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Research Article

Lipopeptide Biosurfactants Enhanced Biohydrogen Evolution from Lignocellulose Biomass and Shaped the Microbial Community and Diversity

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Biohydrogen is a renewable and clean energy source that can be produced from cheap and abundantly available lignocellulose biomass. However, the complex structure of lignocellulose requires various physicochemical and biological pretreatments, as it exhibits significant resistance to microbial degradation. Biosurfactants can play a vital role in facilitating the microbial degradation of lignocellulose and inducing enzymatic hydrolysis. In addition, they can lower the surface tension to impede lignin-cellulase interactions and alter the lignin characteristics. Indeed, the application of lipopeptide biosurfactants to enhance hydrogen production from lignocellulose biomass is poorly studied. Thus, this study investigates the influence of lipopeptide biosurfactants on biohydrogen enhancement from lignocellulose biomass and their impact on short-chain fatty acid generation during anaerobic dark fermentation. Subsequently, Illumina HiSeq 2500 sequencing was employed to analyze the structure of microbial community and diversity significantly affected by the presence or absence of aided biosurfactants. Results revealed that the lipopeptide biosurfactant significantly improved the cumulative biogas and hydrogen production. The maximum cumulative hydrogen yield was achieved in lipopeptide-assisted bioreactors including BioR_3, BioR_2, and BioR_4 (i.e., 4.68, 4.56, and 4.50 mmol/2 g of substrate, respectively), showing an increase of 30.79% to 36.03% higher than BioR_1 (3.44 mmol). In addition, lipopeptide biosurfactants also impacted the short-chain fatty acid generation, where acetic acid, propionic acid, and isobutyric acid were found as major acids. On the other hand, various bacterial phyla, including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetota*, and *Acidobacteriota*, were detected in all bioreactors. Among the phyla, *Firmicutes* were predominated (54.74% to 86.38%) in lipopeptide-assisted bioreactors, indicating that biosurfactants substantially influenced the microbial community structure during hydrogen production. Besides, *Ruminiclostridium* and *Bacillus* were significantly promoted in lipopeptide-assisted bioreactors, representing efficient lignocellulose-degrading and hydrogen-producing genera. Conclusively, this study offers valuable insights into the underlying mechanism through which lipopeptide biosurfactants actively participate in biohydrogen production and illuminates the variations occurring within microbial communities.

1. Introduction

Hydrogen, a highly regarded and prospective energy carrier for the future, is often derived from nonrenewable fossil fuels. The primary methods of production are steam reforming of methane and oxidations of natural gas [1]. Nevertheless, the processes involved in hydrogen production are unavoidably associated with a substantial release of greenhouse gasses (GHG), which contribute to the exacerbation of climate change-related concerns such as global warming [1]. Consequently, these methods are seen as environmentally unfriendly and unsustainable [2]. The feasibility and potential of using

biological techniques for the synthesis of hydrogen from waste biomass render it a viable developing technology that is both sustainable and environmentally beneficial [3]. Dark fermentation (DF) is a biological process used to produce hydrogen. It involves the acidogenic degradation of substrates that are rich in carbohydrates. DF has been shown to possess some notable advantages, such as requiring less energy input, having a simple structure, and offering a cheaper cost of production [1]. Consequently, the use of inexpensive organic materials, such as lignocellulosic agricultural waste, via dark fermentation (DF) is a promising approach for the large-scale production of biohydrogen. This method not only enables the feasibility of H_2 production but also facilitates waste stabilization and ensures environmental safety [4]. To improve the efficiency of dark fermentation, it is necessary to subject the lignocellulosic biomass to a suitable pretreatment prior to biofuel production. This is because the biomass consists of a complex lignocellulosic structure comprising cellulose, hemicellulose, and lignin components, which exhibit significant resistance to microbial degradation [5, 6].

To address this intricate structural obstacle, a variety of physical, physicochemical, and biological pretreatment methods have been used, significantly contributing to the conversion of lignocellulosic biomass [7]. However, some obstacles persist in the process of enzymatic hydrolysis of lignocellulosic biomass, including those related to nonproductive binding, limited total solid loading, and reduced enzyme activity [7]. In order to maximize the effectiveness of the enzymatic hydrolysis of vegetal biomass, it has been suggested that surfactant molecules can lower surface tension and alter the structure of plant biomass and can be employed as effective additives [7]. In a study conducted by Chen et al., the use of Tween 20 resulted in enhanced efficacy of the enzymatic hydrolysis process applied to acid-pretreated wheat straw. This improvement facilitated the attainment of an 80% yield in glucose conversion. The authors observed such enhancement in enzymatic hydrolysis that can be attributed not only due to the surfactant's ability to impede lignin-cellulase interactions but also to the alterations in lignin characteristics (such as hydrophobicity, hydrogen binding capacity, and surface charges) induced by the presence of the surfactant [8].

Although synthetic surfactants are widely used, it is important to note that these compounds are nonbiodegradable and have been identified as environmentally harmful [9]. The use of microbial-derived biological surfactants provides a more sustainable scenario due to their biodegradability, biocompatibility, and low toxicity properties [7, 10]. Hosny and El-Sheshtawy studied to assess the capacity of cellulase-producing bacteria to hydrolyze municipal garbage, with the inclusion of a bacterial lipopeptide (surfactin) biosurfactant as an addition [11]. Upon the addition of the combination to the hydrolysis process, the authors reported 92.2% increase compared to Tween 80. Furthermore, it was discovered that the presence of this biosurfactant resulted in increased cellulase activity. Surfactin is considered as the most powerful compound that can reduce the surface activity of liquid from 72 to 27 mN/m, while critical micelle concentration (CMC) is around 20 $mg{\cdot}L^{-1}$ [12]. Besides, biosurfactant supplementation also can play a significant role to enhance biohydrogen production [13]. For example, Sharma and Melkania [13] invested the effect of two biosurfactants, i.e., surfactin and saponin, on hydrogen evolution from organic fraction municipal solid waste by a coculture bacterial strains (*Enterobacter aerogenes* and *E. coli*). The authors confirmed a substantial improvement in hydrogen production by both species of surfactin and saponin.

At present, very limited reports have been highlighted the role of lipopeptide biosurfactants for biohydrogen enhancement from the lignocellulose bioconversion and their impact on microbial ecosystem. Thus, this study examined lipopeptide biosurfactant derived from *Bacillus subtilis* CW2 strain, and their impact on biohydrogen production from lignocellulose biomass was investigated using anaerobic dark fermentation. The production of short-chain fatty acids in each bioreactor following the fermentation process was analyzed using liquid chromatography and mass spectrometry (LC/MS). Furthermore, Illumina HiSeq 2500 sequencing was employed to analyze the microbial community and diversity, providing insights into the composition of the microbial community.

2. Materials and Methods

2.1. Bacterial Strain for Lipopeptide Biosurfactant Production. The bacterial strain *Bacillus subtilis* CW2 (GenBank accession no. OR431861) was previously isolated from coal bed formation water for lipopeptide production. For biosurfactant screening, the strain was cultivated in LB broth and then incubated for 12 to 18 h at 30°C in a shaking incubator for inoculum preparation. Subsequently, 2% inoculum was transferred into a 100 mL preautoclaved minimal salt medium (MSM). Medium composition was prepared as described in a previous study with minor modifications such as 0.6 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.02 $g{\cdot}L^{-1}$ $FeSO_4{\cdot}7H_2O$, 0.5 $g{\cdot}L^{-1}$ NaCl, 0.02 $g{\cdot}L^{-1}$ $CaCl_2$, 2.2 $g{\cdot}L^{-1}$ Na_2HPO_4, and 1.4 $g{\cdot}L^{-1}$ KH_2PO_4. Trace element 0.1% includes 1.78 $g{\cdot}L^{-1}$ $MnSO_4{\cdot}4H_2O$, 2.32 $g{\cdot}L^{-1}$ $ZnSO_4{\cdot}7H_2O$, 1.0 $g{\cdot}L^{-1}$ $CuSO_4{\cdot}5H_2O$, 0.39 $g{\cdot}L^{-1}$ $NH_4MoO_4{\cdot}2H_2O$, and 0.56 $g{\cdot}L^{-1}$ H_3BO_3. Medium pH was maintained (7.0 ± 0.5). The culture broths were then incubated in a shaking environment at 160 rpm for 4 days at 30°C for lipopeptide production. Thereafter, the cultivated cultures were centrifuged at 11200 g for cell-free supernatants for lipopeptide determination using surface tension measurement and product extraction. Then, the extracted product was characterized using UHPLC-MS for lipopeptide confirmation as described in the previous study [14].

2.2. Seed Sampling for Biohydrogenation. The samples (activated sludge) were collected from municipal wastewater surrounding the University of the Chinese Academy of Sciences (UCAS), main Yanqihu campus, Huairou District, Beijing, China. Before the use of seed cultures, the activated sludge was preheated at 100°C for 20 minutes to suppress the methanogenesis [15]. Then, a 5% of inoculum was enriched in (2%) glucose-rich MSM medium as described previously [15] with minor modification containing 1 $g{\cdot}L^{-1}$ NH_4Cl, 0.3 $g{\cdot}L^{-1}$ KH_2PO_4, 0.3 $g{\cdot}L^{-1}$ K_2HPO_4, 0.25 $g{\cdot}L^{-1}$ $MgCl_2$, 0.1 $g{\cdot}L^{-1}$ $CaCl_2$, 3 $g{\cdot}L^{-1}$ $MgSO_4$, 0.01 $g{\cdot}L^{-1}$ $CuSO_4$, 0.1 $g{\cdot}L^{-1}$ $FeSO_4$,

0.01 g·L^{-1} Na_2MoO_4, and 3·45 g. L^{-1} $NaHCO_3$. The pH of the medium was maintained (7.0 ± 0.5). The seed culture medium was then purged with N_2 gas (99.9% purity) to make an anaerobic environment, and subsequently, the culture broth was covered with aluminum foil to protect it from light and incubated in shaking environment 160 rpm for 24 to 48 hours at 35°C.

2.3. Experimental Setup for Anaerobic Dark Fermentation. The experimental setup was designed in 250 mL Duran reagent bottles with butyl rubber screw caps (Figure 1). A total of four bioreactors (i.e., BioR_01, BioR_02, BioR_03, and BioR_04) with 100 mL working solution were designed. The MSM medium composition was prepared as 1 g·L^{-1} NH_4Cl, 0.3 g·L^{-1} KH_2PO_4, 0.3 g·L^{-1} K_2HPO_4, 0.25 g·L^{-1} $MgCl_2$, 0.1 g·L^{-1} $CaCl_2$, 3 g·L^{-1} $MgSO_4$, 0.01 g·L^{-1} $CuSO_4$, 0.1 g·L^{-1} $FeSO_4$, 0.01 g·L^{-1} Na_2MoO_4, and 3.45 g·L^{-1} $NaHCO_3$. Additionally, a vitamin solution (1%, *v*/*v*) was added to the main production medium as mentioned above. The vitamin solution contained the following: vit. B6, pyridoxine-HCl (10 mg·L^{-1}); vit. B9, folic acid (2 mg·L^{-1}); vit. B7, biotin (2 mg·L^{-1}); vit. B2, riboflavin (5 mg·L^{-1}); vit. B1, thiamine-HCl (5 mg·L^{-1}); vit. B12 (0.1 mg·L^{-1}); vit. B3, nicotinic acid (5 mg·L^{-1}); and lipoic acid (5 mg·L^{-1}). The different concentrations of lipopeptide biosurfactants were prepared (i.e., BioR_01 = 0 mg·L^{-1}, BioR_02 = 25 mg·L^{-1}, BioR_03 = 50 mg·L^{-1}, and BioR_04 = 100 mg·L^{-1}). Thereafter, 2% (*w*/*v*) pretreated wheat straw biomass was transferred into each bioreactor as a sole carbon source. The wheat straw biomass was purchased from Shanghai Jizhi Biochemical Technology Co., Ltd. The pH of the medium was maintained (7.0 ± 0.5). All the bioreactors were then autoclaved at 121°C for 15 psi for sterilization. Afterward, 5% (*v*/*v*) pregrown seed inoculum (day_0) was transferred into each bioreactor, and the N_2 gas (purity 99.9%) was purged for 3-5 minutes to make an anaerobic environment for biohydrogenation. All the bioreactors were covered with aluminum foil for dark fermentation and then incubated at 35°C in static conditions.

2.4. Analytical Analysis. The evolved total biogas in the headspace of each bioreactor was monitored weekly based. H_2, CH_4, and CO_2 contents (%) were determined using gas chromatography (GC-SP7800, Beijing Jing Ke Ruida, China) equipped with a thermal conductivity detector (TCD), a 2.0 m GDX-103 (60/80 mesh column). The GC operational temperatures of the column oven, injection port, and detector were 120°C, 80°C, and 160°C, respectively. The used carrier gas was nitrogen at a flow rate of 25 mL/min, and a 50 μL Agilent syringe was employed to inject the sampled gas in GC. The morphological microstructure of the degraded lignocellulose samples was conducted using a scanning electron microscope (SEM). Short-chain fatty acids including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid, and caproic acid were determined through LCMS. In this experiment, LC-ESI-MS/MS (UHPLC-Qtrap) was used to qualitatively and quantitatively detect targets in the sample. ExionLC AD system and Waters BEH C18 (150∗2.1 mm, 1.7 μm) LC column were used. The temperature 40°C and injection volume of 2 μL were set. Mobile phase A (0.1% formic acid aqueous solution) and mobile phase B (0.1% formic acid acetonitrile) were used. Mass spectrometry conditions are as follows: AB SCIEX QTRAP 6500+, with negative mode detection, curtain gas (CUR) of 35, collision gas (CAD) for medium, IonSpray Voltage (IS) for -4500, temperature (TEM) for 450, ion source gas 1 (GS1) 40, and ion source gas 2 (GS2) 40. The quantitative measurement of short-chain fatty acid content by using standards (Figure S4 and table S1) was calculated as

$$\text{Sample short-chain fatty acid content (ng/mL)} = \frac{(C * \text{DF} * V)}{W}, \quad (1)$$

where C is the concentration measured by the LC-MS machine, V is the volume of fixed volume, DF is the dilution factor, and W is the sampling amount.

2.5. DNA Extraction and Illumina HiSeq 2500 Sequencing. Before (0_day) and at the end of experiment, the bacterial cell biomass (2 mL) was collected from all the bioreactors using centrifugation (11200 g for 10 minutes at 4°C). Afterward, genomic DNA was collected according to manufacturer guidelines through TaKaRa MiniBEST Universal Genomic DNA Extraction Kit (ver. 5.0., Japan). The collected DNA from each bioreactor was measured by an ultramicro spectrophotometer (UV-Vis NanoDrop ND-1000 Technologies, Wilmington, DE, USA) and stored at -80°C for further investigation. Subsequently, the Illumina HiSeq 2500 sequencing platform was used to examine the microbial community. The bacterial region V_3-V_4 of 16S rRNA genes was amplified using a set of universal primers, 338F (5′-3′) and 806R (5′-3′) [16], for the determination of bacterial community analysis. The archaeal region V_4-V_5 of 16S rRNA genes was conducted using universal primers 524–10-ext (5′-3′) and arch958RmodR (5′-3′) [1]. DNA libraries were generated by employing purified PCR products and a TruSeq® DNA PCR-Free Sample Preparation Kit following the manufacturer's guidelines. The created DNA libraries were examined using qPCR and a Qubit before being sequenced on a HiSeq 2500 PE250. Sequence data analysis using phylogenetics Qiime (V1.7.0) was used to process chimeric sequences for quality control [17]. The UPARSE software (v7.0.1001, http://drive5.com/uparse/) was used to establish the OTUs at a 97% cut-off [18]. The corresponding sample sequences of each OTU were subjected to comparison with the small subunit ribosomal RNA (SSUrRNA) sequence database [19].

2.6. Statistical Analysis. In the present study, the data was analyzed in Microsoft Excel (v.2010) and Origin software (v.2023) for basic descriptive statistics and figures. The mothur software package was employed to calculate the microbial diversity indices including Chao1 estimator, goods coverage, and Shannon index. The calculation formula was used as described previously [1].

3. Results and Discussion

3.1. Lipopeptide Biosurfactant Determination. The strain CW2 was previously isolated from coal bed formation water sample and confirmed as *Bacillus subtilis* through 16S rRNA molecular typing. The phylogenetic tree revealed the closest

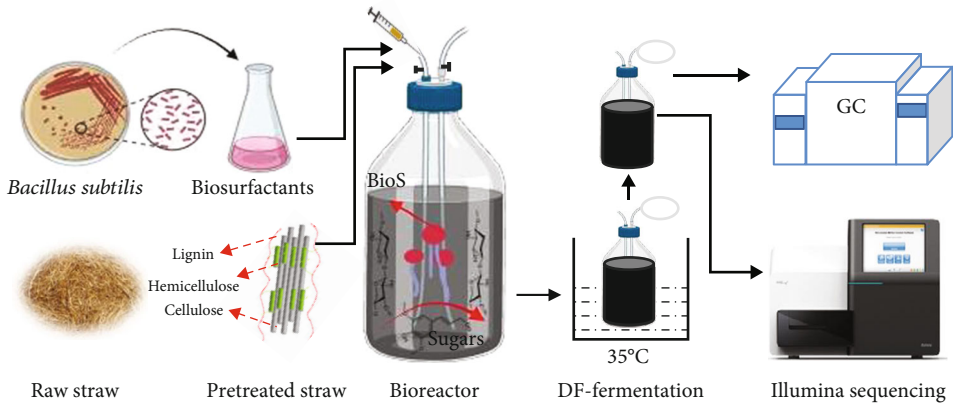


Figure 1: Schematic illustration of designed bioreactors for biohydrogen production from lignocellulose biomass.

relationship with the *Bacillus subtilis* (MT538531.1) strain (Figure S1). The genus *Bacillus* is well known for biosurfactant synthesis, particularly surfactin, the best-known family of cyclic lipopeptides which consisted of seven amino acid peptide rings and β-hydroxy fatty acid chain having a length of 12 to 16 carbon atoms [12, 20]. Generally, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are major lipopeptide producers [12]. In this study, the biosurfactant was collected from *Bacillus subtilis* CW2 in the culture media, and then, the product was characterized using a standard screening methods, i.e., surface tension measurement (SFT) and UHPLC-MS analysis. The lowest SFT reduction of obtained biosurfactants was observed at 32.75 ± 1.63 mN/m in MSM medium containing glucose as a carbon source. The UHPLC-MS results revealed that various surfactin isoforms were detected as depicted in Figure S2. The main surfactin isoforms of lipopeptides at m/z value, i.e., 1008.98 (surfactin A), 1023.00 (surfactin B), and 1037.02 (surfactin C) at the retention time (Rt 13.27), were detected by LC/MS. Similar isoforms were also detected in the standard surfactin as shown in the chromatogram (Figure S2ab).

3.2. Effect of Lipopeptide on H_2, CH_4, and CO_2 Evolution from Lignocellulose. Biosurfactant supplementation can play a significant role to enhance biohydrogen production [13]. At present, very limited reports have been highlighted to investigate the role of lipopeptide biosurfactants for biohydrogen enhancement from lignocellulose biomass. Generally, lignocellulose biomass is hard to degrade by microbes owing to its complex mixture of plant cell wall constituents [6]. Previous studies have elucidated that biosurfactants can facilitate the lignocellulose for microbial degradation [10, 21]. It has been reported that after adding rhamnolipid biosurfactants in the lignocellulose-degrading system, the primary tissues of rice straw were severely destroyed, and a hydrogen bonding was created between biosurfactants and bacteria [21].

In this study, the SEM analysis was conducted to investigate morphological changes in treated straw biomass. Electron microscopy caved significant insights into the degradation of decayed wheat straw and the pattern of lignocellulose degradation [22]. In Figure S3, SEM images of lignocellulose biomass pre- and post-treatment illustrate a significant variation among the control, unaided lipopeptide (BioR_01), and lipopeptide-assisted bioreactors (BioR_02, BioR_03, and BioR_04). The untreated or control wheat straw exhibited fibrils that were rigid, compact, and highly ordered (Figure S3a). In comparison to unaided lipopeptide straw (BioR_01), the SEM image revealed that microbes may significantly destroy the cellulose and hemicellulose-lignin network by penetrating the cell wall of wheat straw in lipopeptide-assisted bioreactors (Figure S3b-e). The biodegradation of wheat straw was expedited by the exposure of its inner structure, which made cellulase more easily accessible. Wheat straw was softer than in the control and BioR_01 groups because microbes thrived on its surface. The inner surface showed the development of tiny, dispersed eroded areas, whereas the exterior and top surfaces showed the presence of distinct, microscopic holes all over the surface. The existence of pores is indicating the increased cellulose surface area available for enzyme action. Furthermore, many fractures, erosion, and holes were discovered on the outer surface of wheat straw, and the fibers seem isolated as a result of secondary wall deterioration. The number and extent of these holes, fissures, and channels grew, and the cell's structure was severely weakened.

Figure 2 illustrates the results of cumulative biogas production in 100 mL reaction volume that exhibited that the maximum biogas was observed in BioR_02 (172 ± 7.77 mL) containing 25 mg·L^{-1} of lipopeptide followed by BioR_03 (170 ± 19.79 mL) (50 mg·L^{-1} of lipopeptide). However, the maximum concentration (100 mg·L^{-1}) of lipopeptide poses a slight negative effect at the initial stage; but later, the biogas production was improved significantly up to 153.5 ± 4.24 mL/2 g of substrate. It might be due to antagonistic activity of lipopeptide biosurfactants against bacteria. On the other hand, BioR_01 (0 mg·L^{-1}) exhibited 107.5 ± 10.60 mL of total cumulative biogas, which was significantly lower compared to other bioreactors (lipopeptide-assisted bioreactors). In current study, the increased biogas production might be due to the addition of lipopeptide-biosurfactants. The biosurfactants may play a significant role in cell surface hydrophobicity that was caused by structural changes in the cell surface microorganisms [13]. The modifications made resulted in an improved level of

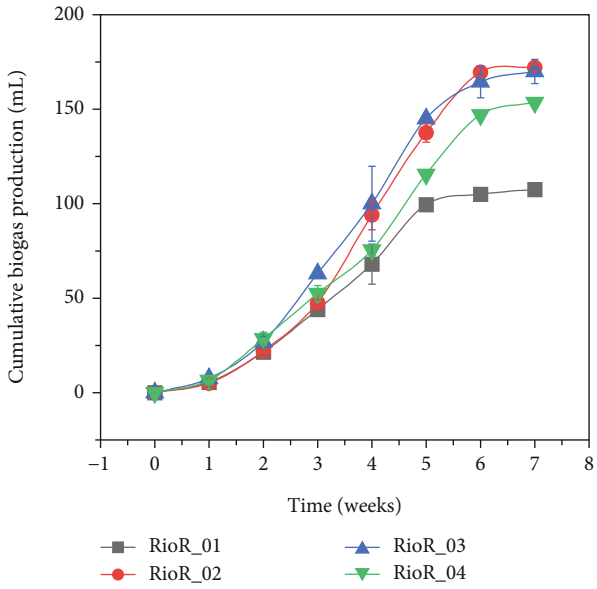


Figure 2: Profile of total biogas generation from lignocellulose biomass in different bioreactors that were investigated with various concentrations of lipopeptide biosurfactants.

accessibility of the organic compounds to the microbial cells [13]. Figures 3(a)–3(d) show the cumulative H_2, CH_4, and CO_2 evolution during the dark fermentation process from lignocellulose. The highest H_2 production was achieved in BioR_03, BioR_02, and BioR_04 bioreactors (i.e., 4.68, 4.56, and 4.50 mmol/2 g of substrate). However, the H_2 production in BioR_01 was observed 3.44 mmol/2 g of substrate, which was significantly lower than lipopeptide-assisted reactors.

Sharma and Melkania investigated the effect of two biosurfactants, i.e., surfactin and saponin, on hydrogen evolution from organic fraction municipal solid waste by culturable coculture bacterial strains (*Enterobacter aerogenes* and *E. coli*). The authors confirmed a substantial improvement in hydrogen production by both species of surfactin and saponin [13]. The use of synthetic surfactants has also been employed as a means to enhance hydrogen generation [23]. Pachapur et al. observed a 1.25-fold increase in hydrogen generation, with a maximum concentration of hydrogen reaching 32.1 ± 0.03 mmol·L^{-1}, when a surfactant known as Tween 80 was present at a concentration of 15 mg·L^{-1} [23]. The increase in hydrogen generation may be ascribed to the surfactant's wetting capabilities, distinctive micelle-forming characteristics, and organic reaction catalysis [23]. The application of surfactant may cause the production of micelles, which creates active sites and improves the coupling of sequential processes that transform polymeric materials into soluble components, fatty acids, and ultimately gasses. Zhou et al. conducted a comparative analysis of two chemosynthetic surfactants, namely, sodium dodecyl sulfate (SDS) and sodium dodecyl benzene sulfonate (SDBS), together with a biosurfactant called rhamnolipid to enhance hydrogen evolution [24]. The authors found that the addition of rhamnolipid resulted in a higher yield of volatile fatty acids compared to samples treated with SDS and SDBS, with an increase of 1.16-fold and 3.63-fold, respectively. Additionally, the highest hydrogen yield of 12.90 mgH2g-VSS was observed in the rhamnolipid-supplemented treatments, surpassing the yields obtained from SDS- and SDBS-treated samples.

Besides H_2 production, the maximum CH_4 evolution was also observed in the lipopeptide-aided bioreactors, particularly in BioR_02 and BioR_03, i.e., 1.30 mmol and 1.24 mmol, respectively, as shown in Figures 3(b) and 3(c). However, the lowest CH_4 was observed in BioR_01 (0.60 mmol) and BioR_04 (0.69 mmol) (Figures 3(a) and 3(d)). Huang et al. reported the potential of biosurfactants to enhance hydrolysate activity, the generation of volatile fatty acids, and suppress the methanation process [25]. In contrast, the current study investigated that low and moderate concentrations of lipopeptide biosurfactants not only improve the H_2 evolution but also promote the CH_4 generation as compared to unaided lipopeptide bioreactors. Zhou et al. also reported that methane generation was improved with a low concentration of biosurfactants (0.05 g g^{-1} TSS), while it was inhibited with >0.10 g g^{-1} TSS dosage [26]. As previously reported, the use of biosurfactants as a pretreatment method has shown a beneficial impact on the hydrolysis and acidogenesis stages in the process of waste-activated sludge (WAS) digestion. Volatile fatty acids (VFAs) are the resultant products of acidogenesis, serving as the substrates for methanogenesis. These VFAs may be readily digested to produce methane [26].

3.3. Effect of Lipopeptide on Short-Chain Fatty Acids. After the completion of dark fermentation experiments, the samples from all the bioreactors were analyzed for short-chain fatty acid determination. Soluble metabolites are critical indicators to determine the fermentation type and evaluate the process performance [27]. Figure 4 illustrates that acetic acid, propionic acid, and isobutyric acid were found to be major acids, while butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid, and caproic acid were observed in minor concentration. The results revealed that the maximum production of acetic acid (3158.899 ng mL^{-1}) followed by propionic acid (2819.022 ng mL^{-1}) and isobutyric acid (1562.608 ng mL^{-1}) was observed in BioR_01 reactor. However, in this study, the lipopeptide-assisted bioreactors including BioR_02 exhibited maximum production of propionic acid (6304.874 ng mL^{-1}) followed by isobutyric acid (1550.112) and acetic acid (1453.606 ng mL^{-1}) as shown in tables S2. A similar trend was also noticed in BioR_03 in the case of propionic and acetic acids, i.e., 5008.711 ng mL^{-1} and 3032.932, respectively, whereas isobutyric acid was found to be decreased even lower than 4-methylvaleric acid (225.045 ng mL^{-1}) and valeric acid (120.384 ng mL^{-1}). In addition, acetic acid, propionic acid, and isobutyric acid were observed to be 3881.34, 3708.71, and 1530.10, respectively, in the BioR_04 reactor.

Previous studies have shown that acetate-type fermentation remains the dominant metabolic pathway even after various pretreatment methods [27]. The metabolic pathway involving acetic acid can be represented by the following equation (Eq. (2)). Bacteria typically convert glucose into pyruvate, resulting in the production of NADH. Specifically, the acetate pathway allows for the metabolism of 1 mol of

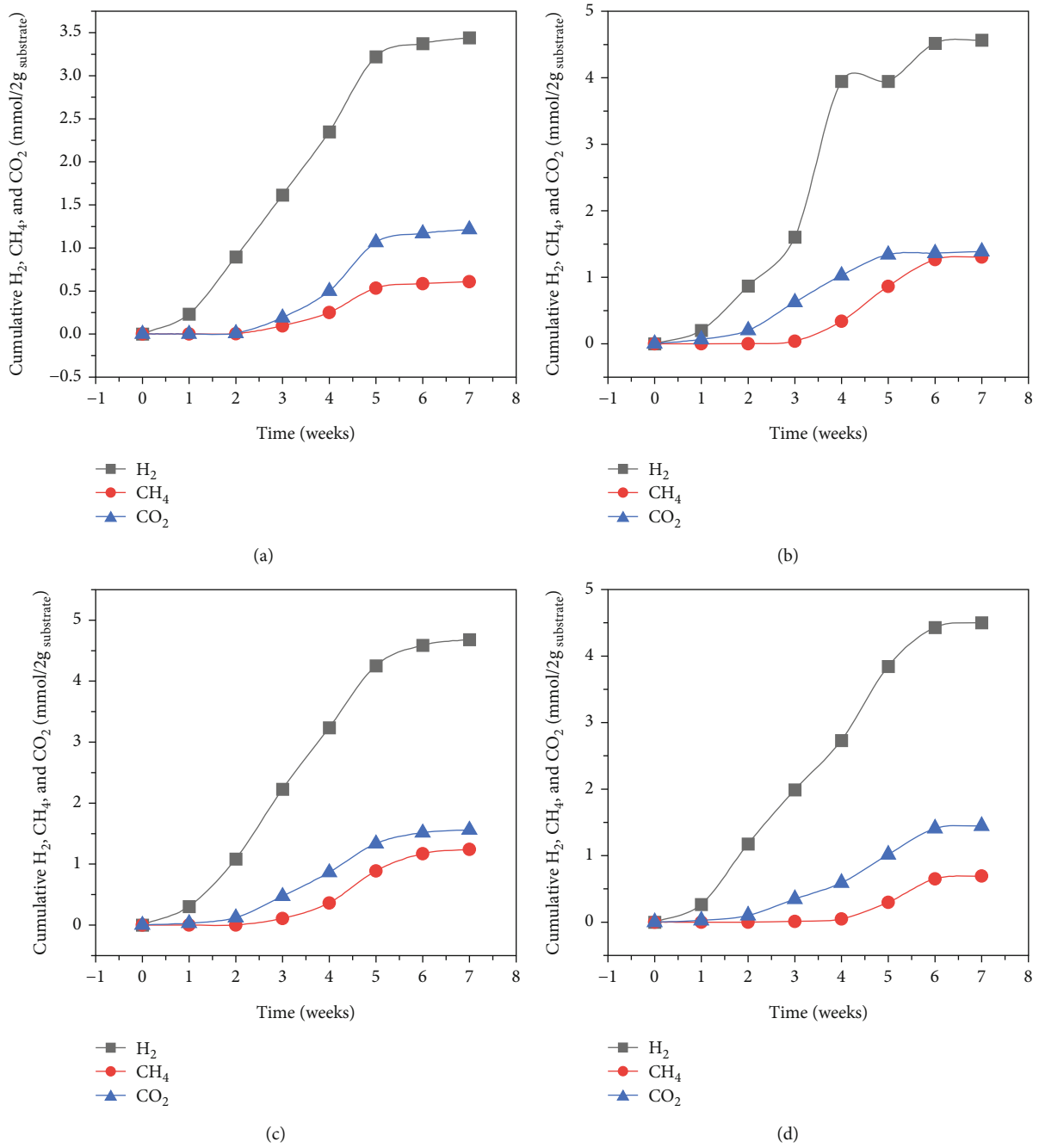


Figure 3: Effect of various concentrations of lipopeptide biosurfactants on H_2, CH_4, and CO_2 evolution (in mmol) from lignocellulose biomass in different bioreactors, i.e., (a) BioR_01, (b) BioR_02, (c) BioR_03, and (d) BioR_04.

glucose to yield 4 mol of NADH [27–29]. Hence, the hydrogen yield per mole of glucose reached 4 mol when the metabolic pathway occurred through acetic acid (Eq. (2)) [27]. However, propionic acid was reported to consume biohydrogen (Eq. (3)) [6].

$$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 = 2CH_3CH_2COOH + 2H_2O \quad (3)$$

In this present study, the propionic acid was significantly enhanced in lipopeptide-assisted bioreactors, whereas the maximum level of H_2 production was also found in these bioreactors compared to BioR_01. It may be attributed due to the H_2 being started to consume by H_2-consuming microbes at the end of fermentation process. Huang et al. examined the impact of biosurfactants, specifically saponin, surfactin, and rhamnolipid, on the production of volatile fatty acids [25]. The researchers found that biosurfactants improved hydrolase activities, increased the production of volatile fatty acids, and suppressed methanation. Saponin exhibited superior performance compared to other biosurfactants

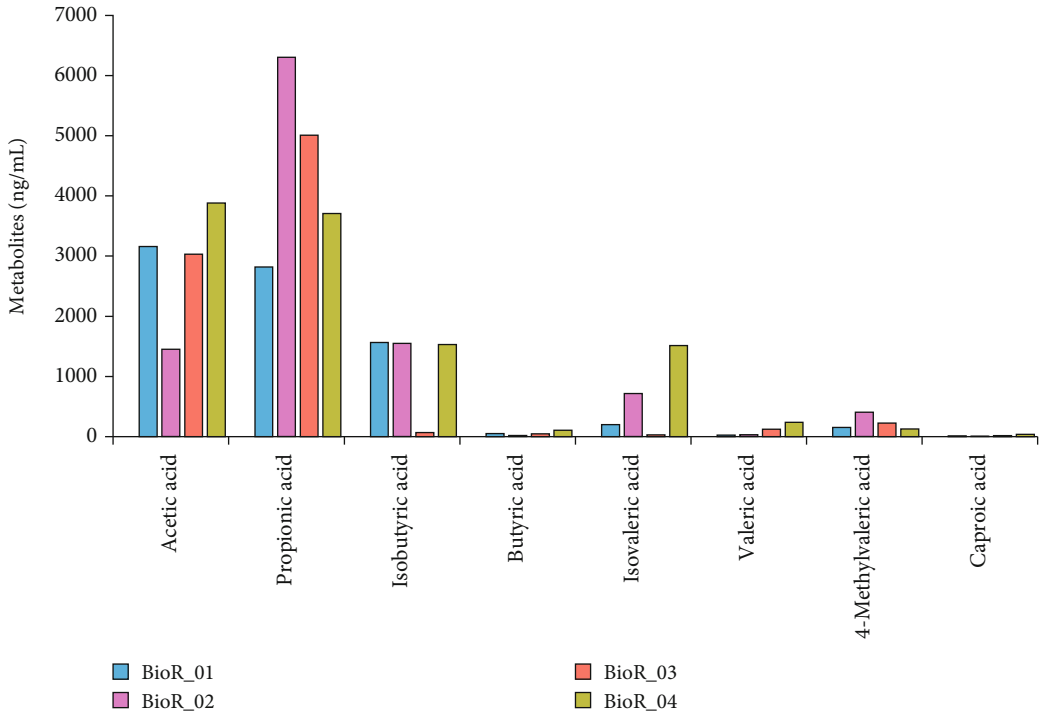


Figure 4: Effect of various concentrations of lipopeptide biosurfactants on short-chain fatty acid generation from lignocellulose biomass at the end of anaerobic-dark fermentation in different bioreactors.

in the conversion of organic material into volatile fatty acids, resulting in the highest yield of 425.2 mg COD/gVSS. Similarly, Zhou et al. observed that the introduction of biosurfactant (specifically rhamnolipid) resulted in an enhancement of volatile fatty acid production. The researchers found that the highest concentration of volatile fatty acids reached 3840 mg COD/L when the biosurfactant concentration was 0.04 g/gTSS. This concentration was 4.24 times more than that of the control group [30]. In another study, it was shown that there was a significant increase in volatile fatty acid synthesis, reaching a maximum fold increase of 4.0, when the saponin concentration was at 0.25 g/gTSS [26]. The improved synthesis of volatile fatty acids resulting from the inclusion of biosurfactants may be attributed to an enhancement in the solubilization of nonaqueous phase substrates in the aqueous phase. This is achieved by reducing the interfacial or surface tension between the two phases.

3.4. Effect of Lipopeptide on Microbial Community and Diversity

3.4.1. Bacterial Community and Diversity Index. The HiSeq 2500 sequencing results demonstrated that a total of 314556 microbial sequences (79585098 bases) were retrieved from all five samples (including day_0, 57072; BioR_01, 64373; BioR_02, 64747; BioR_03, 65485; and BioR_04, 62879). Besides, a total of 1427 OTUs were retrieved (i.e., day_0, 405; BioR_01, 311; BioR_02, 249; BioR_03, 235; and BioR_04, 227). Here, day_0 indicates the inoculum sample which was pregrown for biohydrogen production bioreactors. Table 1 presents the alpha diversity indexing based on the ACE estimator, Chao1 estimator, and Simpson and Shannon measurements. It can be seen that the species richness (ACE and Chao1) estimators in lipopeptide-assisted bioreactors were significantly lower than day_0 and BioR_01 (Table 1). Similarly, Shannon measurements show substantial change among the day_0 and BioR_01 and the BioR_02, BioR_03, and BioR_04 reactors. Conversely, Shannon diversity measurements exhibited higher values in BioR_02, BioR_03, and BioR_04 as compared to day_0 and BioR_01 (Table 1). Moreover, Figures 5(a) and 5(b) illustrate that the microbial communities of all bioreactors were clustered by nonmetric multidimensional scaling (NMDS) and partial least square discriminant analysis (PLS-DA) plots of unweighted UniFrac distance matrices based on OTU level. The microbial communities in BioR_01 and BioR_03 were separated from BioR_02 and BioR_04; however, the communities of day_0 were diverse from all the bioreactors (Figure 5(a)). Similarly, the PLS-DA score plot also illustrates a clear separation degree of the two groups of samples along with the component 1 (75.23%) and component 2 (10.61%) (Figure 5(b)).

3.4.2. Bacterial Community Composition. Various bacterial phyla including *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetota*, *Acidobacteriota*, *Myxococcota*, *Unclassifies_k_norank_d_Bacteria*, *Bacteroidota*, and *Patescibacteria* were detected in all the bioreactors (Figure 5(c)). Previous studies reported that similar type phyla played an indispensable role anaerobic hydrolysis and acidification [25, 31, 32]. Among them, pregrown inoculum (day_0) revealed the *Firmicutes* (95.02%) bacterial phylum followed by *Chloroflexi* (1.50%), *Proteobacteria* (1.04%), *Planctomycetota*

Table 1: The alpha diversity indexing based on ACE estimator, Chao1 estimator, and Simpson and Shannon measurements calculated in all test bioreactors.

Sample\estimators	Ace	Chao1	Shannon	Simpson	Coverage
day_0	414.27	407.55	1.25	0.60	0.99
BioR_01	331.37	326.50	2.82	0.21	0.99
BioR_02	270.02	269.00	3.43	0.06	0.99
BioR_03	259.95	250.52	3.09	0.10	0.99
BioR_04	236.74	233.03	3.46	0.06	0.99

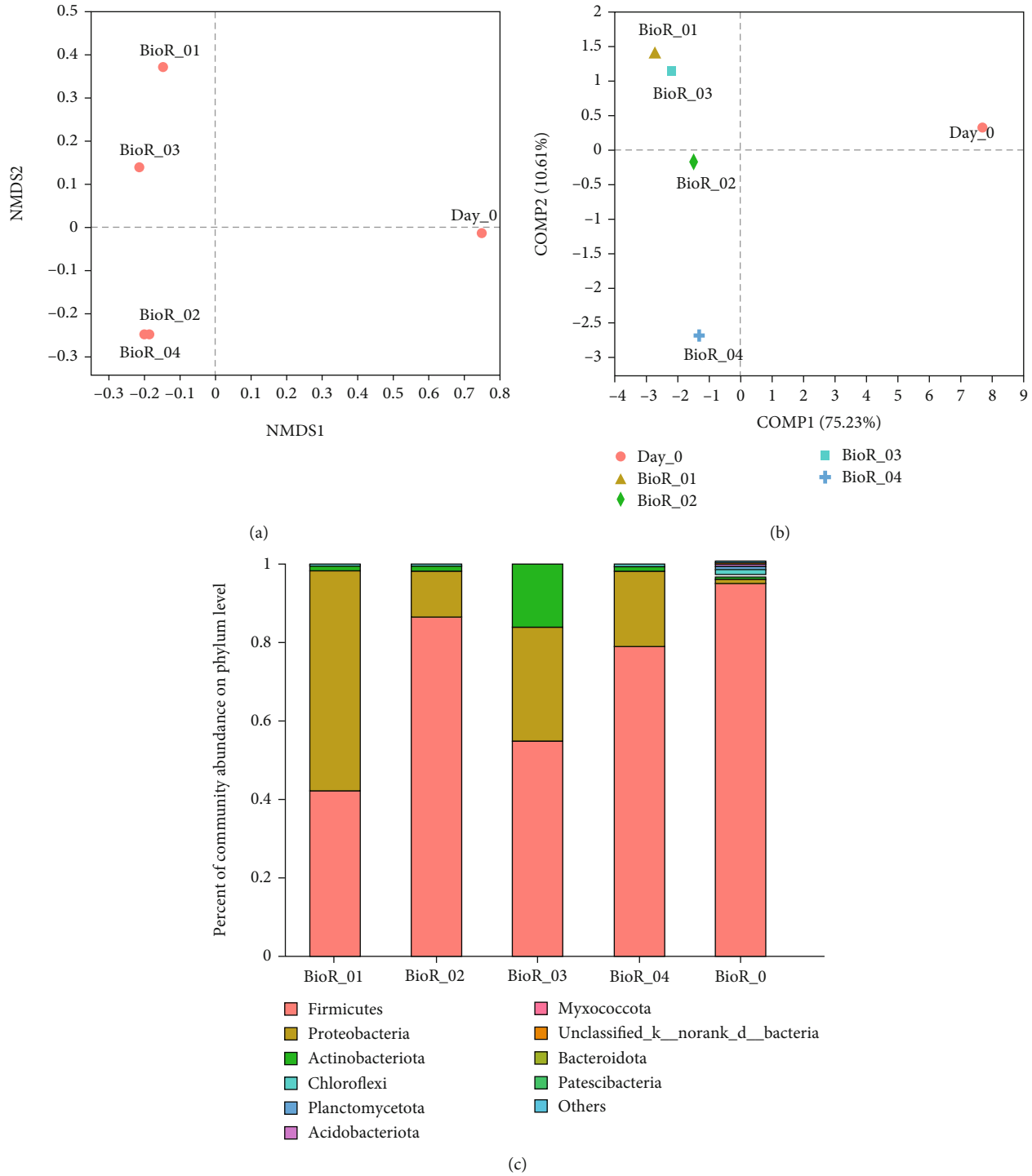
(0.61%), *Acidobacteriota* (0.54%), *Actinobacteriota* (0.50%), *Bacteroidota* (0.32%), *Unclassifies_k_norank_d_Bacteria* (0.14%), *Patescibacteria* (0.06%), *Verrucomicrobiota* (0.05%), and others (0.22%). The use of thermal pretreatment resulted in the initial dominance of *Firmicutes* during the fermentation process. In conditions of elevated temperature, the cells of *Firmicutes* undergo desiccation and subsequently develop into spores. Conversely, bacteria that lack the ability to generate spores, mostly methanogens, are susceptible to elimination from the mixed microbiota [33].

However, BioR_01 unraveled that the bacterial phyla including *Proteobacteria* (56.26%) and *Firmicutes* (42.4%) were dominant, whereas *Actinobacteria* (1.55%), *Planctomycetota* (0.06%), *Unclassifies_k_norank_d_Bacteria* (0.03%), *Chloroflexi* (0.02%), *Myxococcota* (0.02%), *Cyanobacteria* (0.01%), and *Bacteroidota* (0.01%) were found to be minor phyla. On the other hand, lipopeptide-assisted bioreactors such as BioR_02 represented the *Firmicutes* (86.38%), *Proteobacteria* (11.62%), *Actinobacteria* (1.55%), *Chloroflexi* (0.08%), *Planctomycetota* (0.11%), *Unclassifies_k_norank_d_Bacteria* (0.22%), *Bacteroidota* (0.01%), *Patescibacteria* (0.01%), and others (0.02%). The bacterial phyla such as *Firmicutes* (54.74%), *Proteobacteria* (29.11%), *Actinobacteria* (16.10%), *Chloroflexi* (0.02%), *Planctomycetota* (0.02%), and *Bacteroidota* (0.01%) were recovered in BioR_03. The BioR_04 also revealed the maximum abundance of phylum *Firmicutes* (78.83%) followed by *Proteobacteria* (19.26%), *Actinobacteria* (1.20%), *Myxococcota* (0.41%), *Planctomycetota* (0.10%), *Unclassifies_k_norank_d_Bacteria* (0.07%), *Chloroflexi* (0.05%), *Armatimonadota* (0.03%), *Verrucomicrobiota* (0.02%), *Bacteroidota* (0.02%), and others (0.01%) (Figure 5(c)). It can be noticed that the *Firmicutes* was predominant phylum in lipopeptide-assisted fermented bioreactors, suggesting that lipopeptide biosurfactant significantly impacted the microbial community structure as compared to BioR_01 during biohydrogenation processes.

Lipopeptide biosurfactants were widely synthesized by *Bacillus* species belonging to the phylum *Firmicutes* [34]. This study confirms that the lipopeptide biosurfactants support the growth of *Firmicutes*. Moreover, the higher abundance of *Firmicutes* phyla in BioR_02, BioR_03, and BioR_04 resulting in higher biohydrogen production. On the other hand, BioR_01 revealed 56.26% dominance of *Proteobacteria* preceding to *Firmicutes* (42.4%); nevertheless, the H_2 production was observed lowest in this bioreactor. *Firmicutes* phylum has been reported to produce H_2 through the formate hydrogen lyase (FHL) pathway, providing high yields of around 2 molH_2/mol hexose [35, 36].

Figure 5(d) illustrates the genus-level bacterial communities in all test bioreactors. Among them, day_0 unraveled the most dominant bacterial genus *Clostridium_sensu_stricto_1* (79.27%) followed by *Romboutsia* (6.52%), *Bacillus* (4.87%), *unclassified_f_clostrodoaceae* (2.08%), *Clostridium_sensu_stricto_13* (0.99%), *Paraclostridium* (0.62%), *norank_f_norank_o_C10-SB1A* (0.33%), *norank_f_norank_o_norank_c_OLB14* (0.32%), *Terrisporobacter* (0.18%), *norank_f_norank_o_Microtrichales* (0.18%), and others (4.65%). Besides, BioR_01 represented the dominant genera belonging to *Ottowia* (50.44%), *Ruminiclostridium* (10.72%), *Clostridium_sensu_stricto_8* (3.84%), *Pseudomonas* (2.82%), *Bacillus* (2.16%), *Anaerocolumna* (2.12%), *Fonticella* (1.83%), *Clostridium_sensu_stricto_1* (1.76%), *Sedimentibacter* (1.46%), *Christensenellaceae_R-7_group* (1.38%), and others (21.48%). On the other hand, the bacterial genera such as *Ruminiclostridium* (14.13%), *Bacillus* (10.52%), *Brevibacillus* (6.46%), *Aneurinibacillus* (5.76%), *Rummeliibacillus* (4.75%), *Ruminococcus* (4.29%), *Fonticella* (4.18%), *Lachnoclostridium* (3.87%), *Christensenellaceae_R-7_group* (3.49%), *Paenibacillus* (3.23%), and others (39.30%) were retrieved in BioR_02 reactor. BioR_03 exhibited the dominance bacterial genera belonging to the *Ottowia* (26.31%), *Rhodococcus* (15.78%), *Ruminiclostridium* (12.17%), *Bacillus* (5.37%), *Anaerocolumna* (4.99%), *Sedimentibacter* (3.81%), *Acetanaerobacterium* (3.25%), *Sporomusa* (2.87%), *Brevibacillus* (2.69%), *Aneurinibacillus* (2.65%), and others (20.12%). The bacterial genera including *Ruminiclostridium* (22.96%), *Anaerocolumna* (8.86%), *Bacillus* (7.76%), *Brevibacillus* (7.38%), *Ottowia* (4.66%), *Lachnoclostridium* (4.30%), *Paenibacillus* (3.58%), *Aneurinibacillus* (3.57%), *Azospirillum* (3.35%), *Terrisporobacter* (3.32%), and others (30.25%) were determined in BioR_04.

Figures 6(a)–6(d) illustrate that the highest proportion of genus *Clostridium_sensu_stricto* was observed in day_0 (pregrown inoculum) in comparison to other test bioreactors. The *Clostridium_sensu_stricto was* frequently observed in anaerobic processes and well-known genus for H_2 production [33]. However, this genus was significantly decreased in all the fermented bioreactors. It might be that this genus does not possess the active enzymes to degrade the complex substrate such as lignocellulose. The complex composition of lignocellulose contributes to the variability of chemical reactions, the wide range of degrading enzymes, and the intricate nature of enzymatic hydrolysis processes. Cellulose has the potential to undergo degradation, resulting in the formation of polysaccharides, cellobiose, and amino compounds, which all contribute to the formation of a precursor substance known as humic material [37]. Besides,



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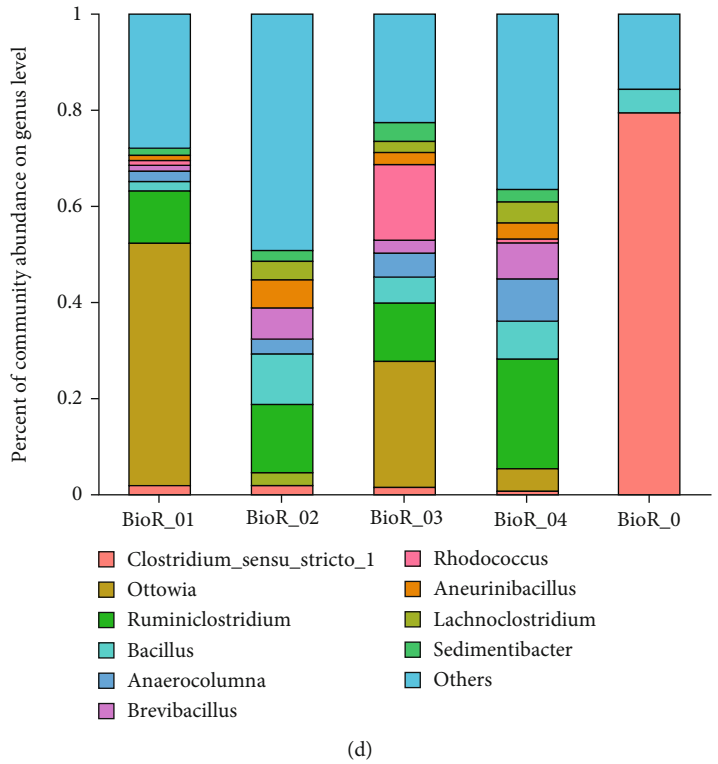


(d)

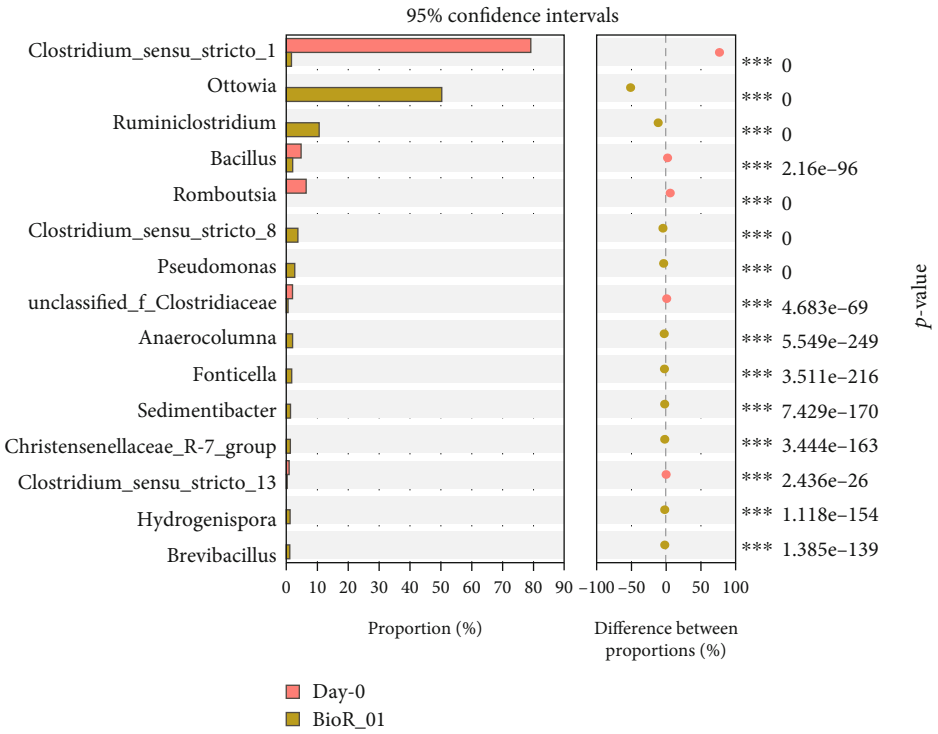
Figure 5: (a) Microbial communities in all bioreactors clustered by nonmetric multidimensional scaling (NMDS) and (b) partial least square discriminant analysis (PLS-DA) plots of unweighted UniFrac distance matrices based on OTU level, which represented that the community of day_0 was diverse from all the fermented bioreactors. (c) Phylum level microbial abundance in all the bioreactors including day_0, BioR_01, BioR_02, BioR_03, and BioR_04. The maximum abundance of phylum *Firmicutes* followed by *Proteobacteria* and *Actinobacteriota* was determined. Besides, microbial communities at the genus level demonstrate that *Clostridium_sensus_stricto_1* was dominant in day_0 (pregrown inoculum). (d) However, the genus *Ottowia* was predominant in BioR_01 (unaided biosurfactant) bioreactor, whereas *Ruminiclostridium* and *Bacillus* genera were frequently dominants in all lipopeptide-assisted bioreactors including BioR_01.

the genera *Ruminiclostridium* and *Bacillus* were frequently observed in all the lipopeptide-assisted bioreactors including BioR_02, BioR_03, and BioR_04. The genus *Ruminiclostridium* is widely recognized for its capacity to produce biohydrogen and can break down cellulose [1, 38].

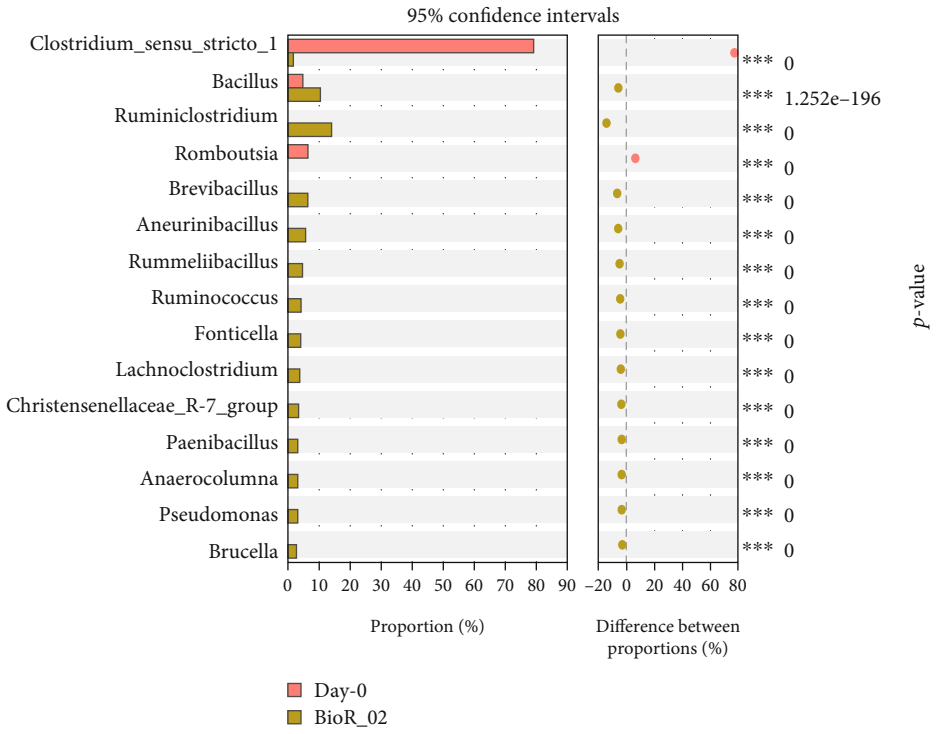
In a previous study, Chen et al. investigated the highest biohydrogen production due to the abundance of *Ruminiclostridium* (54.24%) [38]. The substantial biohydrogen generation may be attributed to the significant presence of cellulose-degrading bacteria, which is mostly due to the challenging destruction process of cellulose [39]. The *Bacillus* genus has been documented as a kind of facultative anaerobic bacterium capable of creating hydrogen during the fermentative process using different waste feedstock materials [36]. In particular, this species thrives in aerobic habitats and can create biohydrogen in both mesophilic and thermophilic environments when anaerobic conditions are present [40]. It grows slowly in anaerobic environments and generates metabolic enzymes like pyruvate formate-lyase and formate dehydrogenase which are crucial for converting pyruvate into hydrogen [41]. Furthermore, it was previously identified that the *Bacillus* genera are promising bacteria capable of degrading lignocellulose [42].

On the other hand, the maximum proportion of genus *Ottowia* was observed in BioR_01 (Figure 6(a)). However, in this bioreactor, the H_2 production was significantly lower than in BioR_02, BioR_03, and BioR_04 (Figures 6(b)–6(d)), indicating that the genus *Ottowia* was hydrogen-consuming genus but it was substantially inhibited in lipopeptide-assisted bioreactors. Nevertheless, the best-known hydrogen-producing bacteria, i.e., *Ruminiclostridium* and *Bacillus*, were also abundant in BioR_01 subsequently to the genus *Ottowia*, while the H_2 production was significantly lower than other bioreactors.

3.4.3. Archaeal Community Composition. The methanogenic archaea are a taxonomically varied group of obligate anaerobic microorganisms that are distinguished by their capacity and reliance on converting simple C1 and C2 chemicals into methane as a means of sustaining their development [43]. The majority of methanogens, which are alternatively referred to as hydrogenotrophic microorganisms, possess the ability to use hydrogen (or formate) in order to enzymatically convert carbon dioxide into methane via a metabolic process often referred to as methanogenesis [1, 44]. The major archaeal phyla such as *Halobacterota*, *Thermoplasmatota*, *Euryarchaeota*, *Crenarchaeota*, and *Euryarchaeota* were detected in all fermented bioreactors; among them, *Halobacterota* and *Euryarchaeota* were predominant in BioR_01, BioR_02, BioR_03, and BioR_04. Table 2 represents the



(a)



(b)

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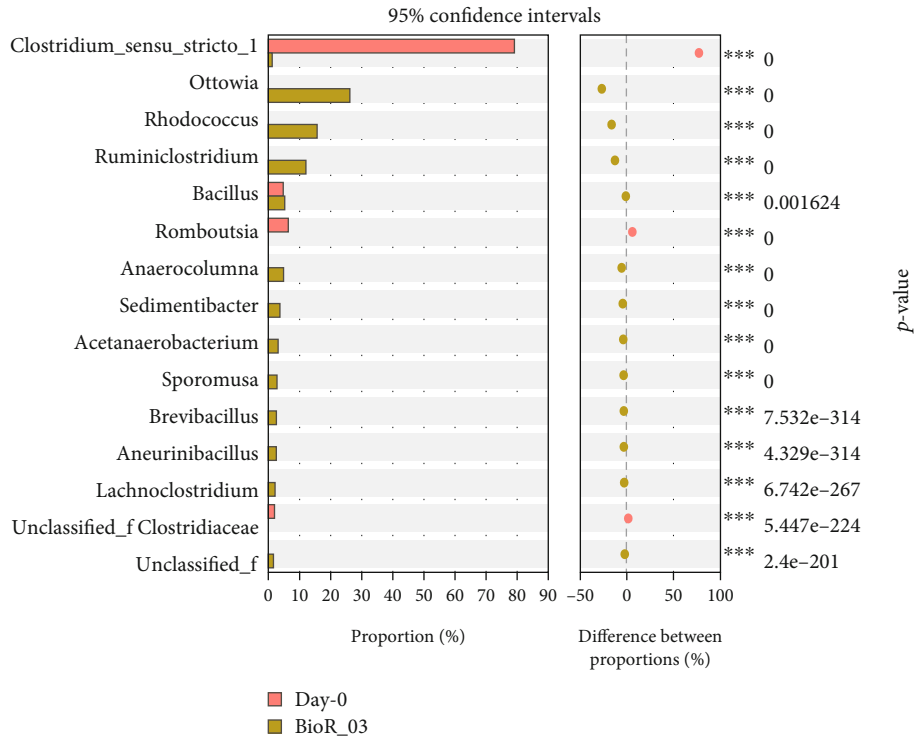
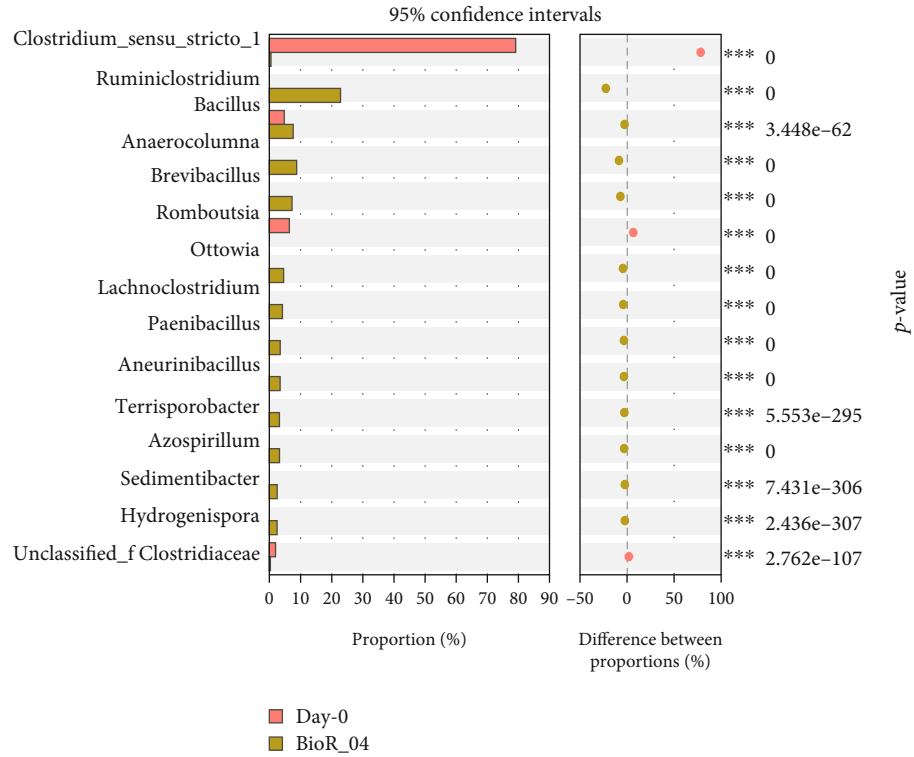


(c)



(d)

Figure 6: The Fisher exact test bar plots show the differences in abundance in the different groups based on genus level. The Y axis of illustrations represents the microbial name, each column corresponding to the species indicates the relative abundance of the species in each sample, and different colors represent different samples. The middle region is within the set confidence interval, the value corresponding to the dot indicates the difference between the relative abundance of species in the two samples, and the dot color is displayed as the sample color with a large proportion of species abundance. The rightmost is the P value; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Table 2: The archaeal community based on genus level and their abundances in various bioreactors during the dark fermentation process.

Genus	BioR_01	BioR_02	BioR_03	BioR_04	day_0
Methanosarcina	1543	5626	2409	1025	2
Methanomassiliicoccus	27	0	2	2	0
Methanosaeta	1	0	0	0	4
Methanobrevibacter	23	3664	0	1	0
Candidatus_Nitrocosmicus	0	1	1	0	0
Methanobacterium	0	0	0	1	2
Methanobacterium	0	0	0	0	4
Methanobacterium	4347	9622	2444	5414	1
norank_f_Methanomassiliicoccaceae	0	0	0	0	4

archaeal community during the dark fermentation process, where BioR_01 reactor revealed the most dominant archaeal genus belonging to *Methanobacterium* (73.00%) followed by *Methanosarcina* (26.00%). However, other genera including *Methanomassiliicoccus*, *Methanobrevibacter*, and *Methanosaeta* were minorly detected. Besides, the archaeal genera such as *Methanobacterium* (51.00%), *Methanosarcina* (30.00%), and *Methanobrevibacter* (19.00%) were also found to be dominant in the BioR_02 reactor. In addition, *Methanobacterium* (50.00%) and *Methanosarcina* (50.00%) were determined in the BioR_03 reactor. The bioreactor BioR_04 exhibited the *Methanobacterium* (84.00%) dominant followed by *Methanosarcina*, *Methanomassiliicoccus*, and *Methanobrevibacter*. However, the very minor archaeal communities of OTUs were also detected in day_0 (pregrown inoculum), for example, *Methanobacterium*, *Methanosaeta*, *norank_f_Methanomassiliicoccaceae*, and *Methanosarcina* (Table 2). Similarly, the archaeal genera including *Methanobacterium*, *Methanosarcina*, and *Methanobrevibacter* were found to be dominant during H_2 production at various stages [38]. *Methanosarcina* consists of irregular clumps of cells that can survive in harsh environments, producing methane mainly by acetic acid and partially by H_2 or CO_2 and ethanol [39]. The optimum range of pH for *Methanosarcina* is reported as 5.0 to 7.0 [45]. The *Methanobacterium* reported for methane generation from H_2/CO_2 in mesophilic conditions, and the optimum pH of *Methanobacterium* is over 6.5 [39, 45]. *Methanobrevibacter genera* were also reported in several studies as hydrogenotrophic methanogens that convert biohydrogen to methane and cannot utilize acetic acid [38, 46, 47].

4. Conclusion

The present study revealed that lipopeptide biosurfactants can play an indispensable role in biohydrogen production from a complex substrate such as lignocellulose biomass through anaerobic dark fermentation. Among the various examined concentrations of lipopeptide biosurfactants, the lowest concentration (25 to 50 $mg{\cdot}L^{-1}$) substantially improved biohydrogen production and significantly impacted the short-chain fatty acid generation. Besides, Illumina HiSeq 2500 sequencing results exhibited that *Firmicutes* was found to be a predominant phylum (54.74 to 86.38%) in lipopeptide-assisted bioreactors, where efficient lignocellulose-degrading and hydrogen-producing genera, i.e., *Ruminiclostridium* and *Bacilli*, were frequently promoted. These findings suggested that it is possible the application of lipopeptide biosurfactants to enhance biohydrogen from lignocellulose waste biomass at the large scale. This can potentially guide the development of a more efficient strategy for sustainable energy from lignocellulose. Lignocellulose-based biohydrogenation is a promising, cheap, renewable, and sustainable energy resource for meeting the global ever-increasing energy need while minimizing environmental issues caused by the combustion of fossil fuel.

Data Availability

Data will be provided upon special request.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

All authors contributed equally to the article.

Acknowledgments

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Supplementary Materials

The supplementary file contains the following: Figure S1: phylogenetic tree analysis of *Bacillus subtilis* CW2 strain. Figure S2: liquid chromatography-mass spectrometry (LCMS) chromatogram of standard surfactin compound and the lipopeptide biosurfactant production by *Bacillus subtilis* CW2. Figure S3: scanning electron microscopy (SEM) images of lignocellulose biomass before and after fermentation in various bioreactors. Figure S4: peaks of each standard short-chain fatty acids based on their retention time (RT) by LCMS. The file also has the following: Table S1: details of standard short-chain fatty acids. Table S2: short-chain fatty acid profile of different bioreactors. *(Supplementary Materials)*

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