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Biorefinery Approach for H_2 and Acids Production Based on Uncontrolled pH Fermentation of an Industrial Effluent

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Abstract: In this work, the feasibility of uncontrolled pH acidogenic fermentation of industrial organic effluent from corn-bioethanol production was studied and modelled by using a Monod-based mathematical model. In order to do that, several tests were carried out at different initial pH values, ranging from 4 to 6. The experimental data showed a pH reduction during the fermentation process due to the generation of short-chain acids. When starting at initial pH of 5.0 and 6.0, the substrates were fully fermented reaching final pH s over 4 units in both cases and a final undissociated fatty acid concentration of about 80 (mmol·L^{-1}) in both cases. Regarding fermentation at an initial pH of 4, the pH decreased to 3.5 units, and the organic substrates were not fully fermented due to the stoppage of the fermentation. The stoppage was caused by the very acidic pH conditions. The biomass showed an uncoupled growth as the operating conditions became more acidic, and, finally, the biomass growth was zero. Regarding the generation of fermentation products, in general terms, the highest economical value of products was obtained when fermenting at an initial pH of 5. More specifically, acetic acid was the acid that presented the highest yield at an initial pH value of 4. Butyric yield showed the highest values at initial pH values of 5 and 6. The highest H_2 yield (1.1 mol H_2·mol^{-1} dextrose) was achieved at an initial pH value of 5. Finally, the experimental data were modelled using a Monod-based model. From this model, the value of the main kinetics and stoichiometric parameters were determined.

Keywords: corn-bioethanol effluent; acidogenic fermentation; uncontrolled pH; modelling

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1. Introduction

The increasing consumption of energy and chemicals has caused undesirable effects on the planet. Regarding the energy aspects, future energy sources should not only be renewable and sustainable, but also versatile [1]. Hydrogen (H_2) has emerged as a potential candidate for energy supply over the past few decades. The advantages of using H_2 include its clean consumption, lack of polluting emissions, and high energy output, i.e., 123 MJ/kg, which is approximately three times that of fossil fuels: gasoline is 47.3 MJ/kg, kerosene is 46.2 MJ/kg, and diesel fuel is 44.8 MJ/kg [2]. The market price of hydrogen depends on its precedence, with the prices of grey, blue, and green H_2 being about 1.5, 2.5, and 5 kEUR/ton, respectively [3,4]. In addition, a significant number of chemicals are produced daily by the industry, among which Volatile Fatty Acids (VFA) is significant. The most known and widely used VFA are acetic, propionic, and butyric acids, which represent the

whole production of close to 4000 kt/year, with a market price of 0.4–0.8 kEUR/ton for acetic acid, 1.7–1.9 for propionic acid, and 1.8–2.0 for butyric acid [5]. These chemicals are usually produced from fossil fuels, with a significant environmental impact, making it necessary to search for more sustainable options [6].

To ensure the sustainability of energy and chemical production, eco-friendly production technologies and sustainable raw materials should be employed. Amongst the sustainable production technologies, biochemical processes stand out. There are different biochemical processes for H_2 and chemical generation, and acidogenic fermentation is one of the most attractive options due to its ability to convert organic waste effluents into H_2 and VFA [7].

Organic-rich effluents can serve as an efficient substrate for H_2 and VFA production, reducing the overall treatment costs [8]. Furthermore, using waste materials as raw materials aligns with the principles of circular economy, providing additional value. In the literature, the generation of industrial wastewater has been described as containing high concentrations of easily degradable organic substrates, which can ensure high material and energy yields. Common examples include the sugar industry [9], the food processing industry [10,11], distilleries [12], chemical industries [13], and pharmaceuticals [14]. Even the energy industry from biofuel production results in the excessive generation of byproducts, which can be effectively utilized as a substrate for H_2 and VFA production [15,16].

One of the most interesting bioenergy industry effluents is corn syrup containing wastewater, which is generated during corn-bioethanol production and typically involves the fermentation and distillation of corn starch to produce bioethanol [17,18]. During this process, enzymes break down corn starch into simpler sugars, primarily dextrose. The dextrose is fermented and then distilled to separate the ethanol from the non-fermentable components, resulting in corn syrup, which is essentially a concentrated solution of dextrose [19]. The wastewaters containing corn syrup can be fermented with the aim of producing chemicals and energy.

Acidogenic fermentation can provide an attractive and efficient process for converting corn syrup substrates into H_2 and VFA, which have broad applications in chemical synthesis [20,21]. In the literature, the influence of the main operational variables on the acidogenic fermentation process has been described, including the influence of the operational pH, temperature, substrate concentration, etc. The pH has been identified as one of the most important variables influencing the metabolic pathways, and therefore, the generation of fermentation products [1,20,22–31]. It is known that there is an optimal operational pH for acidogenic fermentation in which the highest fermentation rate and yield are obtained [32]. This behaviour can be explained by the inhibitory effects of the accumulation of acids generated during fermentation [33]. Acid accumulation reduces pH, which influences the intracellular pH of microorganisms and leads to metabolic disruptions [34]. When the pH falls below the pKa value of the acids, they become protonated and can easily permeate via passive diffusion inside the microorganisms [35]. Once inside the cells, these protonated acids accumulate, resulting in a decrease in the intracellular pH [28]. Therefore, to ensure optimal operational conditions, most fermentation processes are conducted under controlled pH. However, this enhancement of productivity using pH control entails the utilization of control systems and the consumption of acids or alkalis to maintain the pH near the set point. On the one hand, from an environmental perspective, the doses of acids and alkalis lead to an increase in the effluent salinity due to the Cl^-, Na^+, etc. dosage, reducing the sustainability of the generated H_2 and VFA. On the other hand, from an economical perspective, the necessity of control systems and the consumption of acids and alkalis increase the operational costs of the process. Regarding the costs, it must be stated that while pH-controlled fermentative processes could be viable when operated at a laboratory or bench scale, they could become economically challenging when scaled up to full production. The increasing operational costs associated with larger-scale operations can render pH-controlled full-scale fermentative processes economically unsustainable. In any case, the increase in operational cost due to the acid and alkali consumption of the

controlled pH acidogenic fermentation should also be balanced with the lower economic cost of the fermentation product separation due to the higher concentration reached.

Because of this economic drawback, in the literature, some research focused on uncontrolled pH acidogenic fermentation [1,36,37]. In these studies, which were carried out without pH control, the initial pH value is of crucial importance in order to ensure optimum performance. In the literature, different initial pH values have been studied, observing an optimum pH range even broader than that shown by the studies carried out with pH control. For example, the optimal initial pH observed by Khanal et al. [38] and Lin et al. [39] were 4.5 and 5.5, respectively, whereas the optimal initial pH reported by Lee et al. [40] was 9.

In this context, the present work focused on the study of the influence of the initial pH on the uncontrolled pH, mixed culture, and acidogenic fermentation of an industrial organic effluent from corn-bioethanol production. Additionally, a Monod-based mathematical model was proposed with the aim of determining the main kinetic and stoichiometric parameters of substrate fermentation, product generation, and biomass growth.

2. Materials and Methods

2.1. Innoculum and Synthetic Corn-Bioethanol Effluent

The inoculum used in this study was taken from the activated sludge reactor of a conventional municipal wastewater treatment plant. Further details about this facility can be found in the literature [41,42]. To facilitate the acidogenic fermentation of the corn-bioethanol effluent, the inoculum underwent an acclimatization process, following a previously described procedure in the literature [43]. This process involved the utilization of a Sequential Batch Reactor (SBR) operating under strict anaerobic conditions at 35 °C.

In order to ensure the reproducibility of the experiments, corn-bioethanol effluent was used during the batch experiments without pH control, and synthetic corn-bioethanol wastewater was used. According to the literature, the effluent mainly consists of dextrose 80–300 g/L and presents a pH ranging from 4 to 6 [19]. Because of that, a synthetic corn-bioethanol effluent consisting primarily of dextrose, with an initial dextrose concentration of 150 $g \cdot L^{-1}$, and variable initial pH ranging from 4 to 6 was synthesized in the laboratory. The synthetic corn-bioethanol effluent was supplemented with inorganic and trace minerals, as outlined in the literature [19,44]. A detailed composition of the inorganic and trace minerals used in the study is presented in Table 1.

Table 1. Inorganic components and trace minerals used in this study.

Compound	Concentration ($g \cdot L^{-1}$)	Compound	Concentration ($g \cdot L^{-1}$)
$(NH_4)Cl$	4.89	$FeSO_4\ 7H_2O$	11.3×10^{-3}
KH_2PO_4	2.85	$MnCl_2\ 4H_2O$	9.1×10^{-3}
NaCl	1.07	$CuCl\ 2H_2O$	8.0×10^{-3}
Na_2SO_4	0.21	$CoCl_2\ 6H_2O$	3.5×10^{-3}
$MgCl_2\ 6H_2O$	0.44	$CaCl_2$	2.2×10^{-3}
EDTA	0.18	$NiCl_2\ 6H_2O$	1.8×10^{-3}
$ZnSO_4\ 7H_2O$	11.7×10^{-3}		

2.2. Experimental Set-Up

To measure the hydrogen production, batch tests were carried out using Oxitop® reactors. In parallel, liquid samples were extracted from water-sealed serum bottles. Each serum bottle was discarded after sampling, so the number of serum bottles used matched the number of samples taken.

Before the start of the experiments, 1.2 L of acclimatized culture was mixed with 0.2 L of synthetic corn-bioethanol effluent and 1.6 L of demineralised water, resulting in a total mixture volume of 3 L. This mixture yielded an initial dextrose concentration of about 10 $g \cdot L^{-1}$, 50 $mmol \cdot L^{-1}$. Subsequently, the pH of the mixtures was adjusted to the desired initial pH levels of 4, 5, and 6 units using HCl and NaOH. These mixtures were then

distributed among the serum bottles and the Oxitop® reactors. The Oxitop® reactors of 1 L of volume contained 0.3 L of the mixture, whereas the serum bottles of 0.1 L contained 0.03 L of the mixture as they were identical to the ratio liquid/gas.

Once the Oxitop® reactors and the serum bottles were filled, N_2 gas at a flow rate of 5 L·min^{-1} was used to remove the oxygen from the liquid and gas phases. Then, the reactors were hermetically sealed. Throughout the fermentation process, the temperature was maintained at 35 °C using a thermostatic chamber. To ensure homogeneity during the experiments, magnetic stirring was induced in all the Oxitop® reactors and serum bottles.

2.3. Analytical Methods

Total Suspended Solids (TSS) were determined by filtering the samples through a Millipore 0.7-µm membrane filter, followed by overnight drying at 105 °C. To measure Volatile Suspended Solids (VSS), the same sample was subsequently ignited for 2 h at 550 °C. Both TSS and VSS were determined following standard methods [45]. The pH measurements were conducted using a pH probe (Mettler-Toledo, Worthington, EEUU).

Gas production was determined by means of the barometric sensors of the Oxitop® reactors. Continuous recordings of the gas pressure inside these systems were obtained using the sensors located in the Oxitop® reactor heads. To eliminate the contribution of CO_2 to the total pressure, NaOH pearls were placed in the rubber quivers of the Oxitop® reactors.

In order to characterize the substrate and fermentation product concentrations during the fermentative processes, liquid samples were collected and subjected to centrifugation at 12,000 rpm. Then, the supernatant was filtered through a 0.45 µm glass fiber filter. The dextrose concentration was determined from the filtrate using High-Performance Liquid Chromatography (HPLC) with an Agilent refractive index detector (series 1200). A Zorbax Carbohydrate Column (4.6 × 150 mm, 5-micron) was employed to separate the components at a temperature of 35 °C. The mobile phase consisted of 75 vol. % acetonitrile and 25 vol. % water, with a flow rate of 1.5 mL·min^{-1}. The VFA (including acetic, propionic, and butyric acids) were determined from the filtrate samples using a Perkin Elmer flame ionization detector (FID) gas chromatograph. A Crossbond Carbowax Column (15 m × 0.32 mmID, 0.25 mm df) was used. The oven temperature was initially set to 140 °C for 1.5 min, followed by an increase at a rate of 25 °C/min until it reached 190 °C, where it was held for 2 min. The injector and detector temperatures were 200 °C and 230 °C, respectively. Nitrogen was used as the carrier gas. The lactic acid concentration was measured from the filtrate samples by using an HPLC system with an Agilent ultraviolet diode array detection (UV-DAD) detector and a Zorbax SB-Aq column (4.6 × 150 mm, 5 µm). The mobile phase was a pH 2 buffer (0.05 M phosphate) consisting of 99% water and 1% acetonitrile.

2.4. Mathematical Model

In order to model the behaviour of the corn-bioethanol effluent fermentation without pH control, a mathematical model was developed and fitted to the experimental data. The model developed was based on the Monod kinetics and takes into account the inhibition caused by the undissociated acid accumulation described in the literature [46,47]. This kind of inhibition has been described as one of the most relevant when performing fermentation processes [47]. The experimental results of biomass growth and decay, substrate fermentation, and fermentation product generation are described by means of the model presented in the Petersen Matrix of Table 2.

Table 2. Petersen matrix of the processes taking place in this study.

Process \ Components	Dextrose	Acetic	Lactic	Butyric	Propionic	Biomass	CO_2	H_2	*Process Rate*
Acetic production	$-1/Y_A$	1							$q_a \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Lactic production	$-1/Y_L$		1						$q_l \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Butyric production	$-1/Y_B$			1					$q_b \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Propionic production	$-1/Y_P$				1				$q_p \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Biomass growth	$-1/Y_{obs}$					1			$\mu_{max} \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{biomass})}} X - DX$
Carbon dioxide production	$-1/Y_{CO2}$						1		$q_{CO_2} \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Hydrogen production	$-1/Y_{H2}$							1	$q_{H_2} \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Lactic consumption		1	−2		1		1		$q_{lc} \frac{S}{K_s+S} \cdot \frac{1}{1+1.2^{(C_{HA}-K_{substrate})}} X$
Lysis of biomass						−1			D·X

In Table 2, X is the biomass concentration (g SSV·L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is substrate concentration (mmol·L^{-1}), K_s is substrate half-saturation constant (mmol·L^{-1}), D is the decay constant (h^{-1}), and $K_{biomass}$ and $K_{sustrate}$ are the undissociated acid threshold concentrations (mmol·L^{-1}), which caused an important shift in biomass growth and substrate fermentation when those concentrations were reached. Y_{obs} is the observed biomass yield for each experiment (g SSV·$mmol^{-1}$ dextrose). The Y_{obs} can be determined by using Equation (1), where $Y_x{}^{max}$ (g SSV·$mmol^{-1}$ dextrose) is the maximum biomass growth yield and Y_{maint} (g SSV·$mmol^{-1}$ dextrose) is the biomass maintenance.

$$Y_{obs} = Y_x{}^{max} - Y_{maint} \quad (1)$$

The C_{HA}, total undissociated acid concentration (mmol·L^{-1}), can be determined by taking into account that the acids generated can remain dissociated or undissociated as a function of their pKa and the pH of the medium. The pKa values of the obtained acids are as follows: 3.86 (lactic acid); 4.75 (acetic acid); 4.83 (butyric acid); 4.87 (propionic acid). The undissociated acid concentrations were calculated by using Equation (2).

$$C_{HA_i} = \frac{C_{Total}}{1 + 10^{(pH - pKa)}} \quad (2)$$

Therefore, the total concentration of undissociated acids can be calculated using Equation (3).

$$C_{HA} = \sum C_{HA_i} \quad (3)$$

Because of the continuous modification of the pH, the values of the kinetic and stoichiometric parameters changed during the experiment. In this work, according to the experimental results, linear functions were used to describe the pH dependence of the μ_{max} and the Y_{maint}. In the case of the K_s, a potential function was used. In the literature, similar dependencies have been described for these parameters [47,48].

$$\mu_{max} = a \cdot pH + b \quad (4)$$

$$Y_{maint} = c \cdot pH + d \quad (5)$$

$$k_s = e \cdot pH^2 + f \cdot pH + g \quad (6)$$

In Equations (4)–(6), the parameters a–g are the fitting values. Once the set of equations describing the process was defined, the experimental results of biomass growth, dextrose consumption, and fermentation product generation were used to fit the mathematical model.

In order to fit the model to the experimental results, the set of equations presented was solved simultaneously using the Gauss–Newton algorithm. An initial set of values was assigned to the model parameters, and after several iterations, the values of the parameters that minimised the sum of squared errors (SSE) were chosen as the best estimate. The SSE expression used to estimate the values of the model parameters was as follows:

$$\chi(p) = \frac{\sqrt{\sum_{i=1}^{n}(z_{meas,i} - z_i(p))^2}}{z} + \frac{\sqrt{\sum_{i=1}^{n}(y_{meas,i} - y_i(p))^2}}{y} + \frac{\sqrt{\sum_{i=1}^{n}(x_{meas,i} - x_i(p))^2}}{x} \quad (7)$$

where x, y, and z are the model variables: dextrose, biomass, and fermentation product concentration, $x_{meas,i}$, $y_{meas,i}$ and $z_{meas,i}$ are the ith measurement of the dextrose, biomass, and fermentation products concentration, and $x_i(p)$, $y_i(p)$, and $z_i(p)$ are the calculated values of the model variables corresponding to the ith measurement. x, y, and z are the mean value of the measurements and n is the number of data points.

3. Results

3.1. Experimental Results

The first effects observed during the non-controlled pH corn-bioethanol effluent acidogenic fermentation were the dextrose consumption and the pH reduction. The results obtained are presented in Figure 1. Regarding the substrate consumption rate, it was observed that the higher the initial pH, the higher the substrate consumption rate. The time required to complete the fermentation was around 15 and 20 h when the initial pH values were 6 and 5 units, respectively. In the case of the initial pH of 4 units, the substrate was not completely fermented. In the latter case, the substrate concentration only decreased about 10 mmol·L^{-1}, indicating the existence of an inhibition event that stopped the fermentation process when the initial pH was 4.

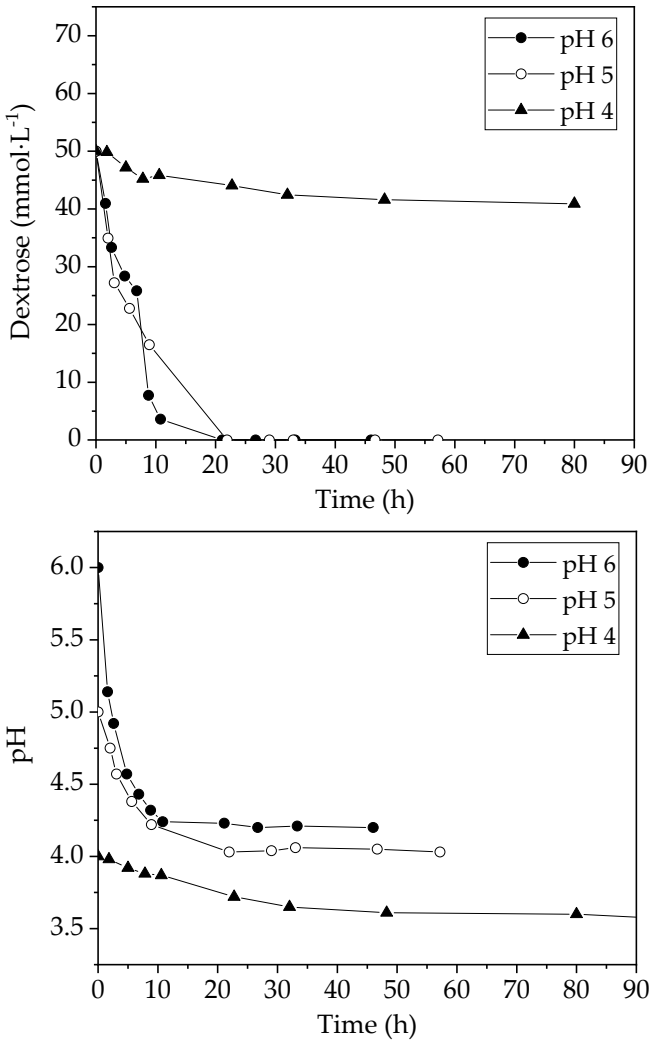

Figure 1. Dextrose and pH evolution during the uncontrolled pH fermentation.

The pH reduction can be explained by the pH values reached and the accumulation of the acids produced as a result of acidogenic fermentation. When the initial pH was 5 or 6, the dextrose was fully consumed and the pH decreased to values close to 4.1 and 4.3 units, respectively. These values remained constant due to the stopping of the fermentation process caused by the full consumption of the dextrose. The higher pH drop that was observed when operating at an initial pH of 6 units could be explained by the effect of the pH on acid dissociation. When operating at a higher pH, a lower fraction of the acids is undissociated due to the relationship between the pKa of the acid and the pH. Taking into account that the undissociated acids are responsible for inhibition events, a

lower undissociated acid concentration leads to a lower inhibitory effect [34]. This lower inhibition could explain the full consumption of the dextrose previously described when fermenting at initial pH values of 5 and 6. When the initial pH was 4, the pH reached a value of 3.5 units, which was a value lower than those obtained at the initial pH of 5 or 6. Once, the system reached the pH of 3.5 the value remained stable. It must be highlighted that the lower pH reached when starting at a pH of 4 could be explained by the inhibition caused by the combined effect of the pH and the undissociated acids accumulated in the medium. Starting at pH 4, a negligible acid concentration was present in the medium; therefore, the concentration of the undissociated acid was also very low. This allowed the fermentation of some substrates in spite of the low operational pH.

The trends of pH and dextrose influenced the kinetic and stoichiometric parameters and, therefore, affected other variables, such as biomass growth and fermentation product generation. Biomass growth, as well as the production of every fermentation product, is defined by the metabolic pathway followed. In this case, due to the evolution of the operational conditions (mainly the pH and the undissociated acid concentration), the biomass growth and fermentation product generation changed during the experiment. Figure 2 presents the experimental results of biomass growth. By comparing the trend of biomass growth with that of dextrose, important differences can be identified.

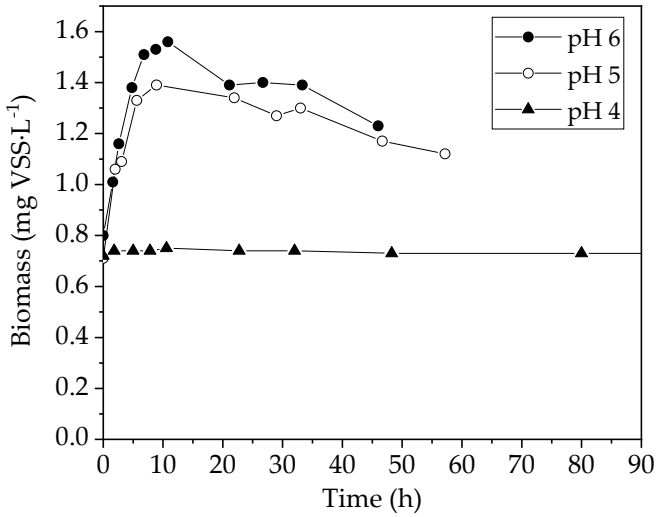

Figure 2. Biomass evolution during uncontrolled pH fermentation.

In Figure 2, it can be observed that when the initial pH was 4 units, the biomass concentration remained stabilized during the experiment. However, the biomass increased during the first hours of fermentation when the initial pH values were 5 or 6 units. At the initial pH values of 5 and 6, it can be seen that the biomass concentration starting at an initial pH of 6 was higher than that achieved at an initial pH of 5. In principle, taking into account that in both cases the substrate was fully consumed, the biomass growth should be identical. However, by comparing these biomass growth results with the substrate consumption trends shown previously, it can be observed that the biomass growth decreased and even stopped before the full consumption of the substrate. This behaviour indicates the difficulties faced by the microorganisms when exposed to the operational conditions reached at the end of fermentation. This biomass growth deviation from theoretical behaviour is called uncoupled growth [35]. Uncoupled growth is caused by the increasing maintenance energy requirements of the cells when developing under hostile conditions [49]. This increase in energy maintenance requirements leads to a decrease in the energy available for biomass growth [50]. As previously stated, hostile conditions could be caused by the accumulation of undissociated acids when operating at a lower

pH. The dissociated and undissociated fractions can be determined by means of the acid dissociation constant (K_a) defined by Equation (8).

$$K_a = \frac{[A^-]\cdot[H^+]}{[AH]} \tag{8}$$

Based on this equation, K_a expresses the strength of an acid (in other words, how easily the acid releases a proton). In addition, the equation shows how the dissociation state of weak acids varies according to the proton's concentration in the solution.

With the aim of investigating the effect of acid accumulation on the behaviour of the system, dissociated and undissociated acid concentrations were determined. Figure 3 shows the total undissociated acid concentrations during the fermentation at initial pH values of 4, 5, and 6. As can be seen at the beginning of fermentation, the undissociated acid concentration decreased as the initial pH increased due to the pH dependence of the acid dissociation. In the fermentations carried out with initial pH values of 5 and 6, the differences between their undissociated acid concentrations decreased during the experiment, and finally, both fermentations achieved a very similar final concentration of undissociated acid, around 80 mmol·L^{-1}. On the other hand, when fermentation was carried out at an initial pH of 4, the total undissociated acid concentration increased slowly due to the very low fermentation rate and conversion caused by the inhibition.

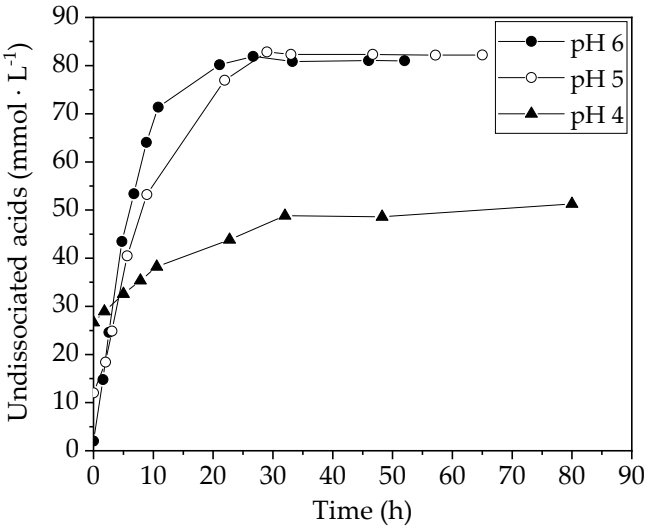

Figure 3. Undissociated acid evolution during uncontrolled pH fermentation.

The total undissociated acid concentration presented a trend different from that of the biomass growth in all the experiments. By comparing these trends, it can be seen that the undissociated acid concentration kept increasing even when the biomass growth stopped. Thus, by comparing Figures 2 and 3, it can be seen that the values of undissociated acid concentration increased the energy maintenance requirements and avoided biomass growth. These values were around 65 mmol·L^{-1} when fermenting at the initial pH of 5 and 6. This value is very similar to that reported in the literature [47,51].

The substrate was completely consumed when the fermentative processes were carried out at the initial pH values of 5 and 6. In both cases, the total undissociated acid concentration reached about 80 mmol·L^{-1} when the metabolism ceased. Regarding the experimental results at an initial pH of 4, it was observed that the total undissociated acid concentrations were significantly lower than 65 mmol·L^{-1} when the metabolism ceased due to the inhibition caused by the undissociated acids. Because of this, it was concluded that a high undissociated acid concentration was not the sole mechanism that stopped the biomass growth and substrate fermentation. By comparing the results obtained at an

initial pH of 4 with those obtained at an initial pH of 5 and 6, it was observed that the only variable that significantly changed was the pH, which reached a value of 3.5 units.

In order to investigate the effect of the initial pH on the generation of fermentation products, the specific production yield of each acid produced was determined. These yields were calculated according to Equation (9), where *P* refers to the product generated and *S* refers to the substrate consumed.

$$Y_X = \frac{P_{Final} - P_{Initial}}{S_{Initial} - S_{Final}} \quad (9)$$

The obtained average fermentation product yields are presented in Figure 4.

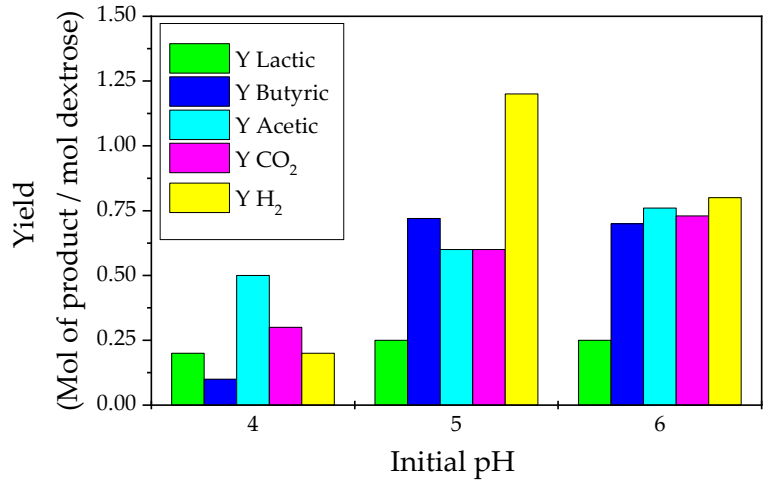

Figure 4. Average experimental yields of the main fermentation products generated.

In Figure 4, it can be observed that the fermentation product yields and distribution changed with the initial pH. In this Figure, it can be seen that acetic and lactic acid yields were higher at initial pH 4. However, the butyric acid showed an opposite trend, achieving the highest yields at the initial pH values of 5 and 6. The highest hydrogen yield (1.1 mol H_2·mol dextrose^{-1}) was achieved at the initial pH of 5, showing a slight decrease at the initial pH of 6 and a significant fall at the initial pH of 4. This H_2 trend is similar to that reported in the literature [27,46]. H_2 production, which is one of the most interesting fermentation products obtained from acidogenic fermentation, has been researched previously by many researchers [3,21]. The H_2 yield results obtained in this work were similar to other results published in the literature. In the literature, similar research studying mixed culture acidogenic fermentation with pH control showed H_2 yields ranging from 1.1 mol H_2·mol dextrose^{-1} [52] to 1.46 mol·H_2·mol dextrose^{-1} [53], and our yield was 1.1 mol·H_2·mol dextrose^{-1}, which is a typical value.

Considering the different substrate conversions obtained when operating at different initial pH values, the net productivities of the acids and H_2 as well as the estimated economic value were determined and are presented in Table 3.

Table 3. Average productivities of the different fermentation products generated at each initial pH.

Fermentation Product	pH 4	pH 5	pH 6
Acetic acid (mmol·L^{-1})	0.52	0.63	0.80
Butyric acid (mmol·L^{-1})	0.07	0.58	0.50
Propionic acid (mmol·L^{-1})	0.03	0.02	0.03
H_2 (mmol·L^{-1})	0.12	0.89	0.56
CO_2 (mmol·L^{-1})	0.23	0.50	0.61
Estimated economic value (EUR·L^{-1})	0.03	0.12	0.11

According to Table 3, the best initial pH from an economic point of view was pH 5 and 6, with the accumulated economic value being approximately the same in both cases. In the cases of initial pH 5 and 6, the main contribution to the economic value was butyric acid due to its higher specific yield and market price. Hydrogen has an even higher market price; however, its lower mass productivity reduced its contribution to the accumulated economic value. Based on these results, it must be stated that from a global economic point of view, it is indifferent to start fermentation at an initial pH of 5 or 6. For hydrogen production, it would be better to ferment the corn-bioethanol effluent at pH 5, and for VFA production, it would be better to ferment at pH 6.

3.2. Mathematical Modelling

With the objective of accurately quantifying all the fermentation products generated during the corn-bioethanol effluent fermentations at different initial pH values, modelling studies were carried out. Before the modelling studies, the quality of the data set obtained was verified by means of a carbon balance, as shown in Figure 5. From the carbon balance, a final carbon recovery of over 80% was obtained in all cases.

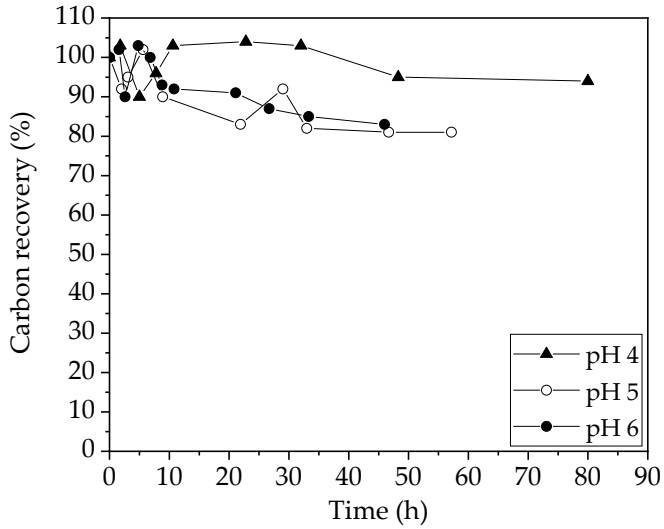

Figure 5. Carbon recovery during uncontrolled pH fermentation.

The lower carbon recovery experienced at the end of the fermentation process can be explained by the loss of carbon compounds such as in the biomass growth. Moreover, the error observed in the carbon balance can be explained by the existence of traces of undetected fermentation products in the liquid bulk. It is remarkable that higher carbon recovery occurs during fermentation at an initial pH of 4. This behaviour could be explained by the lower substrate consumption, and therefore, the lower biomass and fermentation product generation and its associated error. Regarding the carbon balance, it must be highlighted that the SBR operation involves maintaining 1.2 L from the previous cycle to the subsequent ones as an inoculum. This volume contained fermentation products from the previous fermentation cycle; therefore, its carbon content was added to the carbon content of the dextrose added at the beginning of every cycle, 10 $g \cdot L^{-1}$.

Once the quality of the data set was ensured, the main kinetic and stoichiometric parameters when operating at different initial pH values were determined. To do this, the mathematical Monod-based model previously described was fitted to the obtained results.

As previously shown, the pH decreased during the fermentation process due to the generation of short-chain fatty acids. This pH change during the fermentation process could affect the metabolic pathways of the mixed microbial culture used, thereby affecting the kinetic and stoichiometric parameters. In order to take this effect into account, pH-dependent functions were used to describe several kinetic and stoichiometric parameters. In the literature, the mathematical functions describing the stoichiometric and kinetic

parameters are presented as the functions of the pH value [47,48]. In this work, linear and exponential functions were proposed according to Equations (4)–(6).

Linear functions were used to describe the dependence of μ_{max} and Y_{maint} on the pH value. The μ_{max} increased when the pH increased within the range studied in this work, whereas the Y_{maint} decreased as the pH increased. A second-order function was used to describe the dependence of Ks on the pH. This function was selected due to the quick increase in Ks when the pH changed from 5 to 4. This behaviour was also previously described in the literature [48].

To begin the iterative determination of the pH dependence of the model parameters, the values of $Y_x{}^{max}$ (0.023 g SSV·mmol^{-1} dextrose) and the decay coefficient (0.03 h^{-1}, 0.07 h^{-1}, 0.13 h^{-1} at pH 4, 5 and 6) were taken from the literature as initial values [47]. The results obtained from the modelling are presented in Table 4.

Table 4. Modelling values of the kinetic and stoichiometric parameters.

Parameter		pH 4	pH 5	pH 6
$Y_x{}^{max}$ (g VSS·mmol S^{-1})		0.023	0.023	0.023
Decay (h^{-1})		0.001	0.005	0.007
μ_{max} (h^{-1})	a	0.041	00.49	0.046
	b	−0.142	−0.130	−0.133
Y_{maint} (g VSS·mmol S^{-1})	c	0.0040	0.0041	0.0041
	d	0.0271	0.0251	0.0251
K_s (mmol·L^{-1})	e	43.7	40.3	40.3
	f	−472	−472	−472
	g	1295	1285	1288
$K_{susbtrate}$ (mmol·L^{-1})		90	90	90
$K_{biomass}$ (mmol·L^{-1})		70	70	70
Y_a (mol·mol^{-1})		0.50	0.80	0.96
Y_b (mol·mol^{-1})		0.10	0.60	0.60
Y_l (mol·mol^{-1})		0.80	0.20	0.20
Y_p (mol·mol^{-1})		0.01	0.02	0.04
Y_{H2} (mol·mol^{-1})		0.10	1.10	0.69
Y_{CO2} (mol·mol^{-1})		0.32	0.60	0.73

The undissociated acid inhibition parameters, $K_{biomass}$ and $K_{substrate}$, were determined, with their values being 70 and 90 mmol·L^{-1}, respectively. The $K_{biomass}$ value was very similar to the concentration of undissociated acids, resulting in biomass growth detention. The $K_{susbstrate}$ value was higher than the experimental concentration of the achieved undissociated acids. For this reason, it did not cause the detention of the fermentative process, as previously stated. Regarding the parameter values presented in Table 4, they accurately described the process behaviour in spite of the different initial pH values. This demonstrates that the values shown in this Table can be used to describe the corn syrup acidogenic fermentation at any pH value within the range studied.

In the literature, the changes in parameter values when the pH of the medium changes have also been presented. The researchers found that μ_{max} increased from a pH of 6.4 to a pH of 7.8, where it achieved the highest value, after which it started to decrease. In previous studies, the influence of pH on biomass yield in fermentations of rich protein wastewater was carried out by a culture taken from a methanogenic reactor [54]. This study showed that the biomass yield increased when the pH increased from 4 to 6; at this pH, the biomass yield started to decrease. The optimal values of the kinetic parameters for lactose fermentation and lactic acid production were also estimated by using *Lactobacillus plantarum*. The obtained values at different pH values showed an important increase in Ks when the pH changed from 5 to 4, whereas Ks was almost constant in the pH range of 5–7. Similar trends to those obtained in this work for fermenting synthetic corn-bioethanol effluent by using a mixed culture have been described in the literature [48]. This coincidence indicates that these trends are general when fermentation is carried out without pH control. For

example, Figure 5 presents the experimental data and the fitted model. As can be seen in Figure 6, the model accurately predicts the evolution of the main variables, indicating that the fitting values of the kinetic and stoichiometric parameters were adequate for predicting the behaviour during corn syrup fermentation with uncontrolled pH.

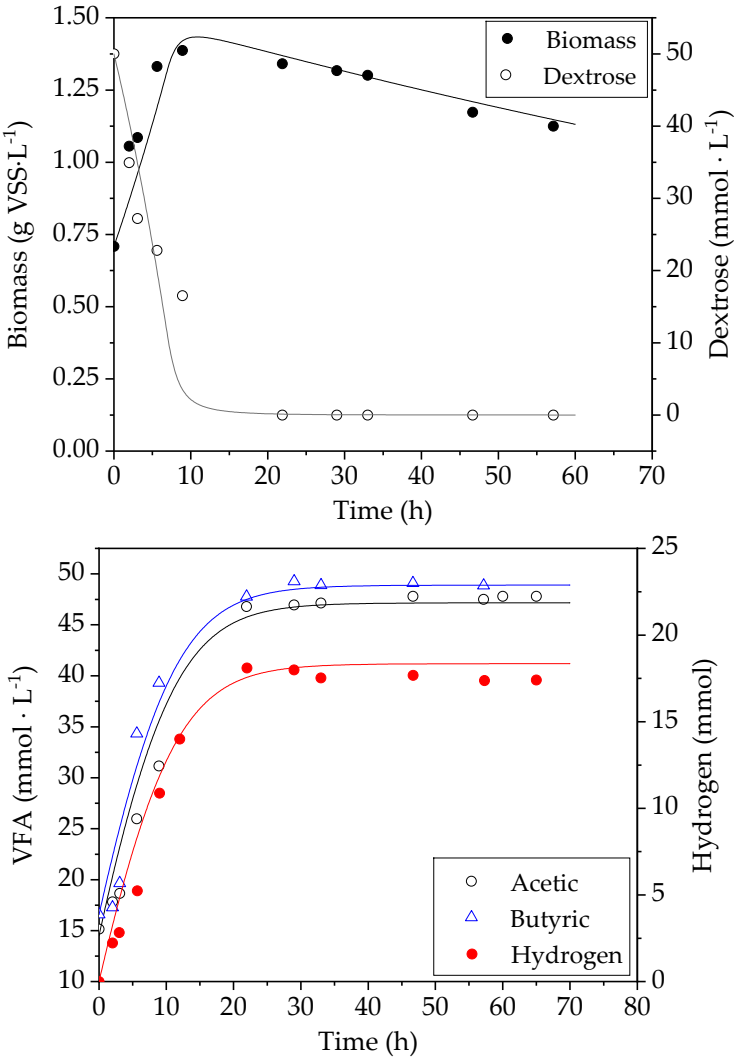

Figure 6. Evolution of the main variables during uncontrolled pH fermentation. Lines correspond to the modelling fit.

4. Conclusions

The fermentation of the substrates contained in the corn-bioethanol effluent without pH control generates a wide spectrum of VFAs, H_2, and CO_2. The decreased pH facilitated acid dissociation, thereby inhibiting the fermentative process. The significance of this inhibition increased as the initial pH decreased and even stopped the fermentation process when the initial pH was 4. The high dissociated acid concentration caused an uncoupled biomass growth when the undissociated acid concentration reached approximately 65 mmol·L^{-1}. The influence of the initial pH and the subsequent evolution of the operational conditions affected the fermentation product distribution. In general, fermentation product yields increased when the initial pH was higher. However, butyric acid presented a maximum yield when fermenting at an initial pH of 5. H_2 and CO_2 were the only gaseous products generated during fermentation. The CO_2 production increased as the pH increased, and H_2 achieved the highest production when fermenting at an initial pH of 5. Based on these results, it can be concluded that corn-bioethanol effluent can be valorised by means of an uncontrolled pH acidogenic fermentation process, especially when starting at an initial pH

of 5 or 6. Finally, a mathematical model was fitted to the experimental data, and the values of the main kinetic and stoichiometric parameters were determined. The parameter values obtained were able to accurately predict the uncontrolled pH of acidogenic fermentation of corn-bioethanol effluent.

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References

1. 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] [PubMed]
2. Baeyens, J.; Zhang, H.; Nie, J.; Appels, L.; Dewil, R.; Ansart, R.; Deng, Y. Reviewing the potential of bio-hydrogen production by fermentation. *Renew. Sustain. Energy Rev.* **2020**, *131*, 110023. [CrossRef]
3. Al-Qahtani, A.; Parkinson, B.; Hellgardt, K.; Shah, N.; Guillen-Gosalbez, G. Uncovering the true cost of hydrogen production routes using life cycle monetisation. *Appl. Energy* **2021**, *281*, 115958. [CrossRef]
4. Spazzafumo, G.; Raimondi, G. Economic assessment of hydrogen production in a Renewable Energy Community in Italy. *e-Prime—Adv. Electr. Eng. Electron. Energy* **2023**, *4*, 100131. [CrossRef]
5. Jankowska, E.; Chwialkowska, J.; Stodolny, M.; Oleskowicz-Popiel, P. Volatile fatty acids production during mixed culture fermentation—The impact of substrate complexity and pH. *Chem. Eng. J.* **2017**, *326*, 901–910. [CrossRef]
6. Pandey, A.K.; Pilli, S.; Bhunia, P.; Tyagi, R.D.; Surampalli, Y.R.; Zhang, C.T.; Kim, S.-H.; Pandey, A. Dark fermentation: Production and utilization of volatile fatty acid from different wastes- A review. *Chemosphere* **2022**, *288*, 132444. [CrossRef] [PubMed]
7. Sarkar, O.; Modestra, J.A.; Rova, U.; Christakopoulos, P.; Matsakas, L. Waste-Derived Renewable Hydrogen and Methane: Towards a Potential Energy Transition Solution. *Fermentation* **2023**, *9*, 368. [CrossRef]
8. Sivagurunathan, P.; Kumar, G.; Mudhoo, A.; Rene, E.R.; Saratale, G.D.; Kobayashi, T.; Xu, K.; Kim, S.H.; Kim, D.H. Fermentative hydrogen production using lignocellulose biomass: An overview of pre-treatment methods, inhibitor effects and detoxification experiences. *Renew. Sustain. Energy Rev.* **2017**, *77*, 28–42. [CrossRef]
9. Jayabalan, T.; Matheswaran, M.; Naina Mohammed, S. Biohydrogen production from sugar industry effluents using nickel based electrode materials in microbial electrolysis cell. *Int. J. Hydrogen Energy* **2019**, *44*, 17381–17388. [CrossRef]
10. Kumar, G.; Bakonyi, P.; Sivagurunathan, P.; Nemestóthy, N.; Bélafi-Bakó, K.; Lin, C.Y. Improved microbial conversion of de-oiled Jatropha waste into biohydrogen via inoculum pretreatment: Process optimization by experimental design approach. *Biofuel Res. J.* **2015**, *2*, 209–214. [CrossRef]
11. 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] [PubMed]
12. Wicher, E.; Seifert, K.; Zagrodnik, R.; Pietrzyk, B.; Laniecki, M. Hydrogen gas production from distillery wastewater by dark fermentation. *Int. J. Hydrogen Energy* **2013**, *38*, 7767–7773. [CrossRef]
13. Venkata Mohan, S.; Vijaya Bhaskar, Y.; Sarma, P.N. Biohydrogen production from chemical wastewater treatment in biofilm configured reactor operated in periodic discontinuous batch mode by selectively enriched anaerobic mixed consortia. *Water Res.* **2007**, *41*, 2652–2664. [CrossRef] [PubMed]
14. Yang, G.; Wang, J.; Shen, Y. Antibiotic fermentation residue for biohydrogen production using different pretreated cultures: Performance evaluation and microbial community analysis. *Bioresour. Technol.* **2019**, *292*, 122012. [CrossRef] [PubMed]

15. Mahapatra, D.M.; Chanakya, H.N.; Ramachandra, T.V. *Euglena* sp. as a suitable source of lipids for potential use as biofuel and sustainable wastewater treatment. *J. Appl. Phycol.* **2013**, *25*, 855–865. [CrossRef]
16. Rossi, D.M.; da Costa, J.B.; de Souza, E.A.; Peralba, M.D.C.R.; Ayub, M.A.Z. Bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol and ethanol by isolated bacteria from environmental consortia. *Renew. Energy* **2012**, *39*, 223–227. [CrossRef]
17. Lu, M.; Li, J.; Han, L.; Xiao, W. High-solids enzymatic hydrolysis of ball-milled corn stover with reduced slurry viscosity and improved sugar yields. *Biotechnol. Biofuels* **2020**, *13*, 77. [CrossRef]
18. Chen, M.H.; Kaur, P.; Dien, B.; Below, F.; Vincent, M.L.; Singh, V. Use of tropical maize for bioethanol production. *World J. Microbiol. Biotechnol.* **2013**, *29*, 1509–1515. [CrossRef]
19. Hafez, H.; Nakhla, G.; El Naggar, H. Biological Hydrogen Production from Corn-Syrup Waste Using a Novel System. *Energies* **2009**, *2*, 445–455. [CrossRef]
20. Eryildiz, B.; Lukitawesa; Taherzadeh, M.J. Effect of pH, substrate loading, oxygen, and methanogens inhibitors on volatile fatty acid (VFA) production from citrus waste by anaerobic digestion. *Bioresour. Technol.* **2020**, *302*, 122800. [CrossRef]
21. Atilano-Camino, M.M.; Luévano-Montaño, C.D.; García-González, A.; Olivo-Alanis, D.S.; Álvarez-Valencia, L.H.; García-Reyes, R.B. Evaluation of dissolved and immobilized redox mediators on dark fermentation: Driving to hydrogen or solventogenic pathway. *Bioresour. Technol.* **2020**, *317*, 123981. [CrossRef] [PubMed]
22. Slezak, R.; Grzelak, J.; Krzystek, L.; Ledakowicz, S. Influence of initial pH on the production of volatile fatty acids and hydrogen during dark fermentation of kitchen waste. *Environ. Technol.* **2020**, *42*, 4269–4278. [CrossRef] [PubMed]
23. Mota, V.T.; Ferraz Júnior, A.D.N.; Trably, E.; Zaiat, M. Biohydrogen production at pH below 3.0: Is it possible? *Water Res.* **2018**, *128*, 350–361. [CrossRef] [PubMed]
24. Ma, H.; Chen, X.; Liu, H.; Fu, B. Improved volatile fatty acids anaerobic production from waste activated sludge by pH regulation: Alkaline or neutral pH? *Waste Manag.* **2016**, *48*, 397–403. [CrossRef] [PubMed]
25. Jiang, J.; Zhang, Y.; Li, K.; Wang, Q.; Gong, C.; Li, M. Volatile fatty acids production from food waste: Effects of pH, temperature, and organic loading rate. *Bioresour. Technol.* **2013**, *143*, 525–530. [CrossRef] [PubMed]
26. Cubillos, G.; Arrué, R.; Jeison, D.; Chamy, R.; Tapia, E.; Rodríguez, J.; Ruiz-Filippi, G. Simultaneous effects of pH and substrate concentration on hydrogen production by acidogenic fermentation. *Electron. J. Biotechnol.* **2010**, *13*, 11–12. [CrossRef]
27. Tang, G.L.; Huang, J.; Sun, Z.J.; Tang, Q.Q.; Yan, C.H.; Liu, G.Q. Biohydrogen production from cattle wastewater by enriched anaerobic mixed consortia: Influence of fermentation temperature and pH. *J. Biosci. Bioeng.* **2008**, *106*, 80–87. [CrossRef] [PubMed]
28. Temudo, M.F.; Kleerebezem, R.; Van Loosdrecht, M. Influence of the pH on (Open) mixed culture fermentation of glucose: A chemostat study. *Biotechnol. Bioeng.* **2007**, *98*, 69–79. [CrossRef]
29. Fang, H.H.P.; Liu, H. Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresour. Technol.* **2002**, *82*, 87–93. [CrossRef]
30. Yu, H.G.; Fang, H.H. Acidogenesis of dairy wastewater at various pH levels. *Water Sci. Technol. A J. Int. Assoc. Water Pollut. Res.* **2002**, *45*, 201–206. [CrossRef]
31. Veeken, A.; Kalyuzhnyi, S.; Scharff, H.; Hamelers, B. Effect of pH and VFA on hydrolysis of organic solid waste. *J. Environ. Eng.* **2000**, *126*, 1076–1081. [CrossRef]
32. Wang, J.; Wan, W. Factors influencing fermentative hydrogen production: A review. *Int. J. Hydrogen Energy* **2009**, *34*, 799–811. [CrossRef]
33. Castro-Villalobos, M.C.; Garcia-Morales, J.L.; Fernandez, F.J. By-products inhibition effects on bio-hydrogen production. *Int. J. Hydrogen Energy* **2012**, *37*, 7077–7083. [CrossRef]
34. Rodríguez, J.; Kleerebezem, R.; Lema, J.M.; Van Loosdrecht, M.C.M. Modeling product formation in anaerobic mixed culture fermentations. *Biotechnol. Bioeng.* **2006**, *93*, 592–606. [CrossRef] [PubMed]
35. Kleerebezem, R.; Rodríguez, J.; Temudo, M.F.; van Loosdrecht, M.C.M. Modeling mixed culture fermentations; the role of different electron carriers. *Water Sci. Technol.* **2008**, *57*, 493–497. [CrossRef] [PubMed]
36. Ribeiro, J.C.; Mota, V.T.; de Oliveira, V.M.; Zaiat, M. Hydrogen and organic acid production from dark fermentation of cheese whey without buffers under mesophilic condition. *J. Environ. Manag.* **2022**, *304*, 114253. [CrossRef] [PubMed]
37. Yuan, Q.; Lou, Y.; Wu, J.; Sun, Y. Long-term semi-continuous acidogenic fermentation for food wastes treatment: Effect of high organic loading rates at low hydraulic retention times and uncontrolled pH conditions. *Bioresour. Technol.* **2022**, *357*, 127356. [CrossRef]
38. Khanal, S.K.; Chen, W.H.; Li, L.; Sung, S. Biological hydrogen production: Effects of pH and intermediate products. *Int. J. Hydrogen Energy* **2004**, *29*, 1123–1131. [CrossRef]
39. Lin, C.-Y.; Chang, R.-C. Fermentative hydrogen production at ambient temperature. *Int. J. Hydrogen Energy* **2004**, *29*, 715–720. [CrossRef]
40. Lee, Y.J.; Miyahara, T.; Noike, T. Effect of pH on microbial hydrogen fermentation. *J. Chem. Technol. Biotechnol.* **2002**, *77*, 694–698. [CrossRef]
41. De Lucas, A.; Rodriguez, L.; Villasenor, J.; Fernandez, F.J. Fermentation of agro-food wastewaters by activated sludge. *Water Res.* **2007**, *41*, 1635–1644. [CrossRef]
42. Rodríguez Mayor, L.; Villaseñor Camacho, J.; Fernández Morales, F.J. Operational optimisation of pilot scale biological nutrient removal at the Ciudad Real (Spain) domestic wastewater treatment plant. *Water Air Soil Pollut.* **2004**, *152*, 279–296. [CrossRef]

43. Fernandez-Morales, F.J.; Villasenor, J.; Infantes, D. Modeling and monitoring of the acclimatization of conventional activated sludge to a biohydrogen producing culture by biokinetic control. *Int. J. Hydrogen Energy* **2010**, *35*, 10927–10933. [CrossRef]
44. Temudo, M.F.; Muyzer, G.; Kleerebezem, R.; Van Loosdrecht, M.C.M. Diversity of microbial communities in open mixed culture fermentations: Impact of the pH and carbon source. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 1121–1130. [CrossRef] [PubMed]
45. American Public Health Association; American Water Works Association; Water Environment Federation. *Standard Methods for the Examination of Water and Wastewater*; APHA-AWWA-WEF: Washington, DC, USA, 2005. (In English)
46. Infantes, D.; Gonzalez del Campo, A.; Villasenor, J.; Fernandez, F.J. Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation. *Int. J. Hydrogen Energy* **2011**, *36*, 15595–15601. [CrossRef]
47. Infantes, D.; González del Campo, A.; Villaseñor, J.; Fernández, F.J. Kinetic model and study of the influence of pH, temperature and undissociated acids on acidogenic fermentation. *Biochem. Eng. J.* **2012**, *66*, 66–72. [CrossRef]
48. Fu, W.; Mathews, A.P. Lactic acid production from lactose by Lactobacillus plantarum: Kinetic model and effects of pH, substrate, and oxygen. *Biochem. Eng. J.* **1999**, *3*, 163–170. [CrossRef]
49. Rodriguez, J.; Premier, G.C.; Guwy, A.J.; Dinsdale, R.; Kleerebezem, R. Metabolic models to investigate energy limited anaerobic ecosystems. *Water Sci. Technol.* **2009**, *60*, 1669–1675. [CrossRef]
50. Elbeshbishy, E.; Dhar, B.R.; Nakhla, G.; Lee, H.S. A critical review on inhibition of dark biohydrogen fermentation. *Renew. Sustain. Energy Rev.* **2017**, *79*, 656–668. [CrossRef]
51. van den Heuvel, J.C.; Verschuren, P.G.; Beeftink, H.H.; de Beer, D. Determination of the critical concentration of inhibitory products in a repeated fed-batch culture. *Biotechnol. Tech.* **1992**, *6*, 33–38. [CrossRef]
52. Lin, C.Y.; Chang, C.C.; Hung, C.H. Fermentative hydrogen production from starch using natural mixed cultures. *Int. J. Hydrogen Energy* **2008**, *33*, 2445–2453. [CrossRef]
53. Davila-Vazquez, G.; Alatriste-Mondragón, F.; de León-Rodríguez, A.; Razo-Flores, E. Fermentative hydrogen production in batch experiments using lactose, cheese whey and glucose: Influence of initial substrate concentration and pH. *Int. J. Hydrogen Energy* **2008**, *33*, 4989–4997. [CrossRef]
54. Yu, H.Q.; Fang, H.H.P. Acidogenesis of gelatin-rich wastewater in an upflow anaerobic reactor: Influence of pH and temperature. *Water Res.* **2003**, *37*, 55–66. [CrossRef]