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/°C, 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 water, hydrocarbons and carbonates (3−5), it has converted the organic compounds 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).

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).

In this study, we report the bio-hydrogen production from dairy wastewater using pretreated landfill leachate sludge (LLS) as an inoculum. LLS was used as inoculum to enhance the H2 production from dairy wastewater. LLS is a byproduct from the leachate facility built to dispose harsh municipal waste under anaerobic conditions (24). In addition, LLS 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 conditions, it has the ability to produce H2. However, the H2 production from LLS is showed low yield. Therefore, this study was conducted to enhance the H2 production from LLS using pretreatment method.

Pretreatment of LLS was performed under anaerobic conditions at 30 °C and pH 6.5 for 24 h. The pretreated LLS was used as an inoculum for H2 production. The initial pH and temperature on bio-hydrogen production from dairy wastewater using pretreated landfills 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/°C, 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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condition, it would possess unique H₂ production performance. Recently, we have reported the performance of leachate sludge for H₂ production using glucose as feedstock. 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 used in the following biotreatment. Since the expired products are unsafe for human consumption, they provide good resources for H₂ production. According to Venkata Mohan et al. 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, LLS has shown a high H₂ production efficiency of 6.43 mol H₂/mol glucose, which outcompete 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 pretreatment. (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 production. Inoculated sludge from the first batch fermentation was re-used 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 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%, and 60%).

**Analysis** Total COD in wastewater 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 biogas production was taken at room temperature. Besides, initial pH for COD and biogas 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 detector 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):

\[
H = H_{\text{max}} \left( 1 - e^{-\frac{A}{h}} \right) \left( e^{-\frac{B}{T}} \right)
\]

\[
H = H_{\text{max}} \left( 1 - e^{-\frac{A}{h}} \right) \left( e^{-\frac{B}{T}} \right)
\]

where \( H_{\text{max}} \) is the cumulative H₂ formation, \( H_{\text{max}} \) is the maximum H₂ production rate, \( r \) is the lag phase time (h), \( t \) is time (h), and \( \frac{A}{h} \) is the rate constant (k).

**Activation enthalpy 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, all like enzymatic reactions, the rate of reaction for the production of bio-H₂ increases with temperature (\( T_{\text{opt}} \)). However, once the temperature exceeds \( T_{\text{opt}} \), bio-H₂ production is subjected to thermal deactivation because of enzyme denaturation and cell death. This is represented by the following equations (26.36):

\[
\ln H_{\text{max}} = \ln(A X Y) - \frac{\Delta H}{R T} T < T_{\text{opt}}
\]

\[
\ln H_{\text{max}} = \ln(A X Y) - \frac{\Delta H}{R T} T > T_{\text{opt}}
\]

where \( H_{\text{max}} \) 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/kmol), T is temperature in Kelvin and \( \Delta H \) 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 (\( \Delta H_{T} \)) and it is determined by Eq. 4.

\[
\Delta H_{T} = \Delta H + |\Delta H'|
\]

where \( \Delta H_{T} \) is the maximum H₂ productivity obtained from modified Gompertz equation, \( \Delta H \) and \( \Delta H' \) are the activation energy of fermentation (\( \Delta S \)) and activation entropy of thermal deactivation (\( \Delta S_{T} \)), respectively (26.36).

**Activation entropy of fermentation and activation entropy of thermal deactivation** The Eyring–Arrhenius equation was used to calculate the activation entropy of fermentation (\( \Delta S \)) and activation entropy thermal deactivation (\( \Delta S_{T} \)).

\[
\Delta S = R \left( \frac{\ln h T}{k_{B} T} \right)
\]

\[
\Delta S_{T} = R \left( \frac{\ln h T}{k_{B} T} \right)
\]

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).

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**TABLE 1. Characteristics of dairy wastewater.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dairy wastewater</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.90 ± 0.26</td>
</tr>
<tr>
<td>Total COD (mg/L)</td>
<td>14150 ± 600</td>
</tr>
<tr>
<td>Soluble COD (mg/L)</td>
<td>9567 ± 293</td>
</tr>
<tr>
<td>Kjeldahl N (mg/L)</td>
<td>306 ± 13</td>
</tr>
<tr>
<td>Soluble carbohydrate (g/L)</td>
<td>84.60 ± 4.75</td>
</tr>
<tr>
<td>Soluble protein (g/L)</td>
<td>3.20 ± 0.77</td>
</tr>
</tbody>
</table>
where ΔΗ is obtained from Eq. 2 and ΔS is obtained from Eq. 5. 

Genomic DNA extraction Prior DNA extraction, sludge was centrifuged at 3600 × 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 × g. The supernatant was completely removed with pipette. 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 × g for 1 min. The supernatants were transferred to clean microcentrifuge tubes and centrifuged at 10,000 × 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 centrifuged at 10,000 × g for 1 min to remove precipitated proteins. Avoiding the pellet, the entire volume of solution S3 was added into the tubes and gently mixed. The volume was loaded onto spin with 0.05% Tween 20. Quantitative PCR (qPCR) was performed to determine DNA concentrations. The V3 region on 16S rRNA was amplified using barcoded primer as follows: forward primer: 5’-CAAGCAGAAGACGGCATACGAGATGA-3’; reverse primer: 5’-AAGCAGGAGACGGTACATTGATCA-3’. The amplification cycle was 95 °C for 3 min; 25 cycles at 98 °C for 20 s, 63 °C for 15 s, 72 °C for 5 s and 72 °C for 1 min. Upon the completion of PCR amplification, the amplified sequence with the size of approximately 300 bp was selected by using 25% gel electrophoresis. The sequence was then recovered using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The extracted DNA sequence was diluted 10,000 × with 0.05% Tween 20. Quantitative PCR (qPCR) was performed to quantify the concentration of amplified sequence at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °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 5.11 (CLC bio, Aarhus, Denmark) was used to trim the raw amplicons to Qscore of 20 and amplicons shorter than 150 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 <1 × 10–8) using USEARCH v6.0 [37]. The output from USEARCH was normalised with MEGAN5 (26). In comparison, no cell growth and H2 productions were detected at initial pH 4. The results indicate that initial pH 6 favours H2 production because this hydrogenase function well in the pH range of 5-9 (38). Hence, the performance of H2 production could be improved with a high diversity of strict anaerobic H2-producing bacteria. As reported in our previous work (26), the sludge originated from landfill shows great potential in H2 production from glucose could be due to the diverse microflora in the inoculum.

Overall, the optimum initial pH for H2 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 H2 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 H2 productions were detected at initial pH 4. The results indicate that initial pH 6 favours H2 production because this hydrogenase function well in the pH between 6 and 6.5 (38). Furthermore, pH 6 also tend to inhibits H2-consuming bacteria including methanogens and acetobacteria (23). This would mean that H2 production could enhance due to suppression of H2 consumption process. Therefore, different initial pH alters bacteria activities which in turn influence H2 production.

The performance of H2 productivity and cell biomass were the highest at 37 °C regardless of the different initial pH (Tables 2 and 3). At temperature beyond 37 °C, the measured cell biomass was not detected.
TABLE 3. Comparison of H₂ yield from this study with other studies.

<table>
<thead>
<tr>
<th>Sludge inoculum</th>
<th>H₂ yield (mmol H₂/g COD)</th>
<th>Sludge pretreatment</th>
<th>Substrate source</th>
<th>Initial pH and temp. (°C)</th>
<th>Substrate consumption</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLS</td>
<td>113.2 ± 2.9</td>
<td>Heat</td>
<td>Dairy wastewater</td>
<td>pH 6; 37.0</td>
<td>81.7 ± 1.2% COD</td>
<td>This study</td>
</tr>
<tr>
<td>H₂ producing reactor</td>
<td>0.0018</td>
<td>Untreated</td>
<td>Dairy wastewater</td>
<td>29.0</td>
<td>79% COD</td>
<td>10</td>
</tr>
<tr>
<td>Palmitic oil mill effluent (POME)</td>
<td>0.12 mmol H₂/g COD</td>
<td>Acid</td>
<td>POME</td>
<td>pH 5.5; 35.0</td>
<td>86% COD</td>
<td>25</td>
</tr>
<tr>
<td>Treatment plant</td>
<td>0.12 mmol H₂/g COD</td>
<td>Untreated</td>
<td>Glucose and peptone</td>
<td>pH 10.5; 37.0</td>
<td>N.A.</td>
<td>26</td>
</tr>
<tr>
<td>Sewage treatment plant</td>
<td>0.32 mmol H₂/g COD</td>
<td>Acid</td>
<td>Heat</td>
<td>pH 6.2; 40.0</td>
<td>90% carbohydrates</td>
<td>27</td>
</tr>
</tbody>
</table>

*H₂ yield was estimated using PV – nRT at standard condition where P = 1 atm; R = 8.21 x 10⁻⁵ m³ atm/mol K and T = 300 K.

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.

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 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 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 ΔH₂ is the threshold energy for deactivation of enzymes and death of bacteria (26,36). The ΔH₂ 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 ΔH₄ 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.

Enthropy measures the randomness of a reaction in which activation entropy of fermentation (ΔS₃) and activation entropy of thermal deactivation (ΔS₄) indicate the randomness during fermentation and thermal deactivation, respectively (26,36). The ΔS₃ 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 ΔS₄ 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 ΔS₄ at initial pH 5, 7 and 8 was higher than the respective value of ΔS₃. This is reasonable because the randomness of the reaction increases with enzymes denaturation (44,45). However, the negative value of ΔS₄ 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).
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. H₂ 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.

Productions of volatile fatty acids and alcohol  Bio-H₂ 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 H₂ yield as illustrated in Eq. (10). In theory, each mole of glucose will be converted to two mole of acetate with four mole of H₂ or one mole of butyrate with two mole of H₂, which can be represented by Eqs. 10 and 11.

Acetate pathway: \[ C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \] (10)

Butyrate pathway: \[ \text{C}_6\text{H}_2\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \] (11)

In contrast to this study, the \( \text{H}_2 \) yield was higher even though Ace:But ratio was less than 1. Let us consider an example at pH 6, \( \text{H}_2 \) 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 \( \text{H}_2 \) productivity. This could be due to the key enzymes involved in the production of \( \text{H}_2 \). It is reported that these genes are parallel regulated in some \( \text{H}_2 \)-producing bacteria such as *Clostridium perfringens* (49). Since \( \text{H}_2 \) 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 \( \text{H}_2 \) production was less efficient, the expression of both enzymes is suppressed, which has led to the lower yield of \( \text{H}_2 \) and butyrate.

Apart from Ace:But ratio, the efficiency of bio-\( \text{H}_2 \) production also monitored by the overall profile of VFA and alcohol. Typically, high concentrations of lactate and ethanol represent a less efficient \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 phosphotransacytase (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 \( \text{H}_2 \) yield. Therefore the fermentation condition is an important factor to control the pathway for VFA and alcohol formation in order to achieve efficient bio-\( \text{H}_2 \) production.

Relationship between microbial community and \( \text{H}_2 \) 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 \( \text{H}_2 \)-consuming bacteria including *Flavobacteriaceae* (22%), *Pseudomonadaceae* (17%) and *Helicobacteraceae* (14%). Top three most abundant genera were *H_2* -consuming bacteria, belonging to genera *Pseudomonas*, *Sulfurimonas* and *Treponema*. Genus *Pseudomonas* and *Sulfurimonas* are facultative anaerobes that oxidize \( \text{H}_2 \) and donate electrons to either oxygen to form water or to produce ATP (53-55). In contrast, genus *Treponema* is obligate anaerobe which oxidizes \( \text{H}_2 \) with oxygen and nitrate forming hydrogen peroxide and nitrogen gas. They can also utilize \( \text{H}_2 \) and carbon dioxide as its sole substrate (56,57). \( \text{H}_2 \)-producing bacteria such as *Clostridiales* and *Peptostreptococcaceae* were only accounted for 6% in the untreated LLS. This suggests that huge amount of \( \text{H}_2 \)-consuming bacteria have restricted the performance of \( \text{H}_2 \) production. Consequently, this revealed that \( \text{H}_2 \) yield is clearly affected by the presence of \( \text{H}_2 \)-consuming bacteria.

As shown earlier, \( \text{H}_2 \) production was significantly increased after heat pretreatment, which also accordence 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 *Clostridiales* (66%) and *Peptostreptococcaceae* (32%). These families represent the source of \( \text{H}_2 \)-producing bacteria (59-62). For this result, we can conclude

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

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>Temp. (C)</th>
<th>Lactate</th>
<th>Formate</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>Butyrate</th>
<th>Ace:But</th>
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<td>5</td>
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<td>13.1 ± 0.2</td>
<td>2.61 ± 0.41</td>
<td>111.8 ± 6.5</td>
<td>14.7 ± 0.9</td>
<td>44.2 ± 1.2</td>
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<td></td>
<td>30</td>
<td>6.2 ± 0.2</td>
<td>2.50 ± 0.24</td>
<td>154.2 ± 4.2</td>
<td>11.1 ± 1.3</td>
<td>71.3 ± 2.6</td>
<td>2.2</td>
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<tr>
<td></td>
<td>37</td>
<td>5.2 ± 0.4</td>
<td>2.36 ± 0.28</td>
<td>88.1 ± 3.5</td>
<td>N.D.</td>
<td>61.6 ± 5.3</td>
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<td></td>
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<td>3.1 ± 2.7</td>
<td>2.39 ± 0.32</td>
<td>137.9 ± 4.8</td>
<td>N.D.</td>
<td>58.1 ± 2.4</td>
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<td>9.5 ± 0.9</td>
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<tr>
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<td>1.9</td>
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</tbody>
</table>

Data for initial pH 4 and 50 °C are not displayed because \( \text{H}_2 \) productions and cell biomass was not detected. N.D., not detected.

FIG. 2. Hydrogen production performance on repeated batch fermentation using sludge of untreated and pretreated at 65 °C.
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 Sporocetigenium, 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
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 *H*₂ 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 *H*₂-producing strain (74). Although these two genera are less studied, their role in *H*₂ 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 *H*₂ yield.

**Conclusions** In summary, dairy wastewater is a potential feedstock for bio-*H*₂ production using LLS as inoculum. The maximum *H*₂ yield was 113.2 ± 2.9 mmol *H*₂/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 system was also in good agreement with the deactivation mechanism of enzymes. Metagenomics by 16S rRNA using Illumina Mi-Seq has successfully shown that the relationship of the microbial community in landfills and the performance of *H*₂ production. The high *H*₂ production was mainly due to the presence of *H*₂-producing bacteria namely *Clostridium* spp. This study provides a framework for further research on bio-*H*₂ 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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**References**


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