Acid-Assisted Organosolv Pre-Treatment and Enzymatic Hydrolysis of *Cynara cardunculus* L. for Glucose Production

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Abstract: Lignocellulosic biomass is a non-edible feedstock that can be used in integrated biorefinery for the production of biochemicals and biofuel. Among lignocellulosic biomass, *Cynara cardunculus* L. (cardoon) is a promising crop thanks to its low water and fertilizer demand. Organosolv is a chemical treatment that uses numerous organic or aqueous solvent mixtures, and a small amount of acid catalyst, in order to solubilize the lignin and hemicellulose fractions, making the cellulose accessible to hydrolytic enzymes. Lignocellulosic residues of cardoon underwent a two-step treatment process to obtain fermentable glucose. In the first step, the milled biomass was subjected to microwave-assisted extraction using an acidified γ-valerolactone (GVL)/water mixture, yielding a solid cellulose pulp. In the second step, the pre-treated material was hydrolyzed by cellulolytic enzymes to glucose. The first step was optimized by means of a two-level full factorial design. The investigated factors were process temperature, acid catalyst concentration, and GVL/water ratio. A glucose production equal to 30.17 g per 100 g of raw material (89% of the maximum theoretical yield) was achieved after conducting the first step at 150 °C using an acidified water solution (1.96% H_2SO_4 *w/w*).

Keywords: *Cynara Cardunculus* L.; organosolv pre-treatment; enzymatic hydrolysis; γ-valerolactone

1. Introduction

Thanks to their availability and no competition with the food chain, lignocellulosic biomass represents an interesting raw material (RM) for the production of energy and chemicals [1]. Lignocellulosic biomass is naturally recalcitrant to enzymatic deconstruction and microbial fermentation, and an effective pre-treatment is required prior to any further conversion into biochemicals and biofuels [2].

Pre-treatment affects the efficiency of enzymatic hydrolysis (EH), improving the overall economics of the conversion process [3]. Pre-treatment technologies and conditions also have an impact on the choice of lignocellulose feedstock, handling and processing, enzyme loading, safety aspects, energy consumption, production cost, and opportunities to generate co-products [4].

Currently, one of the most employed pretreatments for lignocellulosic biomass processing is steam explosion (SE) because of its environmental sustainability, in terms of pollutant generation, and efficiency leading to an increase in enzymatic digestibility [5]. The major disadvantage of SE is its high energy demand for the steam generation step of the process. Among new biomass pretreatments,

microwave heating has been successfully applied to different lignocellulosic biomasses, showing high yields and reduced energy demand when coupled with chemical treatment [6].

The organosolv (OV) pre-treatment represents a high-value opportunity to overcome biomass recalcitrance and to recover relatively pure lignin with high yields as a co-product [7,8]. OV is a chemical treatment that employs numerous organic or aqueous solvent mixtures in order to solubilize both the lignin and hemicellulose fractions, yielding a cellulose-rich residue with increased accessibility to hydrolytic enzymes [9]. In addition, some of the organic solvents, such as methanol, ethanol, acetone, acetic acid, glycerol, and γ-valerolactone (GVL), are derived from renewable sources, enhancing the economic and environmental sustainability of the overall process [10]. GVL is a biomass-derived, renewable, and non-toxic solvent with a high boiling point that provides thermal stability, a key parameter for avoiding high-pressure conditions and reducing the risk usually associated with the volatility and flammability of solvents [11]. By adding a small amount of acid catalyst into GVL, a mild pre-treatment at reduced temperature and reaction times can be performed, obtaining easily accessible cellulose with a low degree of polymerization [12]. As a result, high yields of sugars are achieved after the EH process [13,14]. The liquid fraction could be further processed for the recovery of lignin, hemicellulose derived sugars, and GVL. The GVL can be employed in a new OV cycle, thus decreasing the whole process' environmental pollution impact [15].

Cynara cardunculus L. is a perennial herbaceous species, native to the Mediterranean region, that belongs to the Asteraceae family. Cardoon is an oilseed crop with interesting properties, such as relatively low crop input, large biomass productivity (10–20 t ha^{-1} of dry biomass), and low moisture content. Its properties and ability to grow in marginal lands suggest that it could be useful for biorefinery purposes [16]. Previous studies used cardoon as a feedstock for the production of monosaccharides and biofuels after a physico-chemical pre-treatment, obtaining high process yields [17,18]. On the contrary, very few studies used cardoon as a feedstock in OV processes, and none of these used an acidified GVL/water mixture coupled with microwave heating [19,20]. In this work, lignocellulosic residues of cardoon were treated, through a two-step process, to obtain fermentable glucose. In the first step, the milled biomass was pre-treated, through microwave-assisted extraction, with an acidified GVL/water mixture. In the second step, the cellulose pulp was hydrolyzed by cellulolytic enzymes. Optimization of the first step was carried out using design of experiment (DoE), generating a two-level factorial design. The investigated factors were process temperature, acid catalyst concentration, and GVL/water ratio.

2. Materials and Methods

The RM employed for this study was comprised of dried cardoon stems, kindly provided by Matrica S.p.A (Porto Torres, Italy). The RM was milled to a homogeneous powder, with an average particle size of 1 mm, to be used in both the characterization and pre-treatment phases. All the reagents utilized in this work were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, United States of America). The Cellic® CTec2 enzymatic cocktail was provided by Novozymes (Copenhagen, Denmark).

The RM and the pre-treated material (PM) were characterized according to the National Renewable Energy Laboratory (NREL, Golden, CO, USA) protocols, in order to evaluate their chemical composition [21]. Moisture analysis was performed using a HB43-S Halogen Classic plus (Mettler Toledo, Columbus, OH 43240, USA). Monosaccharide and inhibitor concentrations were analyzed using an ultra-high performance liquid chromatography (UHPLC) Dionex UltiMate 3000 (Thermo Scientific, Sunnyvale, CA, USA) equipped with a Bio-Rad Aminex HPX-87H column (Bio-rad, California, USA) thermo-regulated at 50 °C, and a refraction index detector (ERC RefractoMax 520, Thermo Scientific, Waltham, MA, USA), mobile phase = 0.01 N H_2SO_4, flow 0.6 mL/min. The OV pre-treatment was conducted using an Ethos One microwave-assisted extractor (Milestone S.r.l, Sorisole (BG), Italy).

2.1. Characterization of Raw Material and Pre-Treated Material

The RM showed a water content of 7.98% (*w/w*) and was characterized in terms of cellulose, hemicellulose, lignin, extractives, pectin, acetyl groups, and ash. The PM was characterized for cellulose, hemicellulose, and lignin content [22].

Polymeric cellulose and hemicellulose concentrations were evaluated as C6 and C5 monomers concentrations obtained after acid hydrolysis with H_2SO_4. Monosaccharides concentrations were detected by means of UHPLC analysis. The concentration of polymeric carbohydrates was adjusted for anhydrous polymers correction of 0.88 and 0.90 for C5 and C6 carbohydrates, respectively. The acid-insoluble residue was determined gravimetrically as lignin.

2.2. Design of Experiment

The experimental design generated by the Minitab 17 software and employed in this work was a full factorial design, comprised of 2 levels (−1, +1) for 3 factors, which were temperature of reaction, acid concentration, and solvent composition expressed as the GVL/water ratio. The design comprised 8 runs, which are reported in Table 1. The choice behind each run's reaction conditions was based upon the results observed in a preliminary and unpublished work. The less statistically significant factors of the model were removed using the backward elimination method. The obtained model was used to predict the optimal conditions for maximizing sugar production after EH.

Table 1. The design of experiment (DoE) for the organosolv (OV) pre-treatment of raw material (RM).

Run	T (°C)	Acid (% *w/w*)	GVL/Water
1	180	0.98	0.6
2	160	0.98	0.6
3	180	1.96	0.6
4	180	1.96	0.4
5	160	0.98	0.4
6	160	1.96	0.6
7	180	0.98	0.4
8	160	1.96	0.4

2.3. Process Flowchart

The overall process for the production of fermentable glucose is outlined in Figure 1. The milled RM was added to a PTFE vessel, along with a GVL/water acid solution, using a 1:10 solid to liquid ratio. The GVL/water solution ratio was varied for each different run, as described in the DoE. The reaction was conducted in a microwave extractor and the reaction time was fixed at 30 minutes after reaching the set temperature. The reaction was left to cool for 10 minutes, which is a factory default setting of the microwave extractor. After the reaction, the PM was separated from the solution by vacuum filtration using a Büchner flask. The GVL/water solution, rich in hemicellulose-derived sugars and solubilized lignin, was discarded. The PM was then washed with deionized water (60 °C) to remove all traces of solvent and acid. The washed PM was then subjected to EH. Characterization of cellulose, hemicellulose, and lignin content in the PM, along with cellulose digestibility (CD) and cellulose recovery (CR), was performed for each run. After the OV pre-treatment, the CR was calculated in accordance with the following Equation (1):

$$CR = \frac{C_{pt}}{C_{rm}},\tag{1}$$

where C_{pt} is the cellulose content (g) in the PM and C_{rm} is the cellulose content (g) in the RM prior to the pre-treatment.
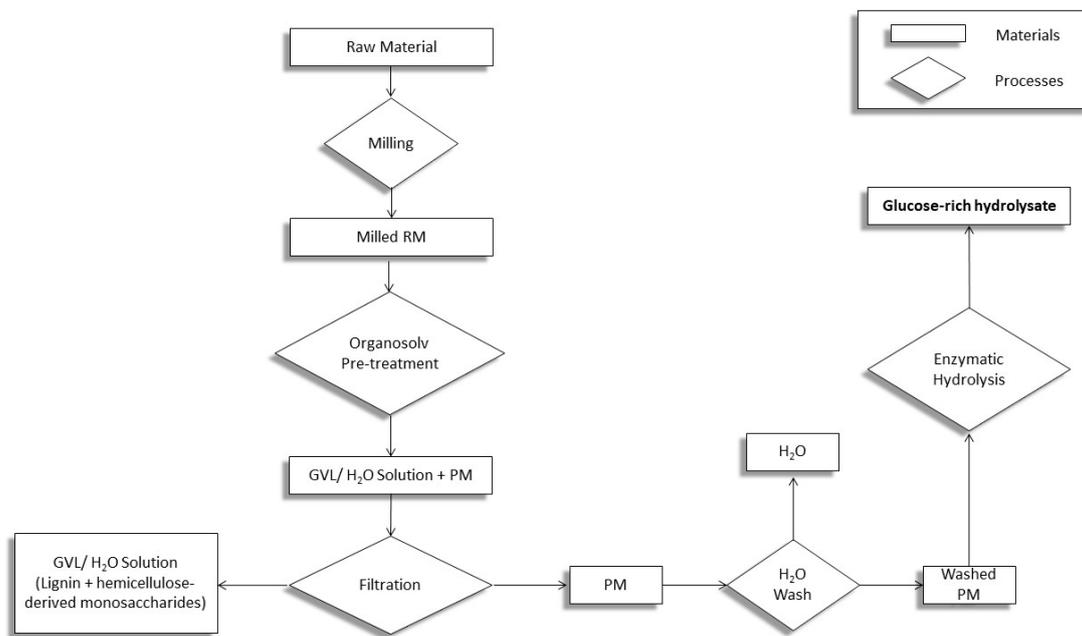
Figure 1. Flowchart of the overall process for the production of fermentable glucose.

2.4. Enzymatic Hydrolysis

The EH of the PM was carried out in 100 mL flasks using a solid loading of 1% (*w/w*), as reported in the analytical procedure for the enzymatic saccharification of lignocellulosic biomass [23]. In order to test the digestibility of cellulose, a high amount of enzyme (0.3 g of enzyme for every g of cellulose) was used. The PM, 0.2 g of sodium azide solution (5%) as an antibacterial agent, and a variable amount of 45 mM citrate buffer solution (pH 5.0) were added to each flask until reaching a total weight of 50 g. The hydrolysis reaction was conducted for 72 h in a thermostatic chamber at 50 °C under shaking condition using an orbital shaker (Phoenix Instrument RS-OS 10) set to 180 rpm. The hydrolysate was then filtered and diluted to 100 mL, in order to normalize the volumes of all runs. The diluted hydrolysates were analyzed for glucose content using the same protocols employed for RM characterization. The CD was calculated in accordance with the following Equation (2):

$$CD = \frac{G_{HY} \times 0.9}{C_{pt}}, \tag{2}$$

where G_{hy} is the glucose content (g) in the hydrolysate, and 0.9 is the anhydrous correction for C6 sugars.

The overall yield (OY), expressed as g of glucose for 100 g of RM, was calculated in accordance with the following Equation (3):

$$OY = \frac{\%C_{rm} \times CR \times CD}{0.9}, \tag{3}$$

where $\%C_{rm}$ is the percentage of cellulose in the RM.

3. Results and Discussion

3.1. Raw Material Characterization

Cardoon was characterized in order to measure its cellulose, hemicellulose, pectin, acetyls, lignin, and extractives content. The results are reported in Table 2.

Table 2. Raw material chemical composition.

Cellulose	Hemicellulose	Pectin	Acetyls	Lignin	Extractives
30.52%	17.17%	4.67%	5.02%	14.21%	7.64%

The results in Table 2 show that cardoon has a reasonable amount of cellulose and a relatively low lignin content, making it an ideal candidate for biorefinery purpose.

3.2. Cellulose Recovery and Digestibility

Table 3 shows the PM characterization and the CR and CD obtained after each run.

Table 3. Pre-treated material (PM) composition, cellulose recovery (CR) and digestibility (CD), and OY.

Run	Cellulose	Hemicellulose	Lignin	CD	CR	OY (g/100 g)
1	92.47%	0.00%	7.33%	76.06%	69.33%	17.87
2	95.71%	0.03%	4.26%	47.17%	89.60%	14.32
3	9.88%	0.00%	9.42%	0.00%	1.27%	0.00
4	78.05%	0.00%	21.95%	94.18%	38.92%	12.42
5	77.82%	2.74%	19.44%	60.67%	92.87%	19.09
6	95.93%	0.00%	4.07%	100%	81.11%	27.47
7	91.09%	0.08%	8.83%	55.58%	67.60%	12.73
8	87.06%	0.06%	12.88%	85.39%	85.29%	24.67

Because of the presence of the acid catalyst, almost all the hemicellulose present in the RM was hydrolyzed into the GVL/water solution, hence the extremely low or null values observed in the PM.

The low CR value observed for run #3 was due to almost complete hydrolysis of the RM, which allowed for the recovery of only a small and carbonized aliquot of sample. The harsh reaction conditions in which the pre-treatment was carried out, along with a higher amount of GVL in the solvent mixture, which further catalyzed the reaction, were the probable culprits of this low yield. The EH was not performed on this sample, since the low mass recovery of hydrolysable material would have produced a negligible quantity of glucose for the CD calculations (hence the 0% in yield). The sample obtained from the run #4 showed the second lowest CD and CR value, again due to the carbonization of the sample during the pre-treatment. This degradation probably hindered the enzymatic attack, lowering the CD. Compared to run #3, the lower amount of GVL generated less harsh pre-treatment conditions, as evidenced by both the higher content in lignin of the final sample and the higher CR value.

Except for these two exceptions, the collected data seemed to confirm the efficiency of the pre-treatment process. The CD was equal to 100% in the sample obtained from run #6, which also showed an excellent value of CR (81.11%). The sample from run #5 had the highest observed CR (92.87%), but a lower CD equal to 60.67%. These achievements suggest that the adequate reaction conditions for the studied biomass were used. Of important notice was also the low amounts of lignin observed in almost all the pre-treated samples, showing the importance of using a lignin coordinating solvent, such as GVL, in the process. A highly delignified substrate could improve the enzymes' activity, as they are not inhibited by the polymeric lignin framework, thus allowing the use of fewer enzymes [24]. In addition, the lignin recovered from organic solvents, such as GVL, is of high quality, due to its low sulfur content, and can used for the production of specialty chemicals [25].

3.3. Design of Experiment

After analyzing the OY response (Table 3) using the backward elimination method with a significance level of 0.1, the linear regression equation generated for the model was (4):

$$\text{OY (g/100 g)} = -129.1 + 0.853 \, T \, (°C) + 160.3 \, \text{Acid} \, (\% \, w/w) - 0.942 \, T \, (°C) * \text{Acid} \, (\% \, w/w), \quad (4)$$

The model explained the total deviance in the OY response with an R^2 equal to 78.96%, which indicates that the model describes the behavior of the data quite well. The residuals versus fits plot showed a non-constant variance (heteroscedasticity) with a decreasing trend, causing a reduction in R^2 value. The higher variance at low fits can be explained by a missing predictor for the model, such as time. The reaction time, which was held fixed in the model, is probably significant at mild pre-treatment conditions, while it becomes non-significant when the other predictor variables of the model assume higher values (high fits).

The *p*-values for the linear term temperature (°C) and the two-way interaction between acid concentration and temperature were significant. The linear term acid concentration (% *w/w*) was also included, since it makes the model hierarchical. Surprisingly, the GVL/water ratio was not statistically significant for the OY response. This result can be explained by the fact that, under the acid concentration investigated, the GVL contribution to CD was negligible. In fact, the acid-catalyzed OV pre-treatment improves the efficiency of EH by both removing the lignin through the organic solvent and boosting the acid catalysis that reduces the degree of cellulose polymerization [26]. Some studies reveal that the improvement in CD is not dependent upon increases in delignification efficiency, but instead the improvement arises from the increase in cellulose chain 'ends', reduced average fiber length, and increased substrate porosity of pre-treated biomass due to the acidic conditions [27–29]. Anyway, the GVL/water ratio becomes statistically significant in the fractionation processes that aim to recover lignin and cellulose as pure as possible [30].

The most important effects and their relative magnitude on the OY response can be observed in the Pareto chart (Figure 2). As previously mentioned, two main effects were statistically significant ($\alpha < 0.1$): temperature (A) and acid concentration by temperature (AB). The largest effect was temperature because it extends the farthest. The interaction effect (temperature by acid concentration) indicates that the relationship between temperature and the OY response depends on the value of acid concentration, as well as the relationship between acid concentration and the OY response depending on the value of temperature.
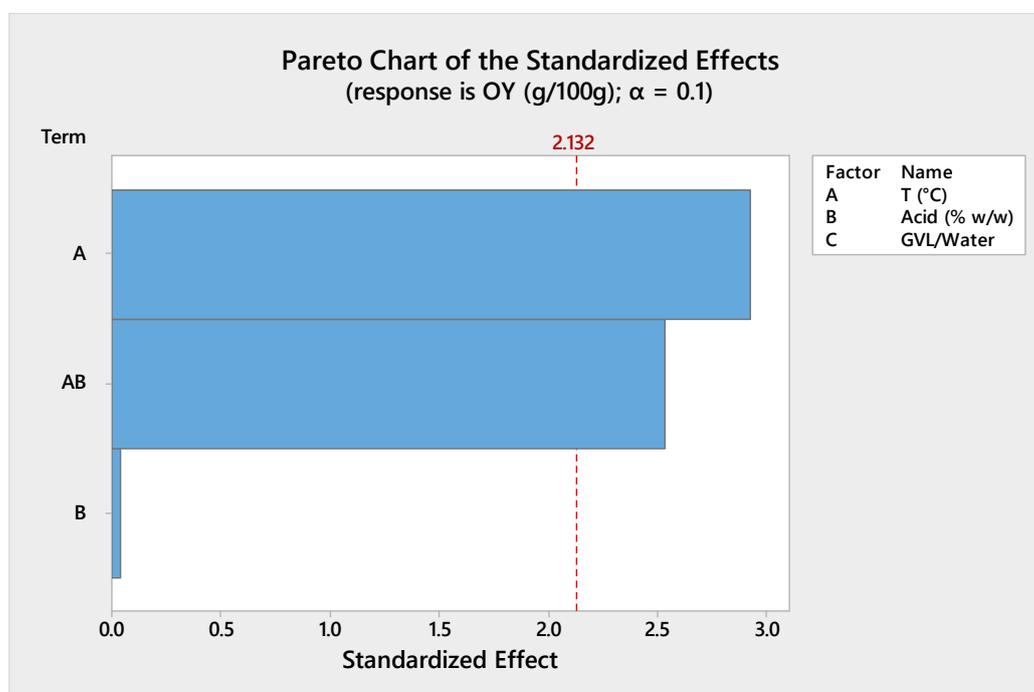

Figure 2. Pareto chart for the OY response, the dashed line indicates the significance limit of the factors on the response.

The contour plot in Figure 3 visualizes in two dimensions the relationship between the OY response and factors. Temperature and acid concentration had positive effects on the OY, but due to the interaction effect, high achievements in terms of OY can obtained by lowering one of the two factors at a time. OY increases as a result of higher CD and CR, which depend on the pre-treatment conditions. Harsh conditions allow for an enhanced CD but at the same time reduce the CR; conversely, mild conditions avoid cellulose degradation, decreasing enzymatic hydrolysis efficiency [18,31].
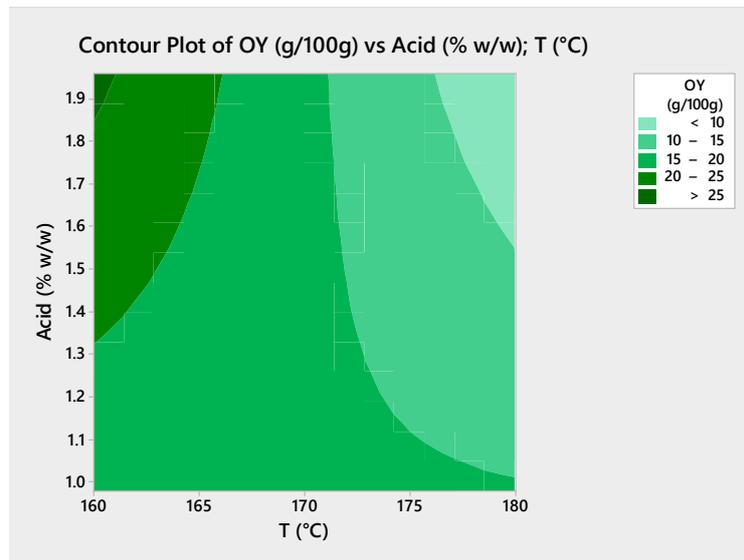

Figure 3. Contour plot of the OY response under different pre-treatment conditions.

In Figure 3, an OY response higher than 25 (g/100 g) can be observed when the lowest temperature (160 °C) and highest acid concentration (1.96%) were used in the pre-treatment process. On the contrary, another respectable OY response, ranging from 20 to 25 (g/100 g), can be obtained by increasing the pre-treatment temperature up to 168 °C and lowering the acid concentration up to 1.3%. These observations are confirmed by some studies where it was shown that the use of an acid catalyst in the pre-treatment reduces the process temperature, delivering an enhanced CD and CR [26,32].

An optimization plot was then generated in order to find the optimal pre-treatment conditions for maximizing the OY response (Figure 4). The optimization plot shows how different experimental settings affect the predicted responses for a stored model.
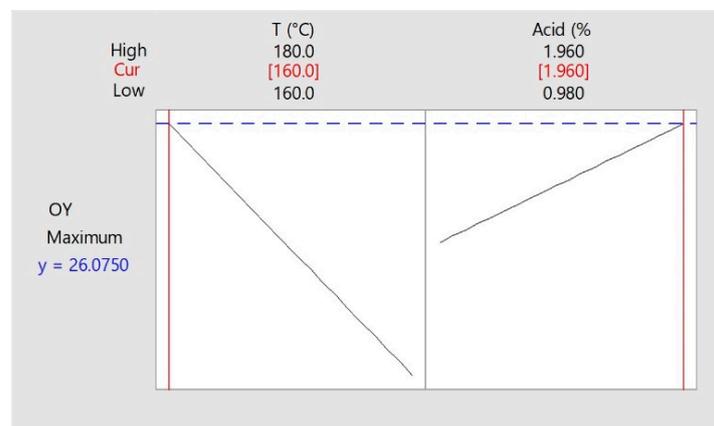

Figure 4. The optimization plot shows the conditions needed to maximize the OY. The vertical red lines on the graph represent the current factors' (temperature and acid concentration) settings.

The optimal conditions predicted by the model possess high desirability for the desired response and predicted an OY of 26.075 ± 2.24 g of glucose per 100 g of RM. The optimization plot shows how high temperatures negatively affect sugar recovery, while a high concentration of acid improves cellulose depolymerization, suggesting the use of the lowest temperature (160 °C) while compensating with the highest acid concentration, in order to enhance carbohydrate production.

The model was validated by performing the pre-treatment under the conditions predicted and using only water as a solvent. The analysis was performed in triplicate in order to obtain statistically significant data and corroborate the robustness of the model. An OY of 26.51 ± 0.75 g of glucose per 100 g of RM (CR 88.37% and CD 88.55%) was found, confirming the value predicted by the model and that the GVL/water ratio is not statistically significant for the OY response. In addition, this achievement validates the idea that at high fits the model variance is low and that the term time is non-significant.

Since the optimization plot (Figure 4) suggested an increasing trend for the OY response at lower temperatures and higher acid concentrations than those investigated, two additional runs using water as solvent were conducted with the scope of testing the gain in OY. The temperature was lowered to 150 °C and 140 °C, respectively, while the acid concentration was set to 1.96% for each run. Since other similar studies on OV processes hardly used acid concentrations higher than 2%, it was decided to leave it at 1.96% [10]. The operational conditions and results are reported in Table 4.

Table 4. Additional runs performed to test the OY response.

Run	T (°C)	Acid (% *w/w*)	CR (%)	CD (%)	OY (g/100 g)
9	140	1.96	95.36	81.65	26.38
10	150	1.96	92.80	95.97	30.17

Run #10 showed an increase in sugar production equal to 30.17 g of glucose per 100 g of RM (89% of the maximum theoretical yield), corroborating the initial hypothesis that lower temperatures are preferred for an efficient sugar extraction. This evidence was also confirmed by the fact that run #10 showed CR and CD values of 92.80% and 95.97%, respectively. Although it is difficult to compare results among different raw materials, the CR and CD values obtained in this study were similar to those reported in an acidified GVL/water solvent system pre-treatment of hardwood (96.5% and 90%) [14] or cotton stalks (87% and 78%) [33].

Despite having the highest CR value (95.36%), run #9 showed a lower value of OY (26.38 g of glucose per 100 g of RM), due to a low CD value (81.65%), indicating that a near maximum yield of fermentable sugars could be achieved at around 150 °C.

Compared to other processes for the production of glucose from cardoon through enzymatic hydrolysis, the combination of this pre-treatment with cellulolytic enzymes led to higher total glucose production. Ballesteros et al. [31] obtained 26.6 g of glucose per 100 g of cardoon using dilute sulfuric acid pre-treatment, while Bertini et al. [18] pre-treated cardoon by acid-catalyzed steam explosion and recovered about 21 g of glucose per 100 g RM.

4. Conclusions

In this work, lignocellulosic residues of cardoon were used as the RM for the production of fermentable sugars from biomass through an OV pre-treatment followed by EH. The biomass was firstly milled and then pre-treated using an acidified solution of water and GVL in a microwave-assisted extractor. The optimal temperature, amount of acid, and the solvent to water ratio to be used in the process was determined using a two-level factorial design of experiment. The generated model was firstly optimized through backward elimination, reducing the number of statistically relevant variables, and then visualizing the most important and influencing factors on a Pareto chart.

Despite its delignification power, GVL resulted to be non-statistically significant in the pre-treatment process that was dominated by the "temperature by acid concentration" interaction

effect, causing a strong depolymerization of cellulose. Under this evidence, the model explained the total deviance in the OY response (g of glucose per 100 g of RM) with an R^2 equal to 78.96%. The highest sugar production, equal to 30.17 g of glucose per 100 g of RM (CD 95.97%), was obtained working at 150 °C with an acid concentration of 1.96%. This value of glucose is equal to 89% of the maximum theoretical yield that can be obtained from 100 g of cardoon. Further development of this study will be focused on the recovery of the liquid fraction resulting from the OV pre-treatment, still rich in high-quality lignin and hemicellulose-derived products.

Author Contributions: A.B. and T.G., carried out experiments on glucose production, M.B. carried out UHPLC analyses, M.G. designed the study and carried out the statistical analyses, G.C. and A.N. drafted the manuscript, and F.C. supervised the research. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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