

The Impact of Enzymatic Hydrolysis of Sewage Sludge as a Pre-treatment for Dark Fermentation

Saleh Al-Haddad, Cynthia Kusin Okoro-Shekwaga, Louise Fletcher, and Miller Alonso Camargo-Valero

Abstract—For many years, sewage sludge has been processed for methane production in anaerobic digestion reactors at wastewater treatment plants around the world. Sewage sludge is produced in large quantities and is rich in biodegradable organic materials, from which sugars (e.g., glucose) can be produced, recovered and used as a substrate to support hydrogen production through the Dark Fermentation (DF) process. DF is one of several methods used for bio-hydrogen production, whereby fermentative bacteria are used to hydrolyse organic substrates to produce hydrogen gas. Carbohydrates (sugars) is one of the main fermentable substrates for hydrogen production, and they are considered the most favourable substrate for fermentative bacteria (e.g., *Clostridium* bacteria). Although sewage sludge is rich in organic materials, still the complexity of its structure and low carbon/nitrogen ratio limits the bio-hydrogen production via DF processes. Therefore, this paper addresses the impact of Enzymatic Hydrolysis (EH) as a pre-treatment of sewage sludge on enhancing the biodegradability and glucose content in sewage sludge. The result shows that using the EH process as pre-treatment for sewage sludge, enhanced the glucose content in sewage sludge and converted some of the macro sewage flocs to easy digestible micro flocs (glucose). Therefore, the substrate being more favourable and easier to digest by bacteria in the DF reactor, enhanced the production of hydrogen and VFAs. More research needs to be done to find the optimum enzyme dosage, initial substrate concentration and operation temperature (especially when the enzyme is used inside the DF reactor).

Index Terms—Anaerobic digestion, dark fermentation, enzymatic hydrolysis, sewage sludge.

I. INTRODUCTION

It is considered more sustainable to produce hydrogen via biological processes than conventional processes such as natural gas decomposition, petroleum oxidation and coal gasification. Biological hydrogen production processes do not require the same fossil-fuel energy inputs, therefore, they emit much less carbon dioxide overall. Bio-hydrogen production can contribute to the net reduction of GHG emissions [1] and provide an alternative and more sustainable option to waste management without any dependency on carbon energy sources. Indeed, bio-hydrogen production can utilise a wide range of substrates and requires relatively low-cost operating conditions, such as ambient temperature,

atmospheric pressure and no need for external energy [2], [3].

DF is one of several methods used for bio-hydrogen production, whereby fermentative bacteria hydrolyse organic substrates to produce hydrogen gas. One of the main fermentable substrates for hydrogen production is carbohydrates (sugars), as they are considered the most favourable substrate for fermentative bacteria (e.g., *Clostridium* bacteria) [4]. Several studies have reported high hydrogen production in DF processes using sugars such as glucose as substrate [5]-[7]. Sugars naturally exist in plants, and they are used extensively in food processing industrial activities [8]. Although biohydrogen can be produced from plant-based sugars using DF processes, this practice would directly compete with food production, just as current biodiesel and bioethanol production competes with soybeans and sugar cane production for food. Therefore, the direct use of plant-based sugars as a substrate for hydrogen production via DF processes is not advised.

As mentioned earlier, DF can utilise organic waste for hydrogen production. Sewage sludge has the potential to be a sustainable source for glucose production as an intermediate product to support hydrogen production: Champagne [9] reported that 6.22 Mt/yr of sugar can be produced from municipal sludge and livestock manures generated in Canada. Although the current route for sewage sludge processing at Wastewater Treatment Works (WWTWs) is via Anaerobic Digestion (AD), there is still an opportunity to move towards sugar production to support biohydrogen production.

Due to the complexity of sewage sludge contents, including a low C/N ratio, fermentative bacteria will find it very difficult to transform the sugars into hydrogen [10]. To overcome this hurdle, the pre-treatment of sewage sludge is an essential step towards efficient biohydrogen production in the DF process. Several studies have reported different pretreatment methods to enhance hydrogen production from sewage sludge [11]. The disintegration of sewage sludge is among the methods that can break down the hard-to-digest macro sewage flocs to more easily digestible micro flocs as shown in Fig. 1. As a result, pre-treated sewage sludge biomass will have a suitable fermentable structure that can be easily utilised by fermentative bacteria for hydrogen production. Disintegration can be achieved by four methods: mechanical, physical, chemical and biological. However, biological (including enzymatic hydrolysis) is preferred to mechanical, chemical and physical pre-treatments, as it requires lower energy inputs than the other three. Moreover, its ability to reduce sludge volume and improve hydrogen production from sewage sludge has been proven [12], [13]. This study aims to determine the potential of increasing glucose (a favourable substrate for fermentative bacteria) content in sewage sludge by the EH process.

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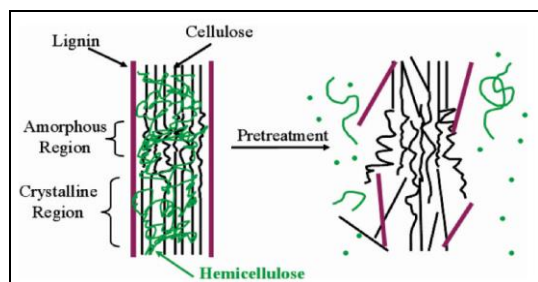


Fig. 1. Effect of enzymatic hydrolysis in breaking down the complex structure of lignocellulosic biomass in sewage sludge, source [14].

II. METHODOLOGY

A. Feedstock Source and Enzyme

Hydrolysed Sewage Sludge (HSS), collected from the effluent of the Hydrothermal Treatment Plant (HTP) at Yorkshire Water's Esholt WWTW, Bradford, UK, was used as the feedstock for the EH test. Fig. 2 shows the steps adopted in Esholt Wastewater Treatment Works to process sewage sludge. The HSS was initially filtered using a 1-mm sieve to remove large particles and then characterized for measuring volatile solids. The HSS sample was stored in a freezer at $-22\text{ }^{\circ}\text{C}$ to be used as feedstock for the EH experiments. Cellulase, an enzyme blend purchased from Sigma-Aldrich was used as the enzyme source for the EH pre-treatment.

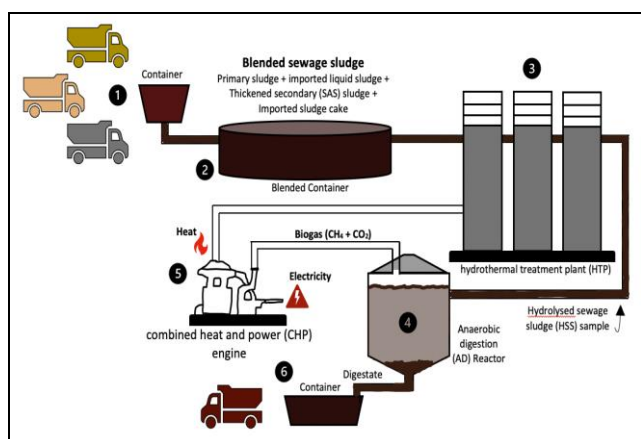


Fig. 2. Sewage sludge processing at Esholt WWTW, Bradford, UK.

B. Analytical Tests and Experimental Set-up

1) Glucose and liquid analysis

Benedict's quantitative method was selected and used to detect and quantify glucose in the liquid. Benedict's quantitative reagent can help to determine and quantify the glucose concentration in a solution by changing from a deep-blue colour (no glucose content) to colours ranging from mid-blue (traceable glucose) to very light blue (high glucose content). The experimental setup was divided into two parts to achieve the aim of this section, as described below.

a) Part 1: Determining the optimum wavelength for Benedict's quantitative reagent

It was critical to find the optimum wavelength, especially when Benedict's reagent was mixed with HSS, as the colour changes to a range of different blue-green colours, which can affect the absorbance reading. A spectrophotometer

(Shimadzu UV1900) was used to measure the absorbance value at wavelengths between 620 nm and 840 nm to find the optimum one for different sample compositions, as shown in TABLE I. The total volume and composition of the liquid affect the final colour; hence, prepared samples were divided into two groups: group A with 6 mL and group B with 7 mL (total volume). These volumes were selected based on the amount of Benedict's reagent required for detecting 10 mg of glucose in a sample [15]. Different volumes were used for each substance to cover the possible scenarios that can happen during an EH test. The composition of the samples consists of Benedict's reagent (B) 5 mL and one or all of the following: Distilled water (DW), HSS and glucose 1% solution (G) (10 g glucose in 1 Litre of DW), as shown in Table I.

TABLE I: COMPOSITION OF SAMPLES USED FOR OPTIMUM WAVELENGTH TEST

ID	1% glucose solution	Distilled water	HSS	Benedict's quantitative reagent	Total volume	Expected glucose concentration added
	mL	mL	mL	mL	mL	mg/mL of 1% glucose solution
A1	1	0	0	5	6	10
A2	0.5	0.5	0	5	6	5
A3	0.1	0.9	0	5	6	1
A4	0	1	0	5	6	0
B1	0	2	0	5	7	0
B2	1	1	0	5	7	10
B3	1	0	1	5	7	10

HSS: hydrolysed sewage sludge

1% glucose standard solution: (10 g of D-glucose powder in 1 litre of DW)

After sample preparation, the following procedures were carried out to calculate the absorbance value for each wavelength:

- 1) All test tubes were mixed well and placed in hot blocks for 25 min at $80\text{ }^{\circ}\text{C}$ to complete the reaction. Then, they were removed from the hot blocks and placed in a tube holder and allowed to cool to room temperature.
- 2) All the samples were centrifuged using Eppendorf 5810 at 4000 rpm for 10 min and then filtered by a $0.45\text{ }\mu\text{m}$ syringe filter.
- 3) The absorbance for all samples was measured against a blank sample (D.W) ($\text{Abs} = 0$) using Shimadzu UV1900 at different wavelengths between 620 nm and 840 nm.

b) Part 2: Standard and modified glucose curve

A glucose standard curve is essential for calculating the glucose concentration in a solution as it explains the relationship between glucose concentration and the absorbance value of the sample at the optimum wavelength. Determining glucose concentration by Benedict's quantitative method requires the absorbance value to be measured, after which the glucose concentration must be found using the glucose standard curve. To maximise the accuracy of Benedict's method, a modified glucose curve was calculated as Benedict's colour is affected by HSS, changing to a range of colours between blue and blue-green. Two Total Chemical Oxygen Demand (TCOD) of HSS (5 and 10 g TCOD/L) were used to calculate different modified glucose curves. All curves (standard and modified) were set up to cover range 0–10 mg glucose/mL of the sample and were calculated by the following procedures:

- 1) Seven glass tubes (50 mL) were used to prepare seven test samples as shown in Tables II and III; the two experiments were run separately but followed the same procedures.
- 2) The seven tubes contained the following:

TABLE II: SET-UP DETAILS FOR DETERMINING STANDARD GLUCOSE CURVE

Glucose standard curve (0–10 mg/mL)	ID	1	2	3	4	5	6	7
mg/mL		10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	6	6	6	6	6	6	6

1% glucose solution: (10 g of D-glucose powder in 1 litre DW)

TABLE III: SET-UP DETAILS TO ESTABLISH MODIFIED GLUCOSE CURVE

Modified glucose curve (0–10 mg/mL)	ID	1	2	3	4	5	6	7
mg/mL		10	8	6	4	2	1	0
Glucose solution (1%)	mL	1	0.8	0.6	0.4	0.2	0.1	0
HSS (5 or 10g TCOD/L)	mL	1	1	1	1	1	1	1
Distilled water	mL	0	0.2	0.4	0.6	0.8	0.9	1
Benedict's reagent	mL	5	5	5	5	5	5	5
Total solution volume	mL	6	6	6	6	6	6	6

1% glucose solution: (10 g of D-glucose powder in 1 litre DW)

- 3) All tubes were mixed well and placed in hot blocks for 25 min at 80 °C to complete the reaction.
- 4) Then, the tubes were removed from the hot blocks, placed in the tube holder and allowed to cool to room temperature.
- 5) All the samples were centrifuged using Eppendorf 5810 at 4000 rpm for 10 min and then filtered by 0.45 µm syringe filter.
- 6) The absorbance of samples was measured against a blank sample (just distilled water, Abs = 0) at wavelength 740 nm (optimum wavelength from the results presented in Part 1).

The liquid samples produced from EH tests were characterised using the following methods and equipment. The pH was measured by a digital pH meter (HACH HQ40D). Soluble chemical oxygen demand (s) and TCOD were measured by AP3900 Laboratory robot with COD kit (LCK 514: COD cuvette test 1000-2000 mg/L O₂). For sCOD, The sample was centrifuged by using centrifuge Eppendorf 5810 at 4,000 rpm for 5 min to separate the solid and liquid.

2) Enzymatic treatment of hydrolysed sewage sludge

Two concentrations of HSS (5g and 10g of TCOD/L), with different enzyme dosages between 1 and 7 mL, were used to assess the effect of enzyme dosage on glucose production during the EH process. Table IV shows the set-up details of this experiment, while Table V summarises the tests conducted to monitor and analyse the EH processes carried out during this experiment.

TABLE IV: EH EXPERIMENT SET-UP DETAILS (TESTS A AND B ARE IN TRIPPLICATE)

	Unit	Blank	Control A	Control B	Test A	Test B
HSS (TCOD)	g/L	None	5	10	5	10
Enzyme (Cellulase blend)	mL	1-7	None	None	1-7	1-7
Working volume	mL	250	250	250	250	250
Operation time	hrs.	4	4	4	4	4

HSS: Hydrolysed sewage sludge

TABLE V: TESTING POINTS DURING THE EH TEST

Parameter/Time	9:30	10:30	11:30	12:30	13:30
Sample test size (mL)	15	8	8	15	8
pH	x	x	x	x	x
TCOD	x				
sCOD	x			x	
Glucose concentration	x	x	x	x	x

TCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand

(x) indicating the parameter was testing at the allocated time

III. RESULT AND DISCUSSION

A. Optimum Wavelength

Each colour has a specific wavelength that exhibits the greatest absorbance; therefore, the wavelength range of 620–840 was selected (according to the deep-blue colour of quantitative Benedict's reagent) and used to determine the optimum wavelength for quantitative Benedict's reagent. According to Fig. (3), it was observed that the optimum wavelength was 740 nm for all the seven different samples tested. A similar result was reported by [16], who tested a wide range of wavelengths (490–890 nm) and also reported 740 nm as the optimum wavelength for Benedict's reagent. Fig. (3) shows that the absorbance value changed with changes in the wavelength and sample composition, but the maximum absorbance was achieved at 740 nm wavelength for all the samples (A1–A4 and B1–B3).

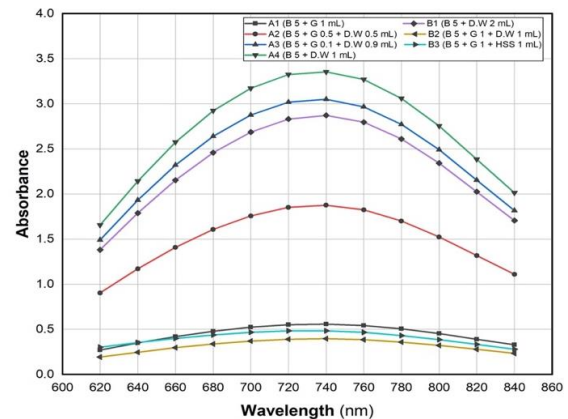


Fig. 3. The optimum wavelength test for different sample compositions (B: Benedict's reagent, G: 1% glucose solution, HSS: hydrolysed sewage sludge and DW: distilled water) (the absorbance is the average value of the duplicate).

The total volume of the sample (6 or 7 mL) also has an impact on the absorbance value, as shown in Fig. (3), where sample B1 (5 mL of B + 2 mL of DW) had a lower absorbance value (2.872) than sample A4 (5 mL of B + 1 mL

of DW) (3.353). Thus, increasing the total volume of the sample by 1 mL DW had a negative impact on the absorbance value: the solution's colour changed, becoming lighter than the A4 sample. Therefore, the final volume of the sample has an effect on the final concentration of glucose due to the relevant dilution. Moreover, the more G a sample contains, the lighter in colour the solution becomes after the reaction is complete, as shown in Fig. 3, where a comparison between samples A1–A4 (total volume = 6 mL) shows the impact of G amount in the solution on the B colour. The A4 sample, which had no G in it, had the maximum absorbance of all four samples, while the A1 sample, with 1 mL of G, had the lowest absorbance value among A samples because the deep-blue colour disappeared due to the reaction between B and G.

Fig. 4 also shows the effect of adding other substances to the solution (such as HSS or DW) on the B colour. HSS and DW had a negative impact by decreasing the absorbance value, which affected the result of the test. Furthermore, the comparison between B2 and B3 samples in Fig. 4 shows that adding a coloured substance, such as HSS (which is yellow-brown), can affect the result of Benedict's test, which could affect the glucose concentration result when the absorbance value is used in a glucose standard curve. Therefore, testing the absorbance for a range of samples with different compositions is an essential step during Benedict's quantitative test.

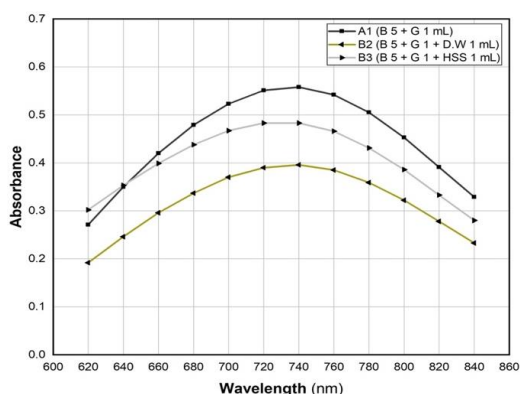


Fig. 4. Optimum wavelength test: the effect of adding HSS or DW to the absorbance value of B colour (B: Benedict's reagent, G: 1% glucose solution, HSS: hydrolysed sewage sludge and DW: distilled water) (the absorbance is the average value of the duplicate).

B. Glucose Standard Curve

After measuring the absorbance of the unknown sample concentration, the standard curve for the known sample concentration was created. For a glucose standard curve, a series of dilutions for 1% glucose solution (G) (10g in 1 Litre of DW) was prepared, as shown in Table II. After the reaction between these samples and Benedict's reagent was complete, the absorbance was measured at the optimum wavelength (740 nm). Fig. 5 shows the negative linear relationship between the absorbance and glucose concentration: the highest absorbance is for the sample without glucose content (deep-blue colour), while the lowest absorbance is for the sample with 1 mL of 1% G (very light-blue colour). Linear regression fitting was applied using OriginPro 2018b software, and the $R^2 = 0.9995$, which is considered an excellent fitting to the test results.

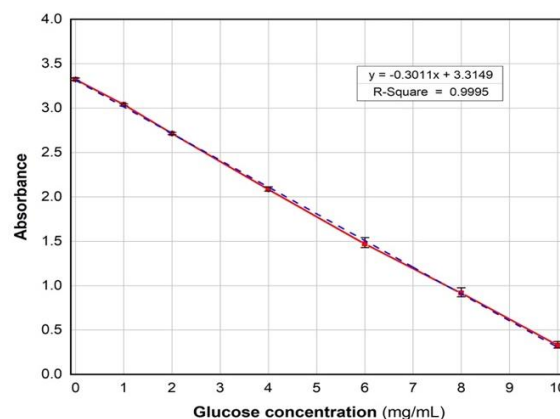


Fig. 5. Glucose standard curve with fitting curve at 740 nm wavelength (average value of triplicate with max/min bar).

C. Modified Glucose Curve

Adding a coloured substance, such as HSS (which is yellow-brown in colour), can affect the result of Benedict's test. Therefore, the glucose concentration result will be affected when the absorbance value is used in the glucose standard curve. Thus, creating a modified glucose curve may enable glucose concentration to be determined more accurately when using Benedict's method. In this study, two concentrations were used for HSS (5 and 10 g of TCOD/L) to create modified glucose standard curves, as shown in Fig. 6 and Fig. 7, both of which had high R^2 . These modified curves were used later in the EH experiments to determine the glucose concentration in a solution that has the same HSS concentration. To ensure accurate results, it is recommended that a new modified glucose curve be created for any specific HSS TCOD concentration before an EH experiment.

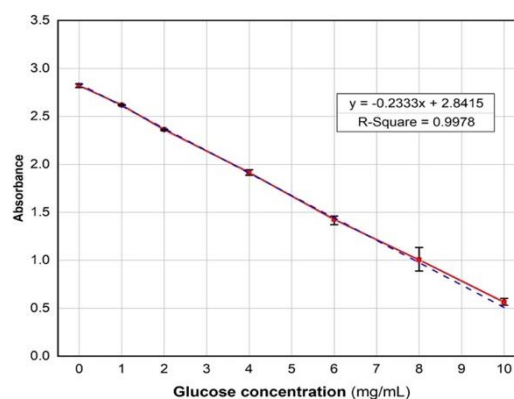


Fig. 6. Modified glucose curve: TCOD of HSS = 5 g/L, with a fitting curve at 740 nm wavelength (average value of triplicate with max/min bar).

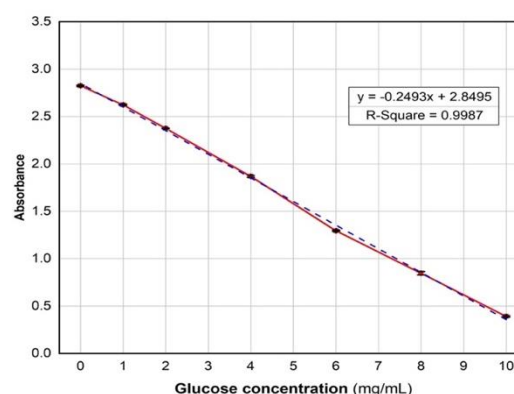


Fig. 7. Modified glucose curve: TCOD of HSS = 10 g/L, with fitting curve at 740 nm wavelength (average value of triplicate with max/min bar).

D. Enzymatic Hydrolysis Test

1) Sugar content in cellulase enzyme

Enzymes can disintegrate hard-to-digest macro sewage flocs to more easily digestible micro flocs in the EH process under specific operating conditions [12], [13]. Some commercial enzymes have glucose in their content, such as the enzyme blend used in this study, cellulase (from Sigma-Aldrich) whereby 26% (average value) of the enzyme was made up of sugars, as shown in Table VI.

TABLE VI: SUGAR CONTENT IN CELLULASE ENZYME

Enzyme ^a dose (mL) /250 mL of D.W	Unit	1	2	3	4	5	6	7
Sugar concentration	mg/L	1364	2474	3788	4837	6328	7312	8196
Sugar mass/250 mL	mg	341	619	947	1209	1582	1828	2049
Sugar	%	28	26	26	25	26	25	24

^aCellulase, enzyme blend (commercial enzyme purchased from Sigma-Aldrich)

Fig. 8 shows sugar concentrations at each enzyme dosage. All reactors were inoculated with a specific enzyme dosage (mL) at the start of the experiment (0 hours), and the curves show a maximum sugar concentration that remains relatively constant after 1 hour of operation, as there were no bacteria to consume the sugar and transfer it to gases and VFAs. Fig. 9 shows the linear relationship between sugar concentration and enzyme dosage with a fitting curve with an R2 value of 0.999.

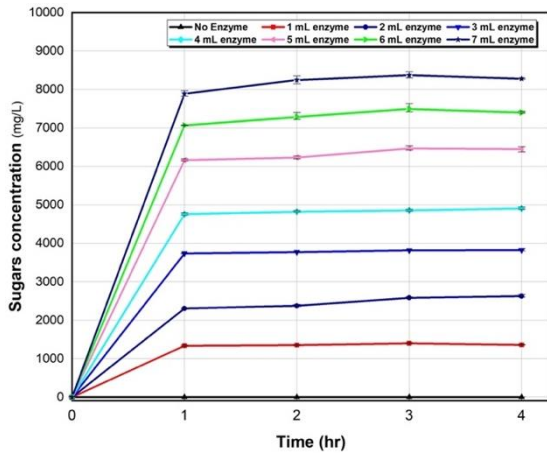


Fig. 8. Sugar concentration for cellulase enzyme vs time (average value of triplicate with max/min bar).

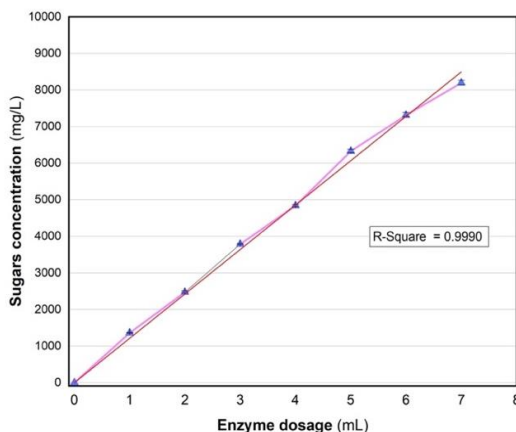


Fig. 9. Sugar concentration vs cellulase enzyme dosage with fitting curve (average value of triplicate with max/min bar).

2) Glucose production

Seven different enzyme dosages (1–7 mL in 250 mL DW) were used to find the optimum enzyme dosage for maximum glucose production from HSS. The results presented in Fig. 10 shows the glucose production having subtracted the yield from the corresponding blank sample containing only the enzyme and DW, as shown in Table VI. As it was necessary to remove the glucose content in cellulase and it was difficult to remove it physically. Fig. 10 shows that cellulase was able to release glucose from HSS. EH is a rapid process, as four hours of operation time was enough to reach the maximum glucose production. Despite the fluctuation of glucose concentration between one and four hours, the maximum glucose concentration occurred at 1 hour, as shown in Fig. 10. On the one hand, this rapid reaction is advantageous for the EH process, as it takes only a short time to convert some of the sewage flocs (cellulose) to glucose, which benefits bio-fuel industries. On the other hand, however, it is difficult to maintain the glucose in the reactor for a long time, as it is highly likely to be consumed by bacteria that exist in HSS and converted to VFAs and/or biogas (which may explain the fluctuation in glucose concentration after 1 hour shown in Fig. 10).

Several parameters can influence enzyme activity and hydrolysis rate in EH. For example, operating temperature and pH are important to maximise glucose production in EH, as enzymes become active in a certain range of temperature and pH. For cellulase enzyme blend that used in this study, the optimal operating temperature is between 45 °C and 55 °C [17], while the optimal range for pH is 4.5–5.0 [18], [19]. In this experiment, 37 °C was used for EH, as this was the temperature used for DF for hydrogen production, as presented in the following chapter. It is challenging to find the optimal temperature for a DF reactor that has enzymes, as the optimal temperature for enzymes is 45–55 °C, while most DF reactors operate at 37 °C, as this is considered the optimal temperature for hydrogen production. Although some DF reactors operate under the thermophilic range (50–65 °C), this is not appropriate for commercial-scale production as it will consume more energy.

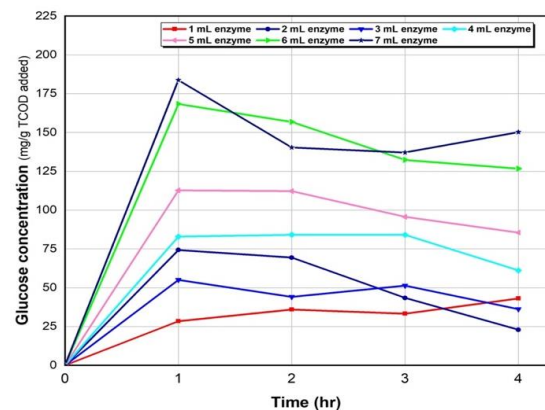


Fig. 10. Glucose production via the enzymatic hydrolysis process for hydrolysed sewage sludge (without blank: enzyme only) (average of triplicate).

Fig. 11 shows the pH behaviour during the EH experiment. At a lower substrate concentration (HSS 5g TCOD/L), the enzyme affected pH: immediately after the inoculation process (after 0 hours), the pH dropped and then started to

recover until 3 hours into the experiments. Thereafter, the pH started to drop again due to the accumulation of VFAs, as there were some bacteria in the HSS that started to consume glucose and transfer it to VFAs. As shown in Fig. 12, the inoculation process for different enzyme dosages did not have a big effect on the pH condition, as the substrate concentration was higher (HSS 10g TCOD/L), which helps to overcome the effect of enzyme pH. For enzyme dosages of 1-3 mL, there was a slight increase in pH, while an enzyme dosage of 4 mL did not change the pH, and enzyme dosages of 5-7 mL caused a slight decrease in the pH. Moreover, the same drop in pH happened again after 3 hours, as the pH started to drop due to the activity of bacteria in degrading the produced glucose.

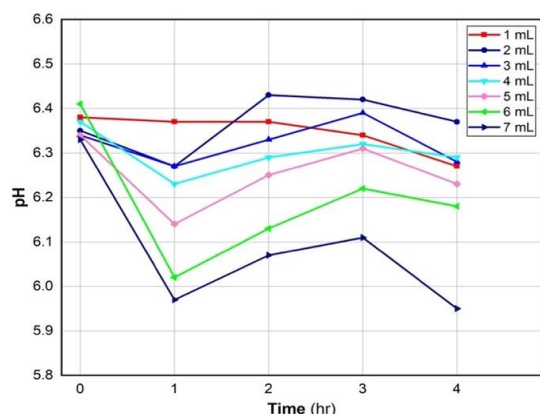


Fig. 11. pH curve for HSS 5g TCOD/L (average of triplicate).

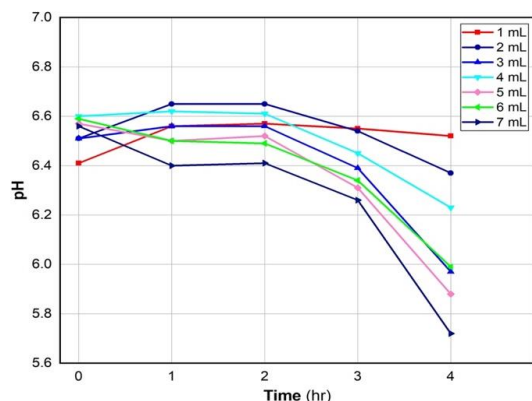


Fig. 12. pH curve for HSS 10g TCOD/L (average of triplicate).

Glucose is the product of the saccharification process for cellulose material [20]. Most studies have reported glucose yield according to cellulose content in the substrate; in this study, however, the glucose yield is reported according to TCOD.

The glucose content for untreated HSS was zero, as shown in Fig. 6 and Fig. 7, where 5g and 10g TCOD HSS were added to Benedict's reagent. The results for both show zero interaction between HSS and Benedict's reagent before and after the EH test, which confirms the absence of glucose in the HSS. The reason for this is that glucose is part of cellulose, which is a linear polymer of cellobiose (glucose-glucose dimer) [20]. Hamelinck, Van Hooijdonk [21] reported that the difficulty in breaking this polymer is due to the rigidity derived from the orientation of the linkages and hydrogen bonding in cellulose. Therefore, glucose cannot react with Benedict's reagent unless the cellulose is treated by the EH process.

Fig. 13 shows that treating HSS by the EH process results in the breakdown of cellulose material in HSS; therefore, glucose starts to be released and detected by Benedict's test. The glucose yield and enzyme dosage were calculated according to TCOD added (5g or 10g/L) of HSS.

After removing the blank value (glucose yield from the reactor that had only enzyme and DW) from the test value (HSS + enzyme + DW), the result in Fig. 13 shows the effect of EH on glucose production. The fitting curve, with an R^2 value of 0.969, shows the linear relationship between enzyme dosage and glucose yield: the more enzyme used, the more glucose was produced.

The fluctuation in the glucose yield curve is due to the influence parameter that affects the EH process and enzyme activity such as substrate concentration.

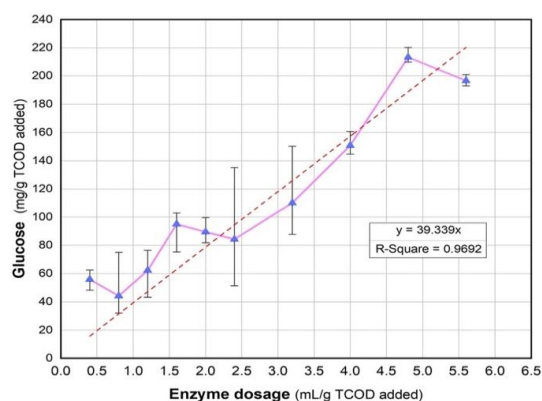


Fig. 13. Glucose yield vs enzyme dosage (without blank: enzyme only) (average of triplicate).

As shown in Fig. 14, the lower HSS concentration (5g TCOD/L) had a higher glucose yield than 10g TCOD/L for enzyme dosage (2-7 mL). The maximum percentage increment was 84% for both enzyme 2 mL and 5 mL at HSS 5g TCOD/L. These results agree with the findings of [20], who reported a 50% increase in glucose yield when the substrate concentration (newspapers and scrap paper) was reduced from 15 g/L to 5 g/L using cellulase enzyme. Moreover, there was an increase in glucose yield for different substrates when the substrate concentration decreased, 43.6% (carrot peeling), 35% (potato peeling), 24.6% (grass) and other substrates [20]. Similar results were reported by [22], who enhanced glucose yields by using a low substrate concentration and concluded that high substrate concentration can inhibit the EH process, although this inhibition is subject to the ratio of total substrate to the total enzyme used [23], [24].

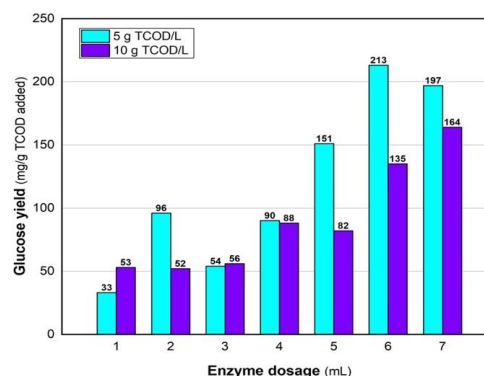


Fig. 14. Substrate concentration (HSS) effect on glucose yield, (without blank: enzyme only) (average of triplicate).

Fig. 14 shows the optimum dose reached for 6 mL enzyme added to a solution containing 5g TCOD/L, with a sugar yield of 213 mg/g TCOD. However, more research is needed to find the optimum HSS concentration to maximise glucose production using cellulase enzyme.

IV. CONCLUSION

Benedict's method has been widely used in laboratories to detect sugars (e.g., glucose) in a solution. This study assessed Benedict's quantitative method to detect and quantify glucose content in a solution that contains HSS. With some modifications (finding the optimum wavelength for a mixed sample and modified glucose curve), Benedict's quantitative method can be more reliable and more accurate than the original Benedict's method, for measuring glucose concentration in HSS samples.

In the work described in this study, the final volume of the sample affected the final concentration of glucose, due to the relevant dilution. Therefore, finding the optimum wavelength should be the first step in any future works that use Benedict's method, as this will give more accurate results. Moreover, creating a modified glucose curve is another approach to ensure accurate glucose concentration measurements in an EH test. The work described in this study demonstrates the effect of the HSS on Benedict's reagent colours and how HSS can negatively impact glucose measurement results.

Using the EH process as pre-treatment for HSS enhanced its glucose content and converted some macro sewage flocs to easily digestible micro flocs (glucose). Therefore, the substrate will be better and more easily digested by bacteria in a DF reactor, which will lead to enhanced production of hydrogen and VFAs.

More research needs to be done to find the optimum enzyme dosage, initial substrate concentration and operation temperature (especially when an enzyme is used inside a DF reactor).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Saleh Al-Haddad is the main writer of the paper, Miller Alonso Camargo-Valero, Cynthia Kusin Okoro-Shekwaga and Louise Fletcher contributed to editing the manuscript. All authors has approved the final version.

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REFERENCES

[1] R. Lukajtis *et al.*, "Hydrogen production from biomass using dark fermentation," *Renewable and Sustainable Energy Reviews*, vol. 91, pp. 665-694, 2018.

[2] A. Singh *et al.*, "Biohydrogen production from lignocellulosic biomass: Technology and sustainability," *Energies*, vol. 8, no. 11, pp. 13062-13080, 2015.

[3] I.K. Kapdan and F. Kargi, "Bio-hydrogen production from waste materials," *Enzyme and Microbial Technology*, vol. 38, no. 5, pp. 569-582, 2006.

[4] B. J. Finlay, *Ecology and Evolution in Anoxic Worlds*, New York: Oxford University Press, 1995.

[5] G. Luo *et al.*, "Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production," *Biotechnology and Bioengineering*, vol. 108, no. 8, pp. 1816-1827, 2011.

[6] S. E. Oh, S. Van Ginkel, and B. E. Logan, "The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production," *Environmental Science & Technology*, vol. 37, no. 22, pp. 5186-5190, 2003.

[7] J. Wang and W. Wan, "Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge," *International Journal of Hydrogen Energy*, vol. 33, no. 12, pp. 2934-2941, 2008.

[8] P. J. Fellows, *Food Processing Technology: Principles and Practice*, Elsevier, 2009.

[9] P. Champagne, "Feasibility of producing bio-ethanol from waste residues: A Canadian perspective: Feasibility of producing bio-ethanol from waste residues in Canada," *Resources, Conservation and Recycling*, vol. 50, no. 3, pp. 211-230, 2007.

[10] A. Xia, J. Cheng, and J. D. Murphy, "Innovation in biological production and upgrading of methane and hydrogen for use as gaseous transport biofuel," *Biotechnology Advances*, vol. 34, no. 5, pp. 451-472, 2016.

[11] G. Yang *et al.*, "A critical review on sludge anaerobic digestion progress from 2012 to 2015," *Current Organic Chemistry*, vol. 20, no. 26, pp. 2762-2779, 2016.

[12] J. Massanet-Nicolau, R. Dinsdale, and A. Guwy, "Hydrogen production from sewage sludge using mixed microflora inoculum: Effect of pH and enzymatic pretreatment," *Bioresource Technology*, vol. 99, no. 14, pp. 6325-6331, 2008.

[13] W. Parawira, "Enzyme research and applications in biotechnological intensification of biogas production," *Critical Reviews in Biotechnology*, vol. 32, no. 2, pp. 172-186, 2012.

[14] S. Schwietzke, Y. Kim, E. Ximenes, N. Mosier, and M. Ladisch, *Molecular Genetic Approaches to Maize Improvement*, Springer, Berlin, Heidelberg, 2009, pp. 347-364.

[15] F. Scientific. (2017). Benedict's quantitative solution. A quantitative test for reducing sugars. [Online]. Available: <https://www.flinnsci.com/api/library/Download/f090bfc6fa7f4d1ab45ce3cf6c4ddaec#:~:text=Benedict's%20Quantitative%20Solution%20allows%20for,blue%20color%20to%20the%20solution>.

[16] A. Hernández-López *et al.*, "Quantification of reducing sugars based on the qualitative technique of benedict," *ACS Omega*, vol. 5, no. 50, pp. 32403-32410, 2020.

[17] D. J. Gregg and J. N. Saddler, "Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process," *Biotechnology and Bioengineering*, vol. 51, no. 4, pp. 375-383, 1996.

[18] M. H. Janssen *et al.*, "Evaluation of the performance of immobilized penicillin G acylase using active-site titration," *Biotechnology and Bioengineering*, vol. 78, no. 4, pp. 425-432, 2002.

[19] M. R. Wilkins, W. W. Widmer, and K. Grohmann., "Enzymatic hydrolysis of grapefruit peel to produce ethanol and other products," presented at ASAE Annual Meeting, 2005.

[20] A. Li, *Bioconversion of Biodegradable Municipal Solid Waste (BMSW) to Glucose for Bio-ethanol Production*, University of London, London, U.K: London, 2008.

[21] C. N. Hamelinck, G. Van Hooijdonk, and A. P. Faaij, "Ethanol from lignocellulosic biomass: Techno-economic performance in short-, middle-and long-term," *Biomass and Bioenergy*, vol. 28, no. 4, pp. 384-410, 2005.

[22] S. W. Cheung and B. C. Anderson, "Laboratory investigation of ethanol production from municipal primary wastewater solids," *Bioresource Technology*, vol. 59, no. 1, pp. 81-96, 1997.

[23] X. Huang and M. H. Penner, "Apparent substrate inhibition of the *Trichoderma reesei* cellulase system," *Journal of Agricultural and Food Chemistry*, vol. 39, no. 11, pp. 2096-2100, 1991.

[24] M. H. Penner and E. T. Liaw, *Enzymatic Conversion of Biomass for Fuels Production*, Oregon State University, USA, 1994, ch. 18, pp 363-371.

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