

Biogas potential of orange peel biomass in anaerobic digestion (AD): Kinetics, Volatile fatty acids, microbiomes, and metabolic pathways

Guozhao jia

Lanzhou University

Nandini Thakur

Lanzhou University

Maha A Alharbi

Princess Nourah bint Abdulrahman University

Sedky H. A. Hassan

Sultan Qaboos University

Roba Alatawy

University of Tabuk

El-Sayed Salama

salama@lzu.edu.cn

Lanzhou University

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Abstract

High production of citrus waste possesses environmental threats that can be tackled by its valorization through anaerobic digestion. Thus, in this study the impact of dried orange peel waste (OPW) at various concentrations from 30 to 120 g was assessed on biogas production, volatile fatty acids (VFAs), and soluble chemical oxygen demand (sCOD). The microbial community (including bacteria and archaea) shifts, as well as the associated functional enzymes and metabolic pathways, were also predicted. The highest cumulative biogas production (117.58 mL g VS) and total VFAs increased up to 11.33 g L, which was observed at 90 g dry weight of OPW addition. Bacterial genera, including , , and , increased by 18%, 1.52%, and 13.45%, respectively, in 90 g OPW reactors. The abundance of acetoclastic and hydrogenotrophic increased up to 53.84% and 11.5%, respectively. The 120 g OPW appears to reduce biogas production by 32 mL g VS, which is 3.67 times lower than that of the 90 g OPW. Thus, the continuous feeding of an optimal amount of substrate and pretreatment can improve biogas production from readily digestible substrates.

1. Introduction

The global orange production reached 47.4 million metric tons in the years 2023–2024, with Brazil and China as the leading producers, each contributing 15.3 million tons [1]. The processing of oranges within juice production industries generates a significant amount of waste, which primarily comprises peels (60–75%), membrane residue (23–33%), and seeds (0–9%) [2]. The quantity and composition of orange waste generated during juice processing vary based on the cultivar and geographical growing parameters, which define the peel's development [3]. A huge portion of this waste is often discarded without proper treatment, resulting in both environmental and economic challenges. Different biological and thermochemical strategies (such as pyrolysis and ultrasonication) were adopted for the treatment of fruit/vegetable waste [4–6].

The implementation of treatment approaches depends on the composition (i.e., energy content) of biomass [7]. The orange peel waste (OPW) contained mono and disaccharides (including glucose, fructose, and sucrose, 6–8%), polysaccharides (pectin, cellulose, and hemicellulose, 1.5–3%), and organic acids (citric and malic acids, 0.5–1.5%) [8]. OPW were used for ethanol production, pectin formation, and polyphenols through thermochemical extractions. However, the high processing costs and energy demand associated with these methods necessitate the development of more efficient and cost-effective management approaches for OPW.

Anaerobic digestion (AD) has emerged as a promising technology for OPW valorization, due to its capacity for high biogas production. The AD process offers several advantages, including high biodegradability, low energy input requirements, a high methane content in biogas (65–70% methane), and the avoidance of odor emissions, making it the best choice [9]. The presence of D-limonene in OPW is responsible for 68–98% of orange's scent/flavor and has antimicrobial activity. This might inhibit microbial activity, resulting in low biogas production. Previous studies have predominantly focused on

co-digestion approaches, combining OPW with other substrates, such as crude glycerol, to mitigate the inhibitory effects of compounds like D-limonene and improve methane yields [10]. The mixed substrate OPW-crude glycerol AD resulted in $501.3 \text{ m}^3 \text{ CH}_4 \text{ ton}^{-1}$ volatile solids (VS), for biochemical methane potential (BMP). A mesophilic continuous AD reactor inhibits biogas production when the concentration exceeds 400 mg L^{-1} [11]. However, there is limited research on optimizing mono-digestion loading rates and process parameters specifically for OPW without co-substrates and pretreatment, which is critical for evaluating its standalone feasibility and biogas potential.

Thus, the biogas potential of OPW in mono-digestion and effect on the microbial community was examined. The biogas production and kinetics at different loadings (30, 60, 90, and 120 g dry weight) were investigated. The trends in volatile fatty acids (VFAs) production and utilization, as well as soluble chemical oxygen demand (sCOD), were monitored to assess substrate degradation. The microbial community (including bacteria and archaea) analysis was conducted to determine the active and synergistic communities at high OPW loading. The major metabolic pathways and functional enzymes of bacteria and archaea involved in OPW digestion were also predicted.

2. Materials and Methods

2.1 Sample, inoculum collection, and characterization

The anaerobic microbial inoculum was obtained from a pilot-scale domestic biogas plant operating at mesophilic temperatures in Yuzhong County, Lanzhou, China. The oranges were purchased from a local fruit market near Lanzhou University, Gansu, China, and then they were peeled. The OPW was first dried at 60°C and then crushed in a blender (Chigo, ZG-L74A, China) operating at a maximum power of 300 W, then passed through a stainless-steel sieve with a 1 mm size. The physicochemical characterization of the finely ground OPW, including moisture content, total solids (TS), volatile solids (VS), and ash content, was conducted following standard procedures described in previous studies. [12, 13]. Moisture content was determined by drying samples in the oven at 105°C overnight until constant weight was achieved following the ASTM E871 standard [14]. The dried samples were then incinerated in a muffle furnace (Kewei yongxing instrument Co., Ltd., Beijing, China) at a temperature of 550°C for four hours to measure the TS, VS, and ash content. The loss on ignition corresponded to the VS, while the remaining residue represented the ash content (Table 1). All samples were stored in the fridge at 4°C for later use.

Table 1
Characterization of inoculum and orange peel waste (OPW) used in this study.

Parameters	Inoculum	OPW
Total solids (TS, %)	2.49 ± 0.31	22.61 ± 0.25
Volatile solids (VS, %)	1.24 ± 0.01	15.70 ± 0.36
Moisture (%)	97.50 ± 0.31	77.39 ± 0.25
Ash (%)	1.24 ± 0.18	6.83 ± 1.16

2.2 Experimental design

The OPW was taken at different loadings (30, 60, 90, and 120 g dry weight) to maintain TS concentrations (2.49–11.53%) for effective mixing with inoculum during mono-digestion. The control reactors were loaded with anaerobic digestion sludge (used as a source of inoculum) only without any substrate (i.e., no OPW) (Table 2). The AD bioreactor was flushed with nitrogen gas (99% pure) for 15 minutes to attain an oxygen-free environment. The bioreactors were closed tightly with butyl rubber septa enclosed in hard plastic caps to achieve an anaerobic condition for 40 days. Each loading rate was tested in triplicate, and a total of 15 reactors were operated under mesophilic conditions at 35°C using a shaker incubator. The total volume of the reactors was 0.5 L in which the working volume and headspace were 0.3 mL and 0.15 mL, respectively. All the reactors were continuously mixed at a rotating speed of 150 rpm (Fig. S1). The initial pH of the substrate was adjusted to 7.0 with 1M NaOH to provide optimal conditions for microbial activity [15].

Table 2
Anaerobic digestion of orange peel waste (OPW) with different loading rates.

OPW (g)	Inoculum (mL)	Total TS (%)	Total VS (%)	ISR
0	300	2.49	1.24	-
30	300	4.751	2.811	1.08
60	300	7.012	4.382	2.2
90	300	9.273	5.953	3.1
120	300	11.534	7.524	4.14

2.3. Analytical analysis

2.3.1. Liquid fraction

Liquid samples were collected after 5 days of incubation to analyze changes in sCOD, VFAs production, and pH. The concentration of sCOD was determined using a commercial LH-DE-500 COD kit (Lianhua

Technology, Beijing). Briefly, a 2.5 mL diluted sample was mixed with 0.7 mL of LH-D500 reagent and 4.8 mL of LH-E500 reagent, then incubated at 165°C for 10 min [16]. After cooling, 2.5 mL of distilled water was added, and sCOD was measured in triplicate using a multi-parameter water quality analyzer (5b-3b, V 8, Lianhua Technology, Beijing).

For VFA analysis, the liquid samples were first filtered through a 0.22 µm filter, then mixed with 200 µL of 1% orthophosphoric acid. The mixture was cooled in ice for 30 min, and centrifuged at 11,800 rpm for 2 min. The resulting supernatant samples were filtered again to remove salt contaminants and aliquoted into vials for analysis. Quantification of VFAs was performed using gas chromatography (9790 plus Fuli, Zhejiang, China), equipped with a flame ionization detector (FID), and a KB-FFAP column (30 m×0.32 mm; 0.25 µm film thickness). The gas chromatography conditions were set as follows: inlet temperature at 250°C, the outlet at 162°C, and the oven at 280°C [16]. The pH of the liquid fraction was measured using a calibrated pH meter (PH 400, Alilas co, Ltd., Beijing, China).

2.3.2. Biogas analyses and kinetics

A 500 mL gas bag was connected to each digester for collecting the headspace biogas. The biogas produced during digestion was collected in the gas bags and quantified by using a calibrated glass syringe [17, 18]. This syringe-based sampling method allowed for the precise quantification of biogas volume, enabling the accurate monitoring of cumulative gas production throughout the retention period. Biogas production was calculated based on the total VS of the reactors, and kinetics were evaluated using various kinetics models. First-order kinetic model (FOM) (Eq. 1) was used to investigate the hydrolysis rate constant [19]. The modified Gompertz model (MGM) (Eq. 2) was adopted for the potential of biogas generation and microbial growth dynamics. MGM exhibits a conventional sigmoidal curve, reflecting the dynamics of microbial growth [20].

$$y(t) = ym * (1 - \exp(-k * t))$$

1

$$y(t) = ym * \exp \left\{ -\exp \left[U * \frac{e}{ym} (\lambda - t) + 1 \right] \right\}$$

2

Where, y(t): cumulative biogas production (mL g⁻¹ VS_{total}) at time t (d), ym: biogas production potential (mL g⁻¹ VS_{total}), U is the maximum biogas generation rate (mL d⁻¹), λ: lag-phase duration (d), t: cumulative time (d) for biogas production, e: mathematical constant (2.718282), k is biogas rate constant (d⁻¹).

2.4 Microbial community analysis

2.4.1 DNA extraction and amplification

The samples were collected in 10 mL Falcon tubes after digestion was completed to identify the microbial communities of all digestors [21]. The samples were washed with a phosphate buffer solution and centrifuged at 4°C for 10 min. A purified DNA sample (50–100 ng) was extracted from digestate and kept at -80°C [22]. The 16S rRNA gene regions (V3-V4) were selected for characterizing the microbial diversity (including bacterial and archaeal species). Bacterial 16S PCR amplification was performed using forward and reverse primers 519F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GACTACHVGGGTATCTAATCC-3'), respectively. While for the amplification of archaeal DNA, the primer Arch-349F (5'-GYGCASCAGKCGMGAAW-3') and Arch-915R (5'-GTGCTCCCCGCCAATTCCT-3') were used [18, 23]. The extraction of DNA from digestate was performed according to the manufacturer's instructions using the MiSeq reagent kit v3 (Illumina, USA). The DNA was run on an agarose gel (2%), and the amplified DNA fragments were purified with the AXYPref DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using Quantus™ Fluorometer (Promega, USA).

2.4.2 Illumina MiSeq sequencing and data processing

The amplicons were purified and sequenced using a paired-end strategy (2×250 bp) by Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Shanghai Genesky Biotechnologies Inc., China). Low quality raw reads (Q score < 20) were filtered using fastp version (0.20.0) and subsequently merged with FLASH (version 1.2.7). Sequences with at least 97% similarity were clustered into operational taxonomic unit (OTUs) using the UPARSE tool (version 7.1) representative sequences for each OTU were compared against the 16S rRNA database using RDP classifier (version 2.2). Functional enzymes involved in OPW degradation and biogas generation attributed to bacterial and archaeal communities were predicted using PICRUSt2, based on the 16S rRNA sequencing data [24].

2.5. Statistical analysis

Spearman correlation analysis was conducted to evaluate the correlations between microbes, functional enzymes, and metabolic pathways, followed by visualization using a combined network heatmap using R software (version 4.4.0).

3. Results and discussion

3.1 Biogas production

The loading of dried OPW at different concentrations (i.e., 30, 60, 90, and 120 g) improved biogas production, primarily due to the presence of readily degradable sugars (Fig. 1). The highest cumulative biogas production (117.58 mL g⁻¹ VS) was observed at 90 g dry weight OPW (Fig. 1d), which was 3.41-fold compared to the control (without OPW). Followed by 60 g OPW that caused improvement in biogas production by 2.86-fold compared with control (Fig. 1c). While the 30 g OPW loading produced less biogas (Fig. 1b), approximately 2.6 times lower than the 90 g OPW. The highest OPW loading (i.e., 120 g dry weight) also appeared to inhibit biogas production, resulting in 3.58 times (32 mL g⁻¹ VS) lower than that of the 90 g OPW (Fig. 1e). The control reactors (without OPW) also exhibited biogas production due

to degradation of simpler organic compounds in the ADS (Fig. 1a). The daily biogas production decreased over time in all reactors, which may be attributed to substrate depletion, pH reduction, and the accumulation of VFAs that inhibit the methanogenic activity.

The FOM exhibited a high curve fit with the experimental biogas production data, and the hydrolytic rate constants were 6.39 and 6.03 for control and 90 g dry weight OPW amended reactors, respectively (Table 3). The low hydrolytic rate constant provided by the FOM in 90 g dry weight OPW reactors indicated greater substrate availability compared to control reactors. The maximum biogas production rate predicted by MGM models for 90 g dry weight of OPW was 17.92 mL g⁻¹ VS, which also indicated increased microbial growth dynamics. Both the FOM and MGM provided the best fit for modeling biogas production from the OPW.

Table 3
Biogas kinetic models for different orange peel waste (OPW) loading.

Parameters	Control	30 g OPW	60 g OPW	90 g OPW	120 g OPW
Modified Gompertz model					
Experimental biogas (mL g ⁻¹ VS)	34.639	35.969	99.143	117.588	32.784
Predicted biogas (mL g ⁻¹ VS)	34.562	35.028	97.405	117.587	32.779
Difference between measured and predicted biogas (%)	0.08	2.61	1.75	0.001	0.01
Maximum biogas potential	6.99	1.54	4.59	17.92	2.98
Lag phase (λ)	1	1	1	1	1
R ²	0.93	0.99	0.99	0.98	0.97
First-order kinetics					
Experimental biogas (mL g ⁻¹ VS)	34.639	35.969	99.143	117.588	32.784
Predicted biogas (mL g ⁻¹ VS)	34.638	31.121	99.142	117.587	32.783
Difference between measured and predicted biogas (%)	0.01	13.47	0.01	0.01	0.01
Hydrolysis rate constant, k (day ⁻¹)	6.39	2.001	3.71	6.03	4.99
R ²	1	0.882	0.978	0.98	0.944
OPW=Orange peel waste, VS=Volatile solids					

The high concentration of OPW loading (120 g dry weight) limited biogas production, due to increased limonene content, which is toxic to methanogens [25]. The rapid degradation of organic content in the

OPW results in the accumulation of VFAs, sCOD, and a decrease in pH, which could inhibit methanogens. Previous studies reported that a higher concentration of limonene (40 mg L^{-1}) can inhibit the biogas production [8]. The addition of 2.1 g of OPW resulted in a cumulative biogas yield of 732 N mL, which is lower than that achieved with 30 g OPW. However, based on VS generated, higher biogas 286 N mL g^{-1} VS. These differences are influenced by characteristics of the inoculum, the substrate properties, retention time, and the composition of the OPW [26].

3.2 Organic compounds degradation

VFAs are critical intermediates that directly influence biogas production and pH stability in the AD [27]. The dynamics of VFAs particularly acetate and propionate, were monitored during AD of the OPW at different substrate loadings (30, 60, 90, and 120 g dry weight) (Fig. 2). The control exhibited 0.59 g L^{-1} of acetate and 0.14 g L^{-1} propionate on day 5 of the digestion (Fig. 2a). The increased acetate concentration on the 5th day (6 g L^{-1}) and the 10th day (9 g L^{-1}) in 30 g OPW showed rapid hydrolysis and acidogenesis of the OPW (Fig. 2b). The VFAs production showed accumulation after day 15 in 60 g OPW reactors with an increase in propionate concentration, compared to the control (Fig. 2c). Similarly, the 90 g OPW loading also exhibited a rapid increase in VFAs, including acetate ($5.51\text{--}8.61 \text{ g L}^{-1}$), propionate ($2.53\text{--}3.29 \text{ g L}^{-1}$), and others (C4-C6) ($3.43\text{--}4.31 \text{ g L}^{-1}$) was noted till day 20 (Fig. 2d). The accumulation of VFA in 90 g dry weight OPW after day 20 resulted in pH drop to 5.8 which reduced biogas yield and disrupted microbial community balance. The 120 g dry weight OPW reactor exhibited the highest acetate accumulation (6.1 g L^{-1}), resulting in a lower biogas yield, which may be attributed to substrate toxicity and pH disruption (Fig. 2e). However, the total VFAs production in 120 g OPW was comparatively lesser than the 90 and 60 g dry weight OPW loadings (Fig. 2f). High substrate loading limits the proper substrate mixing in reactors, thus limiting the substrate availability to microbes and lesser biogas production [28].

The sCOD was relatively low (4.09 g L^{-1}) and stable in control, indicating the minimal organic input, which was decreased to 1.80 g L^{-1} at day 40 (Fig. 3a). During the initial phase (0–15 days) of OPW loading (30–90 g dry weight), a rapid increase in sCOD is observed due to the hydrolysis of complex organic compounds. The initial sCOD of 30 g dry weight OPW was 15.53 g L^{-1} , which was decreased to 6.21 after 40 days, suggesting its conversion to biogas and microbial biomass. The concentration of the sCOD was 14.90 g L^{-1} at zero day for 60 g dry weight OPW, which slightly decreased to 13.36 g L^{-1} , indicating that sCOD is not accumulating in the digester and is utilized as it is generated during the hydrolysis. During 90 g dry weight OPW loading, the sCOD concentration was increased from 14.90 to 19.06 g L^{-1} from day 0 to day 25. Then, a decline of 17.43 was noted at day 30, representing their conversion to biogas. The initial sCOD was 18.32 g L^{-1} for 120 g dry weight of OPW, which was increased to 26.63 g L^{-1} at day 30 (Fig. 3a).

The accumulation of the total VFAs in the bioreactor due to increased substrate loading resulted in a decline in the pH [8]. For 30 g dry weight OPW, the pH slightly increased, reaching 8.2 at day 40. The 60 g,

90g, and 120 g dry weight OPW pH were 6.09, 5.63, and 4.84, respectively, at day 40 (Fig. 3b). Biogas production was stopped at mesophilic conditions when the pH level dropped below 6.4 [29]. The pH (5.6) dropped was observed in the thermophilic digester treating OPW, while for the mesophilic digester, it dropped to 3.55, resulting in total inhibition of biogas production [25]. The VFAs (acetate, propionate, and butyrate) dissociate at low pH due to decreased pKa values and can penetrate cell membranes [30]. The dissociated VFAs divert metabolic energy pathways toward maintaining cellular homeostasis, ultimately compromising process efficacy [29]. The maximum VFAs yield reached 1029.9, 3087.8, 11718.1, and 11996.3 mg COD L⁻¹ at 1:0, 1:5, 1:2, and 1:1 OPW/sludge, respectively [31]. The primary acidification products in AD of carbohydrate-rich substrates such as OPW are acetic and propionic acids, which accumulate at high concentration [32]. The acetate and propionate concentration in the reactors were highly correlated with the different bacterial phyla (Fig. 4).

3.3 Microbial analysis during anaerobic digestion

3.3.1 Bacterial diversity

The richness of bacterial OTUs in the control at day 0, control at day 40, and 90 g OPW was 428, 383, and 286, respectively. A decrease in bacterial diversity in the 90 g OPW was likely due to the substrate induced toxicity. The Shannon index, which accounts for the richness and evenness of OTUs decreased from 3.57 to 2.80 and exhibited a similar pattern to SOBs. However, it was still higher than the critical threshold (Fig. 4). The Simpson index for control at day 0, control at day 40, and 90 g OPW were 0.067, 0.107, and 0.120, respectively, also indicating the diversity reduction (Table 4). The principal component analysis (PCA) revealed cluster differences at day 0, control day 40, and 90 g OPW, indicating a significant shift in the community over time (Fig. 4). The 90 g OPW showed a separate cluster from the control on day 40 and a reduced bacterial diversity due to the presence of limonene compounds [33].

Table 4
Alpha diversity index of bacteria and archaea.

Microbes	Reactors	Sobs	Shannon	Simpson	Ace	Coverage
Bacteria	Control (Day 0)	428	3.57	0.06	471.52	0.99
	Control (Day 40)	383	3.14	0.11	404.17	0.99
	90 g OPW (Day 40)	286	2.79	0.12	318.14	0.99
Archaea	Control (Day 0)	54	2.36	0.15	54	1
	Control (Day 40)	75	1.62	0.33	75.58	0.99
	90 g OPW (Day 40)	29	0.15	0.95	45.37	0.99

The number of unique OTUs in each reactor was as follows: control at day 0, 22.23%; control at day 40, 14.57%; and 90 g OPW, 14.26%. The OTUs shared among all reactors accounted for 21.24% (Fig. 5a). The dominant bacterial composition at the phylum level included Bacillota (formerly known as Firmicutes), Bacteroidota, Synergistota, Pseudomonadota, Spirochaetota, and Actinomycetota. The abundance of Bacillota increased in the 90 g OPW reactor by 10% in the control at day 40 and by 5% compared to the control at day 0 (Fig. 5b). Bacillota abundance was positively correlated with the biogas production and Euryarchaeota population (Fig. 4c). The microorganisms classified under Firmicutes produce hydrolytic enzymes such as xylanase and pectinase that play essential roles in the degradation of the orange peel complex compounds, including pectin, cellulose, and hemicellulose [31]. The Synergistota abundance decreased across the reactors, with values of 3.4% at control day 0, 0.03% at control day 40, and 0.46% in the OPW 90 g dry weight reactor. However, it exhibited a positive correlation with the substrate solubilization and total VFAs. In contrast, Actinomycetota abundance increased by approximately 3.7 to 3.9-fold compared to day 0. Actinomycetota produce polysaccharide hydrolases, which play a crucial role in acetogenesis, the process of producing VFAs from organic matter [34].

The increase in *Enterococcus* from 3% in the control to 18% in the OPW 90 g loading at 40 days can be attributed to several factors related to pH tolerance and biofilm formation. *Enterococcus* species, such as *E. faecalis*, are capable of adapting to acidic environments with pH levels ranging from 2.9 to 4.2, demonstrating resilience against the acids produced by citrus extract [35]. *Clostridium* abundance also increased from 1.24% in the control to 1.52%. *Peptostreptococcaceae* has been also increased from 7.61% to 11.69% in OPW 90 g dry weight reactors (Fig. 5d). *Peptostreptococcus* are responsible for fermenting carbohydrates and generating butyrate and acetate [36].

3.3.2 Archaea diversity

The alpha diversity of archaea was estimated using SOBs, Shannon, and Simpson index. The SOBs were higher at day 40 in control, followed by the control at day 0 and 90 g OPW (Table 4). Richness was decreased in high concentrations of OPW loading, likely due to the presence of D-limonene and pectin that could inhibit certain methanogens. A similar pattern was observed in the Shannon index values, which were 1.62 for the control day 0, 2.36 for the control day 40, and only 0.15 for the OPW 90 g dry weight treatment. The Simpson index for 90 g dry weight OPW loading was 0.96, representing the dominance of specific methanogens (Table 4). PC2 accounted for 93.1% of the variance among the samples due to the addition of the 90 g OPW loading and temporal changes. The minimal separation between control day 0 and day 40 along PC2 indicates minor shifts in the community over time, without substrate perturbation.

The number of shared OTUs among the three samples, such as control day 0, control day 40, and OPW 90 g dry weight, is 28. The OTUs increased by 28% on day 40 compared to day 0. However, a 46.26% reduction in OTUs was observed in the 90 g dry weight loading at day 0. The unique OTUs in the control day 0, control day 40, and OPW 90 g dry weight are 12, 34, and 0, respectively. The reduction in the archaea OTUs was due to a drop in the pH and VFAs accumulation due to D-limonene in the OPW. At the phylum level, Euryarchaeota was the dominant archaeal group, comprising 59.76% of the community on day 40, which was increased by 21% as compared to day 0. Euryarchaeota showed a positive correlation with the reactor pH, which resulted in high biogas production. Unclassified archaea at the phylum level increased by 46% in 90 g dry weight OPW loading compared to day 0.

Archaea composition at the genus level was dominated by *Methanosarcina* (3.12%) and *Methanoculleus* (1.12%) at day 0 (Fig. 6). However, microbial shift was observed with increased abundance in *Methanosarcina* (53.84%) and *Methanobrevibacter* (11.5%) in 90 g OPW reactors at day 40. *Methanosarcina* was reported to tolerate high VFA production during the digestion of readily digestible substrates (i.e., fruit waste) and improve biogas production [37]. The high VFAs production during fruit waste digestion has been reported to stimulate the abundance of strict hydrogenotrophic methanogens such as *Methanobrevibacter* to promote biogas production [38]. *Methanoculleus* (7.8%) and *Methanosphaera* (5.3%) also increased on day 40 in the 90g OPW reactors, indicating adaptation to prolonged AD conditions. The abundance of hydrogenotrophic *Methanoculleus* relies on the higher availability of hydrogen and carbon dioxide produced by the fermentative bacteria during substrate digestion [39]. The presence of methanol-consuming *Methanosphaera* indicated a diverse metabolic community and pathways involved in the digestion of OPW [40]. A slight increase was observed for hydrogenotrophic *Methanospirillum* (1.4%) in 90 g OPW, which was attributed to the VFAs concentration and adaptation of both major methanogenesis pathways. Overall, the addition of dry OPW promoted the abundance of acetoclastic *Methanosarcina* and exhibited a significant shift towards hydrogenotrophic methanogens (including *Methanoculleus*, *Methanobrevibacter*, and *Methanospirillum*).

3.3.3. Major metabolic pathways and enzymes

Changes in metabolic pathways and gene abundances, providing insights into the functional dynamics of the microbial community throughout the digestion of OPW (Fig. 7). The major metabolic pathways that were dominant in bacteria were fermentation, chemoheterotrophy, and nitrate in 90 g OPW loading (Fig. 7a). Fermentation is a key step of the AD process in which organic matter is converted into acids, alcohols, and gases (such as ethanol, lactic acid, acetate, CO₂, and H₂) [41, 42]. It produces NAD⁺ for glycolysis and metabolites for methanogenesis. In 90 g OPW, fermentation gene expression reaches 47.35%, slightly exceeding the 44.7% in the control (day 40) reactors, indicating improved fermentative activity under higher substrate loading. Chemoheterotrophy, involving the oxidation of organic molecules to CO₂, H₂O, and organic acids, was expressed 2.46% more in the 90 g OPW, reflecting increased substrate utilization. The nitrate reduction pathway involved in nitrate (NO₃⁻) reduction to nitrite (NO₂⁻) also upregulated (0.12%) in 90 g OPW reactors, suggesting active nitrogen cycling that may influence microbial redox balance and composition [43, 44]

The major pathways in archaea are methanogenesis, hydrogenotrophic methanogenesis, and dark hydrogen oxidation. The terminal step in anaerobic digestion, where methanogenic archaea produce methane from CO₂ and H₂ (hydrogenotrophic), acetate (acetoclastic), or methylated compounds (methylotrophic). Hydrogenotrophic methanogenesis, methanogenesis, and dark hydrogen oxidation pathways expressed 32.93%, 33.01%, and 32.70%, respectively, in 90 g dry weight OPW loading. These results are substantially higher than those of 17.81%, 18.22%, and 11.76% in the control, indicating that optimum OPW loading (90 g) stimulates methanogenic activity, especially hydrogenotrophic pathways, possibly due to the inhibitory effects of OPW compounds on acetoclastic methanogens [10, 44].

Key enzymes involved in hydrolysis, acidogenesis, and acetogenesis were highly expressed in the 90 g dry weight OPW loading. Bacterial enzymes such as K01200 (pullulanase), K01732 (pectin lyase), degrade starch and pectin, abundant in OPW, facilitating polysaccharide breakdown. K00844 (hexokinase) supports glycolysis, while K00132 (acetaldehyde dehydrogenase), K00925 (acetate kinase), K00656 (formate C-acetyltransferase), K00009 (mannitol-1-phosphate 5-dehydrogenase), K01067 (acetyl-CoA hydrolase) participate in acidogenesis and acetogenesis, converting fermentation products into intermediates for methanogenesis (Fig. 7c). Archaea enzymes such as K00202 (formylmethanofuran dehydrogenase subunit C), K03389 (heterodisulfide reductase subunit B2), K03388 (heterodisulfide reductase subunit A2), K00125 (formate dehydrogenase (coenzyme F420) beta subunit), and K14127 (F420-non-reducing hydrogenase iron-sulfur subunit) were also abundant (Fig. 7d). The abundance of K00202, K03389, and K03388 might be due to the increase in *Methanosarcina* populations, a versatile methanogen capable of both acetoclastic and hydrogenotrophic methanogenesis [45]. These findings demonstrated that optimal OPW loading (90 g) enhanced bacterial hydrolysis, fermentation, and hydrogenotrophic methanogenesis. The coordinated upregulation of these metabolic pathways and enzymes reflects the microbial community's functional plasticity and adaptation to substrate availability, providing a mechanistic basis for improved biogas yields from OPW digestion [10, 46].

Conclusions

The 90 g OPW resulted in the highest biogas production of 117.58 mL g⁻¹ VS. This was driven by efficient hydrolysis and acidogenesis facilitated by hydrolytic bacteria such as *Enterococcus*, *Clostridium*, and *Peptostreptococcaceae*. The 90 g OPW addition improved the predominance of *Methanosarcina* (53.84%) and *Methanobrevibacter* (11.5%). Higher OPW loadings (120 g) caused VFA accumulation and pH drop, which resulted in low biogas yield. However, the excessive production of VFAs disturbed the balanced archaeal community, favoring the hydrogenotrophic methanogens (such as *Methanobacteriaceae*), while inhibiting the acetoclastic pathways. Overall, this study highlighted that a 90 g OPW loading maximizes biogas output, while higher OPW loadings require pretreatment to prevent acidification and maintain process stability. Future research should focus on the application of pretreatment to various OPW loading concentrations to enhance biogas production. The AD of OPW could also be followed after the extraction of value-added components, which can prevent substrate-induced inhibition during biogas production.

Abbreviations

AD	Anaerobic digestion
ADS	Anaerobic digestion sludge
FOM	First-order model
MGM	Modified Gompertz model
OPW	Orange peel waste
OTUs	Operational taxonomic units
sCOD	Soluble chemical oxygen demand
TS	Total solids
VS	Volatile solids
VFAs	Volatile fatty acids

Declarations

Author contribution

Guozhao Jia: Methodology, Validation, Investigation, Data curation, Formal analysis, Writing-original draft. **Nandini Thakur:** Methodology, Resources, Visualization, Formal analysis, Writing-review & editing.

Maha A Alharbi: Resources, Validation, Data curation, Formal analysis, Writing-review & editing, Funding acquisition, Project administration. **Sedky H. A. Hassan:** Resources, Visualization, Formal analysis, Writing-review & editing, Funding acquisition. **Roba Alatawy:** Resources, Visualization, Formal analysis, Writing-review & editing, Funding acquisition, Project administration. **El-Sayed Salama:** Conceptualization, Supervision, Resources, Data curation, Validation, Visualization, Formal analysis, Writing-review & editing, Funding acquisition, Project administration.

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Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

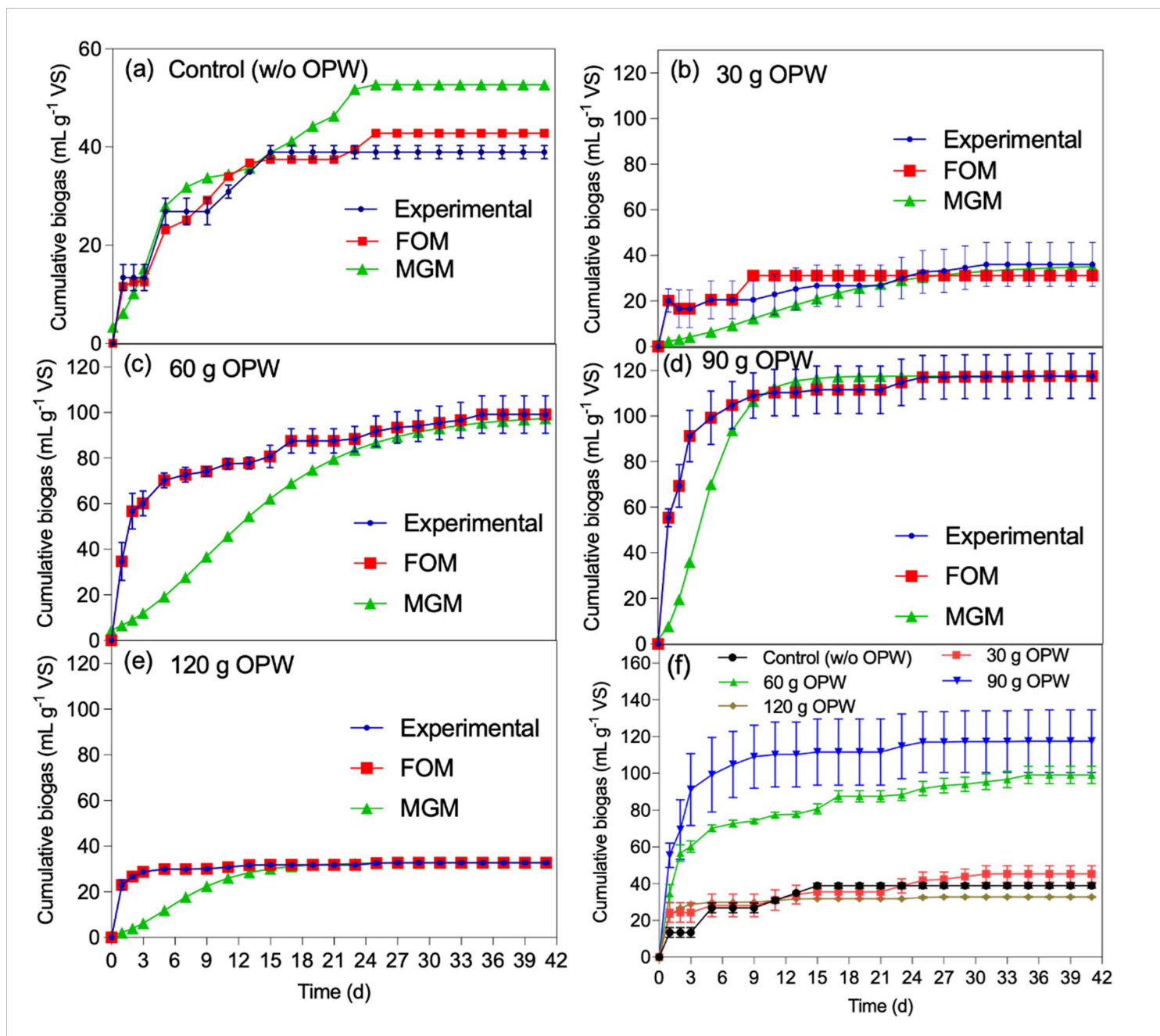


Figure 1

The biogas production under different loadings of orange peel waste (OPW). Control without OPW (a); 30 g OPW (b); 60 g OPW (c); 90 g OPW (d); 120 g OPW (e); and accumulative biogas production (f).

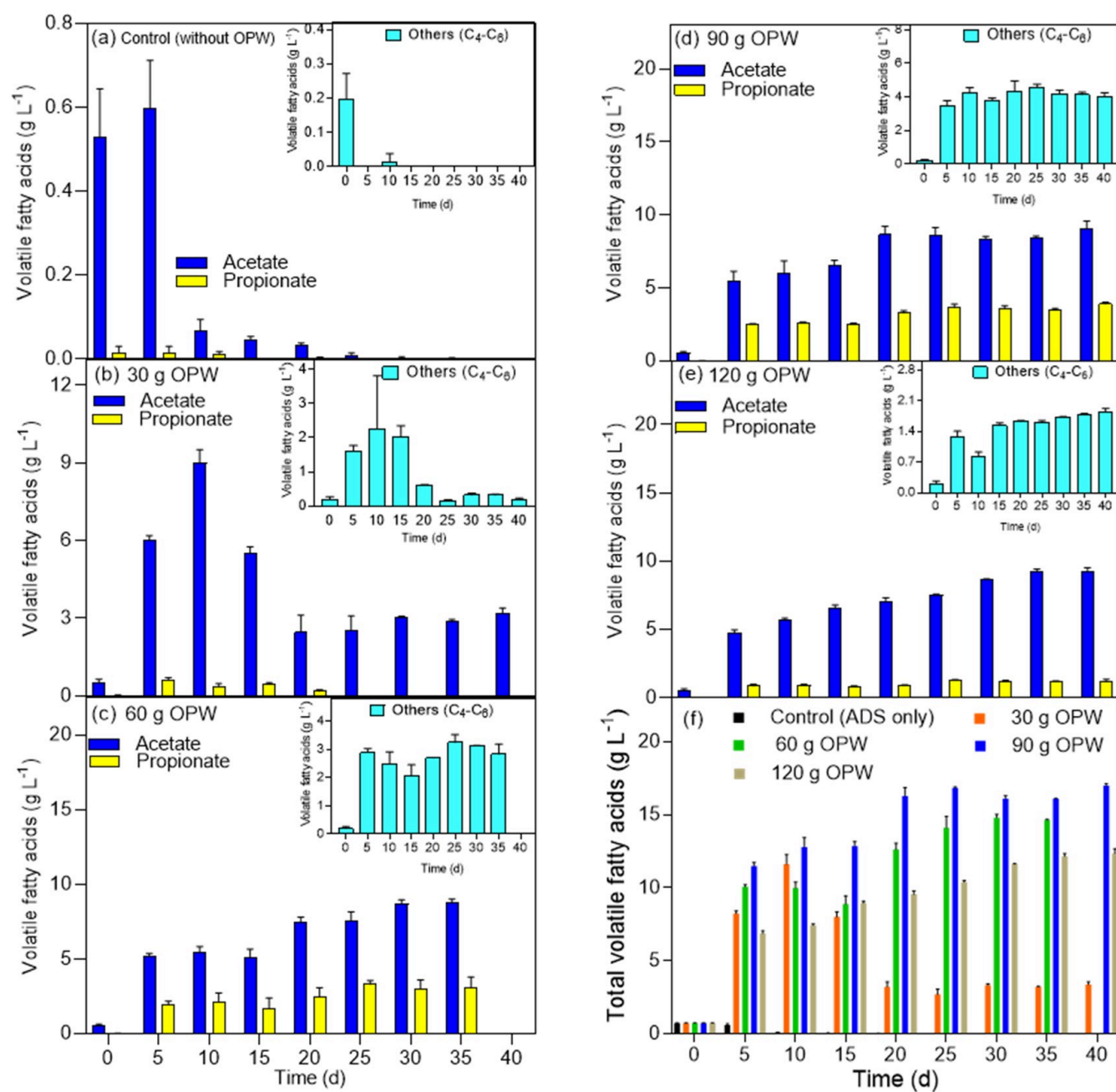


Figure 2

Volatile fatty acids (such as acetic, propionic acid, and others from carbon 4 to carbon 6 (C₄-C₆)) in orange peel waste (OPW) digestion. Control without OPW (a), 30 g OPW (b), 60 g OPW (c), 90 g OPW (d), 120 g OPW (e), and total volatile fatty acids (f).

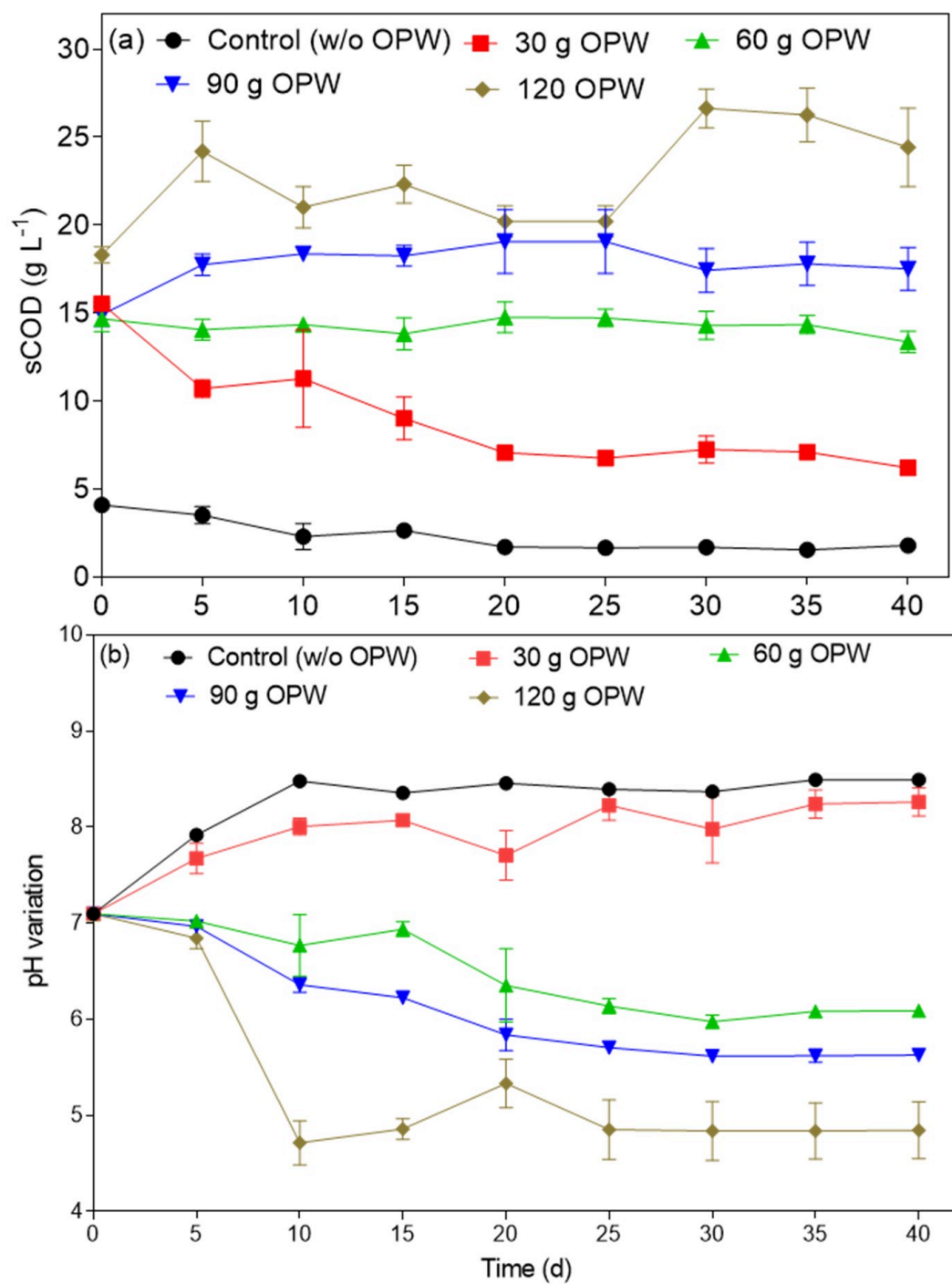


Figure 3

The soluble chemical oxygen demand (sCOD) (a) and pH variations (b) at different loadings of orange peel waste (OPW).

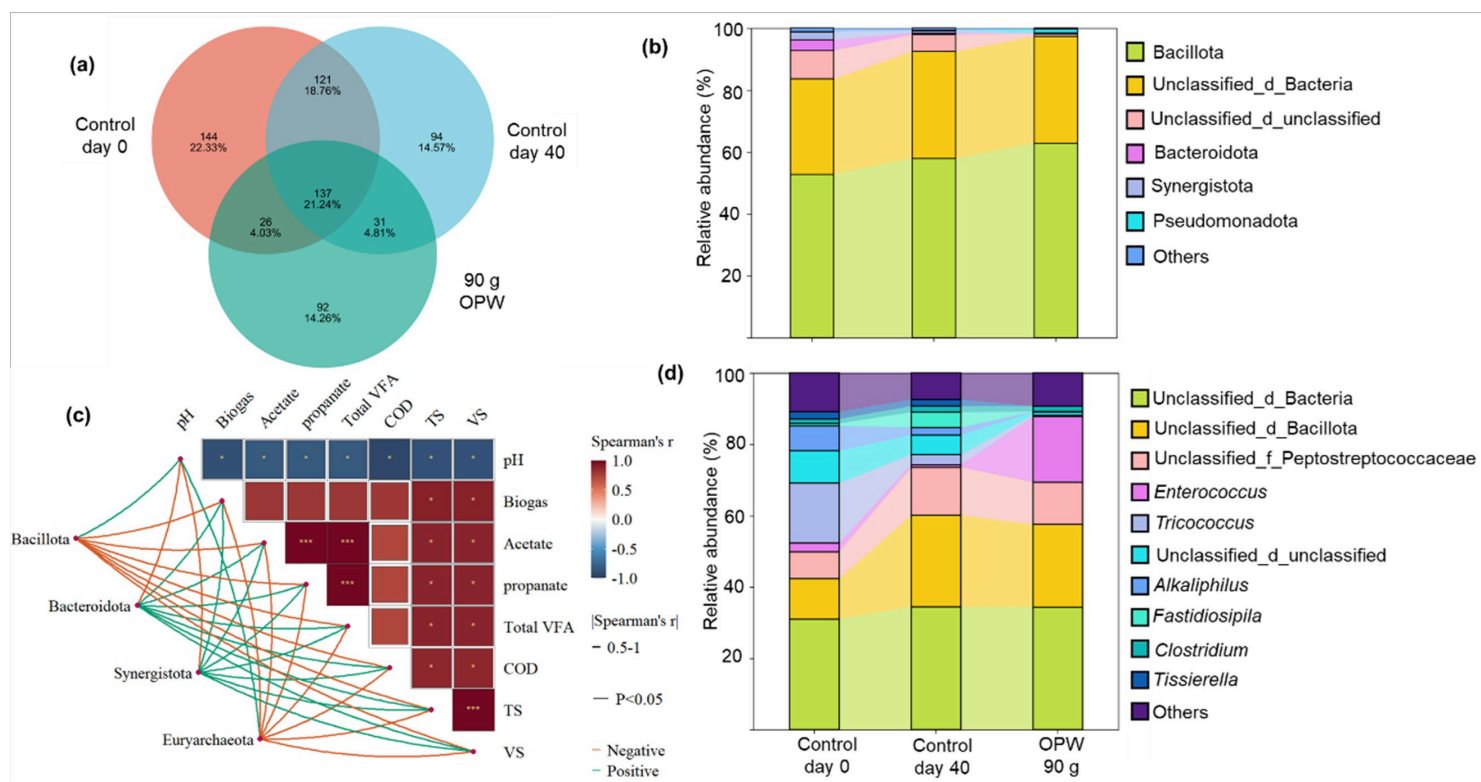


Figure 4

Bacterial community structure in control and orange peel waste (OPW) loaded reactors. Venn plot for OTUs distribution **(a)**; relative abundance of bacterial phyla **(b)**; Spearman's correlation **(c)**; and major bacterial genera **(d)**.

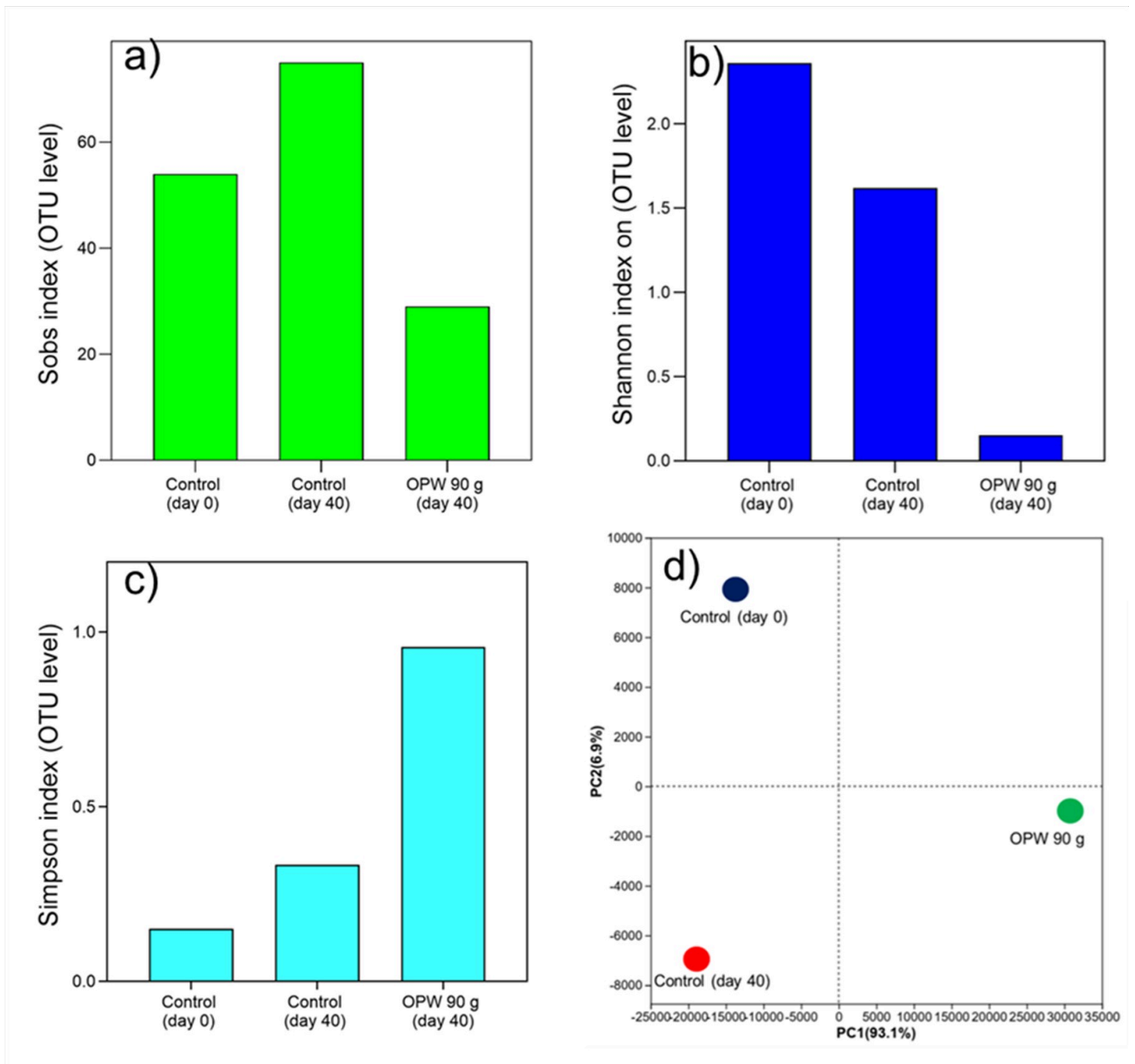


Figure 5

Archaeal diversity during the digestion of orange peel waste (OPW). Sobs index (a). Shannon index (b) and Simpson index (c), and Beta diversity (d).

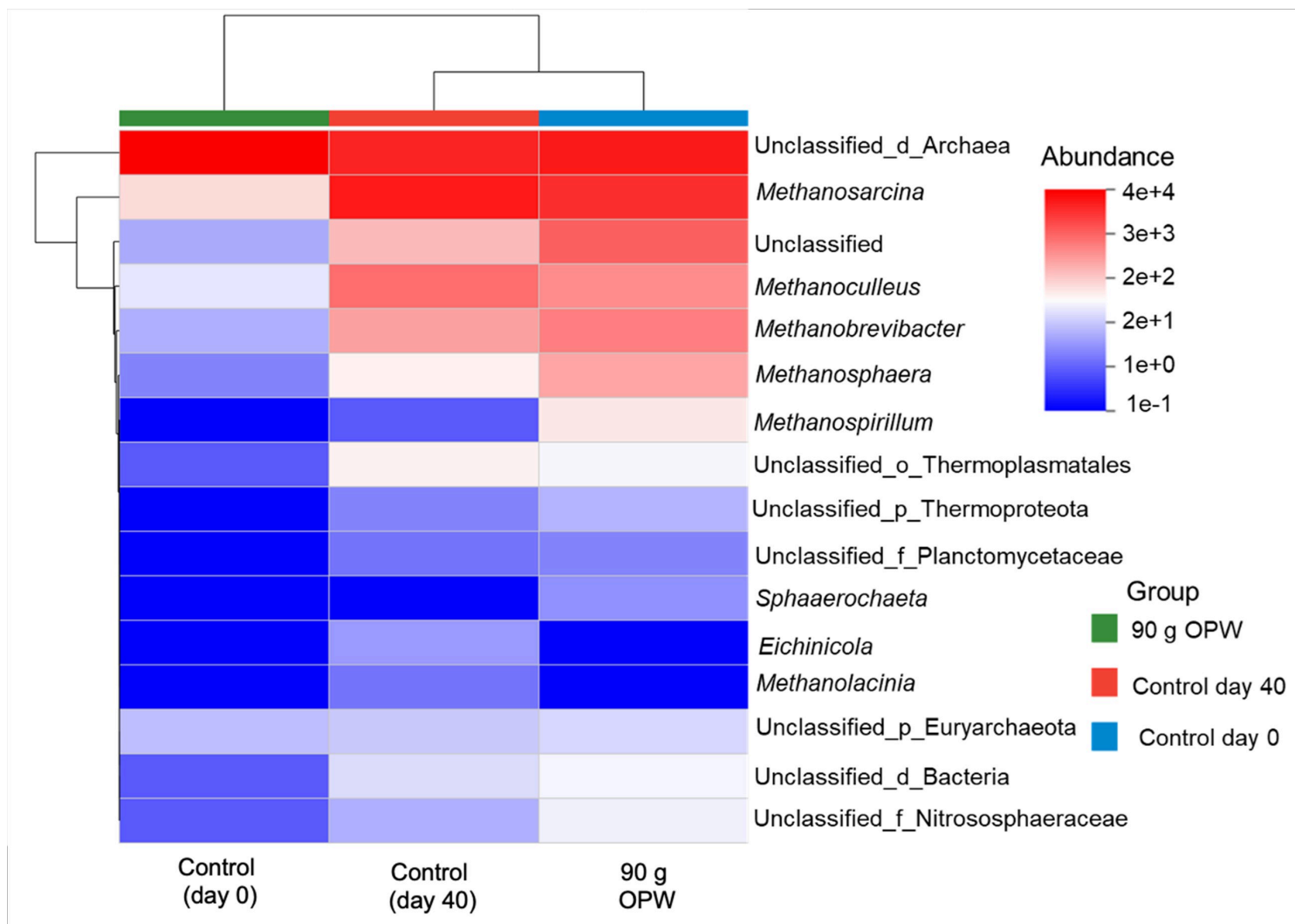


Figure 6

Phylogenetic heat map representing archaeal phyla and genera abundance in different reactors.

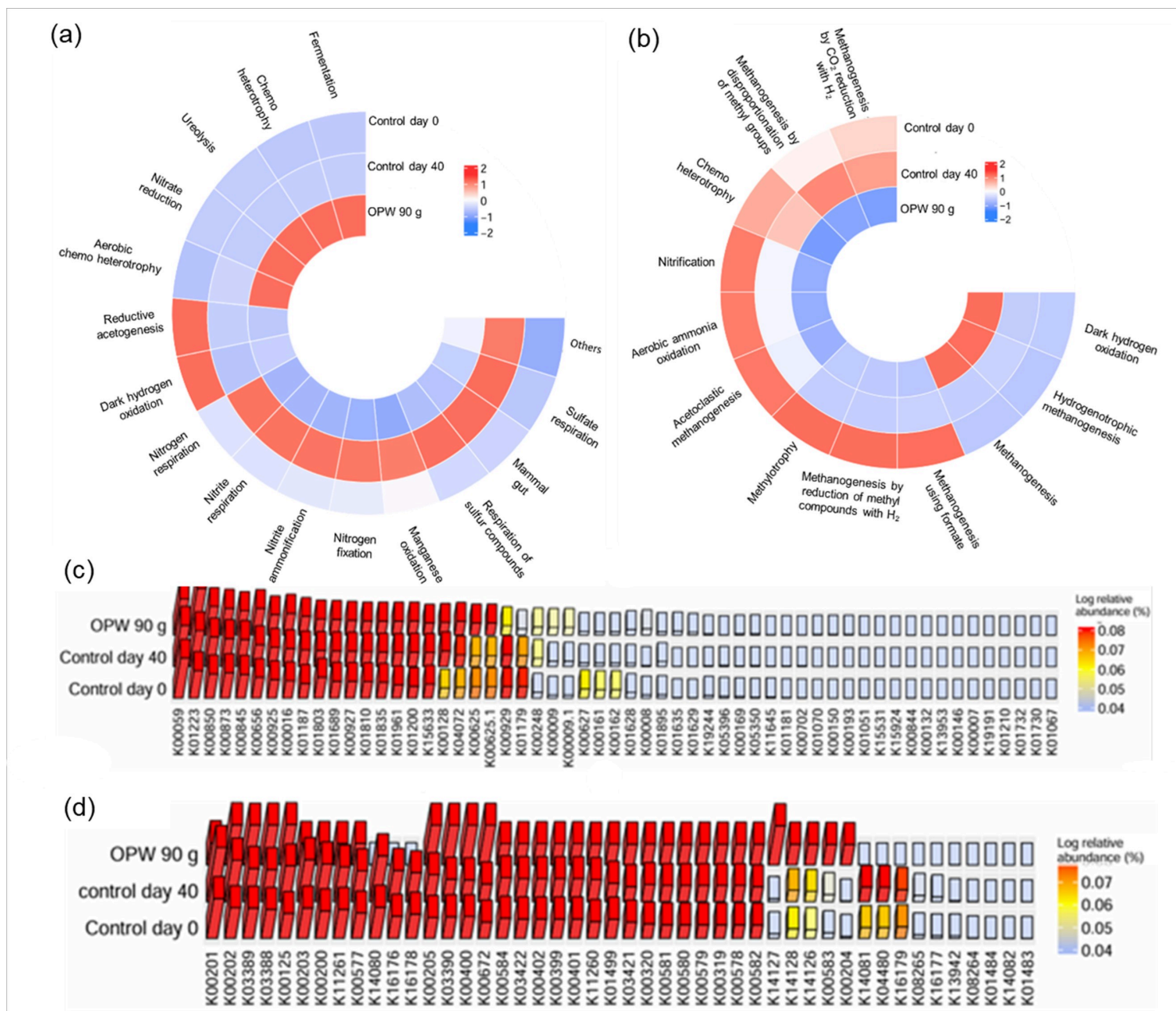


Figure 7

Metabolic pathways and functional enzymes involved in bacterial substrate degradation (a and c) and methanogenic biogas production (b and d).

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