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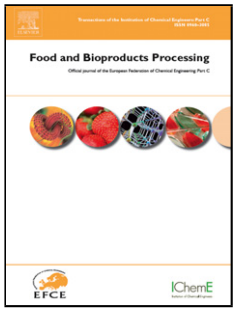

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PII: S0960-3085(21)00017-1

DOI: https://doi.org/10.1016/j.fbp.2021.01.015

Reference: FBP 1402

To appear in: *Food and Bioproducts Processing*

Received Date: 28 October 2020

Revised Date: 25 January 2021

Accepted Date: 28 January 2021

Please cite this article as: Martínez-Avila O, Llimós J, Ponsá S, Integrated solid-state enzymatic hydrolysis and solid-state fermentation for producing sustainable polyhydroxyalkanoates from low-cost agro-industrial residues, *Food and Bioproducts Processing* (2021), doi: https://doi.org/10.1016/j.fbp.2021.01.015

Integrated solid-state enzymatic hydrolysis and solid-state fermentation for producing sustainable polyhydroxyalkanoates from low-cost agro-industrial residues

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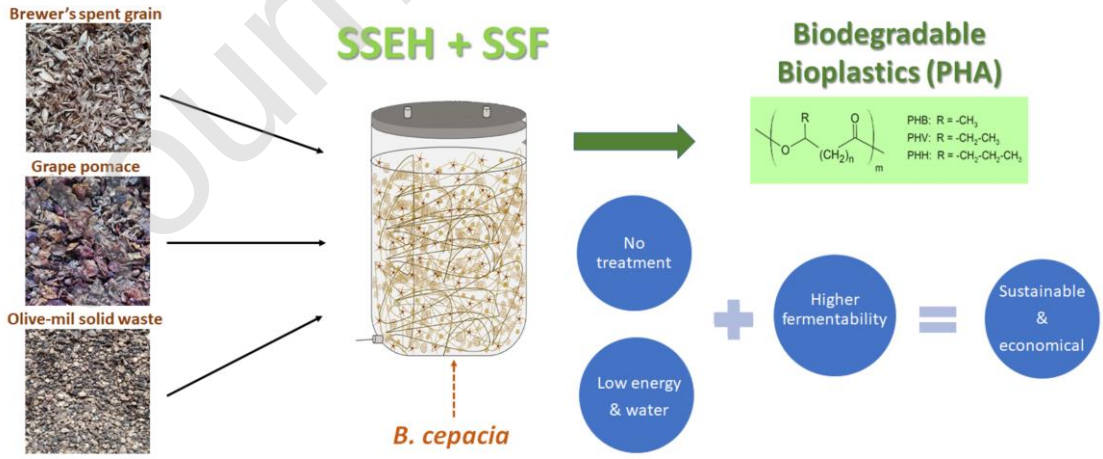
Graphical abstract



Highlights

- Polyhydroxyalkanoates (PHA) were produced from lignocellulosic-derived wastes
- Solid-state hydrolysis and fermentation (SSEH+SSF) were used as alternative strategy
- SSEH served to increase the reducing sugars content of wastes up to 0.16g g^{-1}waste
- Combining SSEH and SSF allowed increasing PHA yield up to 54% compared to SSF alone
- Maximum PHA production reached 12.5mg g^{-1} using brewer's spent grain and *B. cepacia*

Abstract

Polyhydroxyalkanoates (PHA) are biodegradable bioplastics of interest as potential substitutes of petroleum-derived plastics that can be produced starting from lignocellulosic-derived residues. This study presents the combined solid-state enzymatic hydrolysis (SSEH) and solid-state fermentation (SSF) as a sustainable approach for obtaining PHA using the leftovers brewer's spent grain (BSG), grape pomace (GP) and olive-mill solid waste (OSW) as substrates. SSEH performance was influenced by the intrinsic characteristics of each residue, the temperature, and the type of enzymatic extract used. Thus, the maximum sugars release (0.16 g g^{-1} of dry residue (gTS)) was obtained with GP. Furthermore, coupling SSEH and SSF promoted PHA yield increases of up to 54%, 41% and 31% for BSG, GP and OSW respectively, compared to SSF alone. The maximum PHA yield was achieved using hydrolyzed BSG with 12.5 mg g^{-}

[1]TS (0.33 g kg^{-1} h^{-1}). Results show the potential of this approach as an attractive alternative to obtain bioproducts such as PHA sustainably in residue-based systems.

Keywords: PHA, bioplastics, lignocellulosic biomass, solid-state technology, waste valorization*

* Abbreviations:
BSG: Brewer's spent grain
GP: Grape pomace
OSW: Olive-mill solid waste
PHA: Polyhydroxyalkanoates
PHA%: PHA accumulation
P_p: PHA Productivity
RS: Reducing sugars
SmF: Submerged fermentation
SSEH: Solid-state enzymatic hydrolysis
SSF: Solid-state fermentation
TS: Total solids (dry matter)
WHC: Water holding capacity
Y_P: Substrate yield
Y_{rs}: Sugars yield

1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polymers of biological origin accumulated by several microorganisms as energy storage within the cells when facing extreme conditions. In particular, PHA tend to be accumulated under nutrient limiting conditions (nitrogen, phosphorus, magnesium or oxygen) in excess of carbon source (Koller et al., 2010; Raza et al., 2018). In general, gram-positive and gram-negative bacteria constitute the main group of microorganisms capable of producing PHA under both aerobic and anaerobic conditions (Koller, 2018; Raza et al., 2018). Nowadays, more than 150 hydroxyalkanoates (monomers) have been identified as building blocks of PHA. However, the 3-hydroxycarboxylates of 4–14 carbons, 4-hydroxybutyrate, and 4-hydroxyvalerate are the most studied due to their metabolization paths and polymer properties (Kumar et al., 2020). PHA are insoluble in water, possesses excellent

resistance to hydrolytic attack and UV light, and have a melting point in the range 40-180°C (Kumar et al., 2020; Raza et al., 2018). However, one of the traits making PHA more attractive is their non-toxic nature, biocompatibility and biodegradability. Such characteristics allow them to be used in specialized applications such as in medical, pharmaceutical, agricultural and food sectors (Koller, 2018; Kumar et al., 2020). Thus, PHA have gained relevance as potential substitutes of traditional petroleum-derived plastics, since the growing dumping of these materials into the environment, along with the lack of effective recycling strategies, have led to severe environmental issues (Koller et al., 2010).

Although bioplastics are produced at large scale starting from different substrates such as sucrose, glucose, starch or volatile fatty acids, their contribution to the global plastic market is still limited reaching just 1% of the total production in 2019 (European Bioplastics, 2020). One of the main constraints restraining the development of these products is the fermentation cost, mainly influenced by the acquisition of the raw materials, accounting between 30-50% of the total processing costs (Du et al., 2012; Govil et al., 2020; Rodriguez-Perez et al., 2018). Consequently, the search for economic raw materials and effective bioprocess to obtain PHA has served as driving force to develop alternative systems using renewable sources and waste streams (Govil et al., 2020; Kumar et al., 2020; Rodriguez-Perez et al., 2018). Most of the advances for producing PHA in residue-based systems have been performed by using submerged fermentation (SmF) technologies with raw materials of diverse origin such sugarcane molasses (Acosta-cárdenas et al., 2018), municipal wastewater (Mannina et al., 2020), among others. Moreover, different solid organic wastes have also been used to obtain PHA after hydrolysis of these materials, using sugar-rich hydrolysates of residues such

as sugarcane bagasse (Yu and Stahl, 2008), rice husk (Heng et al., 2017), grape waste (Kovalcik et al., 2020), among others.

Alternatively, authors such as Oliveira et al., (2004) have proposed using lignocellulosic-based residues as low-cost raw materials for producing PHA using the solid-state fermentation (SSF) technology as an economical and sustainable approach. SSF presents inherent characteristics that make it an attractive process to valorize solid organic wastes, such as a low energy and water demand, reduced waste generation, and high production rates and yields (Soccol et al., 2017; Yazid et al., 2017). Nevertheless, one of the most interesting traits of SSF is its versatility to process a wide variety of solid wastes to obtain different value-added products through an extensive range of microorganisms (Soccol et al., 2017; Yazid et al., 2017). Accordingly, SSF has been successfully used in combination with several wastes for producing marketable bioproducts such as enzymes (Llimós et al., 2020; Melikoglu et al., 2015), base chemicals (Martínez-Avila et al., 2020), phenolic compounds (Santos da Silveira et al., 2019), among others. Oliveira et al., (2004) first tested the PHA production via SSF using soy cake supplemented with molasses. However, it has also been studied using jackfruit seed extract and polyurethane foam as supporting material (Ramadas et al., 2013), using tapioca waste supplemented with nutrient solutions (Sathiyanarayanan et al., 2013), and using malt complemented with nutrients (Sharma and Bajaj, 2016). Even though, the efficiency of these systems depends on the addition of supplementary carbon sources (most of the times of synthetic origin), limiting their applicability in potential large-scale applications.

An alternative to increase the fermentability of lignocellulosic materials is the solid-state enzymatic hydrolysis (SSEH). In this process, hydrolysis is conducted using

enzymes with high solid loads in the absence or near absence of free water (Chen and Liu, 2017). Similar than SSF, SSEH requires low water and energy inputs, decreasing operating costs while working with small size equipment compared to conventional hydrolysis in a liquid phase (Koppram et al., 2014; Modenbach and Nokes, 2013). Thus, by integrating SSEH with SSF, it would be expected to obtain a solid fraction with higher fermentable sugars in a similar way as performed with their liquid counterparts, which would provide a higher potential for the PHA production via SSF. Besides, this approach would limit the use of additional carbon sources to support PHA production, potentially reducing cost, and increasing the sustainability of the process.

Thus, this study aimed to investigate the feasibility of integrating SSEH and SSF as a potential approach to obtain PHA in a sustainable residue-based system. Three lignocellulosic agro-industrial residues of diverse characteristics (brewer's spent grain, grape pomace and olive-mill solid waste) have been assessed as potential raw materials of the process to achieve such a goal. The SSEH has been evaluated based on the most important factors affecting fermentable sugars' release by using self-produced hydrolytic enzymes (using SSF and *Aspergillus niger*) and commercial enzymes (Viscozyme L). Moreover, the PHA production via SSF was assessed with two well-known PHA producer strains, namely *Burkholderia cepacia* and *Cupriavidus necator* in a batch-SSF system testing both the SSF alone and combined with SSEH. To the best of our knowledge, this is the first report using the proposed integrated approach for producing PHA based on the use of only residues as raw materials.

2. Material and methods

2.1. Microorganisms

Burkholderia cepacia (CCM 2656) was purchased from the Czech Collection of Microorganisms (Brno, Czech Republic), *Cupriavidus necator* (DSM 428) was obtained from the German Culture Collection (Braunschweig, Germany), and *Aspergillus Niger* (ATCC 16888) was acquired from Colección Española de Cultivos Tipo (Valencia, Spain). The strains were preserved at -80°C in cryovials containing saturated pearls with the strain and a glycerol solution. Inoculum preparation for the bacterial strains consisted of putting one saturated pearl into a 100 mL Erlenmeyer flask containing 50 mL of LB media (Lysogeny Broth, Panreac), while for *A. niger*, a glucose-starch media (g L^{-1}) (peptone: 5, starch: 20 and glucose: 20) was used. Cultures were incubated in a rotary shaker at 180 rpm, keeping an aerobic atmosphere at 30°C for 48 h and 96 h respectively. All the reagents and materials have been sterilized by autoclaving at 121°C for 20 min.

2.2. Residues used as substrates

Companyia Cervesera del Montseny (Catalonia, Spain) supplied the brewer's spent grain (BSG). Grape pomace (GP) was collected from the vineyard of Celler Cooperatiu D'Espolla (Catalonia, Spain), and the olive-mill solid waste (OSW) was provided by Davmus Fruits, a local oil producer in Huesca region (Aragon, Spain). Residues were dried overnight at 60°C in an air oven and stored at -20°C until used, keeping their original particle size (Figure S1 supporting material).

2.3. Self-produced enzymes from *A. niger*

Self-produced enzymatic extracts used in the hydrolysis experiments were obtained starting from BSG, as suggested by Llimós et al. (2020). Briefly, the BSG was fermented in solid-state using *A. niger* as inoculum (10% w/w). Fermentation was conducted at 37°C for 48 h using the same reaction system described in section 2.5. At

that point, lignocellulolytic enzymes were extracted by mixing the fermented material with citrate buffer (pH 4.8) in a 1:10 ratio at 30°C and 160 rpm. The flasks were shaken for 30 min, and then, centrifuged at 5000 rpm for 10 min. The obtained supernatant was used as an enzymatic extract for the hydrolysis tests (section 2.4). When required, it was filtered using a 0.45 µm membrane to determine the enzyme activities of the extract.

2.4. Solid-state enzymatic hydrolysis experiments

Enzymatic hydrolysis in solid phase was conducted using the substrates as follows: dried BSG, GP and OSW were pH-adjusted to 4.6-5.0 by using citrate buffer 0.05 M to work at the optimum pH range for this kind of enzymes (Gama et al., 2015), at the same time that setting the initial moisture content (MC_0) levels according to the need of each experiment (section 3.1). The prepared substrates were sterilized by autoclaving at 121°C for 20 min. After cooling, the selected enzymatic agents (Viscozyme L or self-produced from *A. niger*) were added and mixed with the substrates. The initial enzymes activities of both agents were set at 230 ± 10 xylanase activity units (U) per gram of dried residue (gTS) and 5.3 ± 1.3 filter paper unit (FPU) g^{-1}TS equivalent to a 1% dose of Viscozyme L. The SSEH experiments were conducted in 0.6 L glass cylindrical reactors previously sterilized by autoclaving at 121°C for 20 min. Reactors were filled with 200 ± 1 g of prepared substrates and placed in a temperature-controlled incubator at the conditions required for each test (section 3.1). Experiments were followed up to 48 h.

2.5. Solid-state fermentation for producing PHA

Fermentation experiments were conducted using the substrates after preparation as follows: For BSG, OSW and their corresponding hydrolyzed counterparts, the MC and pH of the substrates were adjusted by using different proportions of phosphate buffer

(0.5 M) and water such that the initial pH of the media was 6.0 ± 0.2. For GP and its hydrolyzed fraction, the same pH level was adjusted using NaOH 1 M. The MC of the substrates was set to their respective water holding capacity (WHC) by using the same solutions. Additionally, for OSW, a 10% (w/w) of sponge cloth (Spontex®) was added as a bulking agent to provide enough porosity to the system. The prepared substrates were then sterilized by autoclaving at 121°C for 20 min to limit both the enzyme activity (in the hydrolyzed substrates) and the presence of other potential interferences. After cooling, they were inoculated with 10% (w/w) of the tested bacterial strain at 30°C and supplying air at 0.08 L h^{-1} g^{-1}TS.

The SSF experiments were carried out in 0.5 L glass Erlenmeyers previously sterilized by autoclaving at 121°C for 20 min. The Erlenmeyers were filled with 100 ± 1 g of the prepared substrate (triplicates) and placed in a temperature-controlled water bath in which a mass flow controller (Bronkhorst Hitec) continuously supplied air into each flask as detailed elsewhere (Ponsá et al., 2010). The airtight system allowed the exhausted gases to be individually led to oxygen sensors (αLphase Ltd.) connected to a self-made data acquisition system (Arduino®-based) that recorded oxygen concentrations every five minutes. A respirometric analysis was conducted with the collected data by computing the specific oxygen uptake rate (OUR) as stated by Ponsá et al. (2010). Experiments were followed up to 72 h.

2.6. Analytical methods

2.6.1. Substrates characterization and monitoring

The moisture content (MC), pH, total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total phosphorus, oxidizable carbon (OXC), water holding capacity (WHC) and bulk density (BD) have been determined following standard procedures

(The US Department of Agriculture, 2001). Cellulose, hemicellulose and lignin content were determined by the gravimetric method (Möller, 2009).

2.6.2. Reducing sugars content in the solid phase

Reducing sugars of the solid substrates were estimated following the DNS method (Miller, 1959) on the supernatant obtained after a solid-liquid extraction of the fermented substrate using distilled water in a 1:10 (w/v) ratio at 35°C during 40 min. The supernatant was centrifuged at 4200 rpm for 10 min and adequately diluted before its analysis. Concentrations were computed based on calibration curves using glucose as a reference standard.

2.6.3. Xylanase and cellulase activity in the solid phase

Xylanase and cellulase activities in the solid substrates were determined from the supernatant obtained after a solid-liquid extraction as described in section 2.3 by using the method proposed by Ghose and Bisaria, (1987). The xylose content was determined following the DNS method (Miller, 1959) using xylan from beechwood (Apollo scientific, England) as substrate, and allowing the enzymatic reaction to run for 20 min at 50°C. One unit of xylanase activity (U) was defined as the release of 1 mol of xylose per minute. Xylanase activity was expressed as U g^{-1}TS. Total cellulases activity was measured using the filter paper assay (FPase) according to Ghose, (1987). The substrate was 1x6 cm Whatman filter paper in 0.05 M citrate buffer (pH 4.8), and the enzymatic reaction was run for 1 h at 50°C. One unit of FPase (FPU) was expressed as the amount of enzyme releasing 1 mol of reducing sugars from filter paper per minute. Cellulases activity was expressed as FPU g^{-1}TS.

2.6.4. Biomass production and PHA extraction

Biomass produced in the solid phase was quantified gravimetrically after two-consecutive solid-liquid extractions. The fermented sample (5-8 g) was mixed with distilled water (1:3 ratio) and placed in an orbital shaker at 165 rpm, 30°C for 35 min. Then, the supernatant was vacuum-filtered through AP25 paper and quantitatively recovered in falcon tubes. The filtered was centrifuged at 5000 rpm, 4°C for 15 min. The supernatant was discarded, and the pellet containing the biomass was washed with distilled water and centrifuged again at 5000 rpm for 15 min to recover the solid fraction. The obtained pellet was then dried at 60°C for 24 h, and it was weighted as the biomass or cell dry weight (CDW) contained in the solid sample.

PHA extraction from the dried biomass was conducted following a modified method from Brandl et al., (1988). Briefly, 2 mL vials served to mixed 10-15 mg of dried biomass pellet with 1 mL of chloroform and 0.8 mL of a methanol-sulfuric solution including benzoic acid as the internal standard. Vials were adequately sealed and placed in a thermostatic bloc at 95°C for 3.5 h. After cooling, the vial content was transferred into 4 mL vials and mixed with 0.5 mL of NaOH 0.05 M through inversion. After the phases separation, the organic phase was used to determine the methyl-esters monomers of PHA of the extract.

2.6.5. PHA quantification

PHA content of the extracts was determined by GC-FID. The GC system (Agilent 7820A) consisted of a Flame Ionization Detector with an HP-Innowax column (30mx0.53mmx1µm). Injection port was set at 250°C with a split ratio 1:3, and the column temperature was set at 70°C. A gradient (17°C min^{-1}) allowed reaching 150°C, which was held for 1 min. Finally, the temperature was increased (10°C min^{-1}) until 200°C and maintained for 3 min. Detector temperature was set at 300°C. Identification

and quantification of PHA were performed using calibration curves (internal standard) by comparing retention times of analytical grade standards (Sigma-Aldrich). P(3HB-co-3HV)-12% mol PHV was used to quantify the polyhydroxybutyrate (P3HB) and polyhydroxyvalerate (P3HV), while 2-hydroxycaproic acid was the standard for polyhydroxy-2-methylvalerate (PH2MV). In all cases, they were processed using the same conditions than samples. PHA accumulation (PHA%) was expressed as the total PHA content (P3HB+P3HV+PH2MV) per gram of CDW [g g^{-1}CDW].

2.6.6. Inhibitors quantification in the solid phase

Inhibitors content in the solid phase was determined after a solid-liquid extraction using water. 10-15 g of solid sample was mixed with water (50 mL) and placed in an orbital shaker at 180 rpm, 45°C for 45 min. The supernatant was filtered through a 0.22 µm membrane, and it has served to quantify the inhibitor compounds by HPLC using a modified method from Chen et al., (2006). Briefly, the HPLC consisted of an Agilent 1920 Infinity equipped with a UV-Vis Diode Array Detector and a Nucleosil 120C_{18} (3µmx125mmx4mm) column. The column was set at 30ºC. The mobile phase (1 mL min^{-1}) was a mixture of H_3PO_4 0.05% and acetonitrile:H_2O (90:10). The gradient started with 100% of H_3PO_4 0.05%, and it increased gradually until 100% acetonitrile:H_2O after 38 min. Then it was held for 10 min. Quantification was performed by comparing samples from analytical standards at the same conditions than samples using external standard calibrations.

2.7. Statistical analysis

Statistical differences of the assessed experiments were analyzed using a one-way ANOVA ($p<0.05$ confidence) with the Tukey test. Assays used to monitor the process

have been conducted in triplicates, and the reported values correspond to the mean value and its standard deviation. Minitab 18 (Minitab Inc.) was used to analyze the data.

3. Results and discussion

3.1. Solid-state enzymatic hydrolysis of the residues

These experiments consisted of assessing the potential of SSEH as a tool to increase the reducing sugar content (RS) of the selected residues. It was decided to keep the pH of the solid media between 4.6-5.0 while assessing the effect of temperature, initial moisture content, and type of enzymatic extract as some of the most influencing variables for the process (Canabarro et al., 2017; Lunelli et al., 2014).

Considering that the influence of variables such as pH or temperature could be directly related to the enzymes needs rather than the substrate to be hydrolyzed, the temperature effect was first assessed using BSG as a reference in the range 30°C to 50°C. As Figure 1(a) details, the temperature induced a significant effect on the RS release for both evaluated enzymatic extracts. It can be seen that for the commercial enzymes the range 35-50°C resulted ideal, maximizing the enzyme efficiency as occurred in the hydrolysis of other lignocellulosic materials in liquid media using the same enzymes (Gama et al., 2015; Kumar et al., 2013). Thus, using Viscozyme L, the maximum RS release was obtained at 40°C. On the other hand, when using the self-produced enzymatic extract, a different trend was found. As seen in Figure 1(a), the maximum RS release with these enzymes was achieved at 35°C, and a significant decrease of the efficiency was attained at the other evaluated levels. Although some authors suggest that lignocellulolytic enzymes from *A. niger* can remain active in a range from 30-60°C (Bravo et al., 2000; Uday et al., 2017), there is limited information regarding their efficiency in solid-phase.

Figure 1

These initial tests also allowed noting that MC_0 played a significant role in the RS release but in a different way depending on the type of enzymatic agent and hydrolysis temperature. As detailed in Figure 1(a), for Viscozyme L, the highest RS levels were mainly found at the high MC_0 level, but with a lower effect at temperatures between 45°C and 50°C. On the contrary, for the *A. niger* extract, MC_0 affected the hydrolysis in solid-state differently. While at low temperatures (30-40°C), low MC_0 levels induced a higher RS release, at 45°C and 50°C the highest RS contents were obtained by working at high MC_0. Hence, exploring this variable was further required.

Once identified the temperature effect for each of the used enzymatic extracts, these were further evaluated on the selected residues by setting the hydrolysis temperature at 40°C for Viscozyme L, and 35°C for the *A. niger* extract. In this case, the MC_0 and type of enzymatic extract were evaluated by using a 2^2 factorial design. While the type of enzymatic extract included the self-produced *A. niger* extract and the commercial Viscozyme L, the MC_0 was set using the WHC of each residue as the reference condition. Hence, for BSG it was assessed in the range 50-70%, for GP from 20-40%, and for OSW between 25-50% to agree with the WHC of each residue.

As observed in Figure 1(b), under the assessed conditions, it was clear that the commercial enzymes resulted significantly better to hydrolyze all the selected residues compared from the self-produced extract. Although both extracts' enzymatic activity was adjusted to similar levels at the beginning of the hydrolysis, the inherent differences among extracts promoted these results. As seen, the highest RS levels were obtained from the hydrolysis of GP using the commercial extract with up to 15.6 ± 0.5 g $100g^{-1}$TS. As observed before, the MC_0 resulted significant for the process using Viscozyme L. This time, it was found that for BSG and GP, the higher the MC_0, the higher the RS

release. Although this trend was not such marked for OSW (p 0.08), a slight improvement (not significant) was also reached when working at the higher MC_0 (Figure 1(b)).

When the SSEH was performed using the self-produced extract from *A. niger,* the highest RS levels were found in the GP hydrolysates. However, in this scenario, the maximum reached 12.9 ± 0.2 g $100g^{-1}$TS, 17.3% below the maximum achieved with the commercial enzymes. Here, the MC_0 played a different role depending on the evaluated substrate. As Figure 1(b) details, no significant differences were found for BSG (p 0.07), and OSW (p 0.11), while an increase in MC_0 favored the RS release in GP. The lower results of the self-produced extract from *A. niger* compared to those with the commercial enzymes could be influenced by different variables. However, the limited processing of the extract could significantly influence the hydrolysis performance. Since the self-produced extract contains traces of *A. niger* not removed after the extraction process, these could grow in the solid media during the hydrolysis step (such conditions favor the fungal growth), potentially consuming part of the released sugars. Hence, working at low MC levels would reduce the ability to grow of such traces (by limiting the water requirement for the strain), enhancing the global effect of the hydrolysis with this non-purified enzymatic extract.

Even though there is limited information to identify the global effect of MC_0 on the RS release, dissimilar results have been found for other solid-state hydrolysis systems. While Lunelli et al. (2014) have found that MC_0 has no significant effects on the release of sugars from sugarcane bagasse, Sfalcin et al. (2015) stated that low MC_0 increased the RS content of rice bran. Also, Modenbach and Nokes, (2013) suggest that increasing the solids load constrains the hydrolysis, inducing a lower hydrolysis efficiency.

Although it could be expected that higher MC could benefit the SSEH due to higher water availability and better distribution of enzymes and products, it was not found a clear relationship among the maximum MC the residues could support (related to the WHC) and their performance in solid-state. In this sense, it has to be considered that other variables promote simultaneous changes that cannot be easily distinguished from each other such as those induced by the temperature, the kind of enzymes used, and the same ability of the residue to provide porosity and a suitable mass transfer.

However, the results suggest that, independently of the type of enzymes used, the SSEH has a residue-dependent performance. If the results are analyzed taking in mind the initial RS content of each residue (Table 1) it can be seen that with BSG the maximum increase was almost fivefold, with GP was 1.4-fold, and with OSW it was up to eightfold. However, the hydrolysis yield shows that the RS release tends to be more efficient with BSG (up to 9.5 g $100g^{-1}$TS), followed by GP (up to 8.8 g $100g^{-1}$TS), and OSW (up to 3.3 g $100g^{-1}$TS).

Table 1

Although the self-produced enzymes did not achieve the highest RS levels of the evaluated set, it was remarkable that these were as efficient as the commercial counterparts when working at low MC_0 levels in two out of three residues (Figure 1(b)). Such a result was more evident by analyzing the time-course of the SSEH at low MC_0 levels. As detailed in Figure 2(a), for BSG and GP the release of sugars showed a similar trend by using both enzymatic agents, and the main differences were found during the first hours of hydrolysis. In general, Viscozyme L could reach between 50-65% of the maximum RS release within the first 9 h, while the self-produced extract only reached between 38-45% in the same period. However, in these residues, the

maximum levels achieved were similar, even though, at the end of the hydrolysis, the processes run with the self-produced extracts presented a decrease in the RS levels (Figure 2(a)).

On the other hand, it was found that the remaining xylanase activity of the self-produced extract decreased between 22% and 47% after 24 h of hydrolysis of the processed residues (Figure 2(b)). This trend coincided with a slower release of sugars in the last part of the hydrolysis. As detailed, the xylanase activity at the end of the hydrolysis was far below the initial levels, suggesting that the crude enzymes possess limited performance in time, but good enough to increase the RS levels of the proposed residues. Such an outcome could be derived from the reduced stability of the non-purified enzymes, due to the presence of some inhibitory compounds capable of deactivating the enzymes, but mainly due to the competitive inhibition of the enzymes by the growing content of diverse products (sugars) (López et al., 2005; Sfalcin et al., 2015).

Along with the release of fermentable sugars, some potential inhibitory compounds derived from cellulose, hemicellulose and lignin tend to appear in the solid media during the SSEH affecting the microbial growth and/or the enzyme activity (Chen and Liu, 2017; Modenbach and Nokes, 2013). Inhibitory compounds found in lignocellulosic-derived hydrolysates mainly include furan derivatives, weak acids, and phenolic compounds such as furfuraldehyde, 5-HMF, coumaric acid, vanillic acid, syringaldehyde, acetic acid, among others (Chen and Liu, 2017).

Figure 2

Some authors (Hodge et al., 2008) suggest that the presence of such compounds could become a critical factor affecting the hydrolysis/fermentation of the substrates due to

their increasing concentration in SSEH. Thus, weak acids usually inhibit cell growth and decrease the enzyme activity, furfurals induce larger lag-phases, and phenolic compounds usually act as toxic compounds (Palmqvist and Hahn-Hägerdal, 2000). Even though these compounds are promoted during the enzymatic hydrolysis, they appear naturally in the original residues. As Figure 3 details, some of these compounds were detected in the evaluated residues and their corresponding hydrolysates (complete data in Table S1 supporting material). It can be observed that there was a significant difference in the inhibitors content from the original residues and their hydrolysates. The presence of 5-HFM and furfuraldehyde in GP and Vanillic acid and Vanillin in OSW was particularly high. It was also observed that SSHE induced an increase in the inhibitors content of almost twofold compared to the residues without hydrolysis, and significant levels of acetic acid were found in the different assessed substrates, particularly in BSG hydrolysates.

Figure 3

Although no reports indicate the toxicity levels of such compounds for the tested strains in the solid phase, inhibitors content found in GP and OSW suggest that these residues could require a detoxification step before SSF to limit the effects of such compounds. Some of the most suitable pretreatments for this purpose could include microbial detoxification, adsorption with specific chemicals, or the use of specialized additives such as bovine serum albumin (Kim, 2018; Moreno et al., 2015). On the contrary, for BSG, it is expected that the limitation imposed by these compounds be negligible. Consequently, this trend suggests that applying the SSEH directly on residues (*i.e.*, without any pretreatment) with low lignin content such as BSG, would be an interesting option since the negative effect of lignin on enzyme efficiency is limited. However,

once the lignin content of the residue becomes higher such as with GP and OSW (Table 1), the need for a delignification process turns into crucial.

3.2. Solid-state fermentation for PHA production

In these experiments, the selected residues (with and without previous hydrolysis) were used as substrates for producing PHA via SSF by using *B. cepacia* and *C. necator*. To identify the effect of the SSEH on the PHA production, the hydrolyzed residues obtained using the self-produced enzymes from *A. niger* (section 3.1) were used as the reference. These hydrolysates were selected, having in mind the potential benefits their use could bring to the process's economy and sustainability compared to commercial enzymatic extracts. Although results shown in this section correspond to the total PHA production (P3HB+P3HV+PH2MV), it was found that for *B. cepacia*, in average, P3HB was the main component (92%) followed by PH2MV (7%) and P3HV (1%). Similarly, the average distribution of the products found for *C. necator* was P3HB 95%, PH2MV 5%, P3HV 0%. Figure 4 summarizes the performance indices computed for the evaluated substrates. As can be seen in Figure 4(a), the maximum PHA% was significantly different for each of the tested residues independently of the used strain. While BSG and its hydrolyzed counterpart reached the highest accumulation levels between 0.26 and 0.41 g g^{-1}CDW, with GP, it achieved a maximum between 0.18-0.20 g g^{-1}CDW, and with OSW maximum levels in the range 0.19-0.20 g g^{-1}CDW. Results show that for *B. cepacia*, PHA% was particularly high in the hydrolyzed BSG (up to 0.36 g g^{-1}CDW), but no significant differences were reached with GP (p 0.09) and OSW (p 0.08) compared to their original counterparts. On the contrary, for *C. necator*, it was found that the hydrolyzed residues promoted higher PHA% in GP and OSW, but a significant decrease in BSG (p 0.02).

Figure 4

Figure 4(a) also shows the biomass produced in the solid phase at the point of maximum PHA%. As detailed, the maximum levels were consistently obtained by *B. cepacia* in the different tested substrates, reaching up to 34.5 mgCDW g^{-1}TS with BSG hydrolysate. It can also be observed that there were significant differences in the produced biomass among the selected residues independently of the application of SSEH. Thus, with BSG, it was reached up to threefold the levels found in GP and OSW. Regarding the assessed strains, these results suggest that, in general, *B. cepacia* was better to exploit the available nutrients to grow in the solid media than *C. necator* and to transform these into PHA. Although some previous reports (Oliveira et al., 2004) proposed *C. necator* as a suitable bacterium to produced PHA via SSF, the results obtained here show that *B. cepacia* could be an attractive strain capable of producing high biomass level with consistent PHA% in solid media.

From Figure 4(b), it can be seen the maximum substrate yields (Y_p) (this is the PHA production per gram of dry added residue) obtained from each substrate. It was clear that the best performance was reached with *B. cepacia* and BSG hydrolysate with 12.5 mg g^{-1}TS, 53.7% higher than the yield obtained using BSG alone and the same strain. Although SSEH coupled with SSF produced Y_p increases in GP and OSW of 41.3% and 31.5% respectively, in those cases, Y_p was almost 90% lower than the obtained with BSG hydrolysate.

These results suggest that the fermentable sugars availability was not the only aspect affecting the microbial activity in the solid bed. Although GP and its hydrolysate have had the highest RS availability of the evaluated set, the best performance was achieved with BSG and its hydrolysate. In this sense, it could be stated that the inherent

characteristics of each substrate resulted crucial for the development of the SSF. Apart from the carbon source availability, PHA accumulation is promoted at high C:N ratios and some authors suggest (Annamalai and Sivakumar, 2016; Johnson et al., 2010) that this ratio should be from 10 to 20 to allow a suitable bacterial growth at the same time that a significant PHA%. As detailed in Table 1, under this criteria, BSG has the most suitable C:N from the evaluated residues, while GP and OSW are almost twofold and fourfold from that point, suggesting these residues start with disadvantage to promoting the PHA accumulation.

Moreover, another aspect influencing the process was related to the supporting abilities of each residue. As seen from Table 1, just BSG possesses a high WHC and BD that impart a good porosity to the bed, allowing an appropriate mass transfer from the air stream to the soli-liquid interface. On the contrary, GP and OSW are limited solid materials to provide such traits, even needing bulking agents to supply such porosity. However, one of the main factors defining the performance of the process was the lignocellulosic composition of the residues. Table 1 shows that BSG is a residue with low lignin and high hemicellulose contents, which make it less prone to release inhibitory compounds of lignin origin. Thus, it could be stated that a residue with similar characteristics could be used directly without needing any particular pretreatment for delignification.

A different scenario was found when analyzing the GP. Although the lignin content of this residue was double the found in BSG, such levels were high enough to promote the presence of inhibitory compounds (Figure 3), which likely affected the bacterial growth and PHA accumulation despite the higher sugar availability. Finally, it can be seen that the lignin content of OWS was almost double the one found in GP. Such a condition

allowed the presence of inhibitory compounds (Figure 3) that also influenced the performance of this residue.

From the strains point of view, these results suggest that *B. cepacia* could be a better choice than *C. necator* regarding the ability to withstand the presence of such inhibitory compounds in the solid phase, in agreement with previous results (Obruca et al., 2014). In this sense, the need for resistant strains is gaining attention as an approach to obtain PHA with complex matrices as the solid organic wastes (Kumar et al., 2020). Furthermore, *B. cepacia* appears as an attractive strain for PHA production from solid organic wastes considering that it has shown the ability to use a varied set of hexoses and pentoses typically found in lignocellulosic-derived hydrolysates to accumulate PHA (Heng et al., 2017; Obruca et al., 2014). On the contrary, *C. necator* mainly uses glucose and fructose, limiting their use with waste streams (Heng et al., 2017).

Besides the substrate yield, the performance of the tested substrates can also be analyzed from the efficiency of the used sugars (Figure 4(b)). In this sense, results show that BSG obtained the highest sugars yield (Y_{rs}) (this is the PHA produced per gram of available RS) when using the residue alone (388 mg g^{-1}RS), and it decreased until 172 mg g^{-1}RS for the hydrolyzed residue. However, due to the low RS availability of OSW, this residue and its hydrolysate were able to reach a still competitive Y_{rs} between 251 and 301 mg g^{-1}RS when using *B. cepacia*.

The results obtained in this study show an interesting perspective of the proposed approach when comparing from previous SSF systems for producing PHA. Thus, in the best scenario (using BSG hydrolysate,) Y_p was 155% higher than the previously achieved with soy cake and molasses (4.9 mg g^{-1}TS) (Oliveira et al., 2004), a 44.8% higher than the obtained with tapioca waste (Sathiyanarayanan et al., 2013), and in the

same order of magnitude (13% lower) of the best-reported scenario using malt supplemented with nutrients (Sharma and Bajaj, 2016). Apart from the inherent differences on the substrates tested so far in SSF for obtaining PHA, the main difference that can be highlighted from the proposed integrated approach (SSEH+SSF) is that it induces an improvement in the raw material increasing the potential of the substrates. In the conventional approach (just SSF), such improvement has always been achieved by adding other carbon sources supplementing those provided by the residues. Nevertheless, those sources tend to be other than residues, negatively affecting the economy and sustainability of the approach. Thus, by coupling hydrolysis and fermentation using self-produced enzymes has a positive effect on the PHA production and the economic feasibility of the process.

Furthermore, as shown in Table 2, compared to other systems using similar raw materials to obtain PHA, the proposed approach results in a competitive alternative. As observed, the combined SSEH and SSF of BSG with *B. cepacia* obtained higher productivities (P_p) than several reported SmF systems, without the need of adding supplementary carbon sources nor using a pretreatment step. Similarly, the Y_{rs} of this scenario is still competitive compared to most of the reported SmF systems using similar raw materials.

Table 2

Although comparing SmF and SSF performances is complex due to their different nature, it is evident that SmF systems tend to facilitate the PHA% maybe due to the dilution effect of potential inhibitory compounds, better mass transfer and homogeneous conditions. Nonetheless, the compiled data shows that, despite those disadvantages, SSF

systems tend to reach higher PHA productivities and competitive use of the available sugars.

A detailed analysis of the SSF of BSG shows the process's behaviour run with the residue with and without the SSEH. As observed in Figure 5(a), the fermentation carried out with BSG presented a rapid increase in the bacterial activity (indirectly measured by OUR), reaching the maximum around 18 h. At that point, PHA% also reached the maximum levels, and the strain started to produced biomass rapidly such that the maximum was achieved between 60-70 h. As seen, the maximum PHA production was reached at 48 h. After that point, PHA% started decreasing coinciding with the depletion of the available reducing sugars. This suggests that *B. cepacia* started to consume PHA as a carbon source to remain active in that period.

Figure 5

On the contrary, Figure 5(b) shows that the fermentation run with BSG hydrolysate started with a short lag-phase of almost 2 h (probably due to the higher furfurals and acetic acid contents), a period in which no significant growth was detected. Then, a rapid increase in the bacterial activity was attained, reaching the maximum OUR at 24 h, coinciding again with the maximum PHA%. As seen, during this period, the biomass suffered a drastic change, reaching almost 23 mgCDW g^{-1}TS. From that point, biomass consistently increased almost linearly, reaching up to 51 mg g^{-1}TS at the end of the monitoring. Thus, Y_P has reached the maximum after 38 h of processing, just after the peak of maximum microbial activity, and coinciding with the depletion of the available reducing sugars. Again, PHA% started decreasing at that point, suggesting that these were used by the strain to remain active.

As seen, the higher RS availability of the hydrolysate BSG allowed a faster and higher bacterial growth than in the BSG alone during the first part of the fermentation. This, in turn, conducted to higher PHA production during the same period. Although such production rate was not accompanied by higher bacterial activity, it could be stated that under the evaluated conditions, the fermentation has taken less time. These results are in accordance with previous reports (Oliveira et al., 2004; Sharma and Bajaj, 2016) where the addition of supplementary carbon sources induced an increase in Y_s in SSF processes for producing PHA.

Conclusions

SSEH efficiently increased the reducing sugar content of different lignocellulosic-derived residues without requiring previous pretreatment. Maximum sugars release was achieved using BSG and GP with 0.095gRS g^{-1}TS and 0.088gRS g^{-1}TS, respectively. Besides, by using the hydrolyzed residues in SSF resulted in significant increases of the PHA yield compared from the residues alone, obtaining a maximum of 12.5mg g^{-1}TS (0.33g kg^{-1} h^{-1}) with BSG hydrolysate. Thus, this approach resulted in competitive compared to other conventional PHA production systems using similar raw materials but removing the need for adding supplementary carbon sources. Such improvement was particularly efficient with low-lignin residues such as BSG.

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.

Acknowledgments

This work was supported by the Spanish Ministerio de Economía y Competitividad (CTM2016-81038-R), VALORA Project.

References

Acosta-cárdenas, A., Alcaraz-zapata, W., Cardona-betancur, M., 2018. Sugarcane molasses and vinasse as a substrate for polyhydroxyalkanoates (PHA) production. Dyna 85, 220–225.

Annamalai, N., Sivakumar, N., 2016. Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. J. Biotechnol. 237, 13–17.

Bhatia, S.K., Gurav, R., Choi, T.R., Jung, H.R., Yang, S.Y., Moon, Y.M., Song, H.S., Jeon, J.M., Choi, K.Y., Yang, Y.H., 2019. Bioconversion of plant biomass hydrolysate into bioplastic (polyhydroxyalkanoates) using *Ralstonia eutropha* 5119. Bioresour. Technol. 271, 306–315.

Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., 1988. *Pseudomonas oleovorans* as a source of poly (β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54, 1977–1982.

Bravo, V., Paez, M.P., Aoulad, M., Reyes, A., 2000. The influence of temperature upon the hydrolysis of cellobiose by β- 1,4-glucosidases from *Aspergillus niger*. Enzyme Microb. Technol. 26, 614–620.

Canabarro, N.I., Alessio, C., Foletto, E.L., Kuhn, R.C., Priamo, W.L., Mazutti, M.A., 2017. Ethanol production by solid-state saccharification and fermentation in a packed-bed bioreactor. Renew. Energy 102, 9–14.

Castilho, L.R., Mitchell, D.A., Freire, D.M.G., 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresour. Technol. 100, 5996–6009.

Cesário, M.T., Raposo, R.S., de Almeida, M.C.M.D., van Keulen, F., Ferreira, B.S., da Fonseca, M.M.R., 2014. Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. N. Biotechnol. 31, 104–113.

Chen, H.Z., Liu, Z.H., 2017. Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading. Eng. Life Sci. 17, 489–499.

Chen, S.F., Mowery, R.A., Castleberry, V.A., Walsum, G.P. Van, Chambliss, C.K., 2006. High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates. J. Chromatogr. A 1104, 54–61.

Du, C., Sabirova, J., Soetaert, W., Ki Carol Lin, S., 2012. Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials. Curr. Chem. Biol. 6, 14–25.

European Bioplastics, 2020. Bioplastics market data [WWW Document]. URL https://www.european-bioplastics.org/market/ (accessed 3.28.20).

Gama, R., Van Dyk, J.S., Pletschke, B.I., 2015. Optimisation of enzymatic hydrolysis of apple pomace for production of biofuel and biorefinery chemicals using commercial enzymes. 3 Biotech 5, 1075–1087.

Ghose, T., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 357–268.

Ghose, T., Bisaria, V.S., 1987. Measurement of hemicellulase activities. Part 1: Xylanases. Pure Appl. Chem. 59, 1739–1752.

Govil, T., Wang, J., Samanta, D., David, A., Tripathi, A., Rauniyar, S., Salem, D.R., Sani, R.K., 2020. Lignocellulosic feedstock: A review of a sustainable platform for cleaner production of nature's plastics. J. Clean. Prod. 270, 122521.

Heng, K.S., Hatti-Kaul, R., Adam, F., Fukui, T., Sudesh, K., 2017. Conversion of rice husks to polyhydroxyalkanoates (PHA) via a three-step process: optimized alkaline

pretreatment, enzymatic hydrolysis, and biosynthesis by *Burkholderia cepacia* USM (JCM 15050). J. Chem. Technol. Biotechnol. 92, 100–108.

Hodge, D.B., Karim, M.N., Schell, D.J., McMillan, J.D., 2008. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. Bioresour. Technol. 99, 8940–8948.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 44, 2141–2152.

Kim, D., 2018. Physico-chemical conversion of lignocellulose: Inhibitor effects and detoxification strategies: A mini review. Molecules 23, 1–21.

Koller, M., 2018. A Review on Established and Emerging Fermentation Schemes for Microbial Production of Polyhydroxyalkanoate (PHA) Biopolyesters. Fermentation 4, 30.

Koller, M., Salerno, A., Dias, M., Reiterer, A., Braunegg, G., 2010. Modern biotechnological polymer synthesis: A review. Food Technol. Biotechnol. 48, 255–269.

Koppram, R., Tomás-Pejó, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity: Challenges and perspectives. Trends Biotechnol. 32, 46–53.

Kovalcik, A., Pernicova, I., Obruca, S., Szotkowski, M., Enev, V., Kalina, M., Marova, I., 2020. Grape winery waste as a promising feedstock for the production of polyhydroxyalkanoates and other value-added products. Food Bioprod. Process. 124, 1–10.

Kumar, G., Sen, B., Lin, C.Y., 2013. Pretreatment and hydrolysis methods for recovery of fermentable sugars from de-oiled Jatropha waste. Bioresour. Technol. 145, 275–279.

Kumar, M., Rathour, R., Singh, R., Sun, Y., Pandey, A., Gnansounou, E., Andrew Lin,

K.Y., Tsang, D.C.W., Thakur, I.S., 2020. Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects. J. Clean. Prod. 263, 121500.

Llimós, J., Martínez-Avila, O., Marti, E., Carlos, C.-L., Llenas, L., Gea, T., Ponsá, S., 2020. Brewer's spent grain biotransformation to produce lignocellulolytic enzymes and polyhydroxyalkanoates in a two-stage valorization scheme. Biomass Convers. Biorefinery. https://doi.org/https://doi.org/10.1007/s13399-020-00918-4

López, C., Torrado, A., Guerra, N., Pastrana, L., 2005. Optimization of Solid-State Enzymatic Hydrolysis of Chestnut Using Mixtures of α-Amylase and Glucoamylase. J. Agric. Food Chem. 53, 989–995.

Lunelli, F.C., Sfalcin, P., Souza, M., Zimmermann, E., Dal Prá, V., Foletto, E.L., Jahn, S.L., Kuhn, R.C., Mazutti, M.A., 2014. Ultrasound-assisted enzymatic hydrolysis of sugarcane bagasse for the production of fermentable sugars. Biosyst. Eng. 124, 24–28.

Mannina, G., Presti, D., Montiel-Jarillo, G., Carrera, J., Suárez-Ojeda, M.E., 2020. Recovery of polyhydroxyalkanoates (PHAs) from wastewater: A review. Bioresour. Technol. 297, 122478.

Martínez-Avila, O., Sánchez, A., Font, X., Barrena, R., 2020. 2-phenylethanol (rose aroma) production potential of an isolated *Pichia kudriavzevii* through solid-state fermentation. Process Biochem. 93, 94–103.

Melikoglu, M., Lin, C.S.K., Webb, C., 2015. Solid state fermentation of waste bread pieces by *Aspergillus awamori*: Analysing the effects of airflow rate on enzyme production in packed bed bioreactors. Food Bioprod. Process. 95, 63–75.

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Modenbach, A.A., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids

loadings - A review. Biomass and Bioenergy 56, 526–544.

Möller, J., 2009. Gravimetric determination of acid detergent fiber and lignin in feed: Interlaboratory study. J. AOAC Int. 92, 74–90.

Moreno, A.D., Ibarra, D., Alvira, P., Tomás-Pejó, E., Ballesteros, M., 2015. A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit. Rev. Biotechnol. 35, 342–354.

Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., Marova, I., 2014. Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. Process Biochem. 49, 1409–1414.

Oliveira, F.C., Freire, D.M.G., Castilho, L.R., 2004. Production of poly(3-hydroxybutyrate) by solid-state fermentation with *Ralstonia eutropha*. Biotechnol. Lett. 26, 1851–1855.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: Inhibitors and mechanisms of inhibition. Bioresour. Technol. 74, 25–33.

Ponsá, S., Gea, T., Sánchez, A., 2010. Different indexes to express biodegradability in organic solid wastes. J. Environ. Qual. 39, 706–712.

Ramadas, N. V., Sindhu, R., Binod, P., Pandey, A., 2013. Development of a novel solid-state fermentation strategy for the production of poly-3-hydroxybutyrate using polyurethane foams by *bacillus sphaericus* NII 0838. Ann. Microbiol. 63, 1265–1274.

Raza, Z.A., Abid, S., Banat, I.M., 2018. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. Int. Biodeterior. Biodegrad.

Rodriguez-Perez, S., Serrano, A., Pantión, A.A., Alonso-Fariñas, B., 2018. Challenges of scaling-up PHA production from waste streams. A review. J. Environ. Manage. 205, 215–230.

Santos da Silveira, J., Durand, N., Lacour, S., Belleville, M.P., Perez, A., Loiseau, G., Dornier, M., 2019. Solid-state fermentation as a sustainable method for coffee pulp treatment and production of an extract rich in chlorogenic acids. Food Bioprod. Process. 115, 175–184.

Sathiyanarayanan, G., Kiran, G.S., Selvin, J., Saibaba, G., 2013. Optimization of polyhydroxybutyrate production by marine *Bacillus megaterium* MSBN04 under solid state culture. Int. J. Biol. Macromol. 60, 253–261.

Sfalcin, P., Lunelli, F.C., Maleski, T.P.S., Foletto, V.S., Souza, M., Zimmermann, E., Dal Prá, V., Foletto, E.L., Kuhn, R.C., Mazutti, M.A., 2015. Glucose obtained from rice bran by ultrasound-assisted enzymatic hydrolysis. Ing. e Investig. 35, 61–66.

Sharma, P., Bajaj, B.K., 2016. Economical production of poly-3-hydroxybutyrate by *bacillus cereus* under submerged and solid-state fermentation. J. Mater. Environ. Sci. 7, 1219–1228.

Soccol, C.R., Ferreira da Costa, E.S., Junior Letti, L.A., Karp, S.G., Woiciechowski, A.L., de Souza Vandenberghe, L.P., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71.

The US Department of Agriculture, The US Composting Council, 2001. Test Methods for the Examination of Composting and Compost, First ed. ed. Edaphos international, Houston, Texas.

Uday, U.S.P., Majumdar, R., Tiwari, O.N., Mishra, U., Mondal, A., Bandyopadhyay, T.K., Bhunia, B., 2017. Isolation, screening and characterization of a novel extracellular xylanase from *Aspergillus niger* (KP874102.1) and its application in orange peel hydrolysis. Int. J. Biol. Macromol. 105, 401–409.

Yazid, N.A., Barrena, R., Komilis, D., Sánchez, A., 2017. Solid-state fermentation as a

novel paradigm for organic waste valorization: A review. Sustainability. 9, 1–28.

Yu, J., Stahl, H., 2008. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. Bioresour. Technol. 99, 8042–8048.

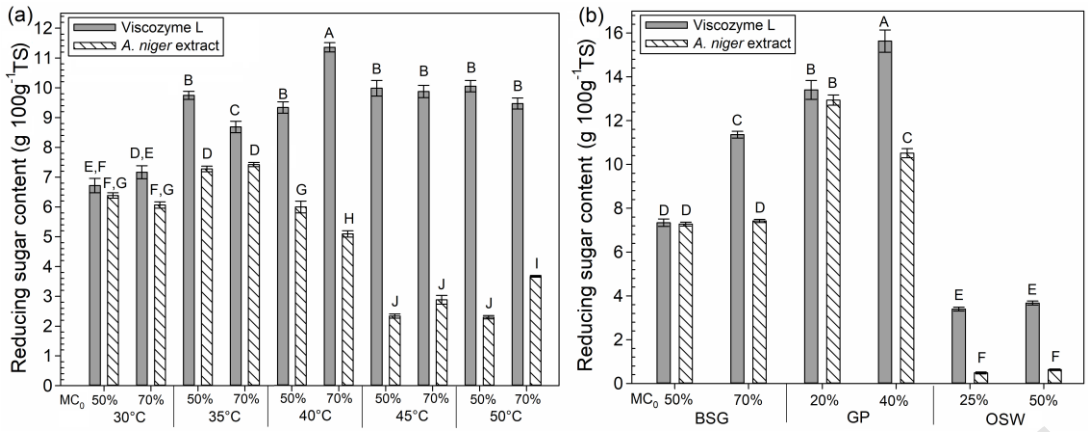


Figure 1. Reducing sugars content (RS) after the solid-state enzymatic hydrolysis of the evaluated residues. (a) Temperature effect using BSG as a reference, and (b) initial moisture content (MC_0) and type of enzymatic extract effects. BSG: Brewer's spent grain; GP: grape pomace; OSW: olive-mill solid waste; TS: total solids base. Different capital letters mean significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

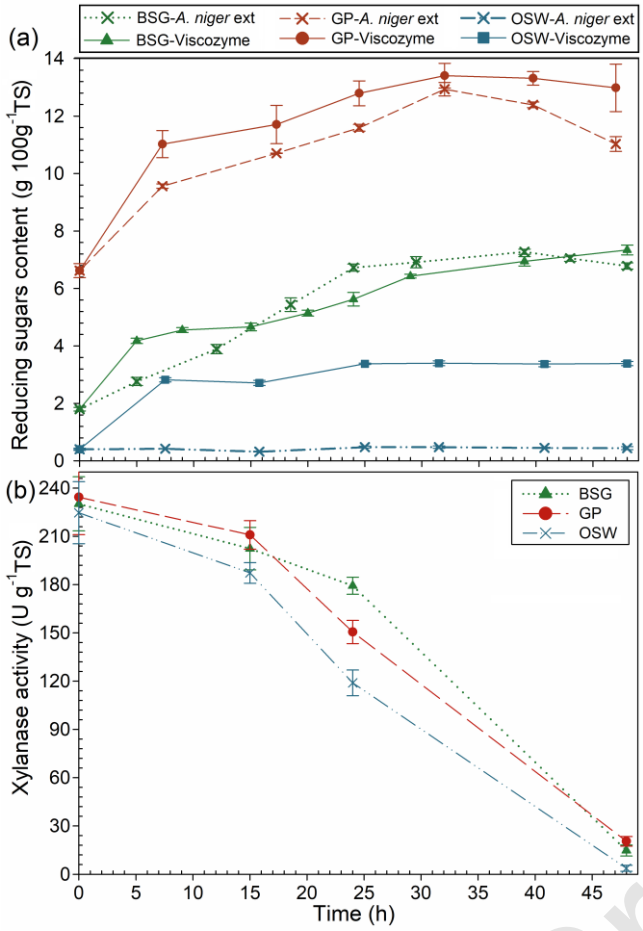


Figure 2. Time-course of the solid-state enzymatic hydrolysis at low MC_0 of the evaluated residues. (a) Reducing sugars content (RS), and (b) remaining xylanase activity (U) of the self-produced enzymatic extract from *A. niger*. MC_0: Initial moisture content; BSG: brewer's spent grain; GP: grape pomace; OSW: olive-mill solid waste; TS: total solids base.

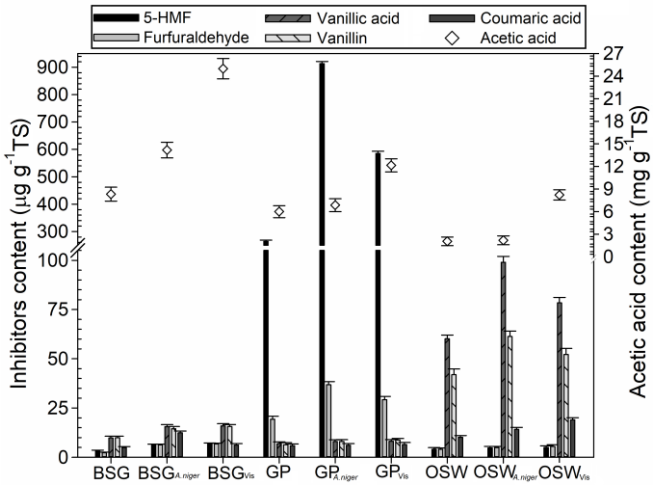


Figure 3. Inhibitor compounds content in the evaluated substrates. BSG: brewer's spent grain; GP: grape pomace; OSW: olive-mill solid waste; *A. niger*: hydrolysate from *A. niger* self-produced extract; Vis: hydrolysate from Viscozyme L; TS: total solids base.

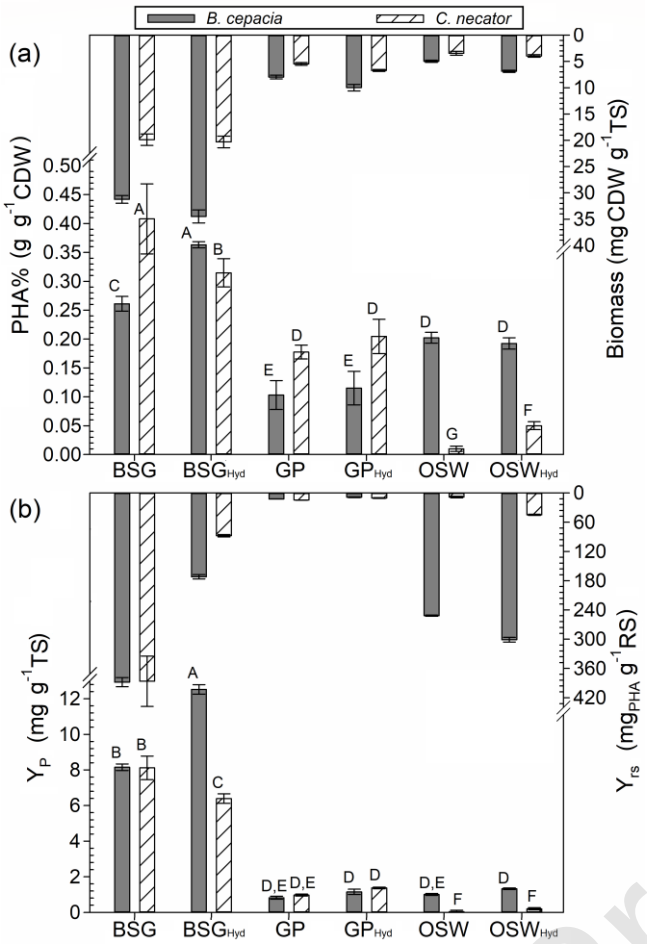


Figure 4. Performance indices of the solid-state fermentation for producing PHA using the evaluated residues. (a) PHA accumulation (PHA%) and produced biomass, (b) substrate yield (Y_p) and sugars yield (Y_{rs}). BSG: brewer's spent grain; GP: grape pomace; OSW: olive-mill solid waste; Hyd: hydrolyzed residue; CDW: cell dry weight; TS: total solids base; RS: reducing sugars. Different capital letters mean significant differences between the evaluated groups ($p < 0.05$) based on the Tukey test.

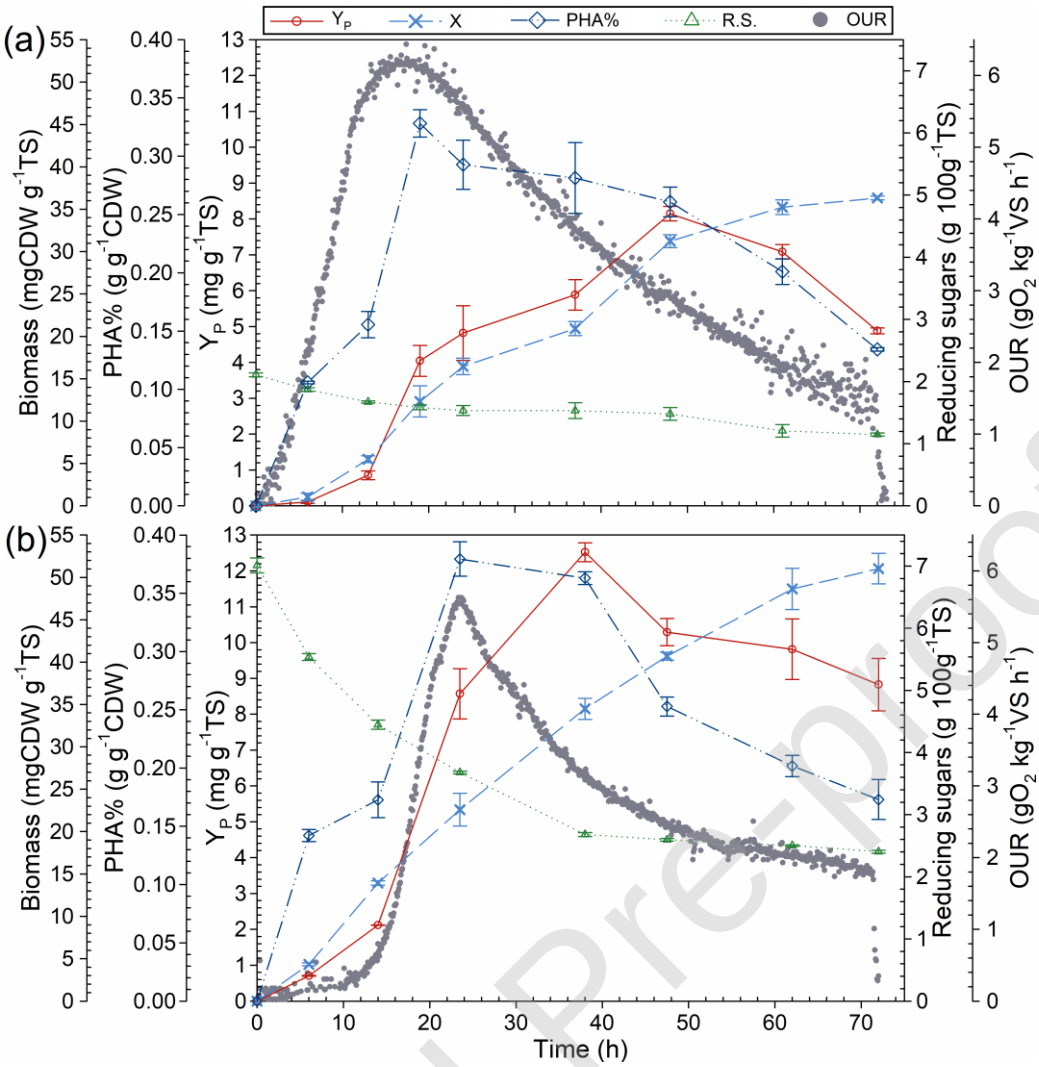


Figure 5. Time-course of the solid-state fermentation for producing PHA using *B. cepacia*. (a) With BSG, (b) with BSG hydrolysate. Y_p: substrate yield; OUR: oxygen uptake rate; X: produced biomass; PHA%: PHA accumulation; RS: reducing sugars; CDW: cell dry weight; TS: total solids base; VS: volatile solids base.

Table 1. Characterization of the evaluated residues.

Parameter	**Brewer's spent grain**	**Grape pomace**	**Olive-mill solid waste**
Total Kjeldahl nitrogen ($g\ kg^{-1}$)	30.4 ± 4.3	8.1 ± 1.5	9.7 ± 2.0
Oxidizable carbon ($g\ kg^{-1}$)	667 ± 23	624 ± 23	437 ± 5
Total phosphorus ($g\ kg^{-1}$)	17.7 ± 0.7	169 ± 13	57.2 ± 10.1
C:N (mass basis)	21.9 ± 2.1	77.0 ± 3.2	45.1 ± 1.5
Total solids (%)	21.9 ± 0.1	54.6 ± 0.8	91.4 ± 0.3
Volatile solids (% dry basis)	92.1 ± 0.6	81.6 ± 0.4	80.7 ± 0.2
Water holding capacity ($gH20\ g^{-1}$)	4.3 ± 0.3	0.64 ± 0.01	0.89 ± 0.05

pH (1:10)	5.5 ± 0.2	3.6 ± 0.3	5.4 ± 0.2
Reducing sugars (g $100g^{-1}$)	1.9 ± 0.3	6.8 ± 0.4	0.38 ± 0.02
Bulk density at WHC ($kg\ L^{-1}$)	0.23 ± 0.03	0.18 ± 0.04	0.50 ± 0.03
Particle size (mm)	1.0-10.0	1.0-15.0	1.0-4.0
Cellulose (%)	21.9 ± 0.2	11.0 ± 0.4	29.5 ± 0.6
Hemicellulose (%)	40.1 ± 0.3	3.5 ± 0.1	17.8 ± 0.7
Lignin (%)	9.1 ± 0.1	16.9 ± 0.6	36.0 ± 0.9

Table 2. Some representative system for producing PHA from lignocellulosic biomasses.

	Strain	Biomass	Treatment	PHA (%)	Y_{rs} (mg g^{-1}sug)	P_p (g kg^{-1} h^{-1})[a]	Reference
SmF	*C. necator*	Sugarcane bagasse	P/H/NS	65.0	NA	0.080	(Yu and Stahl, 2008)
	B. sacchari	Wheat straw	P/H/NS	60.0	190	0.059	(Cesário et al., 2014)
	B. cepacia	Spent coffee	P/H/NS	49.1	270	0.037	(Obruca et al., 2014)
	C. necator	Wheat bran	P/H/NS	62.6	319	0.255	(Annamalai and Sivakumar, 2016)
	B. cepacia	Rice husk	P/H/NS	62.0	118	0.049	(Heng et al., 2017)
	C. necator	Grape waste	P/H/NS	63.0	130	0.176	(Kovalcik et al., 2020)
	C. necator	Miscanthus	P/NS	44.0	140	0.028	(Bhatia et al., 2019)
SSF	*C. necator*	Soy cake	NS	39.2	NA	0.082	(Oliveira et al., 2004)
	B. megaterium	Tapioca waste	NS	56.8	NA	0.180	(Sathiyanarayanan et al., 2013)

B. cereus	Malt	NS	NA	NA	0.300	(Sharma and Bajaj, 2016)
B. cepacia	BSG	NT	26.1	388	0.170	This study
B. cepacia	BSG	SSEH	36.2	172	0.329	This study

SmF: Submerged fermentation systems; SSF: Solid-state fermentation systems; Y_{rs}: sugars yield; P_p: PHA productivity; P: pretreatment for lignin removal; H: hydrolysis in liquid culture; NS: nutrients addition to hydrolysate; SSEH: solid-state enzymatic hydrolysis; NT: no treatment; NA: not available; BSG: brewer's spent grain.

[a]Productivity in SmF was converted into mass base by assuming a cultures density of 1kg L^{-1} as suggested by Castilho et al. (2009) to compare these against SSF properly.