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Acidogenic valorization of agricultural residues and industrial waste streams: substrate composition regulating the microbial community and metabolites

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The global waste crisis is a significant concern driven by urbanization and economic expansion. Untreated waste poses major environmental, economic, and societal challenges, especially affecting agriculture and industry. Addressing this crisis necessitates innovative waste management strategies and sustainable practices to mitigate the impending waste burden on ecosystems and societies worldwide. Recent advancements in biofuels and biochemicals intensified research into the conversion of biogenic waste into bio-carboxylic acid/volatile fatty acids (VFAs), driven by the dual imperatives of sustainable waste management and renewable resource development. This study presents a comparative analysis of three waste streams: cheese whey from the cheese-making industry, lignocellulosic brewery spent grains (BSG), and agricultural by-products like wheat straw (WS) assessing their efficacy in carboxylic acid production by mixed culture fermentation. Each substrate produced a diverse array of carboxylic acids, including acetic, propionic, butyric, valeric, iso-valeric, and caproic acids exhibiting unique fermentation efficiencies in carboxylic acid production. The experimental results reveal distinct fermentation efficiencies, the highest concentration of short-chain carboxylic acids (SCCA) production of 11.84 gCOD per L from CW, alongside a medium-chain carboxylic acid (MCCA) production of 3.95 gCOD per L. Notably, despite the lignocellulosic composition of the substrates, both BSG and WS demonstrated substantial and competitive yields of SCCA and MCCA. Specifically, BSG produced 10.68 gCOD per L of SCCA and 3.54 gCOD per L of MCCA, while WS yielded 11.51 gCOD per L of SCCA and 3.84 gCOD per L of MCCA. These findings highlight the viability of lignocellulosic substrates for carboxylic acid production, suggesting significant opportunities for enhancing bioprocessing strategies in biochemical and industrial applications. Taxonomic analysis of microbial communities showed a significant predominance of *Firmicutes*, *Bacteroidota*, and *Actinobacteriota*. The Clostridiaceae family exhibited dominance across all reactors, with respective abundances of 82.72%, 27.67%, and 61.29%. The BSG uniquely showcased an enrichment of *Lactobacillaceae* (23.86%), *Ruminococcaceae* (7.72%), and *Prevotellaceae* (3.24%). Key genera contributing to carboxylic acid production included *Clostridium sensu stricto 1*, *Romboutsia*, and *Enterococcus*. This diversity highlights the influence of substrate composition on microbial community structure, highlighting the intricate relationships between substrate nature and microbial metabolites suggesting that strategic substrate selection could optimize fermentation efficiency and enhance product yield.

Environmental significance

The escalating global energy demand, driven by population growth, urbanization, and industrialization, poses significant environmental challenges, including greenhouse gas emissions and climate change. To mitigate these issues, a transition to sustainable and renewable energy sources is essential. Agricultural residues and industrial waste, when properly managed, can serve as valuable feedstocks for biofuels and biochemicals. However, mismanagement can lead to severe environmental degradation, including air and water pollution. This study explores microbial valorization of agricultural waste, specifically through acidogenic fermentation of wheat straw, brewery spent grains, and cheese whey, to produce bio-carboxylic acids (platform chemicals) and acidogenic gases. The findings highlight the potential of this approach to generate renewable energy, reduce waste, and minimize environmental impacts, thereby promoting a sustainable energy future.

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1. Introduction

The escalating global population, projected to reach unprecedented levels, presents a formidable challenge in food security and waste management. Rapid urbanization and economic growth are exacerbating waste generation, with estimates indicating that global waste could soar to 2.6 billion tonnes by 2030 and 3.4 billion tonnes by 2050, potentially tripling by 2100.[1] Agricultural waste, primarily composed of lignocellulosic materials such as cellulose and lignin, is increasing at an alarming rate of 5–10% annually. The improper disposal and burning of these materials contribute significantly to environmental degradation, including air, soil, and water pollution.[2] This waste crisis not only threatens ecological balance but also poses severe economic and societal challenges, particularly in agriculture and industry. With a staggering 10% to 50% of waste discarded annually, there is an urgent need for innovative waste management strategies and sustainable practices to foster a circular economy.[3] By utilizing the valuable constituents found in agricultural and industrial by-products, including cellulose, proteins, and lipids, it is possible to reduce environmental impacts while simultaneously unlocking economic opportunities. Therefore, it is essential to address waste management through innovative scientific approaches, as this is crucial for achieving sustainable development in the context of increasing global challenges. Agro-industrial biomass is increasingly recognized as a valuable resource for producing essential industrial enzymes, leveraging its abundant carbon and nitrogen content.[4] The utilization of these renewable residues not only addresses the challenge of waste generated by agricultural industrialization but also promotes sustainable practices. With annual biomass production reaching approximately 5 million metric tonnes, the research trend is towards exploring innovative methods to transform these agricultural by-products into biobased products.[5] This approach not only mitigates environmental pollution but also aligns with the growing global demand for low carbon fuels and biobased chemicals, driven by population growth and industrial needs.[6] Wheat straw, accumulating at approximately 144 million tonnes annually in the EU, presents a significant opportunity for sustainable biochemistry.[7] Researchers aim to convert this agricultural residue, typically left to decompose or used for livestock bedding, into valuable biochemicals through fermentation processes at biorefineries. This innovative approach not only utilizes non-edible biomass but also promotes carbon neutrality by producing biofuels that absorb atmospheric carbon during plant growth, offering a cleaner alternative to fossil fuels like coal, oil, and gas. Utilizing 48 million tonnes of EU wheat straw could yield 21 million tonnes of sugar, supporting 100 biorefineries and replacing 35 million barrels of fossil fuel annually.[7]

Brewery-spent grain (BSG) is the predominant organic by-product of beer production, constituting approximately 85% of brewing waste. For every 100 liters of beer produced, about 20 kg of BSG is generated. In 2017, the EU produced nearly 39.5 million liters of beer, yielding significant economic returns projected to rise to €159.7 billion by 2025.[8] Annually, over 6.4 million tonnes of BSG are produced, yet the traditional use as animal feed is hindered by insufficient local farms, particularly in industrialized regions. This has prompted the need for alternative disposal methods, as landfilling BSG is both unsustainable and costly, releasing approximately 513 kg of CO_2 equivalent per tonne.[8] Currently, around 70% of BSG is utilized as animal feed, 10% for biogas production, and 20% is landfilled.[9,10] The high-water content (80%) of wet BSG complicates transportation, necessitating innovative solutions to manage this substantial waste stream effectively. Cheese whey (CW), a by-product of cheese production, is a nutrient-rich liquid containing lactose, fats, and proteins. The European Union, a major player in the global dairy sector, produced around 160 million tons of milk in 2022, predominantly from cows. Historically, CW disposal was poorly regulated, often leading to environmental degradation due to its high biochemical and chemical oxygen demand. In response, the EU implemented Decision 97/80/EC, classifying CW as a by-product eligible for reuse or disposal. This regulatory shift has opened avenues for utilizing deproteinized whey as a feedstock for biofuels like bioethanol and biogas, as well as for producing value-added products such as polyhydroxyalkanoates, thereby promoting sustainable practices in the dairy industry. Cost-effective technologies are being implemented to cultivate microorganisms that convert lignocellulosic residues into value-added products. Utilizing nutrient-rich agro-industrial biomass presents a sustainable solution for managing the millions of tonnes of agricultural waste generated annually. This approach not only fosters the growth of beneficial microorganisms but also aligns with the principles of the circular economy, promoting waste valorization and reducing environmental impacts. By integrating lignocellulosic waste into bioproduct and bioenergy production, countries can achieve efficient economic growth while advancing environmental sustainability. Mixed culture acidogenic fermentation (AF) is a versatile anaerobic biochemical process that efficiently converts organic waste into valuable bio-based chemicals, specifically short chain carboxylic acids (SCCA)/volatile fatty acids (VFAs) and hydrogen (H_2). VFAs, which are low molecular weight organic acids, with carbon chain lengths ranging from two to five (C2 to C5) such as acetic acid (C2), propionic acid (C3), butyric acid (C4), and valeric acid (C5) (Fig. 1).[11,12]

The production of SCCA occurs as intermediates during the acidogenesis and acetogenesis phases of anaerobic digestion (AD). In mixed culture fermentation, organic substrates (lipids, proteins, and carbohydrates) undergo decomposition into simpler molecules, resulting in an effluent rich in short chain carboxylic acids. The complexities of AF are influenced by various competing microorganisms, biokinetics, catalytic activities, and intermediate syntrophic interactions.[13,14] To enhance VFA production, it is crucial to accelerate both hydrolysis and acidogenesis while simultaneously inhibiting methanogenesis, which can otherwise lead to the consumption of VFAs by methanogenic organisms. One of the significant advantages of mixed culture fermentation is its ability to operate under non-sterile conditions, making it a practical approach for waste treatment.

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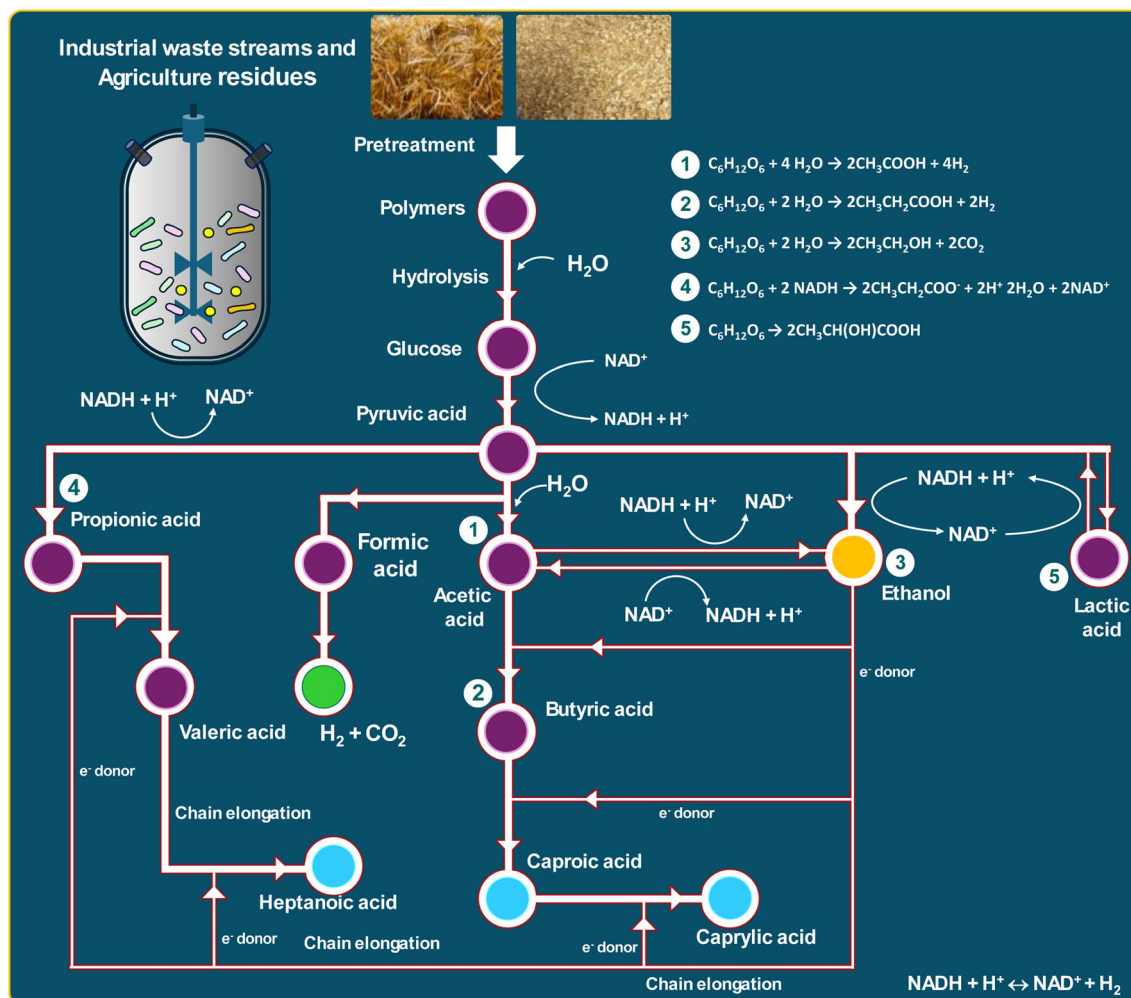
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Fig. 1 The production pathways for acidogenic metabolites include short-chain carboxylic acids (SCCAs) such as acetic acid (C2), propionic acid (C3), butyric acid (C4), and valeric acid (C5), as well as medium-chain carboxylic acids (MCCAs) like hexanoic acid (C6), heptanoic acid (C7), and octanoic acid (C8) from industrial waste streams and agricultural residues.

Chain elongation (CE) *via* reverse β-oxidation represents a subclass of VFA production mechanisms where a VFA of a given chain length is elongated through condensation with Acetyl-CoA.

VFAs are vital precursors for the synthesis of biopolymers, such as polyhydroxyalkanoates (PHAs), and other high-value products, including biofuels, alcohols, aldehydes, and ketones. Each carboxylic acid has distinct applications across various industries. For instance, in the food sector, bio-carboxylic acids are utilized as organic molecules, solvents, flavoring agents, and food additives. In the textile industry, they serve as raw materials for detergents, while in the pharmaceutical and personal care sectors, they are integral to product formulation. MCCA are particularly useful in flavors, fragrances, animal nutrition, biofuels, synthetic lubricants, and chemical synthesis. The current market prices for short-chain carboxylic acids (SCCA) and medium-chain carboxylic acids (MCCA) range from approximately USD 400 to USD 2000 per tonne. For example, acetic acid is priced between USD 400 and USD 800 per tonne, while propionic acid can reach USD 1500 to USD 2000 per tonne. Butyric acid is valued at around USD 2000 to USD 2500 per tonne, and valeric acid ranges from USD 2000

to USD 2600 per tonne.[15] MCCA costs approximately USD 2000 to USD 3000 per tonne.[16–18] Recent advancements highlight the potential for scaling up the utilization of diverse waste streams such as food processing byproducts, dairy manure, and crop residues as feedstocks for producing bio-carboxylic acids and low-carbon hydrogen, thereby enhancing the sustainability and economic viability of the process.

In this study, we conducted a comprehensive comparative evaluation of three distinct types of industrial and agricultural residuals, specifically, brewery spent grains, cheese whey and wheat straw as renewable substrates for the production of bio-carboxylic acids and low-carbon hydrogen (biohydrogen). The study highlights how the composition of these substrates significantly influenced both the fermentation product portfolio and the microbial diversity during mixed culture acidogenic fermentation. By analyzing the metabolic pathways involved, have elucidate how variations in substrate characteristics can lead to shifts in the predominance of specific microbial communities, thus affecting the overall efficiency and yield of target fermentation products. This study emphasizes the potential of tailored substrate selection to optimize acidogenic

fermentation processes, paving the way for more effective strategies in sustainable waste management and biobased products synthesis.

2. Material and methods

2.1. Substrate and inoculum (digested sludge)

This study evaluated the acidogenic fermentation (AF) dynamics of three distinct substrates: lignocellulosic wheat straw (WS), brewery spent grains (BSG), and non-lignocellulosic cheese whey (CW). The BSG and CW were sourced from Skellefte Bryggeri and Norrmejerier in Sweden, respectively, and were preserved at −20 °C to ensure quality prior to use. The WS was obtained from Denmark. Both WS and BSG underwent an air-drying process followed by milling with a Retsch SM 300 knife mill, utilizing a 1-mm screen, and were subsequently stored at ambient temperature. The biocatalyst employed was a mixed culture (digested sludge) from a biogas facility in Luleå, Sweden, which was filtered to eliminate solid particulates and allowed to settle overnight. To enhance the accumulation of acidogenic metabolite and inhibit its methanogenic consumption during fermentation, a heat-shock pretreatment was applied. The sludge was heated at 80–90 °C for one hour to suppress methanogenic activity by reducing methanogens presence. The concentrated sludge, with a volatile solids (VS) content of 0.13 g g^{-1}, was utilized after excess supernatant removal. Analysis of the homogenized BSG indicated a total solids content of 95.1 ± 0.02% w/w, with volatile solids at 92.1 ± 0.02% w/w. The composition revealed cellulose, hemicellulose, and lignin contents of 28.17%, 15.17%, and 12.28% w/w, respectively.

2.2. Organosolv pretreatment and enzymatic hydrolysis

An air-heated multi-digestor system (Haato, Vantaa, Finland) was used for the organosolv fractionation of WS, as previously described.[19] Specifically, WS was treated in a solvent mixture of water, acetone, and ethanol (40 : 30 : 30, % v/v), containing 1% w/w$_{dry\ biomass}$ H$_2$SO$_4$ as an acid catalyst. The liquid-to-solid ratio was set to 10 : 1 (v/w), and the reaction was carried out at 180 °C for 1 h. After treatment, the slurry was vacuum filtered to separate the pretreated solids from the process liquid. The solid fraction was washed with ethanol, air-dried, and stored at room temperature for subsequent use as a substrate for acidogenic fermentation. The liquid phase was further processed to recover lignin and hemicellulose: however, these fractions were not included in this study. Concurrently, BSG underwent acid pretreatment with 0.2% H$_2$SO$_4$, chosen based on optimized conditions (35 ± 2 °C; 3 h). Both the organosolv-pretreated WS and acid-treated BSG were subjected to enzymatic saccharification using a cellulase enzyme blend (Cellic® CTec2, Sigma-Aldrich). Prior to enzymatic saccharification, the pH of the organosolv-pretreated WS and acid-treated BSG were adjusted to 5.5 using 1 M NaOH/H$_2$SO$_4$ (Table 1). The enzymatic hydrolysis was performed at 50 ± 1 °C for 30 hours effectively converting the polysaccharides into fermentable sugars, with enzyme loadings of 6 FPU per g for WS and 5 FPU per g for BSG. Post-hydrolysis, the slurry was evaluated as a substrate for acidogenic fermentation.

2.3. Experimental setup

The study focused on evaluating the acidogenic fermentation (AF) of three distinct substrates: WS, BSG, and CW. WS and BSG were subjected for enzyme hydrolysis prior to use it as substrate. The AF tests was conducted using the Automatic Methane Potential Test System (AMPTS-II) developed by Bioprocess Control AB in Lund, Sweden. Filtered municipal sludge was used as the inoculum. The experimental setup, illustrated in Fig. 2. Each individual reactor, with a total volume of 1 liter, was designed specifically for the AMPTS system and consisted of a glass bottle connected to a top micro-motor for efficient mixing. Substrates and inoculum were loaded into the reactors in specific concentrations, as detailed in Table 1. The reactors operated in batch mode at a temperature of 35 °C for a duration of 8 days with an initial pH of 6.5. Prior to initiating the fermentation process, each reactor was sparged with nitrogen gas for fifteen minutes to ensure anaerobic conditions. Gas production during the fermentation was monitored by connecting the reactor headspaces to a gas-flow meter. Data on volumetric gas production was recorded using the accompanying software, and all experiments were conducted in triplicate to ensure reliability and reproducibility of results.

2.4. Analytical procedures: liquid and gas sample analysis

The chemical composition of the biomass was analyzed by measuring its carbon, hydrogen, and nitrogen contents using a standardized procedure with the Euro EA 3000 Elemental Analyzer. This analysis involved flash combustion at 980 °C in tin vials, utilizing sample weights ranging from 1 to 2 mg (EuroVector, Pavia, Italy). In addition to elemental analysis, the

Table 1 Study assessing the composition of agricultural residues (WS) and industrial waste streams (CW and BSG) influences on acidogenic valorization for the production of bio-carboxylic acids and the generation of biohydrogen

Waste stream	Substrate	Pretreatment	Enzyme hydrolysis	Substrate load (VS gram)	Fermenation time (days)
Industrial waste	Brewery spent grain (BSG)	Mild acidic (0.2% H$_2$SO$_4$)	Cellic CTec2 (cellulase, enzyme blend)	20	8
	Cheese whey (CW)	NA	NA	20	8
Agricultural residue	Wheat straw (WS)	Organosolv	Cellic CTec2 (cellulase, enzyme blend)	20	8

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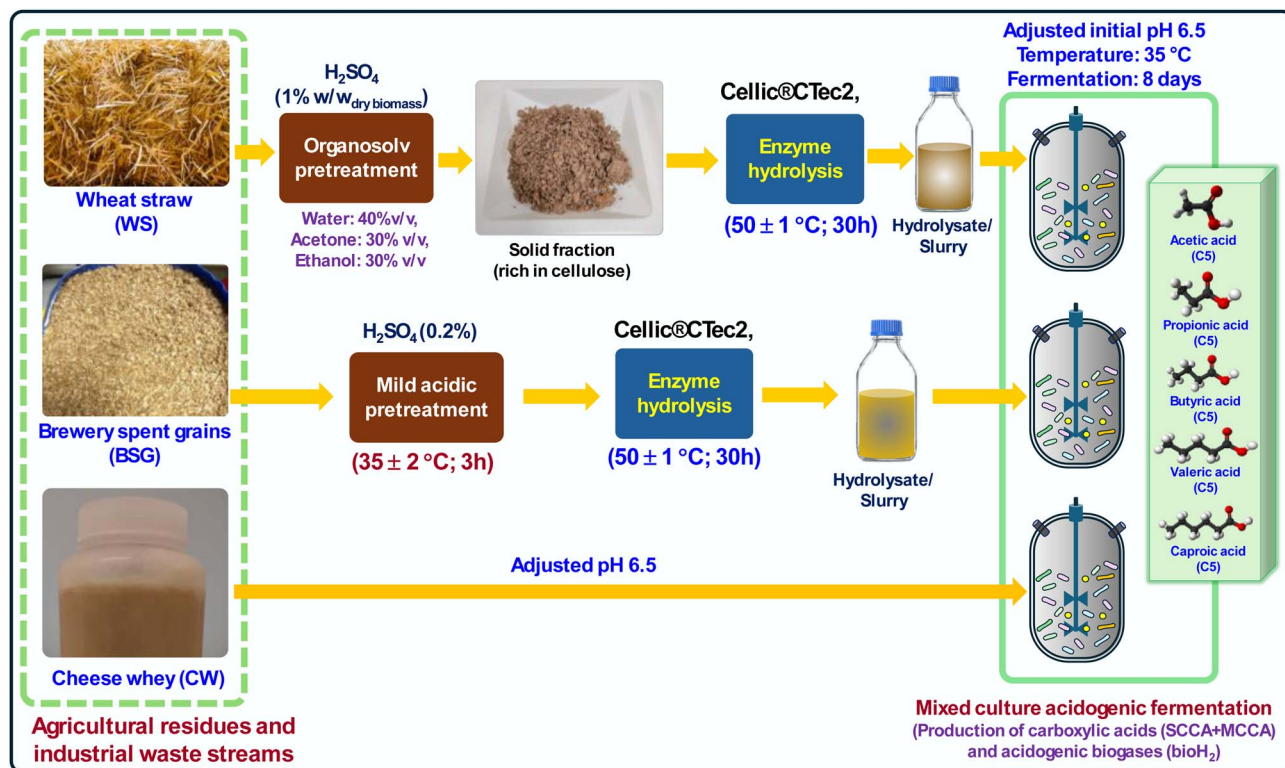*Environ. Sci.: Adv.*, 2026, **5**, 470–484 | **473**

Fig. 2 Acidogenic valorization of brewery spent grains (BSG), cheese whey (CW) and wheat straw (WS) as renewable feedstock for the production of bio-carboxylic acids and biohydrogen.

volatile fatty acids (VFAs) present in the bioreactors, specifically acetic, propionic, butyric, caproic and valeric acids, were quantified through high-performance liquid chromatography (HPLC) with a PerkinElmer system. The HPLC setup included a 410 LC pump and an RID-6A refractive index detector. The separation was achieved using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), measuring 300×7.8 mm, and operated at a consistent temperature of 65 °C. The mobile phase was a 5 mM H_2SO_4 solution, which was eluted at a flow rate of 0.6 mL min^{-1}. For the analysis of acidogenic gas composition, a mass spectrometer (GAM 400; InProcess Instruments, Bremen, Germany) was utilized alongside an Agilent 990 micro-GC equipped with a COX column (COX UM1MX0.8MMID BF, CP-PORABOND Q, 1 MX 0.25 mm $\times$ 3 μm). The operational temperatures were held at 110 °C for the column and 80 °C for the injector, ensuring optimal performance during the analysis. VFAs were quantified using calibration curves derived from commercially available standards (10 mM volatile fatty acid mix; Sigma-Aldrich, St. Louis, MO, USA). Ammonium levels were determined with the Ammonium Test kit (Spectroquant), utilizing photometric analysis on the Spectroquant® Move 100 for accurate measurements. Gravimetric method was employed to quantify total solids (TS) by drying samples at 110 °C for 24 hours, while ash content was determined by combusting samples at 550 °C for 3 hours. Volatile solids (VS) were derived by subtracting ash from TS. Cellulose and hemicellulose in wheat straw were analyzed by established protocols. Furthermore, pH variations during the acidogenic fermentation process were continuously monitored using a pH meter (pHenomenal-pH1100L; VWR, Stockholm, Sweden), ensuring precise tracking of fermentation conditions.

2.5. Microbial diversity: DNA extraction

On day 8, sludge samples were collected from all the reactors to assess microbial diversity. DNA extraction adhered to the FastDNA Spin kit for Soil protocol (MP Biomedicals, USA). Each sample, totaling 500 μL, comprised 480 μL sodium phosphate buffer and 120 μL MT buffer, processed in a Lysing Matrix tube. Bead beating was executed at 6 m s^{-1} for four intervals of 40 seconds to ensure effective cell lysis. The extracted DNA's purity and size validation were conducted using the TapeStation 2200 and genomic DNA screen tapes *via* gel electrophoresis. Subsequently, DNA concentration was quantified utilizing the Qubit dsDNA HS/BR Assay kit (Thermo Fisher Scientific, Waltham, MA, USA), ensuring accurate measurement for downstream applications in microbial analysis.

2.6. Library preparation-DNA sequencing and bioinformatic processing

Amplicon libraries targeting the V4 region of the 16S rRNA gene from bacterial and archaeal communities were developed using an Illumina protocol, employing 10 ng of extracted DNA for PCR amplification.[20] Comprehensive methodologies for DNA sequencing, library preparation, and bioinformatic analyses have been documented in our previous publication, ensuring

reproducibility and accuracy in microbial community characterization.[21]

3. Results and discussion

3.1. Bio-carboxylic acids (SCCA)

In a comparative study of bio-carboxylic acid production from CW, BSG, and WS under a consistent organic loading of 20 gVS, the results indicated distinct efficiencies among the substrates. CW achieved the highest yield of 11.48 gCOD per L, followed closely by WS at 11.05 gCOD per L, while BSG produced 10 gCOD per L. These findings highlight the critical influence of substrate composition on fermentation efficiency and the resultant carboxylic acid yields. The fermentation process exhibited a gradual increase in bio-carboxylic acids (VFAs) over time, with initial high production rates observed by WS and BSG. This rapid accumulation can be attributed to the micro-organisms' ability to efficiently metabolize glucose present in the hydrolysate, contrasting with the more complex substrates in CW, which contain both carbohydrates (lactose and galactose) and proteins. By day three, WS peaked at 7.07 gCOD per L, with CW following at 6.16 gCOD per L, indicating CW's potential for effective VFA generation. BSG lagged with a yield of 4.61 gCOD per L. From day four onward, WS demonstrated a significant increase in carboxylic acid production, likely due to the microbial community's adaptation to the WS substrate. In contrast, BSG and CW experienced slower VFA production, attributed to their complex compositions, which include fibrous materials and proteins respectively that complicate microbial digestion. The reactors reached peak carboxylic acid production on days four and five, after which production stabilized due to acid accumulation affecting pH levels, dropping below 5.5 and creating unfavorable conditions for microbial activity. During the stable phase, pH levels ranged from 6 to 6.5. By day eight, CW recorded the highest concentrations of carboxylic acids, surpassing both WS and BSG, highlighting the delicate balance between acid production and microbial activity in fermentation processes. Overall, this evaluation emphasizes the importance of substrate selection and its composition in optimizing bio-carboxylic acid production, providing insights for future bioprocess engineering applications (Fig. 3).

3.2. Bio-carboxylic selectivity at different substrate composition

The composition of the substrate significantly influenced the distribution and concentration of individual carboxylic acid in the reactors. The total carboxylic acid content comprised five distinct acids, including short-chain acids (acetic, propionic, butyric, and valeric acid) and a medium-chain carboxylic acid (caproic acid). In the reactors, the fastest producing acids were the short-chain carboxylic acids, particularly acetic and butyric acid, followed closely by propionic acid. Conversely, the production of caproic acid, which is a chain-elongated fatty acid, was relatively slower compared to the short-chain fatty acids. Each of the three reactors was operated for a period of 192 hours. Notably, the production of acetic and butyric acid from
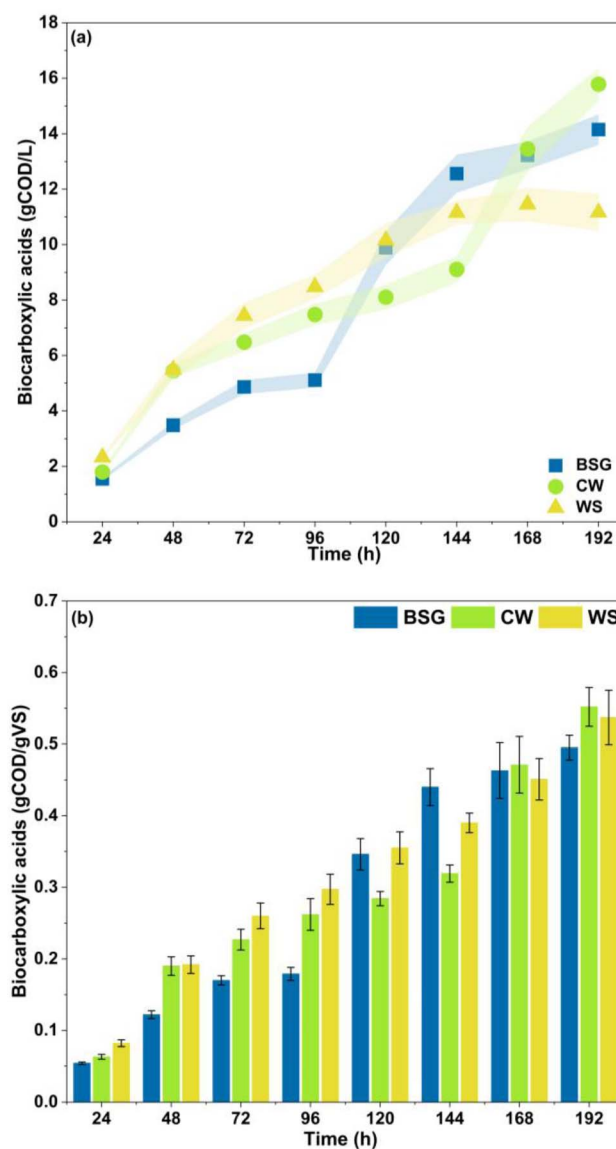

Fig. 3 (a) The cumulative bio-carboxylic (SCCA+MCCA) production from CW and hydrolysate from BSG and WS with respect to fermentation time and (b) bio-carboxylic yield recorded per gram VS load.

the fermentation of WS was significantly higher than that from BSG and CW. Despite the simplicity of the substrates provided by glucose (from enzyme hydrolysis of BSG) and lactose in cheese whey, they were unable to match the fermentation efficiency of WS. The production of acetic acid during the fermentation process was notably rapid, particularly until day 6, leading to an impressive accumulation of 4.68 gCOD per L from the fermentation of WS. In comparison, the fermentation of CW resulted in a slightly lower acetic acid production of 4.71 gCOD per L, while BSG fermentation produced a comparable yield of 4.63 gCOD per L (Fig. 4a–c). As fermentation progressed, significant consumption and utilization of the accumulated VFAs became evident across all reactors. Interestingly, despite the absence of a methanogenic signature, it is anticipated that
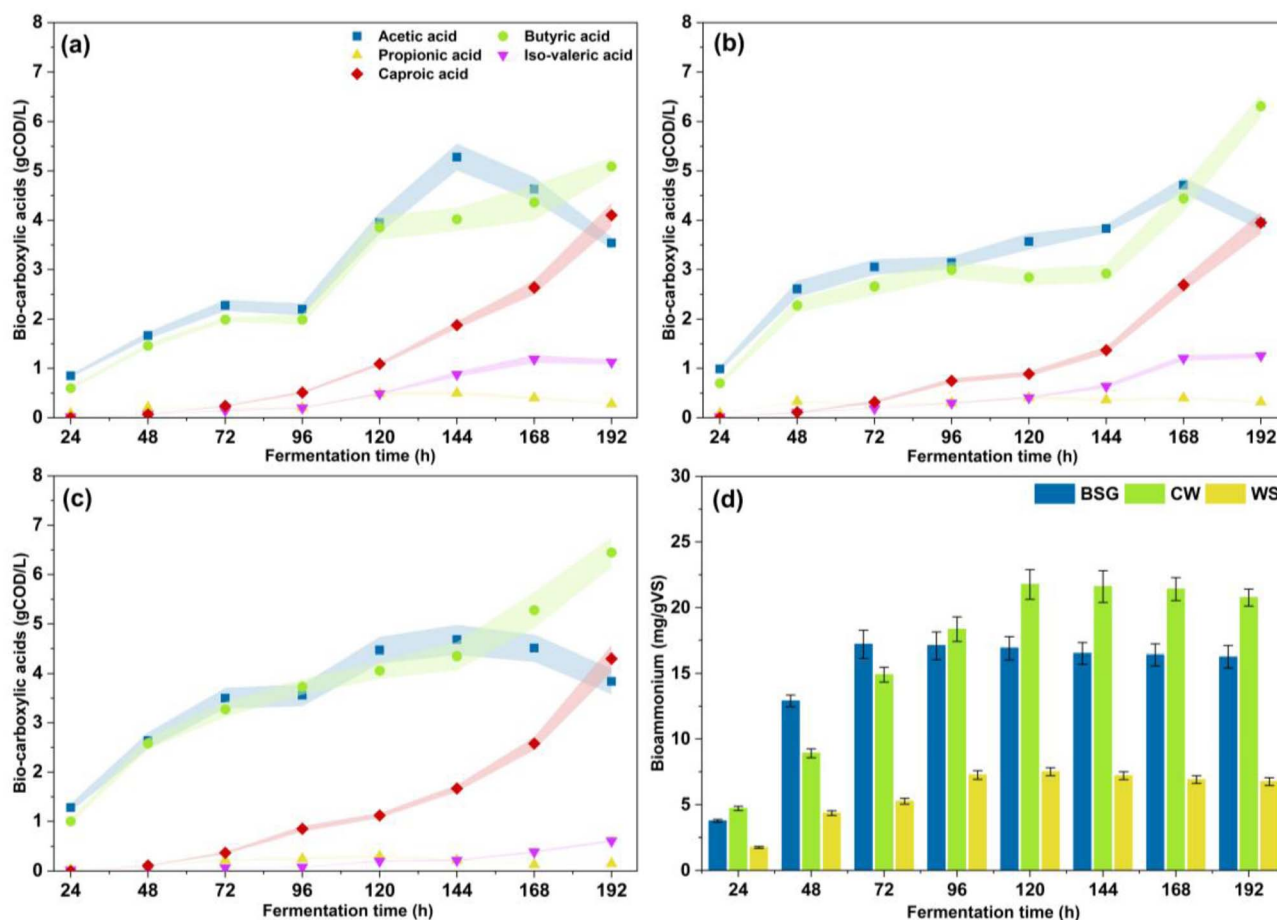
Fig. 4 Bio-carboxylic profile from mixed culture fermentation of (a) BSG (b) CW and (c) WS with respect to fermentation time, (d) bio-ammonium estimated during SCCA and MCCA production from fermentation of BSG, WS and CW.

the utilized VFAs may be converted into alternative metabolites through various biological pathways.

Furthermore, the production of butyric acid reached its peak during days 4 to 5, showcasing the highest yield from WS fermentation at 6.45 gCOD per L. This was closely followed by fermentation of CW, which produced 6.31 gCOD per L, and BSG which yielded 5.09 gCOD per L. This trend illustrates the varying efficacy of different carbon sources in promoting the production of specific fatty acids during fermentation. During the initial two days of fermentation across all reactors, the concentration of propionic acid ranged from 0.1 to 0.33 gCOD per L. Notably, among the various fatty acids produced, propionic acid exhibited a relatively lower concentration compared to its counterparts. Given that all reactors operated under uncontrolled pH conditions, the predominance of propionic acid was somewhat unexpected. In these fermentation processes, acetic and butyric acids consistently emerged as the dominant products, with particularly high concentrations observed in the fermentation of CW. Furthermore, the yields of caproic acid were significantly higher in both lignocellulosic WS and BSG fermentations compared to those achieved with CW as a substrate. This highlights the nutritional composition of these substrates, which may facilitate the production of chain

elongated fatty acids. Ultimately, the fermentation processes led to the nearly complete conversion of all substrates into microbial metabolites, indicating an efficient transformation of organic matter into valuable biochemical products. The concentration of valeric acid across the reactor setups was low, significantly influenced by substrate type. Notably, WS fermentation yielded only 4.3% valeric acid, compared to 9.1% from CW and 9% from BSG. These results emphasize the critical role of substrate composition, its nutrient content and structural properties in shaping microbial metabolic pathways, thereby affecting the production of specific organic acids during fermentation.

Fermentation of CW, a protein-rich substrate, at a low pH has been shown to promote the synthesis of n-valeric acid while not facilitating the generation of iso-caproic acid.[22] The formation of n-valeric acid is hypothesized to occur through a chain elongation process, which serves as an alternative strategy for NADH utilization in contrast to the iso-caproic pathway. This is particularly significant given that protein fermentation is associated with the production of excess reducing equivalents due to the specific amino acid composition of the substrate. The production of caproic acid through the chain-elongation process was observed in all reactors,

exhibiting varying concentrations. In the initial phase, specifically until day 4, the levels of caproic acid were negligible, remaining below one gram in each reactor. However, starting from day 5, there was a noticeable increase in concentration. By this point, the caproic acid levels reached to 4.1 gCOD per L for the reactor utilizing glucose derived from BSG, followed closely by concentrations of 3.95 gCOD per L with CW and slightly lower than the reactor employed with WS (3.84 gCOD per L). This translates to respective contributions of 25 to 29% of the net accumulation of carboxylic acids produced during the experiment. These findings highlight the varying efficacy of different carbon sources in facilitating the production of caproic acid through microbial fermentation processes.

Caproic acid production is predominantly driven by chain-elongating bacteria *via* a reverse β-oxidation pathway, typically necessitating an external electron donor. This study diverges from conventional practices by omitting such a donor in the fermentation media. Notably, during the early fermentation phases (days 2 and 3), all reactors exhibited substantial yields of ethanol and lactic acid. The WS fermentation notably produced 2.7 gCOD per L of lactic acid and 1.1 gCOD per L of ethanol, indicative of vigorous metabolic activity. In contrast, CW fermentation yielded lower outputs of 0.9 gCOD per L lactic acid and 1.4 gCOD per L ethanol, reflecting the distinct compositional and fermentative capabilities of the microorganisms. The BSG reactor achieved 1.8 gCOD per L lactic acid and 1.5 gCOD per L ethanol, demonstrating effective substrate utilization. However, by day 7, lactic acid and ethanol concentrations significantly declined in CW and BSG reactors, suggesting their consumption in the chain elongation process, which facilitates longer-chain fatty acid production. Conversely, the WS reactor exhibited nearly complete ethanol degradation and 74% lactic acid degradation, underscoring the varied utilization of these metabolites based on the carbon source. This result elucidates the intricate dynamics within the fermentation environment and highlights the potential for optimizing conditions to enhance caproic acid production, paving the way for more efficient bioprocessing strategies in the field. The utilization of substrates with higher nitrogen content has significantly enhanced the production of SCCA, particularly MCCAs. Among the three substrates evaluated, fermentation of BSG resulted in caproic acid constituting 29% of the net carboxylic acids produced. In comparison, both CW and WS substrates yielded similar outcomes, with caproic acid selectivity at 25%. These findings indicate that the selection of carbon sources plays a crucial role in determining the profile of carboxylic acids generated, thus emphasizing the potential advantages of employing alternative substrates in bioprocessing applications.

3.3. Ammonium production and consumption during SCCA and MCCA production

The trends in ammonium formation across the three reactors during the operation are illustrated in Fig. 4d. The reactor fermented BSG and CW showed an average ammonium concentration of 292.5 mg L^{-1} and 330.8 mg L^{-1} respectively throughout the experimental period. In contrast, the reactor

with CW showed a low ammonium concentration of 117 mg L^{-1}. During the initial days of the experiment, specifically from day 3 to day 4, ammonium concentration steadily increased in all reactors. The average production value recorded was 274.4 mg L^{-1} for CW, 271.4 mg L^{-1} for BSG, and 105 mg L^{-1} for WS. However, between days 4 and 5, a slight decline in ammonium levels was noted across all reactors. Notably, the reactor utilizing WS exhibited more consumption of ammonium by day 5. In contrast, the reactors fermenting CW and BSG maintained stable ammonium concentrations from day 6 onward. Inorganic nitrogen in water predominantly exists as ammonium ion (NH_4^+).[23] The CW and BSG used as substrate in this study was found to have a total nitrogen of 3% and 4% respectively. The process of hydrolyzing protein from CW, BSG and WS involves extracellular proteases, which break down these proteins into amino acids. Microorganisms then uptake these amino acids for their intracellular metabolism. This enzymatic breakdown leads to the production of ammonium through the deamination of amino acids. While a portion of this ammonium is utilized for the growth of anaerobic microorganisms, the majority remains in the effluent. Limiting the nitrogen content in the fermenting media can adversely impact microbial growth, leading to reduced cell multiplication and slower glycolysis rates.[24,25] Many studies have highlighted the positive effects of amino acids on growth and fermentation rates.[26,27] Additionally, a combination of ammonium and amino acids significantly improves microbial growth and fermentation efficiency, offering a promising strategy for optimizing fermentation processes.[28] The diverse redox properties of amino acids in BSG and CW can play a significant role in providing acetyl-CoA for various anabolic processes. Cysteine can be effectively transformed into acetyl-CoA by the anaerobic acetogenic bacteria, which supports the energy demand for the reduction of CO_2 to acetate.[29] Furthermore, the Stickland reaction and the hydrogen-utilizing pathway, are capable of generating acetyl-CoA.[30] Therefore, given the essential function of acetyl-CoA in chain elongation, amino acids can not only serve as potential electron donors but also vital contributors that facilitate chain elongation, resulting in the production of MCCA in bacteria. Amino acids cannot be directly converted into MCCAs. However, when used alongside substrates like acetate and butyrate, amino acids were found to enhance the abundance of MCCAs. This suggests that amino acids act as electron donors, facilitating chain elongation reactions. Furthermore, the research indicated that amino acids could promote these reactions through *in situ* electron donation, with electro-fermentation playing a key role in MCCA production from both amino acids and SCCAs. The findings also imply that other common electron donors may contribute to chain elongating metabolic processes without directly catalyzing the reactions themselves.[31] The decomposition of amino acids to iso-butyrate and iso-valerate might be inhibited by released hydrogen.[31]

3.4. Acidogenic biogas profile

The fermentation of three different substrates significantly influenced biohydrogen production and yield. Over an 8-day

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period, total gas outputs were 2590 mL from WS, 2553 mL from CW, and 2492 mL from BSG. The cumulative biohydrogen production was comparable between CW and WS, yielding 1608 mL and 1683 mL, respectively. In contrast, BSG fermentation resulted in a slightly lower yield of 1520 mL. Slight lower hydrogen production with BSG might be due to lower carbon content, as the hydrolysate derived from BSG was composed of sugars as well as small fraction of other complex compounds such as lignin. Whereas in the case of CW, most of the substrates were composed of lactose, the glucose fermentation is always found to be simpler form of substrate that is directly utilized by bacteria for biohydrogen production. Glucose in the hydrolysate serves as an efficient and easily metabolized sugar that bacteria can use for biohydrogen production.[13,32,33] It provides a quick energy source through glycolysis and can yield significant hydrogen amounts, especially in bacteria like *Clostridium* species, which can directly metabolize glucose without complex pretreatment. The theoretical maximum yield is 4 moles of hydrogen per mole of glucose, but practical yields are generally lower due to metabolic inefficiencies and by-product formation.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

In this study examining biohydrogen production through fermentation, WS was found to yield hydrogen more rapidly than by using BSG and CW as a substrate. The fermentation process demonstrated that over 90% of the total volumetric biohydrogen production occurred within just two days when WS was utilized. In contrast, the fermentation of CW and BSG achieved only 64% and 70% of net biohydrogen evolution, respectively, within the same timeframe. This highlights the efficiency of glucose derived from WS in biohydrogen production compared to other substrates like CW and BSG. Acidogenic fermentation is recognized for its efficient and rapid biohydrogen production, often achieving notable hydrogen yields within just a few hours. This process employs relatively simple metabolic pathways such as glycolysis and pyruvate fermentation, which allow for quicker hydrogen generation compared to other methods like photo-fermentation or microbial electrolysis. In addition to biohydrogen, acidogenic fermentation also leads to the production of various valuable metabolites, including carboxylic acids/VFAs and carbon dioxide (CO_2). These byproducts are formed during the breakdown of glucose through glycolysis, facilitated by hydrogen-producing enzymes like hydrogenases. This dual production highlights the potential of acidogenic fermentation not only for renewable energy generation but also for the synthesis of useful chemicals.

In terms of biohydrogen yield, WS fermentation demonstrated the highest yield at 140.29 mL per gCOD (84.2 mL per gVS), followed closely by CW at 134.03 mL per gCOD (80.42 mL per gVS) and BSG at 126.68 mL per gCOD (76.01 mL per gVS) (Fig. 5). According to eqn (1), it is expected that higher hydrogen production would correlate with increased volatile fatty acid production. Previous studies have shown a strong correlation between VFA and biohydrogen production, particularly with carbohydrate-rich substrates.[13,14] However, in our current study,

VFA production did not align with hydrogen production levels. This discrepancy may arise from the nature of certain acidogenic bacteria that do not produce hydrogen. Some bacteria may prefer metabolic pathways that generate VFAs without significant hydrogen evolution, such as lactic acid or propionic acid fermentation. Additionally, during mixed culture fermentation, some bacterial species can divert the carbon flow towards non-hydrogen-producing pathways. For example, in lactic acid fermentation, pyruvate is converted into lactate, which does not produce hydrogen. Furthermore, the conversion of pyruvate into propionate and ethanol can consume hydrogen in the process. The presence of compounds like propionate, ethanol, and lactic acid in the reactor suggests that not all substrates are being utilized for hydrogen production, highlighting an area for potential optimization in future research.

Moreover, simple substrates such as glucose are more readily utilized by acidogenic bacteria compared to lignocellulosic biomass, leading to increased production of hydrogen and volatile fatty acids. This enhanced metabolic processing is attributed to structural simplicity and lower energy barriers associated with breaking down monosaccharides like glucose. Additionally, the presence of essential nutrients, specifically nitrogen and phosphorus, plays a crucial role in microbial metabolism, particularly in the fermentation of byproducts like BSG and CW.[34–36] These nutrients can stimulate microbial growth and metabolic pathways, significantly influencing whether the end products will favor VFA or hydrogen production. Such dynamics show the importance of optimizing nutrient composition in fermentation processes to achieve desired output effectively. The hydrolysate derived from BSG is characterized by a diverse array of saccharides, including glucose, arabinose, xylose, and galactose. Generally, the theoretical hydrogen yields from the C5 sugars are lower compared to those derived from C6 glucose.[37] The enzymatic hydrolysis process employed to produce this hydrolysate specifically minimizes the formation of byproducts such as 5-HMF, furfural (originating from hemicellulose), as well as vanillic acid and vanillin (resulting from lignin fractions).[38] This contrasts with hydrothermal or chemical pretreatment methods, where the breakdown of cellulosic materials can lead to a broader spectrum of degradation products. These compounds, including vanillic acid, vanillin, and furfural, are recognized as fermentation inhibitors and can have a negative impact on hydrogen yields. In general, simple glucose fermentation tends to produce higher volumetric hydrogen outputs compared to hydrolysates composed of mixed sugars.[39] Additionally, reduced hydrogen production during the fermentation of BSG and CW may be attributed to the challenges associated with metabolizing both C5 and C6 sugars, along with their oligomers and proteins.

3.5. Substrate utilization

The substrate utilization efficiency was determined based on the residual carbon (COD) present in the fermenting media with relative to fermentation time. This efficiency was directly influenced by the nature of the substrate. The WS demonstrated
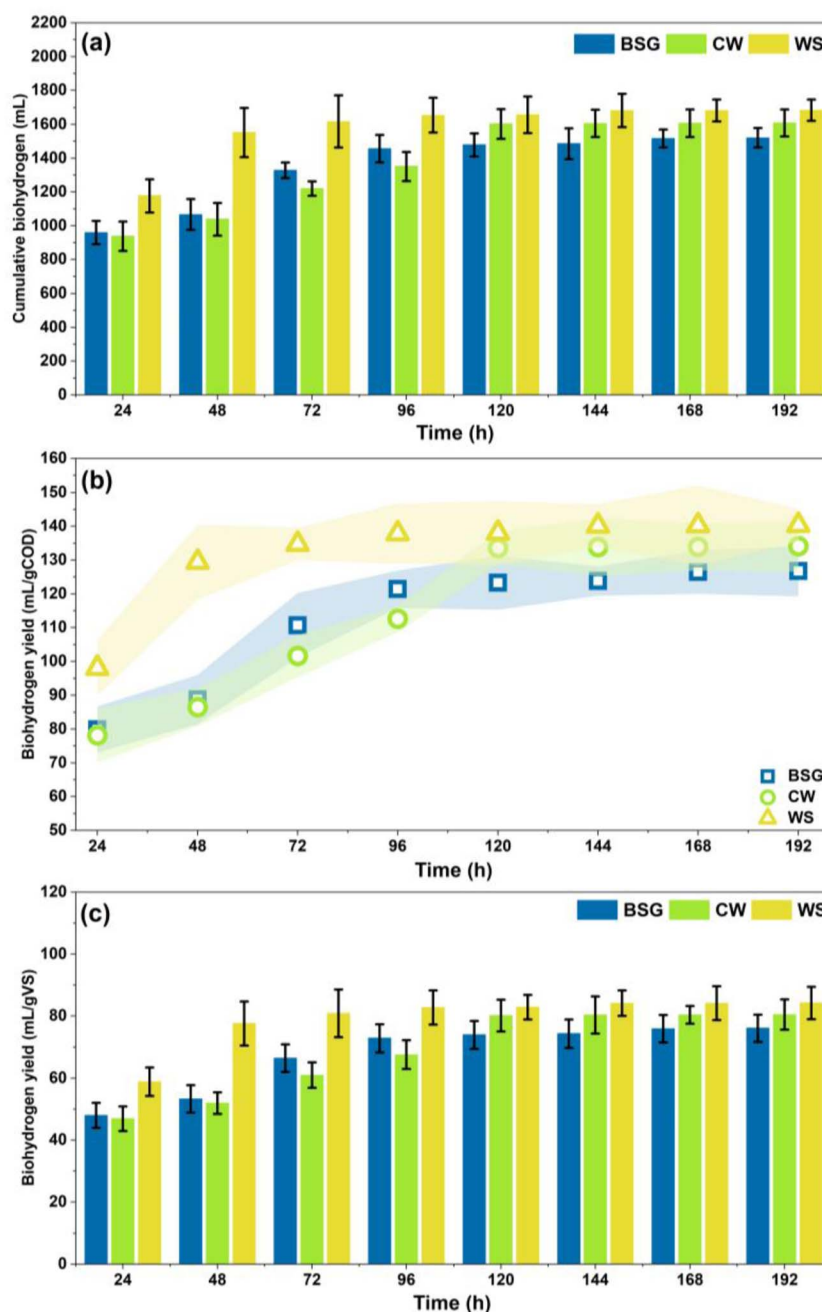
Fig. 5 Acidogenic biogases produced during fermentation of industrial waste stream (cheese whey: CW and brewery spent grains: BSG) and agricultural residues (wheat straw:WS) (a) cumulative biohydrogen and (b) biohydrogen yield in terms of mL per gCOD and (c) biohydrogen yield in terms of mL per gVS.

the highest metabolic flexibility for bacteria, achieving a substrate utilization efficiency of 100% within four days of fermentation. In contrast, CW and BSG exhibited lower efficiencies at 78% and 72%, respectively, after the same four-day fermentation period. Complete substrate utilization of CW and BSG was observed by day six and day seven, respectively. Bacteria demonstrate significant acclimatization to dark fermentation for biohydrogen production, efficiently utilizing WS substrates. However, the decomposition rates of CW and BSG are markedly lower, indicating the difficulties mixed

microbial cultures encounter with these complex carbon sources. The challenge arises from the diverse composition of BSG, containing both C5 and C6 sugars and proteins, while CW primarily consists of proteins and lactose. The metabolic processing of CW, which is rich in lactose and protein, engages distinct metabolic pathways compared to glucose. In the evaluation of BSG hydrolysate within a reactor, it was observed that microbial uptake of glucose significantly surpassed that of xylose and galactose. This phenomenon highlights the inherent preference of fermentative microorganisms for hexose sugars,

leading to expedited glucose fermentation compared to pentoses and other monosaccharides that are less efficiently metabolized.

CW serves as a complex substrate for microbial fermentation, primarily due to its rich lactose and protein composition, which necessitates distinct metabolic pathways compared to standard carbohydrates like glucose. Lactose undergoes hydrolysis into glucose and galactose *via* the action of the enzyme β-galactosidase prior to its entry into the glycolytic pathway.[40] Concurrently, proteins are catabolized into amino acids by proteolytic enzymes, which can also be substrates for fermentation. The efficacy of substrate utilization hinges on the efficient hydrolysis and degradation of lactose and proteins. Once liberated, glucose is readily integrated into glycolysis, yielding pyruvate. The subsequent fate of pyruvate is dependent on the metabolic capabilities of the bacterial strains present and their synergistic interactions within mixed microbial communities, leading to diverse fermentation end products such as lactate, ethanol, or acetate. The experimental results showed that substrate consumption reduction, signaling less efficient conversion to microbial metabolites, is less significant with CW and BSG than with WS. C5 sugars (in BSG) are identified as viable substrates for biohydrogen and VFAs production. However, a clear preference for C6 sugars over C5 sugars emerges when both are present.[41] The hierarchy of substrate utilization from mixed C5 and C6 sugars is influenced by the microbial diversity in the seed sludge. Notably, the mixed microbial culture can effectively metabolize hydrolysate from BSG and WS without a strong preference for any monosaccharide, including glucose, indicating adaptability and versatility in substrate utilization. This emphasizes the importance of microbial diversity in optimizing biofuel production processes. Factors enhancing C5 sugar uptake in mixed cultures include the adaptability of certain microbial strains, such as *Clostridium*, which efficiently process pentose sugars from BSG, highlighting the complexity of microbial interactions in bioprocessing.

3.6. Microbial diversity

To explore the influence of microbial communities on the fermentation of various substrates, specifically brewery spent grains (BSG), cheese whey (CW), and wheat straw (WS), samples were collected from reactors on day 8. Utilizing metagenomic sequencing, the microbial community structure was analyzed, revealing significant insights into alpha diversity as illustrated in Table 2. Key metrics such as the Shannon and Simpson indices were employed to assess microbial diversity and evenness, crucial for evaluating community stability. Notably, BSG (Shannon index: 3.17) and CW (2.61) outperformed WS (2.39) in diversity, while the Simpson index values indicated a strong correlation between substrate type and microbial diversity (0.90 for BSG, 0.85 for CW). Furthermore, the Chao index indicated a decline in richness with CW (329) compared to BSG (708) and WS (499). The Venn diagram illustrates that WS, BSG, and CW each possess largely unique microbial communities, with each community containing 80 to 84 unique OTUs. Only 3 OTUs were

Table 2 Alpha diversity indices during the acidogenic fermentation of cheese whey (CW), brewery spent grains (BSG) and wheat straw (WS)

Substrate	Richness	Shannon	Simpson	Chao1	ACE	Pielou
BSG	711	3.17	0.90	708	687	0.44
CW	332	2.61	0.85	329	346	0.32
WS	500	2.39	0.73	499	534	0.32

shared among all reactors, highlighting their individuality. Pairwise comparisons show limited overlaps as 9 OTUs between WS and BSG, 8 between WS and CW, and 5 between BSG and CW. These findings indicate that while some taxa are common, the overall OTU composition is primarily shaped by nature of substrate (Fig. 6).

The variations in microbial diversity and richness among the substrates reflect the complex interactions present within the fermentation system, demonstrating that substrate composition can exert both stimulatory and inhibitory effects on microorganisms throughout the fermentation process. Understanding these dynamics is essential for optimizing fermentation conditions and enhancing the efficiency of microbial applications in various biotechnological processes. The distribution of sequences at the phylum level within each sample is displayed in Fig. 7a, where the relative abundances of bacterial communities greater than 0.1% in at least one sample are highlighted. The predominant three phyla identified in all three reactors were *Firmicutes*, *Bacteroidota*, and *Actinobacteriota*. Among these, *Firmicutes* exhibited the highest relative abundance (ranging from 85 to 99%) and played a crucial role in the hydrolysis and acidification of high molecular weight carbohydrates. Furthermore, *Firmicutes* are also a vital contributor to the production of acidogenic carboxylic acids and biohydrogen, with all identified strains related to chain elongation belonging to this phylum. Additionally, a small proportion of other phyla were also present in all three reactors (Fig. 6). While their percentages were minimal, phyla such as *Proteobacteria*, *Chloroflexi*, *Halobacterota*, *Caldatribacteriota*, *Acidobacteriota*,
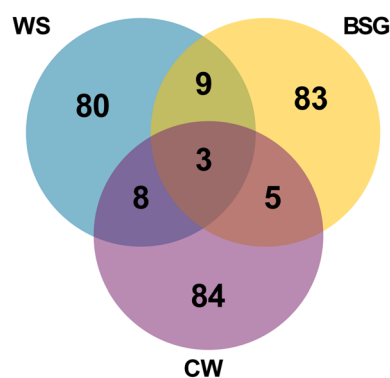
Fig. 6 Venn diagrams of bacterial communities illustrate the number of shared and unique operational taxonomic units (OTUs) among three bioreactors fermenting Brewery Spent Grains (BSG), Cheese Whey (CW), and Wheat Straw (WS) under identical experimental conditions.
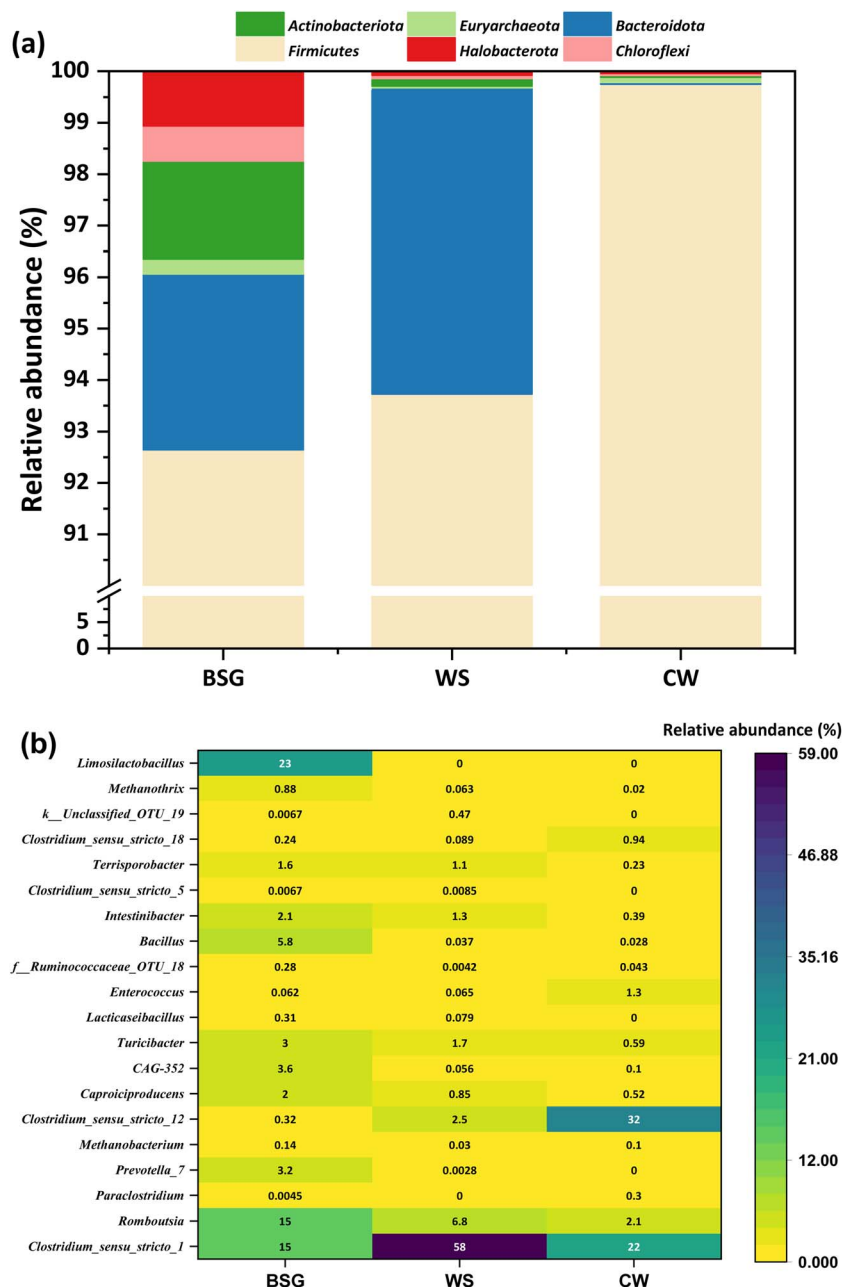
Fig. 7 Microbial community composition (relative abundance) evaluated from the reactors fermented CW, BSG and WS studied on day 8 (a) and heat-map at genus level (b).

and *Euryarchaeota* were noted, suggesting that they may not have been essential to the overall acetogenic process.

Firmicutes were predominantly found and significantly enriched across all reactors, with the highest concentration observed in CW at 99.01%. This was followed by WS fermentation at 90.85% and BSG at 89.45%. *Bacteroidetes* were present in nearly all reactors, showing a relatively higher proportion in BSG (3.3%) and WS (5.77%) compared to CW fermentation, which had only 0.03%. These findings highlight a striking shift in bacterial communities among the three reactors, regardless of the diverse substrates used. The results align with earlier research that investigated the microbial community structure in sludge fermenting CW and BSG, emphasizing how the microbial composition can fluctuate based on the specific experimental conditions.[42] The findings demonstrate that the microbial community present within a reactor system is markedly affected by the operating conditions. The increased prevalence of *Firmicutes* in CW is attributed to its superior fermentation efficiency, particularly in metabolizing lactose and protein. In contrast, the relative abundances of *Actinobacteria* and *Bacteroidota* in BSG and WS are notably higher, at 5.14% and 5.91%, respectively, significantly surpassing the mere 0.06% observed in CW.

Actinobacteria, distinguished as Gram-positive bacteria, exhibit remarkable metabolic versatility, particularly in biohydrogen production and VFAs fermentation. Despite being less studied than *Clostridium* or *Enterobacter* in H_2 generation, *Bacteroidota*, which are Gram-negative and anaerobic, play a crucial role in anaerobic digestion. Predominantly inhabiting the gut microbiome, soil, and wastewater treatment systems, they decompose complex organic substrates into VFAs. These VFAs are then utilized by other microorganisms, such as *Clostridium*, to facilitate the conversion into MCCA, demonstrating a synergistic relationship that highlight's the ecological and biotechnological significance of these microbial communities in renewable energy production. Fig. 7b illustrates the microbial community at the genus level. During WS fermentation, genus *Clostridium sensu stricto 1* was the predominant genus, representing over 50% of the microbial community. Other genera identified in the same reactor during glucose fermentation were present at lower levels, with *Romboutsia* accounting for 6.8%. This was followed by *Clostridium sensu stricto 12* at 2.5%, *Turicibacter* at 1.7%, *Intestinibacter* at 1.3%, and *Terrisporobacter* at 1.1%. In contrast, during CW fermentation, *Clostridium sensu stricto 12* (31.59%) and *Clostridium sensu stricto 1* (22.3%) emerged as the most prevalent genera. Meanwhile, in the fermentation of BSG within the reactor, *Romboutsia* (15.4%) and *Clostridium sensu stricto 1* (15.02%) were the most abundant. *Clostridium butyricum*, a prominent member of the *Clostridium sensu stricto 1* group, has been recognized for its hydrogen-producing capabilities from various substrates. In a fermentation reactor utilizing WS, *C. butyricum* exhibited a remarkable dominance, achieving a peak abundance of 49.91%, significantly surpassing its levels in CW and BSG. This bacterium metabolizes glucose to yield hydrogen and VFAs, primarily through the butyrate pathway (eqn (2)), which generates two moles of hydrogen per mole of glucose.

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (2)$$

Although the acetate pathway (eqn (1)) can produce four moles of hydrogen per mole of glucose, it was less prevalent in this study. The fermentation process was influenced by uncontrolled pH levels, which ultimately dropped below 5.5, favoring butyrate production over other byproducts. This study underscores the potential of *C. butyricum* in biohydrogen production and the importance of pH management in optimizing fermentation processes. The *Clostridium sensu stricto 12* group, a prominent phylogenetic cluster within the *Clostridium* genus, consistently dominated substrate fermentation processes. This group is recognized for its efficient production of biohydrogen and carboxylic acids. Notably, species within this cluster significantly contributed to hydrogen yields, particularly in reactors optimized for VFAs. The primary metabolic pathways facilitating hydrogen production in this context are the acetate and butyrate pathways. The production of acetic and butyric acids exhibited a strong positive correlation with hydrogen generation during this experiment. Conversely, ethanol production did not demonstrate a significant correlation, despite *Clostridium sensu stricto 12* being recognized for its

ethanologenic capabilities. Generally, members of the genus *Clostridium* are associated with enhanced hydrogen production. This observation implies that the complexity of acidification levels in the substrate concentration resulted in a differentiated evolution of the microbial community, and not all variations were associated with hydrogen production. A key factor is that a substantial fraction of hydrogen was sequestered within VFAs, particularly in the forms of propionates and valerates.

Furthermore, recent studies highlighted the significant role of *Romboutsia* and *Caproiciproducens* in hydrogen production within anaerobic reactors. Especially, *Romboutsia* exhibited a remarkable prevalence of 15.4% during the fermentation of BSG, surpassing its presence in WS (6.8%) and CW (2.07%) fermentations. This dominance is likely linked to the abundance of hemicellulose sugars in BSG. Previous studies indicate that *Romboutsia* can reach up to 22% in mixed cultures during BSG fermentation.[20] Additionally, other bacterial taxa, particularly *Bacteroidetes*, have been identified for their cellulose and xylan degradation capabilities in anaerobic systems utilizing organic substrates such as cow manure, Napier grass, and goat manure.[43] These bacteria are known to ferment xylan, producing volatile fatty acids, hydrogen, and carbon dioxide, which serve as precursors for acetogens and methanogens in the anaerobic digestion process.[44] The highest to lowest relative abundance of hemicellulose-degrading bacteria was observed in the reactor that processed BSG, followed by those fermenting WS and CW. This is likely due to the presence of hemicellulosic sugars, such as xylose and galactose, in the substrates. Although the CW fermenting reactor lacks hemicellulose, the proliferation of *Romboutsia* can be attributed to its role in contributing fermentable sugars, amino acids, and lactate to the pool of fermentation intermediates, like acetic and butyric acids. The genus *Turicibacter* is a Gram-positive bacteria nestled within the phylum *Firmicutes*. Currently, *Turicibacter sanguinis* is a sole recognized species, which is known for its production of carboxylic acids. Recently, new strains of *Turicibacter*, isolated from chicken eggshells and the pig ileum.[45,46] In the BSG reactor, *Turicibacter sanguinis* made its mark with a 3% dominance, outpacing WS at 1.73% and making a smaller contribution of 0.59% in the CW fermentation.

Prevotella, particularly the *Prevotella_7* species, is a Gram-negative, anaerobic bacterium that is abundant in the human gut, rumen, and anaerobic digesters. It is primarily recognized for its role in polysaccharide fermentation, biohydrogen production, and the generation of volatile fatty acids.[47,48] Fermenting BSG, *Prevotella* was found to comprise a dominant 3.22% of the microbial community. Members of the *Prevotella* genus are known for their proteolytic activity, which is linked to the presence of peptidases that facilitate the hydrolysis of proteins into amino acids. The degradation of amino acids is associated with the formation of organic acids and ammonia, which correlates well with ammonium production as observed in this study. Recent genomic and metagenomic analyses have revealed that the genus *Prevotella* contains polysaccharide utilization loci (PUL) within its genomes. These gene clusters encode enzymes specifically designed for the hydrolysis of complex carbohydrates.[49] *Prevotella* exhibits an enhanced

ability to adapt to low pH conditions, which arise from higher replacement ratios, and it possesses a significant acidogenesis capacity. This genus greatly influences the production of VFAs. Consequently, the enrichment of *Prevotella* in reactors that specifically digest BSG plays a crucial role in generating acetic acid, propionic acid, and butyric acid through the degradation of polysaccharides and proteins. Furthermore, the genus *Enterococcus* was exclusively detected during the fermentation of CW, where it exhibited a dominance of 1.22%, contributing to the production of biohydrogen and carboxylic acids. While *Enterococcus* is recognized for its ability to utilize hemicellulose sugars for the production of biohydrogen and VFAs, it was also identified in the fermentation of BSG and WS. Additionally, it has been observed in the fermentation of BSG and WS. However, the exclusive presence of *Enterococcus* in CW fermentation in this study may be attributed to the dominance of other genera within the reactor, which likely suppressed the proliferation of *Enterococcus*. Previous studies have linked *Enterococcus* to the fermentation of lignocellulosic waste.[50] It was also reported that *Enterococcus faecalis* can metabolize various sugars.[51] *Caproiciproducens* is a well-known chain-elongating bacterium that can utilize carbohydrates as electron donors to produce medium-chain carboxylic acids.[52] In this study it was found that the pH below 6 significantly enhances caproic acid production. In contrast, pH levels above 6, particularly around 6.5, favor the synthesis of acetic, butyric, and propionic acids by mixed microbial community. It was interesting to note that *Caproiciproducens* has emerged as the leading genera within the *Firmicutes*, with previous studies highlighting its crucial role in the chain elongation process.[20,52,53] In this study, there was a remarkable increase in *Caproiciproducens* abundance in BSG, with rises of 232% and 377% compared to WS and CW fermentation, respectively. Such an enhancement in microbial diversity is poised to significantly boost the biosynthesis of medium-chain carboxylic acids. Furthermore, the shifts in microbial community composition closely corresponded with metabolite patterns observed in the reactors, underscoring the intricate relationship between microbial dynamics and metabolic outputs.

4. Conclusion

The study investigated the role of substrate composition in shaping the fermentation product spectrum and microbial diversity during mixed culture acidogenic fermentation emphasizing the significance of utilizing industrial waste streams and agricultural residues. Acidogenic metabolites reveal notable differences in the distribution and concentration of SCCAs and MCCAs during the fermentation of lignocellulosic *versus* lactose-rich CW, demonstrating the impact of substrate compositions and microbial pathways on fermentation efficiency. Notably, CW, a non-lignocellulosic substrate, yielded the highest SCCA production at 11.84 gCOD per L, surpassing BSG and WS. Conversely, WS and BSG, both lignocellulosic, excelled in MCCA production, with WS achieving 4.30 gCOD per L and BSG 4.1 gCOD per L, while CW produced a competitive 3.95 gCOD per L. Taxonomic analysis revealed a predominance of *Firmicutes*, *Bacteroidota*, and *Actinobacteriota* across all reactors, with the *Clostridiaceae* family being particularly dominant. The study identified key genera, including *Clostridium* and *Enterococcus*, as significant contributors to carboxylic acid production, emphasizing the intricate relationship between substrate composition and microbial community structure. The findings advocate tailored substrate selection to optimize acidogenic fermentation processes, paving the way for sustainable waste management and biobased product synthesis. Despite the challenges posed by the complex structure of lignocellulose, pretreatment can unlock fermentable sugars, transforming these substrates into valuable renewable resources. This study not only emphasizes the potential of lignocellulosic biomass but also illustrates the importance of understanding microbial dynamics to foster a truly circular and sustainable economy.

Conflicts of interest

There are no conflicts to declare.

Data availability

Raw experimental data can be provided upon request.

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