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Optimum Conditions for Enhanced Biohydrogen Production from a Mixture of Food Waste and Sewage Sludge with Alkali Pretreatment

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Abstract: Given the increasing demand for hydrogen, owing to its environmentally friendly nature, it is important to explore efficient methods for hydrogen production. This study investigates dark-fermentative hydrogen production by the co-digestion of food waste and sewage sludge. Both wastes were subjected to alkali pretreatment (at pH 13) to enhance biodegradability. Batch tests were conducted to enhance hydrogen production from food waste and sewage sludge under various volatile solid (VS) concentrations of 1.5–5% and food waste to sewage sludge mixing ratios of 0:100–100:0. We found that alkali pretreatment was effective in increasing hydrogen yields. The maximum specific hydrogen production rate of 163.8 mL H₂/g volatile suspended solid/h was obtained at a VS concentration of 5.0% and food waste composition of 62.5%. Additionally, VS concentration of 2.8% and food waste composition of 100% yielded a maximum hydrogen production potential of 152.1 mL H₂/g VS. Our findings indicate that food waste and sewage sludge with alkali pretreatment are potential substrates to produce biohydrogen.

Keywords: alkali treatment; biohydrogen; food waste; sewage sludge; dark fermentation



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1. Introduction

Net-zero emissions are one of the goals that countries around the world have set for 2050. Hydrogen is one of the keys to achieving this, and its demand has increased substantially because of the development of national hydrogen strategies [1]. Hydrogen is an important fuel as it is a clean environmentally friendly, and sustainable energy source that emits only water. Moreover, it contributes significantly to the reduction in carbon emissions [2,3]. Hydrogen is generally produced using physicochemical and biological methods and is currently almost exclusively produced through physicochemical methods using fossil fuels [3]. The physicochemical methods mainly include coal gasification, hydrocarbon reforming, and hydrocarbon pyrolysis. These methods, however, are unsustainable, as the sources utilized are non-renewable, polluting sources, emitting significant amounts of greenhouse gases, including CO₂ [4]. Furthermore, these methods are unsustainable, as the sources utilized are non-renewable, polluting sources, emitting significant amounts of greenhouse gases. Clean hydrogen needs to be produced through more sustainable methods [5].

Hydrogen can be generated from renewable, sustainable sources using environmentally friendly methods [6,7]. Biological methods are less energy-consuming than physicochemical processes and environmentally friendly. The biological processes include biophotolysis, as well as photo fermentation (PF) and dark fermentation (DF) [8]. Among them, hydrogen production employing DF is regarded as the most practically applicable method because it is not energy intensive, and hydrogen is produced at a much faster rate than when implementing any other biological process. Additionally, it allows the treatment of organic solid wastes such as sewage sludge (SS), food waste (FW), and livestock waste [9–11].

DF is a process of producing biohydrogen as a byproduct during the acidogenesis of carbohydrates, and inoculum is known to greatly affect hydrogen yields [12]. Because homoacetogens and methanogens consume hydrogen to produce acetate and methane, respectively, the activity of hydrogen-producing bacteria needs to be enhanced while that of hydrogen consuming bacteria needs to be suppressed [12]. *Clostridia*, one of the most favorable bacteria for hydrogen production are known to form endospores that are resistant to unfavorable factors such as chemicals, radiation, and heat. Therefore, *Clostridia* can be a favorable choice for hydrogen production via heat treatment [13], as they recorded higher hydrogen yields than *Enterobacter* and *Bacillus* [14,15]. However, they are sensitive to unfavorable environmental conditions because of the metabolic complexity of spore formation.

For DF, organic wastes can be a favorable carbon source because they are nutrient-rich and hydrogen production occurs concurrently with waste reduction. FW is problematic organic solid waste that constitute 15–63% of total municipal solid waste worldwide [3]. Due to the prohibition of direct FW landfill, more competent technologies, in addition to existing recycling technologies, are required to fully explore the potential of high-energy-content FW. In particular, FW is an appropriate substrate for hydrogen production owing to its high carbohydrate content. However, it has relatively low nitrogen content, an essential nutrient for hydrogen-producing microorganisms.

To overcome this limitation, additives and protein-rich waste can be mixed with FW to increase nitrogen content. Among various organic wastes, SS is an optimum choice as a co-substrate to fulfill the nitrogen requirements of the bacteria [11,13,16]. Therefore, the co-digestion of SS and FW will result in inclined hydrogen production due to a more balanced carbon-to-nitrogen ratio in the substrate.

Although SS is a suitable co-substrate for hydrogen generation, it exhibits poor biodegradability due to the low hydrolysis reaction rate of cell-bound organic matter [17]. Hydrolysis is a rate-limiting step in FW degradation [18]; therefore, efficient waste pretreatment, using methods such as thermal, chemical, thermal/chemical, ultrasound, and enzyme treatments, is necessary to enhance biodegradability. As bioconversion generally needs pH adjustment by increasing alkalinity and is highly adequate for enriching basophilic bacteria capable of producing hydrogen, alkaline pretreatment could be used as an efficient pretreatment method [19]. Moreover, alkali addition could be a suitable alternative to counter temporary inhibitory conditions related to the accumulation of volatile fatty acids or free ammonia in bioprocesses [20]. Optimum substrate conditions for an alkali-treated FW and SS mixture for continuous hydrogen production need further investigation.

Therefore, this study investigates the effects of alkali pretreatment on FW and SS to enhance biodegradability, the hydrogen production potential from alkali-treated FW and SS in batch tests, and the optimum substrate conditions by testing various volatile solid (VS) concentrations (1.5–5%) and FW to SS mixing ratios (0:100–100:0) for increased hydrogen generation.

2. Material and Methods

2.1. Preparation of Alkali-Treated Feedstocks

FW and SS were feedstocks for DF. FW was ground using an electrical blender, and SS was obtained from a thickener in a publicly owned treatment work. The substrate was kept in a refrigerator until use and the characteristics are shown in Table 1. Batch kinetic tests were conducted for the two waste streams to establish the optimum pH and reaction time for the alkaline pretreatment. NaOH is known to enhance the solubility of the components of a mixture [21,22]; however, to eliminate the inhibition effect of sodium on DF, KOH (Daejung, Korea) was used instead in this study.

Table 1. Characteristics of food waste and sewage sludge.

Parameter	Unit	Food Waste	Sewage Sludge
Total solids	%	15.9	1.5
Volatile solids	%	15.2	1.2
Total COD	g/L	158.4	19.5
Total carbohydrate	g COD/L	84.9	2.0
Total protein	g COD/L	37.7	8.7
Total Kjeldahl nitrogen	g N/L	4.4	1.0
pH		4.6	7.5

Prior to the hydrogen fermentation batch test, FW and SS were treated with a KOH solution of 6 N at various pH (11.5, 12.0, 13.0, and 13.5) and a constant room temperature (35 °C) for better solubilization and disinfection.

The following batch tests using an alkali-treated mixture of FW and SS were performed under various VS (1.5–5%) and FW to SS mixing ratios (0:100–100:0) conditions. WHEATON® Media 415 mL lab bottles (Wheaton, IL, USA) were used for the batch test of DF hydrogen production. Seed sludge was prepared from an anaerobic digester in the publicly owned treatment work and it was heated at 90 °C for 30 min to select spore-forming bacteria [23]. Subsequently, the seed sludge (40 mL) was added to a serum bottle containing the substrates, and each bottle contained 200 mg KH₂PO₄, 14 mg MgCl₂·4H₂O, 2.5 mg MnCl₂·6H₂O, 2 mg CaCl₂·2H₂O, 2 mg Na₂MoO₄·4H₂O, and 10 mg FeCl₂·4H₂O. The total carbohydrate to alkalinity ratio was set at 1.0 ± 0.1 by adding NaHCO₃. After adding the substrate solution and the seed sludge, the bottles were filled with 200 mL of distilled water. pH was controlled to 6.0 using 1 M HCl (Daejung, Korea) and 1 M KOH. N₂ gas was flushed to the headspaces of the serum bottles, and then we sealed the bottles using screw caps with rubber septa. We incubated the bottles after placing them in a shaker at 35 °C and 100 rpm. Using a glass syringe, we measured the amount of biogas production, and biogas composition was simultaneously determined by a gas chromatograph. The supernatant samples were analyzed for pH and organic concentrations. Throughout the cultivation period, pH was controlled to be in the range of 5.0–6.0 by injecting either 1 M HCl or 1 M KOH. All the experiments were conducted in duplicate. Unless otherwise specified, all the chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Batch Assay

Alkali-based solubilization of FW and SS was calculated in terms of soluble chemical oxygen demand (COD) using Equation (1).

$$COD \text{ solubilization, \%} = \frac{SCOD_t - SCOD_i}{TCOD_i - SCOD_i} \times 100 \quad (1)$$

where $TCOD_i$ is the initial total COD and $SCOD_t$ and $SCOD_i$ are the final and initial soluble CODs, respectively.

The disinfection effect was evaluated using the spread plate technique. After dilution, samples were individually obtained and spread over an agar surface. For this purpose, anaerobic agar of Wilkins–Chalgren (Oxoid company, Basingstoke, UK), with the gelatin peptone of 10 g/L, the tryptone of 10.0 g/L, the yeast extract of 5.0 g/L, the sodium pyruvate of 1.0 g/L, the hemin of 0.005 g/L, the menadione of 0.0005 g/L, and the agar of 10.0 g/L, was used. We incubated the prepared agar plates at 37 °C. After inoculation, the number of colony-forming units was monitored directly from the agar plates.

To describe the hydrogen production process, hydrogen production trends were regressed based on the modified Gompertz equation (Equation (2)) [24].

$$H(t) = P \cdot \exp \left\{ - \exp \left[\frac{R_m \cdot e}{P} (\lambda - t) + 1 \right] \right\} \quad (2)$$

where P (mL) means the ultimate hydrogen production, R_m (mL/day) means the hydrogen production rate, λ (days) means the lag-phase time, H (mL) means the cumulative hydrogen production, and e stands for exponential 1.

The produced hydrogen volume was quantified based on the analyzed gas composition and the total biogas volume produced using the below mass balance equation, at each time interval (Equation (3)).

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (3)$$

where $V_{H,i}$ represent the total cumulative volume of hydrogen gas at the current (i) and $V_{H,i-1}$ represent total cumulative volume of hydrogen gas at the previous ($i - 1$) time intervals, respectively; $V_{G,i}$ represent the total gas volume generated at the current and $V_{G,i-1}$ is the total gas volume generated at the previous time intervals, respectively; $C_{H,i}$ indicates hydrogen fractions in the bottle headspace at the current interval and $C_{H,i-1}$ indicates that of previous intervals, respectively; and V_H is the total volume of the bottle's headspace.

To provide better process design and control, this study adapted response surface methodology which can build a mathematical model. Hydrogen production according to the effects of VS concentrations and FW to SS mixing ratios could be estimated using the below quadratic model (Equation (4)):

$$Y = \beta_0 + \beta_1x + \beta_2y + \beta_{11}x^2 + \beta_{22}y^2 + \beta_{12}xy \quad (4)$$

where x and y are the independent variables, Y is the dependent variable (the predicted response), β_0 , β_1 , β_2 , β_{11} , β_{22} , and β_{12} are the coefficient of each term explaining the overall contribution.

2.3. Analytical Methods

Gas volumes generated were measured using the water displacement method and hydrogen composition was determined by using GOW-MAC Series 580 gas chromatograph (GC) equipped with a thermal conductivity detector. We used a 1.8 m \times 3.2 mm stainless-steel column packed with molecular sieve 5A. The used carrier gas was N_2 . The contents of CO_2 , CH_4 , and N_2 were determined by using the same GC equipped with a 1.8 m \times 3.2 mm stainless-steel column packed with porapak Q (80/100 mesh). The used carrier gas was helium.

Volatile fatty acids were determined by using a high-performance liquid chromatograph (HPLC) (SpectraSYSTEM P2000, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an ultraviolet (210 nm) detector. The used column was a 300 m \times 7.8 mm Aminex HPX-97H. All the samples were pre-treatment with a 0.45 μ m membrane filter. The applied mobile phase was a 0.005 M sulfuric acid at a flow rate of 0.6 mL/min. We determined the contents of alcohols by using an IC (DX-600 Ion Chromatography System, Dionex, CA, USA). It was equipped with an ED 50A electrochemical detector and used a 250 mm \times 4 mm Dionex CarboPac PA10 column after pretreatment with a 0.45 μ m membrane filter.

The contents of VS, volatile suspended solid (VSS), COD, total Kjeldahl nitrogen (TKN), and ammonia were analyzed following standard methods [25]. Using the Dubois method [26], we determined the contents of total/soluble carbohydrates. Based on the prescribed relationship, total protein content was calculated (9.375 g COD/g protein) [27], and soluble protein content was analyzed by using a Lowry method [28].

3. Results and Discussion

3.1. Effect of Alkaline Treatment on Solubilization and Disinfection

Four initial pH values, 11.5, 12.0, 13.0, and 13.5, were assessed to establish an optimum alkaline treatment condition for the solubilization of sludge. KOH amounts of 60.1 meq/L (pH 11.5), 74.5 meq/L (pH 12.0), 120 meq/L (pH 13.0), and 312.3 meq/L (pH 13.5) were

added to adjust the initial pH values. As shown in Figure 1, a higher initial pH value resulted in higher COD solubilization and less pH drop. The COD solubilization increased to 7.8, 29.4, 46.6, and 63.4% at the initial pH values of 11.5, 12.0, 13.0, and 13.5, respectively. However, the soluble to total carbohydrate ratio decreased remarkably at the initial pH of 13.5. As carbohydrate is one of the preferred carbon sources for fermentative hydrogen production [18], pH 13.0 was regarded as the optimum alkaline treatment condition of SS for hydrogen production. This was also validated by the higher soluble to total protein ratio at the initial pH of 13.0 than that at 13.5, given that protein is one of the important sources for a balanced carbohydrate to protein ratio, which increases hydrogen production [1,2]. Moreover, in the case of FW (Figure 2), the experiment was performed only at the initial pH of 13.0 with the addition of 264.3 meq KOH/L. The pH was maintained above 12.5 during the experiment. Results showed that the COD solubilization increased to 31.4%, which was less than that for SS.

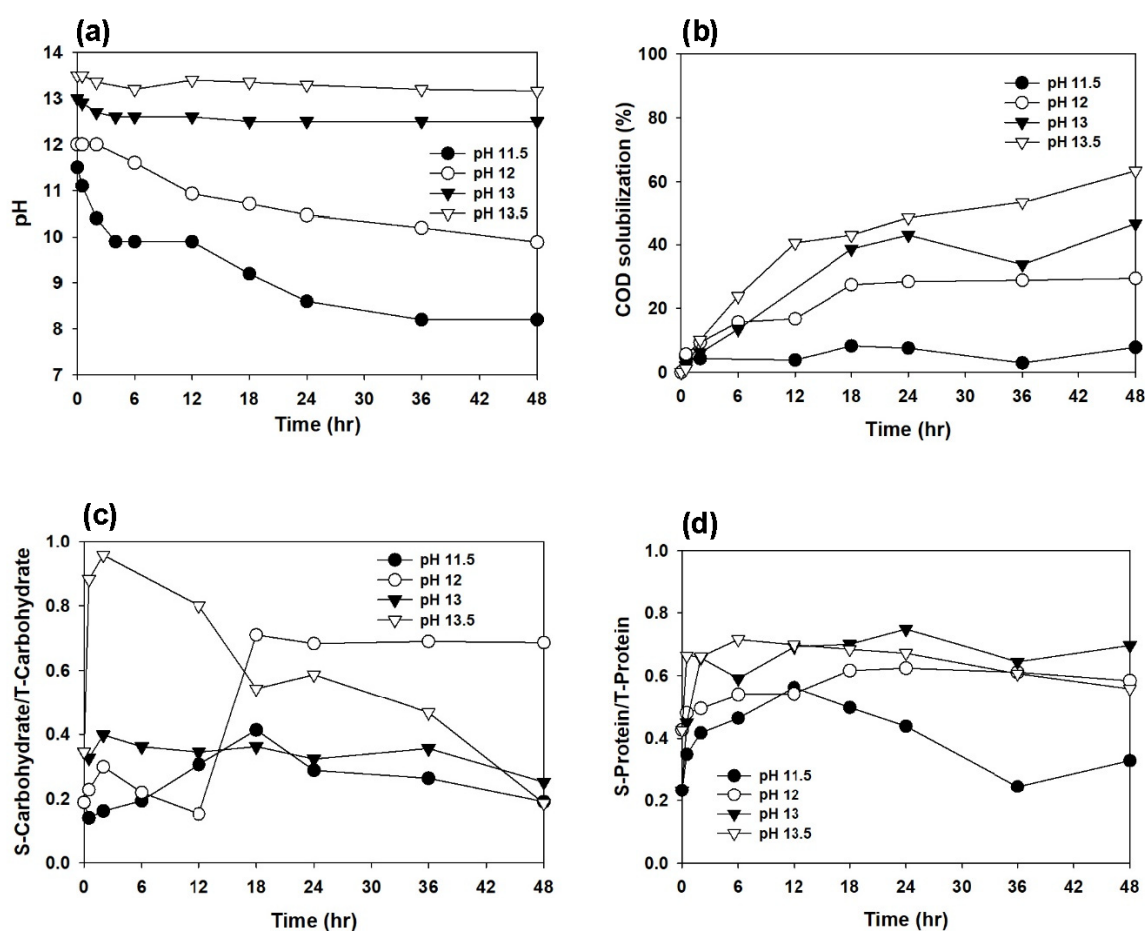


Figure 1. Solubilization of sewage sludge: (a) pH variation, (b) COD solubilization, (c) soluble carbohydrate/total carbohydrate, and (d) soluble protein/total protein.

As shown in Figure 3, alkaline treatment was also effective in disinfecting SS and FW. The bacterial counts of SS and FW dropped rapidly at the beginning of the reactions with KOH. Subsequently, the bacterial counts of alkaline-treated SS and FW decreased at approximately 3.5-log and beyond 7.8-log (CFU, colony-forming unit), respectively, after 48 h. As *Clostridia* species are known to produce spores to survive in extreme environmental conditions [23], alkaline conditions successfully inactivate non-spore-forming bacteria thus the selection of spore-forming bacteria (such as H₂-producing *Clostridia* species) is possible.

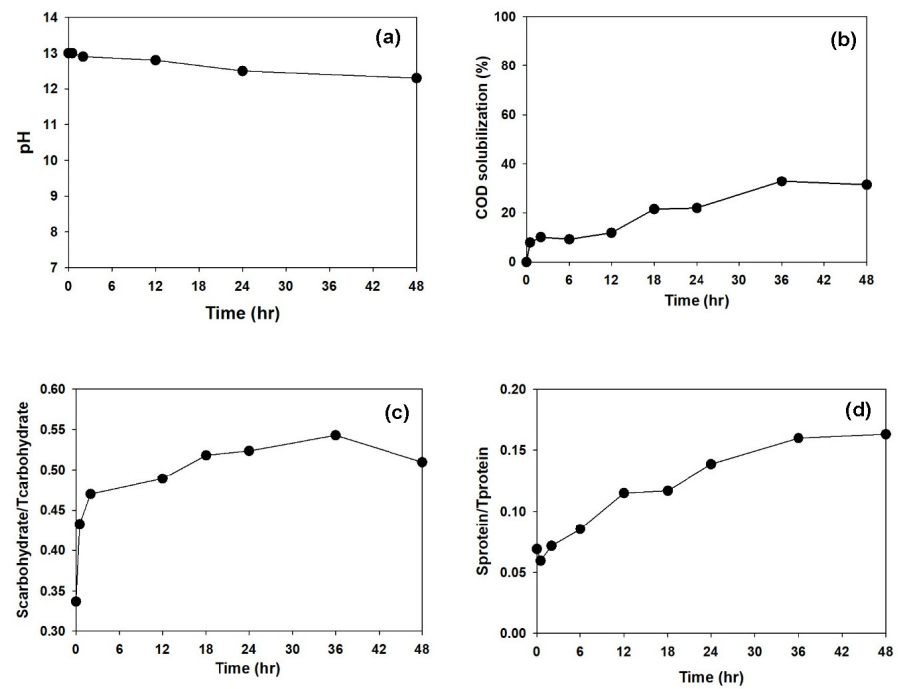


Figure 2. Solubilization of food waste: (a) pH variation, (b) COD solubilization, (c) soluble carbohydrate/total carbohydrate, and (d) soluble protein/total protein.

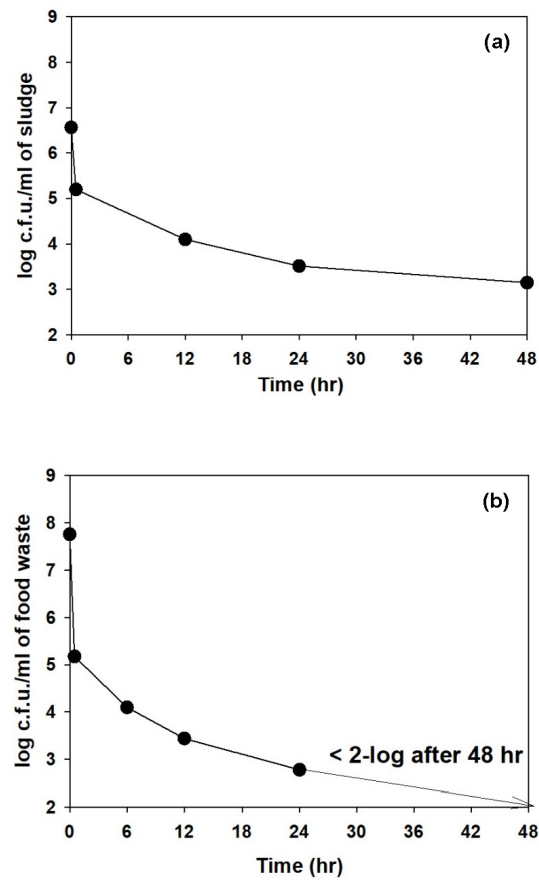


Figure 3. Effect of disinfection at pH 13: (a) Sewage sludge, (b) food waste.

3.2. Enhanced Fermentation of Alkaline-Treated FW and SS for Hydrogen Production

Batch tests of 17 different conditions were performed to determine the optimum VS concentration and FW to SS mixing ratio (Table 2). The trends of cumulative hydrogen production curves were regressed by applying the modified Gompertz equation [24]. Table 2 summarizes the obtained kinetic parameters of the overall hydrogen production potential (P_s) and the specific hydrogen production rate (R_s). For all the experiments, we found that the correlation coefficient R^2 was greater than 0.99 (Table 2). Possibly due to the high percentage of energy-carrying molecules such as polysaccharides, proteins, and lipids, SS must be an abundant source for sustainable energy production. Compared to carbohydrate-rich substrates, however, SS exhibits a lower potential for hydrogen production [29,30]. Our results conform to these observations. When SS (FW:MSW = 0:100) was the sole substrate, hydrogen production was not observed (Table 2).

Table 2. Kinetic parameters of hydrogen production.

VS (%)	FW:MSW (VS Basis)	Carbohydrate (g/L)	Protein (g/L)	R_m (mL/Day)	P (mL H ₂)	λ (Days)	R_s (mL/g VSS/h)	P_s (mL/g VS)	R^2
1.5	100:0	12.9	3.4	844.7	296.0	0.43	113.8	98.7	0.999
1.5	80:20	10.9	5.1	590.4	396.8	0.23	79.5	132.3	0.994
1.5	60:40	8.8	6.5	715.3	172.2	0.29	96.3	57.4	0.999
1.5	40:60	6.7	8.1	453.9	92.4	0.29	61.1	30.8	0.999
2	100:0	16.5	4.9	805.9	634.8	0.40	108.6	158.7	0.997
2	80:20	14.5	6.8	807.1	552.9	0.28	108.7	138.2	0.997
2	60:40	12.4	8.7	871.2	390.2	0.27	117.4	97.6	0.999
2	40:60	9.0	10.8	582.8	130.5	0.29	78.5	32.6	0.999
2	0:100	3.5	13.7	-	-	-	-	-	-
3	100:0	24.8	7.4	717.6	1004.6	0.20	96.7	167.3	0.996
3	80:20	21.7	10.2	804.0	629.3	0.20	108.3	104.9	0.997
3	60:40	22.8	12.7	1075.2	337.8	0.34	150.0	56.3	0.998
3	40:60	13.5	16.1	840.4	215.2	0.29	113.2	35.9	0.999
3	0:100	5.3	20.5	-	-	-	-	-	-
5	100:0	57.5	12.3	964.8	1039.8	0.42	134.6	104.0	0.999
5	80:20	47.8	16.7	1320.0	827.7	0.43	184.2	82.8	0.997
5	60:40	38.1	21.1	1058.4	542.2	0.35	147.7	54.2	0.998

R_s (H₂ mL/g VSS/h) obtained from the regression can systematically evaluate the relationship between FW composition (x) and VS concentration (y), and the following equation (Equation (5)) was generated by using Equation (4):

$$R_s = -64.9237 + 3.5378x + 25.1458y - 0.014x^2 + 0.3149y^2 - 0.1363xy \quad (5)$$

$(R^2 = 0.89, p < 0.0001)$

Analysis of the variance (ANOVA) is tabulated in Table 3. The calculated Fisher value ($F = 18.22$) and a probability value (p) of <0.0001 in ANOVA demonstrate that the regression model was significant. Generally, if the p value < 0.05 , it indicates that the obtained parameter is statistically significant, which means the suggested equation may accurately predict the hydrogen production rate. It was observed from Equation (5) that FW composition ($p = 0.0002$) was the most significant parameter of the model. FW composition must have a complicated effect on the hydrogen production rate since the square term of FW composition also shows statistical significance ($p = 0.0006$). However, VS concentration presents relatively low significance ($p > 0.05$) implying little impact on the hydrogen production rate [13].

Table 3. Analysis of variance for hydrogen production rates.

	DF ⁽¹⁾	SS ⁽²⁾	MS ⁽³⁾	F	<i>p</i>
Regression	5	32,435.61	6487.123	18.2253	<0.0001
Residual	11	3915.343	355.9402		
Total	16	36,350.96	2271.935		

⁽¹⁾ the degrees of freedom; ⁽²⁾ the sum of squares; ⁽³⁾ the mean sum of squares.

The maximum R_s was 163.8 mL H₂/g VSS/h at a VS concentration of 5.0% and FW composition of 62.5% (Figure 4). We found that the addition of alkaline-treated SS increased the R_s . In some cases, a high R_s of 147.4 mL H₂/g VSS/h (i.e., 90% of the maximum R_s) was achieved when a higher composition of SS than that of FW was used. The maximum rate in this study was 1.47 times higher than that in previous research using similar SS and FW without any pretreatment [13]. These results indicate that alkali pretreatment is a suitable option to increase biodegradability and supply temporary inhibitory conditions for hydrogen-producing bacteria against other types of bacteria [20]. Additionally, the SS, a protein-rich waste, was beneficial in increasing the performance of DF. Optimum nitrogen sources and some inorganic materials from alkaline-treated SS could result in a higher metabolic rate, resulting in higher R_s [16,31]. In this study, the optimum C/N ratio was 1.8 g carbohydrate-COD/g protein-COD, and the C/N ratios of FW and SS were 2.25 and 0.23 g carbohydrate-COD/g protein-COD, respectively.

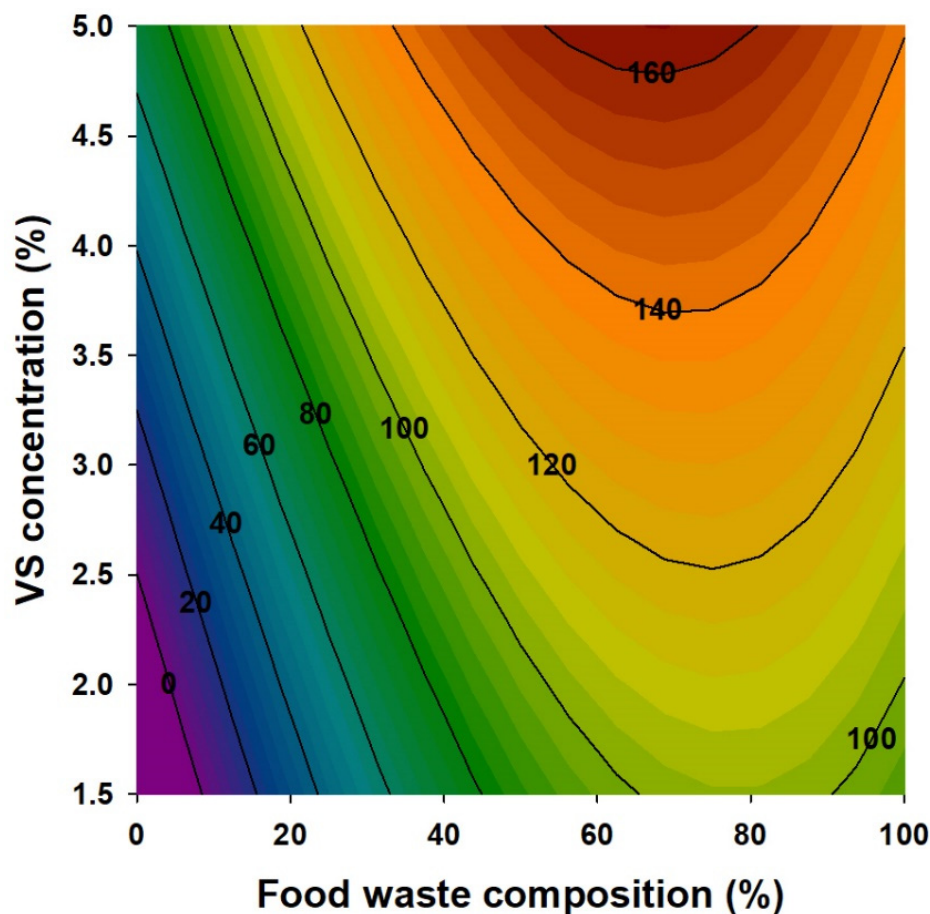


Figure 4. Contour lines of specific hydrogen production rate (R_s , mL H₂/g VSS/h) against volatile solid (VS) concentration and food waste composition.

Unlike R_s , P_s (mL H₂/g VS) increased as FW composition increased. The obtained P_s were subjected to response surface analysis, and the following equation (Equation (6)) was generated:

$$P_s = -59.6055 + 40.6782x + 1.1555y - 7.2369x^2 + 0.0042y^2 - 0.0108xy \quad (6)$$

($R^2 = 0.87, p = 0.0002 < 0.05$)

Figure 5 illustrates P_s contour lines and the ANOVA analysis is shown in Table 4. In particular, SS exhibited relatively low hydrogen production potential due to low carbohydrate content. When SS content was higher than FW content, hydrogen production potential was 0–35.9 mL H₂/g VS (Table 2).

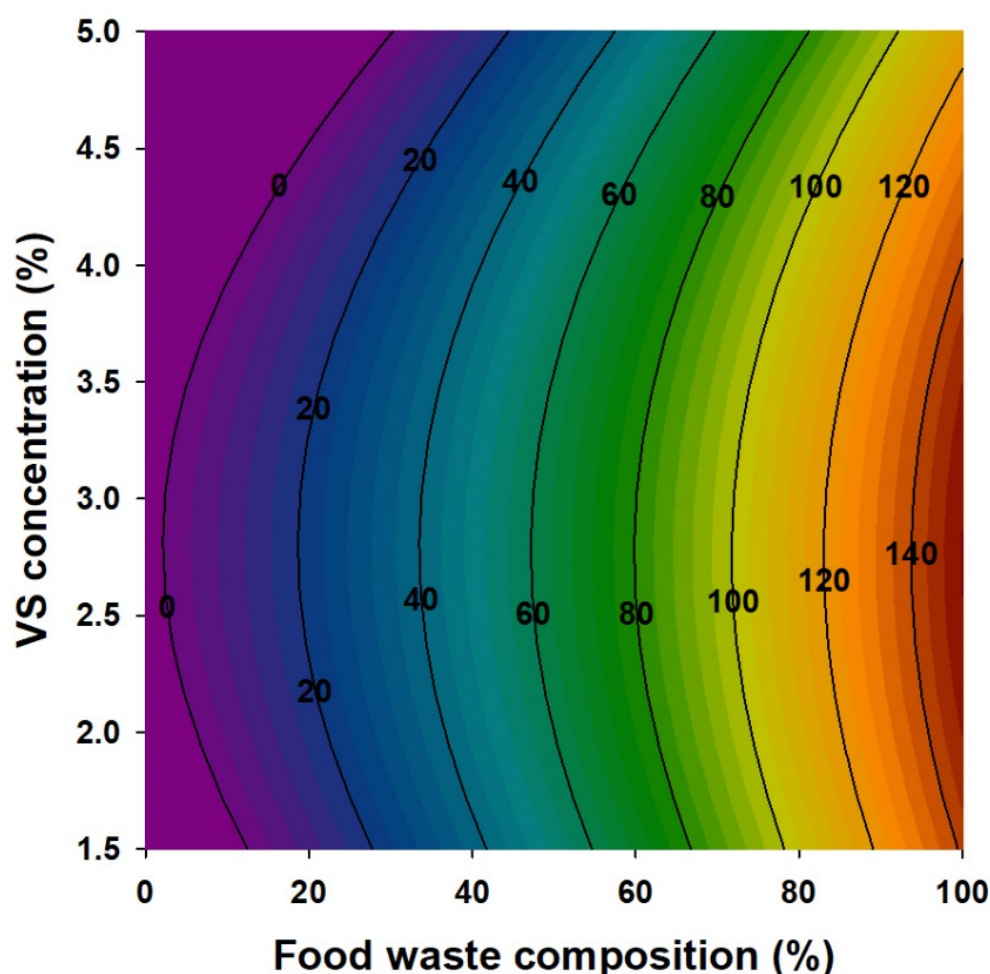


Figure 5. Contour lines of hydrogen production potential (P_s , mL H₂/g VS) against food waste composition and volatile solid (VS) concentration.

Table 4. Analysis of variance for hydrogen production potential.

	DF	SS	MS	F	<i>p</i>
Regression	5	37,269.96	7453.993	14.4392	0.0002
Residual	11	5678.557	516.2325		
Total	16	42,948.52	2684.283		

P_s increased as FW composition increased owing to the high carbohydrate content and the easily hydrolyzable nature of this waste [3]. Maximum P_s was 152.1 mL H₂/g VS at VS concentration of 2.8% and FW composition of 100%, which was higher than those

reported in most previous studies that employed serum bottle tests and organic wastes (40.0–122.9 mL H₂/g VS) [13,31–33].

As previously reported, hydrogen production from alkaline-treated SS as a sole substrate is challenging [10,19]. By contrast, in this study, alkaline-treated FW as the sole substrate proved efficient for hydrogen production. Carbohydrate from FW was considered as the main carbon source for hydrogen production. Figure 6 shows the contour lines of hydrogen production per carbohydrate concentration (COD basis) of FW; a decrease in hydrogen production (solid line) occurred at high carbohydrate concentration (dashed line). The following expression for the hydrogen production per carbohydrate concentration of FW was derived and the model equation presents sufficient statistical significance based on ANOVA (Table 5):

$$\begin{aligned} & \text{(Hydrogen production based on FW carbohydrate}_{\text{added}}) \\ & = -78.3981 + 3.4399x + 62.9690y - 0.0160x^2 - 12.9229y^2 - 0.0047xy \quad (7) \\ & (R^2 = 0.78, p = 0.0021 < 0.05) \end{aligned}$$

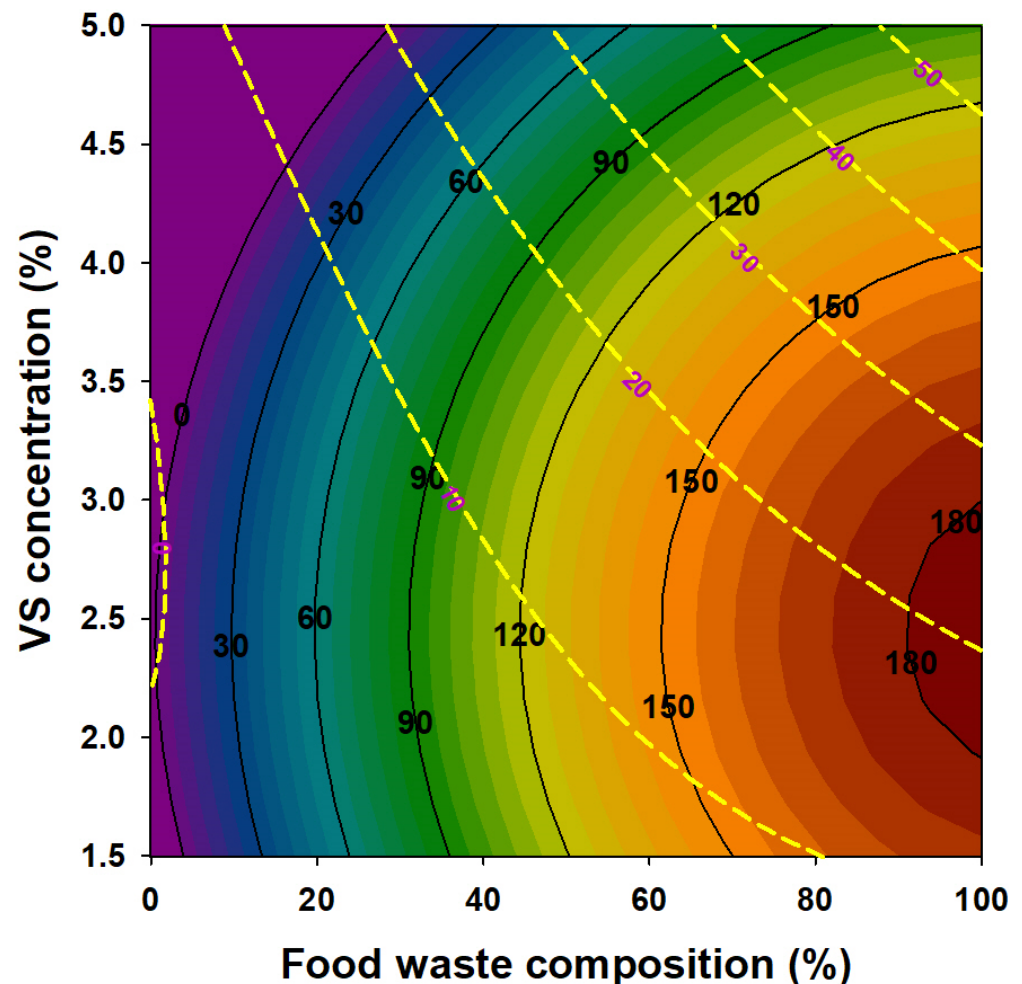


Figure 6. Contour lines of hydrogen production based on food waste carbohydrate_{added} (mL H₂/g food waste carbohydrate_{added}) against food waste composition and volatile solid (VS) concentration (solid line); contour lines of carbohydrate_{added} concentration (COD basis) against food waste composition and VS concentration (g carbohydrate-COD/L) (dashed line).

Table 5. Analysis of variance for hydrogen production based on food waste carbohydrate_{added}.

	DF	SS	MS	F	p
Regression	5	54,520.2	10,904.04	7.9881	0.0021
Residual	11	15,015.39	1365.035		
Total	16	69,535.59	4345.974		

The decrease in hydrogen production (solid line) at high carbohydrate concentration (dashed line) shows that an extremely high carbohydrate concentration inhibited hydrogen production. Hydrogen production at high feedstock concentration would be economical. However, problems may exist with product and/or substrate inhibition at these high concentrations. Substrate concentrations higher than the optimum concentration may result in inhibitory effects through a drop in pH, production of acids, or increase in hydrogen partial pressures [34]. Additionally, high substrate concentrations may cause substrate inhibition of microbial activity [35].

4. Conclusions

In this study, experiments were performed to evaluate the optimum substrate condition for DF and potential hydrogen production from alkali-pretreated FW and SS at various VS concentrations and FW to SS mixing ratios. The biodegradability of FW and SS was enhanced after pretreatment with KOH.

Among various initial pH conditions, the initial pH of 13.0 exhibited the maximum soluble carbohydrate and protein concentrations of 69 and 70%, respectively. Therefore, the initial pH of 13.0 was selected as the optimum alkali pretreatment condition for hydrogen production. Furthermore, the bacterial counts of FW and SS decreased at approximately 3.50-log and beyond 7.80-log (CFU), respectively, indicating that alkaline pretreatment was effective for disinfection and solubilization of organic wastes.

Empirical equations for hydrogen production were obtained through response surface methodology. The maximum R_s of 163.8 mL H₂/g VSS/h was obtained at a VS concentration of 5.0% and FW composition of 62.5% (37.5% of SS). We established that the addition of SS to FW could enhance R_s due to a balanced carbohydrate to protein ratio, resulting in a higher hydrogen production rate, with maximum P_s of 152.1 mL H₂/g VS at a VS concentration of 2.8% and FW composition of 100%.

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

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

References

- van der Spek, M.; Banet, C.; Bauer, C.; Gabrielli, P.; Goldthorpe, W.; Mazzotti, M.; Munkejord, S.T.; Røkke, N.A.; Shah, N.; Sunny, N. Perspective on the hydrogen economy as a pathway to reach net-zero CO₂ emissions in Europe. *Energy Environ. Sci.* **2022**, *15*, 1034–1077. [[CrossRef](#)]
- Moreno-Andrade, I.; Berrocal-Bravo, M.J.; Valdez-Vazquez, I. Biohydrogen production from food waste and waste activated sludge in codigestion: Influence of organic loading rate and changes in microbial community. *J. Chem. Technol. Biotechnol.* **2023**, *98*, 230–237. [[CrossRef](#)]
- Yun, Y.-M.; Lee, M.-K.; Im, S.-W.; Marone, A.; Trably, E.; Shin, S.-R.; Kim, M.-G.; Cho, S.-K.; Kim, D.-H. Biohydrogen production from food waste: Current status, limitations, and future perspectives. *Bioresour. Technol.* **2018**, *248*, 79–87. [[CrossRef](#)] [[PubMed](#)]
- Megía, P.J.; Vizcaíno, A.J.; Calles, J.A.; Carrero, A. Hydrogen Production Technologies: From Fossil Fuels toward Renewable Sources. A Mini Review. *Energy Fuels* **2021**, *35*, 16403–16415. [[CrossRef](#)]
- Ewan, B.C.R.; Allen, R.W.K. A figure of merit assessment of the routes to hydrogen. *Int. J. Hydrogen Energy* **2005**, *30*, 809–819. [[CrossRef](#)]

6. Qyyum, M.A.; Ismail, S.; Ni, S.-Q.; Ihsanullah, I.; Ahmad, R.; Khan, A.; Tawfik, A.; Nizami, A.-S.; Lee, M. Harvesting biohydrogen from industrial wastewater: Production potential, pilot-scale bioreactors, commercialization status, techno-economics, and policy analysis. *J. Clean. Prod.* **2022**, *340*, 130809. [[CrossRef](#)]
7. Yaashikaa, P.R.; Keerthana Devi, M.; Senthil Kumar, P. Biohydrogen production: An outlook on methods, constraints, economic analysis and future prospect. *Int. J. Hydrogen Energy* **2022**, *47*, 41488–41506. [[CrossRef](#)]
8. Akhlaghi, N.; Najafpour-Darzi, G. A comprehensive review on biological hydrogen production. *Int. J. Hydrogen Energy* **2020**, *45*, 22492–22512. [[CrossRef](#)]
9. Balachandar, G.; Khanna, N.; Das, D. Chapter 6—Biohydrogen Production from Organic Wastes by Dark Fermentation. In *Biohydrogen*; Pandey, A., Chang, J.-S., Hallenbecka, P.C., Larroche, C., Eds.; Elsevier: Amsterdam, The Netherlands, 2013; pp. 103–144.
10. El-Qelish, M.; Chatterjee, P.; Dessì, P.; Kokko, M.; El-Gohary, F.; Abo-Aly, M.; Rintala, J. Bio-hydrogen Production from Sewage Sludge: Screening for Pretreatments and Semi-continuous Reactor Operation. *Waste Biomass Valorization* **2020**, *11*, 4225–4234. [[CrossRef](#)]
11. Kim, M.; Yang, Y.; Morikawa-Sakura, M.S.; Wang, Q.; Lee, M.V.; Lee, D.-Y.; Feng, C.; Zhou, Y.; Zhang, Z. Hydrogen production by anaerobic co-digestion of rice straw and sewage sludge. *Int. J. Hydrogen Energy* **2012**, *37*, 3142–3149. [[CrossRef](#)]
12. Bundhoo, M.A.Z.; Mohee, R. Inhibition of dark fermentative bio-hydrogen production: A review. *Int. J. Hydrogen Energy* **2016**, *41*, 6713–6733. [[CrossRef](#)]
13. Kim, S.-H.; Han, S.-K.; Shin, H.-S. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *Int. J. Hydrogen Energy* **2004**, *29*, 1607–1616. [[CrossRef](#)]
14. Lertsriwong, S.; Glinwong, C. Newly-isolated hydrogen-producing bacteria and biohydrogen production by *Bacillus coagulans* MO11 and *Clostridium beijerinckii* CN on molasses and agricultural wastewater. *Int. J. Hydrogen Energy* **2020**, *45*, 26812–26821. [[CrossRef](#)]
15. Tian, H.; Li, J.; Yan, M.; Tong, Y.W.; Wang, C.-H.; Wang, X. Organic waste to biohydrogen: A critical review from technological development and environmental impact analysis perspective. *Appl. Energy* **2019**, *256*, 113961. [[CrossRef](#)]
16. Yang, G.; Wang, J. Biohydrogen production by co-fermentation of sewage sludge and grass residue: Effect of various substrate concentrations. *Fuel* **2019**, *237*, 1203–1208. [[CrossRef](#)]
17. Barber, W.P.F. Thermal hydrolysis for sewage treatment: A critical review. *Water Res.* **2016**, *104*, 53–71. [[CrossRef](#)]
18. Han, W.; Yan, Y.; Shi, Y.; Gu, J.; Tang, J.; Zhao, H. Biohydrogen production from enzymatic hydrolysis of food waste in batch and continuous systems. *Sci. Rep.* **2016**, *6*, 38395. [[CrossRef](#)]
19. Yao, Z.; Su, W.; Wu, D.; Tang, J.; Wu, W.; Liu, J.; Han, W. A state-of-the-art review of biohydrogen producing from sewage sludge. *Int. J. Energy Res.* **2018**, *42*, 4301–4312. [[CrossRef](#)]
20. Cesaro, A.; Belgiorno, V. Pretreatment methods to improve anaerobic biodegradability of organic municipal solid waste fractions. *Chem. Eng. J.* **2014**, *240*, 24–37. [[CrossRef](#)]
21. Ahmed, B.; Kumar Tyagi, V.; Kazmi, A.A.; Khursheed, A. New insights into thermal-chemical pretreatment of organic fraction of municipal solid waste: Solubilization effects, recalcitrant formation, biogas yield and energy efficiency. *Fuel* **2022**, *319*, 123725. [[CrossRef](#)]
22. Mozhiarasi, V. Overview of pretreatment technologies on vegetable, fruit and flower market wastes disintegration and bioenergy potential: Indian scenario. *Chemosphere* **2022**, *288*, 132604. [[CrossRef](#)] [[PubMed](#)]
23. Sinbuathong, N.; Charnnok, B.; Sillapacharoenkul, B.; Sakdaronnarong, C. Preparation of active hydrogen-producing cultures from palm oil mill sludge for biohydrogen production system. *Int. J. Hydrogen Energy* **2021**, *46*, 29100–29107. [[CrossRef](#)]
24. Mahata, C.; Dhar, S.; Ray, S.; Das, D. Effect of thermal pretreated organic wastes on the dark fermentative hydrogen production using mixed microbial consortia. *Fuel* **2021**, *284*, 119062. [[CrossRef](#)]
25. Rice, E.W.; Baird, R.B.; Eaton, A.D.; Clesceri, L.S. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2012.
26. Dubois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. A colorimetric method for the determination of sugars. *Nature* **1951**, *168*, 4265. [[CrossRef](#)] [[PubMed](#)]
27. Miron, Y.; Zeeman, G.; van Lier, J.B.; Lettinga, G. The role of sludge retention time in the hydrolysis and acidification of lipids, carbohydrates and proteins during digestion of primary sludge in CSTR systems. *Water Res.* **2000**, *34*, 1705–1713. [[CrossRef](#)]
28. Brown, M.J.; Lester, J.N. Comparison of bacterial extracellular polymer extraction methods. *Appl. Environ. Microbiol.* **1980**, *40*, 179–185. [[CrossRef](#)]
29. Guo, L.; Lu, M.; Li, Q.; Zhang, J.; She, Z. A comparison of different pretreatments on hydrogen fermentation from waste sludge by fluorescence excitation-emission matrix with regional integration analysis. *Int. J. Hydrogen Energy* **2015**, *40*, 197–208. [[CrossRef](#)]
30. Xiao, B.; Liu, J. Biological hydrogen production from sterilized sewage sludge by anaerobic self-fermentation. *J. Hazard. Mater.* **2009**, *168*, 163–167. [[CrossRef](#)]
31. Shen, M.-Y.; Torre, M.; Chu, C.-Y.; Tratzl, P.; Carnevale, M.; Gallucci, F.; Paolini, V.; Petracchini, F. Green biohydrogen production in a Co-digestion process from mixture of high carbohydrate food waste and cattle/chicken manure digestate. *Int. J. Hydrogen Energy* **2022**, *47*, 40696–40703. [[CrossRef](#)]
32. Wang, W.; Xie, L.; Chen, J.; Luo, G.; Zhou, Q. Biohydrogen and methane production by co-digestion of cassava stillage and excess sludge under thermophilic condition. *Bioresour. Technol.* **2011**, *102*, 3833–3839. [[CrossRef](#)]

33. Zhao, M.; Liu, Z.; Xu, J.; Liu, H.; Dai, X.; Gu, S.; Ruan, W. Dosing effect of nano zero valent iron (NZVI) on the dark hydrogen fermentation performance via lake algae and food waste co-digestion. *Energy Rep.* **2020**, *6*, 3192–3199. [[CrossRef](#)]
34. Eker, S.; Sarp, M. Hydrogen gas production from waste paper by dark fermentation: Effects of initial substrate and biomass concentrations. *Int. J. Hydrogen Energy* **2017**, *42*, 2562–2568. [[CrossRef](#)]
35. Bundhoo, M.A.Z.; Mohee, R.; Hassan, M.A. Effects of pre-treatment technologies on dark fermentative biohydrogen production: A review. *J. Environ. Manag.* **2015**, *157*, 20–48. [[CrossRef](#)] [[PubMed](#)]

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