Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-023-03958-8

ORIGINAL ARTICLE

Effect of bioaugmentation with *Paenibacillus* spp. and thin slurry recirculation on microbial hydrolysis of maize silage and bedding straw in a plug-flow reactor

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Received: 22 December 2022 / Revised: 13 February 2023 / Accepted: 14 February 2023

Abstract
In this work, the effect of bioaugmentation on the hydrolysis and acidogenesis efficiency of bedding straw mixed with maize silage is examined. A plug-flow bioreactor was operated for 70 weeks with maize silage as a reference feedstock and subsequently with an increasing straw content of 30% and 66% (w/w). Bioaugmentation with two *Paenibacillus* species was conducted at each process condition to investigate the impact on hydrolysis of the recalcitrant lignocellulosic feedstock. A stable acidogenic digestion of the substrates was achieved, during which acetic and butyric acid were accumulated as main byproducts. Specific hydrolysis rates between 258 and 264 $gO_2\ kg^{-1}{}_{VS}$ were determined for pure maize silage and maize silage mixed with 30% of straw, while the specific hydrolysis rate decreased to 195 $gO_2\ kg^{-1}{}_{VS}$ when a mixture with 66% of straw was applied. Bioaugmentation with *Paenibacillus* spp. increased the specific hydrolysis rate by up to 41–63% for pure maize silage and the mixture with 30% of straw, while no increase was observed with a mixture of 66% of straw. Acid production, however, was enhanced by 21 to 42% following bioaugmentation for all substrate mixtures. A positive effect on the physiological state of cultures, as recorded with frequency-dispersed polarizability, was seen after bioaugmentation, which remained for two retention times during the continuous fermentation mode. Recirculation of the thin sludge further prolonged the positive effects of bioaugmentation. The results of this work provide a basis to optimize the amount of the bioaugmented microorganisms and hydrolysis of biogenic material with respect to sustainable effects on process performance and costs.

Keywords Microbial hydrolysis · Bioaugmentation · Plug-flow reactor · Anaerobic digestion · Recirculation

Abbreviations

AD	Anaerobic digestion
FDAP	Frequency-dispersed polarizability anisotropy
HRT	Hydraulic retention time
MS	Maize silage
ORP	Oxidation-reduction potential
P. glucanolyticus	*Paenibacillus glucanolyticus*
P. macerans	*Paenibacillus macerans*
PFR	Plug-flow reactor
sCOD	Soluble chemical oxygen demand
SHR	Specific hydrolysis rate
SCCA	Short-chain carboxylic acids
tCOD	Total chemical oxygen demand
TS	Total solids
TVS	Total volatile solids
VS	Volatile solids

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1 Introduction

Hydrolysis poses one of the main challenges during anaerobic digestion (AD) of residual biomass, in particular when lignocellulosic biomass is used. Pretreatment methods to improve hydrolysis efficiency are often energy intensive and account for up to 40% of process costs during AD [1, 2]. Lignocellulosic residues like straw, agricultural waste, or sawmill residues are abundant, but recalcitrant for AD due

Published online: 09 March 2023

to their crystalline lignocellulosic structure. Thus, efficient methods to improve lignocellulose digestion are required [1]. Naturally occurring microorganisms developed complex enzyme systems to break down lignocellulose. However, they might need an acidic environment in order to achieve maximum hydrolysis rates; thus, a separate reactor stage may be needed for a good process performance [3]. In a hydrolysis stage, process conditions can be adjusted to fit the needs of hydrolytic and acidogenic microorganisms better than in a main digester where process conditions have to meet requirements of the more sensitive methanogenic organisms. The separation of hydrolysis and acidogenesis from other phases of full AD has already shown to improve the energy balance of the whole process, which results in a higher net energy production finally [1, 4].

Plug-flow reactors (PFRs) have been applied as reliable AD reactors for dry AD for some time [5]. Higher stability, high removal rates, and low acid contents make them an attractive alternative to a conventional stirred tank reactor [6]. Lately, their characteristic to form gradients along the reactor due to a laminar flow field has got into focus: It has been shown that phase separation into acidogenesis and methanogenesis inside a PFR allows higher substrate flexibility, higher resistance against a low pH value [7], and higher loading rates compared to traditional stirred tanks [8]. The development of certain distinguished process phases inside a PFR has been explored for efficient biogas production of maize silage [8], pineapple peel and pulp [9], food waste with municipal waste water [6], and cattle manure [7], among others. Enrichment of different microbial subpopulations with hydrolytic bacteria in the front and methanogenic bacteria in the late part of a PFR has been shown by Dong et al. [7]. There, recirculation within the acidogenic phase further enriched hydrolytic and cellulose-degrading bacteria and resulted in improved chemical oxygen demand (COD) removal of 25% compared to AD without recirculation. This microbial enrichment helped to digest recalcitrant feedstock-like lignocellulosic residues. However, to the authors' best knowledge, a PFR has not been explored as sole hydrolysis stage yet. In literature, the focus is put on high biogas and methane yields, while pure optimization of hydrolysis and acidogenesis yields remain rare. Operating a PFR separately as hydrolysis stage furthermore opens a variety of biorefinery pathways from intermediate metabolite into valuable products like long-chain fatty acids, biofuels, bioplastics, among others, which can be synthesized from short-chain carboxylic acids [3].

Bioaugmentation is an option to increase yields of certain products in anaerobic processes, especially during AD for methane production. Pure cultures and mixed microbial consortia have been successfully explored to increase hydrolysis, hydrogen production, or the methane yield mostly during batch processes in stirred tank reactors [10–14]. Fewer studies focused on continuous processes, where survival rates and a potential washout of the bioaugmented organisms was investigated. Martin-Ryals et al. studied the effect of a daily bioaugmentation with a cellulolytic consortium on the continuous digestion of sweet corn processing waste in a stirred tank reactor which led to an increase of soluble COD and methane production by 29–68% and 31–34%, respectively, compared to the non-augmented reaction [10]. Comparing one-time and repeated bioaugmentation in a sequencing batch trial, they achieved a 25% higher net soluble COD generation with daily addition while the effects of one-time addition were insignificant after 14 days of fermentation. Similarly, the positive effects of bioaugmentation of a thermophilic consortium on the digestion of corn stalks and cow dung were only notable in the first days, but almost insignificant after 9 days of digestion [15]. The bioaugmentation strategy of Tsapekos et al. of adding *Clostridium thermocellum* over 6 days to the continuous digestion of cow manure and wheat straw enhanced the methane yield significantly during addition (26–33%), but became insignificant after two hydraulic retention times (HRTs) in a steady-state operation mode [16]. In most cases, the reactor conditions are unfavorable to support further growth of the augmented bacteria [16]. The addition of the anaerobic fungus *Orpinomyces joyonii* to the inoculum during AD of rice straw resulted, however, in a long-term increase of cellulose and hemicellulose and reduction of volatile solids, and thus in an increased methane yield (38%). During this continuous digestion with 15 days of HRT, the fungi were able to survive for 122 days inside the reactor [17].

In this study, two *Paenibacillus* spp. were chosen based on their hydrolytic activity under acidic, anaerobic, and mesophilic conditions. *Paenibacillus glucanolyticus* is a facultative anaerobe, Gram-positive, rod-shaped, spore-forming, and mesophilic bacteria that has optimal growth conditions at pH 5.7 [18]. Hydrolysis of various β-glucans like carboxymethylcellulose, hemicellulose, cellulose, starch, arbutin, and cellobiose has been confirmed [18, 19]. Aerobic and anaerobic growth of two strains of *P. glucanolyticus* on lignocellulosic biomass without nutrient supplementation was confirmed by Mathews et al., where anaerobic growth yielded higher cell densities and better lignin degradation at pH 9.0 [19, 20]. *Paenibacillus macerans* exhibits hydrolytic activity on hemicellulose under anaerobic conditions and produces α- and β-glucan-degrading enzymes, including cellulase. It was shown that a hydrolytic activity was maintained until a pH value of 5.5 [21]. The ability to utilize residual oxygen from the substrate and their ability to sporulate probably ensure the survival of both organisms in the reactor under unfavorable conditions.

Maize silage (MS) is one of the most common substrates for AD and provides comparably high yields of biogas [22]. Santi et al. [23] showed, however, that the lignocellulosic

components in MS are often incompletely fermented, with a digestion efficiency of only 40% and 29% for cellulose and hemicellulose, respectively. The application of MS in AD is controversial, due to the competition for arable land for food and feed production and due to soil erosion, nitrate leaching, or pesticide use [22, 24]. For the expansion of a circular economy, the utilization of locally available residual biogenic resources is envisaged. Straw is a lignocellulosic residue connected with grain production and offers potential for AD, but digestion is limited due to its recalcitrant structure [25]. For the investigation of the effect of bioaugmentation on feedstock with different contents of lignocellulosic substrates, MS and local bedding straw were used in different proportions.

To the authors' knowledge, an application of a PFR as a hydrolysis stage in an anaerobic process, combined with bioaugmentation and thin-slurry recirculation, has not yet been investigated. Thus, the aim of our study was, in particular, to (i) describe hydrolysis in a PFR that was operated with MS and straw, (ii) investigate the effect of bioaugmentation with hydrolytic bacteria, and (iii) examine the impact of thin-slurry recirculation on the bioaugmentation efficiency.

2 Materials and methods

2.1 Feedstock

The feedstock that was used in this study was whole plant MS obtained from a farm in Bavaria, Germany, and mixed straw used for bedding in the horse stable of uFA Fabrik Berlin. Due to the continuous operation of the reactors, different batches of feedstock were used throughout the study. Characteristics of the feedstock were analyzed for each batch separately. Data were used for individual calculations correspondingly (Table 1). Upon arrival, both feedstocks were dried and sieved with a particle size exclusion of 50 mm. Bigger parts were crushed with a blender to reach the cutoff size. As storage of the feedstock had to be conducted for several months with limited cooling capacity, feedstock drying was assumed to result in the lowest change of the organic material, although loss of volatile components from the MS could have occurred. The bedding straw also contained small amounts of horse manure, sand, and residues of feed (apples, grains, etc.). Inert residues like plastics and stones were removed, and the residual composition mixed into the substrate. All samples were measured in triplicates. Total solids (TS), volatile solids (VS), and the ash content of the substrate were measured by APHA standards [26]. The soluble and the insoluble acidic lignin content were measured with the two-step acid hydrolysis method as described by Bhagia et al. [27] and Sluiter et al. [28]. For the measurement of non-structural carbohydrates, dry samples were milled

Table 1 Average substrate characteristics of maize silage and bedding straw. Characterization has been conducted for different substrate batches (see supplementary material, Table S1)

Parameter		Maize silage		Bedding straw	
	Unit	Ave	SD	Ave	SD
pH value		4.64	1.07	7.63	0.72
TS	%	29.12	3.41	/	/
Moisture	%	70.88	3.41	/	/
VS	% of TS	96.85	0.70	83.54	8.38
Ashes	% of TS	3.15	0.70	16.46	8.38
tCOD	$gO_2 \cdot g_{TS}^{-1}$	0.85	0.03	0.50	0.27
sCOD	$gO_2 \cdot g_{TS}^{-1}$	0.13	0.03	0.06	0.02
Total nitrogen	% of TS	0.95	0.08	0.62	0.17
Non-structural C content	% of TS	31.60	8.68	12.77	4.72
Acid insoluble lignin	% of TS	15.07	1.70	29.19	2.34
Acid soluble lignin	% of TS	1.48	0.43	1.35	0.40

for 2 min with the IKA A10 laboratory mill (IKA®-Werke GmbH & CO. KG). Fifty milligrams of the milled sample were extracted in 5 mL of 80% ethanol for 10 min according to [29]. The supernatant was removed, and extraction repeated for two times. The supernatants were combined and diluted 1:10 with double-distilled water. The carbohydrate solution was analyzed with the sulfuric-acid-UV method for lignocellulosic feedstock as described by Albalasmeh et al. [30]. For the measurement of the total chemical oxygen demand (tCOD), 50 mg of dried and milled feedstock were mixed with 1 mL double-distilled water in an Eppendorf tube. The sample was vortexed, soaked for 1 h, and afterwards diluted 1:10. Half of the sample was centrifuged for 5 min at $7.5 \times g$ and filtered through 0.45 µm regenerated cellulose filters for the determination of soluble COD (sCOD). Both tCOD and sCOD samples were additionally diluted by a factor of 10, so that the samples were finally diluted by a factor of 100. Samples were analyzed with the Hach Lange Kit LCI 400 (Hach Lange GmbH, Berlin, Germany) for COD content and double-distilled water as reference solution. The tCOD solution was also used to analyze total LATON nitrogen with the Hach Lange Kit LCK 338.

2.2 Plug-flow bioreactor operation

Anaerobic microbial hydrolysis was performed in a PFR with a maximum working volume of 14 L. The PFR was equipped with three sample ports horizontally distributed along the reactor, respectively, at the inlet, center, and outlet, each combined with sets of *online* sensors for conductivity, pH value, oxidation–reduction potential (ORP), and temperature for *online* gradient monitoring. *Online* data points were recorded every

10 min. The reactor scheme is shown in Fig. 1. The reactor was kept at mesophilic temperatures by external hose-heating and isolation. The pH value remained uncontrolled. Continuous stirring at 5 rpm was applied to prevent blockage. Gas composition measurement with BCP-CO_2, BCP-H_2, and BCP-CH_4 sensors (BlueSens gas sensor GmbH, Herten, Germany) was installed in the last experimental phase with a feedstock share of 66% (w/w) of straw. The reactor was inoculated with 1.5 L of sludge from another PFR which was operated with MS in a dark fermentation process mode. Eight hundred grams of dry MS and 12 L of water were added for start-up. The reactor was operated in batch mode and afterwards semi-continuously under dynamic conditions of the HRT and thin sludge recirculation with a constant organic loading rate of 4 kg_{TVS} m^{-3} d^{-1} to achieve stable microbial conditions. From week 15 of the experiment, a constant HRT of 14 days was maintained to achieve comparable conditions for the bioaugmentation trials. Samples from each port were taken two times per week and additionally before and after bioaugmentation. During the bioaugmentation experiments, a HRT of 14 days and a TS content between 12 and 16% (w/w) was maintained. Twenty percent of the weekly harvest of thin sludge was recirculated at each feeding/harvesting event to prolong the retention time of microbial biomass within the reactor. Harvesting and feeding of substrate and water was performed 4 times per week right after sampling. The reactor was operated anaerobically; however, residual oxygen from the dry feedstock entered during the feeding events. The harvest was sieved, and residual solids quantified by dry weight determination ($m_{Harvest}$). Regularly, the solids content of the thin sludge was determined to set up the mass balance of the reactor ($c_{Harvest}$). The total solids content of the PFR was calculated as follows:

$$TS_{PFR}[\%] = \frac{m_{solids(PFR)}}{m_{total(PFR)}} \cdot 100 \quad (1)$$

$$\frac{A \cdot m_{Feed}[kg] - m_{Harvest}[kg] - C_{Harvest}\left[\frac{kg}{L}\right] \cdot V_{Harvest}[L]}{m_{solids}[kg] + m_{H_2O}[kg]} \quad (2)$$

The mass flow of gas was not measured in this setup. In order to account for the organic material that was converted to gas, a percentage of the feedstock was deducted (A). These factors for MS ($A_{MS} = 0.85$) and the mixed straw feedstock ($A_{Straw} = 0.75$) were determined with the average difference of mass inflow and harvest during the total time of operation with the same feedstock. The reactor was operated on three different feedstock mixtures with increasing straw content being (i) MS, (ii) 30% (w/w) of bedding straw with MS, and (iii) 66% (w/w) of bedding straw with MS (see Table 2). After a substrate change, the PFR was allowed to stabilize over 3 HRT before bioaugmentation experiments were started. The benefits of *online* monitoring of gradients in the PFR will be discussed in a subsequent publication. In this report, relevant gradients as in pH value and conductivity are considered briefly. If not otherwise stated, average values of the measurements from all three ports were applied for all figures and calculations.

2.3 Bioaugmentation culture conditions

Two bacterial strains were chosen for bioaugmentation to the microbial hydrolysis process. Robust strains were chosen for hydrolytic activity on lignocellulosic substrates under mesophilic, anaerobic, and acidic conditions. *Paenibacillus glucanolyticus* (DSM 5188, formerly classified as *Bacillus glucanolyticus*) and *Paenibacillus macerans* (DSM 24, formerly *Bacillus macerans*) were both obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). The two strains were grown in full medium containing 10 g L^{-1} yeast extract, 3 g L^{-1} meat extract, 5 g L^{-1} peptone, 10 mg L^{-1} $MNSO_4$, and 10 g L^{-1} glucose, which were set to a pH value of 6.0. As *P. macerans* showed very quick acidification in batch culture, a phosphate buffer was added by amounts of 1.079 g L^{-1} KH_2PO_4 and 3.844 g L^{-1} K_2HPO_4 and the media pH value was adjusted to 8.0 by the addition of 30% NaOH. For cultivations of *P. glucanolyticus*, 25 mL of preculture was inoculated from cryoculture and grown aerobically in batch culture in Ultra-Yield™ flasks

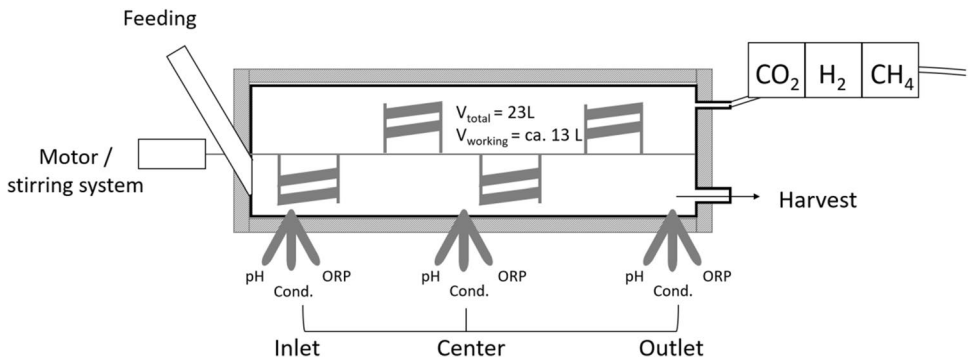

Fig. 1 Schematic design of the plug-flow reactor with sensor triplets and sampling ports at the inlet, center, and outlet

Table 2 Operational parameters of the PFR

Substrate	Recirculation	Bioaugmentation	Period of operation* [weeks]	Non-augmented reference period [weeks]
Maize silage	Operation		1–14	
	None	\	15–16 21–22	15–16
	20%	*P. glucanolyticus*	17–20	
		P. macerans	23–26	
		\	27–30	28–30
30% straw, MS	20%	\	31–35.5	33–35
		P. macerans	35.5–40	
		P. glucanolyticus	43–46	
	None	\	41–42	
66% straw, MS	20%	\	47–54	51–54
		P. glucanolyticus	55–60	
		P. macerans	64–69	
	None	\	61–62	

*Not in chronological order.

with Aerotop™ membranes (Thompson Scientific) at 30 °C and 200 rpm. After 24 h, between 4 and 6 cultures with a volume of 250 mL were inoculated from the preculture and cultivated for 2–3 days until no further growth was observed. Cultivation for *P. macerans* followed the same workflow, while cultures were grown at 37 °C and 200 rpm.

The bacteria were added to the reactor twice, namely at the onset of the cultivation on day 1 and on day 8, which corresponds to $t=0$ and $t=0.5$ of the total HRT. The conditions were maintained between 2 (MS and 30% of straw) and 3 full HRTs (66% of straw), wherein the first HRT is referred to as "addition phase" and the following as "recirculation phase." All bioaugmentation experiments were conducted sequentially in the same reactor. To reduce the mutual influence of bioaugmentation experiments, at least one full HRT of 14 days without recirculation was maintained to wash out residual bacteria. The first addition of *P. glucanolyticus* represents an exemption, as the organism was added three times at $t=0$, 0.5, and 1 HRT due to insufficient growth during the second addition. Detailed information to the bioaugmentation is shown in Table 3.

2.4 Analytical methods

For the measurement of sCOD, samples were centrifuged for 5 min at 7.500 g and filtered through 0.45-µm regenerated

Table 3 Conditions at bioaugmentation with *Paenibacillus* spp. to plug-flow-based hydrolysis

Organism Unit	Substrate in PFR	Addition	HRT passed	Culture volume L	Cell dry weight (DW) g L^{-1}	Cell DW added g_{DW}	Total COD gO_2 L^{-1}
P. glucanolyticus	MS	1	0	1	1.77 ± 0.15	1.77	20.3
		2	0.5	0.27	2.35 ± 0.18	0.63	23.0
		3	1	0.98	2.06	2.02	25.7
	30% straw	1	0	1.36	2.37 ± 0.14	3.22	24.2
		2	0.5	1.4	1.78 ± 0.70	2.50	22.3
	66% straw	1	0	1.68	1.38 ± 0.10	2.32	25.5
		2	0.5	1.4	1.83 ± 0.03	2.56	24.8
P. macerans	MS	1	0	0.94	1.92 ± 0.12	1.80	28.4
		2	0.4	1.2	1.72 ± 0.10	2.06	26.5
	30% straw	1	0	1.34	3.22 ± 0.26	4.31	31.4
		2	0.5	1.42	1.48 ± 0.31	2.10	25.4
	66% straw	1	0	1.18	1.42 ± 0.07	1.67	28.9
		2	0.5	1.13	0.44 ± 0.09	0.5	32.4
		3	1	0.5	1.13 ± 0.40	0.57	34.9

cellulose filters. One sample from each port was measured once per week with the HACH Lange Kit LCI 400 at a dilution of 1:100. Short-chain carboxylic acids (SCCAs) were measured by HPLC (1200 series Agilent Technologies, Waldbronn, DE) with the HyperREZ XP Carbohydrate H+8 µm column (Thermo Fisher Scientific Inc., Waltham, MA) at 65 °C and 0.5 M H_2SO_4 as fluid phase and refractive index detection. HPLC samples were prepared by leaving 2 mL samples at 4 °C overnight for crystallization of lignocellulosic residues. Samples were then centrifuged at 16.000×*g* for 10 min, sterile filtrated (0.2-µm nylon filter, Carl Roth GmbH, Karlsruhe, Germany) and clarified with the Carrez clarification kit (Merck KGaA, Darmstadt, Germany). The physiological state of the mixed culture was determined through the measurement of frequency-dispersed polarizability anisotropy (FDAP) with Elotrace (EloSystems GbR, Berlin, Germany). Firstly, particles were removed by centrifugation for 5 min at 650 rpm. Two milliliters of supernatant were transferred into 18 mL of dH_2O and centrifuged for cell separation (2 min, 6000 rpm). The cell pellet was resuspended in 30 mL of dH_2O in order to achieve an adequate level of initial electrical conductivity (<40 µS cm^{-1}) and measured at frequencies of 200, 400, 900, and 2100 kHz. This was proven to be appropriate for anaerobic fermentation samples [31]. Sugars were determined in selected samples via GC–MS measurement as described by Kielhorn et al. [32].

2.5 Calculation of metabolic activity

Hydrolysis is commonly determined by the measurement of the soluble organic matter versus the total organic matter. This is performed via COD or total organic carbon determinations. Here, the ratio of soluble to total COD was applied (Eq. 3) [33, 34]. During continuous digestion, however, this calculation is not accurate, as the tCOD in the reactor is changing over time and can only be determined by a mass balance. In order to account for that, a COD balance in relation to the feed was applied on a weekly basis. The specific hydrolysis rate (SHR) (Eq. 4) describes the soluble metabolites released from the substrate into the fluid phase (adapted from [35]). Organic material from the recirculation or bioaugmentation was subtracted to obtain the actual release (Eq. 5). The net acid production was calculated correspondingly (Eq. 6). To determine the acidification, the theoretical COD of the acids was calculated and put into proportion with the sCOD (Eq. 7, adapted from [36, 37])).

$$Hydrolysis\ [\%] = \frac{sCOD(t)}{tCOD(t)} \tag{3}$$

$$spec.\ hydrolysis\ rate \left[\frac{g_{O_2 released}}{kg_{vsfed}}\right] = \frac{s\dot{C}OD_{released}}{VS_{in}} \tag{4}$$

$$s\dot{C}OD_{released}\left[\frac{g_{O_2}}{w}\right] = s\dot{C}OD_{out} - \dot{C}OD_{in} - \dot{C}OD_{Recire} \tag{5}$$

$$Acid\ production\ rate \left[\frac{g_{SCCA}}{kg_{vsfed}}\right] = \frac{S\dot{C}CA_{produced}}{VS_{in}} \tag{6}$$

$$Acidification\ [\%] = \frac{COD_{tSCCA}}{sCOD} \tag{7}$$

3 Results and discussion

In order to study the performance of microbial hydrolysis in a PFR, MS mixed with different ratios of bedding straw was digested in a continuous fermentation mode. Bioaugmentation with two *Paenibacillus* spp. and partial thin slurry recirculation was applied to examine the contribution to an improved hydrolysis.

3.1 Influence of bioaugmentation on online measurement of pH value, conductivity, ORP, and gas

During the start-up period under dynamic conditions with MS as single feedstock, gradients of the pH value and conductivity developed. This indicates that the reactor operated in plug-flow mode and as a result, different microenvironmental conditions are present. In this paper, the focus of investigation was put on the influence of bioaugmentation onto hydrolysis efficiency and acid production. However, the possibility of process monitoring and control with *online* gradient monitoring of pH value, OPR, and conductivity in the PFR will be discussed in a separate publication.

After the dynamic operation with MS, a rather stable pH value between 3.8 and 4.0 developed, ensuring the inhibition of methanogenesis in the PFR [38, 39]. Higher straw content in the feedstock increased the pH value and shifted the pH gradient towards higher values at the inlet and decreasing pH along the reactor. Bioaugmentation with *P. glucanolyticus* temporarily increased the pH value during the first addition with MS digestion, whereas bioaugmentation with *P. macerans* increased the pH value in all ports. Estimations with the Henderson-Hasselbach equation showed that the amount of buffer salts in the *P. macerans* medium is too low to shift the pH in 13 L of liquid volume with a SCCA concentration of about 9 g L^{-1}. During all bioaugmentation events, the pH value rose during the addition phases—likely by the higher solubilization of substrate where also alkaline substances were released—and decreased during the recirculation phase of thin sludge due to acid accumulation (see Fig. 2). A pH value between 5.0 and 7.0 is considered best

for hydrolysis in a separate digestion stage while acidogenesis is active in a wide range between 4.0 and 8.0 [3, 40, 41]. Hydrolytic bacteria show higher activity and growth rates at higher to neutral pH value; e.g., degradation of MS was limited below pH 5.5 for *Clostridium* spp. common in AD [42]. While a pH value of 4.0 to 5.0 may inhibit the bacterial growth, enzyme activity of fungal and bacterial cellulases was found to be highest at in this pH range. However, hemicellulases have a high activity between pH 5.0 and 6.0 but possess a higher activity also over a wider pH range of 3.0 to 10.0 [43]. In our study, the pH value was between 3.5 and 4.8, so secreted cellulolytic and hemicellulolytic enzymes should be active at considerably high rates. Moreover, a low pH value below 4.0 can also contribute to hydrolysis of cellulose and hemicellulose as it acts as acid pretreatment [44]. Small organic acids released during AD like acetic acid, butyric acid, and propionic acid have been shown to promote the degradation of lignocellulosic biomass as they act as natural catalysts for the rupture of lignin-carbohydrate complexes (autocatalysis) by increasing the porosity and surface area of the feedstock [45, 46]. Pretreatment of rice straw with 2% acetic acid for 24 h at 80 °C reduced the cellulose and hemicellulose contents by 8.9 and 18.1%, respectively, while the combination of mechanical treatment and 2% acetic acid resulted in up to 29% lignin removal in corn straw [45]. In this study, SCCA concentrations of about 1% were reached (see Section. 3.2) that could have contributed to the rupture of the feedstock.

Conductivity was stable during the reference cultivation with MS (W 15–16/27–30), 30% (w/w) of straw (W 33–36) and 66% (w/w) of straw (W 51–54) but increased significantly after bioaugmentation. A higher straw content increased the baseline conductivity in the reactor, probably due to additional ions that are brought in by the concomitantly added manure in the straw. Conductivity measurements of the different batches of pure feedstocks showed a basal conductivity of 520–540 µS cm^{-1} for MS and 440–621 µS cm^{-1} for the bedding straw. Bioaugmentation with the *Paenibacillus* spp. led to an increased conductivity, especially at the center part of the PFR, likely due to maximal microbial activity there. In any AD process, conductivity is mainly influenced by bicarbonate/carbonate concentrations. During acidic digestion, however, bicarbonate concentration is negligible and linear correlations of conductivity with SCCA production have been found [47, 48]. The same correlation was observed during bioaugmentation in this study, where the total SCCA concentration increased concurrently with the increase in conductivity. From week 60 to 66, a drastic increase in conductivity in the center part of the reactor can be seen. This trend, which starts in the second recirculation period of *P. glucanolyticus* bioaugmentation, is most probably not associated with bioaugmentation or increasing acid concentrations—in fact, SCCA levels decreased from week 60 till 62—but to a new batch of straw substrate with higher basal conductivity of 621 µS cm^{-1} that was fed from the end of week 59 on. In the *online* measurement of conductivity, prompt decreases of conductivity after feeding events are measurable in MS digestion due to dilution with the added water (900–1000 µS cm^{-1}, see supplementary material, Figure S1). However, this effect was quickly compensated by the quick solubilization of easily digestible matter into the liquid phase. The o*nline* measurements also showed a higher instability of conductivity in the inlet with MS feedstock, but a rather stable signal in center and outlet. We assume that the balance of salt uptake for growth and organic solubilization in the inlet is causing this, while the acidogenic processes at the center and outlet are more stable. With higher straw content, larger fluctuations in the center and outlet conductivity can be seen independent of feeding.

In this study, under all conditions of digestion, ORP remained in a favorable range for hydrolysis of lignocellulosic biomass; high ORP would result in complete substrate oxidation and carbon loss [49]. The ORP is naturally low in AD with baselines at − 480 to − 460 mV, whereas an ORP between − 100 and − 300 mV is considered optimal for methane production [40, 49, 50]. No direct influence of the bioaugmentation periods on ORP could be seen in our study. In the recirculation phase of *P. glucanolyticus* (weeks 18–20), the ORP increased abruptly from around − 410 to − 360 mV possibly due to oxygen added during feeding and recirculation or due to the disruption of the phase formation in the PFR. Chetawan et al. [49] found optimal hydrolysis efficiency in piggery waste at ORP of − 420 mV. They hypothesized that a controlled ORP level through microaeration leads to increased activity and enzyme production of facultative bacteria, while carbon loss due to respiration and complete oxidation to CO_2 is diminished. The low ORP in the inlet during MS digestion (Fig. 2(a)) thus could have had positive effects on hydrolysis. The center and outlet ports with higher ORP indicate butyric type fermentation that has been associated with ORP levels between − 300 and − 250 mV [51]. Changing ORP levels in this trial were not related to bioaugmentation but rather to the shifting balance in the redox reactions and the concentration and state of the electron carriers due to the adaption of hydrolysis processes.

Occasional off-gas measurement during the digestion with MS showed expectedly low methane concentrations in the off-gas, namely below 2%. *Online* measurement of the gas phase was conducted from weeks 46 to 63 (Fig. 3). Bioaugmentation with *P. glucanolyticus* in week 55 led to a lasting increase of CO_2 and CH_4 concentration, due to higher metabolic rates in the reactor. Hydrogen concentration was decreased, possibly indicating the substrate concurrence of *P. glucanolyticus* with hydrogen-producing bacteria in the

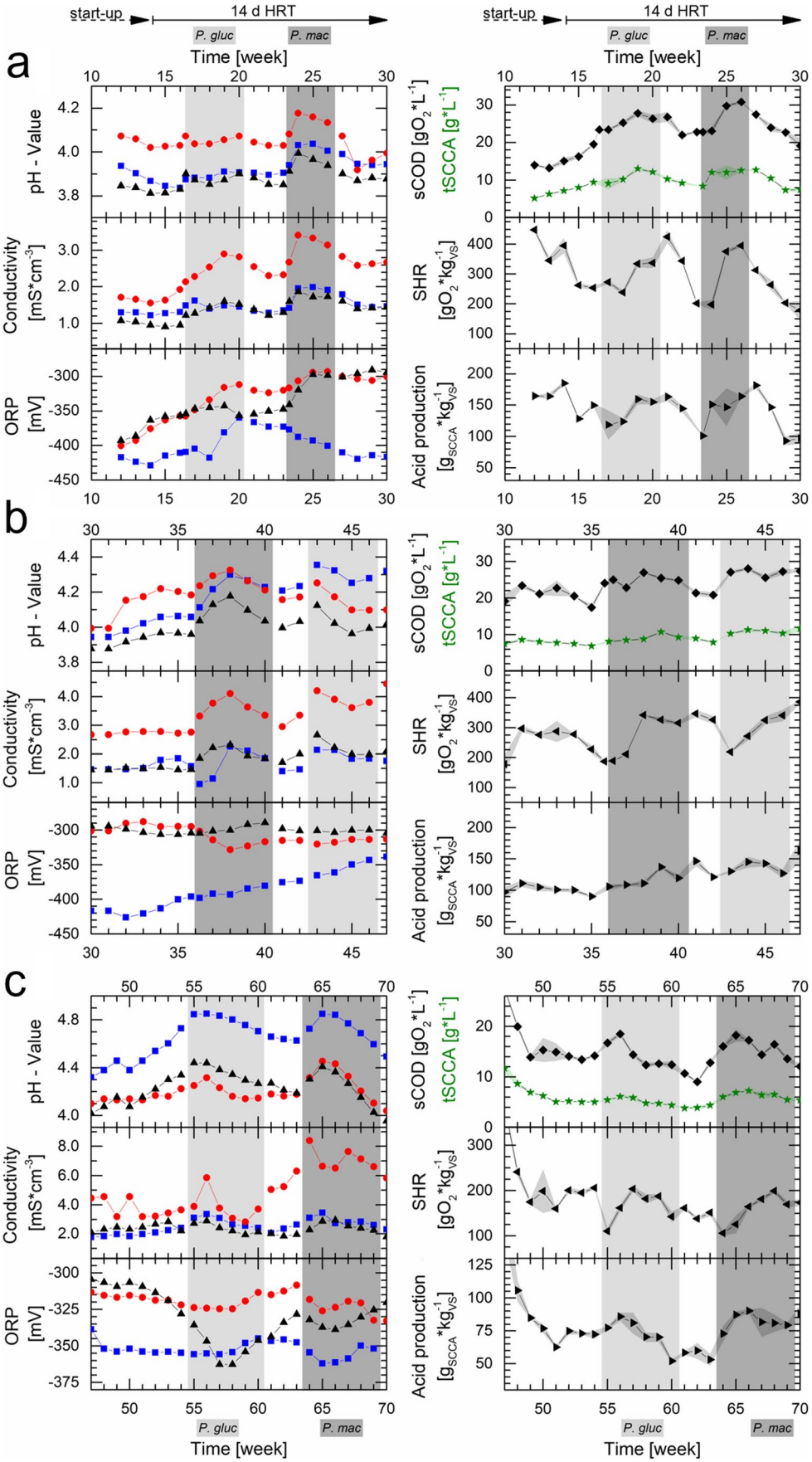

Fig. 2 Data from continuous hydrolysis in a PFR under operation with MS (**a**), 30% straw (**b**) and 66% straw (**c**). Left: *Online* measurement of pH value, conductivity, and oxidation–reduction potential (ORP) at the inlet (blue squares), center (red circles), and outlet (black triangles). Weekly averages are depicted and the periods of bioaugmentation with *P. glucanolyticus* and *P. macerans* are marked in gray. Right: Profiles of soluble COD (black diamonds), total SCCA (green stars), specific hydrolysis rate (SHR), and acid production over time. Shown are the weekly averages, the shades indicate the deviation between sampling ports

first 2 HRTs after addition. Gas concentration of especially hydrogen might be underestimated, due to measurement interferences in mixed gas and its' high volatility. The residual gas volume is largely made up by nitrogen and traces of hydrogen sulfide and ammonia, which both usually appear in AD. Oxygen that might have entered during feeding is consumed immediately by aerobic bacteria (as measured in previous experiments). The low ORP measurement during the experiments confirms the very reductive environment, which is typical for a process with a very low oxygen abundance.

3.2 Effect of bioaugmentation on metabolite accumulation

Under non-augmented operation with 30% and 66% (w/w) of straw, stable concentrations of sCOD and total SCCA were measured in between 1 and 2 HRT of stabilization, indicating a steady state in the reactor (weeks 33–35, 51–54). For MS digestion in the PFR, the more dynamic state before and after bioaugmentation was used as reference. Highest sCOD values with MS were reached under bioaugmentation during the recirculation period being 27.8 $gO_2\ L^{-1}$ in week 19 with *P. glucanolyticus* and 30.8 $gO_2\ L^{-1}$ with *P. macerans* in week 26 (see Fig. 2(a)), which are accordingly 38.9 and 53.9% higher than under non-augmented reference conditions. Correspondingly, higher concentrations of total SCCA with an increase of 47.3 and 43.5% for *P. glucanolyticus* or *P. macerans* addition in the recirculation phase were detected. With the change to 30% (w/w) of straw, sCOD stabilized at 20.2 $gO_2\ L^{-1}$ in the same range as during the non-augmented cultivation with MS. The bioaugmentation increased the sCOD concentration with *P. macerans* by 23.0 and 24.3% during the addition and recirculation period, and for *P. glucanolyticus* by 36.2 and 30.5%, respectively

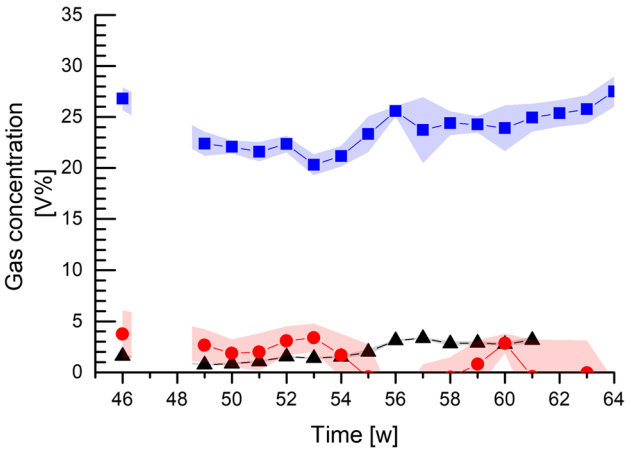

Fig. 3 Average values gained during the *online* measurement of the gas phase of the PFR: CO_2 (blue squares), H_2 (red circles), and CH_4 (black triangles)

(see Fig. 2(b)). Total SCCA increased accordingly from the non-augmented average of 7.4 g L^{-1} to 8.6–10.0 g L^{-1} (+ 17–36%) for *P. macerans* and to 10.7–10.8 g L^{-1} (+ 45–46%) for *P. glucanolyticus.* Differently than with MS, *P. glucanolyticus* showed a faster increase in acid production with 30% straw (w/w). While soluble COD measurements shortly after bioaugmentation showed a higher concentration due to residual sugars in the cultivation medium, this effect did not last long and the weekly average sCOD did not show significant changes within the addition phase. GC–MS analysis of samples taken 4 h after bioaugmentation showed a glucose concentration in the range of 10–90 mg L^{-1}. As shown in Fig. 4, GC–MS measurements of selected samples showed detectable glucose concentrations only in the bioaugmentation samples. A short-term availability of free sugars could thus boost the microbial activity and release of acids. Very small levels of arabinose and xylose between 10 and 40 mg L^{-1} were detected throughout the cultivation, indicating the breakdown of hemicellulosic structures. No other sugars were detectable. During the whole cultivation, hydrolysis was still the limiting factor, as all released sugars remained in a range of the typical saturation constant (the so-called K_S value) of many bacteria with respect to the detectable carbohydrates, and thus were limiting the bacterial metabolism. In the recirculation phase, the sCOD increased significantly for both *Paenibacillus* spp., indicating an increased hydrolytic activity where a higher amount of particulate feedstock is solubilized in the medium. Feedstock adaption and growth of the *Paenibacillus* spp. could explain the delayed increase of sCOD in the recirculation phase.

For both feedstocks, MS and 30% of straw (w/w), the elevated metabolite concentrations decreased, when recirculation was stopped; thus, the benefit of recirculation is directly observable. Therefore, the bioaugmentation tests with 66% (w/w) straw were operated for 3 HRT, including 2 recirculation periods to assess the duration of positive effects on hydrolysis. The change of feedstock to 66% (w/w) of straw decreased the sCOD significantly to an average of 14.2 $gO_2\ L^{-1}$ (Fig. 2(c)). The higher share of recalcitrant substrate reduced the hydrolysis efficiency. Bioaugmentation with *P. glucanolyticus* led to an increase of the sCOD (23.9%) and total SCCA (13.9%) during the addition period but both decreased rapidly in the recirculation periods. The addition of *P. macerans* resulted in an average higher sCOD concentration (16.0 $gO_2\ L^{-1}$) that stayed above the reference level for the two recirculation periods, and correspondingly increased the total SCCA (18–34.2%). The applied bioaugmentation/recirculation strategy was successful in maintaining positive effects for 3 HRT when adding *P. macerans* with recalcitrant feedstock, but *P. glucanolyticus* was less effective. Bioaugmentation, in all feedstocks, increased the acid concentration compared to the prior state (Fig. 4). Judging

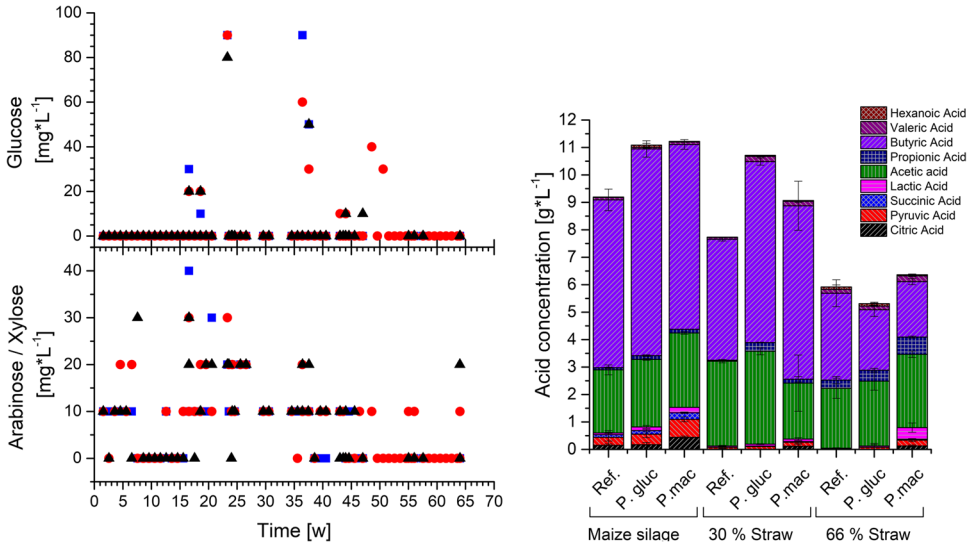

Fig. 4 Left: Soluble sugars of selected samples in the fermentation broth measured by GC–MS in the inlet (blue squares), center (red circles), and outlet port (black triangles) of the PFR. Right: Distribution of SCCAs when different feedstocks and bioaugmentation scenarios were applied

over the whole test period, this was most significant for MS (+29.7 to 31.3%) and 30% straw (w/w) (+26.5 to 45.6%). Even with the higher content of recalcitrant feedstock in the 30% (w/w) straw digestion, bioaugmentation contributed to higher acid concentrations than during the digestion of pure MS. With 66% straw (w/w), only *P. macerans* addition led to an average increase of total SCCAs of 26.5%.

At all different feedstocks, butyrate (31.8–69.7%) and acetate (22.0 to 45.2%) were the dominant SCCAs. Also small proportions of other SCCAs were detected, but their share remained mostly below 5% of all measured components (Figure S2). Bioaugmentation with *P. macerans* increased the concentrations of pyruvate, succinate, citrate and lactate, and both *Paenibacillus* spp. increased the production of butyrate for all feedstock compositions. Although it has been described that *Paenibacillus* spp. do not produce butyrate in high amounts [19, 21, 52], we assume that the hydrolytic enzymes produced by the *Paenibacillus* spp. increased the substrate availability in the PFR and thus contributed to butyrate production by providing the respective precursors. This is in agreement with results from Esquivel-Elizondo et al. [53], who investigated butyrate production in controlled fermentation systems via metagenome prediction analysis and found that non-butyrate-producing bacteria contributed to butyrate production through the generation of acetate, lactate, and succinate; these acids are then converted to butyrate via interconversion reactions. In our study, especially with the addition of *P. macerans*, increased levels of lactate, succinate and pyruvate were detected. Also, concurrent acetate and butyrate production can take place via the butyrate fermentation pathway from sugars released from polysaccharide digestion by microorganisms of the genus of *Clostridium*, *Bacillus*, *Bacteroides*, *Syntrophobacter*, and *Methylobacterium* [54]. Hydrogen is produced as secondary metabolite in this pathway. The drop in hydrogen production during *P. glucanolyticus* bioaugmentation with 66% of straw (w/w) could indicate the metabolic switch from butyrate production from carbohydrates towards the conversion from intermediates to butyrate without hydrogen production. Formerly, it was found that butyrate accumulation is favored at a pH range of 5.0 to 6.0 [3]. In our case, however, a stable production of butyrate was achieved at pH values that were between 3.8 and 4.8. Generally in AD, a low pH value between 4.0 and 4.5 is associated with ethanol and lactate production by Lactobacilli at these lower pH values [51, 55]. In the cultivations studied here, an increased lactate accumulation occurred at a pH value between 3.5 and 3.6 (unpublished data). In dark fermentation, the balance between lactate- and butyrate-producing bacteria highly depends on the pH value. Detman et al. [55] showed that microbial communities from dark fermentation are able to convert acetate and lactate to butyrate. Such communities showed a higher biodiversity than those dominated by lactic acid bacteria. Likely, the gradients in the PFR created microenvironments which contributed to a higher biodiversity at lower pH values; thus, no metabolic shift towards lactate production was observable. Symbiotic cross-feeding on lactate of butyrate producers like Clostridia seems to be common in dark fermentation of complex feedstocks. It is even believed that this is the route that provides major carbon fluxes towards butyrate and hydrogen production [55]. Moreover, in our continuous PFR system, butyrate production might have been favored by several factors, which are a low hydrogen headspace pressure, longer HRT [53], and the availability of pentose sugars [56] (as detected by GC–MS measurements in this study). To the authors' knowledge, no production of butyrate from lignocellulosic residues has been reported at this low pH value so far. Synergistic effects between different microbial species are known to enhance growth and substrate degradation in harsh conditions.

Although partnerships of microbial communities producing butyrate are not well understood [53], these could have ensured a stable production with only minor shifts of the SCCA's accumulation profile in the PFR.

3.3 Effect of bioaugmentation and recirculation on hydrolysis

Hydrolysis (Eq. 3) was about 20% on average during the reference cultivation of MS and 30% straw. A changed composition of the feedstock to 66% straw decreased it to 15.8%, due to the higher amount of recalcitrant material. Bioaugmentation increased the hydrolysis for MS and 30% straw to a maximum of 25.2% for 30% straw using *P. macerans* (Table 4). In case of a digestion of 66% straw (w/w), an increased hydrolysis was only determined for *P. macerans* addition with 19.7%, reaching similar values as with pure MS. It must be noted, however, that using this hydrolysis formula, mass transfer to the gas phase is not included. As mentioned before, there was a decrease in mass flow through the reactor of 15% for MS and 25% for straw substrate caused by substrate conversion to gas, so the hydrolysis rate might have been underestimated.

During MS cultivation, an acidification between 64 and 68% was achieved, which was similar for reference and augmented conditions. For MS, maximal acidification at controlled alkaline conditions reached up to 71% (61% in acidic conditions) as measured by Jankowska et al. [57]. Thus, plug-flow-based digestion in acidic conditions as conducted in our study achieved comparable values as reported in literature. Bioaugmentation increased acidification only in the case of 30% straw (w/w). It can be assumed that the addition of *Paenibacillus* spp. contributed to the solubilization and degradation of polymeric substances, making them better available to the acidogenic bacteria. No significant influence of the *Paenibacillus* spp. could be found for MS and 66% of straw (w/w). Likely, the already-high acidification of MS under non-augmented conditions caused acidic stress and prevented higher acidification by bioaugmentation. Chen et al. [51] only reached a maximal acidification of 36% in the acidogenic digestion of rice straw and food waste; thus, we conclude that our PFR setup is better suited for acid production from lignocellulosic residues like straw. In total, addition of *Paenibacillus* spp. contributed to higher hydrolysis, which was more effective with a higher amount of MS and only slightly contributed to a higher acidification.

In the case of MS, the SHR stabilizes at 258 $gO_2\ kg^{-1}{}_{VSfed}$ in weeks 15 and 16. An SHR of 264 $gO_2\ kg^{-1}{}_{Vsfed}$ was reached when 30% of straw (w/w) was applied. Addition of *P. glucanolyticus* to MS digestion increased the SHR during the addition and recirculation phase by 7.2% and 42%, and by 4.0% and 26.4% for 30% (w/w) of straw accordingly (Fig. 5, Table 5). Recirculation prolonged and increased the positive effects on SHR. Similarly, acid production was enhanced by up to 25% for MS and 42% for 30% (w/w) of straw digestion. SHR with 66% (w/w) of straw was generally lower than that for the other feedstock mixtures, reaching an average value of 195 $gO_2\ kg^{-1}{}_{Vsfed}$ under non-augmented conditions. During the digestion of 66% (w/w) of straw, the addition of *P. glucanolyticus* did not increase the SHR, but decreased it by 15%. This might be attributed to a higher share of carbon sources used for cell maintenance following the bioaugmentation. In the two recirculation phases, SHR fluctuated close around the level under reference cultivation conditions. The addition of *P. glucanolyticus*, however, led to an increased acid production during the addition phase and recirculation phase I. Bioaugmentation with *P. macerans* showed similar effects on metabolic rates. While minor changes in SHR occurred in the addition phase, the SHR was increased by 63.2% and 21.4% for MS and 30% straw (w/w) accordingly during recirculation, while net acid production was increased by up to 23% and 32%, respectively. Equally as with *P. glucanolyticus* addition, *P. macerans* addition to 66% straw (w/w) resulted in a decreased SHR over the whole trial period; however, the net acid production was increased. This indicates that the added

Table 4 Average measurement of FDAP, hydrolysis and acidification depending on substrate and bioaugmentation conditions. Included are all measurements at all ports

Substrate	Condition (Bioaugmentation)	FDAP at 400 kHz	Hydrolysis rate	Acidification
		$5{\cdot}10^{-31}$ F m^{-2}	%	%
MS	Reference	460.2 ± 82.6	20.1 ± 3.1	66.7 ± 8.6
	P. glucanolyticus	519.0 ± 72. 6	23.9 ± 1.4	67.7 ± 7.4
	P. macerans	543.6 ± 107.2	22.7 ± 3.2	64.3 ± 10.2
30% straw	Reference	424.6 ± 61.1	20.2 ± 1.8	54.2 ± 4.6
	P. glucanolyticus	653.9 ± 191.6	24.2 ± 2.5	60.8 ± 4.4
	P. macerans	675.6 ± 87.7	25.2 ± 1.6	56.3 ± 5.9
66% straw	Reference	998.9 ± 78.1	15.8 ± 0.9	52.9 ± 3.5
	P. glucanolyticus	1068.3 ± 123.2	15.6 ± 2.6	49.9 ± 4.9
	P. macerans	1122.7 ± 80.7	19.7 ± 1.7	51.7 ± 3.7

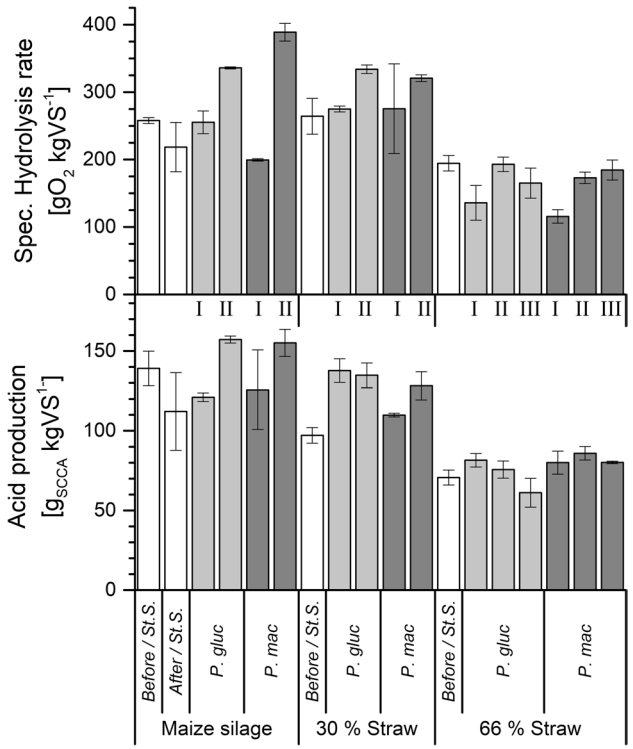

Fig. 5 Average values of SHR (top) and net acid production (bottom) before, with, and after bioaugmentation with *P. macerans* and *P. glucanolyticus*. The columns of bioaugmentation refer to the different phases: addition phase (I), first recirculation phase (II), second recirculation phase (III)

bacteria contributed to the degradation of soluble, polymeric, and oligomeric carbohydrates, making them available for the acidogenic microorganisms.

Maximum values of the net acid production from MS were reached under bioaugmentation (see Fig. 5). Bioaugmentation of both organisms yielded a very similar average acid production of 139.1 and 140.4 $g_{SCCA}\ kg_{VS}^{-1}$ for *P. glucanolyticus* and *P. macerans* correspondingly. Acid production of 310 $g_{SCCA}\ g_{COD}^{-1}$ was reached by Li et al. [58] digesting MS in a leach-bed reactor at controlled pH 8.0. It seems acid production was limited in our study. Since a high acidification of over 65% was achieved, this is likely caused by limited hydrolysis efficiency. Higher straw content decreased acid production to 97.2 and 70.6 $g_{SCCA}\ kg_{VS}^{-1}$ at 30% or 66% (w/w) of straw, respectively. The addition of *Paenibacillus* spp. to 30% straw (w/w) increased the acid production to an average of 116.4 and 136.3 $g_{SCCA}\ kg_{VS}^{-1}$ for *P. macerans* and *P. glucanolyticus*, respectively; the latter being only slightly lower than the corresponding yield reached during MS digestion. As discussed before, *P. macerans* showed a higher impact on the 66% straw digestion lasting for the 3 HRT tested and increased the average acid production by 16.1%.

3.4 Effect of bioaugmentation on culture viability

The electro-optical measurement of cell polarizability by FDAP is an indirect measurement of the cultures' physiological state. High levels of FDAP in a dark fermentation process have been associated with the hydrolytic activity, while a decrease in FDAP might indicate acidic stress conditions [31]. Higher FDAP usually corresponds to cells with a high energy level, a pre-requisite for fast synthesis rates. However, an increased FDAP with higher straw content (see Table 4) is related to a reduction of acidification stress as indicated by the lower acidification, total SCCA concentrations, and higher pH value. In contrast, an increased FDAP during bioaugmentation correlates with a higher metabolic activity due to an increased substrate availability and concomitantly higher metabolite concentration in the media. In the actual study, the average FDAP increased for all bioaugmentation trials in all feedstocks. As it can be seen in Fig. 6, bioaugmentation increased FDAP levels after addition especially when applying 66% straw (w/w). It also contributed to a stabilization of the FDAP values. Positive effects on cell viability of bioaugmentation can be seen during addition and the first recirculation phase, while in the second recirculation phase in weeks 59 and 60 with 66% straw (w/w) stronger fluctuations appeared again, which may be explained by the observed lower effect of *P. glucanolyticus* addition on the hydrolysis due to washout or limited survival. On the contrary, *P. macerans* addition still showed increased FDAP levels in the second recirculation phase (weeks 68 and 69),

Table 5 Percentual change of metabolic rates caused by bioaugmentation. A non-augmented cultivation under the same conditions is serving as reference state. An average of the values of the reference states with MS as feedstock before and after bioaugmentation was used here for easier comparability

Bioaugmentation phases		Change of specific hydrolysis rate [%]			Change of net acid production [%]		
		MS	30% straw	66% straw	MS	30% straw	66% straw
P. glucanolyticus	Addition	7.2	4.07	−30.1	−3.6	41.75	15.4
	Recirc. 1	41.0	26.40	−0.8	25.1	38.74	7.1
	Recirc. 2	–	–	−15.1	–	–	−13.5
P. macerans	Addition	−16.3	4.24	−40.5	0.1	13.09	13.2
	Recirc. 1	63.2	21.38	−11.1	23.5	31.97	21.5
	Recirc. 2	–	–	−5.1	–	–	13.4

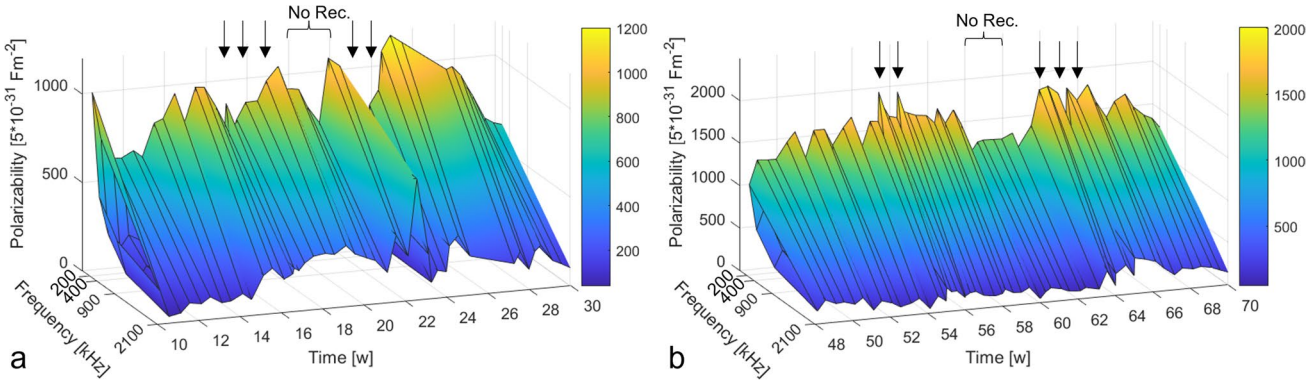

Fig. 6 Measurement of FDAP during the digestion of MS (**a**) and 66% straw (**b**). Sampling points after bioaugmentation are marked with arrows and the phase without recirculation is indicated by "No Rec." The average measurement between the ports of the PFR is depicted

suggesting that it had a longer lasting effect on the cultivation. Our suggestion is supported by the higher increase of sCOD and SCCAs with *P. macerans* addition in 66% straw (w/w). Thin-sludge recirculation apparently also supports cell viability in the PFR, as immediate decreases in FDAP can be seen when it was stopped.

The slope of polarizability across frequencies can also be attributed to performance changes within the culture. Haberman et al. [59] measured FDAP in AD fermenting MS and found a decreasing signal at higher frequencies. A similar pattern was measured in our current study, indicating that this slope is typically for anaerobic, mixed microbial communities. In contrast, Junne et al. [60] found that the increase of polarizability at low frequencies is typical for the acidogenic growth phase under substrate excess in *Clostridium acetobutylicum*. The results of our current study indicate that, in most phases, cells are in a vital state, but not under substrate excess conditions or close to maximum growth rates. This is also not to be expected under the given cultivation conditions anyway. Noteworthy to mention is that bioaugmentation with *Paenibacillus* spp. increased the viability and stability of the whole microbial community, which was further enhanced also over longer time by the application of thin-sludge recirculation.

4 Comparison of effects on the overall process performance

Bioaugmentation studies in AD focus mainly on the direct effect on methane yield, while bioaugmentation of the first stage has been rarely investigated. It was shown that an increase of sCOD and SCCAs in the first-stage results in higher and more stable methane production in AD [10, 61]. As discussed before, bioaugmentation in continuous processes often shows only short-term effects for a few days after addition due to washout and limited survival of the organism. Using the bioaugmentation strategy and recirculation pattern in the PFR as in our current study, positive effects of two microbial additions were seen for 2 HRT with pure MS and 30% (w/w) of straw as feedstock and possibly longer, if recirculation was not stopped. Martin-Ryals et al. [61] achieved 25–38% higher hydrolysis and 25–55% higher acid production with daily bioaugmentation into the acid phase of sewage sludge digestion. Similar results for MS and 30% straw (w/w) could be shown here (26–63% higher hydrolysis rate, 25–42% higher acid production), using only two additions and the recirculation strategy. However, bioaugmentation with any of the *Paenibacillus* strains could not increase hydrolysis of 66% (w/w) of straw with high recalcitrance. The higher pH value during 66% straw digestion should provide a better environment for *Paenibacillus* spp. and enhance activity of extracellular hydrolytic enzymes, but no significant advantage of bioaugmentation was seen at a high straw content in the feedstock. Growth of different *P. glucanolyticus* strains on pure lignin compounds was shown to be possible [20]. However, growth was very slow with generation times exceeding 100 h. Moreover, cinnamic acid, a degradation product of lignocellulose, was suspected to be toxic for *P. glucanolyticus* [20]. Also in the current study, hydrocinnamic acid was detected especially after bioaugmentation by GC measurements (see supplementary material, Figure S3). Hydrocinnamic acids are hydroxy derivatives of cinnamic acids that can be released during lignocellulose hydrolysis, of which many are known to have inhibiting effects on AD [62]. Possibly, higher concentrations of released lignocellulosic compounds at 66% of straw, a low pH value, and very slow growth inhibited further hydrolytic activity of the *Paenibacillus* spp. It has been found that easily degradable polysaccharides like starch and hemicellulose are metabolized before recalcitrant crystalline lignocellulosic compounds in mixed cultures [42]. The same applies for the *Paenibacillus* spp. in our fermentation: a rather increased hydrolysis of MS occurred in parallel with a higher amount of hemicellulose compared to the lignin-rich straw feedstock. Hemicellulose can have a rather small digestion efficiency of 29% in AD of MS [23]; our current study indicates that bioaugmentation may be

a possibility to improve this. The hydrolytic activity of both studied organisms was confirmed on various pure soluble polymers from lignocellulose in laboratory trials. Little is known, however, about their ability to grow on natural crystalline lignocellulosic structures as straw and MS. Decrystallisation of the substrate is a major bottleneck in cellulose hydrolysis and can only be achieved by truly cellulolytic microorganisms like *Clostridium* spp., among others [63]. Clostridia like *C. thermocellum* or *C. cellulolyticum* are ubiquitous and common in AD processes. Their multi-enzyme complex, the cellulosome, can efficiently degrade crystalline lignocellulosic structures, releasing the formerly bound polymers of hemicellulose, lignin, and cellulose into the media. Cellulose degradation through Clostridia, however, is severely inhibited by pH values below 6.0–6.5, mainly due to reduced growth rates [42, 64]. Since SHR decreases at a high straw content and cannot be enhanced by bioaugmentation, it seems that cellulose hydrolysis is limited by an unfavorable pH value below 5.0 in the PFR. While the total hydrolysis and acid yields from the PFR are in a mid-range and could be further optimized, it is likely that destabilization and structural changes occurred in the feedstock making it better accessible for further degradation afterwards.

5 Conclusion

The aim of this study was to evaluate plug-flow-based hydrolysis and the effect of bioaugmentation with *Paenibacillus* spp. The PFR showed to be a suitable system for hydrolysis and acidogenesis of MS with straw addition reaching SHR of 258–264 $gO_2\ kg^{-1}{}_{VS}$ and acid production of 97–135 $g_{SCCA}\ kg^{-1}{}_{VS}$ in continuous operation. Even without pH control, the PFR showed a stable metabolic profile of acid production yielding mainly butyrate and acetate. With a high ratio of 66% straw, an SHR of 195 $gO_2\ kg^{-1}{}_{VS}$ and an acid production of 70.6 to 85.9 $g_{SCCA}\ kg^{-1}{}_{VS}$ were reached. Bioaugmentation with *Paenibacillus* spp. proved to be beneficial for the digestion of MS and 30% straw (w/w), increasing SHR by up to 63% and SCCA production by up to 42%, while minor effects could be seen in the digestion of 66% (w/w) of straw. The net acid production was increased among all feedstock mixtures. Overall, from our results, we can conclude that bioaugmentation with *Paenibacillus* spp. enhanced the fermentation process majorly by three mechanisms:

i) Enhanced solubilization of particulate substrate as shown by higher sCOD concentrations and increased SHR
ii) Enhanced degradation of formerly unused soluble (hemicellulose) polymers resulting in better substrate availability, higher acid production, and higher culture vitality as measured by FDAP
iii) Enhanced butyrate production by the generation of precursors for butyrate-producing bacteria.

Thin-slurry recirculation further increased and prolonged the effects of bioaugmentation for the 2 HRT that were tested and longer lasting effects are probable for MS and 30% straw (w/w). These longer positive effects of bioaugmentation indicate a suitability of the chosen microorganism for the survival and enzyme production in the PFR, because hydrolytic enzymes that were released from the previous *Paenibacillus* sp. Monocultivation would have mostly been deactivated by endogenous proteases in the hydrolysis' culture broth after 24 h [65]. However, long-term survival of the augmented organisms in the PFR with lignin-rich feedstock is not probable, as the wear-off of positive effects can be seen by the time-dependent decrease of FDAP and conductivity measurements, which correlated well with the total SCCA concentration, within 2 HRT after bioaugmentation. Nonetheless, *Paenibacillus* sp. addition boosted microbial hydrolysis and acid production in the PFR by enhancing the indigenous microbial community. Compared to routine bioaugmentation, significantly less culture had to be used to achieve comparable results. This PFR setup can serve as a first stage for two-stage AD processes, in order to broaden the applicable feedstock spectrum and stablize methane synthesis, and thus make methane production more flexible. Bioaugmentation, together with the proposed reactor system, can enhance yields of recalcitrant biogenic residues, and thus pave the way for a good integration of AD and other bioprocesses into a circular bioeconomy.

It remains to be investigated what frequency of addition would be required to maintain enhanced hydrolysis and acidogenesis on a long term and whether bioaugmentation with a mixture of different species would lead to a further improvement when a high content of straw is applied as feedstock. For better insights into microbial changes during bioaugmentation and lignocellulose digestion at a low pH value, a microbial consortium determination via high-throughput 16 s RNA gene amplicon sequencing should be conducted at selected time points.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-023-03958-8.

Author contribution Conceptualization, T.M. and S.J.; writing—original draft preparation, T.M.; writing—review and editing, T.M., S.J., and P.N.; visualization, T.M.; supervision, S.J. and P.N.; funding acquisition, S.J.

Funding Open Access funding enabled and organized by Projekt DEAL. This work was funded by the Fachagentur für Nachhaltige Rohstoffe e.V. (FNR, Agency of Renewable Resources)-22039818.

Data availability Data of graphics as shown in this manuscript are stored on the DepositOnce data storage platform of TU Berlin, accessible at https://depositonce.tu-berlin.de/home. Further datasets used and/or described during the current study are available from the corresponding author on a reasonable request.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

Graphics Graphics were created with Origin (version 2015), ©OriginLab Corp. (Northampton, MA).

References

1. Kucharska K, Rybarczyk P, Hołowacz I, et al (2018) Pretreatment of lignocellulosic materials as substrates for fermentation processes. Molecules 23:. https://doi.org/10.3390/molecules23112937
2. Baruah J, Nath BK, Sharma R, et al (2018) Recent trends in the pretreatment of lignocellulosic biomass for value-added products. Front Energy Res 6:. https://doi.org/10.3389/fenrg.2018.00141
3. Menzel T, Neubauer P, Junne S (2020) Role of microbial hydrolysis in anaerobic digestion. Energ 13:5555. https://doi.org/10.3390/en13215555
4. Carrere H, Antonopoulou G, Affes R et al (2016) Review of feedstock pretreatment strategies for improved anaerobic digestion: from lab-scale research to full-scale application. Bioresour Technol 199:386–397. https://doi.org/10.1016/j.biortech.2015.09.007
5. Van DP, Fujiwara T, Leu Tho B et al (2019) A review of anaerobic digestion systems for biodegradable waste: configurations, operating parameters, and current trends. Environ Eng Res 25:1–17. https://doi.org/10.4491/eer.2018.334
6. Khalil CA, Eraky MT, Ghanimeh S (2021) Localized mixing of anaerobic plug flow reactors. Water Res 204:117588. https://doi.org/10.1016/J.WATRES.2021.117588
7. Dong L, Cao G, Wu J et al (2019) Reflux of acidizing fluid for enhancing biomethane production from cattle manure in plug flow reactor. Bioresour Technol 284:248–255. https://doi.org/10.1016/J.BIORTECH.2019.03.092
8. Veluchamy C, Gilroyed BH, Kalamdhad AS (2019) Process performance and biogas production optimizing of mesophilic plug flow anaerobic digestion of corn silage. Fuel 253:1097–1103. https://doi.org/10.1016/J.FUEL.2019.05.104
9. Namsree P, Suvajittanont W, Puttanlek C et al (2012) Anaerobic digestion of pineapple pulp and peel in a plug-flow reactor. J Environ Manage 110:40–47. https://doi.org/10.1016/j.jenvman.2012.05.017
10. Martin-Ryals A, Schideman L, Li P et al (2015) Improving anaerobic digestion of a cellulosic waste via routine bioaugmentation with cellulolytic microorganisms. Bioresour Technol 189:62–70. https://doi.org/10.1016/j.biortech.2015.03.069
11. Peng X, Börner RA, Nges IA, Liu J (2014) Impact of bioaugmentation on biochemical methane potential for wheat straw with addition of Clostridium cellulolyticum. Bioresour Technol 152:567–571. https://doi.org/10.1016/j.biortech.2013.11.067
12. Poszytek K, Ciężkowska M, Skłodowska A, Drewniak Ł (2016) Microbial Consortium with High Cellulolytic Activity (MCHCA) for enhanced biogas production. Front Microbiol 7. https://doi.org/10.3389/fmicb.2016.00324
13. Ozbayram EG, Kleinsteuber S, Nikolausz M et al (2018) Bioaugmentation of anaerobic digesters treating lignocellulosic feedstock by enriched microbial consortia. Eng Life Sci 18:440–446. https://doi.org/10.1002/elsc.201700199
14. Nzila A (2016) Mini review: update on bioaugmentation in anaerobic processes for biogas production. Anaerobe 46:3–12. https://doi.org/10.1016/J.ANAEROBE.2016.11.007
15. Yu J, Zhao Y, Liu B et al (2016) Accelerated acidification by inoculation with a microbial consortia in a complex open environment. Bioresour Technol 216:294–301. https://doi.org/10.1016/j.biortech.2016.05.093
16. Tsapekos P, Kougias PG, Vasileiou SA et al (2017) Bioaugmentation with hydrolytic microbes to improve the anaerobic biodegradability of lignocellulosic agricultural residues. Bioresour Technol 234:350–359. https://doi.org/10.1016/J.BIORTECH.2017.03.043
17. Shetty D, Joshi A, Dagar SS et al (2020) Bioaugmentation of anaerobic fungus Orpinomyces joyonii boosts sustainable biomethanation of rice straw without pretreatment. Biomass Bioenergy 138:105546. https://doi.org/10.1016/J.BIOMBIOE.2020.105546
18. Alexander B, Priest FG (1989) Bacillus glucanolyticus, a new species that degrades a variety of β-glucans. Int J Syst Bacteriol 39:112–115. https://doi.org/10.1099/00207713-39-2-112
19. Mathews SL, Pawlak JJ, Grunden AM (2014) Isolation of Paenibacillus glucanolyticus from pulp mill sources with potential to deconstruct pulping waste. Bioresour Technol 164:100–105. https://doi.org/10.1016/J.BIORTECH.2014.04.093
20. Mathews SL, Grunden AM, Pawlak J (2016) Degradation of lignocellulose and lignin by Paenibacillus glucanolyticus. Int Biodeterior Biodegradation 110:79–86. https://doi.org/10.1016/J.IBIOD.2016.02.012
21. Williams AG, Withers SE (1985) The production of hemicellulose-degrading enzymes by Bacillus macerans in anaerobic culture. Appl Microbiol Biotechnol 22:318–324. https://doi.org/10.1007/BF00582414
22. Szarka N, Haufe H, Lange N et al (2021) Biomass flow in bioeconomy: overview for Germany. Renew Sustain Energ Rev 150:111449. https://doi.org/10.1016/J.RSER.2021.111449
23. Santi G, Proietti S, Moscatello S et al (2015) Anaerobic digestion of corn silage on a commercial scale: differential utilization of its chemical constituents and characterization of the solid digestate. Biomass Bioenerg 83:17–22. https://doi.org/10.1016/J.BIOMBIOE.2015.08.018
24. Lask J, Martínez Guajardo A, Weik J et al (2020) Comparative environmental and economic life cycle assessment of biogas production from perennial wild plant mixtures and maize (Zea mays L.) in southwest Germany. GCB Bioenergy 12:571–585. https://doi.org/10.1111/GCBB.12715
25. Gao Z, Alshehri K, Li Y et al (2022) Advances in biological techniques for sustainable lignocellulosic waste utilization in biogas production. Renew Sustain Energy Rev 170:112995. https://doi.org/10.1016/J.RSER.2022.112995
26. Baird RB, Eaton AD, Rice EW (2017) Standard methods for the examination of water and wastewater, 23nd edition. American Public Health Association; American Water Works Association; Water Environment Federation
27. Bhagia S, Nunez A, Wyman CE, Kumar R (2016) Robustness of two-step acid hydrolysis procedure for composition analysis of poplar. Bioresour Technol 216:1077–1082. https://doi.org/10.1016/J.BIORTECH.2016.04.138
28. Sluiter A, Ruiz R, Scarlata C, et al (2008) Determination of extractives in biomass: laboratory analytical procedure (LAP); Issue Date 7/17/2005. Natl Renew Energy Lab; Technical Report: NREL/TP-510-42619
29. Chow PS, Landhäusser SM (2004) A method for routine measurements of total sugar and starch content in woody plant tissues. Tree Physiol 24:1129–1136. https://doi.org/10.1093/TREEPHYS/24.10.1129
30. Albalasmeh AA, Berhe AA, Ghezzehei TA (2013) A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. Carbohydr Polym 97:253–261. https://doi.org/10.1016/J.CARBPOL.2013.04.072
31. Gómez-Camacho CE, Pellicer Alborch K, Bockisch A et al (2021) Monitoring the physiological state in the dark fermentation of

maize/grass silage using flow cytometry and electrooptic polarizability measurements. Bioenergy Res 14:910–923. https://doi.org/10.1007/S12155-020-10184-X/FIGURES/6
32. Kiehlhorn E, Neubauer P, Junne S (2013) Determination of carbohydrates based on GC-MS analytics. In: Collection ofMethods for Biogas, vol 7. 2016 p 54–57
33. De La Rubia MA, Raposo F, Rincón B, Borja R (2009) Evaluation of the hydrolytic-acidogenic step of a two-stage mesophilic anaerobic digestion process of sunflower oil cake. Bioresour Technol 100:4133–4138. https://doi.org/10.1016/j.biortech.2009.04.001
34. Zhang B, He PJ (2014) Performance assessment of two-stage anaerobic digestion of kitchen wastes. Environ Technol 35:1277–1285. https://doi.org/10.1080/09593330.2013.866169
35. Guo H, Oosterkamp MJ, Tonin F et al (2021) Reconsidering hydrolysis kinetics for anaerobic digestion of waste activated sludge applying cascade reactors with ultra-short residence times. Water Res 202:117398. https://doi.org/10.1016/J.WATRES.2021.117398
36. De Gioannis G, Muntoni A, Polettini A et al (2017) Energy recovery from one- and two-stage anaerobic digestion of food waste. Waste Manag 68:595–602. https://doi.org/10.1016/j.wasman.2017.06.013
37. Voelklein MA, Jacob A, O' Shea R, Murphy JD (2016) Assessment of increasing loading rate on two-stage digestion of food waste. Bioresour Technol 202:172–180. https://doi.org/10.1016/j.biortech.2015.12.001
38. Rabii A, Aldin S, Dahman Y, Elbeshbishy E (2019) A review on anaerobic co-digestion with a focus on the microbial populations and the effect of multi-stage digester configuration. Energies 12:1106. https://doi.org/10.3390/en12061106
39. Meegoda JN, Li B, Patel K, Wang LB (2018) A review of the processes, parameters, and optimization of anaerobic digestion. Int J Environ Res Public Heal 15:2224. https://doi.org/10.3390/IJERPH15102224
40. Rocha-Meneses L, Zannerni R, Inayat A et al (2022) Current progress in anaerobic digestion reactors and parameters optimization. Biomass Convers Biorefinery 1:1–24. https://doi.org/10.1007/S13399-021-02224-Z/TABLES/8
41. Shrestha S, Fonoll X, Khanal SK, Raskin L (2017) Biological strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion: current status and future perspectives. Bioresour Technol 245:1245–1257
42. Zverlov VV, Hiegl W, Köck DE et al (2010) Hydrolytic bacteria in mesophilic and thermophilic degradation of plant biomass. Eng Life Sci 10:528–536. https://doi.org/10.1002/elsc.201000059
43. De Souza TSP, Haroldo Kawaguti Y (2021) Cellulases, hemicellulases, and pectinases: applications in the food and beverage industry. Food Bioprocess Technolhttps://doi.org/10.1007/s11947-021-02678-z
44. Chen Y, Sharma-Shivappa RR, Chen C (2007) Ensiling agricultural residues for bioethanol production. Appl Biochem Biotechnol 143:80–92. https://doi.org/10.1007/S12010-007-0030-7/TABLES/4
45. Peng J, Abomohra AEF, Elsayed M et al (2019) Compositional changes of rice straw fibers after pretreatment with diluted acetic acid: towards enhanced biomethane production. J Clean Prod 230:775–782. https://doi.org/10.1016/J.JCLEPRO.2019.05.155
46. Ma S, Wang H, Li L et al (2021) Enhanced biomethane production from corn straw by a novel anaerobic digestion strategy with mechanochemical pretreatment. Renew Sustain Energy Rev 146:111099. https://doi.org/10.1016/J.RSER.2021.111099
47. Marín-Peña O, Alvarado-Lassman A, Vallejo-Cantú NA et al (2020) Electrical conductivity for monitoring the expansion of the support material in an anaerobic biofilm reactor. Process 8:77. https://doi.org/10.3390/PR8010077
48. Aceves-Lara CA, Latrille E, Conte T, Steyer JP (2012) Online estimation of VFA, alkalinity and bicarbonate concentrations by electrical conductivity measurement during anaerobic fermentation. Water Sci Technol 65:1281–1289. https://doi.org/10.2166/WST.2012.703
49. Chetawan W, Saritpongteeraka K, Palamanit A, Chaiprapat S (2021) Practical approaches for retrofitting plug flow digester and process control to maximize hydrolysis and methane yield from piggery waste. J Environ Chem Eng 9:105620. https://doi.org/10.1016/J.JECE.2021.105620
50. Nghiem LD, Manassa P, Dawson M, Fitzgerald SK (2014) Oxidation reduction potential as a parameter to regulate micro-oxygen injection into anaerobic digester for reducing hydrogen sulphide concentration in biogas. Bioresour Technol 173:443–447. https://doi.org/10.1016/J.BIORTECH.2014.09.052
51. Chen X, Yuan H, Zou D et al (2015) Improving biomethane yield by controlling fermentation type of acidogenic phase in two-phase anaerobic co-digestion of food waste and rice straw. Chem Eng J 273:254–260. https://doi.org/10.1016/j.cej.2015.03.067
52. Gupta A, Murarka A, Campbell P, Gonzalez R (2009) Anaerobic fermentation of glycerol in Paenibacillus macerans: metabolic pathways and environmental determinants. Appl Environ Microbiol 75:5871–5883. https://doi.org/10.1128/AEM.01246-09
53. Esquivel-Elizondo S, Ilhan ZE, Garcia-Peña EI, Krajmalnik-Brown R (2017) Insights into butyrate production in a controlled fermentation system via gene predictions. mSystems 2. https://doi.org/10.1128/mSystems.00051-17
54. Luo L, Kaur G, Wong JWC (2019) A mini-review on the metabolic pathways of food waste two-phase anaerobic digestion system. Waste Manag Res J a Sustain Circ Econ 37:333–346. https://doi.org/10.1177/0734242X18819954
55. Detman A, Laubitz D, Chojnacka A et al (2021) Dynamics of dark fermentation microbial communities in the light of lactate and butyrate production. Microbiome 9:158. https://doi.org/10.1186/s40168-021-01105-x
56. Chatellard L, Trably E, Carrère H (2016) The type of carbohydrates specifically selects microbial community structures and fermentation patterns. Bioresour Technol 221:541–549. https://doi.org/10.1016/J.BIORTECH.2016.09.084
57. Jankowska E, Chwialkowska J, Stodolny M, Oleskowicz-Popiel P (2017) Volatile fatty acids production during mixed culture fermentation – the impact of substrate complexity and pH. Chem Eng J 326:901–910. https://doi.org/10.1016/J.CEJ.2017.06.021
58. Li Y, Hua D, Xu H et al (2020) Acidogenic and methanogenic properties of corn straw silage: regulation and microbial analysis of two-phase anaerobic digestion. Bioresour Technol 307:123180. https://doi.org/10.1016/J.BIORTECH.2020.123180
59. Habermann B, Ritzi E, Schulze F (2015) Monitoring of biogas plants - experiences in laboratory and full scale. Carbon Sci Technol 7:1–7
60. Junne S, Klein E, Angersbach A, Goetz P (2008) Electrooptical measurements for monitoring metabolite fluxes in acetone–butanol–ethanol fermentations. Biotechnol Bioeng 99:862–869. https://doi.org/10.1002/BIT.21639
61. Martin-Ryals AD, Schideman LC, Ong M (2018) Utilizing bioaugmentation to improve performance of a two-phase AnMBR treating sewage sludge. Environ Technol 41:1322–1336. https://doi.org/10.1080/09593330.2018.1533041
62. Kim D (2018) Physico-chemical conversion of lignocellulose: inhibitor effects and detoxification strategies: a mini review. Mol 23:309. https://doi.org/10.3390/molecules23020309
63. Schwarz WH (2001) (2001) The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol 565(56):634–649. https://doi.org/10.1007/S002530100710
64. Desvaux M (2005) Clostridium cellulolyticum: model organism of mesophilic cellulolytic clostridia. FEMS Microbiol Rev 29:741–764. https://doi.org/10.1016/j.femsre.2004.11.003
65. Odnell A, Recktenwald M, Stensén K et al (2016) Activity, life time and effect of hydrolytic enzymes for enhanced biogas production from sludge anaerobic digestion. Water Res 103:462–471. https://doi.org/10.1016/j.watres.2016.07.064