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Dark Fermentation of Fruit Juice Effluents: Effects of Substrate Concentration

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Abstract: This study explores the impact of initial substrate concentrations on biomass growth, hydrogen production, and acid generation during acidogenic fermentation of a synthetic fruit juice wastewater. Four substrate concentrations, within the range 0.30–2.12 C mol·L^{-1}, were tested using a mixed acidogenic bacterial culture. Batch reactors were employed to conduct the study, and the results were analyzed to determine inhibition effects. The highest hydrogen production (6.1 L H_2·substrate C mol^{-1}) and hydrogen percentage in the gas phase (57%) were achieved at a substrate concentration of 0.30 C mol·L^{-1}. Higher substrate concentrations reduced the hydrogen production due to substrate and product inhibition events. The maximum H_2 potential production was 4.15 Nm^3/m^3 reactor at a substrate concentration of 0.91 C mol·L^{-1}. Biomass growth and VFA production followed exponential trends at low substrate concentrations, 0.30 and 0.091 C mol·L^{-1}, while high concentrations resulted in linear trends due to inhibition effects caused by the substrate. The main acids produced were lactic at low concentrations, and acetic when dealing with high concentrations. The highest final acid concentrations were obtained with the highest initial substrate concentration, but their yields were significantly lower due to the substrate and product inhibitions experienced by the biomass.

Keywords: acidogenic fermentation; hydrogen production; volatile fatty acids; inhibition

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1. Introduction

The fruit juice industry involves significant water consumption during production, leading to the generation of large volumes of wastewater [1,2]. This wastewater is typically discharged into municipal sewage systems and treated at wastewater treatment plants (WWTPs) alongside domestic wastewater. In general, the wastewater produced by the fruit juice industry is characterized by its exceptionally high organic load, including a large proportion of easily biodegradable compounds, low pHs, nutrient imbalances, and seasonal fluctuations in both effluent volume and organic load [3]. These variations are largely due to the seasonal nature of fruit production and processing [4,5]. Given these variable characteristics, it is essential that wastewater from the fruit juice sector undergo pre-treatment to meet quality standards before being discharged into municipal sewage systems, thereby reducing potential environmental impacts.

In recent decades, anaerobic digestion has emerged as an effective method for treating various types of industrial wastewater with a high organic content [3,4,6–9]. The benefits of anaerobic digestion include low sludge production, high conversion efficiency, reduced energy consumption, and the production of valuable byproducts like biogas [3]. A key

phase in anaerobic digestion is acidogenic fermentation, where carbohydrates are converted into volatile fatty acids (VFAs), alcohols, carbon dioxide, and hydrogen [10]. This process not only aids in treating organic waste but also offers the potential for hydrogen production, a clean and renewable energy source. The feasibility of hydrogen production is especially important in today's economy, which is heavily dependent on fossil fuels and faces challenges such as depleting reserves, rising costs, greenhouse gas emissions, and widespread air pollution. Thus, the search for alternative, non-polluting, and sustainable energy sources has become crucial.

Hydrogen offers numerous advantages as an alternative to conventional fossil fuels. These include the absence of harmful emissions—since hydrogen combustion produces only water—higher combustion efficiency, and approximately 50% greater efficiency in internal combustion engines compared to gasoline [11]. Additionally, hydrogen has 2.75 times the energy content of any hydrocarbon, and it can be transported more efficiently through existing natural gas pipelines than electricity via power lines. Hydrogen can be produced through various methods, including thermochemical, electrochemical, and biological processes. However, thermochemical processes often require hydrocarbon feedstocks, mainly derived from fossil fuels, while electrochemical processes demand a continuous electricity supply. In contrast, microbial fermentation presents an attractive alternative as it does not depend on external energy inputs, operates under ambient conditions, and when waste substrates are used, it achieves both renewable energy production and efficient waste treatment [12,13].

Among the biological methods for hydrogen production, acidogenic fermentation is particularly advantageous due to its high hydrogen yield, simple operational control, low production costs, and independence from light [14]. Dark fermentation is usually compared with two-stage anaerobic digestion. On the one hand, dark fermentation converts more efficiently carbohydrate-rich organic substrates into hydrogen and presents the advantage of lower operational costs [15,16]. On the other hand, two-stage anaerobic digestion involves both hydrogen and methane production phases. However, the hydrogen yield alone is typically lower than that of dark fermentation [15]. In the literature, it has been described that numerous operational factors—such as pH, temperature, reactor design, inoculum type, nutrient concentrations, the presence of metal ions, substrate type, and substrate concentration—can significantly influence the efficiency of hydrogen production through acidogenic fermentation [17–19]. Understanding the interactions between these factors is crucial for optimizing both the performance and cost-effectiveness of the system.

As with any biotechnological process, substrate concentration is a critical parameter in acidogenic fermentation. Low substrate concentrations can limit the reaction rate, while high concentrations can lead to substrate and product inhibition [20–22] affecting the generation of valuable products. Therefore, the primary aim of this study was to explore the effect of pollutant concentration in a synthetic fruit juice wastewater on hydrogen production via acidogenic fermentation [20–22]. Additionally, the study examined the hydrogen production potential from out-of-specification fruit juice, contributing to sustainable waste management with renewable energy generation.

2. Materials and Methods

2.1. Mixed Bacterial Culture and Wastewater

The microbial consortium used in this study was a mixed acidogenic bacterial culture, mainly belonging to the genus *Clostridium*, this culture was taken from a sequential batch reactor previously acclimated under strict anaerobic conditions. The acclimation process followed the protocol described in the literature, conducted at a pH of 5 and 26 °C over a 4-month period [23].

To examine the impact of substrate concentration on hydrogen production during acidogenic fermentation, four different substrate concentrations, ranging from 0.30 to 2.12 C mol·L^{-1}, were studied. The concentrations were selected based on studies previously published in the literature [19,24–26]. The synthetic industrial juice wastewaters were

formulated to replicate the effluent characteristics typical of the fruit juice industry [27]. This synthetic effluent consisted primarily of glucose, fructose, and sucrose, reflecting the typical organic carbon substrates found in actual fruit juice wastewater and maintaining their usual ratios [1]. The typical proportions of these substrates in fruit juice wastewaters are 59% glucose, 21% fructose, and 20% sucrose. Trace minerals were also incorporated to ensure the completeness of the synthetic formulation, the concentration of the different trace minerals used are presented in Table 1.

Table 1. Characteristics of the synthetic fruit juice wastewaters used in this study.

Component	0.30 C mol·L^{-1}	0.91 C mol·L^{-1}	1.52 C mol·L^{-1}	2.12 C mol·L^{-1}
Fructose (C mol·L^{-1})	0.18	0.53	0.88	1.24
Glucose (C mol·L^{-1})	0.06	0.18	0.30	0.42
Sucrose (C mol·L^{-1})	0.06	0.20	0.33	0.46
Total organic substrate	*0.30 C mol·L^{-1}*	*0.91 C mol·L^{-1}*	*1.52 C mol·L^{-1}*	*2.12 C mol·L^{-1}*
$(NH_4)Cl$ (mM)	56.46	169.38	282.30	395.21
KH_2PO_4 (mM)	10.10	30.31	50.52	70.73
NaCl (mM)	11.29	33.88	56.47	79.06
Na_2SO_4 (mM)	0.92	2.75	4.58	6.41
$MgCl_2{\cdot}6H_2O$ (mM)	1.33	3.98	6.64	9.30
EDTA (mM)	0.38	1.13	1.88	2.63
$ZnSO_4{\cdot}7H_2O$ (mM)	2.50×10^{-2}	7.51×10^{-2}	1.25×10^{-1}	1.75×10^{-1}
$FeSO_4{\cdot}7H_2O$ (mM)	2.51×10^{-2}	7.53×10^{-2}	1.26×10^{-1}	1.76×10^{-1}
$MnCl_2{\cdot}4H_2O$ (mM)	2.84×10^{-2}	8.53×10^{-2}	1.42×10^{-1}	1.99×10^{-1}
$CuCl{\cdot}2H_2O$ (mM)	2.90×10^{-2}	8.71×10^{-2}	1.45×10^{-1}	2.03×10^{-1}
$CaCl_2$ (mM)	1.22×10^{-2}	3.65×10^{-2}	6.08×10^{-2}	8.52×10^{-2}
$CoCl_2{\cdot}6H_2O$ (mM)	9.12×10^{-3}	2.74×10^{-2}	4.56×10^{-2}	6.39×10^{-2}
$NiCl_2{\cdot}6H_2O$ (mM)	4.75×10^{-3}	1.43×10^{-2}	2.38×10^{-2}	3.33×10^{-2}
$Na_2MoO_4{\cdot}2H_2O$ (mM)	9.30×10^{-4}	2.79×10^{-3}	4.65×10^{-3}	6.51×10^{-3}

Before the experiments, the synthetic juice wastewater was sterilized by autoclaving at 100 °C for 30 min. In this way, the undesired microbial degradation during storage of the wastewater was avoided. Table 1 outlines the composition of the four synthetic wastewater formulations used in this study.

2.2. Experimental Procedure

The experiments were carried out in batch reactors with an effective volume of 3 L. The reactors were equipped with various inlets and outlets, including ports for a pH probe, alkali dosing for pH control, antifoam addition, a heat exchanger for condensing water in the biogas phase, a nitrogen sparger, and a sampling port. The operational temperature was maintained at 26 °C using a water bath connected to the reactor jacket. Reactor content was homogenized with a magnetic stir bar rotating at 500 rpm. The pH was controlled at a stable level of 5 through automatic titration using an ADI 1030 Bio Controller (Delft, The Netherlands), which dosed a 3 M NaOH solution as needed. The operational conditions were selected based on previous studies [19,26].

To minimize foam formation during fermentation, a 2.5% silicone antifoam solution (426R, Prolabo, Radnor, PA, USA) was continuously supplied at a rate of 2.5 µL/min. Anaerobic conditions were ensured by sparging the reactor with nitrogen gas at a flow rate of 40 mL/min [28]. This nitrogen stream also served as a carrier gas, transporting the produced hydrogen to an online gas analyzer (Rosemount Analytical NGA 2000 MLT, Emerson, Ferguson, MO, USA). More information about the experimental procedure can be found in the literature [19,26].

Each batch experiment began with the addition of 1.85 L of synthetic wastewater, containing organic substrate concentrations of 0.30, 0.91, 1.52, 2.12 C mol·L^{-1}, along with

1.15 L of acclimatized mixed acidogenic bacterial culture. Prior to inoculation, the pH of the system was adjusted to 5 using a 3 M HCl or NaOH solution.

2.3. Analytical Methods

A comprehensive set of analyses was conducted to determine the concentrations of substrates and products in both the liquid and gas phases. Liquid samples were immediately centrifuged at 13,000 rpm and filtered through a 0.45 µm membrane. The filtered samples were either analyzed promptly or stored at −4 °C to prevent degradation. Substrate concentrations, including glucose, fructose, and sucrose, were quantified using high-performance liquid chromatography (HPLC, Agilent, Santa Clara, CA, USA) equipped with a refractive index detector (Series 1200). Separation of the components was achieved with a Zorbax Carbohydrate Column (4.6 × 150 mm, 5 µm) at 35 °C, using a mobile phase of 84% acetonitrile and 16% water (v/v) at a flow rate of 1.2 mL/min. Lactic acid was similarly analyzed via HPLC (Agilent) using a UV-DAD detector and a Zorbax SB-Aq column (4.6 × 150 mm, 5 µm), with a mobile phase composed of 99% water and 1% acetonitrile (v/v) in a pH 2 phosphate buffer (0.05 M).

For the analysis of acetic, propionic, and butyric acids, gas chromatography (Perkin Elmer) with a flame ionization detector (FID) and a Crossbond Carbowax Column (15 m × 0.32 mm ID, 0.25 µm df) was employed. The oven temperature was initially set to 140 °C for 1.5 min, followed by a temperature ramp of 25 °C/min up to 190 °C, where it was held for 2 min. The injector and detector temperatures were maintained at 200 °C and 230 °C, respectively, with nitrogen as the carrier gas.

Total Suspended Solids (TSSs) were measured by filtering the liquid samples through a Millipore 0.7 µm membrane, followed by dehydration at 105 °C for 24 h. The filtered samples were then ignited at 550 °C for 2 h to determine dry bacterial biomass.

The composition of gases produced during acidogenic fermentation, specifically H_2 and CO_2, was continuously monitored online using a multi-component gas analyzer (Rosemount Analytical NGA 2000 MLT, Emerson, Ferguson, MO, USA). The gas detection system was connected directly to the bioreactor outlet, with data acquisition and pH control managed via SCADA software, utilizing BioXpert software v1.12 in conjunction with a Biocontroller (ADI 1030 Bio Controller, Delft, The Netherlands).

2.4. Modelling and Statistical Analysis

To accurately determine the behavior of the fermentative system, the experimental results were analyzed using mathematical modeling.

In the literature, the Monod equation is the most straightforward model used in biological systems to describe the relationship between bacterial biomass growth rate and substrate consumption. Equation (1) presents the Monod equation:

$$r_X = \mu_{max} \cdot \frac{S}{K_S + S} \cdot X - b \cdot X \quad (1)$$

where, r_X = bacterial biomass growth rate (C mol·(h·L)$^{-1}$); μ_{max} = maximum specific bacterial biomass growth rate (h^{-1}); S = substrate concentration in the bioreactor C mol·L^{-1}); K_S = saturation constant for substrate (C mol·L^{-1}); b = bacterial biomass decay rate (h^{-1}); and X = bacterial biomass concentration (C mol·L^{-1}).

However, the Monod model is not suitable for fitting experimental data when inhibition events caused by the substrate occurs. Therefore, a mathematical model able to describe the inhibition caused by the substrates, the Haldane–Andrews model, was applied to experimental data. The Haldane model is one of the most often used in the literature [29–31]. The Haldane–Andrews model, shown in Equation (2), is an extension of

the Monod model that can predict the effects of substrate inhibition on bacterial biomass by introducing an inhibition parameter [21,32].

$$r_X = \mu_{max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_i}} \cdot X - b \cdot X \quad (2)$$

where, K_i = inhibition constant for substrate (C mol·L^{-1}).

The Haldane–Andrews model was used to describe the substrate consumption, and the bacterial biomass growth experienced with all the substrate concentrations tested, offering accurate predictions. The substrate inhibition effects over bacterial biomass can be explained by the reduction in the growth rate due to osmotic stress on cells, increase of the medium viscous (affecting the mass transfer) or due to multiple substrate molecules binding to the enzyme, causing a decrease in the reaction rate [30,33,34].

Statistical analyses of variance (ANOVA) of the different process parameters among different runs were conducted. A threshold *p*-value of significant difference was set at 0.05.

3. Results and Discussion

3.1. Biomass Growth and Substrate Fermentation

Substrate fermentation, product generation, and biomass growth were evaluated during the acidogenic fermentation tests conducted with the different initial substrate concentrations. The results obtained in the four experimental series are presented in Figure 1.

As shown in Figure 1, the initial evolution of biomass concentration was similar across all cases. However, a slight decrease in biomass growth rate was observed when increasing the substrate concentration during the first 15 h of fermentation. To accurately determine whether substrate inhibition of bacterial biomass occurred, the experimental results were analyzed using mathematical modeling by using the Haldane–Andrews model [21]. This model described the substrate consumption, and the biomass growth experienced with all the substrate concentrations tested, offering accurate predictions. The results of the fitting are presented in Figure 2, where the Haldane–Andrews modelling results, as well as the experimental data points, are presented. The maximum initial biomass growth rate was observed with a substrate concentration of 0.30 C mol·L^{-1}. This rate slightly decreased when increasing the substrate concentration. From this fitting, the value of the models' parameters were determined, their values being those presented in Table 2.

As shown in Figure 2, and according to the Haldane–Andrews kinetics presented in Figure 3, there is an optimal substrate concentration range of 0.25–0.65 C mol·L^{-1}. When the substrate concentrations in the reactor fall within this range, the maximum substrate conversion rate is achieved, and biomass growth occurs at the maximum growth rate. Lower initial substrate concentrations lead to mass transfer limitations, while higher initial substrate concentrations result in substrate inhibition of bacterial biomass [21,32]. Based on the presented results and modeling data, it can be concluded that, when operating with high substrate concentrations, a slight substrate inhibition of bacterial biomass was experienced within the first 10 h of operation. Similar results have been described in the literature when dealing with high initial substrate concentrations.

However, a markedly different behavior was observed after 40 h of operation. As shown in Figure 1, biomass growth was negligible in all cases after this period. This can be attributed to the complete consumption of the substrate at concentrations of 0.30 and 0.91 C mol·L^{-1}. In contrast, when operating with concentrations of 1.52 and 2.12 C mol·L^{-1}, the substrates were fermented without net biomass growth. The different trend observed after 40 h of operation cannot be solely attributed to substrate inhibition of bacterial biomass. This is because, after 40 h, the substrate concentration was lower than at the beginning. For example, for the initial concentration of 1.52 C mol·L^{-1}, the substrate concentration was about 0.6 C mol·L^{-1}, which falls within the non-substrate inhibition zone according to the Haldane–Andrews model previously fitted. In the literature, this phenomenon has

been explained by product inhibition of biomass [23]. This type of inhibition is caused by the accumulation of fermentation products, which can only occur after several hours of operation. In this case, the undissociated fermentation products can permeate across the membrane increasing the maintenance requirements of microorganisms and reducing the other metabolic routes [12,23,26]. When operating at high initial substrate concentrations, a significant amount of fermentation product is generated. The high concentration of these products can reduce and even stop the fermentative process. The consumption of substrates without biomass growth can be explained by increased maintenance requirements, which can reduce and even make biomass yield negligible. To analyze this effect, the final biomass concentration and the average biomass yield were determined for each test, obtaining biomass yields within the range 0.03–0.13 biomass C mol·substrate C mol^{-1}. Similar results have been reported in the literature [35–37]. The results obtained in this work are presented in Figure 3.

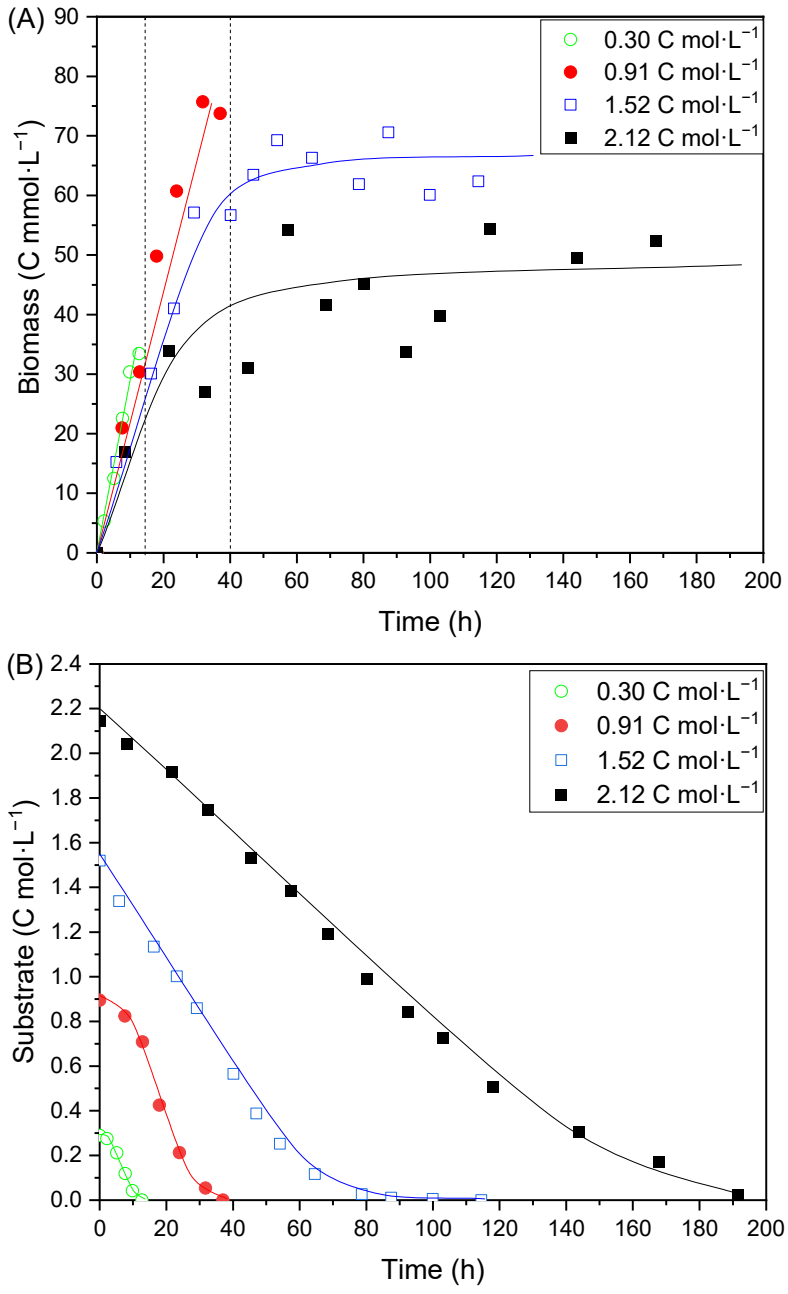

Figure 1. (**A**) Biomass and (**B**) substrate concentration during the experiments. Lines correspond to trends only.

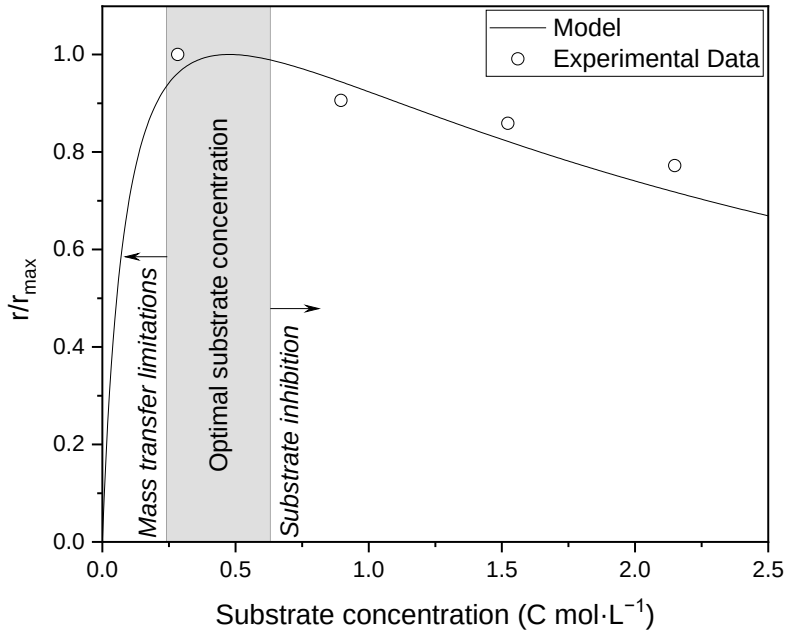

Figure 2. Kinetic evaluation using the Haldane–Andrews model to fit experimental data.

Table 2. Parameter values of the Haldane–Andrews model.

Parameter (mM)	Value
μmax (h^{-1})	0.3840
Ks (C mol·L^{-1})	0.10
Ki (C mol·L^{-1})	1.52

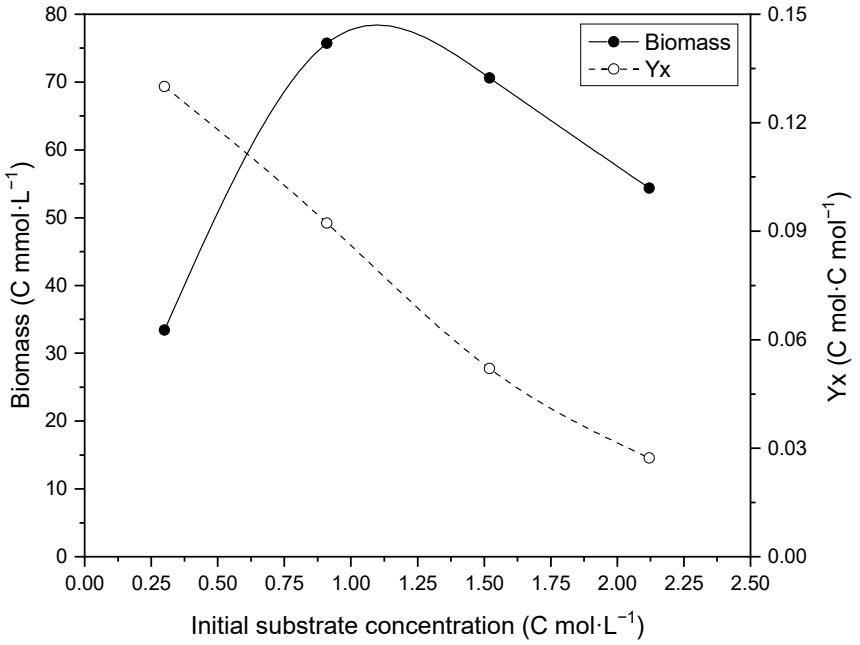

Figure 3. Biomass concentration and biomass yield obtained from wastewater with different initial substrate concentrations.

As shown in Figure 3, different final biomass concentrations and biomass yields were obtained for the different initial substrate concentrations. In this figure, it can be seen that the highest biomass concentration, 76 C mmol·L^{-1}, was achieved when dealing

with a substrate concentration of 0.91 C mol·L^{-1}. However, the maximum biomass yield, 0.78 mol·mol^{-1}, was obtained with a substrate concentration of 0.30 C mol·L^{-1}. As can be seen in Figure 3, the biomass yield decreased as the substrate concentration increased. The results obtained indicate that the microorganisms increased their maintenance requirements, thereby reducing biomass growth, when the substrate concentration exceeded 0.30 C mol·L^{-1}. Similar results have been described in the literature [38]. Consequently, inhibition was more pronounced at higher substrate concentrations. This can be explained by the combination of substrate and products inhibition of the fermentative biomass. In order to ensure the representativity of the results presented in this work, statistical analyses of variance (ANOVA) of the different process parameters among different runs were conducted. The threshold p-value of significant difference was set at 0.05 obtaining that significant differences ($p < 0.05$) between any two groups among the four initial concentrations were observed through ANOVA analysis. These results indicated that the initial concentration significantly affected the fermentation performance.

3.2. Volatile Fatty and Other Acids Production

During acidogenic fermentation, microorganisms metabolize substrates to produce volatile fatty and other acids, hydrogen, and carbon dioxide. Figure 4 presents the VFA and other acids production as well as the substrate concentrations during the tests conducted with different initial substrate concentrations.

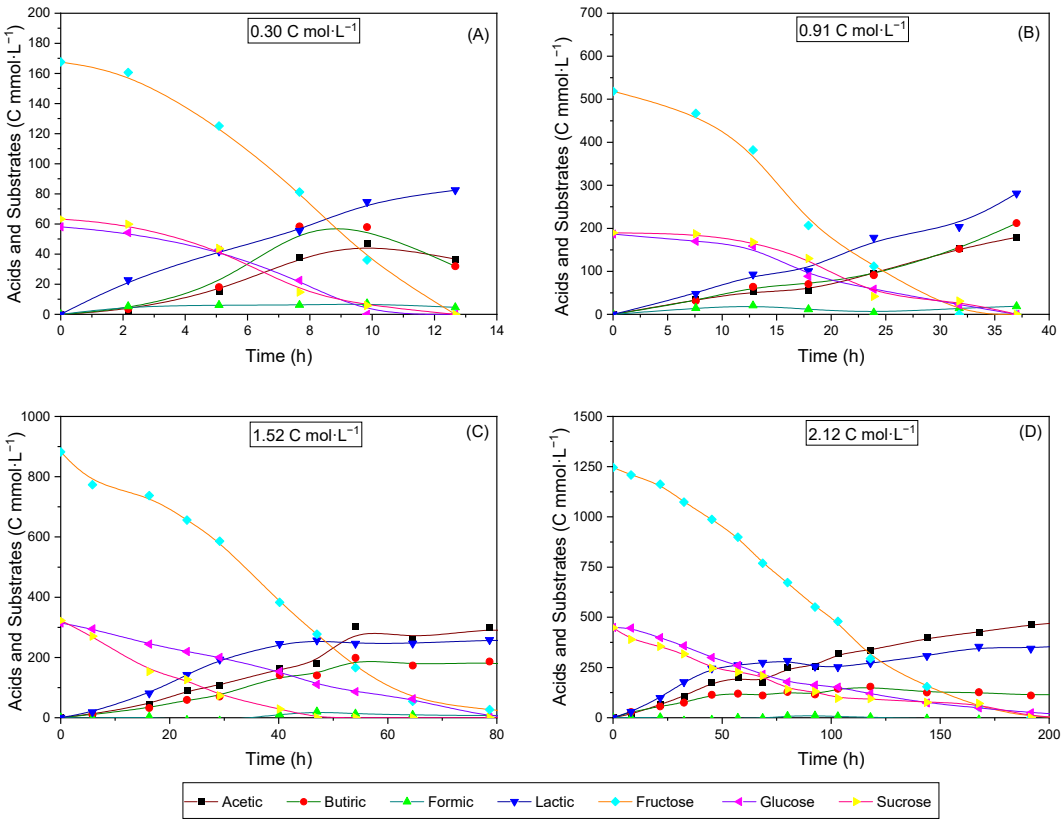

Figure 4. Volatile fatty and other acids and substrate concentrations during acidogenic fermentation of wastewater with different initial substrate concentrations. (**A**) Substrate concentration: 0.30 C mol·L^{-1}; (**B**) substrate concentration: 0.91 C mol·L^{-1}; (**C**) substrate concentration: 1.52 C mol·L^{-1}; and (**D**) substrate concentration: 2.12 C mol·L^{-1}.

As shown in Figure 4, the main acids produced were acetic, butyric, and lactic acids, which is consistent with findings reported in the literature [25]. Formic acid was also produced, but only during the initial stages of the fermentation process, maintaining its concentration thereafter.

In all tests conducted, the organic substrates contained in the synthetic wastewater (fructose, glucose, and sucrose) were fully fermented. When comparing the substrate fermentation rates, it was observed that the fructose fermentation rate was consistently higher than that of glucose and sucrose. This could be attributed to the higher initial concentration of fructose, which facilitates mass transfer, as well as the higher affinity of the microbial culture used in this study for fructose.

It is also important to note that the time required for complete fermentation of the sugars in the different effluents increased exponentially, from 12.67 h to 191.58 h, as the sugar concentration in the wastewater increased linearly from 0.30 to 2.12 C mol·L^{-1}. Thus, higher sugar concentrations in wastewater make sugar fermentation more difficult for the microorganisms. Taking into account that this effect was mainly experienced after 40 h of operation, the inhibition could be mainly explained by a product inhibition event [23].

In the first case, as shown in Figure 4A, when the sugar concentration was 0.30 C mol·L^{-1}, glucose and sucrose were completely fermented simultaneously after approximately 10 h, while fructose required more time, about 12 h, due to its higher concentration. The main acids produced were lactic, acetic, and butyric acids, with final concentrations of 80, 35, and 32 C mmol·L^{-1}, respectively. Thus, lactic acid was the primary product obtained during the acidogenic fermentation when dealing with low initial substrate concentration. As illustrated in Figure 4A, both sugar consumption and acid production followed an exponential trend. The rates of sugar fermentation and acid production were lower during the initial phase due to the low biomass concentration at the early stages, but these rates increased over time. This exponential trend can be explained by the exponential growth of the biomass, which is associated with optimal growth conditions. Therefore, no limitations or inhibitions were detected when dealing with the substrate concentration of 0.30 C mol·L^{-1}.

In the case of feeding wastewater with 0.91 C mol·L^{-1} mM of sugar, as shown in Figure 4B, fructose was completely fermented before glucose and sucrose, taking approximately 34 h. Complete fermentation of glucose and sucrose required 37 h. The production of lactic acid was about 250 C mmol·L^{-1} butyric and acetic concentrations were similar, with final concentrations of about 175 C mmol·L^{-1}. It is important to note that acid production exhibited a slightly linear trend, rather than the expected exponential one, indicating the presence of some sort of inhibition.

Finally, when dealing with wastewaters containing substrate concentrations of 1.52 and 2.12 C mol·L^{-1}, two phases in acid production were observed, as shown in Figure 4C,D. In the first stage, up to 50 h for a sugar concentration of 1.52 C mol·L^{-1}, and up to 60 h for a sugar concentration of 2.12 C mol·L^{-1}, a linear acid production and sugar consumption was observed. During this stage, acetic and lactic acids were produced at similar rates, following linear trends. As previously mentioned, exponential trends are associated with optimal microbial performance, but linear trends are observed when inhibitions or limitations occur, suggesting that the linear trend could be explained by inhibition events. In this case, the high initial substrate concentration could lead to a reduction in the growth rate of cells due to osmotic stress on cells or due to multiple substrate molecules binding to the enzyme, causing a decrease in the fermentation reaction rate [30,33]. Subsequently, in the second stage, lactic and butyric acid production, as well as biomass growth, ceased, and the sugar fermentation rate was significantly reduced. In the literature, this behavior has been attributed to inhibition experienced by microorganisms when product concentrations exceed a certain threshold [39]. In the literature, the products' inhibition effect over the microbial culture has been associated to the presence the undissociated fermentation products. These products can permeate across the membrane increasing the maintenance requirements of the microbial culture and also reducing the other metabolic routes, significantly reducing substrate consumption, and even stopping biomass growth and product generation [12,23,26]. In this scenario, most of the energy obtained from substrate fermentation is used to pump acids out of the cell [37,40]. During the inhibition phase, acetic acid was the only fermentation product obtained. Consequently, the acetic acid concentration at the

end of fermentation was 300 C mmol·L^{-1} for wastewater containing 1.52 C mol·L^{-1} mM of sugar, and 480 C mmol·L^{-1} for wastewater containing 2.12 C mol·L^{-1} of substrate.

3.3. Hydrogen Production

During the acidogenic fermentation of the substrates contained in the synthetic fruit juice wastewaters, H_2 and CO_2 were produced and accumulated in the gas phase. The evolution of cumulative H_2 and CO_2 during experiments with different initial sugar concentrations is presented in Figure 5. Cumulative gas production increased during acidogenic fermentation, but the trend and total amount of gas produced varied depending on the initial substrate concentration. When sugar concentrations were 0.30 and 0.91 C mol·L^{-1}, gas production followed exponential trends, indicating optimal microbial growth. However, with initial substrate concentrations of 1.52 and 2.12 C mol·L^{-1}, gas production exhibited two different trends in a similar way to the other fermentation products previously described.

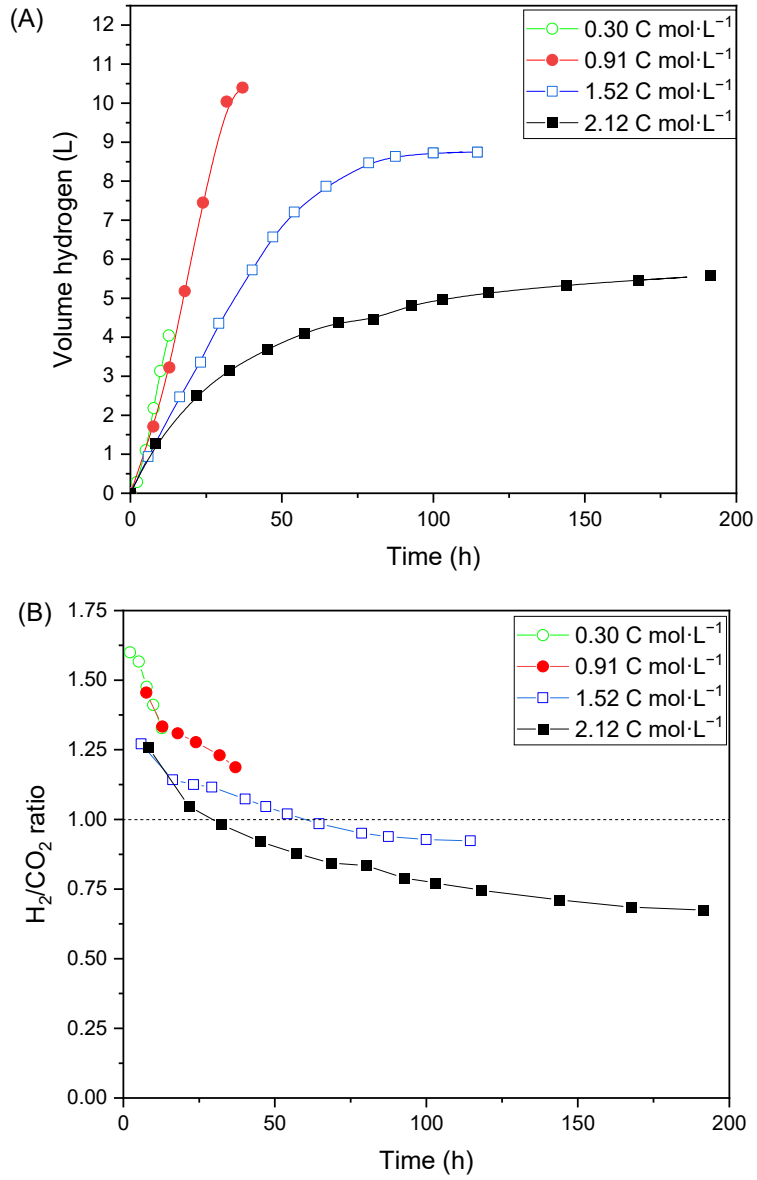

Figure 5. (**A**) Hydrogen production during acidogenic fermentation of synthetic wastewater with different substrate concentrations. (**B**) Hydrogen to carbon dioxide ratio during the fermentation tests.

In the first stage, up to 50 h for the 1.52 C mol·L^{-1} initial concentration and up to 60 h for the 2.12 C mol·L^{-1} initial concentration, gas production rates were approximately linear, with H_2 and CO_2 production being very similar. In the second stage, gas production rates decreased over time, in a similar way to the acid production behavior previously. Additionally, CO_2 production exceeded the H_2 and production during the second phase. The behavior experienced can be explained by a slight initial substrate inhibition of bacterial biomass, taking place in the first stage, and product inhibition due to the accumulation of fermentation products in the second stage.

The highest hydrogen production (6.1 L H_2·substrate C mol^{-1}) and the highest hydrogen percentage in the gas phase (57%) were achieved with a sugar concentration of 0.30 C mol·L^{-1}. These results indicate that increasing the substrate concentration led to lower H_2 production and lower H_2 content in the gas phase. These reductions can be attributed to substrate and product inhibitions experienced in the system. Similar results have been reported in the literature [22,41].

Considering that hydrogen is the most important product of acidogenic fermentation, its production was modeled using the modified Gompertz equation [42,43]. This equation allows for the determination of key kinetic and stoichiometric parameters, as well as H_2 production rates and the potential for H_2 generation, in experiments conducted with different initial substrate concentrations.

$$H(t) = P{\cdot}exp\left\{-exp\left[\frac{R_m{\cdot}e}{P}{\cdot}(\lambda - t) + 1\right]\right\} \quad (3)$$

where, $H(t)$ is the cumulative H_2 production (mL); P is the maximum potential of H_2 production (mL); R_m is the maximum hydrogen production rate (mL·h^{-1}); λ is the duration of the lag phase (h); and t is time (h).

Table 3 summarizes the values of the modified Gompertz equation coefficients for wastewaters with different initial substrate concentrations. Considering the correlation coefficients, the model accurately describes the experimental data in all cases, being the lowest correlation coefficient of 0.9701. This largest error was observed with an initial concentration of 2.12 C mol·L^{-1}, which can be attributed to the significant inhibition caused by substrate and fermentation products experienced by the microorganisms when dealing with this very high initial concentration.

Table 3. Modified Gompertz equation coefficients when dealing with different initial substrate concentrations.

Initial Sugar Concentration (C mol·L^{-1})	P (mL H_2)	R_m (mL H_2·h^{-1})	λ (h)	r^2
0.30	5482.77	444.08	2.71	0.9996
0.91	12,456.73	404.22	4.78	0.9970
1.52	8908.81	163.33	2.60	0.9970
2.12	5217.24	93.06	0	0.9701

Analyzing the results presented in Table 3, it must be highlighted that, on the one hand, the maximum H_2 potential production increased with initial substrate concentrations up to 0.91 C mol·L^{-1}. However, at higher concentrations, the maximum H_2 potential decreased as substrate concentrations increased. This effect can be attributed to a slight substrate inhibition of bacterial biomass, but primarily to product inhibition. As previously mentioned, product inhibition reduces H_2 yield, leading to lower H_2 generation despite consuming the same amount of substrate. Consequently, the highest maximum H_2 potential production, 12.46 L H_2, was achieved with a substrate concentration of to 0.91 C mol·L^{-1}. Taking into account the volume of the reactor, 3 L, the specific hydrogen production was about 4.15 Nm^3 H_2·m^{-3} reactor. A similar value has been reported in the literature by Wang et al., who studied hydrogen production via acidogenic fermentation as a function of organic load (from 0 to 1.6 × 10^3 mM glucose), finding the highest maximum H_2 potential

at a substrate concentration of approximately 0.85 C mol·L^{-1} glucose [31]. This initial substrate concentration closely aligns with the results obtained in this study.

On the other hand, the highest maximum H_2 production rate, 444.08 mL·h^{-1}, was obtained when dealing with a substrate concentration of about 0.30 C mol·L^{-1}. This parameter decreases with increasing initial substrate concentration. However, the highest H_2 production was obtained when dealing with a substrate concentration of about 0.91 C mol·L^{-1}.

Because of the different results obtained with the maximum potential of H_2 production and the maximum hydrogen production rate, a new parameter was selected to establish the comparison. The parameter selected was the Maximum Specific Hydrogen Production Rate (MSHPR). The MSHPR can be defined as the maximum hydrogen production per unit of time and biomass in the reactor and measured as ml H_2·biomass C mol^{-1}·L^{-1}. This parameter was calculated by dividing Rm by the biomass concentration. The results obtained for the MSHPR when feeding different initial substrates concentrations are presented in Figure 6.

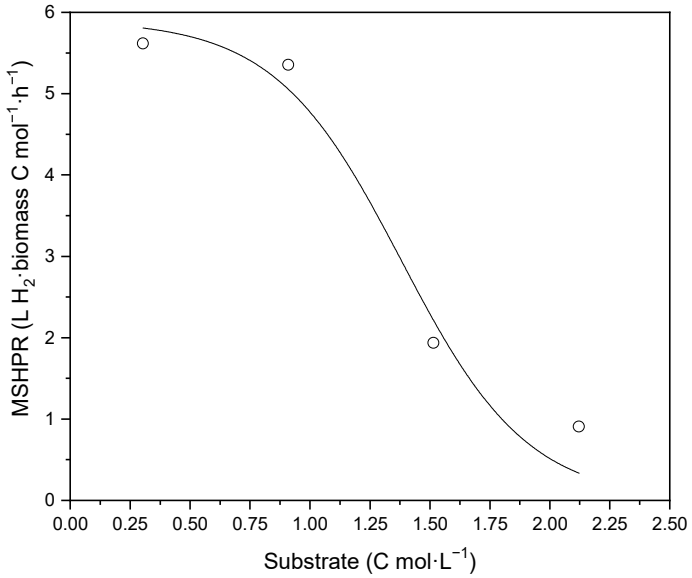

Figure 6. Evolution of maximum specific hydrogen production rates with substrate concentration.

As can be seen in Figure 6, the highest MSHPR was obtained when operating with a substrate concentration of 0.30 C mol·L^{-1}, 5.63 L H_2·biomass C mol^{-1}·h^{-1}. This value was very similar to that obtained with 0.91 C mol·L^{-1}, 5.50 L H_2·biomass C mol^{-1}·h^{-1}. In these cases, almost any substrate or product inhibition was experienced, justifying the similar values obtained. However, the value of the MSHPR significantly decreases when increasing the substrate concentration to 1.52 and 2.12 C mol·L^{-1}. The significantly lower MSHPR values obtained when operating with those concentrations could be explained by the inhibition effects caused by the very high initial substrate concentration as well as by the very high concentration of fermentation products accumulated during the fermentation, as previously described. Based on the results presented, it can be stated that the best initial substrate concentrations to maximize the hydrogen generation were within 0.30 and 0.91 C mol·L^{-1}.

With the aim to extrapolate these results to industrial facilities dealing with fruit juice wastewaters, the potential hydrogen generation was determined. To achieve that, it was taken into account the daily flow and organic load of an average size fruit-juice industry, around 100 m^3/d and 1 C mol·L^{-1} [1–4], and the hydrogen yield previously determined. Based on this information, the hydrogen potential of an average fruit-juice industry was about 11.700 Nm^3 H_2/d, which is a significant amount of energy to be valorized and a renewable energy source that can be framed in the circular economy concept.

4. Conclusions

The results obtained in this work demonstrated that substrate concentration significantly influences the fermentation process, with optimal H_2 production and biomass growth observed at substrate concentrations within 0.30 and 0.91 C mol·L^{-1}. Higher substrate concentrations, in this work 1.52 and 2.12 C mol·L^{-1}, led to substrate and product inhibition effects over the biomass, resulting in reduced H_2 and acid yields. The highest H_2 production (6.1 L H_2·substrate C mol^{-1}) and H_2 percentage in the gas phase (57%) were achieved at a substrate concentration of 0.30 C mol·L^{-1}. The maximum H_2 potential production was 4.15 L H_2·L^{-1} reactor at a substrate concentration of 2.12 C mol·L^{-1}, aligning with literature values. Biomass growth and acids production followed exponential trends at lower substrate concentrations, while higher concentrations resulted in linear trends due to inhibition events. These findings highlight the importance of substrate concentrations to enhance hydrogen production and biomass growth in acidogenic fermentation processes. Anyway, these results require further verification before its industrial use by experimenting with actual fruit juice wastewaters.

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