

1 Article

# 2 Multi-step exploitation of raw *Arundo donax* L. for the 3 selective synthesis of second-generation sugars by 4 chemical and biological route

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15 **Abstract:** Lignocellulosic biomass represents one of the most important feedstocks for future  
16 bio-refineries, being a precursor of valuable bio-products, obtainable ~~obtained~~ ~~from~~ ~~through~~ both  
17 chemical and biological conversion routes. Lignocellulosic biomass has a complex matrix, which  
18 requires the careful development of multi-step approaches for its complete exploitation ~~thus, in~~  
19 ~~order to achieve its complete exploitation, a multi step approach must be developed, optimising~~  
20 ~~the conversion of each fraction~~ to value-added compounds. ~~Based on~~ Under this perspective, the  
21 present work focuses on the ~~investigation~~ valorisation ~~of giant reed (*Arundo donax* L.)~~ hemicellulose  
22 and cellulose fractions of giant reed (*Arundo donax* L.) ~~valorisation~~ to give second-generation sugars,  
23 ~~at the same time minimizing the~~ reducing the ~~formation of reaction~~ by-products ~~formation~~. The  
24 conversion of hemicellulose to xylose was ~~undertaken~~ performed in the presence of the  
25 heterogeneous acid catalyst ~~acid catalyst~~ Amberlyst-70 acid catalyst, under microwave  
26 irradiation. The ~~influence-effect~~ of the main reaction parameters, such as temperature, reaction  
27 time, catalyst and biomass loadings on sugars yield was studied, ~~developing~~ choosing a high  
28 gravity approach ~~the last according to the high gravity approach, on sugars yield was studied~~.  
29 Under the optimised reaction conditions (17 wt% *Arundo donax* L. loading, 160 °C,  
30 Amberlyst-70/*Arundo donax* L. weight ratio 0.2 wt/wt) the xylose yield was 96.3 mol%. In the second  
31 step, ~~t~~ The cellulose-rich solid residue was then ~~exploited~~ through the both chemical and  
32 enzymatic routes, obtaining ~~giving~~ glucose yields of up to 32.5 and 56.2 mol%, respectively. These  
33 ~~present~~ work proves the efficiency of this innovative combination of chemical and biological  
34 catalytic approaches, in order to achieve ~~for~~ the selective conversion of hemicellulose and cellulose  
35 fractions of *Arundo donax* L. to versatile platform products.

36 **Keywords:** *Arundo donax* L.; microwaves; Amberlyst-70; second-generation sugars; high gravity  
37 approach; chemical and enzymatic hydrolysis; cascade biomass exploitation.  
38

## 39 1. Introduction

40 The anticipated depletion of fossil resources in the nearest future together with their associated  
41 unfavourable environmental outcomes ~~The lack of fossil resources expected in the next future~~  
42 together with their contribution to the environmental concerns are driving ~~the~~ industrial research  
43 towards the exploitation of promising alternative renewable resources. These alternative

44 [renewable resources include such as the lignocellulosic biomass \[1,2\], animal manure and human](#)  
45 [sewage \[3,4\], food meat processing waste \[5,6\] and aquatic biomass \[7,8\], -which can be employed](#)  
46 [as biorefinery feedstocks. \(seaweeds, microalgae and aquatic plants\). Among them, such as the](#)  
47 [lignocellulosic biomass. It represents a key is a very promising](#) feedstock, being abundant, [safe and](#)  
48 [cheap and it is](#) mainly composed [of by](#) three biopolymers (cellulose, hemicellulose and lignin), [which](#)  
49 [are all of them](#) precursors of [very valuable bio-products and bio-fuels valuable intermediates](#) [94,102].  
50 For this reason, the selective fractionation of the lignocellulosic biomass is of paramount importance,  
51 in order to optimise the recovery and the valorisation of each biopolymer, adopting a multi-step  
52 [process](#) approach. [However, the conversion of this feedstock is limited by its recalcitrant nature that](#)  
53 [makes indispensable the use of suitable pretreatment steps. They, aimed at improving the efficiency](#)  
54 [of the hydrolysis of the polysaccharides hydrolysis \[113–135\], -thus leading to the release of](#)  
55 [second-generation sugars \(xylose and glucose\), furfural, 5-hydroxymethylfurfural \(HMF\), levulinic](#)  
56 [acid \(LA\) and formic acid \(FA\) \[146–2143\]. However, the conversion of this feedstock is limited by its](#)  
57 [recalcitrant nature that makes indispensable pretreatment steps aimed at decreasing the](#)  
58 [lignocellulosic complex matrix and improving the efficiency of the polysaccharides hydrolysis \[3–5\],](#)  
59 [leading to second generation sugars \(xylose and glucose\), furfural, 5 hydroxymethylfurfural \(HMF\)](#)  
60 [and levulinic acid \(LA\) \[6–13\]. A large number of pretreatments have been reported in the literature](#)  
61 [employing mineral acids \[2214–2446\], inorganic salts \[2517,2648\], alkaline solutions \[2315,2719\],](#)  
62 [ionic liquids \[2820,2924\] and organosolv \[3022,3123\], in addition to physico-chemical techniques,](#)  
63 [such as the steam explosion \[32242\]. The choice of the suitable pretreatment strongly depends on the](#)  
64 [biomass fraction that should be exploited and on the target products. For instance, the organosolv](#)  
65 [treatment is efficient for the recovery of cellulose and lignin, but it is not suitable for that the](#)  
66 [exploitation](#) of hemicellulose [3325]. On the other hand, the pretreatment with mineral acids [Up to](#)  
67 [now, the pretreatment with mineral acids, in particular H<sub>2</sub>SO<sub>4</sub>, is one of is preferred for the](#)  
68 [conversion of both cellulose and hemicellulose fractions, the most adopted because of its high](#)  
69 [efficiency to disrupt the lignocellulosic matrix, thus selectively releasing the monomeric sugars. It](#)  
70 [showed to be highly efficient to disrupt the lignocellulosic matrix releasing the monomeric sugars.](#)  
71 [However, it](#) low acid concentrations and mild reaction conditions must be employed, in order to  
72 limit the [excessive hemicellulose degradation, of the hemicellulose fraction in the liquid phase \[2214\].](#)  
73 [Therefore, the best hemicellulose pretreatment gives back the highest xylose concentration in the](#)  
74 [hydrolysate and, at the same time, the lowest one of inhibitors, avoiding which avoids any additional](#)  
75 [detoxification step before fermentation \[2416,3426\]. At the end of the hemicellulose pretreatment, a](#)  
76 [cellulose-rich solid is recovered, which can be further exploited by a subsequent chemical or](#)  
77 [enzymatic hydrolysis step which is the component of the lignocellulosic biomass mainly affected by](#)  
78 [the acid treatment \[14\], ensuring the realization of an efficient multi step approach. This is very](#)  
79 [important, because the multi step approach affords several benefits, such as the high concentration](#)  
80 [of the xylose solutions, the low amount of inhibitors in the hydrolysate, which makes unnecessary](#)  
81 [any detoxification before fermentation \[16,23\], and the recovery of a cellulose rich solid that can be](#)  
82 [subjected to a subsequent chemical or enzymatic hydrolysis in order to exploit also the cellulose](#)  
83 [fraction \[2517,35274\]. However, the employment of mineral acids has some disadvantages, such as](#)  
84 [the expensive catalyst recovery and the possible corrosion of the stainless steel equipment. About](#)  
85 [the last issue, it is reported that the use of H<sub>2</sub>SO<sub>4</sub> concentration higher than 0.05 wt% can rapidly](#)  
86 [corrode a Type 316 stainless steel \(widely used for the construction of autoclaves\), even at moderate](#)  
87 [severity conditions of temperature and time, which are typical of the hydrothermal pretreatments](#)  
88 [\[3628\]. Therefore, common stainless steels cannot be adopted for the acid hydrothermal treatments,](#)  
89 [which instead require special alloys, such as various grades of Hastelloy, zirconium or other](#)  
90 [corrosion-resistant alloys, which ensure excellent corrosion resistance to acids, even at high severity](#)  
91 [conditions \[3729\]. These issues have driven the research towards the employment of low-cost acid](#)  
92 [solid catalysts, such as styrene-based sulfonic acid resins \(Amberlysts\) -the employment of](#)  
93 [acid corrosion resistant equipment, that have driven the research towards the employment of solid](#)  
94 [acid catalysts \[3830–4436\]. The use of these catalysts can provide a large number of advantages over](#)  
95 [the traditional homogeneous acid catalysts: \(i\) better reaction control, \(ii\) reduced formation of](#)

96 [side-products](#), (iii) [easy and safe operation with minor corrosion problems compared to](#)  
 97 [homogeneous catalysis](#), (iv) [advantageous recovery of the catalyst with less waste disposal](#). In  
 98 [addition, the recyclability of these catalysts is a key requirement for the economics: in fact, despite](#)  
 99 [the higher initial cost of the sulfonic acid resins compared to traditional sulfuric acid, if the acid resin](#)  
 100 [catalyst maintains almost unaltered its starting catalytic performances \(without desulfonation and](#)  
 101 [leaching\)](#), it can be reused repeatedly in additional batch reactions. Therefore, its cost per batch  
 102 [operation can be progressively cut down, even below those of the traditional mineral acids, per unit](#)  
 103 [of productivity](#). Lastly, depending on their thermo-chemical stability, resin catalysts can be used  
 104 [more advantageously in continuous systems, leading to significant economic advantages of](#)  
 105 [productivity on industrial scale](#). However, also heterogeneous catalysts still presents some problems,  
 106 in particular the inefficient solid-solid interaction between the biomass and the catalyst, ~~which that~~  
 107 ~~implies a~~ hampered hydrolysis. For this reason, ~~in the literature~~, the ~~solid-acid solid~~ catalysts, such  
 108 as Amberlyst-15, zeolites and ad-hoc synthesized carbon-based solid acids, have been ~~used mainly~~  
 109 ~~reported~~ in combination with ionic liquids [4133,4335], which are able to dissolve cellulose and even  
 110 raw lignocellulosic biomasses, or ~~as catalysts employed for substrates pretreated by substrates~~  
 111 ~~previously pretreated by~~ ball milled substrates [4234] or ~~pretreated by~~ acid/alkali solutions  
 112 [4032,4436], in this last case not ~~solving/avoiding~~ the corrosion problem. Different raw  
 113 feedstock materials and heterogeneous catalysts have been reported in the literature for the  
 114 [hydrolysis of hemicellulose and cellulose](#), as summarized in Tables 1 and 2, respectively.  
 115

116 **Table 1. Literature review on hemicellulose hydrolysis with based on heterogeneous**  
 117 **catalysts.**

<u>Raw material</u>	<u>Catalyst</u>	<u>Final product</u>	<u>Yield</u>	<u>Process conditions</u>	<u>Reference</u>
<a href="#">Corn cob</a>	<a href="#">HSO<sub>3</sub>-ZSM-5</a>	<a href="#">Xylose</a>	<a href="#">28.7 wt%</a>	<a href="#">120 °C, 6 h, S/L 1/20, Cat/sub 1:1 wt/wt</a>	<a href="#">3830</a>
<a href="#">Corn cob</a>	<a href="#">Carbon based solid acid (C-SO<sub>3</sub>H)</a>	<a href="#">Xylose</a>	<a href="#">78.1 mol%</a>	<a href="#">140 °C, 6 h, S/L 1/100, Cat/sub 1:1 wt/wt</a>	<a href="#">3931</a>
<a href="#">Giant reed</a>	<a href="#">Amberlyst 35DRY</a>	<a href="#">Total reducing sugars</a>	<a href="#">43.0 wt%</a>	<a href="#">IL pretreatment: 120 °C, 3 h, S/L 1/20 [C<sub>4</sub>mim]Cl; acid hydrolysis: 120 °C, 1.5 h, S/L 1/20, Cat/sub 1:5 wt/wt</a>	<a href="#">4133</a>
<a href="#">Rice straw</a>	<a href="#">Carbon based solid acid (-COOH)</a>	<a href="#">Xylose</a>	<a href="#">52.1 mol%</a>	<a href="#">Alkali pretreatment: 120 °C, 4 h, KOH 6 wt%, S/L 1/10; ball-milling treatment: 4 h, 500 r·min<sup>-1</sup>, Cat/sub 1:2 wt/wt; acid hydrolysis: 200 °C, 1 h, S/L 1/100, Cat/sub 1:2 wt/wt</a>	<a href="#">4234</a>
<a href="#">Eucalyptus</a>	<a href="#">Carbon based solid acid (-COOH)</a>	<a href="#">Xylose</a>	<a href="#">83.0 mol%</a>	<a href="#">215 °C, 0.5 h, S/L 1/130, Cat/sub 1:6.5 wt/wt</a>	<a href="#">4436</a>

118 [S/L = solid to liquid weight ratio; Cat/sub = catalyst to substrate weight ratio.](#)  
 119  
 120

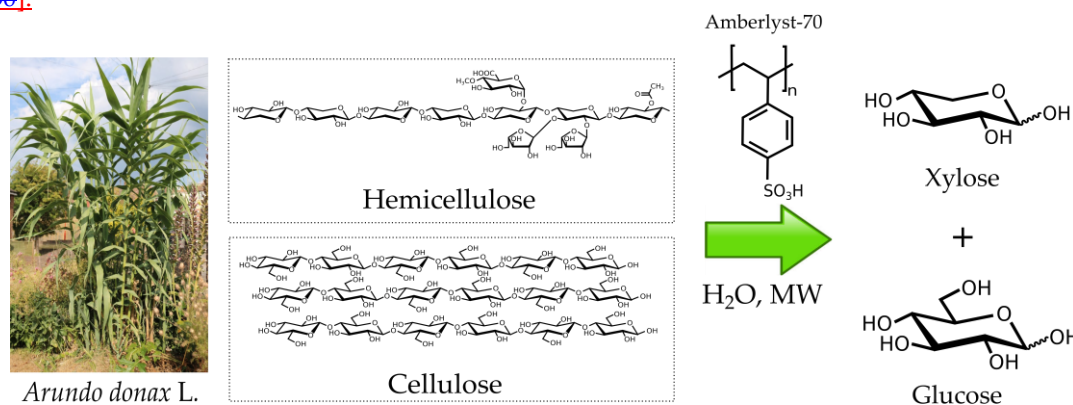
121 **Table 2. Literature review on cellulose hydrolysis with based on heterogeneous catalysts.**

<u>Raw material</u>	<u>Catalyst</u>	<u>Final product</u>	<u>Yield</u>	<u>Process conditions</u>	<u>Reference</u>
<u>Corn cob</u>	<u>HSO<sub>3</sub>-ZSM-5</u>	<u>Glucose</u>	<u>23.0 wt%</u>	<u>120 °C, 6 h, S/L 1/20, Cat/sub 1:1 wt/wt</u>	<u>3830</u>
<u>Corn cob</u>	<u>Cellulase enzyme</u>	<u>Glucose</u>	<u>91.6 wt%</u>	<u>50 °C, 48 h, 40 FPU/g substrate</u>	<u>3931</u>
<u>Rice straw</u>	<u>Amberlyst 15DRY</u>	<u>Total reducing sugars</u>	<u>26.8 wt%</u>	<u>Alkali pretreatment: 120 °C, 1 h, NaOH 2 wt%, S/L 1/7; acid hydrolysis: 140 °C, 4 h, S/L 1/20, Cat/sub 1:1.4 wt/wt</u>	<u>4032</u>
<u>Rice straw</u>	<u>Carbon based solid acid (-COOH)</u>	<u>Glucose</u>	<u>66.5 mol%</u>	<u>ball-milling treatment: 4 h, 500 r·min<sup>-1</sup>, Cat/sub 1:2 wt/wt; acid hydrolysis: 200 °C, 1 h, S/L 1/100, Cat/sub 1:2 wt/wt</u>	<u>4234</u>
<u>Corn stover</u>	<u>PSSA/PIL</u>	<u>Glucose</u>	<u>20.0 wt%</u>	<u>105 °C, 7 h, S/L 1/20, Cat/sub 3:1 wt/wt, [EMIM]Cl/H<sub>2</sub>O 4:1 wt/wt</u>	<u>4335</u>
<u>Eucalyptus</u>	<u>Carbon based solid acid (-COOH)</u>	<u>Glucose</u>	<u>31.0 mol%</u>	<u>215 °C, 0.5 h, S/L 1/130, Cat/sub 1:6.5 wt/wt</u>	<u>4436</u>

S/L = solid to liquid weight ratio; Cat/sub = catalyst to substrate weight ratio. PSSA/PIL = poly(styrene sulfonic acid)/poly(vinyl imidazolium chloride)

Among the lignocellulosic biomasses, giant reed (*Arundo donax* L.) is a suitable feedstock, being a perennial herbaceous plant with a high production yield (up to 30 t ha<sup>-1</sup> year<sup>-1</sup>), ~~growingable to grow~~ also in marginal ~~quality~~ lands. It is rich ~~with of~~ structural C5 and C6 carbohydrates, which represent up to 60 wt% of the dry biomass [45372], ~~and, f~~ For this reason, *Arundo donax* L. ~~it~~ has been widely ~~studied-used~~ as a substrate for the synthesis of important ~~platform-chemicals~~, biofuels and second-generation sugars [146,168,2517,2719,35274,463833–484035]. In particular, the interest towards the synthesis of xylose and glucose is continuously growing, ~~due to their promising applications in both chemical and biological processes to produce alcohols, acids, oils, hydrocarbons, hydrogen and other valuable products~~ [1,157,2214,34263,46383,47394,414936–55483]. However, up to now, only few works have ~~discussedreported~~ the employment of ~~solid-acid~~ solid catalysts for the conversion of *Arundo donax* L. to value-added products, performing the reaction in the presence of ionic liquids as solvents, which have some criticisms related to their high viscosity, toxicity and ~~high~~ cost, ~~which strongly limit the sustainability of this approach.making the process not really sustainable~~. For example, You et al. ~~carried outperformed~~ the hydrolysis of *Arundo donax* L. to second-generation sugars in 1-butyl-3-methylimidazolium chloride, adopting Amberlyst 35DRY as ~~the~~ catalyst. They carried out a pretreatment step without catalyst at 120 °C for 3 h employing the giant reed loading of 5 wt%. Subsequently, the Amberlyst 35DRY, with a catalyst/biomass weight ratio of 0.2 wt/wt, was added to the reaction mixture and the hydrolysis ~~reaction~~ was performed at 120 °C for 1.5 h. Under these reaction conditions, the authors reported the highest yield to total reducing sugars of 43 wt%, ~~based on the starting biomass amount~~ [413328]. ~~Despite the good catalytic performances, the higher manufacturing cost for the Amberlyst-35, ascribed to the over-sulfonation treatment, and its thermal instability (desulfonation and deactivation) at temperatures higher than 150 °C, together with the use of costly ionic liquids, shift the attention~~

148 towards other types of Amberlyst resins. The present work proposes, for the first time, the  
 149 employment of the acid resin Amberlyst-70 for the hydrolysis of hemicellulose and cellulose  
 150 fractions of the untreated giant reed to xylose and glucose, respectively, working in water and under  
 151 microwaves (MW) irradiation (Figure 1). In fact, monosulfonated Amberlyst-70, whose Brønsted  
 152 acidity is due to the sulfonic groups present on its surface, shows acid site strengths similar to  
 153 thatose of the over-sulfonated Amberlysts-35 and -36 [5649], but a higher-and-being-more thermally  
 154 stabilityble than most of the majority-of heterogeneous acid resins, which is a desirable requirement  
 155 for the development of many biomass conversion processes allows us to adopt higher temperatures  
 156 [5750].



157 **Figure 1:** Amberlyst-70 catalysed conversion of the hemicellulose and cellulose fractions of the  
 158 *Arundo donax L.* to xylose and glucose, respectively.  
 159

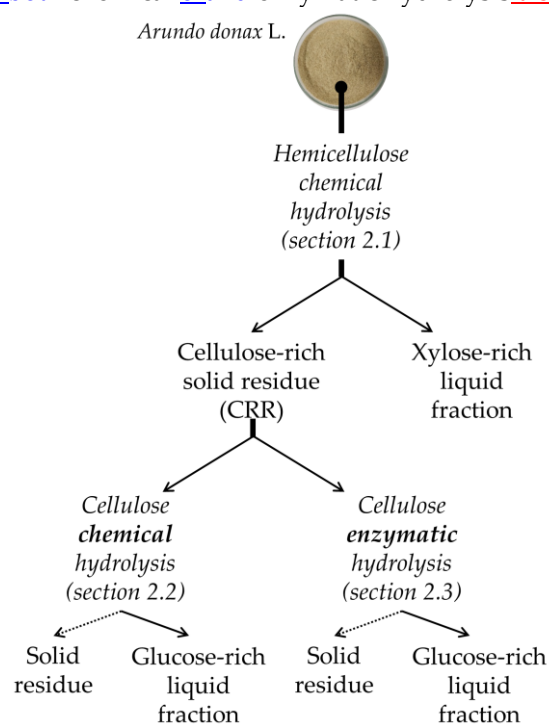
160  
 161 The present work proposes, for the first time, the employment of the acid resin Amberlyst-70  
 162 for the hydrolysis of untreated giant reed in water under microwave irradiation. In fact,  
 163 Amberlyst 70, whose acidity is due to the sulfonic groups present on its surface, is more thermally  
 164 stable than the majority of heterogeneous acid resins, allowing us to study a wider range of  
 165 temperature. Moreover, this resin was already successfully employed by us in a previous research  
 166 where it was adopted as acid catalyst for the conversion of fructose and inulin to  
 167 5-hydroxymethyl-2-furaldehyde (HMF) in water in the presence of microwave heating, resulting an  
 168 active and recyclable system has already proven to be resulted active and recyclable in aqueous  
 169 medium and in the presence of microwave (MW) heating [575044], being the latter representing a  
 170 very important tool for the sustainability of the reaction. In the perspective of the sustainability of  
 171 the reaction, microwaves (MW) represent an important tool because they can reduce the reduction  
 172 time and the energy consumption, thus improving the efficiency of the processes [58-62]. Moreover,  
 173 MW play In fact, (The microwaves (MW), in fact, can improve the efficiency of the process, reducing  
 174 the reaction time and the energy consumption [5145-5549], in addition to the key role of selectively  
 175 breaking the hydrogen bonds during the biomass conversion, decreasing the stability of the  
 176 lignocellulosic matrix [113,63560]. In the first step of the proposed process, the xylose yield washas  
 177 been optimisedoptimized, whilst in the second onestep, the cellulose-enriched solid residue, which  
 178 resulted and the solid residue enriched in cellulose, washas been subsequently subjected to both  
 179 chemical and enzymatic hydrolysis to give glucose. Regarding the chemical hydrolysis, the whole  
 180 solid residue recovered at the end of the first step, containing embedded Amberlyst-70, washas been  
 181 directly reprocessed under more severe reaction conditions, in order to convert the cellulose fraction  
 182 and investigate the recyclability of the catalyst. For the enzymatic hydrolysisOn the other hand, the  
 183 unreacted *Arundo donax L.* was separated from the embedded Amberlyst-70 by sieving, and it was  
 184 converted through the enzymatic hydrolysis-adopting the commercial cellulolytic enzymatic  
 185 preparation Cellic<sup>®</sup>™ CTtec2, which ishas already proved its effectiveness for the hydrolysis of  
 186 cellulose fraction of biomasses pretreated by alkaline and acid treatmentsolutions [64571-66593].  
 187 The present work investigatessuccessfully optimised a novel multi-stage treatment, which consists  
 188 made of an integrated preliminary-chemical and step followed by a biological processone. Lastly, it



189 ~~is important to underline that~~ the catalytic runs ~~were have been~~ carried out employing starting  
 190 concentrations of substrate up to 17 wt%, ~~a value~~ higher than ~~the majority those~~ reported in the  
 191 literature, generally under 8 wt% [~~2,6054,676155~~]. ~~-, The employment of high starting substrate~~  
 192 ~~concentrations is in agreement with adopting~~ the high gravity approach [~~686256~~], which allows the  
 193 production of ~~more~~ concentrated hydrolysates. This approach is particularly promising ~~from their~~  
 194 industrial perspective, because the ~~higher products~~ ~~concentrations of products~~ leads to the increase  
 195 of productivity and the reduction of capital and operating costs, ~~requiring involving~~ easier  
 196 purification steps.

## 197 2. Results and Discussion

198 The multi-step hydrolysis approach is fundamental in order to ~~exploit each enhance the~~  
 199 ~~selective valorisation of each biomass polysaccharide fraction of the biomass.~~ ~~On this basis,~~ in the  
 200 present study, as ~~the~~ first step, the MW-assisted hydrolysis of giant reed hemicellulose into xylose  
 201 was investigated adopting Amberlyst-70 as heterogeneous catalyst and, subsequently, the recovered  
 202 cellulose-rich residue (CRR) was employed as substrate for the conversion of ~~its the~~ cellulose ~~fraction~~  
 203 into glucose, by means ~~of the both~~ chemical ~~and~~ enzymatic hydrolysis ~~treatments~~ (Figure 2).



204  
205 **Figure 2.** Diagram of the multi-step hydrolysis approach of *Arundo donax L.*

### 206 2.1. Microwave-assisted hydrolysis of giant reed hemicellulose to xylose catalysed by Amberlyst-70

207 It is well-known that hemicellulose is more easily hydrolysable than cellulose, ~~requiring~~ the two  
 208 hydrolysis processes ~~involving requiring~~ different optimised reaction conditions. In the first step,  
 209 the selective conversion of the hemicellulose into xylose ~~in the presence of Amberlyst-70~~ was studied  
 210 and ~~optimised optimized, in the presence of Amberlyst-70~~. Before the catalytic investigation, the  
 211 composition of the starting raw *Arundo donax L.* was evaluated according to the NREL protocol  
 212 [~~696357-73674~~], obtaining the following values (wt% on dry matter): glucan  $36.3 \pm 0.4$ , xylan  $17.3 \pm$   
 213  $0.2$ , arabinan  $1.9 \pm 0.1$ , ash  $2.0 \pm 0.0$ , Klason lignin (acid-insoluble residue)  $22.0 \pm 0.0$ , acid-~~soluble~~  
 214 lignin  $0.9 \pm 0.1$ , other compounds  $19.6 \pm 0.9$ . The values ~~represent derived from~~ the mean of 3  
 215 replicates,  $\pm$  standard deviation (SD).

216 The effects of ~~the main~~ process parameters, such as biomass loading, Amberlyst-70/*Arundo*  
 217 *donax L.* weight ratio and temperature, on reducing sugars yield and by-products concentration  
 218 were investigated and the obtained results are reported in Tables ~~34~~ and ~~42~~, respectively. A

219 preliminary investigation regarding the ~~effect influence~~ of the Amberlyst-70/*Arundo donax* L. weight  
 220 ratio on the ~~xylose recovery hemicellulose hydrolysis~~ was carried out at 160 °C for 20 min, keeping  
 221 constant the biomass loading ~~at~~ (5 wt%) and ranging the Amberlyst-70/*Arundo donax* L. weight ratio  
 222 between 0.1–0.3 wt/wt (runs 1–3, Tables ~~31~~ and ~~42~~). These reaction conditions were selected on the  
 223 basis of previous literature researches [~~403227, 413328~~]. As reported in Table ~~31~~, the increase of the  
 224 catalyst/~~substrate to biomass~~ weight ratio from 0.1 to 0.3 wt/wt caused an increase of the biomass  
 225 solubilisation, from 31.5 to 47.7 wt%, together with the improvement of sugars yields. In fact, the  
 226 xylose yield, calculated respect to xylan ~~content~~ in the raw material, ~~rose~~anged from 68.4 to 94.8  
 227 mol%, corresponding to the concentrations of 6.7 and 9.3 g/L, and the glucose yield, calculated  
 228 respect to glucan ~~content~~ in the ~~raw material starting giant reed~~, ~~rose~~anged from 8.5 to 13.0 mol%,  
 229 corresponding to the concentrations of 1.7 and 2.6 g/L. Regarding the xylose yield, the best result of  
 230 97.5 mol% was obtained adopting the catalyst/substrate weight ratio of 0.2 wt/wt (run 2, Table ~~31~~).  
 231 These preliminary tests ensured the complete and selective hydrolysis of *Arundo donax* L.  
 232 hemicellulose to xylose under mild reaction conditions, as confirmed also by the negligible  
 233 by-products concentrations (run 2, Table ~~42~~). Among them, only acetic acid (AA), directly originated  
 234 from the hydrolysis of hemicellulose [~~74682~~], reached concentrations higher than 0.5 g/L. The  
 235 formation of ~~formic acid~~ (FA), LA, HMF and furfural, ~~all which~~ derivinge from the  
 236 ~~degradation hydration~~ pathways of glucose and xylose, ~~was resulted~~ ~~scarcely poorly~~  
 237 ~~promoted favoured~~.

238 **Table 31.** Experimental set-up and results of hemicellulose hydrolysis. Reaction conditions: MW  
 239 heating, 20 minutes.

Run	Biomass loading (wt%)	Cat/Sub <sup>1</sup> (wt/wt)	Temperature (°C)	Biomass solubilisation (wt%)	Xylose yield <sup>2</sup> (mol%)	Glucose yield <sup>3</sup> (mol%)
1	5	0.1	160	31.5	68.4	8.5
2	5	0.2	160	44.5	97.5	12.5
3	5	0.3	160	47.7	94.8	13.0
4	9	0.1	160	30.3	66.7	8.2
5	9	0.2	160	43.8	99.2	11.8
6	9	0.3	160	46.3	94.0	12.0
7	13	0.1	160	29.7	65.3	8.1
8	13	0.2	160	44.3	93.4	11.9
9	13	0.3	160	48.4	95.8	12.2
10	13	0.1	150	29.5	53.3	7.0
11	13	0.2	150	40.9	85.3	10.8
12	13	0.3	150	43.7	83.9	10.9
13	17	0.2	160	42.9	96.3	10.2

240 <sup>1</sup> Weight of Amberlyst-70/dry weight of starting giant reed; <sup>2</sup> Yield respect to moles of xylan in the starting giant  
 241 reed; <sup>3</sup> Yield respect to moles of glucan in the starting giant reed.

242 In order to improve the sustainability of the ~~reaction process~~, the high gravity approach was  
 243 adopted, testing the same experimental conditions (160 °C, 20 min, 0.1–0.3 wt/wt

Amberlyst-70/*Arundo donax* L. weight ratio) at two higher biomass loadings, of 9 wt% (runs 4–6, Tables 31 and 42) and 13 wt% (runs 7–9, Tables 31 and 42). As a first observation, it is important to underline that, keeping constant the Amberlyst-70/*Arundo donax* L. weight ratio and increasing the biomass loading, no significant variations of the biomass solubilisation and the sugars yield occurred, highlighting that these conditions are still favourable to the production of sugars, rather than to that of their degradation products working at the same Amberlyst-70/*Arundo donax* L. weight ratio, the increase of biomass loading did not significantly influence the biomass solubilisation and the sugars yield, contrary to what can be expected due to the fact that the increase of biomass loading generally leads to the decrease of sugars yield because their degradation [686256]. Regarding the sugar concentrations, working with the Amberlyst-70/*Arundo donax* L. weight ratio of 0.2, they increased from 9.6 to 27.5 g/L for xylose and from 2.5 to 7.2 g/L for glucose, adopting 5 and 13 wt% biomass loadings, respectively. This positive trend is due to the very limited by-products formation of by-products, even observed also at these high biomass loadings. In fact, except for AA that derives from hemicellulose hydrolysis, each by-product (FA, LA, HMF and furfural) reached concentrations up to lower than 1.35 g/L for all the performed runs, which are lower than those achieved with traditional typical concentrations achieved in mineral acids pretreatments with the same biomass loadings [35274,46383]. Moreover, the Amberlyst-70/*Arundo donax* L. weight ratio of 0.2 wt/wt produced the optimal best compromise between xylose yield and catalyst amount also working at 9 and 13 wt% of biomass loading, ensuring the very high yields of xylose yields equal to of 99.2 mol% and 93.4 mol%, respectively (runs 5 and 8, Table 31). In order to evaluate the possibility of reaching high xylose yields at milder reaction conditions, and keeping starting from elevated high the biomass loading (13 wt%), the reaction was performed at 150 °C for 20 min with the *Arundo donax* L. loading of 13 wt% and the same catalyst/biomass weight ratios, were investigated (runs 10–12, Tables 31 and 42). Comparing runs 7–9 with runs 10–12, for each value of Amberlyst-70/*Arundo donax* L. weight ratio, the temperature of 150 °C led to lower sugars yields than those obtained at 160 °C and analogous comparable by-products concentrations.

Table 42. Influence of the reaction conditions on by-products concentration formed during the hemicellulose hydrolysis. Reaction conditions: MW heating, 20 minutes.

Run	Biomass loading (wt%)	Cat/Sub <sup>1</sup> (wt/wt)	Temperature (°C)	AA <sup>2</sup> (g/L)	FA <sup>3</sup> (g/L)	LA <sup>4</sup> (g/L)	HMF <sup>5</sup> (g/L)	Furfural (g/L)
1	5	0.1	160	0.8	0.2	0.0	0.1	0.0
2	5	0.2	160	0.9	0.2	0.0	0.1	0.0
3	5	0.3	160	1.1	0.3	0.0	0.2	0.0
4	9	0.1	160	1.6	0.1	0.1	0.4	0.0
5	9	0.2	160	2.1	0.7	0.3	0.7	0.0
6	9	0.3	160	2.8	0.7	0.5	0.7	0.1
7	13	0.1	160	2.9	0.2	0.2	0.8	0.4
8	13	0.2	160	4.1	1.1	0.5	1.1	0.7
9	13	0.3	160	4.2	1.1	0.6	1.0	0.7
10	13	0.1	150	1.0	0.0	0.0	0.3	0.1
11	13	0.2	150	2.9	0.7	0.3	0.9	0.5
12	13	0.3	150	3.1	0.7	0.4	0.8	0.5



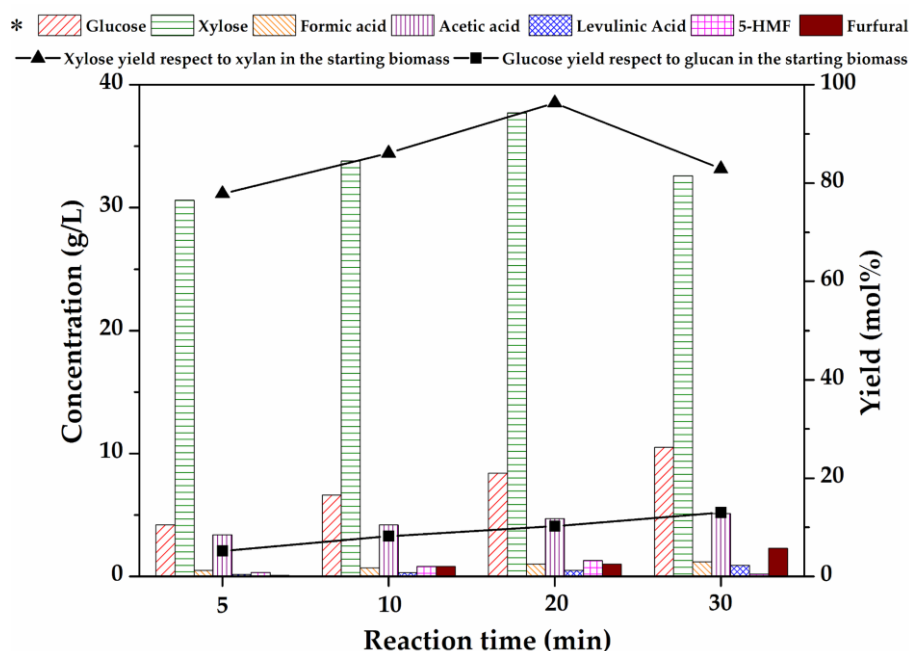
13      17      0.2      160      4.7      1.0      0.5      1.3      1.0

272 <sup>1</sup> Weight of Amberlyst-70/dry weight of starting giant reed; <sup>2</sup> Acetic acid; <sup>3</sup> Formic acid; <sup>4</sup> Levulinic acid; <sup>5</sup>  
273 5-Hydroxymethylfurfural

274 For example, at the Amberlyst-70/*Arundo donax* L. weight ratio of 0.2 wt/wt, ~~the yields of~~ xylose  
275 and glucose yields at 150 °C were 85.3 and 10.8 mol%, respectively, whereas at 160 °C the  
276 corresponding data values were ~~equal to~~ 93.4 and 11.9 mol%, respectively.

277 The biomass loading was then further ~~increased up mented~~ to 17 wt% and the reaction was  
278 carried out at the previously optimised temperature of 160 °C, with the Amberlyst-70/*Arundo donax*  
279 L. weight ratio of 0.2 wt/wt, for 20 min (run 13, Tables 34 and 42). The ~~yields of~~ xylose and glucose  
280 yields were ~~maintained kept~~ almost constant to those achieved with employing lower biomass  
281 loadings (5, 9 and 13 wt%) ~~under the same reaction conditions~~, significantly increasing the xylose  
282 and glucose concentrations up to 37.7 and 8.4 g/L, respectively. Moreover, the formation of  
283 by-products ~~was resulted~~ limited, even working at 17 wt% ~~biomass loading~~, being their  
284 concentrations in the range 0.5–1.3 g/L, except for AA that reached 4.7 g/L. Although the  
285 fermentability of the hydrolysates was not tested in ~~this present~~ work, the by-products  
286 concentrations ~~appeared were to be~~ lower than the typical inhibition thresholds [75693]. ~~This is~~  
287 ~~certainly a positive key aspect, enabling This could enable~~ the direct biological conversion of these  
288 hydrolysates without ~~additional adding further relevant relevant~~ detoxification procedures  
289 [46383,47394]. In fact, in the fermentative route for sugars valorisation, the by-products act as strong  
290 inhibitors for microorganisms [554483]. ~~Therefore, thus~~ the ~~synthesized hydrolysate hydrolysate,~~  
291 characterized by a very low amount poor of by-products and a high quantity of very rich in reducing  
292 sugars (46.1 g/L), represents an ideal substrate for the production of value-added bio-chemicals  
293 through a subsequent biological route.

294 Once ~~optimised having optimised~~ the biomass loading (17 wt%), the temperature (160 °C)  
295 and the Amberlyst-70/*Arundo donax* L. weight ratio (0.2 wt/wt), a kinetic study ~~from 5 to 30 min~~ was  
296 performed ~~at 17 wt% Arundo donax L. loading, at 160 °C, with the catalyst/substrate ratio of 0.2 wt/wt~~  
297 and the obtained results are reported in Figure 34. The xylose concentration ranged from 30.6 to 32.6  
298 g/L prolonging the reaction time from 5 to 30 min, reaching the maximum value of 37.7 g/L,  
299 corresponding to the xylose yield of 96.3 mol%, after 20 min. Differently, glucose concentration  
300 linearly increased prolonging the reaction time, ~~passing from 4.2 g/L to 10.5 g/L, after 5 and 30 min~~  
301 ~~respectively~~, this last corresponding to the glucose yield of 13.0 mol%. Regarding the by-products  
302 concentrations, they showed analogous trends of that found for glucose, except for HMF, which  
303 ~~after 20 min~~ was converted into LA and FA after 20 min. These trends can be rationalized on the  
304 basis of the process severity, which takes into account the combined effect of temperature and time  
305 [2517]. In fact, ~~prolonging the reaction time~~, the process severity increased with time, promoting not  
306 only the xylan and glucan depolymerisation but also, ~~at the same time~~, the by products formation of  
307 by-products. In conclusion, the reaction time of 20 min ensured the highest xylose yield of 96.3  
308 mol%, corresponding to the ponderal yield of 18.9 wt%, calculated respect to the amount of the  
309 ~~respect to the~~ starting *Arundo donax* L.

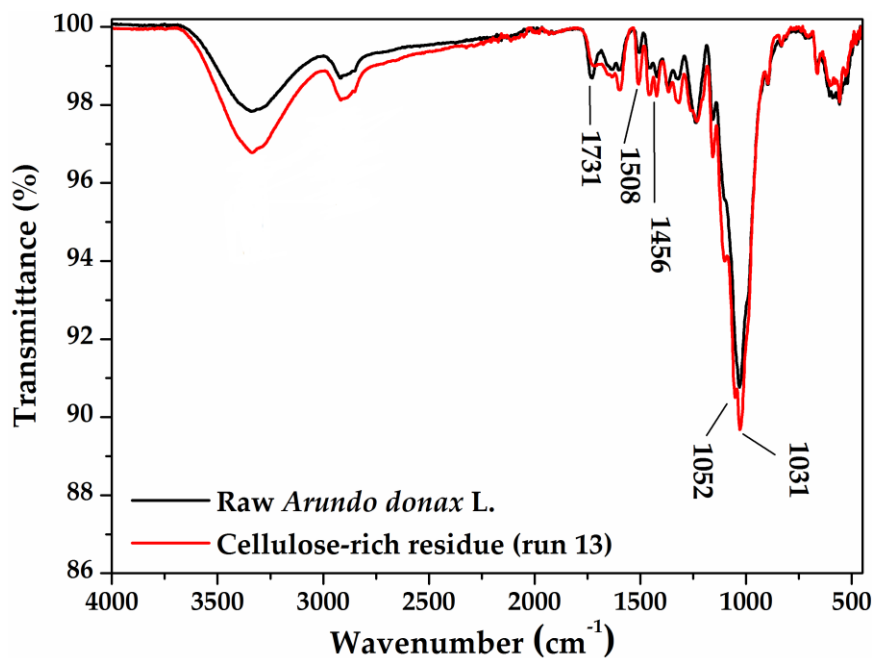


310

311 | **Figure 31.** EffectInfluence of reaction time on xylose and glucose yields (mol%) and on by-products  
 312 | concentration (g/L). Reaction conditions: 17 wt% biomass loading, 160 °C, Amberlyst-70/*Arundo*  
 313 | *donax* L. weight ratio 0.2 wt/wt (\*Compounds concentration).

314 | The cellulose-rich residue recovered at the end of the optimised reaction (run 13) represented  
 315 | the 57.1 wt% of the starting raw *Arundo donax* L. and its chemical composition was the following  
 316 | composition analysis confirmed the complete hydrolysis of hemicellulose, containing (wt% on dry  
 317 | matter): glucan  $52.5 \pm 0.4$ , xylan  $0.4 \pm 0.1$ , arabinan  $0.2 \pm 0.0$ , ash  $2.10 \pm 0.0$ , Klason lignin  
 318 | (acid-insoluble residue)  $35.9 \pm 0.8$ , acid-soluble lignin  $0.4 \pm 0.1$ , other compounds  $8.56 \pm 1.4$ . The  
 319 | values represent the mean of 3 replicates,  $\pm$  standard deviation (SD). Therefore, these data confirm  
 320 | the complete hydrolysis of the hemicellulose fraction.

321 | The CRR was also characterized by FT-IR attenuated total reflection (ATR) spectroscopy and its  
 322 | spectrum is reported in Figure 4, together with that of the raw *Arundo donax* L. are shown in Figure 2,  
 323 | where the most significant bands for the comparison purpose are reported. The comparison between  
 324 | the two spectra shows, in the case of the CRR, the decrease of the intensity of bandpeak at 1731  
 325 |  $\text{cm}^{-1}$ , ascribable assigned to the C=O stretching of the acetyl groups, confirming the occurred  
 326 | depolymerisation of the hemicellulose fraction [146,767064]. Moreover, the absorption bands  
 327 | intensity of the peaks at 1508 and 1456  $\text{cm}^{-1}$ , due to the characteristic respectively of the C=C  
 328 | stretching of benzene rings in the of lignin structure and the bending vibration of the CH<sub>2</sub> groups of  
 329 | cellulose [146,767064], respectively, were more intense higher in the case of CRR respect to the raw  
 330 | Arundo donax L. Also the peaks bands at 1052 and 1031  $\text{cm}^{-1}$ , corresponding assigned to C–O–C  
 331 | stretching of the pyranose ring in cellulose and to the C–O stretching of hydroxyl and ether groups  
 332 | of cellulose, were more intense increased in the CRR [767064]. All these observations confirmed that  
 333 | the quantitative removal of hemicellulose from *Arundo donax* L. caused led to the enrichment in  
 334 | cellulose and lignin in the CRR.

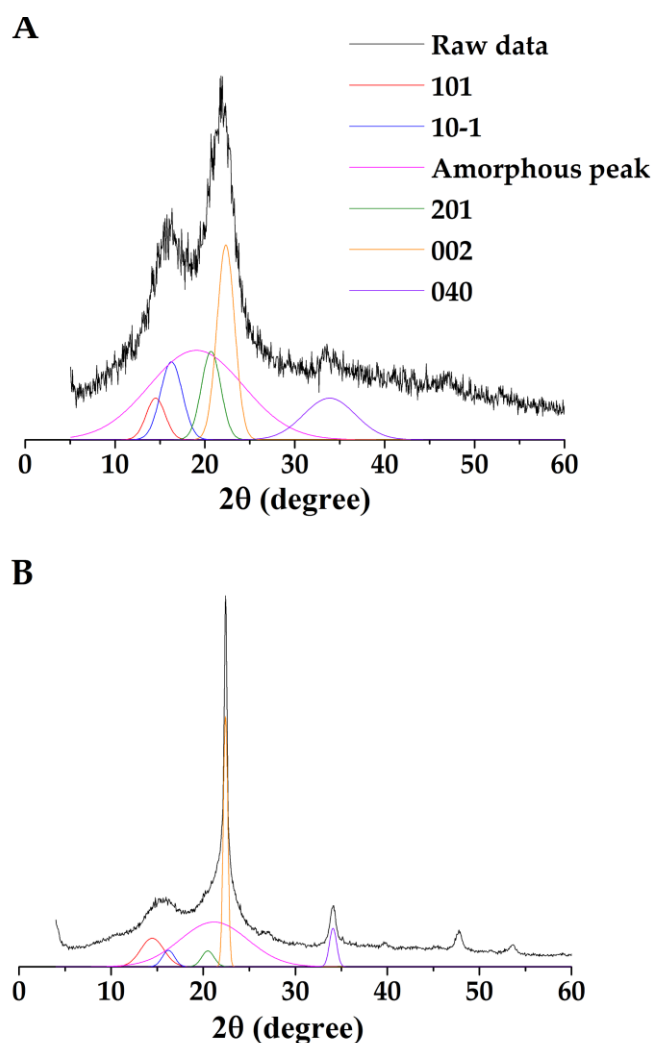


335

336 | **Figure 42.** FT-IR spectra of the starting raw *Arundo donax* L. (black) and the cellulose-rich residue  
 337 | recovered from run 13 (red).

338 | In order to investigate the crystallinity index (CrI) of biomass before and after the hydrolysis of  
 339 | hemicellulose, the solids were characterized by X-ray Diffraction (XRD) analysis. Figure 53 reports  
 340 | the XRD diffractograms of the raw biomass (A) and of CRR after the selective hemicellulose  
 341 | dissolution (B). The CrI of the samples was estimated by the using peak deconvolution method. In  
 342 | particular, five Gaussian curves, each corresponding to each Miller indices of the crystalline  
 343 | cellulose (1 0 1), (1 0 -1), (2 0 1), (0 0 2) and (0 4 0) were used. In addition, and a sixth Gaussian curve  
 344 | for the amorphous phase, due to the presence in the biomass of other amorphous components, such  
 345 | as hemicellulose and disordered cellulose, was included here used [777165,7872]66]. The calculated  
 346 | value of CrI value of raw *Arundo donax* L. giant reed resulted was 53.8%, and this is in agreement with  
 347 | data already reported in the literature on the *Arundo donax* L. for this kind of biomass. [787266]. After  
 348 | complete hemicellulose hydrolysis under the optimised reaction conditions (run 13), the CrI of  
 349 | biomass increased up to, achieving the value of 66.7%. Also this result is in agreement with the  
 350 | literature, highlighting many studies reported in the literature, which have proposed that the mild  
 351 | treatment increases the CrI of biomass, by eliminating removing the hemicellulose and the  
 352 | amorphous phase of cellulose, not limiting the involvement of the crystalline one [787266–807468].  
 353 | The XRD results confirmed that the first step of hydrolysis performed in the first step was selective  
 354 | towards hemicellulose and non-crystalline cellulose fractions, resulting Amberlyst-70 results an  
 355 | effective and selective heterogeneous catalyst for the complete removal of hemicellulose under the  
 356 | adopted reaction conditions, allowing us to achieve concentrated xylose hydrolysates, thanks also to  
 357 | the combined effect of due also to the efficacy of the combination of MW irradiation and with the  
 358 | high biomass loading of 17 wt%.

359 | The recovered CRR represented a good starting feedstock for the cascade valorisation of the  
 360 | cellulose fraction via chemical or enzymatic routes.



361

362 | **Figure 53.** XRD diffractograms of the starting raw *Arundo donax* L. (A) and the CRR recovered from  
 363 | run 13 (B).

### 364 | 2.2. Chemical hydrolysis of cellulose-rich residue (CRR) to glucose

365 | The whole solid residue recovered at the end of the optimised first step of hydrolysis (run 13),  
 366 | ~~which included both~~ composed by CRR ~~and with~~ embedded Amberlyst-70, was ~~reprocessed further~~  
 367 | ~~processed~~, in order to investigate the conversion of cellulose to glucose by chemical hydrolysis  
 368 | catalysed by the ~~same~~ heterogeneous catalyst ~~itself~~. However, ~~as reported in the literature~~, the  
 369 | formation of a solid by-product, ~~named called~~ "humins", can take place during the synthesis of  
 370 | sugars, ~~as a consequence originating from of their~~ degradation ~~pathways and further polymerisation~~  
 371 | [2547]. Humins could be present on the CRR and Amberlyst-70 surfaces, contributing to hampering  
 372 | the conversion of cellulose into glucose. In order to partially remove humins, the whole solid  
 373 | recovered from run 13 was washed with acetone, which ~~is has already proved to be~~ efficient for this  
 374 | purpose [575044,817569], and subsequently dried ~~under vacuum at 50 °C in oven~~ and reprocessed.  
 375 | The reaction was carried out with the CRR loading of 17 wt%, analogously to the conditions of the  
 376 | first step. On the basis of the *Arundo donax* L. solubilisation achieved in the previous step, equal to  
 377 | 42.9 wt%, the embedded Amberlyst-70/CRR weight ratio ~~resulted was~~ 0.35 wt/wt. The obtained  
 378 | results are reported in Table 53. Harsher reaction conditions than those adopted for the  
 379 | hemicellulose conversion were ~~necessary for in this second hydrolysis employed in the second~~ step,  
 380 | due to the higher recalcitrance of cellulose towards the hydrolysis. On this basis, the preliminary run  
 381 | was carried out at 180 °C for 60 min (run 14, Table 53), achieving low biomass solubilisation and  
 382 | glucose yield, ~~probably~~ due to the mild reaction conditions, not ~~appropriate suitable~~ to ~~the~~ promote  
 383 | ~~the~~ hydrolysis.

384  
385**Table 53.** Experimental set-up and results of cellulose hydrolysis. Reaction conditions: MW heating, CRR loading 17 wt%, Amberlyst-70/CRR weight ratio 0.35 wt/wt.

Run	Time (min)	Temperature (°C)	AA <sup>1</sup> (g/L)	FA <sup>2</sup> (g/L)	LA <sup>3</sup> (g/L)	HMF <sup>4</sup> (g/L)	Furfural (g/L)	Biomass solubilisation (wt%)	Glucose yield <sup>5</sup> (mol%)
14	60	180	2.5	1.5	1.9	0.8	0.6	17.2	3.9
15	60	190	2.5	1.1	3.2	1.9	0.8	35.9	26.4
16	20	190	2.2	1.5	1.6	0.6	0.5	27.3	32.5

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<sup>1</sup> Acetic acid; <sup>2</sup> Formic acid; <sup>3</sup> Levulinic acid; <sup>4</sup> 5-Hydroxymethylfurfural; <sup>5</sup> Yield respect to moles of glucan in the CRR solid.

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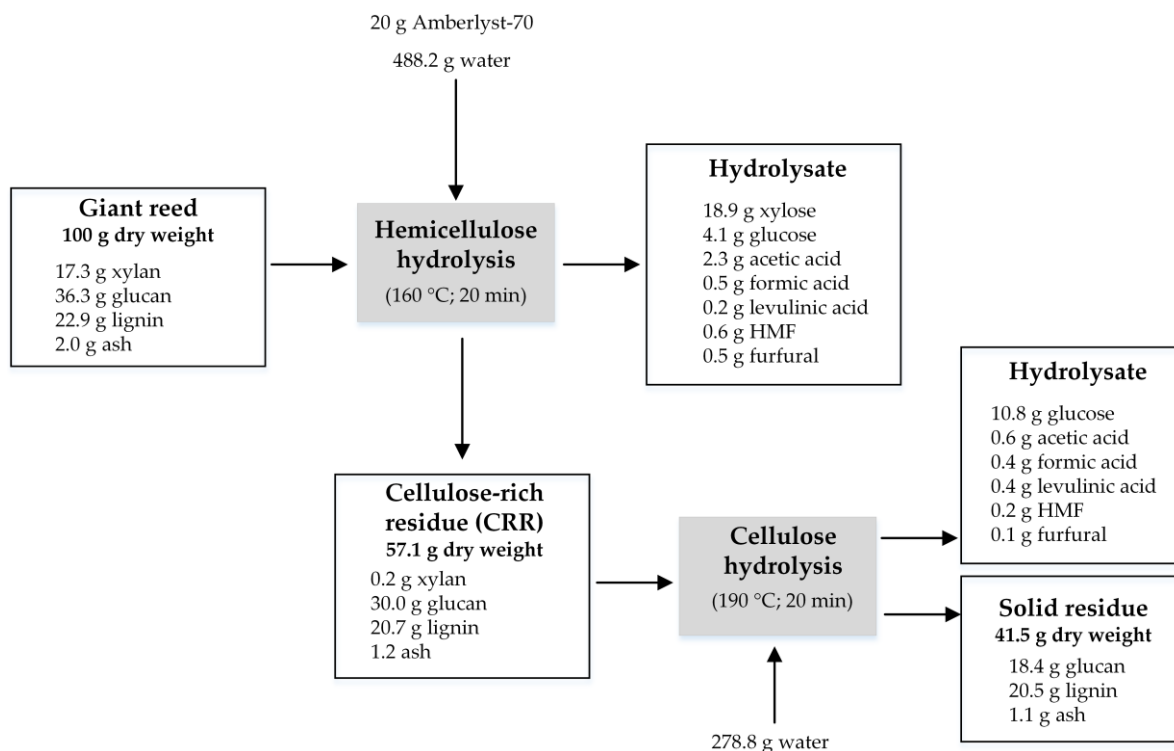
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For this reason, the temperature was increased to 190 °C (run 15, Table 53), which strongly improved the biomass solubilisation and glucose yield, ~~up equal~~ to 35.9 wt% and 26.4 mol%, respectively. However, under these reaction conditions, ~~also also~~ the by-products concentrations, in particular ~~those of~~ LA and HMF, increased, proving that glucose degradation was promoted. The decrease of reaction time from 60 to 20 min (run 16, Table 53) limited the formation of by-products and, despite the biomass solubilisation decreased, ~~led to the a high~~ glucose yield of 32.5 mol% ~~was achieved~~, corresponding to the concentration of ~~387.7~~ 387.7 g/L and the ponderal yield of 18.9 wt% respect to CRR. ~~These are the~~ ~~which is~~ the best results obtained in the present work for the chemical hydrolysis of CRR ~~and they are better than those reported in the literature. In fact, t~~ This glucose ponderal yield was higher than the total reducing sugars yield reported by Meena et al., ~~equal to 15.1 wt%~~, for the conversion of alkali pretreated rice straw with recycled Amberlyst-15, ~~equal to 15.1 wt%~~ [403227]. It is important to underline that ~~they these authors~~ reached this yield working with the biomass loading of 7 wt% and the Amberlyst-15/biomass weight ratio of 1 wt/wt, reaction conditions more favourable towards the hydrolysis than those adopted in the present investigation (17 wt% and 0.35 wt/wt). ~~However, in our case, a higher biomass loading and less amount of catalyst were employed, which~~ resultings more sustainable for an applicative point of view, ~~employing a higher biomass loading and less amount of catalyst~~. In addition, the biomass adopted by Meena et al. was previously pretreated with ~~an~~ alkaline solution, which favours the delignification process, promoting the subsequent hydrolysis, while in the present research Amberlyst-70 was employed as catalyst for the multi-step hydrolysis of unpretreated *Arundo donax* L.

The chemical composition of the solid recovered from the optimised chemical hydrolysis (190 °C and 20 min) of CRR washed with acetone confirmed the exploitation of the *Arundo donax* L. cellulose. In fact, the final residue contained (wt% on dry matter): glucan 44.3 ± 0.3, ash 2.7 ± 0.2, Klason lignin (acid-insoluble residue) 49.2 ± 0.9, acid-soluble lignin 0.2 ± 0.0, other compounds 3.8 ± 0.5. The values represent the mean of 3 replicates, ± standard deviation (SD). The mass balance flow diagram of xylan, glucan, lignin and ash for the optimised multi-step chemical process is reported in Figure 6.

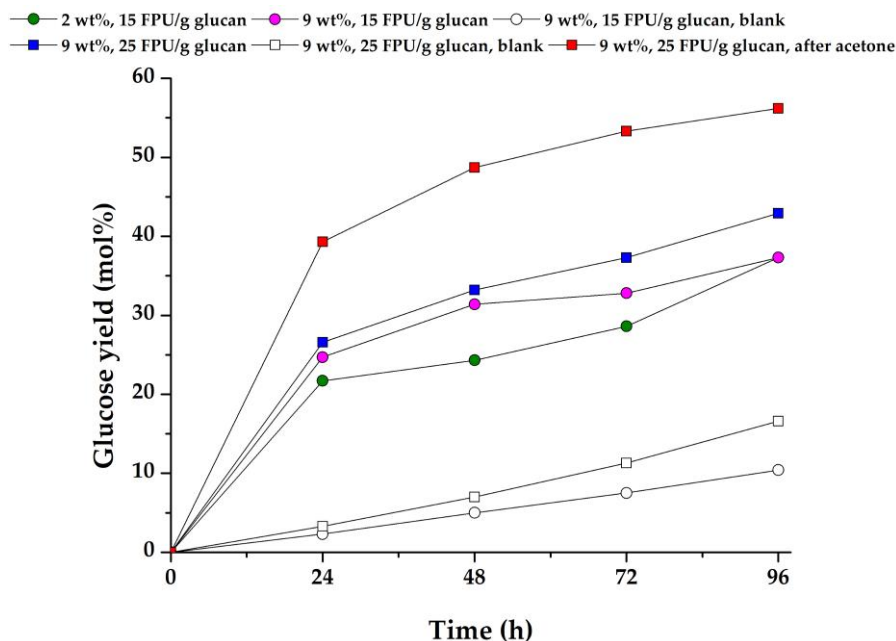




**Figure 6.** Mass balance flow diagram under the optimised reaction conditions for the two-step chemical hydrolysis of both raw giant reed and CRR.

### 2.3. Enzymatic hydrolysis of cellulose-rich residue (CRR) to glucose

Enzymatic hydrolysis represents one of the most important approaches for the selective production of second-generation sugars. Enzyme activities are significantly affected by the biomass structure and by the presence of degradation by-products [82760]. In fact, the biomass structure can influence change the accessibility of enzymes to the polysaccharides the accessibility of enzymes to biomass, which is a crucial step for lignocellulosic biomass hydrolysis [32242]. In particular, the crystallinity of cellulose, the porosity/and the size of fibres of pretreated biomass fibres and the presence of lignin certainly influence the hydrolysis reaction [83]. Lignin inactivates enzymes by forming which forms lignin-enzyme complexes and hampers the enzymes specific adsorption onto the polysaccharides, reducing the efficiency of the considered ineffective for the biomass hydrolysis, certainly influence the hydrolysis also influence the reaction [771,84782]. In the present work, a preliminary study on enzymatic conversion of the recovered CRR to glucose was performed, adopting the commercial cellulolytic enzymatic preparation Cellic<sup>™</sup> CTec2. In order to study the performance of enzymatic hydrolysis of CRR under different reaction conditions, the embedded Amberlyst-70 was previously separated from CRR by sieving. The results of the enzymatic hydrolysis are reported in Figure 74. The effect of biomass loading on enzymatic digestibility was investigated, comparing two different loadings of CRR (2 and 9 wt%) and by using an enzyme dosage of 15 FPU/g glucan, typically lower than those adopted in the literature for the pretreated *Arundo donax* L. hydrolysis (>60 FPU/g glucan) [35274,85793,868074].



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**Figure 74.** Kinetics of enzymatic hydrolysis of the CRR recovered from run 13 in Tables 1 and 2 (coloured curves) and raw *Arundo donax* L. as blank (white curves).

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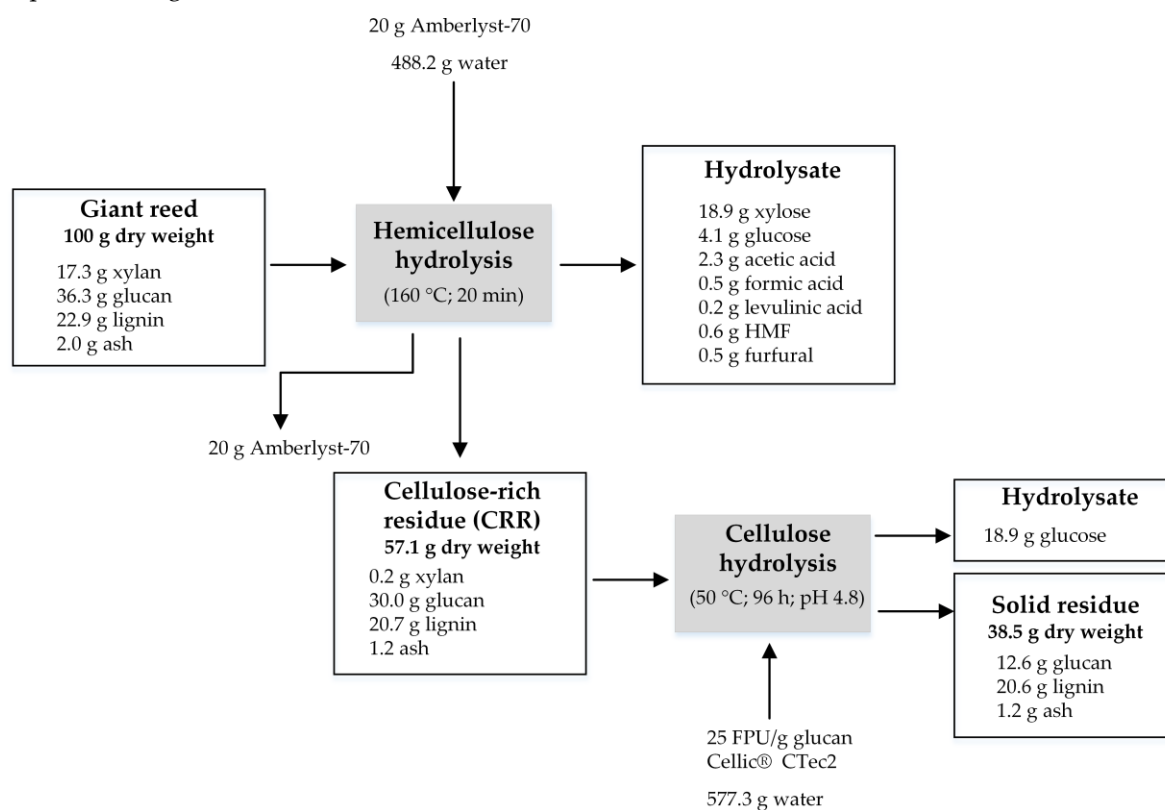
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In both tests, the glucose yield increased during the ~~96 h reaction time~~ entire time range, achieving the same glucose yield of 37.3 mol%, corresponding to the glucose concentration of 21.5 g/L, at the end of the reaction. The implementation of ~~the high biomass loading~~ gravity approach in the enzymatic hydrolysis favors the scale-up of the process and its economic sustainability from an industrial point of view, ~~thanks due~~ to the increase of sugars ~~production concentration~~ and the decrease of the costs related to the downstream workup. The increase of biomass loading did not negatively affect the enzymatic digestibility, thus the higher CRR amount (9 wt%) was adopted in the following tests. In order to demonstrate the beneficial role of the first hydrolytic step on the subsequent enzymatic hydrolysis in this proposed cascade approach, a blank test was performed under the same reaction conditions (9 wt%, 15 FPU/g glucan) adopting the raw *Arundo donax* L. as substrate. As reported in Figure 74, a linear profile was observed, reaching the glucose yield of 10.4 mol%, corresponding to the glucose concentration of 4.2 g/L, after 96 h, which was lower than that achieved starting from the CRR. Subsequently, on the basis of the promising results ascertained working with 15 FPU/g glucan, the enzyme concentration was increased to 25 FPU/g glucan, which represents the typical dosage adopted in literature [32242,817569,878175,888276], and the hydrolysis of both CRR and raw *Arundo donax* L. (blank test) were performed, maintaining the substrate loading at 9 wt%. The glucose yield increased up to 42.9 mol%, corresponding to the glucose concentration of 24.7 g/L, in the enzymatic hydrolysis of the CRR, and up to 16.6 mol%, corresponding to the glucose concentration of 6.7 g/L, in the respective blank test. ~~These values were higher than those achieved working with the enzyme concentration of 15 FPU/g glucan, proving that the increase of enzyme concentration promoted the hydrolysis. As previously proposed, the conversion of cellulose into glucose could be limited by the presence of humins on CRR surface of humins, which could hamper make difficult the contact between enzymes and cellulose, thus acting as inhibitor of the binding between them [8377]. However, Moreover, it is recognized that enzyme performance is reduced during lignocellulose hydrolysis by interaction with lignin or lignin-carbohydrate complex. In particular, the mechanism of enzyme inhibition may involve both the adsorption of the enzyme on the insoluble lignin and interactions with the solubilised low-molecular lignin. Both these interactions can cause the non-specific adsorption of the enzyme onto the polysaccharides of the substrate [8478]. Therefore, the high lignin content of the CRR (36.3 wt%) hampered the complete enzymatic hydrolysis. Moreover, as previously proposed, the conversion of cellulose into glucose could be limited also by the presence of humins on CRR surface, which could hamper the contact~~

471 [between enzymes and cellulose, thus acting as an inhibitor of the binding between them \[89\]](#). On this  
 472 basis, the CRR was washed with acetone, dried in [an](#) oven and employed as substrate in the  
 473 enzymatic hydrolysis (biomass loading of 9 wt% and the enzyme concentration of 25 FPU/g glucan).  
 474 Under these reaction conditions, the glucose yield was further increased to 56.2 mol%,  
 475 corresponding to the concentration of 32.8 g/L, after 96 h, confirming the beneficial role of the  
 476 washing step. These results ~~agree in agreement~~ with those obtained from the enzymatic  
 477 hydrolysis of the steam-pretreated giant reed [performed](#) under the same reaction conditions, being  
 478 the yield of glucose equal to about 56 mol% [32242]. Moreover, the obtained glucose concentration of  
 479 32.8 g/L was comparable with the value of 30.9 g/L reported by Aliberti et al. [85793], who worked  
 480 with the same steam-pretreated giant reed loading (9 wt%), but with higher enzymatic concentration  
 481 (69.6 FPU/g glucan) than that adopted in the present study. [In addition, unlike this cited work, our](#)  
 482 [approach has provided the valorisation of the Amberlyst-70 catalysed hydrolysis of the](#)  
 483 [hemicellulose fraction, thus developing better the biorefinery concept. It is very important to](#)  
 484 [highlight that, not only in this work a lower amount of enzyme was adopted, but also, on the](#)  
 485 [contrary of the one step steam explosion conversion, the valorisation of the hemicellulose fraction](#)  
 486 [was performed, thanks to the preliminary hydrolysis with Amberlyst 70.](#) ~~Moreover,~~ cellulose  
 487 conversion analogous to those reported in the literature for steam-pretreated giant reed was reached,  
 488 leading to similar glucose yields, thus proving the efficiency of the multi-step approach proposed in  
 489 this work.

490 The solid recovered from the optimised enzymatic hydrolysis (9 wt%, 25 FPU/g glucan) ~~of of~~  
 491 ~~CRR washed with~~ ~~acetone-washed CRR~~ was 67.5 wt% of the CRR and 38.5 wt% of initial raw *Arundo*  
 492 *donax* L. and its chemical composition analysis confirmed the ~~exploitation-occurred~~ [fractionation](#)  
 493 of the *Arundo donax* L. cellulose in this innovative process scheme. In fact, the final [solid](#) residue  
 494 contained (wt% on dry matter): glucan 32.7 ± 0.4, ash 3.13 ± 0.2, Klason lignin (acid-insoluble residue)  
 495 53.4 ± 0.8, acid-soluble lignin 0.1 ± 0.0, other compounds 101.76 ± 1.2. The values represent the mean  
 496 of 3 replicates, ± standard deviation (SD). The mass balance flow diagram [of xylan, glucan, lignin](#)  
 497 [and ash for](#) ~~of~~ the optimised multi-step process involving the chemical and biological routes is  
 498 reported in Figure 85.



500 | **Figure 85.** Mass balance flow diagram for chemical hydrolysis of raw giant reed and enzymatic  
501 | hydrolysis of CRR, both of them under the optimised reaction conditions.

502 | Regarding the possible exploitation of the final solid residue, many strategies are available, and  
503 | the choice must be careful, applying the sustainability criteria and considering taking into account its  
504 | real chemical composition. In fact, the final solid residue is a hydrochar, more similar to lignin than  
505 | to the starting lignocellulosic feedstock and, as such, it could be used in energy and environmental  
506 | fields, including the applications as adsorbents, precursor of catalysts, soil amendment, anaerobic  
507 | digestion, composting and electrochemical energy storage materials [9084]. In this work, a  
508 | significant residual fraction of glucan is still present together with lignin. Therefore, the lignin  
509 | exploitation must be synergistically integrated with that of glucans. These last ones could be  
510 | ideally converted into HMF5-hydroxymethylfurfural and/or LAlevulinic acid, but the catalytic  
511 | performances of Amberlyst-70 are weak, due to the presence of the recalcitrant lignin, which  
512 | hampers complete glucan solubilisation and conversion in the water medium. In addition, the  
513 | syntheses of 5-hydroxymethylfurfural or levulinic acid require harsher reaction conditions, which  
514 | are not suitable for the catalyst stability. Instead, more advantageously, in order to overcome the  
515 | recalcitrance of lignin, a very smart approach has been proposed by Antonetti et al. [9185], which  
516 | have used butanol as liquefaction solvents for the conversion of sugars into butyl levulinate,  
517 | allowing the direct production of the more value-added and marketable levulinates and, at  
518 | downstream of the treatment, a more useful hydrochar. On the other hand, in the field of  
519 | bioconversion, Liu et al. [9286] have demonstrated that the co-presence of glucose facilitates both  
520 | lignin solubilisation and the subsequent polyhydroxyalkanoate (PHA) production, carried out in the  
521 | presence of *Pseudomonas putida* KT2440. More than 70% of the residual sugar was released from the  
522 | residue, producing the soluble lignin stream that contains both lignin and residual sugar for  
523 | synergistic bioconversion. By this way, the integrated biorefinery increased the fermentable sugar  
524 | yield and improved the PHA production, thus improving the carbon utilization efficiency.

### 525 | 3. Materials and Methods

#### 526 | 3.1. Materials

527 | *Arundo donax* L. was provided by the Institute of Life Sciences of Scuola Superiore Sant'Anna  
528 | (Pisa, Italy). It was harvested from long term field trials carried out at the Enrico Avanzi  
529 | Interdepartmental Centre for Agro-Environmental Research (CIRAA) of the University of Pisa (San  
530 | Piero a Grado, Pisa) (latitude 43° 68' N, longitude 10° 35' E). The whole raw biomass was ground in  
531 | 1.0 mm average size particles and dried at 105 °C in the oven until constant weight. Amberlyst-70  
532 | (concentration of acid sites: 2.55 meq/g; surface area: 36 m<sup>2</sup>/g; particle size: 0.5 mm; average pore  
533 | diameter: 22 nm; divinylbenzene content: 8 wt%; maximum temperature: 190 °C) was kindly  
534 | provided by Rohm and Haas and used as received. 5-hydroxymethylfurfural (95%) was purchased  
535 | from AVA-Biochem. Xylose (>99%), glucose (>99%), formic acid (99.8%), acetic acid (>99%), levulinic  
536 | acid (98%), furfural (99%) were provided by Sigma-Aldrich and employed as received. The  
537 | enzymatic preparation Cellic<sup>®</sup>™ CTec2 was kindly provided by Novozymes and employed as  
538 | received.

#### 539 | 3.2. Chemical hydrolysis of *Arundo donax* L.

540 | The hydrolysis of *Arundo donax* L. was carried out in the single-mode MW-microwave (MW)  
541 | reactor CEM-Discover S-class System produced by CEM Corporation. This equipment is able to  
542 | work up to the maximum temperature of 300 °C and up to the maximum pressure of 300 psi. The  
543 | reactor is able to adjust the power of the emitted radiation in order to reach the set temperature and  
544 | keep it constant during the reaction, providing the maximum power of 300 W. In a standard reaction,  
545 | the proper amounts of biomass and Amberlyst-70 were weighted and charged in the MW vessel  
546 | together with 20 mL of water. The vessel was placed in the MW reactor and heated at the desired  
547 | temperature for the selected time under magnetic stirring. At the end of the reaction, the vessel was

548 rapidly cooled at room temperature through an external air flow. The mixture was filtered on funnel  
 549 Buckner and the solid fraction was dried in [an](#) oven over the night and weighted. The liquid fraction  
 550 was filtered through a syringe filter (Whatman 0.45 µm PTFE) and analysed [as such](#) by High  
 551 Performance Liquid Chromatography (HPLC) instrument. Each experiment was replicated three  
 552 times with an error less than 5%.

553 The same protocol was also adopted for the chemical hydrolysis of the CRR recovered by the  
 554 hydrolysis of *Arundo donax* L. in the presence of the embedded Amberlyst-70 as catalyst, employing  
 555 a previous washing of the whole solid with acetone (three times, weight ratio of 1:3 solid/acetone).

### 556 3.3. High Performance Liquid Chromatography (HPLC)

557 The HPLC Jasco LC-2000 was employed to analyse the liquid samples. The instrument was  
 558 equipped with the column Benson 2000-0 BP-OA (300 mm x 7.8 mm x 10 µm) kept at 60 °C and the  
 559 0.005 M H<sub>2</sub>SO<sub>4</sub> aqueous solution was used as mobile phase with the flow-rate of 0.6 mL/min. The  
 560 components concentration was evaluated on the basis of the calibration curves obtained from the  
 561 analysis of standard solutions. At least three replicates for each concentration of standards and  
 562 samples were done and the reproducibility of this technique was within 3%.

### 563 3.4. ~~Compositional analysis of the feedstocks~~ *Chemical composition analysis*

564 ~~The chemical composition of the feedstocks, e.g. starting *Arundo donax* L., the recovered~~  
 565 ~~CRR~~~~cellulose rich residue (CRR), and final solid residues, was evaluated through the standard~~  
 566 ~~NREL protocols [6963–7367]. Briefly, the analytical procedure involves two hydrolysis steps,~~  
 567 ~~primary hydrolysis in 72 wt% sulfuric acid at 30 °C for 1 h, followed by secondary hydrolysis of the~~  
 568 ~~slurry in 4 wt% sulfuric acid at 121 °C for 1 h. The slurry was filtered through a ceramic crucible and~~  
 569 ~~the filtered liquid phase was analysed by HPLC in order to get the compositional data related to the~~  
 570 ~~C5 and C6 carbohydrates. Furthermore, this liquid phase was analysed also by UV-Vis spectroscopy~~  
 571 ~~for the quantification of the acid-soluble lignin. Regarding the insoluble residue recovered after the~~  
 572 ~~2<sup>nd</sup> hydrolysis step, it was dried up to constant weight, and its gravimetric quantification gave the~~  
 573 ~~Klason (acid-insoluble) lignin content. Lastly, ash content was determined as the percentage of~~  
 574 ~~residue remaining after dry oxidation of the starting biomass at 575 °C for 24 h.~~

575 ~~The chemical composition of *Arundo donax* L. was evaluated through the standard NREL~~  
 576 ~~protocols [57–61].~~

### 577 3.5. X-ray diffraction method (XRD) *analysis*

578 X-ray powder diffraction was performed using a Bruker D2 Phaser diffractometer (30 kV, 10  
 579 mA) operating in Bragg-Brentano geometry (θ – θ scan mode) and equipped with a 1-dimensional  
 580 Lynxeye detector. Ni-filtered Cu Kα radiation was used. Data were collected in the scan range 4-65°  
 581 in 2θ, with [a](#) scan step of 0.02° and counting times of 0.1 s/step. Data were processed through the  
 582 software Diffrac.Eva (Bruker AXS) and the peak fitting was performed using the software PeakFit  
 583 (Systat Software Inc.; USA). Crystallinity index (CrI) was evaluated according to the peak fitting  
 584 method, considering five diffraction peaks for the crystalline phase at 2θ = 14.4°, 16.2°, 20.5°, 22.4°  
 585 and 33.9°, corresponding respectively to the Miller indices (1 0 1), (1 0 -1), (2 0 1), (0 0 2) and (0 4 0),  
 586 and the peak at approximately 2θ = 19°, for the amorphous phase [64]. The R<sup>2</sup> values for the fitted  
 587 peaks were always greater than 0.95. The peak fitting allows the evaluation of the [peaks](#) area [of the](#)  
 588 [peaks](#) and, on this basis, the CrI was determined through the following equation:

$$\text{CrI} = [1 - (A_{\text{AM}} / A_{\text{TOT}})] \times 100, \quad (1)$$

589 where A<sub>AM</sub> is the area of the peak corresponding to the amorphous cellulose, and A<sub>TOT</sub> is the total  
 590 area of all peaks.

### 591 3.6. *Enzymatic activity*



592 ~~The enzymatic activity of commercial preparation Cellic™ Ctec2 was quantified through the~~  
 593 ~~standard NREL protocol [78] and it was equal to 134.5 FPU/mL.~~

### 594 3.67. Enzymatic hydrolysis of *Arundo donax* L.

595 ~~Before to carrying out the enzymatic hydrolysis of raw *Arundo donax* L. and the CRR, the~~  
 596 ~~enzymatic activity of commercial preparation Cellic® CTec2 was quantified through the standard~~  
 597 ~~NREL protocol [9387]. 0.5 mL of a diluted sample containing enzymes was incubated with 50 mg of~~  
 598 ~~filter paper strip (Whatman No.1, UK) and 1.0 mL of sodium citrate buffer at 50 °C for 1 hour. The~~  
 599 ~~enzymatic reaction was terminated by adding 3.0 mL of 3,5-dinitrosalicylic (DNS) agent at 95 °C for~~  
 600 ~~5 min. Subsequently, the amount of released glucose was measured as reducing sugar at 540 nm~~  
 601 ~~using Varian Cary 300 Scan UV-Visible Spectrophotometer. Glucose standards were prepared and~~  
 602 ~~analysed with the samples to obtain a standard curve. One unit of cellulase (FPU/7mL) was defined~~  
 603 ~~as the amount of enzyme releasing 2.0 mg of glucose in 1 hour. The enzymatic activity of commercial~~  
 604 ~~preparation Cellic® CTec2 was equal to 134.5 FPU/mL.~~ The enzymatic hydrolysis of raw *Arundo*  
 605 *donax* L. and the CRR recovered by the hydrolysis of *Arundo donax* L., previously separated from the  
 606 embedded Amberlyst-70 by sieving, was conducted at pH = 4.8 and 50 °C in a 150 mL flask  
 607 employing 50 mL of the 0.05 M citrate buffer solution and the enzyme Celli~~c~~<sup>®</sup>~~™~~ CT~~e~~<sup>™</sup>tec2, shaking at  
 608 160 rpm. Different substrate loadings and dosages of enzymes were investigated in the present  
 609 study, in agreement with the ranges reported in the literature [32242,7973,8175]. Every 24 hours,  
 610 samples of 2 mL were withdrawn, cooled in ice in order to stop the enzymatic activities, centrifuged  
 611 and analysed by HPLC for determining the glucose concentration. Both hydrolysis and analytical  
 612 determinations were carried out in triplicate and the reproducibility of the reactions was within 5%.

613 The same protocol was also adopted for the enzymatic hydrolysis of the CRR recovered by the  
 614 hydrolysis of *Arundo donax* L., ~~which was~~ previously washed with acetone (three times, weight ratio  
 615 of 1:3 biomass/acetone).

### 616 3.78. Fourier transformation infrared spectroscopy (FT-IR)

617 The Perkin-Elmer Spectrum Two spectrophotometer, equipped with an Attenuated Total  
 618 Reflectance (ATR) apparatus, was used to analyse the *Arundo donax* L. and the CRR. The acquisition  
 619 of each spectrum was provided by 12 scans, with a resolution of 8 cm<sup>-1</sup>, in the wavenumber range  
 620 between 4000-450 cm<sup>-1</sup>.

### 621 3.89. Definitions

622 The substrate loading adopted in all the runs was defined as follows:

$$623 \text{ ~~Biomass-Substrate~~ loading (wt\%) = [m_{\text{substrate}} / (m_{\text{substrate}} + m_{\text{water}})] \times 100, \quad (2)$$

624 where  $m_{\text{substrate}}$  is the weight (g) of the starting substrate and  $m_{\text{water}}$  is the weight (g) of water adopted  
 625 as solvent of the reaction.

626 In all experiments, the masses ( $m_i$ ) of the compounds in the hydrolysate were calculated using  
 the equation:

$$627 m_i = c_i \times V, \quad (3)$$

628 where  $c_i$  (g/L) is the concentration of the compound and  $V$  (L) is the volume of the hydrolysate.

629 The ponderal yield (wt%) of each product (xylose and glucose) respect to the dry weight (g) of  
 the starting substrate ( $m_{\text{substrate}}$ ) was calculated according to the following equation:

$$\text{Product yield (wt\%)} = (m_{\text{product}} / m_{\text{substrate}}) \times 100, \quad (4)$$

630 The molar yield of glucose and xylose respect to the moles of the respective units in the  
 631 polysaccharides (glucan, xylan) in the starting substrate ( $m_{\text{substrate}}$ ) was also determined, according to  
 632 the following equations [948879]:

$$\text{Glu. yield (mol\%)} = (m_{\text{glucose}} \times 0.90) / (m_{\text{substrate}} \times G_f) \times 100, \quad (5)$$

$$\text{Xyl. yield (mol\%)} = (m_{\text{xylose}} \times 0.88) / (m_{\text{substrate}} \times X_i) \times 100, \quad (6)$$

633 where the numbers 0.90 and 0.88 take into account the stoichiometry and the molecular weights in  
634 the hydrolysis of cellulose and hemicellulose, respectively to glucose and xylose; whereas  $G_i$  and  $X_i$   
635 represent the percentage (wt%) of glucan and xylan in the composition of the starting substrate,  
636 respectively.

#### 637 4. Conclusions

638 The proposed cascade biomass exploitation represents a novel promising strategy to maximize  
639 the conversion of *Arundo donax* L. structural carbohydrates into simple sugars, such as [xilose and](#)  
640 [glucose and xylose](#), which are key intermediates in several biorefinery processes. Under the  
641 sustainability and green chemistry perspective, in the first step, for the first time, the heterogeneous  
642 acid catalyst Amberlyst-70 was adopted for the [preliminary](#) hydrolysis of hemicellulose fraction in  
643 combination with MW irradiation and the high gravity approach. The optimised reaction conditions  
644 resulted 160 °C, 20 min, Amberlyst-70/*Arundo donax* L. weight ratio of 0.2 wt/wt with the initial  
645 biomass loading of 17 wt%, which ensured the highest sugars concentration of 46.1 g/L, the xylose  
646 yield of 96.3 mol%, ~~together with~~ the glucose yield of 10.2 mol% and very low concentrations of  
647 by-products, thus underlining the high selectivity of the process. In the second step, the exploitation  
648 of cellulose fraction in the [recovered recovered solid residue-CRR](#) was performed through ~~both the~~  
649 [chemical and](#) biological route. Regarding the chemical conversion, the present study proved the  
650 [feasibility of the cellulose conversion in the presence of activity of](#) the embedded Amberlyst-70 ~~in the~~  
651 [subsequent cellulose exploitation](#), obtaining, [after only 20 min](#), glucose yields up to 32.5 mol%,  
652 corresponding to the concentration of ~~387.7~~ g/L. [The obtained glucose yield was promising and the](#)  
653 [employment of the same catalyst for the two steps allowed the reduction of the process cost,](#)  
654 [however, better results in terms of cellulose exploitation were obtained adopting the enzymatic](#)  
655 [hydrolysis. In fact, On the other hand,](#) the biological route confirmed the efficiency of the  
656 ~~performed adopted~~ preliminary hydrolysis for the successive exploitation of the [CRR cellulose rich](#)  
657 [residue](#) through the enzymatic conversion ~~carried out with performed according to high solids~~  
658 [loading the high gravity approach](#) and with low enzymatic dosage. In fact, the best process reaction  
659 conditions (Cellic<sup>®</sup> CTec2 25 FPU/g glucan, 96 h, biomass loading of 9 wt%) ensured the glucose  
660 yield of 56.2 mol%, corresponding to the concentration of 32.8 g/L, without the ~~co~~-production of  
661 by-products. The proposed approach ~~alloweds~~ us to avoid traditional pretreatments, which ~~wereare~~  
662 replaced by the first reaction step that ~~enables~~ the complete xylose recovery. In conclusion, the  
663 multi-step approach ~~resulteds~~ promising for the valorisation of hemicellulose and cellulose fractions  
664 in raw *Arundo donax* L. to xylose and glucose, underlining that both routes, chemical and biological,  
665 ~~wereare~~ very valuable.

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