Abstract: Biohydrogen production from organic solid waste has shown particular advantages over other methods owing to the combination of waste reduction and bioenergy production. In this study, biohydrogen production from the co-digestion of cattle manure and food waste was optimized in a mesophilic semi-continuous process. To maximize hydrogen production, the effects of the mixing ratio (the proportion of food waste in the substrate), substrate concentration, and hydraulic retention time (HRT) on the co-digestion were systematically analyzed using a Box–Behnken design. The results showed that strong interactive effects existed between the three factors, and they had a direct effect on the responses. Hydrogen was primarily produced via the butyrate pathway, which was accompanied by the competing heterolactic fermentation pathway. Propionate and valerate produced from lipids and proteins, respectively, were obtained along with butyrate. The optimal process parameters included a mixing ratio of 47% to 51%, a substrate concentration of 76 to 86 g L⁻¹, and an HRT of 2 d. Under these optimal conditions, the hydrogen production rate and hydrogen yield were higher than 1.00 L L⁻¹ d⁻¹ and 30.00 mL g⁻¹ VS, respectively, and the predicted results were consistent with the experimental data. The results indicate that the co-digestion of cattle manure and food waste is a practical and economically promising approach for biohydrogen production.

Keywords: biohydrogen; cattle manure; co-digestion; food waste; response surface methodology
equilibrium can be disturbed by the accumulation of volatile fatty acids (VFAs), high ammonia nitrogen concentrations, or trace elements shortage [15–17]. To maintain optimal fermentation conditions, a process adjustment method by which exogenous chemical regulators are added to the substrate has often been applied under lab-scale conditions [18]. However, this approach may not be feasible for full-scale application [19]. Recently, the co-digestion process, which is performed by adding complementary materials to the substrate, has attracted attention because it offers the advantages of better buffer conditions, more balanced nutrients, and the dilution of inhibitors. The co-digestion process has been considered as an economical and feasible approach that can be employed to improve the yield and stability of the hydrogen production process [12,20–23].

For example, cattle manure is rich in alkali and nutrients, and it has been reported to be suitable for co-digestion with carbohydrate-rich and rapidly degradable substrates, such as food waste [24,25]. However, studies on the co-digestion of cattle manure and food waste have been primarily focused on biomethane production [26–29]. Less attention has been given to biohydrogen production via the co-digestion of food waste and cattle manure; thus, available data in this regard are still limited [30]. Compared with methane, hydrogen is a carbon-free clean fuel, and it can be directly used in fuel cells for producing electricity and in the chemical industry for producing fertilizers. Thus, hydrogen has been considered to be the base of future sustainable energy systems [2,8]. However, different from biomethane processes where the VFAs are consumed by acetogenic bacteria and methanogens, dark fermentation processes are unstable because of the continuous production of VFAs [13,14]. For continuous co-digestion application, the equilibrium of biohydrogen production is typically influenced by the mixing ratio as well as by other direct influencing factors, such as the substrate concentration and the hydraulic retention time (HRT). Furthermore, there may be interactions among these factors, and limited information is available on the effect of such factors on the continuous co-digestion process, as related systematic studies are scarce [31–33].

The objective of this work was to investigate the individual and interactive effects of the mixing ratio, substrate concentration, and HRT on the mesophilic semi-continuous co-digestion of cattle manure and food waste. Then, the active metabolic pathways were evaluated based on the Pearson correlation coefficients of the relationship between liquid metabolites and hydrogen production. Finally, the fermentation process was optimized using a Box–Behnken design based on response surface methodology [34,35].

2. Materials and Methods

2.1. Inoculum

Inoculated sludge was obtained from a biogas pilot plant for treating cattle manure located at the Northeast Agricultural University, Harbin, China. To deactivate hydrogen-consuming bacteria, the inoculum was pretreated at 100 °C in a boiling water bath for 30 min immediately before use. The total solid (TS) and volatile solid (VS) contents of the inoculum were 5.19 ± 0.14% (w/w) and 3.51 ± 0.06% (w/w), respectively.

2.2. Substrates

Cattle manure was collected from the experimental farm at the Northeast Agricultural University. Food waste was obtained from the university dining room. The substrate composition is presented in Table 1.

Cattle manure and food waste were homogenized using a blender and stored at −20 °C until use. Before digestion, the cattle manure and food waste were diluted and mixed in accordance with the substrate concentration and mixing ratio defined by the experimental design.
Table 1. Substrate composition. TS: total solid, VS: volatile solid.

| Items          | Solid Contents (% w/w) | pH     | Component (% of TS) | C/N | Total Alkalinity (mg CaCO₃ L⁻¹) |
|----------------|------------------------|--------|----------------------|-----|--------------------------------|
|                | TS VS                  |        | Proteins Lipids Starch Lignocellulose |     |                                 |
| Food waste     | 19.53 ± 0.33 18.20 ± 0.31 5.28 ± 0.54 | 15.72 ± 0.24 29.38 ± 0.13 25.51 ± 0.53 13.16 ± 0.12 | 21.98 ± 0.22 | 225 ± 31 |
| Cattle manure  | 22.15 ± 0.29 18.21 ± 0.28 7.22 ± 0.04 | 11.86 ± 0.78 2.38 ± 0.29 0.51 ± 0.11 | 51.35 ± 0.11 | 18.81 ± 0.18 5171 ± 57 |

2.3. Experimental Design and Statistical Analysis

A three-factor Box–Behnken design was used to evaluate the effects of the operating parameters as well as those resulting from their interactions, on the experimental responses [34]. The three operating parameters chosen as independent variables were as follows: \((x_1)\) the mixing ratio (the proportion of food waste in the substrate, as a percentage), \((x_2)\) the substrate concentration (the total solid per liter of fermentation broth, g L⁻¹), and \((x_3)\) the HRT (d). The hydrogen production performance was characterized by \((y_1)\) the hydrogen production rate (HPR) and \((y_2)\) the hydrogen yield (HY). The HPR, which is indicative of the specific production efficiency of the reactor, was expressed in L L⁻¹ d⁻¹, and HY, which represents the efficiency of the transformation of the substrate, was expressed in mL g⁻¹ VS.

In this experimental design, 15 trials, comprising 12 factorial points and 3 center points, were run. According to the Tenca et al. [23], Cuetos et al. [36], and pre-experiments [37,38], the variation range of the three variables was selected. A summary of the experimental design matrix is presented in Table 2. Design Expert software (6.0.10, Stat-Ease Inc., Minneapolis, MN, USA) was used to analyze the experimental data.

Table 2. Experimental design matrix. HRT: hydraulic retention time.

| Run | Coded Values | Actual Values |
|-----|--------------|---------------|
|     | \(x_1\) \(x_2\) \(x_3\) Mixing Ratio (%) Substrate Concentration (g L⁻¹) HRT (d) |
| 1   | 1  1  1  40  60  2 |
| 2   | 1  1  1  60  60  2 |
| 3   | 1  1  1  40  100 2 |
| 4   | 1  1  1  60  100 2 |
| 5   | 1  1  1  40  80  1 |
| 6   | 1  1  1  60  80  1 |
| 7   | 1  1  1  40  80  3 |
| 8   | 1  1  1  60  80  3 |
| 9   | 1  1  1  50  60  1 |
| 10  | 1  1  1  50  100 1 |
| 11  | 1  1  1  50  60  3 |
| 12  | 1  1  1  50  100 3 |
| 13  | 1  1  1  50  80  2 |
| 14  | 1  1  1  50  80  2 |
| 15  | 1  1  1  50  80  2 |

2.4. Experimental Operating Procedure

The co-digestion experiments were conducted using 500-mL Erlenmeyer flasks in an oscillating incubator maintained at a temperature of 35 ± 1 °C and with an oscillation speed of 150 rpm. To conduct the experiments, 80 mL of pretreated inoculum and 320 mL of substrate were mixed in accordance with the experimental design matrix and poured into the reactor; thus, the operating volume was 400 mL. Reactors were purged with pure nitrogen gas for 60 s and sealed with rubber stoppers before the digestion process. At the beginning of the experiment, the reactors were operated in batch mode for 36 h. After hydrogen production had peaked, the reactor mode was converted into a semi-continuous...
system for 15 d, during which the substrate was fed every 12 h, and biogas and effluents were sampled and measured simultaneously. The average values of the experimental indexes were used to evaluate the hydrogen production performance of the system.

2.5. Analytic Methods

TS, VS, total alkalinity, and pH were determined using the standard methods outlined in American Public Health Association (APHA) [39]. Biogas was collected using sample bags that were connected to the reactor. Biogas volume was measured using the water displacement method and adjusted to the standard conditions (0 °C and 760 mm Hg) [36]. The percentages of H₂, CH₄, CO₂, and N₂ were analyzed using a gas chromatograph (6890N, Agilent Inc., Santa Clara, CA, USA) equipped with a TDX-01 column and a thermal conductivity detector. Argon was the carrier gas, and it flowed at 40 mL/min. The oven and detector temperatures were 170 °C and 220 °C, respectively. The ethanol and VFA (e.g., acetate, propionate, n-butyrate, and n-valerate) contents of the effluent were also analyzed using a gas chromatograph equipped with an infused-silica capillary column and a flame ionization detector. Nitrogen was the carrier gas, and it flowed at 30 mL/min. The temperatures of the injector and detector were 220 °C and 250 °C, respectively. The initial oven temperature was 60 °C. It was increased at 15 °C/min for 5.33 min, after which it was kept at 140 °C for 1.2 min. Acetonitrile and phosphoric acid (at a volume ratio of 2.5:97.5) were employed as the mobile phase to analyze lactate using a high-performance liquid chromatograph (600E-2487, WATERS Inc., Milford, CT, USA) equipped with an ultraviolet detector (210 nm) and a C18 column (250 × 4.6 mm) [38].

3. Results and Discussion

3.1. Overall Performance

The experimental results are summarized in Table 3. Generally, the HPR was in the range of 0–1.13 L L⁻¹ d⁻¹ and the HY was in the range of 0–31.70 mL g⁻¹ VS. The considerable variations in HPR and HY indicate that the metabolic pathways in the co-digestion of cattle manure and food waste were complex. To study the metabolic pathway, the pH and liquid metabolites in the effluent were also analyzed. As shown in Table 3, the pH was in the range of 3.62–5.28, and the liquid metabolites were primarily ethanol, acetate, propionate, n-butyrate, n-valerate, and lactate. The distribution of the liquid metabolites showed that multiple metabolic pathways were active in all the runs.

| Run | Hydrogen Production Rate (L L⁻¹ d⁻¹) | Hydrogen Yield (mL g⁻¹ vs) | pH | Ethanol (mmol L⁻¹) | Acetate | Propionate | Butyrate | Valerate | Lactate |
|-----|------------------------------------|---------------------------|----|------------------|--------|------------|----------|----------|---------|
| 1   | 0.09                               | 3.59                      | 5.34 | 23.65            | 24.50  | 7.61       | 21.62    | 8.07     | 38.03   |
| 2   | 0.05                               | 2.00                      | 4.02 | 85.02            | 23.05  | 3.43       | 2.37     | 1.47     | 119.91  |
| 3   | 0.49                               | 10.99                     | 5.42 | 25.71            | 47.56  | 15.97      | 27.12    | 8.07     | 72.62   |
| 4   | 0                                  | 0                         | 3.79 | 135.02           | 27.05  | 3.43       | 3.37     | 1.47     | 215.67  |
| 5   | 0.67                               | 9.70                      | 5.21 | 60.02            | 27.41  | 20.96      | 39.26    | 12.52    | 44.50   |
| 6   | 0                                  | 0.03                      | 3.88 | 97.82            | 46.63  | 10.28      | 3.33     | 0.93     | 143.59  |
| 7   | 0.01                               | 0.61                      | 5.37 | 14.40            | 47.11  | 9.10       | 18.78    | 5.08     | 85.74   |
| 8   | 0                                  | 0                         | 3.85 | 93.24            | 24.97  | 2.53       | 5.49     | 1.04     | 168.83  |
| 9   | 0.58                               | 10.95                     | 5.06 | 52.29            | 18.68  | 8.18       | 29.47    | 6.68     | 150.81  |
| 10  | 1.13                               | 12.83                     | 5.06 | 80.66            | 28.77  | 22.64      | 63.37    | 20.10    | 51.48   |
| 11  | 0.20                               | 11.41                     | 5.29 | 29.14            | 33.45  | 6.12       | 26.20    | 8.79     | 31.92   |
| 12  | 0.24                               | 8.29                      | 5.2  | 36.78            | 30.58  | 1.51       | 22.03    | 3.71     | 83.24   |
| 13  | 1.10                               | 31.20                     | 5.14 | 39.62            | 31.44  | 19.94      | 33.51    | 13.82    | 49.03   |
| 14  | 1.09                               | 31.70                     | 5.19 | 73.75            | 41.75  | 15.85      | 29.33    | 11.50    | 40.78   |
| 15  | 1.00                               | 26.53                     | 5.20 | 29.00            | 40.01  | 13.04      | 20.56    | 9.47     | 50.23   |

3.2. Hydrogen Production Rate

A quadratic regression model was established to determine the influence of independent variables on the HPR. Analysis of variance (ANOVA) was used to ensure the accuracy of the model. As shown

Table 3. Experimental design matrix and results.
in Table 4, the “Model F value” was 25.18, and the corresponding p value was <0.05, indicating that the model was significant. The “Lack of Fit F value” was 6.47, and the corresponding p value was >0.1, demonstrating that the lack of fit was insignificant.

Table 4. Analysis of variance (ANOVA) for the regression model of hydrogen production rate.

| Source         | Sum of Squares | Degree of Freedom | Mean Square | F Value  | p Value |
|----------------|----------------|-------------------|-------------|----------|---------|
| Model          | 2.78           | 9                 | 0.31        | 25.18    | 0.0012  |
| $x_1$          | 0.18           | 1                 | 0.18        | 14.99    | 0.0117  |
| $x_2$          | 0.11           | 1                 | 0.11        | 8.91     | 0.0306  |
| $x_3$          | 0.46           | 1                 | 0.46        | 37.54    | 0.0017  |
| $x_1x_2$       | 0.05           | 1                 | 0.05        | 4.13     | 0.0978  |
| $x_1x_3$       | 0.11           | 1                 | 0.11        | 8.74     | 0.0317  |
| $x_2x_3$       | 0.06           | 1                 | 0.06        | 5.24     | 0.0708  |
| $x_1^2$        | 1.48           | 1                 | 1.48        | 121.02   | 0.0001  |
| $x_2^2$        | 0.27           | 1                 | 0.27        | 21.91    | 0.0054  |
| $x_3^2$        | 0.24           | 1                 | 0.24        | 19.86    | 0.0067  |
| Residual       | 0.06           | 5                 | 0.01        |          |         |
| Lack of Fit    | 0.06           | 3                 | 0.02        | 6.47     | 0.1368  |
| Pure Error     | 0.01           | 2                 | 0.00        |          |         |
| Cor Total      | 2.84           | 14                | -           | -        | -       |

According to the ANOVA results, Equation (1), with respect to coded factors, had a determinant coefficient of 0.9784, showing that there was a significant correlation between the experimental and predicted values. The optimal conditions for the maximum HPR were a mixing ratio of 47.33%, a substrate concentration of 88.89 g L$^{-1}$, and an HRT of 1.73 d.

$$y_1 = 1.06 - 0.15x_1 + 0.12x_2 - 0.24x_3 - 0.11x_1x_2 + 0.16x_1x_3 - 0.13x_2x_3 - 0.63x_1^2 - 0.27x_2^2 - 0.26x_3^2$$  

(1)

The regression coefficients of the linear effect terms were compared, and the HRT was identified as the factor that contributed the most to the HPR, followed by the mixing ratio, and then the substrate concentration. To investigate the effects of the interaction of these three contributing factors, three-dimensional (3D) response surface and two-dimensional (2D) contour plots were generated based on the experimental data and regression equations. Additionally, the intensities of the interactive effects were reflected by the shape of the contour plots. The effect of the interaction between the mixing ratio and HRT on HPR (at a constant substrate concentration of 80 g L$^{-1}$) is illustrated in Figure 1a, which shows that the contour plot was elliptical, indicating a strong interaction between the two factors.

![Figure 1](link)  
**Figure 1.** Three-dimensional (3D) response surface and two-dimensional (2D) contour plots for hydrogen production rate. (a) Mixing ratio and hydraulic retention time (HRT), (b) substrate concentration and HRT, and (c) mixing ratio and substrate concentration.
When the HRT was fixed, the HPR first increased slowly, and thereafter decreased rapidly as the mixing ratio increased. When the mixing ratio increased to 50%, the proportion of starch, which constituted a greater fraction of the food waste than it did the cattle manure, increased. Reportedly, readily biodegradable carbohydrates, such as starch, are associated with higher HPR than other substrates [40]. When the mixing ratio exceeded 50%, the alkalinity, which was lower in the food waste than it was in the cattle manure, decreased. The readily biodegradable carbohydrates and the lower alkalinity in the digestion system caused the rapid accumulation of undissolved VFAs, which reduced the pH to below the optimal range [18,23].

When the mixing ratio was fixed, the HPR increased slowly at first and thereafter decreased rapidly as the HRT increased. This phenomenon could possibly be attributed to the coupling of the sludge retention time and the HRT in the fully mixed reactor. A longer HRT meant that less substrate was fed into the reactor. However, a longer sludge retention time meant that more hydrogen-producing bacteria were present in the reactor. When the HRT increased from 1 to 2 d, the two effects almost canceled each other out, causing the HPR to increase at a slower pace. Nevertheless, a longer HRT and sludge retention time are beneficial to the growth of hydrogen consumers, such as methanogens [41]. Consequently, when the HRT increased from 2 to 3 d, the HPR decreased rapidly.

Figure 1b illustrates the effects of the interaction between the HRT and substrate concentration on the HPR (at a 50% constant mixing ratio). The contour plot was elliptical, indicating that there is a significant interaction between these two factors. Similar to Figure 1a, as the HRT increased, the HPR initially increased slowly, and thereafter, it decreased rapidly. However, as the substrate concentration increased, the HPR first increased rapidly and thereafter decreased slowly. This can primarily be attributed to the higher substrate concentration, which resulted in a higher organic loading rate. Within an optimum range, the increased organic loading rate enhanced the HPR of the dark fermentation process. However, when it was too high, the accumulation of VFAs and dissolved hydrogen in the digestion broth could potentially inhibit the activity of the hydrogen-producing bacteria [31,42].

Figure 1c shows the effects of the interaction between the mixing ratio and the substrate concentration on the HPR (at 2 d constant HRT). The HPR first increased, and thereafter, it decreased as the mixing ratio and substrate concentration increased. The contour plot was elliptical, indicating a strong interaction between the two factors.

3.3. Hydrogen Yield

ANOVA was also used to evaluate the effects of the mixing ratio, substrate concentration, and HRT on the HY. As shown in Table 5, the quadratic regression model was significant, and the lack of fit was insignificant.

| Source   | Sum of Squares | Degree of Freedom | Mean Square | F Value | p Value |
|----------|----------------|-------------------|-------------|---------|---------|
| Model    | 1740.11        | 8                 | 217.51      | 61.00   | <0.0001 |
| $x_1$    | 65.25          | 1                 | 65.25       | 18.30   | 0.0052  |
| $x_2$    | 2.17           | 1                 | 2.17        | 0.61    | 0.4652  |
| $x_3$    | 21.77          | 1                 | 21.77       | 6.11    | 0.0484  |
| $x_1x_2$ | 22.10          | 1                 | 22.10       | 6.20    | 0.0472  |
| $x_1x_3$ | 20.47          | 1                 | 20.47       | 5.74    | 0.0536  |
| $x_1^2$  | 1006.18        | 1                 | 1106.18     | 310.23  | <0.0001 |
| $x_2^2$  | 300.51         | 1                 | 300.51      | 84.28   | <0.0001 |
| $x_3^2$  | 113.29         | 1                 | 113.29      | 31.91   | <0.0001 |
| Residual | 21.39          | 6                 | 3.57        | -       | -       |
| Lack of Fit | 15.39        | 4                 | 3.90        | 1.34    | 0.4693  |
| Pure Error | 5.81          | 2                 | 2.90        | -       | -       |
| Cor Total | 1761.50        | 14                | -           | -       | -       |
Based on the ANOVA results, the quadratic regression equation was derived using the regression fitting (Equation (2)) with a regression coefficient of 0.9879. The insignificant interaction effect terms of substrate concentration and HRT were removed. However, to enhance the regression model hierarchy, the insignificant linear model term of the HRT was retained. Based on the regression coefficients of the individual factors, the mixing ratio had the most significant impact on the HY, followed by the substrate concentration, and then the HRT.

\[
y_2 = 30.47 - 2.86x_1 + 0.52x_2 - 1.65x_3 - 2.35x_1x_2 + 2.26x_1x_3 - 17.31x_1^2 - 9.02x_2^2 - 10.58x_3^2 \tag{2}
\]

The 3D response surface and 2D contour plots of the HY regression model are shown in Figure 2. With the increasing mixing ratio, HRT, and substrate concentration, the HY increased to an optimal within the optimal pH range, HPR and HY are sensitive to HRT and substrate concentration. Additionally, optimal HY conditions were determined as a mixing ratio of 49.09%, substrate concentration of 80.82 g L\(^{-1}\), and HRT of 1.91 d. The effects of substrate concentration and HRT on HPR and HY were different. Higher substrate concentrations and lower HRTs enhanced the HPR, but not HY. However, the effects of the mixing ratio on HPR and HY were similar, resulting in the same optimal mixing ratio for both indicators.

### 3.4. pH and Liquid Metabolites

HPR and HY as functions of pH are presented in Figure 3, which shows that the HPR and HY were approximately zero when the pH was <4.02. However, as the pH increased, both the HPR and HY peaked within a pH range of 5.06–5.20, and they decreased thereafter. Generally, independent of substrate concentration and HRT, the mixing ratio played a crucial role in determining the pH. When the mixing ratio was 60% (i.e., runs 2, 4, 6, and 8), almost all the pH values observed were below 4. Under such conditions, hydrogen production was low or absent. Additionally, when the mixing ratio was 40% (i.e., runs 1, 3, 5, and 7), the pH was greater than 5.20, and the HPR and HY were less than 0.49 L L\(^{-1}\) d\(^{-1}\) and 10.99 mL g\(^{-1}\) VS, respectively. However, when the mixing ratio was 50%, the pH was maintained within range of 5.06–5.20, and maximum HPR and HY values of 1.10 L L\(^{-1}\) d\(^{-1}\) and 31.70 mL g\(^{-1}\) VS were obtained. Notably, significant deviations in HPR and HY were also observed within a pH range of 5.06–5.20. These results indicate that even within the optimal pH range, HPR and HY are sensitive to HRT and substrate concentration.
were strongly correlated with hydrogen production. This was possibly due to the food waste having produce hydrogen and butyrate, whereas lipid and protein digestions tend to produce propionate and valerate, respectively. These digestion processes occur simultaneously under most conditions [11,44].

Additionally, correlation coefficients of 0.76 and 0.48 for HPR and HY, respectively, were indicative of a weak correlation between acetate and hydrogen production. Furthermore, to estimate the contribution of different liquid metabolites to hydrogen production, Pearson correlation coefficients between liquid metabolites and responses were calculated (Table 6).

As shown in Table 6, the correlation coefficients between the liquid metabolites and HY and HPR coincided. For ethanol and lactate, the correlation coefficients were negative, indicating that the heterolactic fermentation described by Equation (3) was a competing pathway for hydrogen fermentation. Reportedly, zero-hydrogen heterolactic fermentation becomes the dominant metabolic pathway in continuous anaerobic acidogenesis under low pH conditions [43].

\[
\text{C}_6\text{H}_12\text{O}_6 \rightarrow \text{CH}_3\text{CH}_(\text{OH})\text{COOH} + \text{CH}_3\text{CH}_2\text{OH} + \text{CO}_2
\]  

(3)

For acetate, even though high yields were observed in all runs, correlation coefficients of 0.07 and 0.19 for HPR and HY, respectively, were indicative of a weak correlation between acetate and hydrogen production. Additionally, correlation coefficients of 0.76 and 0.48 for HPR and HY, respectively, demonstrated that the main hydrogen production pathway was the butyrate pathway (Equation (4)). However, a large number of by-products, such as propionate and n-valerate, were produced, and they were strongly correlated with hydrogen production. This was possibly due to the food waste having not only a high starch content, but also high lipid and protein contents. Starch digestion tends to produce hydrogen and butyrate, whereas lipid and protein digestions tend to produce propionate and valerate, respectively. These digestion processes occur simultaneously under most conditions [11,44].

\[
\text{C}_6\text{H}_12\text{O}_6 \rightarrow \text{CH}_3\text{COOH} + 1/2\text{CH}_3\text{(CH}_2\text{)}\text{COOH} + 3\text{H}_2 + 2\text{CO}_2
\]  

(4)
3.5. Process Optimization and Verification of the Model

In hydrogen-producing reactors, both HPR and HY should be maximized, because high HPRs offer the possibility of reducing the reactor volume and high HYs offer the possibility of improving the energy conversion efficiency. However, it is difficult to optimize these two responses under the same conditions, because their interest regions differ. Therefore, the optimization region was determined by overlaying the responses according to the defined constraints (Figure 4). The constraints used in the overlaying method were to set the HPR and HY in the range of 1.00 L L$^{-1}$ d$^{-1}$ to its upper amount and 30.00 mL g$^{-1}$ VS to its upper amount, respectively. The optimized processing parameters included a mixing ratio of 47% to 51%, a substrate concentration of 76 to 86 g L$^{-1}$, and an HRT of 2 d. A mixing ratio of 50%, substrate concentration of 80 g L$^{-1}$, and HRT of 2 d were selected as the optimal parameters to verify the models. The optimal responses were predicted as 1.08 L L$^{-1}$ d$^{-1}$ and 30.90 mL g$^{-1}$ VS for the HPR and the HY, respectively. Under the optimum conditions, the verification experiment achieved an HPR of 1.09 L L$^{-1}$ d$^{-1}$ and a HY of 30.22 mL g$^{-1}$ VS, respectively, which agrees well with the experimental data. This indicates that the models can effectively predict the hydrogen fermentation process, and the optimum conditions obtained using the response surface methodology were conducive to the growth and activity of hydrogen-producing microbes.

![Figure 4. Process optimization (the hydraulic retention time is 2 d).](image)

3.6. Comparison of the Hydrogen Production by Different Substrates

A large number of studies have been conducted on the production of biohydrogen with different substrates. Table 7 compares the HPR and HY observed in this study with those obtained from the existing literature.

Even though stable hydrogen production was achieved via the co-digestion of cattle manure and food waste, the optimal HPR and HY did not show obvious advantages compared with the values reported in the literature. The relatively complex nature of the substrates used in this study could be responsible for this observation. On the one hand, cattle manure has a high lignocellulose content, which makes it resistant to biodegradation [45]. Even under thermophilic conditions, the HY of cattle manure was much lower than that of food waste [12,46]. On the other hand, food waste is rich in lipids. Thus, the accumulation of long-chain fatty acids (LCFAs) generated from the lipid degradation process can inhibit hydrogen production [47].
To enhance the efficiency of the process, pretreatments are often required to promote the disintegration and hydrolysis of complex biomass [14]. However, negative effects such as the formation of inhibitors, high energy consumption, and the necessity to add other chemicals are often associated with such pretreatments [48]. Therefore, increased efforts are required to ensure that such pretreatment processes are more efficient in the long term. From the perspective of a full-scale application, simple operations, such as solid–liquid separation for cattle manure and oil–water separation for food waste would be more practical methods in the near future [49,50]. Although further studies are necessary to improve the process, the co-digestion of cattle manure and food waste is a practical and economically promising candidate for hydrogen production from organic solid waste.

Table 7. Biohydrogen production by different substrates.

| Substrates                     | Fermentation Conditions | Hydrogen Production Rate (L L⁻¹ d⁻¹) | Hydrogen Yield (mL g⁻¹ VS) | References |
|--------------------------------|-------------------------|-------------------------------------|-----------------------------|------------|
| Food waste                     | Semi-CSTR (Thermophilic) | 0.4–2.1                             | 20–85                       | [51]       |
| Food waste                     | Batch (Mesophilic)      | -                                   | 120                         | [18]       |
| Cattle manure                  | Batch (Mesophilic/Thermophilic) | -                              | 0–0.73 a                    | [46]       |
| Cattle manure                  | Semi-CSTR(Thermophilic) | 0.41                                | 10.25                       | [12]       |
| Humulus scandens               | Batch (Mesophilic)      | -                                   | 65.12 b                     | [52]       |
| Carbohydrates + Proteins       | Batch (Mesophilic)      | -                                   | 350 c                       | [53]       |
| Swine manure + Pineapple waste | CSTR (Mesophilic)       | 1.49                                | 266.91 c                    | [54]       |
| Food waste + Sewage sludge    | Batch (Mesophilic)      | -                                   | 174.6                       | [22]       |
| Dog food + Cow manure          | Batch (Thermophilic)    | -                                   | 57.67 a                     | [30]       |
| Food waste + Cattle manure     | Semi-CSTR (Mesophilic)  | 1.09                                | 30.22                       | This study |

Note: a calculated from data in literature, b mL g⁻¹ TS, c mL g⁻¹ COD.

4. Conclusions

Biohydrogen production during the mesophilic co-digestion of cattle manure and food waste was optimized using the Box–Behnken design. The optimal process parameters were a mixing ratio of 47% to 51%, a substrate concentration of 76 to 86 g L⁻¹, and an HRT of 2 d. Under these optimal conditions, the HPR and HY were higher than 1.00 L L⁻¹ d⁻¹ and 30.00 mL g⁻¹ VS, respectively, and the predicted results were consistent with the experimental data. Overall, it can be concluded from the current study that biohydrogen production from the co-digestion of cattle manure and food waste is feasible. Additionally, stable biohydrogen production can be obtained using the mixing ratio, substrate concentration, and HRT as the controlling factors. These findings serve as a foundation for improving biohydrogen production from organic solid wastes.

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References

1. Halkos, G.E.; Gkampoura, E.-C. Reviewing Usage, Potentials, and Limitations of Renewable Energy Sources. Energies 2020, 13, 2906. [CrossRef]
2. Kumar, G.; Dharmaraja, J.; Arvindnarayan, S.; Shoban, S.; Bakonyi, P.; Saratale, G.D.; Nemestóthy, N.; Bélafi–Bakó, K.; Yoon, J.J.; Kim, S.H. A comprehensive review on thermochemical, biological, biochemical and hybrid conversion methods of bio-derived lignocellulosic molecules into renewable fuels. Fuel 2019, 251, 352–367. [CrossRef]
3. Sun, Y.; Zhang, Z.Z.; Sun, Y.M.; Yang, G.X. One-pot pyrolysis route to Fe–N-Doped carbon nanosheets with outstanding electrochemical performance as cathode materials for microbial fuel cell. Int. J. Agric. Biol. Eng. 2020. [CrossRef]

4. Jia, W.; Qin, W.; Zhang, Q.; Wang, X.; Ma, Y.; Chen, Q. Evaluation of crop residues and manure production and their geographical distribution in China. J. Clean. Prod. 2018, 188, 954–965. [CrossRef]

5. Cheng, J.; Shi, F.; Yi, J.; Fu, H. Analysis of the factors that affect the production of municipal solid waste in China. J. Clean. Prod. 2020, 259, 120808. [CrossRef]

6. Ranieri, L.; Mossa, G.; Pellegrino, R.; Digiesi, S. Energy Recovery from the Organic Fraction of Municipal Solid Waste: A Real Options-Based Facility Assessment. Sustainability 2018, 10, 368. [CrossRef]

7. Guo, X.M.; Trably, E.; Latrille, E.; Carrère, H.; Steyer, J.-P. Hydrogen production from agricultural waste by dark fermentation: A review. Int. J. Hydrogen Energy 2010, 35, 10660–10673. [CrossRef]

8. Khan, M.A.; Ngo, H.H.; Guo, W.; Liu, Y.; Zhang, X.; Guo, J.; Chang, S.W.; Nguyen, D.D.; Wang, J. Biohydrogen production from anaerobic digestion and its potential as renewable energy. Renew. Energy 2018, 129, 754–768. [CrossRef]

9. Sivagurunathan, P.; Kumar, G.; Mudhoo, A.; Rene, E.R.; Saratale, G.D.; Kobayashi, T.; Xu, K.; Kim, S.-H.; Kim, D.-H. Fermentative hydrogen production using lignocellulosic biomass: An overview of pre-treatment methods, inhibitor effects and detoxification experiences. Renew. Sustain. Energy Rev. 2017, 77, 28–42. [CrossRef]

10. Massaro, V.; Digiesi, S.; Mossa, G.; Ranieri, L. The sustainability of anaerobic digestion plants: A win–win strategy for public and private bodies. J. Clean. Prod. 2015, 104, 445–459. [CrossRef]

11. Kobayashi, T.; Xu, K.-Q.; Li, Y.-Y.; Inamori, Y. Evaluation of hydrogen and methane production from municipal solid wastes with different compositions of fat, protein, cellulosic materials and the other carbohydrates. Int. J. Hydrogen Energy 2012, 37, 15711–15718. [CrossRef]

12. Wang, K.-S.; Chen, J.-H.; Huang, Y.-H.; Huang, S.-L. Integrated Taguchi method and response surface methodology to confirm hydrogen production by anaerobic fermentation of cow manure. Int. J. Hydrogen Energy 2013, 38, 45–53. [CrossRef]

13. Dinesh, G.K.; Chauhan, R.; Chakma, S. Influence and strategies for enhanced biohydrogen production from food waste. Renew. Sustain. Energy Rev. 2018, 92, 807–822. [CrossRef]

14. Saratale, G.D.; Saratale, R.G.; Kim, S.H.; Kumar, G. Screening and optimization of pretreatments in the preparation of sugarcane bagasse feedstock for biohydrogen production and process optimization. Int. J. Hydrogen Energy 2018, 43, 11470–11483. [CrossRef]

15. Elbeshbishy, E.; Dhar, B.R.; Nakhlia, G.; Lee, H.-S. A critical review on inhibition of dark biohydrogen fermentation. Renew. Sustain. Energy Rev. 2017, 79, 656–668. [CrossRef]

16. Carrere, H.; Antonopoulou, G.; Abbès, R.; Passos, F.; Battimelli, A.; Lyberatos, G.; Ferrer, I. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. Bioresour. Technol. 2016, 199, 386–397. [CrossRef] [PubMed]

17. Van Ginkel, S.; Logan, B.E. Inhibition of Biohydrogen Production by Undissociated Acetic and Butyric Acids. Environ. Sci. Technol. 2005, 39, 9351–9356. [CrossRef] [PubMed]

18. Zhu, H.; Parker, W.; Basnar, R.; Proracki, A.; Falletta, P.; Beland, M.; Seto, P. Buffer requirements for enhanced hydrogen production in acidogenic digestion of food wastes. Bioresour. Technol. 2009, 100, 5097–5102. [CrossRef]

19. Ghimire, A.; Frunzo, L.; Pirozzi, F.; Trably, E.; Escudie, R.; Lens, P.N.; Esposito, G. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Appl. Energy 2015, 144, 73–95. [CrossRef]

20. Yang, G.; Wang, J. Co-fermentation of sewage sludge with ryegrass for enhancing hydrogen production: Performance evaluation and kinetic analysis. Bioresour. Technol. 2017, 243, 1027–1036. [CrossRef]

21. Kim, S. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. Int. J. Hydrogen Energy 2004, 29, 1607–1616. [CrossRef]

22. Cheng, J.; Ding, L.; Lin, R.; Yue, L.; Liu, J.; Zhou, J.; Cen, K. Fermentative biohydrogen and biomethane co-production from mixture of food waste and sewage sludge: Effects of physicochemical properties and mix ratios on fermentation performance. Appl. Energy 2016, 184, 1–8. [CrossRef]
23. Tenca, A.; Schievano, A.; Perazzolo, F.; Adani, F.; Oberti, R. Biohydrogen from thermophilic co-fermentation of swine manure with fruit and vegetable waste: Maximizing stable production without pH control. *Bioresour. Technol.*** 2011, *102, 8582–8588. [CrossRef] [PubMed]

24. Perera, K.R.; Nirmalakhandan, N. Evaluation of dairy cattle manure as a supplement to improve net energy gain in fermentative hydrogen production from sucrose. *Bioresour. Technol.*** 2011, *102, 8688–8695. [CrossRef] [PubMed]

25. Lee, J.K.; Patel, S.K.S.; Sung, B.H.; Kalia, V.C. Biomolecules from municipal and food industry wastes: An overview. *Bioresour. Technol.*** 2020, *298, 122346. [CrossRef]

26. Zhang, C.; Xiao, G.; Peng, L.; Su, H.; Tan, T. The anaerobic co-digestion of food waste and cattle manure. *Bioresour. Technol.*** 2013, *129, 170–176. [CrossRef] [PubMed]

27. Maranon, E.; Castrillon, L.; Quiroga, G.; Fernandez-Nava, Y.; Gomez, L.; Garcia, M.M. Co-digestion of cattle manure with food waste and sludge to increase biogas production. *Waste Manag.*** 2012, *32, 1821–1825. [CrossRef] [PubMed]

28. Bi, S.; Hong, X.; Yang, H.; Yu, X.; Fang, S.; Bai, Y.; Liu, J.; Gao, Y.; Yan, L.; Wang, W.; et al. Effect of hydraulic retention time on anaerobic co-digestion of cattle manure and food waste. *Renew. Energy*** 2020, *150, 213–220. [CrossRef]

29. Zarkadas, I.S.; Sofikiti, A.S.; Voudrias, E.A.; Plidis, G.A. Thermophilic anaerobic digestion of pasteurised food wastes and dairy cattle manure in batch and large volume laboratory digesters: Focussing on mixing ratios. *Renew. Energy*** 2015, *80, 432–440. [CrossRef]

30. Hiroshi, Y.; Takahiro, Y.; Akifumi, O.; Mitsuyoshi, I.; Yasuo, T. Hydrogen Fermentation of Cow Manure Mixed with Food Waste. *Ipn. Agric. Res. Q.* 2010, *44, 399–404.

31. Kim, T.H.; Lee, Y.; Chang, K.H.; Hwang, S.J. Effects of initial lactic acid concentration, HRTs, and OLRs on bio-hydrogen production from lactate-type fermentation. *Bioresour. Technol.*** 2012, *103, 136–141. [CrossRef] [PubMed]

32. Pu, Y.; Tang, J.; Wang, X.C.; Hu, Y.; Huang, J.; Zeng, Y.; Ngo, H.H.; Li, Y. Hydrogen production from acidogenic food waste fermentation using untreated inoculum: Effect of substrate concentrations. *Int. J. Hydrogen Energy*** 2019, *44, 27272–27284. [CrossRef]

33. Lu, C.; Wang, Y.; Lee, D.-J.; Zhang, Q.; Zhang, H.; Tahir, N.; Jing, Y.; Liu, H.; Zhang, K. Biohydrogen production in pilot-scale fermenter: Effects of hydraulic retention time and substrate concentration. *J. Clean. Prod.*** 2019, *229, 751–760. [CrossRef]

34. Limkar, M.B.; Pawar, S.V.; Rathod, V.K. Statistical optimization of xylanase and alkaline protease co-production by Bacillus spp using Box-Behnken Design under submerged fermentation using wheat bran as a substrate. *Biocatal. Agric. Biotechnol.* 2019, *17, 455–464. [CrossRef]

35. Mu, Y.; Wang, G.; Yu, H.-Q. Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. *Enzym. Microb. Technol.* 2006, *38, 905–913. [CrossRef]

36. Cuetos, M.J.; Gómez, X.; Escapa, A.; Morán, A. Evaluation and simultaneous optimization of bio-hydrogen production using 32 factorial design and the desirability function. *J. Power Sources*** 2007, *169, 131–139. [CrossRef]

37. Liu, S.; Li, W. Effects of mixing ratio on hydrogen production from anaerobic co-fermentation of cattle manure and food waste. *Trans. Chin. Soc. Agric. Mach.* 2013, *44, 114–118.

38. Liu, S.; Wang, C.Y.; Yin, L.L.; Li, W.Z.; Luo, L.N. Optimization of hydrogen production from agricultural wastes using mixture design. *Int. J. Agric. Biol. Eng.* 2017, *10, 246–254.

39. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater; American Water Work Association, and Water Pollution Control Federation*: Washington, DC, USA, 2004.

40. Lee, K.; Hsu, Y.; Lo, Y.; Lin, P.; Lin, C.; Chang, J. Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *Int. J. Hydrogen Energy*** 2008, *33, 1565–1572. [CrossRef]

41. Chen, C.C.; Lin, C.Y.; Chang, J.S. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. *Appl. Microbiol. Biotechnol.* 2001, *57, 56–64.

42. Das, S.; Calay, R.K.; Chowdhury, R.; Nath, K.; Eregno, F.E. Product Inhibition of Biological Hydrogen Production in Batch Reactors. *Energies*** 2020, *13, 1318. [CrossRef]

43. Itoh, Y.; Tada, K.; Kanno, T.; Horiuchi, J. Selective production of lactic acid in continuous anaerobic acidogenesis by extremely low pH operation. *J. Biosci. Bioeng.* 2012, *114, 537–539. [CrossRef] [PubMed]
44. Ma, H.; Liu, H.; Zhang, L.; Yang, M.; Fu, B.; Liu, H. Novel insight into the relationship between organic substrate composition and volatile fatty acids distribution in acidogenic co-fermentation. *Biotechnol. Biofuels* 2017, 10, 137. [CrossRef] [PubMed]

45. Wang, M.; Li, W.; Li, P.; Yan, S.; Zhang, Y. An alternative parameter to characterize biogas materials: Available carbon-nitrogen ratio. *Waste Manag.* 2017, 62, 76–83. [CrossRef]

46. Yokoyama, H.; Waki, M.; Ogino, A.; Ohmori, H.; Tanaka, Y. Hydrogen fermentation properties of undiluted cow dung. *J. Biosci. Bioeng.* 2007, 104, 82–85. [CrossRef]

47. Yan, B.H.; Selvam, A.; Wong, J.W.C. Bio-hydrogen and methane production from two-phase anaerobic digestion of food waste under the scheme of acidogenic off-gas reuse. *Bioresour. Technol.* 2020, 297, 122400. [CrossRef]

48. Wang, J.; Yin, Y. Fermentative hydrogen production using various biomass-based materials as feedstock. *Renew. Sustain. Energy Rev.* 2018, 92, 284–306. [CrossRef]

49. Wang, H.; Xu, J.; Sheng, L. Study on the comprehensive utilization of city kitchen waste as a resource in China. *Energy* 2019, 173, 263–277. [CrossRef]

50. Aguirre-Villegas, H.A.; Larson, R.A.; Sharara, M.A. Anaerobic digestion, solid-liquid separation, and drying of dairy manure: Measuring constituents and modeling emission. *Sci. Total Environ.* 2019, 696, 134059. [CrossRef]

51. Chu, C.-F.; Xu, K.-Q.; Li, Y.-Y.; Inamori, Y. Hydrogen and methane potential based on the nature of food waste materials in a two-stage thermophilic fermentation process. *Int. J. Hydrogen Energy* 2012, 37, 10611–10618. [CrossRef]

52. Zhang, Y.; Zhang, T.; Zhang, Z.; Tahir, N.; Zhang, Q. Biohydrogen production from Humulus scandens by dark fermentation: Potential evaluation and process optimization. *Int. J. Hydrogen Energy* 2020, 45, 3760–3768. [CrossRef]

53. Tepari, E.A.; Nakhla, G.; Haroun, B.M.; Hafiez, H. Co-fermentation of carbohydrates and proteins for biohydrogen production: Statistical optimization using Response Surface Methodology. *Int. J. Hydrogen Energy* 2020, 45, 2640–2654. [CrossRef]

54. Nguyen, T.-T.; Chu, C.-Y.; Ou, C.-M. Pre-treatment study on two-stage biohydrogen and biomethane productions in a continuous co-digestion process from a mixture of swine manure and pineapple waste. *Int. J. Hydrogen Energy* 2020. [CrossRef]