

Optimization of Operational Parameters for Biohydrogen Production from Waste Sugarcane Leaves and Semi-Pilot Scale Process Assessment

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This study modeled and optimized the operational parameters for biohydrogen production from waste sugarcane leaves and assessed hydrogen production on a semi-pilot scale. A Box-Behnken design with input variables of substrate concentration (8 to 24 g/L), inoculum concentration (10% to 50% v/v), and hydraulic retention time (HRT, 24 to 96 h) was used. A coefficient of determination (R^2) of 0.90 and the predicted optimum operational set-points of 14.2 g/L substrate concentration, 32.7% inoculum concentration, and 62.8 h HRT were obtained. Experimental validation produced a biohydrogen yield of 12.8 mL H₂/g fermentable sugar (FS). A semi-pilot scale process in a 13-L Infors reactor under optimized conditions gave a cumulative hydrogen volume and yield of 3740 mL and 321 mL H₂ g⁻¹ FS, respectively, with a peak hydrogen fraction of 37%. Microbial analysis from the process effluent conducted by Polymerase Chain Reaction cloning indicated the presence of hydrogen-producing bacteria belonging to *Clostridium* sp., *Klebsiella* sp., and *Enterobacter* sp. These findings highlight the feasibility of biohydrogen production from sugarcane waste and provide preliminary knowledge on process scale up.

Keywords: Biohydrogen production; Sugarcane leaves; Bioprocess optimization; Semi-pilot scale process; Dark fermentation

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INTRODUCTION

The growing global population has increased energy demand while increasingly exhausting reserves. The depletions of fossil fuel oil, coal, and gas are estimated in the next 35, 107, and 37 years, respectively (Shafiee and Topal 2009). Moreover, concerns have arisen over climate change from fossil fuel combustion. Thus, alternative energy sources are being pursued (Howaniec and Smoliński 2014). Among the various options, hydrogen (H₂) has been identified as the most favorable because of its high energy content (122 kJ/g) and relatively clean combustion profile (Faloye *et al.* 2013; Seo *et al.* 2015).

Presently, a large fraction of hydrogen is produced from natural gas (Seo *et al.* 2015). The production of hydrogen *via* biological pathways such as dark fermentation, photofermentation, and microbial electrolysis cells has shown immense potential. Dark fermentation is considered a promising technology because of its flexibility, which allows the use of diverse substrates and inocula, adding to its substantial social, economic, and environmental benefits (Ghimire *et al.* 2015). In dark fermentation, substrates are degraded anaerobically by facultative and obligate anaerobic hydrogen-producing microorganisms (Guo *et al.* 2010). Moreover, this method is attractive given that various organic wastes

and wastewater can be used as feedstock, thus significantly enhancing process economics while decreasing environmental degradation.

An estimated 200 billion tons of lignocellulosic biomass is produced annually, and it is considered a low-cost and eco-friendly alternative feedstock to high-value products such as biofuels (Behara *et al.* 2014). In addition, lignocellulosic biomass is rich in fermentable carbohydrates, which makes it an attractive feedstock for biofuel production. Sugarcane is one such example, and it is estimated that 1.6 billion tons are produced annually (Sugarcane.Org 2015). The leaf component of the sugarcane, comprising up to approximately 40% of the plant, is disposed of by burning prior to harvest. This process releases harmful mutagenic polycyclic aromatic hydrocarbons into the atmosphere, which can have severe effects on human health (Silva *et al.* 2010; Prado *et al.* 2012). Because of the complex structure of lignocellulosic materials, a pretreatment is essential because it allows the breakdown of the cross-linked matrix and promotes the release of fermentable sugars (López-Linares *et al.* 2013). The modeling and optimization of fermentable sugar release (xylose and glucose) from waste sugarcane leaves has been reported previously (Moodley and Kana 2015).

The physicochemical parameters for biohydrogen production, including substrate concentration, hydraulic retention time, pH, temperature, and inoculum, impact the cell metabolism fluxes and thus affect hydrogen yield (Mohammadi *et al.* 2012). The optimization of these parameters is essential to achieve higher hydrogen production (Jutakanoke *et al.* 2012; Ghimire *et al.* 2015). The substrate concentration directly affects the formation of volatile fatty acids (VFAs), consequently impacting the process pH and the microbial community composition (Mohammadi *et al.* 2012).

The hydraulic retention time (HRT) can be used in biohydrogen production processes to control the presence of hydrogen-consuming microorganisms in the bioreactor. Values of HRT higher than the growth rate of hydrogen-producing microorganisms prevent washout of the biomass (De Gioannis *et al.* 2013). Longer HRTs, however, allow the accumulation of VFAs, thus lowering the fermentation pH and impeding hydrogen production (Jutakanoke *et al.* 2012).

The inoculum concentration is another key parameter that has been shown to enhance hydrogen production with increasing concentrations while concomitantly inhibiting methanogens (Hallenbeck and Ghosh 2009). Very high inoculum concentrations have been found to increase biomass accumulation, which then has led to rapid nutrient consumption and waste production (Puad *et al.* 2015). The optimization of these parameters is essential to achieve higher hydrogen production (Ghimire *et al.* 2015).

Bioprocess modeling and optimization is required to determine the optimal set-points of key operational parameters. Process modeling can be achieved using mathematical equations to predict or improve a set of conditions. Response surface methodology (RSM) uses polynomial equations to model the relationships between input variables and response outputs (Pan *et al.* 2008). It has been reported in the optimization of various bioprocesses (Pan *et al.* 2008; Chaganti *et al.* 2012; Faloye *et al.* 2013). The present study modeled and optimized the physico-chemical input parameters of the substrate concentration, inoculum concentration, and hydraulic retention time for biohydrogen production from waste sugarcane leaves. Furthermore, it assessed biohydrogen production at a 13-L scale under the optimized operational conditions.

EXPERIMENTAL

Inoculum Preparation

The anaerobic sludge used in this study was collected from the Darvill Wastewater Treatment Facility in Pietermaritzburg, South Africa. The sludge was immediately transported to the laboratory and stored at 4 °C. Prior to fermentation, it was thermally treated at 121 °C for 10 min to promote biohydrogen production by reducing methanogenic activity.

Feedstock Pretreatment

The sugarcane leaves were collected from the South African Sugarcane Research Institute (SASRI), dried at 60 °C for 72 h, and milled using a centrifugal mill (Retsch ZM-1, Durban, South Africa). The sugarcane leaves were optimally pretreated according to the previously established protocol (20 mL of 4.90% HCl added to 9.45 g of milled sugarcane leaves in a 250-mL Schott bottle). The contents were mixed and heated for 84 min at 99 °C using a PolyScience Analogue water bath. Timing was initiated once the set-point temperature of the substrate had been reached. The pretreated solution was adjusted to pH 7 using 10 M NaOH. A process flow diagram illustrating the conversion of sugarcane leaves into biohydrogen is shown in Fig. 1.

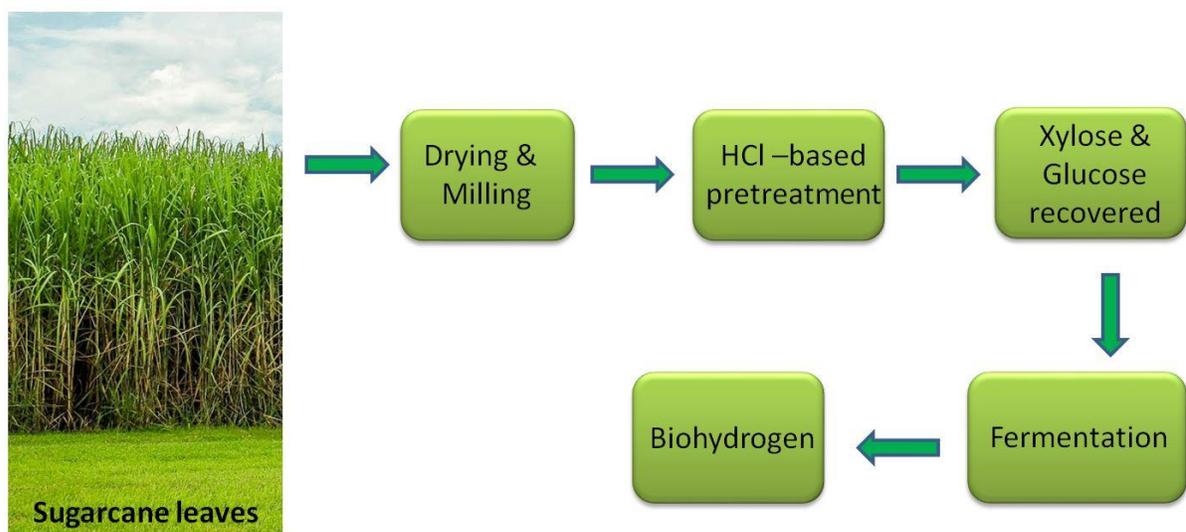


Fig. 1. Overview of the process flow diagram for the conversion of sugarcane leaves into biohydrogen

Experimental Design

The RSM Box-Behnken design using Design Expert (Stat-Ease, Minneapolis, Minnesota, USA) was used to model and optimize three physico-chemical input parameters for the production of biohydrogen.

Three independent variables, namely substrate concentration (A), inoculum concentration (B), and HRT (C), were considered; their ranges were 8 to 24 g/L, 10% to 50% v/v, and 24 to 96 h, respectively. Seventeen experimental runs were generated and carried out in duplicate (Table 1).

Batch Fermentation

Lab scale experiments

Fermentation experiments were carried out in modified 250-mL Erlenmeyer flasks with a working volume of 200 mL. All flasks were inoculated with anaerobic sludge and fed with varied volumes of pretreated sugarcane leaves and mineral salts to obtain a final substrate concentration as specified in the design. The supplemented mineral salts comprised the following (g/L): NH₄Cl 0.5, KH₂PO₄ 0.5, K₂HPO₄ 0.5, NaHCO₃ 4.0, FeCl₃ 0.15, MgCl₂·6H₂O 0.085, ZnSO₄·7H₂O 0.01, MnCl₂·4H₂O 0.03, H₃BO₃ 0.03, CaCl₂·6H₂O 0.01, and Na₂MoO₄·2H₂O 0.03. Prior to fermentation, the initial pH was adjusted to 6.5 using 1 M NaOH and 1 M HCl, and the flasks were flushed with nitrogen gas for 2 min and then tightly capped with rubber stoppers to promote anaerobiosis, as recommended by Van Ginkel *et al.* (2005). The fermentation processes were run at 37 °C and 180 rpm.

Semi-pilot scale

The semi-pilot scale process was carried out in a 13-L bioreactor (Labfors-INFORS HT, Bottmingen, Switzerland) with an 8-L working volume. The vessel was fed with 2600 mL of pretreated WSCL and 2788 mL of mineral salts, and inoculated with 2612 mL of thermally treated anaerobic sludge. The optimum operational set-points obtained in the modeling and optimization phase were applied to the bioreactor. The semi-pilot scale assessment was carried out in duplicate.

Monitoring of the Pilot Scale Experiment

The semi-pilot scale fermentation process was interfaced with the F-Lab Biogas monitoring system (Kana *et al.* 2013); thus, the evolving biogas fractions were monitored in real time at a sampling interval set to 1 min. The cumulative biogas volume was measured using a milligas counter (MGC, Bluesens, Germany). Aliquots from the process were sampled every 6 h and analyzed for pH change and xylose and glucose consumption.

Table 1. Box-Behnken Design with Observed Biohydrogen Yield

Run	Substrate Concentration (g/L)		Inoculum Concentration (% v/v)		HRT (h)		H ₂ yield (mL/g sugar)
	A	Code	B	Code	C	Code	
1	24.00	+1	50.00	+1	60.00	0	0.0098
2	16.00	0	10.00	-1	96.00	+1	5.62
3	16.00	0	10.00	-1	24.00	-1	4.01
4	8.00	-1	30.00	0	24.00	-1	0
5	24.00	+1	30.00	0	96.00	+1	0
6	24.00	+1	10.00	-1	60.00	0	0.073
7	24.00	+1	30.00	0	24.00	-1	0
8	16.00	0	50.00	+1	24.00	-1	5.95
9	16.00	0	30.00	0	60.00	0	12.62
10	16.00	0	30.00	0	60.00	0	13.41
11	16.00	0	30.00	0	60.00	0	12.94
12	16.00	0	30.00	0	60.00	0	13.56
13	16.00	0	30.00	0	60.00	0	8.45
14	16.00	0	50.00	+1	96.00	+1	5.35
15	8.00	-1	10.00	-1	60.00	0	7.34
16	8.00	-1	50.00	+1	60.00	0	9.25
17	8.00	-1	30.00	0	96.00	+1	4.04

Analytical Methods for Batch Experiments

The volume of the evolved gas during flask batch fermentation was monitored using the water displacement technique. In addition, the fraction of biohydrogen was analyzed using a hydrogen sensor (BCP-H₂, Bluesens, Herten, Germany) that employed the thermal conductivity measuring principle with measuring ranges of 0 to 100% vol. The cumulative volume of biohydrogen was calculated according to Eq. 1:

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

$V_{H,i}$ and $V_{H,i-1}$ represent the cumulative biohydrogen gas volumes at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fraction of biohydrogen gas in the headspace of the reactor flask in the current and previous time intervals, and V_H is the total volume of headspace in the reactor. Volatile fatty acids (VFAs) were quantified using gas chromatography–FID, as described by Faloye *et al.* (2013). The concentration of glucose and xylose were monitored using a YSI Biochemistry Analyzer (Model 2700, select-dual configuration, YSI, Yellow Springs, Ohio, USA).

Bacterial Community Analysis

DNA was extracted using a modified protocol previously described by Orsini and Romano-Spica (2001). One milliliter of sample was collected during peak production and suspended in 1 mL of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 0.1% [w/v] SDS, 0.1% [w/v] PVP, pH 8.0). The samples were centrifuged at 8000 rpm for 1 min, followed by supernatant removal and pellet suspension in 500 μ L of lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 3.0% [w/v] SDS, 1.0% [w/v] PVP, pH 8.0). Tubes were then heated at 90 °C for 10 min and rapidly cooled in liquid nitrogen. Pre-warmed (65 °C) extraction solution (500 μ L; 10 mM Tris-HCl, 1 mM EDTA, 300 mM sodium acetate, 1.0% [w/v] PVP) was added to the tube. This was followed by the addition of phenol : chloroform : isoamylalcohol (25:24:1) mixed by inversion, and DNA precipitation was achieved using isopropanol. The DNA pellets were washed with 70% ethanol and re-suspended in 100 μ L of TE buffer (pH 8.0). DNA quantification and purity were checked using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

The 16S rRNA gene fragment of extracted DNA was amplified by Polymerase Chain Reaction using the universal bacterial primer 27-F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492-R (5'-CGGTTACCTTGTTACGACTT-3'), whereas sequencing was performed using 907R (5'-CCGTCAATTCMTTGTGAGTTT-3') (Muyzer *et al.* 1995). PCR was carried out according to the following optimized profile: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 15 s, annealing at 53 °C for 15 s, and elongation at 68 °C for 25 s, with a final extension step of 68 °C for 5 min. Amplicons were resolved on a 2% agarose gel stained with SYBR Safe, and amplicon sizes were verified using a 1 Kb DNA ladder (Thermo Scientific, USA). The PCR products were subsequently ligated into the pMiniT vector and transformed into competent *E. coli* (New England Biolabs 10-beta) cells using an NEB PCR Cloning Kit (New England BioLabs, Ipswich, Massachusetts, USA) per the manufacturer's instructions. Inserts were validated by PCR using the specific forward primer (5'-ACCTGCCAACCAAAGCGAGAAC-3') and reverse primer (5'-TCAGGGTTATTGTCTCATGAGCG-3') with PCR conditions described above. Positive clones were selected for sequence analysis at Inqaba Biotec (Pretoria, South Africa). The sequences were then loaded on the NCBI database using

BLAST online software (NCBI, Bethesda, Maryland, USA) to determine sequence identity.

RESULTS AND DISCUSSION

Model Analysis

The analysis of variance (ANOVA) was performed to assess the significance of fit for the quadratic response surface model (Table 2).

The p-value is an indicator of the significance of each coefficient. Higher significance is inferred with larger F-values and smaller p-values (Chaganti *et al.* 2012). The quadratic model fit was significant given the F-value and p-value of 7.46 and 0.0074, respectively. A large F-value indicates that the aggression equation can account for the regression equation (Chaganti *et al.* 2012). The coefficient of determination (R^2) was 0.90, thereby inferring the model's ability to explain 90% of the variations in the data. As the R^2 value approaches 1.00, the model can predict the response with a higher degree of accuracy for used data. The p-values also indicate a statistically significant fit for the model, as values < 0.01 are generally considered significant (Wang and Wan 2008). The linear and interactive effect of the input parameters on hydrogen is shown by the regression Eq. 2:

$$H_2 \text{ yield} = +12.20 - 2.57A + 0.44B + 0.63C - 0.49AB - 1.01 AC - 0.55BC - 6.13A^2 - 1.90B^2 - 5.06C^2 \quad (2)$$

where A, B, and C represent the coded factors for substrate concentration, inoculum concentration and HRT respectively.

Table 2. Analysis of Variance for the Response Surface Quadratic Model

Source	Sum of Squares	Df	Mean square	F-value	P-value
Model	371.09	9	41.23	7.46	0.0074
A	52.7	1	52.77	9.55	0.0176
B	1.55	1	1.55	0.28	0.6132
C	3.19	1	3.19	0.558	0.4723
AB	0.97	1	0.97	0.18	0.6873
AC	4.08	1	4.08	0.74	0.4186
BC	1.22	1	1.22	0.22	0.6526
A ²	157.97	1	157.97	28.59	0.0011
B ²	15.24	1	15.24	2.76	0.1407
C ²	107.84	1	107.84	19.52	0.0031
Lack of fit	20.58	3	6.86	1.52	0.3395

Interactive Effect of the Input Parameters of Substrate Concentration, Inoculum Concentration, and HRT on H₂ Yield

The biohydrogen yield obtained from experimental runs ranged from 0 to 13.56 mL H₂ g⁻¹ fermentable sugar (FS), thus highlighting the sensitivity of hydrogen production to the considered input parameters (Table 1).

The response surface plots describing the regression model were generated from the deterministic equation (Eq. 2). These graphs visualized the interactive effect of input parameters on hydrogen yield (Figs. 2a-c). Their parabolic shapes indicated that the optimum conditions for hydrogen production were located within the optimization space.

At high substrate concentrations (>24 g/L; runs 1, 5, 6, and 7), low yields of hydrogen (< 0.073 mL H₂ g⁻¹ FS) were obtained, whereas higher yields (> 4.0 mL H₂ g⁻¹ FS) were observed with substrate concentrations in the range of 13 to 20 g/L (Table 1). The 5 repeated center point runs (run 9-13; 16 g/L substrate concentration, 30% inoculum concentration, 60 h HRT) gave yields ranging from 8.45 to 13.56 mL H₂ g⁻¹ FS. The obtained optimized yield value (12.5 mL H₂ g⁻¹ FS) was within this range. Because monomeric sugar can be considered an osmotically active substance, it has a great influence over the osmotic potential of the fermentation medium. Szymanowska-Powałowska (2015) proposed that high substrate concentrations may trigger the cell's defense mechanism, resulting in an intensified production of proteins that ultimately slow or stop the metabolic process. Increasing substrate concentration from 8 to 14 g/L, while maintaining inoculum concentration at 30%, enhanced hydrogen yield from 8.6 to 12.45 mL H₂ g⁻¹ FS. A similar yield pattern can be observed in Fig. 2b. An increase in substrate concentration from 8 to 14 g/L, while maintaining the HRT at 65 h, increased the hydrogen yield from 8.8 to 12.48 mL H₂ g⁻¹ FS. A simultaneous increase in substrate concentration and inoculum concentration from 8 to 16 g/L and 10% to 30%, respectively, resulted in an increase in hydrogen yield from 5.80 to 12.19 mL H₂ g⁻¹ FS. High substrate concentration has been reported to inhibit hydrogen production, while low substrate concentration has caused decreased microbial metabolic activity, thus resulting in a low hydrogen yield (Wang *et al.* 2015). In addition, a high substrate concentration has led to an accumulation of VFAs, considerably decreasing the pH of the medium and thus affecting the activity of the hydrogenase enzyme (Guo *et al.* 2010). The highest hydrogen yield was obtained using 16 g/L substrate concentration (run 12). This result is consistent with the studies by Lin and Cheng (2006). These authors reported an optimal substrate (xylose) concentration of 20 g chemical oxygen demand (COD) per liter when investigating in the range of 10 to 100 g COD/L.

As shown in Fig. 2a, an increase in the inoculum concentration from 10% to 33% while maintaining substrate concentration at 16 g/L resulted in a hydrogen yield increase from 9.85 to 12.21 mL H₂ g⁻¹ FS. Similarly, an increase in inoculum concentration from 10% to 32% (Fig. 2c), while maintaining the HRT at 60 h, increased the hydrogen yield from 9.85 g/L to 12.2 mL H₂ g⁻¹ FS. However, increasing inoculum concentration beyond the 33% threshold resulted in a hydrogen yield lower than the maximum yield of 12.5 mL H₂ g⁻¹ FS (Fig. 2c). Eker and Sarp (2016) found that higher hydrogen yields could be obtained with lower inoculum concentrations, though longer lag times were observed. Similarly, these authors reported that high cell concentrations resulted in higher amounts of VFA formation, thus reducing the pH of the fermentation medium and inhibiting hydrogen production. Furthermore, high inoculum concentrations can negatively affect hydrogen production because the evolving hydrogen would be consumed for acetic acid formation by homo-acetogenic bacteria (Phowan and Danvirutai 2014). A simultaneous increase in the inoculum concentration and the HRT from 10% to 30% and 24 to 65 h, respectively, improved the hydrogen yield from 3.5 to 12.20 mL H₂ g⁻¹ FS, a 4-fold increase (Fig. 2c). These findings agree with those obtained by Puad *et al.* (2015). These authors reported a 20% increase in the hydrogen fraction by increasing the inoculum concentration from 10% to 30%.

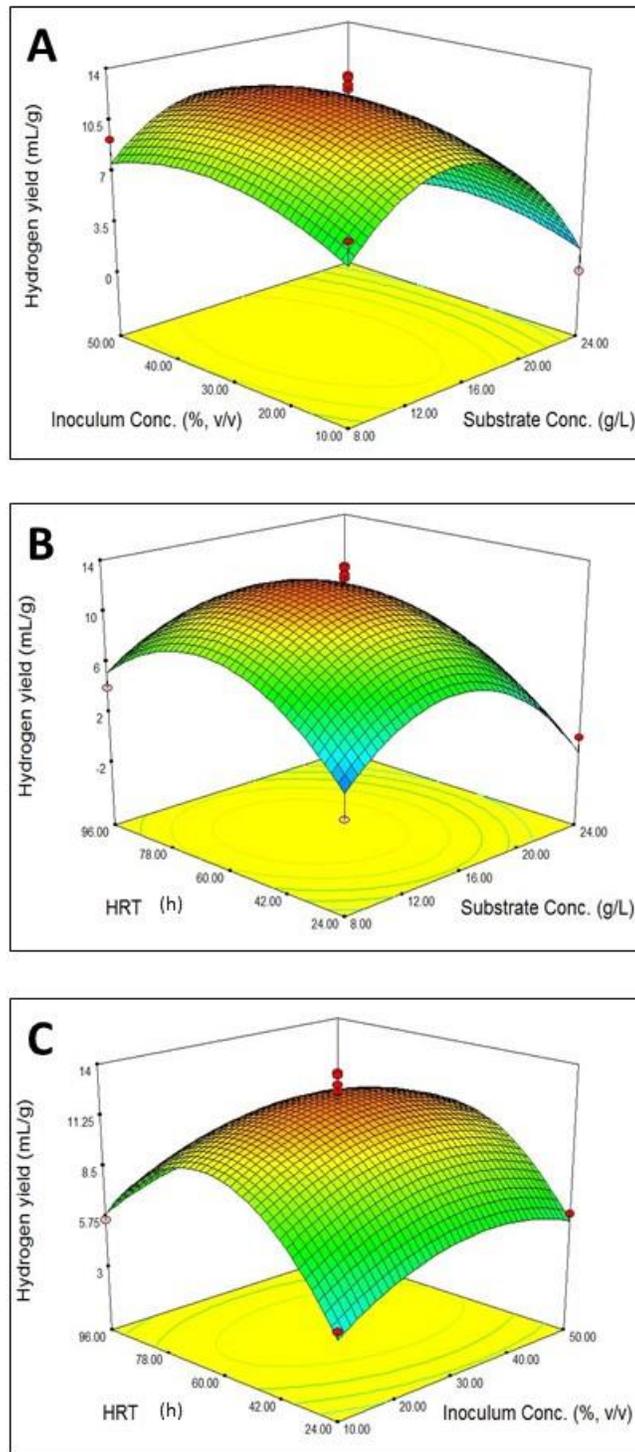


Fig. 2. Response surface plots showing the interactive effect of (a) inoculum and substrate concentration, (b) HRT and substrate concentration, and (c) HRT and inoculum concentration on hydrogen yield

Chaganti *et al.* (2012) obtained an optimal hydrogen yield of 2.4 mol/mol xylose with an inoculum concentration of 1800 VSS (mg/L) and a lower hydrogen yield (1.6 mol H₂/mol xylose) with an inoculum concentration of 1000 VSS (mg/L), thus suggesting that a 44% reduction in inoculum concentration led to a 50% increase in hydrogen yield.

Figure 2b illustrates that by increasing the HRT from 24 to 65 h, while maintaining the substrate concentration at 14 g/L, it was possible to enhance the hydrogen yield from 6.6 to 12.51 mL H₂ g⁻¹ FS. The hydraulic retention time beyond 65 h showed reduced hydrogen production. Similarly, Fig. 2c shows that an increase in the HRT from 24 to 65 h, while maintaining the inoculum concentration at 30%, increased the hydrogen yield from 6.51 to 12.48 mL H₂ g⁻¹ FS. A concomitant increase in HRT and substrate concentration from 24 to 62 h and 8 to 14 g/L, respectively, increased the hydrogen yield markedly, from 2 to 12.52 mL H₂ g⁻¹ FS (Fig. 2b). A low HRT (24 h) showed reduced hydrogen yields, whereas the maximum hydrogen was produced at 65 h. Low hydrogen production rates have been linked to low HRT, as previously reported by Lay (2000). Complex substrates such as food waste and sewage biosolids have been shown to require longer optimum HRTs (36 and 24 h, respectively) compared with glucose and sucrose, which require 2 and 6 h, respectively (Jutakanoke *et al.* 2012). The pretreated slurry used in this study contained monomeric xylose and glucose, as well as complex polymers of cellulose and hemicellulose, the latter of which accounted for the longer HRTs.

Validation of Optimized Process Condition

The model predicted a maximum yield of 12.5 mL H₂ g⁻¹ FS under the optimized operational conditions of 14.23 g/L substrate concentration, 32.68% inoculum concentration, and 62.77 h HRT. The experimental validation of these set-points carried out in triplicate gave 12.76 ± 0.65 mL H₂ g⁻¹ FS; thus, an acceptable prediction error of 5% was observed. The optimized hydrogen yield showed a 45% improvement from the non-optimized hydrogen run. These optimized operational conditions were subsequently used for the semi-pilot scale assessment.

Semi-Pilot Scale-Up of H₂ Production

The semi-pilot process showed a lag phase lasting 23 h, which is similar to the 20-h lag phase observed in the bioconversion of wheat straw to biohydrogen by Fan *et al.* (2006). A lengthy lag phase can be attributed to the reactor size and the nature of the substrate. For instance, Lin *et al.* (2011) observed a lag phase of 9 days using a 400-L reactor with a sucrose medium, whereas Sekoai and Kana (2014) reported a lag phase of 5 h in a 10-L reactor.

Figure 3 shows that glucose and xylose metabolism began simultaneously, with glucose being rapidly depleted within 7 h of fermentation from 1.7 to 0 g/L. Microbial cells have a preferential affinity for glucose substrate over xylose (Moodley and Kana 2015). Xylose degradation occurs either *via* the acetate or butyrate pathway; mixed cultures have, however, been shown to produce hydrogen using both metabolic pathways (Maintinguer *et al.* 2011). Hydrogen production began at 24 h and reached a peak concentration of 37% at approximately 66 h; thus, the exponential phase lasted for 42 h. A total cumulative volume of 3740 mL H₂ was obtained, corresponding to a yield of 321 mL H₂ g⁻¹ FS (49.9 mL H₂ g⁻¹ feedstock). Cui *et al.* (2010) reported a cumulative hydrogen yield of 34 mL with an exponential phase lasting approximately 20 h with acid-pretreated poplar leaves. In another study, hydrogen production from sugarcane bagasse hydrolysate showed an

exponential phase lasting 130 h and a hydrogen yield of 0.8 mol H₂/mol substrate (Fangkum and Reungsang 2011).

Compared with a previous investigation on biohydrogen production from sugarcane leaves with a yield of 248 mL H₂ g⁻¹ FS (Moodley and Kana 2015), the modeling and optimization of operational parameters of substrate concentration, inoculum concentration, and HRT in this study enhanced the hydrogen yield by 29%.

Acetic acid was the major VFA detected, followed by butyric acid at 306 and 78.74 mg/100 mL, respectively (Table 3), indicating that both degradation pathways occurred, with sugar conversion to acetate being the favored pathway. *Clostridium beijerinckii* and *Clostridium bif fermentans* have been shown to produce biohydrogen, accompanied with acetate and butyrate as the major by-products (Evans *et al.* 1998; Wang *et al.* 2003). Moreover, *Enterobacter cloacae* have also been shown to produce both acetic and butyric acid as by-products in hydrogen fermentation, with acetic acid being the major byproduct in lowering the culture's pH (Khanna *et al.* 2004). During hydrogen production, *Klebsiella* sp. has produced succinic and acetic acid with ethanol (Chookaew *et al.* 2014).

Hydrogen production declined when the pH decreased to 5.0 during the 65th hour of fermentation. Similar patterns have been observed by Chaganti *et al.* (2012), who reported a decrease in hydrogen yield from 1.6 to 0.3 mol⁻¹ xylose when the pH dropped from 7.6 to 5.0. A decrease in pH has caused acidogenic processes to shift to solventogenic processes, thus producing mostly acetone, butanol, and ethanol, which inhibit hydrogen production (Khanal *et al.* 2004).

Table 3. Concentration of Measured VFAs (mg/100 mL)

Acetic	Butyric	Iso-butyric	Propionic
306	7.74	71.0	0.00

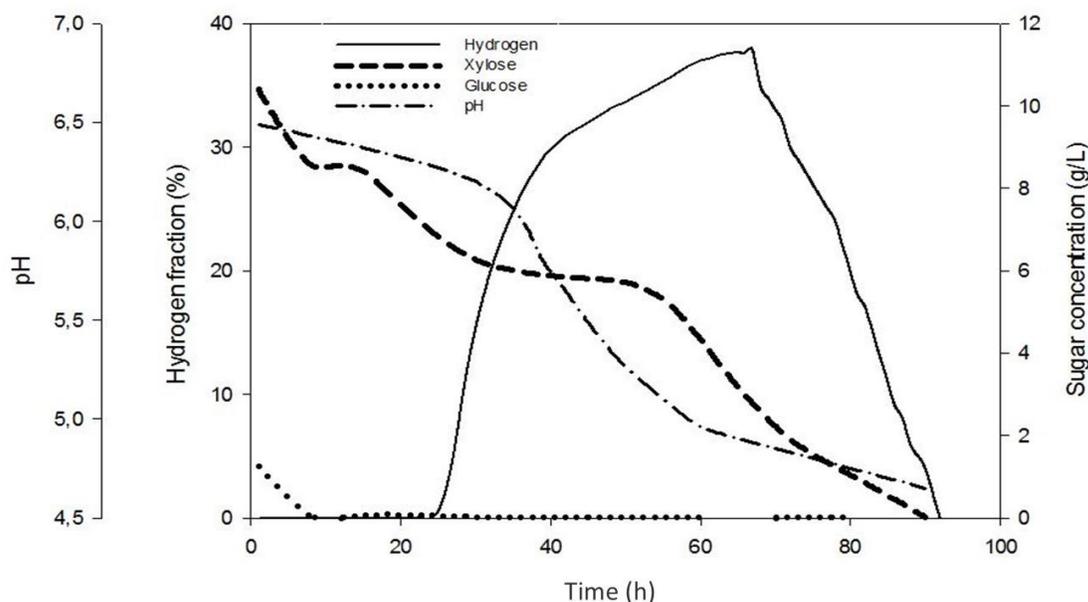


Fig. 3. Time course of hydrogen production, sugar consumption, and pH change for the semi-pilot scale-up process

Microbial Analysis

Analysis of the microbial diversity revealed the presence of *Clostridium* sp., *Klebsiella* sp., and *Enterobacter* sp. (Table 4). These microorganisms have been previously reported as the major hydrogen producers in a sludge inoculum system (Kraemer and Bagley 2007), and the results are similar to those reported by Song *et al.* (2012). These authors employed thermal treatment on a mixed consortium and reported a microbial community dominated by *Clostridium* sp. and *Enterobacter* sp. *Clostridium beijerinckii* and *C. bifermentans* are endospore-forming, Gram-positive bacteria and are likely to survive the heat pretreatment during the inoculum preparation (Baron 1996). Moreover, *C. beijerinckii* and *C. bifermentans* have been reported to produce large amounts of hydrogen (311.3 mL H₂ Lh⁻¹ and 2.1 mmol-H₂/g COD, respectively) using xylose and wastewater sludge substrates (Wang *et al.* 2003; An *et al.* 2014).

Kraemer and Bagley (2007) observed that not all non-spore forming cells were killed by heat treatment, which explains the presence of non-spore formers such as *Klebsiella* sp. and *Enterobacter* sp. The presence of species belonging to the *Enterobacter* and *Klebsiella* genii after heat treatment at 105 °C for 2 h was also observed in a study by Iyer *et al.* (2004). Because of their rapid growth rate and efficient substrate consumption, facultative anaerobes often dominate microbial communities during fermentation, thus out-competing other microbial species (Zhang *et al.* 2015). Their presence may, however, be beneficial for consuming residual oxygen in the reactor (Kraemer and Bagley 2007). It has been proposed that these groups of microorganisms are responsible for hydrogen production *via* the formate cleavage pathway (Zhang *et al.* 2015). The hydrogen-producing efficiency of *E. cloacae* was demonstrated by Sun *et al.* (2015), who reported a yield of 707 mL H₂/L.

Table 4. Phylogenetic Affiliation of 16S rDNA Gene Sequencing from Cloning Analysis

Microorganism	Access number (NCBI)	Similarity %
<i>Clostridium beijerinckii</i>	NR_029230.1	99
<i>Clostridium bifermentans</i>	NR_119066.1	98
<i>Enterobacter cloacae</i>	NR_102794.1	99
<i>Klebsiella oxytoca</i>	NR_102982.1	99
<i>Klebsiella pneumoniae</i>	NR_074913.1	98

Hydrogen Yield Comparison on Selected Lignocellulosic Substrates

A comparative assessment of the yield obtained using other lignocellulosic feedstocks is presented in Table 5. Zheng *et al.* (2014) reported on the enzymatic pretreatment of poplar leaves and obtained 44.9 mL H₂/g feedstock. Enzymes, however, incur additional costs, thus reducing commercial feasibility. Considerably lower yields were observed for rice straw and barley hulls (24.8 and 29.2, respectively), which could be attributed to the low release of fermentable sugar because of the non-pretreatment of the feedstock with a higher and lower content of lignin and cellulose, respectively. These data indicated that more hydrogen was produced from low-cost sugarcane leaves than other agricultural lignocellulosic residues, as shown in Table 5.

Table 5. Hydrogen Yield from Various Lignocellulosic Feedstocks

Feedstock	Hydrogen Yield (ml H ₂ /g feedstock)	Reference
Rice Straw	24.8	Chen <i>et al.</i> 2012
Barley hulls	29.2	Magnusson <i>et al.</i> 2008
Soybean straw	60.2	Han <i>et al.</i> 2012
Grass	39.5	Cui and Shen 2012
Poplar leaves	44.92	Cui <i>et al.</i> 2010
Sugarcane leaves	49.87	This study

CONCLUSIONS

1. This study modeled and optimized the key operational parameters of substrate concentration, inoculum concentration, and HRT for biohydrogen production using pretreated waste sugarcane leaves.
2. The generated model showed a good fitness and generated the optimal operational conditions of 14.2 g/L substrate concentration, 32.7% inoculum concentration, and 62.8 h HRT.
3. The scalability of this process was demonstrated at a semi-pilot scale, where a biohydrogen yield of 321 mL H₂ g⁻¹ FS was obtained, indicating a 23% improvement compared to the non-optimized process.
4. Sugarcane leaves were shown to be a suitable feedstock for biohydrogen production with higher hydrogen yields compared with other selected agricultural wastes. These findings highlighted an alternative option for managing waste sugarcane leaves through dark fermentative hydrogen production.

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