



Innovative char-sparger for improving volatile fatty acids (VFA) production in homoacetogenic fermentation of H₂/CO₂ with microbial mixed cultures (MMC)

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ABSTRACT

The homoacetogenic fermentation of CO₂ and H₂ allows the production of renewable volatile fatty acids (VFA) using microbial mixed cultures (MMC). The main challenges of such approach, which would open a reliable power-to-X pathway, are the low product concentration and low volumetric productivity. To address such key features, which are due to product inhibition and low solubility of H₂, biomass-derived biochar and polystyrene foam were used to manufacture a porous monolith (sparger) that allows the delivery of H₂/CO₂ to bacterially colonisable surfaces. Such device was used within a novel char-based biofilm sparger reactor (CBSR), which was tested for acetogenic fermentation of H₂/CO₂ with variable dilution rates. Noticeably, a low dilution rate (0.1 d⁻¹) achieved the highest VFA concentration ever obtained (58 gCOD L⁻¹ and 52 g/L acetic acid) with MMC, whereas a higher dilution rate (0.8 d⁻¹) provided 15 gCOD L⁻¹ VFA with a volumetric productivity of 18.5 gCOD L⁻¹ d⁻¹. According to the 16S rRNA sequencing of the biofilm microbiota, *Acetobacterium* was the dominant genus with a 90% relative abundance ratio. Such performances, which are close to that obtainable with optimised axenic fermentation, suggest that the innovative char-sparger could enhance mass transfer rate, shield from product inhibition and/or improve the growth of biofilm.

1. Introduction

The power-to-X concept proposes the use of renewable power (e.g., photovoltaic, wind, hydropower), H₂O and CO₂ to produce commodity chemicals, materials, feed, and food [1].

Green hydrogen, and carbon dioxide obtained by either carbon capture technologies, biogas plants or industrial off gases (e.g. emissions from steelmaking, cement production or waste incineration) can be transformed by strictly anaerobic homoacetogens, which are capable of autotrophic assimilation of inorganic H₂/CO₂ and CO and ferment them into volatile fatty acids (VFA) [2], which can be used in one or two stages of biological conversions to obtain biopolymers, feed/food or

fuels [3].

Amongst the numerous homoacetogens identified and isolated, *Acetobacterium woodii*, *Clostridium junghdalii* and *Moorella* sp. are the microorganisms which were more extensively studied for four decades [4–13]. The anaerobic conversion of H₂/CO₂ into VFA is thermodynamically favourable [14,15], implying that yields can be close to that expected by stoichiometry. Nonetheless, given that H₂ is poorly soluble (C_{aq}*: 1.62 mg/L at 21 °C and 1 bar) [16,17] and VFA are relatively toxic (50% inhibition concentration, IC₅₀ of acetic acid is between 5 and 12 g/L [18]), the two main challenges of gas fermentation are the relatively low volumetric productivity (V_p) and the low concentration of produced VFA [19]. Given the high cost of axenic reaction volume and complex

Abbreviations: CBSR, Char-based biofilm sparger reactor; VFA, Volatile fatty acids; MMC, Microbial mixed culture; DR, Dilution rate; DIET, Direct interspecies electron transfer.

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VFA recovery, such features are especially critical for economics of processes based on single strains. The use of microbial mixed culture (MMC), which has received increased attention in the last five years, allows the use of standard wastewater treatment approaches and simplifies the process integration (e.g., inlet solution can be nonsterile and VFA can be recovered by simple overflow) [15]. According to reliable arguments, such MMC features could lower the bar of acceptable volumetric productivity (V_p) and VFA concentration by at least one order of magnitude.

Homoacetogens are ubiquitous in natural anaerobic consortia [20], as several studies demonstrated that methanogen-deprived MMC can convert H_2/CO_2 mixtures into VFAs. Omar *et al.* tested the capability of MMC (mesophilic digestate treated with bromoethanesulfonate, BES) for conversions of H_2/CO_2 , showing VFA accumulation in a batch test performed with serum bottles [21]. The same configuration was tested by He *et al.*, who used thermally treated anaerobic sludge as inoculum, achieving a production of 8.5 gCOD L^{-1} from H_2/CO_2 and V_p equal to $0.5 \text{ gCOD L}^{-1} \text{ d}^{-1}$ [22].

Modestra *et al.* and Katakajwala *et al.* evaluated the effect of increased pressure (2 and 15 bar, respectively) on the performance of a similar system (CSTR with moderate 100 rpm mixing), obtaining an increased V_p and/or VFA concentration [23,24]. As expected by the increase in H_2 solubility with increased pressure, the maximum performance obtained with CSTR was achieved at 15 bar, i.e., 4.5 gCOD L^{-1} VFA with a V_p of $1.1 \text{ gCOD L}^{-1} \text{ d}^{-1}$. Through the adoption of similar intensification approaches that were successful for single-strain studies, Steger *et al.* [25] and Baleeiro *et al.* [26] showed that increasing the cell concentration (e.g., biofilms) and K_La (e.g., gas bubbling or trickling) allows a slight increase in V_p ($1.3\text{--}2.1 \text{ gCOD L}^{-1} \text{ d}^{-1}$) and achieves a VFA concentration ($21\text{--}35 \text{ gCOD L}^{-1}$) comparable with that of single strains.

The largest improvement in V_p was certainly achieved by Wang *et al.* [27,28] and Zhang *et al.* [29,30], who used an innovative hollow fibre membrane reactor, achieving an increase in VFA concentration and productivities of one order of magnitude above any previous study. The best performance achieved with such system at 55°C allowed to produce $48\text{--}44 \text{ gCOD L}^{-1}$ of VFA with $0.8\text{--}2.1 \text{ gCOD L}^{-1} \text{ d}^{-1}$ rate [28]. Such outstanding results showed that, providing the gas onto a large and bacterially colonisable area, drastically increases V_p . Such improvement was probably due to the combined effect of increased mass transfer (due to small-semipermeable channels) and high cell concentration as biofilm, which was previously proven successfully in top performing single-strain fermentations [5,6]. In principle, a similar effect can be obtained by trickle beds, monoliths or gas spargers with attached-grown bacteria. Starting from this idea and considering the natural tendency of gas spargers to be colonised by biofilm, we designed a new type of reactor. Such reactor is equipped with a monolith made of biochar composite, which is tailored in accordance with gas fermentation needs. Amongst different materials that can be used for char-sparger manufacturing, biochar (namely the solid residue of biomass pyrolysis) has been previously shown to possess several features that could be beneficial to gas transfer [31], microbial activity [32,33], gas fermentation [34,35] and acidogenic performance [36–38]. To assess the use of biochar as growth media and gas fermentation enhancer, a novel biochar/polystyrene composite was used within a char-based biofilm sparger reactor (hereafter called CBSR), which was tested for MMC fermentation of H_2/CO_2 , thereby evaluating the performance (yield, VFA concentration and V_p) for 101 days of operation.

2. Materials and methods

2.1. Preparation of biochar–polystyrene monolith

The manufacturing process of the novel biochar-made monolith was optimised prior to this study and it is shared in detail elsewhere as a method article (hereafter referred to as MethodsX).

The final sparging device consisted of a cylinder with a coaxial

cavity, which was obtained from about 22 g biochar and 11 g of polystyrene. Biochar was ground and dry sieved to 1.0 mm to obtain powdered biochar. Polystyrene (PS) foam was softened with acetone and mixed with ground and sieved biochar. The heterogenous biochar and PS mixture was harshly kneaded with subsequent additions of acetone until the obtainment of a soft dough-like material. The latter was casted into a conical 50 mL Falcon™ test tube to provide the external shape, and a cylindrical glass rod was replaced into the centre, which provided the internal channel for gas delivery. The entire assembly was then dried at 80°C for 2 h; afterwards, the monolith was removed from the cavity. Finally, the inner glass tube was removed from the monolith and substituted with an 8 mm polyamide tubing, which was sealed with a fresh biochar–PS dough and dried again. The resulting monolith consisted of a cylinder with a truncated cone head with the following geometrical–physical characteristics: total length of 11 cm, outer diameter of 27.0 mm, wall thickness of $21.0 (\pm 2.0)$ mm, bulk volume of 63 cm^3 and 50% porosity (Fig. 1).

2.2. Char-based biofilm sparger reactor (CBSR manufacturing and test)

The CBSR system was designed on the basis of a previously developed ‘biochar-packed anaerobic bioreactor’ [39], with several modifications which were related to the specific requirements for gas fermentation at bench scale (Fig. 2). The system, whose detailed information are provided in the MethodsX article, is a 700 mL reactor (500 mL working volume + 200 mL total headspace) kept at $36 \pm 0.3^\circ\text{C}$ (measured in the middle of the liquid) with heating pads. A gas circulation pump was used to pump the gas from the bioreactor headspace (connected to a laminated gas bag) into the biochar-based sparger. Liquid and gas feedings to the CBSR system and subsequent discharging were conducted automatically six times per day; these processes were controlled by a mini-PLC system explained in detail elsewhere (MethodsX).

To allow the formation of a biofilm over the char-sparger and the release of VFA into the surrounding environment, the CBSR was operated through three consecutive phases, namely (i) squeezing, (ii) gas sparging and (iii) soaking, which are detailed in Fig. 3. The squeezing phase starts when the gas circulation pump delivers a positive pressure to the liquid-filled sparger, causing the expulsion of liquid from the channels thereof to the surrounding broth. The subsequent gas-sparging phase starts as soon as the liquid is removed from the channels, and the gas fills the sparger and makes direct contact with the microbial biofilm until it bubbles on the external wall. Finally, the gas recirculation pump is stopped, causing the soaking of the monolith channels by the surrounding broth. In practice, such cyclic operations are obtained by turning on the gas pump for 2.5 s every 10 s. Such timing allows a complete squeezing phase and minimal (<1 sec, <20 mL/cycle) bubbling time.

2.3. Gas fermentation

CBSR was inoculated with 50 mL of digestate from an industrial anaerobic digester (details in [36]), 50 mL of basal medium (according to [36]), 20 g of NaHCO_3 and 400 mL of distilled water. The pH of this suspension was 8.5, and the total chemical oxygen demand (COD) was $5.4 \pm 0.3 \text{ gCOD L}^{-1}$ with a negligible amount of soluble COD content ($<0.35 \text{ COD L}^{-1}$).

Throughout the experiment, the liquid input consisted of distilled water containing 40 g/L NaHCO_3 buffer and 10% v/v basal medium (as nutrient source for microbial growth). Such composition provided a stable pH within the target neutral-alkaline conditions (pH 6.8–8.8) without an active pH control system, as previously shown in [24]. According to the stoichiometry of homoacetogenesis, the $H_2:CO_2$ (mol: mol) ratio (Eq. 1 in MethodsX) in the gas bag was kept above two (2.4 ± 0.4) during the entire experimental period. An excess amount of H_2/CO_2 mixture, equal to 5.4 L/d, was provided continuously, and nonconverted

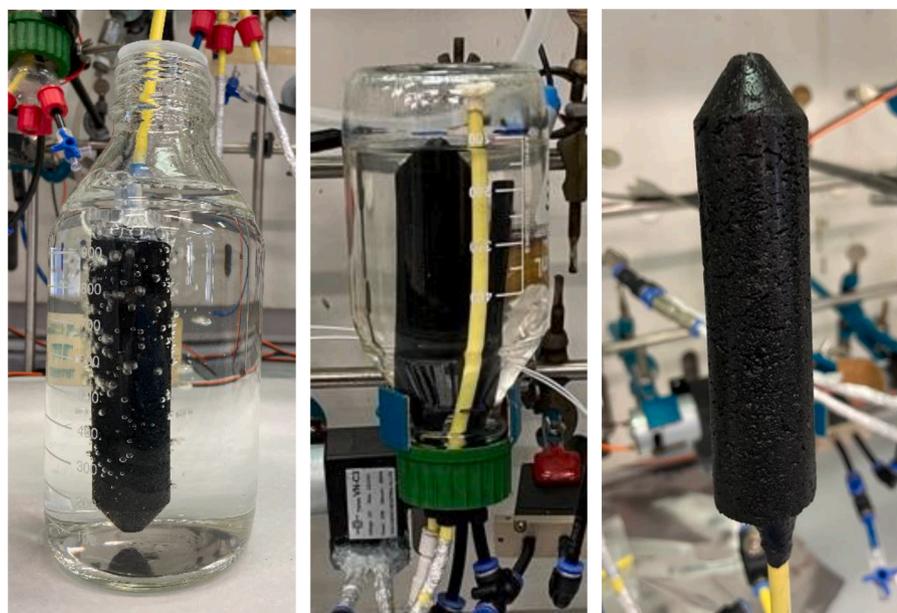


Fig. 1. Biochar-polystyrene monolith used in this study during the initial tests (left), prior to the fermentation test inside the bioreactor (middle) and after 101 days of continuous operation (right).

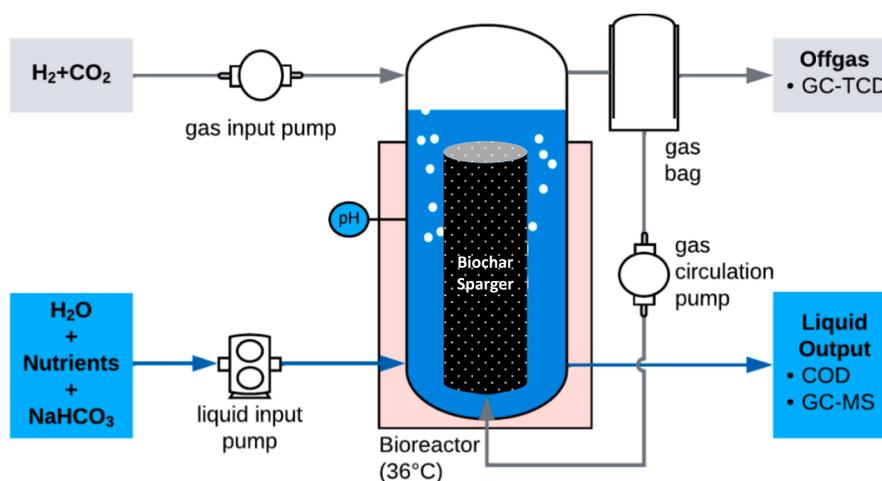


Fig. 2. Methodological scheme of the CBSR experiment.

H_2/CO_2 (and minimal amount of CH_4) were measured, analysed and manually vented on a daily basis. To suppress methanogenic activity, when more than 1% v/v methane was detected, the liquid input was spiked with sodium 2-bromoethanesulfonate (BES) to achieve a 25 mM concentration in the bioreactor wet volume.

Quantitation of H_2 , CO_2 and CH_4 was performed with GC-TCD (7820A, Agilent Technologies), as previously described [40]. The COD concentration ($gCOD L^{-1}$) and VFA ($gCOD_{VFA} L^{-1}$) of fermentation effluents were obtained on a daily basis. COD was quantified using a Quick-COD analyser (LAR Process Analyser AG), following the ASTM D6238-98 method based on thermal oxidation at 1200 °C. VFA was quantified via solvent extraction and GC-MS in accordance with a previously published method [41].

2.4. 16S rRNA gene sequencing, microbial analysis and SEM

Samples for the characterisation of sparger microbiota, namely, gene sequencing and SEM analysis mentioned in Section 3.3, were obtained by removing the sparger at the end of the fermentation experiment,

freeze drying the entire item at -65 °C and 1.0 mbar and manually grinding the freeze-dried sparger. This sample was gold coated before SEM (Philips XL30S-FEG) and subjected to DNA extraction and analysis. Total DNA was extracted from about 500 mg of freeze-dried samples using the E.Z.N.A.® SOIL DNA Kit (Omega Bio-Tek) following the manufacturer's instructions. DNA extractions from negative controls based on laboratory aerosol (Eppendorf opened on the workbench during the extraction procedure) were conducted at the end of each extraction following the same procedure as the real samples. DNA yield was assessed using the Qubit dsDNA HS Assay Kit with a Qubit 2.0 fluorometer (Invitrogen).

16S sequencing libraries were generated using the 16S Barcoding Kit (SQK-16S024) from Oxford Nanopore Technologies (ONT), Oxford, UK, following the manufacturer's instructions. About 10 ng of DNA was used for PCR amplification, where 30 PCR cycles were chosen instead of 25 to increase reaction yield. The entire PCR process was composed of initial denaturation at 95 °C for 1 min, denaturation at 95 °C, annealing at 55 °C and extension at 65 °C for 30 cycles, followed by a final extension at 65 °C for 1 min. Negative PCR controls were also included for each

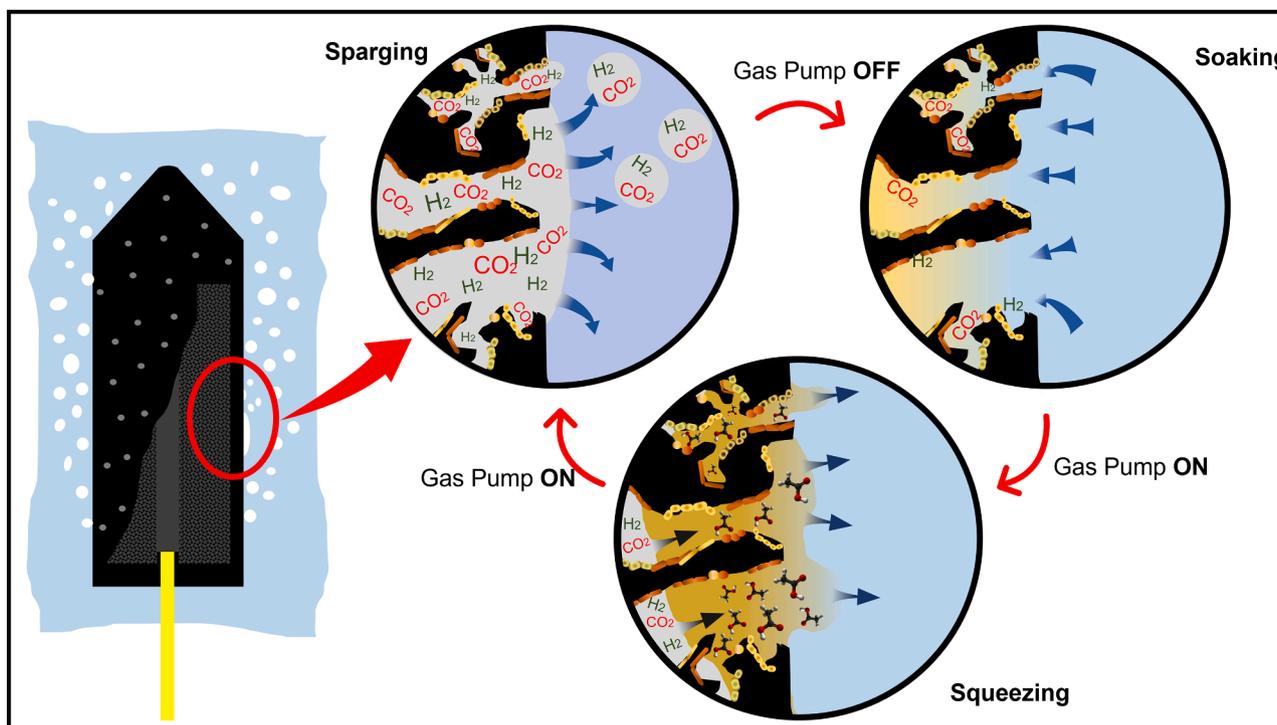


Fig. 3. Visual description of the cyclic operating principle of the biofilm-colonised sparger for H_2/CO_2 fermentation into acetic acid.

batch of PCRs. Targeted samples were pooled in equimolar ratios, and the pooled samples were loaded onto a MinION flow cell (R10.3, FLO-MIN111). The flow cell was placed into the MinION for sequencing and controlled using ONT's MinKNOW 4.3.12 (Oxford Nanopore Technologies, Oxford, UK) software. The use of long-read 16S rRNA amplicon in Nanopore MinION brought the accuracy of taxa identification to $\sim 95\%$ [42].

The base-called data (fastq) were further processed using the 16S-workflow available in the cloud-based data analysis platform EPI2ME with 'Fastq 16S Analysis' and the average quality of about 85% for demultiplexing. The reads were clustered at different taxa levels. The relative abundance of each taxon within each sample was calculated, and the taxa were sorted in descending order by relative abundance, retaining only the taxa with a relative abundance higher than 0.1 %.

2.5. Rationale of the study and calculations

The system was operated by changing the dilution rate (DR) as defined in [6] to highlight the performance of the system at different concentrations of VFA:

$$DR = \frac{Q_{in} \left(\frac{L}{d} \right)}{V_{sparger}(L)}$$

where Q_{in} is the liquid input/output daily flow (L/d), and $V_{sparger}$ is the volume of the biologically colonisable sparging device (Section 2.1), namely 0.063 L.

For the scope of this study, 1 g of COD (otherwise named as gCOD, gO or gO_2) is defined as the amount of organic matter that needs 1 g of oxygen to be completely oxidised. For theoretical reasons detailed elsewhere [43] and in accordance with a common practice in MMC studies, all concentrations and amounts of organics were reported as gCOD L^{-1} and gCOD, respectively. All mass-to-COD conversions were performed in accordance with stoichiometry, therefore considering 7.94 gCOD per gram of hydrogen and 1.07, 1.51, 1.81 and 2.04 gCOD per gram of acetic, propionic, butyric and valeric acid, respectively.

On the basis of the above, the performance of the system was

measured as hydrogen fixation rate ($gCOD_{H_2} L^{-1} d^{-1}$), VFA concentration ($gCOD_{VFA} L^{-1}$) and volumetric productivity ($gCOD_{VFA} L^{-1} d^{-1}$), whose calculations are detailed below.

H_2 fixation rate ($gCOD_{H_2} L^{-1} d^{-1}$) was calculated from gas volume measurement as

$$HFR \left(\frac{gCOD_{H_2}}{L \cdot d} \right) = \frac{1}{V_{sparger}} \cdot \frac{\Delta V_{H_2}}{\Delta t} \cdot 0.67$$

where $\Delta V_{H_2}/\Delta t$ is the daily volume of hydrogen consumed (L/d) and 0.67 is the $gCOD L^{-1}$ of hydrogen gas at room temperature and pressure.

Concentration of VFA (C_{VFA} , $gCOD_{VFA} L^{-1}$) was calculated from the measured concentration of each detected VFA as follows:

$$C_{VFA} \left(\frac{gCOD_{VFA}}{L} \right) = A \cdot 1.07 + P \cdot 1.51 + B \cdot 1.81 + V \cdot 2.04$$

where A, P, B and V are the mass concentration (g/L) of acetic, propionic, butyric and valeric acid, respectively. V_p ($gCOD_{VFA} L^{-1} d^{-1}$) for a certain day was calculated from a variation of VFA in the broth and the VFA removed from the system due to liquid output, as described by the equation below:

$$V_p \left(\frac{gCOD_{VFA}}{L \cdot d} \right) = \frac{\left(\frac{\Delta C_{VFA}}{\Delta t} \right) \cdot V_{liquid} + Q \cdot C_{VFA}}{V_{sparger}}$$

where Q is the liquid input/output flow (L/d), $\Delta C_{VFA}/\Delta t$ is the daily increase in VFA concentration ($gCOD_{VFA} L^{-1} d^{-1}$), C_{VFA} is the VFA concentration ($gCOD_{VFA} L^{-1}$) and V_{liquid} is the total wet volume of the reactor bottle (0.5 L).

3. Results and discussion

3.1. Substrate consumption rate (H_2) and product (VFA) profiles

To initiate the sparger colonisation, the CBSR was inoculated and subjected to a DR of $0.8 d^{-1}$, which was expected to keep the VFA concentration to a moderate level and ease the biofilm growth [6]. As

shown in Fig. 4, H_2/CO_2 conversion started almost immediately, with an increase in H_2 fixation rate ($29 \text{ gCOD}_{H_2} \text{ L}^{-1} \text{ d}^{-1}$ at day 3) and a sharp increase in VFA (almost exclusively, namely 98%, acetic acid) concentration. Given the relatively large amount of volume that surrounds the sparger and the amount of inoculum used, such prompt start of fermentation could be due to the initial biofilm formation and the activity of suspended biomass. On day 5, to highlight the performance of biochar attached biofilm, the entire liquid suspension was centrifuged for 10 min at 5000 RPM ($\approx 4000 \text{ g}$), thus removing all the suspended biomass. After this operation, suspended matter was negligible ($<3\%_{\text{COD}}$) until the end of the experiment; thus, all observed biological activity was assumed to occur in the biofilm attached to the sparger.

After removal of suspended biomass, H_2 fixation rate first slightly decreased and subsequently became variable (15 to $48 \text{ gCOD}_{H_2} \text{ L}^{-1} \text{ d}^{-1}$) for the first 18 days. Later, a $29 \text{ gCOD}_{H_2} \text{ L}^{-1} \text{ d}^{-1}$ (± 4) more stable H_2 fixation rate was observed. VFA (mainly acetic acid) concentration was roughly correlated with H_2 fixation in previous days, with a variable concentration (12 – $20 \text{ gCOD}_{\text{VFA}} \text{ L}^{-1}$) that finally stabilised around $24 \text{ gCOD}_{\text{VFA}} \text{ L}^{-1}$ in the end of the experimental stage.

During this first stage of experiment, the average H_2 fixation rate was estimated as $25 \text{ gCOD} \text{ L}^{-1} \text{ d}^{-1}$, whereas the final soluble concentration and volumetric productivity for VFA were equal to $28.6 \text{ gCOD} \text{ L}^{-1}$ and

$17.9 \text{ gCOD}_{\text{VFA}} \text{ L}^{-1} \text{ d}^{-1}$, respectively. Interestingly, at such stage, 85% ($\text{gCOD}_{\text{VFA}}/\text{gCOD}$) of soluble products consisted in VFA, with a production of non-negligible concentration of ethanol, butanol and other metabolites which were not investigated in this study (e.g., exopolysaccharides). This phenomenon was previously observed within the initial stages of MMC fermentation of H_2/CO_2 [21,27,44] and, given that these products are less thermodynamically favourable than VFA [14,15], this could suggest a non-equilibrium state of microbial ecology, especially during the initial stage of biofilm growth.

No considerable methane production was observed, suggesting an adequate suppression of methanogens via the on-demand BES addition. From the COD balance of this stage, the average difference between H_2 fixed and soluble products generated was equal to 26% of total H_2 fixed, thus confirming that biofilm formation occurred during this stage.

After 20 days, the H_2 fixation rate plateaued to an average of $27 \text{ gCOD}_{H_2} \text{ L}^{-1} \text{ d}^{-1}$, and the difference between H_2 fixed and soluble products generated decreased to $<14\%$, suggesting a completion of biofilm formation. To explore the potential of obtaining higher acetic acid titer and investigate the tolerance of biofilm to VFA, the liquid input was minimised, and the system was operated almost as a batch mode with minimal DR (0.1 d^{-1}). Such approach aimed to push for adaptation to higher VFA concentration (and consequential higher inhibition) and

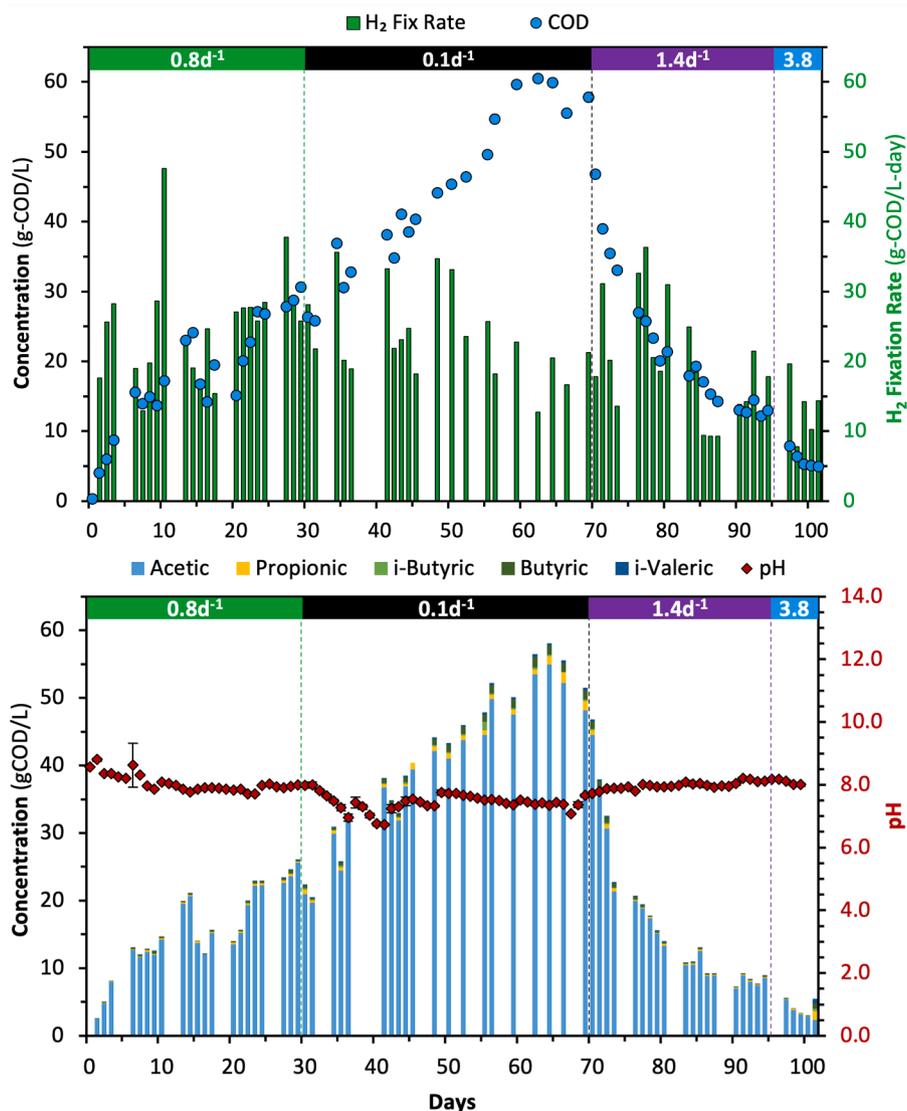


Fig. 4. Substrate (H_2) consumption rate and dissolved organic concentration (COD) profiles (up-graph) and VFA/pH profile (below-graph). Error bars on pH values corresponds to the intraday standard deviation ($n = 3$).

Table 1
Performance parameters for CBSR operations with different DR.

Days	0–30	31–69	70–94	95–101	0–101
DR (d ⁻¹)	0.8	0.1	1.4	3.8	n.a.
V _P (gCOD L ⁻¹ d ⁻¹)	17.9	12.5	9.1	10.7	13.1
%VFA/Solubles ^a	85 ± 0	95 ± 6	63 ± 5	64 ± 6	82 ± 13 ^a
Solubles (gCOD _{SOL} L ⁻¹) ^a	28.6 ± 2	57.4 ± 3	13.2 ± 1	5.1 ± 0.1	26.5 ^a
VFA (gCOD _{VFA} L ⁻¹) ^a	24.4 ± 2	55.0 ± 3	8.4 ± 1	3.3 ± 0.5	23.1 ^a

^a Average value of the last three measures obtained at the relevant operational phase.

^a Averaged value through all the study.

to establish when product inhibition becomes unsustainable for the CBSR microorganisms.

After the decrease in DR, a linear increase in VFA concentration was observed without any considerable effect on H₂ fixation rate. VFA and soluble COD peaked after day 60 and reached 58 gCOD_{VFA} L⁻¹ and 60 gCOD L⁻¹ as the highest values obtained during the study. At this point, the VFA consisted almost exclusively of acetic acid (55 gCOD L⁻¹) with a minor amount of propionic and butyric acid (both between 1.3 and 1.6 gCOD L⁻¹).

The peak VFA concentration was achieved at such stage; to the best of the authors' knowledge, it is the highest VFA concentration ever achieved by anaerobic acidogenic MMC [45], and it is almost identical to the maximum achieved with *Acetobacterium woodii* in single-strain fermentation [5,6].

Although H₂ fixation rate values were not indicating a stable trend throughout this period, after a certain level of VFA concentration (40–50 gCOD L⁻¹) achieved in the system, a nonreversible decreasing performance trend was observed in terms of CBSR productivity. For instance, V_P for the last 12 days of this period (DR: 0.1 d⁻¹), when VFA

values were between 50 and 58 gCOD L⁻¹, was halved (8 gCOD L⁻¹ d⁻¹). Moreover, the selectivity toward VFA, which was close to 100%, decreased to 63% (gCOD_{VFA}/gCOD) which is lower to that observed during system startup. Such phenomena could be interpreted as an impairment of microbial biofilm growth/maintenance due to VFA concentration close to the absolute inhibition concentration.

To relieve VFA inhibition and evaluate the actual relationship between V_P and VFA concentration for the biochar-attached biofilm, the DR was stepwise increased to 1.4 and 3.8 d⁻¹. In principle, such change in DR should lower the VFA concentration by dilution and consequentially improve V_P. As expected, upon dilution, VFA concentration decreased to approximately 11–13 gCOD L⁻¹ in 15 days; however, unexpectedly, actual V_P values did not show an instant increase. Such V_P values below 10 gCOD L⁻¹ d⁻¹ were observed until DR was further increased to 4 d⁻¹ and VFA concentration decreased to 5 gCOD L⁻¹ levels. Under these conditions, V_P was raised back to a level above 10 gCOD_{VFA} L⁻¹ d⁻¹ with a subsequent increasing trend, suggesting a relief of VFA inhibition and/or a biofilm re-establishment.

To examine further the relationship between the seven-day-average

Table 2
Comparison of H₂/CO₂ fermentation performance between CBSR and the literature based on MMC and single strain fermentation.

Reactor type	Strain	T (°C)	P (Bar)	pH	C _{VFA} [*]	V _P ^{**}	Ref
Serum bottle	MMC	37	1.0	6.0	0.3	< 0.1	[21]
Stirred	MMC	30	2.0	6.5	2.5	0.8	[23]
Serum bottle	MMC	25	1.8	6.0	8.4	0.5	[22]
HfMBR ^a	MMC	25	1.4	7.5	6.9	0.3	[47]
Stirred	MMC	28	2.0	6.5	3.7	0.9	[24]
Stirred	MMC	28	2.0	7.5	4.0	1.0	[24]
Stirred	MMC	28	2.0	8.5	4.5	1.1	[24]
Bubbled	MMC	25	1.0	4.5	2.6	0.7	[48]
Bubbled	MMC	32	1.0	5.9	35	2.1 ^b	[26]
Bubbled	MMC	23	1.0	6.5	4.9	0.7	[49]
TBR	MMC	30	1.0	7	23	1.2	[25]
HfMBR	MMC	35	1.0	4.5	13.6	0.5	[29]
HfMBR	MMC	35	1.0	4.5	3.9	0.5	[29]
HfMBR	MMC	35	1.0	6.0	11.2	0.2	[29]
HfMBR	MMC	25	1.0	6.0	46.2	0.5	[27]
HfMBR	MMC	25	1.0	6.0	48.2	0.8	[27]
HfMBR	MMC	55	1.0	6.0	44.9	2.1	[28]
HfMBR	MMC	55	1.0	6.0	20.7	8.2	[28]
HfMBR	MMC	55	1.0	6.0	11.2	11	[28]
ICR	<i>A. woodii</i>	35	1.0	7.2	4.3	4.8	[6]
RTR	<i>A. woodii</i>	35	1.0	7.2	2.9	75	[6]
RTR	<i>A. woodii</i>	35	2.7 ^c	7.2	–	160	[6]
RTR	<i>A. woodii</i>	35	1.0	7.2	55	–	[6]
Bubbled	<i>A. woodii</i>	30	1.0	–	1.2	23	[7]
Serum Bottle	<i>Clostridium</i> sp.	30	1.0	–	13	3	[10]
Bubbled	<i>Moorella</i> sp.	55	1.0	6.2	24	16	[9]
Bubbled	<i>A. woodii</i>	30	1.0	7.0	54	19	[4]
RTR	<i>A. woodii GM</i>	30	0.67	7.2	54	31	[11]
RTR	<i>A. woodii</i>	30	0.85	7.0	19	160	[5]
RTR	<i>A. woodii</i>	30	0.85	7.0	64	20	[5]
ICR	<i>A. woodii</i>	30	0.5	6.8	16	1.3	[12]
Stirred	<i>C. ljungdahlii</i>	37	1.0	5.9	6	1	[13]
CBSR	MMC	36	1.0	7.8 ^c	23	13 ^c	This study

HfMBR: Hollow fiber membrane reactor; TBR: Trickle Bed Reactor; ICR: Immobilised Cells Reactor; RTR: Rushton Turbine Reactor with cell retention; C_{VFA}: concentration of VFA delivered in the effluent; V_P: VFA volumetric productivity; * gCOD L⁻¹; ** gCOD L⁻¹ d⁻¹; ^a CO₂ provided as bicarbonate; ^b gas fermentation volumetric productivity calculated from H₂ uptake in codigestion with acetic acid and lactic acid; ^c time weighted average during the study.

V_p and the actual VFA concentration of each week, comparable results were found in the 0–50 gCOD L⁻¹ range, whereas V_p was halved above 50 gCOD_{VFA} L⁻¹ and became minimal (≤ 5.5) at more than 55 gCOD L⁻¹. Interestingly, after the VFA peak, V_p was mainly correlated to the level of exposure to VFA in the 10 days before. In practice, the biofilm shows consistent performance when the VFA concentration is < 50 gCOD L⁻¹ (before day 56 and after day 70), with poorer performance when the VFA concentration is above that concentration and shortly thereafter such VFA peaking event (up to 10 days after). Such effect can be attributed to the intrinsic hysteresis of a system based on immobilised bacteria, which require some weeks to grow or re-establish after a toxicity shock.

3.2. Comparative evaluation of CBSR performance in H₂/CO₂ fermentation

Table 1 provides a summary of the average results obtained during the study, highlighting some performance indicators for CBSR. VFA (mainly acetic acid) were the main fermentation products irrespective to the DR used, as expected for methanogen-deprived MMC at alkaline pH [14]. Nonetheless, a considerable production of other non-quantified soluble products (e.g., ethanol) was observed in the last two phases (DR equal to 1.4 and 3.8 d⁻¹) after the extremely high VFA concentrations achieved in the previous phase with 0.1 d⁻¹ DR; this result suggests a change of fermentation product profile after steep changes of DR.

The average V_p ranged between a minimum of 8.1 gCOD_{VFA} L⁻¹ d⁻¹ (obtained at the end of batch mode when the system was stressed by a VFA concentration equal to 55 gCOD_{VFA} L⁻¹) and a maximum of 17.9 gCOD_{VFA} L⁻¹ d⁻¹, obtained during the first phase of operations performed with a DR equal to 0.8 d⁻¹ and 24 gCOD L⁻¹ equilibrium VFA concentration. Given the entire 101 days of study, the system showed an average VFA concentration equal to 23 gCOD_{VFA} L⁻¹ and demonstrated an average V_p equal to 13 gCOD_{VFA} L⁻¹ d⁻¹. For the sake of comparison, Table 2 shows the V_p and VFA titers related to the H₂/CO₂ fermentation studies previously performed. Even when comparing studies with different VFA concentrations, the maximum (1–30 days) and average (1–101 days) V_p obtained with CBSR were the highest ever obtained in conversion of H₂/CO₂ to VFA with MMC, with absolute values that approach those obtained with a single strain at similar VFA

concentrations. Even when the VFA concentration was close to the maximum ever achieved with *Acetobacterium woodii* (days 60–70 with DR equal to 0.1 d⁻¹), a still acceptable V_p (6.3 gCOD_{VFA} L⁻¹ d⁻¹) was demonstrated for the first time.

In sake of comparison, it should be noted that this study was performed within a pH range (7.8 ± 0.4) which was slightly higher than most of the previous works. Therefore, the use of high pH can be one of the drivers of high V_p . It is known that undissociated VFA are more toxic than VFA salts, thus the increase of pH usually increases the V_p and the maximum VFA titer of homoacetogenic fermentation. Nonetheless, from Henderson–Hasselbalch equation, above pH 6.8 the amount of undissociated VFA becomes negligible and, from the literature, there is not clear pH effect on acidogenesis [45]. Therefore, we can compare the VFA titer and V_p obtained with most of the previous works performed in neutral and alkali pH.

Observed improvement of V_p and mitigation of VFA toxicity could be considered remarkable for an MMC system and, according to literature, could be due to peculiar microbiota–biochar interactions [46]. To gather some clues about these phenomena, the sparger-attached biofilm was subjected to preliminary investigation, which is detailed in the subsequent section.

3.3. Characterisation of sparger-supported biofilm

To understand the degree of colonisation of the char–sparger, additional analyses were performed on the monolith after the 101-day operation.

The char–sparger was removed, lyophilised, weighted and subjected to elemental analysis, SEM and DNA sequencing. Weight difference before and after the experiment revealed a slight increase in dry mass (123 ± 1 mg) and nitrogen content ($0.3 \pm 0.2\%$), which could suggest the growth of biofilm over the sparger. SEM photos of biochar and manufactured biochar/PS composite shows the typical xylem-derived structure (Fig. 5, left and middle pictures), with 10–20 μ m channels which, in principle, can provide support for growth of prokaryotes and Direct Interspecies Electron Transfer, abbreviated as DIET [50,51]. The SEM picture of the biochar/PS monolith after the 101-day experiment (Fig. 5, right) shows rod-like and cocci-like shapes. Even if sample preparation and lyophilization can affect the appearance of biological

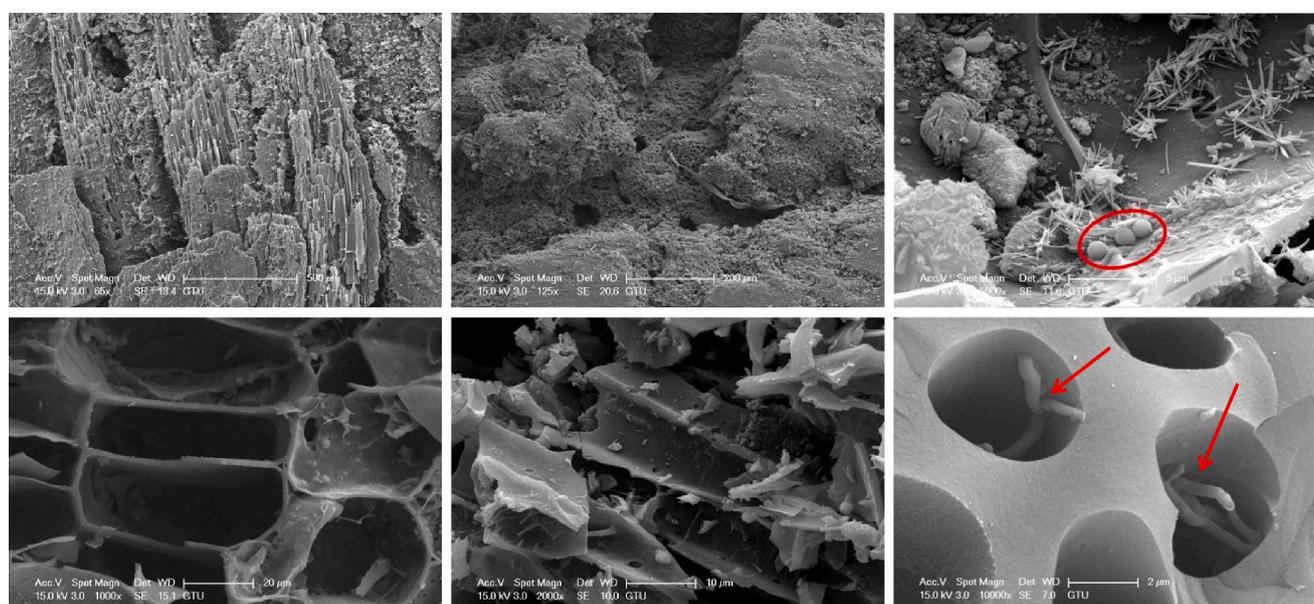


Fig. 5. SEM photographs of biochar as raw material (left), internal structure of char-based sparger prior to the fermentation (middle), and microbially colonised sparger after the experiment (right).

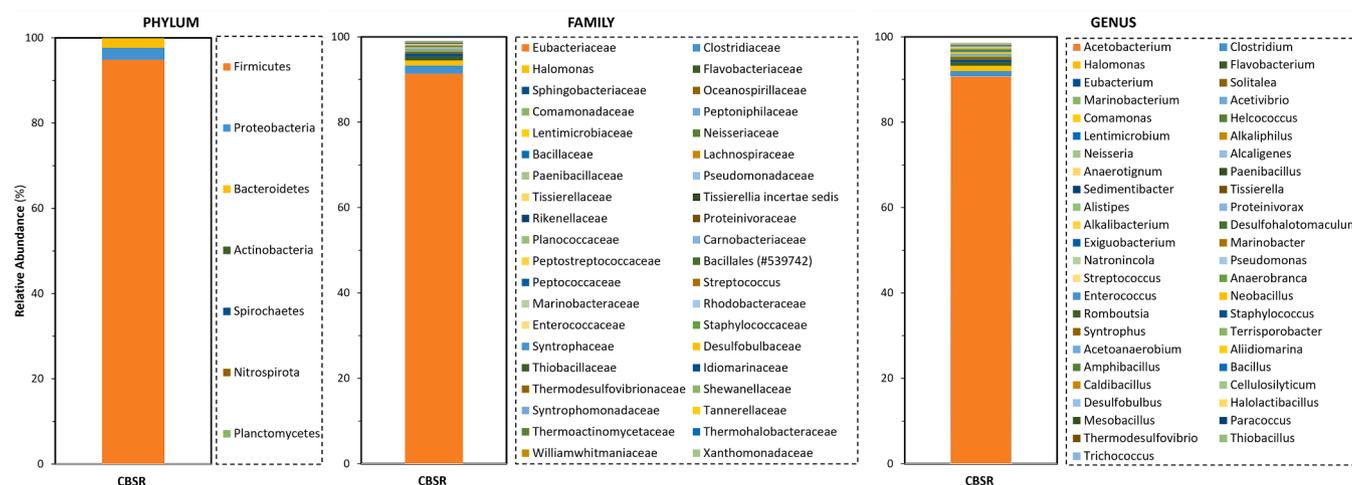


Fig. 6. Taxonomic analysis of microbial communities found in biochar-based sparger after 101 days of operation (only phylum with an r.a. greater than 0.5% or genus with an r.a. greater than 0.1% were shown).

samples and hamper the identification of microorganisms, according to the size and regularity, such elements are comparable with that observed in previous SEM investigations about biochar microbiota [52].

To highlight the main microorganisms involved in H_2/CO_2 fermentation within the CBSR, the microbially colonised char-sparger was subjected to 16S rRNA gene sequencing. Such procedure provided the taxonomic composition of the microbiota of the CBSR biofilm, which is shown in Fig. 6. Such taxonomic analysis shows that even starting from a biodiverse inoculum as previously shared elsewhere [36], homoacetogenic conversion within CBSR selects one genus, namely *Acetobacterium*, over a long period. *Acetobacterium* includes some of the most known gas fermenting strains, namely *Acetobacterium woodii*, which is one of the best characterised strains that can use the Wood-Ljungdahl pathway or CO_2 reduction [53]. Such *Acetobacterium* enrichment fits well with the presence of rod-like shapes in the SEM pictures [54].

Although comparable conditions (e.g., slight alkaline pH regulated with sodium bicarbonate buffer) and study length are difficult to find, a certain degree of selection of a single genus was previously shown by several authors, who observed an increased dominance of *Clostridium* [22,27,44] or *Acetobacterium* [47] upon feeding with H_2/CO_2 as sole feedstock. Nonetheless, the degree of selection observed during this study was more marked than those previously observed, with just one dominant genus, namely, *Acetobacterium*, which accounts for more than 90% of the biofilm microbiota.

Some reasons for *Acetobacterium* selection can be tentatively found in some peculiar interactions between *Acetobacterium* and biochar. For instance, *A. woodii* is known to use aromatic compounds as electron acceptor [55], as electron donor [56] and/or obtain electrons from conductive materials (within microbial electrosynthesis cell [57]). Such capability of *Acetobacterium* could be advantageous in the peculiar environment of biochar-based sparger, providing a way for indirect (through a chemical mediator) and/or direct (through polyaromatic structures) interspecies electron transfer. An intriguing hypothesis is that when microbiota can perform interspecies electron transfer (namely, DIET or transfer through chemical mediator), a portion of the bacteria, which reduces CO_2 to VFA, is more subjected to the adverse effect of VFA, whereas the H_2 oxidation can be performed in a spatially different location. With the H_2 solubilisation/oxidation as the limiting step within the H_2/CO_2 fermentation [58], if the H_2 oxidating biofilm would be shielded from VFA, then the overall rate of homoacetogenic consortium could increase. In addition, given that biochar contains free radicals which could react chemically with H_2 [59], the bacteria that can use biochar as source of reducing power could circumvent H_2 solubilisation steps. Although establishing the exact type/extent of mechanism for biochar enhancement of H_2/CO_2 fermentation is beyond the scope of

this study, several interesting aspects of microbiota-biochar interaction could be explored in the future.

4. Conclusion

A novel CBSR was proposed, designed, manufactured and tested for H_2/CO_2 acidogenic fermentation experiment with MMC. CBSR sustained remarkable productivities in the 9–18 $gCOD_{VFA} L^{-1} d^{-1}$ range, and allowed to achieve VFA concentration equal to 58 $gCOD_{VFA} L^{-1}$ (almost entirely acetic acid) which is, to the best of the authors' knowledge, the highest ever obtained by anaerobic mixed microbial communities.

SEM observation and 16S rRNA sequencing demonstrated that biochar-based sparger stimulated the growth of bacteria belonging to the *Acetobacterium* genus, which colonises the xylem derived cavities and constitutes more than 90% of the biofilm formed. Given that some species in that genus can interact with biochar structure (through DIET and other indirect electron donation/withdrawal), it could be inferred that the interaction between *Acetobacterium* and biochar could provide ecological advantages within a VFA-stressed system.

In accordance with the results obtained, CBSR can be considered a novel bioreactor with interesting gas fermentation performance. Given the low cost of biochar (by-product of biomass pyrolysis) and polystyrene foam (obtainable from waste packaging) and simple sparger preparation, the CBSR reactor can already be proposed as a replicable strategy for gas fermentation approaches to obtain building block chemicals and/or commodity chemicals.

CRedit authorship contribution statement

Yusuf Küçükkağa: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Andrea Facchin:** Conceptualization, Methodology, Software, Writing – review & editing, Visualization. **Vittoria Stefanelli:** Investigation. **Federica Costantini:** Formal analysis, Investigation, Resources. **Serdar Kara:** Writing – review & editing, Supervision. **Cristian Torri:** Conceptualization, Methodology, Resources, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yusuf Küçükkağa reports financial support was provided by Scientific and Technological Research Council of Turkey.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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