



Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum

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Bio-hydrogen production from wastewater using sludge as inoculum is a sustainable approach for energy production. This study investigated the influence of initial pH and temperature on bio-hydrogen production from dairy wastewater using pretreated landfill leachate sludge (LLS) as an inoculum. The maximum yield of 113.2 ± 2.9 mmol H₂/g chemical oxygen demand (COD) (12.8 ± 0.3 mmol H₂/g carbohydrates) was obtained at initial pH 6 and 37 °C. The main products of volatile fatty acids were acetate and butyrate with the ratio of acetate:butyrate was 0.4. At optimum condition, Gibb's free energy was estimated at -40 kJ/mol, whereas the activation enthalpy and entropy were 65 kJ/mol and 0.128 kJ/mol/l, respectively. These thermodynamic quantities suggest that bio-hydrogen production from dairy wastewater using pretreated LLS as inoculum was effective and efficient. In addition, genomic and bioinformatics analyses were performed in this study.

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[**Key words:** Bio-hydrogen; Enthalpy; Entropy; Gibb's free energy; Arrhenius; Modified gompertz model]

Hydrogen (H₂) is a potential alternative green energy carrier because only water and energy are generated from the complete combustion (1,2). H₂ does not naturally exist as gas but more commonly found in combination with other elements and form a variety of compounds such as water, hydrocarbons and carbonates (3–5). Hence, it can be extracted from these compounds to be used as an energy carrier. Currently, most of the H₂ production derived from fossil fuels as raw material via water gas swift (WGS) reaction (6). When considering environmental problems tied to fossil fuels such as emission of CO₂ as greenhouse gas (1,2), there is an urgency to explore green new renewable sources.

Recently, biological H₂ (bio-H₂) has attracted many researchers interests because H₂ can be harvest from organic rich waste via dark fermentation with anaerobic bacteria (7). The advantages of dark fermentation are as follows: there is no requirement for light and thus fermentation can be conducted day and night with simpler and cheaper bioreactor design, and (8) various type of substrate can be utilized in particularly renewable resources that are organically rich such as wastewater, stillage, sludge, pomace, stalks and bagasse (9–15). Therefore, H₂ production via dark fermentation will be potentially integrated into waste management in order to turn waste into energy (16).

Many wastewater treatment plants incorporates biological process to breakdown organic compounds in wastewater (17). Although this conventional treatment method has successfully reduced the level of biological oxygen demand (BOD) or chemical oxygen demand (COD), it has converted the organic compounds (pollutants) into carbon dioxide which is a potent greenhouse gas (18–20). To improve the sustainability of biological wastewater treatment and bio-H₂ production, mixed microbial community from various type of sludge could be integrated into wastewater management (20). Sludge is a byproduct from wastewater treatment, which usually contain abundant of bacteria. These bacteria may have unique features to treat the wastewater and also generate H₂ because it normally feeds on various type organic compounds under anaerobic condition (21,22). Furthermore, sludge represent the natural symbiotic interaction within the mixed microbial community, which might potentially enhance H₂ production (23).

H₂-producing microbial community naturally subsists in wastewater sludge under anaerobic condition is a convenient source of inoculum for H₂ production (7). This led us to emphasis on landfill leachate sludge because it was understood that landfill leachate facility built to dispose harsh municipal waste under anaerobic condition (24). Bacteria that survive in such harsh environment usually have a better adaption to harsh living conditions such as poor nutrients and pH. This microbial community usually has special synergistic interactions to improve food and nutrients availability through decomposing organic matters (24,25). Since microbial community in leachate sludge survives under anaerobic

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condition, it would possess unique H₂ production performance. Recently, we have reported the performance of leachate sludge for H₂ production using glucose as feedstock (26). The H₂ yield was 6.43 mol H₂/mol glucose with Gibbs free energy of -34 kJ/mol at pH 6 and 37 °C. Further, it is crucial to analyse the relationship between microbial community and H₂ production in wastewater.

In this study, bio-H₂ was produced from dairy wastewater as organic feedstock using landfill leachate sludge (LLS) as an inoculum. Dairy products are the important source of proteins, vitamins and minerals but they have a short shelf life. The expired products are often returned and then followed by biological treatment. Since the expired products are unsafe for human consumption, they provide good resources for H₂ production. According to Venkata Mohan et al. (27), sludge from bioreactor managed to produce 1.8 mmol H₂/g COD by using dairy wastewater as feedstock. Therefore, dairy wastewater can be potentially integrated with bio-H₂ production to achieve the goal of waste-to-energy technology. Based on our previous study (26), LLS has shown a high H₂ production efficiency of 6.43 mol H₂/mol glucose, which outcompetes the conventional yield. In this study, we have attempted to use the same sludge inoculum to investigate on the H₂ production from dairy wastewater.

The role of microbial diversity of LLS inoculum in bio-H₂ production carried important information. Currently, the most common NGS methods used to study the microbial community in biogas productions are 454 pyrosequencing and SOLiD (Thermo Fisher Scientific, Waltham, MA, USA; sequencing by oligo ligation and detection) (28–32). Bio-H₂ producing microbial communities has not been analysed by Illumina Mi-Seq (Illumina, Inc., San Diego, CA, USA) yet. It was reported that the sequencing cost of Illumina for every megabase is 50- and 12,000-fold cheaper than 454 pyrosequencing and Sanger sequencing, respectively (33). Moreover, this approach adopted paired-end sequencing which is rapid, comprehensive, and reproducible with Illumina Mi-Seq (33). This study provides a better understanding on the relationship between the effect of microbial diversity and bio-H₂ production. This will allow us to better understand the dynamic and synergism effect of the microbial community of the LLS inoculum. The importance of this study represents the practical application of H₂ production from dairy wastewater using LLS as inoculum.

MATERIALS AND METHODS

Inoculum and treatment conditions The sanitary landfill was located in Jeram, Selangor, Malaysia. Fresh sludge was obtained from the leachate collection pond. Large particles were removed through a 400 µm screen and stored at 4 °C. The LLS was heated at 65 °C for 30 min to eliminate H₂-consuming bacteria and enriched at 37 °C for 24 h with Reinforced Clostridial Medium (CM0149, Oxoid, Thermo Fisher Scientific) to standardise cell count inoculated into the reactors (26). The features of this sludge are as follows: (i) Untreated LLS was raw LLS collected from landfill leachate pond without any pre-treatment. (ii) Pretreated LLS was pretreated with heat at 65 °C for xx hours. (iii) Recycled LLS was a pretreated LLS after third cycle in the dark fermentation.

Wastewater collection Dairy wastewater was collected from a manufacturer of dairy products in Selangor, Malaysia. It was collected freshly from wastewater discharge point where hot wastewater is channelled into the treatment plant. The characteristics of the dairy wastewater are summarized in Table 1. The bioavailable substrates for H₂ production in the dairy wastewater composed of a mixture of glucose, sucrose, lactose and fructose which was represented by concentration of soluble carbohydrate.

Experimental setup The batch fermentation and biogas analysis was carried out as reported in our previous work (26) with minor modification. Repeated batch fermentation in triplicate was conducted to examine the sustainability of H₂ yield. Inoculated sludge from the first batch fermentation was reused as the inoculum for the next fermentation and hence forth. To reuse the sludge, the media were centrifuged at 5000 rpm for 10 min upon the end of fermentation. The pellet was rinsed three times with saline to ensure no residue was carried forward to the subsequent fermentation. A 150 mL of dairy wastewater was placed into each 200 mL serum bottle. The pellet was re-suspended in saline in order to readjust

TABLE 1. Characteristics of dairy wastewater.

Parameter	Dairy wastewater
pH	5.90 ± 0.26
Total COD (mg/L)	14150 ± 600
Soluble COD (mg/L)	9567 ± 293
Kjeldahl N (mg/L)	306 ± 13
Soluble carbohydrate (g/L)	84.60 ± 4.75
Soluble protein (g/L)	3.20 ± 0.77

the sludge concentration to 2% v/v. This recycled sludge was re-inoculated into the fresh medium to resume fermentation. The H₂ production from dairy wastewater was tested in the effect of organic load (dilution factor: 0%, 20%, 40%, 60% and 80%), initial pH (pH 4, 5, 6, 7 and 8) and temperature (25, 30, 37, 45, and 50 °C).

Analysis Total COD in wastewaters was measured using Hach method 8000 with COD digestion reagent vials, high range (20–1500 mg/L) (Hach Company, Loveland, CO, USA). Soluble carbohydrates in wastewater were measured using phenol/sulphuric acid method (34). The COD and carbohydrate reading was taken at room temperature. Besides, initial pH for COD and carbohydrate were also analysed before and after dark fermentation. This is a measurement problem. Kjeldahl nitrogen was tested using Hach Total Kjeldahl Nitrogen Method 8075.

The concentration of soluble metabolites was analysed with the Agilent HPLC (1200 series, Agilent Technologies, Santa Clara, CA, USA), using refraction index detection at 55 °C and Animex Hi-Pex H column at 65 °C (26). Water displacement method was adopted for biogas collection. The biogas composition was examined with Agilent gas chromatography, using thermal conductivity detector with Hayesep Q column. The temperature of injector, detector and oven was set at 100, 150 and 60 °C, respectively. The flow rate for carrier gas was set as 2 mL/min (26).

Kinetic analysis with modified Gompertz model Cumulative H₂ production was analysed with the modified Gompertz equation using OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA) (35):

$$H = H_{\max} \left\{ -e \left[\frac{R_{\max} \cdot e}{H_{\max}} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where H is the cumulative H₂ production, H_{\max} is the maximum H₂ production, R_{\max} is the maximum H₂ production rate, λ is the lag phase time (h), t is time (h), and $\frac{R_{\max} \cdot e}{H_{\max}}$ is the rate constant (k).

Activation entropy of fermentation and activation enthalpy of thermal deactivation Modified Arrhenius equation was used to determine the enthalpies. The production of Bio-H₂ involves a series of enzymatic reactions. Therefore, like all enzymatic reactions, the rate of reaction for the production of bio-H₂ increases with temperature (T_{opt}). However, once the temperature exceeds T_{opt} , bio-H₂ production is subjected to thermal deactivation because of enzyme denaturation and cell death. This is represented by the following equations (26,36):

$$\ln H_{\max} = \ln(A \cdot X \cdot Y) - \frac{\Delta H}{RT}, \quad T < T_{\text{opt}} \quad (2)$$

$$\ln H_{\max} = \ln(B \cdot X \cdot Y) - \frac{\Delta H^*}{RT}, \quad T > T_{\text{opt}} \quad (3)$$

where H_{\max} is the maximum H₂ productivity obtained from modified Gompertz equation, A and B are the Arrhenius pre-exponential factors, X is the cell biomass concentration, Y is the H₂ yield per unit cell biomass, R is the ideal gas constant (8.3144621 J/K/mol), T is temperature in Kelvin and ΔH is the fermentation activation enthalpy (26,36). The threshold energy for deactivation of enzymes and death of bacteria is represented by the activation enthalpy of thermal deactivation (ΔH_d) and it is determined by Eq. 4.

$$\Delta H_d = \Delta H + |\Delta H^*| \quad (4)$$

Activation entropy of fermentation and activation entropy of thermal deactivation The Eyring–Arrhenius equation was used to calculate the activation entropy of fermentation (ΔS) and activation entropy thermal deactivation (ΔS_d) (26,36).

$$\Delta S = R \left(\ln \frac{Ah}{k_b T} \right) \quad (5)$$

$$\Delta S_d = R \left(\ln \frac{Bh}{k_b T} \right) \quad (6)$$

where A and B are the Arrhenius pre-exponential factors, h is the Planck's constant (6.63 × 10⁻³⁴ J s) and k_b is the Boltzmann's constant (1.38 × 10⁻²³ J/K).

Gibbs free energy Gibbs free energy is calculated with Eq. 7 (26).

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

where ΔH is obtained from Eq. 2 and ΔS is obtained from Eq. 5.

Genomic DNA extraction Prior DNA extraction, sludge was centrifuged at $3600 \times g$ to remove excess liquid. Bacterial genomic DNA in sludge was extracted using UltraClean Soil DNA Isolation Kit (MoBio Inc., Beijing, China) with modifications. To remove water from sludge, 0.25 g of sludge was added to bead tube for 30 s at $10,000 \times g$. The supernatant was completely removed with pipet. Subsequently, the bead and bead solution were added back into the tube and vortex to mix. To lyse bacterial cells, 60 μL of lysis buffer S1 was added to the tube. After brief vortex, 200 μL of inhibitor removal IRS was added. The tubes were secured horizontally on a vortex and then vortex at maximum speed for 20 min. Upon the end of vortex, the tubes were centrifuged at $10,000 \times g$ for 1 min. The supernatants were transferred to clean microcentrifuge tubes and centrifuged at $10,000 \times g$ for 30 min to completely remove fine sludge particles. The supernatants were transferred to clean tubes. Proteins from the extractions were removed by adding 250 μL solution S2, gently mixed and incubated in ice for 5 min. The tubes were centrifuge at $10,000 \times g$ for 1 min to remove precipitated proteins. Avoiding the pellet, the entire volume of supernatant was transferred to clean collection tubes. 1.0 mL of DNA binding salt solution S3 was added into the tubes and gently mixed. The volume was load onto the spin filter and centrifuged at $10,000 \times g$ for 1 min. The flow through was discarded. The spin filters were washed with 300 μL ethanol based wash solution S4 and centrifuge at $10,000 \times g$ for 1 min. This step was repeated with 150 μL of solution S4 to completely remove inhibitors. The spin filters were dried by centrifugation min at $10,000 \times g$ for 5 min. To elute extracted DNA, 20 μL of nuclease free water was added to the spin filter and centrifuged at $10,000 \times g$ for 1 min. The elution was stored at $-4 \text{ }^\circ\text{C}$ for downstream applications.

16S rRNA-metagenomics sequencing with next generation sequencing The concentration of gDNA was quantified using Qubit 2.0 Fluorometer. The V3 region on 16S rRNA was amplified using KAPA HiFi PCR kit with barcoded primer as follows: forward primer: 5'-AATGATACGGCACCACATCTACA-3'; reverse primer: 5'-CAAGCAGAAGACGGCATACGAGATGA-5'.

The amplification cycle was $95 \text{ }^\circ\text{C}$ for 3 min; 25 cycles at $98 \text{ }^\circ\text{C}$ for 20 s, $63 \text{ }^\circ\text{C}$ for 15 s, $72 \text{ }^\circ\text{C}$ for 5 s and $72 \text{ }^\circ\text{C}$ for 1 min. Upon the completion of PCR amplification, the amplified sequence with the size of approximately 300 bp was selected by using 2% gel electrophoresis. The sequence was then recovered using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The extracted DNA sequence was diluted $10,000\times$ with 0.05% Tween 20. Quantitative PCR (qPCR) was performed to quantify the concentration of amplified sequence at $95 \text{ }^\circ\text{C}$ for 5 min; 35 cycles at $95 \text{ }^\circ\text{C}$ for 30 s, $60 \text{ }^\circ\text{C}$ for 30 s, and $72 \text{ }^\circ\text{C}$ for 10 s. The sample preparation kit and sequencing kit used were Nextera XT DNA Sample Preparation Kit (Illumina Inc.) and MiSeq Reagent Kits v2 (Illumina Inc.), respectively. Whole genome sequencing was performed using the Illumina MiSeq Benchtop Sequencer (2×150 bp paired-end sequencing).

Bioinformatics analysis CLC Genomic Workbench 5.1.1 (CLC bio, Aarhus, Denmark) was used to trim the raw amplicons to Qscore of 20 and amplicons shorter than 50 bp were eliminated and minimum length was set at 400 bp. The trimmed amplicons were aligned against the 16S rRNA in NCBI GenBank (e-value of $<10^{-20}$) using USEARCH v6.0 (37). The output from USEARCH was normalised with MEGAN5 by the "use normalised count" option and analysed with biodiversity and abundance assessment (28). The threshold was set at 97% for taxonomic classification at genus level. The taxonomy distributions were represented using Krona interactive visualization program (29).

RESULTS AND DISCUSSION

Effects of initial pH and fermentation temperature on H_2 production from dairy wastewater

Initial pH and temperature are the most important factors because they influence the bacterial metabolism. As shown in Table 2, the highest H_2 production performance was observed at pH 6 and $37 \text{ }^\circ\text{C}$. This falls within the reported range of pH (pH 6–8) and temperature ($35\text{--}39 \text{ }^\circ\text{C}$) for H_2 production (30,31). At optimum condition, the maximum H_2 production was 113.2 ± 2.9 mmol H_2/g COD or 12.8 ± 0.3 mmol H_2/g carbohydrates at production rate of 3.73 ± 0.01 mmol H_2/g COD/h (0.42 ± 0.00 mmol H_2/g carbohydrates/h) with lag time of 25.9 ± 0.8 h. The H_2 production from dairy wastewater using LLS as inoculum was more efficient as compared to other inoculums (Table 3). The efficient H_2 production is commonly related to the type and diversity of H_2 -producing bacteria present in the inoculum. The performance could be efficient if the inoculum contains little or no H_2 -consuming bacteria. In addition, H_2 -producing bacteria belonging to the family of strict anaerobes *Clostridiaceae* have the greatest potential in H_2 production via dark fermentation (32), as compared to facultative H_2 -producing bacteria such as the family of *Enterobacteriaceae*. Hence, the performance of H_2 production could be improved with a high diversity of strict anaerobic H_2 -producing bacteria. As reported in our previous work (26), the sludge originated from landfill shows great potential in H_2 production from glucose could be due to the diverse microflora in the inoculum.

Overall, the optimum initial pH for H_2 production was 6 and reduced by 22% at initial pH of 8. Nevertheless, the cell biomass at initial pH 6 was 6.6% higher and faster growth lag time of 24.2 ± 1.2 h (Supplementary data Table S1). This suggested that more cell biomass instead of H_2 was produced from the carbohydrates at higher initial pH 8, which was well agreed with our previous study (26). In comparison, no cell growth and H_2 productions were detected at initial pH 4. The results indicate that initial pH 6 favours H_2 production because this hydrogenase function well in the pH between 6 and 6.5 (38). Furthermore, pH 6 also tend to inhibits H_2 -consuming bacteria including methanogens and acetobacteria (23). This would mean that H_2 production could enhance due to suppression of H_2 consumption process. Therefore, different initial pH alters bacteria activities which in turn influence H_2 production.

The performance of H_2 productivity and cell biomass were the highest at $37 \text{ }^\circ\text{C}$ regardless of the different initial pH (Tables 2 and 3). At temperature beyond $37 \text{ }^\circ\text{C}$, the measured cell biomass was

TABLE 2. Kinetic parameters of production H_2 from dairy wastewater using modified Gompertz equation.

Initial pH	Temp (C)	H_{\max}		R_{\max}		k	λ (h)	R^2
		mmol H_2/g COD	mmol H_2/g carb	mmol H_2/g COD/h	mmol H_2/g carb/h			
5	25	10.9 \pm 0.3	1.2 \pm 0.0	0.48 \pm 0.00	0.05 \pm 0.00	0.120 \pm 0.007	49.4 \pm 0.3	0.999
	30	39.5 \pm 1.1	4.5 \pm 0.1	1.10 \pm 0.00	0.12 \pm 0.00	0.076 \pm 0.004	35.6 \pm 0.4	0.999
	37	54.1 \pm 4.1	6.1 \pm 0.5	1.40 \pm 0.01	0.16 \pm 0.00	0.071 \pm 0.008	38.4 \pm 0.7	0.996
	45	30.3 \pm 2.1	3.4 \pm 0.2	0.73 \pm 0.00	0.08 \pm 0.00	0.066 \pm 0.006	38.3 \pm 0.6	0.997
6	25	39.8 \pm 0.7	4.5 \pm 0.1	1.40 \pm 0.00	0.16 \pm 0.00	0.096 \pm 0.004	40.1 \pm 0.3	0.999
	30	78.9 \pm 2.1	8.9 \pm 0.2	2.99 \pm 0.01	0.34 \pm 0.00	0.103 \pm 0.007	37.9 \pm 0.5	0.998
	37	113.2 \pm 2.9	12.8 \pm 0.3	3.73 \pm 0.01	0.42 \pm 0.00	0.090 \pm 0.007	25.9 \pm 0.8	0.997
	45	43.0 \pm 2.6	4.9 \pm 0.3	1.01 \pm 0.01	0.11 \pm 0.00	0.064 \pm 0.008	24.3 \pm 1.3	0.992
7	25	20.2 \pm 0.6	2.3 \pm 0.1	0.74 \pm 0.00	0.08 \pm 0.00	0.100 \pm 0.005	45.4 \pm 0.3	0.999
	30	42.4 \pm 1.1	4.8 \pm 0.1	1.73 \pm 0.00	0.20 \pm 0.00	0.111 \pm 0.008	37.3 \pm 0.6	0.998
	37	88.5 \pm 3.2	10.0 \pm 0.4	2.66 \pm 0.01	0.30 \pm 0.00	0.082 \pm 0.009	21.1 \pm 1.2	0.993
	45	32.8 \pm 0.5	3.7 \pm 0.1	1.41 \pm 0.00	0.16 \pm 0.00	0.117 \pm 0.007	29.4 \pm 0.5	0.998
8	25	37.7 \pm 3.5	4.2 \pm 0.4	0.90 \pm 0.01	0.10 \pm 0.00	0.065 \pm 0.009	36.9 \pm 0.9	0.994
	30	38.2 \pm 1.6	4.3 \pm 0.2	3.00 \pm 0.01	0.16 \pm 0.00	0.098 \pm 0.012	31.1 \pm 1.1	0.993
	37	89.2 \pm 3.7	10.1 \pm 0.4	2.57 \pm 0.01	0.29 \pm 0.00	0.078 \pm 0.008	24.2 \pm 1.2	0.993
	45	39.0 \pm 1.4	4.4 \pm 0.2	1.43 \pm 0.01	0.16 \pm 0.00	0.100 \pm 0.011	30.3 \pm 1.0	0.994

Data for initial pH 4 and $50 \text{ }^\circ\text{C}$ are not displayed because H_2 productions and cell biomass was not detected.

TABLE 3. Comparison of H₂ yield from this study with other studies.

Sludge inoculum	H ₂ yield	Sludge pretreatment	Substrate source	Initial pH and temp. (°C)	Substrate consumption	Ref.
LLS	113.2 ± 2.9 mmol H ₂ /g COD 12.8 ± 0.3 mmol H ₂ /g carbohydrates	Heat	Dairy wastewater	pH 6; 37.0 °C	81.7 ± 1.2% COD 85.6 ± 0.9% carbohydrates	This study
H ₂ producing reactor	0.0018 mmol H ₂ /g COD 1.8 mmol H ₂ /g COD	Untreated Acid	Dairy wastewater	29.0 °C	79% COD 63% COD	10
Palm oil mill effluent (POME) treatment plant	0.0122 mmol H ₂ /g COD	Heat	POME	pH 5.5; 35.0 °C	69% COD	25
	0.0317 mmol H ₂ /g COD	BES			87% COD	
	0.41 mmol H ₂ /g COD	Heat			86% COD	
Sewage treatment plant	0.32 mmol H ₂ /g COD	Acid	Glucose and peptone	pH 10.5; 37.0 °C	51% COD	26
	0.23 mmol H ₂ /g COD	Chloroform			51% COD	
	0.12 mmol H ₂ /g COD	Untreated			66% COD	
Sewage treatment plant	^a 0.42 mmol H ₂ /g COD	Base	Stillage of ethanol plant	pH 5.05; 37.0 °C	N.A. 90% carbohydrates	27

^a H₂ yield was estimated using $PV = nRT$ at standard condition where $P = 1 \text{ atm}$; $R = 8.21 \times 10^{-5} \text{ m}^3 \text{ atm/mol} \cdot \text{K}$ and $T = 300 \text{ K}$.

reduced along with H₂ production. The H₂ production and cell growth was totally ceased at 50 °C. Temperature could manipulate growth of H₂-producing bacteria by changing the permeability of cell membrane. The membrane solidify at low temperature whereas liquefy at high temperature and this may inactivate integral proteins for transport of nutrients and ions (39). Moreover, H₂ is the product of deprotonation from a series of enzymatic reactions. Since enzymatic activities are susceptible for thermal deactivation (36), hydrogenase in the microbial community was also temperature sensitive. Furthermore, most of the H₂-producing bacteria are reported to be mesophiles such as *Clostridium* spp., *Enterobacter* spp., and *Bacillus* spp. (40,41). They survive in a wide range of temperature from 15 to 45 °C but most commonly fermentative H₂ production was reported at a narrow range of 35–39 °C (7). Based on the metagenomics analysis, the pretreated LLS in this study mostly comprised of *Clostridium* spp.

Thermodynamics of H₂ production from dairy wastewater

Table 4 shows that the Gibbs free energy at initial pH 5, 6, 7, and 8 was –17, –40, –20 and –16 kJ/mol, respectively. It was observed that highest Gibbs free energy was belonged to the highest H₂ yield at initial pH 6. This demonstrated that H₂ production at initial pH 6 was more thermodynamically favourable as compared to that of other pH values. The modified Arrhenius plot described by Eqs. 2 and 3 shows a good regression (Fig. 1). The optimum operation temperature represents by the intersection point. It is interesting that the linear lines intersect at the same point at all initial pH and hence the optimum fermentation temperature was 38.4 °C. The thermodynamic analysis indicated that H₂ production from dairy wastewater using LLS as inoculum is thermodynamically favourable.

ΔH represents the thermodynamic potential of this reaction, it measured the amount of heat released or absorbed in the reaction (26,36). The ΔH at initial pH 5, 6, 7 and 8 was 99, 65, 62 and 57 kJ/mol, respectively. It was interesting to note that ΔH of this dark fermentation was endothermic reaction which showed that

energy was absorbed during H₂ production. This is because ATP was hydrolysed to drive enzymatic conversions of substrate into molecular H₂ (26,42). The ΔH obtained in this study falls within the range for microbial growth and enzymatic reaction of 54–71 and 18–83 kJ/mol, respectively (26,36). However, it is surprising that the ΔH at initial pH 5 was out of the range for microbial growth and enzymatic reaction. This phenomenon occurred because more energy was required to drive H₂ production. In other words, bacteria in LLS need more ATP to drive H₂ production at pH 5 and thus it was thermodynamically less favourable as compared to other pH. On the contrary, the ΔH_d is the threshold energy for deactivation of enzymes and death of bacteria (26,36). The ΔH_d at initial pH 5, 6, 7 and 8 was 236, 282, 268 and 267 kJ/mol, respectively. These values fall within the 290–380 kJ/mol for bacterial cell death (43) and therefore it is less sensitive to thermal deactivation. In comparison with the ΔH_d reported by Wong et al. (26) and Fabiano and Perego (36), the H₂ production was 118 and 113 kJ/mol, respectively. This clearly showed that H₂ production from dairy wastewater by using LLS as inoculum was less sensitive to thermal deactivation.

Entropy measures the randomness of a reaction in which activation entropy of fermentation (ΔS) and activation entropy of thermal deactivation (ΔS_d) indicate the randomness during fermentation and thermal deactivation, respectively (26,36). The ΔS was 0.054, 0.128, 0.265 and 0.052 kJ/mol/K at initial pH 5, 6, 7 and 8, respectively. This indicated that H₂ production from dairy wastewater was a random reaction. The ΔS_d initial pH 5, 6, 7 and 8 was 0.424, –1.17, 0.521 and 0.516 kJ/mol/K, respectively. It is noteworthy that the value of ΔS_d at initial pH 5, 7 and 8 was higher than the respective value of ΔS_d . This is reasonable because the randomness of the reaction increases with enzymes denaturation (44,45). However, the negative value of ΔS_d at initial pH 6 shows a reduced randomness which was also observed by others (26,36,44). This phenomenon was worth to be further investigated because the implication of this negativity value has not been well explained yet (44,45).

Effect of organic load on H₂ production from dairy wastewater

The initial organic load plays an essential role on the production of H₂. The performance of H₂ production was enhanced with increasing dilution factor. Based on H₂/g COD H₂, the yield for 80% dilution was 1.5 times higher than that of undiluted wastewater (Table 5). According to law of mass action, fermentation rate increases with substrate concentration (46). In this study, the fermentation rate increase with decrease of substrate concentration, which is contradict with law of mass action. This was due to enzymatic reaction of hydrogenase that governed H₂ production was prone to inhibition by high organic

TABLE 4. Parameters of thermodynamic for H₂ production from dairy wastewater using LLS inoculum.

Thermodynamic parameter	pH 5	pH 6	pH 7	pH 8
ΔG (kJ/mol)	–17	–40	–20	–16
ΔH (kJ/mol)	99	65	62	57
ΔH_d (kJ/mol/K)	0.054	0.128	0.265	0.052
ΔS (kJ/mol)	236	282	268	267
ΔS_d (kJ/mol/K)	0.424	–1.17	0.521	0.516

Data for initial pH 4 and 50 °C were not displayed because H₂ productions and cell biomass was not detected.

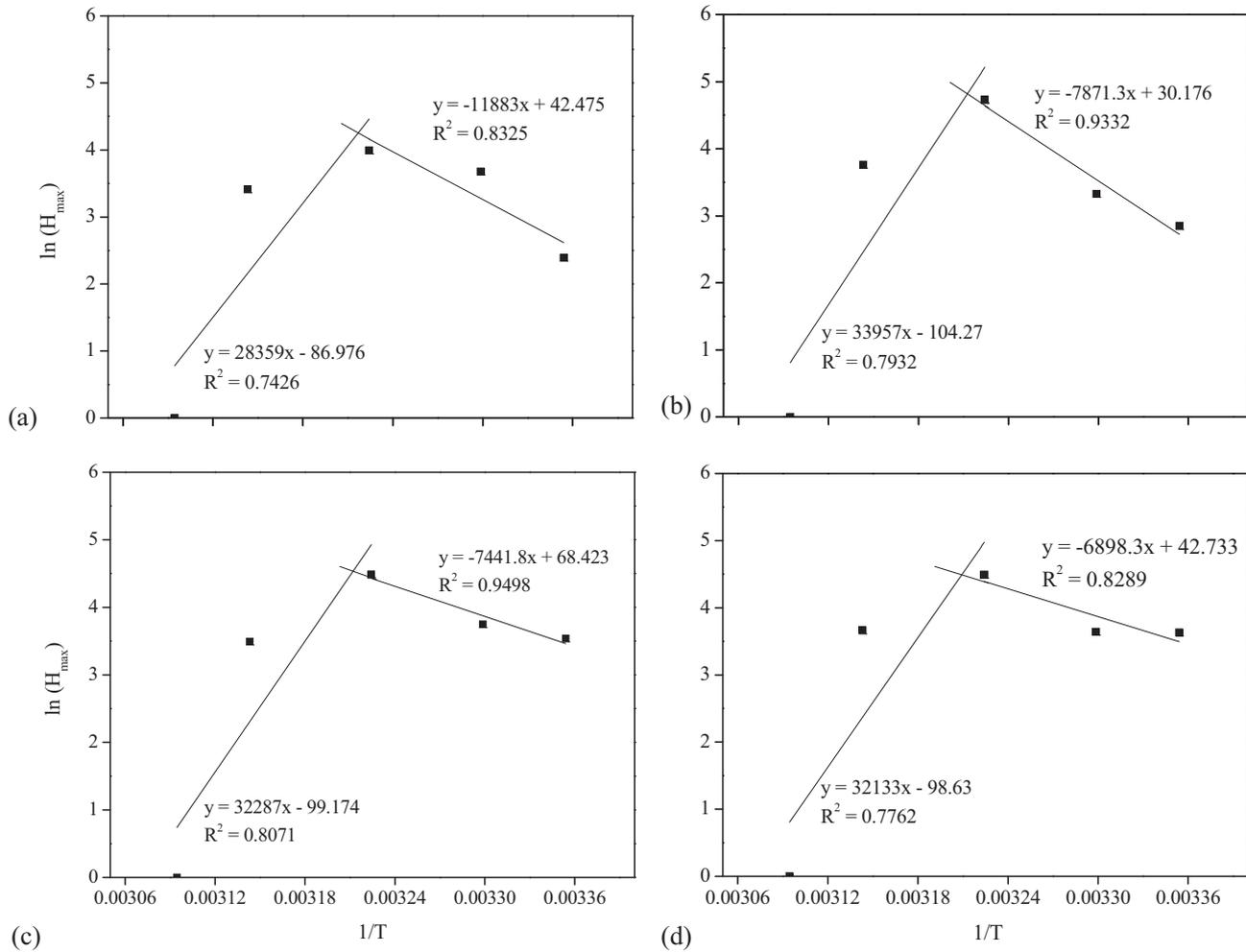


FIG. 1. The plot of modified Arrhenius at different initial pH: (a) pH 5, (b) pH 6, (c) pH 7 and (d) pH 8. The optimum fermentation temperature is 38.4 °C at all initial pH which represents by the intersection of the line.

load (26). This results were accordance with an early study conducted by Roychowdhury et al. (47) which found that high carbohydrate concentrations counteract fermentation performance. H_2 production was inhibited due to the unavailability of the subsequent metabolites like reduced-ferredoxin that involves in proton reduction (26). High amount of carbohydrates in undiluted wastewater led to a product or feedback inhibition that triggered the reduction of carbohydrate consumption.

TABLE 5. H_2 production at different organic load and characteristics of wastewater after fermentation.

Dilution factor	0%	20%	40%	60%	80%
H_2 yield					
mmol H_2 /g COD	41.7 ± 0.4	55.4 ± 1.6	60.6 ± 0.9	84.0 ± 2.3	113.2 ± 2.9
mmol H_2 /g carbohydrates	4.71 ± 0.05	6.26 ± 0.18	6.85 ± 0.10	9.49 ± 0.26	12.8 ± 0.3
Final pH	3.85 ± 0.06	3.81 ± 0.02	3.71 ± 0.03	3.62 ± 0.04	3.54 ± 0.01
COD reduction					
tCOD (%)	83.7 ± 0.6	85.0 ± 0.3	86.1 ± 0.6	84.5 ± 0.6	81.7 ± 1.2
sCOD (%)	75.9 ± 0.9	77.8 ± 0.4	87.2 ± 0.8	88.4 ± 1.2	83.3 ± 1.6
Soluble carbohydrate consumption (%)	11.1 ± 1.7	19.3 ± 1.4	31.3 ± 1.9	43.3 ± 1.5	85.6 ± 0.9

Fermentation conditions: Initial pH of 6, 37 °C, 3 days. tCOD, total chemical oxygen demand; sCOD, soluble chemical oxygen demand.

Productions of volatile fatty acids and alcohol Bio- H_2 production was accompanied by the production of volatile fatty acids (VFA) and alcohol. The concentration of VFA and alcohol different fermentation conditions is displayed in Table 6. It is notable that the profile of VFA and alcohols was different at various fermentation conditions, in which the predominated VFA were acetate and butyrate. The minor by-products consist of lactate, formate and ethanol. At the optimum condition (pH 6 and 37 °C), the ratio of acetate to butyrate (Ace:But) was 0.4. The concentration of lactate increases with temperature and initial pH, for example the lactate concentration increased 79 % from when initial pH reduced from 6 to 5 at 37 °C and 148 % from when temperature increased from 37 to 45 °C at pH 6 (Table 6).

The ratio of Ace:But indicates the direction of fermentation pathway. If the Ace:But ratio is smaller than one, it represents the reaction was prone towards butyrate production whereas when the Ace:But ratio is larger than one, it favour towards acetate formation (48). It was commonly known that the production of acetate usually represents a higher H_2 yield as illustrated in Eq. 10. In theory, each mole of glucose will be converted to two mole of acetate with four mole of H_2 or one mole of butyrate with two mole of H_2 , which can be represented by Eqs. 10 and 11.

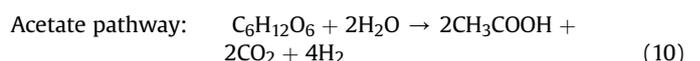
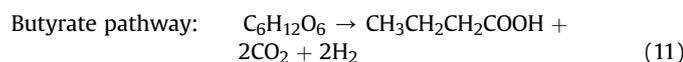


TABLE 6. Profile of soluble metabolites at different fermentation conditions.

Initial pH	Temp. (C)	Concentration (mmol/L)					Ace:But
		Lactate	Formate	Acetate	Ethanol	Butyrate	
5	25	13.1 ± 0.2	2.61 ± 0.41	111.8 ± 6.5	14.7 ± 0.9	44.2 ± 1.2	2.5
	30	6.2 ± 0.2	2.50 ± 0.24	154.2 ± 4.2	11.1 ± 1.3	71.3 ± 2.6	2.2
	37	5.2 ± 0.4	2.36 ± 0.28	88.1 ± 3.5	N.D.	61.6 ± 5.3	1.4
	45	3.1 ± 2.7	2.39 ± 0.32	137.9 ± 4.8	N.D.	58.1 ± 2.4	2.4
6	25	6.6 ± 0.5	2.72 ± 0.45	145.9 ± 6.4	9.5 ± 0.9	71.6 ± 4.5	2.0
	30	4.1 ± 0.3	2.30 ± 0.20	87.0 ± 5.3	6.5 ± 0.6	92.0 ± 3.4	0.9
	37	2.9 ± 0.1	2.22 ± 0.17	54.6 ± 0.8	N.D.	125.3 ± 1.0	0.4
	45	7.2 ± 0.2	2.42 ± 0.22	140.4 ± 6.0	N.D.	78.4 ± 3.0	1.8
7	25	9.8 ± 0.8	2.39 ± 0.21	122.0 ± 7.6	10.2 ± 1.3	50.6 ± 2.3	2.4
	30	7.0 ± 0.2	2.62 ± 0.20	138.6 ± 3.0	6.3 ± 0.7	76.9 ± 4.0	1.8
	37	3.3 ± 0.2	2.34 ± 0.13	99.7 ± 9.7	N.D.	98.0 ± 3.4	1.0
	45	7.6 ± 0.3	2.57 ± 0.10	145.4 ± 4.2	N.D.	62.8 ± 3.2	2.3
8	25	6.4 ± 0.3	2.46 ± 0.14	158.8 ± 2.1	10.6 ± 0.9	69.8 ± 0.7	2.3
	30	6.1 ± 0.4	2.54 ± 0.17	149.2 ± 6.8	7.9 ± 0.7	71.7 ± 2.5	2.1
	37	3.3 ± 0.2	2.53 ± 0.34	99.3 ± 7.8	N.D.	98.8 ± 7.2	1.0
	45	6.4 ± 0.4	2.52 ± 0.14	146.8 ± 4.4	N.D.	75.3 ± 6.1	1.9

Data for initial pH 4 and 50 °C are not displayed because H₂ productions and cell biomass was not detected. N.D., not detected.



In contrast to this study, the H₂ yield was higher even though Ace:But ratio was less than 1. Let us considered an example at pH 6, H₂ yield at 37 °C with Ace:But ratio of 0.4 was about 2.8 times higher than that of 25 and 45 °C with Ace:But ratio of 2.0 and 1.8, respectively (Tables 2 and 6). This demonstrated that butyrate pathway can also produce high H₂ productivity. This could be due to the key enzymes involved in the production of H₂. It is reported that these genes are parallel regulated in some H₂-producing bacteria such as *Clostridium perfringens* (49). Since H₂ production increased with higher expression of hydrogenase gene, the production of butyrate will also increase due to the parallel expression of both key enzymes. In contrast, when H₂ production was less efficient, the expression of both enzymes are suppressed, which has led to the lower yield of H₂ and butyrate.

Apart from Ace:But ratio, the efficiency of bio-H₂ production also monitored by the overall profile of VFA and alcohol. Typically, high concentrations of lactate and ethanol represent a less efficient H₂ production. As shown in Table 6, the amount of lactate only represents about 1.5% of the overall VFA and alcohol production as compared to 7% from fermentation occurred at initial pH 5 and 25 °C. Furthermore, ethanol was only detected at 25 and 30 °C,

which will also inhibit H₂ production. Therefore, formation of VFA and alcohol are highly related to fermentation conditions because the pH and temperature will alters bioactivity of the enzymes. For example, phosphotransbutyrylase (butyrate formation) (50), NAD-independent lactate dehydrogenase (pyruvate formation) (51) and phosphotransacetylase (acetate formation) (51) was only physiologically active at pH 6, 7.5 and 5, respectively. Besides that pH also influences the electron flow in the fermentation (52). When there are excess protons and substrates, fermentation pathways are shifted to produce more reduced metabolites including ethanol and lactate which eventually reduces H₂ yield. Therefore the fermentation condition is an important factor to control the pathway for VFA and alcohol formation in order to achieve efficient bio-H₂ production.

Relationship between microbial community and H₂ production from different sludge samples Taxonomic distributions of the three sludge samples are display in Fig. 3 and the distribution at genus level are listed in Table 7. It is obvious that there is a vast difference in the three types of sludge samples namely untreated LLS, pretreated LLS, and recycle LLS. In the untreated LLS, microbial community was very diverse. It harboured over 100 different families of bacteria and the most abundant were belonged to the family of H₂-consuming bacteria including *Flavobacteriaceae* (22%), *Pseudomonadaceae* (17%) and *Helicobacteraceae* (14%). Top three most abundant genera were H₂-consuming bacteria, belonging to genera *Pseudomonas*, *Sulfurimonas* and *Treponema*. Genus *Pseudomonas* and *Sulfurimonas* are facultative anaerobes that oxidize H₂ and donate electrons to either oxygen to form water or to produce ATP (53–55). In contrast, genus *Treponema* is obligate anaerobe which oxidizes H₂ with oxygen and nitrate forming hydrogen peroxide and nitrogen gas. They can also utilize H₂ and carbon dioxide as their sole substrate (56,57). H₂-producing bacteria such as *Clostridiaceae* and *Peptostreptococcaceae* were only accounted for 6% in the untreated LLS. This suggests that huge amount of H₂-consuming bacteria have restricted the performance of H₂ production. Consequently, this revealed that H₂ yield is clearly affected by the presence of H₂-consuming bacteria.

As shown earlier, H₂ production was significantly increased after heat pretreatment, which also accordance with previous reports (26,42,58). In this study, we found out that the microbial community in pretreated sludge was greatly reduced to less than 60 families but was dominated by family *Clostridiaceae* (66%) and *Peptostreptococcaceae* (32%). These families represent the source of H₂-producing bacteria (59–62). For this result, we can conclude

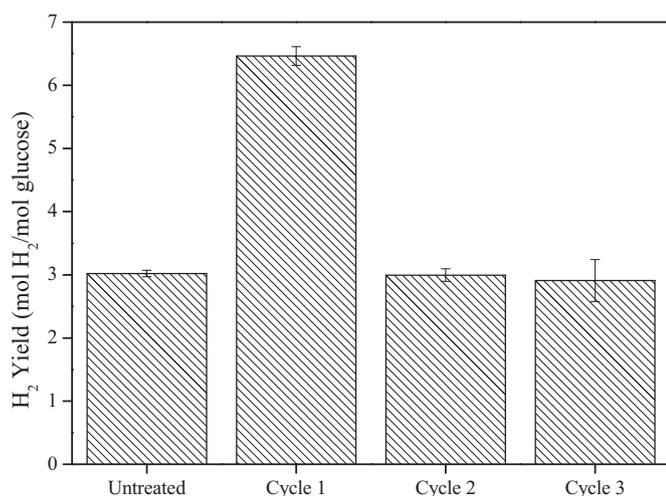


FIG. 2. Hydrogen production performance on repeated batch fermentation using sludge of untreated and pretreated at 65 °C.

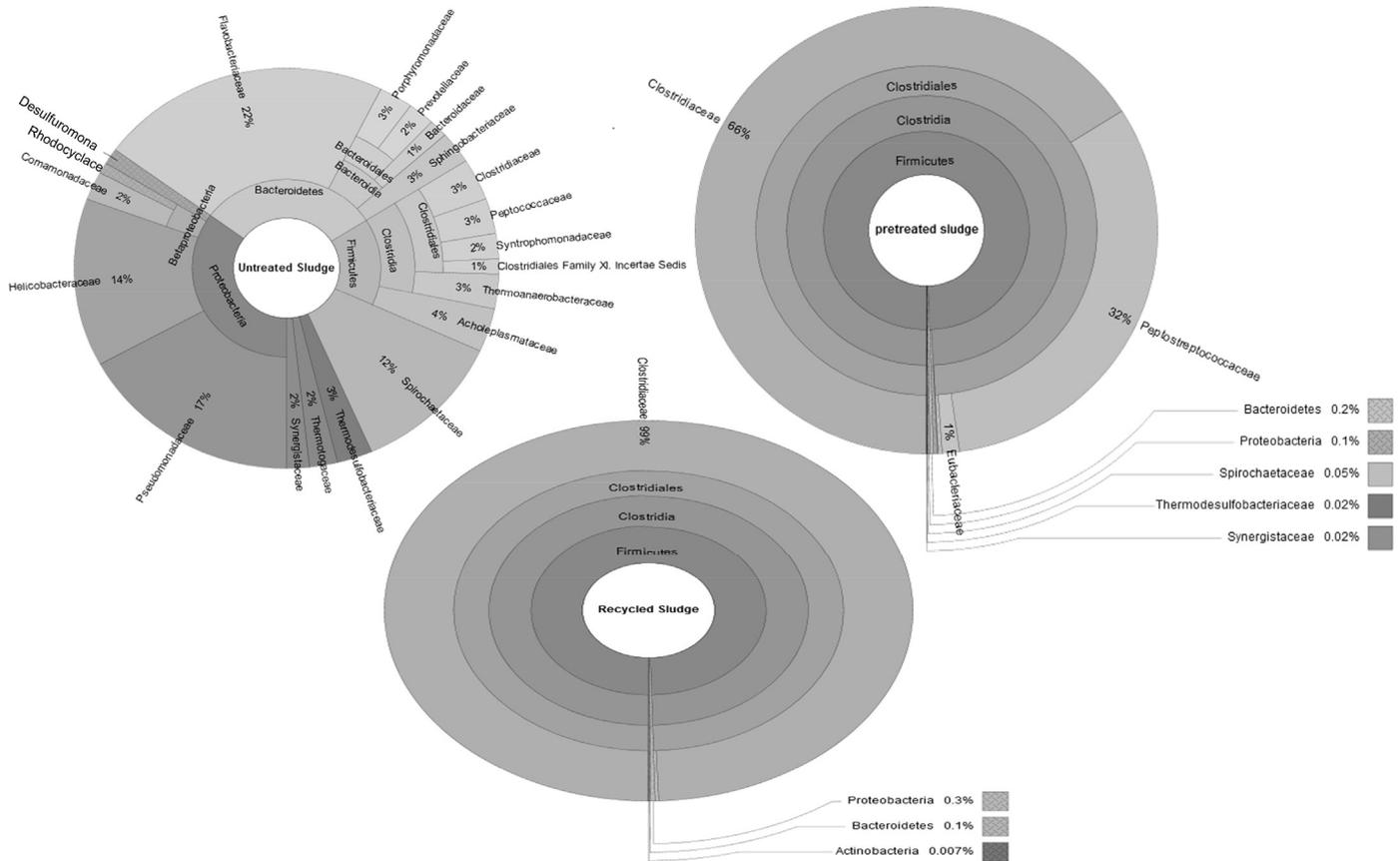


FIG. 3. Taxonomic distribution of untreated, pretreated and recycled landfill leachate sludge up. The figure was prepared using data from the 20 most abundant families. The innermost ring represents the phylum level and the outermost ring represents the family level.

that the pretreatment method has successfully eliminated H₂-consuming bacteria and also enriched H₂-producing bacteria. The Illumina Mi-Seq revealed heat pretreatment is a simple method but yet is highly effective to enrich H₂-producing bacteria. The principle of this method is that H₂-producing bacteria such as the genus *Clostridia* survived during heat pretreatment due to the sporulation characteristics (7). Likewise, H₂-consuming bacteria such as the genus *Pseudomonas* did not sporulate and easily deactivated. Therefore, H₂ yield has improved by 110% due to enrichment of H₂-producing microbial community in the heat pretreated LLS.

In the recycled LLS, the H₂ production reduced 55% because recycled LLS only contain limited diversity of H₂-producing bacteria and mainly consist of *Clostridiaceae* family (99%). The other families detected in the first cycle were no longer surviving in subsequent cycles. It was obvious that the diversity of microbial community has drastically reduced after several cycles of repeated fermentation. Microbial diversity can be reduced by the variation of doubling time of different H₂-producing bacteria. The fast growing bacteria has short doubling time. Hence, they will displace the slow growing bacteria and eventually dominate the microbial community in the recycled sludge. The possibility of this phenomenon might be due to reduce diversity of H₂-producing microbial community after recycled. It was reported that the diversity of H₂-producing microbial community assists high H₂ production performance because of the synergistic interaction amongst various type of H₂-producing bacteria (53,58,63–67). Besides that, the most efficient H₂-producing bacteria might be outcompeted by the less efficient H₂-producing bacteria after the fermentation.

Hydrogen producing microbial community in landfill leachate sludge H₂-producing bacteria in landfill leachate

sludge belong to four main genera, namely which are *Clostridium*, *Bacillus*, *Eubacterium* and *Sporacetigenium*, respectively (Table 7). Pretreated sludge was found to contain abundant genus *Clostridium*. This genus is reported as the most popular H₂ producers which is spore-forming obligate anaerobe (54). Generally, they are found in the environment rich in decaying plant materials. Therefore, they are capable of hydrolysing a wide range of carbohydrates including monosaccharide, disaccharides, xylan, cellulose, starch, chitin, pectin and others (55). The main enzyme that is responsible for H₂ production is hydrogenase, which triggered H₂ production by proton reduction. Commonly, *Clostridia* spp. contains multi-subunits hydrogenase including the [FeFe] hydrogenases and [NiFe] hydrogenases. Three species of H₂-producing bacteria have been successfully isolated from the pretreated sludge, namely *C. perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* strain Ade.TY, which possess unique genomic characteristics and high H₂ production (56,57,64). Therefore, the abundance of *Clostridia* in LLS could be the key factor for the high H₂ production.

H₂ producers from other genus especially facultative species are less popular, e.g., genus *Bacillus* is a facultative H₂ producer. The presence of facultative bacteria in H₂-producing sludge acts as the defence mechanism for strict anaerobic H₂-producer. Facultative H₂-producer was able to consume oxygen rapidly which accidentally enters the fermentation medium and recover the activity of anaerobic H₂-producer before the inhibition effect become permanent (68–70). In contrast, genus *Eubacterium* was found in H₂-producing sewage sludge (15) but the capacity of H₂ production from individual isolates was not reported. The plausible reason is that the identity of genus *Eubacterium* is often

TABLE 7. The 20 most abundant genera in untreated, pretreated and recycled landfill leachate sludge.

Untreated landfill leachate sludge			Pretreated landfill leachate sludge			Recycled landfill leachate sludge		
Family	Genus	Number of reads	Family	Genus	Number of reads	Family	Genus	Number of reads
Pseudomonadaceae	Pseudomonas	12848	Clostridiaceae	Clostridium	76400	Clostridiaceae	Clostridium	137368
Helicobacteriaceae	Sulfurimonas	11325	Peptostreptococcaceae	Unclassified Peptostreptococcaceae	11400	Rhodobacteriaceae	Rosevivax	112
Spirochaetaceae	Treponema	4101	Eubacteriaceae	Eubacterium	1603	Rhodobacteriaceae	Yangia	82
Spirochaetaceae	Sphaerochaeta	3393	Bacillaceae	Bacillus	364	Eubacteriaceae	Eubacterium	59
Flavobacteriaceae	Capnocytophaga	2656	Flavobacteriaceae	Capnocytophaga	124	Pseudomonadaceae	Pseudomonas	52
Acholeplasmataceae	Acholeplasma	2611	Pseudomonadaceae	Pseudomonas	102	Hyphomicrobiaceae	Rhodoplanae	51
Sphingobacteriaceae	Parapedobacter	2085	Syntrophomonadaceae	Syntrophomonas	61	Prevotellaceae	Prevotella	41
Thermoanaerobacteriaceae	Thermoanaeromonas	2060	Peptococcaceae	Desulfotomaculum	53	Clostridiales Family XI. Incertae Sedis	Peptoniphilus	36
Thermotogaceae	Petrologa	1747	Acholeplasmataceae	Acholeplasma	49	Flavobacteriaceae	Flavobacterium	28
Syntrophomonadaceae	Syntrophomonas	1321	Clostridiales Family XI. Incertae Sedis	Soehngenia	49	Bacteroidaceae	Bacteroides	20
Porphyromonadaceae	Proteiniphilum	1259	Thermoanaerobacteriaceae	Thermoanaeromonas	47	Porphyromonadaceae	Proteiniphilum	18
Bacteroidaceae	Bacteroides	1215	Spirochaetaceae	Treponema	46	Xanthobacteriaceae	Ancylobacter	17
Peptococcaceae	Desulfotomaculum	1157	Clostridiaceae	Natronincola	41	Xanthobacteriaceae	Nubsella	13
Thermodesulfobacteriaceae	Thermodesulfobacterium	1110	Prevotellaceae	Prevotella	40	Xanthobacteriaceae	Xanthobacter	11
Desulfobulbaceae	Desulfuromonas	1070	Peptostreptococcaceae	Sporacetigenium	35	Brevibacteriaceae	Brevibacterium	10
Thermodesulfobacteriaceae	Caldimicrobium	1061	Clostridiaceae	Caloramator	32	Ruminococcaceae	Ruminococcus	9
Clostridiaceae	Thermohalobacter	880	Clostridiaceae	Thermohalobacter	29	Clostridiaceae	Caloramator	8
Oceanospirillaceae	Marinospirillum	686	Brevibacteriaceae	Brevibacterium	24	Helicobacteriaceae	Sulfurimonas	0
Cloacimonetes	Candidatus Cloacimonas	676	Helicobacteriaceae	Sulfurimonas	21			

confused with other genus, typically saccharolytic species of *Eubacterium* share similar phenotypic features as *Clostridium*. Moreover, the genera *Eubacterium* and *Clostridium* are phylogenetically related (71). Another less common H₂ producer found in landfill leachate sludge belong to the genus *Sporacetigenium*, family *Peptostreptococcaceae*. They produce volatile fatty acids such as acetic and propionic acids (72,73). Based on literature, *Sporacetigenium mesophilum* strain ZLJ115T is the only studied H₂-producing strain (74). Although these two genera are less studied, their role in H₂ production using landfill leachate sludge appears to be important. As observed from Figs. 2 and 3, the absence of these genera from families *Eubacteriaceae* and *Peptostreptococcaceae* in the recycled sludge may be the contributing factor to the reduced H₂ yield.

Conclusions In summary, dairy wastewater is a potential feedstock for bio-H₂ production using LLS as inoculum. The maximum H₂ yield was 113.2 ± 2.9 mmol H₂/g COD at initial pH 6 and 37 °C. It tend to follows butyrate pathway because the ratio of Ace:But was 0.4. The kinetic and thermodynamic analysis revealed that the optimum dark fermentation is thermodynamically favourable with Gibbs free energy of -40 kJ/mol. Besides, the complex activated theory was also in good agreement with the deactivation mechanism of enzymes. Metagenomics by 16S rRNA using Illumina Mi-Seq has favourably shown the relationship of the microbial community in landfill leachate and the performance of H₂ production. The high H₂ production was mainly due to the presence of H₂ producing bacteria namely *Clostridium* spp. This study provides a framework for further research on bio-H₂ production from dairy wastewater using LLS as inoculum.

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jbiosc.2018.07.012>.

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