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Brewer's spent grain as a no-cost substrate for polyhydroxyalkanoates production: Assessment of pretreatment strategies and different bacterial strains

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are polyesters of significant interest due to their biodegradability and properties similar to petroleum-derived plastics, as well as the fact that they can be produced from renewable sources such as by-product streams. In this study, brewer's spent grain (BSG), the main by-product of the brewing industry, was subjected to a set of physicochemical pretreatments and their effect on the release of reducing sugars (RS) was evaluated. The RS obtained were used as a substrate for further PHA production in *Burkholderia cepacia, Bacillus cereus,* and *Cupriavidus necator* in liquid cultures. Although some pretreatments proved efficient in releasing RS (acid-thermal pretreatment up to 42.1 gRS L^{-1} and 0.77 gRS g^{-1} dried BSG), the generation of inhibitors in such scenarios likely affected PHA production compared with the process run without pretreatment (direct enzymatic hydrolysis of BSG). Thus, the maximum PHA accumulation from BSG hydrolysates was found in the reference case with 0.31 ± 0.02 g PHA per g cell dried weight, corresponding to 1.13 ± 0.06 g L^{-1} and a PHA yield of 23 ± 1 mg g^{-1} BSG. It was also found that *C. necator* presented the highest PHA accumulation of the tested strains followed closely by *B. cepacia*, reaching their maxima at 48 h. Although BSG has been used as a source for other bioproducts, these results show the potential of this by-product as a no-cost raw material for producing PHAs in a waste valorization and circular economy scheme.

Introduction

Petroleum-derived plastics are used in numerous applications in modern society, becoming indispensable to our daily life. Their production has reached approximately 322 M tons per year [1], but their disposal poses significant environmental issues due to their limited recycling rate and nonbiodegradability [2]. Thus, the search for more sustainable and biodegradable substitutes for these plastics is of growing concern. Polyhydroxyalkanoates (PHAs) are among the most studied bio-based plastic substitutes. Generally, the most widespread and best studied member of the PHA family is the homopolymer 3-hydroxybutyrate, poly(3-hydroxybutyrate) (P3HB) [3]. PHAs are polyesters produced by numerous microorganisms that use them as a carbon source and energy storage, and that can be biodegraded to CO_2 and H_2O [4,5].

Along with their biocompatibility, biodegradability and green credentials, PHAs resemble most of the properties of petroleum-derived plastics. Such traits make them attractive for multiple purposes, including specialized applications in the medical, pharmaceutical and energy sectors. [6,7]. PHAs are intracellular products derived from the secondary metabolism of several prokaryotes (over 300 species, eubacteria, and archaea) [8,9]. Their synthesis is typically affected by different aspects such as the type and concentration of the carbon source, temperature, nutrient availability, pH or electron acceptor availability [8]. However, their synthesis occurs under unfavorable

Abbreviations: BSG, brewer's spent grain; CDW, cell dry weight; OXC, oxidizable carbon; PHA, polyhydroxyalkanoates; P3HB, poly-3-hydroxybutyrate; RS, reducing sugars; SmF, submerged fermentation; TKN, total Kjeldahl nitrogen; WHC, water holding capacity.

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growth conditions inducing environmental stress of at least one essential nutrient for growth other than the carbon source (*e.g.* nitrogen, phosphorus or oxygen limitation) [6,7].

Among the bioprocesses used to obtain PHAs, the most developed are those performed in submerged fermentation systems (SmF) [9]. Although significant efforts have been made to enhance their production via SmF, the current trend is the development of efficient bioprocesses capable of reducing the fermentation costs [10,11]. In general, a significant fraction of the production cost (between 30 and 50 %) can be attributed to substrate acquisition. Such a situation implies that using costly raw materials like glucose, sucrose or starch, becomes less attractive (1.3 $ kg^{-1} P3HB using glucose as carbon source compared to 0.34$ kg^{-1} P3HB using hemicellulose hydrolysate) [3,12,13]. Hence, this factor has served as a driving force to explore more economical substrates as alternative sources for PHA production, including by-product streams such as cheese whey [14], biodiesel by-products [15], brewery wastewater [16] or activated sludge from municipal wastewater [17]. Similarly, the use of no-cost solid substrates as the source of fermentable sugars to produce PHAs have been assessed with different lignocellulosic-based waste, including spruce sawdust [4], sugar maple [5], sugarcane bagasse [18] and waste office paper [19]. Consequently, several large companies have started considering such streams as raw materials for other processes instead of referring to them as 'wastes' [20].

One of the lignocellulosic materials with higher potential for biotechnological applications is brewer's spent grain (BSG) [21,22]. BSG represents up to 85 % of the waste generated in the beer industry [21,23], comprising mainly lignin, lipids, polysaccharides (cellulose and hemicellulose) and proteins. However, to access the polysaccharide fractions entrapped within the lignin matrix and to release the desirable fermentable sugars, a suitable pretreatment is often required [22] and generally, after the disruption of the complex structure of lignocellulose, enzymatic hydrolysis can be conducted [24]. Although hydrolysis mainly produces fermentable sugars, the nature of each pretreatment could induce inhibitory compounds that can affect the fermentability of the hydrolysates and therefore the efficiency of the fermentation stage [25]. The methods most frequently used to pretreat lignocellulosic-derived materials include acid or alkaline methods, steam explosion, oxidative methods using strong oxidants such as H_2O_2, chemical pulping processes, hydrothermal processing or the use of alternative solvents to dissolve the lignocellulosic components [25,26]. Although BSG has been used previously as a substrate for different applications [27,28], and some pretreatments have been tested to obtain fermentable sugars starting from this by-product [24,29,30], there are no reports of its use as a source for PHA production. Thus, it is crucial to determine the effects of different BSG pretreatments, not only on the release of fermentable sugars, but also on the desired PHA accumulation.

This study proposes a valorization strategy to recover value-added PHAs through the use of BSG as a sustainable raw material. The release efficiency of fermentable sugars from BSG through a selected set of pretreatments (thermal, acid-thermal, alkaline-thermal and microwave-assisted alkaline) was assessed and the released sugars were used as the source for PHA production via SmF. In the latter stage, some of the bacterial strains that have demonstrated acceptable performance in the accumulation of PHAs using similar substrates were analyzed. While *Burkholderia cepacia* was selected as the model strain thanks to its ability to utilize different sugar sources (including those present in lignocellulose materials) and its tolerance towards different inhibitory compounds [31–33], other characteristic PHA-producing bacteria, namely *Bacillus cereus* and *Cupriavidus necator* were also assessed [47]. As far as we are aware, this is the first report using BSG as a raw material for PHA production in a valorization scheme.

Material and methods

Microorganisms and inoculum preparation

B. cepacia (CCM 2656) was from the Czech Collection of Microorganisms, Brno, Czech Republic. *B. cereus* (DSM 31), and *C. necator* (DSM 428) were obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) and all were stored at −80 °C preserved with glycerol (20 % v/v). Inoculum preparation for each strain consisted of adding 500 µL of the preserved strain into a 100 mL Erlenmeyer containing 50 mL of LB media (Lysogeny Broth), (PanReac Applichem, Madrid, Spain), and then placing in an orbital shaker at 30 °C and 120 rpm for 24 h. All reagents and materials were previously sterilized at 121 °C for 15 min.

Substrate

BSG was kindly provided by Companyia Cervesera del Montseny (Catalunya, Spain). It was dried at 60 °C for 48 h and stored at room temperature (RT) until used.

Pretreatments and hydrolysis experiments

Thermal pretreatment (TP)

TP consisted of mixing 9 g of dried BSG with distilled water (5% BSG w/v) in a 250 mL Erlenmeyer flask and autoclaved at 121 °C for 15 min [35]. After cooling, the mixture was used to perform the enzymatic hydrolysis.

Acid-thermal pretreatment (A)

The acid-thermal pretreatment consisted of mixing 9 g of dried BSG (5% w/v) with diluted H_2SO_4 (1%, 2% and 3% v/v) in 250 mL Erlenmeyer flasks [24] and autoclaved at 121 °C for 15 min. After cooling, the mixture was used to perform either the enzymatic hydrolysis or diverse detoxification strategies.

Three strategies were applied to reduce the content of inhibitors produced during the pretreatment. First, a washing stage (A + W) [29] consisting of adding 40 mL of distilled water per g initial dried BSG to flush the solid substrate. After a short mixing, 20 mL of distilled water per g initial dried BSG were used to carry out the enzymatic hydrolysis. Alternatively, after enzymatic hydrolysis of the pretreated material, a detoxification process was conducted using over-liming (OL) [36] by adjusting the pH to 10 using $Ca(OH)_2$, and then placing the flasks at 50 °C in a water bath for 30 min. Finally, a detoxification including Active Charcoal (AC) and Amberlite XAD4 (AM) was tested by adding 5 g of these adsorption materials into 100 mL of hydrolyzed samples in 250 mL Erlenmeyer flasks [5] and shaking at RT for 24 h at 120 rpm. After detoxification, mixtures were filtered through a 0.25 mm membrane (Merck Millipore, Darmstadt, Germany), and filtrates were used for PHA production.

Alkaline-thermal pretreatment (B)

This consisted of mixing 9 g of dried BSG with 1% (w/v) NaOH solution using 5% (w/v) of BSG in 250 mL Erlenmeyer flasks [24] and autoclaving at 121 °C for 15 min. After cooling, the mixture was used for the enzymatic hydrolysis.

Microwave-assisted alkaline pretreatment (MAA)

MAA pretreatment was carried out with 9 g of dried BSG 5% (w/v) in NaOH 1% (w/v). The mixture was heated in a Rommer:721 Microwave (Rommer, Terrassa, Spain) at power levels 231 W/10 min, 385 W/10 min, and 539 W/1.5 min [24]. After cooling, the mixtures were used for the enzymatic hydrolysis.

Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated BSG was carried out by adjusting

the pH of the solid-liquid mixture to 5 with 5 N NaOH [4]. 0.2 mL of Viscozyme L g^{-1} dried BSG (an enzymatic mixture of *Aspergillus sp*. of cellulases, xylanases and arabinases, (Sigma Aldrich, Darmstadt, Germany; cat. no. V2010)) was added. The Erlenmeyer containing the mixture was placed in an orbital shaker at 145 rpm and 45 °C for 24 h. After hydrolysis, the liquid culture was filtered through a 0.25 mm membrane (Merck Millipore), and the liquid fraction used to produce PHAs via SmF. As a reference condition for the pretreatments, a solid-liquid extraction (9 g of the dried BSG and distilled water (5% BSG: w/v)) was conducted in 250 mL Erlenmeyer flasks (no pretreatment (NP)). The mixture was shaken at 120 rpm and 25 °C for 30 min, and processed by enzymatic hydrolysis as described above.

Submerged fermentation for PHA production

Extracts obtained after each pretreatment and the subsequent enzymatic hydrolysis served as substrates to produce PHAs via SmF. 100 mL of the extracts were placed in 250 mL Erlenmeyer flasks, and were supplemented by addition of two mineral media [4]. The first was composed of $(NH_4)_2SO_4$ 1 g L^{-1}, $Na_2HPO_4 \cdot 12H_2O$ 9.02 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.1 g L^{-1}, $C_6H_8FeNO_7$ 0.06 g L^{-1}, and $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}. The second (Trace Element Solution, 1 mL L^{-1}) comprised: $ZnSO_4 \cdot 7H_2O$ 0.1 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.03 g L^{-1}, H_3BO_3 0.3 g L^{-1}, $CoCl_2 \cdot 6H_2O$ 0.2 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.02 g L^{-1}, $NiCl_2 \cdot 6\ H_2O$ 0.02 g L^{-1}, and $NaMoO_4 \cdot 2H_2O$ 0.03 g L^{-1}. Finally, the pH of the media was adjusted to 7 using 5 N NaOH. The prepared substrate and materials were sterilized at 121 °C for 15 min. After cooling, the liquid substrate was inoculated with 5% (v/v) of the corresponding strain. Fermentation was conducted at 30 °C and 120 rpm in an orbital shaker assuring aerobic conditions. The system was monitored for up to 72 h, as suggested by [4].

Analytical methods

Solid substrate characterization

BSG was characterized using standard procedures [37] for total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), ammonia ($NH4^+$), oxidizable carbon (OXC) and water holding capacity (WHC) [38]. Cellulose, hemicellulose and lignin content were determined by gravimetrically [39]. The different hemicellulosic fractions (arabinan and xylan) were taken from literature [40].

Reducing sugar (RS) quantification

RS content of the liquid substrates was quantified by the dinitrosalicylic acid (DNS) method as described by [41]. Briefly, 0.5 mL of sample was mixed with 0.5 mL of DNS reagent and heated at 70 °C for 10 min; 10 mL of distilled water were added, and absorbance measured at 540 nm. Glucose was used as the standard for the calibration curve.

PHA extraction and quantification

The cell biomass produced through the fermentation was quantified gravimetrically by centrifuging 10 mL of the fermented sample at 7000 RCF for 5 min. The cell biomass was washed with distilled water and centrifuged again at 7000 RCF for 5 min. While the supernatant was collected and used to determine the RS content, the biomass was dried at 60 °C (for 48 h) and weighed for the cell dry weight (CDW) contained in the sample. The dried pellet was used to determine PHA content after extraction using a modified method [4]. Briefly, 8−12 mg of the dried pellet was mixed in a 2 mL vial with 1 mL of chloroform and 0.8 mL of methanol-sulfuric solution (used for PHA extraction and derivatization). Benzoic acid (0.2 *g* L^{-1}) (Sigma Aldrich, Darmstadt, Germany) was also added as the internal standard. Vials were adequately sealed and placed in a thermostatic bloc at 94 °C for 3 h. After cooling, the content was transferred into 4 mL vials and mixed with 0.5 mL of 0.05 N NaOH through inversion. After 5 min, phase separation was completed. The lower organic phase was used to determine the methyl-esters monomers of PHAs contained in the extract. PHA quantification was performed by gas chromatography with flame ionization detector GC-FID. The GC system (Agilent 7820A, California, USA) consisted of a,FID with an HP-Innowax column (30mx0.53mmx1 μm). The injection port was set at 250 °C in splitless mode, and the column temperature was initially set at 70 °C for 2 min, then temperature increased to 190 °C at 10 °C min^{-1} and held for 7 min. The detector temperature was set at 300 °C. Identification and quantification were performed using calibration curves (internal standard) by comparing retention times of analytical grade standards (Poly-3-hydroxybutyrate (P3HB)-co-3-hydroxyvalerate (P3 HV) (Sigma-Aldrich, Darmstadt, Germany) P(3HB-co-3 HV) 12 % mol P3 HV processed using the same conditions than the samples.

Inhibitory compound determination

Inhibitory compounds in the liquid hydrolysates were determined by high-performance liquid chromatography (HPLC) using a method modified from [42]. Briefly, the HPLC consisted of an Agilent 1920 Infinity UHPLC equipped with a UV–vis Diode Array Detector G4212A and a Nucleosil $120C_{18}$ (3μmx125 mm x 4 mm) column. The column temperature was set at 30 °C. The mobile phase (1 mL min^{-1}) was a mixture of 0.05 % H_3PO_4 and acetonitrile:H_2O (90:10). The gradient started with 100 % 0.05 % H_3PO_4, and increased gradually until 100 % acetonitrile: H_2O after 38 min, and then was held for 10 min. Quantification was performed by comparing samples from the analytical standards of the selected inhibitory compounds under the same conditions by using external standard calibrations.

Statistical analysis

Statistical differences of the pretreatments were analyzed using one-way ANOVA ($p < 0.05$) with the Tukey test. Experiments were conducted in triplicate, and the reported values are presented as mean ± standard deviation (SD). SPSS version 21 was used to analyze the data.

Results and discussion

BSG characterization

As can be seen in Table 1, BSG is a by-product with approximately 77 % moisture content and 90 % volatile solids. It has a relatively high WHC and its physical appearance is that of moistened barley. However, the potential for biotechnological application lies in its high cellulose and hemicellulose content (> 60 % of the dry matter), low lignin content (8% of the dry matter), and the presence of significant levels of macro- and micronutrients that can support microorganism growth [21]. Although some reports [43] suggest that BSG could possess higher lignin content, some authors [21,44,45] have found similar lignin content to those reported here. It can be stated that BSG composition depends on

Table 1
Physicochemical characterization of the brewer's spent grain used.

Characteristic	Value
Total solids (g kg^{-1})	230 ± 22
Volatile solids (g kg^{-1})	206 ± 0
WHC (dry basis) (g g^{-1})	5.9 ± 1.0
TKN (g kg^{-1}) (dry basis)	30.4 ± 4.3
Ammoniacal nitrogen (g kg^{-1}) (dry basis)	3.6 ± 0.4
OXC (g kg^{-1}) (dry basis)	585 ± 92
Cellulose (g kg^{-1}) (dry basis)	220 ± 10
Hemicelluloses (g kg^{-1}) (dry basis)	400 ± 11
Xylan hemicellulose fraction (dry basis)	0.28*± 0.00
Arabinan hemicellulose fraction (dry basis)	0.12*± 0.00
Lignin (g kg^{-1}) (dry basis)	80.1 ± 9.6
C/N ratio	19.2

Values are presented as mean value ± standard deviation. *Data taken from [40].

the specific processing used in the brewery. Since these industrial processes could include different malting and mashing conditions, as well as the addition of diverse adjuncts to prepare the wort, the resulting BSG would present significant variations depending on such factors [44].

Pretreatment assessment

The initial experiments consisted of testing each pretreatment for its ability to render the cellulose and hemicellulose fraction of the BSG more available. RS release after pretreatment/enzymatic hydrolysis was used as the efficiency index in each case. In general, after pretreatment, RS content was not appreciably high, and varied between 0.36 and 1.63 g L^{-1} for the conditions evaluated. However, once the enzymatic hydrolysis step was completed, the RS increased significantly, as expected, reaching the values reported in Fig. 1, where it is seen that the RS content varied between 11.7 and 42.1 g L^{-1}. The maximum RS release, in the range 39.5–42.1 g RS L^{-1}, was consistently achieved from hydrolysates with acid-thermal pretreatments. It can also be seen that such pretreatments released up to 61 % more RS than the reference without pretreatment (26.2 ± 0.6 g RS L^{-1}). Fig. 1 also shows that applying detoxification steps after acid pretreatment was not efficient in keeping the RS at the original levels. As detailed, A + W reduced the RS to 12.0 ± 0.3 g RS L^{-1}, suggesting that apart from potential inhibitory compounds, some sugars were washed away during the process. Similarly, adsorbent materials such as activated charcoal (AC) and amberlite (AM) promoted RS reduction by almost 42 %, but using overliming (OL) such reduction reached 14 %. Nevertheless, in this case, the RS content was still 38.1 % higher than that the reference control without pretreatment.

The results shown in Fig. 1 are in line with others' findings, suggesting that the most effective pretreatments to obtain fermentable sugars use acids [24,25]. However, the presence of potential inhibitors in the hydrolysates remains a challenge for these pretreatments [25]. Regarding the thermal pretreatment (Fig. 1), the final RS levels were identical to those using the enzymatic step alone (p 1.00). As suggested [46], thermal pretreatments tend to be less efficient than other methods, requiring high temperatures and exposures times to reach equivalent RS levels to acid-thermal pretreatments, where the added acids act as a catalyst. Similar behavior is found in alkaline-thermal pretreatments, where the catalytic activity is achieved with a strong base such as NaOH. Here, it was found that by using such a pretreatment, the RS increased 36.8 % compared to the reference (Fig. 1), even though it was less efficient than using H_2SO_4 (15 % lower), considering that alkalis usually lead to lower solubilization of hemicelluloses than strong acids [25]. Finally, MAA pretreatment was found to have an intermediate performance (26.0–31.8 g RS L^{-1}) compared to the others. Although some authors [24] suggest that MAA pretreatment could yield high RS levels starting from BSG, here the maximum RS content using MAA was 31.8 ± 5.2 g L^{-1} with 385 W, which was 21.7 % higher than the reference without pretreatment, but 24.5 % lower compared to the acid-thermal treatment, which gave the best RS yield.

Although it was expected that the direct hydrolysis of BSG (NP) would produce negligible RS, the levels obtained were comparable to those reached in some of the evaluated pretreatments. The results could be explained by the lignin content of the raw material (Table 1), which may be low enough to allow the added enzymes to act on the cellulose and hemicellulose fractions without significant adverse effects. Moreover, it must be kept in mind that lignin content in BSG could also be affected by the malting and mashing conditions applied in the brewery process [47], which could explain why processing BSG without pretreatment appeared suitable for direct enzymatic hydrolysis. In addition, these results could be explained by the fact that BSG is prone to contain residual starch (from brewery processing) that remains unreleased from the BSG matrix. For instance, others [47] have found that BSG could contain starch in the range 16−130 mg g^{-1} dry BSG, and [45] suggested that residual starch could be up to 5% (w/w). It was proposed that residual starch remains attached to the BSG matrix, and that once physicochemical pretreatments temperatures (around 121 °C) are applied, starch is dissolved and can be broken down [45].

The maximum RS content released using the acid-thermal pretreatment corresponded to a yield of 0.77 g RS g^{-1} dried BSG, which was higher than that obtained in previous studies such as in [24] (0.23 g RS g^{-1} dried BSG) or [30] (0.36 g RS g^{-1} dried BSG) using MAA treatment with HCl.

PHA production via submerged fermentation

After pretreatments, the set of hydrolysates were used as substrates to test the ability of *B. cepacia* to produce PHAs via SmF. It was found that the only type of PHA produced after fermentation was P3HB. Fig. 2

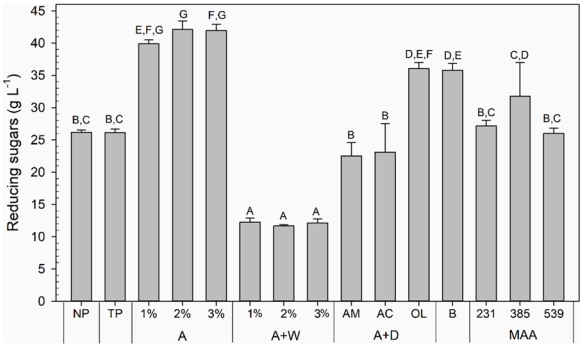

Fig. 1. Final reducing sugar content of brewer's spent grain hydrolysates. NP: no pretreatment; TP: thermal pretreatment; A: acid-thermal pretreatment; A + W: acid-thermal pretreatment with wash; A + D: acid-thermal pretreatment with detoxification; 1, 2, 3% (1, 2, 3% v/v H_2SO_4); AM: amberlite; AC: active charcoal; OL: overliming; B: alkaline-thermal pretreatment 1% (w/v) NaOH; MAA: microwave-assisted alkaline pretreatment; 231: 231 W power, 385 W power and 539 W power. Different capital letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

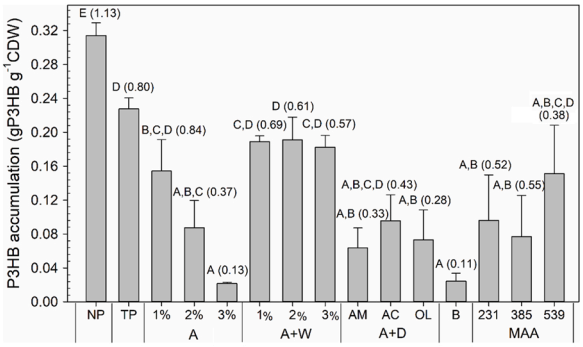

Fig. 2. Poly-3-hydroxybutyrate (P3HB) accumulation after 72 h fermentation using *B. cepacia* and brewer's spent grain hydrolysates. NP: no pretreatment; TP: thermal pretreatment; A: acid-thermal pretreatment; A + W: acid-thermal pretreatment with wash; A + D: acid-thermal pretreatment with detoxification; 1, 2, 3% (1, 2, 3% v/v H_2SO_4); AM: amberlite; AC: active charcoal; OL: overliming; B: alkaline-thermal pretreatment 1% (w/v) NaOH; MAA: microwave-assisted alkaline pretreatment; 231: 231 W power, 385 W power and 539 W power. CDW: cell dry weight. Values in brackets correspond to the P3HB content in the liquid culture (g L^{-1}). Different capital letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

summarizes P3HB accumulation (g P3HB g^{-1} CDW) and P3HB production (g P3HB L^{-1}) after 72 h of fermentation on the assessed hydrolysates. The maximum accumulation was reached in the reference without pretreatment (0.31 ± 0.02 g P3HB g^{-1} CDW), significantly higher than any of the tested pretreatments, independent of initial RS availability. Accumulation decreased by 27.5 % in TP and was between 0.06−0.19 g P3HB g^{-1} CDW in the acid-thermal pretreatments. However, among the latter, the best result was reached when using a washing step. On the other hand, B and MAA resulted in P3HB accumulations <0.16 P3HB g^{-1} CDW. Fig. 2 also shows that P3HB concentration in the liquid culture followed a similar trend to that of accumulation, reaching a maximum of 1.13 g P3HB L^{-1} in the reference condition.

In general, these results suggest that there was no direct relationship between the RS released in each pretreatment and the P3HB produced from those sugars. As previously discussed, when a lignocellulose material is subjected to physicochemical pretreatments, some inhibitors are likely to be generated, which could affect the fermentability of the released sugars [25]. Table 2 shows that although acid-thermal pretreatment (1% v/v H_2SO_4) promoted the highest RS yield, those sugars were not completely exploited for P3HB production, reaching a maximum sugars transformation of 22.2 ± 0.1 mg P3HB g^{-1} RS. In contrast, the reference condition with an RS yield of 0.52 g RS g^{-1} dried BSG, produced the highest P3HB levels, with a sugar transformation of 44.2 ± 1.9 mg P3HB g^{-1} RS. Similarly, the different hydrolysate detoxification strategies resulted in low P3HB accumulation. Despite the high RS yield of some pretreatments, the transformation of such sugars only reached from 7.9 to 14.2 mg P3HB g^{-1} RS. Such a trend changed significantly when a washing step was applied. The transformation of sugars (56.8 ± 0.5 mg P3HB g^{-1} RS) was the highest from the acid-thermal pretreatments leading to a significant PHA accumulation (0.19 g P3HB g^{-1} CDW) despite the drop in RS yield.

Table 3 shows that the previous results are in line with the presence of potential inhibitors in the acid-thermal pretreatments. It was found that the concentration of some of these compounds (acetic acid, furfuraldehyde, and 5-HMF) was particularly high. Others, such as [32], have suggested that concentrations of > 0.1 g L^{-1} of furfuraldehyde, could negatively influence PHA accumulation (% of CDW) reducing the PHA content by up to 20 % compared to control levels.

From Fig. 2, it was evident that the higher the H_2SO_4 addition, the lower the P3HB accumulation, in agreement with the higher probability of producing more furfurals and consequently lower PHA production [48]. Although washing and detoxification approaches have induced a loss of RS, P3HB accumulation was higher in those cases using washing compared to the acid-thermal and acid-thermal with detoxification treatments, suggesting a particular efficiency in removing potential inhibitors that affect PHA production. Furthermore, it was remarkable that despite the relatively low sugar consumption (Fig. 3) in these conditions (A + W), P3HB accumulation reached up to 19.1 % of the CDW (Fig. 2). Such differences suggest that it could be better to use a non-selective strategy (*e.g.* washing with abundant water) than to apply particular adsorbent materials capable of retaining some of the potential inhibitors, even though it resulted in higher RS losses. As some others suggest [4,18], PHA production in liquid cultures does not require a high initial sugar content, as the cultures are capable of reaching acceptable levels when starting in the range 10−20 g L^{-1}, in agreement with the levels found in procedures with a washing step.

On the other hand, for the alkaline and microwave-assisted alkaline pretreatments, it was found that the P3HB accumulation ranged from 3 to 17% of the CDW. In these cases, sugar consumption reached between 10.3–21.9 g L^{-1}, suggesting that the presence of inhibitors could also interfere with the P3HB production. Hence, from Table 3, it can be seen that different phenolic compounds (vanillic acid, syringic acid, vanillin, syringaldehyde, and coumaric acid) were present in significant amounts when using these pretreatments. As some authors point out [4], the

Table 2
Pretreatment effect on sugar release and PHA production using *B. cepacia.*

Strategy	RS yield (g RS g^{-1} BSG)	P3HB yield (mg P3HB g^{-1} BSG)	Transformation of sugars (mg P3HB g^{-1} RS)
NP	0.52 ± 0.00	23.0 ± 1.0	44.2 ± 1.9
A 1%	0.77 ± 0.01	17.1 ± 0.3	22.2 ± 0.1
A + W 1%	0.25 ± 0.01	14.2 ± 0.7	56.8 ± 0.5
A-AM 1%	0.45 ± 0.04	6.4 ± 0.2	14.2 ± 0.7
A-AC 1%	0.46 ± 0.08	8.8 ± 3.6	19.1 ± 3.8
A-OL 1%	0.72 ± 0.01	5.7 ± 3.5	7.9 ± 4.6

Values presented as the mean value ± the standard deviation. BSG: Brewer's spent grain; RS: reducing sugars; NP: no pretreatment; A 1%: acid-thermal pretreatment (1% v/v H_2SO_4); A + W: acid-thermal pretreatment with wash; A-AM: acid-thermal pretreatment + detoxification with amberlite; A-AC: acid-thermal pretreatment + detoxification with: active charcoal; A-OL: acid-thermal pretreatment + detoxification with overliming.

Table 3
Potential inhibitory compound content (mg L^{-1}) derived from different pretreatments on brewer's spent grain.

Inhibitory compounds	Pretreatments				
	A-3%	NP	TP	385	B
Formic acid	<50	<50	<50	<50	<50
Acetic acid	354 ± 11	150 ± 1	85 ± 2	<50	<50
Levulinic acid	<100	<100	<100	<100	<100
Furfuryl alcohol	<0.5	<0.5	0.5 ± 0.0	<0.5	<0.5
5-HMF	64 ± 0	0.5 ± 0.0	1.2 ± 0.0	<0.2	<0.2
Furfuraldehyde	160 ± 1	4.9 ± 0.1	6.4 ± 0.0	5.4 ± 0.2	2.1 ± 0.1
Vanillic acid	0.7 ± 0.1	<0.5	<0.5	3.0 ± 0.2	2.6 ± 0.1
Syringic acid	<0.5	<0.5	<0.5	1.1 ± 0.1	1.8 ± 0.0
Vanillin	1.1 ± 0.0	<0.5	<0.5	3.6 ± 0.1	4.5 ± 0.4
Syringaldehyde	<0.2	<0.2	<0.2	0.2 ± 0.0	1.5 ± 0.2
Cinnamic acid	<0.5	<0.5	<0.5	<0.5	<0.5
Coumaric acid	3.5 ± 0.0	0.9 ± 0.0	<0.4	38 ± 0	35 ± 0

Values presented as the mean value ± the standard deviation. "<" means below the detection limit of the method. A3: Acid-thermal pretreatment (3% v/v H_2SO_4); NP: No pretreatment; TP: thermal pretreatment; 385; B: alkaline pretreatment.

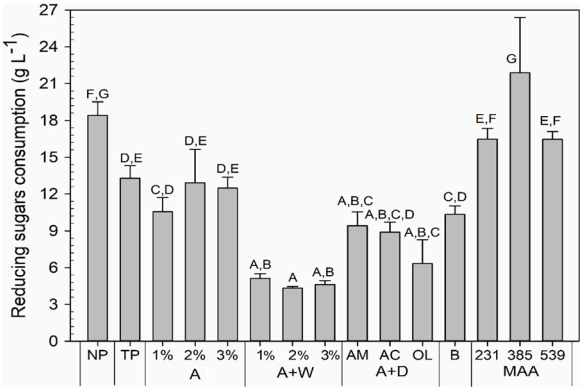

Fig. 3. Consumption of reducing sugars (72 h) of *B. cepacia* in different brewer's spent grain hydrolysates. NP: no pretreatment; TP: thermal pretreatment; A: acid-thermal pretreatment; A + W: acid-thermal pretreatment with wash; A + D: acid-thermal pretreatment with detoxification; 1, 2, 3% (1, 2, 3% v/v H_2SO_4); AM: amberlite; AC: active charcoal; OL: overliming; B: alkaline-thermal pretreatment 1% (w/v) NaOH; MAA: microwave-assisted alkaline pretreatment; 231: 231 W power, 385 W power and 539 W power. Different capital letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

influence of phenolic compounds in the P3HB synthesis could be significant, such that reducing the total phenol content from 1 to 0.02 g L^{-1} can induce a P3HB increase from 9.8%–74.7% CDW.

With the thermal pretreatment, although the initial sugar concentrations were similar to the reference (Fig. 1), significant amounts of furfuraldehyde and furfuryl alcohol were found. These compounds have been previously reported to affect enzymatic activities, preventing cell replication [49]. However, although the concentration of acetic acid found in the hydrolysate without pretreatment was higher than that after the thermal pretreatment, it was innocuous for the P3HB production. Acetic acid in low concentrations could be used as an alternative carbon source for some bacteria such as *B. cepacia* [5].

Based on these results, it can be concluded that the evaluated pretreatments were more effective in releasing RS compared to no pretreatment. Their main drawback was the significant presence of potential inhibitors, which probably limited the fermentation step. Although some detoxification approaches were tested, their particular limitations induced lower PHA production as well. Thus, the maximum P3HB accumulation achieved was in the reference case without pretreatment, corresponding to a production of 1.13 ± 0.06 g P3HB L^{-1} and a yield of 23 ± 1 mg P3HB g^{-1} dried BSG. Compared to other processes using alternative raw materials, the maximum production obtained here was a 7.1 % increase over that obtained by [4], not only without pretreatment but without detoxification with activated charcoal or overliming; however, it was 35 % lower than that achieved with *B. cepacia* and the use of a culture media supplemented with glucose [34].

Future developments using BSG could be focused on finding pretreatments able to release significant fermentable sugars while lowering inhibitory content. The efficiency of other inhibitor-resistant PHA-producing microorganisms could also be evaluated. These results shine a new light on the use of BSG as a raw material; thus far, BSG has been used as a source of energy (through incineration), as animal feed [44], as a substrate for anaerobic digestion to produce biogas [50], and as a raw material in pyrolysis processes [51]. A valorization strategy to obtain high value-added products from this by-product has not yet been proposed.

PHA production from BSG with other bacterial strains

In these experiments, the optimal conditions found using *B. cepacia* (NP) were replicated with two other PHA-producing bacterial strains (*B. cereus* and *C. necator)* that have presented PHA production capabilities [52]. Fermentation was monitored for 72 h, while also taking samples at 24 h and 48 h. The experiments were conducted using a second batch of BSG (from a different type of beer from that used in the previous experiments). Fig. 4(a) shows the P3HB accumulation obtained for the three strains tested. The maximum was reached after 48 h by *C. necator* followed closely by *B. cepacia* (p 1.00), reaching values around 0.17 g P3HB g^{-1} CDW for both and suggesting that *C. necator* and *B. cepacia* can reach their maximum at 48 h (this maximum is also maintained after 72 h). Both demonstrated high potential for the processing of similar hydrolysates, in line with previous reports [4,53]. In contrast, *B. cereus* only reached a third of the P3HB accumulation obtained with the other strains tested, suggesting that it was unable to use the available sugars to produce PHAs efficiently. As Fig. 4(b) illustrates, fermentation started with 11.0 ± 0.1 g RS L^{-1}, and although *B. cereus* consumed 74 % of the available RS, P3HB production was limited. In contrast, *C. necator* was able to reach the highest accumulation by consuming only 6.8 ± 0.6 g RS L^{-1}, showing a higher efficiency than the others evaluated.

It can also be noted that there were differences among the results shown in the previous section and those found here when using *B. cepacia*. Hence, it must be kept in mind that the different characteristics (mainly the fiber content) of the BSG batches were key factors influencing RS release. For this batch, RS release was lower (11.6 g L^{-1}) than that obtained from the previous batch (26.2 g L^{-1}). However, for enzymatic hydrolysis, the ratio of P3HB to available RS was maintained at 40.5–44.2 mg P3HB g^{-1} RS, suggesting a direct relationship between available sugars and P3HB produced when no pretreatment is used.

Conclusion

These results show the potential of using BSG as a no-cost raw material for production of value-added PHAs in a by-product valorization and circular economy scheme. Although acid-thermal and alkaline-thermal pretreatments released significant levels of RS, they were accompanied by the generation of inhibitory compounds such as furfural (with the acid-thermal pretreatments) and phenols (with alkaline pretreatments) that probably limited the P3HB production. Hence, the highest P3HB accumulation (0.31 ± 0.02 P3HB g^{-1} CDW) with *B. cepacia* was obtained in the reference condition without pretreatment. It was also found that, compared to *B. cereus, B. cepacia*, and *C. necator* presented the highest PHA accumulation, reaching their maxima at ~48 h. These observations are encouraging for the use of BSG as an alternative carbon source to obtain PHAs, as well as the development of

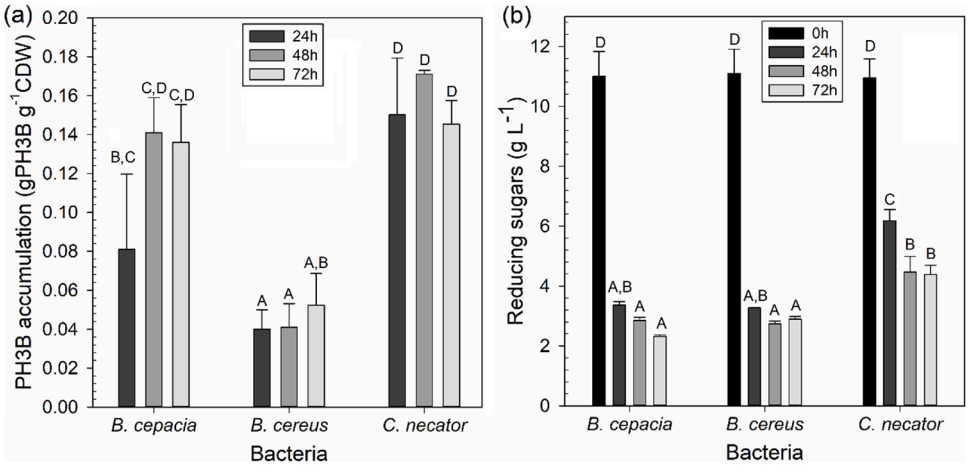

Fig. 4. Performance of different representative PHA-producing bacteria to obtain poly-3-hydroxybutyrate (P3HB) from non-pretreated brewer's spent grain hydrolysates. (a) P3HB production, (b) reducing sugars content during the fermentation. Different capital letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

alternative pretreatment approaches.

Declaration of Competing Interest

The authors reported no declarations of interest.

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