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Hydrothermal pretreatment of brewer's spent grain: A pathway to sustainable biogas production and waste valorization

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ABSTRACT

Brewer's spent grain (BSG), a by-product of the brewing industry, faces significant waste management challenges but holds potential as a substrate for biogas production via anaerobic digestion (AD). However, its lignocellulosic complexity limits microbial degradation and methane yield. In previous studies by our research group, various treatment strategies for BSG were explored, including approaches aimed at recovering high-value compounds. Building upon this foundation, the present work evaluates the potential of hydrothermal pretreatment to enhance methane production from BSG via AD. The novelty lies in the combination of a continuous hydrothermal pretreatment reactor with meta-transcriptomic analysis of the anaerobic digestion process, enabling a direct correlation between operational performance and microbial functional shifts. Hydrothermal pretreatment was applied to partially hydrolyze cellulose and hemicellulose into fermentable sugars, improving biodegradability while minimizing the formation of inhibitory compounds. Results showed that hydrothermally pretreated BSG supported methane productions over 1500 mL CH_4/L_reactor·day, markedly higher than the 100–500 mL CH_4/L_reactor·day observed for untreated BSG. Volatile solids degradation efficiency improved by 30 %, while methane content in biogas increased from 30 % to 65 %. Genomic analysis of the microbial consortium revealed enhanced activity of methanogenic archaea and fermentative bacteria associated with the increased methane production. This integrated approach not only disrupts lignocellulosic barriers more effectively but also provides deeper insights into microbial functionality, reinforcing hydrothermal pretreatment as a viable strategy for boosting biogas yield and advancing sustainable waste-to-energy solutions.

1. Introduction

After water and tea, beer is the third most popular beverage in the world and is a historic low-alcohol beverage. Approximately 30 million tons of brewer's spent grain (BSG) are produced annually by the global brewing industry as a by-product of making beer. Indeed, 15–20 kg of BSG are produced for every 100 L of beer [1,2]. Currently, three primary methods exist for treating BSG: utilization as animal feed, landfill disposal, and an ingredient in human food due to its high moisture content, fiber, and protein [3,4]. However, as global priorities shift towards sustainability, bioeconomy, and waste valorization, BSG has emerged as an important raw material in the search for renewable energy sources and sustainable waste management solutions. BSG's lignocellulosic structure, rich in cellulose and hemicellulose, makes it an attractive substrate for biofuel production through anaerobic digestion (AD), while lignin, although typically considered a recalcitrant fraction, was shown under our hydrothermal pretreatment and digestion conditions to undergo partial structural modification, reducing its inhibitory effect and thereby contributing to enhanced biodegradability [3]. Despite its potential, the use of BSG in AD processes faces significant challenges. The complex lignocellulosic composition of BSG resists enzymatic breakdown, limiting the efficiency of biogas production [5]. Current research focuses on optimizing bioconversion pathways, particularly through pre-treatment methods that enhance substrate availability. Hydrothermal pretreatment, particulary under subcritical water, offer several advantages for processing lignocellulosic biomass,

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breaking down complex polysaccharides into simpler sugars, enhancing microbial accessibility during anaerobic digestion and increasing biogas yield [6]. This process involves subjecting BSG to high temperatures between 100 °C and 374 °C and pressures sufficient to maintain water in its liquid state, promoting the hydrolysis of hemicellulose and cellulose and partial depolymerization of cellulose into soluble sugars while minimizing the formation of degradation products typically associated with harsher chemical treatments [7]. Hydrothermal pre-treatment not only improves methane yields but also aligns with bioeconomy goals by enabling the use of lignocellulosic waste as a renewable energy source. Recent studies, such as that by Świechowski et al. (2023), have demonstrated that biochar addition and thermal treatments significantly influence the bioconversion efficiency of BSG [7]. By enhancing the digestibility of BSG, these methods create a more consistent substrate that allows for faster and more efficient biogas production, particularly under anaerobic conditions [8]. This process targets the recalcitrant lignocellulosic bonds, converting them into fermentable sugars and improving microbial access during anaerobic digestion. Additionally, hydrothermal pretreatment minimizes the formation of inhibitors, such as furfurals hydroxymethylfurfurals and phenolics, which can hinder microbial activity in subsequent AD processes. By adjusting parameters like temperature, pressure, and reaction time, the process can be optimized to achieve high conversion rates without compromising other valuable compounds in the BSG matrix.

However, there is a critical gap in current research regarding the scalability and operational efficiency of hydrothermal pretreatment systems for industrial applications. Most studies have been limited to batch reactors, which often lack the precise control over reaction time and temperature required to maximize hydrolytic efficiency while minimizing the formation of undesired by-products. This limitation has implications for both the economic feasibility and environmental impact of biogas production on a large scale. Our study addresses this gap by employing a continuous reactor system, which allows for precise control of reaction parameters. This technology not only improves BSG hydrolysis efficiency but also reduces the risk of forming inhibitory compounds, thereby enhancing the overall yield and quality of biogas. Moreover, the continuous reactor system facilitates scalability, offering a more cost-effective and energy-efficient solution compared to traditional batch processes.

To further advance innovation in anaerobic digestion, this study combined for the first time a continuous hydrothermal pretreatment reactor with advanced genomic profiling of microbial communities. This integrated approach enhances pretreatment efficiency while simultaneously revealing how specific microbial taxa and metabolic pathways contribute to methane production. Through metagenomic analysis of the anaerobic sludge digesting BSG, we identified the diversity and metabolic capabilities of the microbial community, and how these influence the breakdown of complex substrates. This synergy between process optimization and microbial insight provides a deeper understanding of AD dynamics and offers a foundation for developing targeted strategies to maximize methane yields in industrial applications.

This study compared biogas production from untreated BSG and hydrothermally pretreated BSG to evaluate the efficacy of hydrothermal hydrolysis in enhancing methane yield. By implementing a continuous hydrothermal pretreatment reactor and characterizing the microbial population structure genomically, this work bridges the gap between lab-scale studies and industrial applications. This work underscores the importance of advanced pretreatment technologies in realizing the full potential of BSG as a substrate for renewable energy production. By addressing the challenges of substrate recalcitrance, optimizing reactor design for scalability, and investigating the microbial ecology of AD systems, this study aims at contributing to the development of sustainable waste-to-energy technologies within the framework of a circular bioeconomy.

2. Material and methods

2.1. Seed sludge and substrate

The inoculum sludge was sourced from the mixed sludge anaerobic digester of Valladolid Wastewater Treatment Plant and introduced into the lab-scale anaerobic digester without further conditioning. The anaerobic inoculum was characterized by determining the total solids (TS), volatile solids (VS) and microbial population structure. “Compañía cervecera de Canarias” brewery (Canary Islands, Spain) kindly provided the BSG used in this research. BSG was cleaned with distilled water and further dried at 80 °C overnight (to minimize microbial growth and moisture without significantly altering lignocellulosic structure). The dried BSG was grinded using an IKA® MF10 basic microfine grinder to a particle size of 0.4 mm and then stored at room temperature until further processing or analysis.

2.2. Experimental setups

The hydrothermal treatment setup for BSG followed the general methodological framework described by Cantero et al. (2015) for continuous systems, although the process conditions applied in this study differed substantially from theirs. Specifically, a 11.3 % w/w BSG suspension in water was fed into the hydrothermal treatment system (Fig. 1) along with a stream of water at subcritical conditions (15 MPa and 280 °C). Both streams were mixed at a 2.2:1 water/biomass ratio, yielding reaction conditions of 210 °C and 15 MPa, with a residence time of approximately 2.0 s. These operational parameters are consistent with previous batch hydrothermal pretreatment studies on lignocellulosic residues, including BSG, which identified 180–220 °C as an optimal range for enhancing biodegradability prior to anaerobic digestion [9–11]. At the reactor outlet, an expansion valve allowed a sudden reduction in temperature and pressure to instantly stop further reactions. The anaerobic digester consisted of a 5 L transparent PVC stirred tank reactor with magnetic agitation (Selecta, Spain), working volume of 4 L, equipped with a biogas counter and sampling port. It was maintained at 37 °C in a thermostatic chamber, and both feeding of BSG and withdrawal of digestate were conducted with a Watson Marlow 323 peristaltic pump (Watson Marlow, UK).

Finally, the reactor was completely filled with the inoculum and hydrothermally pretreated BSG slurry, leaving no headspace. No nitrogen purge was performed; however, the full-volume filling and hermetic sealing ensured that any residual oxygen was negligible. The biogas flow rate was monitored using a pulse flow meter, previously calibrated under laboratory conditions to ensure measurement accuracy. Calibration results indicated that a single pulse corresponded to 19 mL of biogas, with the calibration process performed prior to the experimental phase to optimize measurement reliability. The pulse signals were continuously recorded, and cumulative gas volumes were calculated by summing the individual pulses and converting them to volume using the calibrated conversion factor.

2.3. Experimental conditions for anaerobic digestion

The reactor was operated in a mesophilic temperature range (38 ± 1 °C) in a thermostatic chamber. The reactor was assembled with 4 mm poliamide tubes in the liquid and gas streams, a port for biogas sampling and a biogas flowrate counter (Fig. 1). The bioreactor was agitated continuously using a magnetic stirrer (Agimatic-HS, Selecta®, Spain) at 100 rpm.

The BSG was resuspended at a total solid concentration of 25 g TS L^{-1} in a mineral salt medium containing (g L^{-1}): $NaHCO_3$ (0.75), $(NH_4)_2SO_4$ (2.05) as a nitrogen source, KH_2PO_4 (0.278) and K_2HPO_4 (0.219). A 1 mL of two trace element solutions (M1 and M2) was added to 1 L of mineral salt medium. M1 had the following composition (g L^{-1}): $FeSO_4.7H_2O$ (15.0), $ZnSO_4.7H_2O$ (5.0), $MnSO_4.H_2O$ (3.0), $CuSO_4.5H_2O$

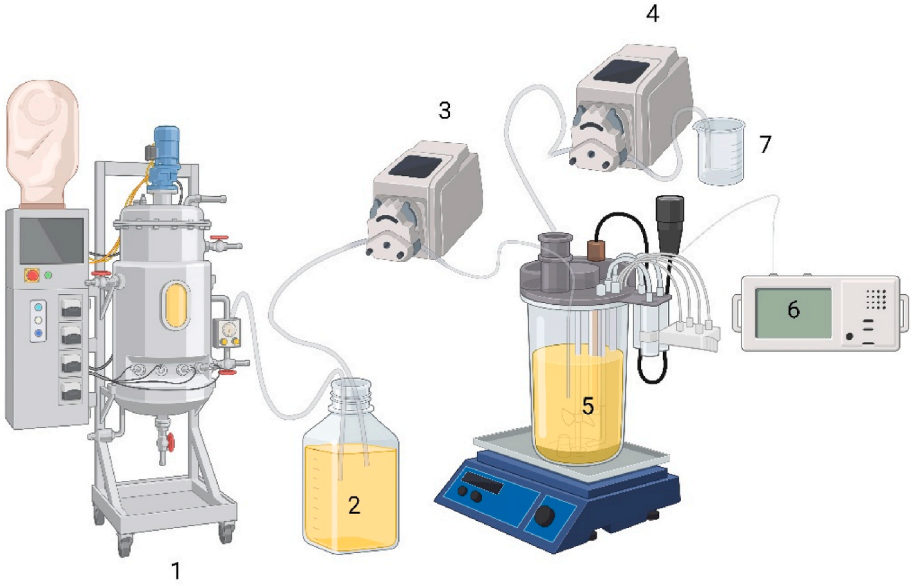

Fig. 1. Experimental setup for the continuous hydrothermal pretreatment and anaerobic digestion of BSG [1]. Hydrothermal pretreatment reactor [2], Collection flask for hydrolyzed BSG [3,4], Peristaltic pumps for controlled feeding and effluent transfer [3]; Anaerobic digestion bioreactor [5] Gases pulse counter [6] Sampling beaker for analytical characterization. This configuration allowed precise control of pretreatment conditions, continuous feeding to the anaerobic digester, and real-time monitoring of system performance.

(0.75), $CoCl_2.6H_2O$ (0.15), citric acid (6.0). M2 was composed of (g L^{-1}): $(NH_4)\ 6Mo_7O_{24}.4H_2O$ (0.5), BO_3H_3 (0.1), IK (0.1).

2.4. Experimental design

The reactor was completely filled with the inoculum and hydrothermally pretreated BSG slurry, leaving no headspace. No nitrogen purge was performed; however, the full-volume filling and hermetic sealing ensured that any residual oxygen was negligible. The anaerobic digester was inoculated with 4 L of 12 g TS L^{-1} fresh anaerobic sludge. The potential of raw BSG (Stage 1) to generate biogas was compared with that of hydrothermally pre-treated BSG (Stage 2). The raw BSG was resuspended in mineral salt medium (MSM) at a concentration of 25 g TS L^{-1}. The digester was daily fed with 260 mL of raw or pretreated substrate at a pH of 7, which entailed a hydraulic retention time (HRT) of the digester was 15 days. Process operation with raw BSG was conducted for 90 days, while process operation with pretreated BSG was also performed for 90 days. The collected biogas volumes were expressed under standard conditions (0 °C and 1 atm). The gas temperature was measured directly at the gas collection point to ensure accurate standardization. Following volumetric quantification, the composition of the produced biogas was analyzed using gas chromatography (Bruker, Palo Alto, USA), allowing for precise determination of methane and carbon dioxide concentrations. The sampling procedure with a gas-tight syringe might have entailed the presence of N_2 in the gas chromatogram due to air intrusion in the needle during sample transportation. Liquid samples were periodically collected from the effluents of the pretreatment unit and anaerobic digester to monitor the concentrations of VS, TS, TSS, VSS, alkalinity, pH, the organic acid profile, and ammonium ($N–NH_4^+$), total nitrogen (TN) and total organic carbon (TOC) concentrations. Key performance indicators included CH_4 concentration in the biogas, stability index, and CH_4 productivity and yield. Three samples were collected for analysis of the microbial community composition: INOCULUM from the inoculum, BSG1 at the end of Stage 1 and BSG2 at the end of stage 2.

2.5. Analytical methods

Alkalinity, pH, VS, TS, Total Suspended Solid (TSS) and Volatile Suspended Solid (VSS) concentrations were determined using standard methods [12]. Gas composition was periodically monitored using a gas chromatograph Bruker 430 GC-TCD (Bruker, Palo Alto, USA). The stability index, CH_4 productivity, and yield were reported according to the methodology of García-Depraect et al. [13] Briefly, the volumetric methane production rate (VMPR), in $mLCH_4/L/d$, was calculated using Eq. (1), where QCH_4 is the daily methane production ($mLCH_4/d$) and V the volume of reactor (L). Dissolved TOC, IC and TN concentrations were analyzed using a TOC-V_{CSH} analyzer equipped with a TNM^{-1} module (Shimadzu, Japan). Ammonium concentration was quantified applying the Nessler analytic technique in a UV-2550 spectrophotometer (Shimadzu, Japan) at 425 nm.

$$VMPR = \frac{Q_{CH_4}}{V} \quad (1)$$

2.6. Meta-transcriptomics of RNA samples

2.6.1. Sample collection and preparation

RNA samples (INOCULUM, BSG1 and BSG2) were prepared following standard protocols to ensure high purity and integrity, meeting Novogene's submission criteria. The samples were shipped on dry ice to Novogene (UK) Company Limited for meta-transcriptomic sequencing. The samples underwent rigorous quality control to evaluate concentration, purity, and RNA integrity before library preparation using a meta-transcriptome-specific protocol ensuring high-quality nucleic acids for downstream analyses. Primers used were: P5 adapter in direction P5→P7′(5'→3′)AATGATACGGCGACCACCGAGATCTACAC [i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT and P7 adapter as P5→P7′(5'→3′) GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[i7] ATCTCGTATGCCGTCTTCTGCTTG.

2.6.2. Meta-transcriptomic sequencing

RNA samples were shipped on dry ice to Novogene (UK) Company Limited for meta-transcriptomic sequencing. Rigorous quality control was performed to assess RNA concentration, purity, and integrity before library preparation using a meta-transcriptome-specific protocol. The sequencing was conducted on the NovaSeq X Plus platform with a paired-end 150 bp (PE150) strategy, generating a minimum of 12 Gb of raw data per sample, with a Q30 quality score of ≥85 %. RNA libraries for three samples were prepared and sequenced using Illumina HiSeq/MiSeq.

2.6.3. Quality control and preprocessing

Raw sequencing data underwent stringent preprocessing using Fastp (https://github.com/OpenGene/fastp) to trim adapters, remove low-quality reads (quality score <5), and discard sequences containing >10 % unknown nucleotides. For RNA samples, Novogene's bioinformatics pipeline included additional steps such as rRNA depletion and de novo assembly using Trinity. For RNA samples, host contamination was removed by mapping reads using Bowtie2 with sensitive end-to-end alignment settings.

2.6.4. Functional annotation and taxonomic profiling

Functional annotation of genes was performed using databases including KEGG, eggNOG/COG, and CAZy, with DIAMOND (https://github.com/bbuchfink/diamond/) used for high-speed sequence alignment (e-value threshold: 1e-5). Gene Ontology (GO) terms and KEGG pathway mappings were used to analyze functional enrichments, with ClusterProfiler utilized for statistical and visual analyses. Taxonomic profiling of rRNA and mRNA was carried out using MetaStat analysis to provide insights into microbial community composition and activity.

2.6.5. Differential expression and comparative analyses

Differential expression analyses were conducted across INOCULUM, BSG1, and BSG2 samples using DESeq2. Enrichment studies utilizing GO terms and KEGG pathways were performed to identify significant metabolic shifts. Comparative analyses, including functional clustering and taxonomic profiling, highlighted microbial adaptations to substrate complexity and pretreatment. Functional shifts were visualized through PCA, Bray-Curtis clustering, and heatmaps.

2.6.6. CAZyme and meta-functional profiling

To evaluate carbohydrate-active enzymes (CAZymes) in microbial communities, genes were annotated using the CAZy database, categorized into families such as glycoside hydrolases (GH), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), auxiliary activities (AA), and polysaccharide lyases (PL). Differences in CAZyme abundances were compared across sample groups to understand enzymatic contributions to biomass degradation.

2.6.7. Data delivery and analysis timeline

The processed data, including functional and taxonomic profiles, were securely delivered via Novogene's cloud platform. The meta-transcriptomic analysis process, from quality control to data delivery, was completed within 48 working days after QC approval. Final analyses were conducted using [Insert Software for Statistical Validation], ensuring reproducibility and accuracy of results.

2.7. Statistical analysis

Data processing was performed using Microsoft Excel 2016® (Redmond, WA, USA) and SPSS version 22.0® (IBM Corp., NY, USA). GraphPad Prism 9 (GraphPad Software Company, CA, USA) was used for graph design. Data were expressed as the mean ± standard deviation (SD) unless otherwise stated. Statistical analysis was carried out using Student's *t*-test for simple comparisons, and one-way ANOVA followed by Tukey post-hoc test for multiple comparisons Assumptions of normality and homogeneity of variances were considered in all analyses.

3. Results and discussion

3.1. Effect of hydrothermal pretreatment on carbon and nitrogen removal from BSG

Previous studies have shown that subcritical water treatment promotes hemicellulose solubilization and partial lignin depolymerization, while improving cellulose accessibility [14]. These mechanisms are consistent with the enhanced methane yields and volatile solids degradation observed in our experiments. In particular, the reduction in lignin recalcitrance under the applied conditions allowed the microbial consortium to efficiently metabolize the hydrolyzed substrate without the accumulation of inhibitory byproducts.

The analysis of TS, TSS, VS and VSS of the effluent of the anaerobic digester over the 181 days of operation revealed a decreasing trend, indicating a gradual reduction in the organic material present in the bioreactor (Fig. 2). During the first experimental phase, referred to as stage 1 (0–90 days), where non-hydrolyzed BSG was used as a substrate, a progressive stabilization in anaerobic digestion was observed, with average effluent concentrations of 8 g TSS L^{-1} and 8.5 g TS L^{-1}. This suggests that the microbial consortium gradually adapted its metabolic activity to the degradation of the non-hydrolyzed BSG.

On day 90, the original substrate was replaced with hydrolyzed BSG treated via hydrothermal pretreatment. This modification allowed for a greater accessibility of the organic compounds, reflected in a more pronounced reduction in TS and VS concentrations during the initial days of stage 2. This behavior could be attributed to the increased availability of soluble sugars and easily fermentable compounds in the hydrolyzed substrate, which likely promoted microbial activity. It is important to note that the BSG used in this study had been previously characterized in earlier stages of our research, including its compositional profile after mashing, and was evaluated under different treatments (chemical and enzymatic hydrolysis), confirming its low residual sugar content. Although washing with distilled water may have removed part of the remaining free sugars, potentially influencing the formation of 5-hydroxymethylfurfural (HMF) and secondary chars during hydrothermal processing [15], the solids obtained after HTC were directly fed into the anaerobic digestion process, minimizing possible pore-blocking effects from char deposition [15]. Furthermore, other fermentable compounds present in the HTC liquid fraction such as acetic and formic acids [16] could also have contributed to enhancing microbial metabolism. From day 140 onwards, the solids concentrations plateaued at approximately 4–5 g SST L^{-1} and 6-5 g VS L^{-1}, indicating that the system reached a new steady state. This behavior may be associated with a

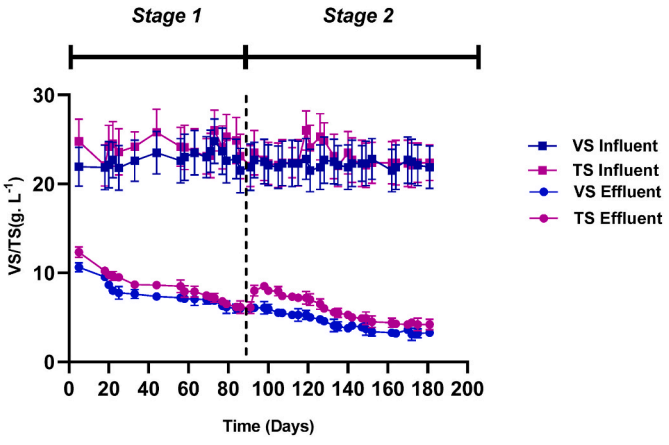

Fig. 2. Time course of ST and SV during Stage1 (non-hydrolyzed BSG) and Stage 2 (hydrolyzed BSG).

limitation in easily biodegradable compounds or the accumulation of inhibitory metabolites. Overall, the transition from non-hydrolyzed to hydrolyzed BSG exerted a significant impact on degradation dynamics. The hydrolyzed substrate increased VS degradation efficiency by 30 % compared to the original substrate, highlighting the potential of pretreatments as a strategy to enhance anaerobic process efficiency.

During stage 1, when non-hydrolyzed BSG was used as the primary substrate, ammonium levels in the influent remained constant at approximately 400–500 mg NH_4^+-N L^{-1} (Fig. 3). This reflects a relatively stable initial substrate load, consistent with the typical characteristics of BSG. In the effluent, ammonium levels were significantly lower, ranging between 50 and 150 mg NH_4^+-N L^{-1}, showing an efficient conversion into simpler nitrogen compounds and low ammonium accumulation in the system.

From day 90 onwards, a change in substrate (Stage 2) was introduced, replacing conventional BSG (non-hydrolyzed BSG) with hydrolyzed substrate. This change did not significantly affect the influent ammonium concentration, which remained stable within the range of 400–500 mg NH_4^+-N L^{-1}. However, the effluent showed a slight decrease in ammonium concentrations, possibly due to the higher biodegradability of the hydrolyzed substrate, which could be more accessible to the anaerobic microbial consortium and allow for more efficient nitrogen assimilation. These findings imply that both before and after the hydrolyzed substrate was added, the process was stable in terms of ammonium concentration and no ammonium inhibition was recorded.

The TC (data no showed) and IC concentrations recorded reflect an initial composition rich in easily accessible carbon and inorganic compounds. As shown in Fig. 4 (a), when non-hydrolyzed BSG was used during Stage 1, TC concentrations remained high at 1200–1400 mg C L^{-1}, with a predominant contribution of IC (approximately 600–800 mgC L^{-1}) and lower TOC concentrations (300–500 mgC L^{-1}).

During the first 85 days of operation, the TOC concentrations progressively decreased until stabilization. This suggested an efficient degradation of organic compounds in the BSG by the anaerobic microbial consortium, a trend similar to that observed in the nitrogen profile. The shift in substrate to hydrolyzed BSG on day 90resulted in a further reduction in TOC concentrations, reaching concentrations of ~200 mg $L^{-\cdot 1}$. This indicates that hydrothermal treatment increased the biodegradability of BSG by breaking down its structure and transforming its organic compounds into simpler and more soluble forms, such as sugar and organic acids. This facilitated their utilization by the microbial consortium during anaerobic digestion, promoting a more efficient conversion into methane and other end-products. On the other hand, IC concentrations did not show significant variations after the change in substrate, suggesting that the inorganic carbon fraction remained constant, while the organic fraction was processed more efficiently. The apparent predominance of IC over TOC arises from the carbonate/bicarbonate buffer present in the medium and produced during microbial activity, which keeps IC relatively high throughout the run. Methanogenic activity can indeed be linked to the consumption of inorganic carbon in the form of bicarbonate/CO_2 by hydrogenotrophic methanogens, while TOC consumption reflects conversion of solubilized organics by fermentative and acetoclastic pathways. The transient increase in TOC observed after hydrothermal pretreatment and at early digestion times is consistent with lignocellulose solubilization (release of fermentable sugars and soluble organics) that temporarily elevates the measured organic-carbon pool before it is metabolized. Overall, the carbon pools represent a dynamic balance between (i) BSG solubilization and hydrolysis increasing TOC and (ii) microbial conversion decreasing both TOC (to intermediates and CH_4) and TIC (via hydrogenotrophic uptake), which is typical of AD systems treating lignocellulosic feeds. Finally, the TN values in the effluent remained low (~80–100 N L^{-1}) and stable throughout the operational period, evidencing effective nitrogen management in the system likely mediated by assimilation by the microbial consortium. This also confirmed that system stability was not compromised, even after introducing the modified substrate.

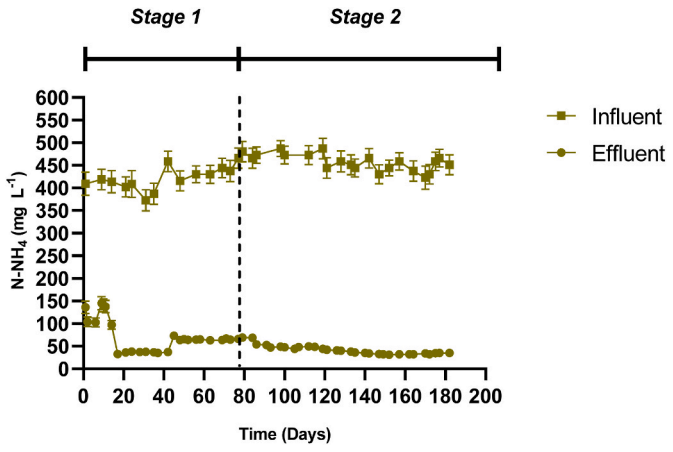

Fig. 3. Time course of the N-NH_4^+concentrations (g L^{-1}) in the influent and anaerobic effluent during Stage 1 (non-hydrolyzed BSG) and Stage 2 (hydrolyzed BSG).

3.2. Effect of hydrothermal pretreatment of BSG on alkalinity and pH

The alkalinity exhibited stable behavior in the influent and effluent throughout stage 1 and 2 (Fig. 5A). In stage I, the influent alkalinity values remained at ~2200 mg $CaCO_3$ L^{-1}, while higher levels but no significant in this process were observed in the effluent between 3000 and 3500 mg $CaCO_3$ L^{-1}. This increase was mediated by the release of intermediate products from anaerobic degradation. Process supplementation with hydrothermally hydrolyzed BSG by day 90 resulted in no significant variations in the effluent alkalinity of 3500 mg $CaCO_3$ $L^{-1\cdot}$ However, slight fluctuations were observed in the influent concentrations, which increased up to 2500 mg $CaCO_3$ L^{-1} by day 120 before stabilizing again. These fluctuations could be related to the higher content of easily degradable compounds in hydrolyzed BSG in stage 2, which could generate volatile fatty acids and require greater buffering capacity. During both stages, the constant difference between influent and effluent values reflects the microbial consortium's ability to buffer the acids generated during the process. This could be crucial to prevent adverse conditions that could inhibit methanogenic activity, especially after substrate change.

pH values fluctuated between 7.0 and 7.5 over the 180 days of operation and confirmed the optimal environmental conditions prevailing during BSG biodegradation in both stage 1 and 2. During stage 1, pH stability reflected a proper balance between the generation of volatile compounds from the fermentation of non-hydrolyzed BSG and the system's buffering capacity as evidenced by the alkalinity levels (Fig. 5A). Indeed, after the introduction of hydrothermally hydrolyzed BSG, the pH remained within the same range, which suggested that the system rapidly adapted to the new substrate without compromising its chemical balance. This is consistent with the observed alkalinity levels, which ensured adequate buffering against possible increases in organic acid production, typical of more easily biodegradable substrates. Maintaining stable pH levels is critical to avoid inhibition of methanogenic archaea, particularly after a substrate change. The conditions prevailing in this experiment ensured that the microbial consortium could operate under ideal conditions, maximizing the degradation of organic matter and its conversion to organic compounds such as methane.

3.3. Effect of hydrothermal pretreatment of BSG on biogas production

The dynamics of gases generated during the anaerobic digestion process (Fig. 6) reflect the metabolic activity of the microbial

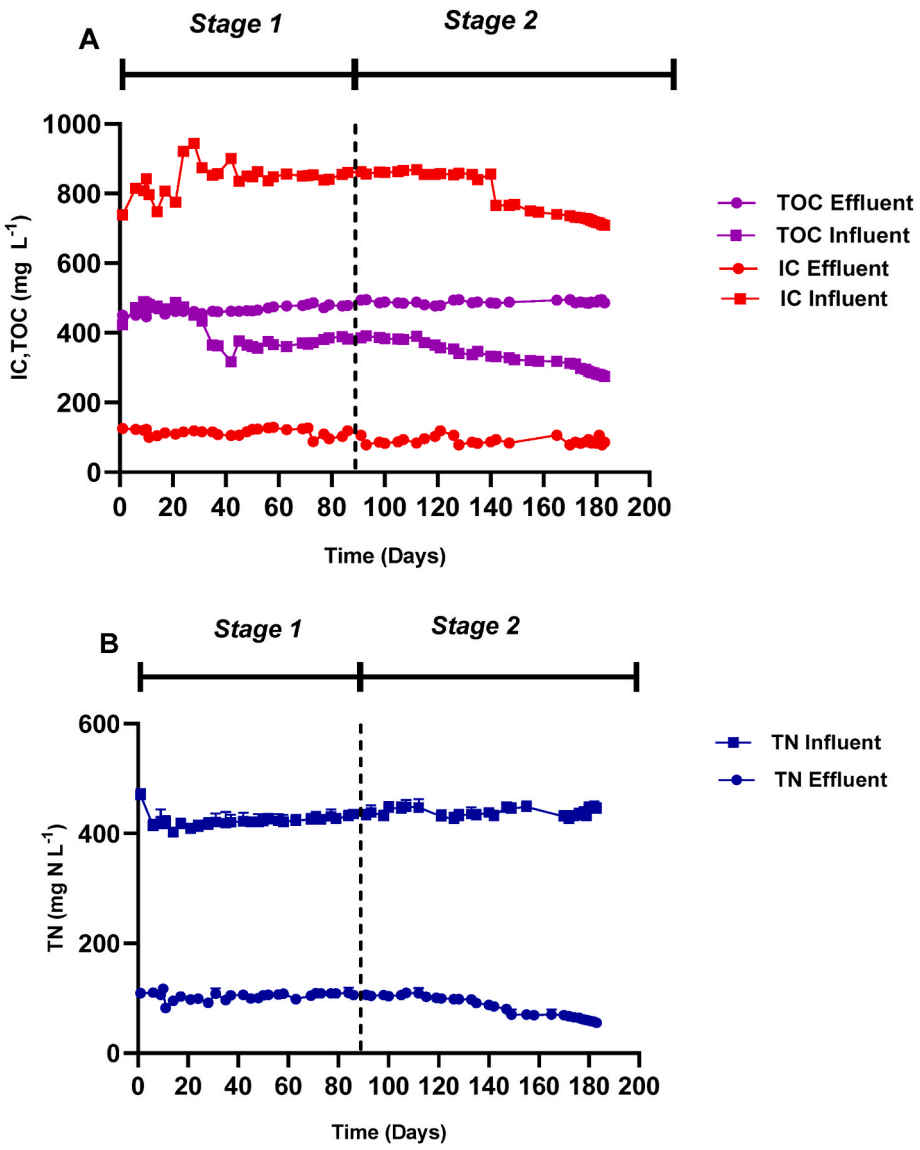

Fig. 4. Time course of the TOC and IC (A) and TN (B) concentrations (mg L^{-1}) in the influent and anaerobic effluent during Stage 1 (non-hydrolyzed BSG) and Stage 2 (hydrolyzed BSG).

consortium and its response to substrate changes. During stage 1, when non-hydrolyzed BSG was used, the biogas produced exhibited an average composition of 30–40 % CH_4 and 50–60 % CO_2. This behavior is characteristic of an anaerobic system under acclimation, where the degradation of more complex organic compounds is limited. O_2 and N_2 levels remained consistently low, which confirmed the occurrence of proper anaerobic conditions.

After the substrate change to hydrolyzed BSG on day 90, a gradual increase in CH_4 content was observed, reaching approximately 65 % by day 120 (Fig. 6). This increase was attributed to the higher availability of easily fermentable compounds in the hydrolyzed BSG, likely promoting a greater activity of methanogenic archaea [17]. Simultaneously, CO_2 levels decreased slightly, stabilizing at ~30 %, which supported an efficient system converting carbon to methane. From day 120 onwards, the system reached a new stability in biogas composition, with an average CH_4 content of 65 % and 30 % CO_2. This suggests that the degradation of more easily hydrolysable compounds was completed, leaving more recalcitrant compounds in the system.

The evolution of solids (Fig. 2) and gases (Fig. 6) shows an inverse correlation between the degradation of VS and CH_4 production. During stage 1, the CH_4 production rate was low, consistent with the limited degradation of more complex organic material. Process supplementation with hydrolyzed BSG entailed a significant improvement in system efficiency, as evidenced by an increased CH_4 generation and a faster reduction of solids, which was attributed to the conversion of degraded organic matter. These results suggest that hydrothermal BSG pretreatment could improve the degradation rate and methane production.

On the other hand, Fig. 7 shows the specific methane production (mL CH_4 (L (reactor day)$^{-1}$) over 180 days of operation of the anaerobic digester. During stage 1, CH_4 production was relatively low and stable, ranging between 100- and 500-mL CH_4 (L (reactor day)$^{-1}$), with a slightly increasing trend towards the end of the stage. This could be attributed to the structural characteristics of non-hydrolyzed BSG, rich in lignocellulosic compounds that are difficult to degrade. The limited availability of simple sugars and the low accessibility of organic matter for the anaerobic microbial consortium could have restricted biogas production.

Following the introduction of hydrolyzed BSG, a significant and sustained increase in methane production was observed, reaching values exceeding 1500 mL CH_4 (L_reactor·day)$^{-1}$ by day 100. This change reflects the positive impact of hydrothermal pretreatment, which breaks down the lignocellulosic matrix, releasing easily biodegradable

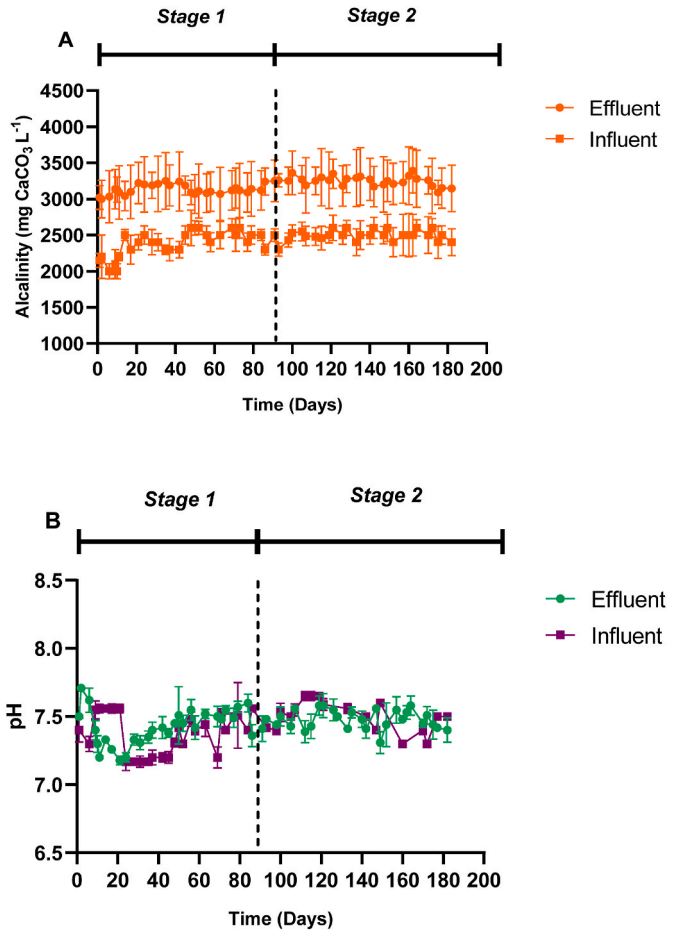

Fig. 5. Time course of alkalinity concentrations (mg $CaCO_3$ L^{-1}) (A) and pH (B) in the influent and anaerobic effluent during Stage 1 (non-hydrolyzed BSG) and Stage 2 (hydrolyzed BSG).

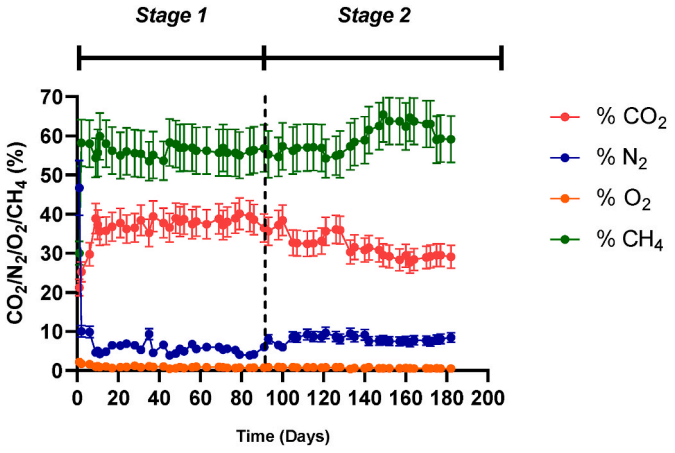

Fig. 6. Time course of biogas concentrations in the influent and anaerobic effluent during Stage 1 (non-hydrolyzed BSG) and Stage2 (hydrolyzed BSG).

compounds such as soluble sugars and organic acids. These products could be rapidly metabolized by the anaerobic consortium, resulting in increased methanogenic activity.

The change in substrate entailed a critical shift in the efficiency of the anaerobic digestion process, resulting in a two-to three-fold increase in CH_4 production. This supports the hypothesis that hydrothermal pretreatment is an effective strategy for improving the digestibility of BSG,

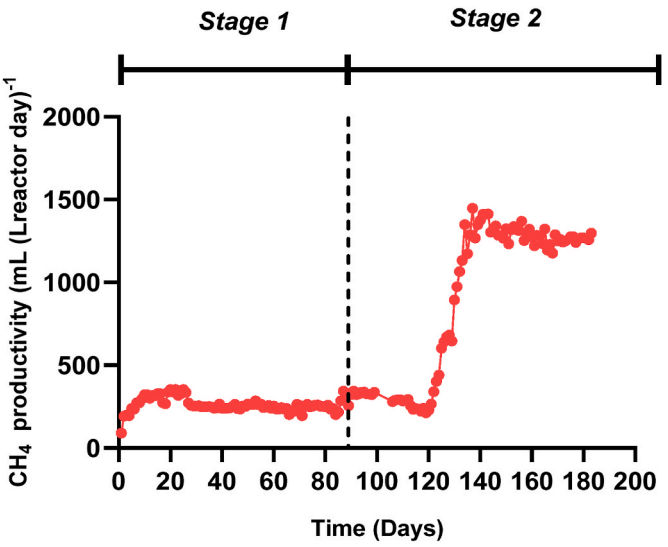

Fig. 7. Methane production rate during anaerobic digestion of non-hydrolyzed and hydrolyzed BSG.

leading to enhanced biogas yield. Furthermore, the results support the implementation of pretreatment technologies for lignocellulosic residues as a feasible approach to optimize renewable energy production in the form of biogas.

The results here obtained aligned with findings in similar studies demonstrating the enhanced biodegradability of lignocellulosic materials after hydrothermal pretreatment. Hydrothermal processes disrupt lignocellulosic structures, improving the accessibility of cellulose and hemicellulose to enzymatic degradation, as reported in previous research. For instance, Liu et al. [18] observed a significant reduction in solid residues and an increase in methane yield when hydrothermal pretreatment was applied to wheat straw. In contrast, chemical pretreatments, such as alkali or acid hydrolysis, could also enhance BSG biodegradability but often result in the production of inhibitory byproducts like furfural and HMF (hydroxymethylfurfural), which may impair microbial activity. A comparative study by Zhang et al. [19] highlighted that hydrothermal pretreatment led to a 20 % higher methane yield compared to alkali pretreatment when applied to corn stover, primarily due to the absence of toxic inhibitors. In this context, other lignocellulosic residues such as rice straw, corn stover, and sugarcane bagasse have shown similar patterns when pretreated hydrothermally. For example, Fernandes et al. [20] demonstrated that hydrothermally pretreated sugarcane bagasse achieved a 30 % higher biodegradability efficiency compared to untreated bagasse, with a corresponding increase in methane production.

In this study, the stabilization of solids at the end of stage 2 might suggest a depletion of readily fermentable substrates. This observation underscores the importance of optimizing substrate loading strategies to sustain microbial activity and prevent inhibitory metabolite accumulation. Such strategies might include co-digestion with nitrogen-rich substrates or periodic substrate supplementation to maintain system performance.

3.4. Comparison with alkali and chemical pretreatment methods

Alkali and acid pretreatments are frequently employed to enhance the biodegradability of lignocellulosic residues, but they often generate undesirable byproducts. Alkali treatments, such as those based on sodium hydroxide (NaOH), can break down lignin and hemicellulose, increasing substrate accessibility to microbial enzymes; however, they may also release phenolic compounds that inhibit microbial activity and reduce biogas yields [14]. Acid hydrolysis, while effective in increasing

cellulose accessibility, can produce furfural and hydroxymethylfurfural (HMF), both of which impair methane production.

Hydrothermal pretreatment, in contrast, effectively disrupts lignocellulosic structures while minimizing the formation of toxic compounds such as phenolics, furans, and HMF. This advantage has been demonstrated in comparative studies, where alkali treatment of corn stover showed faster degradation rates but lower methane yields due to inhibitor formation, whereas hydrothermal pretreatment maintained stable microbial activity and higher biogas production [14–16]. For example, Zerback et al. [17] reported a 20 % improvement in biomethane potential (BMP) for wheat straw, and studies with sugarcane bagasse have shown a 30 % increase in biodegradability efficiency, both attributed to enhanced hydrolysis of hemicellulose and cellulose into fermentable sugars [18].

In this study, the hydrothermal pretreatment of BSG not only increased methane production but also improved the stability of the anaerobic digestion process. The microbial consortium in the reactor degraded hydrolyzed BSG more efficiently, as evidenced by faster volatile solids (VS) reduction and higher methane content (from 30 % to 65 %). Building on previous findings by Bucci et al. [9], which identified hydrothermal pretreatment as an efficient method for ferulic acid release without chemical additives, our work extends its application to continuous anaerobic digestion.

This integration bridges biomass valorization and renewable energy production, while the continuous reactor configuration ensures operational stability and scalability, reducing batch-to-batch variability common in laboratory studies. Furthermore, coupling this continuous hydrothermal pretreatment system with meta-transcriptomic monitoring offers a distinct technological advantage: it allows both scalable operation and a mechanistic understanding of microbial shifts during AD. This dual approach not only maximizes substrate conversion and methane output but also provides actionable insights for optimizing microbial performance in industrial applications.

From a process engineering standpoint, the observed 30 % improvement in VS degradation efficiency, combined with higher methane yields, has significant implications for the energy recovery and economic feasibility of BSG-based AD plants. Although hydrothermal pretreatment requires thermal energy input, integrating waste heat recovery systems could offset these costs. Overall, this study demonstrates that a continuous hydrothermal pretreatment system, enhanced by functional microbial profiling, represents a novel and scalable solution for sustainable waste management in the brewing sector.

3.5. Meta-transcriptomics analysis

A meta-transcriptomic analysis was conducted to explore the microbial composition and functional diversity of the microbial consortium involved in the anaerobic digestion of hydrothermally pretreated BSG, named as BSG2, comparing its structure with that of the initial inoculum (INOCULUM) and the consortium adapted to the untreated BSG substrate (named as BSG1).

Fig. 8 represents the most relative abundance of different microbial phyla in the three samples analyzed: INOCULUM, BSG1 and BSG2. Significant differences in the microbial composition were observed across the analyzed samples, with the dominant phyla being Bacteroidota, Euryarchaeota, and Pseudomonadota. Other phyla, including Bacillota, Actinomycetota, Candidatus Cloacimonetes, Chloroflexota, and Thermotogota, were present in smaller proportions. The remaining phyla were grouped under the "Others" category (data not shown), highlighting the substantial microbial diversity beyond the primary phyla. These observations suggest a notable variation in microbial community structure, likely influenced by the different substrates and pretreatment processes.

A high abundance of Pseudomonadota (>15 %) was observed in the microbial consortium of the INOCULUM sample, followed by Bacteroidota (~5 %) and Actinomycetota (~3 %). The BSG1 sample (BSG without pretreatment) exhibited a similar diversity pattern to the INOCULUM, but with a significant predominance of Bacteroidota (>20 %). However, the relative abundance of Pseudomonadota decreased markedly (~2 %), while Bacillota (~5–6 %) became more prominent in comparison to the initial inoculum. Additionally, this sample showed a reduction in the "Others" category, suggesting that the new substrate may favor microbial selection, leading to a concentration of specific phyla.

On the other hand, a significant increase in Bacteroidota (>28 %) was observed in BSG2 sample, suggesting that hydrothermal pretreatment may have induced the formation of certain recalcitrant compounds available for metabolism by these microorganisms, promoting their growth and activating their microbial metabolism. Similarly, a reduction in the "Others" category was recorded, with Bacteroidota dominating but with Bacillota (~3 %), Pseudomonadota (~2 %), and Candidatus Actinomycetota (~4 %) also present, albeit in smaller

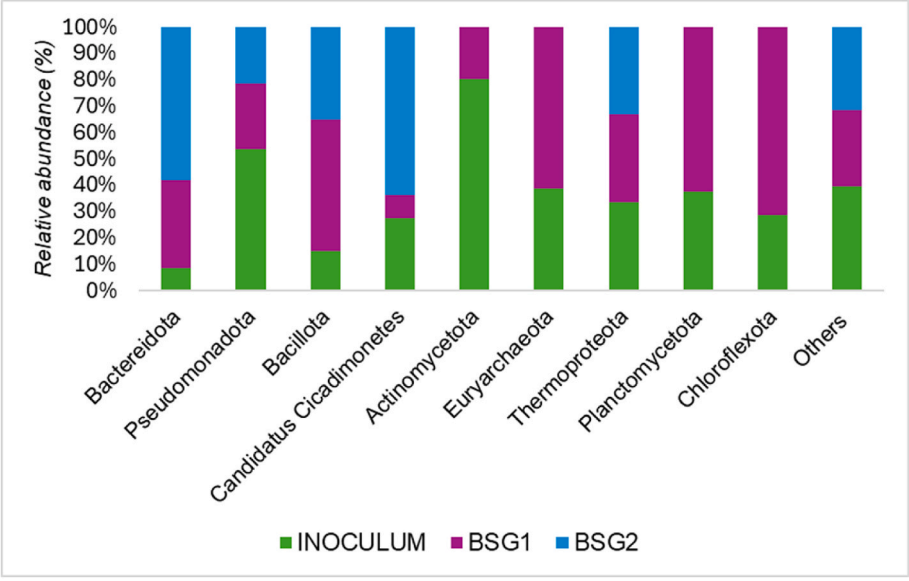

Fig. 8. Relative species abundance statistics: Taxonomic distribution at the phylum level, displaying the relative abundance of the most prevalent microbial phyla across the analyzed samples, with less abundant phyla grouped as "Others."

proportions. This diversity analysis suggests that hydrothermal pretreatment in BSG2 may have led to a reduction in overall microbial diversity (as indicated by the lower proportion of "Others"), while simultaneously increasing the relative abundance of key functional phyla such as Bacteroidota, Actinomycetota, and Bacillota. These groups are known for their ability to degrade complex polymers and participate in fermentation processes [19].

Bacteroidota, Actinomycetota, and Bacillota play crucial roles in the breakdown of lignocellulosic compounds, such as those found in BSG. Bacteroidota are primarily involved in hemicellulose degradation, with studies indicating that they contribute significantly to its breakdown during the later stages of plant residue decomposition. They produce essential enzymes such as cellulases and xylanases, which facilitate lignocellulosic material degradation. Actinomycetota, particularly members of the genus Streptomyces, are well known for their capacity to degrade lignin and cellulose, the main structural components of lignocellulose. Meanwhile, Bacillota has been identified as key players in cellulose degradation, particularly during the early stages of plant residue decomposition. This shift in microbial composition suggests that hydrothermal pretreatment enhances the bioavailability of lignocellulosic substrates, favoring microbial populations with specialized metabolic functions for efficient organic matter degradation. On the other hand, the Candidatus phylum includes organisms that have not yet been cultivated in laboratory conditions, and their taxonomic classification remains variable [20]. However, some bacteria within this group may play a role in degradation processes, although available information is still limited [21].

These findings reinforce the idea that Bacteroidota, Actinomycetota, and Bacillota play a fundamental role in the decomposition of lignocellulosic components in both natural environments and industrial applications, such as the anaerobic digestion of BSG. Their activity enhances substrate availability and acts as a selective filter, favoring microorganisms with specialized metabolic capacities for the degradation and utilization of pretreated BSG.

Notably, these dominant genera in BSG2 were already present in the initial inoculum, suggesting that the microbial consortium was previously adapted to metabolize complex organic compounds such as those present in BSG. However, when comparing the samples BSG1 and BSG2, it becomes evident that this dominance not only persists but increases significantly. This indicates that hydrothermal pretreatment does not substantially alter the microbial community structure but rather improves substrate bioaccessibility, facilitating its utilization by the present microorganisms.

On the other hand, secondary genera, although less abundant, play an essential role in maintaining microbial ecosystem stability. These include taxa such as Euryarchaeota, Chloroflexota, and Thermotogota, which, despite representing a smaller fraction, may be involved in auxiliary functions such as by-product detoxification or the recycling of intermediate compounds [22–24]. Their relative stability across the three conditions suggests coexistence without direct competition with the dominant genera, thereby contributing indirectly to the overall efficiency of the system.

These findings demonstrate that hydrothermal pretreatment, in combination with anaerobic reactor design, not only enhances anaerobic digestion efficiency but also preserves an optimal microbial balance to maximize BSG conversion into methane. This supports the development of more sustainable technologies and provides a solid foundation for future optimizations focused on improving yields and industrial scalability [23].

The clustering analysis based on Bray-Curtis distance and relative abundance distribution at the taxonomic order level shows that BSG1 and BSG2 are more similar to each other, suggesting that the use of BSG as a substrate induces similar changes in the microbial community structure. In contrast, the greater distance between INOCULUM and the other two treatments indicates that the addition of BSG, whether pretreated or untreated, significantly alters the initial microbial community [21–23] (Fig. S1). INOCULUM showed orders such as Cytophagales, Kosmotogales, Flavobacteiales, Burkholderiales, Chromatiales, Acidimicrobiales, and Xanthomonadales. This basal community reflects a generalist profile adapted to anaerobic digestion without prior exposure to BSG. However, orders such as Cytophagales and Flavobacteriales are associated with the degradation of simple organic polymers, indicating a generalist functionality in the inoculum [24]. In this context, Kosmotogales and Acidimicrobiales are known for their adaptability to extreme environments, as may be found in initial anaerobic digestion conditions. Burkholderiales and Chromatiales participate in nutrient cycling and organic compound conversion, although their role in methanogenesis is limited [23,25].

The dominant orders in the BSG1 sample include Methanobacteriales, Sphingobacteriales, Lactobacillales, Spirochaetales, Methanotrichales, Methanomicrobiales, and others. This profile demonstrates greater diversity, indicating that the microbial community works to break down the complex lignocellulosic structure of untreated BSG. Methanobacteriales and Methanotrichales are relevant to the methanogenesis process, although their efficiency in BSG1 may be limited by competition with hydrolytic bacteria for available substrates [26]. Orders such as Sphingobacteriales and Bacteroidales are associated with the degradation of complex polysaccharides, reflecting the need to break down lignocellulosic structures into simpler compounds, albeit less efficiently without pretreatment. Lactobacillales, fermentative bacteria responsible for organic acid production, may have a limited role due to the availability of fermentable sugars in untreated BSG [27,28]. Spirochaetales and Anaerolineales contribute to the degradation of recalcitrant compounds and primary fermentation, highlighting their importance in a more complex environment like BSG1. This suggests that the BSG1 sample presents a diverse microbial community working on the initial degradation of lignocellulose but with less specialization in methanogenesis due to the complexity of the substrate [29].

Finally, the profile observed in the BSG2 sample suggests that this condition supported the highest methane generation potential due to its lower relative diversity, which may indicate a more specialized microbial community. Dominant orders such as Hyphomicrobiales, Bacillales, and Pseudomonadales play key roles in the breakdown of simple organic compounds released after hydrothermal pretreatment. The substrate simplification through hydrothermal pretreatment likely facilitates the production of key metabolic precursors (acetate and hydrogen), essential for methanogenic archaea. In comparison to BSG1 and INOCULUM, BSG2 lacks specialization for handling lignocellulosic substrates and its community is geared towards general metabolic maintenance.

FPKM values within specific intervals for the three conditions were studied (Fig. S2). In the inoculum, most genes fall into low intervals (0.1–0.3 FPKM), reflecting a low initial metabolic activity. In BSG1, a notable increase was observed in medium intervals (3.57–15 FPKM), indicating an initial gene response to the untreated lignocellulosic material. On the other hand, BSG2 shows a significant shift towards higher intervals (>60 FPKM), indicating greater gene activation and expression of specialized metabolic pathways, such as those related to the efficient degradation of simpler substrates and methane production. This increase in gene expression levels supports the hypothesis that hydrothermal pretreatment enhances substrate availability and promotes a more efficient metabolic response in the microbial community [30,31].

The heatmap with KO identifiers (Fig. S3) highlights the enzymatic pathways involved in anaerobic digestion under the three conditions (https://www.genome.jp/kegg/ko.html). In BSG2, the higher abundance of KO entries such as K07497 (methanogenesis), K01153 (cellulase activity), and K12132 (hemicellulose breakdown) indicates greater availability of complex polysaccharides for microbial metabolism due to hydrothermal pretreatment. Additionally, the reduction of KO identifiers related to adaptive stress responses (e.g., K07133, oxidative stress-related enzymes) compared to BSG1 suggests more efficient metabolic activity when the substrate was pretreated. [32]. The elevated presence of K03427 (hydrogenase activity) in BSG2 correlates with improved

hydrogen turnover, a precursor for methanogenesis. Similarly, KO identifiers linked to pathways for fermentation intermediates, such as K01338 (acetate kinase) and K01156 (formate dehydrogenase), are notably enriched in BSG2. This reinforces the hypothesis that hydrothermal pretreatment not only enhances the digestibility of the substrate but also facilitates the activation of downstream metabolic pathways critical for methane production [33]. Interestingly, in BSG1, KO identifiers such as K02035 (transport proteins for recalcitrant sugars) and K01955 (oxidative enzymes for lignin breakdown) were more prevalent, reflecting the need for more diverse enzymatic activities to initiate the degradation of untreated bagasse. This diversity is reduced in BSG2, where the microbial community focuses on more specialized pathways for converting simple sugars into methane, as evidenced by the enrichment of K04763 (methanogen-specific methyltransferases) [34]. Additionally, KO identifiers such as K01869 (glucose-6-phosphate isomerase) and K01153 (cellulase) in INOCULUM represent baseline metabolic activity. However, the introduction of BSG1 prompts an increase in pathways targeting lignocellulosic complexity, as indicated by K01955 (lignin-peroxidase) and K02035 (sugar transporters). These pathways reflect the microbial community's effort to manage the structural challenges of untreated BSG. In BSG2, hydrothermal pretreatment simplifies the substrate and reduces the need for diverse enzymatic activity, allowing for a concentration on methanogenic pathways. The increased abundance of K03427 (hydrogenase activity) and K01338 (acetate kinase) supports the efficient conversion of accessible intermediates into methane. This functional shift highlights the hydrothermal pretreatment's role in reducing redundancy and directing microbial activity towards streamlined energy recovery.

These results underscore the critical role of hydrothermal pretreatment in reducing metabolic redundancy and directing microbial activity towards efficient energy recovery. The observed enrichment of key enzymatic pathways in BSG2 supports the conclusion that pretreatment enhances both the accessibility of lignocellulosic substrates and the metabolic efficiency of microbial consortia.

4. Conclusion

The study demonstrated the potential of BSG hydrothermal pretreatment as an effective strategy to overcome lignocellulosic barriers that limit biogas production through anaerobic digestion. This process has been shown to significantly improve the degradation of volatile solids and methane production, with efficiency increases of up to 30 % and an increase in methane from 30 % to 65 %. The integration of a continuous reactor for pretreatment not only optimized operational parameters but also reduced the formation of inhibitory compounds, enabling greater scalability and economic feasibility for industrial applications. Advanced genomic analysis revealed how BSG pretreatment enhances the activity of methanogenic archaea and fermentative bacteria. This established a direct correlation between microbial composition and biogas yield. This dual approach, combining hydrothermal pretreatment technology with metagenomic tools, opens new possibilities for customizing and improving anaerobic digestion processes based on substrate characteristics and microbial communities. This work not only validated the potential of BSG as a renewable source for clean energy production but also laid the foundation for advancing toward a more sustainable circular bioeconomy. The combination of innovative technologies and in-depth microbiological analysis positions hydrothermal pretreatment as a key solution for large-scale industrial waste valorization.

CRediT authorship contribution statement

Paula Bucci: Writing – original draft, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis. **Danilo Cantero:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Data curation, Conceptualization. **Andrea Casas:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Enrique Marcos:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Enkeledo Menalla:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Raúl Muñoz:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2025.108399.

Data availability

No data was used for the research described in the article.

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