Exploring the feasibility of biological hydrogen production using seed sludge pretreated with agro-industrial wastes

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A B S T R A C T

The effect of applying agro-industrial waste (AIW), such as potash extract (PE), cassava-steep wastewater (CSWW), and corn-steep liquor (CSTL), as an alternative material to pretreat digested cattle slurry (DCS) for biological hydrogen production was examined. In this study, the pretreated (PT) DCS was employed for H_2 fermentation in batch cultures utilising glucose and sucrose as substrates. The result showed that, at 55 °C and pH 5.5, the pretreated DCS's daily volumetric hydrogen production (VHP) was higher than the untreated DCS. Although heat-shocked DCS produced a higher daily VHP of 135 NmL H_2 g^{-1} VS on the second day using glucose as substrates, it is followed by PE-PT DCS, which gave a peak daily VHP of 115 NmL H_2 g^{-1} VS but at a shorter time. When sucrose was the carbon source, the highest peaks were recorded in all the laboratory reactors on day two, with the highest daily VHP of 211 NmL H_2 g^{-1} VS achieved in PE-PT DCS digesters. After the different DCS PT studies, the dominant phylum *Firmicutes,* represented by the *Clostridium* and *Ruminococcus,* were the most abundant bacteria compared to the untreated DCS, which was more diverse. Further research is required to optimise the conditions for AIW DCS pretreatment.

1. Introduction

There is a search for renewable and more environmentally friendly energy sources prompted by the rise in environmental issues caused by continuous greenhouse gas emissions such as CO_2 and CH_4. Subsequently, the over-reliance on fossil fuels for global energy production has led to a severe decline and increased cost of traditional fossil fuels [1–3]. However, alternative renewable energy sources such as wind, hydro, solar, geothermal, and oceanic are capital-intensive and require substantial infrastructure layouts. On the other hand, biomass, the fourth largest energy source after coal, oil, and natural gas [4], is a promising energy source due to its availability and renewability. In addition, it is known that energy can be generated by burning, combustion, gasification, and pyrolysis from biomass, or biomass can be refined to produce cleaner fuels (biofuels) in solids, liquid, or gaseous form [4–6]. Moreover, biomass is converted into a gaseous state mainly by anaerobic digestion (AD), which is eco-friendly and more economical.

The high energy content, increased energy efficiency, and environmental friendliness of production are reasons hydrogen could be termed

as the future energy and alternative to fossil fuels [1]. Moreover, hydrogen produced via dark fermentation (DF) can be a sustainable and clean fuel [6,7] and offers solutions to agricultural wastes [8]. Despite this, biological hydrogen production is affected, among other factors, by the quality of seed sludge in the DF process, which is because seed sludge contains diverse populations of microorganisms that can produce hydrogen using the AD pathway [9]. Thus, the main benefits of the use of seed sludge over pure cultures are its affordability, and that bacteria genera participate in synergistic interactions with other microbes, and its resistance and adaption to environmental stresses [1,10–13]. However, hydrogen-consuming bacteria (HCB) existing together with hydrogen-producing bacteria (HPB) in seed sludge presents a challenge to seed sludge use in the efficient production of hydrogen from organic matter as untreated sludge generally produces a low H_2 yield of about <1.0 mol H_2 mol^{-1} of glucose [12,14]. The low production of H_2 is because molecular hydrogen is used for energy by hydrogenotrophic methanogens [10], producing other products such as methane, ethanol, and volatile fatty acids. In mixed cultures, hydrogen yield is within the range of 0.28 and 0.57 mol H_2 mol^{-1} glucose from the 4 mol H_2 mol^{-1} glucose that is biologically possible under DF technology, which perhaps

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is a direct consequence of hydrogen consumption by HCB [15].

Several conventional methods to enhance sludge solubility, enrich hydrogen producers and eliminate hydrogen consumers' activities using chemical agents (chloroform, extreme acids or alkali) or physical procedures (heat-shock, ultrasonication, irradiation, aeration) or a combination of both have been reviewed [7,12,14,16–31]. In general, the heat-shock conditions vary from 80 to 121 °C with the duration of exposure between 15 and 120 min in the literature [18,20,23]. In addition, for acid and alkali HPB enrichment, the pretreatment is carried out by subjecting the seed sludge to extreme pH values; that is, for acidic PT, pH is adjusted to 2–4, while for base pretreatment method, pH is altered to 11–12 [16]; Show et al., 2004; [18,23,30]. Commonly used acids like HCl, H_2SO_4, and HNO_3 have 0.1–6.0 M concentrations. NaOH, KOH, and $Ca(OH)_2$ are widely used for alkali pretreatment at 1.0–8.0 M [18,30,32]. However, these traditional approaches are energy consuming, require high costs, especially in chemicals purchase, affect the synergistic interaction of sludge microbes, decimate some HPB and hydrolytic microorganisms' population and have related environmental issues [12,33,34].

Research on less energy-intensive and environmentally friendly options has been reported. For example, [35] started hydrogen fermentation using glucose and sucrose substrates from untreated sewage sludge. They obtained a hydrogen yield of 1.63 mol H_2 mol^{-1} glucose when glucose was used as the substrate and a hydrogen yield of 4.45 mol H_2 mol^{-1} sucrose when sucrose was employed as the feeder in less than 60 days of incubation. Nevertheless, the accumulation of short-chain fatty acids (SCFA), mainly acetic and butyric acids, from the two sugars and the use of soluble sugars for seed sludge enrichment could be expensive and unstainable. [36] reported that in untreated sludge at a carbon-to-nitrogen (C/N) ratio of 25, hydrogen production was 33% higher than that of heat-shocked, with a stable hydrogen content of 58% in their work on hydrogen production by anaerobic co-digestion of rice straw (RS) and sewage sludge. They adduced that the reason for the low hydrogen yield of the heat-treated sludge was due to the inhibition of the microbial community that could be involved in RS decomposition for bio-hydrogen production. The problem with this study is the reduced utilisation of biomass from the recalcitrant nature of RS and low hydrogen yield (18 mL H_2 g-added^{-1} straw), which was reported as the maximum cumulative hydrogen yield [36]. Although the maximum hydrogen yield increased from 7.96 to 19.40 mL H_2 g^{-1} VS after waste-activated sludge (WAS) was pretreated by freezing in the presence of nitrite [7], this approach might be both cost and energy intensive. Furthermore, the added nitrite can be easily converted to ammonia, affecting fermentation. Similarly, [9,14] reported that combining K_2FeO_4/PH 9.5, cobalt, and iron nanoparticles could promote hydrogen yield from WAS. While it is true that potassium ferrate, an oxidant, is non-polluting to the environment, and Co is required in Coenzyme B12, an essential enzyme in DF, the procedures can affect the total cost of the fermentation. In a recent [31] study, the potential of freezing coupled with calcium hypochlorite pretreatment for enhancing biohydrogen production from sludge was explored. The results showed a hydrogen yield of 18.18 + 0.43; however, it was noted that the process is unstainable due to its reliance on energy and chemical inputs, which would inevitably increase the cost of the process.

A greener and more promising approach is proposed, which is novel to the best of the author's knowledge. It entails the application of agro-industrial waste (AIW), such as potash extract (PE), cassava-steep wastewater (CSWW), and corn-steep liquor (CSTL), as alternative materials that can enrich HPB levels and inhibit HCB populations in digested cattle slurry (DCS). Potash, widely available and locally produced in Nigeria, is leftover ash from utilising the empty palm fruit bunches (EPFB) as a heat energy source for cooking. In contrast, the CSTL is usually produced as wastewater during the wet-milling of corn in the production of corn-related products such as *akamu* (pap), a local infusion widely consumed in Nigeria and some African countries. Finally, the CSWW is the spent wastewater used in cassava fermentation.

In oil palm processing and extraction, EPFB is one of the most abundant produced palm biomasses (4.42 t ha^{-1} per y) [37,38]. As documented by Ref. [39], about 5–7 L of wastewater of CSWW is generated from 1 kg of fresh tubers, which means that the wastewater from cassava processing can amount to millions of litres depending on the quantity of raw cassava processed. Therefore, it can be inferred that about 295–413 million kilolitres of CSWW are produced annually in Nigeria (about 59.5 million tonnes of cassava are produced yearly [40]). Although there are inadequate available statistics on the amount of CSTL generated, many industries using corn as raw materials thrive globally, and these companies create a lot of corn wastewater. Having said that and using available China statistics, more than 20 million tonnes of CSTL are produced by over 600 companies [41].

Potash extract contains some heavy metals [42] that can be utilised to inhibit or slow down hydrogenotrophic microbes' activities in seed sludge. Highly alkaline PE can enhance sludge solubility and create extremely alkaline conditions for HCB, while HPB will form spores and survive [19,23,29]. Similarly, CSWW and CSTL are highly acidic due to the concentration of SCFA. The SCFA can also induce effects like HCl pretreatment on seed sludge for HPB enrichment. Therefore, in this study which is novel to the best of the authors' knowledge, a) agro-industrial waste was investigated as an inexpensive and energy-saving technique for enriching HPB in DCS for maximum hydrogen production. In addition, b) Microbial community diversity after pretreatments and acidogenic processes was examined.

2. Materials and methods

2.1. Agro-industrial wastes collection and preparation

2.1.1. Potash collection and preparation of potash extract

The potash or ash from empty palm fruit was collected from a heap of burnt empty palm bunches at a palm oil processing factory in Anara Town, Imo State, Nigeria. The PE was prepared by mixing 500 g potash with 1L of deionised water (1:2 ratio). The mixture was appropriately stirred and allowed to settle at room temperature for 48 h. After, the potash mixture was vacuum filtered using Whatman filter paper (0.45 μm), and the residue was rinsed with 500 mL of distilled water to bring the total ratio of potash to distilled water to 1:3. The pH of the filtrate was observed to be 11.05 ± 0.25, which is highly alkaline. The PE colour was found to be deep brown, and then the PE was kept at a refrigerating temperature of 4 °C after the metal analysis.

2.1.2. Corn seed collection and preparation of corn-steep liquor

The corn was purchased from an African shop at Westgate Road, Newcastle Upon Tyne, UK. The CSTL was prepared to represent the ones obtainable in Nigeria's corn-based industries. The dried corn was coarsely crushed and imbued into plastic containers containing sulphuric-added deionised water (0.1%) for 2 days at room temperature, with the wastewater changed every 24 h. The wastewater, termed corn-steep liquor, was placed in a container and kept at a temperature of −25 °C until used. The SCFA and the COD contents of the CSTL were analysed before use (Table 1).

2.1.3. Cassava collection and preparation of cassava-steep wastewater

Fresh cassava root tubers were purchased from a Hutchinson fruit shop in Fenham Newcastle Upon Tyne, UK. The CSWW is the spent water generated during the processing of the raw cassava into edible forms through anaerobic fermentation. In the fermentation procedure, small-sized peeled cassava was incubated in a water-filled airtight plastic container for 4 days at RT. The SCFA and COD contents of the CSWW were determined (Table 1) before application as a pretreatment agent.

2.2. Digested cattle slurry collection and preparation

The DCS was collected from Cockle Park Farm, Newcastle University,

Table 1
Characterisation of cassava-steep wastewater and corn-steep liquor.

Sample	Acetic Acid (mg L^{-1})	Butyric Acid (mg L^{-1})	Formic Acid (mg L^{-1})	Lactic Acid (mg L^{-1})	Total COD (mg L^{-1})	Soluble COD (mg L^{-1})	pH
CSWW	2329.89	2679.71	NA	NA	13640.00	5680.00	3.81
CSTL	1595.32	1201.69	NA	NA	7520.00	3600.00	3.45

NA: Not available.

Newcastle Upon Tyne, UK, which processes cattle and pig slurry and then stored at 4 °C until further use. Before use, the sludge was degassed to remove any remaining indigenous biomass by incubating it for 30 days at 55 °C.

2.3. Digested cattle slurry pretreatments for bio-hydrogen production

The DCS was sieved through a 2.0 mm screen to filter out impurities and afterwards pretreated to deactivate hydrogen consumers using AIW (PE, CSTL, and CSWW) as pretreatment agents. In addition, an amended method of seed sludge acid pretreatment by Refs. [18,32], and [23] was applied. The pretreatment was done by adjusting DCS pH values employing each waste. Specifically, the DCS enrichment was performed by altering the DCS's pH with CSTL or CSWW to 3.53 ± 3.0 and maintaining this pH value for 48 h at RT on a magnetic stirrer at 200 rpm. The CSTL/CSWW-PT DCS was then acclimatised for a certain period (7 days) at RT. Before use, the pH was adjusted to 5.7 ± 0.3 using 5 M NaOH.

Similarly, in DCS pretreatment with PE, [22] revised protocol was employed. The DCS was adjusted to pH 11.26 ± 0.4 with PE and mixed at 200 rpm for 24 h at room temperature. The PE-PT DCS was then acclimatised for a month at RT, and the pH was adjusted to pH 5.7 ± 0.2 using 5 M HCl before application on batch cultures.

A conventional heat-shock procedure of applying heat on the DCS at 100 °C for 1 h was also used as a positive control for comparison. Prior to laboratory studies, pretreated inocula were acclimatised using glucose or sucrose for 3 or 4 days at pH 5.5 and 55 °C before solid characterisation was done (Table 2).

2.4. Experimental design of the hydrogen fermentation process

The acidogenesis design and process were as outlined as follows. The batch cultivation was done using 500 mL grade 3.3 borosilicate glass Duran bottles (VWR 215-1594) with a working volume of 400 mL, and the C/N ratio was maintained at 25. In detail, the pretreated and untreated DCS were added into the fermenting bottles and supplemented with a 5% mineral medium. The mineral medium contained the following per litre: (NH$_4$HCO$_3$ 6.72 g; KH$_2$PO$_4$ 0.125 g; Na$_2$HPO$_3$ 5.24 g CaCl$_2$ 0.3 g; MgCl$_2$·4H$_2$O 0.1 g; FeSO$_4$·7H$_2$O 0.025 g; CoCl$_2$·6H$_2$O 0.0001 g; ZnSO$_4$·7H$_2$O 0.0024 g; CuSO$_4$·5H$_2$O 0.005 g, peptone 0.75 g; distilled water 1000 mL). The respective substrates – glucose or sucrose- were added to the digester to bring the substrate-to-microbial ratio to 0.75. The digesters were labelled CSTL, CSWW, PE-PT DCS and heat-shocked DCS, reflecting their respective agents applied during DCS pretreatments. At the same time, the control reactor was identified as an untreated DCS. The fermenters were flushed with nitrogen for 3 min, and the fermentations were done in triplicates in an automated stirred incubator system with an initial pH of 5.8. Nonetheless, the operational

pH of 5.5 was maintained using 5M HCl and 5M NaOH. The cultivation temperature of the incubator system was 55 °C, while the rotational speed was set at 120 rpm. Batch incubation was done for 3 days when glucose was employed as the substrate and 4 days when sucrose was used as the carbon source. The total biogas volume produced by each fermenter was measured daily. Finally, the hydrogen concentration (%) was determined using a thermal conductivity detector gas chromatography-Trace GC Ultra (Thermo Scientific, UK) with argon as the carrier gas and calculated with Verein Deutscher Ingenieure [43].

2.5. Genetic extraction and microbial community analysis

The samples for microbial analysis were taken at the end of individual experiments and stored at −20 °C in a sterile 50 mL centrifuge tube (VWR, 525-0402) before genomic DNA extraction. The DNA extraction was performed using the FastDNA^{TN} SPIN Kit for Soil (MP Biomedicals LLC., 116560200) and according to the manufacturer's protocol, except for the sample preparations where 500 μL of seed sludge or digestate was used. In addition, a blank tube containing 500 μL of microbiological grade sterilised water (Microzone, UK) was also used as the control to checkmate the presence or absence of kit contaminants. After the genomic DNA extraction, the DNA concentrations and quality were determined using a Qubit® 2.0 Fluorometer. The samples' DNA quantity and specificity were also improved by following the cleaning protocol of QIAquick® Nucleotide Removal Kit (Qiagen, 2016) and ensuring the acceptable range of 1.8–2.2 for the DNA quality ratios 260:280 and 260:230 is sustained. The samples were then stored at −20 °C until when ready for genomic sequencing. For sequencing, about 100 μL of the DNA extracts were placed in polymerase chain reaction (PCR) tubes and sent to NU-OMICS laboratory, Northumbria University, UK, where the PCR amplification, library preparation and high-throughput 2 X 250 amplicon sequencing of the V4 region of the 16S rRNA gene using the Illumina MiSeq Personal Sequencer Protocol [44] were carried out. Whereas the amplicon sequencing of taxonomic marker genes such as the 16S rRNA gene in bacteria provides an efficient characterisation of bacteria communities [45], the PCR process involves the amplification of the extracted DNA V4 hypervariable region of 16S rRNA using the universal reverse primer 806R (GGACTACHVGGGTWTCTAAT) and the forward primer 515F (GTGCCAGCMGCCGCGGTAA) primers to analyse the bacterial and archaeal communities [46].

After each sample was entirely sequenced from the Illumina MiSeq, the resulting raw sequenced data (FastQ files) was denoised and quality filtered using DADA2, a publicly-available R package (https://github.com/benjjneb/dada2) that extends and improves the Divisive Amplicon Deionising Algorithm (DADA) model [47]. Quality filtration involves the trimming and truncating of low-quality regions, especially the first 10 bases for both forward and reversed reads, often known to

Table 2
Characterisation of raw and pretreated digested cattle slurry.

Substrate	TS (g mL^{-1})	TSS (g mL^{-1})	VS (g mL^{-1})	VSS (g mL^{-1})	Ash (g mL^{-1})	VS (%)	Ash (%)	NH$_4^+$-N (g L^{-1})	Alkalinity (mg CaCO$_3$ L^{-1})	Moisture Content	SCOD (mg L^{-1})
DCS	0.095	0.069	0.073	0.052	0.022	77	23	0.30	4014.50	95.00	520.00
PE-PT DCS	0.045	0.036	0.030	0.021	0.015	67	33	0.19	3015.00	96.00	1760.00
CSTL-PT DCS	0.014	0.010	0.011	0.006	0.003	79	21	0.15	2030.00	97.10	1160.00
CSWW-PT DCS	0.010	0.008	0.009	0.007	0.001	90	10	0.16	2540.00	98.50	4680.00

contain pathological errors [45]. The "derep" function then dereplicated the reads in each sample in DADA2 to identify the unique amplicon sequence variants (ASV) from redundant sequences contained in the data set. Whilst the chimeric sequences from each sample were removed from each sequence, the non-chimeric sequences from the samples were taxonomically assigned using MIDAS 2.0 reference database [47–49] within the Quantitative Insights Into Microbial Ecology (QIIME2) pipeline (https://qiime2.org/ [50]. Afterwards, a feature table was produced for data visualisation and statistical analysis containing the unique ASVs and their relative abundance per each sequenced DNA digestate sample.

The statistical analysis, especially the Local Contributions of Beta Diversity (LCBD), which is a comparative indicator of the degree of the uniqueness of digestate samples about the local community composition, was conducted on these data to generate figures and pictographs using the MicrobiomeSeq in R packages [51] built from existing packages such as vegan (Oksanen et al., 2007), phyloseq [52] and DESeq2 [53].

2.6. Determination of the elemental composition of PE, untreated and pretreated DCS

The procedure was performed using a modified description of [54]. The wet ash process involves the digestion of 1.0 mL of seed sludge in a conical flask placed in a fume cupboard. Approximately 10 mL of the concentrated H_2SO_4 and HNO_3 acids were poured into the flasks. Following this, the mixture of biomass and acid was placed on a hot plate and heated at 120 °C for 15 min. During this heating process, effervescence occurred along with the release of NO_2 gas, depicted by reddish-brown gas production. The addition of more acids sustains the digestion process until all the biomass is entirely digested, evidenced by the formation of a light-yellow solution. At this juncture, no further release of NO_2 gas. Subsequently, the digested mixture was transferred to a 50 mL standard volumetric flask and was made up to 50 mL using deionised water. A sample of this dilute solution and a sample of PE were used to determine the elemental composition using an inductively coupled plasma atomic emission spectrometer (Vista-MPX) with a CCD detector, Newcastle University, UK, according to the analytical process outlined in the Standard Methods for the Examination of Water and Wastewater 20th Edition (APHA 3120C) [55]. The anionic composition of the PE sample was analysed using HPLC Thermo-scientific DIONEX AQUION, Newcastle University, UK.

2.7. Analytical measurements and data analysis

The seed sludge solids, the alkalinity test, the nitrogen content, and chemical oxygen demand were measured according to standard methods 2540 B, 2320, 4500 and 5220 B, respectively (ALPHA standard, 2005), while the concentration of SCFA such as acetate, butyrate, formate and propionate were determined using an HPLC Thermo-scientific DIONEX AQUION equipped with Dionex IonPac™ ICE-ASI columns.

The hydrogen production and accumulation rate kinetic model was determined using a modified Gompertz equation (Equation (1)) and Matlab software (MATLAB R2016a). At the same time, the data calculations were analysed using the statistical Excel software (Microsoft Corporation, USA). The R software packages (R version 3.3.2; R Core Team, 2013) were employed for the statistical microbial analysis. Values are presented based on a 5% statistical significance level, and results were displayed at ± 2 SD.

$$H(t) = P.exp\left\{ -exp\left[\frac{R_m * e}{P} (\lambda - t) + 1 \right] \right\} \qquad \text{Equation 1}$$

Where H (t) is the cumulative H_2 production (mL); *P* is the H_2 production potential (mL): R_m is the maximum H_2 production rate (mL/d); *e* is

2.71828; λ is the lag phase time (d), and t is the fermentation time (d).

3. Results and discussion

3.1. Seed sludge characterisation

The physicochemical attributes of the seed sludge employed in the study are characterised in Table 2. The carbon/nitrogen ratio of the seed sludge was within an acceptable range of 5–200 [36]. The DCS pH was 8.56 before pretreatments. After DCS enrichment with AIW, the elemental composition of PE and the various pretreated DCS is shown in Table 3.

It can be seen in Table 3 that pretreating DCS with PE increased the concentration of metal ions, especially the earth metals, even after 30 days of adaptation compared with the ionic distribution of other PT DCS and raw DCS. This increase in earth metals, especially potassium, magnesium, and calcium, will create unfavourable conditions for microorganisms' metabolic activities, especially the HCB. In addition, PE pretreatment ensures the growth of HCB is inhibited while HPB, although also affected by the increased levels of metal ions, will survive and form spores [19,23,29].

3.2. Characterisation of agro-industrial wastes

The elemental concentrations (cations and anions) of PE in Table 3 show that the earth metals - potassium (8.74 g L^{-1}), calcium (1.96 g L^{-1}), and magnesium (1.25 g L^{-1}) were the highest among the cations, while chloride (0.77 g L^{-1}) was the highest among the anions. These results were slightly consistent with those of [42] on potash extract. Therefore, it can also be said that based on the elemental composition of PE in Table 3, PE can be used as a buffer depending on the aims and circumstances. Similarly, Table 1 shows the chemical attributes of CSWW and CSTL. In both samples, the pH was within 3.0 and 4.0, which was acidic. The acidity is due to organic acids, predominantly acetic and butyric acid. In addition, both samples had high concentrations of chemical oxygen demand, mainly from carbohydrates or starch molecules.

Table 3
Elemental composition of potash extract and various digested cattle slurry.

Cations	PE (mg L^{-1})	PE-PT DCS (mg L^{-1})	CSTL-PT DCS (mg L^{-1})	CSWW-PT DCS (mg L^{-1})	Heat-shocked DCS (mg L^{-1})	Raw DCS (mg L^{-1})
Calcium	1957.84	630.13	256.00	270.93	208.65	205.00
Magnesium	1246.41	670.48	223.39	179.77	206.88	200..18
Sodium	280.22	182.22	89.05	75.05	66.22	76.22
Potassium	8741.55	2919.35	607.31	639.39	375.08	365.88
Zinc	44.37	6.33	3.26	4.62	2.97	2.00
Nickel	0.49	0.27	0.23	0.24	0.20	0.20
Aluminium	513.89	115.34	27.19	20.36	21.95	15.87
Iron	913.08	427.56	149.96	147.77	131.37	135.13
Manganese	26.37	5.56	6.57	4.83	5.26	4.26
Copper	10.48	2.3	3.46	2.78	3.11	2.75
Silicon	41.32	14.75	1.3	1.70	1.21	1.00
Arsenic	0.36	0.21	0.21	0.21	1.10	0.56
Chromium	0.63	0.21	0.18	0.27	0.29	0.29
Lead	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Strontium	13.19	3.07	3.87	4.34	3.74	2.89
Barium	0.70	0.28	0.41	0.39	0.47	0.50
Selenium	5.88	3.28	0.21	19.41	0.71	0.67
Anions						
Chloride	766.86	60	Na	Na	Na	Na
Nitrite	26.09	Na	Na	Na	Na	Na
Bromide	4.43	Na	Na	Na	Na	Na
Sulphur	274.90	98.48	25.88	21.71	27.09	25.00
Nitrate	2.50	Na	Na	Na	Na	Na
Phosphor	1.50	Na	Na	Na	Na	Na

3.3. Effects of pretreatments technologies on fermentative hydrogen production

3.3.1. Hydrogen production on glucose reactors

After acclimatisation, the enriched seed sludge, CSTL, CSWW and PE-PT DCS were tested for their capabilities to produce hydrogen using glucose or sucrose. The hydrogen production using glucose or sucrose as a substrate by different DCS pretreatments is illustrated in Figs. 1 and 2. Whereas the hydrogen production profile was modelled from the Gompertz equation and presented in Table 4, the figures showed hydrogen production activities from all the samples, including the control, the seed sludge without pretreatment. Similarly, an R-square value of 0.999 indicated that the fermentations were stable and efficient in all the reactors (Table 4). Nonetheless, the results showed that the cumulative and hydrogen production rates (HPR) from the various pretreated DCS were much higher than those of untreated DCS using glucose (Fig. 1) and sucrose (Fig. 2) substrates, which tallies with [21] findings. Furthermore, the high hydrogen production showed that these pretreatments could inhibit HCB while preserving the activities of HPB in DCS, unlike untreated DCS, where the daily volumetric hydrogen production (VHP) and accumulation are reduced.

The variation in daily VHP is due to HCB using molecular hydrogen for energy [10]. When glucose was the medium, the heat-shocked DCS produced the most daily VHP of 135 NmL H_2 g^{-1} VS on the second day compared to other pretreated DCS, followed by PE-PT DCS, which gave the highest daily VHP of 115 NmL H_2 g^{-1} VS within 24 h (Fig. 1). The heat-shocked DCS reactor also had the highest hydrogen accumulation of 229 NmL H_2 g^{-1} VS, followed by PE-PT DCS digester, which has 222 NmL H_2 g^{-1} VS as the volumetric hydrogen yield (VHY) (Table 4). In contrast, the other pretreated DCS (CSWW and CSTL-PT) and untreated DCS (control) gave 202, 143 and 77 NmL H_2 g^{-1} VS, respectively, as VHY. These findings agreed with [18,21]. They demonstrated that the hydrogen yield from the heat-shocked seed sludge was the highest among all the tested samples in their study. However, AIW-PT DCSs have shortened lag phases from 0.2 (5.0 h) to 0.4 (10 h) day (Table 4) compared to heat-shocked DCS when glucose is used as the medium (Fig. 1). The high hydrogen production and the shortened lag phases, mainly from PE-PT DCS digesters, can be attributed to trace elements in PE-PT DCS (especially potassium, iron, manganese, cobalt, copper, zinc chromium, and barium) that favours acidogenesis and thus, more evolution of hydrogen gas [56]. The high accumulation of ions in the PE extracts can also act as a soluble buffer, thereby maintaining the effect of total volatile fatty acids (TVFA) in the fermentation medium, ensuring

the traditional function of fermentative microbial metabolism [57,58]. In addition, the presence of soluble monosaccharides and other organic constituents in CSWW and CSTL-PT DCS could act as start-up substrates for hydrogen fermentation and biochemical productions such as bio-alcohols, organic acids production by HPB and HCB [59,60].

On the other hand, the extended lag phase from heat-repressed sludge may be due to the inhibition of some microbial communities involved in the start-up of the hydrogen production process [36]. A shortened lag phase was also observed in untreated DCS (Table 4), possibly because the microbial consortia were better in hydrogen fermentation in terms of adaptability and tolerance to sudden changes in the environmental and nutritional shocks [61]. Nevertheless, hydrogen is rapidly consumed by HCB in untreated sludge for energy in metabolic activities [10]. In addition, HCB in untreated sludge can alter hydrogen production's biochemical pathway to the production of SCFA, solvents, and alcohols, thereby reducing the net bio-hydrogen yield [25,34,85]. This alteration of metabolism is confirmed by the highest daily VHP of 37 NmL H_2 g^{-1} VS produced on the second day by the untreated DCS sample (Fig. 1). Other pretreated DCS (CSWW-PT DCS and CSTL-PT DCS) had lower daily VHP of 92 and 82 NmL H_2 g^{-1} VS, respectively, when compared to heat-shocked and PE-PT DCS (Fig. 1).

Using the kinetic parameters derived from the Gompertz equation (3-9) (Table 4), the maximum HPR of 240 mL d^{-1} was obtained from a heat-shocked DCS digester, followed by CSWW-PT DCS (151 mL d^{-1}), PE-PT DCS (144 mL d^{-1}), and CSTL-PT DCS (144 mL d^{-1}) when glucose was used as the energy source. The least HPR of 67 mL d^{-1} was observed from untreated DCS. These results differ from the outcomes of [23]. They obtained a maximum HPR of 21.02 mL h^{-1} (504.48 mL d^{-1}) from heat-shocked sludge. The difference could be from the mode of treatment of sludge, the source of activated sludge applied, and the gas measurement time. While [23] experiment was monitored hourly, the gases in this study were measured daily for three to four days.

3.3.2. Hydrogen production on sucrose digesters

The result of hydrogen production using sucrose as the carbon source (Fig. 2) differed from fermentation with glucose as a substrate. The lag phase attained when sucrose was employed as the medium was almost the same in all the pretreated DCS digesters except for CSWW-PT DCS (Table 4). This development is contrary to the lag phase when glucose was used as a substrate and can be explained by the different solubility rates of the two carbohydrates in the reactors. The highest daily VHP peaks were recorded in all laboratory reactors on the second day, with the highest peak of 211 NmL H_2 g^{-1} VS recorded at the PE-PT DCS
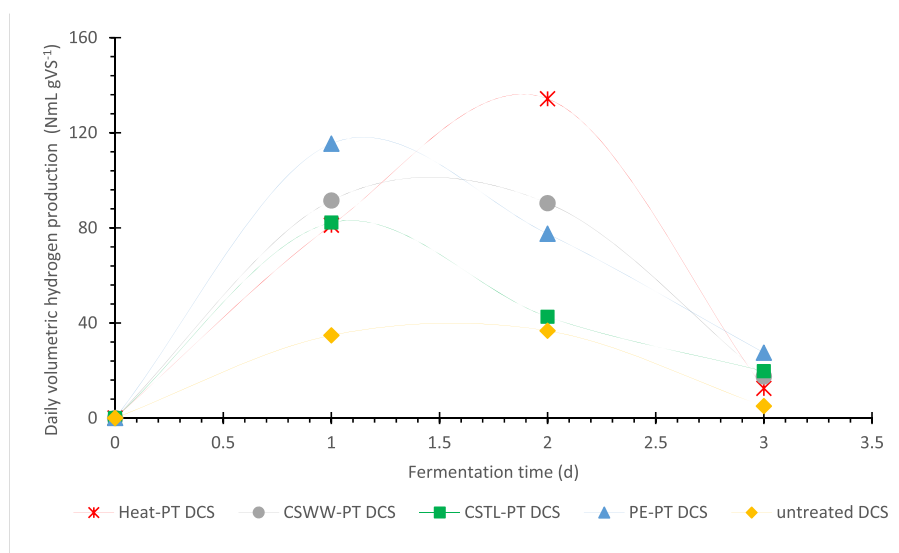

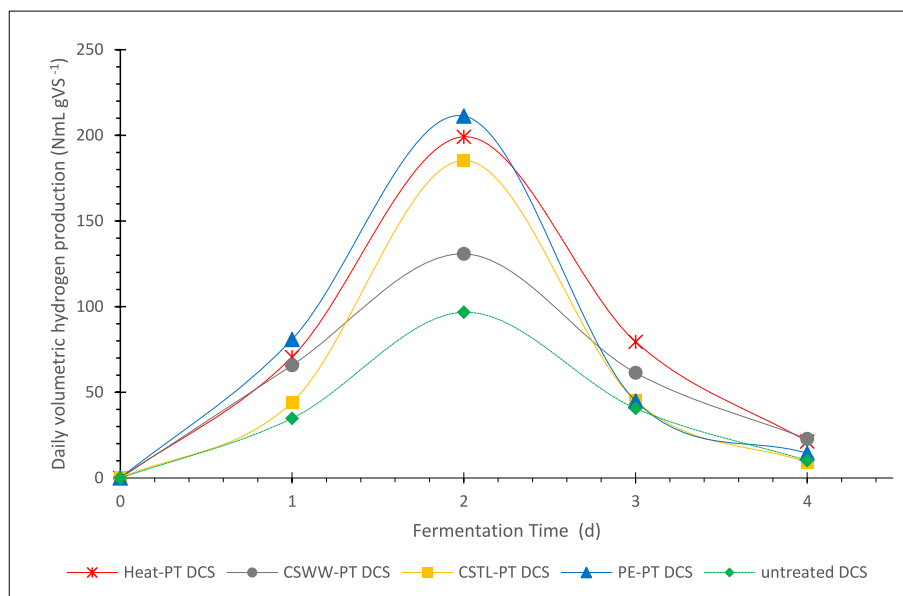**Fig. 1.** Hydrogen production using glucose as substrates by various pretreated DCS.

Fig. 2. Hydrogen production using sucrose as substrates by different pretreated DCS.

Table 4
Kinetic parameters of hydrogen production from various pretreated DCS.

PT DCS	Gompertz Data							
	P (NmL H_2 g^{-1} VS)		λ (d)		R_m (mL d^{-1})		R-Square	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
Heat	229	374	0.7	0.7	240	223	0.999	0.999
PE	222	349	0.2	0.7	144	268	0.998	0.999
CSWW	202	287	0.4	0.5	151	142	0.999	0.999
CSTL	143	283	0.2	0.8	97	226	0.998	0.999
Untreated	77	185	0.5	0.7	67	108	0.999	0.999

reactor (Fig. 2). The subsequent higher VHP peak was obtained from a heat-shocked DCS sample with 199 NmL H_2 g^{-1} VS as the highest peak value. The values of 185 and 131 NmL H_2 g^{-1} VS were obtained as the highest daily VHPs for CSTL-PT and CSWW-PT DCS samples, while for untreated DCS, 97 NmL H_2 g^{-1} VS was produced as the highest daily VHP.

In contrast, the highest hydrogen accumulation of 374 NmL H_2 g^{-1} VS was achieved from the heat-shocked DCS reactor, followed by the PE-PT DCS reactor digester, which had 349 NmL H_2 g^{-1} VS as the volumetric hydrogen yield (VHY) compared to CSWW, CSTL-PT and untreated DCS. The other PT DCS and control gave 287, 283 and 185 NmL H_2 g^{-1} VS as the VHY, respectively (Table 4). The result obtained agreed with the review of [20] and the studies of [17,24], and [27] on heat-shock processes for seed sludge enrichment.

The result of the HPR affirmed the outcome of the PE-PT DCS fermenter as the highest daily hydrogen producer when sucrose was employed as substrate (Table 4). The highest maximum HPR of 268 mL d^{-1} was observed from the PE-PT DCS digester, followed by CSTL-PT DCS (226 mL d^{-1}), heat-shocked DCS (223 mL d^{-1}), and CSSW-PT DCS (142 mL d^{-1}). Untreated DCS gave the least HPR of 108 mL d^{-1}.

The disparities in the reactor samples regarding VHY, daily VHP and HPR when either substrate was employed as the medium could not be explained. Still, glucose is presumed more readily metabolised than sucrose, confirmed by the degradation profiles (Figs. 1 and 2). Therefore, HPB is much more reactivated under a glucose medium in heat-shocked DCS than sucrose, favouring PE-PT DCS owing to trace elements (Table 3) [56].

The mechanism or strategy of PE on sludge enrichment is not fully understood. However, it is believed that suppression of HCB could be from the highly alkaline condition of the PE and the presence of earth and trace metals, particularly potassium and calcium. The alkaline state could accelerate sludge solubilisation and inhibition of methanogenesis and other HCBs [14,16,62]. Furthermore, the presence of these earth metals (Table 3) could lead to the formation of earthly salts such as potassium ferrate that can lyse the microbial cells of HCB, causing the release of essential nutrients required for metabolism and hydrogen fermentation [14,56]. PE contains trace elements supporting acidogenesis [56] and hydrogen fermentation processes. The element "iron" is of great focus as it is essential in anaerobic fermentation for hydrogen production [9,63,64]. The hydrogenase enzyme responsible for hydrogen evolution from sugar monomer requires reduced ferredoxin to be oxidised, and this compound is usually Fe^{2+} complexed [64–66]. In the same vein, the iron-sulphur protein – ferredoxin is involved in (1) pyruvate oxidation to acetyl-CoA and CO_2 under the pyruvate ferredoxin oxidoreductase pathway in dark fermentation processes for H_2 production, (2) acts as an electron carrier, (3) in proton reduction to molecular hydrogen in anaerobic fermentative hydrogen production where they assist in the formation of hydrogenase and (4) reduction of inhibition due to sulphide [9,67,68]. Potassium ions in high PE concentrations could also complement the proton and K^+ deficiency caused by increased free ammonia levels [69,70]. Therefore, the hydrogen fermentation process is stabilised. The presence of these earth metals in PE also has flocculation abilities paramount for the formation of biological linkages at cellular levels, in the separation and disintegration at sludge levels, and in the oxidation of organic compounds [14,56,71,72]. These physiological activities are essential for the AD process.

3.3.3. Biogas/hydrogen yield and average hydrogen content of the reactor samples

The result of the biogas/hydrogen yield and average hydrogen content of the different DCS from glucose (Fig. 3) and sucrose (Fig. 4) are presented here. At a mean hydrogen content of 46%, the heat-shocked DCS produced the highest biogas and hydrogen accumulation of 494 NmL g^{-1} VS and 229 NmL H$_2$ g^{-1} VS correspondingly when glucose was employed as substrates (Fig. 3). Similarly, when sucrose was the carbon source, heat-shocked DCS, at average hydrogen content of 55%, produced the highest biogas and hydrogen accumulation of 674 NmL g^{-1} VS and 374 NmL H$_2$ g^{-1} VS, respectively (Fig. 4). Nonetheless, the PE-PT DCS with an average hydrogen content of 52% had the highest biogas yield of 676 NmL g^{-1} VS in all the enriched samples (Fig. 4) when sucrose was used as the substrates. The VHY of 222 and 349 NmL H$_2$ g^{-1} VS of glucose and sucrose from PE-DCS reactors were comparable to heat-shocked DCS outcomes. Untreated DCS had the least VHY (77 and 185 NmL H$_2$ g^{-1} VS) and the mean hydrogen content (17 and 28%) when glucose and sucrose were applied as carbon sources, respectively (Figs. 3 and 4).

While the CSWW-PT DCS digester with mean hydrogen content (42%) produced 202 NmL H$_2$ g^{-1} VS as the cumulative hydrogen yield, the CSTL-PT DCS digester, which has mean hydrogen content (33%), generated the VHY of 143 NmL H$_2$ g^{-1} VS when glucose was the medium (Fig. 3). On the other hand, in sucrose reactors, CSTL-PT DCS samples had a slightly higher hydrogen content (49%) and VHY of 283 NmL H$_2$ g^{-1} VS than CSSW-PT DCS samples, which had mean hydrogen content (45%) and VHY of 287 NmL H$_2$ g^{-1} VS (Fig. 4). Compared to heat-shocked DCS, the VHY and the average hydrogen content of other agro-industrial PT DCS were lower in glucose and sucrose fed reactors (Figs. 3 and 4-5). However, the biogas yield of all pretreated DCS and the untreated except CSTL-PT DCS using sucrose as a medium was approximately within the same range for glucose and sucrose systems.

The close volumetric biogas production of agro-industrial PT DCS with heat-PT DCS shows the fermentation process was excellent and stable despite disparities in hydrogen content and yield (Figs. 3 and 4). These differences could be from liberated hydrogen consumption, where molecular hydrogen serves as an energy source for some HCB [10] and thus, the shifting of fermentation pathways following lactic and solvent production (Table 5) [73], which explains the reason for the low mean hydrogen content observed in some agro-industrial PT digesters. In addition, it is believed that the mechanisms of action for CSWW and CSTL-PTs are 1) suppressing microbes with weak acids, mainly acetic acids, and 2) from nutrient shock due to the presence of carbohydrates. And as such, there will be more evolution of VFA (Table 5), which will

also lead to more biogas production. Hence, CSWW and CSTL can create an unfavourable environment for acidogenic microorganisms. However, HCB can survive and further alter the acidogenic pathway. In affirming this view, [25] reported that HCB in seed sludge could rival HPB for nutrients. Therefore, HCB can alter the biochemical pathways, reducing net hydrogen yield and producing unwanted products. On the other hand, heat-shocked and PE-PT DCS could produce higher mean hydrogen content because the various pretreatments could repress HCB, while HPB survives the harsh treatments.

3.4. Total volatile fatty acids production from various digested cattle slurry

Typically, bio-hydrogen is produced during the acidogenic stage in an AD process [1,23] together with SCFA and CO$_2$. The HPR was also confirmed by the SCFA produced during the hydrogen production process (Table 5). Thus, TVFA concentration from the different PT DCS reactors is a valuable indicator for monitoring hydrogen fermentation. Table 5 illustrates the distribution of associated SCFA concentrations from DCS samples. It can be seen from the table that there was increased production of SCFA in CSWW-PT DCS and PE-DCS, which could be due to more carbohydrate compounds in CSWW, which were degrading either during acclimatisation or along with the added substrates.

On the other hand, the metallic ions content in PE (Table 3) could have influenced the accumulation of SCFA in PE-PT DCS. Whereas acetic and butyric acid accounted for the most soluble fermentation products, butyrate was almost twice the acetate amount in the fermentation products for glucose and sucrose. The more butyrate concentration shows that the fermentation process was primarily due to the butyric-acid type pathway [18,23]. Although the result was consistent with findings for acids and base pretreatments by Refs. [18,74], the established pathway was different for various methods applied by Ref. [20], where the acetate-type fermentation pathway was recorded to be the most dominant in all samples. The production of formate was insignificant in glucose samples. However, when sucrose was applied as a carbon source, there was a minimal concentration of formic acids, with CSTL and CSWW-PT reactors having about 102–106 mg L^{-1} levels indicating a continuous hydrogen fermentation process from dissolved but complex carbohydrates molecules contained in CSTL and CSWW-PT DCS.

Furthermore, the gradual increase in the formation of lactate in both the glucose and sucrose reactors (especially from CSTL-PT and CSWW-PT DCS (Table 5)), which agrees with the findings of [39], could either be from a) the consumption of liberated hydrogen and alteration
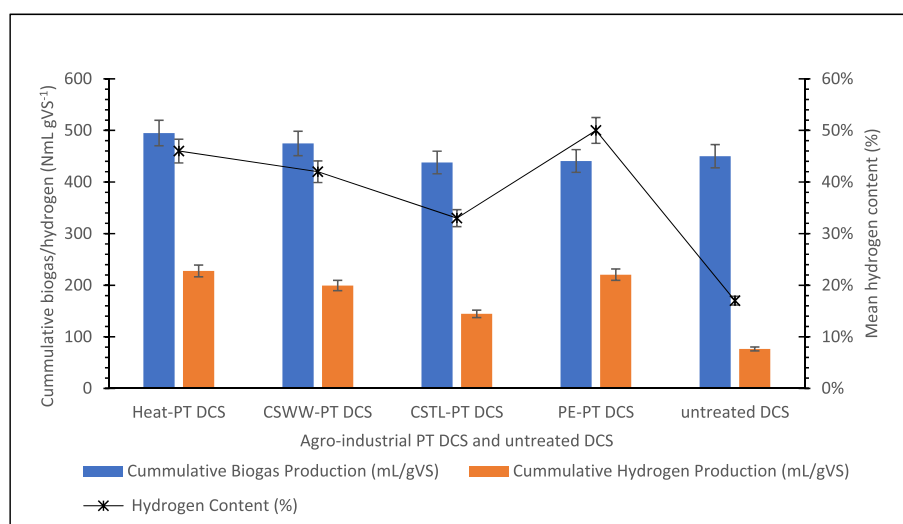

Fig. 3. Biogas/hydrogen yield and average hydrogen content of the various DCS from glucose.
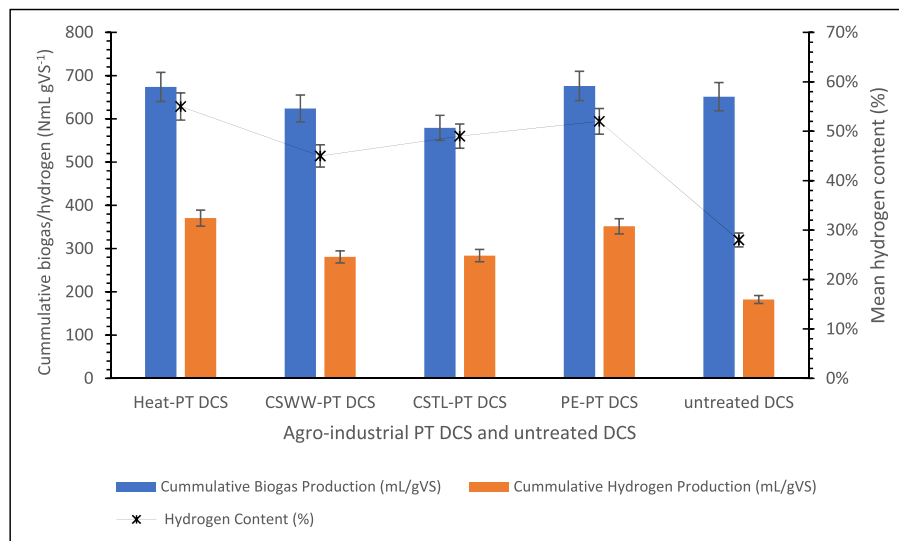
Fig. 4. Biogas/hydrogen yield and average hydrogen content of the various DCS from Sucrose.

Table 5
The distribution of associated SCFA concentrations from DCS samples.

Sludge Enrichments	Acetic (mg L^{-1})		Butyric (mg L^{-1})		Formic (mg L^{-1})		Lactic acid (mg L^{-1})	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
Heat-shocked DCS	520.00	698.57	748.68	969.72	2.85	2.83	NA	NA
CSTL-PT DCS	788.90	988.60	1061.34	1241.34	NA	105.70	180	340
CSWW-PT DCS	848.80	1048.80	2567.01	2707.01	NA	101.50	185	350
PE-PT DCS	722.03	992.04	1149.15	1249.15	NA	40	90	150
Untreated DCS	435.80	546.70	578.90	795.00	NA	60	Na	25
Raw DCS (control)	320.15		205.00		NA		NA	

of hydrogen fermentative pathways by HCB especially methanogens or b) nutrient shock [25,58,73]. This argument is supported by the relative abundance of the most dominant microbial community at the genus and phylum level (Fig. 5), where the principal genus *Ruminococcus* products (H$_2$, acetate, and CO$_2$) are easily used as substrates for methanogens.

Additionally, lactic acid bacteria are known for their anti-microbial activities, which are inhibitory to HPB [75–77] and affect the hydrogen production yield. Therefore, the change of fermentative medium pH caused by lactic acid and anti-microbial products such as hydrogen peroxide and polypeptide antibiotics (bacteriocins) can inhibit hydrogen fermentation pathways.

3.5. Microbial community composition

The bacteria community's relative abundance and taxonomic distribution in each sample were analysed at the genus and phylum levels (Fig. 5). It can be seen from the graph that a total of 25 phyla across all samples were identified, with 24 phyla classified and 1 unclassified. *Firmicutes* were the most dominant phylum, accounting for ~70–100% in the pretreated samples, followed by *Actinobacteria* (~1–15%), *Chloroflexi* (~1–5%), *Proteobacteria* (~1–4%) and *Bacteroidetes* (~1–3%). Heat-shocked DCS also contains *Planctomycetes* (~1%). In addition, PE-PT DCS digestates had *Thermotogae* (3.5%), which produce hydrogen, acetate, and CO$_2$ from sugar. *Thermotogae* also produces sulphide in the presence of sulphur compounds or hydrogenotrophic sulphate-reducers such as *Desulfovibrio Vulgaris* [46]. This argument is definite as PE contains varying amounts of sulphur (Table 3). While most of the mentioned phylum are well-known hydrogen producers from simple to complex substrates producing variable fatty acids, *Planctomycetes* and *Chloroflexi* are known for degrading organic matter [78]. The high population of *Firmicutes* across pretreated samples compared to

the control could be from the impacts of the DCS pretreatments, which enriched the hydrogen producers and eliminated the activities of hydrogen consumers.

Nonetheless, the control DCS sample had *Firmicutes* (~20%) and *Actinobacteria* (~20%) as the predominant phylum, followed by *Bacteroidetes* (~12%), *Euryarchaeota* (~10%), *Chloroflexi* (~5%), *Actinobacteria* (~4%), *Spirochaetes* (~2.5%), *Planctomycetes* (~2%), *Fibrobacteres* (~2%) and others (~1–1.5%). The uneven distribution of the microbial composition in the control samples compared to pretreated samples confirmed the effectiveness of DCS pretreatment in the hydrogen fermentation process. Furthermore, the high inhabitants of HCB observed in the microbial configuration in the DCS control digestates confirmed that untreated DCS produce lower hydrogen yield than DCS enriched by either physical or chemical methods.

Ruminococcus, Bacillales, Clostridium, Thermoanaerobacterium and *Rhodococcus* dominated the enriched DCS samples' microbial community at the genus level. Other notable genera were *Bacillus* and *Coprococcus* (Fig. 5). The identified genera were reported among the HPB by Refs. [10,65,79]. While the reported organisms were mesophiles and thermophiles, *Thermoanaerobacterium* is a strict hyperthermophile that thrives better at temperatures above 70 °C. However, it has adapted to the operating temperature of 55 °C employed in this study. In contrast, *Bacillales, Bacteroidales, Methanobacterium, Idiomarinaceae, Acholeplasma Methanosarcina, Clostridium, Thermoacetogenium* and *Carnobacterium* were among the most dominant communities in control DCS digestates. The genera *Ruminococcus* and *Coprococcus* found in the mammalian gut can degrade recalcitrant substrates producing varying SCFA [80]. The organisms, notably *Ruminococcus,* also contributed to cellulose and cellobiose degradation to hydrogen, acetic acid, and CO$_2$, providing direct soluble substrates for methane production [46]. This idea explains why the CSWW-PT reactor produced low hydrogen yield than PE and
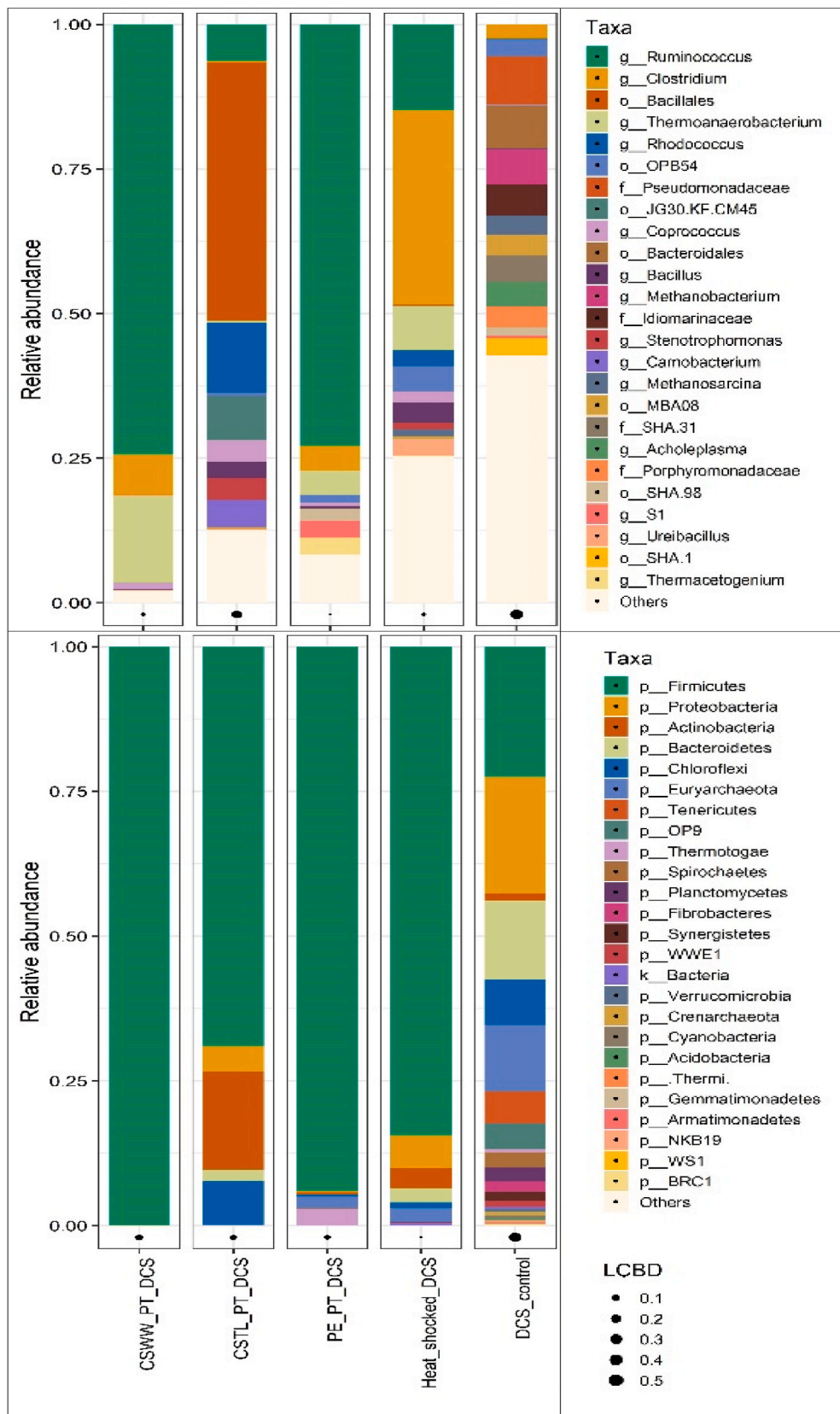
Fig. 5. The relative abundance of the top 25 most abundant microbial communities at the genus and phylum level of pretreated DCS samples. While the bars correspond to taxa that are most dominant within the sample, the black points whose diameter relates to the magnitude of the LCBD value of the digestates that is higher LCBD mean the sample has more unique species than others.

heat-shocked DCS, even though it has a comparable HPB community (Fig. 5). Many thermophilic *Clostridium* grows optimally at 55 - 65 °C and can degrade a broad complex of carbohydrates such as cellulose, hemicellulose and xylans producing hydrogen and SCFA [81]. The most popular *Clostridium* is C. buytricum, C. thermolacticum, C. pasteurianum, C. paraputrificum M-21 and C. bifermentants [65].

Additionally, the order Bacillales, represented by Geobacillus and Thermobacillus, is a facultative endospore-forming bacteria that can degrade lignocellulose under aerobic and anaerobic conditions employing highly thermostable enzymes such as α-amylase and β-xylosidase [81]. Another most dominant genus, *Thermoanaerobacterium,* can utilise complex substrates such as xylan, starch, cellulose, hemicellulose, and its degraded products and transform them to acetic acids, hydrogen, and CO_2 at an optimal temperature of 65–70 °C [81,82]. This knowledge explains why *Thermoanaerobacterium* was the second most abundant genus in CSWW-PT DCS. The CSWW employed in the DCS pretreatment has high COD, mainly of starch-related compounds (Table 1). Even though there was no alcohol detection in all the reactors, it has been reported that *Thermoanaerobacterium* may contribute to ethanol production during the AD process [83]. The genus *Bacillus,* which also belongs to the phylum *Firmicutes* is known to produce hydrogen and fatty acids from the consumption of a wide variety of substrates in aerobic conditions [10,65,84].

The genus *Ruminococcus* was the principal bacteria in CSWW (~75%), and PE-PT DCS (~73%) (Fig. 5). On the other hand, *Bacillales* (~45%) and *Clostridium* (~35%) were the predominant genera in CSTL-PT and heat-shocked DCS, respectively. Their different pretreatments could explain the differences in the most abundant bacteria across the enriched samples. In CSWW and PE enrichments, the *Ruminococcus* is popular in the stomach of rumen animals, and thus the digested cattle slurry strived better when enriched with CSWW and PE. Nevertheless, in heat-shocked DCS, the genus *Clostridium* survived better the heat treatment applied. At the same time, the HCB, as seen in DCS control samples and other HPB, were eliminated, inactivated, or reduced in population. Even so, there was no unequivocal explanation for the increased dominance of *Bacillales* in CSTL-PT DCS; they may have been favoured more than the other HPB during CSTL DCS enrichment processes due to the presence of assimilable and soluble substrates.

4. Conclusions

The feasibility of biology hydrogen production using seed sludge pretreated with agro-industrial waste (PE, CSWW and CSTL) was explored. Based on the result obtained, it is concluded that agro-industrial waste can enrich hydrogen producers while inhibiting hydrogen consumers in DCS. Although heat-shocked DCS produced the highest daily VHP of 135 NmL H_2 g^{-1} VS on the second day when compared to other pretreated DCS using glucose as substrates, it is followed by PE-PT DCS, which gave the highest daily VHP of 115 NmL H_2 g^{-1} VS but at a shorter time (24 h). The other PT DCS - CSWW-PT DCS and CSTL-PT DCS had lower daily VHP of 92 and 82 NmL H_2 g^{-1} VS, respectively, while the untreated DCS had 37 NmL H_2 g^{-1} VS as its highest daily VHP. Similar results were obtained when sucrose was the medium. However, the highest peaks were recorded in all the laboratory reactors on day two, with the highest daily VHP of 211 NmL H_2 g^{-1} VS achieved in PE-PT DCS digesters.

The mechanism of PE, CSTL, and CSWW on sludge enrichment is not fully understood. However, it is believed that suppression of HCB by PE could be from the extremely alkaline condition of the PE and the presence of earth and trace elements, particularly potassium, magnesium, calcium, and iron. In the same vein, the strategy of microbial inhibition from CSWW and CSTL-PTs could be from the high concentration of SCFA and nutrient shock. Finally, after the various DCS PT studies, the dominant phylum *Firmicutes,* represented by the *Clostridium* and *Ruminococcus,* were the most abundant bacteria compared to the untreated DCS (control), which was more diverse. The results suggest that using

AIW as a pretreatment agent can improve biological hydrogen production efficiency from DCS, an abundant and underutilised waste material. The findings of this study could contribute to the development of sustainable and cost-effective methods for enriching hydrogen-producing bacteria. Further research is required to optimise the conditions for AIW DCS pretreatment.

CRediT authorship contribution statement

Emeka Boniface Ekwenna: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Shamas Tabraiz:** Writing – review & editing, Software, Data curation. **Yaodong Wang:** Writing – review & editing. **Anthony Roskilly:** Conceptualization, Resources, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

AD	Anaerobic digestion
AF	Anaerobic fermentation
AIW	Agro-industrial wastes
ASV	Amplicon sequence variant
C/N	Carbon-to-nitrogen
COD	Chemical oxygen demand
CSTL	Corn-steep liquor
CSWW	Cassava-steep wastewater
DCS	Digested cattle slurry
DADA	Divisive amplicon deionising algorithm
DF	Dark fermentation
EPFB	Empty palm fruit bunch
HCB	Hydrogen-consuming bacteria
HPB	hydrogen-producing bacteria
HPR	Hydrogen production rates
H_2	Hydrogen gas
LCBD	Local Contributions of Beta Diversity
N mL	Millilitres in normal condition (gas volumes at 0 °C and an atmospheric pressure of 101.3 kPa) OLR Organic loading rate
PCR	polymerase chain reaction
PE	Potash extract
PT	Pretreated
QIIME	Quantitative Insights into Microbial Ecology
RS	Rice straw
SCFA	short-chain fatty acid (s)
TS	Total solid
TSS	Total suspended solid
VFA	Volatile fatty acid (s)
VHP	Volumetric hydrogen production (N mL)
VHY	Volumetric hydrogen yield (N mL)
VDI	Verein Deutscher Ingenieure
VS	Volatile solids
VSS	Volatile suspended solids
WAS	waste-activated sludge

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