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Hydrogen Production from Gelatin, Cotton, Wheat Straw, and Sour Cabbage and Their Mixtures—Short Communication

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Abstract: The influence of microaeration, pH, and substrate during dark fermentation of sour cabbage, gelatin, and wheat straw was investigated, and the results of dark fermentation of these three substrates and their mixtures are presented in this research. The fermentation of cabbage, gelatin, and wheat straw was investigated under varying pH and aeration conditions. We investigated concentrations of volatile suspended solids (VSS) of 20 g VSS/L of a substrate at a stable pH of 6.0 and a not aligned pH value. Sour cabbage resulted in the highest volume of hydrogen for 450 mL/g VSS with a pH of 6.0. The mixing of substrates caused lower hydrogen production than sour cabbage or wheat straw alone.

Keywords: dark fermentation; anaerobic digestion; hydrogen; sour cabbage; gelatin; wheat straw

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1. Introduction

Dark fermentation is a microbial conversion process of organic matter, especially carbohydrates. The most investigated substrates for this process are carbohydrate-rich materials like potatoes, molasses, or lignocellulose, but fats and proteins, e.g., gelatin, have also been used [1]. According to Khandelwal et al. [2], the dark fermentation of sugars, like confectionary waste, is an optimal method for upgrading methane fermentation. The process of hydrogen production initially could be troublesome. It occurs naturally in tissues infected by *Clostridium perfringens* as a sickness named gangrene, and can even cause explosions after the death of the host (as was reported for the body of King Henry VIII of England in the XVIth century), or in affected teeth, sometimes causing their decay [3]. Both of these phenomena result from a high content of hydrogen and even 16% is affected by bacteria part of organs [4].

The European Union has set a target to reach climate neutrality by the year 2050 [4], and an important component of this goal is the generation of green hydrogen [5]. Wu and Strezov [6] outlined the challenges of green technology in connecting the concept of 'zero waste' with maintaining the current lifestyle. Technology needs to solve recent degradation and pollution by using this waste via biotechnological methods such as anaerobic digestion and algae. Monitoring of changes and control requires modification of algorithms without limitation to artificial neural network (ANN). In addition to waste generation and disposal, and transferring to energy or green chemistry, environmental engineering needs to overcome obstacles associated with devices and tool preparation. Photovoltaics and wind power apparatus are built mostly with fossil fuel sources and should be replaced with more sustainable methods. Proper lignocellulose pretreatment can allow obtaining material suitable for those purposes. Dark fermentation digests some parts of lignocellulose waste, and the remainder usually shows changes in some properties, like lower crystallinity, and short organic compounds that can be raw materials for green polymers.

Biohydrogen is an excellent energy carrier with previously designed methods of storage [7]. The huge demand for hydrogen to fulfill the global demand requires the planning of various sources [8]. Corn wastes processed into hydrogen can fulfill only a small fraction of the needs of both Poland [9] and China [10]. The extension of wide uses of algae as biophotolysis factors in dark fermentation after curtailing carbon dioxide and oil extraction and pharmacy demands was also insufficient [11,12]. Kora et al. [13] and Okurowska et al. [12–14] designed a plant using every potential carbon dioxide capturer, then used the remainder as biofuels and substrates for dark fermentation and biopolymers, closing the loop. According to the Iranian life cycle project [15,16], by 2050 both dark fermentation and anaerobic digestion will be part of gas power plants focusing on the utilization of waste. Designs of renewable resource facilities require inexpensive and fast solutions. Checking pure compounds is suitable for a pilot plant extension, although segregation generates costs and demands for legislative change [17]. Therefore, an important part of research is seeking mixtures for fermentations [18] that also allow continuous processes in such cases. Continuous dark fermentation is usually applied as a source of methane upgrading [19]. The application of dark fermentation as supplementation for anaerobic digestion is a relevant step for producing biomethane of a quality that is suitable to meet the requirements of gas stations and chemical industries [20]. Therefore, in this research, we doubled concentrations and mixed recently investigated substrates. In methanotrophic anaerobic digestion, the biogas methane potential (bmp) is a standardized approach for testing various feeds. We decided to modify bmp into biogas hydrogen potential (bhp) to implement the batch method of checking different substrates for hydrogen production.

Lignocellulose waste is generated from daily life, food, agriculture, and textile industries [21] and is one of the most troublesome wastes. The difficulties of landfilling this waste lead to the necessity of utilizing it. Thermal degradation approaches like pyrolysis and gasification are fast and require high-energy-demanding and expensive catalysts, and in gasification, they generate highly toxic compounds [22,23]. Lignocellulose is easier to decompose compared to synthetic polymers or tires [24]. Thus, it is often mixed with highly toxic compounds. The addition of glucose helped to utilize asbestos [25] and ethylene glycol [26].

Cotton is a model plant, as it contains no lignin [27]. Unfortunately, bacteria hardly digest cotton wastes due to feed structure [28,29]. Cotton depolymerization is still a problem that can be solved by genetically modified enzymes [30]. The high hydrogen potential of cotton can be applied after solving the strength problem of the material [31].

Dark fermentation is much more stable compared to biophotolysis or photofermentation but still has much lower efficiency and requires more space for the production of one liter of hydrogen compared to pyrolysis [32]. The microbial electrolysis cells can split only ammonia and low-carbonic acids ($C < 4$) without external charging [33,34]. Extending the applicability for wide feeds of microbial electrolysis cells (like water) requires the design of photovoltaic cells or wind power energy [35]. Both wind power energy and photovoltaic cells require mostly synthetic materials primarily produced from conventional sources, so the loop is not closed [36]. Therefore, it is necessary to design a method of producing material for solar and wind energy from lignocellulosic waste using algae, yeasts, and bacteria (cyano-, photo-fermentative, and fermentative). Biotechnological approaches also include bioaugmentation and biosorption with some thermal technologies for closing the loop [37]. The closing loop needs adamant updating of a growing population. Hydrogen production development is necessary for developing space exploration and translocating some populations to reduce the overpopulation of Earth. However, if the population surpasses 13 billion, then there is no method for maintaining the current lifestyle except overcoming that green energy generation is obligative [38].

According to Abiev et al. [39], bioprocessing, including dark fermentation, has scarcely scaled up due to problems with continuity, dependence on unknown variables, and the selection of proper statistical methodologies. Dark fermentation is a process of limited efficiency (only 33%), but requires little energy and reduces a wide variety of wastes [39].

The Thauer limit was only omitted by genetic modification of bacteria extending enzyme potentials [40]. The acetic pathway naturally occurring is hard to obtain, forcing it to follow a less efficient butyric pathway [41]. Bacteria in the process can utilize animal, plant, and mushroom wastes after daily life, food, pharmacy, and industries with much lower energy demand compared to pyrolysis [42]. The process is independent of light intensity like biophotolysis or photofermentation [14,43] and can reduce a wider group of wastes without supplementation of extra energy, like in microbial electrolysis cells [14]. Due to the discontinuity of the process for a single bacteria unit [44], there were established models of growth using arc trigonometric functions or exponentials [45]. There are also still doubts about the scaling-up process in concentration due to different results showing that fewer concentrations are more efficient than higher [46] or reverse [47]. The profitability of dark fermentation requires planning seasonal feeds throughout the year similar to a biogas plant. In the research, different waste and mixing show potential arrangements that can respond to the selected time. All selected waste appears with different frequencies on the year and periods. Bioprocessing for cost-efficiency requires maintenance all the time; thus, such a plan is necessary for planned waste management. The potential utilization of dark fermentation allows for supporting the waste management of the selected area with other waste management utilities and local demands.

Therefore, there is necessary testing of the doubling of concentrations in cotton, sour cabbage, wheat straw, gelatin, potatoes, and mixtures for checking the growing load impact. We aimed to check the hydrogen and hydrogen sulfite ratio occurring in previous research on pure compounds [1].

2. Materials and Methods

Description of Experiments

The materials preparations for dark fermentation were selected similarly to previous successful experiments [1]. The methods and analyses were unchanged other than increasing concentration from 5 g VSS/L to 20 g VSS/L.

Inoculum originated from the biogas plant in Darżyno. The inoculum stayed in a plastic bucket, firstly in the dry room at 36 °C for one month for degassing to avoid biogas production of the previous substrate. Then, it was kept in the fridge at 5 °C. Then, the container with inoculum was stored in a cool room at room temperature for the next month. Then, the inoculum was pretreated using heating for 15 min at 121 °C, like in [1], in a pot. Heat shock was chosen as the pretreatment with the highest conversion of substrate [48]. The sour cabbage, gelatin, and wheat straw originated from local bar 3 Smaki.

We analyzed the inoculum and substrate content of the carbon, nitrogen, and sulfur ratio using Elementar Analyzer Flash 2000 (Thermo Scientific, Waltham, USA). Determining the C:N ratio consisted of catalytic combustion in a proper amount of oxygen.

We used the characteristics of VSS of mixtures for evaluation of conversion of waste such as cotton, wheat straw, collagen, and sour cabbage. The conversion of feed was according to NREL [49,50], using Formula (1).

$$\omega\% = \frac{VSS_1 - Vss_2}{VSS_1} * 100\% \quad (1)$$

where ω is the conversion factor, VSS_1 initial volatile solid, and VSS_2 is the final volatile solid degree.

We placed feed and heat-shocked bacteria in glass reactors of 1.2 L placed in a water bath. The temperature kept in reactors was 36 °C ± 0.5 and higher compared to the outside water bath of 35 °C ± 0.5; all fermentations were endothermic. The reactors were connected with propylene ducts of 1.5 m to cylinder reactors filled with water placed also into water (see Figure 1). The biogas produced in reactors was transferred by these ducts to cylinders and replaced water of some height allowing for evaluations of gas volume measurement by the Owen method [51]. The samples were kept until the growth of biogas was below 0.5% by day, lower compared to what was proposed in [52]. This level was considered the end

of the assay and the test. The reading was facilitated by filling the top of the cylinder with diesel oil Ludwik® detergent mixture for marking the level of gas, also preventing from solving gas in water. Then, we analyzed gas content by two methods if the gas height was above 0.45 dm in the two-stage method, firstly, by opening the valve on top of the cylinder and connecting it with the duct of the gas analyzer (GA5000, Geotech QED Environmental Systems, Inc., Coventry, UK). The analyzer poses ATEX II 2G Ex ib IIA T1 Gb (Ta from −10 °C to +50 °C), IECEx and CSA quality certifications, and UKAS ISO 17,025 calibration certificate. The equipment allowed measurements of CH_4, CO_2, O_2, H_2, and H_2S in the ranges 0–100%, 0–100%, 0–25%, 0–1000 ppm, and 0–5000 ppm, respectively. If the hydrogen fraction was above 1000 ppm, we corrected the measurement by taking 1 mL of the sample and putting it into a Todlar bag for chromatograph detection. Then, biogas content was assessed using a gas chromatograph (GC) GC SRI 8060 with a thermal conductivity detector (SRI) and argon as a carrier (gas flowrate was 0.6 mL/h), as recommended by Hitit and Hallenbeck [53]. A silica packed column Restek® with characteristics of 2 m/2 mm ID 1/8″ OD silica was used. The detector temperature was between 46 °C and 196 °C. The oven was working at a temperature from 23 °C to 200 °C. The injection temperature (splitless mode) method was 45 °C. The error of the apparatus was 0.1%. The experiments were tripled. The statistical error follows [54–56].

The mean values were calculated after the result triplication ($n = 3$). One-way ANOVA followed by the Bonferroni post hoc test was approached obtaining error regression at the level of $p < 0.05$.

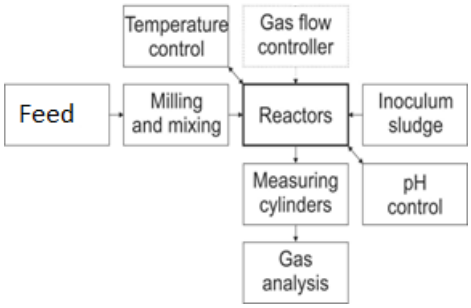

Figure 1. Sketch of the experimental procedure for biogas production by dark fermentation of sour cabbage [44] (reproduced with permission from Sołowski Heliyon Elsevier 2021).

3. Results and Analysis

The biogas was emitted depending on feed until 63 days and contained hydrogen, methane, carbon dioxide, and hydrogen sulfide. Looking at Figure 2, we observed that the highest hydrogen production was for fine sour cabbage with slight microaeration and pH control of 6.0 taken in [57]. In other cases, dark fermentation gas volumes were significantly lower. It seems that lactic acid bacteria stimulated hydrogen generation. A mixture of sour cabbage and cotton worked better compared to only cotton [29]. The straw curtailed the time of hydrogen production three times in comparison to gelatin [1] and twice in comparison to sour cabbage and wheat straw [58]. This is related to the earlier conversion of bacterial rest of which hydrogen sulfide is the source. Every feed produced much higher hydrogen than in unstressed cases [28,40,59]. These differences were twice higher than in glucose [48]. Gelatin and straw hydrogen emissions were shorter but twice higher than the only wheat straw assay and four times as in the single gelatin [1]. Cotton without pH control was the least fermentative to hydrogen. The mixture of sour cabbage and wheat straw was more 'unattractive' for stressed inoculum compared to pure feeds of sour cabbage but more compared to wheat straw [60]. The data was using unmodified genetic inoculum without nutrients and, thus, was of lower efficiency. The biogas hydrogen potential was revealed like in methane [61], with only an initial positive response that in continuous mode could be inefficient due to toxic effects, or unsuitable hydraulic retention time.

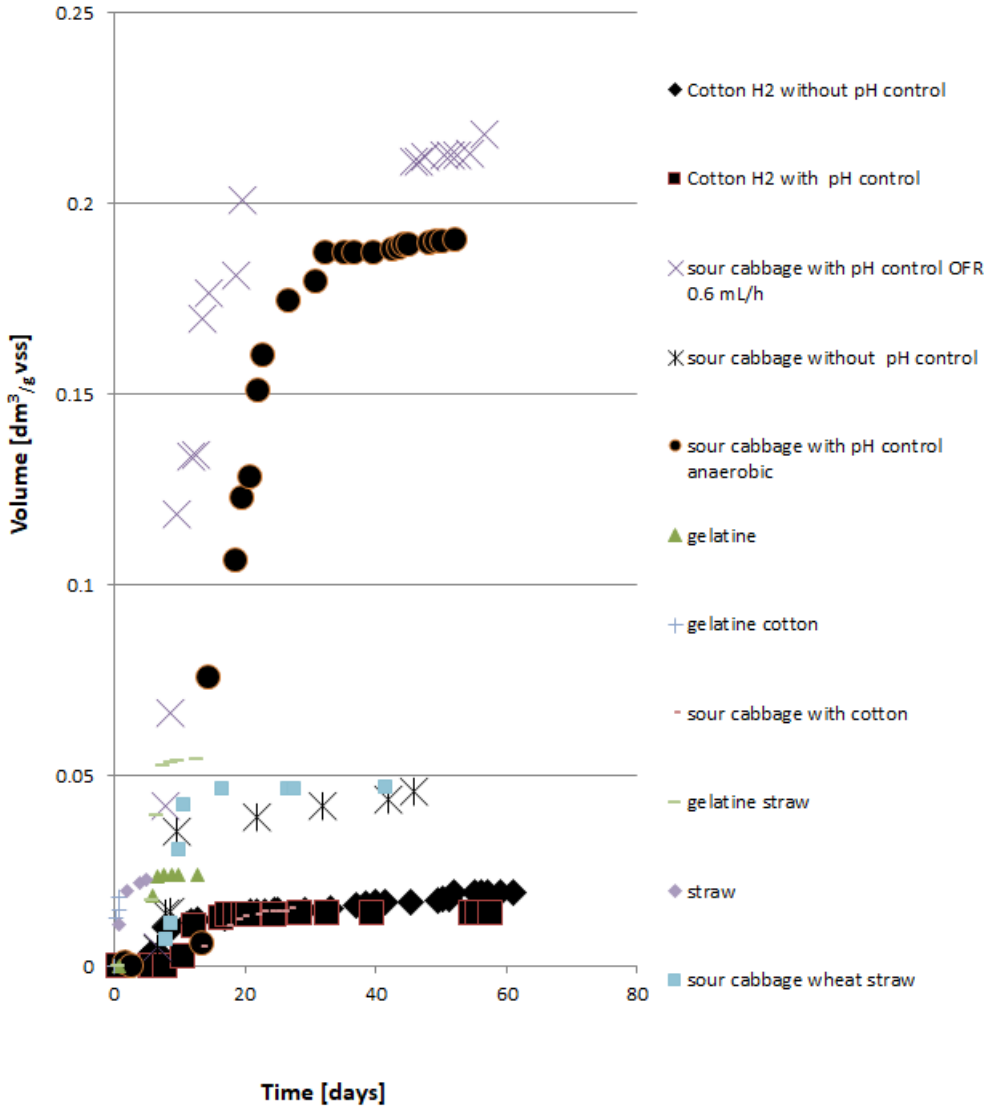

Figure 2. Comparison of different substrates' hydrogen yield production for a feed of 20 g VSS/L and compared with data from [51].

Figure 3 confirmed earlier observations. In every substrate hydrogen and hydrogen sulfide cogeneration occurred. Since the inoculum was degassed, the hydrogen sulfide originated from bacterial rests like in earlier cases [1]. Gelatin with straw produced twice more hydrogen sulfide than pure gelatin [1], but was higher than in the case of wheat straw [57]. Reviewing this data, we concluded that bacteria prefer substrate shifts to complex material, supplementing it with simple feed [62]. Therefore, the conversion of straw was higher without enhancing significantly hydrogen production. Because the biological layers were properly prepared earlier, it can be concluded that hydrogen sulfide had bacterial rests origin since was higher compared to sulfur in feeds [58].

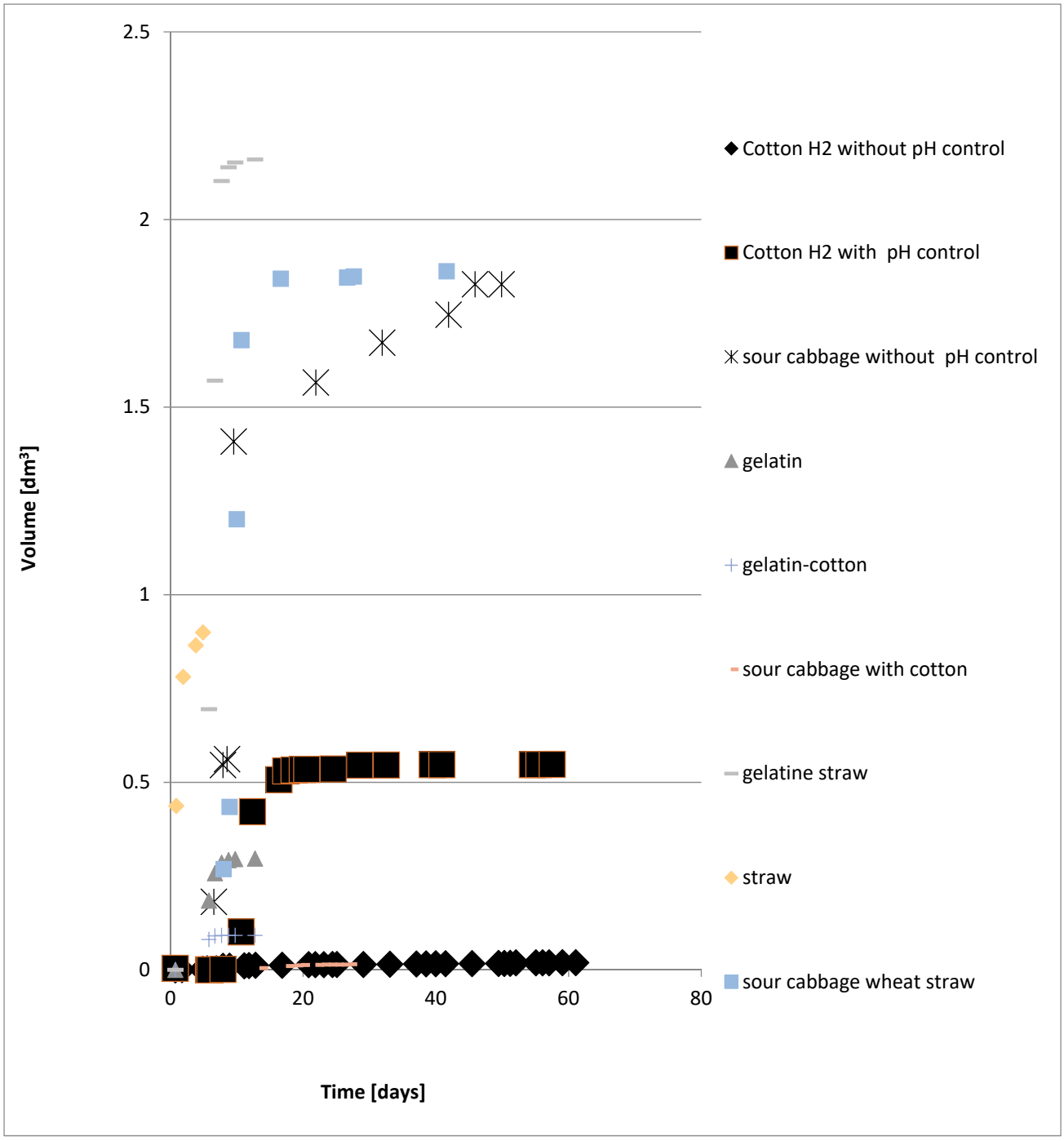

Figure 3. Hydrogen sulfide cumulative emission yield from selected feeds.

The ratio in mixtures is indiscernible as in pure compound fermentations [63,64]. Parallel production of hydrogen and hydrogen sulfide in a stable manner occurred (see Figure 4). Therefore, the effect observed earlier [29] occurred in a more chaotic and less linear manner [1]. The value of ratios doubled with the growth of concentration in comparison to [65].

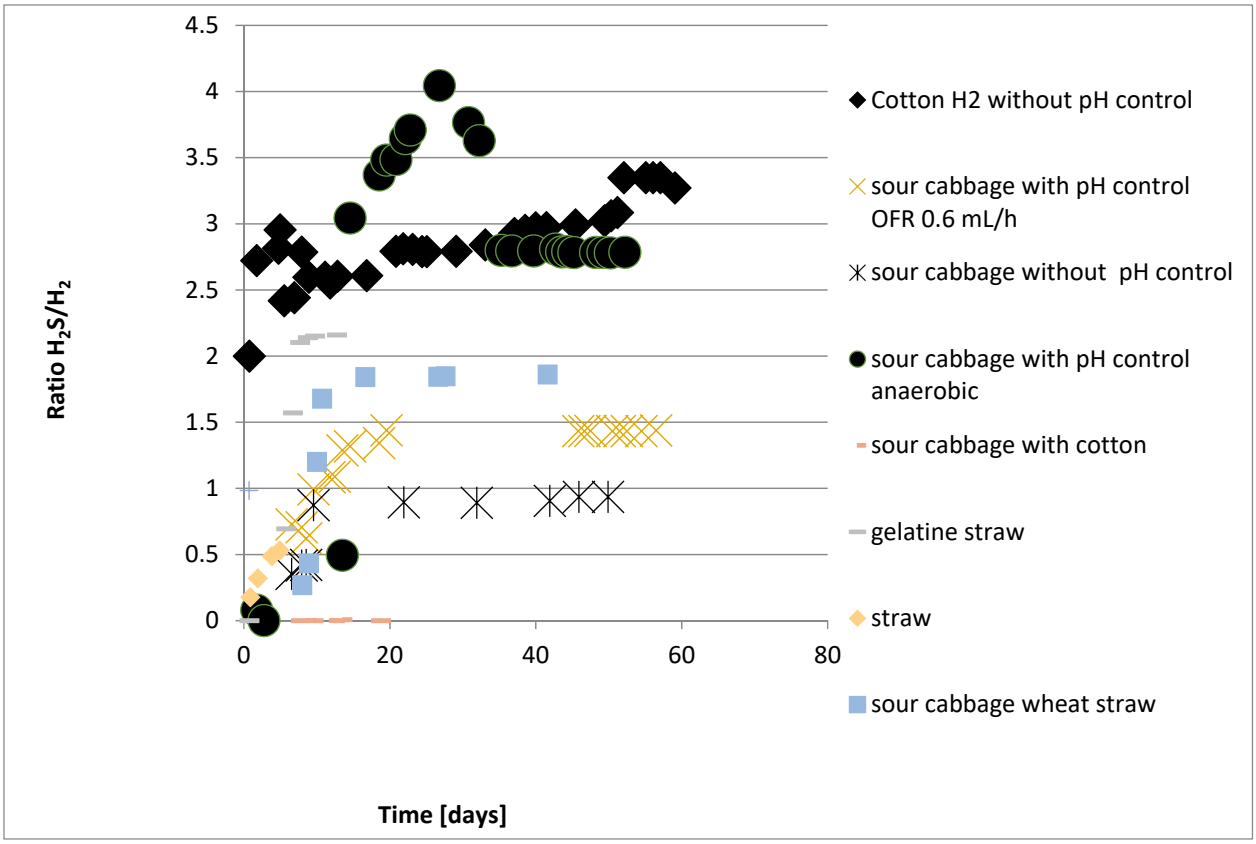

Figure 4. The ratio of hydrogen and hydrogen sulfide emission.

The pretreatment stopped methane and after 20 days returned as restored methanogenesis, Figure 5. The methane was significantly smaller than in unstressed cases [30]. The pretreating efficiently blocks anaerobic digestion as aimed. The mixing effect blocks restoration more than in pure compounds. We showed methane production to illustrate the efficiency of heat shock, which illustrates the potential to upgrade for designs that include mixed dark fermentation, anaerobic digestion, algae, or other compounds.

The methane was less compared to anaerobic digestion [66]. The methane was the highest in gelatin and straw in reverse than in alone straw. Therefore, it is worth adding gelatin waste for shifting processes like agar addition [67]. The methane data allow for the design of potential approaches in biohythane process design. Thus, it allows for planning packing in dark fermentation preventing washouts of bacteria with feed flowing to a methanotrophic reactor. The highest methane production is for the gelatin higher than in mixtures. The highest gelatin production was obtained in gelatin higher than in [1], proving the influence of shock conditions for obtaining enough repeatability. Wheat straw and sour cabbage fermentations obtained higher production of methane so it resulted in higher gas volume production proving that there are good additives for improving methane, like pickled cucumbers [66]. The methane production in mixed forms was much less than hydrogen, unlike [46,67]. The bacterial layer for mixtures needed less stress magnitude compared to pure compounds.

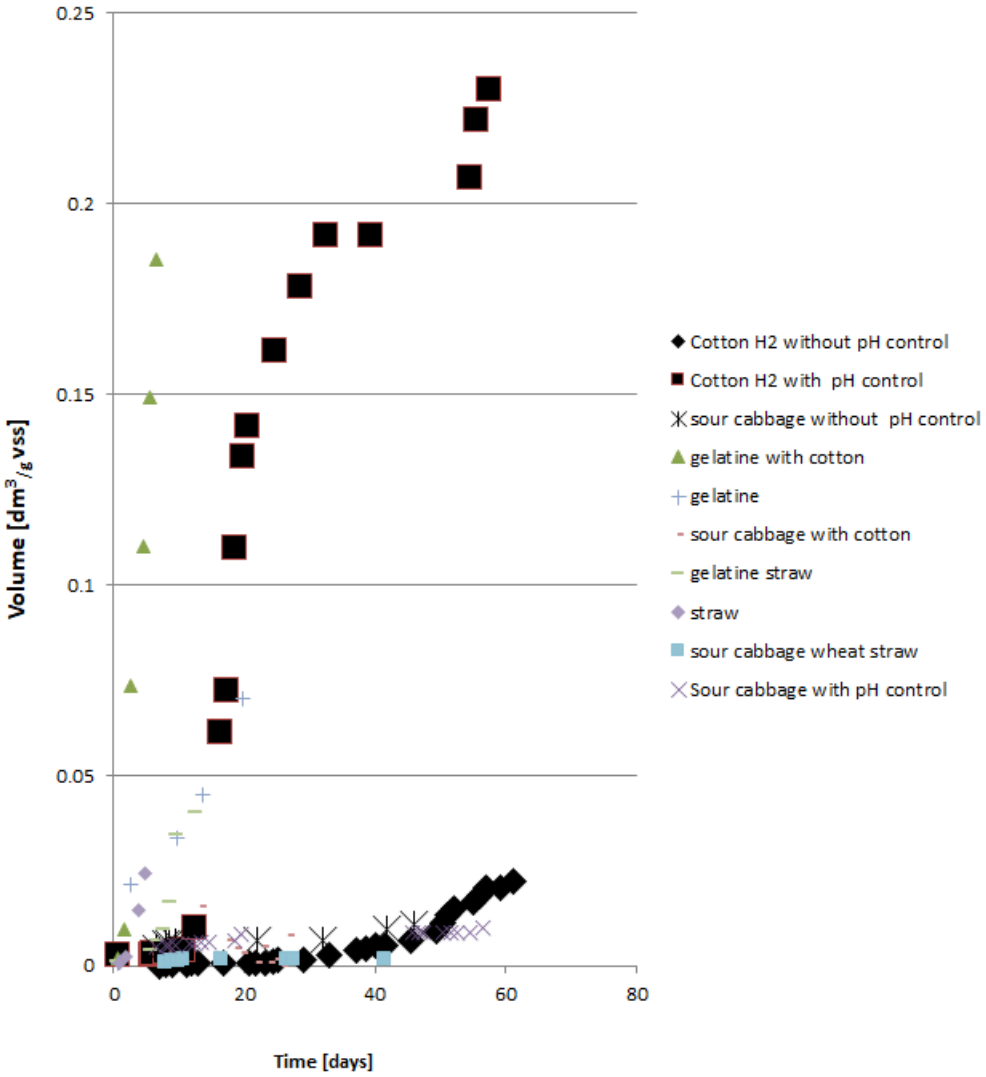

Figure 5. Methane production yield from different substrates.

Conversion and General Discussion

It can be observed that the addition of gelatin stimulated the digestion of wheat straw and cotton helped more compared to sour cabbage. The addition of protein in gelatin increased hydrogen production slightly from only wheat straw, but still far from sour cabbage production. Tables 1 and 2 showed differences in feed characteristics before and after fermentation. The pretreatment of lignocellulose waste by dark fermentation showed the efficiency of this approach in their conversions, and thus, utilizations. This is relevant for designing processes with an expected effluent for combining with other techniques like pyrolysis, photofermentation, and algal pretreatment [14].

Table 1. Physicochemical characteristics of the inoculum and substrates used in various tests.

TS	VSS	C: N	Carbon (% TS)	Nitrogen (% TS)	Sulfur (% TS)
1.49% ± 0.03%	41.61% TS ± 3.03%	10.53	20.1% ± 1.23	1.88% ± 0.03	0.4% ± 0.02
6.99% ± 0.02%	89.32% TS ± 2.2%	11.8	34.2% ± 1.43	2.88% ± 0.02	0.61% ± 0.02
13.43% ± 0.02%	98.52% TS ± 1.02%	22.22	74% ± 1.62	3.33% ± 0.02	0.3% ± 0.02
35.43% ± 0.02%	98.43% TS ± 1.04%	18.1	76.4% ± 1.57	4.19% ± 0.02	0.3% ± 0.02
89.89 ± 0.03%	96.5 TS ± 1.06%	12.8	39.2% ± 1.63	3.18% ± 0.02	0.41% ± 0.02

Table 2. Conversion of biomass during two months of digestion and yields.

Substrate	Sample Weight g/20 mL	Dry Mass g/20 mL	Dry Mass %	Ashes g/20 mL	VSS	VSS g/20 mL %	Reduction %
Straw	11.93 ± 0.01	0.33 ± 0.01	0.0277	1.106 ± 0.02	0.089 ± 0.02	0.269 ± 0.02	−0.011 ± 0.02
Cotton after gelatin	2.92 ± 0.02	1.23 ± 0.02	0.4212	15.106 ± 0.02	1.22 ± 0.02	0.993 ± 0.02	−0.713 ± 0.02
Straw after gelatin	2.26 ± 0.03	0.78 ± 0.01	0.3451	16.106 ± 0.03	0.765 ± 0.02	0.981 ± 0.02	−0.701 ± 0.02
Cotton after sour cabbage	11 ± 0.01	0.59 ± 0.02	0.054 ± 0.02	3.106 ± 0.02	0.32 ± 0.02	0.542 ± 0.02	−0.398 ± 0.02
Sour cabbage after cotton	11.05 ± 0.02	0.41 ± 0.02	0.037 ± 0.02	11.105 ± 0.02	0.19 ± 0.02	0.463 ± 0.02	−0.292 ± 0.02
Sour cabbage	18.47 ± 0.02	0.58 ± 0.02	0.0314 ± 0.02	14.106 ± 0.02	0.23 ± 0.02	0.397 ± 0.02	−0.359 ± 0.02

Therefore, the gelatine addition worked less efficiently as agar plates in Pan et al. [48] for glucose. The feeds were more complex material compared to model feeds. The gelatine application was more feasible due to its more common availability in the market compared to agar plates. The gelatine is powdered collagen waste, which is troublesome for some industries like tannery fishing or butchery. Thus, it would allow for more waste generated by those sectors with the formation of energy carriers. The bacteria after heat shock regenerated and blocked the fast loss of hydrogenotrophic potential for methane production. Hydrogen production elevated with higher concentrations similar to [68]. Table 2 showed the results of the conversion of biomass during two months of digestion. The bioprocessing significantly changed the parameters of cotton with gelatine more than compared to other combinations.

Reduction of dry matter was observed in every case, but the highest in assays with additions of gelatine. The cotton and sour cabbage enhanced the digestion of both wastes. Hydrogen production in a mixture of sour cabbage and cotton increased slightly with significant utilization of both wastes. The gelatine improved both the digestion of straw and cotton. The pretreatment showed the economic way to increase cotton or straw utilization. Gelatin addition of fermentation mixture of lignocellulose waste improved utilization similarly to glucose of asbestos [25,69] or fuel rests [26]. In Table 3, there is a comparison of gaseous phase product yield from different products. As can be observed in the tables, the highest hydrogen was for sour cabbage and then from gelatine with wheat straw. The least hydrogen production was for cotton and sour cabbage unless it was high substrate conversion. Gelatin increased both methane and hydrogen yields.

Table 3. Comparison of yields of methane and hydrogen of different substrates of 20 g VSS/L.

Substrate	Yield CH_4 L/g VSS ± 0.01	Yield H_2 L/g VSS ± 0.01
Sour cabbage 20 g VSS/L	0.02	0.44
Sour cabbage cotton 1:1 20 g VSS/L	0.09	0.0068
Wheat straw 20 g VSS/L	0.17	0.038
Cotton 20 g VSS/L	0.46	0.027
Sour cabbage wheat straw 1:1 20 g VSS/L	0.028	0.093
Gelatin	0.029	0.047
Gelatin:cotton 1:1	0.12	0.035
Gelatin wheat straw 1:1	0.08	0.1

The differences between pure compounds and mixtures proved the relevance of empirical checking at batch mode for further planning of industrialization of dark fermentation. The design for making process economical needs predict changes of feed for long-term functioning. The substrates possessing high methane potential can obtain high hydro-

gen potential and reverse. This is caused because anaerobic digestion also uses liquid substrates of acidogenesis in methanogenesis [70] uninvolved in dark fermentation [71]. Carbohydrates can digest in methanogenic anaerobic digestion in an exothermic manner causing fewer energy demands compared to other compounds. Protein and fats in both anaerobic digestion and dark fermentation are endothermic. Although thermophilic dark fermentation is slightly more productive compared to mesophilic [72], the endothermicity of the process favors the second option [73]. Therefore, the design of dark fermentation shifts to mesophilic conditions compared to thermophilic with a wide regional analysis of the potential feed. The rapid climate and political changes and droughts forces designers to the prediction of replacement substrate. Tests of dark fermentations should include a variety of wastes, and models can give hints for selections [74].

Recently, combining many renewable plants, like algae farms [75,76] with dark fermentation or biohythane plants is an excellent solution for limitations of organisms in comparison to conventional methods or thermal decompositions [44]. The combinations of thermal and biological are also suitable for replacing raw materials for polymer industries. The extension of dark fermentation for many substrates and mixtures enforces applicability for local communities, less strict legislation, and more attraction for funding institutions. Low pH industries like dark fermentation should mix in the production of different products other than hydrogen and also low organic acids suitable for nutrients. Other benefits for waste management can be obtained from pretreatment rests [77], like lignin rests, which can be suitable for green synthesis [78]. The cotton waste disadvantage is caused by the strength that made wide use of plants and huge landfill problems in addition to high water resource demands [79]. The Busswell formulas [80] and their dark fermentation substitutes [8] are important hints for developing dark fermentation and anaerobic digestion without the complexity of substrate consideration. Therefore, relevant steps are empirical tests of many mixtures, especially with recently emerging wastes (for example, COVID-19 medication rests). The researchers developing processes should follow natural solutions for providing industrially available models. The gangrene-affected vessels can contain even 15% of hydrogen which is enough for recent standards [81]. Therefore, researchers should watch diseases caused by *Clostridium perfingens* and antibacterial mouth infections causing tooth decay for solving dark fermentation problems [2]. The obtained data showed relations between protein additions and lignocellulose, textile, and food waste utilization. The approaches of protein additions increased the 'edibility' of lignocellulose wastes by heat-shocked bacteria.

4. Conclusions

We found gelatin and sour cabbage to be useful supplements for improving the conversion of both cotton and wheat straw as examples of lignocellulosic waste. The highest hydrogen production for mixtures of concentration 20 g VSS/L was sour cabbage giving a volume of 8.9 l. Mixtures of feeds improved the conversion of substrates without improving general cumulative hydrogen production. The mixing of feed data is necessary for designing a profitable process of dark fermentation. The results allow for the planning process combining dark fermentation with other bioprocess technologies with cotton, sour cabbage, wheat straw, and gelatin. Utilization of organic waste in addition to replacing conventional sources of energy carriers and green chemistry and fermentation of feed with gelatin or sour cabbage is a tool for improving the efficiency and economics of the process.

Author Contributions: G.S. performed all aspects of the research, supervised by F.A.O. and M.S.S. Conceptualization, G.S.; methodology, G.S.; software, G.S.; validation, G.S., F.A.O., M.S.S. and G.S.; formal analysis, G.S.; investigation, G.S.; resources, G.S., F.A.O.; data curation, G.S.; writing—original draft preparation, G.S.; writing—review and editing, F.A.O., M.S.S.; visualization, G.S.; supervision, F.A.O., M.S.S.; project administration, F.A.O.; funding acquisition, M.S.S. All authors have read and agreed to the published version of the manuscript.

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