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

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ABSTRACT

Carrot discard was evaluated as a raw material for acetone-butanol-ethanol (ABE) fermentation. Different strategies based on hydrothermal pretreatment and/or enzymatic hydrolysis were compared for biobutanol production from carrot discard pulp. In addition, the use of different types of enzymes and diverse enzyme mixtures were evaluated. In this way, total sugar recoveries of up to 76%, and butanol and ABE concentrations of 7.4 and 11.5 g/L, respectively (74 g butanol and 115 g ABE/kg carrot pulp), were achieved when the carrot discard pulp was enzymatically hydrolyzed, without pretreatment, using a mixture of enzymes of Cellic CTec2 and Viscozyme L at a dosage of 0.1 and 0.2 g/g, respectively. When a hydrothermal pretreatment was applied, a total sugar recovery of 88%, 6.9 g/L butanol and 10.1 g/L ABE (69 g butanol and 101 g ABE/kg carrot pulp) were attained using the same mixture of enzymes. In this way, no hydrothermal pretreatment would be necessary to produce ABE from carrot discard, which is very interesting for the profitability of the process. Furthermore, the carrot discard juice yielded 6.4 and 9 g/L butanol and ABE, respectively, showing that all the carrot discards could be used for ABE production.
Keywords: carrot discard; hydrothermal pretreatment; enzymatic hydrolysis; biofuels; biobutanol; *Clostridium beijerinckii*.

1. Introduction

One of the most important economic sectors in Spain is the agro-food industry. The fruit and vegetable sector contributes 1.7% of Spain’s gross domestic product (GDP) and is responsible for up to 300,000 jobs. In addition, Spain is regarded as the first country in the EU in the production of fruit and vegetables, generating up to 28 million tons per year (Mt/y) (25% of European production), while worldwide it ranks sixth [1].

As a result of this activity, a great amount of organic residue is obtained, such as surplus, non-conformity fruit and vegetables, as well as by-products generated in the processing activities [2]. These residues have in common a high content in organic matter and a perishable character that makes their valorization difficult. Although fruit and vegetable residues can be used for animal feed, this application has some limitations; for instance, there are some residues which cannot be taken by some animals and some diseases could be transmitted due to the presence of toxic substances. The high transportation and conservation costs usually make this alternative unfeasible [3]. On the other hand, dumping fruit and vegetable waste in landfills is associated with the production of greenhouse gases (CH₄, CO₂), due to the degradation of the organic matter under anaerobic conditions. Methane from landfills accounts for about 800 million tons of CO₂-equivalent and is the third largest source of methane emissions [3].

The Directive (EU) 2018/850 aims to ensure a progressive reduction in the landfilling of biodegradable waste, especially that which is suitable for recycling or recovery. Therefore, in order to improve the efficiency of the food sector and achieve a circular economy, it is necessary to convert fruit and vegetable residues into a resource.
Due to the interesting composition of this type of residue (carbohydrates, pectin, lipids, proteins, phytochemicals, and none or very low lignin content) [4–6], fruit and vegetable residues could have a great potential for the production of such high value-added chemicals as bioactive compounds (polyphenols, polysaccharides, proteins) [7], commodity chemicals, and/or biofuels [8].

Biobutanol is considered a promising advanced biofuel due to its high energy density, low vapor pressure, and its lower volatility, explosiveness, hygroscopicity and solubility in water compared to bioethanol [9]. Moreover, biobutanol could directly replace gasoline, thus also being regarded as a drop-in-fuel [10]. What is more, it can also be used as a solvent and a chemical commodity (for instance, in varnish, lac, or in the pharmaceutical and food industries, among others) [11,12]. Biobutanol production can therefore be very interesting. According to Ibrahim et al. [13], around 5 million tons of biobutanol are generated worldwide, its price being about 0.9-1.4 USD/kg.

Butanol-producing microorganisms are not able to directly ferment carbohydrates in agro-industrial biomass; it must first undergo a pretreatment to improve the saccharification of the complex polysaccharides to render fermentable sugars. In this way, biobutanol could be produced biologically from fruit and vegetable residues through a process which involves three main stages: pretreatment and enzymatic hydrolysis to release the fermentable sugars, and acetone-butanol-ethanol (ABE) fermentation by Clostridia strains; this process being carried out anaerobically [14]. It is worth mentioning that the pretreatment is the more expensive and one of the most important stages of the process, since it is required to disrupt the structure of the organic waste, dividing its different components and enhancing the access of the enzymes to the glucan in the subsequent enzymatic hydrolysis stage [15]. In this context, there is a great variety of pretreatments, such as hydrothermal, microwave, dilute acid, alkaline,
organosolv or biological, among others [16,17]. Hydrothermal processing or autohydrolysis is considered a sustainable and environmentally friendly process [18]. It is based on the application of water as a solvent at high temperature and pressure ranges to generate hydronium ions that cause the dissolution of the hemicelluloses. The advantages of autohydrolysis include the requirement of no chemicals other than water, its non-corrosivity to equipment, a higher enzymatic hydrolysis rate, and milder reaction conditions [19]. Hydrothermal processing has proved to be efficient as a pretreatment for the production of biobutanol from potato peel [20], apple pomace [21] and tomato waste [22].

The carrot is one of the most widely produced vegetables in the world. Its global annual production is estimated to be 36 Mt, of which 0.4 Mt were produced in Spain in 2020 [23]. Approximately 25-30% of carrots are discarded because they do not meet market specifications due to physical defects [24]. Carrot discards could be valorized through recovery of cellulose (used, for example, to obtain nanofibrillated cellulose and nanocrystalline cellulose, which are employed to make films), hemicellulose (mainly arabinogalactans), pectins, and carotenoids [25]. Although it could be a valuable feedstock for the production of biofuels (such as bioethanol) by fermentation of free sugars and structural carbohydrates [26], to the best of our knowledge, the production of butanol has not previously been reported.

The objective of this study was to analyze the production of biobutanol from carrot discard, evaluating the different alternatives for the efficient saccharification of carrot discard pulp. Diverse strategies based on hydrothermal pretreatment and/or enzymatic hydrolysis were compared to evaluate the recovery of fermentable sugars and the global butanol and ABE yields obtained after the fermentation of hydrolysates with *Clostridium beijerinckii*, selecting the most suitable strategy for valorizing carrot
discard as a feedstock for biobutanol production. The production of ABE from carrot discard juice was also evaluated.

2. Materials and Methods

2.1. Raw material

Carrot discards (CD) were kindly donated by Horcaol, a vegetable company located in Olmedo (Valladolid, Spain). The particle size of carrot discards was reduced to 1-3 mm with a domestic grinder and stored at 4 °C before being used. The CD were processed by a juice extractor (Kenwood JMP-400/WH) resulting in two fractions: a liquid fraction (juice from CD, CDJ) rich in free sugars, which was directly fermented to produce ABE; and a solid fraction (carrot discard pulp, CDP; 73% of humidity), which was subjected to different process alternatives based on a hydrothermal process and/or enzymatic hydrolysis before ABE fermentation (Figure 1). All experimental runs were performed in duplicate and mean values and standard deviation were calculated.

2.2. Hydrothermal pretreatment

The hydrothermal pretreatment of the CDP was carried out in an autoclave (Model MED-12, Selecta, Barcelona, Spain) at 121 ºC for 15 min, using 1000 mL ISO bottles at a CDP concentration of 10% w/w. The slurry attained after pretreatment was either directly fed to ABE fermentation (Figure 1, Process (b)) or subjected to enzymatic hydrolysis followed by ABE fermentation (Figure 1, Process (c)).

2.3. Enzymatic hydrolysis

The enzymatic hydrolysis was carried out in 100 mL Erlenmeyer flasks in an orbital shaker (Optic Ivymen Systems, Comecta, Spain) using CDP as substrate (Figure 1, Process (a)) or the slurry from the CDP pretreatment (Figure 1, Process (c)). The enzymatic hydrolysis conditions were: substrate loading 10% (w/w), 50 °C, 150 rpm, 24
h, and pH 4.8, employing water as the solvent (the initial pH was adjusted to 4.8 when necessary with NaOH 10 M or H$_2$SO$_4$ 1 M). The efficiencies for saccharification of the three enzymes, Cellic CTec2 (C), Viscozyme L (V), and Shearzyme (S), were compared. The enzymes were added individually or combined and were kindly donated by Novozymes A/S (Denmark). Samples were taken at 2, 4, 8 and 24 h, and centrifuged (Mini Spin, Eppendorf), and their sugar and inhibitor content were analyzed. In order to consider the sugar content in commercial enzymes, tests were carried out with enzyme blanks. Monosaccharide recoveries in enzymatic hydrolysis were calculated regarding the total monosaccharide content in CDP.

Figure 1. Configurations for the valorization of carrot discards by ABE fermentation; (a) enzymatic hydrolysis of carrot discard pulp and fermentation; (b) hydrothermal pretreatment of carrot discard pulp and fermentation of the slurry; (c) hydrothermal pretreatment of carrot discard pulp, enzymatic hydrolysis of the slurry and fermentation.

2.4. Microorganism
The microorganism, *Clostridium beijerinckii* DSM 6422, was obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The strain was maintained on Reinforced Clostridial Medium (RCM) in 100 mL serum bottles in spore form and cold stored at 4 °C under anaerobic conditions. The inoculum was grown in 100 mL serum bottles with rubber septum under anaerobic conditions, using RCM and flushed with free O\textsubscript{2} nitrogen. A thermal shock was performed at 80 °C for 2 min, then at 4 °C for another 2 min and repeated twice to stimulate the germination of the spores. The inoculum was grown in an orbital shaker (Optic Ivymen Systems, Comecta, Spain) at 35 °C and 135 rpm for 48 h.

2.5. ABE fermentation

The CDJ and CDP hydrolysates obtained after the different saccharification alternatives were subjected to ABE fermentation with *C. beijerinckii* (Figure 1). ABE fermentations were carried out in 100 mL serum bottles at 35 °C, initial pH of 5.5 (without pH adjustment during the fermentation process) and 50 rpm for 48 h under anaerobic conditions (flushing O\textsubscript{2} free nitrogen into the serum bottles before inoculation). The fermentation media were pasteurized at 90 °C for 15 min, and vitamin, salt and acetate buffer solutions were also added under the same conditions as described by López-Linares et al. [27]. The inoculum was also added at a concentration of 10% (v/v). Samples were taken after 48 h, centrifuged (Mini Spin, Eppendorf), and the sugar and ABE (acetone, butanol and ethanol) contents were determined.

2.6. Analytical methods

The National Renewable Energy Laboratory (NREL) analytical methodology was applied to determine the content of extractives [28], ash [29], structural carbohydrates [30], and lignin [30] in CD. High Performance Liquid Chromatography (HPLC) was the
technique employed to analyze the content of galacturonic acid, sugars (glucose, galactose, fructose, and arabinose) and inhibitors (acetic and formic acids, furfural and hydroxymethylfurfural (HMF)), using an Aminex HPX-87H column and a refractive index detector (Waters 2414). 0.01 N H₂SO₄ (0.6 mL/min) was used as the mobile phase, at 30 °C (solvents) or 60 °C (sugars and inhibitors). Samples were centrifuged (13400 rpm, 10 min) and filtered (using 0.2 μm nylon filters) before being measured by HPLC.

3. Results and discussion

3.1. Characterization of the raw material

The carrot discards were processed using a juice extractor, resulting in a juice and a solid fraction (pulp). The carrot juice was rich in carbohydrates, with up to 33.7 g/L total sugars (glucose, 16.6 g/L; and fructose, 17.1 g/L), thus being of great interest for butanol production by ABE fermentation. The pulp fraction presented the following composition (% w/w, dry matter): glucan, 28.3; hemicellulose, 16.5 (galactan, 13.5; arabinan, 3.0); extractives, 43.1 (water extractives, 33.6; ethanol extractives, 9.5; glucose in water extractives, 1.3; galactose in water extractives, 1.3); ash, 5.0; insoluble acid lignin, 1.8; soluble acid lignin, 0.1; and protein, 1.5.

Regarding the CDP composition, a very high extractive content (43.1%) was found compared to other agro-industrial residues, such as carrot press cake (17%) [31], apple industry waste (3.1%) [32] and spent coffee grounds (12.4%) [33]. In addition, it is worth highlighting that a very low presence of lignin (insoluble and soluble) was detected in the CDP, unlike other fruit and vegetable residues: for example, carrot press cake, 6.9% [31]; apple industry waste, 23.5% [32]; pea pod waste, 21.6% [34]; and spent coffee grounds, 39.2% lignin [33]). So CDP may be enzymatically hydrolyzed
without a pretreatment process, which is very important for the profitability of the
global butanol production process from an agro-industrial residue. Moreover, CDP
contains a high total carbohydrate content, 47.5% (44.9 and 2.6% of structural and non-
structural carbohydrates, respectively), mainly glucose and galactose. Considering
hemicellulosic sugars in CDP, there is a high presence of galactose, which involves
81.8% of the total hemicellulosic sugars.

On the other hand, by comparing the composition of the CDP observed in this work
with the composition of carrot discards previously reported in the literature [26,31,35],
the residue studied in this work has a similar or relatively lower carbohydrate content, a
higher extractive content and a similar ash content. Ramos-Andrés et al. [25]
determined the composition of CDP, reporting a much higher content of water
extractives (63.9% vs 33.6%) and lignin (7.8 vs 1.9%), but a lower cellulose (10.7 vs
28.3%) and hemicellulose (8.4 vs 16.5%) content, and a similar protein content (2.3 vs
1.5%).

3.2. Sugars recovery from carrot discard pulp

3.2.1. Configuration (a): enzymatic hydrolysis of CDP

In order to enzymatically hydrolyze pulp from carrot discard, different enzymatic
hydrolysis tests were carried out (Figure 1, configuration (a)), using different types of
enzymes (Cellic CTec2, Viscozyme L, and Shearzyme, at 0.15, 0.3 and 0.3 g/g
substrate, respectively) as well as diverse mixtures (g/g substrate) of Cellic CTec2 and
Viscozyme L enzymes (C+V (0.075+0.15), C+V (0.1+0.1), C+V (0.1+0.2), and C+V
(0.15+0.3)).

First of all, regarding the use of individual enzymes (C, V and S, at 0.15, 0.3 and 0.3
g/g substrate, respectively), as can be seen in Figure 2a, the highest monosaccharides
content in enzymatic hydrolysates were achieved at 24 h of enzymatic hydrolysis when
the Viscozyme L enzyme was used, obtaining an enzymatic hydrolysate with up to 46.3
g/L of total sugars (glucose, 29.0 g/L; galactose, 13.8 g/L; and arabinose, 3.5 g/L),
corresponding to a total sugars recovery of 72.8% (Table 1). Cellic CTec2 led to an
enzymatic hydrolysate with a significantly lower sugars content, 33.6 g/L (glucose, 25.2
g/L; galactose, 7.3 g/L; and arabinose, 1.1 g/L) (52.8% total sugars recovery, Table 1).
The use of Viscozyme L led to higher recoveries of glucose, galactose and arabinose
than Cellic CTec2. In this context, different studies have also shown a high
saccharification activity for Viscozyme L when applied to different lignocellulosic
residues, such as seaweed biomasses [36], Okara (a soybean residue from soymilk and
tofu manufacture) [37] and Salix viminalis cv. Q683 (a bioenergy crop) [38]. This can
be due to the fact that Viscozyme L is a blend of β-glucanases (which hydrolyzes the
β(1,3)- and β(1,4)-linkages in β-D-glucans), pectinases, cellulases, hemicellulases and
xylanases [39,40], unlike Cellic CTec2 or Shearzyme. It is, therefore, able to
enzymatically hydrolyze both cellulosic and hemicellulosic sugars. However, in general,
a much lower monosaccharides content was attained by using the Shearzyme enzyme
(17.6 g/L of total sugars) (Figure 2a), with only 27.7% of total sugars recovery (Table
1). What is more, as shown in Figure 2a, glucose was the predominant sugar in
enzymatic hydrolysates, involving 63% and 75% for the V and C enzymes, respectively.
Furthermore, no presence of arabinose was detected when the S enzyme was used. It is
worth highlighting that S is a xylanase that mainly releases galactose from the
hemicellulosic fraction (Figure 2a).
Figure 2. Concentration of monosaccharides during enzymatic hydrolysis of CDP using (a) Cellic CTec2 (C), Viscozyme L (V) or Shearzyme (S); (b) after 24 h using mixtures of the enzymes Cellic CTec2 and Viscozyme L. Enzyme dose in brackets (g/g DM).
Table 1. Recovery of monosaccharides (%) from carrot pulp after 24 h of enzymatic hydrolysis. Comparison of different types and concentrations of enzymes. Cellic CTec2 (C), Viscozyme L (V), and Shearzyme (S). Enzyme dose in brackets (g/g DM).

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

Figure 2a also shows the time courses of the monosaccharides content on enzymatic hydrolysates obtained using individual enzymes (C, V and S, at 0.15, 0.3 and 0.3 g/g substrate, respectively). As can be seen, Viscozyme L was able to hydrolyze 90% of total monosaccharides in an enzymatic hydrolysis time as short as 4 h. Cellic CTec2 and Shearzyme enzymes were also able to rapidly hydrolyze the monosaccharides, achieving 90.3% and 82.7%, respectively, of the total monosaccharides content at 8 h of enzymatic hydrolysis. In this way, it can be said that the monosaccharides in carrot discard can be quickly released in enzymatic hydrolysis, unlike other agro-industrial residues, such as orange peel [41], brewer’s spent grain [42], lettuce residues [43], spent coffee grounds [33] or oil palm empty fruit bunch [44], where up to 48 or 72 h enzymatic hydrolysis were necessary, due to the higher lignin content.

On the other hand, considering the use of enzyme mixtures (g/g substrate) (C+V (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 hydrolysis process (Figure 2b), the highest sugars content in enzymatic hydrolysates was achieved for the mixture C+V (0.15+0.3), obtaining a total sugar concentration as high as 55.5 g/L (corresponding to a total sugars recovery of 87.2% (Table 1)).
Moreover, it is worth mentioning that this behavior was observed for both glucose and galactose sugars, while the arabinose content was similar for all the cases studied (ranging between 3.3 and 3.7 g/L). In this way, the use of the C+V (0.15+0.3) enzymes mixture allows an increase of 20% and 65% to be attained in the total sugar concentration compared to the use of individual V and C enzymes (using 0.3 and 0.15 g/g substrate, respectively) (Figure 2a). Therefore, very high sugar concentrations and recoveries can be obtained by enzymatic hydrolysis from CDP without the use of any pretreatment process, which is very important for the profitability of the global butanol production process from agro-industrial residues (for instance, CD). Previous studies have also shown that the use of a mixture of enzymes (Viscozyme L and Celluclast-1.5 L) is more effective than the single-enzyme treatment for the enzymatic hydrolysis of red seaweed (1.2 and 8.4 U/mL, respectively) [39] and Gracilaria verrucosa (4-32 U/mL) [40].

High carbohydrate recoveries (90-100%) from enzymatic hydrolysis (without pretreatment of the raw material) were also reported from apple pulp residues [45], using Pulpzyme HC and Novozym 188 enzymes (24.7 FPU and 121.7 CbU (Cellobiase Unit)/g substrate) after 5 h enzymatic hydrolysis; oil palm empty fruit bunch fiber [44], using a cocktail of Cellic CTec2 and Cellic HTec2 after 72 h enzymatic hydrolysis; and spent coffee grounds [33], using Cellic CTec2 as enzyme (15 FPU/g substrate) and 72 h of enzymatic hydrolysis. Aimaretti et al. [46], employing pulp from carrot discard, were able to attain a total sugars recovery in enzymatic hydrolysis (at 2.5 h of process) of up to 78.5%, using the Optimase CX255L enzyme (0.05% v/v), which is a thermostable xylanase. Similar glucose recoveries on enzymatic hydrolysis (of about 90%) to those achieved in this work were also reported by De Vrije et al. [47] at 42h of enzymatic hydrolysis, also using carrot pulp and GC 220 enzyme (15 mL/100 g dry raw material).
3.2.2. Configurations (b) (pretreatment) and (c) (pretreatment and enzymatic hydrolysis)

In addition to enzymatic hydrolysis, two different strategies to recover the carbohydrates contained in carrot discard pulp were a hydrothermal pretreatment (Figure 1, configuration (b)) or a sequential pretreatment and enzymatic hydrolysis of pretreated CDP slurry (Figure 1, configuration (c)), in this case using different types of single enzyme (C (0.15) and V (0.3)) as well as enzyme mixtures (C+V (0.1+0.2)). The hydrothermal pretreatment in autoclave (121 ºC) employed in this work is usually used to pretreat lignocellulosic biomass for biofuel production, such as bioethanol and biobutanol. In this way, the hydrothermal pretreatment has been successfully applied to sugarcane trash [48], grape marc [49], and carrot pomace [50] to produce bioethanol; as well as for the production of biobutanol from potato peel [20], apple pomace [21] and tomato waste [22].

Figure 3 shows the monosaccharide concentration achieved for both configurations ((b) and (c)). In this way, the carbohydrate concentration achieved in the pretreated CDP slurry (configuration (b) (20.9 g/L total sugars) was much lower than those attained in configuration (c) (42.4-55.9 g/L total sugars). Moreover, the total sugars recovery was also much higher when a sequential pretreatment and enzymatic hydrolysis process of pretreated CDP slurry (configuration c) was carried out (up to 88 vs 33% obtained for configuration (b) (Figure 3). A low glucose yield (< 20%) was also reported for carrot peelings after dilute acid pretreatment (H₂SO₄, HNO₃, or HCl) (60 ºC, 180 min and 4% acid) [35]. Procentese et al. [43] only achieved about 7 g/L of total sugars after the alkaline pretreatment (121 ºC, 30 min and 200 kg/m³ NaOH) of lettuce residues. These data show that an enzymatic hydrolysis is usually necessary after the pretreatment of vegetable waste to release the fermentable sugars.
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).

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
total sugars recovery (88%) was also obtained using enzyme mixtures (C+V (0.1+0.2)),
compared to the use of single enzymes (67% and 77% for C (0.15) and V (0.3),
respectively) (configuration c, Figure 3). Furthermore, it is worth highlighting that
glucose was the main sugar obtained in both configurations b and c (involving 61.7%
and 54.2-62.8% of total sugars, respectively). Yoon et al. [51] also reported 61.7 g total
sugars/100 g raw material after 24 h of enzymatic hydrolysis from NaOH and acetic
acid pretreated carrot pomace, using a cellulase-rich enzyme isolated from Achatina
fulica (0.25 mL/g solid).

Finally, by comparing the three configurations (Figure 1) studied in this work, the
highest total sugars concentration 55.5-55.9 g/L (87-88% total sugars recovery) was
obtained for configurations (a) (enzymatic hydrolysis, C + V (0.15+0.3) and (c)
(pretreatment followed by enzymatic hydrolysis (C + V (0.1+0.2)); while only 20.9 g/L
(33% total sugars recovery) was attained for configuration (b) (only pretreatment).
Considering the presence of inhibitors, formic acid, furfural and HMF were not detected
in the enzymatic hydrolysates in any of the experimental runs, even when the
hydrothermal pretreatment was applied. The concentration of acetic acid in the
enzymatic hydrolysates ranged from 0.7-1 g/L, this being a beneficial compound for the
subsequent ABE fermentation tests. In this way, no inhibition was observed in the
experimental runs. Therefore, in conclusion, no hydrothermal pretreatment may be
necessary for the saccharification of carrot pulp. In addition, it is worth highlighting that
the whole slurry from carrot pulp was used in this work, which is very interesting for
the profitability of the process in a biorefinery context.

Similar results (80 and 92%, respectively) for enzymatic hydrolysis were reported
from the literature using carrot peel, but in these cases the use of a hydrothermal
pretreatment (121 °C and 60 min) [52], or sequential dilute sulfuric acid (4%, 180 min)
and steam (121°C, 15 min) pretreatment [35], was necessary. Other vegetable residues also required the application of a pretreatment to obtain good saccharification yields. For instance, apple pomace (autohydrolysis, 10% solid-liquid ratio, 142.4 °C and 12 min) [21], potato peel (autohydrolysis 10% solid-liquid ratio, 140.2 °C and 56.1 min) [20], and tomato pomace (hydrothermal process, 20% solid-liquid ratio, 121 °C and 20 min) [22].

3.3. Fermentation process

3.3.1. Configuration (a): enzymatic hydrolysis

The different enzymatic hydrolysates obtained in the configuration (a) were subjected to ABE fermentation. Figure 4 shows the concentration of sugars consumed and the butanol and ABE concentrations produced.

![Figure 4. ABE fermentation of the enzymatic hydrolysates obtained from CDP (configuration (a)). Consumption of monosaccharides and concentrations of butanol and](image)

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

Enzyme dose in brackets (g/g DM).

In this way, as can be seen, except for S (0.3), high sugar concentrations (> 30 g/L) were used by C. beijerinckii in all cases, the sugars being consumed almost entirely in all the fermentation tests (sugar consumption 96.4-98.3%). On the other hand, also except for the S enzyme (0.3), high butanol (5.4-7.9 g/L) and ABE (8.4-12.3 g/L) concentrations were achieved for the different enzymatic hydrolysates; the highest butanol and ABE levels (7.9 and 12.3 g/L, respectively) being attained when the C+V (0.15+0.3) enzyme mixture was employed.

In addition, as can be appreciated in Table 2, in general, high butanol and ABE yields ($Y_{\text{BUT}}$: 0.20-0.21 g/g; $Y_{\text{ABE}}$: 0.32-0.33) and productivities ($P_{\text{BUT}}$:0.13-0.16 g/L·h; $P_{\text{ABE}}$: 0.21-0.25 g/L·h) were achieved in the fermentation of the enzymatic hydrolysates obtained using mixtures of enzymes (Table 2). Therefore, it is worth highlighting that up to 79 g butanol and 123 g ABE per kg carrot pulp could be achieved from CDP by enzymatic hydrolysis with C+V (0.15+0.3) (Table 2).

Table 2. ABE fermentation of the hydrolysates obtained from carrot pulp. Butanol and ABE yields ($Y_{\text{BUT}}$/sugars, $Y_{\text{ABE}}$/sugars expressed as g/g sugars consumed); butanol and ABE productivities (expressed as g/(L·h) at 48 h); and butanol and ABE global yields (expressed as g/kg carrot pulp (DM)). Cellic CTec2 (C), Viscozyme L (V), Shearzyme (S). Enzyme dose in brackets (g/g DM).

<table>
<thead>
<tr>
<th>Configuration</th>
<th>$Y_{\text{BUT}}$ (g/g)</th>
<th>$Y_{\text{ABE}}$ (g/g)</th>
<th>$P_{\text{BUT}}$ (g/L·h)</th>
<th>$P_{\text{ABE}}$ (g/L·h)</th>
<th>$g_{\text{BUT}}$/kg pulp (DM)</th>
<th>$g_{\text{ABE}}$/kg pulp (DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (0.15)</td>
<td>0.18</td>
<td>0.27</td>
<td>0.11</td>
<td>0.17</td>
<td>54 ± 3</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>V (0.3)</td>
<td>0.15</td>
<td>0.25</td>
<td>0.13</td>
<td>0.22</td>
<td>63 ± 2</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>S (0.3)</td>
<td>0.22</td>
<td>0.34</td>
<td>0.07</td>
<td>0.11</td>
<td>35 ± 1</td>
<td>54 ± 2</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Configuration (b)</th>
<th>Slurry</th>
<th>0.22</th>
<th>0.12</th>
<th>0.09</th>
<th>0.12</th>
<th>42 ± 3</th>
<th>58 ± 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Configuration (c)</td>
<td>Slurry + C (0.15)</td>
<td>0.22</td>
<td>0.33</td>
<td>0.15</td>
<td>0.23</td>
<td>72 ± 2</td>
<td>112 ± 4</td>
</tr>
<tr>
<td>Slurry + V (0.3)</td>
<td>0.20</td>
<td>0.31</td>
<td>0.15</td>
<td>0.23</td>
<td>71 ± 1</td>
<td>111 ± 2</td>
<td></td>
</tr>
<tr>
<td>Slurry + C (0.1) + V (0.2)</td>
<td>0.21</td>
<td>0.30</td>
<td>0.14</td>
<td>0.21</td>
<td>69 ± 1</td>
<td>101 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

By comparing the results obtained in this work in the ABE fermentation of the different enzymatic hydrolysates (configuration (a)) (Figure 4 and Table 2) with those reported in the literature for other agro-industrial residues; López-Linares et al. [33] achieved similar butanol and ABE concentrations (7.7 and 11.4 g/L, respectively), yields (0.23 and 0.34 g/g, respectively) and productivities (0.160 and 0.238 g/L-h, respectively) in the ABE fermentation of the enzymatic hydrolysate obtained from unpretreated SCG (spent coffee grounds), using also C. beijerinckii. Therefore, no pretreatment is necessary with some agro-industrial residues (i.e., CD and SCG) in order to obtain high butanol yields, which is an important advantage compared to the conventional lignocellulosic residues.

### 3.3.2. Configurations (b) (pretreatment) and (c) (pretreatment and enzymatic hydrolysis)

The slurry generated after CDP hydrothermal pretreatment (configuration (b)), as well as the different enzymatic hydrolysates produced from sequential pretreatment and enzymatic hydrolysis of pretreated CDP slurry (configuration (c)), were all fermented by C. beijerinckii.

Figure 5 shows the concentration of sugars consumed, as well as butanol and ABE concentrations obtained during the different fermentation tests. In this way, the lowest sugar concentrations consumed (19 g/L) and the lowest butanol and ABE production
(4.2 and 5.8 g/L, respectively) were achieved when CDP was only hydrothermally pretreated (configuration (b)) and directly fed to fermentation. Then, an ABE yield as low as 0.12 g/g, and butanol and ABE productivities of only 0.09 and 0.12 g/L·h, respectively, were reported in this case (Table 2). Nimbalkar et al. [34] also reported butanol and ABE productions as low as 2.31 and 4.80 g/L, respectively (ABE yield = 0.15 g/g), from hydrothermal pretreated pea pod waste slurry (121 ºC and 15 min) by *C. acetobutylicum* B 527. On the other hand, although carrot pulp has not previously been used for butanol and ABE production, Survase et al. [53] used carrot waste as a supplement for ABE solvent production (9.96 g/L ABE) by *C. acetobutylicum* DSM 792, from the spent liquor of spruce wood chips, which was fractionated by SO₂–ethanol–water, evaporation, steam stripping, liming and catalytic oxidation methods, but without any enzymatic process.
Figure 5. ABE fermentation of the hydrolysates obtained from carrot pulp after hydrothermal pretreatment (slurry, configuration (b)) and after hydrothermal pretreatment and enzymatic hydrolysis of the slurry using different enzymes (configuration (c)). Consumption of monosaccharides and concentrations of butanol and ABE (g/L) after 48 h fermentation. Cellic CTec2 (C), Viscozyme L (V). Enzyme dose in brackets (g/g DM).

Nevertheless, as can be seen in Figure 5, high sugar concentrations consumed (> 33.3 g/L) and high butanol and ABE levels (> 6.9 and 10.1 g/L, respectively) were attained when CDP was both hydrothermally pretreated and enzymatically hydrolyzed (configuration (c)). On the other hand, regarding the configuration (c), although the highest sugar content consumed (36.2 g/L) was obtained when Viscozyme L (V (0.3)) was used, the butanol and ABE concentrations were similar (butanol: 6.9-7.2; and ABE: 10.1-11.2 g/L) for the three enzymatic hydrolysates (C (0.15), V (0.3), and C+V (0.1+0.2)) (Figure 5). In this context, similar butanol and ABE yields (0.20-0.22 and 0.30-0.33 g/g, respectively), and butanol and ABE productivities (0.14-0.15 and 0.21-0.23 g/L·h), were also observed (Table 2). In this way, considering the ABE fermentation, when CDP was subjected to a pretreatment, no considerable differences between the C (0.15) and V (0.3) enzymes, or even the enzyme mixtures (C+V (0.1+0.2)), were appreciated (Figure 5 and Table 2).

By comparing the results obtained in this study for configuration (c) (sequential hydrothermal pretreatment and enzymatic hydrolysis of CDP) (Figure 5 and Table 2) with those reported in the literature for other agro-industrial residues, similar results (about 7 g/L butanol and 10 g/L ABE) to those obtained in configuration (c) were obtained after the autohydrolysis of potato peel (10% solid-liquid ratio, 140.2 °C and 56.1 min) [20] or tomato pomace (20% solid-liquid ratio, 121 °C and 20 min) [22],
enzymatic hydrolysis and fermentation using *C. saccharobutylicum* DSM 13864 or *C. beijerinckii* DSM 1820, respectively. Slightly lower ABE concentrations (8.3 g/L) and butanol yields (0.17 g/g) were achieved from the autohydrolysis of pretreated apple pomace (10% solid-liquid ratio, 142.4 °C and 12 min) followed by the enzymatic hydrolysis (15 mg Cellic CTec2/g glucan) and fermentation using *C. beijerinckii* CECT 508 [21]. Much lower butanol and ABE levels (1.1 and 1.44 g/L, respectively) were obtained from lettuce residues using *C. acetobutylicum* DSMZ 792 [43]. Although lettuce residues were pretreated under alkaline conditions and enzymatically hydrolyzed by Cellic CTec2, low sugar concentrations were released, leading to considerably lower butanol concentrations.

In short, using the configuration (c), up to 72 g butanol and 112 g ABE per kg carrot pulp could be achieved from sequential hydrothermally pretreated and enzymatically hydrolyzed CDP, using for instance, the Cellic CTec2 enzyme and an enzymatic loading of 0.15 g/g CDP (Table 2). In this context, only 26.7 g butanol and 29.4 g ABE per kg tomato pomace were obtained by *C. beijerinckii* DSM 6423 after hydrothermal pretreatment (20% solid-liquid ratio, 121 °C and 20 min) and enzymatic hydrolysis [22]. López-Linares et al. [54] was also able to recover up to 81 kg butanol and 126 kg ABE per ton of SCG, which was pretreated by microwave dilute sulfuric acid and enzymatically hydrolyzed.

By comparing the three configurations analyzed in this work, it is worth highlighting that the highest butanol and ABE productions (79 g butanol and 123 g ABE per kg carrot pulp) were attained for the configuration (a), where CDP was only enzymatically hydrolyzed (C+V (0.15+0.3)). Therefore, the results reported in this work show that no hydrothermal pretreatment would be necessary for carrot pulp, unlike other agro-industrial residues, such as brewer’s spent grain [27], where a microwave-assisted dilute
acid pretreatment was required due to their higher lignin content. This fact is of great interest, since it has been reported from the literature that, in general, a pretreatment stage involves up to 30% of the total cost of the biofuel production process from lignocellulosic residues [55].

Furthermore, the CDJ obtained from CD was also subjected to ABE fermentation, 96% of the sugars content being consumed, and resulting in 6.35 and 9.04 g/L butanol and ABE, respectively. This allows the use of all the carrot discards for ABE fermentation, thus proving highly interesting in a biorefinery context.

4. Conclusions

Different strategies based on hydrothermal pretreatment and/or enzymatic hydrolysis were evaluated for efficient biobutanol production from carrot discards by C. beijerinckii. Saccharification times were considerably shorter (about 6 h) compared to lignocellulosic residues with a higher lignin content. Global yields of 79 g butanol and 123 g ABE/kg carrot discard pulp were achieved after enzymatic hydrolysis using a blend of enzymes, Cellic CTec2 and Viscozyme, at 0.15 and 0.3 g/g substrate. The results demonstrate that the pretreatment step is not necessary to achieve a proper saccharification of the residue, which is very interesting for the economic profitability of the process. Furthermore, the carrot juice can also be used for butanol production without the presence of inhibition, which is very interesting in a biorefinery context.

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References


[7] K. Kumar, S. Srivastav, V.S. Sharanagat, Ultrasound assisted extraction (UAE)


[28] A. Sluiter, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of

[29] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton,
Determination of ash in biomass. National Renewable Energy Laboratory,

Determination of structural carbohydrates and lignin in biomass. National
(2011).

Claassen, E.G. Koukios, Technical suitability mapping of feedstocks for

LeBihan, G. Buelna, M. Verma, C.R. Soccol, Hydrolytic pre-treatment methods
for enhanced biobutanol production from agro-industrial wastes, Bioresour.

production by acetone-butanol-ethanol fermentation from spent coffee grounds
with microwave assisted dilute sulfuric acid pretreatment, Bioresour. Technol.

[34] P.R. Nimbalkar, M.A. Khedkar, P. V. Chavan, S.B. Bankar, Biobutanol
production using pea pod waste as substrate: Impact of drying on saccharification

[35] A. Li, B. Antizar-Ladislao, M. Khraisheh, Bioconversion of municipal solid


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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: