fermentation

MDPI

Article

q-PCR Methodology for Monitoring the Thermophilic Hydrogen Producers Enriched from Elephant Dung

Khamanitjaree Saripan [1,2], Chonticha Mamimin [2], Tsuyoshi Imai [3], Sureewan Sittijunda [4] and Alissara Reungsang [2,5,6,*]

[1] Environmental Science Program, Faculty of Science and Technology, Thepsatri Rajabhat University, A. Muang, Lopburi 40002, Thailand
[2] Department of Biotechnology, Faculty of Technology, Khon Kaen University, A. Muang, Khon Kaen 40002, Thailand
[3] Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Yamaguchi 755-8611, Japan
[4] Faculty of Environment and Resource Studies, Mahidol University, Nakhon Pathom 73170, Thailand
[5] Academy of Science, Royal Society of Thailand, Bangkok 10300, Thailand
[6] Research Group for Development of Microbial Hydrogen Production Process from Biomass, Khon Kaen University, A. Muang, Khon Kaen 40002, Thailand
* Correspondence: alissara@kku.ac.th; Tel.: +66-43-362121

Abstract: This study aims to create a quantitative polymerase chain reaction (q-PCR) methodology for monitoring the hydrogen-producing mixed cultures enriched from elephant dung using alpha-cellulose as a carbon source through five generations of repetitive sub-culture. The enriched thermophilic mixed cultures from the fifth cultivation cycle gave the highest hydrogen yield of 170.3 mL H_2/g cellulose and were used to generate hydrogen from sawdust. *Clostridium* sp. and *Thermoanaerobacterium* sp. were the dominant bacteria in thermophilic mixed cultures with high hydrogen yield, according to polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE). q-PCR primers Chis150F and ClostIR, TherF and TherR, and BacdF and BacdR were developed to amplify the 16S rRNA genes of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp., respectively, for the quantification of hydrogen-producing bacteria in biohydrogen fermentation. Similar q-PCR analysis of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. 16S rRNA gene amplification during hydrogen production from cellulose and sawdust revealed increasing gene copy number with time. The molecular approaches developed in this study can be used to monitor microbial communities in hydrogen fermentation processes efficiently.

Keywords: PCR-DGGE; microbial community; thermophilic hydrogen production; sawdust; lignocellulosic biomass

check for updates

Citation: Saripan, K.; Mamimin, C.; Imai, T.; Sittijunda, S.; Reungsang, A. q-PCR Methodology for Monitoring the Thermophilic Hydrogen Producers Enriched from Elephant Dung. *Fermentation* **2022**, *8*, 506. https://doi.org/10.3390/fermentation8100506

Academic Editor: Alessio Siciliano

Received: 14 August 2022
Accepted: 28 September 2022
Published: 1 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Recent years have seen a greater focus of research efforts on the production and utilization of alternative fuels to reduce dependency on fossil fuels. Hydrogen production via biological processes that utilize organic materials is a sustainable alternative source of renewable energy that is cost-effective [1] and environment-friendly [2]. Biological hydrogen production processes are broadly classified into biophotolysis, photo fermentation, and dark fermentation processes. The latter process produces hydrogen from organic waste and thus has the double advantage of potentially reducing waste disposal problems and decreasing the cost of raw materials [3]. Thus, biological hydrogen production promotes the waste-to-energy concept, rapidly increasing commercial hydrogen applications as biofuels globally [1]. Organic waste materials such as lignocellulosic waste are potential low-cost renewable raw material sources for biological hydrogen production [4]. Lignocellulosic biomass contains a high content of cellulose, hemicellulose, and lignin, which serve as

carbon sources for the release of hydrogen by microorganisms. Various lignocellulosic waste has been widely used as feedstock for hydrogen production via dark fermentation. These include barley hulls, rice straw, corn stalks, and oil palm residues with hydrogen yields of 27.8 mL $H_2/g_{substrate}$ [5], 24.8 mL $H_2/g_{substrate}$ [6], 142.9 mL $H_2/g_{substrate}$ [7] and 59.2 mL $H_2/g_{substrate}$ [8], respectively. However, the recalcitrance of lignocellulosic biomass is a major challenge in its conversion to bio-hydrogen by microorganisms. Consequently, the successful use of lignocellulosic biomass as a substrate for bio-hydrogen production requires the utilization of microorganisms with enhanced digestive abilities. In this study, elephant dung was used as a source of microorganisms capable of utilizing lignocellulosic biomass to produce hydrogen. In general, elephants consume lignocellulosic biomass as their primary diet; thus, the microorganisms in the digestive tract may contain the enzyme for lignocellulosic biomass digestion.

Many microbial species, including *Clostridia* and *Thermoanaerobacterium* species, are efficient hydrogen producers under thermophilic conditions via the degradation of various types of carbohydrates. *Clostridium* sp. is the most researched bacterial genera in dark fermentative hydrogen production [3]. *Thermoanaerobacterium* sp. has been established as a highly efficient hydrogen producer in thermophilic hydrogen production [9–11]. In addition, various studies have reported using mixed microorganism cultures from anaerobic sludge, hot spring sediment, compost, and animal dung as inoculum for fermentative hydrogen production [12,13]. These approaches are more practical than the utilization of pure cultures since they result in more efficient substrate degradation [14]. However, properly comprehending the microbial community and its various functions is essential to improving the efficiency and stability of the hydrogen production process. To this end, numerous approaches, including molecular approaches, have been developed and applied [15].

Several molecular approaches based on 16S rRNA genes, including fluorescence in situ hybridization (FISH) [16], denaturing gradient gel electrophoresis (DGGE) [17], and shotgun sequencing of total DNA metagenomics [18] have been used to study hydrogen producers. Even though these techniques provide information that aids the characterization of microbial communities in mixed cultures, they are time-consuming and labor-intensive, rendering them impractical for high sample throughput and real-time assays [19]. The rapid development of quantitative polymerase chain reaction (q-PCR) has successfully overcome these limitations and may be considered a potential technique to monitor process performance. To our knowledge, the q-PCR methodology for quantifying thermophilic hydrogen producers utilizing lignocellulosic biomass to produce hydrogen is still underdeveloped.

Therefore, the present study aimed to develop a quantification methodology using q-PCR approaches to quantify and monitor thermophilic hydrogen-producing bacteria enriched from the elephant dung. Our findings can be applied to other research studies aiming to produce and develop alternative sources of green fuel, especially biohydrogen.

2. Materials and Methods

2.1. Substrate

Sawdust was used as the substrate for hydrogen production and was collected from a local wood factory in Nongkai province, Thailand, air dried, and stored in a plastic box until further use. The sawdust composition was determined to be 40.06% cellulose, 27.00% hemicelluloses, and 32.95% lignin.

2.2. Inoculum and Culture Enrichment

Hydrogen-producing bacteria were enriched from elephant dung collected from an elephant village in Surin province, Northeastern Thailand. The elephant dung was chopped with a knife and heat-treated in a hot air oven at 105 °C for 2 h to inhibit the activity and growth of methanogens. Subsequently, it was enriched with 5 g/L alpha-cellulose as a carbon source in a basic anaerobic medium (BA) containing in g/L 1.0 NH_4Cl, 0.1 NaCl, 0.1 $MgC_{l2}{\cdot}6H_2O$, 0.05 $CaCl_2{\cdot}2H_2O$, 0.4 $K_2HPO_4{\cdot}3H_2O$, 2.6 $NaHCO_3$, 1.0 Yeast extract,

1.0 Peptone, 0.25 Na_2S, and 1 mL/L trace element solution. The trace element solution comprised in g/L 0.002 $FeC_{l2}{\cdot}4H_2O$, 0.00005 H_3BO_3, 0.00005 $ZnCl_2$, 0.000038 $CuCl_2{\cdot}2H_2O$, 0.00005 $MnCl_2{\cdot}4H_2O$, 0.00005$(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 0.00005 $AlCl_3$, 0.00005 $CoC_{l2}{\cdot}6H_2O$, 0.000092 $NiCl_2{\cdot}6H_2O$, 0.0005 ethylenediaminetetraacetate, 0.0001 $Na_2SeO_3{\cdot}5H_2O$, and 1 mL concentrated HCl [20]. Serum bottles with a total volume of 120 mL and a working volume of 70 mL were used for enrichment. The initial pH of the medium in the serum bottles was adjusted to 7.0 with 2 M HCl or NaOH and flushed with N_2 gas for 5 min to obtain anaerobic conditions, followed by incubation at 55 °C for 4 days. The enriched cultures were diluted at each enrichment cycle to 50% v/v in a fresh BA medium. This process was repeated five times to establish a stable microbial community. Each cycle of enriched cultures was monitored for hydrogen gas generation, and all samples were analyzed for soluble metabolite products (SMPs) and microbial community. The final enrichment cultures (fifth batch cycle) were used further as inoculum for hydrogen production from sawdust.

2.3. Bio-Hydrogen Fermentation from Sawdust

Bio-hydrogen fermentation was performed in serum bottles with a total volume of 80 mL and a working volume of 40 mL, using 12 mL final enrichment cultures as inoculum and 12.5 g/L sawdust as substrate. The initial pH was adjusted to 7.0 with 3 M NaOH and 3 M HCl. All bottles were subsequently purged with nitrogen gas for 5 min to ensure anaerobic conditions. Later, the serum bottles were sealed with a rubber septum and aluminum crimp caps and placed in a 55 °C incubator for 7 days. All treatments were conducted in triplicate. The BA medium without sawdust was used as a control to account for the background production of hydrogen. In addition, the biogas volume and composition were routinely monitored. The hydrogenic effluent was collected and analyzed for SMPs at the end of the fermentation process. Simultaneously, sludge was collected and analyzed for the microbial community responsible for hydrogen production using polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE).

2.4. Analytical Methods

The volume of biogas in the headspace was measured with a wetted glass syringe. The hydrogen content was analyzed with gas chromatography (GC-8APT, Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a Shin Carbon column. The GC operational conditions followed were identical to those reported by O-Thong et al. [11]. For the SMPs (ethanol (EtOH), acetic acid (Hac), propionic acid (HPr), butyric acid (HBu), lactic acid (HLa)) analysis, hydrogenic effluent was centrifuged at 10,000 rpm for 5 min, acidified with 0.2 N oxalic acid, and filtered through a 0.2 µm nylon membrane. The resulting filtrate was analyzed on a GC instrument (GC-8APF, Shimadzu, Japan) with a flame ionization detector (FID) and a Unisole F-200 glass column. The GC operation conditions were set as previously reported by O-Thong et al. [11]. The HLa concentration was analyzed by high-performance liquid chromatography (HPLC; LC-10AD, Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column and a UV detector. The HPLC operating conditions were based on those reported in a previous study [21]. Cumulative hydrogen production was calculated using the mass balance equation [22] to measure headspace gas composition and the total volume of biogas produced during each time interval. The hydrogen production yield was calculated as the cumulative hydrogen production divided by the amount of cellulose or sawdust added (mL $H_2/g_{cellulose}$ or mL $H_2/g_{sawdust}$). The hydrogen production rate was calculated as the cumulative hydrogen production divided by the fermentation time (mL H_2/L·d).

2.5. PCR-DGGE Analysis

Triplicate sludge samples were collected from the end of bio-hydrogen fermentation to investigate the microbial community structure. DNA quality was assessed on a 1% agarose gel before PCR-DGGE analysis. The 16S rRNA gene was amplified from genomic DNA of

sludge samples using the universal bacterial primers, 518r (5′ ATTACCGAGCTGCTGG 3′) and 357f (5′ CCTACGGGAGGCAGCAG 3′ with 40 bp GC-clamp). [23]. Amplification mixtures for bacteria were TopTaq™ Master Mix Kit (Qiagen, Hilden, Germany) with a final volume of 25 µL. The reaction mixture has a final concentration of TopTaq DNA Polymerase, PCR Buffer, dNTP, and primers of 1.25 units, 1×, 200 µM, and 0.2 µM, respectively. PCR amplification of bacterial DNA began with an initial denaturation of 94 °C for 3 min followed by 34 cycles of three steps: 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis before DGGE analysis.

The DGGE analysis of PCR products was performed using a vertical Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) with 8% (*v*/*v*) polyacrylamide gels and a denaturant gradient of 30–60% (100% denaturing solution containing 7 M of urea and 40% formamide). The gel was run at 60 °C in 0.5X tris-acetate EDTA (TAE) buffer at 70 V for 16 h. DGGE gels were stained with SYBR Green for 15 min and analyzed on a GelDoc XR 1708170 system (Bio-Rad Laboratories, Hertfordshire, UK). DGGE profiles were compared using the Quantity One software package (version 4.6.0; Bio-Rad Laboratories). Most bands were excised from the gel and re-amplified with the forward primer 357f without a GC clamp and the reverse primer 518r. After re-amplification, PCR products were purified using Takara SUPRECTM-PCR (Takara Bio, Shiga, Japan). A Sanger sequencing was performed with an automated DNA sequencer using the BigDye Terminator v3.1 cycle sequencing kit. Then Sanger sequencing products were purified by using traditional ethanol precipitation. Subsequently, the closest matches for partial 16S rRNA gene sequences were identified in the GenBank database using the web-based basic local alignment tool (BLAST).

2.6. q-PCR Analysis

The DNA standard for q-PCR was extracted from pure cultures of *Thermoanaerobacterium* sp., *Clostridium* sp., and *Bacillus* sp. Cells were harvested in microcentrifuge tubes by centrifugation at 10,000 rpm for 5 min. The cell pellets were re-suspended in 1 mL of tris-EDTA (TE) buffer. Total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). Triplicate sludge samples were collected from elephant dung in each repetitive sub-culture (C1-C5) and cell culture of biohydrogen fermentation (3, 6, and 9 days) to quantify the *Thermoanaerobacterium* sp., *Clostridium* sp., and *Bacillus* sp. in it. Specific degenerate primers were designed to amplify a nucleotide sequence of the bacterial 16S rRNA gene from hydrogen-producing bacteria [21]. Specific degenerated primers were designed to amplify a nucleotide sequence of the 16S rRNA gene according to the o nucleotide sequence of *Thermoanaerobacterium thermosaccharolyticum* (accession number AF247003) and *Bacillus licheniformis* (accession number AF516176) using NCBI Primer-BLAST Designers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed on 17 September 2022). Based on the alignment, the primer set was designed to conserve sequences region of genes from the two strains. The pairs of primer gave PCR product range from 70–110 base pair and were selected to perform real-time PCR system. The primer name and sequence were used in this study as shown in Table 1. Primer specificities towards 16S rDNA of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. were evaluated using arb-silva (https://www.arb-silva.de/search/testprime/ accessed on 17 September 2022), an in-silico PCR analysis tool which uses 16S/18S rDNA non-redundant reference dataset, SSURef 108 NR [24]. q-PCR was performed using Chromo 4 real-time PCR (Bio-Rad, Hercules, CA, USA) in 96-well PCR plates. Standard curves were constructed with ten-fold dilutions of genomic DNA from 16S rDNA PCR amplification. Stock concentrations (gene copies µ/L) were determined via PicoGreen measurement and freshly prepared ten-fold dilutions were used in order to build the calibration curve. The wells were sealed with optical flat cap strips (Bio-Rad), and each 25 µL q-PCR reaction mixture contained 12.5 µL SYBR Green (IQ, Biorad, Hercules, CA, USA), 0.25 µL each of 0.25 µM forward and reverse primers, 11 µL sterile water, and 1 µL genomic DNA as a template. The q-PCR program began with initial denaturing at 95 °C

for 3 min, followed by 39 cycles of denaturation at 95 °C for 45 s, annealing for 1 min at 57 °C for all primers set, and extension at 72 °C for 7 min. The PCR was completed with a melting analysis starting from 60 °C to 99 °C with temperature increments of 0.2 °C and a transition rate of 5 s to check for product specificity and primer dimer formation. The purity of PCR products was also checked by 1% agarose gel, the presence of a single band of the expected 540 (*Clostridium* sp.), 92 (*Thermoanaerobacterium* sp.), and 82 (*Bacillus* sp.) base.

Table 1. Primers targeted to 16S rRNA were used for q-PCR in this study.

Organism Name	Primer Name	Sequence (5′→3′)	Coverage (%)	Specificity (%)	Sequence Length (bp)	References
Clostridium sp.	Chis150F	AAAGGRAGATTAATACCGCATAA	88.6	99.6	540	[25]
	ClostIR	TTCTTCCTAATCTCTACGCA				
Thermoanaerobacterium sp.	TherF	GTGGAGAACACGGAGGAAGG	21.3	100	93	This study
	TherR	CCCTCTGTTCAGGCCATTGT				
Bacillus licheniformis	BacdF	TGGCTCAGGACGAACGCTG	100	100	82	This study
	BacdR	CCGCTGACCTAAGGGAGCAA				

3. Results and Discussion

3.1. Enrichment of Thermophilic Hydrogen-Producing Bacteria

Thermophilic hydrogen-producing bacteria were enriched from elephant dung using alpha-cellulose as a carbon source by repetitive sub-culture for five generations. Hydrogen production was highest during the third cycle. Stable through the third to the fifth cycle (Figure 1). The first and second cultivation cycles (C1 and C2) gave hydrogen yields of 20.2 and 23.5 mL H_2/g cellulose, respectively. In contrast, the third, fourth, and fifth cultivation cycles (C3) yielded 198.3, 169.1, and 170.3 mL $H_2/g_{cellulose}$, respectively. These results further suggest that multiple transfers of enriched mixed cultures result in highly reproducible hydrogen production [26]. Furthermore, stable hydrogen production indicates the significance of hydrogen-producing bacteria in enriched mixed cultures [3]. Additionally, these repeated transfers confer an environmental adaptive advantage to microorganisms in the culture, resulting in increased hydrogen yields compared to single batch cultivations [11]. Consequently, the enriched thermophilic mixed cultures from the fifth cultivation cycle were employed for hydrogen production from sawdust.

The SMPs of each cultivation cycle is shown in Figure 2. The generation of HAc and HBu accompanies the formation of hydrogen. The total volatile fatty acid (TVFAs) concentration in the five cultivation cycles (C1–C5) was 4597.39 mg/L. The TVFAs (HAc, HBu, HPr, and HLa) showed an increasing tendency in the fourth cycle (C4) and then declined to 1080 mg/L in the fifth cycle. In contrast, when ethanol production increased, the concentrations of HLa increased (Figure 2). It is well established that carbohydrate fermentation by mixed bacterial cultures can produce ethanol and lactic acid [27]. The type of microorganisms involved in the fermentation process has a major impact on this [28]. HLa is generated by competing strains present in the microbial consortium at the beginning of the process, resulting in the alteration of metabolic pathways [29]. Lactic acid bacteria (LAB) have been shown to compete with hydrogen-producing consortia for substrates, resulting in lower hydrogen yields and lower hydrogen production [30]. The dominance of hydrogen-producing bacteria (*Clostridium* sp. and *Thermoanaerobacterium* sp.) in C4 and C5 cultivations indicates that they are highly adapted to the hydrogen production process. Thus, enriched mixed cultures from elephant dung can be expected to enhance hydrogen yield and may be employed as suitable inocula for hydrogen production from sawdust.

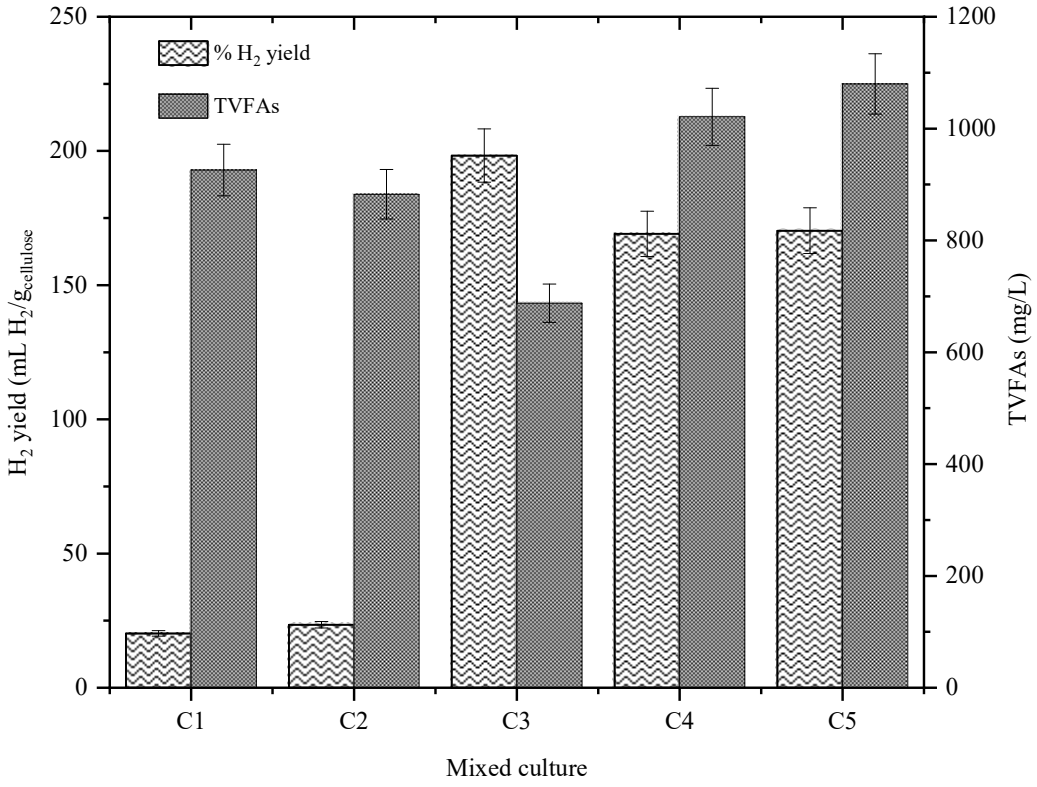


Figure 1. Hydrogen yield from alpha-cellulose by enriched mixed cultures from elephant dung in each repetitive sub-culture for five generations (Cycle (C) 1–Cycle (C) 5).

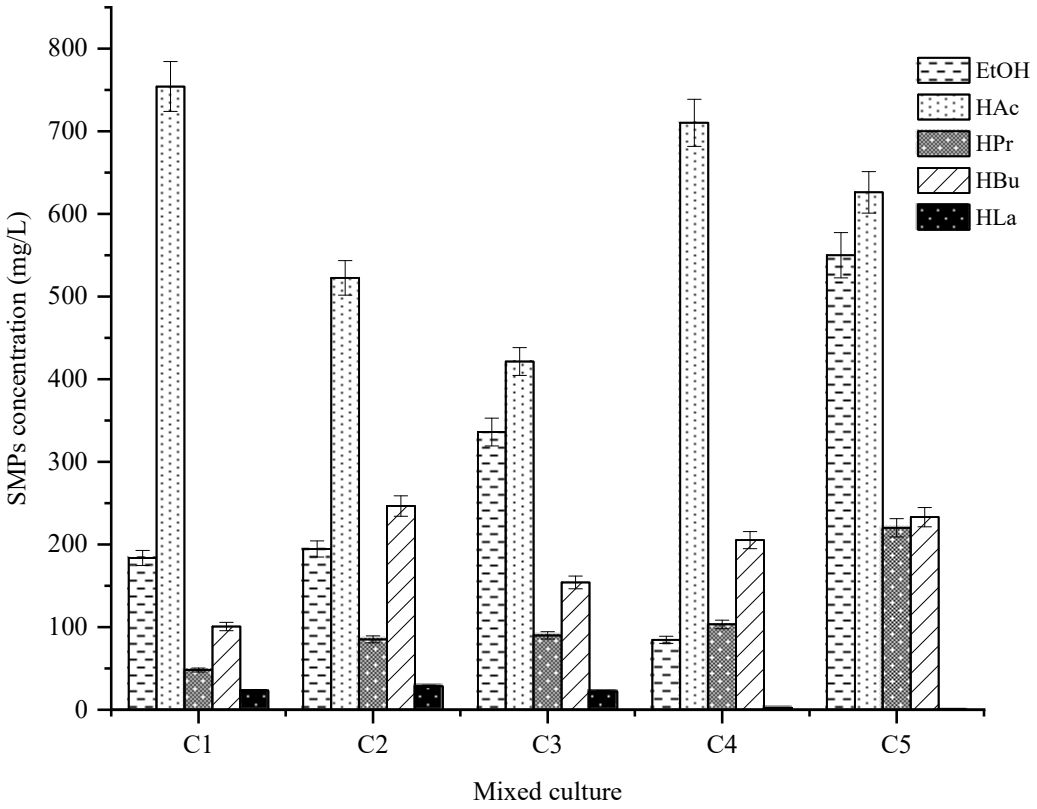


Figure 2. Soluble metabolite product (SMP) in each repetitive sub-culture for five generations (Cycle (C) 1–Cycle (C) 5).

3.2. Thermophilic Bio-Hydrogen Production from Sawdust

Figure 3 depicts the cumulative hydrogen production from sawdust under thermophilic conditions to measure the efficiency of enriched cultures derived from elephant dung. Our study observed a maximum cumulative hydrogen production of 179 mL H_2/L, corresponding to a hydrogen yield of 37.1 mL $H_2/g_{cellulose}$. In concurrence with the report by Mamimin et al. [8], the major SMPs formed during dark fermentation of lignocellulosic material by mixed cultures were HAc and HBu (Figure 2). There was no significant difference between alpha-cellulose and sawdust in hydrogen production, indicating that mixed cultures of elephant dung contained bacteria capable of converting lignocellulose into hydrogen. This is because elephants predominantly consume lignocellulosic plant materials. Thus, their dung is abundant in cellulolytic bacteria that digest cellulose into glucose and hydrogen-producing bacteria that convert this glucose into hydrogen. Therefore, the utilization of elephant dung as inocula for mixed-culture systems has the dual advantages of cellulose degradation and hydrogen production [14]. However, previous research reported that hydrogen production of unpretreated sawdust is less efficient than that from pretreated sawdust [31], possibly due to lower biodegradability on account of the complex structure of cellulose, hemicellulose, and lignin in plant cell walls [32]. While pretreatment of the substrate increases hydrogen yield, it also raises production costs. On the other hand, enrichment cultures derived from elephant dung can efficiently produce hydrogen from sawdust without pretreatment, providing a low-cost benefit.

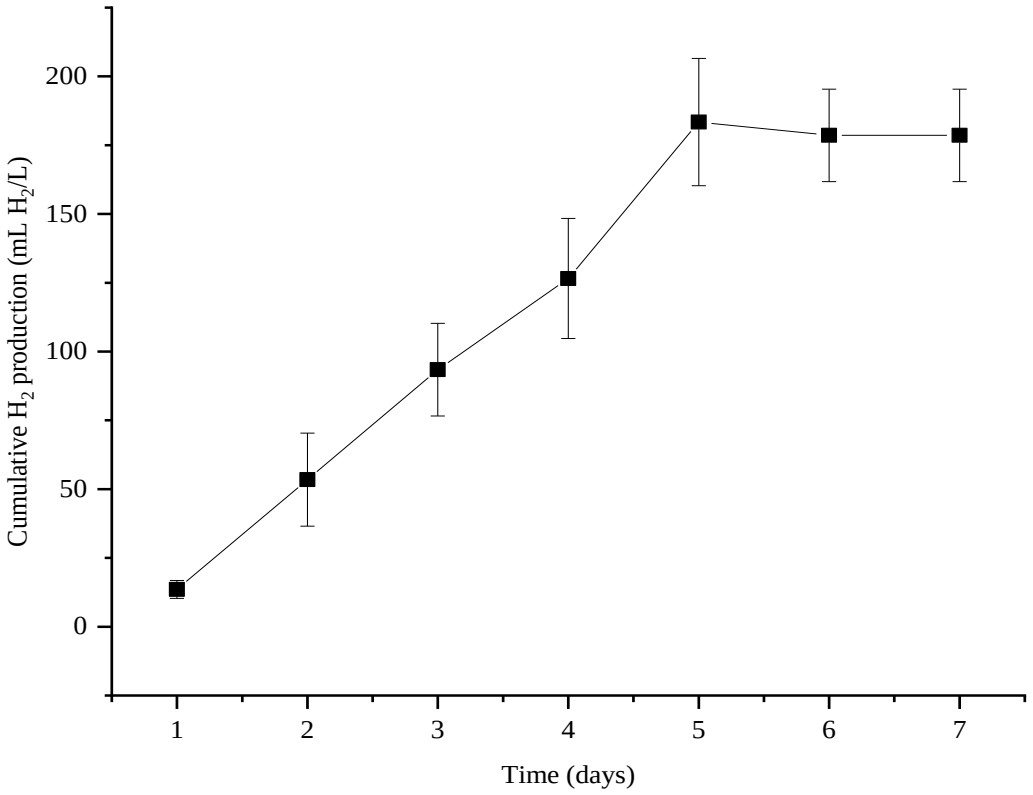
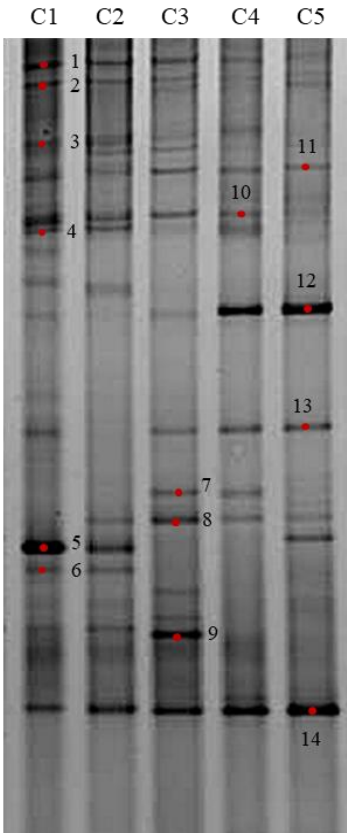


Figure 3. Cumulative hydrogen production from sawdust.

3.3. Microbial Community Structure

The microbial community present in enriched mixed cultures from each sub-culture of elephant dung as well as sawdust hydrogen fermentation were analyzed by PCR-DGGE that targeted the 16S rRNA gene (Figure 4). Repetitive sub-culture of elephant dung was shown to affect the diversity of the bacterial community (Figure 4a). These communities from each repetitive sub-culture (C1–C5) were identified as a mixture of *Clostridium* sp., uncultured bacterium, uncultured rumen bacteria, uncultured *Firmicutes* bacterium, uncul-

tured *Lachnospiraceae* bacterium, *Bacillus* sp., *Geobacillus* sp., *Tissierella* sp., *Streptomyces* sp., uncultured compost bacterium, *Clostridium cellulolyticum*, and *Thermoanaerobacterium* sp. Species diversity decreased from the first cycle (C1) to subsequent cultivations (C2–C5), as evident from prominent band patterns. The first cycle (C1) and second cycle (C2) had slight differences in microbial community structure, with uncultured *Lachnospiraceae* bacterium appearing as strong bands. At the same time, the third transfer (C3) displayed strong bands that indicated *Tissierella* sp. However, bands corresponding to *Clostridium* sp., and *Thermoanaerobacterium* sp. responsible for hydrogen production under thermophilic conditions became most dominant after the third transfer (C3). *Thermoanaerobacterium* species, a well-established thermophile with optimal growth at 60°C that converts carbohydrates to hydrogen with butyrate as the end soluble product [33], remained the abundant species throughout the repetitive mixed culture. *Clostridium* sp. and *Thermoanaerobacterium* sp. have been reported as potential hydrogen producers during the acetogenesis stage of hydrogen production [34], which is in concordance with other studies that report animal dung as a rich source of hydrogen-producing bacteria [26]. From these results, it may be concluded that hydrogen-producing bacteria, such as *Clostridium* sp. and *Thermoanaerobacterium* sp., were present in all cultivation cycles and are the major hydrogen-producing species in enriched mixed cultures from elephant dung detected by PCR-DGGE. Additionally, the quantity of *Clostridium* sp. detected by q-PCR in the C3, C4, and C5 cultivations directly correlated with the high hydrogen yield obtained during these transfers (See Section 3.4).

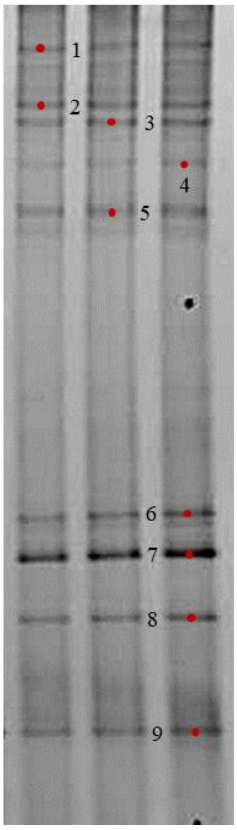


(**a**)

Band	Genus	C1	C2	C3	C4	C5
1	*Clostridium* sp.					
2	Uncultured bacterium					
3	Uncultured rumen bacterium					
4	Uncultured *Firmicutes* bacterium					
5	Uncultured *Lachnospiraceae* bacterium					
6	*Bacillus* sp.					
7	*Geobacillus* sp.					
8	*Clostridium* sp.					
9	*Tissierella* sp.					
10	*Streptomyces* sp.					
11	Uncultured compost bacterium					
12	*Clostridium* sp.					
13	*Clostridium cellulolyticum*					
14	*Thermoanaerobacteriaceae bacterium*					

low high

Figure 4. *Cont.*



(**b**)

Band	Genus	SD1	SD2	SD3
1	*Clostridium* sp.			
2	*Clostridium* sp.			
3	Clostridiales bacterium			
4	Unidentified rumen bacterium			
5	*Streptomyces* sp.			
6	*Lachnospiraceae* sp.			
7	Uncultured *Lachnospiraceae* bacterium			
8	Uncultured compost bacterium			
9	*Thermoanaerobacterium* sp.			

low high

Figure 4. Bacteria DGGE profile of (**a**) elephant dung enrichment in each repetitive sub-culture for five generations and (**b**) sludge from hydrogen reactor of sawdust at different loading.

As shown in the DGGE profile in Figure 4b, microbial communities had similarities in numbers (band density) and diversity (number of the band) in most hydrogen fermentation (SD1, SD2, and SD3). The microbial community structure of sludge from sawdust hydrogen fermentation at different DNA loading of 1.18 ug/uL (SD1), 1.58 ug/uL (SD2) and 2.37 ug/uL (SD3) comprised *Clostridium* sp., Clostridiales bacterium, uncultured rumen bacteria, *Streptomyces* sp., *Lachnospiraceae* sp., uncultured compost bacterium, *Thermoanaerobacterium* sp., and strong bands of uncultured *Lachnospiraceae* bacterium. The DGGE profile further showed that the hydrogen-producing bacteria primarily comprised *Clostridium* sp. and *Thermoanaerobacterium* sp., which was previously reported as the predominant species in hydrogen reactor sludge [9]. Furthermore, these species have been shown to drive hydrogen production from a wide range of lignocellulosic substances [4,7].

3.4. q-PCR

This study developed a quantitative monitoring protocol for *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. during the hydrogen fermentation process by q-PCR. The q-PCR technique provides results within a day and has been increasingly used for identifying and quantifying specific microorganisms within complex microbial communities [35–37]. The linear detection range was determined using a series of standard dilutions of genomic DNA extracted from *Clostridium* sp., *Thermoanaerobacterium thermosaccharolyticum*, and *Bacillus* sp. Threshold cycles were calculated for each sample based on the threshold value after each q-PCR run, and standard curves for *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. were generated by plotting the threshold cycle. The slope and y-intercept were evaluated using linear regression analysis, and gene copy numbers were analyzed using the standard curves. The high R-squared values obtained in Standard curves (*Thermoanaerobacterium* sp. = 0.98; *Bacillus* sp. = 0.99; *Clostridium* sp. = 0.98)

confirm that the reactions were consistent with an absence of any non-specific product. Both *Clostridium* sp. and *Thermoanaerobacterium* sp. were detected by PCR-DGGE and q-PCR in the enriched mixed cultures from each sub-culture of elephant dung (C1–C5). Gene copy numbers from the q-PCR analysis revealed *Bacillus* sp. (1.5×10^{16} to 4.39×10^{14} gene copy number) as the dominant genus in the first cultivation (C1) that demonstrated a decreasing trend in subsequent sub-cultures. In contrast, *Clostridium* sp. (4.66×10^{14} to 5.63×10^{15} gene copy number) and *Thermoanaerobacterium* sp. (5.03×10^{14} to 3.62×10^{15} gene copy number) became dominant genera during repetitive sub-culture, concomitant with a high hydrogen yield in later cycles (Figure 5). A similar correlation between hydrogen yield and 16S rRNA gene copy number of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. was obtained during hydrogen production from saw dust (Figure 6), thus suggesting these strains play an important role in the hydrogen fermentation process. These findings also show that heat treatment of elephant dung prior to forming mixed cultures inhibits non-spore-forming bacteria while not affecting hydrogen-producing bacteria [38]. A recent report by Okonkwo et al. supports our findings by demonstrating the use of q-PCR for quantitative monitoring of hydrogen-producing bacteria [39]. As a result of these findings, molecular approaches for monitoring microbial communities and fermentation processes in mixed cultures were developed to improve hydrogen production.

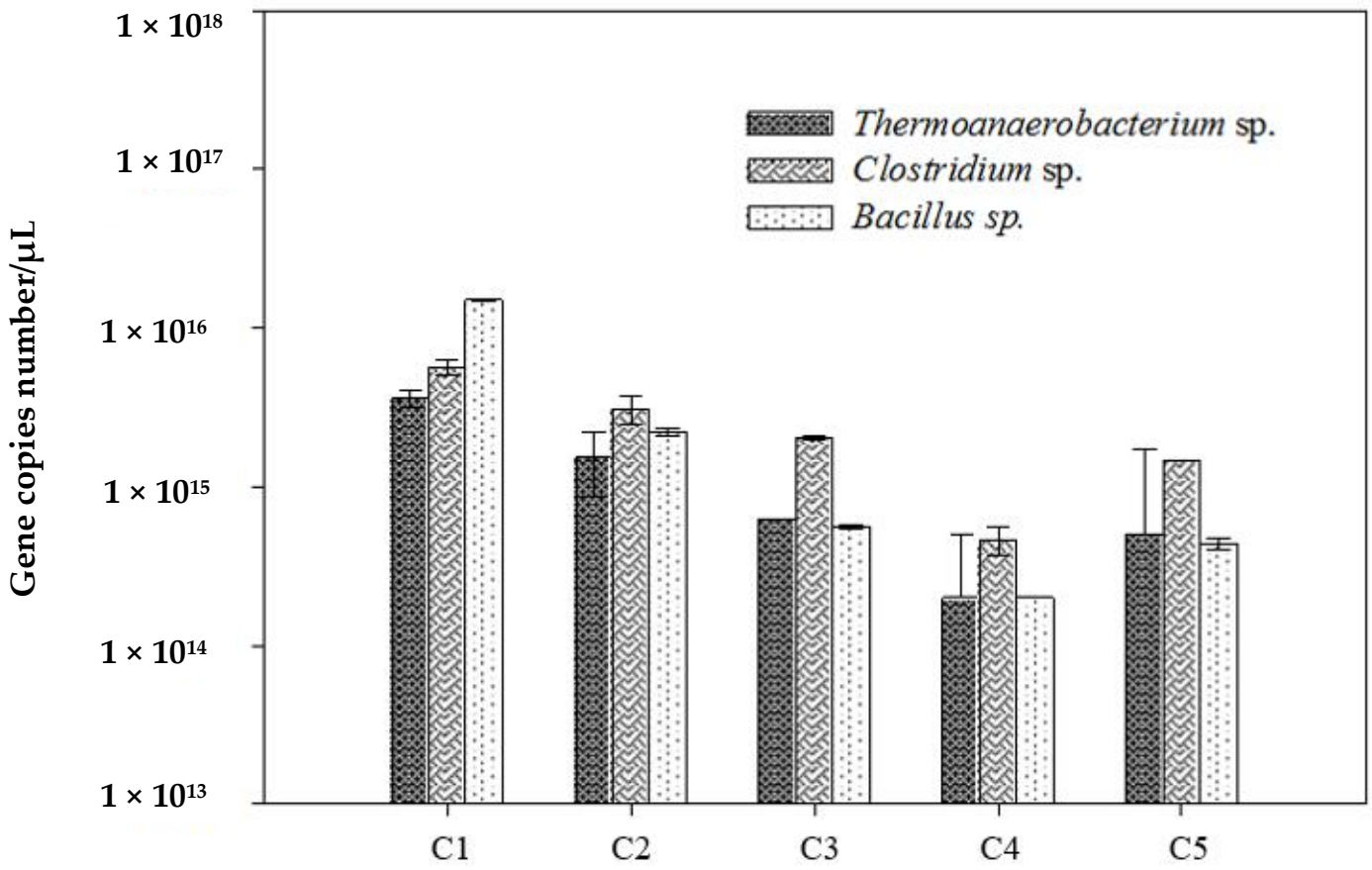


Figure 5. The 16S rRNA amplicons of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. in each repeated repetitive sub-culture.

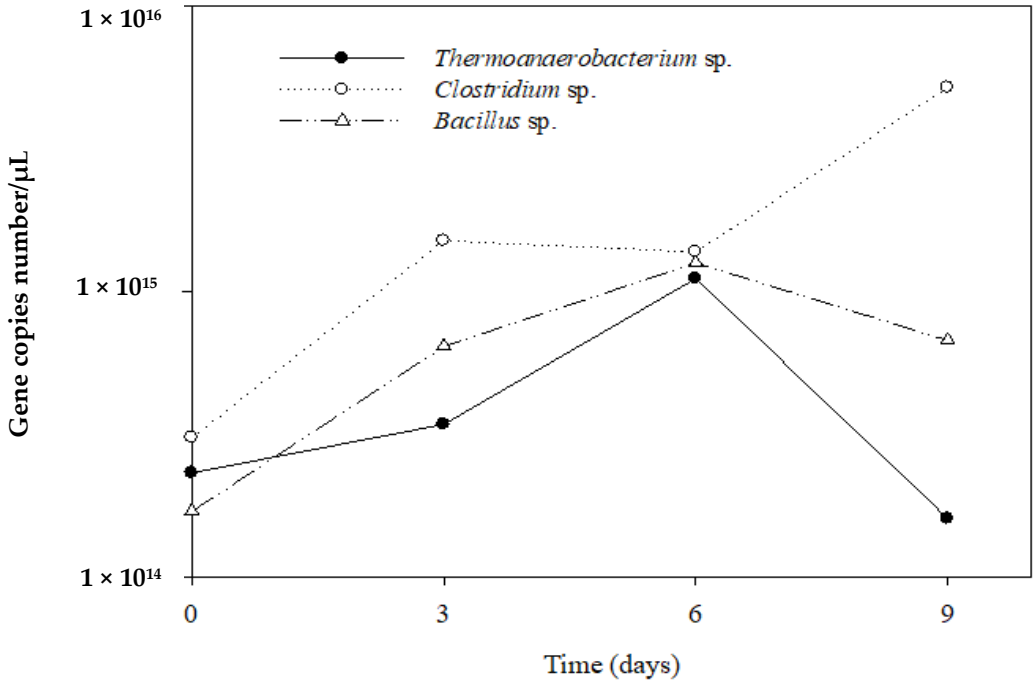


Figure 6. Real-time monitoring of *Clostridium* sp., *Thermoanaerobacterium* sp., and *Bacillus* sp. genome amplification during hydrogen production from sawdust.

4. Conclusions

Hydrogen-producing mixed cultures were efficiently enriched from heat-treated elephant dung and subsequently selected as inocula for hydrogen production from sawdust. Maximum cumulative hydrogen production of 179 mL H_2/L was observed with HAc (1141.64 mg/L) and HBu (563.63 mg/L) generated as major SMPs. The PCR-DGGE profile identified *Clostridium* sp. and *Thermoanaerobacterium* sp. as potential hydrogen producers in the acetogenesis stage of hydrogen production. Quantitative results from the q-PCR analysis provided vital information about the biohydrogen production process in mixed cultures, which will be helpful for further improvement of hydrogen production. The primary hydrogen producer was *Clostridium* sp. in both the enrichment culture and the hydrogen fermentation process of sawdust.

Author Contributions: Conceptualization, K.S. and A.R.; methodology, K.S. and A.R.; formal analysis, K.S. and A.R.; investigation, K.S. and A.R.; resources, A.R. and T.I.; data curation, K.S., C.M., S.S. and A.R.; writing—original draft preparation, K.S.; writing—review and editing, K.S., C.M. and A.R.; visualization, K.S. and C.M.; supervision, T.I. and A.R.; funding acquisition, A.R.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by TSRI Senior Research Scholar, grant number RTA6280001.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: All the authors of the manuscript are immensely grateful to their respective Division of Graduate School of Science and Engineering, Yamaguchi University, Research Group for the Development of Microbial Hydrogen Production Processes from Biomass, the Graduate and Research Studies Department, Khon Kaen University for their technical assistance and valuable support in the completion of this research project.

Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

References

1. Ali, M.; Elreedy, A.; Ibrahim, M.G.; Fujii, M.; Tawfik, A. Hydrogen and methane bio-production and microbial community dynamics in a multi-phase anaerobic reactor treating saline industrial wastewater. *Energy Convers. Manag.* **2019**, *186*, 1–14. [CrossRef]
2. Gomez-Flores, M.; Nakhla, G.; Hafez, H. Hydrogen production and microbial kinetics of *Clostridium termitidis* in mono-culture and co-culture with *Clostridium beijerinckii* on cellulose. *AMB Express* **2017**, *7*, 84. [CrossRef]
3. Rosa, D.; Medeiros, A.B.P.; Martinez-Burgos, W.J.; do Nascimento, J.R., Jr.; de Carvalho, J.C.; Sydney, E.B.; Soccol, C.R. Biological hydrogen production from palm oil mill effluent (POME) by anaerobic consortia and *Clostridium beijerinckii*. *J. Biotechnol.* **2020**, *323*, 17–23. [CrossRef] [PubMed]
4. Zhang, K.; Cao, G.L.; Ren, N.Q. Bioaugmentation with *Thermoanaerobacterium thermosaccharolyticum* W16 to enhance thermophilic hydrogen production using corn stover hydrolysate. *Int. J. Hydrogen Energy* **2019**, *44*, 5821–5829. [CrossRef]
5. Magnusson, L.; Islam, R.; Sparling, R.; Levin, D.; Cicek, N. Direct hydrogen production from cellulosic waste materials with a single-step dark fermentation process. *Int. J. Hydrogen Energy* **2008**, *33*, 5398–5403. [CrossRef]
6. Chen, C.C.; Chuang, Y.S.; Lin, C.Y.; Lay, C.H.; Sen, B. Thermophilic dark fermentation of untreated rice straw using mixed cultures for hydrogen production. *Int. J. Hydrogen Energy* **2012**, *37*, 15540–15546. [CrossRef]
7. Sheng, T.; Gao, L.; Zhao, L.; Liu, W.; Wang, A. Direct hydrogen production from lignocellulose by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* strain DD32. *RSC Adv.* **2015**, *5*, 99781–99788. [CrossRef]
8. Mamimin, C.; Kongjan, P.; O-Thong, S.; Prasertsan, P. Enhancement of biohythane production from solid waste by co-digestion with palm oil mill effluent in two-stage thermophilic fermentation. *Int. J. Hydrogen Energy* **2019**, *44*, 17224–17237. [CrossRef]
9. Mamimin, C.; Jehlee, A.; Seengenyoung, J.; Saelor, S.; Prasertsan, P.; O-Thong, S. Thermophilic hydrogen production from co-fermentation of palm oil mill effluent and decanter cake by *Thermoanaerobacterium thermosaccharolitycum* PSU-2. *Int. J. Hydrogen. Energy* **2016**, *41*, 21692–21701. [CrossRef]
10. O-Thong, S.; Mamimin, C.; Prasertsan, P. Effect of temperature and initial pH on biohydrogen production from palm oil mill effluent: Long-term evaluation and microbial community analysis. *Electron. J. Biotechnol.* **2011**, *14*, 9. [CrossRef]
11. O-Thong, S.; Hniman, A.; Prasertsan, P.; Imai, T. Biohydrogen production from cassava starch processing wastewater by thermophilic mixed cultures. *Int. J. Hydrogen Energy* **2011**, *36*, 3409–3416. [CrossRef]
12. Lay, C.H.; Wu, J.H.; Hsiao, C.L.; Chang, J.J.; Chen, C.C.; Lin, C.Y. Biohydrogen production from soluble condensed molasses fermentation using anaerobic fermentation. *Int. J. Hydrogen Energy* **2010**, *35*, 13445–13451. [CrossRef]
13. Kan, E. Effects of pretreatments of anaerobic sludge and culture conditions on hydrogen productivity in dark anaerobic fermentation. *Renew. Energy* **2013**, *49*, 227–231. [CrossRef]
14. Pachapur, V.L.; Kutty, P.; Pachapur, P.; Brar, S.K.; Le Bihan, Y.; Galvez-Cloutier, R.; Buelna, G. Seed pretreatment for increased hydrogen production using mixed-culture systems with advantages over pure-culture systems. *Energies* **2019**, *12*, 530. [CrossRef]
15. Salakkam, A.; Sittijunda, S.; Mamimin, C.; Phanduang, O.; Reungsang, A. Valorization of microalgal biomass for biohydrogen generation: A review. *Bioresour. Technol.* **2021**, *322*, 124533. [CrossRef] [PubMed]
16. Amann, R.I.; Fuchs, B.M.; Behrens, S. The identification of microorganisms by fluorescence in situ hybridization. *Curr. Opin. Microbiol.* **2001**, *12*, 231–236. [CrossRef]
17. Muyzer, G.; de Waal, E.C.; Uitterlinden, A.G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **1993**, *59*, 695–700. [CrossRef] [PubMed]
18. O-Thong, S.; Khongkliang, P.; Mamimin, C.; Singkhala, A.; Prasertsan, P.; Birkeland, N.-K. Draft genome sequence of *Thermoanaerobacterium* sp. strain PSU-2 isolated from thermophilic hydrogen producing reactor. *Genom. Data* **2017**, *12*, 49–51. [CrossRef] [PubMed]
19. Tolvanen, K.E.; Koskinen, P.E.; Ylikoski, A.I.; Ollikka, P.K.; Hemmilä, I.A.; Puhakka, J.A.; Karp, M.T. Quantitative monitoring of a hydrogen-producing *Clostridium butyricum* strain from a continuous-flow, mixed culture bioreactor employing real-time PCR. *Int. J. Hydrogen Energy* **2008**, *33*, 542–549. [CrossRef]
20. Angelidaki, I.; Sanders, W. Assessment of the anaerobic biodegradability of macropollutants. *Rev. Environ. Sci. Bio/Technol.* **2004**, *3*, 117–129. [CrossRef]
21. Fangkum, A.; Reungsang, A. Biohydrogen production from mixed xylose/arabinose at thermophilic temperature by anaerobic mixed cultures in elephant dung. *Int. J. Hydrogen Energy* **2011**, *36*, 13928–13938. [CrossRef]
22. Zhang, X.J.; Yu, H.Q. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. *J. Environ. Manag.* **2005**, *74*, 65–70. [CrossRef] [PubMed]
23. Keyser, M.; Witthuhn, C.; Lamprecht, C.; Coetzee, M.; Britz, T. PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. *Syst. Appl. Microbiol.* **2006**, *29*, 77–84. [CrossRef]
24. Klindworth, A.; Pruesse, E.; Schweer, T.; Peplies, J.; Quast, C.; Horn, M.; Glöckner, F.O. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* **2013**, *41*, e1. [CrossRef]
25. Hung, C.H.; Cheng, C.H.; Cheng, L.H.; Liang, C.M.; Lin, C.Y. Application of Clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. *Int. J. Hydrogen Energy* **2008**, *33*, 1586–1592. [CrossRef]
26. Sivagurunathan, P.; Sen, B.; Lin, C.-Y. Batch fermentative hydrogen production by enriched mixed culture: Combination strategy and their microbial composition. *J. Biosci. Bioeng.* **2014**, *117*, 222–228. [CrossRef]

27. Cardoso, V.; Romao, B.B.; Silva, F.T.M.; Santos, J.G.; Batista, F.R.X.; Ferreira, J.S. Hydrogen production by dark fermentation. *Chem. Eng. Trans.* **2014**, *38*, 481–486. [CrossRef]
28. Darwin; Cord-Ruwisch, R.; Charles, W. Ethanol and lactic acid production from sugar and starch wastes by anaerobic acidification. *Eng. Life Sci.* **2018**, *18*, 635–642. [CrossRef]
29. Moreira, F.S.; Machado, R.G.; Romão, B.B.; Batista, F.R.X.; Ferreira, J.S.; Cardoso, V.L. Improvement of hydrogen production by biological route using repeated batch cycles. *Process. Biochem.* **2017**, *58*, 60–68. [CrossRef]
30. Sikora, A.; Błaszczyk, M.; Jurkowski, M.; Zielenkiewicz, U. Lactic acid bacteria in hydrogen-producing consortia. In *Lactic Acid Bacteria—R & D for Food, Health and Livestock Purposes*; Kongo, M., Ed.; IntechOpen: London, UK, 2013; pp. 487–514.
31. Dimitrellos, G.; Lyberatos, G.; Antonopoulou, G. Does acid addition improve liquid hot water pretreatment of lignocellulosic biomass towards biohydrogen and biogas production. *Sustainability* **2020**, *12*, 8935. [CrossRef]
32. Morsy, F.M.; Elbadry, M.; Elbahloul, Y. Semidry acid hydrolysis of cellulose sustained by autoclaving for production of reducing sugars for bacterial biohydrogen generation from various cellulose feedstock. *PeerJ* **2021**, *9*, e11244. [CrossRef] [PubMed]
33. O-Thong, S.; Prasertsan, P.; Karakashev, D.B.; Angelidaki, I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. *Int. J. Hydrogen Energy* **2008**, *33*, 1204–1214. [CrossRef]
34. Su, X.; Zhao, W.; Xia, D. The diversity of hydrogen-producing bacteria and methanogens within an in-situ coal seam. *Biotechnol. Biofuels* **2018**, *11*, 245. [CrossRef]
35. Burrell, P.C.; O'Sullivan, C.; Song, H.; Clarke, W.P.; Blackall, L.L. Identification, detection, and spatial resolution of Clostridium populations responsible for cellulose degradation in a methanogenic landfill leachate bioreactor. *Appl. Environ. Microbiol.* **2004**, *70*, 2414–2419. [CrossRef]
36. Rasolofo, E.A.; St-Gelais, D.; LaPointe, G.; Roy, D. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *Int. J. Food Microbiol.* **2010**, *138*, 108–118. [CrossRef]
37. Smith, C.J.; Nedwell, D.B.; Dong, L.F.; Osborn, A.M. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcription numbers in environmental samples. *Environ. Microbiol.* **2006**, *8*, 804–815. [CrossRef]
38. Chang, J.; Wu, J.; Wen, F.; Hung, K.; Chen, Y.; Hsiao, C.; Lin, C.; Huang, C. Molecular monitoring of microbes in a continuous hydrogen-producing system with different hydraulic retention time. *Int. J. Hydrogen Energy* **2008**, *33*, 1579–1585. [CrossRef]
39. Okonkwo, O.; Lakaniemi, A.-M.; Santala, V.; Karp, M.; Mangayil, R. Quantitative real-time PCR monitoring dynamics of *Thermotoga neapolitana* in synthetic co-culture for biohydrogen production. *Int. J. Hydrogen Energy* **2018**, *43*, 3133–3141. [CrossRef]