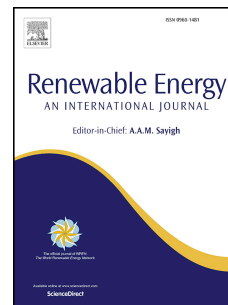# Journal Pre-proof

Waste-to-fuel technologies for the bioconversion of carrot discards into biobutanol

Juan C. López-Linares, Mónica Coca, Pedro E. Plaza, Susana Lucas, María Teresa García-Cubero

CRediT author statement

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Project administration; Funding acquisition. **Pedro E. Plaza**: Methodology;

Investigation. **M.Teresa García-Cubero**: Methodology; Funding acquisition;

Project administration**. Susana Lucas**: Conceptualization; Supervision; Writing

- Review & Editing; Funding acquisition.

1 **Waste-to-fuel technologies for the bioconversion of carrot discards into biobutanol**

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9 ABSTRACT

10 Carrot discard was evaluated as a raw material for acetone-butanol-ethanol (ABE)

11 fermentation. Different strategies based on hydrothermal pretreatment and/or enzymatic

12 hydrolysis were compared for biobutanol production from carrot discard pulp. In

13 addition, the use of different types of enzymes and diverse enzyme mixtures were

14 evaluated. In this way, total sugar recoveries of up to 76%, and butanol and ABE

15 concentrations of 7.4 and 11.5 g/L, respectively (74 g butanol and 115 g ABE/kg carrot

16 pulp), were achieved when the carrot discard pulp was enzymatically hydrolyzed,

17 without pretreatment, using a mixture of enzymes of Cellic CTec2 and Viscozyme L at

18 a dosage of 0.1 and 0.2 g/g, respectively. When a hydrothermal pretreatment was

19 applied, a total sugar recovery of 88%, 6.9 g/L butanol and 10.1 g/L ABE (69 g butanol

20 and 101 g ABE/kg carrot pulp) were attained using the same mixture of enzymes. In

21 this way, no hydrothermal pretreatment would be necessary to produce ABE from carrot

22 discard, which is very interesting for the profitability of the process. Furthermore, the

23 carrot discard juice yielded 6.4 and 9 g/L butanol and ABE, respectively, showing that

24 all the carrot discards could be used for ABE production.

25   **Keywords:** carrot discard; hydrothermal pretreatment; enzymatic hydrolysis; biofuels;

26   biobutanol; *Clostridium beijerinckii*.

27

28   **1. Introduction**

29   One of the most important economic sectors in Spain is the agro-food industry. The

30   fruit and vegetable sector contributes 1.7% of Spain's gross domestic product (GDP)

31   and is responsible for up to 300,000 jobs. In addition, Spain is regarded as the first

32   country in the EU in the production of fruit and vegetables, generating up to 28 million

33   tons per year (Mt/y) (25% of European production), while worldwide it ranks sixth [1].

34   As a result of this activity, a great amount of organic residue is obtained, such as

35   surplus, non-conformity fruit and vegetables, as well as by-products generated in the

36   processing activities [2]. These residues have in common a high content in organic

37   matter and a perishable character that makes their valorization difficult. Although fruit

38   and vegetable residues can be used for animal feed, this application has some

39   limitations; for instance, there are some residues which cannot be taken by some

40   animals and some diseases could be transmitted due to the presence of toxic substances.

41   The high transportation and conservation costs usually make this alternative unfeasible

42   [3]. On the other hand, dumping fruit and vegetable waste in landfills is associated with

43   the production of greenhouse gases (CH_4, CO_2), due to the degradation of the organic

44   matter under anaerobic conditions. Methane from landfills accounts for about 800

45   million tons of CO_2-equivent and is the third largest source of methane emissions [3].

46   The Directive (EU) 2018/850 aims to ensure a progressive reduction in the landfilling of

47   biodegradable waste, especially that which is suitable for recycling or recovery.

48   Therefore, in order to improve the efficiency of the food sector and achieve a circular

49   economy, it is necessary to convert fruit and vegetable residues into a resource.

2

50   Due to the interesting composition of this type of residue (carbohydrates, pectin,

51   lipids, proteins, phytochemicals, and none or very low lignin content) [4–6], fruit and

52   vegetable residues could have a great potential for the production of such high value-

53   added chemicals as bioactive compounds (polyphenols, polysaccharides, proteins) [7],

54   commodity chemicals, and/or biofuels [8].

55   Biobutanol is considered a promising advanced biofuel due to its high energy

56   density, low vapor pressure, and its lower volatility, explosiveness, hygroscopicity and

57   solubility in water compared to bioethanol [9]. Moreover, biobutanol could directly

58   replace gasoline, thus also being regarded as a drop-in-fuel [10]. What is more, it can

59   also be used as a solvent and a chemical commodity (for instance, in varnish, lac, or in

60   the pharmaceutical and food industries, among others) [11,12]. Biobutanol production

61   can therefore be very interesting. According to Ibrahim et al. [13], around 5 million tons

62   of biobutanol are generated worldwide, its price being about 0.9-1.4 USD/kg.

63   Butanol-producing microorganisms are not able to directly ferment carbohydrates in

64   agro-industrial biomass; it must first undergo a pretreatment to improve the

65   saccharification of the complex polysaccharides to render fermentable sugars. In this

66   way, biobutanol could be produced biologically from fruit and vegetable residues

67   through a process which involves three main stages: pretreatment and enzymatic

68   hydrolysis to release the fermentable sugars, and acetone-butanol-ethanol (ABE)

69   fermentation by Clostridia strains; this process being carried out anaerobically [14]. It is

70   worth mentioning that the pretreatment is the more expensive and one of the most

71   important stages of the process, since it is required to disrupt the structure of the organic

72   waste, dividing its different components and enhancing the access of the enzymes to the

73   glucan in the subsequent enzymatic hydrolysis stage [15]. In this context, there is a

74   great variety of pretreatments, such as hydrothermal, microwave, dilute acid, alkaline,

75   organosolv or biological, among others [16,17]. Hydrothermal processing or

76   autohydrolysis is considered a sustainable and environmentally friendly process [18]. It

77   is based on the application of water as a solvent at high temperature and pressure ranges

78   to generate hydronium ions that cause the dissolution of the hemicelluloses. The

79   advantages of autohydrolysis include the requirement of no chemicals other than water,

80   its non-corrosivity to equipment, a higher enzymatic hydrolysis rate, and milder reaction

81   conditions [19]. Hydrothermal processing has proved to be efficient as a pretreatment

82   for the production of biobutanol from potato peel [20], apple pomace [21] and tomato

83   waste [22].

84   The carrot is one of the most widely produced vegetables in the world. Its global

85   annual production is estimated to be 36 Mt, of which 0.4 Mt were produced in Spain in

86   2020 [23]. Approximately 25-30% of carrots are discarded because they do not meet

87   market specifications due to physical defects [24]. Carrot discards could be valorized

88   through recovery of cellulose (used, for example, to obtain nanofibrillated cellulose and

89   nanocrystalline cellulose, which are employed to make films), hemicellulose (mainly

90   arabinogalactans), pectins, and carotenoids [25]. Although it could be a valuable

91   feedstock for the production of biofuels (such as bioethanol) by fermentation of free

92   sugars and structural carbohydrates [26], to the best of our knowledge, the production of

93   butanol has not previously been reported.

94   The objective of this study was to analyze the production of biobutanol from carrot

95   discard, evaluating the different alternatives for the efficient saccharification of carrot

96   discard pulp. Diverse strategies based on hydrothermal pretreatment and/or enzymatic

97   hydrolysis were compared to evaluate the recovery of fermentable sugars and the global

98   butanol and ABE yields obtained after the fermentation of hydrolysates with

99   *Clostridium beijerinckii*, selecting the most suitable strategy for valorizing carrot

100   discard as a feedstock for biobutanol production. The production of ABE from carrot

101   discard juice was also evaluated.

102

103   **2. Materials and Methods**

104   *2.1. Raw material*

105   Carrot discards (CD) were kindly donated by Horcaol, a vegetable company located

106   in Olmedo (Valladolid, Spain). The particle size of carrot discards was reduced to 1-3

107   mm with a domestic grinder and stored at 4 ℃ before being used. The CD were

108   processed by a juice extractor (Kenwood JMP-400/WH) resulting in two fractions: a

109   liquid fraction (juice from CD, CDJ) rich in free sugars, which was directly fermented

110   to produce ABE; and a solid fraction (carrot discard pulp, CDP; 73% of humidity),

111   which was subjected to different process alternatives based on a hydrothermal process

112   and/or enzymatic hydrolysis before ABE fermentation (Figure 1). All experimental runs

113   were performed in duplicate and mean values and standard deviation were calculated.

114   *2.2. Hydrothermal pretreatment*

115   The hydrothermal pretreatment of the CDP was carried out in an autoclave (Model

116   MED-12, Selecta, Barcelona, Spain) at 121 ℃ for 15 min, using 1000 mL ISO bottles at

117   a CDP concentration of 10% w/w. The slurry attained after pretreatment was either

118   directly fed to ABE fermentation (Figure 1, Process (b)) or subjected to enzymatic

119   hydrolysis followed by ABE fermentation (Figure 1, Process (c)).

120   *2.3. Enzymatic hydrolysis*

121   The enzymatic hydrolysis was carried out in 100 mL Erlenmeyer flasks in an orbital

122   shaker (Optic Ivymen Systems, Comecta, Spain) using CDP as substrate (Figure 1,

123   Process (a)) or the slurry from the CDP pretreatment (Figure 1, Process (c)). The

124   enzymatic hydrolysis conditions were: substrate loading 10% (w/w), 50 °C, 150 rpm, 24

125 h, and pH 4.8, employing water as the solvent (the initial pH was adjusted to 4.8 when

126 necessary with NaOH 10 M or H_2SO_4 1 M). The efficiencies for saccharification of the

127 three enzymes, Cellic CTec2 (C), Viscozyme L (V), and Shearzyme (S), were

128 compared. The enzymes were added individually or combined and were kindly donated

129 by Novozymes A/S (Denmark). Samples were taken at 2, 4, 8 and 24 h, and centrifuged

130 (Mini Spin, Eppendorf), and their sugar and inhibitor content were analyzed. In order to

131 consider the sugar content in commercial enzymes, tests were carried out with enzyme

132 blanks. Monosaccharide recoveries in enzymatic hydrolysis were calculated regarding

133 the total monosaccharide content in CDP.
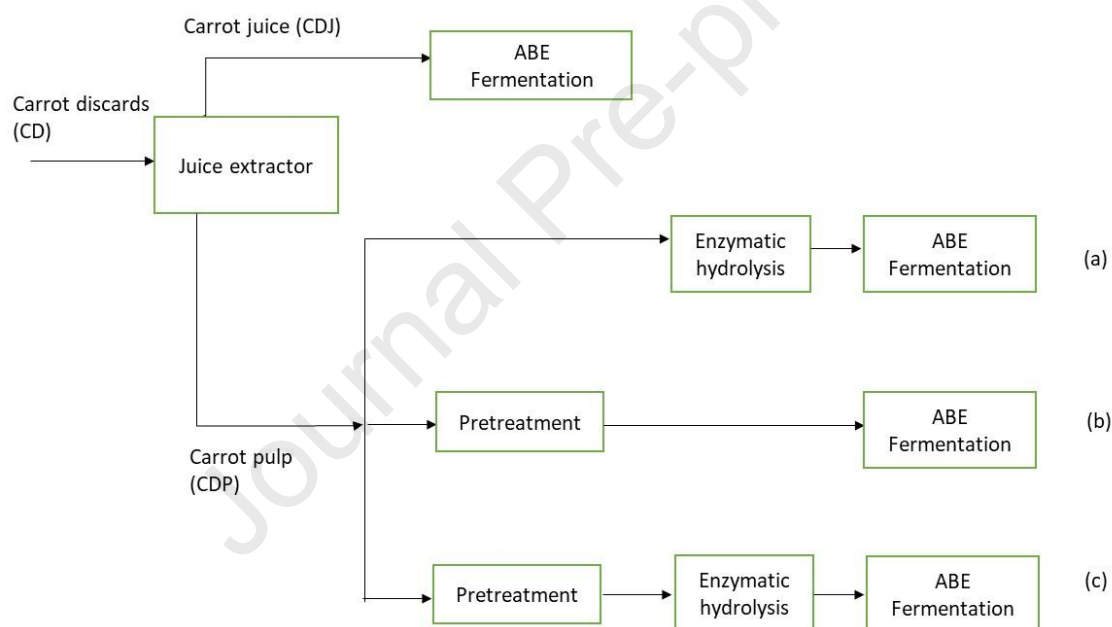
134

135 **Figure 1.** Configurations for the valorization of carrot discards by ABE fermentation;

136 (a) enzymatic hydrolysis of carrot discard pulp and fermentation; (b) hydrothermal

137 pretreatment of carrot discard pulp and fermentation of the slurry; (c) hydrothermal

138 pretreatment of carrot discard pulp, enzymatic hydrolysis of the slurry and fermentation.

139

140 *2.4. Microorganism*

141   The microorganism, *Clostridium beijerinckii* DSM 6422, was obtained from the

142   German collection of microorganisms (DSMZ, Leibniz, Germany). The strain was

143   maintained on Reinforced Clostridial Medium (RCM) in 100 mL serum bottles in spore

144   form and cold stored at 4 °C under anaerobic conditions. The inoculum was grown in

145   100 mL serum bottles with rubber septum under anaerobic conditions, using RCM and

146   flushed with free O_2 nitrogen. A thermal shock was performed at 80 °C for 2 min, then

147   at 4 °C for another 2 min and repeated twice to stimulate the germination of the spores.

148   The inoculum was grown in an orbital shaker (Optic Ivymen Systems, Comecta, Spain)

149   at 35 ºC and 135 rpm for 48 h.

150

151   *2.5. ABE fermentation*

152   The CDJ and CDP hydrolysates obtained after the different saccharification

153   alternatives were subjected to ABE fermentation with *C. beijerinckii* (Figure 1). ABE

154   fermentations were carried out in 100 mL serum bottles at 35 ºC, initial pH of 5.5

155   (without pH adjustment during the fermentation process) and 50 rpm for 48 h under

156   anaerobic conditions (flushing O_2 free nitrogen into the serum bottles before

157   inoculation). The fermentation media were pasteurized at 90 ºC for 15 min, and vitamin,

158   salt and acetate buffer solutions were also added under the same conditions as described

159   by López-Linares et al. [27]. The inoculum was also added at a concentration of 10%

160   (v/v). Samples were taken after 48 h, centrifuged (Mini Spin, Eppendorf), and the sugar

161   and ABE (acetone, butanol and ethanol) contents were determined.

162   *2.6. Analytical methods*

163   The National Renewable Energy Laboratory (NREL) analytical methodology was

164   applied to determine the content of extractives [28], ash [29], structural carbohydrates

165   [30], and lignin [30] in CD. High Performance Liquid Chromatography (HPLC) was the

166   technique employed to analyze the content of galacturonic acid, sugars (glucose,

167   galactose, fructose, and arabinose) and inhibitors (acetic and formic acids, furfural and

168   hydroxymethylfurfural (HMF)), using an Aminex HPX-87H column and a refractive

169   index detector (Waters 2414). 0.01 N H_2SO_4 (0.6 mL/min) was used as the mobile

170   phase, at 30 °C (solvents) or 60 °C (sugars and inhibitors). Samples were centrifuged

171   (13400 rpm, 10 min) and filtered (using 0.2 μm nylon filters) before being measured by

172   HPLC.

173

174   **3. Results and discussion**

175   *3.1. Characterization of the raw material*

176   The carrot discards were processed using a juice extractor, resulting in a juice and a

177   solid fraction (pulp). The carrot juice was rich in carbohydrates, with up to 33.7 g/L

178   total sugars (glucose, 16.6 g/L; and fructose, 17.1 g/L), thus being of great interest for

179   butanol production by ABE fermentation. The pulp fraction presented the following

180   composition (% w/w, dry matter): glucan, 28.3; hemicellulose, 16.5 (galactan, 13.5;

181   arabinan, 3.0); extractives, 43.1 (water extractives, 33.6; ethanol extractives, 9.5;

182   glucose in water extractives, 1.3; galactose in water extractives, 1.3); ash, 5.0; insoluble

183   acid lignin, 1.8; soluble acid lignin, 0.1; and protein, 1.5.

184   Regarding the CDP composition, a very high extractive content (43.1%) was found

185   compared to other agro-industrial residues, such as carrot press cake (17%) [31], apple

186   industry waste (3.1%) [32] and spent coffee grounds (12.4%) [33]. In addition, it is

187   worth highlighting that a very low presence of lignin (insoluble and soluble) was

188   detected in the CDP, unlike other fruit and vegetable residues: for example, carrot press

189   cake, 6.9% [31]; apple industry waste, 23.5% [32]; pea pod waste, 21.6% [34]; and

190   spent coffee grounds, 39.2% lignin [33]). So CDP may be enzymatically hydrolyzed

191   without a pretreatment process, which is very important for the profitability of the

192   global butanol production process from an agro-industrial residue. Moreover, CDP

193   contains a high total carbohydrate content, 47.5% (44.9 and 2.6% of structural and non-

194   structural carbohydrates, respectively), mainly glucose and galactose. Considering

195   hemicellulosic sugars in CDP, there is a high presence of galactose, which involves

196   81.8% of the total hemicellulosic sugars.

197   On the other hand, by comparing the composition of the CDP observed in this work

198   with the composition of carrot discards previously reported in the literature [26,31,35],

199   the residue studied in this work has a similar or relatively lower carbohydrate content, a

200   higher extractive content and a similar ash content. Ramos-Andrés et al. [25]

201   determined the composition of CDP, reporting a much higher content of water

202   extractives (63.9% vs 33.6%) and lignin (7.8 vs 1.9%), but a lower cellulose (10.7 vs

203   28.3%) and hemicellulose (8.4 vs 16.5%) content, and a similar protein content (2.3 vs

204   1.5%).

205

206   **3.2. Sugars recovery from carrot discard pulp**

207   **3.2.1. Configuration (a): enzymatic hydrolysis of CDP**

208   In order to enzymatically hydrolyze pulp from carrot discard, different enzymatic

209   hydrolysis tests were carried out (Figure 1, configuration (a)), using different types of

210   enzymes (Cellic CTec2, Viscozyme L, and Shearzyme, at 0.15, 0.3 and 0.3 g/g

211   substrate, respectively) as well as diverse mixtures (g/g substrate) of Cellic CTec2 and

212   Viscozyme L enzymes (C+V (0.075+0.15), C+V (0.1+0.1), C+V (0.1+0.2), and C+V

213   (0.15+0.3)).

214   First of all, regarding the use of individual enzymes (C, V and S, at 0.15, 0.3 and 0.3

215   g/g substrate, respectively), as can be seen in Figure 2a, the highest monosaccharides

9

216 content in enzymatic hydrolysates were achieved at 24 h of enzymatic hydrolysis when

217 the Viscozyme L enzyme was used, obtaining an enzymatic hydrolysate with up to 46.3

218 g/L of total sugars (glucose, 29.0 g/L; galactose, 13.8 g/L; and arabinose, 3.5 g/L),

219 corresponding to a total sugars recovery of 72.8% (Table 1). Cellic CTec2 led to an

220 enzymatic hydrolysate with a significantly lower sugars content, 33.6 g/L (glucose, 25.2

221 g/L; galactose, 7.3 g/L; and arabinose, 1.1 g/L) (52.8% total sugars recovery, Table 1).

222 The use of Viscozyme L led to higher recoveries of glucose, galactose and arabinose

223 than Cellic CTec2. In this context, different studies have also shown a high

224 saccharification activity for Viscozyme L when applied to different lignocellulosic

225 residues, such as seaweed biomasses [36], Okara (a soybean residue from soymilk and

226 tofu manufacture) [37] and Salix viminalis cv. Q683 (a bioenergy crop) [38]. This can

227 be due to the fact that Viscozyme L is a blend of β-glucanases (which hydrolyzes the

228 β(1,3)- and β(1,4)-linkages in β-D-glucans), pectinases, cellulases, hemicellulases and

229 xylanases [39,40], unlike Cellic CTec2 or Shearzyme. It is, therefore, able to

230 enzymatically hydrolyze both cellulosic and hemicellulosic sugars. However, in general,

231 a much lower monosaccharides content was attained by using the Shearzyme enzyme

232 (17.6 g/L of total sugars) (Figure 2a), with only 27.7% of total sugars recovery (Table

233 1). What is more, as shown in Figure 2a, glucose was the predominant sugar in

234 enzymatic hydrolysates, involving 63% and 75% for the V and C enzymes, respectively.

235 Furthermore, no presence of arabinose was detected when the S enzyme was used. It is

236 worth highlighting that S is a xylanase that mainly releases galactose from the

237 hemicellulosic fraction (Figure 2a).
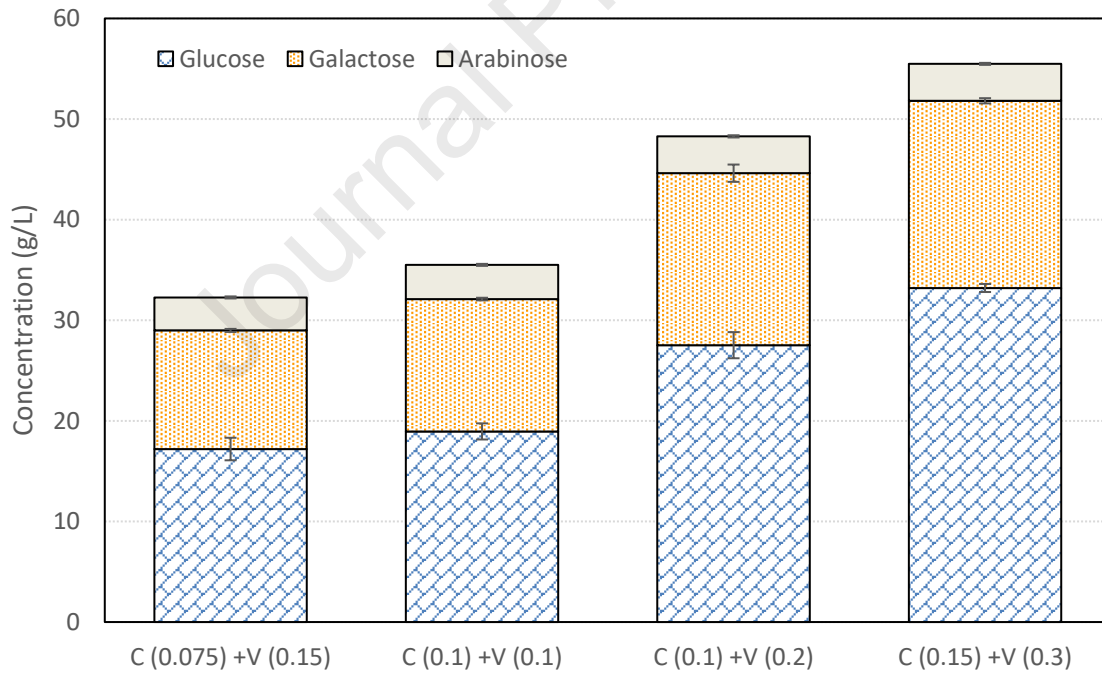
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241 a)
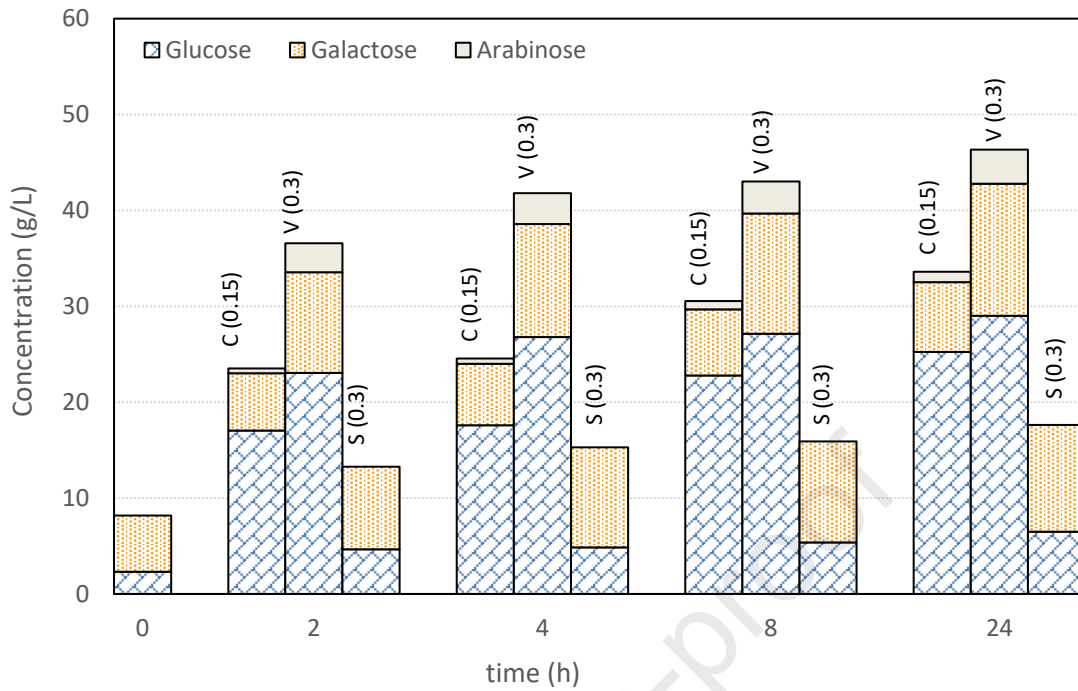
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243 b)

244

245 **Figure 2.** Concentration of monosaccharides during enzymatic hydrolysis of CDP using

246 (a) Cellic CTec2 (C), Viscozyme L (V) or Shearzyme (S); (b) after 24 h using mixtures

247 of the enzymes Cellic CTec2 and Viscozyme L. Enzyme dose in brackets (g/g DM).

248

249 **Table 1.** Recovery of monosaccharides (%) from carrot pulp after 24 h of enzymatic

250 hydrolysis. Comparison of different types and concentrations of enzymes. Cellic CTec2

251 (C), Viscozyme L (V), and Shearzyme (S). Enzyme dose in brackets (g/g DM).

252

Enzyme (g/g DM)	Glucose (%)	Galactose (%)	Arabinose (%)	Total sugars (%)
C (0.15)	65.3 ± 2.8	34.4 ± 1.8	28.5 ± 6.9	52.8 ± 2.7
V (0.3)	75.1 ± 2.9	64.9 ± 0.7	93.6 ± 0.6	72.8 ± 1.5
S (0.3)	16.8 ± 1.3	52.5 ± 2.2	0.0 ± 0.0	27.7 ± 1.0
C (0.075) + V (0.15)	44.5 ± 2.1	55.6 ± 0.6	86.5 ± 1.1	50.7 ± 1.0
C (0.1) + V (0.1)	49.0 ± 3.3	62.0 ± 4.1	90.0 ± 3.1	55.8 ± 4.3
C (0.1) + V (0.2)	71.2 ± 1.0	80.6 ± 1.2	96.6 ± 0.4	75.8 ± 1.0
C (0.15) + V (0.3)	85.9 ± 0.1	87.7 ± 0.2	96.9 ± 1.0	87.2 ± 0.2

253

254 Figure 2a also shows the time courses of the monosaccharides content on enzymatic

255 hydrolysates obtained using individual enzymes (C, V and S, at 0.15, 0.3 and 0.3 g/g

256 substrate, respectively). As can be seen, Viscozyme L was able to hydrolyze 90% of

257 total monosaccharides in an enzymatic hydrolysis time as short as 4 h. Cellic CTec2 and

258 Shearzyme enzymes were also able to rapidly hydrolyze the monosaccharides,

259 achieving 90.3% and 82.7%, respectively, of the total monosaccharides content at 8 h of

260 enzymatic hydrolysis. In this way, it can be said that the monosaccharides in carrot

261 discard can be quickly released in enzymatic hydrolysis, unlike other agro-industrial

262 residues, such as orange peel [41], brewer's spent grain [42], lettuce residues [43], spent

263 coffee grounds [33] or oil palm empty fruit bunch [44], where up to 48 or 72 h

264 enzymatic hydrolysis were necessary, due to the higher lignin content.

265 On the other hand, considering the use of enzyme mixtures (g/g substrate) (C+V

266 (0.075+0.15), C+V (0.1+0.1), C+V (0.1+0.2), and C+V (0.15+0.3)) in the enzymatic

267 hydrolysis process (Figure 2b), the highest sugars content in enzymatic hydrolysates

268 was achieved for the mixture C+V (0.15+0.3), obtaining a total sugar concentration as

269 high as 55.5 g/L (corresponding to a total sugars recovery of 87.2% (Table 1)).

270   Moreover, it is worth mentioning that this behavior was observed for both glucose and

271   galactose sugars, while the arabinose content was similar for all the cases studied

272   (ranging between 3.3 and 3.7 g/L). In this way, the use of the C+V (0.15+0.3)) enzymes

273   mixture allows an increase of 20% and 65% to be attained in the total sugar

274   concentration compared to the use of individual V and C enzymes (using 0.3 and 0.15

275   g/g substrate, respectively) (Figure 2a). Therefore, very high sugar concentrations and

276   recoveries can be obtained by enzymatic hydrolysis from CDP without the use of any

277   pretreatment process, which is very important for the profitability of the global butanol

278   production process from agro-industrial residues (for instance, CD). Previous studies

279   have also shown that the use of a mixture of enzymes (Viscozyme L and Celluclast-1.5

280   L) is more effective than the single-enzyme treatment for the enzymatic hydrolysis of

281   red seaweed (1.2 and 8.4 U/mL, respectively) [39] and *Gracilaria verrucosa* (4-32

282   U/mL) [40].

283   High carbohydrate recoveries (90-100%) from enzymatic hydrolysis (without

284   pretreatment of the raw material) were also reported from apple pulp residues [45],

285   using Pulpzyme HC and Novozym 188 enzymes (24.7 FPU and 121.7 CbU (Cellobiase

286   Unit)/g substrate) after 5 h enzymatic hydrolysis; oil palm empty fruit bunch fiber [44],

287   using a cocktail of Cellic CTec2 and Cellic HTec2 after 72 h enzymatic hydrolysis; and

288   spent coffee grounds [33], using Cellic CTec2 as enzyme (15 FPU/g substrate) and 72 h

289   of enzymatic hydrolysis. Aimaretti et al. [46], employing pulp from carrot discard, were

290   able to attain a total sugars recovery in enzymatic hydrolysis (at 2.5 h of process) of up

291   to 78.5%, using the Optimase CX255L enzyme (0.05% v/v), which is a thermostable

292   xylanase. Similar glucose recoveries on enzymatic hydrolysis (of about 90%) to those

293   achieved in this work were also reported by De Vrije et al. [47] at 42h of enzymatic

294   hydrolysis, also using carrot pulp and GC 220 enzyme (15 mL/100 g dry raw material).

295 **_3.2.2. Configurations (b) (pretreatment) and (c) (pretreatment and enzymatic_**

296 **_hydrolysis)_**

297   In addition to enzymatic hydrolysis, two different strategies to recover the

298 carbohydrates contained in carrot discard pulp were a hydrothermal pretreatment

299 (Figure 1, configuration (b)) or a sequential pretreatment and enzymatic hydrolysis of

300 pretreated CDP slurry (Figure 1, configuration (c)), in this case using different types of

301 single enzyme (C (0.15) and V (0.3)) as well as enzyme mixtures (C+V (0.1+0.2)). The

302 hydrothermal pretreatment in autoclave (121 ºC) employed in this work is usually used

303 to pretreat lignocellulosic biomass for biofuel production, such as bioethanol and

304 biobutanol. In this way, the hydrothermal pretreatment has been successfully applied to

305 sugarcane trash [48], grape marc [49], and carrot pomace [50] to produce bioethanol; as

306 well as for the production of biobutanol from potato peel [20], apple pomace [21] and

307 tomato waste [22].

308   Figure 3 shows the monosaccharide concentration achieved for both configurations

309 ((b) and (c)). In this way, the carbohydrate concentration achieved in the pretreated

310 CDP slurry (configuration (b) (20.9 g/L total sugars) was much lower than those

311 attained in configuration (c) (42.4-55.9 g/L total sugars). Moreover, the total sugars

312 recovery was also much higher when a sequential pretreatment and enzymatic

313 hydrolysis process of pretreated CDP slurry (configuration c) was carried out (up to 88

314 vs 33% obtained for configuration (b) (Figure 3). A low glucose yield ($< 20\%$) was also

315 reported for carrot peelings after dilute acid pretreatment (H_2SO_4, HNO_3, or HCl) (60

316 ºC, 180 min and 4% acid) [35]. Procentese et al. [43] only achieved about 7 g/L of total

317 sugars after the alkaline pretreatment (121 ºC, 30 min and 200 kg/m^3 NaOH) of lettuce

318 residues. These data show that an enzymatic hydrolysis is usually necessary after the

319 pretreatment of vegetable waste to release the fermentable sugars.

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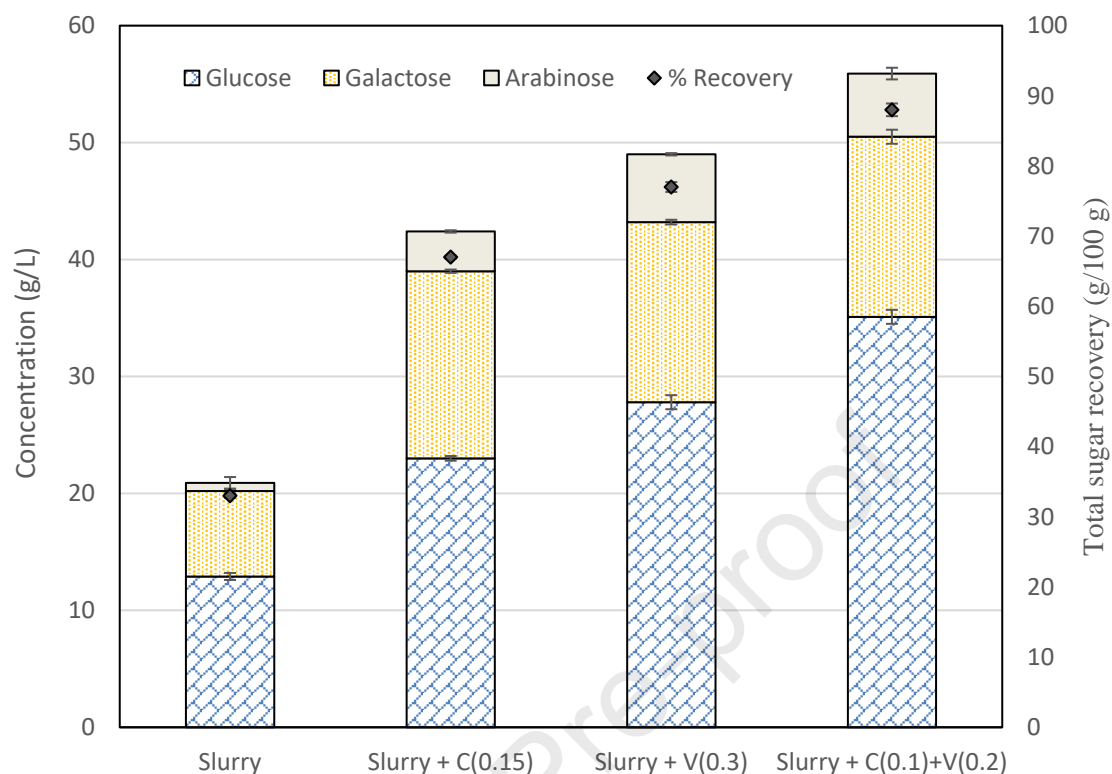
Figure 3. Concentrations of monosaccharides and total sugar recoveries (g/100 g sugars

in carrot pulp) after CDP hydrothermal pretreatment (slurry, configuration (b)) and

carrot pulp hydrothermal pretreatment followed by enzymatic hydrolysis (24 h) of the

slurry using different enzymes (configuration (c)). Cellic CTec2 (C), Viscozyme L (V).

Enzyme dose in brackets (g/g DM).

327

   On the other hand, by comparing the use of single enzymes (C (0.15) and V (0.3))

and enzyme mixtures (C+V (0.1+0.2)), as can be seen in Figure 3; the highest

carbohydrate concentrations were achieved when enzyme mixtures were employed

(55.9 g/L total sugars: 35.1 g/L glucose, 15.4 g/L galactose and 5.4 g/L arabinose). In

this way, the use of enzyme mixtures (C+V (0.1+0.2)) in the enzymatic hydrolysis of

hydrothermally pretreated CDP slurry allowed the total sugars content to increase by up

to 31.8% compared to the use of single enzymes (for example, C (0.15)). The highest

335   total sugars recovery (88%) was also obtained using enzyme mixtures (C+V (0.1+0.2)),

336   compared to the use of single enzymes (67% and 77% for C (0.15) and V (0.3),

337   respectively) (configuration c, Figure 3). Furthermore, it is worth highlighting that

338   glucose was the main sugar obtained in both configurations b and c (involving 61.7%

339   and 54.2-62.8% of total sugars, respectively). Yoon et al. [51] also reported 61.7 g total

340   sugars/100 g raw material after 24 h of enzymatic hydrolysis from NaOH and acetic

341   acid pretreated carrot pomace, using a cellulase-rich enzyme isolated from *Achatina*

342   *fulica* (0.25 mL/g solid).

343   Finally, by comparing the three configurations (Figure 1) studied in this work, the

344   highest total sugars concentration 55.5-55.9 g/L (87-88% total sugars recovery) was

345   obtained for configurations (a) (enzymatic hydrolysis, C + V (0.15+0.3) and (c)

346   (pretreatment followed by enzymatic hydrolysis (C + V (0.1+0.2)); while only 20.9 g/L

347   (33% total sugars recovery) was attained for configuration (b) (only pretreatment).

348   Considering the presence of inhibitors, formic acid, furfural and HMF were not detected

349   in the enzymatic hydrolysates in any of the experimental runs, even when the

350   hydrothermal pretreatment was applied. The concentration of acetic acid in the

351   enzymatic hydrolysates ranged from 0.7-1 g/L, this being a beneficial compound for the

352   subsequent ABE fermentation tests. In this way, no inhibition was observed in the

353   experimental runs. Therefore, in conclusion, no hydrothermal pretreatment may be

354   necessary for the saccharification of carrot pulp. In addition, it is worth highlighting that

355   the whole slurry from carrot pulp was used in this work, which is very interesting for

356   the profitability of the process in a biorefinery context.

357   Similar results (80 and 92%, respectively) for enzymatic hydrolysis were reported

358   from the literature using carrot peel, but in these cases the use of a hydrothermal

359   pretreatment (121 ºC and 60 min) [52], or sequential dilute sulfuric acid (4%, 180 min)

360 and steam (121°C, 15 min) pretreatment [35], was necessary. Other vegetable residues

361 also required the application of a pretreatment to obtain good saccharification yields.

362 For instance, apple pomace (autohydrolysis, 10% solid-liquid ratio, 142.4 °C and 12

363 min) [21], potato peel (autohydrolysis 10% solid-liquid ratio, 140.2 °C and 56.1 min)

364 [20], and tomato pomace (hydrothermal process, 20% solid-liquid ratio, 121 °C and 20

365 min) [22].

366 *3.3. Fermentation process*

367 *3.3.1. Configuration (a): enzymatic hydrolysis*

368 The different enzymatic hydrolysates obtained in the configuration (a) were

369 subjected to ABE fermentation. Figure 4 shows the concentration of sugars consumed

370 and the butanol and ABE concentrations produced.
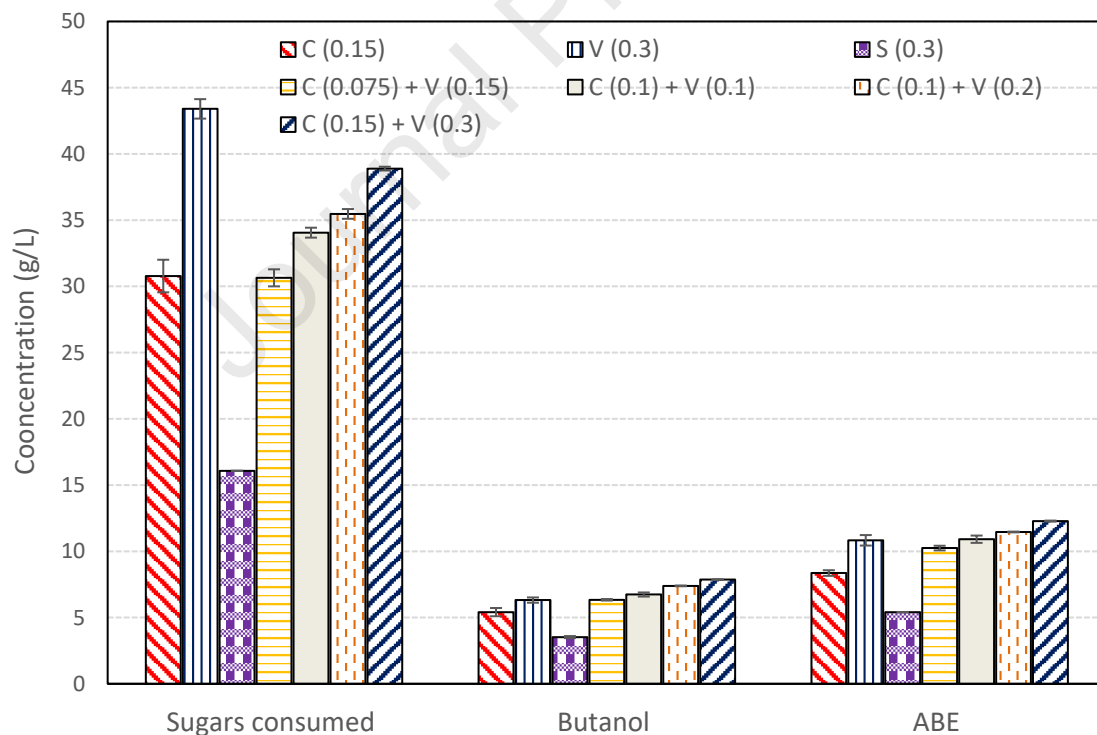
371

372 **Figure 4.** ABE fermentation of the enzymatic hydrolysates obtained from CDP

373 (configuration (a)). Consumption of monosaccharides and concentrations of butanol and

374 ABE (g/L) after 48 h fermentation. Cellic CTec2 (C), Viscozyme L (V), Shearzyme (S).

375 Enzyme dose in brackets (g/g DM).

376

377 In this way, as can be seen, except for S (0.3), high sugar concentrations (> 30 g/L)

378 were used by *C. beijerinckii* in all cases, the sugars being consumed almost entirely in

379 all the fermentation tests (sugar consumption 96.4-98.3%). On the other hand, also

380 except for the S enzyme (0.3), high butanol (5.4-7.9 g/L) and ABE (8.4-12.3 g/L)

381 concentrations were achieved for the different enzymatic hydrolysates; the highest

382 butanol and ABE levels (7.9 and 12.3 g/L, respectively) being attained when the C+V

383 (0.15+0.3) enzyme mixture was employed.

384 In addition, as can be appreciated in Table 2, in general, high butanol and ABE

385 yields (Y_{BUT}: 0.20-0.21 g/g; Y_{ABE}: 0.32-0.33) and productivities (P_{BUT}:0.13-0.16 g/L·h;

386 P_{ABE}: 0.21-0.25 g/L·h) were achieved in the fermentation of the enzymatic hydrolysates

387 obtained using mixtures of enzymes (Table 2). Therefore, it is worth highlighting that

388 up to 79 g butanol and 123 g ABE per kg carrot pulp could be achieved from CDP by

389 enzymatic hydrolysis with C+V (0.15+0.3) (Table 2).

390

391 **Table 2.** ABE fermentation of the hydrolysates obtained from carrot pulp. Butanol and

392 ABE yields (Y_{BUT}/sugars, Y_{ABE}/sugars expressed as g/g sugars consumed); butanol and

393 ABE productivities (expressed as g/(L·h) at 48 h); and butanol and ABE global yields

394 (expressed as g/kg carrot pulp (DM)). Cellic CTec2 (C), Viscozyme L (V), Shearzyme

395 (S). Enzyme dose in brackets (g/g DM).

	Y_{BUT} (g/g)	Y_{ABE} (g/g)	P_{BUT} (g/L·h)	P_{ABE} (g/L·h)	g $_{BUT}$/kg pulp (DM)	g $_{ABE}$/kg pulp (DM)
Configuration (a)						
C (0.15)	0.18	0.27	0.11	0.17	54 ± 3	84 ± 2
V (0.3)	0.15	0.25	0.13	0.22	63 ± 2	108 ± 4
S (0.3)	0.22	0.34	0.07	0.11	35 ± 1	54 ± 2

C (0.075) + V (0.15)	0.21	0.33	0.13	0.21	63 ± 1	102 ± 2
C (0.1) + V (0.1)	0.20	0.32	0.14	0.23	67 ± 2	109 ± 3
C (0.1) + V (0.2)	0.21	0.32	0.15	0.24	74 ± 1	115 ± 1
C (0.15) + V (0.3)	0.20	0.32	0.16	0.25	79 ±1	123 ± 1
Configuration (b)						
Slurry	0.22	0.12	0.09	0.12	42 ± 3	58 ± 2
Configuration (c)						
Slurry + C (0.15)	0.22	0.33	0.15	0.23	72 ± 2	112 ± 4
Slurry + V (0.3)	0.20	0.31	0.15	0.23	71 ± 1	111 ± 2
Slurry + C (0.1) + V (0.2)	0.21	0.30	0.14	0.21	69 ± 1	101 ± 2

396

397   By comparing the results obtained in this work in the ABE fermentation of the

398   different enzymatic hydrolysates (configuration (a)) (Figure 4 and Table 2) with those

399   reported in the literature for other agro-industrial residues; López-Linares et al. [33]

400   achieved similar butanol and ABE concentrations (7.7 and 11.4 g/L, respectively),

401   yields (0.23 and 0.34 g/g, respectively) and productivities (0.160 and 0.238 g/L·h,

402   respectively) in the ABE fermentation of the enzymatic hydrolysate obtained from

403   unpretreated SCG (spent coffee grounds), using also *C. beijerinckii*. Therefore, no

404   pretreatment is necessary with some agro-industrial residues (i.e., CD and SCG) in

405   order to obtain high butanol yields, which is an important advantage compared to the

406   conventional lignocellulosic residues.

407

408   *3.3.2. Configurations (b) (pretreatment) and (c) (pretreatment and enzymatic*

409   *hydrolysis)*

410   The slurry generated after CDP hydrothermal pretreatment (configuration (b)), as

411   well as the different enzymatic hydrolysates produced from sequential pretreatment and

412   enzymatic hydrolysis of pretreated CDP slurry (configuration (c)), were all fermented

413   by *C. beijerinckii*.

414   Figure 5 shows the concentration of sugars consumed, as well as butanol and ABE

415   concentrations obtained during the different fermentation tests. In this way, the lowest

416   sugar concentrations consumed (19 g/L) and the lowest butanol and ABE production

417   (4.2 and 5.8 g/L, respectively) were achieved when CDP was only hydrothermally

418   pretreated (configuration (b)) and directly fed to fermentation. Then, an ABE yield as

419   low as 0.12 g/g, and butanol and ABE productivities of only 0.09 and 0.12 g/L·h,

420   respectively, were reported in this case (Table 2). Nimbalkar et al. [34] also reported

421   butanol and ABE productions as low as 2.31 and 4.80 g/L, respectively (ABE yield =

422   0.15 g/g), from hydrothermal pretreated pea pod waste slurry (121 ℃ and 15 min) by *C.*

423   *acetobutylicum* B 527. On the other hand, although carrot pulp has not previously been

424   used for butanol and ABE production, Survase et al. [53] used carrot waste as a

425   supplement for ABE solvent production (9.96 g/L ABE) by *C. acetobutylicum* DSM

426   792, from the spent liquor of spruce wood chips, which was fractioned by SO_2–

427   ethanol–water, evaporation, steam stripping, liming and catalytic oxidation methods, but

428   without any enzymatic process.
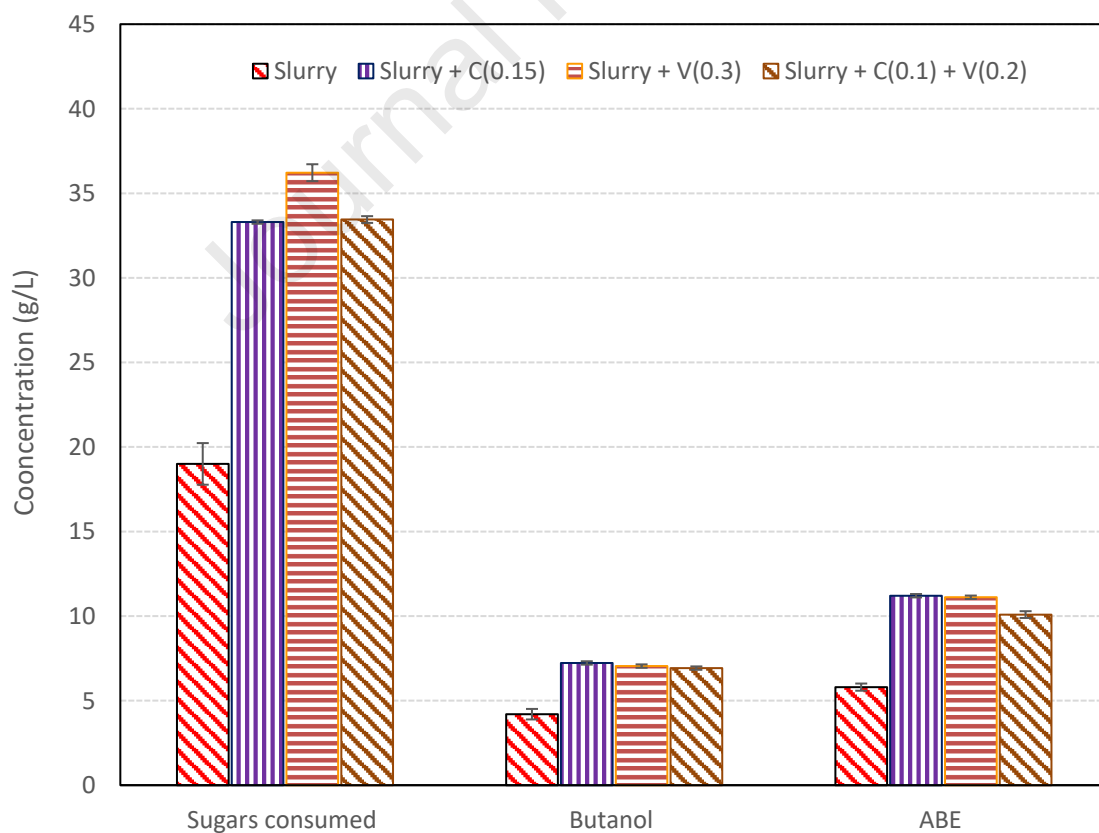
429

430   **Figure 5.** ABE fermentation of the hydrolysates obtained from carrot pulp after

431   hydrothermal pretreatment (slurry, configuration (b)) and after hydrothermal

432   pretreatment and enzymatic hydrolysis of the slurry using different enzymes

433   (configuration (c)). Consumption of monosaccharides and concentrations of butanol and

434   ABE (g/L) after 48 h fermentation. Cellic CTec2 (C), Viscozyme L (V). Enzyme dose

435   in brackets (g/g DM).

436   Nevertheless, as can be seen in Figure 5, high sugar concentrations consumed (> 33.3

437   g/L) and high butanol and ABE levels (> 6.9 and 10.1 g/L, respectively) were attained

438   when CDP was both hydrothermally pretreated and enzymatically hydrolyzed

439   (configuration (c)). On the other hand, regarding the configuration (c), although the

440   highest sugar content consumed (36.2 g/L) was obtained when Viscozyme L (V (0.3))

441   was used, the butanol and ABE concentrations were similar (butanol: 6.9-7.2; and ABE:

442   10.1-11.2 g/L) for the three enzymatic hydrolysates (C (0.15), V (0.3), and C+V

443   (0.1+0.2)) (Figure 5). In this context, similar butanol and ABE yields (0.20-0.22 and

444   0.30-0.33 g/g, respectively), and butanol and ABE productivities (0.14-0.15 and 0.21-

445   0.23 g/L·h), were also observed (Table 2). In this way, considering the ABE

446   fermentation, when CDP was subjected to a pretreatment, no considerable differences

447   between the C (0.15) and V (0.3) enzymes, or even the enzyme mixtures (C+V

448   (0.1+0.2)), were appreciated (Figure 5 and Table 2).

449   By comparing the results obtained in this study for configuration (c) (sequential

450   hydrothermal pretreatment and enzymatic hydrolysis of CDP) (Figure 5 and Table 2)

451   with those reported in the literature for other agro-industrial residues, similar results

452   (about 7 g/L butanol and 10 g/L ABE) to those obtained in configuration (c) were

453   obtained after the autohydrolysis of potato peel (10% solid-liquid ratio, 140.2 ºC and

454   56.1 min) [20] or tomato pomace (20% solid-liquid ratio, 121 ºC and 20 min) [22],

455 enzymatic hydrolysis and fermentation using *C. saccharobutylicum* DSM 13864 or *C.*

456 *beijerinckii* DSM 1820, respectively. Slightly lower ABE concentrations (8.3 g/L) and

457 butanol yields (0.17 g/g) were achieved from the autohydrolysis of pretreated apple

458 pomace (10% solid-liquid ratio, 142.4 ºC and 12 min) followed by the enzymatic

459 hydrolysis (15 mg Cellic CTec2/g glucan) and fermentation using *C. beijerinckii* CECT

460 508 [21]. Much lower butanol and ABE levels (1.1 and 1.44 g/L, respectively) were

461 obtained from lettuce residues using *C. acetobutylicum* DSMZ 792 [43]. Although

462 lettuce residues were pretreated under alkaline conditions and enzymatically hydrolyzed

463 by Cellic CTec2, low sugar concentrations were released, leading to considerably lower

464 butanol concentrations.

465   In short, using the configuration (c), up to 72 g butanol and 112 g ABE per kg carrot

466 pulp could be achieved from sequential hydrothermally pretreated and enzymatically

467 hydrolyzed CDP, using for instance, the Cellic CTec2 enzyme and an enzymatic

468 loading of 0.15 g/g CDP (Table 2). In this context, only 26.7 g butanol and 29.4 g ABE

469 per kg tomato pomace were obtained by *C. beijerinckii* DSM 6423 after hydrothermal

470 pretreatment (20% solid-liquid ratio, 121 ºC and 20 min) and enzymatic hydrolysis [22].

471 López-Linares et al. [54] was also able to recover up to 81 kg butanol and 126 kg ABE

472 per ton of SCG, which was pretreated by microwave dilute sulfuric acid and

473 enzymatically hydrolyzed.

474   By comparing the three configurations analyzed in this work, it is worth highlighting

475 that the highest butanol and ABE productions (79 g butanol and 123 g ABE per kg

476 carrot pulp) were attained for the configuration (a), where CDP was only enzymatically

477 hydrolyzed (C+V (0.15+0.3)). Therefore, the results reported in this work show that no

478 hydrothermal pretreatment would be necessary for carrot pulp, unlike other agro-

479 industrial residues, such as brewer's spent grain [27], where a microwave-assisted dilute

480 acid pretreatment was required due to their higher lignin content. This fact is of great

481 interest, since it has been reported from the literature that, in general, a pretreatment

482 stage involves up to 30% of the total cost of the biofuel production process from

483 lignocellulosic residues [55].

484   Furthermore, the CDJ obtained from CD was also subjected to ABE fermentation,

485 96% of the sugars content being consumed, and resulting in 6.35 and 9.04 g/L butanol

486 and ABE, respectively. This allows the use of all the carrot discards for ABE

487 fermentation, thus proving highly interesting in a biorefinery context.

488

489 4. Conclusions

490 Different strategies based on hydrothermal pretreatment and/or enzymatic hydrolysis

491 were evaluated for efficient biobutanol production from carrot discards by *C.*

492 *beijerinckii*. Saccharification times were considerably shorter (about 6 h) compared to

493 lignocellulosic residues with a higher lignin content. Global yields of 79 g butanol and

494 123 g ABE/kg carrot discard pulp were achieved after enzymatic hydrolysis using a

495 blend of enzymes, Cellic CTec2 and Viscozyme, at 0.15 and 0.3 g/g substrate. The

496 results demonstrate that the pretreatment step is not necessary to achieve a proper

497 saccharification of the residue, which is very interesting for the economic profitability

498 of the process. Furthermore, the carrot juice can also be used for butanol production

499 without the presence of inhibition, which is very interesting in a biorefinery context.

500

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508

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: