processes

MDPI

Article

Enhancing Biobased Volatile Fatty Acids Production from Olive Mill Solid Waste by Optimization of pH and Substrate to Inoculum Ratio

Yasmim A. da Fonseca [1], Adonai B. de Camargos [1], Gustavo S. M. Gomes [1], P. Fregulia [2], Silvana Q. Silva [3], Leandro V. A. Gurgel [1,*] and Bruno E. L. Baêta [1,*]

[1] Environmental and Chemical Technology Group, Department of Chemistry, Federal University of Ouro Preto, Campus Universitário Morro do Cruzeiro, Bauxita, s/n, Ouro Preto 35400-000, MG, Brazil
[2] U.S. Dairy Forage Research Center, Agricultural Research Service (ARS), U.S. Department of Agriculture (USDA) 1925 Linden Drive, Madison, WI 53706, USA
[3] Department of Biological Sciences, Federal University of Ouro Preto, Campus Universitário, Morro do Cruzeiro, Bauxita, s/n, Ouro Preto 35400-000, MG, Brazil
* Correspondence: legurgel@ufop.edu.br (L.V.A.G.); brunobaeta@ufop.edu.br (B.E.L.B.); Tel.: +55-31-3559-1744 (L.V.A.G)

Abstract: The *pH* and substrate-to-inoculum ratio (*S/I*) are important parameters in the anaerobic fermentation of agroindustrial residues, and therefore the optimization of these two parameters is needed for a stable, efficient, and sustainable reactor operation. In this work, the parameters *pH* (5–9) and *S/I* (0.5–3 gVS gVS^{-1}) were optimized to produce biobased volatile fatty acids (VFAs) from hydrothermally pretreated olive mill solid waste (HPOMSW). The response variables evaluated in the Doehlert design were total VFAs concentration (*tVFAs*) (mg L^{-1}) and amounts (%) of isobutyric, butyric, isovaleric, and valeric acids on the VFAs profile. The *pH* was the variable that most influenced the mixed culture fermentation of HPOMSW, proving to be a key parameter in the process. Microbial community analyses of conditions 1 (*S/I* = 3 gVS gVS^{-1} and *pH* = 7) and 4 (*S/I* = 1.13 gVS gVS^{-1} and *pH* = 5) showed that Proteobacteria and Firmicutes accounted for more than 87% of the total microorganisms identified for both conditions. In addition, the second-order model best fitted the experimental data for the VFAs production at the desirable condition (*S/I* = 3 gVS gVS^{-1} and *pH* = 8).

Keywords: biomass; carboxylic acids; anaerobic digestion; microbial community; kinetics

Citation: da Fonseca, Y.A.; de Camargos, A.B.; Gomes, G.S.M.; Fregulia, P.; Silva, S.Q.; Gurgel, L.V.A.; Baêta, B.E.L. Enhancing Biobased Volatile Fatty Acids Production from Olive Mill Solid Waste by Optimization of pH and Substrate to Inoculum Ratio. *Processes* **2023**, *11*, 338. https://doi.org/10.3390/pr11020338

Academic Editor: Chyi-How Lay

Received: 10 December 2022
Revised: 13 January 2023
Accepted: 16 January 2023
Published: 20 January 2023

1. Introduction

The increased global demand for energy and food, global warming and dependence on fossil fuels have shifted the linear economy towards a biobased circular economy. This model is the pillar of future global economic development, providing circularity, sustainability, and progress [1]. Among the value-added products that can be obtained in a biorefinery, volatile fatty acids (VFAs) produced by anaerobic digestion are highlighted for their universality and versatility of applications, which include food (flavorings, pH regulators, additives, and preservatives), pharmaceutical (production of perfumes and moisturizing agents), and chemical (synthesis of polymers and solvents) industries [2,3]. In another application, VFAs can also be used for the production of polyhydroxyalkanoates or polyhydroxybutyrates, biobased polymers that have shown important material properties such as biocompatibility and thermoplasticity [4].

Currently, VFAs are produced from fossil fuels. Acetic acid, for example, is produced through the carbonylation of methanol, while propionic acid is synthesized by the hydrocarboxylation of ethylene in the presence of a catalyst such as nickel carbonyl or rhodium. In another route of oxo synthesis, butyric acid has been produced by the oxidation of butyraldehyde. Although well-established, these chemical routes have some drawbacks,

such as low efficiency and the use of expensive catalysts [5]. Moreover, dependence on fossil resources encourages the search for alternative and sustainable biochemical processes for the production of VFAs [2].

Carboxylic acids can be produced from renewable raw materials through microbial fermentation, as these acids are the end products of various fermentation routes. An alternative to reduce carbon source costs in acidogenic fermentation processes is the use of lignocellulosic residues as substrates. This scenario benefits from the use of renewable raw materials and the possibility of adding greater value to them, besides mitigating the environmental problems generated by the inadequate disposal of waste materials [6].

Among the agroindustrial residues studied as substrates to produce VFAs, the olive mill solid waste (OMSW) produced during the extraction of olive oil is a renewable and promising biomass, considering its availability and potential for application in circular production chains. The OMSW is a problematic residue due to high organic matter concentration that includes polyphenols and lipids, transforming them into phytotoxic materials with negative effects on soil microbiota and aquatic ecosystems [7]. Although many studies aiming to add value to OMSW through bioproducts obtained by producing other compounds from alternative and sustainable processes, such as phenolic compounds, ethanol, furfurals, and coals, among others [8–10], have been published, its potential for VFAs production is still little exploited, with few studies available in the literature [11,12].

In our previous work [13], the hydrothermal pretreatment of OMSW for VFAs production was investigated for different anaerobic digestion systems. The evaluation of the effect of hydrothermal pretreatment severity and typology of anaerobic digestion (AD) in liquid (L-AD), semi-solid (Ss-AD), and solid phase (S-AD) was important to understand the performance of fermentative systems concerning the profile of VFAs produced. However, to make the VFAs platform from OMSW more attractive and consolidated in the future, the optimization of anaerobic digestion parameters is essential to achieve greater process efficiency.

In this way, this work aimed to optimize the *pH* and substrate-to-inoculum ratio (*S/I*) to produce volatile fatty acids from the anaerobic digestion of OMSW using a mixed culture of anaerobic bacteria. In addition to *pH*, *S/I* is recognized as one of the main parameters affecting the anaerobic digestion of lignocellulosic residues [14]. Besides the effects on microbial communities, metabolic pathways, and kinetic parameters (biodegradation rate and lag time), the concentration of microorganisms is very important for the start-up of anaerobic reactors and also for the stability of anaerobic digestion systems [15]. It has been reported that a high *S/I* ratio can be toxic to bacteria, while a very low *S/I* ratio can prevent the induction of enzymes necessary for biodegradation [16].

The novelty of this work lies in the optimization of the anaerobic digestion of hydrothermally pretreated OMSW to produce VFAs. As the production of VFAs from agroindustrial residues is still incipient when it comes to industrial plants, the optimization of process parameters is essential for designing a process that is more likely to be competitive and scalable. In addition to process optimization, this work provides important information to clarify the OMSW fermentation from a mixed culture, such as the analysis of the microbial community and its metabolic pathways and the kinetic study of the fermentative process.

2. Material and Methods

2.1. Chemicals

Chromatography grade sulfuric acid (99.999%) and chromatography standards (acetic acid, formic acid, propionic acid, isobutyric acid, butyric acid, valeric acid, and isovaleric acid) were purchased from Sigma-Aldrich. Sodium bicarbonate and hydrochloric acid (36–37 wt.% in H_2O) were bought from Synth (Brazil). Nitrocellulose membranes were acquired from Unifil (Brazil).

2.2. Hydrothermally Pretreated Olive Mill Solid Waste

The raw olive mill solid waste (OMSW) collected in the 2018/2019 harvest was kindly provided by the "Serra que Chora" olive oil mill (Barbacena, MG, Brazil). Prior to its use, OMSW was stored under freezing conditions (<0 °C) to avoid undesirable fermentation processes. Hydrothermal liquefaction of OMSW was performed at 162 °C and 61 min. This reaction condition was described by Fonseca et al. [13] as the most suitable condition to produce VFAs from OMSW. The heating of the reactor was carried out by a thermostatically controlled 25 L glycerol bath (Marconi®, model MA 159). At the end of the reaction, the 470 cm^3 stainless steel reactor vessel (8 cm O.D. × 6 cm I.D. × 16.6 cm H) was rapidly cooled in an ice bath, and the reactor content was collected at room temperature. Chemical oxygen demand (COD) and *pH*, as well as total and volatile solids (TS and VS) of the pretreated material, were determined according to Standard Methods for the Examination of Water and Wastewater [17].

2.3. Statistical Analysis: Response Surface Methodology (RSM)

A Doehlert experimental design (DED) was used to determine the influence of fermentation conditions on VFAs production from hydrothermally pretreated OMSW. The DED matrix was composed of 9 unique experiments with replicates at the center point, as shown in Table 1.

Table 1. Doehlert design matrix was used for the optimization of the independent variables *pH* and *S/I* ratio.

Experiment Number	Independent Variable (Coded)		Independent Variable (Uncoded)		Dependent Variable	
	S/I	***pH***	***S/I***	***pH***	***tVFAs* (mg L^{-1}) (Y_1)**	***C4 and C5 VFAs* (%) (Y_2)**
1	1	0	3	7	8543.5 ± 531.0	27.2 ± 2.7
2	0.5	0.866	2.4	9	11302.3 ± 168.1	19.0 ± 1.5
3	−1	0	0.5	7	5228.6 ± 636.6	36.4 ± 3.2
4	−0.5	−0.866	1.13	5	3623.6 ± 262.0	11.9 ± 1.2
5	0.5	−0.866	2.4	5	5527.9 ± 168.1	8.8 ± 0.2
6	−0.5	0.866	1.13	9	8816.4 ± 945.2	13.4 ± 4.2
7	0	0	1.75	7	7605.3 ± 182.6	30.3 ± 1.5
8	0	0	1.76	7	6517.7 ± 431.8	27.9 ±2.9
9	0	0	1.77	7	7880.9 ± 319.5	30.2 ± 1.1

The independent variables evaluated were *S/I* (0.5–3 gVS gVS^{-1}) (X_1) and *pH* (5–9) (X_2). The response variables chosen for the statistical analyses were the *tVFAs* (Y_1, mg L^{-1}), i.e., the sum of the concentrations of formic, acetic, propionic, butyric, valeric, and isovaleric acids on the 15th day of fermentation; and the amount of C4 and C5 fatty acids (isobutyric, butyric, isovaleric, and valeric acids) (Y_2, %). The variable Y_2 represents the ratio of C4 and C5 acids in the *tVFAs* profile and was calculated according to Equation (1):

$$Y_2\ /\ (\%) = \left[\frac{(\text{isobutyric acid} + \text{butyric acid} + \text{isovaleric acid} + \text{valeric acid}) \left(\text{mg L}^{-1}\right)}{(\text{formic acid} + \text{acetic acid} + \text{propionic acid} + \text{isobutyric acid} + \text{butyric acid} + \text{isovaleric acid} + \text{valeric acid}) \left(\text{mg L}^{-1}\right)} \right] \times 100 \quad (1)$$

The choice of the second variable is due to the higher market value of acids with four or more carbon atoms [2]. Experimental results were evaluated with the Statistica 12.0 (StatSoft, Inc.) routines for regression coefficients and graphical analysis. Statistical analysis was performed at a 95% confidence level and according to the pure error.

The second-degree polynomial equation used to model the experimental data obtained from the DED is shown in Equation (2). The most complex model, including interactions between the independent variables, was used to model the responses as follows:

$$Y = b_0 + b_1\ X_1 + b_2\ X_2 + b_{11}\ (X_1)^2 + b_{22}\ (X_2)^2 + b_{12}\ X_1\ X_2 \quad (2)$$

where Y is the response variable, X_1 and X_2 are the independent variables *S/I* and *pH*, respectively; b_0 is the model constant, b_1 and b_2 are the estimated linear regression coefficients, b_{11} and b_{22} are the estimated quadratic regression coefficients, and b_{12} is the interaction coefficient.

The desirability function approach was used to optimize multiple responses simultaneously. In this approach, each response is converted into individual desirability values (d_i) ranging from 0 to 1 (lowest to highest). Then, multivariate numerical optimization was performed to identify the combination of factors that maximizes the overall desirability of the process, calculated according to Equation (3) [18]:

$$D = (d_1\ d_2\ \ d_3 \ldots\ d_k)^{1/k} \quad (3)$$

The scores (s) were set at 1.0 (highest value) for responses Y_1 and Y_2 since the desirable condition is the one with the highest production of total VFAs and a higher amount of longer-chain VFAs. The factor grid was set at 40 interactions (Statistica 12.0).

2.4. Batch Fermentation Experiments

Batch fermentation experiments were performed in duplicate using glass bottle reactors with 60 mL working volume. The bottles were loaded with 12 wt% total solids (TS). Thus, inoculum and OMSW were fed in sufficient amounts to satisfy the TS content and the *S/I* ratio of each experimental condition (Table 1). The inoculum used in the tests consisted of a mixture of anaerobic sludge (AS) and fresh bovine manure (FBM) in the proportion of 1 g VS_{AS}: 1 g VS_{FBM}, as defined by Lima et al. [19]. The FBM was collected from local farms (Ouro Preto, MG, Brazil), and the anaerobic sludge was collected from a pilot-scale mesophilic upflow anaerobic sludge blanket reactor (UASB) fed with sewage at the Centre for Research and Training in Sanitation (CePTS) (UFMG/Copasa, Arrudas Wastewater Treatment Plant, Belo Horizonte, MG, Brazil). A thermal treatment using an autoclave (Prismatec®,Itu, SP, Brazil, model CS 50) at 100 °C for 120 min was used to enrich the inoculum with spore-forming VFA-producing microorganisms and eliminate some VFA consumers, such as methanogenic archaea from the microbial consortium [20].

A micronutrient solution was added to each bottle, according to Fonseca et al. [13]. $NaHCO_3$ was also added to provide an alkalinity of 2.5 g L^{-1} as $CaCO_3$ in order to avoid a sudden drop in *pH* [21]. The initial *pH* of the anaerobic reactors was measured with a *pH* meter (Meta® Química, Jaraguá do Sul, SC, Brazil, model Meta 210) and adjusted by adding drops of 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl aqueous solutions. After adjusting the *pH*, all bottles were sealed and purged with N_2 for 3 min at a flow rate of 109.3 mL min^{-1} to ensure anaerobic conditions. The reactors were placed in a thermostatic orbital shaker at 35 ± 1 °C (mesophilic condition) and stirred at 150 rpm for 15 days. At the end of the tests, liquid samples were collected from the reactors, centrifuged for 30 min at 14,000 rpm (relative centrifugal force of 12,709× *g*) (Eppendorf®, Hauppauge, NY, USA), model 5410), and filtered on nitrocellulose membranes (0.22 µm). The VFAs were quantified by high-performance liquid chromatography (HPLC), as described in Section 2.5.

2.5. Volatile Fatty Acids Quantification

The VFAs (formic, acetic, propionic, isobutyric, butyric, valeric, and isovaleric acids) were quantified using a Shimadzu® HPLC system (Chiyoda-ku, Tokyo, Japan) equipped with a pre-column (Cation H Refill Cartridge Microguard column, Bio-Rad®) and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad®) kept at 55 °C. Aqueous sulfuric acid (5 mmol L^{-1}) solution was used as eluent at a flow rate of 0.6 mL min^{-1}. The detection of organic acids was performed by a UV-Vis detector (Shimadzu®, model SPD-10AV) at a wavelength of 210 nm.

2.6. Microbial Community Analysis

Microbial community analysis was performed by Neoprospecta Microbiome Technologies, Inc. (Florianópolis, SC, Brazil). The libraries were prepared and paired sequenced (2 × 300 bp) on the Miseq sequencing platform (Miseq®, Illumina Inc., San Diego, CA, USA). The sludge was collected on the 15th day of fermentation. Samples were washed with phosphate buffer solution (pH = 7.2 ± 0.2) and centrifuged for 5 min at 3500 rpm. Subsequently, the samples were stored at −20 °C until DNA extraction [22]. The genetic material was extracted from one gram of the biomass sample using the DNeasy PowerSoil (QIAGEN®) kit, following the manufacturer's instructions. The polymerase chain reaction (PCR) was carried out in triplicate using Platinum Taq (Invitrogen, USA) under the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45 s, 55 °C for the 30s and 72 °C for 45 s, and a final extension of 72 °C for 2 min for PCR 1 in the conditions: 95 °C for 5 min, 10 cycles of 95 °C for 45 s, 66 °C for 30 s and 72 °C for 45 s, and a final extension of 72 °C for 2 min for PCR 2 [23]. The final PCR reaction was cleaned up using AMPureXP beads (Beckman Coulter, Brea, CA, USA), and samples were pooled into sequencing libraries for quantification using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems®, Wilmington, MA, USA). After sequencing, the bioinformatics pipeline performed sequence demultiplexing, adaptor, and primer trimming, as described by Christoff [23]. The sequences were clustered into operational taxonomic units (OTUs) considering at least 99% identity in the reference 16S rRNA database (NeoRefdb, Neoprospecta Microbiome Technologies, Florianópolis, SC, Brazil).

Metabolic pathways were estimated from the 16S rRNA using the tool Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) version 2.0.0 [24]. PICRUSt is an alternative to previous expensive and complex metatranscriptomic analyses and is useful for predicting the functional composition of bacterial communities from 16S rRNA amplicon sequencing data. The prediction of MetaCyc pathways is based on marker gene data and a database of reference genomes, such as the Cluster of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [24,25].

2.7. Kinetics of VFAs Production

The optimized condition (S/I = 3 gVS gVS^{-1}; pH = 8) was validated by upscaling the process to 500 mL glass bottle reactors (Schott Duran®, model GL 45, Mainz, Rhineland-Palatinate, Germany). Thus, the same protocol described in Section 2.4 was maintained, but proportional amounts were added to the working volume (250 mL). To assess the change in the concentration and profile of VFAs over time, 1 mL aliquots were collected daily for analysis of organic acids, as described in Section 2.5. The experimental data for VFAs production were fitted according to different kinetic models (First-order, Second-order, Fitzhugh, Monomolecular, Modified Gompertz, Logistic, Transference, and Richards), as described in Table 2. The Matlab® R2016b (The MathWorks, Natick, MA, USA) software was employed to fit the models to the experimental data using the Least Squares Method (LSM) through the function fminsearch.

The selection of the model that best described the VFAs production was based on the following functions: coefficient of determination (R^2); adjusted coefficient of determination (R^2_a); Root Mean Square Error ($RMSE$); Normalized Root Mean Square Error ($NRMSE$), and Akaike Information Criterion (AIC) [27,28].

The R^2 value was calculated using Equation (4).

$$R^2 = 1 - \frac{\sum_i \left(Y_{i,exp} - Y_{i,est}\right)^2}{\sum_i \left(Y_{i,exp} - \overline{Y}\right)^2} \quad (4)$$

where $Y_{i,exp}$ is the observed data point, $Y_{i,est}$ is the data point predicted by the model, and $\overline{Y}$ is the mean of the observed data.

The R_a^2 value was calculated using Equation (5).

$$R_a^2 = 1 - \frac{(1 - R^2)(N - 1)}{N - k - 1} \quad (5)$$

where N is the number of experimental data points (observations), and k is the number of model parameters (variables).

The $RMSE$ value was calculated using Equation (6).

$$RMSE = \sqrt{\frac{\sum_i \left(Y_{i,exp} - Y_{i,est}\right)^2}{N}} \quad (6)$$

The $NRMSE$ value was calculated using Equation (7).

$$NRMSE = \left[\frac{RMSE}{(Y_{max} - Y_{min})}\right] \times 100 \quad (7)$$

where Y_{max} and Y_{min} are the maximum and minimum observed values for the VFAs production.

The AIC value was calculated using Equation (8).

$$AIC = N \ \ln\left(\frac{SS}{N}\right) + 2k \quad (8)$$

where SS is the squared sum of residuals.

Table 2. Kinetic models selected to describe VFAs production.

Kinetic Model	Model Equation
First-order	$CA_t = CA_f[1 - \exp(-k_{CA}t)]$
Second-order	$CA_t = \frac{k_{CA}''(CA_f)^2 t}{1 + k_{CA}''(CA_f)t}$
Fitzhugh	$CA_t = CA_f\left[1 - \exp(-k_{CA}t)^n\right]$
Monomolecular	$CA_t = CA_f[1 - \exp(-k_{CA}(t - \lambda))]$
Modified Gompertz	$CA_t = CA_f \exp\left\{-exp\left[\frac{\mu_m e}{CA_f}(\lambda - t) + 1\right]\right\}$
Logistic	$CA_t = \frac{CA_f}{1 + exp\left[\frac{4\mu_m(\lambda - t)}{CA_f} + 2\right]}$
Transference	$CA_t = CA_f\left\{1 - exp\left[-\frac{\mu_m(t - \lambda)}{CA_f}\right]\right\}$
Richards	$CA_t = CA_f\left\{1 + \nu\, exp(1 + \nu)\, exp\left[\frac{\mu_m}{CA_f}(1 + \nu)\left(1 + \frac{1}{\nu}\right)(\lambda - t)\right]\right\}^{\left(-\frac{1}{\nu}\right)}$

CA: carboxylic acids; CA_t: *CA* concentration over time; CA_f: final concentration of *CA*; k_{CA}: first-order *CA* production rate constant, k_{CA}'': second-order *CA* production rate constant, t: digestion time, n: form constant; e: Euler number; λ: lag phase time; μ_m: maximum *CA* productivity; ν: shape coefficient. [26]

3. Results and Discussion

3.1. Fermentation Assays

The optimization of the independent variables *pH* and *S/I* ratio in the acidogenic fermentation of hydrothermally pretreated OMSW (162 °C; 62 min) in a semi-solid phase (12 wt% TS) aimed to understand the effects of these parameters on the production of VFAs from this pretreated biomass. The pretreated slurry presented the following physico-chemical characteristics: *pH* = 4.84, total solids = 0.22 g gOMSW^{-1}, volatile solids = 0.21 g gOMSW^{-1}, and COD = 1.64 gO_2 gOMSW^{-1}. The full characterization of the pretreated OMSW can be found in the study by Fonseca et al. [13].

The analysis of variance (ANOVA) for the dependent variables evaluated in the Doehlert experimental design is shown in Table 3. The adjusted model showed R^2 values higher than 0.97. This means that the adjusted regression model was able to explain more

than 97% of the variability in the experimental data. Furthermore, the quadratic models showed a significant regression ($p_{regression} < 0.05$) without lack of fit ($p > 0.05$).

Table 3. Analysis of variance (ANOVA) for the dependent variables evaluated in the Doehlert experimental design.

	***tVFAs Concentration* (mg L^{-1}) (Y_1)**					***C4 and C5 VFAs* (%) (Y_2)**				
	Sum of Squares	**Degrees of Freedom**	**Mean Square**	***F***	***p***	**Sum of Squares**	**Degrees of Freedom**	**Mean Squares**	***F***	***p***
S/I	**10,120,033**	**1**	**10,120,033**	**19.479**	**0.048**	20.718	1	20.718	12.267	0.073
$(S/I)^2$	241,472	1	241,472	0.465	0.566	6.487	1	6.487	3.841	0.189
pH	**30,069,869**	**1**	**30,069,869**	**57.880**	**0.017**	**34.357**	**1**	**34.357**	**20.344**	**0.046**
pH^2	19,279	1	19,279	0.037	0.865	**603.865**	**1**	**603.865**	**357.559**	**0.003**
S/I x pH	84,565	1	84,565	0.163	0.726	19.018	1	19.018	11.261	0.079
Lack of fit	192,712	1	192,712	0.371	0.605	22.950	1	22.950	13.589	0.066
Pure error	1,039,048	2	519,524			3.378	2	1.689		
Total SS	41,806,271	8				762.282	8			
	R^2		R^2_a		$p_{regression}$	R^2		R^2_a		$p_{regression}$
	0.970		0.921		0.0167	0.965		0.908		0.066

The values in bold correspond to statistically significant regression coefficients ($p < 0.05$).

As the second-order polynomial equations fitted well to the experimental data, the mathematical models in terms of coded factors are described in the following Equations (9) and (10):

$$Y_1/(\text{mg L}^{-1}) = 7334.6 + 1836.7\ (S/I) + 2741.8\ (pH) - 448.6\ (S/I)^2 + 95.06\ (pH)^2 + 290.8\ (S/I)\ (pH) \quad (9)$$

$$Y_2/(\%) = 29.5 - 2.6\ (S/I) + 2.9\ (pH) + 2.3\ (S/I)^2 - 16.8\ (pH)^2 + 4.4\ (S/I)\ (pH) \quad (10)$$

The response surfaces for the response variables *tVFAs* concentration and *C4 and C5 VFAs* are shown in Figure 1. The total concentration of VFAs is an important variable for the industrial production of biobased VFAs since low concentrations can be an economic challenge for the recovery of these acids by separation techniques such as adsorption and membrane separation [29,30]. As can be seen in Figure 1a, higher *tVFAs* concentration can be achieved by using a *S/I* ratio higher than 2 gVS gVS^{-1} and pH higher than 8.

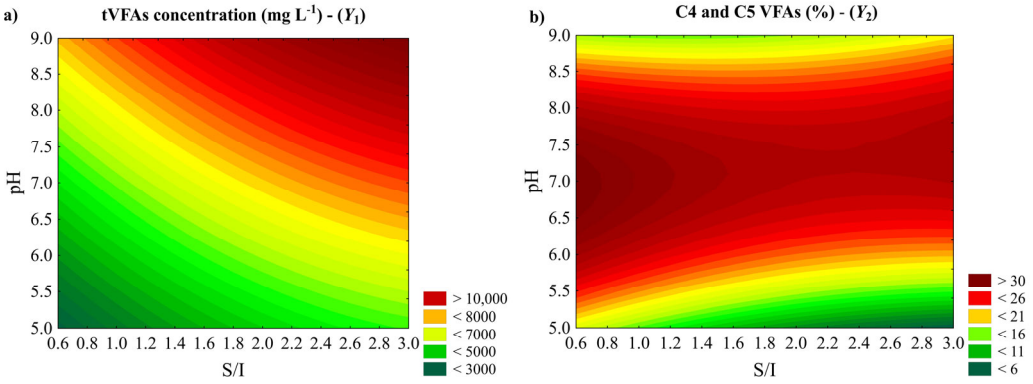

Figure 1. Fitted response surfaces generated from quadratic models for (**a**) total VFAs (formic, acetic, propionic, isobutyric, butyric, valeric, and isovaleric acids) concentration (Y_1, mg L^{-1}) and (**b**) amount of C4 and C5 VFAs (Y_2, %).

The linear terms of *pH* and *S/I* ratio had a significant positive effect on the variable concentration of *tVFAs* (Y_1), as shown in the Pareto chart of standardized effects (Figure 1). In addition, *pH* has a greater influence on VFAs concentration as it has a greater effect (ef = 7.60) on the response Y_1 when compared to the *S/I* ratio (ef = 4.41).

The analysis of the experimental conditions confirms a greater influence of *pH*, compared to the *S/I* ratio, on the total production of VFAs from OMSW. Conditions 1 (*S/I* = 3 and *pH* = 7) and 3 (*S/I* = 0.5 and *pH* = 7), both at *pH* 7, showed that a six-fold

increase in the *S/I* ratio led to an increase of 58% in the concentration of VFAs. On the other hand, experimental conditions 4 (*S/I* = 1.13 and *pH* = 5) and 6 (*S/I* = 1.13 and *pH* = 9), both at an *S/I* ratio of 1.13 gSV gSV^{-1}, showed that increasing the *pH* from 5 to 9 resulted in a 144% increase in *tVFAs* concentration in the system.

Some authors who focused on the production of organic acids applied a *pH* of around 5.5 to reduce the influence of archaea [6,10]. However, recent studies focused on the production of VFAs from agroindustrial residues have reported that an alkaline medium may favor the accumulation of VFAs, as shown in this work [31,32].

A reason for the low performance of fermentative processes at acidic *pH* is that when the *pH* of the system is below the pK_a of carboxylic acids (from 4.76 to 4.88), these species are protonated and can easily penetrate the bacterial cell through passive diffusion, dissociating in the cytosol. Therefore, the accumulation of carboxylates in bacterial cells can reach concentrations that are toxic to microorganisms, due to mechanisms such as membrane rupture and enzymatic inhibition [6,33].

In addition to the effects of *pH* on the enzymatic and metabolic activities of microorganisms, the effect of this parameter on the solubility and hydrolysis of substrates may explain the lower total concentration of VFAs at acidic *pH*. According to Dahiya et al. [32], compared to an acidic medium, an alkaline medium favors the hydrolysis of carbohydrates, proteins, and lipids by causing the ionization of the acidic groups, thereby facilitating the availability of readily available soluble substrates for microorganisms.

Some authors using different substrates, such as granular sludge and food residues, concluded that the highest concentrations of carboxylic acids were favored by alkalinity [12,21,34,35]. Regarding OMSW, Cabrera et al., 2019 studied the accumulation of VFAs in methanogenic systems at different *pH* values (5.7 and 9) and reported that the highest accumulation of VFAs (3.69 $gDQO_{VFA}$ L^{-1}) was reached at *pH* 9, where the profile of generated acids was mainly represented by acetic acid (79.3%).

As well as concentration, the profile of VFAs plays a significant role in their subsequent application and therefore deserves attention. Long-chain carboxylic acids have a higher market price and can still facilitate further recovery for hydrophobicity-based techniques [2,36]. The linear (*pH*) and quadratic (pH^2) terms of *pH* showed statistical significance in the variable response C4 and C5 VFAs. However, as observed in the Pareto chart of standardized effects (Figure 2), the linear term (*pH*) had a positive effect (ef = 4.51), while the quadratic term (pH^2) had a negative effect (ef = −18.9) on the dependent variable Y_2.

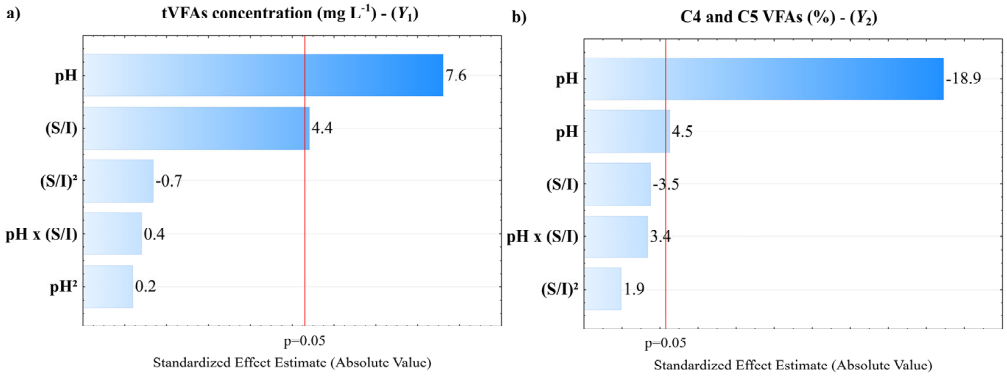

Figure 2. Pareto chart of the standardized effects for the substrate to inoculum (*S/I*) ratio, *pH*, and their interaction on (**a**) total VFAs concentration (Y_1, mg L^{-1}) and (**b**) amount of C4 and C5 VFAs yielded during anaerobic production of organic acids from olive mill solid waste (Y_2, %).

For the experimental *pH* range evaluated (5–9), *pH* values close to the extreme *pH* range (~5, ~9) promoted a decrease in the amount of C4 and C5 VFAs. Therefore, as can be seen in Figure 1, the most suitable conditions to obtain a profile with a higher amount of C4 and C5 VFAs are those with *pH* between 6 and 8 and extreme *S/I* ratio conditions (~0, 5, ~3 gSV gSV^{-1}). Even though neutral *pH* favors the production of VFAs, this range is also

optimum for acetolactic methanogens, which can lead to a reduction in the accumulation of VFAs. [32]. It is worth mentioning that in the present study, the methane composition in the gas fraction was <5 % in all conditions, showing that the thermal pretreatment of the inoculum was efficient for the suppression of methanogenic activity.

3.2. VFAs Composition

The production profile of VFAs for all conditions studied is shown in Figure 3. The experimental conditions 1, 2, 3, 6, 7, 8, and 9 ($pH = 7$ and $pH = 9$) presented a more diversified profile regarding the variety of acids produced when compared to conditions 4 and 5 ($pH = 5$). When considering the influence of pH on the system, the VFAs profile obtained under experimental conditions 2 and 5 (2.4 gVS gVS^{-1}) and 4 and 6 (1.13 gVS gVS^{-1}) suggests that increasing the pH from 5 to 9 is related to a higher amount of isobutyric acid.

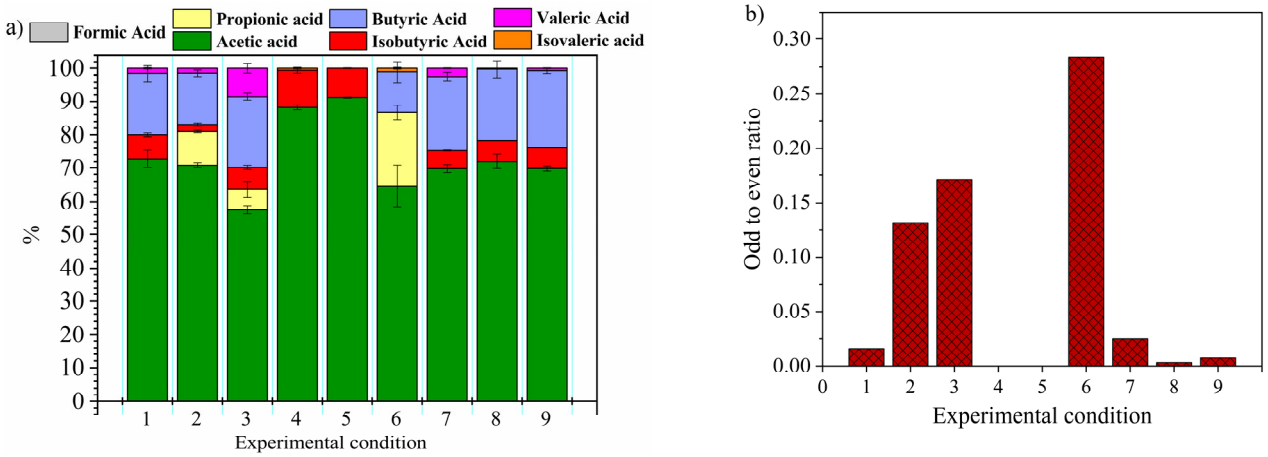

Figure 3. (**a**) Profile of VFAs obtained in each experimental condition of the Doehlert experimental design; (**b**) Odd-to-even ratio and acidification efficiency obtained in each experimental condition of the Doehlert experimental design.

When considering the influence of the S/I ratio on the acid profile of the system, it can be inferred that this parameter did not influence at pH 5 since the profile of VFAs under conditions 4 (S/I = 2.4 gSV gSV^{-1} and $pH = 5$) and 5 (S/I = 1.13 gSV gSV^{-1} and $pH = 5$) were similar and had no significant changes in the amount of isobutyric acid ($p > 0.05$ by the Tuckey test). On the other hand, the S/I ratio proved to be important at pH 9, considering that the comparison of conditions 2 and 6 showed a decrease in the amount of propionic acid when the S/I ratio increased from 1.13 to 2.4 gVS gVS^{-1}.

The fermented stream of organic acids as they come out of the process is of little value. However, if VFAs can be concentrated and recovered individually or if the concentrated mixture of acids can be converted into a final product that can be easily separated from the liquid phase, an attractive biorefinery can be developed [37]. In this way, the production of polyhydroxyalkanoates (PHA), biopolymers that can be used as substitutes for petroleum-derived plastics, is one of the potential applications of VFAs, whose profile strongly affects the type of PHA that is produced in the next step [34].

The odd-to-even ratio is defined as the sum of the odd-equivalent carboxylic acids produced (propionic and valeric acids) divided by the sum of the even-equivalent carboxylic acids produced (acetic, isobutyric, butyric, and isovaleric acids [21]. This parameter is used to assess the potential of an acidified stream to produce 3-hydroxybutyrate or 3-hydroxyvalerate. In general, an even acid tends to produce 3-hydroxybutyrate, while an odd acid tends to produce 3-hydroxyvalerate [38].

The odd-to-even ratio of all experimental conditions tested is shown in Figure 3b. Among experimental assays, condition 6 (S/I = 1.13 gSV gSV^{-1} and $pH = 9$) presented the highest proportion of acids containing an odd number of carbons in the chain. This is because higher pH ranges favored propionic acid production and therefore increased

the odd-to-even VFA ratio. Propionic and valeric acids are odd acids, and their formation favors the potential valorization and consequent conversion of OMSW into polyhydroxyalkanoates [39]. Thus, it would be expected that in technological routes to produce PHAs, the profile of VFAs obtained at *pH* 9, and low *S/I* ratio would favor the production of hydroxyvalerate (HV).

The *pH* had a greater influence on the acidogenic fermentation of OMSW for the response variables evaluated (tVFAs concentration and amount of C4 and C5 VFAs), proving to be a key parameter in the process. By studying the parameters influencing the production of VFAs using wastewater obtained from olive oil production to produce polyhydroxyalkanoates, Gameiro et al. [40] reported the same trend observed in the present study, i.e., alkalinity was the most important parameter for the production of VFAs from olive mill solid waste, showing a greater effect on acidification when compared to the *S/I* ratio applied to the system under study.

3.3. Microbial Community

The bioprocess parameters have a great influence on the selection of the microbial community active in the process. Understanding these effects in a mixed microbial community is very important to clarify the bacteria involved in the fermentation for a more robust and efficient bioprocess. The microbial analysis was performed for conditions 1 (*S/I* = 3 gSV gSV^{-1}, *pH* = 7) and 4 (*S/I* = 1.13 gSV gSV^{-1}, *pH* = 5), as shown in Figure 4.

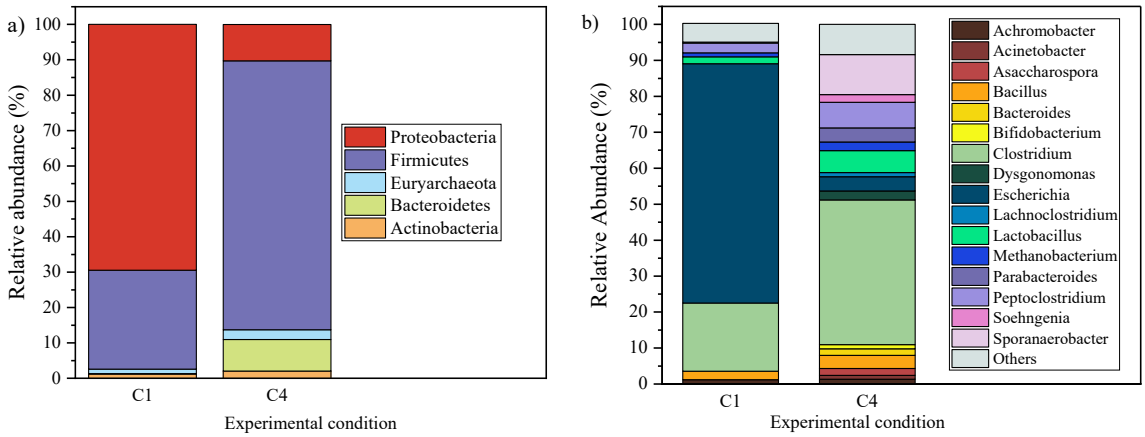

Figure 4. (**a**) Microbial community composition at the phyla level in fermentative systems under conditions 1 (C1) (*S/I* = 3 gSV gSV^{-1}, *pH* = 7) and 4 (C4) (*S/I* = 1.13 gSV gSV^{-1}, *pH* = 5. (**b**) genera with a relative abundance higher than 1% in the acidogenic fermentation systems (conditions 1 and 4) fed with pretreated OMSW substrate.

Experimental condition 1 (*SI* = 3 gVS gVS^{-1}, *pH* = 7) was chosen because it was the most similar to the desirable condition (*SI* = 3 gVS gVS^{-1}, *pH* = 8) among the experimental conditions evaluated. On the other hand, condition 4 had the worst performance for the total concentration of *tVFAs* (3623.6 mg L^{-1}), as well as a low amount of C4 and C5 VFAs (11.9%). Therefore, this condition was chosen to compare the microbial community in reactors with different performances to produce precursors for the biosynthesis of longer chain-length VFAs.

The sequences of the bacterial community were assigned to 6 phyla, 14 classes, and 60 genera for condition 1 and 8 phyla, 18 classes, and 129 genera for condition 14. As shown in Figure 4a, Proteobacteria and Firmicutes were the dominant phyla in the reactors, together representing over 87% of the microbial community for both conditions. Firmicutes have a thick cell wall, and their ability to produce endospores makes them able to survive in extreme conditions [25]. Also, this taxon is closely involved in the hydrolysis of complex organic matter by producing proteases, cellulases, lipases, and other extracellular enzymes [21]. However, in this study, the dominance of Firmicutes phylum observed

for condition 4 was not related to the high production of VFAs reported for this reactor. Therefore, it seems that members of Proteobacteria phylum, which prevailed in condition 1, may be responsible for the amount and composition of VFA production.

The relative abundance of the most representative genera identified in conditions 1 (SI = 3 gVS gVS^{-1}, pH = 7) and 4 (S/I = 3 gSV gSV^{-1}, pH = 7) is shown in Figure 4b. It can be seen that in such conditions, the microbial community is less diverse compared to condition 4 and is dominated by members of the *Escherichia* genus (66.6%), followed by Clostridium sp. (19.0%). On the other hand, *Clostridium* (40.3%), *Sporanaerobacter* (11.2%), *Lactobacillus* (5.1%), and *Peptoclostridium* (7.1%) species, among others, were detected in condition 4.

According to the microbial results, the experimental conditions used in condition 1 (S/I = 3 gVS gVS^{-1}, pH = 7) favored the *Escherichia* genus over the others. It is noteworthy that condition 1 promoted a high concentration of *tVFAs* and a high amount of C4 and C5 VFAs. Thus, the genus *Escherichia* may be associated with the positive results obtained in this condition. According to Wilks et al. [40], species of the genus *Escherichia*, such as *Escherichia* coli, can grow in a *pH* range of 4.5–9, over which bacteria preserve enzymatic activity, as well as protein and nucleic acid stability, maintaining the cytoplasmic *pH* in the *pH* range of 7.2–7.8 [41].

In condition 1, butyric acid corresponded to 18.4% of the VFAs production profile. Considering that butyric acid was not detected in condition 4, this suggests a correlation between the genus *Escherichia* and the production of butyrate. In fact, *Escherichia* species are well known to perform mixed acid fermentation and can simultaneously generate acetate, butyrate, succinate, lactate, and formate during their metabolism. Several studies in the literature have studied engineered *E. coli* to exclusively produce butyrate by excluding major NADH-dependent oxidation/reduction processes, re-hypothesizing the metabolic pathway for butyryl-CoA, and overexpressing the fermentation genes as a requirement to remove acetate synthesis pathways and transform butyryl-CoA to butyrate [6].

The genus *Clostridium*, which accounted for 19.0% and 35.9% in conditions 1 and 4, respectively, is recognized for its hydrolytic function, accelerating the hydrolysis of polysaccharides for further fermentation of sugar in acids and solvents. Therefore, *Clostridium* species may be involved in the substrate availability for all fermentative bacteria. In condition 4, where *Clostridium* species dominated, acetic and isobutyric acids accumulated in the reactor; however, their concentrations were lower in comparison to condition 1. In the literature, *Clostridium* has been identified as playing an important role in the production of VFAs in anaerobic systems using lignocellulosic residues as substrates. For instance, Adarme et al. [22] identified that *Clostridium* corresponded to 63% of the bacterial community present in an acidogenic reactor using vinasse, hemicelluloses hydrolysate (HH), yeast extract (YE), and sugarcane bagasse fly ash (SBFA) as substrates. In another study, *Clostridium* was the dominant bacteria genus in the digestion of swine manure under anaerobic and microaerobic conditions (SM), with relative abundances of 44.8% and 42.7%, respectively [42]. Moreover, *C. butyricum* is known to be one of the most important producers of butyric acid due to its high productivity and relatively greater stability, and it has been used in bioaugmented experiments to enhance butyric acid production [43].

Aiming to further explore the mechanisms underlying the VFAs production from pretreated OMSW, PICRUSt analysis based on 16S rRNA of the identified genera was used. In this way, a total of 6230 MetaCyc pathways were predicted, and the twenty main pathways for conditions 1 and 4 are shown in Figure 5. Metabolic pathways included signaling and cellular processes (C1 = 51% and C4 = 60%), genetic information processing (C1 = 25% and C4 = 21%), and metabolism (C1 = 17% and C4 = 14%).

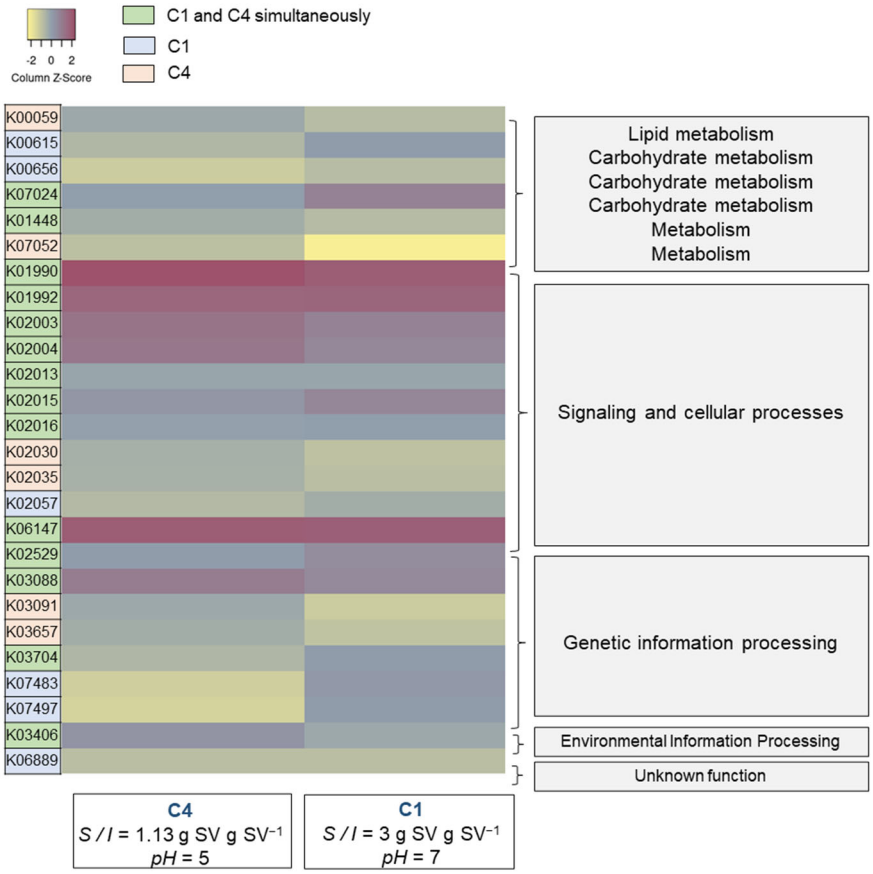

Figure 5. Heatmaps of the main functional pathways involved in VFAs production using conditions 1 (C1) and 4 (C4).

Signaling and cellular processes accounted for the highest proportion in conditions 1 and 4 (Figure 5). Although these conditions (1 and 4) presented different concentrations and profiles of VFAs after fermentation, 70% of the top twenty functional pathways were the same. The substrate composition also played an important role in mixed culture fermentation. As raw olive pomace is composed of lipids (16.81%) and carbohydrates (21.73%) [13], enzymes such as sucrose-6-phosphatase [EC:3.1.3.24], sucrose-6-phosphatase [EC:3.1.3.24], transketolase [EC:2.2.1.1], formate C-acetyltransferase [EC:2.3.1.54], and 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100] might be related to the conversions of these molecules. In addition to these enzymes, other gene expressions linked to the metabolism of substrates (e.g., glucokinase and 6-posphofructokinase) and biosynthesis of VFAs (e.g., butyrate kinase, acetate kinase, pyruvate kinase) were predicted in the analysis [44].

The main benefit of PICRUSt is its evolutionary models that infer functions for the complete bacterial community. However, some drawbacks, such as focusing only on the 16S rDNA marker and using only Greengenes taxonomy, are reported in the literature [45]. Despite these limitations, when PICRUSt is used properly, it is a reliable tool that has been widely used to predict the functional genes involved in the microbial community of acidogenic fermenters for the production of carboxylic acids [44,46–48].

3.4. Simultaneous Optimization and Kinetic Studies

Aiming to achieve better production of VFAs from OMSW, with high concentrations of carboxylic acids and a high amount of longer chain VFAs, the desirability function was used to verify which combination of *pH* and *S/I* ratio would be best suited to provide this scenario. The variables Y_1 and Y_2 were set to fully desirable levels (s = 1). The response of the desirability function indicated that the best scenario to produce VFAs

from OMSW is S/I = 3 gVS gVS^{-1} and pH = 8. The global desirability function value was 0.79. Since this value can vary from 0 to 1 and the closer this value is to unity, the better the simultaneous optimization, the statistical tool was successfully implemented for the proposed objective [18].

Kinetic analysis and data modeling of carboxylic acid production were carried out for the desirable condition (S/I = 3 gVS gVS^{-1}, pH = 8). The kinetic parameters estimated by fitting the First-order, Second-order, Fitzhugh, Monomolecular, Modified Gompertz, Logistic, Transference, and Richards models to the experimental data are shown in Table 4.

Table 4. Kinetic parameters were estimated by modeling the VFAs production from anaerobic digestion of pretreated OMSW (S/I = 3 gVS gVS^{-1}, pH = 8).

Kinetic Model	Parameters									
	k_{CA} (d^{-1})	n	λ (d)	μ (g L^{-1} d^{-1})	ν	R^2	$R^2{}_a$	RMSE	NRMSE (%)	*AIC*
First-order	0.44					0.81	0.80	2.69	11.10	41.56
Second-order	0.04					0.89	0.89	2.03	8.38	30.31
Fitzhugh	1.07	0.41				0.81	0.79	2.69	11.10	43.56
Monomolecular	0.44		0.00			0.81	0.79	2.69	11.10	43.56
Modified Gompertz			0.00	6.24		0.77	0.75	2.93	12.08	46.94
Logistic			0.00	5.85		0.78	0.75	2.91	12.03	46.77
Transference			0.00	9.92		0.81	0.79	2.69	11.10	43.56
Richards			0.00	2.22	0.17	0.77	0.73	2.93	12.08	48.94

The R^2 values estimated by kinetic models ranged from 0.77 to 0.89. As the production of carboxylic acids by mixed culture is a complex bioprocess involving many metabolic pathways, these results are considered satisfactory. Morais et al., 2020 modeled the carboxylic acid production from swine wastewater, and except for the Richards model, they reported R^2 values ranging from 0.73 to 0.87.

The production of VFAs from hydrothermally pretreated olive mill solid wastes in mixed culture was best described by exponential growth models, as evidenced by the higher R^2 values and lower AIC values, such as for the First-order model (R^2 = 0.81 and AIC = 41.56), the Second-order model (R^2 = 0.89 and AIC = 30.31), and the Monomolecular model (R^2 = 0.81 and AIC = 43.56) compared to Logistic growth kinetics, such as for the Richards model (R^2 = 0.77 and AIC = 48.94) and the Logistic model (R^2 = 0.78 and AIC = 46.77).

In exponential models, the production of VFAs increased until a maximum concentration was reached. As shown in Figure 6, the maximum concentration of VFAs was reached on the eighth day of the experiment. In addition, the amount of acetic acid decreased, and a profile with a higher amount of butyric acid (25.5–36.2%) and propionic acid (5.3–11.1%) was observed until the 12th day of the test. One of the hypotheses for the reduction in acetic acid concentration and the increase in propionic and butyric acid concentration over the days is the metabolic shift of the bacteria in response to the accumulation of acetic acid in the reactor. Therefore, a shift in the metabolic pathway probably occurred to ensure the internal recycling of electron-carrying species such as NAD [13]. In the butyrate-type metabolic pathway, butyrate is synthesized by the reduction and decarboxylation of pyruvate with the consumption of acetate. Furthermore, the low yield of propionate compared to butyrate during anaerobic fermentation of OMSW can be attributed to the fact that propionate-producing bacteria are strongly inhibited by undissociated propionate, especially when the pH is below 6 [49].

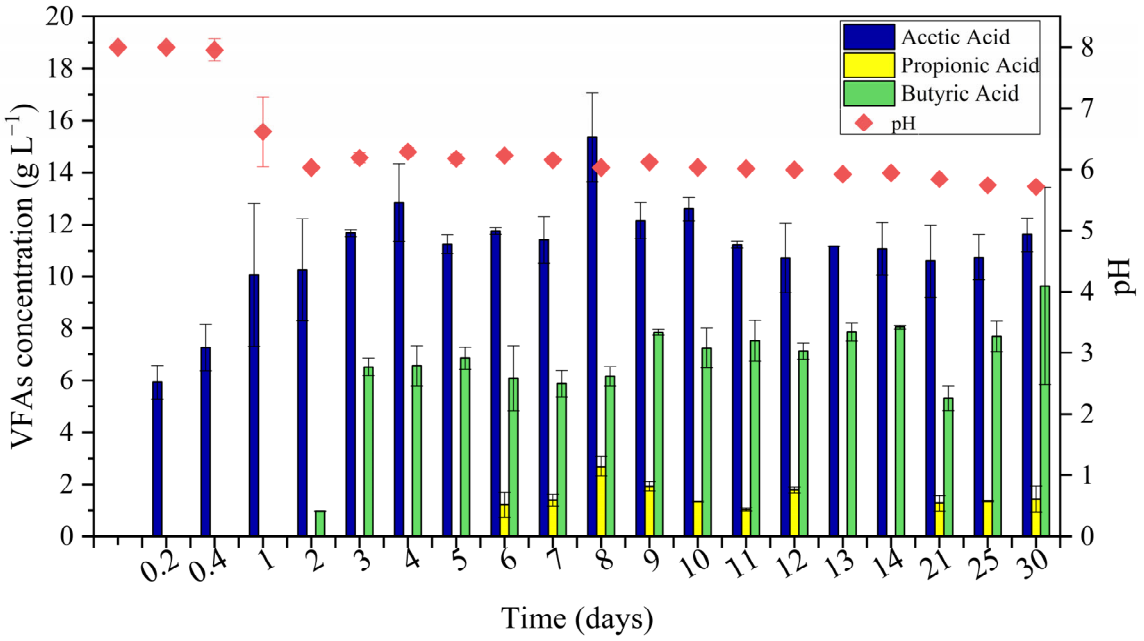

Figure 6. The concentration of carboxylic acids and *pH* of the fermentation medium on the monitored days.

In addition to the mathematical meaning, data modeling also brings physical meaning to the system [50]. The lag phase time (λ = 0) predicted by the modified Gompertz, Monomolecular, Transfer, Logistic, and Richards models, and the value of $n < 1$ (n = 0.41) predicted by the Fitzhugh model suggested that the adaptation of the microbial community to the fermentative environment was not a bottleneck for fermentation reactions. This information reinforces that the hydrothermal pretreatment fulfilled its function of making the biomass more accessible to microorganisms [13]. Similarly, in the work of Fonseca et al., 2021, the authors observed that the steam explosion of the coffee husk under more severe conditions contributed to improving the reduction of the lag phase of methane production, which was also equal to zero when the biomass was treated at 180 °C and 60 min and 210 °C and 15 min.

It is noteworthy that kinetic data from reliable experimental data are essential for modeling biotechnological systems. This is particularly important because process simulation needs to be strongly supported by data collection for practical validation purposes [51]. In addition, this information supports prospective techno-economic and life-cycle assessments of early-stage technologies, which are important tools to guide development efforts and investment decisions, since they allow identifying sustainability hurdles and opportunities [52,53].

4. Conclusions

A total VFA concentration of 22.7 g L^{-1} and an amount of longer chain VFAs of 42% were obtained in the optimized condition in this work (*S/I* = 3 gVS gVS^{-1} and *pH* = 8). *Clostridium*, *Escherichia*, and *Lactobacillus* strengthened microbial cooperation to enhance metabolic functions (e.g., carbohydrate and lipid metabolism) for VFA production. It can be concluded that OMSW can be a valuable resource for biorefineries, thus generating a business opportunity for the olive oil industry. Future studies should focus on the chain elongation of carboxylic acids and the upscaling of the technological roadmap, with the aim of advancing the technology readiness level.

Author Contributions: Conceptualization, Y.A.d.F. and B.E.L.B.; methodology, A.B.d.C. and Y.A.d.F.; software, P.F.; Y.A.d.F. and G.S.M.G.; validation, A.B.d.C. and Y.A.d.F.; formal analysis, P.F., Y.A.d.F. and G.S.M.G.; investigation, Y.A.d.F.; writing—original draft preparation, Y.A.d.F.; writing—review and editing, L.V.A.G., S.Q.S. and B.E.L.B.; visualization, L.V.A.G., B.E.L.B., S.Q.S.; supervision, B.E.L.B. and L.V.A.G.; funding acquisition, B.E.L.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Minas Gerais State Research Funding Agency, grant number APQ-00325-17 and the Brazilian National Council for Scientific and Technological Development (CNPq), grant number 438527/2018-6. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brazil (CAPES)—Finance Code 001.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the authors.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. European Commission. *Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*; European Commission: Brussels, Belgium, 2015.
2. Atasoy, M.; Owusu-Agyeman, I.; Plaza, E.; Cetecioglu, Z. Bio-Based Volatile Fatty Acid Production and Recovery from Waste Streams: Current Status and Future Challenges. *Bioresour. Technol.* **2018**, *268*, 773–786. [CrossRef] [PubMed]
3. Zacharof, M.; Lovitt, R. Complex Effluent Streams as a Potential Source of Volatile Fatty Acids. *Waste Biomass Valorization* **2013**, *4*, 557–581. [CrossRef]
4. Chandrasekhar, K.; Kumar, S.; Lee, B.D.; Kim, S.H. Waste Based Hydrogen Production for Circular Bioeconomy: Current Status and Future Directions. *Bioresour. Technol.* **2020**, *302*, 122920. [CrossRef] [PubMed]
5. Murali, N.; Srinivas, K.; Ahring, B.K. Biochemical Production and Separation of Carboxylic Acids for Biorefinery Applications. *Fermentation* **2017**, *3*, 22. [CrossRef]
6. Wainaina, S.; Lukitawesa; Kumar Awasthi, M.; Taherzadeh, M. Bioengineering of Anaerobic Digestion for Volatile Fatty Acids, Hydrogen or Methane Production: A Critical Review. *Bioengineered* **2019**, *10*, 437–458. [CrossRef]
7. Roig, A.; Cayuela, M.L.; Sa, M.A. An Overview on Olive Mill Wastes and Their Valorisation Methods. *Waste Manag.* **2006**, *26*, 960–969. [CrossRef]
8. Rodríguez, G.; Lama, A.; Rodríguez, R.; Jiménez, A.; Guillén, R.; Fernández-Bolaños, J. Olive Stone an Attractive Source of Bioactive and Valuable Compounds. *Bioresour. Technol.* **2008**, *99*, 5261–5269. [CrossRef]
9. Serrano, A.; Fermoso, F.G.; Rodríguez-Gutierrez, G.; Fernandez-Bolaños, J.; Borja, R. Biomethanization of Olive Mill Solid Waste after Phenols Recovery through Low-Temperature Thermal Pre-Treatment. *Waste Manag.* **2017**, *61*, 229–235. [CrossRef]
10. Yarimtepe, C.; Oz, N.; Ince, O. Volatile Fatty Acid Production Dynamics during the Acidification of Pretreated Olive Mill Wastewater. *Bioresour. Technol.* **2017**, *241*, 936–944. [CrossRef]
11. Rincón, B.; Borja, R. Assessment of the Anaerobic Acidogenesis of Wet Olive Cake from a Two-Phase Olive Oil Mill. *J. Environ. Sci. Health* **2012**, *47*, 1439–1445. [CrossRef]
12. Cabrera, F.; Serrano, A.; Torres, Á.; Guillermo, R.-G.; Jeison, D.; Fermoso, F. The Accumulation of Volatile Fatty Acids and Phenols through a pH- Controlled Fermentation of Olive Mill Solid Waste. *Sci. Total Environ.* **2019**, *657*, 1501–1507. [CrossRef]
13. Da Fonseca, Y.A.; Silva, N.C.S.; De Camargos, A.B.; Silva, S.D.Q.; Wandurraga, H.J.L.; Gurgel, L.V.A.; Baêta, B.E.L. Influence of Hydrothermal Pretreatment Conditions, Typology of Anaerobic Digestion System, and Microbial Profile in the Production of Volatile Fatty Acids from Olive Mill Solid Waste. *J. Environ. Chem. Eng.* **2021**, *9*, 105055. [CrossRef]
14. Angelidaki, I.; Alves, M.; Bolzonella, D.; Borzacconi, L.; Campos, J.L.; Guwy, A.J.; Kalyuzhnyi, S.; Jenicek, P.; Van Lier, J.B. Defining the Biomethane Potential (BMP) of Solid Organic Wastes and Energy Crops: A Proposed Protocol for Batch Assays. *Water Sci. Technol.* **2009**, *59*, 927–934. [CrossRef]
15. Li, Y.; Chen, Z.; Peng, Y.; Huang, W.; Liu, J.; Mironov, V.; Zhang, S. Deeper Insights into the Effects of Substrate to Inoculum Ratio Selection on the Relationship of Kinetic Parameters, Microbial Communities, and Key Metabolic Pathways during the Anaerobic Digestion of Food Waste. *Water Res.* **2022**, *217*, 118440. [CrossRef]
16. Elbeshbishy, E.; Nakhla, G.; Hafez, H. Biochemical Methane Potential (BMP) of Food Waste and Primary Sludge: Influence of Inoculum Pre-Incubation and Inoculum Source. *Bioresour. Technol.* **2012**, *110*, 18–25. [CrossRef]
17. APHA. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2005.
18. Derringer, G.; Suich, R. Simultaneous Optimization of Several Response Variables. *J. Qual. Technol.* **1980**, *12*, 214–219. [CrossRef]

19. Lima, D.; Adarme, O.; Baêta, B.; Gurgel, L.; Aquino, S. Influence of Different Thermal Pretreatments and Inoculum Selection on the Biomethanation of Sugarcane Bagasse by Solid-State Anaerobic Digestion: A Kinetic Analysis. *Ind. Crop. Prod.* **2018**, *111*, 684–693. [CrossRef]
20. Baêta, B.; Lima, D.; Filho, J.; Adarme, O.; Gurgel, L.; Aquino, S. Evaluation of Hydrogen and Methane Production from Sugarcane Bagasse Hemicellulose Hydrolysates by Two-Stage Anaerobic Digestion Process. *Bioresour. Technol.* **2016**, *218*, 436–446. [CrossRef]
21. Iglesias-Iglesias, R.; Campanaro, S.; Treu, L.; Kennes, C.; Veiga, M.C. Valorization of Sewage Sludge for Volatile Fatty Acids Production and Role of Microbiome on Acidogenic Fermentation. *Bioresour. Technol.* **2019**, *291*, 121817. [CrossRef]
22. Adarme, O.; Eduardo Lobo Baêta, B.; Cardoso Torres, M.; Camilo Otalora Tapiero, F.; Vinicius Alves Gurgel, L.; de Queiroz Silva, S.; Francisco de Aquino, S. Biogas Production by Anaerobic Co-Digestion of Sugarcane Biorefinery Byproducts: Comparative Analyses of Performance and Microbial Community in Novel Single-and Two-Stage Systems. *Bioresour. Technol.* **2022**, *354*, 127185. [CrossRef]
23. Christoff, A.; Fernanda, A.; Sereia, R.; Rodrigues, D.; Lucio, R.; De Moraes, V.; Felipe, L.; De Oliveira, V. Bacterial Identification through Accurate Library Preparation and High-Throughput Sequencing. *Neoprospecta Microbiome Technol.* **2017**, *25*, 1–5.
24. Langille, M.G.I.; Zaneveld, J.; Caporaso, J.G.; McDonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Vega Thurber, R.L.; Knight, R.; et al. Predictive Functional Profiling of Microbial Communities Using 16S RRNA Marker Gene Sequences. *Nat. Biotechnol.* **2013**, *31*, 814–821. [CrossRef] [PubMed]
25. Zhang, Q.; Zhao, X.; Li, W.; Chen, H.; Zhu, X.; Zhu, H.; Zhang, P. Responses of Short-Chain Fatty Acids Production to the Addition of Various Biocarriers to Sludge Anaerobic Fermentation. *Bioresour. Technol.* **2020**, *304*, 122989. [CrossRef] [PubMed]
26. Morais, N.W.S.; Coelho, M.M.H.; Silva, A.D.S.E.; Pereira, E.L.; Leitão, R.C.; dos Santos, A.B. Kinetic Modeling of Anaerobic Carboxylic Acid Production from Swine Wastewater. *Bioresour. Technol.* **2020**, *297*, 122520. [CrossRef] [PubMed]
27. Akaike, H.A.I. A New Look at the Statistical Model Identification. *IEEE Trans. Automat. Contr.* **1974**, *19*, 716–723. [CrossRef]
28. Motulsky, H.; Christopoulos, A. *Fitting Models to Biological Data Using Linear and Nonlinear Regression*; GraphPad Software: San Diego, CA, USA, 2003.
29. Aghapour Aktij, S.; Zirehpour, A.; Mollahosseini, A.; Taherzadeh, M.; Tiraferri, A.; Rahimpour, A. Feasibility of Membrane Processes for the Recovery and Purification of Bio-Based Volatile Fatty Acids: A Comprehensive Review. *J. Ind. Eng. Chem.* **2020**, *81*, 24–40. [CrossRef]
30. Reyhanitash, E.; Kersten, S.R.A.; Schuur, B. Recovery of Volatile Fatty Acids from Fermented Wastewater by Adsorption. *ACS Sustain. Chem. Eng.* **2017**, *5*, 9176–9184. [CrossRef]
31. Sarkar, O.; Rova, U.; Christakopoulos, P.; Matsakas, L. Influence of Initial Uncontrolled pH on Acidogenic Fermentation of Brewery Spent Grains to Biohydrogen and Volatile Fatty Acids Production: Optimization and Scale-Up. *Bioresour. Technol.* **2021**, *319*, 124233. [CrossRef]
32. Dahiya, S.; Sarkar, O.; Swamy, Y.V.; Venkata Mohan, S. Acidogenic Fermentation of Food Waste for Volatile Fatty Acid Production with Co-Generation of Biohydrogen. *Bioresour. Technol.* **2015**, *182*, 103–113. [CrossRef]
33. López-Garzón, C.S.; Straathof, A.J.J. Recovery of Carboxylic Acids Produced by Fermentation. *Biotechnol. Adv.* **2014**, *32*, 873–904. [CrossRef]
34. Strazzera, G.; Battista, F.; Garcia, N.H.; Frison, N.; Bolzonella, D. Volatile Fatty Acids Production from Food Wastes for BioreFi Nery Platforms: A Review. *J. Environ. Manag.* **2018**, *226*, 278–288. [CrossRef]
35. Atasoy, M.; Eyice, O.; Schnürer, A.; Cetecioglu, Z. Fatty Acids Production via Mixed Culture Fermentation: Revealing the Link between pH, Inoculum Type and Bacterial Composition. *Bioresour. Technol.* **2019**, *292*, 121889. [CrossRef]
36. Wu, Q.; Bao, X.; Guo, W.; Wang, B.; Li, Y.; Luo, H.; Wang, H.; Ren, N. Medium Chain Carboxylic Acids Production from Waste Biomass: Current Advances and Perspectives. *Biotechnol. Adv.* **2019**, *37*, 599–615. [CrossRef]
37. Kleerebezem, R.; Joosse, B.; Rozendal, R.; Van Loosdrecht, M.C.M. Anaerobic Digestion without Biogas? *Rev. Environ. Sci. Biotechnol.* **2015**, *14*, 787–801. [CrossRef]
38. Li, D.; Yin, F.; Ma, X. Towards Biodegradable Polyhydroxyalkanoate Production from Wood Waste: Using Volatile Fatty Acids as Conversion Medium. *Bioresour. Technol.* **2020**, *299*, 122629. [CrossRef]
39. Gameiro, T.; Sousa, F.; Silva, F.C.; Couras, C.; Lopes, M.; Louros, V.; Nadais, H.; Capela, I. Olive Oil Mill Wastewater to Volatile Fatty Acids: Statistical Study of the Acidogenic Process. *Water Air Soil Pollut.* **2015**, *226*, 115. [CrossRef]
40. Wilks, J.C.; Slonczewski, J.L. pH of the Cytoplasm and Periplasm of Escherichia Coli: Rapid Measurement by Green Fluorescent Protein Fluorimetry. *J. Bacteriol.* **2007**, *189*, 5601–5607. [CrossRef]
41. De Jonge, R.; Takumi, K.; Ritmeester, W.S.; Van Leusden, F.M. The Adaptive Response of Escherichia Coli O157 in an Environment with Changing pH. *J. Appl. Microbiol.* **2003**, *94*, 555–560. [CrossRef]
42. Cao, Q.; Zhang, W.; Lian, T.; Wang, S.; Yin, F.; Zhou, T.; Zhang, H.; Zhu, J.; Dong, H. Roles of Micro-Aeration on Enhancing Volatile Fatty Acids and Lactic Acid Production from Agricultural Wastes. *Bioresour. Technol.* **2022**, *347*, 126656. [CrossRef]
43. Atasoy, M.; Cetecioglu, Z. Butyric Acid Dominant Volatile Fatty Acids Production: Bio-Augmentation of Mixed Culture Fermentation by Clostridium Butyricum. *J. Environ. Chem. Eng.* **2020**, *8*, 104496. [CrossRef]
44. Liang, J.; Fang, W.; Chang, J.; Zhang, G.; Ma, W.; Nabi, M.; Zubair, M.; Zhang, R.; Chen, L.; Huang, J.; et al. Long-Term Rumen Microorganism Fermentation of Corn Stover in Vitro for Volatile Fatty Acid Production. *Bioresour. Technol.* **2022**, *358*, 127447. [CrossRef]

45. Djemiel, C.; Maron, P.A.; Terrat, S.; Dequiedt, S.; Cottin, A.; Ranjard, L. Inferring Microbiota Functions from Taxonomic Genes: A Review. *Gigascience* **2022**, *11*, 1–30. [CrossRef] [PubMed]
46. Douglas, G.M.; Langille, M.G.I. A Primer and Discussion on DNA-Based Microbiome Data and Related Bioinformatics Analyses. *Peer Community J.* **2021**, 1. [CrossRef]
47. Shao, Q.; Zhang, Q.; Fang, S.; Huang, W.; Li, Z.; Fang, X. Upgrading Volatile Fatty Acids Production from Anaerobic Co-Fermentation of Orange Peel Waste and Sewage Sludge: Critical Roles of Limonene on Functional Consortia and Microbial Metabolic Traits. *Bioresour. Technol.* **2022**, *362*, 127773. [CrossRef] [PubMed]
48. Liang, J.; Fang, W.; Wang, Q.; Zubair, M.; Zhang, G.; Ma, W.; Cai, Y.; Zhang, P. Metagenomic Analysis of Community, Enzymes and Metabolic Pathways during Corn Straw Fermentation with Rumen Microorganisms for Volatile Fatty Acid Production. *Bioresour. Technol.* **2021**, *342*, 126004. [CrossRef]
49. Zhou, M.; Yan, B.; Wong, J.W.C.C.; Zhang, Y. Enhanced Volatile Fatty Acids Production from Anaerobic Fermentation of Food Waste: A Mini-Review Focusing on Acidogenic Metabolic Pathways. *Bioresour. Technol.* **2018**, *248*, 68–78. [CrossRef]
50. Ware, A.; Power, N. Modelling Methane Production Kinetics of Complex Poultry Slaughterhouse Wastes Using Sigmoidal Growth Functions. *Renew. Energy* **2017**, *104*, 50–59. [CrossRef]
51. Hartmann, F.S.F.; Udugama, I.A.; Seibold, G.M.; Sugiyama, H.; Gernaey, K.V. Digital Models in Biotechnology: Towards Multi-Scale Integration and Implementation. *Biotechnol. Adv.* **2022**, *60*, 108015. [CrossRef]
52. Cordero-Lanzac, T.; Ramirez, A.; Navajas, A.; Gevers, L.; Brunialti, S.; Gandía, L.M.; Aguayo, A.T.; Mani Sarathy, S.; Gascon, J. A Techno-Economic and Life Cycle Assessment for the Production of Green Methanol from CO_2: Catalyst and Process Bottlenecks. *J. Energy Chem.* **2022**, *68*, 255–266. [CrossRef]
53. Thomassen, G.; Van Dael, M.; Van Passel, S.; You, F. How to Assess the Potential of Emerging Green Technologies? Towards a Prospective Environmental and Techno-Economic Assessment Framework. *Green Chem.* **2019**, *21*, 4868–4886. [CrossRef]