CHAPTER 3: MATERIALS AND METHODS

3.1. Materials and processes

3.1.1. Waste Sources

Different sources of waste were used throughout the Ph.D. Thesis.

1) OFMSW. In section I, two different OFMSW were selected based on their volumetric importance in real-scale plants. Samples were collected from an urban waste treatment facility located in Madrid (Spain), one immediately after fresh dumping from vehicles coming from a selective organic fraction (Pre-Sorted by the citizens), and the other one previously sorted by the waste treatment plant (Selective). Different macroscopic parameters were obtained in the OFMSW used in section 3, showing the significant heterogeneity of this waste.

2) FW and LW were used In section II. The origin of the FW samples is the Mercamadrid food wholesale market, composed mainly of fruit and vegetables. LW samples correspond mostly to prune and gardening residues. Both were collected from a solid waste plant in Madrid (Spain).

The waste was always blended and homogenized with an electric mixer grinder to achieve less than 10 mm particle size and then stored at 4 ^{o}C until further use. Table 3.1 summarizes the main macroscopic characteristics of the organic wastes used as feedstock during the Ph.D. Thesis.

3.1.2. Growth media

In this Ph.D. Thesis, two synthetic cultivation media were used for inoculum adaptation and control tests when necessary. The carbon source used was:

1) Acidogenic Fermentation Medium (AFM): For the acidogenic fermentation, a complex synthetic feed of starch, sucrose, peptone, and frying oil in a ratio of 1:1:1:0.1 g kg^{-1} was used as a carbon source.

2) Modified Ormerod Medium (MOM): For PPB photoheterotrophic tests, a synthetic substrate mixture of HAc:HPr:HBu:EtOH on a 1:1:1:1 COD basis (2 gCOD L^{-1} in total) was used as a carbon source.

The culture's composition was adapted from Ormerod *et al.* (1961), as follows: Macro nutrients (g L^{-1}): 1.086 $K_2HPO_4 \cdot 3H_2O$; 0.666 K_2HPO_4; 0.4 NH_4Cl; 0.075

Table 3.1. Average values with 95% confidence intervals for the macroscopic characteristics of the organic solid waste used.

	Section 1		Section 2	Section 1 and 2	Section 3
Waste	OFMSW Pre-sorted	OFMSW Selective	FW	LW	OFMSW
TS (g kg^{-1})	367 ± 51	343 ± 42	115 ± 12	950 ± 12	185 ± 21
VS (g kg^{-1})	298 ± 46	297 ± 19	99 ± 6	911 ± 11	159 ± 13
TKN (gN $kgTS^{-1}$)	3.7 ± 1.7	3.5 ± 0.8	3.2 ± 0.5	2.5 ± 0.8	2.9 ± 0.3
TCOD (g L^{-1})	143.9 ± 12.5	135.5 ± 6.7	122 ± 5	1075±8*	185 ± 10
SCOD (g L^{-1})	11.2 ± 0.2	10.5 ± 0.2	9.5 ± 0.6	-	2.3 ± 1.1

*g gTS^{-1}

$CaCl_2 \cdot 2H_2O$; 0.2 $MgSO_4 \cdot 7H_2O$ and 0.007 $FeCl_2$ and 0.02 of yeast extract. Micro nutrients (mg L^{-1}): 2.81 H_3BO_3; 2.02, 2.05 $CoCl_2$; 2.02 $MnCl_2 \cdot 4H_2O$; 0.16 $Na_2SeO_3 \cdot 5H_2O$; 0.09 $NiCl_2$; 0.55 $(NH)_6Mo_7O_{24} \cdot 4H_2O$; 0.114 $ZnCl_2$; 0.028 $CuCl_2 \cdot 2H_2O$; 0.015 Biotin and 2.01 EDTA.

3.1.3. INOCULA

Different types of inocula were used during this Thesis. They are summarised in three main sources of inocula:

1) For the anaerobic digestion process, a fresh methanogenic anaerobic inoculum was obtained from the urban wastewater treatment plant placed in Mostoles (Madrid).

2) For the acidogenic fermentation process (batch and continuous tests), the methanogenic sludge was inoculated into an anaerobic fermenter and maintained at 55 °C and 5.5 pH to avoid methanogenic activity. This reactor was fed with AFM and operated for more than 2 years, resulting in a highly acclimatized anaerobic acidogenic thermophilic sludge that produced butyrate predominantly (40% of total SCCA).

Figure 3.1 SCCA composition of the effluent obtained during continuous incubation in an anaerobic fermenter with an inoculum for non-pretreated waste. Hac: Acetic acid, HPr: Propanoic Acid, HBu: Butiric acid, HisoBut: Isobutiric acid, HisoVa: Isovaleric acid, HVa: Valeric acid, HHe, Hexanoic acid and HLa: Lactic acid

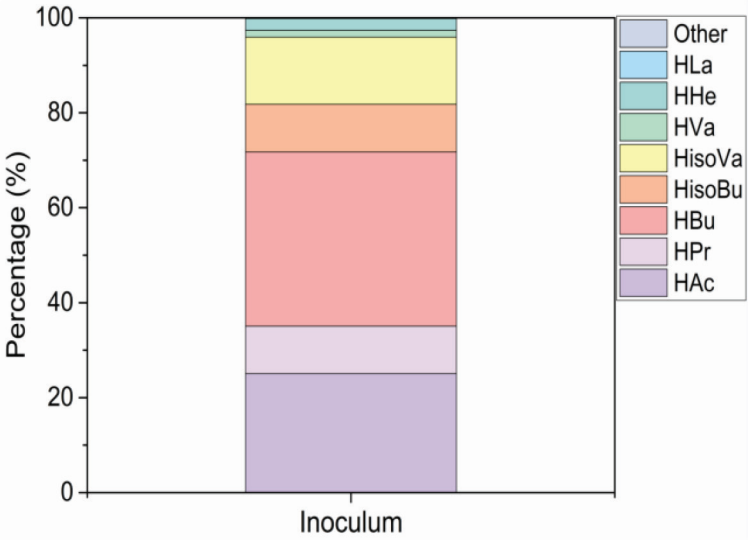


Figure 3.1 shows the SCCA composition of the effluent obtained from this reactor.

3) For the phoheterototrophic process (both batch and continuous processes), the active biomass of the mixed culture of PPB was obtained from an MPBR described elsewhere (de las Heras *et al.*, 2020). This inoculum was fed with the MOM and grown in batch culture for almost three years in an incubator illuminated with IR lamps (Philips, BR125 IR, España) at around 45 W m^{-2} and covered with a UV/VIS filtering foil. Every week, the culture media was refreshed (90% of the volume).

3.1.4. Thermal pretreatments

Two thermal hydrolysis reactors were used: A laboratory-scale autoclave reactor and a pilot-scale steam explosion reactor. Each of these reactors is detailed below:

1) Section I. Hydrothermal pretreatments for the proof of concept were performed in a 1-L autoclave (Parker, Autoclave Engineers, USA) at 150 rpm with a mass solid-to-liquid ratio of 1:5 and under autogenous pressure

conditions (Westerholm *et al.*, 2019). The autoclave was heated to the target temperature with an average heating rate of 6 °C min^{-1}. Upon the treatment, the samples were cooled with ice until room temperature to minimize the volatilization of organic compounds. After that, the samples were centrifuged at 6000 rpm for 10 min to separate solid and liquid phases. Both fractions were stored at 4 °C until further use.

2) Section II and III. The pilot-scale steam explosion reactor consists of a steam boiler (Certuss E56, Krefeld, Germany) and a 20-L total volume hydrolysis reactor (working volume of 10 L) connected to a flash tank (100 L). The reactor load was 6 kg of waste per batch. Steam input at 10 bar achieved a heating rate of 18±4 °C min^{-1} up to the target temperature. Upon the reaction, a sudden opening of the reactor caused a steam explosion in the flash tank, and the treated wastes were extracted and cooled to ambient temperature. The hydrolysis reaction was set at 150 °C for 40 min. These parameters were selected based on an optimization carried out by our research group within the framework of the Deep Purple project, but whose data are not shown in this Ph.D Thesis.

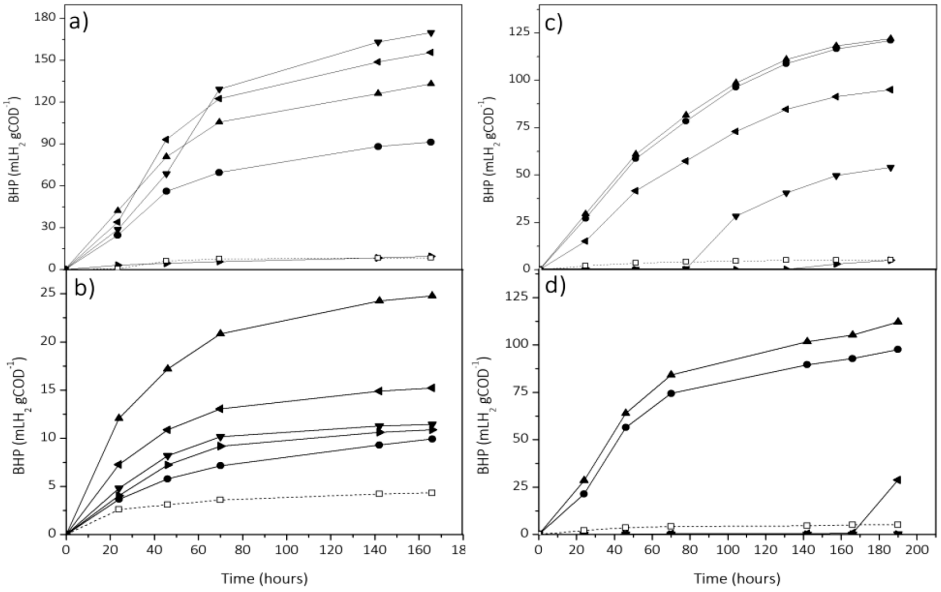
Water was added to LW to reach 20% TS, to match it to FW or OFMSW. The phases were separated by centrifugation at 6000 rpm and 10 min after the hydrolysis reaction if needed. The complete hydrolysate was stored at 4 °C until further use if not needed.

3.1.5. Thermophilic acidogenic fermentation

Batch experiments (Section II). Each biochemical hydrogen potential (BHP) test was performed in sextuplicate, allowing a fermentation period of 8 d. One triplicate served for measuring daily biogas production at a constant liquid level, while a second triplicate was used to measure daily COD dynamics. Experiments were conducted in a 160 mL Pyrex flask. A thermostatic incubator maintained a temperature of 55 °C and kept the bottles in constant shaking during the reaction time. The addition of 1 M HCl and KOH solutions allowed the setting of the pH at 5.5 for all conditions.

To correctly adjust the inoculum-substrate ratio, preliminary tests were performed with both untreated and pretreated substrates (Figure 3.2). The inoculum was added at 5% by volume to prevent a high initial SCCA presence that could falsify the results. Different substrate-to-biomass loadings (gCOD $gVSS^{-1}$) were tested, and conditions were chosen based on hydrogen production as it is a fast and easy-to-measure response indicator. Its high output is related to the metabolic pathways of acetic and butyric acid formation, which is widely reported (Hoelzle *et al.*, 2014). Consequently, a ratio of 25 gCOD $gVSS^{-1}$ was chosen for the untreated wastes and 5 gCOD

Figure 3.2 Hydrogen potential in preliminary tests (BHP) at the different FW (a), LW (b) and pretreated FW (c) and LW (d) concentrations (gCOD $gVSS^{-1}$). On all panels □ represents the inoculum while •, ▲, ▼,□ , ▶ represents 10, 25, 40 75 and 120 (gCOD $gVSS^{-1}$) on panels (a) and (b) and 2, 5, 10, 20 and 30 (gCOD $gVSS^{-1}$) on panels (c) and (d), respectively.



$gVSS^{-1}$ for the pretreated ones. The bottles were filled to a volume of 120 mL with milli-Q water. Negative control tests were also performed using both the inoculum alone (considering the endogenous production of hydrogen and SCCA) and the substrates without inoculum (eliminating interferences in the estimations).

Before conducting the experiments, all the elements used, such as the flask bottles, tubes, substrates, or milli-Q water, were heated up to 55°C to avoid a lag phase. The bottles were sealed with rubber stoppers and flushed for 5 min with Argon to remove any residual oxygen. Liquid samples were extracted from one triplet of bottles and filtered through a cellulose-ester filter of 0.45 µm of pore size (Advantech) to monitor the pH evolution and determine the SCOD and the SCCA composition of the fermentation broth. Gas samples were extracted from the other triplet to analyze the H2 production and gas composition.

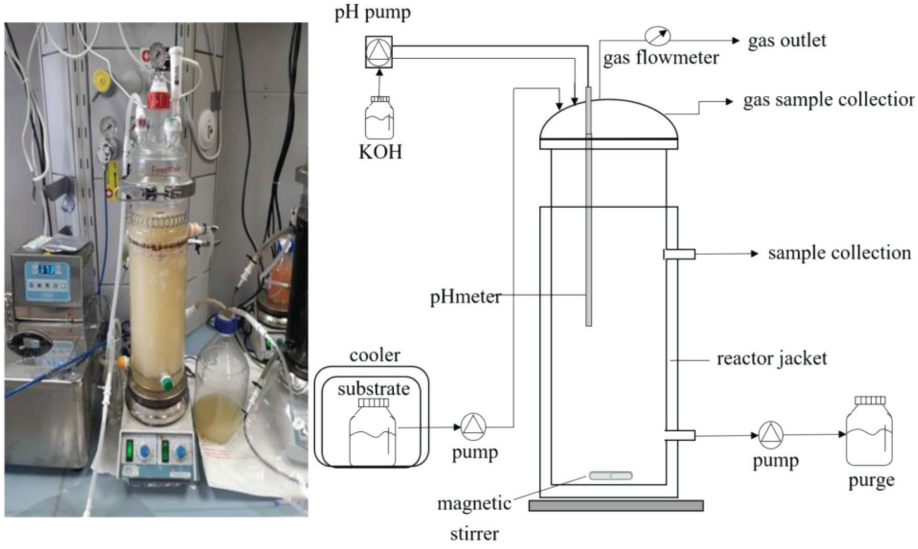
Continuous experiments (Section III). This process was carried out in an acidogenic CSTR reactor with a total volume of 2.5 L (working volume of 2 L),

as shown in Figure 3.3. The thermophilic temperature (55 °C) was controlled with an external water bath connected to an external jacket of the reactor. The HRT was fixed at 5 d, and the pH was set at 5.5 with a PLC. One outlet on the headspace was connected to a flowmeter (Ritter, Germany), while the other was sealed with a rubber stopper to take biogas samples with a gas syringe. The anaerobic state of the reaction was ensured both in the reactor and in the feed and outlet bottle. Anaerobic conditions were established in the substrate by sparging the medium with Ar and reducing it with Na2S + 9H2O (1 mL per liter). No more than 0.5% O2 was observed in the reactor headspace during the experimental period.

3.1.6. Anaerobic digestion process

All anaerobic digestion processes studied were carried out through standard biochemical methane potential (BMP) tests. The tests were performed by triplicate, yielding $mLCH_4$ gVS^{-1}, in 160 mL serum bottles at mesophilic conditions (37 ± 0.5 °C) following Angelidaki *et al.* (2009). The organic substrate used was the solid fraction after centrifugation of the hydrolysate. An inoculum to substrate (I/S) ratio of 2:1 (as VS) and an initial concentration of 10 $gVS\ L^{-1}$ were set up. A triplicate control of the inoculum was used to subtract the methane production coming from its endogenous digestion.

Figure 3.3 Picture (left) and schematic representation (right) of the continuously-fed thermophilic acidogenic fermentation reactor.



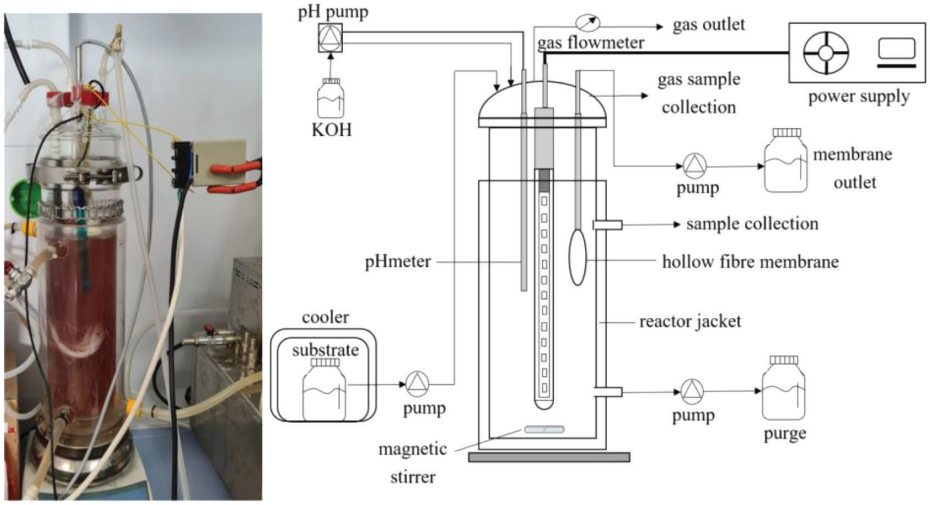
3.1.7. PPB photoheterotrophic process

Batch tests (Section I). The activity of the phototrophic biomass was determined by Specific Phototrophic Activity (SPA) batch tests following the indications in Hülsen *et al.* (2016). Triplicate experiments were performed in 160 mL anaerobic serum bottles in a temperature-controlled incubator (at 30 ^{0}C) with an initial pH of 6.5. The incubator was illuminated with IR lamps (Philips, BR125 IR, España) at around 45 W m^{-2} and covered with a UV/VIS filtering foil. An inoculum was added at 10 mg VSS L^{-1}, and the organic substrate (the liquid fraction obtained after centrifugation of the hydrolysate pretreated through thermal hydrolysis) was diluted with Milli-Q water to a concentration of 1 gCOD L^{-1}. Control tests fed with the MOM described before were used to compare with the experimental tests.

The light absorption spectra of the culture over the visible and near-infrared range (VIS-NIR) it was checked to verify that the biomass has two prominent adsorption peaks at 805 and 865 nm, which corresponds to the absorption maxima for the bacteriochlorophyll *a*, indicating an evident enrichment in PPB (Hülsen *et al.*, 2016). Biomass samples were also extracted at the end of the experiments and fixed with formaldehyde at 0.2% volume for 1 h and 4°C to measure PHA content, thus calculating the PHA production yield.

Continuous tests (Section III). This process was carried out in a MPBR inoculated with a mixed culture of PPB. Figure 3.4 shows the scheme of the MPBR. A 2.5-L reactor (working volume of 2 L) with a submerged LED lamp emitting

Figure 3.4 Picture (left) and schematic representation (right) of the MPBR.



at 805nm (Idea Bioprocess technology, Italy) that provides a volumetric irradiance of 2.2 W L^{-1}. A submerged hollow fiber membrane (Zena s.r.o, Czech Republic) was also used to discharge the filtered output. A pH-meter with a control system was used to maintain the pH above 6.5 by dosing KOH (0.1 M). The reactor head had two gas outlets, one sealed with a rubber stopper dedicated to taking biogas samples and the other connected to a flowmeter (Ritter, Germany) to measure the volume of gas produced. The MPBR head was opened every other day to clean the submerged LED lamp and to avoid biofilm formation. Each time this happened, the reactor headspace was purged with Ar to ensure anaerobic conditions. As in the acidogenic fermentation process, the anaerobic state of the reaction was ensured in the reactor and the feed and outlet bottle. The feed bottle was kept in a cooler at 4 °C.

3.2. Methods

3.2.1. Analytical methods

Analytical determination of pH, total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), total Kjeldahl Nitrogen (TKN), total phosphorus (TP) and COD (TCOD and SCOD) are carried out following Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Ammonia (NH_4+), and phosphate (PO_4^{3-}) concentrations dissolved in the aqueous phase are determined using Smartchem 140 (AMS Alliance), following APHA-AWWA Standard Method 4500-NO2 B and 4500-P E, respectively (APHA, 2005). Liquid samples are filtered through a cellulose-ester filter of 0.45 µm of pore size prior to the analysis (Advantech, Japan). Elemental analysis (C, H, N, and S) is performed by an elemental analyzer (Vario EL III, Elemental Analysis System GMHB, Germany). SCCA and monosaccharides are analyzed using an ion-exclusion RazexTM ROA-Organic Acid H+ HPLC column (Phenomenex, USA), coupled to a refractive index detector (Agilent, USA) and operated at 65 °C and 1 mL min^{-1}, with 0.005 M H2SO4 as the eluent.

Gas pressure is measured using a Boyle-Mariotte apparatus. The composition of each reactor's headspace is analyzed using a 7820A gas chromatography (GC-TCD) system (Agilent Technologies, Santa Clara, CA, USA). The mobile phase is Argon at a 5 mL min^{-1} flow rate. The temperature of the oven and the detectors are 45 °C and 220 °C, respectively.

The metal content is determined using inductively coupled plasma-optical emission spectrometry ICP-OES (Varian Vista AX Pro, USA). First, 0.5 g of the sample is measured, and 10 mL of Nitric acid and 4 mL of Hydrochloric acid are added. Then it is digested for 2 h at 175 °C and diluted at 1:10 before being measured. The determination of the concentration of the metals involves the creation of calibration curves corresponding to each metal, with

six calibration points for each metal. The solutions are prepared from certified standard solutions for atomic emission analysis of 1000 mg L^{-1} in nitric acid medium. The correlation coefficient r^2 obtained for all cases are 0.999.

The effect of thermal pretreatment over solid samples is evaluated through the characterization of the samples using mid-infrared spectroscopic (FTIR) and X-ray diffraction (XRD) tests. Firstly, the samples are dried at 100 °C in an oven. For XRD, samples as flat as possible are correctly distributed on a glass holder that does not produce diffraction effects. A X'Pert PRO diffractometer (Malvern Panalytical, Netherlands), with θ / 2θ geometry, is used, using Cu-Kα radiation. The data are collected from 5 to 90° (2θ) with a resolution of 0.02°.

The crystallinity index is calculated according to Eq. [3.1]:

$$CrI = \frac{I_{002} - I_{amorphous}}{I_{002}} \times 100 \qquad \text{Eq. [3.1]}$$

Where CrI is the crystallinity index, I_{002} is the maximum intensity of the 002 peaks at 2θ = 22.5°, and $I_{amorphous}$ is the intensity at 2θ = 18.7°

The FTIR spectra are recorded between 400 and 4000 cm^1 using a Spectrum 100 system (Perkin Elmer, US). Samples are prepared by grinding the solid fractions on an agate mortar and pressed at 10 MPa.

For PHA content quantification, biomass samples are collected, and 0.2% (by vol.) formaldehyde is added to stop the microbial activity. The samples are then lyophilized overnight to remove water. Commercial standards of PHA are used to make calibration curves: one containing 88% of hydroxybutyrate (HB) and 12% of hydroxyvalerate (HV) and another with 100% Methyl 3-hydroxyhexanoate (Sigma-Aldrich, USA). Dried biomass samples of 1-2 mg are weighted using an analytical balance (Radwag, Poland) and transferred into round base glass tubes. Volumes of 2 mL of ultra-pure trichloromethane (CHCh) and 2 mL of a standard solution (97% methanol, 3% H_2SO_4, and 200 mg L^{-1} sodium benzoate) are added. Tubes are heated for 20 h at 100 °C and then cooled at room temperature. 3 mL of milliQ water is added to each sample, and two phases (aqueous and organic) are formed. The organic phase is analyzed in a gas chromatograph coupled to a Flame Ionization Detector (GC-FID Varian CP-3800). When an unidentified peak is discovered in the GC-FID, a check is done using a 320-MS GC Triple Quadrupole Mass Spectrometer with a Rxi-5Sil Colum to identify this peak.

The method used for EPS quantification is adapted from Felz *et al.* (2016). For each measurement, 1 L of biomass culture is collected from the reactor outlet. The biomass is centrifuged at 4000 x g for 20 min, and the supernatant was

removed. The biomass pellets are collected, and the TS and VS are measured. The formaldehyde extraction method is used. First, at least 1 g (wet weight) of biomass is measured in an ISO bottle and filled to 50 ml with demineralized water. Then, 0.3 ml of 37% formaldehyde is added, and the bottle was shaken and left in a refrigerator at 4°C for one h. Subsequently, 20 mL 1M NaOH is added, and the bottle is shaken again and kept in a refrigerator for 3 h. Once finished, the sample is centrifuged at 4000 x g for 20 min, and the supernatant is collected. This supernatant is dialyzed (3500 Da) for 24 h in 1 liter of ultrapure water. Finally, TS and VS are measured to the dialyzed supernatant to calculate the dry weight of the EPS.

The glycogen measurement is based on a method adapted from Lanham *et al.* (2012). Briefly, after overnight lyophilization, 1 to 2 mg of dry biomass are weighed, then digested with 2 ml of 0.9M HCl and dissolved in a thermoblock for 3 h at 100°C. Subsequently, samples are quickly cooled on ice and filtered with a 0.45 cellulose-ester filter. Finally, glycogen is quantified by HPLC measurement using the same method described for SCCA quantification. The calibration curve is performed with standard glycogen from oysters (Sigma-Aldrich, USA).

3.2.2. Biological methods

Total DNA is extracted with a Microbial DNA isolation kit (Soil DNA Isolation Kit, CANVAX, Cordoba, Spain) to perform the molecular identification of prokaryotes and frozen to -20 °C for 4 d until use. The rest of the analysis is carried out by the FISABIO (Valencia) Sequencing and Bioinformatics Service research center. A brief summary of the protocol used is described below:

The quantification, amplification (PCR) and 16S rRNA gene measurements, and the amplicon taxonomic annotation and comparative analysis are outsourced to Instituto ISABIAL-FISABIO, Hospital General Universitario de Alicante, Alicante, Spain. Single molecule real time sequencing sequencing is performed on a PacBio RS II instrument. The preparation of the library is carried out through the 27F: AGRGTTYGATYMTGGCTCAG and 1492R: RGYTACCTTGTTACGACTT universal primer set to amplify the full-length 16S rRNA gene from the genomic DNA. Both the forward and reverse 16S primers are tailed with sample-specific PacBio barcode sequences to allow for multiplexed sequencing. Those barcoded primers are chosen because this reduces chimera formation compared to the alternative protocol in which primers are added in a second PCR reaction. The KAPA HiFi Hot Start DNA Polymerase (KAPA Biosystems) is used to perform 23 cycles of PCR amplification, denaturing at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 60 s. Post-amplification quality control is performed on a Fragment analyzer

(Agilent Technologies, Santa Clara, CA, USA). Amplified DNA from the samples is then pooled in equimolar concentration before loading.

Bioinformatics analysis is performed employing Pacific Bioscience system. Data are demultiplexed, and Circular Consensus Sequences (CCS) are called using the SMRT-Link analysis software (v9). The obtained CCS are submitted to the quality check pipeline following the DADA2 R Statistics package (v1.20.0) (Callahan *et al.*, 2016), which reorients the sequences, removes forward (27F, AGRGTTYGATYMTGGCTCAG), and reverse (1492R, RGYTACCTTGTTACGACTT) primers, filters and trims the sequences by length and average quality, dereplicates and estimates sequencing errors using PacBio error's model, and infers the compositions of the samples. The non-redundant sequences data set and the features abundance table are obtained using the previous steps entered the QIIME2 pipeline (v2021.4) (Bolyen *et al.*, 2019). Sequences that do not match any 16S rRNA are filtered out using the VSEARCH algorithm (Rognes *et al.*, 2016) against the Silva138 reference. Features of less than ten reads among all samples are also filtered out. The remaining sequences are annotated using the sklearn algorithm against the Silva138 QIIME2 classifier reference database (Quast *et al.*, 2013) (clustered at 99% of similarity). The taxonomic annotations are then used to compile contingency tables at every taxonomic rank.

3.3. Calculations

3.3.1. Data handling and statistical analysis

Unless otherwise stated, error bars and ± symbols are always 95% confidence intervals. Statistical analyses, modeling equations, and performance parameters used in each of the technologies in this Thesis are detailed below.

a) Performance parameters of thermal hydrolysis pretreatment

VS destruction in the thermal hydrolysis was expressed according to Eq. [3.2]:

$$VS\ destruction\% = \frac{(VS_{in} - VS_{out})}{VS_{in}} x\ 100 \qquad \text{Eq. [3.2]}$$

, where VS_{in} and VS_{out} are the VS concentration before and after the thermal hydrolysis in the solid, respectively. For steam explosion, the steam entering the hydrolysis reactor is considered the same as the steam leaving the flash tank. The organic matter and nutrients recovery in the liquid fraction (solubilization of COD, N, and P) was calculated according to Eq. [3.3]:

$$Solubilization\% = \frac{(C_{To} - C_{Sol})}{C_{To}} \times 100 \qquad \text{Eq. [3.3]}$$

, where C_{To} and C_{Sol} are the COD, N, or P concentrations (dry basis) on the initial raw waste and the remaining solid fraction after centrifugation, respectively.

The obtaining of comparably and reliably effects of hydrolysis on the treatment of solids due to time and temperature is usually carried out through the calculation of the severity factor, log R_0 (Overend and Chornet, 1987). This factor, though subjected to some limitations, enables a more straightforward comparison among different tested conditions (Eq. [3.4]).

$$R_0 = \int_0^t exp\left(\frac{T(t) - T_{Ref}}{14.75}\right) dt \qquad \text{Eq. [3.4]}$$

In this instance, *t* is heating time (min), *T* is the target temperature (°C), T_{Ref} is the reference temperature of 100 °C, and 14.75 is based on the activation energy when assuming pseudo-first-order kinetics.

b) Performance parameters of acidogenic fermentation

To better understand the results of acidogenic fermentation, we use several parameters like the extent of hydrolysis (H_E), which delimits the overall efficiency of acidogenic fermentation. The acidification extent determines the percentage of SCCA (as COD) compared to the released SCOD. Both are calculated according to Eq. [3.5] and [3.6]:

$$H_E = \frac{COD_{H_2} + SCODf - SCODi}{TCOD - SCODi} \times 100 \qquad \text{Eq. [3.5]}$$

$$Acidification\ extent = \frac{COD_{SCCA}}{SCOD_f} \times 100 \qquad \text{Eq. [3.6]}$$

, where COD_{H2} represents the amount of COD converted into hydrogen gas, expressed as COD equivalents, considering the ratio of 8 gCOD gH_2^{-1}. Total SCCA production (COD_{SCCA}) is the sum of each SCCA.

The conversion yield of the total COD added from the substrate (TCOD) to the SCCA in the fermentation process is determined as the SCCA yield (Y_{SCCA}), according to Eq. [3.7]:

$$Y_{SCCA} = \frac{COD_{SCCA}}{tCOD} \times 100 \quad \text{Eq. [3.7]}$$

Finally, the synergistic effect (φ) produced by the co-fermentation of FW and LW can be determined mathematically by dividing the measured variable by the calculated theoretical variable according to Eq. [3.8]:

$$\varphi = \frac{He_i}{(He_{FW} \times \alpha_i + He_{LW} \times \beta_i)} \quad \text{Eq. [3.8]}$$

, where the individual H_E of each waste (H_{EFW} and H_{ELW}) are multiplied by each mixing ratio from the co-fermentation mixture (α and β). Obviously, φ values over 1 would mean a presence of a synergistic effect.

c) Performance parameters of photoheterotrophic process

Microbial protein estimation is calculated based on NH4-N and TKN content (solid TKN x 6.25) following Eding *et al.* (2006), according to Eq. [3.9]:

$$Protein\ (mgN\ L^{-1}) = (TKN_T - TKN_S) * 6.25 \quad \text{Eq. [3.9]}$$

, where TKN_T is the TKN from the culture broth and TKN_S is the TKN from the soluble fraction (after filtration).

The percentage of intracellular/extracellular compounds accumulated (Y_{PHA}, Y_{GLY}, Y_{EPS}) is expressed as dry weight (wt %) and calculated according to Eq. [3.10]:

$$Accumulation\ (wt\ \%) = \frac{M}{VSS} * 100 \quad \text{Eq. [3.10]}$$

, where M is the measured mass of PHA, Glycogen or EPS (g), and VSS is the dry mass of Volatile Suspended Solids in the medium (g).

For the statistical analysis of the evolution of the bacterial composition determined by PacBIO, a principal component analysis (PCA) is performed to explore the variation of the communities over time. Distance-based redundancy (RDA) is conducted to reveal potential associations and multivariate relationships between environmental factors (OLR, sludge retention time (SRT), PHA, Glycogen, VSS, and hydrogen production) and the relative abundance of phylotypes at the genre level. Both PCA and RDA are carried out

using the "Vegan" Package in RStudio (R Development Core Team). Relationships between samples, bacterial communities, and environmental parameters are examined using multiple linear regression analysis to rule out linear variables (>95%).

3.3.2. Modeling

a) Modeling and statistical analysis of BMP

Kinetic parameters are obtained by fitting the data to a first-order model simplified from the IWA Anaerobic Digestion Model no. 1 (ADM1) (Batstone *et al.*, 2002), thereby calculating the first-order hydrolysis constant *kH* (d^{-1}) and the biodegradability, *Bo* (mLCH4 gVS^{-1}). The following equations describe the model (Eqs. [3.11]-[3.12]):

$$\frac{dS}{dt} = -k_h * S \qquad \text{Eq. [3.11]}$$

$$\frac{dS}{dt} = \frac{-B_o * dS}{dt} = k_h * B_o * S \qquad \text{Eq. [3.12]}$$

, where k_H: First-order constant (d^{-1}), *S*: Biodegradable substrate (kgVS L^{-1}), *B* methane production (LCH_4 $kgVS^{-1}$), *Bo*: *B* at infinite time (LCH_4 $kgVS^{-1}$) and t: time (d).

Statistical significance is analyzed by calculating the confidence intervals (at 95%) for all the experimental data and the estimated parameters, when possible. Parameter uncertainty surface is obtained as previously described (Batstone *et al.*, 2003), and confidence intervals are also calculated based on two-tailed t-tests from parameter standard error for statistical representative comparison. All parameter uncertainty analyses are performed using Matlab.

b) Modeling of the photoheterotrophic process

Apparent biomass yield ($Y_{X/S}$ in gVSS $gCOD^{-1}$) is calculated as the ratio between the total biomass growth measured as VSS and the total consumed COD according to Eq. [3.13].

$$Y_{X/S} = \frac{gVSS_{biomass}}{gCOD_{consumed}} \qquad \text{Eq. [3.13]}$$

The specific substrate rate, k_M (gCOD $gVSS^{-1}$ h^{-1}) is estimated by fitting the experimental data to the differential equation specified in Eq. [3.14] by using the software Aquasim 2.1d, as in Puyol *et al.* (2017).

$$\frac{dS}{dt} = k_M * X_{ph} * \frac{S}{K_s + S} \quad \text{Eq. [3.14]}$$

, where S is the concentration of soluble substrate (mgCOD L^{-1}), K_s is the saturation constant for S consumption (mgCOD L^{-1}), X_{ph} is the concentration of photoheterotrophic biomass (mgVSS L^{-1}), and t is time (d).

The specific production rates of H_2 (q_{H2}, mlH_2 L^{-1} d^{-1}), PHA (q_{PHA} , $gVSS_{PHA}$ L^{-1} d^{-1}) and glycogen (q_{GLY}, $gVSS_{Glycogen}$ L^{-1} d^{-1}) and consumption rates of substrate ($-q_s$, mgCOD L^{-1} d^{-1}) are determined by multiplying the amount of compound produced in the reactor (PHA, glycogen, H_2) to the substrate consumed by the dilution rate of the reactor (Outflow rate divided by reactor volume).

3.3.3. Energy and mass balance assessments

An energy evaluation is performed to check the scalability of the proposed photobiorefinery. The energy balance of the process is assessed to ensure the sustainability and economic viability of the process (Avellar and Glasser, 1998). Energy balances are carried out simulating a commercial Cambi® CHP plant. Recovery of heat from the flash vapors (saturated steam at 105 °C) to the preheating stage of the substrate leads to considerable saving in energy consumption. This way, steam requirements have been estimated with energy and mass balances considering 20% vapor losses in the pre-heating stage according to *Scenario three* in Cano *et al.* (2014). Biogas generation are extrapolated from laboratory scale BMP tests, but steam consumption and equipment's characteristics has been determined theoretically considering typical design values. Briefly:

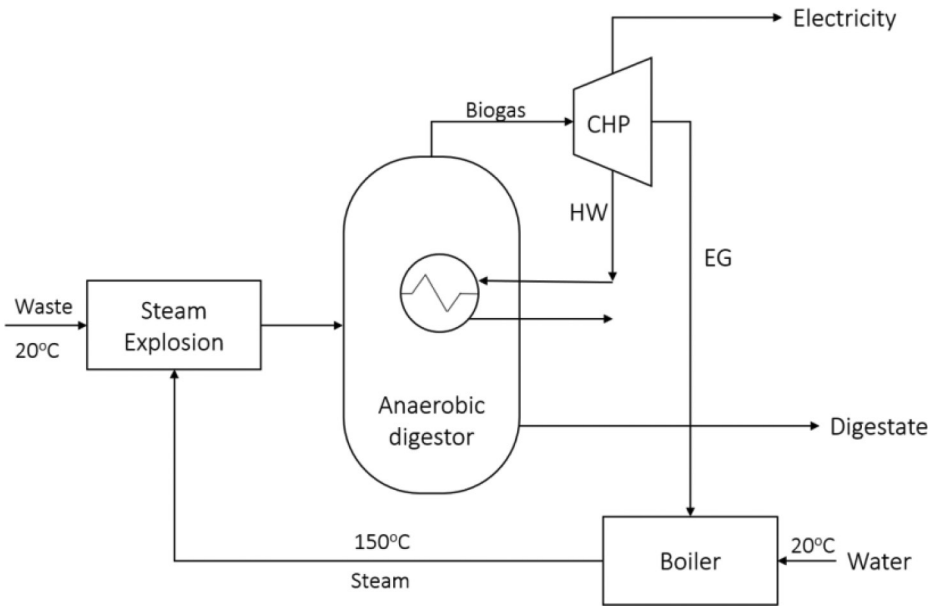
The biogas is burned in a combined heat and power system (CHP) providing three main streams (Figure 3.5):

Electrical green energy: to be sold, providing net benefits.

Hot exhaust gases (EG): residual stream in which heat can be recovered in a boiler to produce steam for the energy requirements.

Hot water (HW): it can be used to heat the digester, if necessary.

Figure 3.5 Simplified flow diagram with the considerations for the energy balance.



Thermal energy is not necessary for the process that uses directly raw waste since it does not involve a pretreatment process. The total energy of biogas is calculated based on 1 tonne of feedstock (20% TS) and then calculating the amount of biogas produced per kg of TS obtained during the BMP tests and multiplied by the calorific value of methane. The electrical and thermal balances are estimated by subtracting the thermal needs of the hydrothermal reactor and anaerobic digestor and the electrical needs of the centrigugation, pump and mixing. An overview of all parameters is summarized in Table 3.2. Combining the experimental, theoretical calculations, and published references used in these estimations (Table 3.2), preliminary mass balances were constructed to demonstrate the potential benefits of the proposed process and to estimate the mass yields to the target products (mainly PHA and H_2).

Table 3.2. Mass and energy assessment parameters

Parameter	Unit	Value	Reference
Electrical Efficiency	%	30	(Cano *et al.*, 2014)
Thermal Efficiency	%	55	(Cano *et al.*, 2014)
Energy loses	%	15	(Cano *et al.*, 2014)
Boiler efficiency	%	100	(Cano *et al.*, 2014)
Anaerobic digestion Temperature	°C	37	This study
Steam Explosion Temperature	°C	120-180	This study
Specific heat of water	kJ kg°C^{-1}	4.18	(Safoniuk, 2004)
Heat recovery	%	85	(Lu *et al.*, 2008)
Pumping energy consumption	kJ m^{-3}	1800	(Lu *et al.*, 2008)
Mixing energy consumption	kJ m^{-3}	300	(Lu *et al.*, 2008)
Calorific value of methane	kWh Nm^{-3}	11	(Cano *et al.*, 2014)
Membrane retention	%	100	(Davis *et al.*, 2016)
Centrifugation retention	%	72	(Davis *et al.*, 2016)
Pretreatment rejection	%	10	(Andreasi Bassi *et al.*, 2021)
PHA extraction efficiency	%	90	(Andreasi Bassi *et al.*, 2021)
Prices of electrical energy	€ kWh^{-1}	0.15	(Cano *et al.*, 2014)

3.4. References

Andreasi Bassi, S., Boldrin, A., Frenna, G., and Astrup, T. F. (2021). An environmental and economic assessment of bioplastic from urban biowaste. The example of polyhydroxyalkanoate. Bioresource Technology, 327, 124813. https://doi.org/10.1016/j.biortech.2021.124813

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J. L., Guwy, A. J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J. B., and Angelidaki, A. I. (2009). Defining the biomethane potential (BMP) of solid organic wastes and energy

crops: a proposed protocol for batch assays. Water Sci Technol., 59(5), 927-934. https://doi.org/10.2166/wst.2009.040

APHA. (2005). Standard Methods for the Examination of Water and Wastewater, 21 ed. In American Journal of Public Health and the Nations Health (Vol. 51, Issue 6). American Public Health Association. https://doi.org/10.2105/AJPH.51.6.940-a

Avellar, B. K., and Glasser, W. G. (1998). Steam-assisted biomass fractionation. I. Process considerations and economic evaluation. Biomass and Bioenergy, 14(3), 205-218. https://doi.org/10.1016/S0961-9534(97)10043-5

Batstone, D. J., Keller, J., Angelidaki, I., Kalyuzhnyi, S. V, Pavlostathis, S. G., Rozzi, A., Sanders, W. T. M., Siegrist, H., and Vavilin, V. A. (2002). The IWA Anaerobic Digestion Model No 1 (ADM1). Water Science and Technology, 45(10), 65-73. https://doi.org/10.2166/wst.2002.0292

Batstone, D. J., Pind, P. F., and Angelidaki, I. (2003). Kinetics of thermophilic, anaerobic oxidation of straight and branched chain butyrate and valerate. Biotechnology and Bioengineering, 84(2), 195-204. https://doi.org/10.1002/bit.10753

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodriguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology, 37(8), 852-857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods, 13(7), 581-583. https://doi.org/10.1038/nmeth.3869

Cano, R., Nielfa, A., and Fdz-Polanco, M. (2014). Thermal hydrolysis integration in the anaerobic digestion process of different solid wastes: Energy and economic feasibility study. Bioresource Technology, 168, 14-22. https://doi.org/10.1016/j.biortech.2014.02.007

de las Heras, I., Molina, R., Segura, Y., Hülsen, T., Molina, M. C., Gonzalez-Benítez, N., Melero, J. A., Mohedano, A. F., Martínez, F., and Puyol, D. (2020). Contamination of N-poor wastewater with emerging pollutants does not affect the performance of purple phototrophic bacteria and the subsequent resource recovery potential. Journal of Hazardous Materials, 385, 121617. https://doi.org/10.1016/jjhazmat.2019.121617

Eding, E. H., Kamstra, A., Verreth, J. A. J., Huisman, E. A., and Klapwijk, A. (2006). Design and operation of nitrifying trickling filters in recirculating aquaculture: A review. Aquacultural Engineering, 34(3), 234-260. https://doi.org/10.1016/j.aquaeng.2005.09.007

Felz, S., Al-Zuhairy, S., Aarstad, O. A., van Loosdrecht, M. C. M., and Lin, Y. M. (2016). Extraction of structural extracellular polymeric substances from aerobic granular sludge. Journal of Visualized Experiments, 2016(115), 1-8. https://doi.org/10.3791/54534

Hoelzle, R. D., Virdis, B., and Batstone, D. J. (2014). Regulation mechanisms in mixed and pure culture microbial fermentation. Biotechnology and Bioengineering, 111(11), 2139-2154. https://doi.org/10.1002/bit.25321

Hülsen, T., Barry, E. M., Lu, Y., Puyol, D., Keller, J., and Batstone, D. J. (2016). Domestic wastewater treatment with purple phototrophic bacteria using a novel continuous photo anaerobic membrane bioreactor. Water Research, 100, 486-495. https://doi.org/10.1016/j.watres.2016.04.061

Lanham, A. B., Ricardo, A. R., Coma, M., Fradinho, J., Carvalheira, M., Oehmen, A., Carvalho, G., and Reis, M. A. M. (2012). Optimisation of glycogen quantification in mixed microbial cultures. Bioresource Technology, 118, 518-525. https://doi.org/10.1016Zj.biortech.2012.05.087

Ormerod, J. G., Ormerod, K. S., and Gest, H. (1961). Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Archives of Biochemistry and Biophysics, 94(3), 449463. https://doi.org/10.1016/0003-9861(61)90073-X

Overend, R. P., and Chornet, E. (1987). Fractionation of lignocellulosics by steam-aqueous pretreatments. Philos.Trans.R.Soc.Lond.A, 321(1561), 523-536. https://doi.org/https://doi.org/10.1098/rsta.1987.0029

Puyol, D., Barry, E. M., Hülsen, T., and Batstone, D. J. (2017). A mechanistic model for anaerobic phototrophs in domestic wastewater applications: Photo-anaerobic model (PAnM). Water Research, 116, 241-253. https://doi.org/10.1016/j.watres.2017.03.022

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research, 41(Database issue), D590-D596. https://doi.org/10.1093/nar/gks1219

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. PeerJ, 2016(10), 1-22. https://doi.org/10.7717/peerj.2584

Westerholm, M., Castillo, M. D. P., Chan Andersson, A., Jahre Nilsen, P., and Schnürer, A. (2019). Effects of thermal hydrolytic pre-treatment on biogas process efficiency and microbial community structure in industrial- and laboratory-scale digesters. Waste Management, 95, 150-160. https://doi.org/10.1016/j.wasman.2019.06.004