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Bioconversion of Giant Cane for Integrated Production of Biohydrogen, Carboxylic Acids, and Polyhydroxyalkanoates (PHAs) in a Multistage Biorefinery Approach

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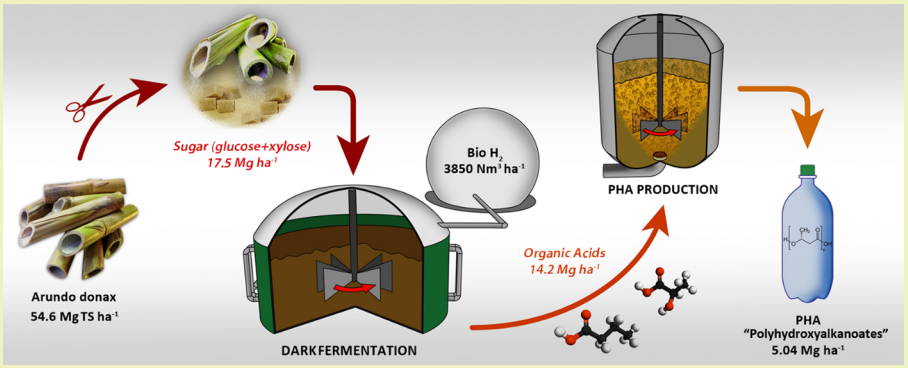
Supporting Information



ABSTRACT: A highly productive *Arundo donax* L. clone (Clone AD-20) was produced at full field to give 54.6 Mg total solids biomass Ha^{-1}. Biomass was chemically and enzymatically pretreated, recovering 13.9 Mg Ha^{-1} of glucose and 3.6 Mg Ha^{-1} of xylose, i.e., 3.5−4.5 more than yield typically obtained from corn stover or switchgrass. The subsequent fermentation of the liberated sugars to organic acids (OA) by dark fermentation generated yields of 3850 Nm^3 Ha^{-1} of biohydrogen and 14.2 Mg Ha^{-1} of OAs. OAs were then used as a feed to produce polyhydroxyalkanoates (PHA), with 3-hydroxybutyrate the major monomer present (PHB > 95% PHA), from a biological process using mixed microbial culture producing 5.04 Mg Ha^{-1} of PHA. An initial economic analysis indicated that this multistage biorefinery approach would result in a net revenue of 10,415 € Ha^{-1}, which is approximately 9-fold greater than that obtained by a traditional biorefinery producing bioethanol.

KEYWORDS: *Arundo donax L., Biohydrogen, Biorefinery, Organic acids, Polyhydroxyalkanoates, Sustainability*

INTRODUCTION

A biorefinery is defined as the sustainable processing of biomass feedstock into a spectrum of marketable products and energy using a multistep processing approach.[1−3] Different kinds of feedstocks, such as residues from agricultural, industrial, and urban sectors can be used in the biorefinery,[4,5] but in any case, the biorefinery should be designed to guarantee its sustainability in terms of environment, economy, and societal benefit in all of the processes involved.[5]

Certain lignocellulosic feedstock are now becoming more attractive because of the lack of competition with the food production sector[6] and also due to the possibility of valorizing the entire aerial biomass.[7] Lignocellulosic biomasses are known to be sources of lignin, hexoses (C6), and pentoses (C5) that can be converted into large amounts of chemicals and energy carriers.[2,8]

Arundo donax L., or giant cane, is arousing interest in the scientific community for its use as a feedstock for energy sources and chemical products in a biorefinery model.[9] *A. donax* is a perennial herbaceous plant widespread in different environments, and it can grow on many types of soils.[10] One of the most interesting characteristics of this plant is the amount of biomass achieved per cultivated surface area, which is much higher than that of other traditional crops. An average

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production of 37.7 Mg total solid (TS) Ha^{-1} was reported for a long-term monitoring experiment in central Italy.[11] This data was recently exceeded by newer studies reporting yields of 60−70 Mg TS Ha^{-1} obtained in an experimental field in northern Italy.[12,13] The limited investment and the low maintenance costs necessary for *A. donax* cultivation, due to low agronomic interventions, low water, and fertilizer requirements and no phytosanitary product applications,[13] makes this crop a suitable candidate for the development of an economically sustainable biorefinery.

Many studies have highlighted the possibilities of using giant cane biomass for the production of bioenergy, biocombustibles, chemicals, and other products. Biogas[12−15] and bioethanol[16] production, and energy from combustion processes,[17] have been previously investigated for this crop. In addition, the production of chemical compounds with an economic value, such as xylo-oligosaccharides,[18] levulinic acid, γ-valerolactone,[19,20] *p*-hydroxyphenyl-propane,[21] and several alkaloids,[22] have all been reported for *A. donax*, as well as paper[23] and activated carbon[24] production from the raw biomass. Nevertheless, there are very few available examples of the biorefinery approach using *A. donax*. An interesting example of a biorefinery from this crop is represented by the simultaneous recovery of lignin, hemicellulose, cellulose nanocrystals, and silica by using sequential steps of chemical treatments and then separating the solid and liquid fractions.[25] Again, coupled with chemical and biological treatments of giant cane in order to deconstruct fibers is obtaining furfural and levulinic acids and recycling the solid residues.[26]

The cellulose and hemicellulose contents in *A. donax*, coupled with the high crop yield, represent a significant potential source of glucose, xylose, arabinose, and lignin.[15] Overcoming the natural recalcitrance of biomass by chemical pretreatment, the production of 11 Mg Ha^{-1} of glucose plus 4.84 Mg Ha^{-1} of xylose was reported.[15] This large amount of sugar can then be used for different purposes, such as the production of organic molecules. Carboxylates are dissociated organic acids, which are characterized by the presence of at least one carboxyl group. Dark fermentation (DF) has been proposed as a low-cost bioprocess[27] to produce short length organic acids (OA) from wastes or biomass, i.e., the carboxylate platform;[28] together with H_2 this latter is to be valorized as coproducts of OAs. The demand for polymers is continuously growing (e.g., 50 million Mg in Europe in 2016), and its negative impact due to its accumulation in the environment is well documented in the literature.[29] Recycling could limit the environmental impact of these plastics, but only a small portion is currently recycled.[30] As a result, bioplastic began to be substituted for fossil-fuel-derived plastic in the market,[30] and although it represents only 1% of the total plastic currently produced in the world, demand is expected to grow from around 2.05×10^6 Mg in 2017 to approximately 2.44×10^6 Mg in 2022.[31] Biopolymers such as polylactic acid (PLA) and polyhydroxyalkanoate (PHA) are the main drivers of this growth.[31]

Polyhydroxyalkanoates are biodegradable polyesters of microbial origin having chemical−physical properties that are not very different from those of traditional plastics,[32] and they can replace common plastics for several applications, such as packaging films, disposable bulk materials, and paper coatings. PHA production currently has high costs due to the substrate used and the use of pure microbial cultures.[33] Cost reductions can be achieved through the use of mixed microbial cultures (MMC)[34] that convert short-length organic acids to produce PHAs.[35]

This work investigates the potential for producing PHAs from organic acids via dark fermentation using sugars obtained from *A. donax* and evaluates the overall yields and productivities achieved by converting this crop in comparison with those achieved from more traditional bioenergy crops. A complete mass balance referring to the cropped surface (Ha), i.e., m^3 of H_2 Ha^{-1}, Mg of OAs Ha^{-1}, and Mg of PHA Ha^{-1}, and a rough economic (revenue, € Ha^{-1}) analysis are presented as useful data to investigate the sustainability and viability of a such biorefinery.

EXPERIMENTAL SECTION

Biomass Production and Characterization. *Arundo donax* L. biomass was cultivated and harvested in the A. Menozzi experimental farm (University of Milan, Landriano, PV, Italy, N 45°18′; E 9°15′). The clone used in this work (denominated AD 20 clone, UNIMI) was chosen both because of its optimal agronomic performance and its aptitude to be pretreated and subsequently saccharified.[13] The clone was harvested at the end of the growing season (October 2016), in the fourth year from transplantation (April 2012). Harvested biomass was air-dried at 60 °C and then milled to 2 mm particle size for further analyses.

Biomass was characterized using the standard National Renewable Energy Laboratory (NREL) procedures for compositional analysis.[36] Biomass sugar determination was performed by a high-pressure liquid chromatography binary pump (Binary pump 1525, Waters) equipped with a 300 mm × 7.8 mm Aminex HPX-87H column and refractometer (Refractive Index 2410, Waters). A 0.004 mol L^{-1} H_2SO_4 mobile phase was used with an operational temperature of 60 °C at a flow rate of 0.6 mL min^{-1}. Data treatment was carried out with Breeze Software (Waters, Milford, MA, USA).

Biomass Pretreatment and Hydrolysis. In order to convert the biomass into "ready-to-use" sugars for the subsequent steps, ionic liquid (IL) pretreatment and enzymatic saccharification processes were performed by using 1-ethyl-3-methylimidazolium acetate ($[C_2C_1im][OAc]$) at a temperature of 160 °C for 3 h (severity factor $logR_0$ of 4.02).[15] This pretreatment and IL were chosen as the gold standard as previously reported,[15] taking into consideration that this no-cost-effective IL was comparable, in terms of sugar yield efficiency, with recently proposed low-cost ILs that were reported to be effective and competitive with the cheapest chemical pretreatment.[37]

Enzymatic hydrolysis was carried out as previously reported;[15] in brief, the enzymatic hydrolysis was performed at 50 °C and at 300 rpm in an incubator. Biomass was diluted to a glucan loading of 2% (w/w) in a total batch volume of 2 mL with a 50 mmol L^{-1} sodium citrate solution at pH 4.8. The enzyme loading was of 20 mg of protein g^{-1} glucan. The enzymes used were cellulase CTec2 and hemicellulase HTec2 mixtures (Novozymes, Franklinton, NC, USA), and the respective protein concentrations were 186.6 and of 180.1 mg mL^{-1}. The ratio of the two enzymes was 10:1 of cellulase and hemicellulase, respectively.

After hydrolysis, the biomass−buffer mixture was filtered in order to separate the insoluble fraction; the recovered liquid fraction was characterized by determining the principal chemical parameters with particular reference to the sugars content as above described. In total, 37 L of filtered hydrolyzed AD20 (AH) was produced and stocked in a freezer for the subsequent dark fermentation process.

Dark Fermentation for Biohydrogen Production. Dark fermentations were carried out in a continuously stirred tank reactor (CSTR) with a total capacity of 4 L and a working volume of 1.5 L under continuous agitation (200 rpm). Biological processes were performed at thermophilic conditions (55 °C) since this choice allowed better slurry mixing, lower slurry viscosity, and microbial kinetics improvement that made for faster sugar fermentation.[38] The pH range was set to 5.5−5.8 and automatically controlled and

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maintained by adding KOH 3 mol L^{-1}. Before biological processes were initiated, the headspace was fluxed with N_2 to ensure anaerobic conditions.

The inoculum used was a digestate coming from a full-scale anaerobic digester (55 °C and hydraulic retention time, HRT, of 40 d) fed mainly with corn silage. Before its use, the digestate was thermally pretreated (1 h at 100 °C) in order to eliminate the methane-producing microorganisms and to isolate the spore-forming bacteria. The pH of the inoculum was adjusted to 5.5−5.8 with the aim of inactivating bacterial consumers of hydrogen.[39]

Four CSTR reactors were prepared, and they were initially operated for 3 days in batch mode by feeding them pure glucose in order to acclimate the hydrogen-producing bacteria. Each reactor was set up with one liter of inoculum and the remaining half-liter was integrated with the feeding solution containing an organic substrate (glucose), macronutrients, and micronutrients.[40,41] After the batch-mode period, two reactors were started to be continuously fed (continuous mode) with the hydrolyzed *Arundo donax* (AH) substrate and the other two with glucose (Control).

Glucose was used as the "standard control substrate" in order to get data about dark fermentation performances by comparing H_2 yield (mol H_2 mol^{-1} glucose) obtained in this work with those from the literature.

Process parameters were as follows: organic loading rate (OLR) of 13 g substrate (glucose for the control) d^{-1} (OLR = 8.6 g L^{-1} d^{-1}) and hydraulic retention time (HRT) of 2 days.[42,43] In addition, 50 mL d^{-1} of fresh inoculum, i.e., 10% of the total work volume (v/v), was added to stimulate the microbial activity.[44] Processes were brought to stability; i.e. H_2 production was observed to be constant (8th day), and then monitoring started for the next 25 days, for a total of 33 days.

The effluents produced daily were sampled and analyzed. Total solids (TS), pH, and ammonia content (NH_3-N) were detected using standard methods.[45] Organic acid concentration and speciation were determined by using high-pressure liquid chromatography (HPLC) equipped with a UV-detector (UV detector 2487, Waters) in isocratic mode (flow rate of 0.60 mL min^{-1} and λ of 210 nm). A Rezex ROA−organic Acid H+ (8%) Lc column, 300 mm × 7.8 mm, was used. Total sugar content in the media was daily determined by HPLC, as previously reported.

The gas produced was collected in a dallying gas sampling bags (Supel-Inert multi-layer foil), and gas volume was measured with a Ritter drum (Gas Meter TG0.5/5, Germany). The percentage of H_2 (v/v) was measured by gas chromatography (3000A-μGC, AGILENT-SRA Instruments, USA) with a thermal conductivity detector (TCD); samples were analyzed in triplicate for each reactor.

Spent liquid fractions (SLF) rich in organic acid (OAs) coming from the dark fermentations were retained, stocked, and mixed.

All data were statistically analyzed by one-way ANOVA to compare means with a level of significant difference set at $p < 0.05$; the Tukey test was used as the method to compare means. All statistical analyses were performed by using SPSS software (SPSS Statistics v21.0, IBM, Armonk, NY, USA).

Polyhydroxyalkanoates (PHA) Production. SLF coming from the dark fermentation of AH formed the substrate to produce PHA. Before its use, SLF was submitted to a treatment consisting of centrifugation at 20,000 g for 15 min to reduce suspended organic carbon. The supernatant produced was used for PHA-storing bacteria selection after the C:N ratio adjustment to 10 by using NH_4Cl and its dilution with deionized water to a final COD of 1900 mg L^{-1} .[46]

The substrate used for PHA accumulation was the same as that used for bacterial selection, but in this case, no NH_4Cl was added since it has been reported that N starvation can lead to a greater conversion of carbon into PHA because of cell growth limitation.[47] Moreover, the substrate used for PHA accumulation was less diluted than that used for bacterial selection to avoid an excessive dilution of the biomass during the accumulation tests, with a final COD of ∼7500 mg L^{-1}.[48]

The PHA-producing bacteria selection (mixed microbial culture) (MMC) was performed by using an inoculum constituted by an activated sludge (8 g of total suspended solids L^{-1}) collected from the secondary sedimentation tank of a wastewater treatment plant (5.2×10^5 equivalent inhabitants) located at Peschiera Borromeo (Milan, Italy). The selection process, lasting for 50 days, was done by carrying out 10 sludge retention time cycles (SRT) of 5 d each, and it was carried out in a sequencing batch reactor (SBR) (Tecnovetro, Monza, Italy) with a working volume of 1 L and by adopting an aerobic dynamic feeding (ADF) strategy as previously reported.[46] The selection trend was monitored by determining the duration of the feast phase achievable by using the dissolved oxygen (DO) concentration in the selection media measured by an optical probe (FDO 925, WTW, Germany).[48]

In particular, the feast (h) to famine (h) ratio (F/F ratio) was calculated as the ratio between the lengths (h) of the two phases. For a correct selection of the PHA-storing bacteria, the F/F ratio had to be equal or less than 0.33.[49] The organic loading rate (OLR) was kept close to 1900 mg COD L^{-1} d^{-1}, and the C:N:P ratio was about 100:10:4 mmol C:mmol N:mmol P. For every SRT, a cycle was monitored in order to evaluate the performance of the selected culture.

The ability of the MMC to accumulate PHA was assessed by feed-batch tests carried out in a 500 mL working volume flask, with continuous aeration and stirring.[46] Six accumulation tests were performed taking selected biomass from SBR between the 7th and the 10th SRT. The biomass collected after PHA accumulation tests was treated, and PHA was extracted as previously described.[35] The substrates fed during the selection and accumulation processes were characterized in terms of pH, TS, VS, chemical oxygen demand (COD), organic acids content (acetate, butyrate, lactate, propionate and valerate), TKN, N-NH_4^+, and phosphorus (P) content.

During the selection, samples were taken during the cycle once in each SRT; every sample was characterized in terms of total suspended solids (TSS), volatile suspended solids (VSS), soluble COD, organic acids content, N-NH_4^+ content, and PHA content.

During accumulation trials, samples were taken continuously in order to measure TSS, VSS, soluble COD, organic acids content, and PHA content. Biomass concentration was calculated as VSS according to the standard methods.[48]

TSS and VSS were determined as previously reported.[50] Organic acid concentrations measured on filtered samples (filter diameter of 0.45 μm) were determined by high performance liquid chromatography (HPLC) as previously described.[46] PHAs were determined by GC MS:[46] in this work hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and another for HV using standards (1.5−15 g L^{-1}) of a commercial P(HB-HV) (88%/12%) (Sigma Aldrich, Germany), and corrected using heptadecane as the internal standard (concentration of approximately 0.1 g L^{-1}) (Sigma Aldrich, Germany).

The PHA content in cells was referred to VSS on a mass basis [PHA= (g kg^{-1} VSS)], considering VSS to be constituted by both active biomass (X) and PHA.[48] PHA was converted into COD according to the following oxidation stoichiometry: 1.67 mg COD mg^{-1} HB monomer and 1.92 mg COD mg^{-1} HV monomer.[49]

Acetate, butyrate, and lactate were considered as HB precursors, valerate and propionate as HV precursors.[48] X was calculated on a COD basis considering that 1 g of X contains 1.42 g of COD.[49]

For SBR, the specific COD consumption rate (q_{COD}, mg COD mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of COD consumed during the feast phase and the time needed to deplete it per unit of active biomass. The specific PHA storage rate (q_{PHA}, mg COD_{PHA} mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of PHA stored during the feast phase and the time needed to deplete the COD per unit of active biomass. Specific growth rate (q_X, mg COD_X mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of new active biomass produced during the feast phase and the time needed to deplete the COD per unit of active biomass.[49]

For SBR, PHA yield was calculated as the ratio between the amount of PHA stored expressed as COD and the amount of both

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Table 1. Dark Fermentation: H_2 Production and Chemical Characterization of Feeding and Effluent

		Control		Hydrolyzed *A. donax* (AH)	
		Feeding	Effluent	Feeding	Effluent
Daily Gas Volume	NL H_2 L^{-1} d^{-1}	–	1.92 ± 0.21a[a]	–	1.9 ± 0.2a
H_2	% v/v	–	47.6 ± 2.5a	–	44.6 ± 3.7b
H_2 molar yield	mol H_2 mol^{-1} sugars	–	1.78 ± 0.08a	–	1.70 ± 0.06a
Feeding solution					
OLR	g sugars d^{-1}	13	0	13	0
Sugars					
Glucose	g L^{-1}	17.3 ± 0.05	0	13.9 ± 0.8	0
Xylose	g L^{-1}	0	0	3.5 ± 0.54	0
Arabinose	g L^{-1}	0	0	0	0
Total sugars	g L^{-1}	17.3 ± 0.05a	0	17.4 ± 0.12a	0
Organic acids					
Citrate	mmol L^{-1}	0	0	7.85. ± 0.9	6.47 ± 0.87
Formate	mmol L^{-1}	0	0.41 ± 0.08	0	0.11 ± 0.05
Acetate	mmol L^{-1}	0	37.5 ± 1	0	37.3 ± 2.30
Propionate	mmol L^{-1}	0	0	0	0
Isobutyrate	mmol L^{-1}	0	0.12 ± 0.07	0	0
n-butyrate	mmol L^{-1}	0	38.3 ± 2.9	0	36.5 ± 3.1
Isovalerate	mmol L^{-1}	0	0	0	0
Lactate	mmol L^{-1}	0	9.14 ± 0.7	0	16.5 ± 1
Total OA	mmol L^{-1}	0	85.5 ± 15.8	0	96.9 ± 10.2
pH	–	6.25 ± 0.15a	5.76 ± 0.6a	4.37 ± 0.11b	5.78 ± 0.11a
NH_3	mg L^{-1}	216 ± 12[a]	240 ± 24a	158 ± 15b	232 ± 45a
TKN	g L^{-1}	0.24 ± 0.02a	0.55 ± 0.11a	0.30 ± 0a	0.68 ± 0.08a
C:N	–	29 ± 0a	12 ± 0a	20 ± 0b	13 ± 1.5a

[a]Averages followed by the same letter are not statistically different for a $p < 0.05$.

organic acids (expressed as COD) and COD depleted, i.e. mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$ and mg COD_{PHA} mg^{-1} $COD_{cons.}$). PHA yield was also calculated on organic acids fed expressly COD (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$) and on COD fed (mg COD_{PHA} mg^{-1} COD_{in}). Again, the growth yield was calculated as the ratio between the new biomass produced during the feast phase on a COD basis and the amount of COD depleted (mg COD_X mg^{-1} $COD_{cons.}$), as reported previously.[49]

In the accumulation batches, the specific rates and yields, except for q_X and the growth yield, that were not considered during the accumulation tests were calculated as described before for each pulse. In order to compare different accumulation tests, the average values for the first four pulses and for each parameter were considered.

PHAs produced were characterized by both ^{13}C- and ^{1}H NMR spectroscopies. In particular, solid-state ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at 75.47 MHz, using a 4 mm × 21 mm cylindrical zirconium rotor spun at 11,000 Hz to avoid the side bands. The ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectra were acquired using a recycle delay of 15 s, ^{1}H 90 pulse length of 3.5 μs, 1 m contact time, acquisition time of 35 ms, and from 1 to 2 K scans. The ^{13}C single pulse excitation (SPE) NMR spectra were recorded with delays of 2 s and 1−2 K scans. The chemical shifts were recorded relative to tetramethylsilane via benzene as a secondary reference. NMR experiments were performed on a Bruker 500 MHz AVANCE III NMR spectrometer (Bruker GmbH, Germany) with a 5 mm TCI cryoprobe. Deutered chloroform (99.6%, Sigma Aldrich) was used as the solvent.

^{1}H NMR spectra were recorded at 303 K using a recycle delay of 10 s, 64 K fid size, and 16 scans. 2D COSY were recorded at 303 K with a recycle delay of 2 s and 8 scans. Multiple bond ^{1}H-^{13}C heteronuclear correlated experiment (HSQC) data were collected as 340 TD experiments each with 2000 complex data points and 24 scans.

Molecular weights were detected by HP-SEC/TDA. The HPLC equipment consisted of a Viscotek system (Malvern Instruments Ltd., Malvern, UK) equipped with a Knauer HPLC pump K501 and a Biotech Degasi GPC degassing device. The detector system was a Viscotek model 302 triple detector array (TDA), which is composed of a laser light scattering detector (90° and 7°; wavelength 670 nm), refractive index (RI) detector (cell volume of 12 μL; light emitting diode (LED) at 660 nm wavelength), and viscosimeter detector (four capillaries with a differential Wheatstone bridge configuration). A PL GEL 20 um MIXED A column (7.5 mm × 300 mm) was used. THF (tetrahydrofuran for liquid chromatography LiChrosolv, Sigma-Aldrich) was used as the mobile phase at a flow rate of 1 mL min^{-1}. Columns, injector, and detectors were maintained at 35 °C. Samples were dissolved in chloroform at concentrations of 2−6 mg mL^{-1} and filtered on a 0.2 μm membrane before injection; injection volume was 100 μL. The system was calibrated with the PS narrow standard of known Mw, polydispersity, and intrinsic viscosity (Malvern PolyCAL PS std 105,000). Using extracted PHA (Arundo) at different concentrations (1.3, 2.6, 5.2 mg mL^{-1}), the differential refractive index increment (dn/dc) value was found to be equal to 0.047 and used for further calculations.

RESULTS AND DISCUSSION

Biomass Pretreatment, Enzymatic Saccharification, and Chemical Characterization. Chemical characterization of *A. donax* biomass revealed a glucose, xylose, arabinose, and lignin content of 36.1 ± 0.2%, 19.1 ± 0.3%, 1.9 ± 0.2%, and 24.3 ± 0.7% TS, respectively (Table S1), in line with data reported in previous work.[15] Taking into consideration the conversion factors to be applied to convert sugars into polymers, i.e., 0.9,[51] cellulose and hemicellulose contents were of 32.5 ± 0.2% and 17.2 ± 0.3% TS, respectively, in agreement with previous data.[52]

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Table 2. Organic Acids Yield Obtained in This Work by Dark Fermentation in Comparison with Data from the Literature

Feedstock	Culture	HRT (d)	Operation modality	OLR	Temperature (°C)	Total OA[a] (g L^{-1})	Yield (g OA g-1 COD)	Refs
FW[b]	Mixed	3−3.3	continuous	16.8−17 kg VS m^{-3} d^{-1}	55	12.3−13.7	0.22−0.23	63
WAS[c]	Mixed	4.6−5.9	continuous	1.2−1.9 kg VS m^{-3} d^{-1}	35	3.2−7.5	0.21−0.33	63
FW	Mixed	2	continuous	15 kg COD m^{-3} d^{-1}	28	6.3	0.42	64
AH[d]	Mixed	2	continuous	13 g_{sugar} d^{-1} (8.6 kg_{sugar} m^{-3} d^{-1})	55	7	0.64	This study

Feedstock	Culture	HRT (d)	Operation modality	OLR	Temperature (°C)	Total OA[a] (g L^{-1})	Yield (g OA g^{-1} VS)	Refs
WAS+FW	Mixed	4	continuous	14.4 g L^{-1}	20	8.33	0.57	65
FW	Mixed	8	continuous	13 g L^{-1}	35	28.9−30	0.29−0.3	66
CS[e]/PM[f]	Mixed	6	continuous	2%−5% TS	37	31.8	0.55	67
KW[g]	Mixed	4	batch	48.2 g VS L^{-1}	37	9.81	0.2	68
WAS[h]+HPB	Mixed	21	continuous	19.4 g VS L^{-1}	35	5.3	0.27	69
FW	Mixed	5	continuous	11 g TS L^{-1} d^{-1}	35	21.4	0.41	70
Xylose	Pure	22	continuous	50 g L^{-1}	36	59	0.99	71
AH	Mixed	2	continuous	13 g_{sugar} d^{-1} (8.6 g_{sugar} L^{-1} d^{-1})	55	7	0.81	This study

[a]OA: organic acids. [b]FW: food waste. [c]WAS: waste activated sludge. [d]AH: arundo hydrolyzed. [e]CS: corn stover. [f]PM: pig manures. [g]KW: kitchen wastes. [h]HPB: henna plant biomass.

IL pretreatment determined a TS loss because of the partial hemicellulose and lignin solubilization due to the pretreatment.[53] As consequence of that, pretreated biomass showed an increase in glucose concentration of 45.8 ± 1% TS and a slight decrease in xylose of 18.7 ± 1.3% TS. Lignin content reduced to 19.4 ± 3.1% TS (Table S1).[15] The efficiency of the pretreatment was highlighted by both the high percentage of lignin removal after IL pretreatment, i.e., 45.6 ± 1.8% lignin and cellulose conversion to simple glucose after enzymatic hydrolysis, i.e., 71.2 ± 11.5% cellulose present in the *A. donax*. This result underlined a high performance of the pretreatment performed, in agreement with our previous findings and comparisons made with other lignocellulose crops (e.g., switchgrass).[15] Yield obtained was in line and in the range with data previously reported for *A. donax* obtained by using different pretreatment approaches, although higher sugar yields have been reported in the literature. For example a maximum glucose yield of 74.6% cellulose was obtained by using dilute oxalic acid pretreatment,[54] and yields ranging from 48.41% to 93.77% of cellulose were reported for a crop pretreated with ammonia .[55] These results were obtained, generally, by adopting less severe conditions than that considered in this work, indicating that a consideration on pretreatment must be made adopting different strategies (testing different pretreatments) able to enhance sugar yield and preventing xylan losses. Hemicellulose conversion to xylose, in fact, was less efficient, i.e., 32.8 ± 3.6% hemicelluloses (xylan losses of 67.22% hemicelluloses) due to the loss of part of this polymer during pretreatment (Table S2).[15] There was not any direct evidence for xylan degradation able to explain this trend. However, probably xylans were partially solubilized because of the IL and high temperature used, and both might be responsible for the disruption of intra/intermolecular H-bonding within cellulose and hemicellulose as previously reported.[15]

After biomass pretreatment and saccharification, the liquid fraction containing the solubilized sugars was used as feed for the subsequent dark fermentation to produce organic acids and contained 13.9 ± 0.8 g L^{-1} of glucose and 3.5 ± 0.5 g L^{-1} of xylose.

Monitoring the Dark Fermentation Process. The gas produced during dark fermentation was primarily composed of H_2 and CO_2. The average H_2 content for control and AH tests was 47.6 ± 2.5% (v/v) and 44.6 ± 3.7% (v/v), respectively; no CH_4 production was detected during tests in any reactors. Gas production was stable and similar for all substrates tested, with an average H_2 production of 1.64 ± 0.26 and 1.57 ± 0.18 NL L^{-1} d^{-1} for the control and AH reactors, respectively (Table 1). The control experiment presented an H_2 molar yield of 1.78 ± 0.08 mol H_2 mol^{-1} sugars. This yield was higher than that previously reported, on average, for fermented sugar-based substrates (1.48 ± 0.66 mol H_2 mol^{-1} sugars; n = 18) (Table S2), indicating that the dark fermentation performed was well designed. Hydrogen yield obtained for hydrolyzed *A. donax* was observed to be 1.7 ± 0.1 mol H_2 mol^{-1} sugars, in agreement with that obtained for the control, and they were not statistically different.

Substrates fed during DFs trial showed differences for the pH, NH_3, and TKN contents and C:N ratio. Parameters were in the optimal range. It seemed they did not affect biological processes, as was confirmed by both the similar H_2 yields obtained and by considering that after the dark fermentation all these parameters were similar, and no statistical differences were registered (Table 1).

Dark fermentation in this work was designed not only to produce H_2, but also OAs to be used as the substrate to produce PHAs. For the control, the main OAs produced were n-butyrate (38.3 ± 2.9 mmol L^{-1}) and acetate (37.5 ± 1.0 mmol L^{-1}), followed by lactate (9.14 ± 0.7 mmol L^{-1}). Hydrolyzed *A. donax* gave similar OAs profile after DF; i.e., acetate was the main OA (37.3 ± 2.3 mmol L^{-1}) followed by n-butyrate (36.5 ± 3.1 mmol L^{-1}) and lactate (16.5 ± 1.0 mmol L^{-1}). The butyrate and acetate concentrations obtained in this study were comparable with those found previously, i.e., 30 and 40 mmol L^{-1} of butyrate and acetate, respectively.[56]

A high concentration of organic acids has been reported to inhibit DF, and an OA content between 33 and 63 mmol L^{-1} has been reported to reduce gas production,[57] with particular reference to the presence of butyrate.[58] Nevertheless, good results obtained in this work, i.e., high H_2/sugar molar ratio (Table S2), seemed to exclude any toxicity effect by OAs on the fermentation processes. A restricted amount of formate was observed both for the control test (0.41 ± 0.08 mmol L^{-1}) and

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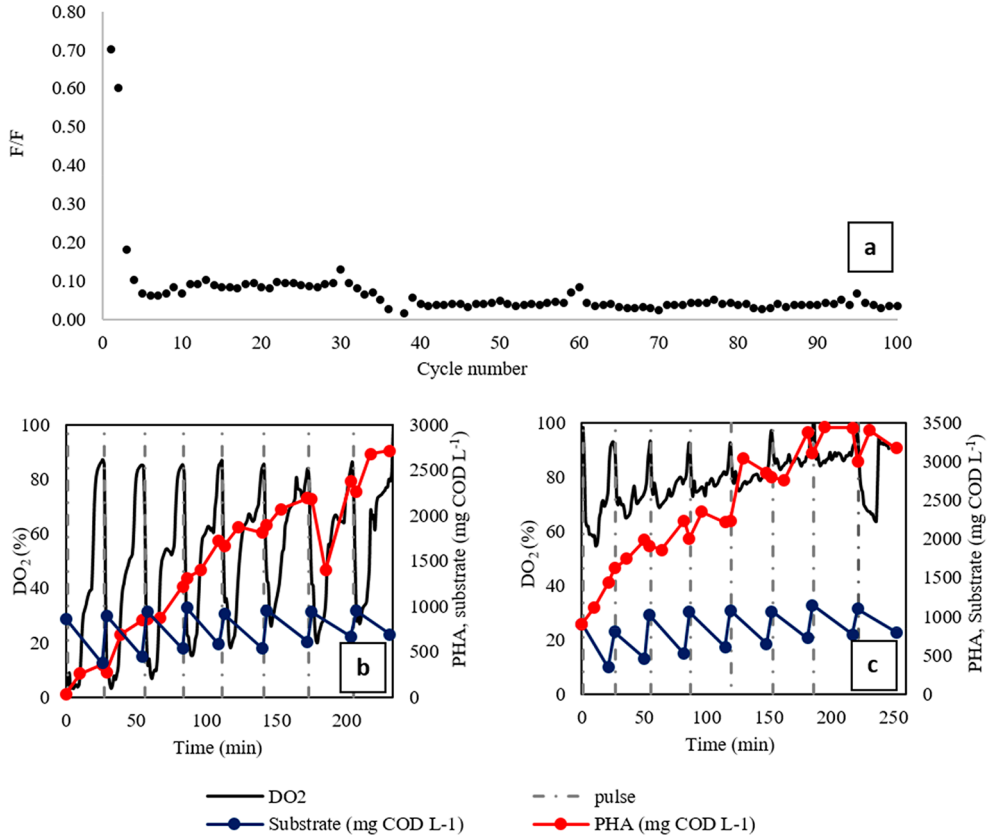

Figure 1. Feast to famine ratio (F/F) during all the duration of the selection process (a). DO_2, substrate, and PHA trend during two accumulation tests performed between the 7th SRT (b) and 10th SRT (c).

for the AH test (0.11 ± 0.05 mmol L^{-1}), probably due to the pathway involving H_2 consuming bacteria present.[57] Citrate (6.47 ± 0.87 mmol L^{-1}) was present only in AH due to the buffer used during biomass pretreatment. Taking into consideration metabolites produced during DF, it was possible to speculate that a mixed acid and butyric fermentation occurred.[59,60] In terms of ammonia production during DF, during the stable phase, both glucose and AH generated similar values, i.e., 240 ± 24 and 232 ± 45 mg L^{-1}, respectively. These data are lower than those reported to induce microbial toxicity.[61,62] Total OAs yield obtained from DF of the hydrolyzed *A. donax* was significant, i.e., 0.64 g OA g^{-1} COD (i.e., 0.81 g OA g^{-1} VS) (Table 2) and much higher than OAs yields typically obtained by DF that are reported in the literature (Table 2). However, it was difficult comparing data coming from pure sugar fermentation with those coming from different substrates that are sometimes complex organic matrices such as food wastes, agricultural residues, etc. (Table 2).

The only exception was for OA generated by DF of pure sugar (xylose) using pure microbial culture and a long retention time (22 d).[71] This data is important as it indicated that working with high sugar concentration could lead to high OAs yield. Regarding this point, DF performed by us with OLR ranged from 12 to 22.7 g sugar d^{-1} (this experiment considers 13 g d^{-1}) indicated that H_2 yield (mol H_2 mol^{-1} glucose) remained quite stable but that OAs concentration increased in the final slurry (data not shown); this result showed that DF potentially could be performed at a higher sugar concentration. Nevertheless, the necessity to dilute successively the fermented slurry to produce PHA suggested that it may be better to keep to lower sugar concentration, avoiding subsequent concentration steps.

PHA Production from Dark Fermentation Effluent. PHA-storing bacteria selection performed on the spent liquid fraction coming from the dark fermentation of hydrolyzed Arundo biomass showed good performance; i.e., the average feast to famine ratio (F/F) was equal to 0.06 with a standard deviation of 0.03 (Figure 1). This result was much lower than 0.33; this value is assumed as the limit up to which a good selection is obtainable.[49] Microbial growth yield referred to COD consumed was equal to 0.38 ± 0.03 mg COD_X mg^{-1} $COD_{cons.}$, clearly lower than that reported for PHA storage, i.e., 0.54 ± 0.11 mg COD_{PHA} mg^{-1} $COD_{cons.}$, indicating a preferred utilization of the carbon consumed for polymer storage rather than for microbial growth during the feast phase (Table 3).

The PHA stored in the microbial cells at the end of the accumulation tests (Figure 1) was, on average, equal to 543 ± 106 g PHA kg^{-1} VSS, with PHA being primarily composed of HB (96%, w/w) (Table 3). PHA content observed in this work was in agreement with the average data reported in the literature by other authors using fermented vegetable substrates (581 ± 193 g PHA kg^{-1} VSS) (Table S3). The higher PHA content reported in Table S3 are attributed to both longer selection processes performed (5 to 8 months vs 50 days adopted in this work) and the higher temperature used (30 vs 23 °C). Taking into consideration that about 77% of the COD consumed ($COD_{cons.}$) consisted of organic acids (COD_{OAcons}), it can be deduced that PHA was mainly produced starting from OAs, of which the yield, referred to COD_{OA}, resulted in 1.15 ± 0.21 mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$; that is, 0.85 ± 0.21 mg COD_{PHA} mg^{-1} $COD_{cons.}$

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Table 3. Parameters Characterizing MMC Selection (SBR) and PHA Accumulation Processes Performed in This Study

Stage	PHA content (g PHA kg^{-1} VSS)	Polymer composition (ΔHB/ΔHV) (%, w/w)	PHA characterization	PHA Yield[a] (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$)	PHA Yield[b] (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$)	PHA Yield[c] (mg COD_{PHA} mg^{-1} $COD_{cons.}$)	PHA Yield[d] (mg COD_{PHA} mg^{-1} COD_{in})	PHA Yield[e] (g PHA kg^{-1} OA_{in})	PHA Yield[f] (g PHA kg^{-1} COD_{in})	Growth Yield[g] (mg COD_X mg^{-1} $COD_{cons.}$)
SBR	132.3 ± 23.2[h]	95.3 ± 0.6/ 4.7 ± 0.6	–	0.87 ± 0.31	0.35 ± 0.14	0.54 ± 0.11	0.26 ± 0.08	–	–	0.38 ± 0.03
Accumulation	543 ± 106[i]	96 ± 1/4 ± 1	1 × 10^6 kDa (MW)[j] 2.1 (Polydispersity) 30 nm (Rh)[k] 0.8 (a)[l] −4.5 (log K)[m]	1.15 ± 0.21	0.65 ± 0.11	0.85 ± 0.21	0.41 ± 0.13	450 ± 89	241 ± 75	–

[a]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}cons.}$. [b]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}in}$. [c]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{cons.}$. [d]PHA storage yield expressed as COD (COD_{PHA}) referred to COD_{in}. [e]PHA produced referred to OA_{in}. [f]PHA produced referred to COD_{in}. [g]Growth yield during feast phase expressed as COD (COD_X) referred to $COD_{cons.}$. [h]PHA stored at the end of the feast phase referred to VSS. [i]PHA accumulated at the end of the accumulation test referred to VSS. [j]MW: molecular weight. [k]Rh: hydrodynamic radius. [l]a: Mark–Houwink parameter. [m]log K: Mark–Houwink parameter.

(Table 3). Both values reported were much higher than those cited in the literature for similar substrates (Table S3).

These data confirm that OAs were the preferred carbon sources for PHA production by mixed microbial cultures (MMC).[72,73] Again, the good PHA yield obtained in this work, i.e., 290 g PHA kg^{-1} of sugars, can be ascribed to the use of OAs as feed, as OAs have been reported to be direct precursors of PHA monomers.[34,74,75] The use of sugars instead of OAs as feed generally leads to a microbial population (MMC) enriched in other bacteria than just the PHAs-storing ones (i.e., glycogen-storing bacteria), reducing final PHA content.[75,76]

Considering the total substrate fed to the system, PHA yield was 0.65 ± 0.11 mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$ and 0.41 ± 0.13 mg COD_{PHA} mg^{-1} COD_{in} (Table 3). These results indicate that about half of the COD was not used to produce PHA. This means that the OAs present in the feed (71% of the total COD fed) were not completely consumed because of the reduced culture performance observed during the production runs. In fact, when the bacteria reached values close to the maximum theoretical polymer content, they started to reduce their ability to consume OAs. In any case, PHA storage yield on COD fed obtained in this study was, on average, 241 ± 75 g PHA per kg of COD, which was in agreement with results reported by using fermented paper mill wastewater (containing lignocelluloses) as substrate (Table S3).[73]

From the above discussion, it is possible to underline that fermenting sugar to OAs allowed us to get a very good substrate to select an MMC able, subsequently, to perform PHA accumulation with high performance. On the other hand, the shorter length of the selection process adopted and lower temperature used than those of previous work resulted in a lower PHA content (Table SI). This fact should be considered in the future to improve total PHA production performance.

Taking into consideration PHA storage yield in relation to COD fed, and dark fermentation yield in terms of COD produced in relation to sugars fed, a total PHA production of 290 g per kg of sugars was reached, i.e., 90 g of PHA kg^{-1} TS *A. donax*. This yield was in agreement with values previously reported by us, i.e., 92[35] and 65 g PHA kg^{-1} TS,[46] by using the organic fraction of municipal solid waste (OFMSW) and cheese whey, respectively. These results seem to indicate that it is not the complexity of the raw biomass used that affect final PHA yield, but the efficacy of the processes that preceded PHA production, i.e., biomass pretreatment (deconstruction), hydrolysis (enzymatic), and fermentation (dark fermentation).

Solid state ^{13}C NMR was used to characterize the extracted PHA polymers. The ^{13}C cross-polarization NMR technique, based on the carbon magnetization transferred from ^{1}H protons, was optimized for the identification of major signals related to PHA polymers. The obtained ^{13}C CP-MAS NMR of reference $PHA^{88/12}$ standard (3-hydroxyvalerate, 3HV, content 12% mol) was compared to extracted PHA (Arundo) as reported in Figure 2 (top left). Notably, PHA extracted from Arundo was mainly composed by the 3-hydroxybutyrate monomer (3HB). In order to provide more detailed structural features of the extracted PHA polymers solution, ^{1}H NMR was applied along with solid state ^{13}C NMR.

Reference $PHA^{88/12}$ was used for signal assignment in the ^{1}H NMR spectra. 3-Hydroxybutyrate and 3-hydroxyvalerate related signals were assigned by ^{1}H and COSY NMR (Figure 2, top right). The results obtained were in agreement with the previously published data.[77] ^{1}H, COSY NMR, and 2D HSQC

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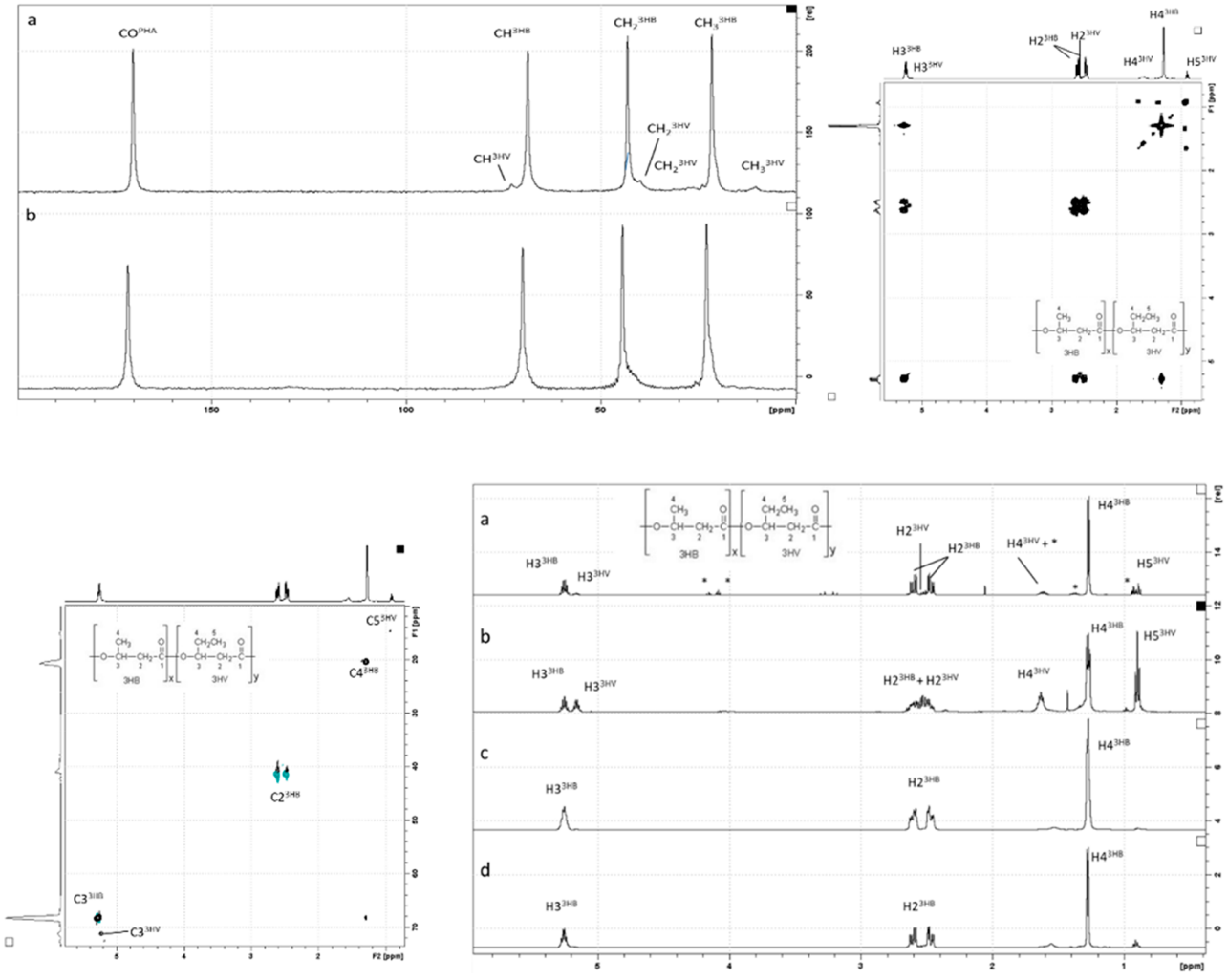

Figure 2. CP-MAS ^{13}C NMR spectra of reference $PHA^{88/12}$ (a) and fermented-Arundo-PHA (b) (top left). COSY NMR spectra of the fermented-Arundo-PHA in $CDCl_3$ (top right). HSQC NMR spectra of the fermented-Arundo-PHA in $CDCl_3$ (bottom left). ^1H NMR spectra of the reference $PHA^{88/12}$ (a), PHA obtained from OFMSW[35] (b), and PHA obtained from fermented cheese whey[46] (c) and the fermented-Arundo-PHA (d) in $CDCl_3$ (bottom right). Asterisks indicate signals related to impurities present in reference PHA sample.

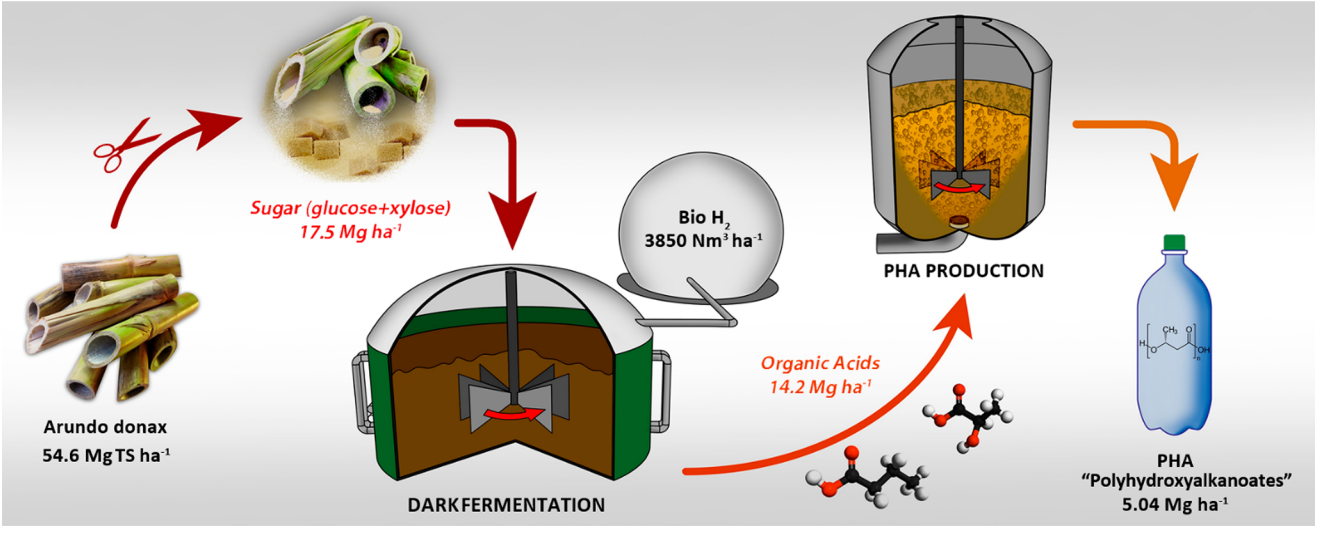

Figure 3. *A. donax* biorefinery concept and mass balance.

NMR (Figure 2, top right and bottom left) recorded on PHA extracted from Arundo confirmed that 3-hydroxybutyrate (3HB) represented the mayor monomer in the structure (>95%), while the amount of 3-hydroxyvalerate (3HV) was

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Table 4. Comparison of Hydrogen and PHA Production with Biomethane and Ethanol in Terms of Product Yield, Energy Recovered, Production Costs, and Estimated Net Revenue

	Product yield	Energy recovered (GJ Ha^{-1})	Unitary production cost	Total costs per hectare (€ Ha^{-1})	Unitary selling price	Total revenue (€ Ha^{-1})	Net revenue (€ Ha^{-1})
A. donax Biomethane	9518[a] Nm^3 CH_4 Ha^{-1}	301[a]	−0.57[a] € Nm^{-3} CH_4	5425	0.19[b] € Nm^{-3}	1808	−3617
Ethanol	11,324[c] L Ha^{-1}	239[d]	−0.26[e] € L^{-1}	2944	0.35[f] € L^{-1}	4012	1068
			This work				
A. donax Crop	54.6 Mg TS Ha^{-1}			1000[g]			
Hydrogen	3850 Nm^3 H_2 Ha^{-1}	42.4[h]	−0.82[i] € Nm^{-3} H_2	3157	0.63[j] € Nm^{-3}	2426	−731
PHA	5.04 Mg Ha^{-1}	111[k]	−1000[l] € Mg^{-1}	5040	3410[m] € Mg^{-1}	17,186	12,146
Whole biorefinery (sums of H_2 and PHA productions)		153		−9197		19,612	10,415
Corn stover							
Crop	8.6[n] Mg TS Ha^{-1}			535[o]			
Hydrogen	1036 Nm^3 H_2 Ha^{-1}	11.4[h]	−0.82[i] € Nm^{-3} H_2	850	0.63[j] € Nm^{-3}	653	−197
PHA	1.3 Mg Ha^{-1}	30[k]	−1000[l] € Mg^{-1}	1360	3410[m] € Mg^{-1}	4638	3278
Whole biorefinery (sums of H_2 and PHA productions)		41.4		−2745		5291	3081
Switchgrass							
Crop	10[p] Mg TS Ha^{-1}			433[q]			
Hydrogen	1164 Nm^3 H_2 Ha^{-1}	12.8[h]	−0.82[i] € Nm^{-3} H_2	954	0.63[j] € Nm^{-3}	733	−221
PHA	1.52 Mg Ha^{-1}	33.4[k]	−1000[l] € Mg^{-1}	1524	3410[m] € Mg^{-1}	5183	3659
Whole biorefinery (sums of H_2 and PHA productions)		46.2		−291		5916	3438

[a]Biomethane production yield, total energy produced and biomethane production cost.[12] [b]Biomethane selling price as reported by Cucchiella et al.[78] [c]Ethanol production yield calculated considering sugars conversion as 0.51 g ethanol g^{-1} sugar.[79] [d]Total energy produced with ethanol calculated by considering a LHV of 26.84 MJ kg^{-1}. [e]Ethanol production cost as reported by Cheng and Anderson.[80] [f]Ethanol selling price as reported by Zhang.,[81] in agreement with ethanol price commodity on 5/10/2018. [g]Biomass production costs as previously reported.[12] [h]Total energy produced by H_2 calculated by considering LHV of 11 MJ Nm^{-3}.[82] [i]Hydrogen production cost composed by cost for pretreatment and cost in performing the dark-fermentation.[82,83,84] [j]Hydrogen selling price as reported by FCH.[85] [k]Total energy produced calculated by considering a PHA-LHV of 22 MJ kg^{-1}.[86] [l]PHA/PHB production cost, referred just to polymer production without considering substrate cost (already considered), recalculated from CalRecycle.[87] [m]PHA/PHB selling price reported by CalRecycle.[87] [n]Biomass yield of corn-stover as previously reported.[79] [o]Corn-stover production cost as reported by Thompson and Tyner.[88] [p]Biomass yield of switchgrass as reported by Shi et al.[89] [q]Switchgrass production cost as reported by Khanna et al.[90]

lower than 5%. Quantitative estimation of PHA monomers was performed by ^{1}H NMR analysis using the ratio of signals related to H3 of 3HB and 3HV at 5.25 and 5.16 ppm, respectively. The same calculation was made for reference PHA$^{88/12}$ and biosynthesized PHA from different sources. From the comparison of the ^{1}H NMR spectra (Figure 2, bottom right), it can be seen that the different composition of the polymers was due mainly to the different organic acids contained in the substrate fed to the PHA storing bacteria. In particular, the carbon sources used in this study and in a previous work (fermented cheese whey)[46] contained 100% of HB precursors, which led to primarily HB found in the polymer stored. In contrast, by using a substrate composed of 50% of HB precursors (percolate of OFMSW), a previous study reported only 50% of HB in the polymer stored.[35]

The molecular weight distribution of PHA samples was analyzed by HP-SEC-TDA. Separation and detection conditions, including *dn/dc* values, were optimized and tested using an extracted PHA (Arundo) sample. Average molecular weight (Mw), polydispersity (M_w/M_n), and hydrodynamic radius (Rh) determined for the commercial reference PHA$^{88/12}$ and PHA extracted from *A. donax* were found to be 2 × 10^5 Da, 1.3 and 12 nm, and 1 × 10^6 Da, 2.1 M_w/M_n, and 30 nm, respectively. The biosynthesized PHA was characterized by higher molecular weight and hydrodynamic radius than the commercial reference sample. Mark−Houwink parameters *a* and log K, reflecting conformational behavior of polymers in solution were detected as well. The *a* values obtained for reference PHA$^{88/12}$ (a = 0.80; log K = −4.5) and extracted PHA from *A. donax* (a = 0.70; log K = −3.9) are consistent with the values attributed to flexible polymers in solution. Interestingly, these properties did not depend on the differences in molecular weight between the two samples analyzed.

Mass Balance and Economic Evaluations. The *Arundo donax* L. clone produced 54.6 Mg Ha^{-1} of dry biomass, and these high levels of productivity are well documented in the literature.[13,15] This biomass led, after pretreatment and hydrolysis processes, to the production of 13.9 Mg Ha^{-1} of glucose and 3.6 Mg Ha^{-1} of xylose (Figure 3). These values are

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comparatively significant as *A. donax* was able to produce about 3−5 times more sugar Ha^{-1} than those obtained, for example, from switchgrass and corn stover,[13] and confirm the potential of this crop to enable the realization of a sustainable and viable lignocellulosic biorefinery. The subsequent use of the sugars mixture to sustain the dark fermentation process gave an amount of bio-H_2 that was quantifiable as 3850 Nm^3 H_2 Ha^{-1} and 14.2 Mg Ha^{-1} of OAs (Figure 3). The OAs recovered after DF were then used as feed to produce PHAs by using MMC. Taking into consideration both crop yield and subsequent transformation of biomass into PHA (Table 3), a potential production of 5.04 Mg Ha^{-1} of PHA (Figure 3) was calculated.

Now, taking into consideration potential productivity of *A. donax* in terms of H_2 and PHA per surface area obtained in this work, the costs both to produce biomass and to convert it into final products, and the products' selling price (Table 4), a brief economic analysis was made of the proposed biorefinery.

Economic evaluation is very complex, as it requires an analytical approach with particular reference to the acquisition of data regarding processes' cost, yield, energy, etc. at full scale. This approach was not possible for this new biorefinery concept because many data were missing; moreover, this was not within the aim of this paper. So in order to attempt a first assessment of the economic sustainability of the biorefinery proposed, a different approach was considered. In doing so, crops and bioprocesses' yields obtained in this work have been considered together with data regarding bioprocesses from the literature (Table 4, see footnotes). The references selected were those considered most accurate in providing data which would be capable of resuming the entire bioprocess at full scale for lignocellulose feedstocks. In particular, the costs for producing the unit products (e.g., bioethanol, biogas, H_2, PHA) were considered as integral parts of the entire process (Table 4, see footnotes). Obviously, economic evaluation needs to be thoroughly investigated in order to be optimized.

The results from this economic analysis were compared with alternative and documented uses of *A. donax*, i.e., biogas and bioethanol production, representing simple biorefinery approaches (Table 4).

Results were interesting: bioethanol appeared to be sustainable with a total revenue of 73.4 € Mg^{-1} TS biomass, i.e., net revenue of 19.5 € Mg^{-1} TS biomass (calculated from Table 4). The large amount of biomass produced per Ha generated a total revenue per surface area of 4012 € Ha^{-1}, (net revenue of 1068 € Ha^{-1}), significantly higher than those obtained from corn stover, i.e., 1032 € Ha^{-1} (total biomass revenue of 120−130 € Mg^{-1} TS biomass[81] and corn stover production of 8.6 Mg Ha^{-1}[1,15]). Biomethane production from *A. donax* gave a negative economic balance, although the total energy produced per Ha^{-1} was higher than that of bioethanol. The low methane price was responsible for that and confirmed that biogas/biomethane production needs to be supported by government benefits or other income such as for example a waste tariff.

Increasing the number of products derived from lignocellulosic biomass by a partial or complete biorefinery process has been reported to increase total and net revenue.[81] In this work where comparing a simple biorefinery (bioethanol) (Table 4) with the partial multistage biorefinery proposed (H_2 plus PHA), total revenue increased by 4.9 fold and the net revenue by 9.7-fold.

The sustainability of the *A. donax* biorefinery was more evident when this feedstock was compared with the most common feedstock proposed to develop lignocellulose biorefinery, i.e., corn stover and switchgrass (Table 4).[88−90] The high biomass yield reported for *A. donax* assured a total PHA production 3.87 and 3.31 higher than that estimated for corn stover and switchgrass, respectively (Table 4). This fact allowed getting a net revenue that increased with respect to those calculated for these two feedstock by 3.38- and 3.02-fold (Table 4). These results indicated that the choice of crop becomes important for the development of a sustainable biorefinery and that *A. donax* is an excellent candidate to get this because of high biomass (and products) production performance and low crop cost and environmental impact.[91]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b03794.

Compositional analysis and mass balance of *Arundo donax* L. pretreatment and hydrolysis, literature data for biohydrogen production and biohydrogen yield obtained adopting different operating conditions, and comparison among parameters characterizing MMC selection (SBR) and PHA accumulation processes performed in this study and in other studies carried out with fermented vegetable effluents. (PDF)

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Notes

The authors declare no competing financial interest.

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