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Production of biogas from sugarcane wastes: an assessment of microbial community dynamics for an efficient process

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The disposal of large amounts of waste still containing energetic value is a central challenge in the waste management of the Brazilian sugarcane industry. As a sustainable solution, the biogas process appears to be a suitable technology for treating sugarcane waste products and for providing valuable commodities such as energy-rich biogas and digestate with fertilizer properties. Additionally, the proper treatment of the four major waste types (straw, bagasse, filter cake and vinasse) would avoid greenhouse gas emissions, air pollution and environmental contamination of soil and water. In order to investigate the feasibility and reliability of biogas production from sugarcane wastes, the microbial community dynamics of laboratory-scale reactors were assessed under different start-up strategies. Despite the promising results of the methane potential for all the waste products, chemical and physical pre-treatments were applied successfully to increase the methane yield of straw, bagasse and filter cake. The microbial community dynamics observed during co-digestion of filter cake and bagasse showed, together with the process parameters, that cattle manure can be effectively used as an inoculum for the start-up of a biogas process in the remote-located sugarcane industry. Monitoring methanogenic community dynamics at high organic loading rate of filter cake and bagasse demonstrated that the genera *Methanosarcina* and *Methanobacterium* are the major methanogens that produce biogas, even under process imbalances. Moreover, the results obtained from the process parameters and methanogenic community analyses revealed that the stable isotope fingerprinting technique may be a potential monitoring tool for quickly identifying changes in the methanogenic pathway, which indicates process disturbances. In conclusion, these studies established techniques for the efficient substrate processing and start-up procedure of a biogas process designed for the anaerobic digestion of sugarcane wastes, and by these means provided a highly detailed profile of the microbial community in relation to process parameters.

‘*alles is overal*: maar *het milieu selecteert*’

(Lourens Gerhard Marinus Baas Becking, 1934)

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Summary

Through the production of bioethanol and sugar in the Brazilian sugarcane industry, a large amount of waste is generated. Unfortunately, poor waste management contrasts with Brazil's successful bioethanol history and also with the country's long tradition as a global sugar producer. The four major waste products generated by the Brazilian sugarcane industry pose a direct or indirect threat to the environment and human health. Straw (the leaves of the sugarcane) is either left or burned on the fields, the latter causing air pollution. Bagasse (the crushed stalk) is usually burned to produce heat and electricity but sometimes it is simply put aside due to the fact that it is in surplus. Filter cake (the press-mud) and vinasse (the distillery residue) are spread onto the fields as fertilizers, contributing to soil and water contamination. In addition, during the temporary storage of vinasse in open lagoons (before spreading), a great amount of greenhouse gases are emitted. These harmful waste disposal options squander the considerable energy content of these wastes, which could be exploited. As a sustainable solution for the energy recovery of sugarcane wastes, biogas production via anaerobic digestion (AD) might be a suitable treatment technology. Energy-rich biogas has many potential applications, e.g. vehicle fuel, and heat and power generation. Moreover, the digestate could be used as a valuable fertilizer. In order to conduct a feasible and reliable start-up procedure and later a stable operation of the biogas process in the sugarcane industry, the wastes were initially characterized and then the biogas production, in a semi-continuous feeding regime, was conducted in several laboratory-scale reactors. Additionally, the microbial community dynamics was assessed by molecular and stable isotope fingerprinting techniques.

Straw, bagasse, filter cake and vinasse were subjected in the first study to physico-chemical analyses and batch tests in order to determine the biochemical methane potential (BMP). Furthermore, other characteristics of sugarcane wastes, including total solids, volatile solids (VS), chemical oxygen demand (COD), pH value, total Kjeldahl nitrogen, raw fat and protein content, total carbohydrate and lignin content, and organic acid concentration, were assessed. This information was fundamental to the design of a semi-continuously fed biogas process, especially in regard to the selection of reactor type, trace element additive requirement, identification of potential inhibitors, assessment of substrate pre-treatment options, and evaluation of the potential for co-digestion with other waste products from the sugarcane process.

The BMP results, of around 215 $mL_N \cdot g_{VS}^{-1}$ for straw, 275 $mL_N \cdot g_{VS}^{-1}$ for bagasse, 250 $mL_N \cdot g_{VS}^{-1}$ for filter cake, and 260 $mL_N \cdot g_{COD}^{-1}$ for vinasse, were promising compared to those of energy crops such as maize silage. However, in order to increase the degree of conversion, physical and chemical pre-

treatments were applied to the lignocellulosic materials (straw, bagasse and filter cake). Submitting straw to only a physical pre-treatment (reduction to a particle size of under 2 mm) yielded a 26% higher BMP. Alkaline (NaOH) pre-treatment of filter cake resulted in a BMP increase of 22%. When the reduced particle size of bagasse (below 10 mm) was treated with calcium hydroxide solution, its BMP rose drastically by more than 50%.

Based on the newly described characteristics of sugarcane wastes, experiments using continuously stirred tank reactors were designed and carried out to investigate the AD of filter cake as a single substrate and also its co-digestion with bagasse. Filter cake was the most interesting and promising waste product, mainly due to its unique characteristic of being suitable for long-distance transportation, its current disposal status and its elemental composition. The co-digestion of filter cake with bagasse at a feeding ratio of 7:3 (based on fresh mass) was carried out with the intention of using part of the remaining bagasse deposited by the industry, and also to complement the AD of the filter cake by adding bagasse as an additional carbon source. In the continuous feeding experiments, the microbial community dynamics was investigated in order to determine feasible and reliable start-up options for an efficient biogas process. The microbial community structure was assessed using terminal restriction fragment length polymorphisms and next-generation 16S rRNA amplicon sequencing. The microbial activity of the methanogens was monitored by stable isotope fingerprinting of the produced methane and carbon dioxide. Both molecular and stable isotope techniques were compared in terms of their value as indicators of process imbalances.

In the second study, the effect of the gradual increase of the organic loading rate (OLR) from start-up to overload on the mono-digestion of filter cake and its co-digestion with bagasse was investigated. This was an important factor for the process design, since it allowed the determination of an appropriate reactor volume and, consequently, investment costs. In this second study, a high OLR was desired because it implies a reduction of reactor volume. However, it often results in process imbalance and acidification due to the accumulation of organic acids. Therefore, the biogas reactors operated until high OLR levels were monitored systematically in order to identify at early stage indicators of process failure. Furthermore, an additional challenge for continuous biogas production from filter cake and bagasse was the seasonality of the sugarcane industry, which operates only around 200 days per year. Hence, due to the short substrate availability, a quick start-up of the biogas reactors by means of a fast OLR increase was preferred.

The adaptation period of the inoculum in the reactors for testing the gradual OLR increase (eight stepwise increases of 0.5 $g_{vs} \cdot L^{-1} \cdot day^{-1}$) showed a very similar methanogenic community structure in the initial start-up phases of the mono-digestion of filter cake compared to its co-digestion with

bagasse. At this stage, the predominant genera were *Methanosarcina*, *Methanosaeta* and *Methanoculleus*. The biogas process was stable up to an intermediate OLR of 2.5 $g_{vs}\cdot L^{-1}\cdot day^{-1}$. *Methanosaeta* and *Methanoculleus* were not any longer detected at an OLR of 2.5 and 3.0 $g_{vs}\cdot L^{-1}\cdot day^{-1}$, respectively. Co-digestion system suffered strong process imbalances at lower OLR (3.0 $g_{vs}\cdot L^{-1}\cdot day^{-1}$) compared to mono-digestion system (3.5 $g_{vs}\cdot L^{-1}\cdot day^{-1}$). The methanogenic community changed in a very similar way in both digestion set-ups up to an OLR of 3.0 $g_{vs}\cdot L^{-1}\cdot day^{-1}$, when *Methanosarcina* became the most abundant (around 80% relative abundance). During reactor acidification, the methanogenic community structure changed differently in the mono- and co-digestion processes. The aceticlastic genus *Methanosaeta* was detected again in the mono-digestion system, while the abundance of hydrogenotrophic genus *Methanobacterium* increased drastically in the co-digestion set-up (about 76% relative abundance). Process imbalance was also indicated by stable isotope fingerprinting, as the strong depletion of methane in ^{13}C revealed a shift towards hydrogenotrophic methanogenesis. All these findings regarding the monitoring of microbial community structure and activity together with the process parameters showed that an intermediate reactor volume operated up to an OLR of 3.0 $g_{vs}\cdot L^{-1}\cdot day^{-1}$ is appropriate for the mono-digestion of filter cake and its co-digestion with bagasse.

The third study was devoted to the investigation of different inoculation strategies for biogas production in the co-digestion of filter cake and bagasse. Inoculating the biogas reactors was a very important step investigated at the start-up procedure, since the functionality and adaptability of the microbial communities in the inoculum were supposed to determine the efficiency of the biogas process. In regions where full biogas plants are widespread and well established, new biogas systems are commonly inoculated with digestate from the already existing plants. However, this scenario was not considered feasible in Brazil, since the few biogas plants currently in the country are located very far from the sugarcane industries. In order to overcome this deficiency, fresh cattle manure from regional farms surrounding the sugarcane industry was considered as a potential alternative inoculum for the start-up of biogas reactors. To test fresh cattle manure as an inoculum for the co-digestion process, two parallel biogas reactors were inoculated and compared to two other parallel reactors inoculated with mixed inoculum containing different digestate from various biogas systems. Furthermore, the mono-digestion of filter cake and its co-digestion with bagasse was compared in terms of their efficiency using the mixed inoculum in five times up-scaled reactors (compared to the previous study) with longer microbial adaptation times during the new feeding load.

The process parameters showed that both mono- and co-digestion systems inoculated with mixed inoculum were mainly stable throughout the operation time of the reactors. Still, strong accumulation

of organic acids was observed during the start-up phase of the reactors inoculated with cattle manure. This drawback lasted for the first twenty operational days until the process was under stable running conditions. Although the C:N ratio of the mono- and co-digestion systems were in the appropriate range (24:1 and 41:1, respectively) for the AD process, the nitrogen bioavailability of filter cake and bagasse was very low. This influenced the buffer capacity and, consequently, the pH values, mainly at the steady-state. Despite the fact that the mono-digestion set-up presented higher biogas yield compared to co-digestion, further assessment demonstrated that, in a large-scale plant, the co-digestion set-up would be able to produce about 58% more biogas than the mono-digestion of filter cake. The reason for that regards the availability of the bagasse, which is approximately seven times more produced than filter cake (in relation to one ton of processed sugarcane).

The bacterial community structure at the start-up phase presented a higher diversity at phylum level in the reactors inoculated with cattle manure compared to those inoculated with mixed inoculum. The phyla Firmicutes and Bacteroidetes were dominant from start-up to steady-state. While the relative abundance of Bacteroidetes gradually increased towards the steady-state (reaching around 70%), the abundance of Firmicutes decreased. At the final steady-state of the experiment, all reactors in co-digestion process, independently of the inoculation strategy, displayed very similar bacterial community structure. Another phylum that showed significant abundance at steady-state was Synergistetes, which, together with the other two former phyla, comprised around 94% of the entire community. The major bacterial families involved in the AD of filter cake and bagasse were Bacteroidaceae, Prevotellaceae and Porphyromonadaceae (phylum Bacteroidetes), Ruminococcaceae, Clostridiaceae and Lachnospiraceae (phylum Firmicutes) and Synergistaceae (phylum Synergistetes). Following the same trend as the bacterial community, at initial start-up phase the methanogenic community structure was distinctive in the reactors with different inoculation strategies and later, at the final steady-state, was very similar independent of the inoculation strategy. The predominance of hydrogenotrophic genera (represented mostly by *Methanospirillum* and *Methanobacterium*) at the initial start-up phase was indicated by the strong depletion of ^{13}C in the isotope composition of the methane produced. Later, during the steady-state phase, the methanogenic pathways in all reactors shifted towards aceticlastic methanogenesis, as evident by the lower depletion of ^{13}C. Furthermore, the H_2-dependent methylotrophic genus *Methanomassiliicoccus* played an important role at the later steady-state phase in the co-digestion system. Comparing mono- to co-digestion performances, the up-scaling and adaptation period also revealed the contribution of the genera *Methanosarcina* and *Methanobacterium* to the mono-digestion of filter cake and its co-digestion with bagasse. Due to the high similarity of the microbial community in both inoculation strategies at final steady-state, cattle

manure can be successfully used as an inoculum for the start-up of the biogas process for co-digestion of filter cake and bagasse.

In a forth study, investigations into the AD of filter cake were carried out by assessing the production of volatile fatty acids (VFA) instead of biogas. This experiment was conducted because despite VFA being important intermediates during biogas production, they can also be utilized for the production of chemicals. The semi-continuous experiment was divided into 5 different phases: start-up, steady-state, urea implementation, urea plus nitric acid supplementation, and sodium hydroxide pre-treatment. Due to the low levels of ammonium nitrogen concentration during the steady-state, urea was added to the reactors as a nitrogen source in order to increase the VFA yield. However, the addition of urea drastically raised the pH values to around neutral, which favoured VFA consumption by methanogens. Consequently, urea was added together with nitric acid to maintain pH values around 5.5. However, under this new supplementation, the VFA yield did not recover to the same level as it was in the steady-state. Thus, the addition of urea did not yield any improvement in VFA production. The efficiency of alkaline pre-treatment was first investigated under various concentrations of NaOH solution applied to the filter cake in batch essays. Based on the finding that the highest BMP was achieved with 6 g NaOH per 100 g filter cake (fresh mass), this concentration was subsequently used to pre-treat the filter cake in a semi-continuous feeding regime. In this case, the alkaline pre-treatment increased the VFA yield by 37%.

In summary, the four major waste products (straw, bagasse, filter cake and vinasse) from the Brazilian sugarcane industry are suited to an efficient biogas production process. Batch and semi-continuous feeding experiments were carried out systematically in order to link so-far theoretically based research to future practical applications. Pre-treatment options to enhance methane yield, and nutritional supplementation assessment for the continuous biogas production process of straw, bagasse, filter cake and vinasse were established. Monitoring microbial community dynamics from the start-up to the steady-state phase of the mono-digestion of filter cake and its co-digestion with bagasse revealed the reliability of using cattle manure as an inoculum for biogas production in the sugarcane industry. Moreover, the assessment of the methanogenic community dynamics during the gradual increase of OLR showed the feasibility of using an intermediate reactor volume by defining an OLR at which an efficient biogas production from filter cake and bagasse was performed. In addition, the assessment of the microbial community dynamics in the experiments performed provided insights regarding possible microbial resource management during biogas production from sugarcane wastes. The stable isotope fingerprinting was used as a potential monitoring tool for indicating process imbalances through changes in the methanogenic pathway. Nevertheless, further investigation of the stable isotope

fingerprinting with multiple sample analyses under different process conditions should be conducted in order to develop predictable models integrating the process parameters and microbial community dynamics. Lastly, the continuous biogas production from sugarcane wastes can be efficiently performed, ensuring its feasibility and reliability as an energy source. Based on the final results, the biogas produced from sugarcane wastes would have been able to supply alone around 12% of Brazil's electricity consumption in 2016. However, the governmental incentives are crucial for promoting the biogas sector in the sugarcane industry and for triggering the necessary changes towards sustainable development.

Zusammenfassung

Bei der Herstellung von Bioethanol und Zucker in der brasilianischen Zuckerrohrindustrie entstehen großen Mengen Abfälle. Leider steht die schlechte Abfallwirtschaft der langen Tradition Brasiliens als größter Zuckerrohrproduzent der Welt und erfolgreicher Bioethanol-Geschichte gegenüber. Die vier Hauptabfälle, die von der brasilianischen Zuckerrohrindustrie erzeugt werden, stellen eine direkte oder indirekte Bedrohung für die Umwelt und die menschliche Gesundheit dar. Stroh (die Blätter des Zuckerrohres) wird entweder auf den Feldern liegen gelassen oder verbrannt, wodurch die Luft verschmutzt wird. Bagasse (die ausgequetschten Stiele) wird hauptsächlich verbrannt, um Hitze und Elektrizität zu produzieren. Aufgrund der hohen Mengen an anfallender Bagasse bleibt sie oft aber auch ungenutzt. Filterkuchen (der Pressschlamm) und Vinasse (der Destillationsrückstand) werden direkt als Dünger auf dem Feld verteilt und verunreinigen damit den Boden und Gewässer. Außerdem wird bei der vorübergehenden Lagerung von Vinasse in offenen Lagunen (vor der Ausbreitung) eine große Menge Treibhausgase emittiert. Darüber bleibt die in den Abfällen enthaltene Energie ungenutzt. Als nachhaltige Lösung für die Energierückgewinnung aus Zuckerrohrabfällen erscheint die Biogasproduktion über anaerobe Vergärung als geeignete Behandlungstechnologie. Das energiereiche Biogas hat viele Anwendungsmöglichkeiten in Form von Kraftstoff, Wärme und Strom. Zusätzlich kann der Gärrest als Dünger verwendet werden. Um ein plausibles und zuverlässiges Inbetriebnahmeverfahren und später einen stabilen Betrieb des Biogasprozesses in der Zuckerrohrindustrie durchzuführen, wurden die Abfälle zunächst charakterisiert. Anschließend wurden Versuche im Labormaßstab zur Biogasproduktion in einem halbkontinuierlichen Fütterungsregime durchgeführt. Zusätzlich wurde die mikrobielle Gemeinschaftsdynamik durch molekulare Mikrobiologie und Analyse von stabilen Isotopen bewertet.

In der ersten Studie wurden Stroh, Bagasse, Filterkuchen und Vinasse physikalisch-chemischen Analysen und Batchversuchen zur Bestimmung des biochemischen Methanpotentials (BMP) unterzogen. Außerdem wurden weitere Eigenschaften der Zuckerrohrabfälle wie Trockensubstanzanteil und Anteil der organischen Trockensubstanz (oTS), chemischer Sauerstoffbedarf (CSB), pH-Wert, Gesamtstickstoffgehalt (nach Kjeldahl), Rohfett- und Rohproteingehalt, Gesamtkohlenhydrat- und Ligningehalt sowie Konzentration an flüchtigen organischen Säuren bewertet. Diese Informationen war von grundlegender Bedeutung für die Gestaltung des kontinuierlichen Biogasverfahrens, insbesondere hinsichtlich der Auswahl des Reaktortyps, der Anforderung der Spurenelementzugabe, der Identifizierung potentieller Inhibitoren, der Abschätzung der Substratvorbehandlungsoptionen und der Bewertung der möglichen Co-Vergärung von mehreren Abfallprodukten aus der Zuckerrohrindustrie.

Die Ergebnisse des BMP-Tests von circa 215 $mL_N \cdot g_{oTS}^{-1}$ für Stroh, 275 $mL_N \cdot g_{oTS}^{-1}$ für Bagasse, 250 $mL_N \cdot g_{oTS}^{-1}$ für Filterkuchen und 260 $mL_N \cdot g_{CSB}^{-1}$ für Vinasse waren vielversprechend im Vergleich zu denen von Energiepflanzen wie Maissilage. Nichts desto trotz wurden physikalische und chemische Vorbehandlungen der lignocellulosehaltigen Materialien Stroh, Bagasse und Filterkuchen zur Erhöhung des Abbaugrades angewendet. Eine physikalische Vorbehandlung des Strohs (Partikelgröße unter 2 mm) ergab ein 26% höheres BMP. Eine alkalische Vorbehandlung mit NaOH des Filterkuchens führte zu einer Erhöhung des BMPs um 22%. Eine Reduzierung der Partikelgröße von Bagasse (unter 10 mm) und eine Vorbehandlung mit Calciumhydroxidlösung erhöhte das BMP erheblich um mehr als 50%.

Basierend auf den neu beschriebenen Eigenschaften der Zuckerrohrabfälle wurden Experimente in kontinuierlichen Rührkesselreaktoren entworfen und durchgeführt, um die anaerobe Vergärung des Filterkuchens als einzelnes Substrat und dessen Kovergärung mit Bagasse zu untersuchen. Filterkuchen war das interessanteste und vielversprechendste Abfallprodukt aufgrund seiner einzigartigen Eigenschaften wie guter Transportfähigkeit, derzeit schlechten Entsorgungsstatus und geeigneter elementarer Komposition. Die Kovergärung von Filterkuchen mit Bagasse im Verhältnis von 7:3 bezogen auf frische Masse wurde im Hinblick darauf durchgeführt, einen Teil der verbleibenden Bagasse in der Industrie zu verwenden und auch die anaerobe Vergärung des Filterkuchens mit Bagasse als zusätzliche Kohlenstoffquelle zu verbessern. In den kontinuierlichen Fütterungsexperimenten wurde die mikrobielle Gemeinschaftsdynamik untersucht, um durchführbare und zuverlässige Inbetriebnahmeoptionen für einen effizienten Biogasprozess zu ermitteln. Die mikrobielle Gemeinschaftsstruktur wurde unter Verwendung von terminalen Restriktionsfragmentlängenpolymorphismen und 16S-rRNA-Amplikonsequenzierung untersucht. Die mikrobielle Aktivität der Methanogenen wurde durch die Analyse der stabilen Isotope des erzeugten Methans und Kohlenstoffdioxids verfolgt. Sowohl die molekularbiologischen Methoden als auch die Analyse der stabilen Isotope wurden hinsichtlich ihres Wertes als Indikatoren für Prozessstörungen verglichen.

In der zweiten Studie wurde die Wirkung der Zunahme der Raumbelastung von der Inbetriebnahme bis zur Überlastung auf die Monovergärung von Filterkuchen und dessen Kovergärung mit Bagasse untersucht. Dies war ein wichtiger Faktor für die Prozessgestaltung, da es die Bestimmung eines geeigneten Reaktorvolumens und damit der Investitionskosten erlaubte. In dieser zweiten Studie wurde eine hohe Raumbelastung erwünscht, weil sie eine Reduktion des Reaktorvolumens impliziert. Allerdings führt es oft zu Prozessstörungen einschließlich Versäuerung aufgrund der Anhäufung von organischen Säuren. Daher wurden die betriebenen Biogasreaktoren unter hoher Raumbelastung

systematisch überwacht, um im Frühstadium Indikatoren für ein Prozessversagen zu identifizieren. Außerdem war die Saisonalität der Zuckerrohrindustrie, die nur rund 200 Tage im Jahr betreibt, eine zusätzliche Herausforderung für die kontinuierliche Biogasproduktion aus Filterkuchen und Bagasse. Aufgrund der kurzen Substratverfügbarkeit wurde daher eine schnelle Inbetriebnahme der Biogasreaktoren mittels einer schnellen Zunahme der Raumbelastung bevorzugt.

Die Anpassungsperiode des Inokulums in den Reaktoren zum Testen der allmählichen Raumbelastungserhöhung (in acht Schritten zu je 0,5 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$) zeigte eine sehr ähnliche mikrobielle Gemeinschaftsstruktur in der Anfangsphase der Monovergärung und der Kovergärung. In dieser Phase waren die vorherrschenden Gattungen *Methanosarcina*, *Methanoculleus* und *Methanosaeta*. Bis zu einer mittleren Raumbelastung von 2,5 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$ war das Biogasverfahren stabil. Im Vergleich zur Monovergärung (bei Raumbelastung von 3,5 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$) zeigte die Kovergärung schon früher eine starke Prozessunausgewogenheit (bei einer Raumbelastung von 3,0 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$). Die methanogene Gemeinschaft änderte sich entsprechend dem Anstieg der Raumbelastung in sehr ähnlicher Weise in beiden Vergärungsansätzen. Allerdings wurde diese Entwicklung nur bis Raumbelastung von 3,0 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$ beobachtet, bei der *Methanosarcina* am häufigsten vorkam (ca. 80% relative Häufigkeit). Während der Reaktorversäuerung veränderte sich die methanogene Gemeinschaftsstruktur in beiden Vergärungsansätzen. Die acetoklastische Gattung *Methanosaeta* wurde in der Monovergärung erneut nachgewiesen, während die Häufigkeit der hydrogenotrophen Gattung *Methanobacterium* bei der Kovergärung drastisch anstieg (ca. 76% relative Häufigkeit). Die methanogene Aktivität wurde durch die Analyse der stabilen Isotope nachgewiesen, insbesondere durch die Isotopenzusammensetzung von Kohlenstoff im Methan. Eine starke Abreicherung von ^{13}C im Methan während der Reaktorüberlastung zeigte die Tendenz zu hydrogenotropher Methanogenese, was auf eine Prozessunausgewogenheit hinweist. Die Änderungen der mikrobiellen Struktur und Aktivität zusammen mit den Prozessparametern zeigten, dass ein mittleres Reaktorvolumen, das bis zu einer Raumbelastung von 3.0 $g_{oTS} \cdot L^{-1} \cdot d^{-1}$ betrieben wird, für die Monovergärung von Filterkuchen und dessen Kovergärung mit Bagasse am besten geeignet ist.

Die dritte Studie widmete sich der Untersuchung verschiedener Inokulationsstrategien für die Biogasproduktion bei der Kovergärung von Filterkuchen und Bagasse. Die Inokulation der Biogasreaktoren war ein sehr wichtiger Schritt bei der Inbetriebnahme, da die Funktionalität und Anpassungsfähigkeit der mikrobiellen Gemeinschaften im Inokulum die Effizienz des Biogasprozesses bestimmen. In Regionen, in denen Biogasanlagen weit verbreitet und gut etabliert sind, werden neue Biogasreaktoren häufig mit Gärresten aus den bereits bestehenden Anlagen inokuliert. Allerdings ist dieses Szenario in Brasilien unrealistisch, da die wenigen Biogasanlagen derzeit im Land sehr weit von

den Zuckerrohrindustrien entfernt sind. Als Alternative wurde frische Rindergülle aus landwirtschaftlichen Betrieben, die die Zuckerrohrindustrie umgeben, als potenzielles Inokulum für die Inbetriebnahme von Biogasreaktoren erwogen. Um Rindergülle als Inokulum für den Kovergärungsprozess zu untersuchen, wurden zwei parallele Biogasreaktoren inokuliert und mit zwei anderen parallel betriebenen Reaktoren, die mit gemischtem Inokulum (d. h. Gärrestemischung aus verschiedenen Biogasreaktoren) inokuliert wurden, verglichen. Weiterhin wurde die Monovergärung von Filterkuchen und dessen Kovergärung mit Bagasse im Hinblick auf die Leistung verglichen, indem beide Vergärungsansätze unter Verwendung des gemischten Inokulums in fünfmal so großen Reaktoren (verglichen mit der vorherigen Studie) mit längerer mikrobieller Adaptionszeit untersucht wurden.

Die Prozessparameter zeigten, dass sowohl die Monovergärung als auch die Kovergärung, die mit gemischtem Inokulum inokuliert wurden, während der gesamten Betriebszeit der Reaktoren stabil waren. Dennoch wurde eine starke Anhäufung von organischen Säuren während der Anlaufphase der mit Rindergülle inokulierten Reaktoren beobachtet. Diese Beeinträchtigung hielt die ersten zwanzig Betriebstage an, bis der Prozess sich stabilisierte. Obwohl das C:N-Verhältnis der Monovergärung und der Kovergärung für die anaerobe Vergärung im empfohlenen Bereich (24:1 bzw. 41:1) lag, war die Bioverfügbarkeit von Stickstoff sehr gering. Dies beeinflusste die Pufferkapazität und damit den pH-Wert, vor allem im stationären Zustand. Trotz der Tatsache, dass der Monovergärungsaufbau im Vergleich zur Kovergärung eine höhere Biogasausbeute aufwies, zeigte eine Abschätzung, dass in einer großskaligen Biogasanlage der Co-Vergärungsaufbau etwa 58% mehr Biogas als die Monovergärung von Filterkuchen produzieren könnte. Der Grund dafür ist die verfügbare Menge der Bagasse, die etwa siebenmal mehr produziert wird als Filterkuchen (bezogen auf eine Tonne verarbeiteten Zuckerrohr).

Bei der Inbetriebnahme umfasste die bakterielle Gemeinschaftsstruktur in den mit Rindergülle inokulierten Reaktoren eine höhere Diversität auf Phylumebene im Vergleich mit den Reaktoren, die mit gemischtem Inokulum inokuliert wurden. Die Phyla Firmicutes und Bacteroidetes waren von der Inbetriebnahme bis zum stationären Betriebszustand dominant. Während die relative Häufigkeit von Bacteroidetes um 70% zunahm, nahm die Häufigkeit an Firmicutes ab. Im abschließenden stationären Zustand des Experiments zeigten alle Reaktoren in Kovergärung, unabhängig von der Inokulationsstrategie, eine sehr ähnliche bakterielle Gemeinschaftsstruktur. Ein weiteres im stationären Zustand abundantes Phylum war Synergistetes. Zusammen mit den beiden anderen dominanten Phyla umfasste Synergistetes etwa 94% der gesamten Gemeinschaft. Die wichtigsten Bakterienfamilien, die an der anaeroben Vergärung von Filterkuchen und Bagasse beteiligt waren, waren Bacteroidaceae,

Prevotellaceae und Porphyromonadaceae (Phylum Bacteroidetes), Ruminococcaceae, Clostridiaceae und Lachnospiraceae (Phylum Firmicutes) und Synergistaceae (Phylum Synergistetes). Ähnlich wie die bakterielle Gemeinschaft war die methanogene Gemeinschaftsstruktur nach Inbetriebnahme mit unterschiedlichen Inokulum zunächst verschieden und später, im stationären Endzustand, sehr ähnlich. Die Aktivität der dominanten hydrogenotrophen Gattungen (repräsentiert vor allem durch *Methanospirillum* und *Methanobacterium*) bei der Inbetriebnahme wurde durch die starke Abreicherung von ^{13}C in der Isotopenzusammensetzung von Methan angezeigt. Im stationären Zustand wurde die acetoklastische Methanogenese zum dominanten Weg der Methanbildung, angezeigt durch eine stärkere Anreicherung des ^{13}C. Ansonsten spielte die H_2-abhängige methylotrophe Gattung *Methanomassiliicoccus,* vor allem bei der Kovergärung im späteren stationären Zustand, eine wichtige Rolle. Die Gattungen *Methanosarcina* und *Methanobacterium* nahmen eine Schlüsselposition in der Monovergärung von Filterkuchen und dessen Kovergärung mit Bagasse ein. Dies wurde sowohl in den kleinen als auch in den großen Reaktoren beobachtet. Beide Gattungen dominierten dabei die methanogene Gemeinschaft unabhängig von der Adaptationszeit. Schließlich wurde aufgrund der hohen Ähnlichkeit der mikrobiellen Gemeinschaft im stationären Endzustand bei beiden Inokulationsstrategien nachgewiesen, dass Rindergülle erfolgreich als Inokulum für die Inbetriebnahme des Biogasprozesses für Kovergärung von Filterkuchen und Bagasse verwendet werden kann.

Neben der Biogasproduktion wurde die anaerobe Vergärung von Filterkuchen in einer vierten Studie bis zur Produktion flüchtiger Fettsäuren (FFS) durchgeführt. In diesem Fall wurde der Versuch durchgeführt, weil die FFS als wertvolles Zwischenprodukt sowohl für die Biogas- als auch für die Chemieproduktion eingesetzt werden können. Ein semi-kontinuierliche Experiment wurde in 5 verschiedene Phasen aufgeteilt: Inbetriebnahme, stationärer Betriebszustand, Harnstoff-Umsetzung, Harnstoff plus Salpetersäure-Supplementierung und Natriumhydroxid-Vorbehandlung. Aufgrund der geringen Konzentration an Ammoniumstickstoff während des stationären Zustands wurde den Reaktoren Harnstoff als Stickstoffquelle zugesetzt, um die FFS-Ausbeute zu erhöhen. Allerdings steigerte die Zugabe von Harnstoff drastisch die pH-Werte, was den FFS-Verbrauch durch Methanogene begünstigte. Folglich wurde Harnstoff zusammen mit Salpetersäure zugegeben, um einen pH-Werte von 5,5 zu halten. Dadurch erholte sich die FFS-Ausbeute wieder, aber nicht bis zu dem Niveau wie im stationären Betriebszustand. So ergab die Zugabe von Harnstoff keine Verbesserung der FFS-Produktion. Die Effizienz der Vorbehandlung des Filterkuchens mit Natriumhydroxid wurde zunächst unter Verwendung realistischer NaOH-Konzentrationen in Batchversuchen untersucht. Da eine Vorbehandlung mit 6 g NaOH pro 100 g Filterkuchen (frische Masse) den höchsten BMP ergab, wurde diese Konzentration zur Vorbehandlung des Filterkuchens im

semi-kontinuierlichen Experiment angewandt. Durch diese Vorbehandlung konnte die FFS-Ausbeute um 37% erhöht werden.

Zusammenfassend sind die vier bedeutenden Abfallprodukte Stroh, Bagasse, Filterkuchen und Vinasse aus der brasilianischen Zuckerrohrindustrie für eine effiziente Biogaserzeugung geeignet. Batch und semi-kontinuierliche Fütterungsexperimente wurden systematisch durchgeführt, um die soweit theoretische Forschung mit einer zukünftigen praktischen Anwendung zu verknüpfen. Vorbehandlungsoptionen zur Verbesserung der Methanausbeute und Zusatzstoffe für den kontinuierlichen Biogasherstellungsprozess von Stroh, Bagasse, Filterkuchen und Vinasse wurden untersucht. Die Überwachung der mikrobiellen Gemeinschaftsdynamik von der Inbetriebnahme bis zur stationären Phase der Monovergärung von Filterkuchen und dessen Kovergärung mit Bagasse zeigte die Zuverlässigkeit der Verwendung von Rindergülle als Inokulum. Darüber hinaus zeigte die Einschätzung der methanogenen Gemeinschaftsdynamik während des allmählichen Anstiegs der Raumbelastung die Durchführbarkeit, ein mittleres Reaktorvolumen zu verwenden, indem eine Raumbelastung für eine effiziente Biogasproduktion aus Filterkuchen und Bagasse definiert wurde. Außerdem lieferte die Beurteilung der mikrobiellen Gemeinschaftsdynamik in den durchgeführten Experimenten Erkenntnisse über das mikrobielle Ressourcenmanagement bei der Biogasproduktion aus Zuckerrohrabfällen. Die Analyse der stabilen Isotope wurde als potentielles Überwachungsinstrument zur Anzeige von Prozessungleichgewichten durch Veränderungen der Methanogenesewege herangezogen. Trotzdem sollte eine weitere Untersuchung der stabilen Isotope mit mehreren Probenahmen unter verschiedenen Prozessbedingungen durchgeführt werden, um vorhersagbare Modelle, die die Prozessparameter und die mikrobielle Gemeinschaftsdynamik integrieren, zu entwickeln. Die effiziente Biogasproduktion aus Zuckerrohrabfällen könnte eine realisierbare und zuverlässige Energiequelle darstellen. Auf der Grundlage der vorgestellten Ergebnisse hätte das aus Zuckerrohrabfällen hergestellte Biogas im Jahr 2016 rund 12% des brasilianischen Stromverbrauchs decken können. Allerdings sind die staatlichen Anreize entscheidend für die Förderung des Biogassektors in der Zuckerrohrindustrie und für die Auslösung der notwendigen Veränderungen in Richtung einer nachhaltigeren Entwicklung.

Resumo

Durante a produção de bioetanol e açúcar na indústria brasileira de cana-de-açúcar é gerada uma grande quantidade de resíduos. Infelizmente a má gestão dos resíduos se contrasta com a bem sucedida história do bioetanol no Brasil e sua longa tradição como grande produtor de açúcar no mundo. Os quatro principais resíduos gerados pela indústria brasileira de cana-de-açúcar apresentam uma ameaça direta ou indireta ao meio ambiente e a saúde humana. A palha (conjunto de folhas da cana-de-açúcar) é descartada ou queimada nos campos, causando o último poluição do ar. O bagaço (cana moída) é principalmente queimado para produzir calor e eletricidade, mas em alguns casos é simplesmente depositado em grandes áreas a céu aberto devido ao excedente. A torta de filtro (pasta da prensa) e a vinhaça (resíduo da destilaria) são espalhados diretamente no campo como fertilizante, contribuindo para a poluição das águas e do solo. Ademais, durante o armazenamento temporário da vinhaça em lagoas abertas (antes de ser usada na fertirrigação), uma grande quantidade de gases de efeito estufa são emitidos. Além destes tipos de descarte final dos resíduos serem nocivos ao meio ambiente, desperdiça consideravelmente o conteúdo energético que ainda poderia ser melhor explorado. Como uma solução sustentável para a recuperação energética de resíduos da cana-de-açúcar, a produção de biogás via digestão anaeróbia pode ser uma tecnologia de tratamento adequada. O biogás, rico em energia, tem potencial para muitas aplicações, como por exemplo combustível de veículos e geração de calor e eletricidade. Além disso, o digestato poderia ser usado como um fertilizante valioso. Para conduzir um procedimento de inicialização viável e confiável, e posteriormente uma operação estável do processo de biogás na indústria de cana-de-açúcar, os resíduos foram inicialmente caracterizados e a produção de biogás, em regime de alimentação semicontínua, foi realizada em vários reatores de escala laboratorial. Além disso, a dinâmica da comunidade microbiana foi avaliada através de técnicas da biologia molecular e da análise de isótopos estáveis.

Em um primeiro estudo, a palha, o bagaço, a torta de filtro e a vinhaça foram submetidos a análises físico-químicas e testes em batelada para a determinação do potencial bioquímico de metano (PBM). Também concomitante a isso, foram avaliadas algumas outras características dos resíduos da cana-de-açúcar como sólidos totais, sólidos voláteis (SV), demanda química de oxigênio (DQO), pH, nitrogênio total (segundo Kjeldahl), teor de gordura e proteína bruta, teor total de carboidratos e lignina, e concentração ácidos orgânicos. Estas informações foram fundamentais para a concepção do processo de biogás com alimentação semicontínua, especialmente no que se refere à seleção do tipo de reator, adição de elementos nutricionais, identificação de potenciais inibidores, avaliação das opções

de pré-tratamento do substrato e análise do potencial de codigestão dos resíduos da indústria sucroalcooleira.

Os resultados do teste de PBM de aproximadamente 215 $mL_N \cdot g_{SV}^{-1}$ para a palha, 275 $mL_N \cdot g_{SV}^{-1}$ para o bagaço, 250 $mL_N \cdot g_{SV}^{-1}$ para a torta de filtro e 260 $mL_N \cdot g_{DQO}^{-1}$ para a vinhaça eram muito promissores comparados com aqueles de culturas energéticas como a silagem de milho. No entanto, para aumentar a taxa de degradação, foram aplicados pré-tratamentos físicos e químicos no caso dos materiais lignocelulóticos: palha, bagaço e torta de filtro. Submeter a palha apenas a um pré-tratamento físico (tamanho abaixo de 2 mm) forneceu um PBM 26% maior. O pré-tratamento alcalino (NaOH) da torta de filtro resultou num aumento do PBM em 22%. Quando o bagaço com tamanho reduzido (abaixo de 10 mm) foi tratado com solução de hidróxido de cálcio, o seu PBM aumentou drasticamente em mais de 50%.

Com base nas características recém descritas dos resíduos da cana-de-açúcar, experimentos em reatores contínuos de tanque agitado foram projetados e operados para a digestão anaeróbia da torta de filtro como único substrato e também para sua codigestão com bagaço. A torta de filtro foi o resíduo mais interessante e promissor a ser investigado devido as suas características peculiares quanto a sua adequação para transporte de longa duração, sua situação atual de destino final na indústria e sua composição elementar. A codigestão da torta de filtro com bagaço em uma proporção alimentar de 7:3 (com base na massa fresca) foi realizada com a intenção de utilizar parte do bagaço que se acumula na indústria e também de complementar a digestão anaeróbia da torta de filtro com a adição de bagaço como uma fonte de carbono adicional. Nos experimentos de alimentação contínua foi investigada a dinâmica da comunidade microbiana nos reatores, a fim de revelar opções viáveis e confiáveis de procedimento de inicialização para um processo de biogás eficiente. A estrutura das comunidades microbianas foi avaliada utilizando o polimorfismo de comprimento de fragmentos terminais de restrição e o sequenciamento de próxima geração de amplicons 16S rRNA. A atividade microbiana das arqueas metanogênicas foi investigada através da análise de isótopos estáveis do metano e do dióxido de carbono produzido. Ambas as técnicas de microbiologia molecular e de análise de isótopos estáveis foram comparadas em relação ao seus potenciais de indicação de desequilíbrio no processo.

Em um segundo estudo, investigou-se o efeito do aumento gradual da taxa de carga orgânica (TCO), da inicialização do processo à sobrecarga, na monodigestão da torta de filtro e na sua codigestão com bagaço. Este foi um fator importante para a concepção do processo, uma vez que permitiu a determinação de um volume de reator apropriado e, consequentemente, uma estimativa de custos de investimento. Neste segundo estudo, foi desejado uma TCO alta porque isto implica numa redução do

volume do reator. No entanto, muitas vezes isto também resulta em desequilíbrio do processo e acidificação devido ao acúmulo de ácidos orgânicos. À vista disso, os reatores de biogás, operados até altos níveis de TCO, foram monitorados sistematicamente a fim de identificar, em fase inicial, indicadores de falha do processo. Ademais, um desafio adicional para a produção contínua de biogás a partir da torta de filtro e bagaço foi a sazonalidade da indústria de cana-de-açúcar, que opera apenas cerca de 200 dias por ano. Assim, devido à curta disponibilidade de substrato, foi preferida um rápido procedimento de inicialização dos reatores de biogás por meio de um aumento acelerado da TCO.

O período de adaptação do inóculo nos reatores do experimento com aumento gradual da TCO (oito aumentos escalonados de 0,5 $g_{SV}\cdot L^{-1}\cdot d^{-1}$) mostrou uma estrutura de comunidade microbiana muito similar na fase inicialização do processo, tanto da monodigestão quanto da codigestão, com predominância dos gêneros *Methanosarcina*, *Methanoculleus* e *Methanosaeta*. Até a metade do experimento, quando a TCO era de 2,5 $g_{SV}\cdot L^{-1}\cdot d^{-1}$, o processo de biogás estava estável. *Methanosaeta* e *Methanoculleus* não foram mais detectados em uma TCO de 2,5 e 3,0 $g_{SV}\cdot L^{-1}\cdot d^{-1}$, respectivamente. Um vigoroso desequilíbrio de processo foi primeiro observado na codigestão (em uma TCO de 3,0 $g_{SV}\cdot L^{-1}\cdot d^{-1}$) e depois na monodigestão (em uma TCO de 3,5 $g_{SV}\cdot L^{-1}\cdot d^{-1}$). A comunidade metanogênica mudou de maneira muito semelhante em ambos os sistemas de digestão até uma TCO de 3,0 $g_{SV}\cdot L^{-1}\cdot d^{-1}$, quando *Methanosarcina* se tornou a mais abundante (cerca de 80% de abundância relativa). Durante a acidificação dos reatores, a estrutura da comunidade metanogênica em ambos sistemas de digestão sofreu mudanças em sentidos diferentes. O gênero acetoclástico *Methanosaeta* foi detectado novamente na monodigestão, enquanto a abundância do gênero hidrogenotrófico *Methanobacterium* aumentou drasticamente na codigestão (cerca de 76% de abundância relativa). O desequilíbrio do processo também foi indicado pela análise de isótopos estáveis, uma vez que a forte diminuição do ^{13}C do metano revelou uma mudança em direção a metanogênese hidrogenotrófica. Todas estas descobertas sobre o monitoramento da estrutura e atividade da comunidade microbiana juntamente com os parâmetros do processo mostraram que um volume intermediário de reator, operado até uma TCO de 3,0 $g_{SV}\cdot L^{-1}\cdot d^{-1}$, é apropriado para a monodigestão da torta de filtro e a sua codigestão com bagaço.

Um terceiro estudo foi dedicado à investigação de diferentes estratégias de inoculação para a produção de biogás em codigestão de torta de filtro e bagaço. A inoculação dos reatores de biogás foi um passo muito importante a ser investigado no início do procedimento de inicialização, uma vez que a funcionalidade e adaptabilidade das comunidades microbianas no inóculo deveriam determinar a eficiência do processo de biogás. Em regiões onde as plantas de biogás são comuns e bem estabelecidas, applica-se comumente a prática de inoculação de novos sistemas de biogás a partir do

digestato de plantas já existentes. No entanto, esse cenário não é considerado realista no Brasil, já que as poucas plantas de biogás do país estão muito distantes das indústrias de cana-de-açúcar. Para superar esta questão, o estrume fresco de gado gerado nas fazendas localizadas na região da indústria foi considerado como um inóculo com grande potencial para o procedimento de inicialização da produção de biogás. Para testar o estrume de gado como inóculo para a codigestão, dois reatores de biogás em paralelo foram inoculados e comparados com outros dois reatores paralelos inoculados com um chamado inóculo misto, contendo diferente digestatos de vários sistemas de biogás. Ademais, a monodigestão da torta de filtro e a sua codigestão com bagaço foi comparada em termos de eficiência utilizando o inóculo misto em reatores de escala cinco vezes superior (em comparação com o estudo anterior) e com tempo de adaptação microbiana mais longo sob nova carga de alimentação.

Os parâmetros do processo mostraram que ambos os sistemas de monodigestão e codigestão inoculados com inóculo misto estavam predominantemente estáveis ao longo do tempo de operação dos reatores. Durante a fase de inicialização dos reatores inoculados com estrume bovino, uma forte acumulação de ácidos orgânicos foi observada. Este inconveniente durou pelos primeiros vinte dias operacionais até que o processo estivesse sob condições de funcionamento estáveis. Embora a razão C:N dos sistemas de monodigestão e codigestão estivesse na gama apropriada (24:1 e 41:1, respectivamente) para o processo de digestão anaeróbia, a biodisponibilidade de nitrogênio da torta de filtro e do bagaço era muito baixa. Isso influenciou a capacidade tampão e, consequentemente, os valores de pH, principalmente no estado estacionário. Apesar do sistema de monodigestão ter apresentado maior produtividade de biogás em comparação com o da codigestão, uma nova avaliação demonstrou que, em uma planta em larga escala, o sistema de codigestão seria capaz de produzir cerca de 58% mais biogás que o da monodigestão da torta de filtro. A razão para isso é a disponibilidade do bagaço, que é aproximadamente sete vezes maior do que da torta de filtro (em relação a uma tonelada de cana-de-açúcar processada).

A estrutura da comunidade bacteriana na inicialização do processo de biogás apresentou uma maior diversidade a nível de filo nos reatores inoculados com estrume de gado em comparação com aqueles inoculados com o inóculo misto. Os filos Firmicutes e Bacteroidetes foram dominantes da inicialização do processo ao estado estacionário. Enquanto a abundância relativa do filo Bacteroidetes aumentou gradualmente em direção ao estado estacionário (atingindo cerca de 70%), a abundância do filo Firmicutes diminuiu. No estado estacionário final do experimento, todos os reatores em codigestão, independentemente da estratégia de inoculação, apresentaram uma estrutura de comunidade bacteriana muito semelhante. Outro filo que mostrou abundância representativa no estado estacionário foi o Synergistetes que juntamente com os outros dois outros filos corresponderam cerca de 94% de toda a

comunidade. As principais famílias bacterianas envolvidas na digestão anaeróbia da torta de filtro e bagaço foram Bacteroidaceae, Prevotellaceae e Porphyromonadaceae (filo Bacteroidetes), Ruminococcaceae, Clostridiaceae e Lachnospiraceae (filo Firmicutes) e Synergistaceae (filo Synergistetes). Seguindo a mesma tendência que a comunidade bacteriana, a estrutura da comunidade metanogênica na inicialização do processo era distinta nos reatores com diferentes estratégias de inoculação, entretanto mais tarde no estado estacionário final era muito semelhante independentemente da inoculação aplicada. A predominância dos gêneros hidrogenotróficos (representados principalmente por *Methanospirillum* e *Methanobacterium*) na inicialização do processo foi indicada pela baixa presença do ^{13}C na composição isotópica do metano produzido. Mais tarde, no estado estacionário, a via metanogênica em todos os reatores foi deslocada em direção à acetoclástica com mais enriquecimento do ^{13}C. Ademais, o gênero metilotrófico, H_2 dependente, *Methanomassiliicoccus* representou um papel importante no estado estacionário no caso da codigestão. Comparando-se o desempenho da monodigestão com a codigestão, o aumento da escala dos reatores e o longo processo de adaptação microbiana deste estudo mostrou mais claramente a importante contribuição dos gêneros *Methanosarcina* e *Methanobacterium* para a monodigestão da torta de filtro e sua codigestão com bagaço. Portanto, devido à alta similaridade da comunidade microbiana em ambas as estratégias de inoculação no estado estacionário final, o estrume de gado pode ser utilizado com sucesso como inóculo para a inicialização do processo de biogás na codigestão da torta de filtro com bagaço.

Além da produção de biogás, a digestão anaeróbia da torta de filtro foi investigada em um quarto estudo no intuito de produzir ácidos graxos voláteis (AGVs). Este experimento foi conduzido porque além dos AGVs serem intermediários importantes durante a produção de biogás, eles também podem ser usados para a produção de produtos químicos. O experimento semicontínuo foi dividido em 5 fases diferentes: inicialização do processo, estado estacionário, implementação de ureia, suplementação de ureia e ácido nítrico, e pré-tratamento com hidróxido de sódio. Devido aos baixos níveis de concentração de nitrogênio durante o estado estacionário, a ureia foi adicionada aos reatores como uma fonte de nitrogênio para aumentar o rendimento de AGVs. Contudo, a adição de ureia aumentou drasticamente os valores de pH, favorecendo o consumo de AGVs pelas arqueas metanogênicas. Consequentemente, ureia foi adicionada juntamente com ácido nítrico para manter os valores de pH em torno de 5,5. No entanto, sob esta nova suplementação, o rendimento de AGVs não se recuperou ao mesmo nível que no estado estacionário. Assim, a adição de ureia não produziu qualquer melhoria no rendimento de AGVs. A eficiência do pré-tratamento da torta de filtro com hidróxido de sódio foi investigada primeiro sob concentrações distintas em testes de batelada. Após a melhor performance do teste de PBM sob 6 g de NaOH por 100 g de torta de filtro (massa fresca), esta concentração foi

utilizada para pré-tratar a torta de filtro num regime de alimentação semicontínua. Neste caso, o pré-tratamento alcalino aumentou o rendimento dos AGVs em 37%.

Em resumo, os quatro principais resíduos (palha, bagaço, torta de filtro e vinhaça) da indústria brasileira de cana-de-açúcar são adequados para um eficiente processo de produção de biogás. Experimentos de alimentação em batelada e semicontínua foram realizados sistematicamente, a fim de vincular a, até então, pesquisa teórica de base às futuras aplicações práticas. Foram estabelecidas algumas opções de pré-tratamento para aumentar o rendimento de metano, e também uma avaliação da suplementação nutricional para o processo contínuo de produção de biogás de palha, bagaço, torta de filtro e vinhaça. O monitoramento da dinâmica da comunidade microbiana desde o procedimento de inicialização até a fase estacionária da monodigestão da torta de filtro e da sua codigestão com bagaço revelou a confiabilidade do uso de estrume bovino como inóculo para a produção de biogás na indústria de cana-de-açúcar. Além disso, a avaliação da dinâmica da comunidade metanogênica durante o aumento gradual da TCO mostrou a viabilidade de usar um volume intermediário de reator, definido por uma TCO na qual foi realizada uma produção eficiente de biogás a partir da torta de filtro e do bagaço. Ademais, a avaliação da dinâmica da comunidade microbiana nos experimentos realizados forneceu informações sobre um possível gerenciamento de recursos microbianos durante a produção de biogás a partir de resíduos da cana-de-açúcar. A análise de isótopos estáveis foi usada como uma potencial ferramenta de monitoramento para indicar desequilíbrios no processo através de alterações na via metanogênica. No entanto, uma investigação mais aprofundada das análises de isótopos estáveis, com múltiplas amostragens em diferentes condições de processo, deve ser conduzida a fim de desenvolver modelos previsíveis que integrem os parâmetros do processo e a dinâmica da comunidade microbiana. Por fim, a produção contínua de biogás a partir de resíduos da cana-de-açúcar pode ser realizada de forma eficiente, garantindo sua viabilidade e confiabilidade como fonte de energia. Com base nos resultados finais, o biogás produzido a partir de resíduos da cana-de-açúcar teria sido capaz de suprir sozinho cerca de 12% do consumo de eletricidade do Brasil em 2016. Todavia, os incentivos governamentais são cruciais para promover o setor de biogás na indústria de cana-de-açúcar e para desencadear as mudanças necessárias ao desenvolvimento sustentável.

Abbreviations

AD - Anaerobic digestion

BMP - Biochemical methane potential

CHP - Combined heat and power

COD - Chemical oxygen demand

CSTR - Continuous stirred tank reactor

GC-IRMS - Gas chromatograph isotope ratio mass spectrometry

GHG - Greenhouse gas

HRT - Hydraulic retention time

mcrA - methyl coenzyme-M reductase α-subunit

OLR - Organic loading rate

PCR - Polymerase chain reaction

SRT - Solid retention time

T-RF - Fluorescent-labelled terminal restriction fragment

T-RFLP - Terminal restriction fragment length polymorphism

UASB - Upflow anaerobic sludge blankets

VFA - Volatile fatty acids

VOA - Volatile organic acids

1. Introduction

1.1. Brazilian sugarcane wastes

Brazil is the world leader in sugarcane production. The tradition of sugarcane cultivation goes back to the sixteenth century. The development of the sugarcane industry was mainly based on sugar processing and since the seventies, also on bioethanol production (Figure 1). Brazilian sugar currently accounts for around 20% of global production and 43% of world exports (USDA, November, 2015). Brazil is the greatest bioethanol exporter worldwide and the second largest bioethanol producer, with a share of about 38% (Balat & Balat, 2009; Valdes, 2011). Bioethanol is consumed by 100% of passenger cars in Brazil either as a single fuel or blended with gasoline (up to 27%) (van den Wall Bake et al., 2009).

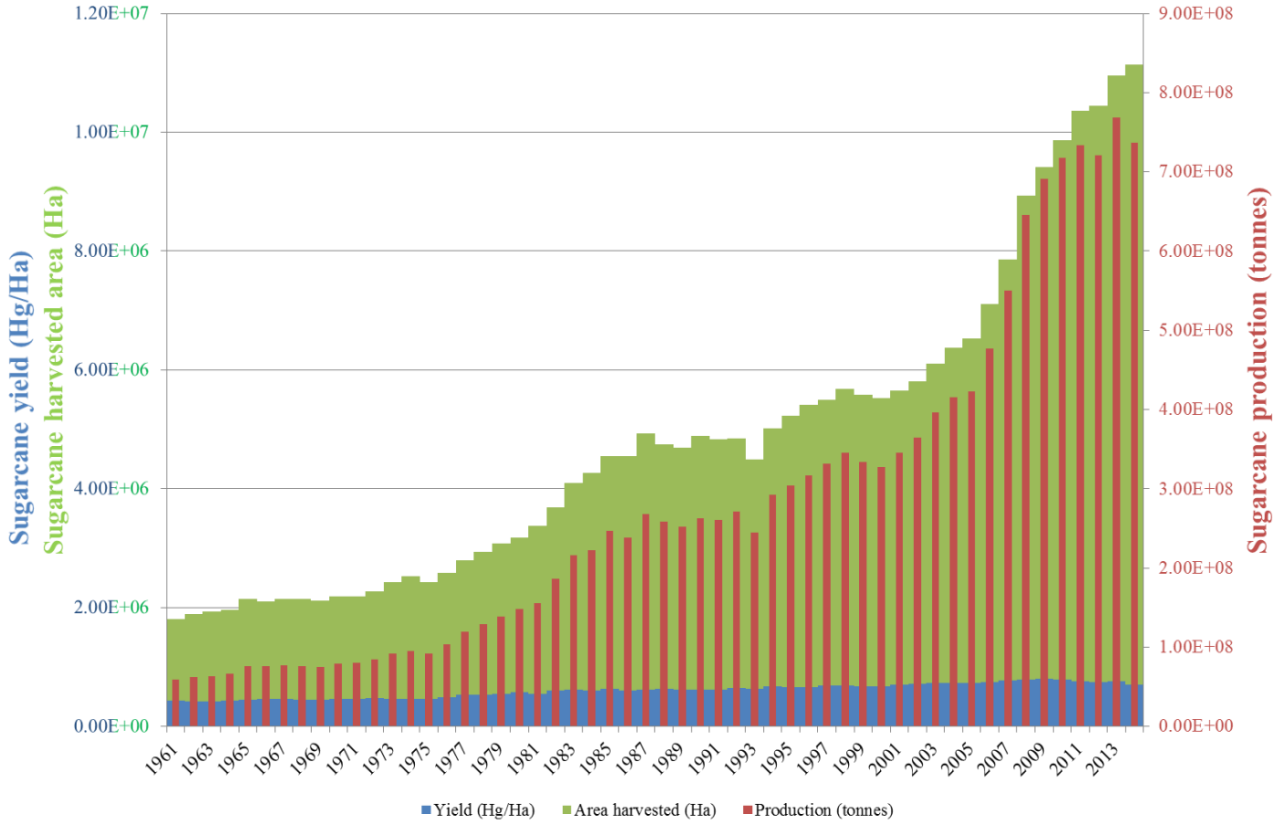

Figure 1. Historical progression of Brazilian sugarcane yield per hectare, total harvested area and total production according to FAO (2016).

The Brazilian biofuel program is considered to be the most developed and integrated in the world. Brazil's successful bioethanol industry is the result of investment and incentives from the government, which launched the program "ProÁlcool" following the world oil crisis in 1973 with the aim of reducing dependency on oil imports and high fuel prices. Over the decades, Brazil has significantly improved its bioethanol program by using new sugarcane varieties, improving soil fertility and

applying novel agricultural and processing technologies, among others (Goes et al., 2011). Nowadays, bioethanol from sugarcane in Brazil is considered to be produced at a better cost-efficiency compared to other crops such as corn, sugarbeet or cassava (Balat & Balat, 2009; Pippo & Luengo, 2013).

During the sugar and bioethanol processes, four major waste types are produced: straw, bagasse, filter cake and vinasse (Figure 2). Straw consists mainly of sugarcane leaves from harvesting. Bagasse is the crushed sugarcane stalk from milling. Filter cake comprises the doughy part from filtering the sugarcane juice. Vinasse is a liquid effluent from the distilleries that is produced during the final bioethanol separation step. In view of the large sugar and bioethanol production in Brazil, a huge amount of waste is produced. For example, in the initial process phase, 1000 kg of sugarcane stalks generate as waste 140 kg of straw (dry basis), 280 kg of bagasse (wet basis) and 40 kg of filter cake (wet basis) (de Campos et al., 2015; Leal et al., 2013; Moraes et al., 2014; Zancaner & de Souza Santos, 2013). In autonomous plants where only bioethanol is produced from sugarcane juice, about 900 L of vinasse and 80 L bioethanol are generated (Cerqueira Leite et al., 2009; Fuess & Garcia, 2014; Moraes et al., 2014). In annexed plants, where bioethanol is produced from both sugarcane juice and/or molasses (i.e. waste from sugar production), approximately 50 kg of sugar, 50 L of bioethanol and 550 L vinasse are obtained (Cavalett et al., 2011; Moraes et al., 2014). Thus, based on the 2015/2016 season, in which 667×10^6 tons of sugarcane were processed (UNICA, 2016), approximately 93×10^6 tons of straw, 190×10^6 tons of bagasse, 27×10^6 tons of filter cake and 300×10^9 L of vinasse were generated.

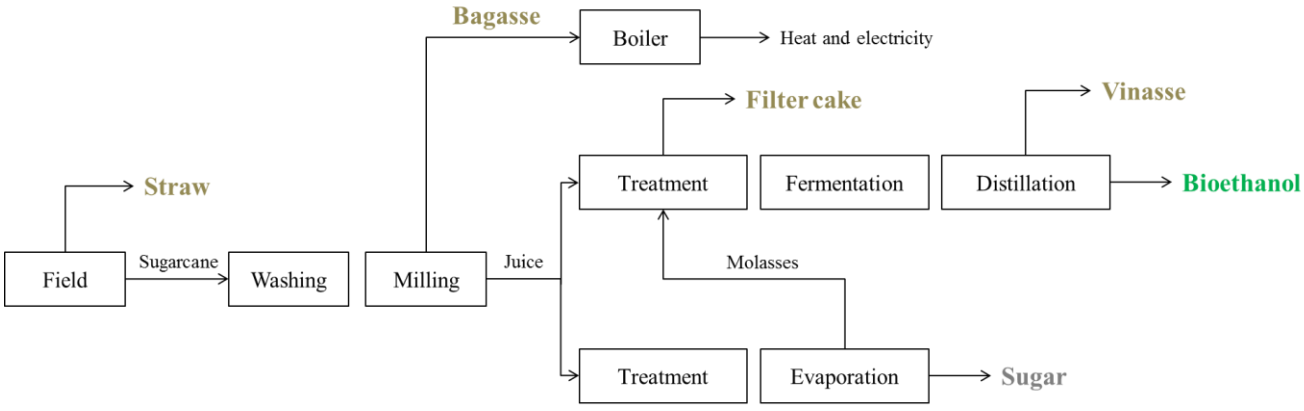

Figure 2. Simplified flowchart of the sugarcane industry. The final commercial products are represented in green for bioethanol and grey for sugar. The waste products are shown in brown.

1.1.1. Waste management: challenges and opportunities

Waste management is a global issue that requires stronger efforts and initiatives from all nations for sustainable development (Finnveden et al., 2013; Halvorsen, 2012). Unfortunately, waste management

has achieved a steady-state in which the improvements are counterbalanced by increasing waste generation and pollution (Giusti, 2009). While very few developed countries put a lot of effort into waste management, most developing and emerging economies still face many challenges due to rapid urbanization, population growth and industrialization (Khalili et al., 2015; Marshall & Farahbakhsh, 2013; Zhang et al., 2010).

For instance, Germany and Sweden have achieved great results in the development of their waste management (Nelles et al., 2016; Sun, 2015). Nevertheless, countries such as Brazil have over the past years faced many challenges regarding the regulation of solid waste management, hindering sustainable development and thus continuing to contribute to climate change (Jabbour et al., 2014). In this context, developing countries have the chance to overcome certain waste-related issues by learning from developed countries that have already been dealing with the problem of waste in a systematic and sustainable way for decades.

Besides governmental investments on concepts that involve recycling, reducing and reusing of materials (Zaman & Lehmann, 2011), some countries such as Austria, Germany, Finland, Sweden and the United Kingdom also focus on the biomass wastes as an energy source (Demirbas et al., 2009). Their strategy includes the use of technologies for converting many agricultural and industrial waste products as well as the organic fraction of municipal solid waste into valuable commodities such as fuel, electricity and heat, among others. Within these technologies, biogas process has been extensively applied as crucial component in the waste management (Laurent et al., 2014). Despite the production of the energy-rich gas methane, the digestate as a waste product generated during the biogas process is also used as a well-balanced fertilizer, closing thus the material flow cycle.

Along these lines, the Brazilian sugarcane industry with its large amount of biomass waste has a spectrum of opportunity to apply already developed technologies for its own waste management. Considering that the sugarcane wastes are disposed of directly into the environment, mostly without any treatment, a proper waste management would not only overcome certain issues related to climate change and environmental pollution, but also produce commercialised products providing further profit to the industry. In this scenario, based on the biogas process applied successfully in some countries such as Germany and Sweden (Lantz, 2013; Lebuhn et al., 2014; Weiland, 2010), the anaerobic digestion (AD) process appears to be the most suitable treatment technology for the waste management in the sugarcane industry. Thus, the energy potential of sugarcane waste products could be exploited to supplement the energy demand in the country.

At the beginning of the sugarcane supply chain, straw is usually left on the sugarcane fields after harvesting in order to maintain soil moisture and nutrient cycle (Carvalho et al., 2013). However, there are often cases in which straw is just burned on the field to facilitate manual harvesting. This practice causes severe consequences in the environment, with large amounts of greenhouse gas (GHG) emissions and air pollutants threatening people's health in the surrounding area (Martinelli & Filoso, 2008). Soil erosion and great losses of soil nutrients are also described as being common in straw burning regions (Leal et al., 2013). States with large shares in sugarcane production, such as São Paulo and Minas Gerais, have already shifted harvesting to mechanical forms. However, the Brazilian Federal law established a time schedule to stop straw burning by 2018 only where mechanical harvesting is possible with current technology (less than 12%), with a date for other areas yet to be defined (Leal et al., 2013; Modesto et al., 2016).

Bagasse is used to produce heat and electricity in so-called co-generation plants associated with the sugarcane industry. Unfortunately, bagasse is not burned in a cost-efficient way that could exploit most of its energy potential. Moreover, not all bagasse is used for the incineration process. In fact, large amounts of bagasse are simply stored at the perimeters of the sugarcane industry areas (Figure 3). Considerable research aimed at using bagasse as a substrate to produce so-called second-generation biofuels has been carried out (Damaso et al., 2014; Pippo & Luengo, 2013; Valdes, 2011).

Figure 3. Temporary bagasse storage at the co-generation plant on the left, and long-term bagasse storage on the right. Photos by Athaydes F. Leite Jr.

Filter cake and vinasse are used as a fertilizer and applied directly to the sugarcane fields; no previous treatment of these wastes to avoid environmental contamination is carried out. Vinasse is collected in open channels and storage lagoons, and later spread through pumps throughout the sugarcane fields (Figure 4). In the sediments of these channels and lagoons, large amounts of GHG are emitted,

especially in form of methane, which is an approximately 21 times more potent GHG than carbon dioxide. In addition, these waste products often contaminate rivers and underground water reservoirs.

Considering the current infrastructure of most Brazilian sugarcane industry facilities, the challenge of applying a proper waste management would appear to be more complicated for straw. Machinery harvesting of sugarcane would be the best option to collect and transport straw to the industry, where this waste could be further processed into final valuable products. However, machinery harvesting has a negative effect on root development and consequently, sugarcane productivity due to soil compaction, which changes the natural dynamics of water and gas (Walter et al., 2014). Bagasse, filter cake and vinasse could easily be used or complemented (in case of bagasse) for biogas production, considering that they are already generated within industrial settings where a biogas plant could be directly placed.

Figure 4. Vinasse channel going through a sugarcane field (upper picture) and vinasse being spread on new growing plants (lower picture). Photos by Athaydes F. Leite Jr.

The introduction of a new approach is challenging and is often held back by fears of failure. However, considering the energetic potential of waste products and the benefits of the biogas process, sustainable waste management should be seen as an opportunity to make the sugarcane industry an even stronger business. With global warming already affecting our climate worldwide, many countries would be willing to import a more sustainable bioethanol in order to mitigate their GHG emissions. Brazil would have the capacity to respond to greater bioethanol demand. Cerqueira Leite et al. (2009) showed that the Brazilian bioethanol industry has the potential to expand to displace 5% of the world's projected gasoline demand in 2025. Therefore, in order to ensure further development of the bioethanol sector and to avoid drastic effects of global warming, the challenges of applying biogas technology to waste management in the sugarcane industry should be solved quickly.

1.2. Biogas production

Biogas is produced during the AD process of various organic matters such as crops (e.g. maize, grass), agricultural waste, sewage sludge, industrial wastewater and food waste. Biogas contains mainly methane (55-75%) and carbon dioxide (25-45%). The energy-rich methane is a versatile renewable gas with considerable potential to replace fossil fuels. For instance, the direct combustion of biogas in a combined heat and power (CHP) unit can generate heat and electricity. Upgrading the biogas to pure methane (often called biomethane) extends its range of use. Biomethane can be injected into the gas grid and thus replace non-renewable natural gas or be used as vehicle fuel or cooking gas.

The digestate from the biogas process can be used as fertilizer on agriculture fields, contributing to the reduction of mineral fertilizer consumption and consequently, GHG emissions. The digestate is already well balanced, with different nutrients that are essential for microorganisms' development during AD. The application of digestate as fertilizer increases agricultural productivity through the presence of more soluble and bioavailable nutrients, such as nitrogen forms that can easily be taken up by plants (Weiland, 2010). Avoiding the accumulation and/or storage of organic materials by producing biogas reduces GHG emissions that would have been otherwise released to the atmosphere during anaerobic processes in the environment, e.g. open wastewater lagoons that are used to store manure and industrial wastewater.

The use of biogas as an energy source has increased in recent years worldwide, mainly in developed countries interested in mitigating their GHG emissions (Lebuhn et al., 2014). Within the European Union, there are more than 14,000 biogas plants in operation according to EurObserv'ER (2014). The type of organic material used for biogas production varies depending on availability and on the political strategies regarding legislation and subsidies. Germany is the largest biogas producer in the

world, with around 8,900 plants corresponding about 4,100 MW installed electric capacity (Fachverband Biogas, 2016). Figure 5 shows the strong development of the biogas market in Germany over the past 20 years. Most of the biogas produced in Germany comes from energy crops (75%), especially from ensilaged maize (60%) (EurObserv'ER, 2014). However, this situation has been changed recently with the new German renewable energy law (Bundesgesetzblatt, 2014), which states that biogas should be more oriented to flexible production according to demand peaks and should be produced from a more diverse substrate range.

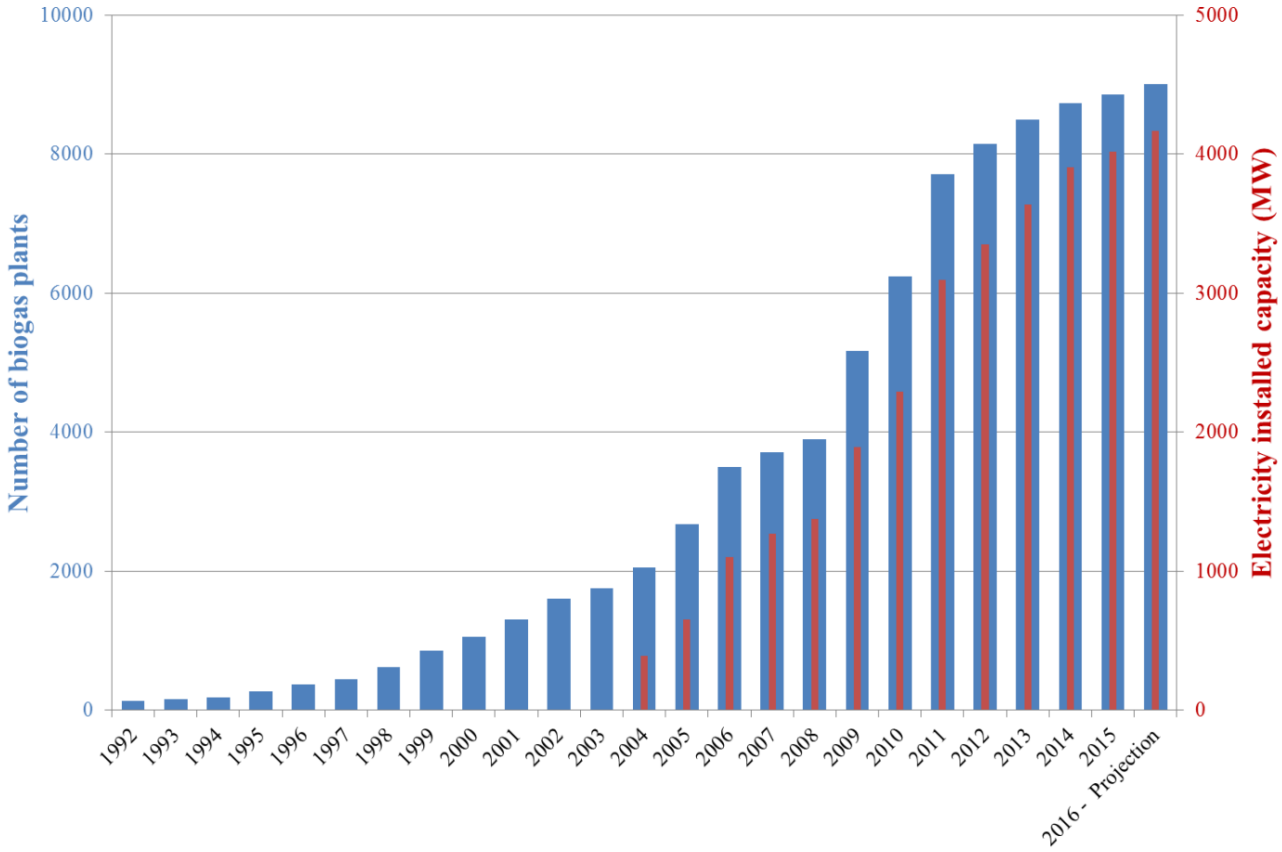

Figure 5. Biogas sector progression in Germany according to Fachverband Biogas (2016).

1.2.1. Microbiology of anaerobic digestion

In the AD process, a complex microbial consortium consisting of bacteria and methanogenic archaea converts the organic materials to biogas in four main metabolic steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 6). Some steps can be rate limiting under distinct conditions, particularly hydrolysis and methanogenesis (Batstone & Jensen, 2011). An efficient and stable AD process is essentially based on the concerted syntrophic activity of various microbes. Thanks to recent advances in molecular biology techniques, new opportunities have arisen that help fully understand microbial community dynamics, interactions and functionality in AD (Vanwonterghem et al., 2014).

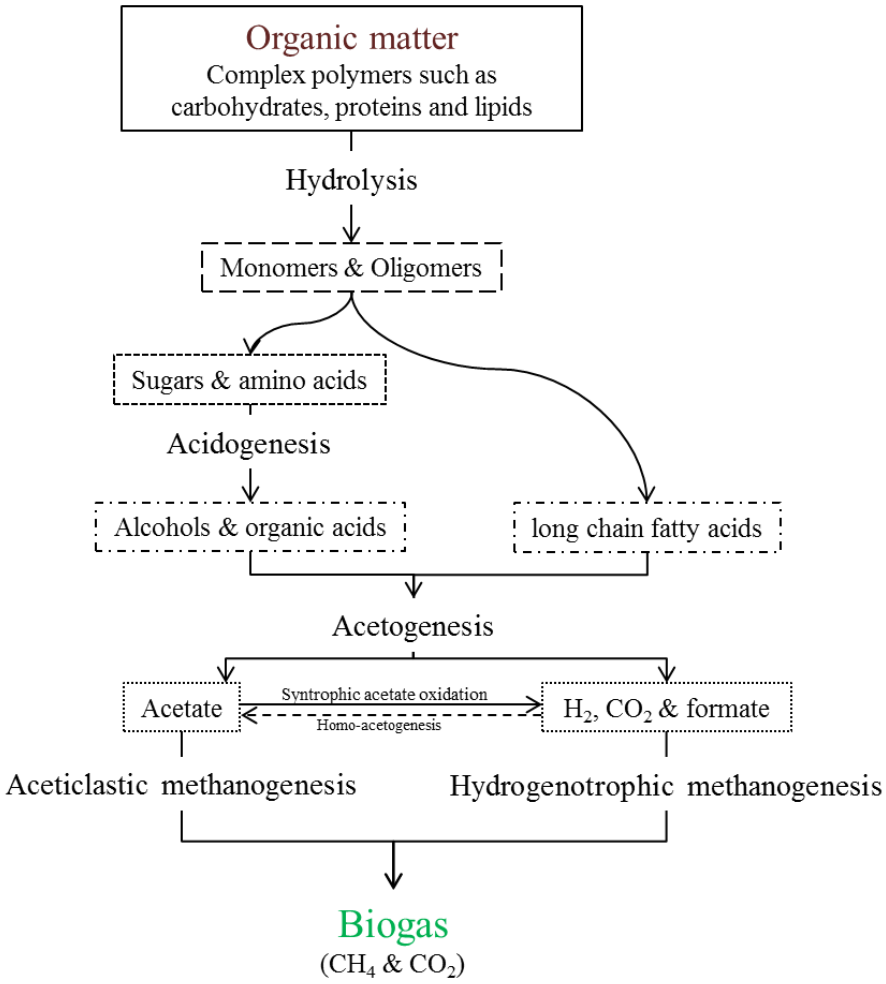

Figure 6. Simplified diagram showing the main metabolic steps in AD. Adapted from Batstone and Jensen (2011) and Schink and Stams (2013).

The microbial communities in the AD are considered to be one of the phylogenetically and functionally most diverse among engineered microbiomes (Vanwonterghem et al., 2014). The bacterial phyla Firmicutes and Bacteroidetes, and the methanogenic archaeal orders Methanosarcinales, Methanobacteriales, Methanomicrobiales and Methanococcales are commonly found to be dominant in biogas reactors (Krober et al., 2009; Lucas et al., 2015; Strauber et al., 2015; Ziganshin et al., 2013).

In the first conversion step, complex organic matter, including carbohydrates, proteins and lipids, is converted to soluble monomers and oligomers (e.g. sugar, amino acids and long-chain fatty acids). In this step, the microorganisms cannot directly use the solubilized particles or substrates. Three main pathways are involved in extracellular enzymatic hydrolysis: (1) enzymes are excreted by the microorganisms into the bulk liquid, where the enzymes diffuse into the particle; (2) adsorption processes occur intensively when the microorganisms attach to the particles and secrete enzymes into the surrounding area; (3) the end products of the enzymatic reactions are then released into the bulk liquid (Batstone & Jensen, 2011; Lynd et al., 2002).

Hydrolysis is generally considered to be the rate limiting step of AD, specifically for particulate or slowly degradable materials (Batstone & Jensen, 2011). For this reason, most of the large-scale biogas plants have to operate with low organic loading rates (OLR) and very long hydraulic and solid retention times (HRT and SRT, respectively) (Schmidt et al., 2014). Therefore, this requires biogas reactors with a higher volume in order to avoid the washout of the microbial communities and potential process failures. However, a larger reactor size implies higher costs during implementation and maintenance of the plant.

The most common substrates fed into biogas plants are carbohydrates derived from lignocellulosic materials of plants. The major fractions presented in lignocellulosic materials are lignin and the complex carbohydrates cellulose and hemicellulose. However, the content of each fraction varies depending on the plant material and harvesting time. In the plant cell wall, these three fractions are combined in a complex network form that limits microbial hydrolytic attack. Cellulose consists of a linear biopolymer with β(1→4) linked D-glucose units. The cellulose chains are aligned in microfibrils that are generally incorporated in a matrix of specific polymers such as hemicellulose and lignin (Leschine, 1995). Hemicellulose, which is present in almost all plant cell walls, is composed of randomly branched polysaccharides, which comprise minor sugars. In contrast to the low hydrolytic degradability of cellulose, hemicellulose is easily hydrolysed due to its low-strength amorphous structure. Lignin is a hydrophobic aromatic polymer that provides strength to the cell wall and restricts or prevents hydrolysis of the cellulosic material (Batstone & Jensen, 2011). Together, hemicellulose and lignin are considered to be responsible for the structure and tenacity of biomass (Bergman et al., 2005).

Approximately 5 to 10% of cellulose is anaerobically degraded in environments such as soil, water and animal guts (Leschine, 1995). Among the microorganisms involved in hydrolysis, the phyla Actinobacteria (aerobic order Actinomycetales) and Firmicutes (anaerobic order Clostridiales) present most cellulolytic capacity (Lynd et al., 2002), specifically the genera *Clostridium*, *Ruminococcus*, *Caldicellulosiruptor*, *Acetivibrio*, *Butyrivibrio*, *Halocella*, *Fibrobacter*, *Bacteroides* and *Spirochaeta*. For example, in an animal's rumen, the predominant bacterial genera are *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Prevotella* and *Eubacterium* (Sun, 2015).

Many pre-treatments have been applied to lignocellulosic biomass in order to improve the efficiency of biogas production by increasing microbial degradability (Lynd et al., 2002). The various pre-treatment methods currently used in AD can be briefly classified into three major groups: (1) chemical pre-treatment with strong acids such as H_2SO_4 and strong bases such as NaOH and $Ca(OH)_2$; (2) physical pre-treatment, for instance, milling, liquid hot water, irradiation and steam explosion; (3) biological

pre-treatment by fungal attack or enzymatic hydrolysis (Hendriks & Zeeman, 2009). Each pre-treatment option has certain advantages and disadvantages, but generally they improve hydrolysis during AD by changing the substrates' properties to a form that is more suitable for microbial and enzymatic access. Hence, the use of a specific pre-treatment depends on the cost-efficiency of the method in terms of providing more benefits for biogas production.

In the second step of the AD process, acidogenic bacteria convert the hydrolytic products (sugar and amino acids) into simpler compounds, mainly alcohols and organic acids. Through the Embden-Meyerhof-Parnas pathway, sugars are converted to pyruvate, and subsequently to C3 products (i.e. propionate and lactate), or C2-C6 products (e.g. acetate, butyrate and ethanol) via acetyl-CoA (Batstone & Jensen, 2011). Examples of sugar fermenting bacteria are *Petrimonas sulfuriphila*, which produces acetate but can also use sulphur or nitrate as an electron acceptor (Grabowski et al., 2005), and *Paludibacter propionicigenes*, which generates propionate, acetate, and succinate (Ueki et al., 2006).

In the third step, acetogenesis take place, which is the conversion of short chain fatty acids (mainly propionate, butyrate and valerate) into acetate and carbon dioxide. During acetogenesis, hydrogen and formate are also produced by microbial use of hydrogen ions and bicarbonate ions, respectively (Batstone & Jensen, 2011). Oxidation reactions require a very low concentration level of hydrogen or acetate in order to occur. The commonly found acetogens during propionate and butyrate degradation are *Syntrophobacter* and *Syntrophomonas*, respectively (Christy et al., 2014; Müller et al., 2010).

Biogas is finally produced by methanogens in the last step of AD through two main pathways: aceticlastic and hydrogenotrophic methanogenesis. The methanogens are a much less diverse microbial group than the bacteria involved in the previous steps. They belong to the domain of archaea and specifically to the strictly anaerobic phylum Euryarchaeota. Methanogens are also found in extreme environments such as hot springs and salt lakes, and in human and ruminant guts. Generally, methanogens are used in biogas production, sewage treatment, and among other biotechnology applications such as enzyme generation.

The hydrogenotrophic methanogens reduce carbon dioxide to methane using hydrogen as the primary electron donor. Some members of this group are also able to use formate as an electron donor. Hydrogenotrophic methanogenesis is the main pathway responsible for electron removal, allowing syntrophic acetogenesis to proceed. However, these methanogens are not the only microbial group acting as a sink for electrons. During reduction of nitrate, sulphate and iron, hydrogen is also required as an electron donor. This therefore causes competition for the available hydrogen in the AD of

substrates containing nitrate, sulphate and/or iron (in oxidized form). In the case of sulphate, further undesirable consequences may occur after sulphate reduction: (1) the presence of the resulting hydrogen sulphide in the biogas process causes several problems due to the toxicity and corrosive properties of this gas; moreover, its removal from biogas is rather expensive; (2) the formation of sulphide in the AD process induces the precipitation of specific metals that otherwise could have been used by the microorganisms (Isa et al., 1986).

Furthermore, homo-acetogenic bacteria are also able to convert hydrogen and carbon dioxide into acetate and vice versa. According to the Gibbs free energy under standard conditions, methanogenic hydrogen oxidation requires more energy than homo-acetogenesis, resulting in a higher chance for the homo-acetogenic bacteria to outcompete methanogens for hydrogen at limiting concentrations (Schink & Stams, 2013). Homo-acetogenic bacteria have been commonly observed under lower temperatures, which favours more their thermodynamics (Batstone & Jensen, 2011).

The aceticlastic methanogens cleave acetate into methyl and carboxyl groups, which are further converted into methane and carbon dioxide (Zinder & Koch, 1984). Only two taxa of the archaea are responsible for aceticlastic methanogenesis: the strictly acetate-cleaving genus *Methanosaeta* and the versatile genus *Methanosarcina*, which is capable of using different substrates such as CO_2/H_2, acetate and methyl groups. Whereas *Methanosaeta* is sensitive to process changes (such as pH or ammonia increase), *Methanosarcina* is considered to be more robust and tolerant to specific inhibitors and changing process conditions (De Vrieze et al., 2012).

Under certain inhibitory conditions (e.g. high ammonia concentration), acetate can also be consumed by syntrophic acetate oxidation, which is otherwise thermodynamically not favourable under standard condition. Hydrogen and carbon dioxide produced by syntrophic acetate-oxidising bacteria are then converted into methane by the hydrogenotrophic methanogens (Schnürer & Nordberg, 2008). *Tepidanaerobacter acetatoxydans, Clostridium ultunense, Syntrophaceticus schinkii* are some of the species belonging to the syntrophic acetate-oxidising bacteria community observed in biogas reactors with high levels of ammonia and volatile fatty acids (VFA) (Sun et al., 2014).

Methanogenesis is generally described as another rate-limiting step due mainly to its sensitivity to specific inhibitors, lack of functional redundancy, slow growth and low microbial diversity (Batstone & Jensen, 2011; Demirel, 2014). Taking these aspects into consideration, methanogens are commonly considered the most susceptible microorganisms in the AD process under stress (inhibition) conditions. Therefore, the monitoring of methanogenesis provides essential information on the overall process stability (Lucas et al., 2015).

1.3. Microbial performance

Although knowledge about microbial ecology has increased greatly in the last decades along with the advances of molecular biology (Volmer et al., 2015), biogas production nowadays is still carried out under suboptimal process conditions. Considering the broad range of opportunities to further develop the efficiency of the biogas process, some of the most important factors regulating the efficiency in terms of microbial performance include: (a) operating parameters such as temperature, substrate composition and HRT; and (b) monitoring parameters such as methane yield, potential inhibitors (e.g. ammonia, sulphide and VFA at high concentration levels), and pH value.

Temperature has a strong influence on biochemical and physico-chemical processes. In biogas systems, temperature can be classified into three different ranges according to reactor operability and predominance of microbial communities: psychrophilic (10 to 30 °C), mesophilic (30 to 40 °C) and thermophilic (40 to 70 °C). Reactions rates rise with increasing process temperature in accordance with the Arrhenius equation. The increased temperature causes a further increase in volumetric gas production, solubility of solid particles, gas transfer rates, biogas water content, liquid viscosity and pathogen deactivation (Batstone & Jensen, 2011).

Most biogas plants operate at mesophilic temperatures of around 40 °C. There are only a few plants that operate under thermophilic conditions of around 55 °C (Weiland, 2010). Mesophilic processes are more stable compared to thermophilic ones and are, therefore, more popular in AD facilities. Although the thermophilic process provides several advantages, the efficiency of biogas production is very risky due to process imbalances resulting often from higher microbial metabolic rate and higher VFA concentration (Chen et al., 2008). Moreover, a thermophilic process requires additional energy to keep the reactor at a high temperature. In this regard, an appropriate choice for operating either under mesophilic or thermophilic condition depends on many aspects, including substrate composition, final biogas use (CHP or biomethane), local infrastructure and others.

Substrate composition determines primarily the potential of a specific organic material to produce biogas and consequently, energy-rich methane. Substrate properties vary widely and determine the type of reactor suitable for microbial digestion. For instance, the AD of industrial wastewater requires biogas reactors that provide relatively short HRT such as ones with high biomass retention: fixed bed reactors and upflow anaerobic sludge blanket (UASB) reactors. By contrast, solid waste products need reactors that allow slow substrate degradation while the organic material is solubilized. An example of such a reactor type is the commonly used continuous stirred tank reactor (CSTR), which operates with a long HRT and SRT in the range of 10 to 60 days.

Information about the nutritional composition of the substrate is fundamental to ensure microbial growth and development during the biogas process. Among the nutrients, the most important macroelements are carbon, nitrogen, phosphor and sulphur, while the essential microelements are iron, nickel, cobalt, selenium, molybdenum and tungsten. When any of these nutrients are in short supply, a supplementary addition must be introduced into the reactor in order for the process to be efficient. Thus, the nutritional content in the AD process should be appropriately balanced. Excesses may lead to process failure; for example, due to ammonia accumulation during the AD of substrates with high nitrogen content. To prevent such conditions, the recommended carbon to nitrogen ratio should be between 15 and 30 (Weiland, 2010).

In solid organic materials, the composition of lignin and carbohydrates determine microbial performance. Since lignocellulosic matter is relatively resistant to microbial degradation during hydrolysis, the application of pre-treatments is required for better process efficiency as mentioned earlier. However, among the pre-treatments applied to the substrate for AD, there are some that release by-products, which cause inhibition of the biogas process. The most common inhibitory compounds in such conditions are furfural and phenols, produced during thermo-chemical pre-treatment (Chen et al., 2008; Monlau et al., 2014). For instance, phenols in the AD process have been documented as inhibitory for aceticlastic methanogenesis (Levén et al., 2012).

The microbial communities may also suffer inhibition by weak acids (e.g. VFA) and bases (e.g. ammonia). Weak acids such as VFA and hydrogen sulphide are considered to have an inhibitory effect around neutral and low pH respectively, while for weak bases such as ammonia, inhibition occurs at higher pH (Batstone & Jensen, 2011). To solve this issue with acid or base inhibition in the AD process, many chemicals are used to adjust the pH; however, this practice may incur high costs. Ammonia in its free form is the most common inhibitory factor in biogas reactors, especially in AD processes with proteinaceous or urea-rich substrate feeding such as animal manure. Karakashev et al. (2005) reported for example that *Methanosarcina* and other hydrogenotrophic methanogens are more tolerant to high ammonia concentration compared to *Methanosaeta*.

Monitoring pH values is also fundamental for controlling process efficiency. In a well-balanced AD process, the microorganisms metabolise intermediate products (such as organic acids) in a synchronised way such that no considerable accumulations are detected that cause drastic pH changes. In other cases, many chemical reactions performed by the microorganisms are strongly influenced by pH variations (Franke-Whittle et al., 2014; Sträuber et al., 2012; Veeken et al., 2000). For instance, low pH can inhibit the biological activity by deactivating particular enzymes.

Moreover, microbial growth varies greatly among the different microorganisms. The optimum range of pH values generally considered for the growth of hydrolytic and acidogenic bacteria is lower compared to methanogens (normally 6.5 to 8.5) (Weiland, 2010). Since the bacterial communities are less sensitive to pH changes, biogas reactors are usually operated under the optimum pH range for methanogens; alkalinity is usually buffered by bicarbonates that keep the pH quite constant, around neutral values.

Gas-liquid transfer during AD is also an important physico-chemical parameter for monitoring process stability. In essence, a biogas reactor consists typically of a mixture of different physical states such as solid particles, gas bubbles and liquid phase. The mass transfer of dissolved gas to gas bubbles is very intense during biogas production. Carbon dioxide and hydrogen sulphide are relatively soluble, whereas hydrogen and methane are nearly insoluble under process conditions. Due to this particularity, carbon dioxide and hydrogen have important effects on the stability of the biogas process.

1.3.1. Methods to investigate microbial communities

Physico-chemical process parameters such as pH, volatile organic acids (VOA) and VFA concentration do not always reliably indicate unbalanced process conditions. Often, the high buffer capacity of the system causes a misinterpretation of the parameters. The solution for this can be the monitoring of microbial activity. Intermediate compounds produced by microbes are measured as process parameters and therefore changes in microbial activity take place earlier than variations in the concentration of process parameters (Lebuhn et al., 2014). Thus, changes detected in microbial activity serve as an early warning indicator, considering that in some cases a distinctive microbial community may process the same intermediate products. Therefore, the monitoring of microbial activity in the biogas process is crucial for keeping the process under stable conditions and for eventually counteracting inhibitors.

The introduction of molecular techniques into microbial ecology has revolutionized the investigation of complex microbial communities of environmental and engineered systems. In recent decades, significant development in microbial community analysis has taken place, ranging from methods based on molecular techniques (e.g. characterization of non-cultivable microorganisms) to current next-generation sequencing technologies. Molecular fingerprinting techniques enable a fast comparison of community structures of numerous samples, while novel sequencing techniques allow a deeper investigation of those communities with more precise taxonomic affiliation. Due to the large amount of sequencing data generated by many laboratories and genome sequencing centres, a sequence

database was required to store all information and to make it easily accessible to search with online programs such as BLAST.

Terminal restriction fragment length polymorphism (T-RFLP)

This technique has been frequently used to monitor changes in the microbial community structure and dynamics in response to operational variations in the biogas process (Lv et al., 2014b; Nikolausz et al., 2013; Schmidt et al., 2014; Westerholm et al., 2011). In this approach, the polymerase chain reaction (PCR) method is conducted with primers labelled with a fluorescent dye to amplify, from the community DNA, the target genes. As an example of targeted genes, the 16S ribosomal RNA gene for bacteria and the methyl coenzyme-M reductase α-subunit (*mcrA*) gene for methanogenic archaea are targeted. The PCR products are then digested with a restriction enzyme, which cuts the DNA amplicons at specific sequences. After that, the digested products are separated according to the size on a capillary gel-electrophoresis system (automated DNA sequencer), and only terminally-labelled fragments are detected by laser-induced fluorescence (Madigan et al., 2010). Thus, T-RFLP allows researchers to assess the diversity and relative abundance of the microbial community members by analysing the peak areas of single fluorescent-labelled terminal restriction fragments (T-RFs). Single T-RFs may be assigned to a specific taxon following further analyses, i.e. cloning and sequencing. In this case, copies of the target gene are inserted into a simple genetic element such as a plasmid (also referred to as a vector). The vector usually carries an antibiotic resistance gene, and is taken up by competent cells at very low probability (transformation). Only cells containing the vector will grow on the selective agar (containing antibiotics) and a single colony (clone) contains only one type of vector with a single gene fragment type. During the replication of the vectors, the cloned DNA is also replicated (Madigan et al., 2010). Thus, the cloned genes can be identified individually from the colonies by Sanger sequencing. Unfortunately, as with T-RFLP analysis, the clone library approach for *mcrA* and 16S rRNA presents some limitations for detection of relatively low abundance microbes in the biogas reactor digestate. In addition, genetic analysis through cloning and sequencing is time-consuming, which consequently reduces the number of comparable samples.

454 Pyrosequencing

This technology, developed by 454 Life Science Corporation, revolutionized DNA sequencing with rapid sample processing compared to conventional techniques. The 454 pyrosequencing system has been used for the massive parallel analysis of DNA sequences, e.g. amplicon sequencing, genome sequencing and metagenomics (Iacono et al., 2008; Petrosino et al., 2009). In this approach, each DNA fragment, comprising around 400 to 600 base pairs, is attached to a microscopic bead. After DNA

amplification by PCR, the beads, carrying full of amplicons, pass through a fibre-optic plate containing over a million wells, where finally the sequencing reactions take place simultaneously (Madigan et al., 2010). Although both the Sanger sequencing and 454 pyrosequencing approaches use DNA polymerase for the synthesis of a complementary strand, the final detection method is different. Instead of chain termination as in the Sanger sequencing, pyrophosphate release upon nucleotide incorporation is detected in the 454 method (Ronaghi, 2001). Compared to the time-consuming clone library and Sanger sequencing processes, next-generation sequencing systems provide much faster and larger sequence libraries of complex communities.

Stable isotope fingerprinting

Compound-specific stable isotope analysis, also called stable isotope fingerprinting, is a frequently used tool for the identification of chemical, physical or biological processes that are involved in the conversion of substrate to products. In the biogas field, the stable isotope compositions of methane and carbon dioxide allow an approximate identification of the dominant metabolic pathway during methanogenesis. In biochemical reactions, molecules with lighter isotopes react much faster compared to those with heavier isotopes. The kinetics for carbon and hydrogen isotope fractionation is different in the two major methanogenic pathways, resulting thus in distinctive isotope patterns of CH_4 from aceticlastic vs. hydrogenotrophic methanogenesis (Whiticar, 1999). The investigation of the predominant methanogenic pathways as a function of the differences in the isotope composition of carbon from CH_4 and CO_2, and hydrogen from CH_4 has been reported in recent decades (Sugimoto & Wada, 1995; Whiticar, 1999; Whiticar et al., 1986). These authors used a number of models, including a scheme combining the isotope composition of carbon and hydrogen from methane, and the apparent fractionation factor, αC, calculated from the isotope composition of carbon from CH_4 and CO_2, to show whether methane in natural systems came predominately from the aceticlastic or hydrogenotrophic pathways.

With the recent intensification of biogas research, many studies have been devoted to investigate the isotope composition of biogas in engineered systems, including biogas plants or laboratory-scale reactors (Keppler et al., 2010; Laukenmann et al., 2010; Nikolausz et al., 2013; Polag et al., 2014; Polag et al., 2015). They revealed that the stable isotope approach may be used as a monitoring tool for engineered AD processes. In biogas reactors, methanogens are the most sensitive microorganisms, reacting rapidly to process changes. Thus, shifts in the predominance of the methanogenic pathway generally reflect process imbalances, which can lead to the collapse of the system. For instance, aceticlastic methanogenesis is strongly inhibited under high ammonia concentration. In this case, a

shift from aceticlastic to hydrogenotrophic methanogenesis is a sign that the process is under stress and therefore prompt readjustment is required to avoid process failure.

The measurement of stable isotope ratios is usually performed by gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS). This technique exhibits high precision for the detection of stable isotopes of carbon (^{13}C and ^{12}C) and hydrogen (^{2}H and ^{1}H) found at natural abundance level. The GC-IRMS carbon measurement method comprises basically three steps: (1) individual separation of the compounds on a gas chromatograph; (2) conversion of each compound to carbon dioxide in a high temperature combustion oven; (3) water removal and introduction of CO_2 originated from each compound into the isotope ratio mass spectrometer (Hunkeler et al., 2008). In the first step of the mass spectrometer, the CO_2 is subjected to ionization. Due to the difference in mass-to-charge ratios, the ionised compounds are then magnetically separated and detected simultaneously with fixed Faraday cups. This whole instrumentation description of the GC-IRMS is represented in Figure 7.

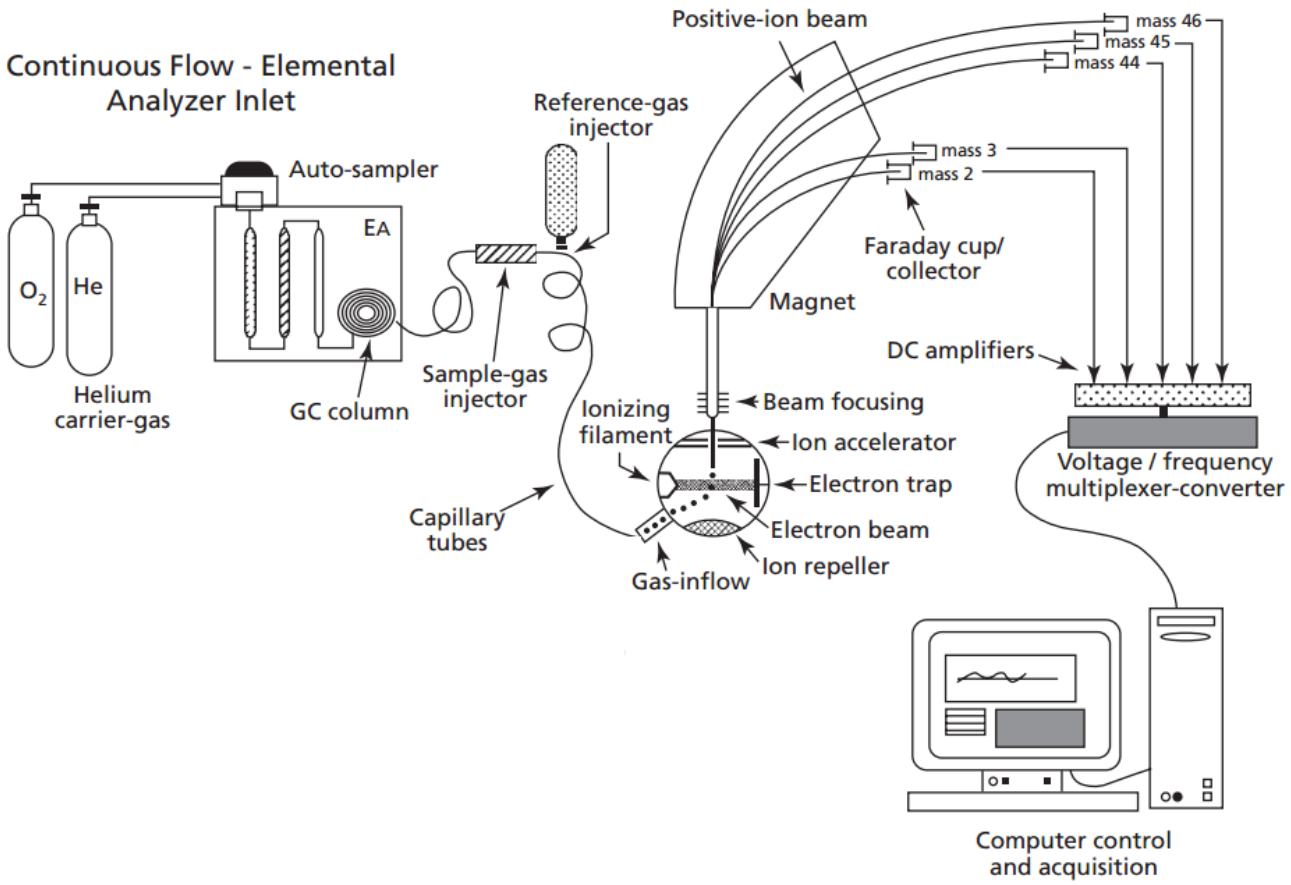

Figure 7. Schematic of the GC-IRMS instrumentation. Partially adapted from Dawson and Brooks (2001) as cited in Michener and Lajtha (2008).

The international standards for carbon (Vienna Pee Dee Belemnite - VPDB) and hydrogen (Vienna Standard Mean Ocean Water - VSMOW) are used to ensure accuracy and comparable results between

different laboratories. The relative abundance of the ions is converted by specific software into an isotope ratio, which is finally reported as $\delta^{13}C$ for carbon (Eq. 1) and $\delta^{2}H$ for hydrogen (Eq. 2) in the following equations:

$$\delta^{13}C(‰) = \left[\frac{\left(\frac{^{13}C}{^{12}C}\right)sample - \left(\frac{^{13}C}{^{12}C}\right)standard}{\left(\frac{^{13}C}{^{12}C}\right)stardand}\right] \times 1000 \qquad \text{Eq. 1}$$

$$\delta^{2}H(‰) = \left[\frac{\left(\frac{^{2}H}{^{1}H}\right)sample - \left(\frac{^{2}H}{^{1}H}\right)standard}{\left(\frac{^{2}H}{^{1}H}\right)stardand}\right] \times 1000 \qquad \text{Eq. 2}$$

During hydrogenotrophic methanogenesis, the lighter carbon is more often metabolized than during aceticlastic methanogenesis. Hence, more negative $\delta^{13}C_{CH_4}$ values indicate depletion of the ^{13}C product (and enrichment in ^{12}C) and reveal that hydrogenotrophic methanogenesis is the major pathway for methane formation.

1.4. Outline of the thesis

The conversion of organic waste produced in our houses, or by industry, agriculture and forestry into energy in the form of biogas is a promising approach to change our fossil-fuel-based economy into a bioeconomy based on sustainable flow of matter and energy. Unfortunately, biogas technology for transforming waste to energy is still not very efficient, requiring further process enhancement to increase methane production and its use as an energy source. In this context, complex microbial interactions, under the influence of their reactor environment, are keys to improve the cost-efficiency of biogas production. As described previously, there are many factors that drive the AD process under stable conditions, which consequently provide higher energy yields and higher digestate quality as a mineralized fertilizer. Substrate characteristics reveal primarily the potential for biogas production and provide fundamental information for the design of the AD process. In a continuously running biogas reactor, systematic monitoring of the reactor parameters should be performed intensively in order to avoid process imbalances and possible failures. In addition, the monitoring of microbial communities, especially more sensitive methanogens, is essential to understand the microbial interactions in the reactor and thus be able to develop strategies for a cost-efficient biogas production.

Hence, this PhD thesis describes the performance of various laboratory-scale biogas reactors under different conditions with a focus on the microbial community structure involved in the AD of sugarcane wastes. In order to provide crucial information on the process optimization of biogas

production, the correlations between operating parameters and community structures were thoroughly assessed. The specific objectives were designed to:

- characterise the physico-chemical properties of sugarcane wastes and to determine their biogas potential in batch tests (subsections 2.1. and 2.2.);
- follow biogas production from sugarcane wastes according to their variations in physico-chemical characteristics during an industrial season (subsection 2.1.);
- propose a potential process design for biogas production of each sugarcane waste (subsection 2.2.);
- operate continuous feeding reactors for mono-digestion of filter cake and its co-digestion with bagasse and to evaluate their biogas processing without any nutritional supplementation (subsections 3.1., 3.2., 3.3. and 3.4.);
- investigate the co-digestion set-up as a complementary source of nutrients for microorganisms (subsections 3.1., 3.2. and 3.3.);
- assess the volume capacity for substrate loading of biogas reactors fed with filter cake and bagasse by gradual increase of the OLR (subsection 3.1., 3.2. and 3.3.);
- apply different pre-treatments to the sugarcane wastes to obtain higher methane yield (subsections 2.1., 2.2. and 3.4.);
- investigate VFA yield during AD of filter cake and to evaluate its performance by nitrogen supplementation (subsection 3.4.);
- analyse cattle manure as a potential inoculum for the start-up of a biogas process as a solution for remotely located sugarcane industries (subsection 3.2. and 3.3.);
- assess microbial community dynamics throughout continuous operation of biogas reactors fed with filter cake and bagasse with molecular and stable isotope fingerprinting techniques (subsection 3.1. and 3.3.).

2. Characterization of the sugarcane wastes

2.1. Assessment of the variations in characteristics and methane potential of major waste products from the Brazilian bioethanol industry along an operating season

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Assessment of the Variations in Characteristics and Methane Potential of Major Waste Products from the Brazilian Bioethanol Industry along an Operating Season

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ABSTRACT: Anaerobic digestion appears to be a favorable option to optimize the energetic exploitation and reduce the environmental impacts of bioethanol waste products. Some analytical characteristics of these waste products are available in various sources. However, these data are too incomplete and unsystematic to be compared among the bioethanol industries. Design of biogas processes based on such data has to deal with considerable unknowns regarding the technical feasibility and operating costs. Therefore, to better understand and assess the applicability of these bioethanol waste products in anaerobic digestion, the micro- and macro-element concentrations, the physicochemical parameters, and the methane potential were analyzed. In addition to the assessment of seasonal variations, the effect of alkaline and mechanical treatments was also investigated for lignocellulosic bagasse samples. Possible deficiencies of the trace elements Ni, Co, Mo, Se, and W in vinasse as a substrate for anaerobic digestion were recorded. The correlation between the gradual increase in methane yields of vinasse and filter cake along the bioethanol operating season and the dynamic changes in the substrate characteristics was shown. Moreover, the methane yield of raw bagasse increased by 50% after applying both treatments in combination.

1. INTRODUCTION

Bioethanol has been produced as a biofuel in many countries, such as the U.S.A. and Brazil. A consequence of the recent expansion of bioethanol is an increase of waste products from the bioethanol industry. In some countries, the management of such waste is not well established. Brazil, for example, as one of the leaders in bioethanol production, has not yet implemented a waste management strategy, which takes into consideration the energy potential of these residues.

Worldwide, Brazil is considered to have the largest and most successful biofuel program. Brazilian bioethanol is produced from sugar cane at higher efficiency compared to other agricultural feedstocks, such as corn. In 2013, the total bioethanol produced in Brazil was about 14.7 billons liters, 18% more than in 2012.[1] Nowadays, in Brazil, 100% of the passenger cars run with (blended) bioethanol, including the flex-fueled vehicles, which are designed to run with hydrated and anhydrous ethanol with blending rates in gasoline up to 26%.[2]

The three major waste products from the sugar-cane-based bioethanol industry are bagasse, filter cake, and vinasse. Bagasse is generated in the milling process of sugar cane and has a high dry content. Filter cake, often referred to as press mud, is derived from the physicochemical sugar cane juice treatment and contains small solid particles from milling as well as non-fiber carbohydrates in the form of dissolved sugars. Vinasse, also termed as stillage, is a dark brown waste water that comes from the distillation process after bioethanol is separated. Approximately 280 kg of bagasse, 40 kg of filter cake, 1000 L of vinasse, and 85 L of bioethanol are produced per ton of processed sugar cane.[3,4]

The bioethanol waste management in Brazil is mainly destined to co-generation and field applications as fertilizer. Bagasse is burned to produce heat and electricity, whereas filter cake and vinasse are directly spread on sugar cane crop fields. Most co-generation facilities are working with low efficiency. Vinasse and filter cake are often applied on the fields without any previous treatment or proper dosage. The vinasse is temporarily stored in large open lagoons, which potentially release large amounts of methane because of microbial activity under anoxic conditions. Proper treatment and further energetic utilization of these waste products thus would not just improve the overall energy balance of the process but would help to avoid uncontrolled release of a large amount of greenhouse gas, mainly in the form of methane, during storage and upon waste disposal on land.[5,6]

Considering the large amount of waste products generated by the Brazilian bioethanol industry and the energy potential remaining in it, biogas production via anaerobic digestion appears to be a suitable treatment technology producing additional commodities. Anaerobic digestion offers a broad variety of usage of its main products: biogas and digestate.

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The biogas can be used for the production of heat and electricity, kitchen gas, and as a vehicle fuel. The digestate is a valuable fertilizer, because it has balanced elemental composition and a lower carbon/nitrogen ratio than its precursors.[7]

The potential of biogas production from the bioethanol waste products was evaluated in only a few studies.[8−13] However, the impact of seasonal variations of these waste products on biogas production has not been investigated thus far according to our best knowledge. Variations in characteristics originated from such factors as soil, crop diversity, climate, harvest time, and industrial process have significant impact on the biogas yield, as reported by other studies using different types of biomass for biogas production.[14,15] A previous study revealed substantial variation in vinasse composition; however, the factors that led to it and its influence on biogas production were not discussed.[16]

Process design for anaerobic digestion and technical feasibility assessment based on such limited and variable data would be unreliable.[17] Therefore, we believe that the assessment of changes in the characteristics of bioethanol waste products is essential for the design of an economically viable and sustainable anaerobic digestion strategy. Because Brazil is a big country extending over different climate zones, we focused on one region of the Brazilian state Goiás to have an isolated and common view on the seasonal variations of the bioethanol waste products on biogas production along an operating year. In addition to the detailed characterization of the substrate, the methane potential and the effect of mechanical and alkaline treatment in the case of the lignocellulose-rich bagasse were also evaluated.

2. MATERIALS AND METHODS

2.1. Biomass Sampling. The biomass used in the physicochemical analyses and as substrate for anaerobic digestion was collected at various times along the 2013 industrial operating season (nearly 7 months a year) of a bioethanol plant, located in the Brazilian state of Goiás. Bagasse was taken directly after sugar cane milling. Filter cake samples were taken from the sugar cane juice rotary vacuum-drum filter. Vinasse was taken directly from the distillation discharge. All samples had high temperature at the collecting points, around 50 °C for bagasse and filter cake and nearly 90 °C for the vinasse. The samples were transported for 4 h at ambient temperature in sealed 15 L hard plastic canisters to a refrigerator and stored there at 4 °C for about 30 days until further transportation. Subsequently, samples were transported by air freight to Germany, where they were kept in a cold room at 4 °C for a maximum of 5 and 30 days until analytical analyses and batch tests in an anaerobic system were carried out, respectively.

2.2. Physicochemical Analyses. Total solids (TS) and volatile solids (VS) were determined according to the standard methods DIN 12880[18] and DIN 12879,[19] respectively. The pH values were characterized using a pH-211 pH meter (Hanna Instruments, Woonsocket, RI). Chemical oxygen demand (COD) analysis was carried out by a COD test kit (Hach Lange, Düsseldorf, Germany). Nessler's reagent (Merck, Darmstadt, Germany) was used to analyze the ammonium nitrogen (NH_4^+-N) concentration with a DR/2000 spectrophotometer (Hach Company, Loveland, CO) at 425 nm. Reducing sugars were determined according to standards.[20] Acetate, propionate, isobutyrate, and other volatile organic acids (VOA) were measured after acidification using a 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA). An elution of the dissolved filter cake in distilled water (1 g of filter cake to 10 mL of distilled water for 24 h) was performed to analyze VOA and pH. Cellulose, lignin, hemicellulose, non-fibrous carbohydrate, raw protein, raw fat, and total Kjeldahl nitrogen (TKN) values were measured according to standard analytical procedures.[20,21]

2.3. Biomass Treatments. The lignocellulosic sugar cane bagasse was subjected to two different types of treatment. A physical treatment was applied to the raw bagasse for a reduction of particle size to 10 or 1 mm. The 10 mm milled bagasse was additionally subjected to an alkaline treatment, which was carried out for 24 h after the addition of 0.1 g of $Ca(OH)_2$/g bagasse in 24 mL of distilled water.

Table 1. Characteristics of Vinasse Samples Taken during the Operating Season of a Bioethanol Industry

parameter	unit	May	September	November
pH		4.21	4.06	3.81
COD	g L^{-1}	16.40	20.96	23.57
TS	g L^{-1}	16.10	17.00	19.40
VS	g L^{-1}	11.54	13.21	16.10
NH_4N	g L^{-1}	0.01	nd[a]	0.02
TKN	mg L^{-1}	218.56	105.06	77.95
sulfate (SO_4^{2-})	mg L^{-1}	66.30	1959.09	584.00
aluminum (Al)	mg L^{-1}	10.77	18.64	29.80
cobalt (Co)	mg L^{-1}	0.00	0.03	0.01
copper (Cu)	mg L^{-1}	0.19	0.38	0.24
iron (Fe)	mg L^{-1}	12.02	24.27	27.80
manganese (Mn)	mg L^{-1}	1.63	10.59	4.72
molybdenum (Mo)	mg L^{-1}	0.01	0.04	0.01
nickel (Ni)	mg L^{-1}	0.01	0.02	0.03
silicon (Si)	mg L^{-1}	13.05	69.09	64.10
tungsten (W)	mg L^{-1}	nd	nd	nd
zinc (Zn)	mg L^{-1}	0.19	1.61	9.60
calcium (Ca)	mg L^{-1}	55.32	459.09	259.00
carbon (C)	mg L^{-1}	14384.00	8338.00	6552.00
magnesium (Mg)	mg L^{-1}	39.84	307.27	116.00
nitrogen (N)	mg L^{-1}	855.60	638.00	275.40
phosphorus (P)	mg L^{-1}	44.03	242.27	48.00
potassium (K)	mg L^{-1}	466.13	3072.73	1070.00
sodium (Na)	mg L^{-1}	1.01	15.14	4.94
sulfur (S)	mg L^{-1}	304.42	279.40	212.40
C/N ratio		17	13	24
reducing sugar	g L^{-1}	1.39	2.30	2.58
glucose	mg L^{-1}	60.13	na[b]	27.84
alcohol	mg L^{-1}	451.99	na	365.24
acetic	mg L^{-1}	114.99	306.41	1380.25
propionic	mg L^{-1}	6.15	5.83	6.43
isobutyric	mg L^{-1}	1.10	0.99	3.09
n-butyric	mg L^{-1}	3.91	1.20	6.81
isovaleric	mg L^{-1}	0.00	0.00	2.39
hexanoic	mg L^{-1}	0.75	0.46	3.64
BMP	mL_N/g_{COD}	220.84	260.74	269.72

[a]nd = not detected. [b]na = not analyzed.

2.4. Anaerobic Digestion Tests. Mesophilic anaerobic digestion of the bioethanol waste products was carried out in a multi-channel analyzer, the Automatic Methane Potential Test System II (AMPTS II, Bioprocess Control, Sweden). Six different batch tests for anaerobic digestion were setup in the AMPTS II, resulting in a total of 90 reactor experiments.

The inoculum used for the batch tests was from a standard mesophilic biogas reactor operated under a minimal feeding regime of digestate from a biogas plant with co-digestion of maize silage and cow manure. Representative inoculum samples were collected from this standard reactor 2 days before the batch test setup for TS and VS analyses. The TS and VS values of inoculum together with those of each waste product were the basis for calculating the amount of substrate applied in the anaerobic batch systems. This calculation followed the AMPTS II producer instructions. The inoculum/substrate ratio chosen for this experiment was 2 (on the basis of VS). The substrate amount was calculated based on the TS and VS determined after the treatment in the case of alkaline-treated bagasse.

AMPTS II was setup and run under the same conditions in all 15 reactors in a batch system. Each reactor had 400 mL working volume and 250 mL headspace. The anaerobic digestion was carried

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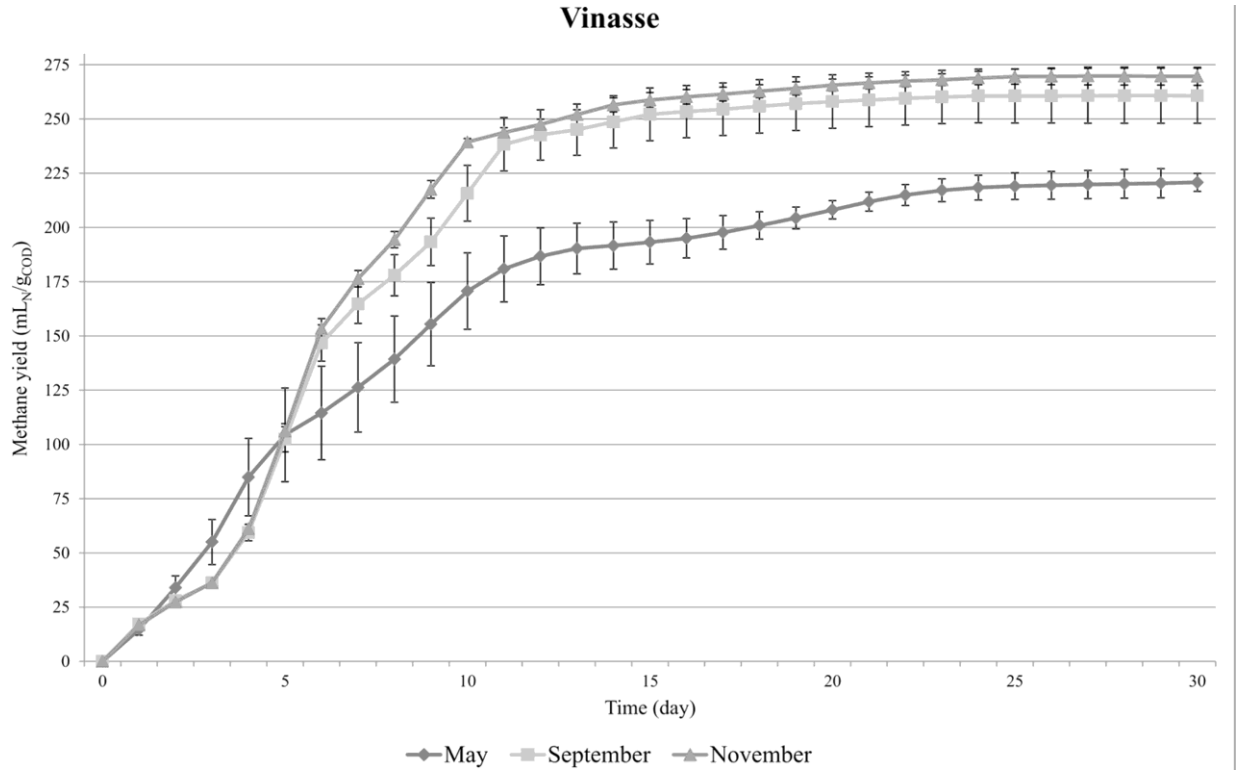

Figure 1. Methane yield during anaerobic digestion of vinasse samples collected in three different periods along the operating season of a bioethanol industry. Two independent batch tests were run with May samples: one with six and the other with three reactors for vinasse. Separate batch tests were run for September and November samples with three reactors for vinasse, each. The average of all daily methane measurements for each particular vinasse setup is represented on the graph, with the average of methane production from the blank reactors already subtracted. The bars show the standard deviation.

out in triplicates, and all reactors were filled with the same amount of inoculum. The reactors that had the substrate with the lowest VS value were filled just with inoculum and substrate itself. To have the same volume in the reactors with other substrates, distilled water was added up to 400 mL. Crystalline cellulose was used as a carbon source in the positive control reactors. The buffer capacity of inoculum was sufficient for keeping the biogas process at an optimum pH level, even with the very acidic vinasse or the basic alkaline-treated bagasse. Before anaerobic digestion, the pH values of all batch reactors were measured.

During the entire experiment, the stirrers had the configuration of 30 s on and 60 s off at 50 rpm. A further technical setup of the batch tests was conducted according to the manual of the manufacturers. Methane production was determined automatically in real time for each single reactor by the AMPTS II software. The experiment time of the reactors varied from 30 to 40 days depending upon the degradation rate. At the end of the experiment, pH values were measured again.

3. RESULTS AND DISCUSSION

3.1. Vinasse. The variation of vinasse composition was determined at three different sampling times in May, September, and November (Table 1), thus covering the entire operating season. The COD, VS, and TS values increased during the year. In November, these parameters had the highest values, whereas the pH was the lowest.

The TKN represents the sum of ammonia (NH_3), organic, and reduced nitrogen. The vinasse sample collected in May had a higher TKN than later samples. An increase in nitrogen content of the substrate can result in ammonia accumulation, which may lead to process inhibition.[22] Therefore, the monitoring of the TKN levels in the substrate is fundamental.

Although the sulfate concentration fluctuated during the sampling season, it was relatively high in all vinasse samples. This must be taken into consideration, because sulfate can hinder the biogas process in different ways. Sulfate-reducing bacteria use sulfate as an electron acceptor to oxidize fermentation products into carbon dioxide. This affects both acetogenic and methanogenic microorganisms, once sulfate-reducing bacteria compete with methanogenic archaea for hydrogen as well as with acetogenic bacteria for propionate and butyrate.[23] In addition to this direct negative impact on biogas production, reduced sulfur in the form of H_2S may cause corrosion in the treatment facilities.[24,25]

The nutritional elements are essential for the metabolism of microorganisms in the biogas process. Deficiency of trace elements limits microbial growth, thereby affecting the performance of an anaerobic treatment plant.[26,27] The macro-elements calcium, magnesium, phosphorus, sodium, and sulfur varied moderately, whereas carbon, nitrogen, and potassium varied more. Because vinasse contains generally very low concentrations of trace elements, anaerobic digestion of this waste product might possibly require amendments of Ni, Co, Mo, Se, and W.[27,28]

The variation in the carbon/nitrogen ratio directly influences the biogas production. The optimal C/N ratio for the biogas process ranges from 10 to 40.[7,29] The low carbon/nitrogen ratio, such as in the vinasse samples, might indicate the requirement of the addition of other carbon-rich substrates for effective co-digestion.

Figure 1 shows the methane yield of various vinasse samples during 30 days of anaerobic digestion in independent batch

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Table 2. Characteristics of Filter Cake Sampled during the Operating Season of a Bioethanol Industry

parameter	unit	May	September	November
TS	%	22.85	22.02	23.70
VS	$\%_{TS}$	75.86	81.43	75.33
cellulose	%	29.51	33.25	30.39
lignin	%	24.31	27.13	28.48
hemicellulose	%	26.32	29.15	30.23
extractives[a]	%	19.86	10.47	10.89
carbohydrates and lignin	%	77.02	80.03	83.19
raw protein[b]	%	17.99	14.33	11.89
raw fat[c]	%	4.99	5.64	4.92
TKN	mg/g	95.15	84.78	60.47
aluminum (Al)	mg/g	46.22	81.33	165.98
cobalt (Co)	mg/g	0.03	0.02	0.05
copper (Cu)	mg/g	0.37	0.29	0.18
iron (Fe)	mg/g	39.02	102.49	117.43
manganese (Mn)	mg/g	1.35	0.99	1.11
molybdenum (Mo)	mg/g	0.01	0.01	0.00
nickel (Ni)	mg/g	0.04	0.05	0.07
silicon (Si)	mg/g	0.60	2.07	209.54
tungsten (W)	mg/g	nd[d]	nd	nd
zinc (Zn)	mg/g	2.45	0.66	0.57
calcium (Ca)	mg/g	71.11	40.04	18.38
carbon (C)	mg/g	91800.00	102907.00	96641.00
magnesium (Mg)	mg/g	22.00	5.27	4.12
nitrogen (N)	mg/g	3870.00	3277.60	2337.70
phosphorus (P)	mg/g	27.82	10.75	7.88
potassium (K)	mg/g	162.67	6.27	13.15
sodium (Na)	mg/g	16.53	0.17	0.37
sulfur (S)	mg/g	1833.75	1648.44	134.96
C/N ratio		24	31	41

[a]Non-fibrous carbohydrates, such as sugar, fructan, starch, pectins, and organic residues. [b]Raw protein is the sum of all compounds containing nitrogen [c]Raw fat content that dissolves in fat solvents. [d]nd = not detected

systems. Two independent batch systems were run with May samples: the first one included six reactors with vinasse, three with cellulose as the control and three just with inoculum as the blank (the inoculum had pH 8.00, 3.33% TS, and 63.95% VS of TS); the second system had three reactors with vinasse and the same number of reactors used as the control and blank as in the first system (the inoculum had pH 8.62, 4.22% TS, and 63.14% VS of TS). In these first and second batch systems, the accumulated methane production of 15.37 and 2.20 mL_N for blank reactors and 830.20 and 867.28 mL_N for positive control reactors were observed, respectively.

A distinct batch system for September and November samples consisted of three reactors for each vinasse sample, controls, and blanks, in a total of 12 reactors (the inoculum had pH 7.81, 6.42% TS, and 59.29% VS of TS). This last batch system had on average an accumulated methane production of 21.10 mL_N for the blank reactors and 965.80 mL_N for the control reactors.

The results obtained with the blank and control reactors showed that the systems were reliable and demonstrated that the inocula without substrate were barely producing methane, although they were active when substrate was added. The pH values of the reactors with vinasse samples before anaerobic digestion ranged from 6.89 to 7.26 and at the end of the experiment from 7.37 to 7.54. Therefore, they remained in an optimal range for anaerobic digestion.

The vinasse samples were rapidly degraded in the reactors without a lag phase. There was a pronounced log phase during the first 16 days, until almost all organic matter was digested and more than 88% of the total accumulated methane had been produced. After this period, the gas production became stationary and only little further methane was produced. This was indicative of easily degradable organic material, mostly in the form of acetate and reducing sugars in vinasse.

The methane yields of the various vinasse samples followed a similar trend along the operating season. Differences between cumulative methane yields were due to variation in the vinasse composition. The main contributing factors for such variation were the gradual increase of reducing sugars and acetate. The alcohol and glucose concentrations in May and November were not considered to be responsible for changes in the methane yield because of their low concentrations.

It is not realistic to assume that inhibition by sulfate and macro-, and micro-elements took place in the reactors because the greater inoculum part of the operated batch systems should have suppressed such effects. Inhibition would rather be observed in continuous systems, where the accumulation of sulfate or other elements, such as potassium, could affect the process.

It is excluded that the lack of macro- and trace elements influenced the assessments in our batch systems because sufficient amounts were introduced with the inoculum. For that reason, further experiments in continuous biogas processes are required to further study the impacts of elemental composition.

3.2. Filter Cake. General characteristic parameters of filter cake from different countries were found in the literature.[8,12,30,31] However, these data vary dramatically as a result of differences in the sugar cane composition and conditions applied in bioethanol processes. Even in a specific industry using the same cultivar and process, as shown in Table 2 for our case study, filter cake characteristics vary during the operating season.

TS and VS values of filter cake samples changed slightly during the operating season. However, even small variations of TS and VS affect the feeding regime in the anaerobic digestion. For that reason, it is important to analyze TS and VS periodically. Alteration in the VS values may be related to variations in the raw fat, raw protein, carbohydrate, and lignin contents.

Between the sampling months, the raw fat oscillated, reaching the highest value in September. In the course of the operating season, the amount of raw protein decreased. In contrast, the total carbohydrate and lignin contents increased. The cellulose and extractives values varied distinctively within the sampling time, whereas the lignin and hemicellulose contents rose gradually.

The filter cake elution for the September sample had an acidic pH of 5.04, which might pose a problem in the case of continuous operation mode with filter cake and might require further process control efforts. The low COD concentration of filter cake elution was expected, because most of the organic carbon present in the raw filter cake is in a non-soluble form.

During the operating season, the TKN concentration in filter cake samples decreased along with total elemental nitrogen content. Consequently, the C/N ratio rose across the year, although the carbon concentration somewhat fluctuated. A high variation in the elemental sulfur concentration was observed between the two first sampling months and November. A high sulfur concentration might effect the anaerobic digestion, once sulfur may be oxidized to sulfate. Potassium concentrations also varied largely among the different samples. It was much higher

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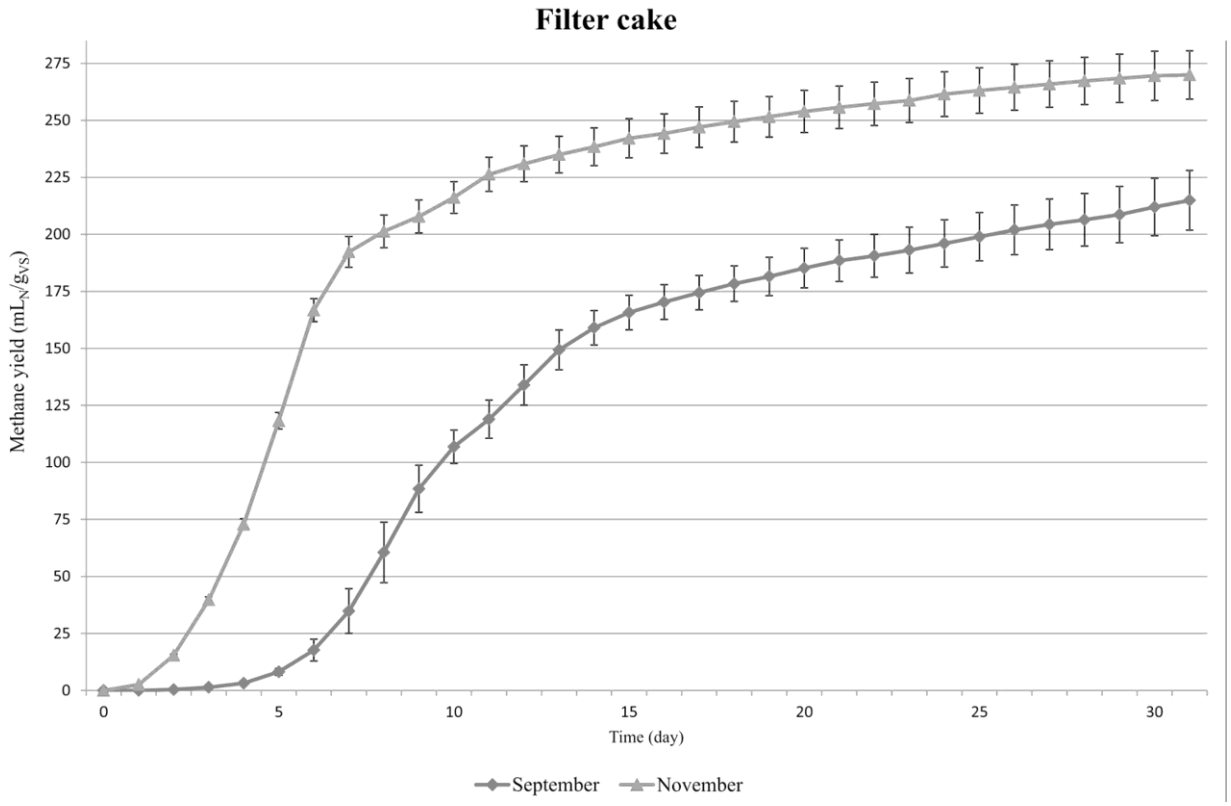

Figure 2. Methane yield during anaerobic digestion of filter cake samples collected along the operating season of a bioethanol industry. Independent batch systems for each sample were run with three reactors for filter cake and triplicates for the blank and control. The daily average of all methane measurements for each particular filter cake setup is represented on the graph, with methane production from the blank reactors already subtracted. The bars show the standard deviation.

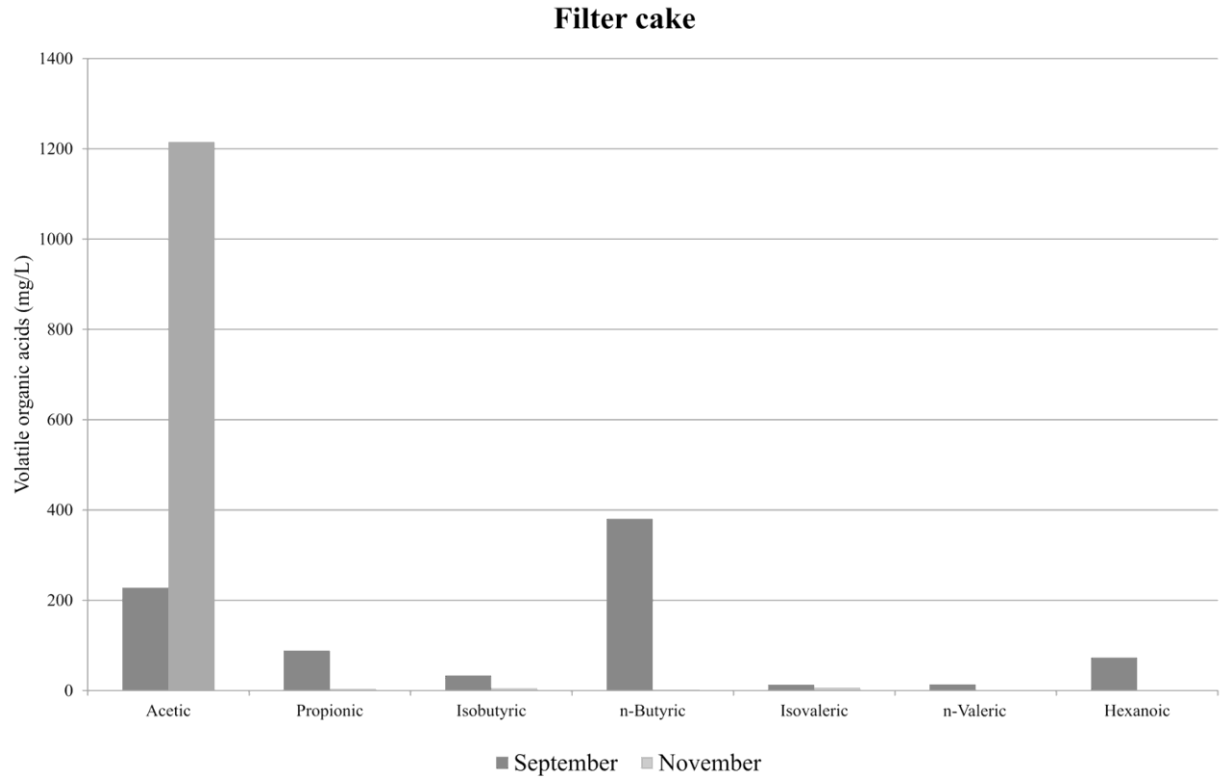

Figure 3. Concentration of VOA in filter cake samples collected along the operating season of a bioethanol industry. The elution of each filter cake sample was analyzed separately by gas chromatography. The columns on the graphic show the VOA concentration levels of September and November samples.

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Table 3. Characteristics of Bagasse Samples Taken during the Operating Season of a Bioethanol Industry

parameter	unit	May	November
TS	%	57.13	45.62
VS	$\%_{TS}$	97.84	96.91
cellulose	%	45.96	43.01
lignin	%	15.60	17.22
hemicellulose	%	30.01	37.32
extractives[a]	%	8.43	2.45
carbohydrates and lignin	%	98.02	97.92
raw protein[b]	%	1.15	1.20
raw fat[c]	%	0.83	0.88
TKN	mg/g	3.15	4.04
aluminum (Al)	mg/g	1.15	5.73
cobalt (Co)	mg/g	0.00	0.00
copper (Cu)	mg/g	0.00	0.02
iron (Fe)	mg/g	1.44	7.29
manganese (Mn)	mg/g	0.05	0.12
molybdenum (Mo)	mg/g	0.00	0.00
nickel (Ni)	mg/g	0.01	0.01
silicon (Si)	mg/g	0.49	29.90
tungsten (W)	mg/g	nd[d]	nd
zinc (Zn)	mg/g	0.02	0.03
calcium (Ca)	mg/g	0.81	3.89
carbon (C)	mg/g	278817.00	189448.00
magnesium (Mg)	mg/g	0.42	1.51
nitrogen (N)	mg/g	3085.50	1870.60
phosphorus (P)	mg/g	0.34	1.11
potassium (K)	mg/g	3.05	8.37
sodium (Na)	mg/g	0.02	nd
sulfur (S)	mg/g	920.04	nd
C/N ratio		90	101

[a]Non-fibrous carbohydrates, such as sugar, fructan, starch, pectins, and organic residues. [b]Raw protein is the sum of all compounds containing nitrogen [c]Raw fat content that dissolves in fat solvents. [d]nd = not detected

for May. The most abundant trace elements filter cake samples were aluminum, iron, and silicon. Their concentrations gradually increased toward the end of the operating season.

The variation in methane potential of filter cake was analyzed in batch tests for September and November sampling months. Figure 2 shows the methane yield of two filter cake samples obtained in two independent batch systems within 31 days. Each batch system consisted of three reactors for filter cake samples, three reactors for cellulose as the control, and three reactors only with inoculum as the blank. The blank reactors for September and November samples had inocula with pH 8.00 and 7.81, TS 4.22 and 6.42%, and VS of TS 63.14 and 59.29%, respectively. The pH values ranged from 7.71 to 8.60 before the experiment and from 7.20 to 7.29 at the end. The accumulated methane production in September and November batch systems was 2.30 and 21.20 mL_N for the blanks and 868.13 and 966.17 mL_N for the controls, respectively.

Samples from November were digested faster without a lag phase in the batch test compared to those from September. There was a 4 day lag phase in the case of samples collected in September. The observed differences were attributed to the bioavailability of easily degradable organic matter in the process and the inoculum activity.

As shown in Figure 3, filter cake in November contained much more acetic acid than in September. On the contrary, other longer chain VOA, such as butyric acid, reached a much higher concentration in September. Available acetate can be converted directly into methane by aceticlastic methanogens, whereas other VOA had to be transformed first into acetate for further degradation.

As for vinasse, the evaluation of influences of micro- and trace elements on the anaerobic digestion was beyond the scope of this investigation.

3.3. Bagasse. In Table 3, the variation in bagasse composition is evaluated for May and November sampling months, boundaries of the operation season. TS and VS values for bagasse were the highest among the three bioethanol waste products. Bagasse had 57.13% TS and 97.84% VS in May. On the contrary, it had much lower TS (45.62%) in November, but the VS was kept nearly constant (96.91%). Such variation mainly relates to the weather and harvesting and milling in the bioethanol process.

Most of the VS content in bagasse was carbohydrate and lignin, 98.02% in May and 97.92% in November, and only minor parts were raw protein (1.15% for May and 1.20% for November) and raw fat (0.83% for May and 0.88% for November). From May to November, cellulose and extractives contents decreased, while lignin and hemicellulose increased. Literature information supports substantial variation of the carbohydrate and lignin contents of bagasse.[11,32] The characterization of carbohydrate and lignin is crucial as a determining factor for the microbial degradability and, consequently, the design and operation of the biogas process.

TKN of bagasse increased from 3.15 mg/g in May to 4.04 mg/g in November. In contrast, total elemental nitrogen decreased. A drop in carbon and nitrogen concentrations from May to November went along with a rising C/N ratio. Elemental sulfur had a high concentration in May but, surprisingly, was not detected in November. The other macro- and trace elements were present in very low concentrations.

Figure 4 shows the methane yield of bagasse for different treatment setups during 33 days of anaerobic digestion. Bagasse samples were collected in August and then subjected to a specific treatment. A batch system was run with three reactors for each sample (raw bagasse, alkaline-treated 10 mm bagasse, and 1 mm bagasse) and triplicates with cellulose as the control and with inoculum only as a blank (the inoculum had pH 8.33, 4.22% TS, and 63.14% VS of TS). The final accumulated methane production was 146.80 mL_N for the blanks and 2033.30 mL_N for the controls.

At the beginning of the batch test, the pH values in the reactors ranged for raw bagasse from 8.26 to 8.32, for 1 mm cut bagasse from 8.15 to 8.29, and for alkaline-treated 10 mm bagasse from 8.38 to 8.43. At the end of the experiment, the pH values ranged for raw bagasse from 7.49 to 7.57, for 1 mm cut bagasse from 7.46 to 7.61, and for alkaline-treated 10 mm bagasse from 7.51 to 7.56. All reactors, thus, were in a pH range considered to be optimal for anaerobic digestion.

The combined alkaline and 10 mm milling treatment achieved the best performance, increasing the methane yield of raw bagasse by 50%. During the initial log phase, from the second to seventh day, more than 62% of the final methane yield was obtained. After that, the methane yield increased steadily.

There are various factors, such as the crystallinity of cellulose, available surface area, and lignin content, that limit the biodegradability of lignocellulosic biomass.[33] Many studies have tested specific treatments to make such substrate more accessible to microorganisms.[11,13,33−36] Our method choice was

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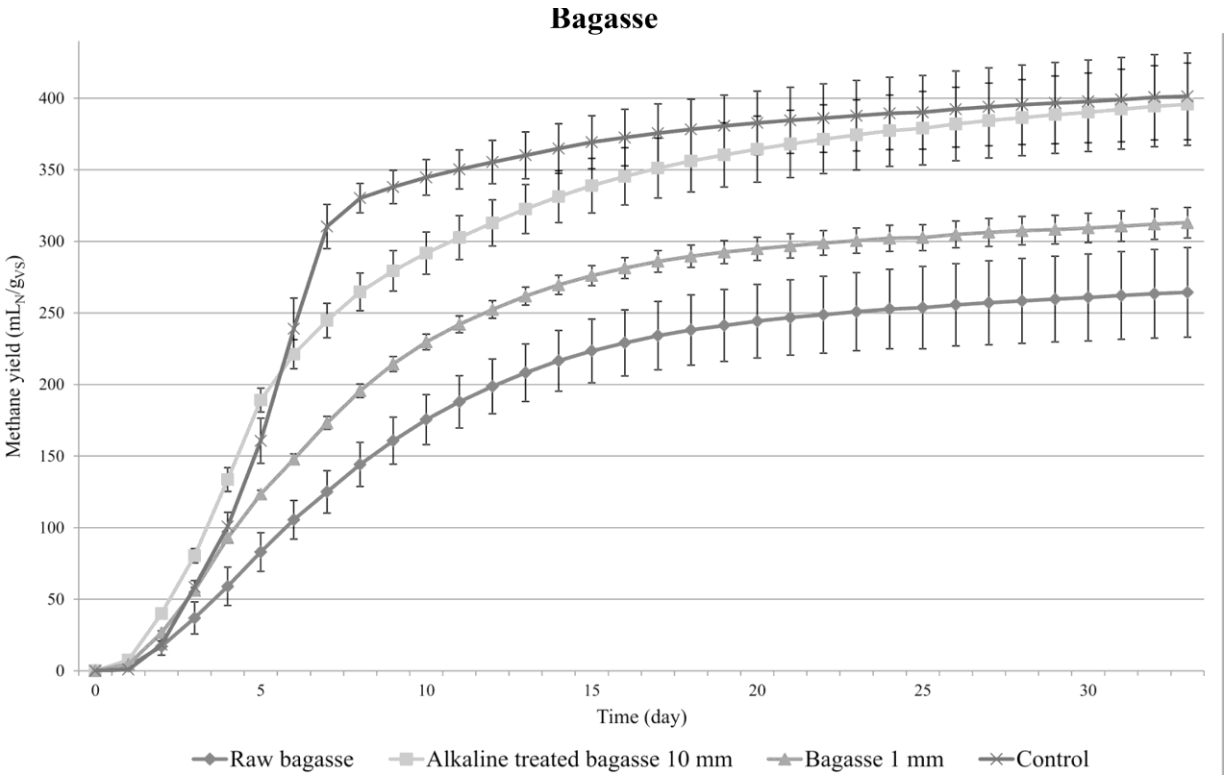

Figure 4. Methane yields from differentially treated bagasse as a function of the incubation time. The samples collected in August were subjected to a milling process to obtain particle sizes of 10 or 1 mm. The 10 mm bagasse was subjected to an alkaline lime treatment. Independent batch systems were run with three reactors for each bagasse configuration and with triplicates for the blank and control, each. The daily average of all methane measurements for each particular bagasse setup is represented on the graph, with the methane production from the blank reactors already subtracted. The bars show the standard deviation.

based on the high methane yield achieved when milling[33] and lime[11] treatment were applied, and the fact that alkaline treatment is economically feasible provided that the residual alkali water is recycled.[34]

4. CONCLUSION

Waste products from the Brazilian bioethanol industry showed satisfactory biogas potential for energy production. The seasonal dynamic changes on the characteristics of bioethanol waste products resulted in methane yields varying by 22% for vinasse and 26% for filter cake. Even more dramatic year to year variations in the substrate composition are expected, which should also be considered. Therefore, repeated analysis of the chemical composition of these substrates is recommended to estimate their expected methane yields as a basis for optimal process operation. It turned out that the methane production from lignocellulose-rich bagasse could be increased more than 50% by milling and alkaline treatment. Co-digestion of the nitrogen-poor bagasse together with nitrogen-rich vinasse is an option for achieving an increased overall efficiency of biogas production. Moreover, the co-digestion of the acidic vinasse together with the alkaline-treated bagasse may solve the pH optimization problem of the process. However, the availability of bagasse needs to be assessed along with the economic and energetic consequences of its use in the biogas process, instead of thermal energy generation. Further investigations should focus on microbiological aspects of the use of bioethanol waste products. In particular, a more systematic investigation of the influences of the elemental balance to avoid limitation or inhibition would be desirable.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Empresa de Pesquisa Energética (EPE). *Análise de Conjuntura dos Biocombustíveis Ano 2013*; EPE, Ministério de Minas e Energia do Brasil: Brasília, Brazil, 2014.

(2) van den Wall Bake, J. D.; Junginger, M.; Faaij, A.; Poot, T.; Walter, A. Explaining the experience curve: Cost reductions of Brazilian ethanol from sugarcane. *Biomass Bioenergy* **2009**, *33* (4), 644−658.

(3) Fuess, L. T.; Garcia, M. L. *Qual o Valor da Vinhaça Mitigação de Impacto Ambiental e Recuperção de Energia por Meio da Digestão Anaeróbia*; UNESP/Cultura Acadêmica/PROGRAD: São Paulo, Brazil, 2013.

(4) Cerqueira-Leite, R. C. *Estudo sobre as Possibilidades e Impactos da Produção de Grandes Quantidades de Etanol Visando à Substituição Parcial*

de Gasolina no Mundo – Fase 1. Relatório Final; Projeto CGEE-Nipe, Unicamp: São Paulo, Brazil, 2005.

(5) Janke, L.; Leite, A.; Wedwitschka, H.; Schmidt, T.; Nikolausz, M.; Stinner, W. Biomethane production integrated to the Brazilian sugarcane industry: The case study of São Paulo state. *Proceedings of the 22nd European Biomass Conference and Exhibition*; Hamburg, Germany, June 23−26, 2014; pp 1295−1299.

(6) de Oliveira, B. G.; Carvalho, J. L. N.; Cerri, C. E. P.; Cerri, C. C.; Feigl, B. J. Soil greenhouse gas fluxes from vinasse application in Brazilian sugarcane areas. *Geoderma* **2013**, *200−201*, 77−84.

(7) Weiland, P. Biogas production: Current state and perspectives. *Appl. Microbiol. Biotechnol.* **2010**, *85* (4), 849−860.

(8) Rouf, M. A.; Bajpai, P. K.; Jotshi, C. K. Optimization of biogas generation from press mud in batch reactor. *Bangladesh J. Sci. Ind. Res.* **2010**, *45* (4), 371−376.

(9) Schaefer, S. H.; Sung, S. Retooling the ethanol industry: Thermophilic anaerobic digestion of thin stillage for methane production and pollution prevention. *Water Environ. Res.* **2008**, *80* (2), 101−108.

(10) Salomon, K. R.; Silva Lora, E. E. Estimate of the electric energy generating potential for different sources of biogas in Brazil. *Biomass Bioenergy* **2009**, *33* (9), 1101−1107.

(11) Rabelo, S. C.; Carrere, H.; Maciel Filho, R.; Costa, A. C. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. *Bioresour. Technol.* **2011**, *102* (17), 7887−7895.

(12) Agrawal, K. M.; Barve, B. R.; Khan, S. S. Biogas from pressmud. *J. Mech. Civ. Eng.* **2010**, 37−41.

(13) Badshah, M.; Lam, D. M.; Liu, J.; Mattiasson, B. Use of an automatic methane potential test system for evaluating the biomethane potential of sugarcane bagasse after different treatments. *Bioresour. Technol.* **2012**, *114*, 262−269.

(14) Kandel, T. P.; Sutaryo, S.; Møller, H. B.; Jorgensen, U.; Laerke, P. E. Chemical composition and methane yield of reed canary grass as influenced by harvesting time and harvest frequency. *Bioresour. Technol.* **2013**, *130*, 659−666.

(15) Bruni, E.; Jensen, A. P.; Pedersen, E. S.; Angelidaki, I. Anaerobic digestion of maize focusing on variety, harvest time and pretreatment. *Appl. Energy* **2010**, *87* (7), 2212−2217.

(16) União da Agroindústria Canavieira do Estado de São Paulo (UNICA). *Sugar Cane's Energy – Twelve Studies on Brasilian Sugar Cane Agribusiness and Its Sustainability*; Berlendis and Vertecchia, UNICA: São Paulo, Brazil, 2007.

(17) Wilkie, A. C.; Riedesel, K. J.; Owens, J. M. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass Bioenergy* **2000**, *19* (2), 63−102.

(18) Deutsches Institut für Normung e.V. (DIN). *Charakterisierung von Schlämmen – Bestimmung des Trockenrückstandes und des Wassergehaltes*; DIN: Berlin, Germany, 2001.

(19) Deutsches Institut für Normung e.V. (DIN). *Charakterisierung von Schlämmen – Bestimmung des Glühverlustes der Trockenmasse*; DIN: Berlin, Germany, 2001.

(20) Naumann, C.; Bassler, R. *VDLUFA Methodenbuch: Die Chemische Untersuchung von Futtermitteln*; VDLUFA-Verlag: Darmstadt, Germany, 2006.

(21) VanSoest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, 3583−3597.

(22) Drosg, B. *Process Monitoring in Biogas Plants*; IEA Bioenergy: Paris, France, 2013.

(23) Stams, A.; Plugge, C.; Bok, F.; van Houten, B.; Lens, P.; Dijkman, H.; Weijma, J. Metabolic interactions in methanogenic and sulfate-reducing bioreactors. *Water Sci. Technol.* **2005**, *52* (1−2), 13−20.

(24) Ahammad, S. Z.; Davenport, R. J.; Read, L. F.; Gomes, J.; Sreekrishnan, T. R.; Dolfing, J. Rational immobilization of methanogens in high cell density bioreactors. *RSC Adv.* **2013**, *3* (3), 774.

(25) Lauterbock, B.; Nikolausz, M.; Lv, Z.; Baumgartner, M.; Liebhard, G.; Fuchs, W. Improvement of anaerobic digestion performance by continuous nitrogen removal with a membrane contactor treating a substrate rich in ammonia and sulfide. *Bioresour. Technol.* **2014**, *158*, 209−216.

(26) Qiang, H.; Lang, D. L.; Li, Y. Y. High-solid mesophilic methane fermentation of food waste with an emphasis on iron, cobalt, and nickel requirements. *Bioresour. Technol.* **2012**, *103* (1), 21−27.

(27) Demirel, B.; Scherer, P. Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. *Biomass Bioenergy* **2011**, *35* (3), 992−998.

(28) Schattauer, A.; Abdoun, E.; Weiland, P.; Plöchl, M.; Heiermann, M. Abundance of trace elements in demonstration biogas plants. *Biosyst. Eng.* **2011**, *108* (1), 57−65.

(29) Fachagentur Nachwachsende Rohstoffe e.V. (FNR). *Guide to Biogas – From Production to Use*; FNR: Gülzow, Germany, 2012.

(30) Radjaram, B.; Saravanane, R. Assessment of optimum dilution ratio for biohydrogen production by anaerobic co-digestion of press mud with sewage and water. *Bioresour. Technol.* **2011**, *102* (3), 2773−2780.

(31) Meunchang, S.; Panichsakpatana, S.; Weaver, R. W. Co-composting of filter cake and bagasse; by-products from a sugar mill. *Bioresour. Technol.* **2005**, *96* (4), 437−442.

(32) Aguiar, M. M.; Ferreira, L. F. R.; Monteiro, R. T. R. Use of vinasse and sugarcane bagasse for the production of enzymes by lignocellulolytic fungi. *Braz. Arch. Biol. Technol.* **2010**, *53* (5), 1245−1254.

(33) Hendriks, A. T.; Zeeman, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **2009**, *100* (1), 10−18.

(34) Pavlostathis, S. G.; Gossett, J. M. Alkaline treatment of wheat straw for increasing anaerobic biodegradability. *Biotechnol. Bioeng.* **1985**, *27*, 334−344.

(35) Taherzadeh, M. J.; Karimi, K. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *Int. J. Mol. Sci.* **2008**, *9* (9), 1621−1651.

(36) Carrier, M.; Neomagus, H. W.; Görgens, J.; Knoetze, J. H. Influence of chemical pretreatment on the internal structure and reactivity of pyrolysis chars produced from sugar cane bagasse. *Energy Fuels* **2012**, *26* (7), 4497−4506.

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2.2. Biogas production from sugarcane waste: Assessment on kinetic challenges for process designing

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Article

Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing

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Abstract: Biogas production from sugarcane waste has large potential for energy generation, however, to enable the optimization of the anaerobic digestion (AD) process each substrate characteristic should be carefully evaluated. In this study, the kinetic challenges for biogas production from different types of sugarcane waste were assessed. Samples of vinasse, filter cake, bagasse, and straw were analyzed in terms of total and volatile solids, chemical oxygen demand, macronutrients, trace elements, and nutritional value. Biochemical methane potential assays were performed to evaluate the energy potential of the substrates according to different types of sugarcane plants. Methane yields varied considerably (5–181 $Nm^3 \cdot ton_{FM}^{-1}$), mainly due to the different substrate characteristics and sugar and/or ethanol production processes. Therefore, for the optimization of AD on a large-scale, continuous stirred-tank reactor with long hydraulic retention times (>35 days) should be used for biogas production from bagasse and straw, coupled with pre-treatment process to enhance the degradation of the fibrous carbohydrates. Biomass immobilization systems are recommended in case vinasse is used

as substrate, due to its low solid content, while filter cake could complement the biogas production from vinasse during the sugarcane offseason, providing a higher utilization of the biogas system during the entire year.

Keywords: sugarcane waste; nutritional requirements; methane potential; degradation rates; process designing

1. Introduction

Although the combined production of sugar and ethanol based on sugarcane is recognized as one of the most efficient systems for biofuels production (yield per hectare) [1], the Brazilian sugarcane industry is responsible for the generation of different types of organic waste which, in most cases, are still not being properly treated, especially from the energy point of view [2]. Based on the amount of sugarcane processed during the 2013–2014 season (653×10^6 tons of cane) [3], the generation of 91×10^6 tons of straw (dry basis), 169×10^6 tons of bagasse (wet basis), 22×10^6 tons of filter cake (wet basis), and $286–678 \times 10^6$ m^3 of vinasse are estimated.

While some of these organic wastes are directly applied as organic fertilizers on the sugarcane fields for nutrient recycling without previous energetic utilization (*i.e.*, vinasse and filter cake), the other part of the residues are mostly used as fuel in low-efficiency cogeneration systems (*i.e.*, bagasse) or even left to decay on the fields (*i.e.*, straw) due to a lack of incentives to produce bioenergy from them [4–6]. Non-controlled digestion of such waste on the fields may lead to the release of large amounts of methane, which may hinder the positive effect of bioenergy utilization on the climate change mitigation.

The State of São Paulo, a major sugarcane producer in Brazil, is responsible for more than 56% of the sugarcane processed in the 2013–2014 season. The state is committed to an ambitious plan to further increase the share of renewable energy from 55% to 69% until 2020, as well as to reduce the CO_2 emissions by 20% in comparison to 2005 levels. To achieve these targets, among other measures, a biogas program was launched in 2012 to stimulate and increase the sustainable use of biomass for biogas production, including a future mandatory share of biomethane into the natural gas grid [7–9].

The anaerobic digestion (AD) of sugarcane waste can be considered a promising strategy, since the digestate could still be used to partially replace the mineral fertilizers on the sugarcane fields and the produced biogas could be upgraded to biomethane and sold as a new energy product by the sugarcane plants [10–12]. However, before being implemented on a large-scale, the AD process should be carefully evaluated, especially regarding the substrates' characteristics, as organic matter and nutritional value, macronutrients, trace elements, and specific biogas production. Those parameters directly influence some other important process parameters, such as the pH, accumulation of inhibitors, potential macronutrients, and trace elements deficiencies, as well degradation rates.

Several authors [10,13,14] suggested that for a proper AD process a balance among the main nutrients: carbon, nitrogen, phosphorous and sulfur is necessary. If a certain substrate has a too high C:N ratio, or in another words, has nitrogen deficiency, it may negatively influence the microbial community functioning. That means a direct influence on the ability to produce enzymes that are needed to the carbon utilization, causing an incomplete conversion of the carbon contained in the

substrates, resulting in lower methane yields. On the other hand, substrates that contain high levels of nitrogen can cause inhibition to the AD process via accumulation of toxic ammonia (NH_3) produced from protein degradation or by urea conversion [15].

According to Britz *et al.* [16] and Scherer *et al.* [17], phosphorus and sulfur are also considered essential nutrients for the AD process. While sulfur is an important constituent of amino acids, phosphorus is needed for microbial growth during the formation of energy carriers ATP (adenosine triphosphate) and NADP (nicotinamine adenine dinocleotide phosphate), an important constituent of nucleic acids, as well as it plays an important role in the maintenance of an optimum pH. However, when sulfur is found in high concentrations, sulfates are reduced to sulfide by the so-called sulfate-reducing bacteria, leading to two possible inhibitions. On the one hand, due to thermodynamic advantages sulfate-reducing bacteria outcompetes methanogens for hydrogen and acetate [18,19]. On the other hand, hydrogen sulfide (H_2S), an end product of sulfate reduction, has a toxic effect on various groups of bacteria [20]. In addition to that, when found in high concentrations, H_2S can cause corrosion to some biogas plant parts, such as the combined heat and power units (CHP), biogas upgrading systems, and metal pipes and tanks, leading to high costs of maintenance [21].

The importance of trace elements during AD is relatively well known, especially for important enzymes and cofactors involved in different steps of the process, where special metal ions are required. Several authors [22,23] reported higher gas yields, improvements on process stability and reaching higher organic loading rates (OLR) through the supplementation of cobalt, copper, iron, molybdenum, nickel, selenium, tungsten, and zinc. While animal waste (e.g., bovine and swine manure) are expected to require less amendments, AD of energy crops, plant residues, and agro-industrial waste faces more trace element deficiencies. Therefore, it is expected that the substrate composition plays a major role for the trace elements requirements during the AD process, being an important parameter to be considered during the development of stable process with novel substrates. Additional aspects also need to be taken into account, especially regarding the trace elements' availability for microbial uptake, mainly driven by metal speciation, pH and process temperature, applied organic loading rate, as well as the chemical processes of precipitation and complexation [23–25].

Furthermore, another aspect that should be taken into account when a biogas concept based on agroindustry waste is under development, is the seasonality of the crops and the feasibility of minimizing the negative effects by conserving and storing part of the substrates to be used during the offseason period. Therefore, allowing a higher utilization of the biogas system during the entire year.

To provide guidance during the designing of an AD system based on sugarcane waste, the present research performed an extensive evaluation of vinasse, filter cake, bagasse, and straw generated by plants with different production systems in the Center-South Region of Brazil, including autonomous plants, where ethanol is produced exclusively from sugarcane juice, and annexed plants, where ethanol is produced by a mixture of sugarcane juice and/or molasses (*i.e.*, waste from sugar production).

Through the analysis of several parameters, as total solids (TS), volatile solids (VS), macronutrients, trace elements, nutritional value, and biochemical methane potential (BMP). It was possible to assess the main kinetic challenges of the sugarcane waste, in terms of nutritional imbalances, energy potential, degradation rates, and proper hydraulic retention time (HRT) for the substrates' conversion in a continuous stirred-tank reactor (CSTR) system. Moreover, in order to

demonstrate how the sugarcane seasonality could be overcome, the energy synergies among the substrates were demonstrated based on the two different types of sugarcane plants.

2. Results and Discussion

2.1. Physical-Chemical Composition

2.1.1. Basic Characteristics

The basic characteristics of the sugarcane waste types are presented in Table 1. Considerable differences among vinasse samples derived from sugar and/or ethanol production were found. Samples from the autonomous plant presented lower values of organic matter content in terms of COD, TS, and VS (22.1 $g \cdot L^{-1}$, 1.15% and 76.0% of TS), while samples from the annexed plants presented higher values for the same parameters (32.4 $g \cdot L^{-1}$, 3.44% and 70.6% of TS). That fact is explained, among other reasons, by the use of different feedstocks (molasses and/or sugarcane juice) during the ethanol production in these two different types of sugarcane plants. Similar values were also found previously [4] in an extensive survey about water uses in the sugarcane industry.

Filter cake presented intermediate values for TS and VS (28.9% and 74.2% of TS) in comparison to vinasse, bagasse, and straw, having an appearance similar to sludge, once it is derived from a physical-chemical treatment process that removes soluble and insoluble impurities from the sugarcane juice. Similar values were also reported during co-digestion experiments for bio-hydrogen production [26].

Table 1. Main characteristics of the sugarcane waste.

Parameters	Units	Vinasse				Filter Cake		Bagasse		Straw		Recommendation	
		Autonomous (*n* = 6)		Annexed (*n* = 15)		(*n* = 9)		(*n* = 9)		(*n* = 12)			
		AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	[13]	[14]
TS [a]	% FM [d]	1.15	±0.12	3.44	±1.11	28.9	±3.77	55.4	±4.19	76.7	±21.6	-	-
VS [b]	% TS	76.0	±6.99	70.6	±3.84	74.2	±10.8	96.0	±2.70	86.3	±11.9	-	-
COD [c]	$g \cdot L^{-1}$	22.1	±0.46	32.4	±10.0	-	-	-	-	-	-	-	-
C	% TS	37.0	±4.24	39.0	±8.61	42.7	±6.95	47.6	±2.69	43.4	±4.78	C:N	-
N	% TS	2.94	±0.35	2.31	±0.35	1.76	±0.24	0.41	±0.04	0.52	±0.21	20–40:1	-
P	% TS	0.16	±0.05	0.35	±0.12	0.60	±0.25	0.04	±0.02	0.06	±0.03	C:N:P:S	-
S	% TS	0.87	±0.49	2.12	±0.27	0.18	±0.02	0.05	±0.03	0.21	±0.06	600:15:5:3	-
Ca	$mg \cdot L^{-1}$	77.4	±24.3	655	±211	4139	±1667	704	±215	2981	±1656	-	100–200
Na	$mg \cdot L^{-1}$	16.4	±9.39	24.5	±9.58	7.75	±5.98	11.3	±8.83	37.1	±25.1	-	100–200
K	$mg \cdot L^{-1}$	1306	±708	6021	±565	740	±280	1651	±1036	5002	±2344	-	200–400
Mg	$mg \cdot L^{-1}$	173.6	±72.4	771	±177	971	±259	409	±173	1140	±404	-	75–150

[a] total solids; [b] volatile solids; [c] chemical oxygen demand; [d] fresh matter; *n* = number of repetitions (each sample was analyzed in triplicate); AV = average values; SD = standard deviation.

Bagasse and straw presented relatively similar average TS and VS values (55.4% and 96.0% of TS for bagasse; 76.7% and 86.3% of TS for straw), due the fact of being mainly formed by sugarcane fibers. The only exception was one sample of straw that presented different values of TS and VS

(92.4% and 68.6% of TS), possibly due to the inorganic soil particles attached to its fibers during harvesting.

2.1.2. Macronutrients

The macro element contents of the sugarcane waste types are also presented in Table 1. The carbon to nitrogen ratio (C:N) of vinasse samples derived from the autonomous plant presented slightly lower average value (12:1) in comparison to the samples from the annexed plants (16:1). For both cases the values found are lower than the optimal value recommended for AD (20–40:1) [13], and could lead, in extreme cases, to process inhibition if the surplus nitrogen is converted into ammonia.

Samples of filter cake presented an average C:N ratio considered proper for AD (24:1), meanwhile bagasse and straw presented different C:N ratio profiles of 116:1 and 83:1, respectively. From the carbon and nitrogen content, a lack of nitrogen in bagasse and straw is clearly noted. However, it is noteworthy that not all of the carbon content in these lignocellulosic substrates will be bioavailable because of the high content of non-degradable lignin.

When the other macronutrients are also considered, the high sulfur content in both types of vinasse was around four, up to 10, times higher than what would be considered an optimum concentration [13]. Such high sulfur content can cause several undesirable effects as the competition of sulfate-reduction with methanogenesis, reducing the conversion of organic acids into biogas, besides also negatively influencing the bioavailability of trace elements inside the bioreactors [23,24,27].

For the other sugarcane waste, sulfur and phosphorus contents were rather low. Scherer *et al.* [17] observations demonstrated that the addition of phosphate and sulphate increased considerably the degradation rate and biogas production from fodder beet silage as a mono-substrate, supporting the role of P and S as limiting macro nutrients for certain AD systems.

The development of a co-digestion strategy to balance the macronutrients of the sugarcane waste apparently could make sense from the economic point of view, once it could partially replace the addition of high cost chemicals as, for example, urea that usually would be used to balance the C:N ratio during AD of bagasse and straw. However, the co-digestion of vinasse with other lignocellulosic waste can also provide undesirable effects as increasing the production of H_2S that could lead to the necessity of costly biogas desulfurization.

Moreover, another aspect that should be taken into account is the degradation rates of the substrates (see Subsection 2.2.2 and 2.2.3). The eventually co-digestion of waste with different degradation profiles could lead to a lower biomass conversion if the HRT is too short compared to the other co-substrate, or lead to an unnecessary increase in the reactor size if the HRT is based on the substrate with the slowest degradation rate.

2.1.3. Trace Elements

A lack of some important trace elements can be seen from the composition of autonomous- and annexed-derived vinasse (Table 2); especially nickel is critical. Meanwhile, the concentrations of other trace elements, as tungsten, manganese, selenium, zinc, cobalt, molybdenum, and copper, are close to the lowest limit recommended by Kayhanian *et al.* [22] and Oechsner *et al.* [28].

Table 2. Trace elements content of the sugarcane waste.

Parameters	Units	Vinasse				Filter Cake		Bagasse		Straw		Recommendation	
		Autonomous (*n* = 6)		Annexed (*n* = 15)		(*n* = 9)		(*n* = 9)		(*n* = 12)			
		AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	[22]	[28]
Fe	$mg \cdot kg_{TS}^{-1}$	200	±177	488	±142	27,267	±24,625	2012	±1530	14,949	±23,435	100–5000	750–5000
Ni	$mg \cdot kg_{TS}^{-1}$	0.49	±0.03	2.30	±0.92	14.3	±5.81	4.04	±3.17	7.17	±5.22	5–20	4–30
Co	$mg \cdot kg_{TS}^{-1}$	0.55	±0.02	0.62	±0.46	3.36	±1.34	0.52	±0.25	2.87	±3.73	<1–5	0.4–10
Mo	$mg \cdot kg_{TS}^{-1}$	0.48	±0.01	0.84	±0.20	1.03	±0.74	0.58	±0.37	0.71	±0.25	<1–5	0.05–16
W	$mg \cdot kg_{TS}^{-1}$	Nd	-	0.08	±0.04	0.29	±0.50	0.19	±0.05	0.24	±0.05	<1	0.1–30
Mn	$mg \cdot kg_{TS}^{-1}$	59.6	±5.94	194	±49.2	566	±188	43.4	±11.1	177	±85.1	-	100–1500
Cu	$mg \cdot kg_{TS}^{-1}$	3.62	±0.14	7.96	±4.03	43.8	±4.04	4.82	±1.93	10.7	±12.7	-	10–80
Se	$mg \cdot kg_{TS}^{-1}$	Nd	-	0.08	±0.06	0.01	±0.02	0.83	±0.12	0.19	±0.24	-	0.05–4
Zn	$mg \cdot kg_{TS}^{-1}$	36.8	±10.5	32.6	±4.45	132	±7.21	17.2	±10.1	10.1	±14.9	-	30–400

From the filter cake composition, less trace elements were observed below the recommendation values (*i.e.*, tungsten and selenium) in comparison to vinasse, as well as elements close to the lowest limit (*i.e.*, molybdenum). This occurs, probably, because at the industrial phase where filter cake is generated, metal based coagulants and flocculants are added during the physical-chemical process that removes impurities from sugarcane juice.

Trace element deficiencies were also found for bagasse and straw, as manganese, copper, zinc, tungsten, and selenium, as well as nickel, were found in a concentration close to the lowest recommended limit.

2.1.4. Nutritional Values

Carbohydrates were the main constituents found in all analyzed samples, though differing considerably into its components (Table 3). Filter cake, bagasse, and straw presented higher average lignin content, 116, 124, and 162 $g \cdot kg_{TS}^{-1}$, respectively, while samples of vinasse had much lower values, 34.3 up to 56.5 $g \cdot kg_{TS}^{-1}$. According to previous studies [29–32] the presence of lignin in certain substrates can negatively influence the microbial access to cellulose and hemi-cellulose during the AD process. Such type of substrate is frequently submitted to physical, chemical or biological pre-treatment procedures to accelerate and enhance degradation in large-scale biogas plants.

Non-fiber carbohydrates (NFC), which are easy accessible to microbial degradation, were found in different concentrations among two of the lignocellulosic waste types (*i.e.*, filter cake and bagasse). The high variation found in bagasse (181 ± 216 $g \cdot kg_{TS}^{-1}$) is explained by different milling efficiencies during the extraction of the juice at the sugarcane plants, whereas the differences found among the three analyzed samples of filter cake (118 ± 136 $g \cdot kg_{TS}^{-1}$) can be explained by the use of different technologies (*i.e.*, rotary vacuum-drum filter or filter press) to recover sucrose during the process of juice treatment.

Table 3. Nutritional values of the sugarcane waste.

Parameters		Units	Vinasse				Filter Cake		Bagasse		Straw	
			Autonomous (*n* = 6)		Annexed (*n* = 6)		(*n* = 9)		(*n* = 6)		(*n* = 9)	
			AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
Raw protein	-	$g \cdot kg_{TS}^{-1}$	137	±5.17	159	±11.5	124	±12.8	17.9	±3.89	27.7	±8.36
Raw fat	-	$g \cdot kg_{TS}^{-1}$	0.72	±0.85	0.12	±0.10	39.5	±5.11	7.20	±3.27	9.18	±1.55
Carbohydrate	NFC [a]	$g \cdot kg_{TS}^{-1}$	372	±44.7	263	±69.9	118	±136	181	±216	107	±41.2
	Cellulose	$g \cdot kg_{TS}^{-1}$	34.9	±11.7	60.9	±13.8	126	±19.2	357	±96.1	311	±88.1
	Hemi-cellulose	$g \cdot kg_{TS}^{-1}$	101	±27.5	243	±176	164	±16.3	233	±93.1	227	±14.5
Lignin	-	$g \cdot kg_{TS}^{-1}$	56.5	±15.5	34.3	±3.03	116	±27.1	124	±38.2	162	±26.2
Raw ash	-	$g \cdot kg_{TS}^{-1}$	296	±82.0	238	±101	309	±125	78.3	±10.1	154	±120
TKN [b]	-	$g \cdot kg_{TS}^{-1}$	22.2	±0.78	49.6	±32.1	20.0	±2.09	2.90	±0.65	4.48	±1.36

[a] non-fiber carbohydrates; [b] Total Kjeldahl nitrogen.

2.2. Biochemical Methane Potential

2.2.1. Methane Yields

The results of BMP assays are presented in Figure 1 and Table 4. It can be observed that vinasse from the autonomous plant (V-1) achieved the lowest methane yield after 35 days (246 ± 15 $NmL \cdot g_{COD}^{-1}$), in comparison to the two samples from the annexed plants (273 ± 02 and 302 ± 06 $NmL \cdot g_{COD}^{-1}$). However, when these results are given in methane production per fresh matter, the differences among samples of autonomous and annexed plants are even higher. Sample V-1 achieved the methane yield of 5 $Nm^3 \cdot ton_{FM}^{-1}$, while samples V-2 and V-3 achieved higher values, 7 and 11 $Nm^3 \cdot ton_{FM}^{-1}$, respectively. The reason for such differences is due to the lower COD content found in the sample from the autonomous plant (23.1 $g \cdot L^{-1}$) in comparison to the samples from the annexed plants (24.9–39.5 $g \cdot L^{-1}$).

The methane yield achieved by the three filter cake samples did not present any major difference in terms of final methane yield, varying between 245–281 $NmL \cdot g_{VS}^{-1}$. When the results are converted to methane production per fresh matter (50 up to 58 $Nm^3 \cdot ton_{FM}^{-1}$), it becomes clear that filter cake has a higher energy content than vinasse from the autonomous plant (around 10 times higher), or even from the annexed plant (around 7 times higher).

However, in comparison to vinasse it seems that the higher lignin content of filter cake could have hampered the utilization of the cellulose and hemi-cellulose during the biogas conversion. This fact is even clearer in sample (FC-3), where the lower NFC content also influenced the lag phase during its degradation.

The methane yields obtained from two different samples of bagasse varied considerably between 236–326 $NmL \cdot g_{VS}^{-1}$ (119 to 181 $Nm^3 \cdot ton_{FM}^{-1}$), possibly due to different NFC concentrations found in these samples (181 ± 216 $g \cdot kg^{-1}$ TS). In a different way, samples of straw did not vary considerably into its nutritional content, however presented slightly different methane yields from 199 to 234 $NmL \cdot g_{VS}^{-1}$ (101 to 126 $Nm^3 \cdot ton_{FM}^{-1}$). Nevertheless, submitting the straw to a physical pre-treatment by grinding to 2 mm, resulted in 26% higher methane yield.

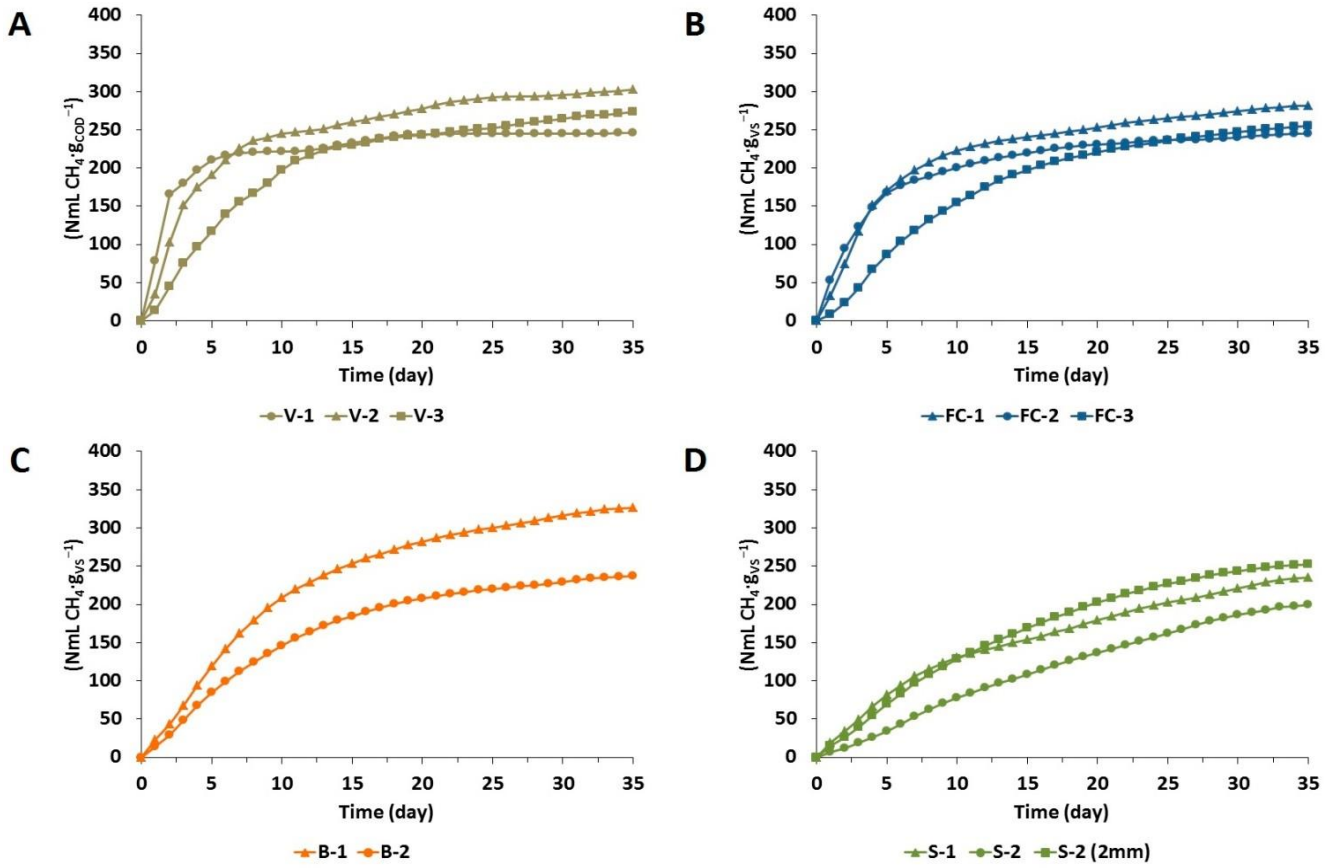

Figure 1. Cumulative methane yield of the sugarcane waste during 35 days of BMP assays. (A) vinasse from autonomous (V-1) and annexed plants (V-2 and V-3); (**B**) filter cake (FC-1, FC-2 and FC-3); (**C**) bagasse (B-1 and B-2); (**D**) straw (S-1 and S-2), and S-2 ground to 2 mm.

Therefore, the theory that smaller particle sizes increases the specific surface area of the biomass, providing greater possibility for enzymatic attack [33] is reinforced. Thus, if the straw would have undergone a more intensive physical pre-treatment, it could have produced higher biogas yields.

Table 4. Biochemical methane potential of the sugarcane waste after 35 days of assay.

Substrates	Samples	Methane Yield * [NmL·g_{VS}^{-1} or NmL·g_{COD}^{-1}]	Methane Yield [$Nm^3 \cdot ton_{FM}^{-1}$]	*K* [day^{-1}]
Straw	S-1	234 ± 03	101 ± 01	0.091
	S-2	199 ± 23	126 ± 15	0.075
	S-2 (2mm)	252 ± 02	160 ± 01	0.102
Bagasse	B-1	326 ± 04	181 ± 02	0.119
	B-2	236 ± 05	119 ± 02	0.124
Filter cake	FC-1	281 ± 04	58 ± 01	0.159
	FC-2	245 ± 01	50 ± 01	0.178
	FC-3	254 ± 08	54 ± 02	0.091
Vinasse	V-1	246 ± 15	05 ± 01	0.413
	V-2	302 ± 06	08 ± 01	0.209
	V-3	273 ± 02	11 ± 01	0.107

***** Methane yield of vinasse is given in NmL·g_{COD}^{-1}.

2.2.2. Reaction Rates

The decay constant (*k*-value) calculated with Equation (1) (see Subsection 3.3) is presented in Table 4. Samples of vinasse achieved the highest *k*-value (0.107–0.413), mainly by the lower lignin and higher NFC content found in this material. Interestingly, the sample from the autonomous plant (V-1) presented a higher *k*-value (0.413) than samples from the annexed plants. Vinasse from the annexed plant that produces ethanol from a mix of sugarcane juice and molasses (V-2) presented an intermediate *k*-value (0.209), while vinasse from the annexed plant that produces ethanol exclusively from molasses presented a lower *k*-value (0.107). According to Wilkie *et al.* [34] the concentration of sugars in molasses, through crystalinization and evaporation of cane juice, increases the content of more recalcitrant organics which remain in the vinasse after fermentation. This fact supports the presented results, since vinasse from molasses-based ethanol resulted in longer lag phases in comparison to vinasse from sugarcane juice-based ethanol.

Samples of filter cake achieved intermediate *k*-values (0.091–0.159), however, as well as for vinasse, one of the samples achieved a completely different performance. In that case, sample FC-3 was from a sugarcane plant that applies a different strategy during the juice treatment, where small pieces of bagasse are intentionally added to the filter cake to increase its permeability, enhancing the recovery of residual sucrose at the rotary vacuum-drum filter. Therefore, the longer lag phase can be explained by the lower NFC content found in this sample.

Although the samples of bagasse presented considerably different methane yields, both displayed similar *k*-values (0.119–0.124). In the meantime, the two different straw samples presented the slowest reaction rates (0.075–0.091) of all analyzed materials, even always presenting similar values of TS, VS, and nutritional values in comparison to bagasse. Fact that explained by the longer length of its fibers (±6 cm) in comparison to bagasse (±3 cm), once when the sample of straw (S-2) was submitted to a mechanical pre-treatment, the *k*-value increased from 0.075 to 0.102.

2.2.3. Substrate Conversion in a CSTR System

The substrate conversion in a hypothetical CSTR system was simulated with Equation (2) and data presented in Table 5. Depending on the feedstock used for ethanol production, the HRT needed to achieve 80% of the vinasse degradation would vary considerably (10–40 days). In this case, the vinasse derived from an autonomous plant would demand a shorter HRT. On the other hand, the vinasse derived from an annexed plant that was using the entire sugarcane juice for sugar production at the time of sampling—that is, only molasses mixed with water was used for ethanol production—would demand a longer HRT. However, for the Brazilian conditions, where, in most cases, the ethanol is produced by a mix of molasses and juice, the most likely scenario would be the values found for vinasse (V-2).

In this way, due to economic reasons, one of the main concerns during the choice of an AD system is to reach the expected methane yield with maximum organic loading rate (OLR) and, consequently, minimum hydraulic retention time (HRT) that can be applied under stable process conditions.

Considering the low TS content of vinasse and the absence of clogging materials, it seems to be clear that the utilization of a biomass immobilization system would be the most suitable for this substrate, once it would permit a lower HRT in combination with higher solid retention times (SRT),

considerably reducing the size of the reactors in comparison to the conventional continuous stirred-tank reactor (CSTR) system.

Therefore, the AD using vinasse as a substrate for biogas production should focus on these types of reactors, such as fixed bed reactors, fluidized bed reactors or granular sludge systems, especially the upflow anaerobic sludge blanket (UASB) reactor, as this system is already being successfully used in Brazil for the treatment of different types of wastewater. Thus, the process of technology scaling up could be facilitated, once the basic reactor concept and operation is already widespread in the region [35].

The results from simulating the filter cake samples also presented a wide range of HRT (25–45 days). This is justified since one of the samples (FC-3) has presented a longer lag phase and, consequently, lower *k*-value. The two bagasse samples presented the same conversion profile in general, demanding longer HRT in comparison to vinasse and filter cake, in a similar way to straw, that would need between 45–55 days to achieve 80% of its degradation. Nevertheless, the effect of a physical pre-treatment could increase the methane yield, or alternatively reduce the HRT from 55 to 40 days as demonstrated in the straw S-2 (2 mm).

Table 5. Simulation of the hypothetical substrates conversion using CSTR system.

HRT	Vinasse			Filter Cake			Bagasse		Straw		
(Days)	V-1	V-2	V-3	FC-1	FC-2	FC-3	B-1	B-2	S-1	S-2	S-2 (2 mm)
5	0.67	0.51	0.35	0.45	0.47	0.31	0.37	0.38	0.31	0.27	0.34
10	**0.80**	0.68	0.52	0.62	0.64	0.48	0.54	0.55	0.48	0.43	0.50
15	0.86	0.76	0.62	0.71	0.73	0.58	0.64	0.65	0.58	0.53	0.60
20	0.89	**0.81**	0.68	0.77	0.78	0.65	0.70	0.71	0.64	0.60	0.67
25	0.91	0.84	0.73	**0.80**	**0.82**	0.70	0.75	0.76	0.69	0.65	0.72
30	0.93	0.86	0.76	0.83	0.84	0.73	0.78	0.79	0.73	0.69	0.75
35	0.94	0.88	0.79	0.85	0.86	0.76	**0.81**	**0.81**	0.78	0.72	0.78
40	0.94	0.89	**0.81**	0.87	0.88	0.79	0.83	0.83	0.78	0.75	**0.80**
45	0.95	0.90	0.83	0.88	0.89	**0.80**	0.84	0.85	**0.80**	0.77	0.82
50	0.95	0.91	0.84	0.89	0.90	0.82	0.86	0.86	0.82	0.79	0.84
55	0.96	0.92	0.86	0.90	0.91	0.83	0.87	0.87	0.83	**0.80**	0.85
60	0.96	0.93	0.87	0.91	0.91	0.85	0.88	0.88	0.84	0.82	0.86
65	0.96	0.93	0.87	0.91	0.92	0.86	0.89	0.89	0.85	0.83	0.87
70	0.97	0.94	0.88	0.92	0.93	0.86	0.89	0.90	0.86	0.84	0.88

HRT = hydraulic retention time; The results highlighted in bold correspond to approximately 80% of the substrate conversion at different HRT.

2.3. Process Design

2.3.1. Energy Potential

BMP results combined with specific waste generation from two different sugarcane plants (Figure 2A) were used to assess the energy potential in a hypothetical plant with a capacity to process 4 million tons of cane (TC) per year (Figure 2B).

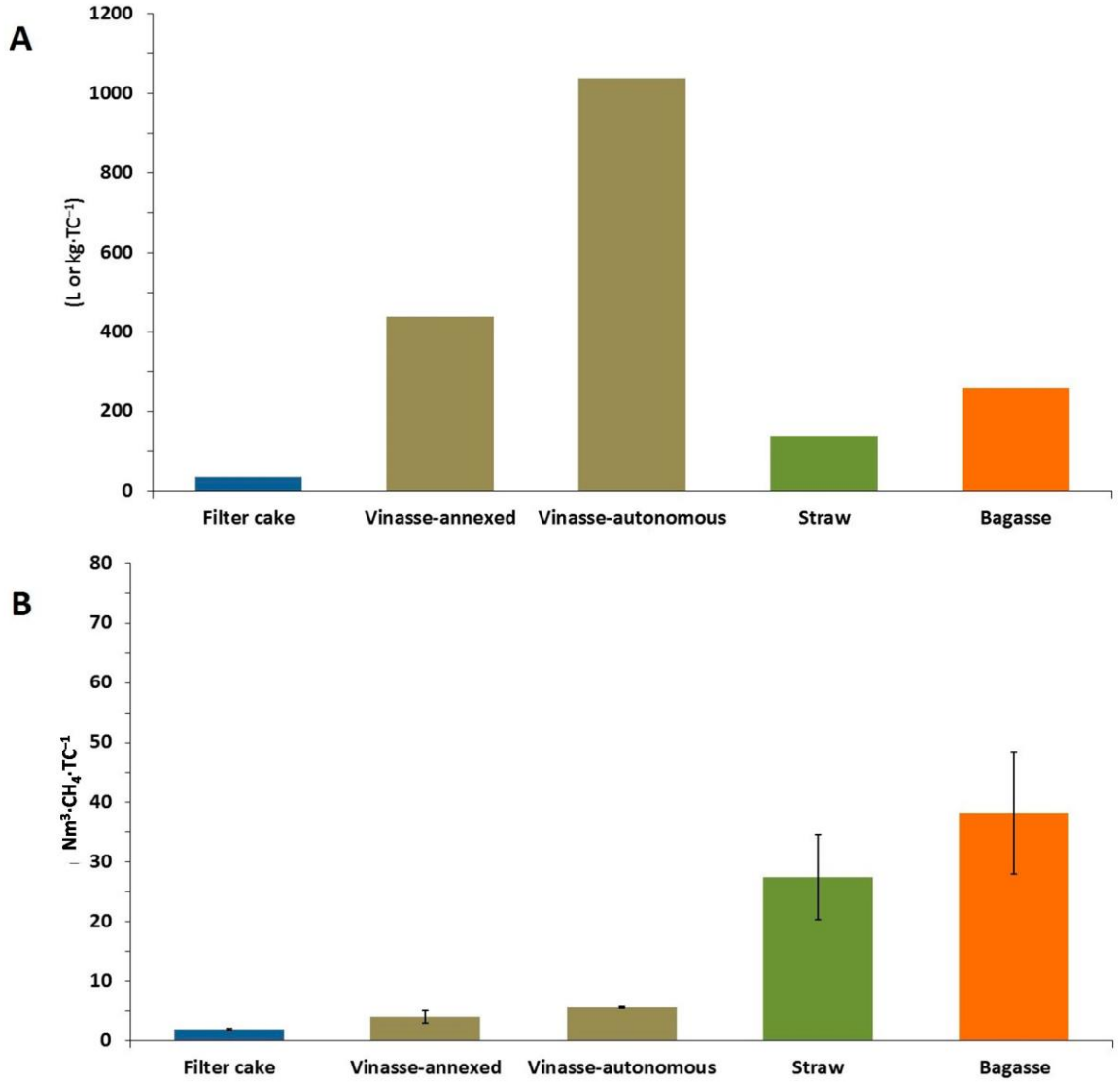

Figure 2. Mass flows VS energy potential of the sugarcane waste. (**A**) The values liters or kilogram per ton of cane (L or kg·TC^{-1}) refer to fresh matter according to data collected in two different sugarcane plants, except straw (dry matter) according to previous study [4]; (**B**) The values normal cubic meter of methane per ton of cane ($Nm^3 \cdot CH_4 \cdot TC^{-1}$) were calculated based on the BMP results and data presented in Figure2A.

Although vinasse from the autonomous plant has a lower methane yield per fresh matter, an autonomous plant could produce 40% more energy from vinasse in comparison to an annexed plant. This is possible due to differences in ethanol production between these two types of plants, from 30 to 71 L·TC^{-1}, directly influencing the specific vinasse generation of them (Figure 2A). Even filter cake being produced in clearly lower amounts in comparison to vinasse derived from autonomous and annexed plants, the energy potential of filter cake is close to the vinasse potential.

Although bagasse presented the highest methane potential, its entire utilization as a substrate for biogas production may suffer restrictions due to the fact that it is already being used for co-generation purposes by the thermochemical conversion system. Straw would not suffer the same restriction, since

nowadays, in most cases, this material is left to decay on the fields due to the low incentives to produce bioelectricity from it.

Nevertheless, the dissemination of second-generation biofuels in the future can lead to an extreme competition for biomass among different available energy conversion pathways, whereas it is expected that the system providing not only higher energy production, but also higher environmental benefits, will have better possibilities to play an important role in the market.

2.3.2. Energy Complementarities

In order to understand how the AD process could be designed to enhance the utilization capacity of a biogas plant integrated to the sugar and/or ethanol production processes (Table 6), it is reasonable to develop a storage system that would permit the use of filter cake, or even parts of bagasse and straw, during the sugarcane offseason, in the same way as is already practiced for the maize ensiling in Germany, for example.

The Figure 3 presents the daily methane generation that could be produced using vinasse during the 236 days of sugarcane season (±8 months, from April to November) and filter cake during the 129 days of offseason period (±4 months, from December to March) in the main sugarcane producing region in Brazil (*i.e.*, Center-South Region).

By applying this concept to an annexed plant, the daily methane production that filter cake during the offseason period could provide is 14.4% lower than the daily methane production of vinasse during the sugarcane season. The remaining methane production needed to compensate for such difference during the sugarcane offseason (6398 $Nm^3 \cdot CH_4 \cdot day^{-1}$) could be provided by converting in methane 2.1% ± 0.5% of the total straw generated or, alternatively, 1.8% ± 0.4% of bagasse. In the meantime, when an autonomous plant is considered (Figure 3B), the remaining residual energy (23,925 $Nm^3 \cdot CH_4 \cdot day^{-1}$) would be equivalent to 7.9% ± 1.9% of the energy potential straw or 6.8% ± 1.7% of bagasse.

For those plants where a fraction of straw or bagasse would not be available for biogas production, two different possibilities could be explored to keep methane production constant during the entire year.

Considering that, in Brazil, there is no environmental obligation that mandates the treatment of vinasse before fertirrigation on the fields, the biogas plant does not necessarily need to operate as a treatment facility and the amount of vinasse used for methane production could be reduced by 14.4% in the case of an annexed plant, and by 38.6% in the case of an autonomous plant. However, obviously in this case the final energy produced by the biogas plant would be reduced.

Another option could be the utilization of a pre-treatment process on filter cake to increase its specific methane production, since such an alternative has already been tested before. Gonzalez *et al.* [36,37] evaluated two different pre-treatment methods to increase the methane yields of filter cake (named as press mud) under mesophilic conditions (±37 °C). The liquid hot water pre-treatment method was able to increase the methane yield by 63% after 20 min of exposure at 150 °C, while the thermo-alkaline pre-treatment method was able to increase the methane yield by 72% by adding 10 g $Ca(OH)_2$ per 100 $g \cdot TS^{-1}$ for 1 h. However, questions regarding the application of such methods at the large-scale still remain unclear, especially whether these types of pre-treatments would be able to provide net profit gain to the biogas system.

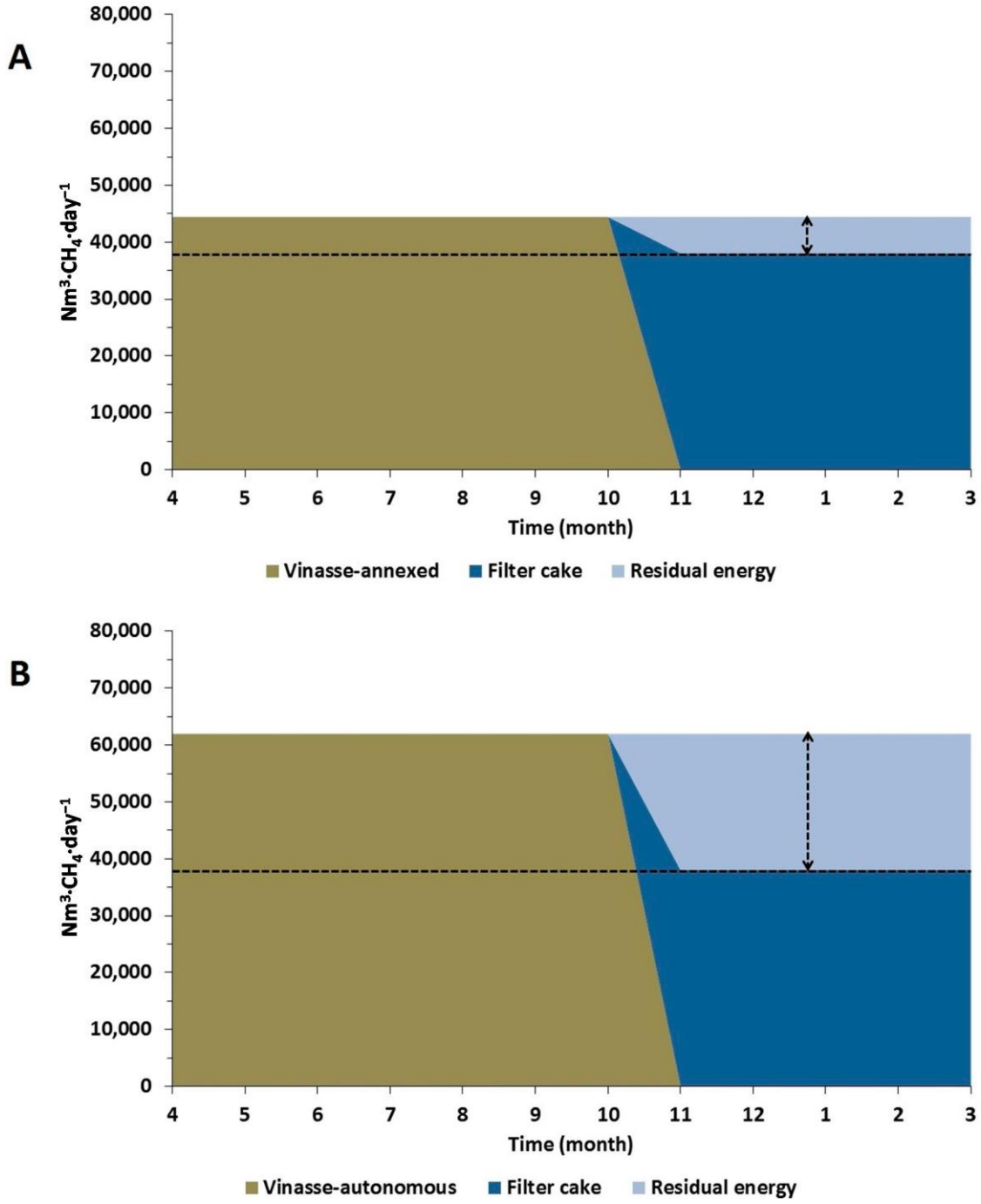

Figure 3. Energy potential of the vinasse and filter cake calculated for a hypothetical sugarcane plant. (**A**) Energy potential according to an autonomous sugarcane plant; (**B**) Energy potential according to an annexed sugarcane plant. Residual energy (highlighted by the dotted lines) is the difference in daily methane production between the potential energy of vinasse and filter cake.

Table 6. Summary of the kinetic challenges for designing an anaerobic digestion process applied to the sugarcane industry.

Aspects	Vinasse	Filter cake	Bagasse	Straw
Reactor type	Biomass immobilization system (e.g., UASB)	CSTR or combination with biomass immobilization system	CSTR with high HRT (>35 days)	CSTR with high HRT (>40 days)
Pre-treatment	Not necessary	Recommended	Highly recommended	Highly recommended
Macronutrients	Phosphorous addition to balance C:P ratio at autonomous plant	Sulfur addition to balance C:S ratio	Nitrogen, sulfur and phosphorus addition to balance C:N:P:S ratio	Nitrogen, sulfur and phosphorus addition to balance C:N:P:S ratio
Trace elements	Lack of Fe, Ni, Co, Mo, W, Mn, Cu, Se, and Zn	Lack of Mo, W, and Se	Lack of Fe, Ni, Co, Mo, W, Mn, Cu, Se, and Zn	Lack of Fe, Ni, Co, Mo, W, Mn, Cu, Se, and Zn
Major challenge	High sulfur content, especially at annexed plants	Storage in case of use during sugarcane offseason	Low biomass availability; High lignin content;	Substrate logistic; High lignin content

3. Experimental Section

3.1. Substrates

Sugarcane waste derived from one autonomous plant, where ethanol is produced exclusively from the sugarcane juice, and sugarcane waste derived from two annexed plants, where ethanol is flexibly produced from molasses (*i.e.*, by-product from sugar production), sugarcane juice, or in most cases by a mix of both, were utilized in order to provide an extensive evaluation of the different existing sugarcane industrial processes found in Brazil. Therefore, samples of vinasse, filter cake, bagasse, and straw were collected from those sugarcane plants in the States of Goiás and São Paulo, Brazil during different seasons (2012–2013 and 2013–2014), transported to Germany in cooled boxes and kept under low temperature (*i.e.*, 4 °C) until its use.

3.2. Analytical Methods

For all samples, TS and VS were analyzed according to VDI 4630 [38]. For vinasse samples, chemical oxygen demand (COD) was also analyzed through LCK 014 COD kit (Hach-Lange, Düsseldorf, Germany) according to the manufacturer's protocol.

Nutritional content of the substrates was determined according to Weender, followed by Van Soest methods. By the Weender method raw protein, raw fat, NFC, and raw fiber are determined. Van Soest method allows the determination of the remaining carbohydrates and lignin fractions from the neutral detergent fiber (NDF), which represents hemicellulose, cellulose, lignin and ash, acid detergent fiber (ADF) represented by cellulose, lignin and ash, and the lignin content depicted by the acid detergent lignin (ADL). Detailed description of the methods were previously published by Liebetrau [39].

To determine the major and trace elements contained in each sugarcane waste, dried samples were pretreated with a mixture of $HNO_3/H_2O_2/HF$, followed by neutralization with H_3BO_3, and the resulting

clear solution was analyzed by inductively-coupled plasma atomic spectrometry (ICP-OES, ThermoFischer iCAP6200) according to standard procedures [40–42].

3.3. Biochemical Methane Potential

The biogas yield of each sugarcane waste was obtained through BMP assays according to VDI 4630 [38] using eudiometer systems under mesophilic temperature (38 °C) for 35 days, and corrected to normal conditions, considered 273.15 K and 101.325 kPa. Methane concentration in biogas was measured by using a GA2000 Landfill Gas Analyzer (Geotechnical Instruments Ltda., Warwickshire, UK). The inoculum used for the BMP assays was originally from a large scale biogas plant, which uses maize silage and cattle manure as substrates. The decay constant (*k*-value) was calculated based on the results from BMP assays by using a first-order kinetic model (Equation (1)), which equates biogas production with organic mass reduction [43]:

$$St = So \times e^{-kt} \quad (1)$$

where, *So*: initial mass of the substrate, substrate input (gVS); *St*: mass of the substrate at time *t*, after degradation (gVS); *k*: conversion rate (day^{-1}); *t*: time (day).

In order to eliminate the lag phase and use only the log phase of the test, which better represent the first order kinetics, data between days 0–5, 0–12, 3–25, and 3–25 were considered for vinasse, filter cake, bagasse, and straw, respectively. The high values of coefficient of determination (R^2), from 0.93 to 0.99, demonstrate the congruence of the model and experimental data.

The simulation of the substrates conversion using a CSTR system was performed by transferring the *k*-values previously calculated into the CSTR kinetics presented in Equation (2):

$$\mathrm{St} = \frac{So}{1 + k \times \theta} \quad (2)$$

where, *So*: initial mass of the substrate, substrate input (gVS); *St*: mass of the substrate at time *t*, after degradation (gVS); θ: hydraulic retention time (day); *k*: conversion rate (day^{-1}); *t*: time (day).

3.4. Energy Assessment

The energy potential of each sugarcane waste type was calculated based on the obtained BMP results, together with the specific waste production of annexed and an autonomous sugarcane plants. The energy complementarity between vinasse and the other types of waste was performed considering a hypothetical sugarcane plant with capacity to process 4×10^6 tons of cane per year ($TC \cdot year^{-1}$), considering the same average days of operating season (236 days) as the two sugarcane plants previously analyzed.

4. Conclusions

The organic waste generated during the sugar and/or ethanol productions have different characteristics that should be taken into account during the design of the anaerobic digestion process. The challenges of straw and bagasse utilization as substrates for biogas production are clear as several potential nutritional deficiencies were identified for these substrates. Urea supplementation could not

only balance the C:N ratio, but also increase the buffer capacity of the system and enhance the quality of the digestate for further use as fertilizer on the sugarcane fields. Although the higher methane yields of straw and bagasse (based on fresh matter), respectively, were 129 and 150 $Nm^3 \cdot ton_{FM}^{-1}$), the high lignin content of these substrates suggests that a pre-treatment process can enhance their degradability. The lower methane yield of vinasse (5–11 $Nm^3 \cdot ton_{FM}^{-1}$), together with its huge specific generation (438–1038 $L \cdot TC^{-1}$) suggests that future developments should be focused on a biomass immobilization reactor to allow higher OLR and lower HRT. Filter cake can play an important role to reduce the negative effects of sugarcane seasonality in the biogas system, if utilized as an alternative substrate to vinasse during the offseason. Such a concept still needs to be technically proven, especially regarding the feasibility of filter cake storage and the net energy gain that a pre-treatment procedure could provide to the system.

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Author Contributions

Designed the experiments: Leandro Janke and Walter Stinner; Performed the experiments: Leandro Janke and Athaydes Leite. Analyzed the data: Marcell Nikolausz and Thomas Schmidt; Supervised the study: Jan Liebetrau, Michael Nelles and Walter Stinner; Wrote the paper: Leandro Janke.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Elbehri, A.; Segerstedt, A.; Liu, P. *Biofuels and the Sustainability Challenge: A Global Assessment of Sustainability Issues, Trends and Policies for Biofuels and Related Feedstocks*; FAO: Rome, Italy, 2013; p. 15.
2. De Carvalho Macedo, I. *Sugar Cane's Energy—Twelve Studies on Brazilian Sugar Cane*, 2nd ed.; UNICA: São Paulo, Brazil, 2007; pp. 119–137.
3. UNICA (União da Indústria da Cana-de-açúcar) Unicadata: Brazilian sugarcane crushing during 2013/2014 season. Available online: http://www.unicadata.com.br (accessed on 11 August 2015).
4. ANA (Agência Nacional de Águas). *Manual de Conservação e Reuso de Água na Agroindústria Sucoenergética*; Universidade de Brasília: Brasília, Brazil, 2009. (In Portuguese)
5. De Paoli, F.; Bauer, A.; Leonhartsberger, C.; Amon, B.; Amon, T. Utilization of by-products from ethanol production as substrate for biogas production. *Bioresour. Technol.* **2011**, *102*, 6621–6624.

6. Leal, M.R.L.V; Galdos, M.V.; Scarpare, F.V.; Seabra, J.E.A; Walter, A.; Oliveira, C.O.F. Sugarcane straw availability, quality, recovery and energy use: A literature review. *Biomass Bioenergy* **2013**, *53*, 11–19.
7. UNICA (União da Indústria da Cana-de-açúcar). Moagem de cana-de-açúcar no Estado de São Paulo. Available online: http://www.unicadata.com.br/ (accessed on 15 February 2015). (In Portuguese)
8. CEPE (Conselho Estadual de Política Energética). *Plano Paulista de Energia—PPE 2020*; Governo do Estado de São Paulo: São Paulo, Brazil, 2012. (In Portuguese)
9. Governo do Estado de São Paulo. *Programa Paulista de Biogás-Decreto n. 58.659*. Available online: http://www.legislacao.sp.gov.br/legislacao/dg280202.nsf/5fb5269ed17b47ab83256cfb00501469/0250b268dd46ba4c83257acb004382ef?OpenDocument (accessed on 18 January 2015). (In Portuguese)
10. Al Seadi, T.; Rutz, D.; Prassl, H.; Köttner, M.; Finsterwalder, T. *Biogas Handbook*; University of Southern Denmark: Funen, Denmark, 2008.
11. Moraes, B.S.; Junqueira, T.L.; Pavanello, L.G.; Cavalett, O.; Mantelatto, P.E.; Bonomi, A.; Zaiat, M. Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? *Appl. Energy* **2014**, *113*, 825–835.
12. Janke, L.; Leite, A.F.; Wedwitschka, H.; Schmidt, T.; Nikolausz, M.; Stinner, W. Biomethane production integrated to the Brazilian sugarcane industry: The case study of São Paulo state. In Proceedings of the 22nd European Biomass Conference and Exhibition, Hamburg, Germany, 23–26 June 2014; pp. 23–26.
13. FNR (Fachagentur Nachwachsende Rohstoffe e.V.). *Guide to Biogas—From Production to Use*; Fachagentur Nachwachsende Rohstoffe e.V. (FNR): Gülzow, Brazil, 2010; p. 24.
14. Mccarty, P.L. Anaerobic Waste Treatment Fundamentals. *Public Work* **1964**, *95*, 107–112.
15. Lv, Z.; Hu, M.; Harms, H.; Richnow, H.H.; Liebetrau, J.; Nikolausz, M. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. *Bioresour. Technol.* **2014**, *167*, 251–259.
16. Britz, T.; Noeth, C.; Lategan, P. Nitrogen and phosphate requirements for the anaerobic digestion of a petrochemical effluent. *Water Res.* **1988**, *22*, 163–169.
17. Scherer, P.; Neumann, L.; Demirel, B.; Schmidt, O.; Unbehauen, M. Long term fermentation studies about the nutritional requirements for biogasification of fodder beet silage as mono-substrate. *Biomass Bioenergy* **2009**, *33*, 873–881.
18. Oremland, R.S.; Polcin, S. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. *Appl. Environ. Microbiol.* **1982**, *44*, 1270–1276.
19. Raskin, L.; Rittmann, B.E.; Stahl, D.A. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Appl. Environ. Microbiol.* **1996**, *62*, 3847–3857.
20. Lauterböck, B.; Nikolausz, M.; Lv, Z.; Baumgartner, M.; Liebhard, G.; Fuchs, W. Improvement of anaerobic digestion performance by continuous nitrogen removal with a membrane contactor treating a substrate rich in ammonia and sulfide. *Bioresour. Technol.* **2014**, *158*, 209–216.
21. Ramos, I.; Peña, M.; Fdz-Polanco, M. Where does the removal of H_2S from biogas occur in microaerobic reactors? *Bioresour. Technol.* **2014**, *166*, 151–157.
22. Kayhanian, M.; Rich, D. Pilot-scale high solids thermophilic anaerobic digestion of municipal solid waste with an emphasis on nutrient requirements. *Biomass Bioenergy* **1995**, *8*, 433–444.

23. Schmidt, T.; Nelles, M.; Scholwin, F.; Pröter, J. Trace element supplementation in the biogas production from wheat stillage—Optimization of metal dosing. *Bioresour. Technol.* **2014**, *168*, 80–85.
24. Gustavsson, J.; Shakeri Yekta, S.; Sundberg, C.; Karlsson, A.; Ejlertsson, J.; Skyllberg, U.; Svensson, B.H. Bioavailability of cobalt and nickel during anaerobic digestion of sulfur-rich stillage for biogas formation. *Appl. Energy* **2013**, *112*, 473–477.
25. Shakeri, S.; Svensson, B.H.; Björn, A.; Skyllberg, U. Thermodynamic modeling of iron and trace metal solubility and speciation under sulfidic and ferruginous conditions in full scale continuous stirred tank biogas reactors. *Appl. Geochem.* **2014**, *47*, 61–73.
26. Radjaram, B.; Saravanane, R. Assessment of optimum dilution ratio for biohydrogen production by anaerobic co-digestion of press mud with sewage and water. *Bioresour. Technol.* **2011**, *102*, 2773–2780.
27. Shakeri Yekta, S.; Lindmark, A.; Skyllberg, U.; Danielsson, Å.; Svensson, B.H. Importance of reduced sulfur for the equilibrium chemistry and kinetics of Fe(II), Co(II) and Ni(II) supplemented to semi-continuous stirred tank biogas reactors fed with stillage. *J. Hazard. Mater.* **2014**, *269*, 83–88.
28. Oechsner, H.-W.; Lemmer, A.; Hamhold, D.; Mathies, E.; Mayrhuber, E.; Preißler, D. Method for Producing Biogas in Controlled Concentrations of Trace Elements. Patent US20100304457 A1, 2 December 2008.
29. Leite, A.F.; Janke, L.; Harms, H.; Zang, J.W.; Fonseca-Zang, W.A.; Stinner, W.; Nikolausz, M. Assessment of the variations in characteristics and methane potential of major waste products from the Brazilian bioethanol industry along an operating season. *Energy Fuels* **2015**, *29*, 4022–4029.
30. Janke, L.; Leite, A.; Batista, K.; Weinrich, S.; Sträuber, H.; Nikolausz, M.; Nelles, M.; Stinner, W. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. *Bioresour. Technol.* **2015**, doi:10.1016/j.biortech.2015.07.117.
31. Zheng, Y.; Zhao, J.; Xu, F.; Li, Y. Pretreatment of lignocellulosic biomass for enhanced biogas production. *Prog. Energy Combust. Sci.* **2014**, *42*, 35–53.
32. Zhu, J.; Wan, C.; Li, Y. Enhanced solid-state anaerobic digestion of corn stover by alkaline pretreatment. *Bioresour. Technol.* **2010**, *101*, 7523–7528.
33. Montgomery, L.F.R.; Bochmann, G. *Pretreatment of Feedstock for Enhanced Biogas Production*; IEA Bioenergy: Dublin, Ireland, 2014; p. 4.
34. Wilkie, A.C.; Riedesel, K.J.; Owens, J.M. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. *Biomass Bioenergy* **2000**, *19*, 63–102.
35. Fang, H.H.P. *Environmental Anaerobic Technology: Applications and New Developments*; Imperial College Press: London, UK, 2010; p. 59.
36. López González, L.M.; Vervaeren, H.; Pereda Reyes, I.; Dumoulin, A.; Romero Romero, O.; Dewulf, J. Thermo-chemical pre-treatment to solubilize and improve anaerobic biodegradability of press mud. *Bioresour. Technol.* **2013**, *131*, 250–257.

37. López González, L.M.; Pereda Reyes, I.; Dewulf, J.; Budde, J.; Heiermann, M.; Vervaeren, H. Effect of liquid hot water pre-treatment on sugarcane press mud methane yield. *Bioresour. Technol.* **2014**, *169*, 284–290.
38. *VDI 4630-Fermentation of Organic Materials: Characterisation of the Substrate, Sampling, Collection of Material Data, Fermentation Tests*; VDI-Gesselschaft Energietechnik, Beuth Verlag: Berlin, Germany, 2006; p. 11.
39. Liebetrau, J.; Pfeiffer, D.; Thrän, D. *Messmethodensammlummg Biogas—Methoden zur Bestimmung von analytischen und prozessbeshreibenden Parametern im Biogasbereich*; Liebetrau, J., Pfeiffer, D., Thrän, D., Eds.; DBFZ Deutsches Biomasseforschungszentrum: Leipzig, Germany, 2015; pp. 38–50.
40. *DIN EN ISO 15587-2-2002: Water Quality—Digestion for the Determination of Selected Elements in Water—Part 2: Nitric Acid Digestion (ISO 15587-2:2002)*; DIN Deutsches Institut für Normung e. V.: Berlin, Germany, 2002; p. 13.
41. *DIN EN 16170: Schlamm, Behandelter Bioabfall und Boden—Bestimmung von Spurenelementen Mittels Optischer Emissionsspektrometrie mit Induktiv Gekoppeltem Plasma (ICP-OES)*; DIN Deutsches Institut für Normung e. V.: Berlin, Germany, 2011; p. 7.
42. DIN *DIN EN 15104: Feste Biobrennstoffe—Bestimmung des Gesamtgehaltes an Kohlenstoff, Wasserstoff und Stickstoff—Instrumentelle Verfahren*; DIN Deutsches Institut für Normung e. V.: Berlin, Germany, 2011; p. 8.
43. Schumacher, B.; Wedwitschka, H.; Hofmann, J.; Denysenko, V.; Lorenz, H.; Liebetrau, J. Disintegration in the biogas sector—Technologies and effects. *Bioresour. Technol.* **2014**, *168*, 2–6.

3. Biogas from filter cake and bagasse: special focus on microbial ecology

3.1. Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: Effects of increasing organic loading rate on methanogenic community dynamics

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Article

Improved Monitoring of Semi-Continuous Anaerobic Digestion of Sugarcane Waste: Effects of Increasing Organic Loading Rate on Methanogenic Community Dynamics

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Abstract: The anaerobic digestion of filter cake and its co-digestion with bagasse, and the effect of gradual increase of the organic loading rate (OLR) from start-up to overload were investigated. Understanding the influence of environmental and technical parameters on the development of particular methanogenic pathway in the biogas process was an important aim for the prediction and prevention of process failure. The rapid accumulation of volatile organic acids at high OLR of 3.0 to 4.0 $g_{vs} \cdot L^{-1} \cdot day^{-1}$ indicated strong process inhibition. Methanogenic community dynamics of the reactors was monitored by stable isotope composition of biogas and molecular biological analysis. A potential shift toward the aceticlastic methanogenesis was observed along with the OLR increase under stable reactor operating conditions. Reactor overloading and process failure were indicated by the tendency to return to a predominance of hydrogenotrophic methanogenesis with rising abundances of the orders Methanobacteriales and Methanomicrobiales and drop of the genus *Methanosarcina* abundance.

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Keywords: sugarcane waste; biogas reactor overloading; methanogenic pathways; stable isotope fingerprinting; monitoring tool

1. Introduction

Sugarcane is widely used in Brazil for bioethanol and sugar production. Bioethanol is well established as fuel primarily in Brazil, whereas sugar supplies the national and international markets. However, the waste generated by the bioethanol/sugar industry consisting mainly of filter cake, vinasse, and bagasse is not managed adequately, since it is still rich in energy when disposed. For the treatment of these waste products, the anaerobic digestion (AD) process was chosen as a promising technology for energy recovery, since the digestate could still be used to fertilize the sugarcane fields, as discussed in our previous study [1–4].

Despite the methane potential of sugarcane waste, the use of such novel substrates in AD requires research to achieve a process of practical and economic viability. Applying a continuous feeding biogas process along the entire year is a challenge due to the temporal availability of the substrate during approximately 200 operating days of the Brazilian bioethanol/sugar industry. A rapid start-up of the biogas process for more productivity reaching high levels of OLR at the beginning of the operation season may be the solution. Furthermore, the organic loading rate (OLR) is a factor of interest as it determines how much substrate can be treated and converted into biogas per time and reactor volume. However, at high OLR there is a risk of acidification by overloading, potentially followed by process failure. The tightrope walk between exploiting the reactor potential and maintaining a stable process requires cost effective monitoring which allows predicting and assessing process instabilities/failure particularly when the feeding regime is changed. It has been shown that stable isotope fingerprinting of the produced biogas can provide information about the most sensitive functional guild in the AD, the methanogens [5].

In AD the organic matter degradation into biogas proceeds in four major steps, *i.e.*, hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which are carried out by the complex consortia of various bacteria and methanogenic archaea [6]. Methanogenesis, as the terminal phase for methane production, to which two major pathways (aceticlastic and hydrogenotrophic methanogenesis) contribute, is a major target for biogas process optimization [7].

Acetate, H_2, and CO_2 (or formate) are the products of the digestion of macromolecules by hydrolytic, acidogenic, and acetogenic bacteria. Aceticlastic methanogens convert acetate into methane and carbon dioxide, whereas hydrogenotrophic methanogens catalyze the conversion of H_2 and CO_2 (or formate) to methane. In a stable AD process these complex microbial consortia cooperate and self-regulate their abundances and activities. However, overproduction of organic acids by the bacterial community, e.g., triggered by substrate overload, may overexert the downstream consumption and result in drastic acidification which inhibits the methane production [8]. Thus, to establish and optimize a biogas process with novel substrates such as waste products from the bioethanol/sugar industry, it is essential to study the propensity for acidification and its effect on the methanogenic communities.

Several research studies using different substrates have assessed the microbial diversity in laboratory- and large-scale biogas reactor [9–11] and the effect of reactor parameters such as organic acid accumulation on the methanogenic diversity and pathway dominance [12–14]. However, to our best knowledge, the effect of acidification by overloading of biogas reactors fed with sugarcane waste has not been investigated. Furthermore, it is known that the susceptibility of AD to overloading depends on the substrate, reactor type, and temperature, thus motivating the present investigation with a novel substrate.

The assessment of the methanogenic community in biogas reactors requires appropriate methods. Variations of the methanogenic community can be analyzed by molecular techniques [15,16], whereas analysis of the biogas isotope composition provides information about the relative contributions of the methanogenic pathways [5,17–19]. While molecular biological analyses appear very time-consuming and costly for routine process monitoring, biogas isotope composition appears to be a promising monitoring parameter for industrial biogas processes [20].

Laboratory-scale, semi-continuously-fed, stirred digesters were established to observe the changes of the activity of methanogens as a function of reactor acidification triggered by overloading. Two parallel reactors with mono-digestion of filter cake as substrate, and two other parallel reactors with co-digestion of filter cake and bagasse were monitored by molecular and isotopic techniques to determine the contributions of the methanogenic pathways. Statistical analyses served to correlate isotope signatures and community structures.

2. Results and Discussion

2.1. Biogas Reactor Performance

In Table 1 the technical parameters and reactor performance during the eight phases of operation are shown. An average value was calculated for each of the two reactor pairs performing mono- and co-digestion. Due to the high volatile organic acids (VOA) concentration (2.45 $g \cdot L^{-1}$) of the digestate mixture used as inoculum, an acclimation of 10 days was required for degradation of the remaining organic matter from the previous reactors. The OLR increased from 0.5 to 4.0 $g_{vs} \cdot L^{-1} \cdot day^{-1}$ within nine weeks. As a consequence, the hydraulic retention time (HRT) decreased from 36 to 7 and from 37 to 12 days for the mono- and co-digestion reactors, respectively. Along the experiment until Phase 5, the biogas yield was lower for mono- than for co-digestion, whereas the methane content, in mono-digestion reactors was higher. After exceeding the OLR of 2.5 (Phase 5) and 3.0 $g_{vs} \cdot L^{-1} \cdot day^{-1}$ (Phase 6) for co- and mono-digestion, respectively, the biogas yield decreased drastically. In the co-digestion reactors, the biogas production was inhibited earlier because the pH and the buffering capacity were lowered already in Phase 6. In the following phases, the buffer capacity was insufficient to neutralize the VOA accumulation. The acidification led to decreased biogas yield followed by process failure in both digestion set-ups. Reactor overload and imbalance were already noticed in Phase 6, when the propionate-to-acetate ratios rose from 0.043 to 1.434 (33×) and 0.037 to 1.999 (54×) for mono- and co-digestion, respectively. The results from this phase for mono-digestion were consistent with some other studies, thus confirming that the overload effect is seen earlier from the propionate-to-acetate ratio than from changes in pH or in biogas yield [12,14,21]. Prochazka, *et al.* [22]

reported that low ammonium nitrogen (NH_4-N) concentration (0.5 $g \cdot L^{-1}$) caused low methane yield, loss of biomass and loss of aceticlastic methanogenic activity, and further presented lower buffer capacity and less stable pH. However, this statement does not corroborate our results until Phase 5, indicating that these findings were circumstantial, *i.e.*, depending on the substrate and microbial adaptation. Although the low NH_4-N concentration during mono-digestion in Phase 6 did not influence negatively the methane yield, foaming was observed in both parallel reactors, which necessitated liquid volume reduction for mono-digestion. The foaming can be ascribed to non-degraded soluble organics, which result in the surface tension reduction of reactor content [23]. At high volatile organic acids per total inorganic carbonate buffer (VOA/TIC) values (3.1 $g_{VOA} \cdot g_{CaCO3}^{-1}$) in Phase 7, the total- (TS) and volatile solids (VS) values also increased due to the lack of further degradation of the organic matter. This indicated that not just the methanogenesis, but the whole process was inhibited eventually.

2.2. Methanogenic Community Dynamics

The diversity and structure of the methanogenic communities from the mono- and co-digestion were investigated by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of the *mcrA*/*mrtA* gene (Figure 1) and further validated by sequence analysis of clone libraries (Supplementary Table S1). Immediately before the first feeding on day 10, the reactors displayed similar *mcrA*/*mrtA* profiles for both reactor types, but a slight difference in the relative T-RF abundances was observed, indicating distinct acclimatization of the inoculum mixture. In Phase 1, the strictly aceticlastic genus *Methanosaeta* and the versatile genus *Methanosarcina* were more abundant in mono- than in co-digestion set-ups, whereas the strictly hydrogenotrophic genus *Methanoculleus* was more predominant in the co-digestion reactors. In both digestions, the abundance of *Methanosarcina* increased gradually from Phase 2 to Phase 6, reaching a proportion of approximately 80%. *Methanosaeta* was not detected after the acetic acid concentration started to increase in the process in Phase 4. The high affinity of *Methanosaeta* for acetate is a competitive advantage over *Methanosarcina* at low acetic acid concentrations [24], but at higher concentrations *Methanosarcina* is outcompeting *Methanosaeta*. Our observation of *Methanosaeta* at low acetic acid concentration is consistent with the findings of other studies [12,25,26]. To our surprise, at very high acetic acid concentrations during the Phase 7 and Phase 8 of mono-digestion, *Methanosaeta* was detected again, whereas the abundance of *Methanosarcina* dropped. Chen and He [27] also demonstrated competitiveness of *Methanosaeta* with *Methanosarcina* at high acetate levels. In the co-digestion reactors *Methanobacterium* predominated mainly in Phases 7 and 8. Sequences affiliated to the hydrogenotrophic genus *Methanoregula* were relatively abundant in the last phases of the experiment, when the propionate-to-acetate ratio drastically increased and the pH decreased. According to Yashiro, *et al.* [28] the genus *Methanoregula* includes acid-tolerant strains.

Table 1. Major reactor parameters along the eight phases of the experiment set-up.

Reactor' Parameters	Phase 1 (sampling Day: 19)		Phase 2 (sampling day: 26)		Phase 3 (sampling day: 33)		Phase 4 (sampling day: 40)		Phase 5 (sampling day: 55)		Phase 6 (sampling day: 61)		Phase 7 (sampling day: 68)		Phase 8 (sampling day: 75)	
	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion
Biogas yield * ($mL \cdot g_{VS}^{-1}$)	1086.3	1198.2	506.9	541.0	283.9	409.6	292.8	329.2	368.3	410.9	397.6	251.2	127.9	58.3	69.7	33.7
CH_4 § (%)	57.7	55.6	57.4	56.6	60.3	53.9	57.8	54.9	61.3	54.9	61.1	na	na	na	na	na
CO_2 § (%)	42.3	44.4	42.6	43.4	39.7	46.1	42.2	45.1	38.7	45.1	38.9	na	na	na	na	na
Acetic acid ($mg \cdot L^{-1}$)	55.7	37.3	26.7	25.4	46.1	69.6	160.3	120.5	240.6	155.6	145.1	141.9	1003.0	914.2	1370.0	1334.1
Propionic acid ($mg \cdot L^{-1}$)	10.7	6.9	6.7	4.9	5.3	4.6	13.1	8.8	10.4	5.8	208.0	283.7	537.6	550.7	433.9	391.8
n-Butyric acid ($mg \cdot L^{-1}$)	4.7	2.5	3.1	2.0	2.2	1.8	19.5	12.1	5.3	1.8	26.0	6.7	428.9	403.3	1144.8	1193.0
VOA ($g \cdot L^{-1}$)	0.8	0.8	0.6	0.7	0.6	0.6	0.5	0.5	1.0	0.8	0.6	0.6	2.2	2.1	na	na
VOA/TIC ($g_{VOA} \cdot g_{CaCO3}^{-1}$)	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.7	0.6	3.1	3.1	na	na
pH *	7.5	7.5	7.4	7.3	7.5	7.5	7.2	7.1	7.2	6.9	7.0	6.5	6.3	5.7	5.4	5.2
NH_4-N ($g \cdot L^{-1}$)	1.0	1.1	0.8	0.8	0.6	0.9	0.5	0.5	0.3	0.3	0.1	0.1	0.2	0.1	0.1	0.2
TS (%)	3.1	2.9	2.7	2.6	2.5	2.7	2.3	2.8	2.1	2.3	2.1	2.1	5.6	na	6.9	7.6
VS (%)	2.1	2.0	1.8	1.7	1.7	1.8	1.6	1.9	1.5	1.7	1.6	1.5	3.8	na	4.5	5.0

* Only for these parameters an average of all measurements during each specific Phase was done, since these parameters were analyzed almost every day; § Trace gases were not detected in our measurements with the applied technique, therefore we rounded our CH_4 and CO_2 values to 100%; "sampling day" corresponds to the last Phase day, when the samples were analyzed; "na" refers to not analysed due to technical operation problems: the very low biogas production on the last two phases hindered the GC measurement for gas composition; the low pH values detected on the last phase hindered the titration of sample for measuring VOA and VOA/TIC; and the TS and VS measurement was hindered by technical mistake while handling the samples.

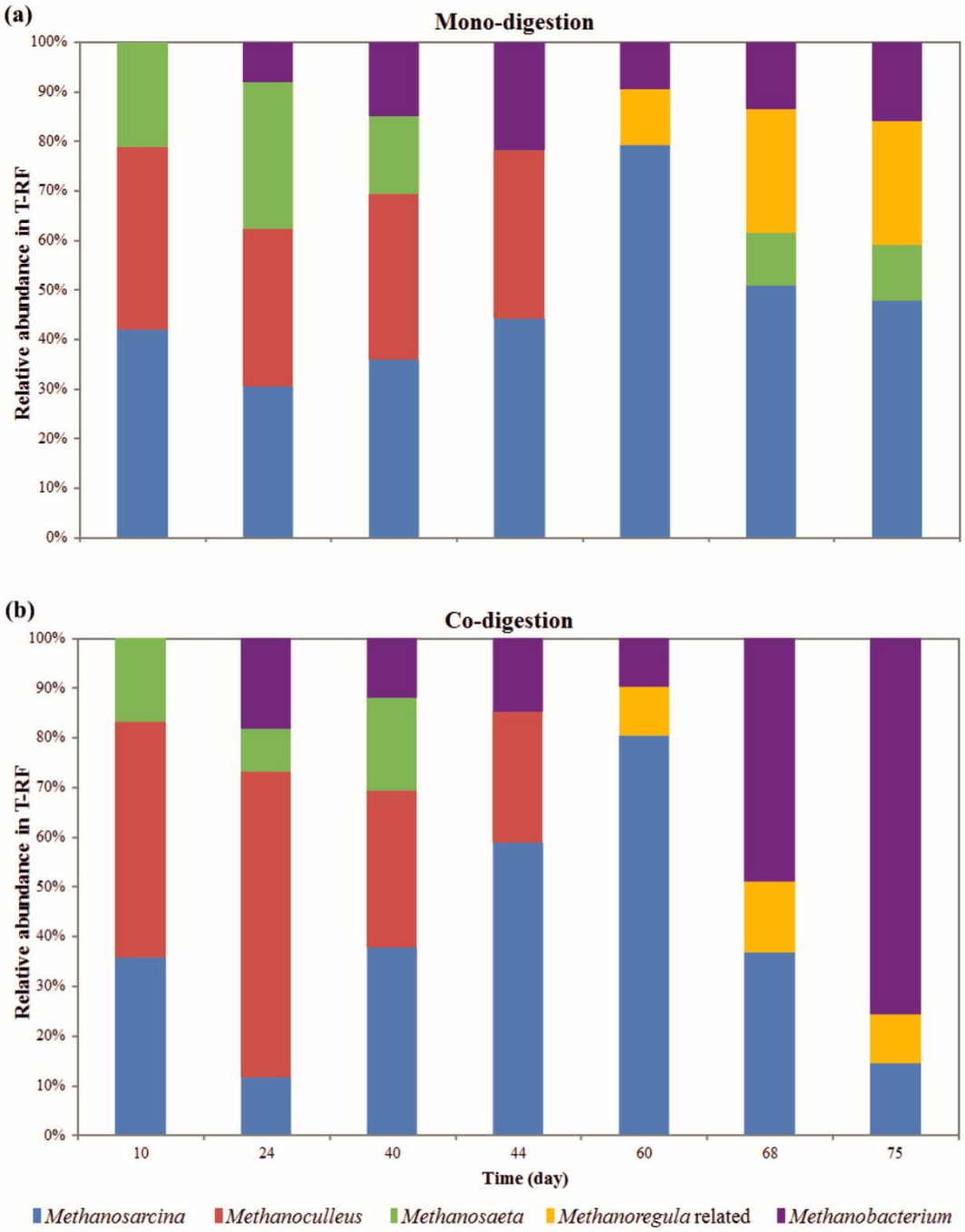

Figure 1. Methanogenic community dynamics in the mono- (**a**) and co-digestion (**b**) reactor. The relative T-RF abundance of methanogens in the digestate samples are given as function of experiment time. For each of the parallel reactors in the specific digestion set-up, two samples were analyzed, that in total four samples were analyzed for each, mono- and co-digestion. All samples belonging to the same digestion set-up had similar methanogenic community based on the relative T-RF abundances. Therefore, each bar on the graphic represents the T-RFLP profile calculated by the average of the four analyzed samples in each digestion set-up. The supporting clone libraries and sequence analysis of the selected clones allowed the taxonomic affiliation of the T-RFs from the community T-RFLP profiles of the complex reactor samples.

2.3. Isotopic Changes of the Produced Biogas

Carbon-stable isotope compositions of filter cake and bagasse samples were analyzed since they influence the final isotope composition of the produced methane [5]. Filter cake and bagasse had isotope signatures of −14.30‰ and −13.64‰ $\delta^{13}C$, respectively, which are in the typical range for C4 plants ($\delta^{13}C$ values between −12‰ and −16‰) [29].

The gradual overload of the mono- and co-digestion reactors resulted in process changes that were monitored via the isotope composition of the biogas in terms of $\delta^{13}C_{CH4}$, $\delta^{13}C_{CO2}$, and $\delta^{2}H_{CH4}$ (Figure 2). Both digestion set-ups had very similar dynamics. The $\delta^{13}C_{CH4}$ became enriched from −52‰ to about −32‰ along the gradual OLR increase until Phase 5 at 2.5 $g_{vs}\cdot L^{-1}\cdot day^{-1}$ (Figure 2a). Following, the Phase 6 had similar isotope values as the previous phase. This stationary isotope signature around −32‰ is consistent with former studies that found similar isotope fractionation of biogas samples from continuous stirred tank reactors (CSTRs) fed with C4 plant maize silage [5,19]. The inhibition of biogas production in co-digestion in Phase 6 also coincided with the isotopic depletion of the methane associated with slightly lighter $\delta^{13}C_{CH4}$ values. In the last two phases of the experiment, when the process was clearly inhibited, depletion of $\delta^{13}C_{CH4}$ values was observed.

The $\delta^{13}C$ of carbon dioxide in the produced biogas in the mono- and co-digestion presented also similar trends (Figure 2b) with an enrichment from 4‰ to about 15‰. However, only in Phase 6 the tendency between both digestion set-ups differed. In this case, the biogas production inhibition in co-digestion may have resulted in abrupt $\delta^{13}C_{CO2}$ depletion. Phase 7 had the most enriched $\delta^{13}C_{CO2}$ composition, followed by drastic depletion in ^{13}C values in Phase 8. However, the observed isotope effect is at certain extent due to the decreasing pH and the associated fast degassing of the CO_2 from the bicarbonate in the liquid.

The hydrogen isotope composition of methane ($\delta^{2}H_{CH4}$) in the mono- and co-digestion shows similar trends (Figure 2c). The hydrogen isotope compositions showed an opposite tendency to carbon isotope composition regarding enrichment and depletion periods. After the feeding regime has stated at Phase 1 the isotope values depleted from around −327‰ to −342‰ at the end of the stable operation phases. In the final phases, $\delta^{2}H_{CH4}$ enriched to about −322‰ when OLR drove the methanogenic process to collapse.

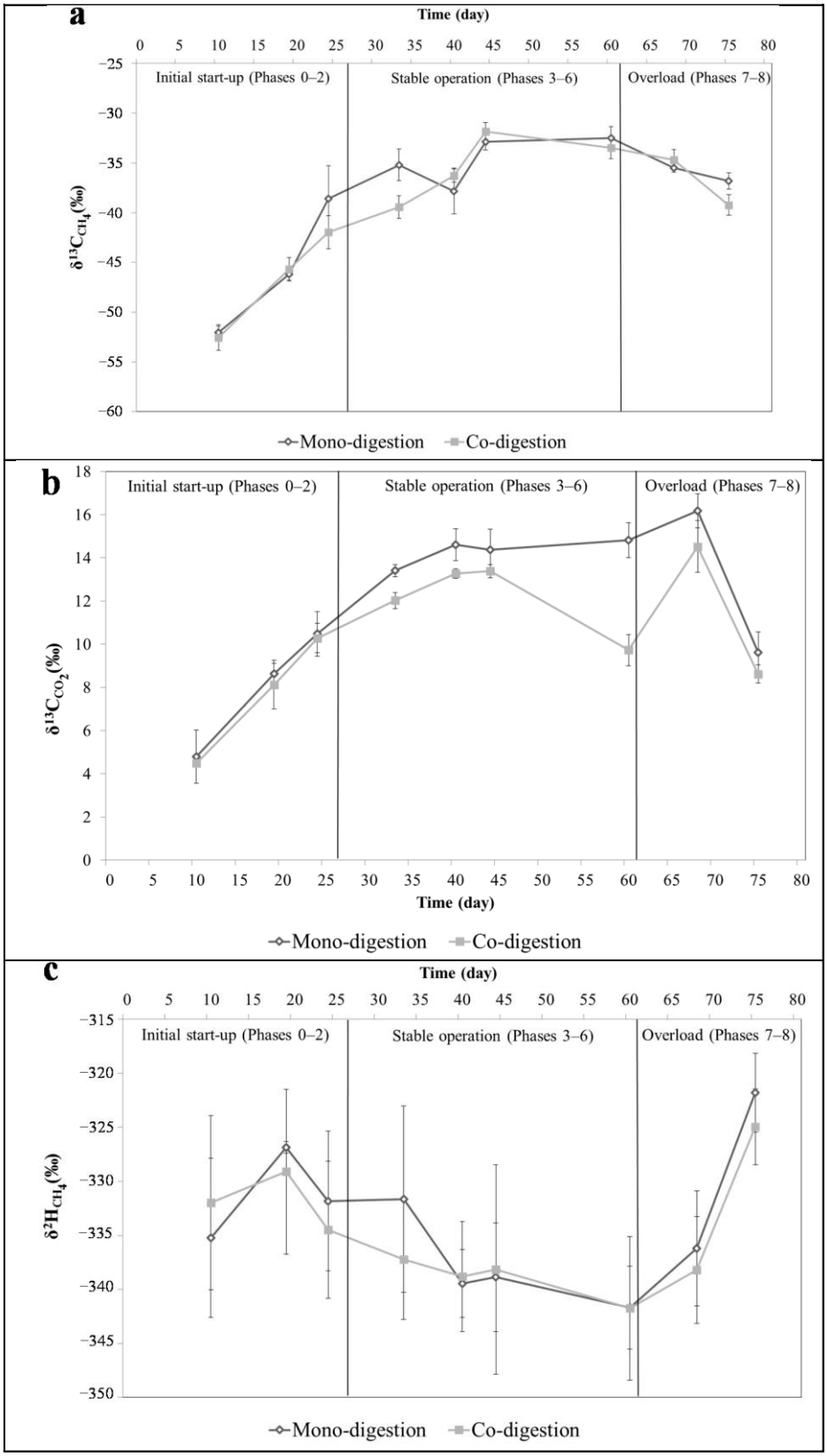

Figure 2. Isotopic dynamics of $\delta^{13}C_{CH4}$ (**a**); $\delta^{13}C_{CO2}$ (**b**); and $\delta^{2}H_{CH4}$ (**c**) along gradual OLR increase in biogas reactors fed with sugarcane waste products. Isotope data of CO_2 during the last overload phase contains data uncorrected regarding the pH shift induced degassing.

2.4. Methanogenic Pathways

The apparent fractionation factor (αC) calculated based on $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$ composition as previously described [30–32] was used to identify a predominance of hydrogenotrophic and aceticlastic methanogenesis (Figure 3a). An intermediate αC value ranging between 1.065 (>for hydrogenotrophic) and 1.025 (<for aceticlastic) was found in our experiment, indicating that the methane produced during increasing OLR was derived similarly from both methanogenic pathways. This agrees with the broad spectrum of methanogenic genera (Figure 1) which included the versatile genus *Methanosarcina*, the strictly aceticlastic genus *Methanosaeta* and the strictly hydrogenotrophic order Methanomicrobiales (*Methanoculleus* and *Methanoregula*-related microorganisms) and the genus *Methanobacterium*. However, the high αC values 1.060 and 1.056 at the beginning of the experiment, in Phase 0 (at day 10 just before the first feeding) and Phase 1, respectively, indicated a predominance of hydrogenotrophic methanogenesis, suggesting that *Methanosarcina* was using this pathway together with *Methanoculleus* and *Methanobacterium*. The substrates from other AD processes in the mixture of digestate inoculated in our reactors may have also contributed to the initial isotope composition. Along the experiment, the relative abundance of *Methanosarcina* gradually increased and may have slightly shifted the methanogenesis from the hydrogenotrophic towards the aceticlastic pathway until the OLR of 3.0 $g_{vs}\cdot L^{-1}\cdot day^{-1}$ was reached. In this case, the composition of sugarcane waste and the added water favored a tendency towards aceticlastic methanogenesis, though the strictly aceticlastic genus *Methanosaeta* was no longer abundant after Phase 4 in both digestion set-ups. However, in case of mono-digestion in the inhibition-characterized Phases 7 and 8, sequences affiliated with the genus *Methanosaeta* were detected again. A similar finding was described by Schmidt, *et al.* [33] who observed that decreasing HRT may favor the genus *Methanosaeta* under certain conditions. Nikolausz, *et al.* [5] described that more depleted $\delta^{13}C_{CH4}$ values in biogas reactors potentially indicate a shift toward the dominance of hydrogenotrophic methanogenesis. This observation was also supported by our results. Since the first feeding with the sugarcane waste products *Methanobacterium* became abundant with minor changes along mono-digestion and with increase in dominance during overload of co-digestion reactors. This was shown by the increase of the αC values at reactor overload, which also indicated a shift towards hydrogenotrophic methanogenesis. In addition, the relative abundance of the other hydrogenotrophic taxon related to the genus *Methanoregula* also increased during reactor overload in both digestion set-ups.

The combination plot of $\delta^{13}C_{CH4}$ and $\delta^{2}H_{CH4}$ as function of increasing ORL is shown in Figure 3b, where the dotted, dashed, and lined hulls represent the beginning, middle, and end of the experiment, respectively. Phases 0 and 1 are represented in the dotted hull with higher αC values as described earlier. The dashed hull area is covering most of the phases (from Phase 2 to Phase 7), which had similar ranges and trends of αC values for mono-digestion (1.049–1.055) and co-digestion (1.045–1.055). Samples from Phase 8 are grouped into the lined hull, representing the period when the mono- and co-digestion reactors were overloaded, imbalances were clearly observed and less depleted δD values were measured. The isotope effect associated with aceticlastic methanogenesis is significantly larger in case of hydrogen derived from the water, but it affects only one out of four hydrogen atoms of the methane, while the other three atoms are influenced by the δD of the organic matter. In natural environments aceticlastic methanogenesis results in more depleted δD values of

methane, which is in agreement with our data where depleted values were observed during the stable reactor operating conditions and explained by the predominance of *Methanosarcina*.

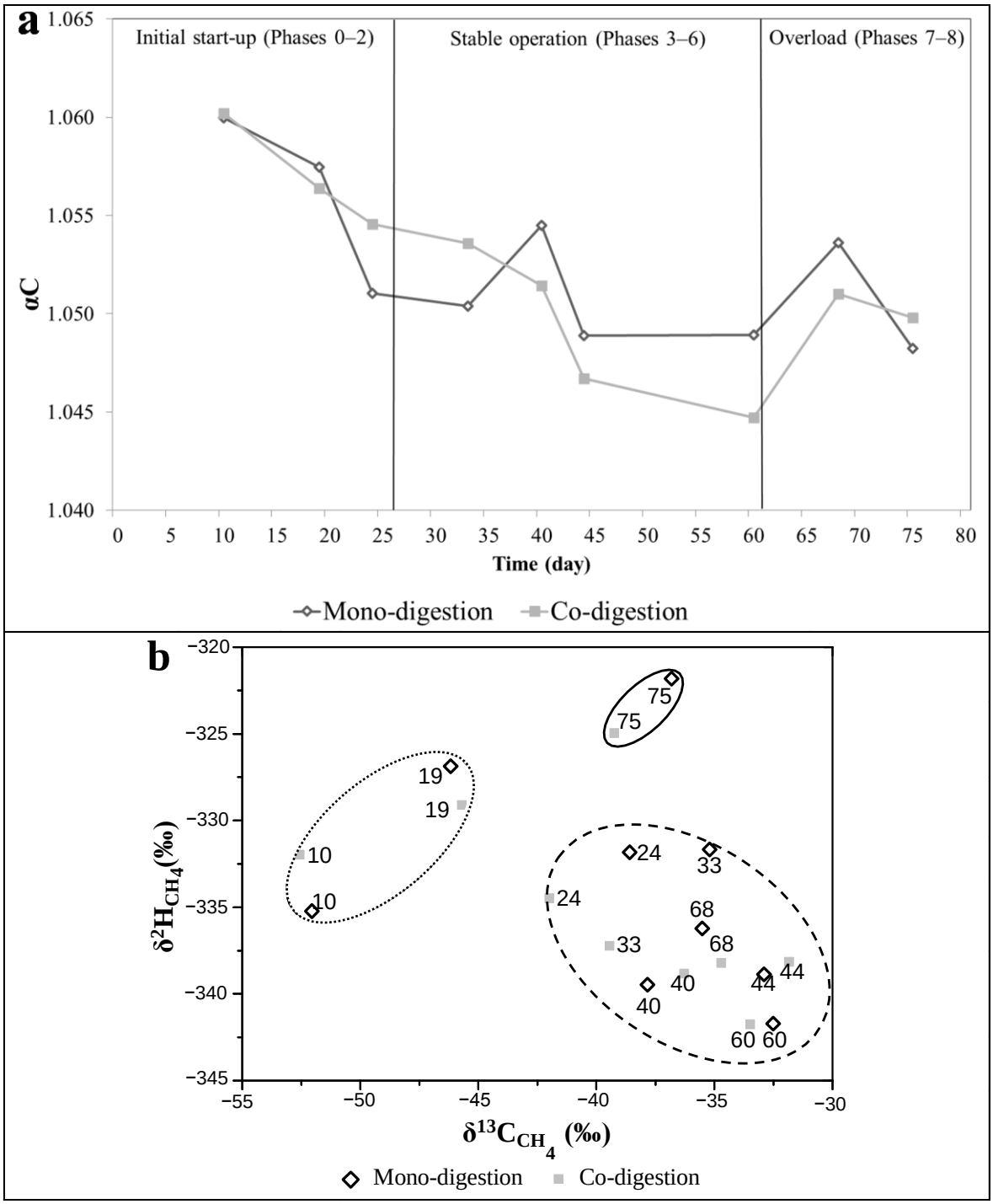

Figure 3. Characterization of the potential predominant methanogenic pathway along gradual OLR increase in biogas reactors fed with sugarcane waste products in mono- and co-digestion. In diagram (**a**) the dynamic shift of αC values are shown, while diagram (**b**) presents the correlation of δ^2H_{CH4} and $\delta^{13}C_{CH4}$; In (**b**) the dotted, dashed and lined hulls represent the beginning, middle, and end of the experiment, respectively. The numbers in the graphic indicate the experiment day.

In Figure 4 the correlation between the T-RFLP profile dynamics and the isotope composition of biogas is shown in a non-metric multidimensional scaling (NMDS) plot. The methanogenic pathway shift can be viewed in the NMDS plot as shifts of T-RFLP profile clusters (dashed hulls) during the different phases of the ORL increase. The methanogenic community most significantly correlates with the isotopic fractionation of $\delta^{13}C_{CH4}$ as indicated by the vector converted to the larger grey arrow in the NMDS plot. The reactor overload in Phases 7 and 8 for mono-digestion was characterized by a strong correlation of the hydrogenotrophic taxon related to the genus *Methanoregula* and less depleted isotopic values of $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$, whereas for co-digestion it was characterized by a significant correlation of the strict hydrogenotrophic genus *Methanobacterium* and $\delta^{2}H_{CH4}$. This corroborates the increase of the αC values for both digestion set-ups at reactor overload in Phase 7 indicating the shift towards hydrogenotrophic methanogenesis and higher αC values of co-digestion compared to mono-digestion at the Phase 8, when the relative abundance of *Methanobacterium* was around 75%.

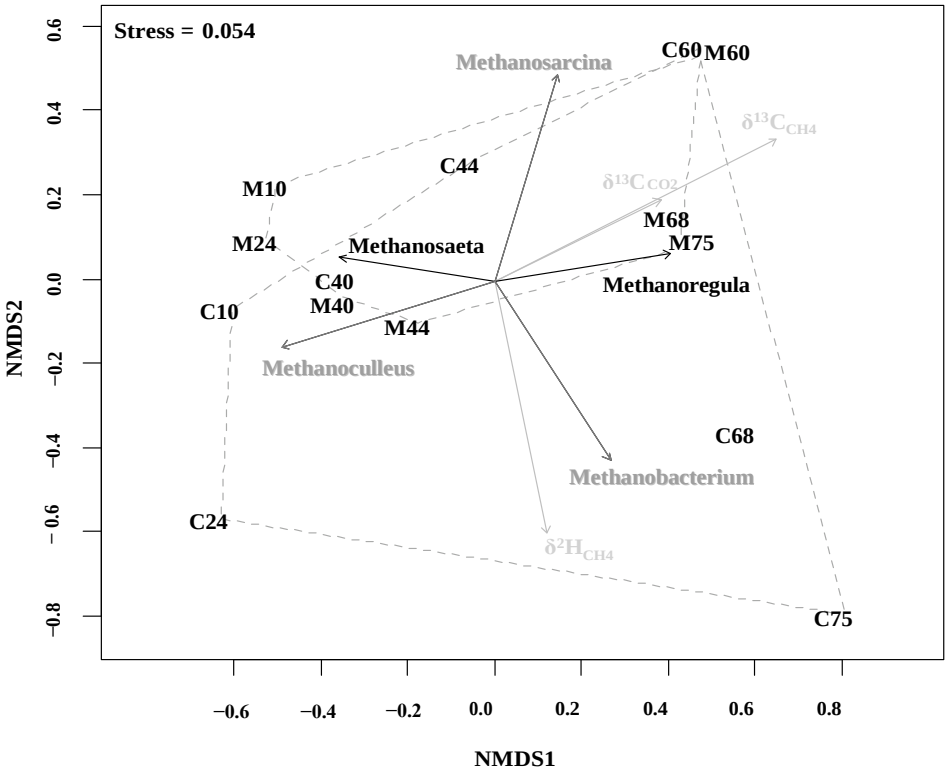

Figure 4. NMDS analysis plot for correlating the T-RFLP profile of methanogens with the isotope composition of produced biogas. The smaller and bigger hull in the diagram represents the mono- and co-digestion in several sampling time, respectively. The letter M stands for mono-digestion and C for co-digestion set-up and the following numbers correspond to the sampling day. The dim grey and black arrows indicate the highly significant ($p < 0.001$) and significant ($p < 0.05$) correlations, respectively. Grey arrows indicate the correlation vectors of community differences and the isotope composition at lower significance ($p < 0.5$). Monte-Carlo permutation was used to test the significance against 999 random data sets. The direction of the arrows show the correspondence to the community structures and the length of the arrow indicate the strength of the correlation with the ordination axis.

3. Experimental Section

3.1. Biogas Reactors, Operation and Analytical Methods

The experiment was carried out in four CSTRs under mesophilic conditions at 38 °C. In order to provide a diverse microbial start-up community as inoculum, each reactor was inoculated with a mixture of digestates from several CSTRs, which had been fed daily with either maize silage, dried distillers grains with soluble (DDGS), straw or chicken manure. In our experiment, filter cake and bagasse, two solid waste products from the bioethanol industry (Goiás, Brazil), were used as substrate. Bagasse was cut to 1 mm pieces by milling to increase the accessible surface area and to facilitate reactor feeding and stirring. The TS and VS were 28% and 17% for filter cake and 57% and 55% for bagasse, respectively. Mono-digestion was performed with filter cake, whereas co-digestion reactors were fed at a substrate ratio of 70% filter cake and 30% bagasse (based on fresh mass), corresponding to a VS-based filter cake to bagasse ratio of 1:0.74. All four reactors were fed every day according to the digestion set-up.

Table 2 shows further technical parameters of the operation. The experiment was divided into eight phases according to the gradual increase of the OLR. Biogas production was monitored, by counting biogas bubbles in a liquid-filled pipe via digital imaging and size recognition [34]. Biogas composition was measured with a thermal conductivity detector Chrompack Micro GC CP-2002P (Middelburg, The Nederland). The TS and VS, the pH values, the NH_4-N concentration, acetate, propionate, and *n*-butyrate were determined as described previously by Leite, *et al.* [1]. The total VOA concentration and the VOA/TIC were analyzed as described earlier by Ziganshin, *et al.* [35].

3.2. Methanogenic Community Analysis

Duplicate digestate samples were collected in 2-mL test tubes and immediately stored at −20 °C for further analysis. Total DNA isolation was carried out using NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany). Methanogen-specific methyl coenzyme-M reductase (*mcrA*) gene fragments were amplified by polymerase chain reaction (PCR) using the forward primer mlas and the reverse primer mcrA-rev labeled with 6-carboxyfluorescein (FAM) for T-RFLP analyses. Non-labeled primers were used for molecular cloning and sequencing as in a previous study [5]. Further, T-RFLP screening and partial sequencing of purified PCR products were performed as described by Nikolausz, *et al.* [5]. The BLASTN and BLASTX tools were used to search for similar sequences in public databases. The *mcrA*/*mrtA* gene sequences obtained in this study were deposited in the European Bioinformatics Institute (EMBL-EBI) database under the accession numbers LN847074-LN847091. The NMDS analyses were conducted as described by Sträuber, *et al.* [36].

Table 2. Technical parameters during the experiment set-up of the mono- and co-digestion of filter cake and bagasse.

Set-up-Technical Parameters	Phase 1		Phase 2		Phase 3		Phase 4		Phase 5		Phase 6		Phase 7		Phase 8	
	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion	Mono-Digestion	Co-Digestion
Experiment phase (day)	11–19		20–26		27–33		34–40		41–55		56–61		62–68		69–75	
Substrate ($g_{\text{fresh mass}}$)	2.4	1.5	4.9	3.0	7.3	4.5	9.7	6.0	12.2	7.5	14.6	9.0	10.7	10.5	12.2	12.0
Water mixed with substrate (mL)	20		25		30		35		45		45		50		55	
Working volume (L)	0.8		0.8		0.8		0.8		0.8		0.8		0.5	0.8	0.5	0.8
VS ($g \cdot day^{-1}$)	0.4		0.8		1.2		1.6		2.0		2.4		1.8	2.8	2.0	3.2
OLR ($g_{VS} \cdot L^{-1} \cdot day^{-1}$)	0.5		1.0		1.5		2.0		2.5		3.0		3.5		4.0	
HRT (day)	35.7	37.2	26.8	28.6	21.4	23.2	17.9	19.5	15.3	15.2	13.4	14.8	8.2	13.2	7.4	11.9

3.3. Stable Isotope Analysis

The carbon isotope composition of the solid waste products (filter cake and bagasse) were measured in a continuous flow system consisting of an elemental analyser (Euro EA, HEKAtech GmbH, Wegberg, Germany) connected to an isotope ratio mass spectrometer (Finnigan MAT 253, Thermofinnigan, Bremen, Germany).

Biogas from the reactor headspace was sampled with a syringe at the same time as digestate was sampled. Twenty mL biogas was transferred and stored in gas-tight pre-evacuated vials until further analysis. Isotope measurements were performed as described by Feisthauer, *et al.* [37]. Briefly, an isotope ratio mass spectrometry system (Finnigan MAT 253, Thermofinnigan, Bremen, Germany) was coupled to a gas chromatograph (HP 6890 Series, Agilent Technology, Santa Clara, CA, USA) either via a combustion device for carbon analysis or via a pyrolysis unit for hydrogen analysis. Fifty μL of biogas sample from the vials were injected into a helium carrying tube at the split ratio of 1:50 for carbon and 1:5 for hydrogen analysis.

4. Conclusions

Strong dynamics of community structure and pathway shifts in methanogens were observed by molecular and stable isotope fingerprinting during gradual increase of OLR. The overloading effect in both digestion set-ups was observed beginning at an OLR of 2.5 $g_{vs} \cdot L^{-1} \cdot day^{-1}$ from the increase of the propionate-to-acetate ratio. However, the co-digestion processes suffered process failure earlier (at OLR 3.0) than mono-digestion (at OLR 3.5). Until process-overload *Methanosarcina* became gradually predominant, shifting the methanogenic pathway towards aceticlastic. The change towards hydrogenotrophic methanogenesis during reactor overload might be taken as an indicator for process failure. Monitoring of the methanogenic pathways by stable isotope composition of biogas can be an excellent tool to control and predict process failure.

Supplementary Materials

Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/10/23210/s1.

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Author Contributions

Designed the experiments: Athaydes Francisco Leite, Marcell Nikolausz and Leandro Janke; Performed the experiments: Athaydes Francisco Leite, Leandro Janke and Zuopeng Lv. Analyzed and

interpreted the data: Athaydes Francisco Leite, Marcell Nikolausz and Hans-Hermann Richnow; Supervised the study: Marcell Nikolausz and Hauke Harms; Wrote the paper: Athaydes Francisco Leite.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Leite, A.F.; Janke, L.; Harms, H.; Zang, J.W.; Fonseca-Zang, W.A.; Stinner, W.; Nikolausz, M. Assessment of the variations in characteristics and methane potential of major waste products from the brazilian bioethanol industry along an operating season. *Energy Fuels* **2015**, *29*, 4022–4029.
2. Janke, L.; Leite, A.; Batista, K.; Weinrich, S.; Strauber, H.; Nikolausz, M.; Nelles, M.; Stinner, W. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. *Bioresour. Technol.* **2015**, doi:10.1016/j.biortech.2015.07.117.
3. Janke, L.; Leite, A.; Wedwitschka, H.; Schmidt, T.; Nikolausz, M.; Stinner, W. Biomethane production integrated to the brazilian sugarcane industry: The case study of são paulo state; In Proceedings of the 22nd European Biomass Conference and Exhibition, Hamburg, Germany, December 2014; pp. 1295–1299.
4. Janke, L.; Leite, A.; Nikolausz, M.; Schmidt, T.; Liebetrau, J.; Nelles, M.; Stinner, W. Biogas production from sugarcane waste: Assessment on kinetic challenges for process designing. *Int. J. Mol. Sci.* **2015**, *16*, 20685–20703.
5. Nikolausz, M.; Walter, R.F.; Strauber, H.; Liebetrau, J.; Schmidt, T.; Kleinsteuber, S.; Bratfisch, F.; Gunther, U.; Richnow, H.H. Evaluation of stable isotope fingerprinting techniques for the assessment of the predominant methanogenic pathways in anaerobic digesters. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 2251–2262.
6. Weiland, P. Biogas production: Current state and perspectives. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 849–860.
7. Demirel, B.; Scherer, P. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. *Rev. Environ. Sci. Biol. Technol.* **2008**, *7*, 173–190.
8. Chen, Y.; Cheng, J.J.; Creamer, K.S. Inhibition of anaerobic digestion process: A review. *Bioresour. Technol.* **2008**, *99*, 4044–4064.
9. Briones, A.; Raskin, L. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr. Opin. Biotechnol.* **2003**, *14*, 270–276.
10. Karakashev, D.; Batstone, D.J.; Angelidaki, I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl. Environ. Microbiol.* **2005**, *71*, 331–338.

11. Lucas, R.; Kuchenbuch, A.; Fetzer, I.; Harms, H.; Kleinsteuber, S. Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. *FEMS Microbiol. Ecol.* **2015**, *91*, doi:10.1093/femsec/fiv004.
12. Blume, F.; Bergmann, I.; Nettmann, E.; Schelle, H.; Rehde, G.; Mundt, K.; Klocke, M. Methanogenic population dynamics during semi-continuous biogas fermentation and acidification by overloading. *J. Appl. Microbiol.* **2010**, *109*, 441–450.
13. Franke-Whittle, I.H.; Walter, A.; Ebner, C.; Insam, H. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste Manag.* **2014**, *34*, 2080–2089.
14. Marchaim, U.; Krause, C. Propionic to acetic acid ratios in overloaded anaerobic digestion. *Bioresour. Technol.* **1993**, *43*, 195–203.
15. Steinberg, L.M.; Regan, J.M. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. *Appl. Environ. Microbiol.* **2008**, *74*, 6663–6671.
16. Talbot, G.; Topp, E.; Palin, M.; Masse, D. Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Res.* **2008**, *42*, 513–537.
17. Chidthaisong, A.; Chin, K.-J.; Valentine, D.L.; Tyler, S.C. A comparison of isotope fractionation of carbon and hydrogen from paddy field rice roots and soil bacterial enrichments during CO_2/H_2 methanogenesis. *Geochim. Cosmochim. Acta* **2002**, *66*, 983–995.
18. Lv, Z.; Leite, A.F.; Harms, H.; Richnow, H.H.; Liebetrau, J.; Nikolausz, M. Influences of the substrate feeding regime on methanogenic activity in biogas reactors approached by molecular and stable isotope methods. *Anaerobe* **2014**, *29*, 91–99.
19. Lv, Z.; Hu, M.; Harms, H.; Richnow, H.H.; Liebetrau, J.; Nikolausz, M. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. *Bioresour. Technol.* **2014**, *167*, 251–259.
20. Keppler, F.; Laukenmann, S.; Rinne, J.; Heuwinkel, H.; Greule, M.; Whiticar, M.; Lelieveld, J. Measurements of 13C/12C methane from anaerobic digesters: Comparison of optical spectrometry with continuous-flow isotope ratio mass spectrometry. *Environ. Sci. Technol.* **2010**, *44*, 5067–5073.
21. Nielsen, H.; Uellendahl, H.; Ahring, B. Regulation and optimization of the biogas process: Propionate as a key parameter. *Biomass Bioenerg.* **2007**, *31*, 820–830.
22. Prochazka, J.; Dolejs, P.; Maca, J.; Dohanyos, M. Stability and inhibition of anaerobic processes caused by insufficiency or excess of ammonia nitrogen. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 439–447.
23. Jenkins, D.; Richard, M.G.; Daigger, G.T. Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Separation Problems; IWA Publishing: London, UK, 2003; pp. 131–161.
24. Zinder, S. Physiological ecology of methanogens. In *Methanogenesis*; Ferry, J., Ed.; Springer: New York, NY, USA, 1993; pp. 128–206.

25. Griffin, M.E.; McMahon, K.D.; Mackie, R.I.; Raskin, L. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. *Biotechnol. Bioeng.* **1998**, *57*, 342–355.
26. Yu, Y.; Kim, J.; Hwang, S. Use of real-time PCR for group-specific quantification of aceticlastic methanogens in anaerobic processes: Population dynamics and community structures. *Biotechnol. Bioeng.* **2006**, *93*, 424–433.
27. Chen, S.; He, Q. Persistence of methanosaeta populations in anaerobic digestion during process instability. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1129–1137.
28. Yashiro, Y.; Sakai, S.; Ehara, M.; Miyazaki, M.; Yamaguchi, T.; Imachi, H. *Methanoregula formicica* sp. nov., a methane-producing archaeon isolated from methanogenic sludge. *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 53–59.
29. O'Leary, M.H. Carbon isotopes in photosysnthesis. *BioScience* **1988**, *38*, 328–336.
30. Conrad, R. Quantification of methanogenic pathways using stable carbon isotopic signatures: A review and a proposal. *Org. Geochem.* **2005**, *36*, 739–752.
31. Galand, P.E.; Yrjälä, K.; Conrad, R. Stable carbon isotope fractionation during methanogenesis in three boreal peatland ecosystems. *Biogeosciences* **2010**, *7*, 3893–3900.
32. Whiticar, M.J.; Faber, E.; Schoell, M. Biogenic methane formation in marine and freshwater environments: CO_2 reduction *vs.* Acetate fermentation—Isotope evidence. *Geochim. Cosmochim. Acta* **1986**, *50*, 693–709.
33. Schmidt, T.; Ziganshin, A.M.; Nikolausz, M.; Scholwin, F.; Nelles, M.; Kleinsteuber, S.; Pröter, J. Effects of the reduction of the hydraulic retention time to 1.5 days at constant organic loading in CSTR, ASBR, and fixed-bed reactors—Performance and methanogenic community composition. *Biomass Bioenerg.* **2014**, *69*, 241–248.
34. Tauber, T.; Berta, B.; Szabo, Z.; Kovacs, J.; Marialigeti, K.; Toth, E.M. A simple and novel volumetric method to metre low gas flows from laboratory-scale bioreactors and its application on laboratory sludge digesters. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1453–1461.
35. Ziganshin, A.M.; Schmidt, T.; Scholwin, F.; Il'inskaya, O.N.; Harms, H.; Kleinsteuber, S. Bacteria and archaea involved in anaerobic digestion of distillers grains with solubles. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 2039–2052.
36. Sträuber, H.; Schröder, M.; Kleinsteuber, S. Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. *Energy Sustain. Soc.* **2012**, *2*, 13, doi:10.1186/2192-0567-2-13.
37. Feisthauer, S.; Siegert, M.; Seidel, M.; Richnow, H.H.; Zengler, K.; Gründger, F.; Krüger, M. Isotopic fingerprinting of methane and CO_2 formation from aliphatic and aromatic hydrocarbons. *Org. Geochem.* **2010**, *41*, 482–490.

3.2. Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse

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Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse

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ABSTRACT

The anaerobic digestion of sugarcane filter cake and the option of co-digestion with bagasse were investigated in a semi-continuous feeding regime to assess the main parameters used for large-scale process designing. Moreover, fresh cattle manure was considered as alternative inoculum for the start-up of biogas reactors in cases where digestate from a biogas plant would not be available in remote rural areas. Experiments were carried out in 6 lab-scale semi-continuous stirred-tank reactors at mesophilic conditions (38 ± 1 °C) while the main anaerobic digestion process parameters monitored. Fresh cattle manure demonstrated to be appropriate for the start-up process. However, an acclimation period was required due to the high initial volatile fatty acids concentration (8.5 g L^{-1}). Regardless the mono-digestion of filter cake presented 50% higher biogas yield (480 mL gVS^{-1}) than co-digestion with bagasse (320 mL gVS^{-1}) during steady state conditions. A large-scale co-digestion system would produce 58% more biogas (1008 m^3 h^{-1}) than mono-digestion of filter cake (634 m^3 h^{-1}) due to its higher biomass availability for biogas conversion. Considering that the biogas production rate was the technical parameter that displayed the most relevant differences between the analyzed substrate options (0.99–1.45 m^3 biogas m^3 d^{-1}). The decision of which substrate option should be implemented in practice would be mainly driven by the available construction techniques, since economically efficient tanks could compensate the lower biogas production rate of co-digestion option.

1. Introduction

The production of sugar and ethanol based on sugarcane as feedstock is responsible for generation of different types of organic waste, which in most cases are still not being properly managed from the energy point of view (De Carvalho Macedo, 2007). In this context, bagasse, a solid waste derived from the extraction of sugarcane juice, is generated in large amounts (260 kg per ton of cane), and usually used as fuel in low-efficiency cogeneration systems or sold by the sugarcane plants to another end-uses (e.g. animal feeding) (Bressan Filho, 2011; Nogueira et al., 2008). On the other hand, filter cake, produced during the clarification (physical–chemical process) of the sugarcane juice, is generated in lower amounts than bagasse (35–40 kg per ton of cane), however it is mostly applied as organic fertilizer on the sugarcane fields without any previous energy recovery (Janke et al., 2015a).

Anaerobic digestion (AD) is a promising strategy to manage such type of waste, since as a result of the biochemical process in which complex organic matter is degraded to CH_4 and CO_2 by

Abbreviations: AD, anaerobic digestion; C:N, carbon to nitrogen; C:P, carbon to phosphorus; C:S, carbon to sulfur; FCM, fresh cattle manure; FM, fresh matter; HRT, hydraulic retention time; MIX, mixture of digestates; NFC, non-fiber carbohydrates; NH_4-N, ammonium-nitrogen; OLR, organic loading rate; SBP, specific biogas production; SCSTR, semi-continuous stirred-tank reactor; SUC, specific upgrading cost; TBP, theoretical gas potential; TS, total solids; VFA, volatile fatty acids; VOA, volatile organic acids; VOA/TIC, ratio of volatile organic acids and total inorganic carbonate to calcium carbonate; VS, volatile solids.

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various types of microorganisms biogas could be produced and used as fuel to improve the energy balance of the sugarcane plants (Janke et al., 2015b; Leite et al., 2015a). However, several factors such as temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT), balance of nutrients and presence of inhibitors must be considered for an efficient AD process. Furthermore, the microbial community and the quality of the inoculum used for the start-up of an anaerobic reactor are also considered decisive factors for a successful biogas production (Cho et al., 2013; Moset et al., 2014).

In previous studies (Janke et al., 2014; Leite et al., 2015b) our group has already assessed the possibility of using these type of waste for biogas production in batch tests. However, the feed regime and high proportion of inoculum used during batch tests do not allow an adequately assessment of anaerobic reactors start-up, neither possible process inhibition during digestion of the substrates. Therefore, understanding the reactor's behavior during the start-up phase can be attained only by using a similar feeding regime applied in large-scale applications (semi-continuous system).

For large-scale applications, it is well known that using digestate taken from a stable working digester could be a good strategy to overcome the start-up challenges (Kobayashi et al., 2009). However, in countries where biogas technology is not established in the market yet, plant operators must find alternative sources of inoculum suitable for the start-up period of an anaerobic reactor. In this case, the utilization of animal waste, such as cattle manure, could be a useful strategy, since such material is rich in microorganisms from animal digesting system, as well important macronutrients and trace elements (Seadi et al., 2008).

According to earlier studies (Kayhanian and Rich, 1995; Mccarty, 1964), another factor that can influence the performance of the AD process is the nutrient content of the substrates. If a certain substrate has too high C:N ratio and consequently nitrogen deficiency, it may negatively affect the functioning of the microbial community. Thus, a direct effect on their ability to produce enzymes that are needed for the carbon utilization, causing an incomplete conversion of the substrates, resulting in lower CH_4 yields. On the other hand, substrates that contain high levels of nitrogen can cause inhibition to the AD process via accumulation of toxic ammonia (NH_3) produced from protein degradation or by urea conversion (Lv et al., 2014).

The characteristics of different sugarcane waste assessed by Leite et al. (2015b), showed that bagasse has a C:N variation of 90–101:1 along an operating season, which is higher than the range of values (20–40:1) recommended by others (FNR, 2012). Meanwhile, previous studies (López González et al., 2013) showed that filter cake has a C:N ratio of 26:1, that is around the lowest recommended limit.

Considering that filter cake is a waste stream that currently is not used for any energy purpose, it makes sense to use such type of biomass on the AD process to produce biogas. This would enhance the energy balance of sugarcane plants without losing the essential nutrients for the sugarcane cultivation. Additionally, bagasse that is the major solid waste produced on-site by the sugarcane plants, could be an interesting co-substrate to balance the C:N of filter cake and improve energy production in the biogas system. Thus, the objectives of the present study were to (i) assess cattle manure as alternative inoculum for the start-up phase of semi-continuous anaerobic reactors; (ii) compare the process performance during semi-continuous mono-digestion of filter cake versus the option of co-digestion with bagasse; and (iii) analyze both substrate options (mono-digestion and co-digestion) on the main parameters used for the AD process design integrated to a large-scale sugarcane plant.

2. Materials and methods

2.1. Substrates and inocula

Samples of sugarcane filter cake and bagasse were collected from a distillery plant in the State of Goiás (Brazil) during the 2012/2013 season, transported to Germany in sealed plastic bags and kept under low temperature (i.e. 4 °C) until its use. A large-scale biogas plant that uses maize silage and fresh cattle manure (FCM) as substrate provided FCM that was used for the start-up of two semi-continuous reactors. A mixture of several digestates (hereafter referred as MIX) from mesophilic lab-scale reactors were used for the start-up of four other semi-continuous reactors. To avoid inlet and outlet pipes from clogging, both inocula were sieved prior to inoculation in the reactors. Tap water was utilized to keep the total solids of the feed below 15% for the wet fermentation process.

2.2. Semi-continuous feeding experiments

Six lab-scale semi-continuous stirred-tank reactors (SCSTR) with 5 L total volume and 3 L working volume were carried out in these experiments. The reactors were continuously stirred (100 rpm) using a central stirrer with helix shaped blades located in the lower part of the reactors. The operation temperature was kept under mesophilic conditions (38 ± 1 °C) by recirculating hot water through the double-walled reactors.

Each of the three following experiments performed in our study was carried out in duplicate with the same feeding regime (once per day). Reactors R3.3 and R3.4 were fed with filter cake and MIX for start-up. Reactors R3.5 and R3.6 were fed in a co-digestion system with filter cake (70%) and bagasse (30%) on fresh matter basis, also using MIX for start-up. Reactors R3.7 and R3.8 were fed with the same co-digestion proportion, however using FCM as inoculum. Detailed information about the different feeding rates, OLR and HRT are listed in Table 1.

2.3. Analytical methods

For all samples, total solids (TS) and volatile solids (VS) were analyzed according to VDI 4630 (2006). Nutritional values were determined according to Weender followed by Van Soest methods. By the Weender method raw protein, raw fat, non-fiber carbohydrates (NFC) and raw fiber are determined. Van Soest method allows the determination of the remaining carbohydrates and lignin fractions from the neutral detergent fiber (NDF), which represents hemicellulose, cellulose, lignin and ash, acid detergent fiber (ADF) represented by cellulose, lignin and ash, and the lignin content depicted by the acid detergent lignin (ADL). Detailed description of the methods was previously published by Liebetrau et al. (2015). To determinate the major elements contained in each sugarcane waste, dried samples were pre-treated with a mixture of HNO_3/H_2O_2/HF and latter neutralized with H_3BO_3, and the resulting clear solution was analyzed by inductively coupled plasma atomic spectrometry (ICP-OES, ThermoFischer iCAP6200) according to standard procedures (DIN, 2011a, 2011b, 2002).

The daily biogas production in each of the semi-continuous reactors was measured by a milligascounter type MGC-10 (Ritter, Bochum, Germany), and corrected to standard temperature and pressure conditions (273.15 K and 101.325 kPa), and the specific biogas production (SBP) was presented in norm milliliters per g of VS (mL gVS^{-1}). The composition of the produced biogas (CH_4, CO_2 and O_2) was measured twice a week in the headspace of the reactors by using a GA2000 Landfill Gas Analyzer (Geotechnical Instruments Ltda., UK).

Table 1
Overview of the semi-continuous feeding experiment.

Reactors	Inoculum	C:N ratio	Phase	Period (day)	Filter cake Input (g day^{-1})	Bagasse Input (g day^{-1})	Water Input (mL day^{-1})	HRT (days)	OLR (gVS L d^{-1})
R3.3	MIX	24:1	Start-up	0–41	36.5	–	50	34.7	2.0
R3.4				42–69	45.7	–	50	31.4	2.5
			Steady I	70–113	54.8	–	50	28.6	3.0
			Steady II	114–137	54.8	–	75	23.1	3.0
R3.5	MIX	41:1	Start-up	0–41	15.78	6.78	50	41.4	2.0
R3.6				42–69	19.73	8.45	50	38.4	2.5
			Steady I	70–113	23.67	10.15	50	35.8	3.0
			Steady II	114–137	23.67	10.15	75	27.6	3.0
R3.7	FCM	41:1	Start-up	0–3	15.78	6.78	50	41.4	2.0
R3.8				4–20	7.89	3.39	25	82.7	1.0
				21–41	15.78	6.78	50	41.4	2.0
				42–69	19.73	8.45	50	38.4	2.5
			Steady I	70–113	23.67	10.15	50	35.8	3.0
			Steady II	114–137	23.67	10.15	75	27.6	3.0

Five days per week pH was measured immediately in fresh digestate of each reactor with a pH-electrode (WTW type pH 3310 Sentix 41, Germany). Once a week fresh digestate samples were also centrifuged in 10,000 rpm during 10 min at 10 °C and supernatant liquid was used after filtration to subsequent analysis. Filtered samples (10 mL) were used for the quantification of all volatile organic acids (VOA) and a ratio of total inorganic carbonate to calcium carbonate (VOA/TIC, g_{VOA}/g_{CACO3}) measurement in a Titration Excellence T90 titrator (Mettler-Toledo GmbH, Switzerland). The concentrations of the major volatile fatty acids (VFA), including acetic- and propionic acid, were determined by gas chromatography using a 5890 series II gas chromatograph (Hewlett Packard, USA) equipped with a HS40 automatic headspace sampler (Perkin Elmer, USA) and an Agilent HP-FFAP column (30 m × 0.32 mm × 0.25 μm). Ammonium-nitrogen (NH_4-N g L^{-1}) was determined from 500 μL filtered samples diluted with deionized water in a proportion of 1:1000 during the start-up phase and 1:500 during the steady phase with the Nessler method using a benchtop spectrophotometer (Hach-Lange DR 3900, Loveland, US).

2.4. Degradation index

A degradation index was calculated to compare specific biogas production achieved during semi-continuous feeding experiments to the theoretical biogas potential of the substrates, according to Eq. (1).

$$D_{index} = SBP/TBP \quad (1)$$

where,

D_{index}: degradation index (fraction);
SBP: specific biogas production (mL gVS^{-1});
TBP: theoretical biogas potential (mL gVS^{-1});

Theoretical biogas potential was calculated according to Eq. (2) considering average values for carbohydrates, proteins and lipids published by Weißbach (2009).

$$TBP = (P_{TBP} + L_{TBP} + C_{TBP}) \quad (2)$$

where,

P_{TBP}: theoretical biogas potential of proteins (mL gVS^{-1});
L_{TBP}: theoretical biogas potential of lipids (mL gVS^{-1});
C_{TBP}: theoretical biogas potential of carbohydrates (mL gVS^{-1});

2.5. Preliminary assessment for large-scale process designing

Results from semi-continuous digestion were used to assess the effects of different substrate options on the main parameters used for large-scale process designing. Considering that for the Brazilian conditions depending on the energy balance of the sugarcane plants a surplus of bagasse is expected (Nogueira et al., 2008), which corresponds to an average value of 8.5% of total bagasse generated (Bressan Filho, 2011). The co-digestion proportion used in the laboratory experiments represents less than 6% of the total bagasse generated by a sugarcane plant. Table 2 presents the main characteristics of the reference sugarcane plant used in this study.

Table 2
Main characteristics of the sugarcane plant used as reference to assess the large-scale biogas application.

Characteristic	Value	Unit
Working days	232	days year^{-1}
Sugarcane processed	2.0×10^6	TC year^{-1}
Specific filter cake generation	35	kg$_{FM}$ TC^{-1}
Total filter cake generation	70×10^3	ton$_{FM}$ year^{-1}
Specific bagasse generation	260	kg$_{FM}$ TC^{-1}
Total bagasse generation	520×10^3	ton$_{FM}$ year^{-1}

TC: tons of cane.

3. Results and discussion

3.1. Substrate composition

The main characteristics of substrates used during semi-continuous feeding experiments are presented in Table 3. Filter cake, having an appearance similar to sludge, showed lower TS and VS content once is derived from a physical–chemical treatment process that removes soluble and insoluble impurities from

Table 3
Composition of sugarcane waste used during semi-continuous feeding experiments.

Parameters		Filter cake	Bagasse	Units
TS		21.9	54.7	% FM
VS		75.5	97.7	% TS
Raw protein		135.9	11.2	g kg^{-1} TS
Raw fat		37.7	8.2	g kg^{-1} TS
Carbohydrate	NFC	115.5	80.8	g kg^{-1} TS
	Cellulose	171.7	440.4	g kg^{-1} TS
	Hemi-cellulose	153.1	287.6	g kg^{-1} TS
Lignin		141.4	149.4	g kg^{-1} TS
Raw ash		244.6	22.4	g kg^{-1} TS
Carbon		40.8	49.7	% TS
Nitrogen		1.72	0.55	% TS
Phosphorus		0.6	0.01	% TS
Sulfur		0.7	0.02	% TS
Total Kjeldahl nitrogen		21.8	1.82	g kg^{-1} TS
C:N ratio		24:1	90:1	–

the sugarcane juice. Bagasse presented higher carbohydrate content, especially in form of cellulose and hemicellulose, since it is basically composed by sugarcane fibers. In this way, although both substrates displayed similar lignin content, it is expected that the negative effects of lignin during AD would be more significant in bagasse than filter cake because bagasse has 124% higher carbohydrates in fibrous form.

Both substrates presented large differences in terms of macronutrients. The carbon to nitrogen ratio of filter cake (24:1) is within the optimum range for AD (20–40:1), while bagasse presented much lower nitrogen content, resulting in higher C:N ratio of 90:1. If phosphorus and sulfur are also considered, the unbalance of nutrients is even more evident. Filter cake presented relatively low C:P and C:S ratios (68:1 and 58:1), what could lead in extreme cases to competition of sulfate-reduction with methanogenesis, reducing the conversion of organic acids into biogas, and also negatively influencing the bioavailability of trace elements inside the bioreactors (Gustavsson et al., 2013; Schmidt et al., 2014a).

Meanwhile, bagasse presented much higher values for C:P (4970:1) and C:S 2485:1, supporting the idea of a co-digestion strategy to balance those nutrients in order to improve the degradability and energy production from both substrates.

3.2. Start-up during the semi-continuous experiment

3.2.1. Mixture of digestates (MIX)

All reactors that used MIX as inoculum performed a relatively stable process during the start-up phase (days 0–69), except in a short period between days 20–30 for co-digestion with bagasse (reactors R3.5–3.6) and days 30–40 for mono-digestion of filter cake (reactors R3.3–3.4). In both cases acetic acid was the predominant accumulated VFA (around 90% of total VFA) suggesting a temporarily failure of the acetoclastic methanogenesis pathway during the adaptation of the microbial community to the substrate (Banks et al., 2012).

However, the same amount of accumulated acids caused different negative effects when both substrate options are compared. The VOA/TIC increased higher in mono-digestion than in co-digestion, since different concentrations of NH_4-N, at the moment when VFA started to accumulate, were found (Figs. 1 and 2). The higher NH_4-N concentration found during process instability of co-digestion (0.80 g L^{-1}) could have helped to buffer the system against the more severe pH drop found during the process instability of mono-digestion (NH_4-N of 0.57 g L^{-1}). Moreover, the process of inoculum washing-out that usually occurs immediately after the start-up of SCSTRs seems to be the reason why the concentration of NH_4-N was higher in co-digestion (instability at 20–30 days) than in mono-digestion (instability at 30–40 days) because the trend of gradually NH_4-N depletion can be seen in all reactors during the entire experiment period.

Regarding biogas production, the mono-digestion showed a 41% lower specific biogas production (SBP) than the theoretical biogas potential calculated based on substrate nutritional values (see Eq. (2)). In general, the SBP did not vary considerably during the entire phase, except during the process instability occurrence described previously. During this short period, a lower than average SBP was observed for both mono-digestion reactors, caused by VFA accumulation, followed by a rapidly increase over than the average SBP. This can be explained by the normal substrate feeding without any interruption or reduction in OLR, which has provided more VFA to biogas conversion at the moment when reactors recovered normal performance. The CH_4 content found in biogas was around 60% (v/v) during stable conditions and 55% (v/v) during the short unstable process period (Table 4).

When those results are compared to the co-digestion with bagasse, the SBP was 12.8% lower than in case of the mono-digestion, which in turn represents 50% lower than the theoretical biogas potential calculated based on substrate nutritional values. During the unstable period in the process, the co-digestion reactors behaved similarly to the mono-digestion, decreasing the SBP below average as result of the VFA accumulation and increasing above average afterward due to the same reasons previously described.

Additionally, the CH_4 content found in biogas from the co-digestion was lower than in mono-digestion during stable conditions and lower as well during the unstable period of the process. This can be explained by different substrate compositions, since according to Weißbach (2009) carbohydrate-based substrates would produce around 50–55% (co-digestion of filter cake and bagasse) of CH_4 in biogas, while a share of protein could increase CH_4 content up to 60–65% (mono-digestion of filter cake).

Furthermore, the higher cellulose and hemicellulose content found in bagasse might have been another factor that has influenced the differences in SBP between the mono-digestion and co-digestion with bagasse. According to Montgomery and Bochmann (2014) the recalcitrant presence of lignin may have hampered the hydrolysis of such fibrous materials resulting in a lower conversion of the volatile solids into biogas.

3.2.2. Fresh cattle manure (FCM)

A completely different situation was found during the start-up of co-digestion reactors inoculated with FCM (reactors R3.7–3.8). Due to the high VFA concentration in the inoculum, composed by 60% of acetic acid, 20% of propionic acid and 10% of butanoic acid, among other minor acids, both reactors were subjected to a greater process instability demanding a temporary feed reduction (OLR at 1.0 gVS L d^{-1}) during days 4–20, until VFA levels stabilized and VOA/TIC returned to the safe ratio of <0.5.

Therefore, the biogas production monitored during this period cannot be considered realistic from the substrate potential because most of the performed SBP were derived from the inoculum VFA conversion into biogas, which after further observation showed a drastic SBP reduction immediately after the consumption of VFA originated from the inoculum (Fig. 3).

Despite of the high VFA concentration, the measured pH during the entire start-up phase was in an optimum range considered for AD, mainly due to the high buffer capacity provided by FCM, of which the initial concentration of NH_4-N was around 117% higher than found in the other co-digestion system inoculated with MIX.

On one hand the higher NH_4-N concentration provided by FCM can be considered an advantage over the other evaluated inoculum, since in case of process instability, the reactor inoculated with FCM could withstand higher unbalances caused by VFA accumulation without major changes in the pH, which usually can happen during start-up of anaerobic reactors due to process instabilities caused by OLR increase and slow adaptations of microorganisms to the substrate.

On the other hand, the high VFA concentration found in FCM would demand a proper acclimation of inoculum to the reactor environment to reduce the VFA and VOA/TIC ratio to a safe level, which could delay the normal start-up of a large-scale biogas plant by at least 20 days. On the contrary, if the acclimation period is not respected, the VFA concentration could reach a certain level where the NH_4-N would not be able to keep an optimum pH value for methanogenesis (>6.5), leading to a possible failure of the AD process.

To compare the SBP of two co-digestion systems, it is appropriate to consider values achieved after the acclimation period because until day 20 most part of SBP in reactors R3.7–3.8 was derived from VFA degradation of the inoculum. In this way, the co-digestion system that used FCM as inoculum produced a similar SBP than observed in reactors inoculated with MIX. In contrast, CH_4 in reactors R3.7–3.8 has presented a slightly higher concentration in comparison to reactors R3.5–R3.6.

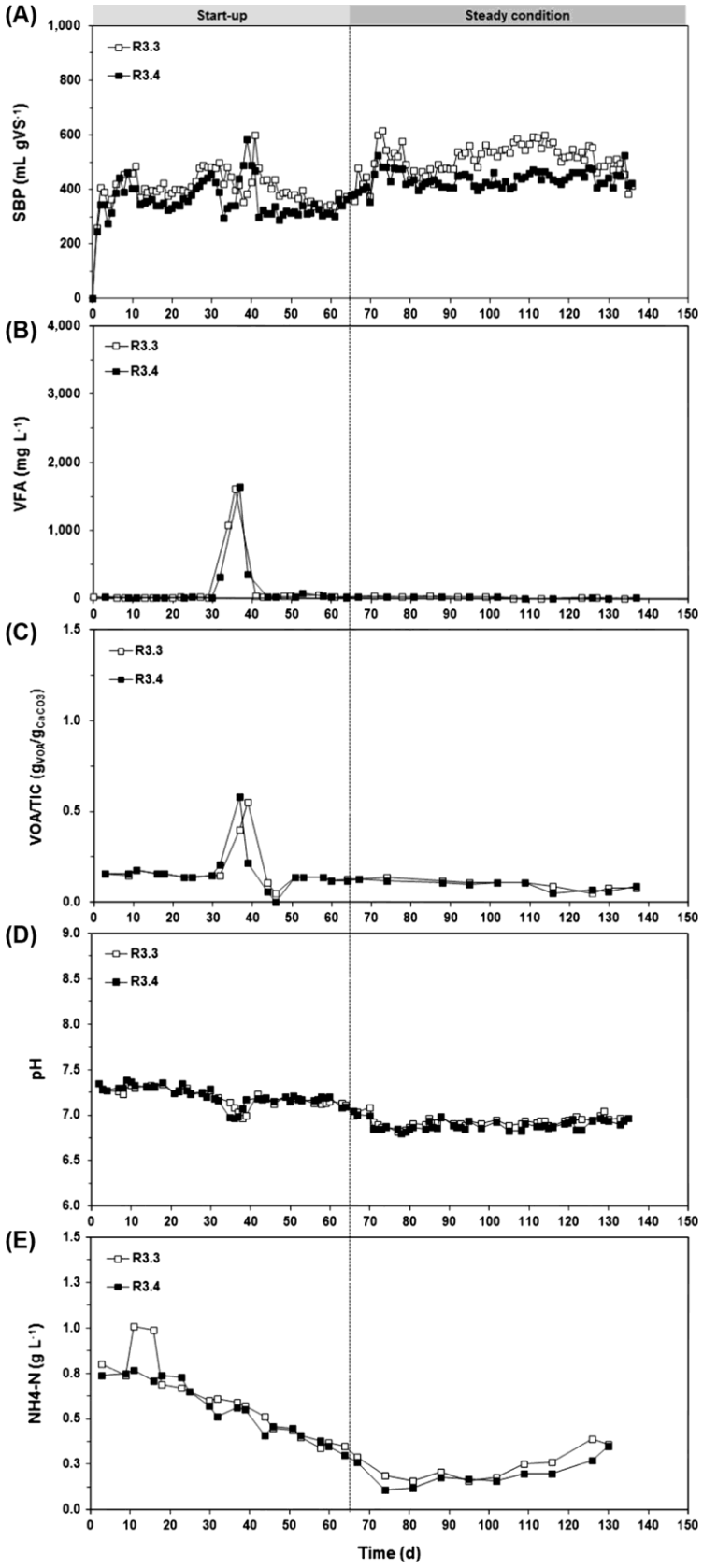

Fig. 1. Results during semi-continuous feeding experiment of filter cake using mix of inocula (Reactors R3.3 and R3.4). (A) Specific biogas production (SBP); (B) total volatile fatty acids (VFA); (C) ratio of volatile organic acids with total inorganic carbonate to calcium carbonate (VOA/TIC); (D) pH; (E) Ammonium-nitrogen concentration (NH_4-N).

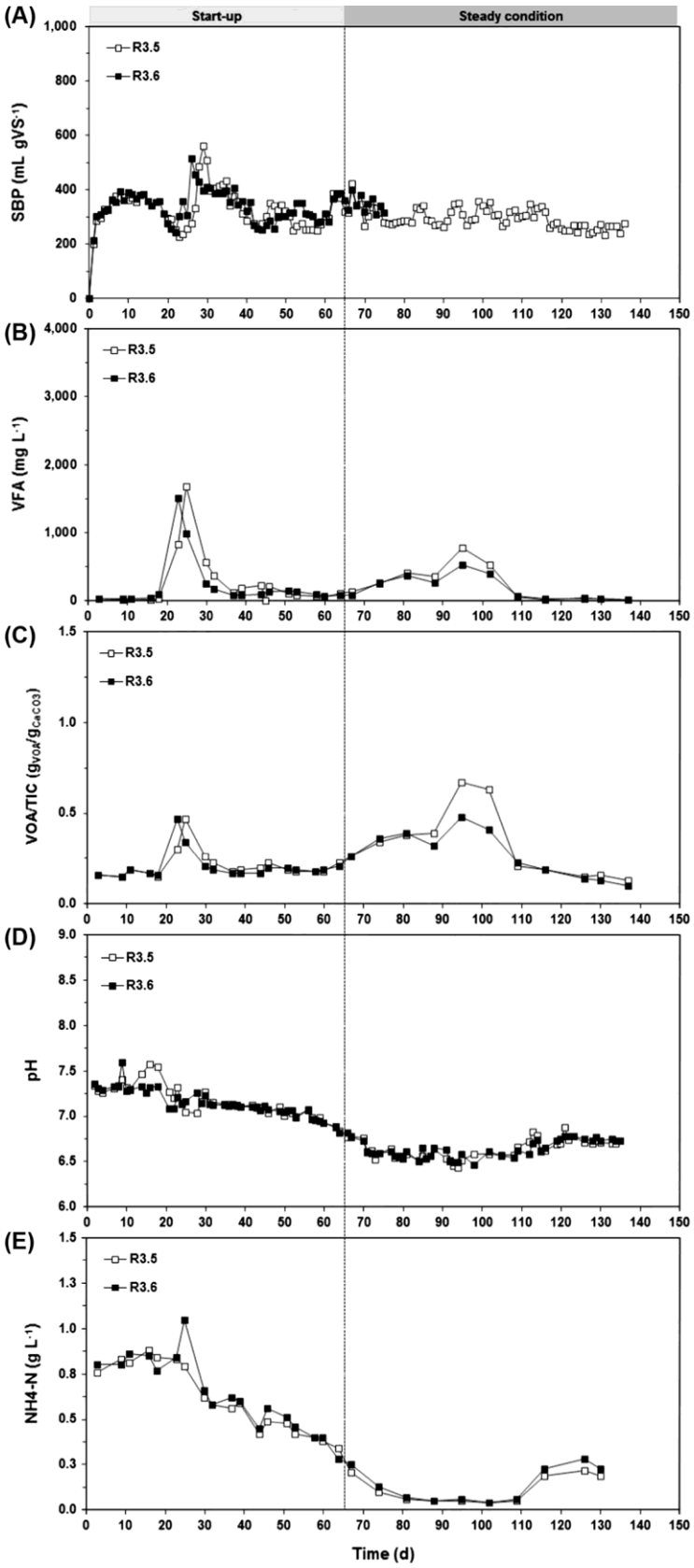

Fig. 2. Results during semi-continuous feeding experiment of filter cake and bagasse using mix of inocula (Reactors R3.5 and R3.6). (A) Specific biogas production (SBP); (B) total volatile fatty acids (VFA); (C) ratio of volatile organic acids with total inorganic carbonate to calcium carbonate (VOA/TIC); (D) pH; (E) Ammonium-nitrogen concentration (NH_4-N).

Table 4
Specific biogas production during start-up and steady condition.

Reactors	Phase	SBP (mL gVS^{-1})	CH_4 (% v/v)	TBP (mL gVS^{-1})	D_{index}
R3.3–R3.4	Start-up	382	60.0		0.59
	Steady I	480	57.9	650	0.74
	Steady II	471	58.7		0.72
R3.5–R3.6	Start-up	333	55.0		0.50
	Steady I	320	53.0		0.48
	Steady II	277	54.8		0.41
R3.7–R3.8	Start-up	326	54.4	671	0.49
	Steady I	320	52.3		0.48
	Steady II	277	55.6		0.41

SBP: specific biogas production.
TBP: theoretical biogas potential.
D_{index}: degradability index.

These findings are considered unexpected since the higher availability of nitrogen in the form of NH_4-N provided by FCM should have increased the conversion of the co-digestion substrate (C:N of 41:1) into biogas. This means that the type of inoculum has not influenced significantly the final energy production for the co-digestion with bagasse, leading to the conclusion that utilization of FCM did not result in any advantage over MIX, at least from the energy point of view.

3.3. Steady condition during the semi-continuous experiment

3.3.1. Mono-digestion of filter cake

After the start-up phase and inoculum wash-out, the OLR of all reactors was increased to 3.0 gVS L d^{-1} at day 70 and stayed until the end of the experiment at day 137. During this period the mono-digestion of filter cake (reactors R3.3–3.4) did not present any sign of process unbalance, even when the NH_4-N and pH achieved their lowest values observed during the entire experiment.

The mono-digestion system achieved a SBP of 480 mL gVS^{-1} (D_{index} of 0.74) during the period when the HRT was kept in 28.6 days (70–113 days), and slightly reduced when the HRT was changed to 23.1 days during the days 114–137. Such higher biogas production found during steady phase (+25%) in comparison to the start-up phase is explained by a higher biomass concentration inside the reactors found at day 137 (TS of 10.6% and VS 6.07%) in comparison to the biomass found during the beginning of experiment (TS of 4.02% and VS of 1.42%).

3.3.2. Co-digestion with bagasse

In contrast to the results obtained during the mono-digestion of filter cake, all reactors used for the co-digestion with bagasse (R.3.5–3.8) presented an acid accumulation immediately after the OLR increase from 2.5 to 3.0 gVS L d^{-1} at day 70 until day 105, this time mainly composed of propionic acid (90% of total VFA). According to previous studies conducted by Schmidt et al. (2014b), propionate oxidizing bacteria are sensitive to the lack of trace elements, especially iron and nickel depletion, which could be the reason of such VFA accumulation, since at this time of the experiment most part of the macronutrients and trace elements contained in MIX and FCM were already washed-out from the reactors.

Moreover, due to the lower buffer capacity as a result of the decreasing NH_4-N levels in comparison to the start-up phase, the VOA/TIC ratio achieved its highest value during the entire experiment (0.57). As a consequence, the pH dropped to its lowest observed level (6.45), which could have caused an inhibition of methanogenic activity leading to complete process failure.

In the meantime, the SBP of the co-digestion system (biogas production was measured only in reactors R3.5, R3.7 and R3.8 due to a technical failure of the milligascounter in reactor R3.6) reached the value of 320 mL gVS^{-1} (D_{index} of 0.48) when the HRT was kept in 35.8 days, and reduced by 13.2% when the HRT decreased to 27.6 days. However, as opposed to the mono-digestion, in both co-digestion systems the SBP observed during the steady phase was not higher than found during the reactors start-up (excluding the days 4–20 in reactors R3.7–R3.8), even considering an average increase in the biomass inside the reactors TS from 4% to 8%. In this case, the higher dependency of additional nutrients necessary for the co-digestion with bagasse (poorer in macronutrients) was possible supplied by both inocula during the reactor's start-up.

Indeed, the substrates composition played a major role during the semi-continuous experiments. The additional carbon supplied by bagasse in the co-digestion system was not able to improve the overall AD performance, mainly due to the form in which carbon was provided (fibrous carbohydrates). Moreover, the low concentration of phosphorus and sulfur found in bagasse has also negatively influenced the biogas production, since both elements are important during microbial growth (Kayhanian and Rich, 1995). In this case, a supplementation strategy based on phosphate and sulfate are recommended (Scherer et al., 2009), however demanding special attention to avoid excessive costs for the anaerobic system.

3.4. Preliminary assessment for large-scale process designing

In order to analyze the positive and negative aspects of different substrate options, an assessment of the main parameters used for designing the AD process integrated to a sugarcane plant with the capacity to process 2 million tons of cane per year is presented in Table 5.

Considering the same substrate proportions used during our semi-continuous digestion experiments, the co-digestion option would have 27,606 tons of VS y^{-1} to be used as substrate for biogas conversion, while mono-digestion would have 11,574 tons of VS y^{-1}. Such a difference is not only explained by higher substrate utilization during co-digestion (35 kg of filter cake per ton of cane + 15 kg of bagasse per ton of cane), but also by higher TS and VS content found in bagasse in comparison to filter cake.

Therefore, even considering that mono-digestion presented higher SBP during continuous digestion, the hourly biogas production of co-digestion (1008 m^3 h^{-1}) would be 58% higher than mono-digestion (634 m^3 h^{-1}).

However, such higher biogas production during co-digestion could only be achieved if the HRT would be 35.8 days, which is 25% longer than the HRT needed for mono-digestion (28.6 days). Thus, when the sizes of the reactors are calculated, the differences between the substrate options are even larger, and co-digestion would need a 24,303 m^3 reactor, while mono-digestion a 10,499 m^3 reactor. Nevertheless, differences on biogas production rate

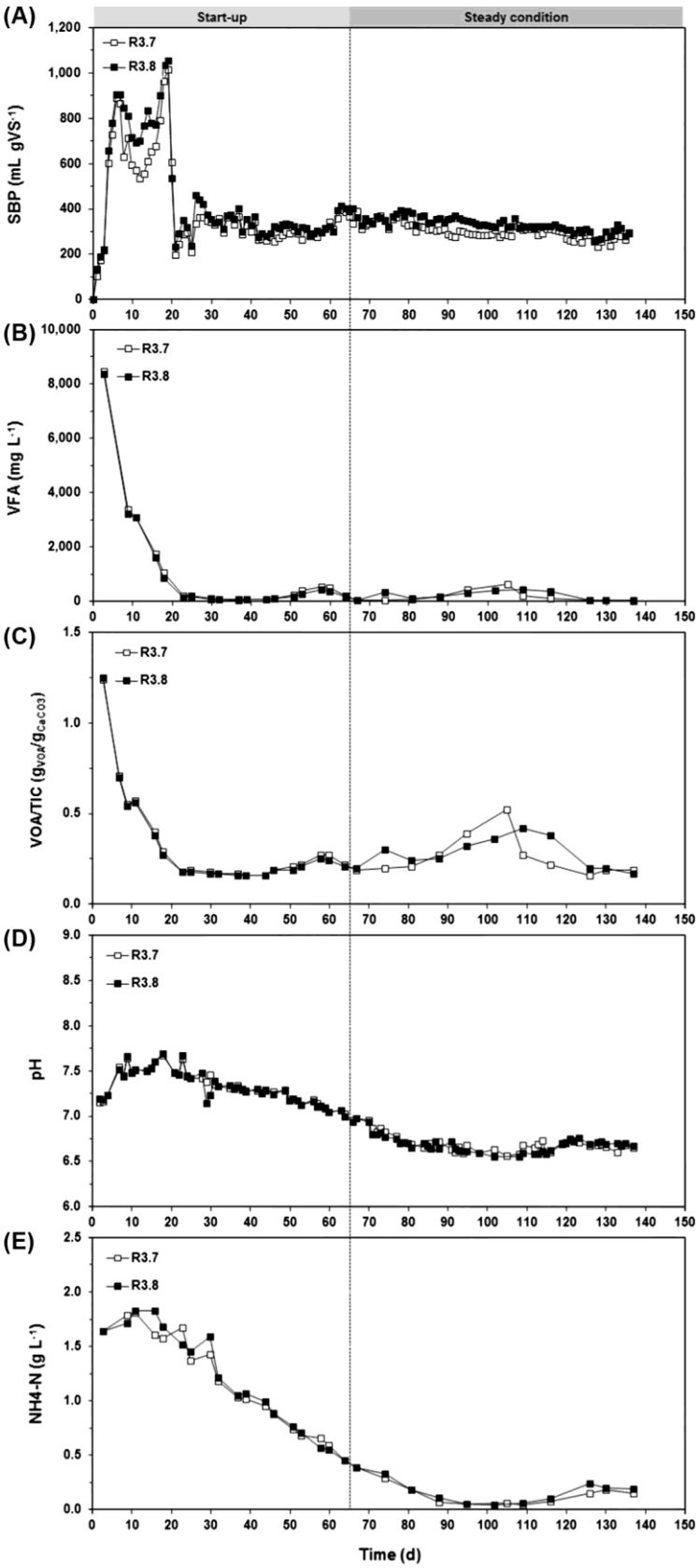

Fig. 3. Results during semi-continuous feeding experiment of filter cake and bagasse using fresh cattle manure as inoculum (Reactors R3.7 and R3.8). (A) Specific biogas production (SBP); (B) total volatile fatty acids (VFA); (C) ratio of volatile organic acids with total inorganic carbonate to calcium carbonate (VOA/TIC); (D) pH; (E) Ammonium-nitrogen concentration (NH_4-N).

Table 5
Main parameters used for designing the AD process in large-scale.

Substrates	Filter cake	Bagasse	Co-digestion[a]	Unit
Fresh matter	70,000	30,000	100,000	ton y^{-1}
TS	15,330	16,410	31,740	ton y^{-1}
VS	11,574	16,032	27,606	ton y^{-1}
SBP	480	–	320	mL gVS^{-1}
CH_4 content	60	–	55	% v/v
OLR	3.0	–	3.0	gVS L d^{-1}
HRT[b]	28.6	–	35.8	days
Biogas production	634	–	1008	$m^3 h^{-1}$
Size of reactor	10,499	–	24,303	m^3
Biogas production rate	1.45	–	0.99	m^3 biogas m^3 d^{-1}
SUC[c]	1.60	–	1.34	EUR ct kW h^{-1}

[a] Calculated based on the same substrate proportion used during semi-continuous feeding experiment: 70% of filter cake + 30% of bagasse on fresh matter.
[b] Calculated based on the fresh matter input + water necessary to adjust the TS below 15% according to semi-continuous feeding experiment.
[c] Specific upgrading cost calculated based on FNR (2014).

would be lower, since co-digestion would produce 0.99 m^3 biogas m^3 d^{-1} and mono-digestion 1.45 m^3 biogas m^3 d^{-1}.

On the other hand, if biogas is intended to be upgraded to biomethane, the specific upgrading cost decreases as the amount of biogas processed increases (scale effect). According to a market survey considering different upgrading technologies (FNR, 2014), the specific upgrading cost calculated for the co-digestion option would be 1.34 EUR ct kW h^{-1}, which is 16% lower than the upgrading cost for mono-digestion (1.60 EUR ct kW h^{-1}), thus in this case would give an advantage for co-digestion option over mono-digestion.

Finally, the application of a pre-treatment technique to breakdown the lignocellulosic structure of the substrates making cellulose and hemicellulose more accessible to microbial degradation could lead to different perspectives in terms of SBP and HRT (De Paoli et al., 2011; Schumacher et al., 2014), which would directly influence the main parameters used for process designing. However, questions regarding the application of pre-treatment techniques in large-scale still remain unclear, especially whether these types of pre-treatment would be able to provide net profit gain to the biogas system.

4. Conclusion

The present study demonstrated that fresh cattle manure is an alternative inoculum suitable for the start-up of anaerobic reactors if a proper acclimation period of at least 20 days to reduce the initial VFA concentration is respected. However, despite of presenting a higher availability of nitrogen in form of NH_4-N, fresh cattle manure was not able to improve the biogas production of filter cake co-digested with bagasse (C:N ratio of 41:1), possibly due to the recalcitrant lignocellulosic fraction found in bagasse.

Although the mono-digestion of filter cake displayed 50% higher specific biogas production in comparison to the option of co-digestion with bagasse during the steady condition phase of the semi-continuous experiment. If both substrate options are assessed for a large-scale sugarcane plant with capacity to process 2.0×10^6 tons of cane per year, the co-digestion with bagasse would produce 58% more biogas in comparison to the mono-digestion due to the higher biomass availability. In this case, the decision of which substrate option should be used would be mainly driven by the different construction techniques available in the market, since the biogas production rate of co-digestion with bagasse was 31% lower than the mono-digestion of filter cake.

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References

Banks, C.J., Zhang, Y., Jiang, Y., Heaven, S., 2012. Trace element requirements for stable food waste digestion at elevated ammonia concentrations. Bioresour. Technol. 104, 127–135. http://dx.doi.org/10.1016/j.biortech.2011.10.068.
Bressan Filho, A., 2011. A Geração Termoelétrica com a Queima do Bagaço de Cana-de-Açúcar no Brasil: Análise do Desempenho da Safra 2009–2010. Companhia Nacional de abastecimento, CONAB, Brasília.
Cho, K., Lee, J., Kim, W., Hwang, S., 2013. Behavior of methanogens during start-up of farm-scale anaerobic digester treating swine wastewater. Process Biochem. 48, 1441–1445. http://dx.doi.org/10.1016/j.procbio.2013.04.016.
De Carvalho Macedo, I., 2007. Sugar Cane's energy – Twelve Studies on Brazilian Sugar Cane, pp. 119–37.
De Paoli, F., Bauer, a., Leonhartsberger, C., Amon, B., Amon, T., 2011. Utilization of by-products from ethanol production as substrate for biogas production. Bioresour. Technol. 102, 6621–6624. http://dx.doi.org/10.1016/j.biortech.2011.03.045.
DIN, 2011a. DIN EN 15104 – Feste Biobrennstoffe – Bestimmung des Gesamtgehaltes an Kohlenstoff, Wasserstoff und Stickstoff – Instrumentelle Verfahren. DIN Deutsches Institut für Normung e. V., Berlin.
DIN, 2011b. DIN EN 16170 – Schlamm, behandelter Bioabfall und Boden – Bestimmung von spurenelementen mittels optischer Emissionsspektrometrie mit induktiv gekoppeltem Plasma (ICP-OES). DIN Deutsches Institut für Normung e. V., Berlin.
DIN, 2002. DIN EN ISO 15587-2 – Water quality – Digestion for the determination of selected elements in water – Part 2: Nitric acid digestion (ISO 15587-2:2002). DIN Deutsches Institut für Normung e. V., Berlin.
FNR, 2014. Leitfaden Biogasaufbereitung und – Einspeisung, 1st ed. Fachagentur Nachwachsende Rohstoffe e.V (FNR), Gülzow.
FNR, 2012. Guide to Biogas: From Production to Use.
Gustavsson, J., Shakeri Yekta, S., Sundberg, C., Karlsson, A., Ejlertsson, J., Skyllberg, U., Svensson, B.H., 2013. Bioavailability of cobalt and nickel during anaerobic digestion of sulfur-rich stillage for biogas formation. Appl. Energy 112, 473–477. http://dx.doi.org/10.1016/j.apenergy.2013.02.009.
Janke, L., Leite, A., Batista, K., Weinrich, S., Sträuber, H., Nikolausz, M., Nelles, M., Stinner, W., 2015a. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: effects of urea supplementation and sodium hydroxide pretreatment. Bioresour. Technol. http://dx.doi.org/10.1016/j.biortech.2015.07.117.
Janke, L., Leite, A., Nikolausz, M., Schmidt, T., Liebetrau, J., Nelles, M., Stinner, W., 2015b. Biogas production from sugarcane waste: assessment on kinetic challenges for process designing. Int. J. Mol. Sci. 16, 20685–20703. http://dx.doi.org/10.3390/ijms160920685.
Janke, L., Leite, A.F., Wedwitschka, H., Schmidt, T., Nikolausz, M., Stinner, W., 2014. Biomethane production integrated to the Brazilian sugarcane industry: the case study of São Paulo State. In: 22nd European Biomass Conference and Exhibition, pp. 23–26.
Kayhanian, M., Rich, D., 1995. Pilot-scale high solids thermophilic anaerobic digestion of municipal solid waste with an emphasis on nutrient requirements. Biomass Bioenergy 8, 433–444. http://dx.doi.org/10.1016/0961-9534(95)00043-7.
Kobayashi, T., Yasuda, D., Li, Y.Y., Kubota, K., Harada, H., Yu, H.Q., 2009. Characterization of start-up performance and archaeal community shifts during anaerobic self-degradation of waste-activated sludge. Bioresour. Technol. 100, 4981–4988. http://dx.doi.org/10.1016/j.biortech.2009.05.043.
Leite, A., Janke, L., Lv, Z., Harms, H., Richnow, H.-H., Nikolausz, M., 2015a. Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: effects of increasing organic loading rate on methanogenic community dynamics. Int. J. Mol. Sci. 16, 23210–23226. http://dx.doi.org/10.3390/ijms161023210.
Leite, A.F., Janke, L., Harms, H., Zang, J.W., Fonseca-Zang, W.a., Stinner, W., Nikolausz, M., 2015b. Assessment of the variations in characteristics and methane potential of major waste products from the Brazilian bioethanol industry along an operating season. Energy Fuels. http://dx.doi.org/10.1021/ef502807s, 150318103543009.
Liebetrau, J., Pfeiffer, D., Thrän, D., 2015. Messmethodensammlummg Biogas – Methoden zur Bestimmung von analytischen und prozessbeshreibenden Parametern im Biogasbereich. DBFZ Deutsches Biomasseforschungszentrum, Leipzig.

López González, L.M., Vervaeren, H., Pereda Reyes, I., Dumoulin, A., Romero Romero, O., Dewulf, J., 2013. Thermo-chemical pre-treatment to solubilize and improve anaerobic biodegradability of press mud. Bioresour. Technol. 131, 250–257. http://dx.doi.org/10.1016/j.biortech.2012.12.167.

Lv, Z., Hu, M., Harms, H., Richnow, H.H., Liebetrau, J., Nikolausz, M., 2014. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. Bioresour. Technol. 167, 251–259. http://dx.doi.org/10.1016/j.biortech.2014.06.029.

Mccarty, P.L., 1964. Anaerobic waste treatment fundamentals. Public Works 95, 107–112.

Montgomery, L.F.R., Bochmann, G., 2014. Pretreatment of feedstock for enhanced biogas production. IEA Bioenergy.

Moset, V., Bertolini, E., Cerisuelo, a., Cambra, M., Olmos, a., Cambra-López, M., 2014. Start-up strategies for thermophilic anaerobic digestion of pig manure. Energy 74, 389–395. http://dx.doi.org/10.1016/j.energy.2014.07.003.

Nogueira, L., Seabra, J., Best, G., Leal, M., Poppe, M., 2008. Bioetanol de cana-de-açúcar: energia para o desenvolvimento sustentável. BNDES, Rio de Janeiro.

Scherer, P., Neumann, L., Demirel, B., Schmidt, O., Unbehauen, M., 2009. Long term fermentation studies about the nutritional requirements for biogasification of fodder beet silage as mono-substrate. Biomass Bioenergy 33, 873–881. http://dx.doi.org/10.1016/j.biombioe.2009.01.011.

Schmidt, T., Nelles, M., Scholwin, F., Pröter, J., 2014a. Trace element supplementation in the biogas production from wheat stillage – optimization of metal dosing. Bioresour. Technol. 168, 80–85. http://dx.doi.org/10.1016/j.biortech.2014.02.124.

Schmidt, T., Ziganshin, A.M., Nikolausz, M., Scholwin, F., Nelles, M., Kleinsteuber, S., Pröter, J., 2014b. Effects of the reduction of the hydraulic retention time to 1.5 days at constant organic loading in CSTR, ASBR, and fixed-bed reactors – performance and methanogenic community composition. Biomass Bioenergy 69, 241–248. http://dx.doi.org/10.1016/j.biombioe.2014.07.021.

Schumacher, B., Wedwitschka, H., Hofmann, J., Denysenko, V., Lorenz, H., Liebetrau, J., 2014. Disintegration in the biogas sector – technologies and effects. Bioresour. Technol. 168, 2–6. http://dx.doi.org/10.1016/j.biortech.2014.02.027.

Seadi, T. Al, Rutz, D., Prassl, H., Köttner, M., Finsterwalder, T., 2008. Biogas Handbook.

VDI, 2006. VDI 4630 – Fermentation of organic materials: Characterisation of the substrate, sampling, collection of material data, fermentation tests. VDI-Gesselschaft Energietechnik.

Weißbach, F., 2009. Die Bewertung von nachwachsenden Rohstoffen für die Biogasgewin- nung. Teil I: Das Gasbildungspotenzial der fermentierbaren Nährstoffe. Pflanzenbauwissenschaften 13, 72–85.

3.3. Lessons learned from the microbial ecology resulting from different inoculation strategies for biogas production from waste products of the bioethanol/sugar industry

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Biotechnology for Biofuels

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Lessons learned from the microbial ecology resulting from different inoculation strategies for biogas production from waste products of the bioethanol/sugar industry

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Abstract

Background: During strategic planning of a biogas plant, the local availability of resources for start-up and operation should be taken into consideration for a cost-efficient process. Because most bioethanol/sugar industries in Brazil are located in remote areas, the use of fresh cattle manure from local farms could be a solution for the inoculation of the biogas process. This study investigated the diversity and dynamics of bacterial and archaeal communities and the performance of biogas reactors inoculated with manure and a mixed inoculum from different biogas reactors as for a controlled start-up until steady state.

Results: Laboratory-scale biogas reactors were fed semi-continuously with sugarcane filter cake alone (mono-digestion) or together with bagasse (co-digestion). At the initial start-up, the reactors inoculated with the mixed inoculum displayed a less diverse taxonomic composition, but with higher presence of significant abundances compared to reactors inoculated with manure. However, in the final steady state, the communities of the differently inoculated reactors were very similarly characterized by predominance of the methanogenic genera *Methanosarcina* and *Methanobacterium*, the bacterial families *Bacteroidaceae*, *Prevotellaceae* and *Porphyromonadaceae* (phylum *Bacteroidetes*) and *Synergistaceae* (phylum *Synergistetes*). In the mono-digestion reactors, the methanogenic communities varied greater than in the co-digestion reactors independently of the inoculation strategy.

Conclusion: The microbial communities involved in the biogas production from waste products of the Brazilian bioethanol/sugar industry were relatively similar and stable at the reactor's steady phase independently of the inoculum source (manure or mixed inoculum). Therefore, the locally available manure can be used as inoculum for start-up of the biogas process, since it also contains the microbial resources needed. The strong fluctuation of methanogenic communities in mono-digestion reactors indicates higher risk of process instability than in co-digestion reactors.

Keywords: Inoculation, Biogas process, Cattle manure, Bioethanol/sugar waste, 454 Pyrosequencing, Methanogens

Background

The Brazilian bioethanol/sugar industry has been previously reported in our studies to have a big potential to improve the local bioeconomy while reducing greenhouse gas emission by applying biogas technology to the treatment of its waste products [1 – 3]. However, to make the biogas technology profitable and reliable for the Brazilian bioethanol/sugar industry, the reactor design and start-up requires strategic considerations.

In regions where biogas plants are widespread and well developed, a biogas reactor can be started with inocula from already established processes. Contrary to this scenario, in Brazil there are only very few plants applying the anaerobic digestion (AD) process to treat waste at large scale and these plants are spread across the

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country and separated by long distances. Therefore, we have addressed the possibility of reactor inoculation with fresh cattle manure (FCM) as a locally available, potential inoculum. For comparison in terms of microbial robustness, we have also prepared an engineered mixed inoculum (referred to as MIX) originating from the digestate of different biogas reactors fed mainly with energy crops and agricultural wastes such as maize silage, thin stillage, straw and chicken manure. The first results regarding biogas production and feasibility of these two different inoculation strategies were recently reported by Janke et al. [4]. Nevertheless, the microbiological background of this experiment remained to be investigated.

In the biogas process, a complex metabolic network of microorganisms is responsible for organic matter degradation, which proceeds in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. While bacteria are involved in the first three steps, methanogenic archaea are responsible for the last step. Methanogens are very sensitive to process changes due to the relative lack of functional redundancy and low diversity [5]. Besides that, the methanogens are very important for the AD process stability, because they are directly involved in the removal of fermentation product acetate or makes the syntrophic oxidation of acetate and other fermentation products thermodynamically feasible by keeping hydrogen partial pressure low [6]. Therefore, the methanogenic community requires particular regard for the development of an efficient and robust AD process.

The methane formation by the methanogens is carried out either via direct acetate conversion (aceticlastic methanogenesis) or reduction of CO_2 with H_2 (hydrogenotrophic methanogenesis). The determination of different methanogenic pathways has been described to have crucial implications for the design and operation of biogas reactors [5, 7 – 10], since the aceticlastic and hydrogenotrophic methanogens may have different growth rates depending on the reactor' s conditions [11]. However, the factors controlling the balance of methanogenic pathways is still not clear and seems to depend on the substrate used and process conditions such as organic loading rate, reactor type and temperature [12 – 14].

Knowledge of microbial adaptation to environmental conditions is also very important to understand the complex interplay between bacteria and methanogens [15, 16], particularly during acclimatization leading to successful and efficient reactor operation [17]. Moreover, correlations of methanogenic community data with process parameters have been reported to contain decisive information about shifting pathway dominance during biogas production [9, 18, 19].

In a comprehensive study, we investigated the impact of different inoculation strategies and digestion setups (mono-digestion of filter cake and its co-digestion with bagasse) on the microbial community composition and dynamics along the operation of six mesophilic laboratory-scale continuously stirred tank reactors. In addition, the bacterial and methanogenic communities were assessed to identify key microorganisms of indicator value for the AD of the waste products from the bioethanol/sugar industry. Moreover, this study evaluated the links between microbial composition and reactor performance as a basis for an efficient future microbial resource management. The microbial communities were analyzed by DNA-based fingerprinting techniques and next-generation sequencing. The correlation between the microbial communities and reactor parameters was established by multivariate data analysis. In addition, the dominant methanogenic pathway was determined by the stable isotope fingerprinting of the produced biogas, following approaches applied to various anaerobic systems [20] and biogas reactors with several substrates [7, 21, 22].

Methods

Biogas reactors

Six identical laboratory-scale continuously stirred tank reactors with working volumes of 3 L under mesophilic conditions (38 ± 1 °C) were established to operate three experiments in duplicate: R3.3 and R3.4 were inoculated with MIX and fed with a single substrate (mono-digestion of filter cake); R3.5 and R3.6 obtained the same inoculum, but the filter cake was co-digested with bagasse; R3.7 and R3.8 were inoculated with FCM and performed co-digestion similarly to R3.5 and R.3.6. Figure 1 shows the major process parameters for each reactor along the whole experiment. The analytical methods and detailed description of the start-up and performance of the reactors are reported in our previous study [4]. Briefly, the experiments of 137 days duration were divided into two phases: start-up (until day 69) and steady state (from day 70 on), as shown in Table 1. In our experiments, the day 0 does not represent the inoculation with MIX and FCM, but the end of an acclimatization time of around 24 h. On day 0, the feeding of the reactors was started. During the start-up phase, the organic loading rate (OLR) varied from 1.0 (only R3.7 and R3.8) or 2.0 (the other four reactors) to 2.5 $g_{vs}\,L^{-1}\,days^{-1}$ (all reactors), whereas during the steady state the OLR was constant at 3.0 $g_{vs}\,L^{-1}\,days^{-1}$. The hydraulic retention times (HRT) at the final stage of the steady state were 23 and 28 days for mono- and co-digestion, respectively.

Microbial community analysis

Duplicate digestate samples from each reactor were taken for molecular analysis on specific days and stored at −20 °C until further analysis. Simultaneously, biogas

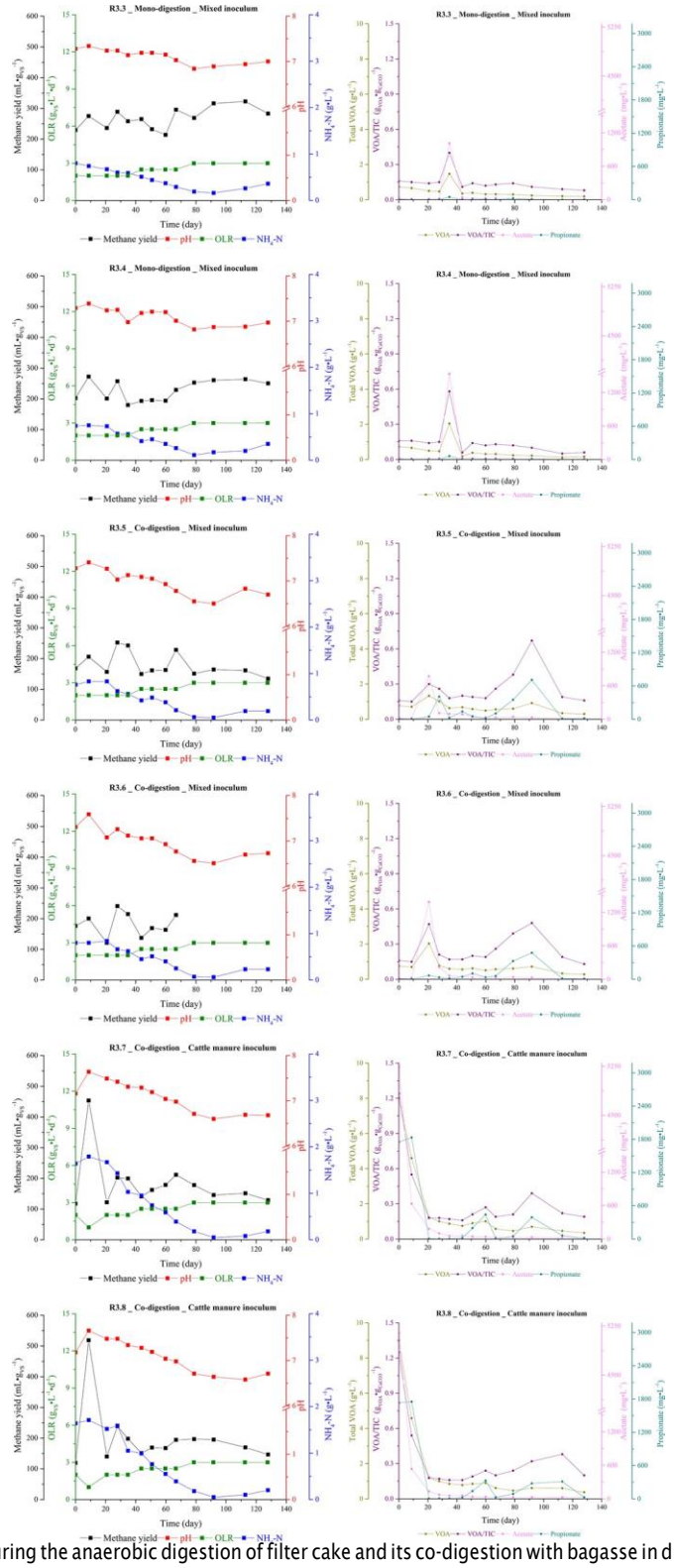


Fig. 1 Major process parameters during the anaerobic digestion of filter cake and its co-digestion with bagasse in different inoculation strategies (MIX and FCM). The data showed in this figure was adapted from our previous study [4]. The process parameters data corresponds to the same sampling time of the digestate and the biogas for the molecular and isotopic analyses, respectively

Table 1 Feeding regime divided into start-up and steady state according to variations on HRT and OLR. This table was adjusted from our previous study [4]

Reactors	Inoculum	C:N ratio	Phase	Period (day)	Filter cake input (g day^{-1})	Bagasse input (g day^{-1})	Water input (mL day^{-1})	HRT (days)	OLR (g_{vs} L^{-1} d^{-1})
R3.3 R3.4	MIX	24:1	Start-up	Initial (0–41)	36.5	–	50	34.7	2.0
				Final (42–69)	45.7	–	50	31.4	2.5
			Steady	Initial (70–113)	54.8	–	50	28.6	3.0
				Final (114–137)	54.8	–	75	23.1	3.0
R3.5 R3.6	MIX	41:1	Start-up	Initial (0–41)	15.78	6.78	50	41.4	2.0
				Final (42–69)	19.73	8.45	50	38.4	2.5
			Steady	Initial (70–113)	23.67	10.15	50	35.8	3.0
				Final (114–137)	23.67	10.15	75	27.6	3.0
R3.7 R3.8	FCM	41:1	Start-up	Initial I (0–3)	15.78	6.78	50	41.4	2.0
				Initial II (4–20)	7.89	3.39	25	82.7	1.0
				Initial III (21–41)	15.78	6.78	50	41.4	2.0
				Final (42–69)	19.73	8.45	50	38.4	2.5
			Steady	Initial (70–113)	23.67	10.15	50	35.8	3.0
				final (114–137)	23.67	10.15	75	27.6	3.0

samples were taken from the reactors' headspace for stable isotope analysis. The total genomic DNA of the bacterial and methanogenic communities was extracted with the 'NucleoSpin Soil' kit (Macherey – Nagel) as recommended by the supplier. The buffers SL2 and SX were used.

PCR amplifications for terminal restriction fragment length polymorphism (T-RFLP) screening of the methanogenic community were targeting the *mcrA* genes using the forward primer mlas and the reverse primer mcrA-rev and following the PCR protocol of Steinberg and Regan [23]. T-RFLP analysis of purified PCR products was conducted after digestion with the restriction enzyme *Bst*NI using the fragment size standard GeneScan-500 ROX (Applied Biosystems GmbH, Weiterstadt, Germany). T-RFLP electropherograms were processed as described by Lucas et al. [24]. During statistical analysis in R, signals with low peak areas were removed using a cutoff of 12 times the standard deviation of the data sets. The reproducibility of the T-RFLP was validated by comparing the results with duplicate samples from each reactor at a particular sampling day (Additional file 1: Figure S1). The *mcrA*-derived T-RFs were assigned taxonomically using cloned *mcrA* amplicons database from anaerobic digester sample analyses performed in our laboratory [21, 24 – 27].

The bacterial community analysis was performed only for the four co-digestion reactors inoculated with either FCM or MIX. Samples from two times (days 0 and 44) in the start-up phase and from one time (day 113) of the steady state were processed based on the 16S ribosomal RNA genes and further analyzed on the 454-pyrosequencing platform GS Junior (Roche) as described by Ziganshin et al. [28]. The variable regions V1 – V3 of the bacterial 16S rRNA gene fragments were amplified with the primers Bac27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and Bac519R (5′-GWATTACCGCGGCKGCTG-3′) using the Phire Hot Start II DNA Polymerase (Thermo Scientific). The raw sequence data were assessed with the QIIME 1.8.0 Virtual Box release [29]. Further data processing was performed according to Lucas et al. [24] and Sun et al. [14]. In summary, the dataset was firstly quality filtered by excluding sequences that were shorter than 150 and longer than 590 bp in lengths, comprised an average quality score below 25, held 50 bp at the end section below the quality score threshold of 25, comprised ambiguous bases, held a homopolymer run with more than 6 bp, or did not comprise any primer or barcode sequence. The USEARCH pipeline was applied on the sequences for further quality filtering based on non-chimeric sequences and for clustering into operational taxonomic units (OTUs) consisting of 97 % identity threshold [30]. The taxonomic classification based on representative sequences was performed using the Greengenes core set (gg_13_8) [31] and the Ribosomal Database Project classifier 2.2 [32]. For the taxonomic alignment, the Infernal algorithm with default setting was used [33]. Finally, the summarized OTU tables were constructed according to their taxonomy and abundance. Further, the visualization of the OTU tables was

processed via the spreadsheet program. De-multiplexed sequences of the 12 samples were deposited under the EMBL-EBI accession number PRJEB12073 (http://www.ebi.ac.uk/ena/data/view/PRJEB12073). The ecological data analyses leading to chao1, Shannon and Simpson indices [34, 35] and rarefaction curve were also performed with the QIIME software based on alpha diversity. Due to the differences in sequencing library size between the samples, we have used QIIME further to subsample (rarefy) the libraries down to 8000 sequences per sample for comparative diversity analyses and to calculate the beta diversity (pairwise sample dissimilarity).

The ordination of the dissimilarity matrices achieved by non-metric multidimensional scaling (NMDS) was processed as reported by Lucas et al. [24]. Shortly, the variability of the microbial communities was evaluated by the Bray – Curtis dissimilarity index based on the presence and relative abundance. Thus, e.g., highly similar community composition is indicated by tiny distances. The correlation of reactor parameters and microbial communities based on the relative abundance was analyzed with the 'envfit' function and its significance was tested by a Monte Carlo test with 999 permutations. The significance threshold was set to a maxima of 0.001 and 0.05 for the methanogenic and the bacterial communities, respectively.

Stable isotope fingerprinting

The carbon and hydrogen stable isotope compositions of CH_4 and CO_2 from each reactor were measured in triplicate biogas samples collected in 20-mL gas-tight pre-evacuated vials. For analysis, an isotope ratio mass spectrometry system (Finnigan MAT 253, Thermofinnigan Bremen) coupled to a gas chromatograph (GC) (HP 6890 Series, Agilent Technology, USA) via a combustion device and a pyrolysis unit (with a water-removal assembly) was used for carbon and hydrogen measurements, respectively. Fifty-microliter biogas sample was injected into the inlet tube of the GC instrument equipped with a CP-Porabond Q column (50 m × 0.32 mm ID Varian, USA) held at a constant temperature of 40 °C. Helium was used as a carrying gas at the split ratio of 1:50 for carbon and 1:5 for hydrogen analysis. The isotope ratios of all samples are given in delta notation ($\delta^{13}C$ and $\delta^{2}H$) in per mil (‰) units according to the standards VPDB (Vienna Pee Dee Belemnite) for carbon and VSMOW (Vienna Standard Mean Ocean Water) for hydrogen.

Results and discussion

Bacterial community succession

The microbial profiles of anaerobic digesters have been reported to be very specific for each type of reactor and substrate feeding [12, 28, 36]. Thus, to investigate the shaping forces of novel substrates (filter cake and bagasse) on the inocula (FCM and MIX), the bacterial community in the co-digestion reactors (R3.5, R3.6, R3.7 and R3.8) was assessed by amplicon pyrosequencing at three sampling times, i.e., days 0 and 44 in the start-up phase and day 113 during steady state. The bacterial community succession of each reactor is shown on phylum level in Fig. 2. The parallel reactors R3.5 and R3.6, and R3.7 and R3.8 had very similar bacterial profiles. The community similarity between the reactor samples based on the beta diversity are shown in Additional file 1: Table S1 and Figure S3. All OTUs obtained from the 12 samples are presented in Additional file 2: Table S2, and the respective rarefaction curves are shown in the Additional file 1: Figure S2.

We obtained on average around 12,000 high-quality sequence reads per reactor, varying from circa 9000 to 13,000. Along the operation of the co-digestion reactors during start-up and steady state, the taxonomic composition of our analyzed reactors was very diverse with a total of 1137 OTUs, but only 18 core OTUs (1.6 %), which were found in all reactors at all sampling times (Fig. 3), accounting for 18 % of all sequence reads. Thus, the variation of bacterial communities was very high with only a few microorganisms being significantly abundant in all reactors throughout the experiment. Examples were the families *Porphyromonadaceae* and *Synergistaceae* accounting for 8 and 4 % of all sequence reads, respectively.

Figure 3 also shows the variation of the numbers of core OTUs and phyla along the experiment. Day 0 samples contained the highest numbers of unique OTUs shared either only by the MIX parallels R3.5 and R3.6 (242, 21 % of all OTUs) or by the FCM parallels R3.7 and R3.8 (313, 28 % of all OTUs). The parallel FCM reactors R3.7 and R3.8 were more diverse in terms of OTUs, but only few microorganisms of high relative abundance were detected. On the other hand, parallel MIX reactors R3.5 and R3.6 presented lower diversity comprising microorganisms with high relative abundances (Fig. 2). On day 44, the number of total shared OTUs and phyla for all reactors was significantly increased, whereas the amount of unique OTUs per parallel reactors was drastically decreased (57 for R3.5 and R3.6 and 90 for R3.7 and R3.8). On day 113, the number of total OTUs and phyla shared by all reactors was decreased to 110 and 16, respectively. Moreover, the number of unique OTUs shared by parallel reactors was much lower.

These variations indicate that despite different inoculation, the bacterial communities were already very similar after 44 days of reactor operation. Nevertheless, the consolidation and stabilization of the bacterial communities proceeded until the steady state. Figure 3 shows that there was uniqueness in terms of OTUs and phyla of

	R3.5			R3.6			R3.7			R3.8		
	Day			Day			Day			Day		
	0	44	113	0	44	113	0	44	113	0	44	113
Unclassified bacteria	0.0001	0.0002	0.0002	0.0000	0.0001	0.0003	0.0000	0.0000	0.0003	0.0000	0.0000	0.0001
Other bacteria	0.0323	0.0278	0.0009	0.0295	0.0349	0.0013	0.0107	0.0223	0.0010	0.0079	0.0235	0.0029
Acidobacteria	0.0003	0.0025	0.0027	0.0005	0.0066	0.0048	0.0002	0.0064	0.0028	0.0000	0.0042	0.0028
Actinobacteria	0.0186	0.0059	0.0028	0.0166	0.0070	0.0081	0.0296	0.0027	0.0039	0.0294	0.0013	0.0076
Armatimonadetes	0.0015	0.0076	0.0034	0.0006	0.0052	0.0053	0.0000	0.0027	0.0013	0.0000	0.0019	0.0018
BRC1	0.0000	0.0000	0.0011	0.0000	0.0001	0.0006	0.0000	0.0005	0.0003	0.0000	0.0003	0.0000
Bacteroidetes	0.1784	0.3514	0.7030	0.1776	0.3442	0.6364	0.2409	0.3266	0.6298	0.2571	0.2856	0.6749
Chlorobi	0.0000	0.0017	0.0007	0.0001	0.0009	0.0017	0.0003	0.0006	0.0031	0.0003	0.0003	0.0042
Chloroflexi	0.0054	0.0047	0.0226	0.0062	0.0046	0.0378	0.0031	0.0072	0.0053	0.0039	0.0032	0.0027
Cyanobacteria	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Elusimicrobia	0.0041	0.0048	0.0055	0.0070	0.0029	0.0058	0.0001	0.0011	0.0061	0.0000	0.0011	0.0061
Fibrobacteres	0.0123	0.0063	0.0003	0.0164	0.0056	0.0005	0.0012	0.0079	0.0011	0.0006	0.0110	0.0016
Firmicutes	0.4356	0.3600	0.1083	0.4455	0.3248	0.1476	0.6151	0.4119	0.2270	0.5939	0.4525	0.1894
Lentisphaerae	0.0063	0.0015	0.0002	0.0059	0.0013	0.0002	0.0036	0.0027	0.0002	0.0037	0.0025	0.0003
NKB19	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0001	0.0003	0.0002
OD1	0.0001	0.0081	0.0000	0.0001	0.0043	0.0000	0.0001	0.0095	0.0000	0.0003	0.0106	0.0004
OP3	0.0012	0.0001	0.0000	0.0007	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
OP9	0.0005	0.0020	0.0002	0.0006	0.0022	0.0002	0.0001	0.0007	0.0001	0.0002	0.0015	0.0003
Planctomycetes	0.0056	0.0032	0.0007	0.0057	0.0055	0.0006	0.0056	0.0035	0.0004	0.0072	0.0085	0.0001
Proteobacteria	0.0061	0.0112	0.0083	0.0058	0.0132	0.0091	0.0128	0.0113	0.0036	0.0156	0.0089	0.0089
SR1	0.0034	0.0070	0.0000	0.0035	0.0026	0.0000	0.0001	0.0043	0.0000	0.0001	0.0091	0.0000
Spirochaetes	0.0612	0.0024	0.0024	0.0562	0.0034	0.0052	0.0367	0.0078	0.0044	0.0363	0.0095	0.0039
Synergistetes	0.0506	0.0478	0.1344	0.0475	0.0653	0.1320	0.0154	0.0461	0.1050	0.0153	0.0232	0.0837
TM6	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004
TM7	0.0005	0.0000	0.0000	0.0006	0.0000	0.0000	0.0006	0.0003	0.0000	0.0008	0.0002	0.0000
Tenericutes	0.0330	0.0070	0.0000	0.0325	0.0092	0.0000	0.0212	0.0068	0.0003	0.0197	0.0112	0.0004
Thermotogae	0.0728	0.0020	0.0000	0.0775	0.0052	0.0002	0.0000	0.0002	0.0000	0.0000	0.0003	0.0000
Verrucomicrobia	0.0012	0.0099	0.0000	0.0014	0.0145	0.0000	0.0005	0.0034	0.0002	0.0039	0.0040	0.0002
WPS-2	0.0061	0.0022	0.0000	0.0059	0.0009	0.0000	0.0000	0.0007	0.0000	0.0000	0.0003	0.0002
WS1	0.0000	0.0018	0.0023	0.0001	0.0020	0.0019	0.0000	0.0008	0.0034	0.0001	0.0007	0.0066
WS4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0003	0.0000	0.0000	0.0001
WWE1	0.0629	0.1209	0.0000	0.0561	0.1333	0.0000	0.0011	0.1117	0.0000	0.0029	0.1244	0.0000
[Thermi]	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0013	0.0000	0.0000	0.0008	0.0000	0.0000

Fig. 2 Phylogenetic composition and succession of the bacterial communities. Heatmap displaying the relative abundance of the bacterial communities at the phylum level along the experiment using different inoculation strategies (MIX and FCM) for the co-digestion of filter cake and bagasse. The relative abundances were based on the 454-pyrosequencing of 16S ribosomal RNA gene amplicons

individual reactors along the experiment, although R3.5 and R3.6 as well as R3.7 and R3.8 were operated in parallel under the same conditions.

The estimated richness of the bacterial community in all samples is shown in Table 2. The number of OTUs decreased slightly during start-up, whereas at steady state a drastic drop was observed. The same trend was reflected by the OTU richness estimator chao 1 and the Shannon index. The Simpson diversity was stable between the two samplings during start-up, while it decreased during steady state. In MIX reactors R3.5 and R3.6, the Simpson index dropped more than in the FCM reactors R3.7 and R3.8, indicating higher community evenness in the former. Generally, the bacterial species richness was lower during steady state with more pronounced predominance of some microorganisms.

The phyla *Firmicutes* and *Bacteroidetes* dominated the bacterial communities in all analyzed reactors along the entire operation time (Fig. 2). Both together comprised 72 % of all sequence reads. The presence of most OTUs affiliated with these two phyla known to utilize carbohydrates has frequently been reported in studies about AD of different substrates, mainly maize silage and manure, in laboratory- and full-scale biogas reactors [12, 24, 28, 36, 37].

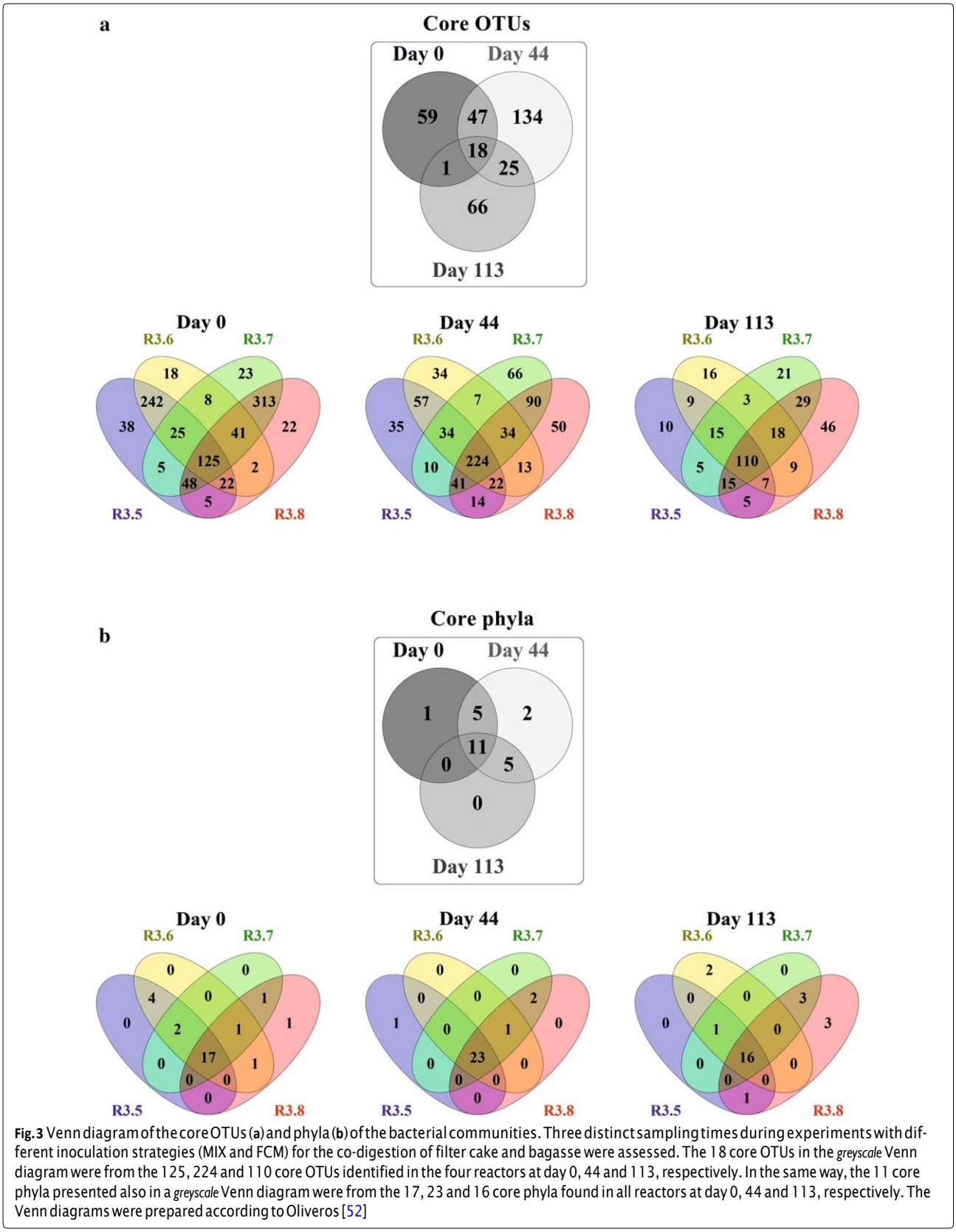


Fig. 3 Venn diagram of the core OTUs (**a**) and phyla (**b**) of the bacterial communities. Three distinct sampling times during experiments with different inoculation strategies (MIX and FCM) for the co-digestion of filter cake and bagasse were assessed. The 18 core OTUs in the *greyscale* Venn diagram were from the 125, 224 and 110 core OTUs identified in the four reactors at day 0, 44 and 113, respectively. In the same way, the 11 core phyla presented also in a *greyscale* Venn diagram were from the 17, 23 and 16 core phyla found in all reactors at day 0, 44 and 113, respectively. The Venn diagrams were prepared according to Oliveros [52]

Although the succession toward steady state was not favorable for *Firmicutes*, the phylum was the most diverse taxonomic group in the reactors with 620 OTUs (54 %), represented mainly by the order *Clostridiales* (485 OTUs), in which the families *Ruminococcaceae* (153 OTUs), *Clostridiaceae* (53 OTUs) and *Lachnospiraceae* (41 OTUs) were predominant. Following the succession of these families during our experiment, *Ruminococcaceae* was the most constant family, without much variation in its abundance (around 5 % in all reactor samples). *Clostridiaceae* had also a constant abundance around 0.8 % in the reactors inoculated with MIX. However, the same family was the most variable one in the reactors inoculated with FCM, presenting 21.8 and 1.3 % relative abundances at the beginning (day 0) and end of the experiment (day 113), respectively. The family *Lachnospiraceae* was relatively constant at abundances around 1.8 %, except for the reactors at the beginning of the experiment with MIX. Hence, our results with sugarcane waste products were in agreement with former studies that also investigated the bacterial communities in the AD of plant-based biomass [37, 38], especially in terms of the prevalence of *Clostridiales*. Members of this order are equipped with the cellulosome, a multienzyme complex, which enables them to efficiently hydrolyze recalcitrant cellulosic and hemicellulosic structures in the plant cell wall [39]. Our feeding substrates from the sugarcane industry contain high percentages of cellulose and hemicellulose, both together representing 75 and 55 % of the total carbohydrate and lignin content of bagasse and filter cake, respectively [1]. Sequences affiliated to the cellulosome-producing bacterium *Ruminococcus flavefaciens* were detected in our reactors. These type of sequences related to the anaerobic cellulolytic rumen bacterium contributed significant proportions of sequence reads in our experiment (0.79 % of all), especially at the end at day 113 (0.34 %), which suggests that this phylotype was one of the specialists degrading bagasse and filter cake, since both inocula sustained its presence. Members of the genus *Ruminococcus* are also known to produce hydrogen and acetate, thus supplying the hydrogenotrophic methanogenic genus *Methanobacterium* [40].

Bacteroidetes as the second most diverse phylum with 128 OTUs showed a gradual increase in relative abundance toward steady state. Within this phylum, OTUs affiliated with the genera *Bacteroides* (4 OTUs, ca. 9.4 % of all sequence reads) and *Prevotella* (4 OTUs, ca. 7.2 % of all reads) and the family *Porphyromonadaceae* (39 OTUs, ca. 9.8 % of all reads) were predominant. Since most of these sequence reads were detected at the end of the experiment, we assume that these microorganisms are crucial for the hydrolysis and fermentation of the filter cake and bagasse. The genus *Bacteroides* represented by the species *B. cellulosolvens* [41] is also known for its cellulosome. Within the genus *Prevotella*, there are some species notably involved in the degradation of hemicellulose, e.g., *Prevotella paludivivens* [42].

On day 0, the phyla *Firmicutes* and *Bacteroidetes* together represented about 85 and 62 % abundance in the reactors inoculated with FCM and MIX, respectively (Fig. 2). In R3.5 and R3.6 (MIX), other phyla such as *Actinobacteria*, *Fibrobacteres*, *Spirochaetes*, *Synergistetes*, *Tenericutes*, *Thermotogae* and the candidate WWE1 were also presented in significant relative abundances. Most of these phyla are involved in the degradation of lignocellulose-rich substrates [14, 28, 36]. However, the ecophysiological role of some phyla such as WWE1 is still unclear [24].

Table 2 Ecological index showing the estimated richness of the bacterial communities

Reactor	Day	OTUs		Chao1		Shannon		Simpson	
R3.5	0	510	(441)	590	(562)	6.35	(6.35)	0.96	(0.96)
R3.6		483	(433)	548	(545)	6.33	(6.34)	0.96	(0.96)
R3.7		588	(554)	635	(621)	6.63	(6.64)	0.97	(0.97)
R3.8		578	(545)	607	(620)	6.69	(6.71)	0.97	(0.97)
R3.5	44	437	(400)	528	(521)	6.03	(6.03)	0.96	(0.96)
R3.6		425	(412)	538	(519)	6.24	(6.24)	0.96	(0.96)
R3.7		506	(462)	632	(597)	6.52	(6.48)	0.97	(0.97)
R3.8		488	(414)	595	(502)	6.36	(6.34)	0.97	(0.97)
R3.5	113	176	(156)	200	(194)	3.80	(3.76)	0.82	(0.82)
R3.6		187	(169)	227	(209)	4.06	(4.05)	0.86	(0.86)
R3.7		216	(181)	258	(222)	4.33	(4.32)	0.90	(0.90)
R3.8		239	(226)	316	(283)	4.58	(4.58)	0.90	(0.90)

Values in parentheses originated from all randomly subsampled (without replacement) libraries down to the lowest number of sequences per sample

On day 44, the bacterial communities were already very similar in all reactors independent of the inoculation source. The relative abundance of the phylum *Firmicutes* decreased, whereas that of the *Bacteroidetes* increased. While the relative abundance of *Synergistetes* was kept constant in R3.5 and R3.6 (MIX), it increased slightly in the other co-digestion reactors (R3.7 and R3.8). The third most abundant phylum at this time was the candidate WWE1 (12 %), which had been found in many other studies dealing with mesophilic AD in the frame of wastewater treatment [43, 44]. Moreover, this candidate phylum was recently reported in large-scale continuously stirred tank reactors digesting the cellulose-rich substrate maize silage [24].

The steady state on day 113 was characterized by a less diverse community with three major phyla, *Bacteroidetes*, *Firmicutes* and *Synergistetes*, comprising nearly 94 % of the entire community. The relative abundance of the phylum *Bacteroidetes* of approximately 66 % in all reactors was even greater than on day 44. On the contrary, the abundance of *Firmicutes* dropped further to about 16 %. *Synergistetes* were present in all reactors along the experiment and their increasing abundance (to ca. 11 %) demonstrated their participation in the degradation of the lignocellulosic bagasse and filter cake. Within this phylum, the class *Synergistia* has reported to degrade fiber-rich feedstock [36]. Whereas all reactors had a very similar overall community structure, the phylum *Chloroflexi* (specifically, class *Anaerolineae*) was significantly abundant (3 %) only in R3.5 and R3.6 (MIX). This phylum had also been found in similar proportion in a biogas plant co-digesting maize silage, green rye and liquid manure [37].

The plot in Fig. 4 shows an NMDS analysis of bacterial 16S rRNA genes. The bacterial community compositions notably converged toward steady state (day 113). It is also visible that duplicate FCM reactors presented more diverse bacterial communities than MIX reactors. The correlation between the bacterial communities and the reactor parameters is indicated by the most significant vectors represented as arrows in the NMDS plot. The abundance of the phylum *Synergistetes* was strongly correlated with increasing OLR, whereas the abundance of the candidate phylum TM7 grew with the decrease of NH_4 − N. The more enriched values for $\delta^{13}C$ and $\delta^{2}H$ of methane were inversely correlated with NH_4 − N and positively with the bacterial community presented after the second sampling point (day 44). Furthermore, the decrease of pH influenced the bacterial communities primarily in the start-up phase. As already mentioned, the presence of phylum *Bacteroidetes* was strongly correlated with the steady state (at day 113).

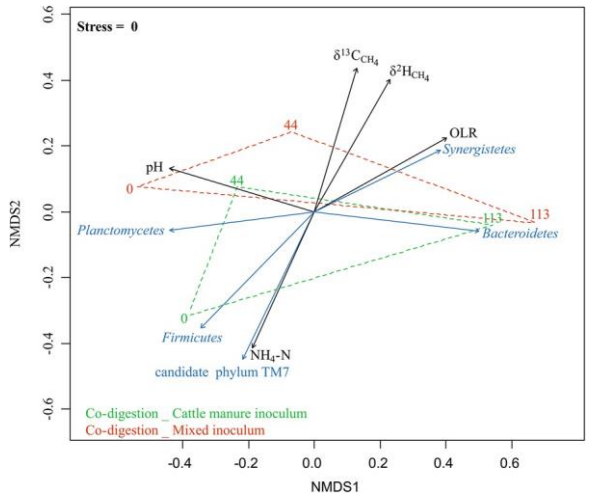
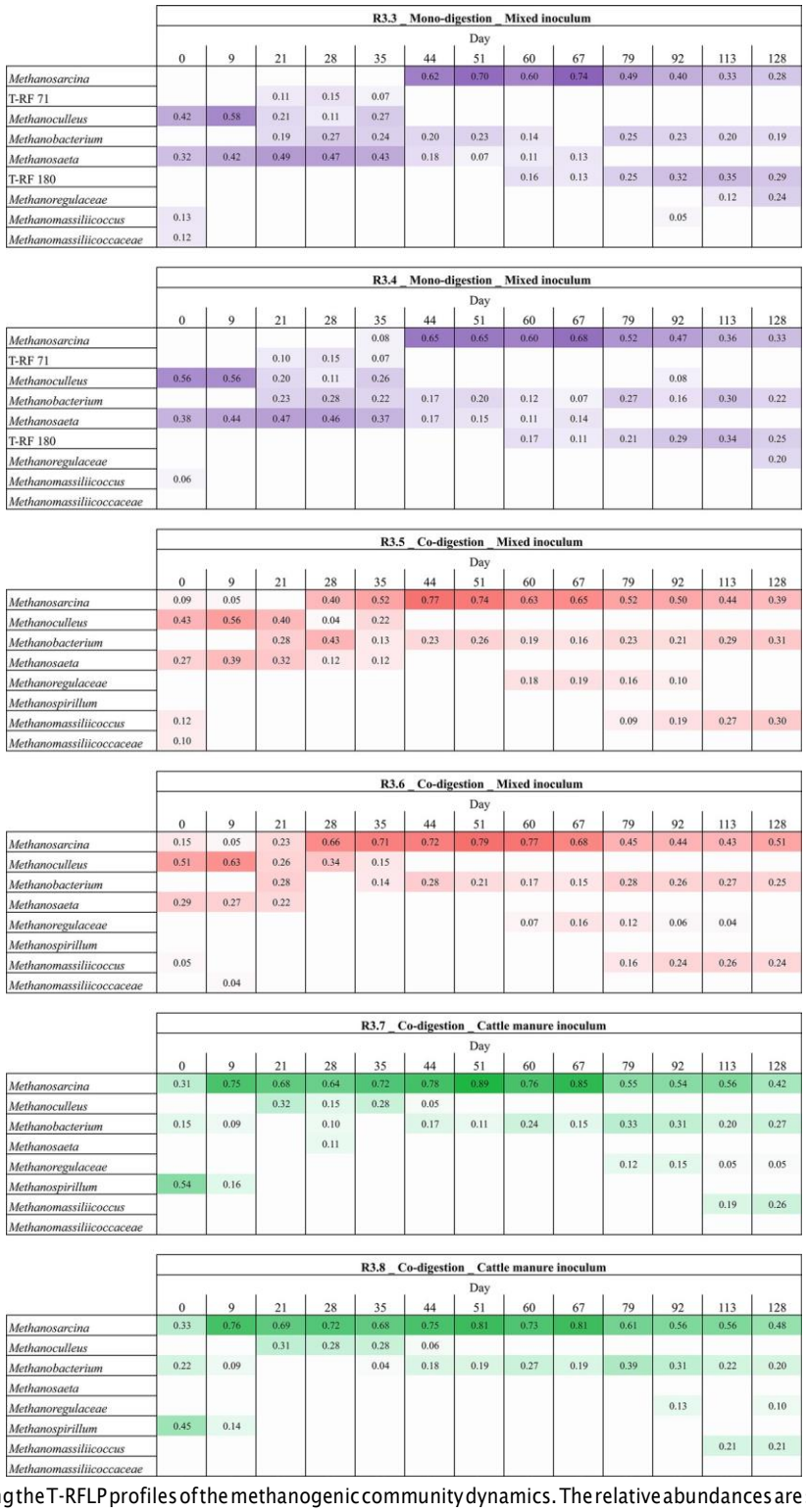


Fig. 4 The NMDS plot analyses of the bacterial communities (phylum level). The results were based on the pyrosequencing data of the 16S ribosomal RNA genes. The data points are numbered according to specific sampling days. The *blue arrows* indicate the highly significant ($p < 0.05$) bacterial phyla, whereas the *black arrows* represent the highly significant ($p < 0.05$) reactor parameters as correlation vectors of the bacterial community succession. The arrow length shows the correlation with the ordination axis, while the arrow direction corresponds to the community structures

Dynamics of the methanogenic community

The phylogenetic composition and dynamics of the methanogenic community were analyzed in all six reactors by *mcrA*-based T-RFLP fingerprinting. A heatmap presenting the relative abundance of the methanogenic communities along the whole experiment is shown in Fig. 5. Duplicate reactors (R3.3 and R3.4, R3.5 and R3.6, R3.7 and R3.8) had very similar methanogenic community compositions at each sampling time (Additional file 1: Figure S4). However, some non-abundant microorganisms, for examples *Methanomassiliicoccaceae* at the initial phase and *Methanomassiliicoccus* and *Methanoregulaceae* at the steady phase were occasionally detected in one of the duplicate reactors. Except during start-up, microorganisms affiliated with the genus *Methanosarcina* were the most abundant methanogens across all analyzed samples, reaching as much as 90 % T-RF abundance at day 51 in the final stage of the start-up phase.

The first sampling on day 0 was characterized by different methanogens in the MIX and FCM inocula. MIX-inoculated reactors were dominated by the genera *Methanoculleus* (ca. 50 %) and *Methanosaeta* (ca. 30 %) and less by *Methanomassiliicoccus* (ca. 8 %). *Methanosarcina* and *Methanomassiliicoccaceae* were not detected simultaneously in all four reactors at day 0. In the FCM-inoculated reactors, the most abundant genera were *Methanospirillum* (ca. 50 %), *Methanosarcina* (ca. 30 %)



R3.3 _ Mono-digestion _ Mixed inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina						0.62	0.70	0.60	0.74	0.49	0.40	0.33	0.28
T-RF 71			0.11	0.15	0.07								
Methanoculleus	0.42	0.58	0.21	0.11	0.27								
Methanobacterium			0.19	0.27	0.24	0.20	0.23	0.14		0.25	0.23	0.20	0.19
Methanosaeta	0.32	0.42	0.49	0.47	0.43	0.18	0.07	0.11	0.13				
T-RF 180								0.16	0.13	0.25	0.32	0.35	0.29
Methanoregulaceae												0.12	0.24
Methanomassiliicoccus	0.13										0.05		
Methanomassiliicoccaceae	0.12												

R3.4 _ Mono-digestion _ Mixed inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina					0.08	0.65	0.65	0.60	0.68	0.52	0.47	0.36	0.33
T-RF 71			0.10	0.15	0.07								
Methanoculleus	0.56	0.56	0.20	0.11	0.26						0.08		
Methanobacterium			0.23	0.28	0.22	0.17	0.20	0.12	0.07	0.27	0.16	0.30	0.22
Methanosaeta	0.38	0.44	0.47	0.46	0.37	0.17	0.15	0.11	0.14				
T-RF 180								0.17	0.11	0.21	0.29	0.34	0.25
Methanoregulaceae													0.20
Methanomassiliicoccus	0.06												
Methanomassiliicoccaceae													

R3.5 _ Co-digestion _ Mixed inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina	0.09	0.05		0.40	0.52	0.77	0.74	0.63	0.65	0.52	0.50	0.44	0.39
Methanoculleus	0.43	0.56	0.40	0.04	0.22								
Methanobacterium			0.28	0.43	0.13	0.23	0.26	0.19	0.16	0.23	0.21	0.29	0.31
Methanosaeta	0.27	0.39	0.32	0.12	0.12								
Methanoregulaceae								0.18	0.19	0.16	0.10		
Methanospirillum													
Methanomassiliicoccus	0.12									0.09	0.19	0.27	0.30
Methanomassiliicoccaceae	0.10												

R3.6 _ Co-digestion _ Mixed inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina	0.15	0.05	0.23	0.66	0.71	0.72	0.79	0.77	0.68	0.45	0.44	0.43	0.51
Methanoculleus	0.51	0.63	0.26	0.34	0.15								
Methanobacterium			0.28		0.14	0.28	0.21	0.17	0.15	0.28	0.26	0.27	0.25
Methanosaeta	0.29	0.27	0.22										
Methanoregulaceae								0.07	0.16	0.12	0.06	0.04	
Methanospirillum													
Methanomassiliicoccus	0.05									0.16	0.24	0.26	0.24
Methanomassiliicoccaceae		0.04											

R3.7 _ Co-digestion _ Cattle manure inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina	0.31	0.75	0.68	0.64	0.72	0.78	0.89	0.76	0.85	0.55	0.54	0.56	0.42
Methanoculleus			0.32	0.15	0.28	0.05							
Methanobacterium	0.15	0.09		0.10		0.17	0.11	0.24	0.15	0.33	0.31	0.20	0.27
Methanosaeta				0.11									
Methanoregulaceae										0.12	0.15	0.05	0.05
Methanospirillum	0.54	0.16											
Methanomassiliicoccus												0.19	0.26
Methanomassiliicoccaceae													

R3.8 _ Co-digestion _ Cattle manure inoculum

	Day 0	9	21	28	35	44	51	60	67	79	92	113	128
Methanosarcina	0.33	0.76	0.69	0.72	0.68	0.75	0.81	0.73	0.81	0.61	0.56	0.56	0.48
Methanoculleus			0.31	0.28	0.28	0.06							
Methanobacterium	0.22	0.09			0.04	0.18	0.19	0.27	0.19	0.39	0.31	0.22	0.20
Methanosaeta													
Methanoregulaceae											0.13		0.10
Methanospirillum	0.45	0.14											
Methanomassiliicoccus												0.21	0.21
Methanomassiliicoccaceae													

Fig. 5 Heatmap composing the T-RFLP profiles of the methanogenic community dynamics. The relative abundances are shown according to the sampling days of the experiments with different inoculation strategies (MIX and FCM) and digestion setups with filter cake and bagasse. Color intensity increases with relative abundance. Two digestate samples for each reactor were analyzed on every sampling day. Due to the high similarity of these samples, an average was calculated to show a more representative T-RFLP profile of each reactor

and *Methanobacterium* (ca. 20 %). In case of both inoculation strategies, the predominant methanogens were affiliated to either strictly hydrogenotrophic taxa or to the metabolically flexible *Methanosarcina* genus. Nevertheless, strict aceticlastic methanogens were also present at usually minor abundances.

The addition of bagasse as a co-substrate in MIX-inoculated reactors had an influence on the methanogenic community. The T-RF profiles of the mono- and co-digestion on day 28 were already significantly distinct. On the other hand, the methanogenic community structures of the co-digestion reactors (MIX and FCM) were similar after sampling day 21. In this latter case, the dynamics of the microbial community also followed the same trend toward the predominance of the *Methanosarcina*.

The genus *Methanosarcina* has been described to outcompete *Methanosaeta* at elevated acetate levels, due to its faster growth kinetics [45, 46]. However, recently *Methanosaeta* was reported to dominate over *Methanosarcina* even at high acetate concentration in the AD of dairy waste with poultry waste as co-substrate [47]. In accordance with this recent finding, the high abundance of the strictly aceticlastic *Methanosaeta* was stable during volatile organic acids (VOA) accumulation (1273 mg L^{-1} of acetate and 55 mg L^{-1} of propionate) at day 35. This corroborates our previous study [26], in which *Methanosaeta* was also detected at high VOA concentrations.

In the final part of the start-up phase (days 42 – 69), *Methanosarcina* was the most abundant methanogen reaching the highest T-RF relative abundance in all reactors. The presence of *Methanosarcina* in AD is regarded to be advantageous. As discussed by De Vrieze et al. [48], it is very robust against many stress conditions, e.g., tolerate process overloading, sudden pH changes and high levels of ammonium and salt. In addition, *Methanosarcina* has high growth rates, can use both the aceticlastic and the hydrogenotrophic pathway and utilize methanol to produce methane.

In the late start-up stage, *Methanoculleus* and *Methanospirillum*, which had been dominant in the MIX and FCM inocula, respectively, were no longer abundant. The taxon represented by T-RF 180 in the mono-digestion (MIX) and *Methanoregulaceae* in the co-digestion (MIX) became abundant until steady state.

The relative abundance of *Methanosarcina* decreased at steady state to around 30 and 50 % in the mono- and co-digestion reactors, respectively. In the mono-digestion, the taxon represented by T-RF 180 and *Methanobacterium* became more abundant, while *Methanoregulaceae* appeared first in the process. In the four co-digestion reactors, the methanogenic communities were very similar during steady state. Generally, the abundances of *Methanomassiliicoccus* and *Methanobacterium* increased, whereas *Methanoregulaceae* was not abundant.

Figure 6 shows the results of a multivariate statistical analysis based on the correlation between T-RLFP data and reactor parameters such as biogas yield and composition (CH_4 and CO_2), volatile fatty acids (acetate, propionate and butyrate), pH, total VOA, volatile organic acids per total inorganic carbonate buffer (VOA/TIC), HRT, ammonium – nitrogen (NH_4 – N) and stable isotope data ($\delta\,^{13}C_{CH4}$, $\delta\,^{13}C_{CO2}$ and $\delta\,^{2}H_{CH4}$). The effect of the inoculation strategy (MIX vs. FCM) using the co-digestion setup and the effect of the substrate (mono vs. co-digestion) applying MIX inoculum were analyzed separately for more comprehensible results.

Figure 6a compares the different inoculation strategies (MIX and FCM) in the co-digestion setup. The main difference between them was observed at the beginning of the start-up phase. After day 21, both setups, independent of the inoculum, had attained similar methanogenic community patterns with minor temporal compositional shifts. *Methanoculleus* and *Methanosaeta* were strongly correlated with the MIX inoculum at the beginning of the experiment, whereas *Methanosarcina* was predominantly present in the second part of the start-up phase. The methanogenic community dynamics was most significantly correlated ($p < 0.001$) with the pH, which gradually decreased during the start-up phase and kept constant values during steady state. On day 128, the T-RFLP profiles clustered together, indicating very high similarity of the methanogenic community structures, independent of the inoculum source.

Mono- and co-digestion with the same inoculum (MIX) were compared in an independent analysis (Fig. 6b). Although the substrate regimes differed, methanogenic community patterns were similar. Bigger changes in the structure of the methanogenic community were observed during mono-digestion, visible as a more extended dashed hull, while the samples from the co-digestion reactor clustered more closely together. The reactor parameters with most significant correlation ($p < 0.001$) to the community structures of the investigated samples were OLR, $\delta\,^{13}C_{CH4}$, NH_4 – N and pH. Whereas OLR and $\delta\,^{13}C_{CH4}$ values rose along the reactor operation, NH_4 – N concentration and pH value decreased gradually, mainly in the start-up phase.

Stable isotope characterization of the produced biogas

Isotopic variations as a function of inoculation strategies (MIX and FCM) and substrate regimes are shown in Fig. 7. Samples for $\delta\,^{13}C_{CH4}$, $\delta\,^{13}C_{CO2}$ and $\delta\,^{2}H_{CH4}$ measurements were taken together with samples for the analyses of microbial profiles and reactor parameters. Substantial isotopic changes were mainly observed in

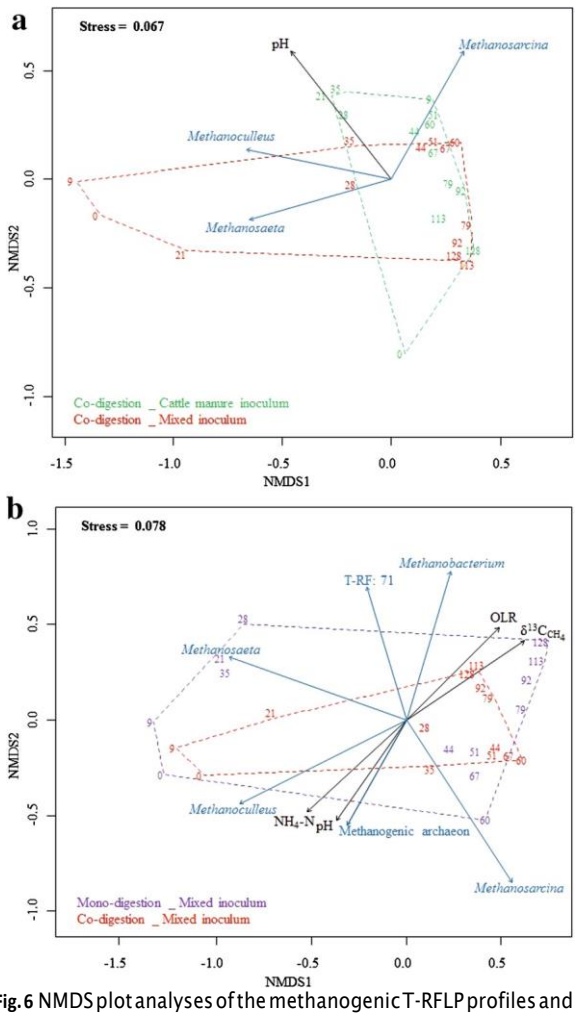


Fig. 6 NMDS plot analyses of the methanogenic T-RFLP profiles and its correlation with reactor parameters. The hulls show the effect of the inoculation strategy (MIX vs. FCM) using the co-digestion setup (**a**), and the effect of the substrate (mono vs. co-digestion) applying MIX inoculum (**b**). The data points are numbered according to specific sampling days. The *blue arrows* indicate the highly significant ($p < 0.001$) methanogens, whereas the *black arrows* represent the highly significant ($p < 0.001$) reactor parameters as correlation vectors of the methanogenic community dynamics. The *arrow length* shows the correlation with the ordination axis, while the *arrow direction* corresponds to the community structures

the initial start-up phase (days 0 – 41). Thereafter, the isotope values followed very similar trends independent of the inoculum. Initially, the isotope values were strongly depleted, particularly in reactors inoculated with FCM (−64 ‰ for $\delta^{13}C_{CH4}$, −6 ‰ for $\delta^{13}C_{CO2}$ and −352 ‰ for $\delta^{2}H_{CH4}$).

Figure 7a shows that the $\delta^{13}C_{CH4}$ values during the initial start-up increased until day 44. As an exception on day 9 in the FCM-inoculated reactors, the $\delta^{13}C_{CH4}$ values changed drastically to −25 ‰, but from day 21 onward the $\delta^{13}C_{CH4}$ values followed the same trend as in the other reactors inoculated with MIX. A similar isotope signature for $\delta^{13}C_{CH4}$ of about −32 ‰ on day 44 had also been observed in our previous study of co-digestion of filter cake and bagasse at gradual OLR increase [26] and in some other studies with C4 plant biomass, namely maize silage [7, 21]. Afterward, all reactors displayed constant isotope signatures also indicating stable methanogenic process.

The effect of different inocula and substrates on the $\delta^{13}C_{CO2}$ composition is clearly observed in Fig. 7b. The reactors inoculated with MIX had very similar isotope values on days 0 and 9. But after that, the isotopic pattern diverged between mono- and co-digestion. Enriched $\delta^{13}C_{CO2}$ values were found for mono-digestion (up to about 15 ‰). The reactors inoculated with FCM had much more depleted $\delta^{13}C_{CO2}$ values on days 0 and 9. On day 79 during steady state, the $\delta^{13}C_{CO2}$ values for all co-digestion reactors were very similar (around 11 ‰).

The $\delta^{2}H_{CH4}$ values shown in Fig. 7c presented a similar tendency among the reactors at specific sampling times. After start feeding the freshly inoculated reactors (from day 9 on), the $\delta^{2}H_{CH4}$ values indicated enrichment of methane in deuterium. Similarly to the dynamics of $\delta^{13}C_{CO2}$ values, the influence of the co-digestion was also visible from the variation of the $\delta^{2}H_{CH4}$ isotopic signatures, mainly at the last samplings. In co-digestion reactors, lower $\delta^{2}H_{CH4}$ values were observed.

The $\delta^{13}C$ composition of filter cake and bagasse were −14.30 and −13.64 ‰, respectively [26]. The isotopic signature of the substrates has also influenced the trends observed in Fig. 7, in which the $\delta^{13}C$ values became more enriched. The daily added tap water affects as well the $\delta^{2}H_{CH4}$ isotopic signatures. Nikolausz et al. [7] reported that the $\delta^{2}H$ of the tap water in Leipzig corresponds to about −64.7 ± 0.8 ‰. Therefore, more enriched $\delta^{2}H_{CH4}$ values were found after the addition of water to the substrates.

Methanogenic pathways

The dominance of a specific methanogenic pathway along the reactor operation was assessed using the apparent fractionation factor (αC) (Fig. 8a), which was calculated based on the isotopic composition ($\delta^{13}C$) of methane and carbon dioxide. As previously reported [20, 49, 50], a predominance of hydrogenotrophic methanogenesis is indicated by $\alpha C > 1.065$, whereas $\alpha C < 1.025$ indicates the predominance of aceticlastic methanogenesis. Intermediate αC values mostly found along the experiment showed that both methanogenic pathways were involved in methane production. This is also in agreement with the methanogenic community profiles composed of hydrogenotrophic and aceticlastic methanogens (Fig. 5).

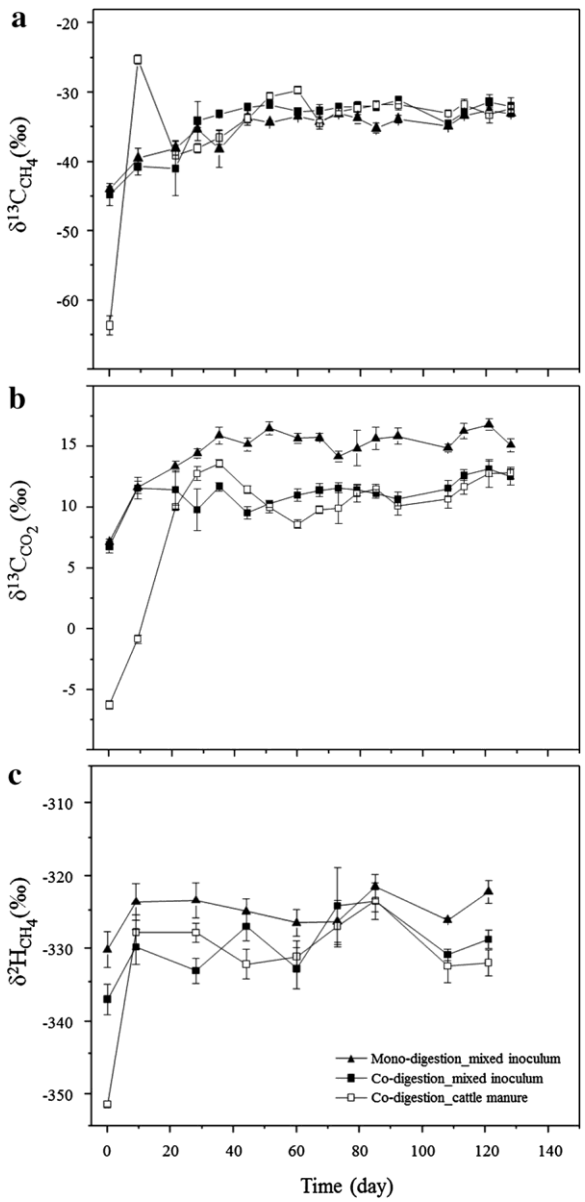


Fig. 7 Biogas isotope composition dynamics. The isotope signatures were plotted according to $\delta^{13}C$ (**a**) and $\delta^{2}H$ (**c**) of methane and $\delta^{13}C$ of carbon dioxide (**b**) along the experiment with different inoculation strategies and digestion setups. The *error bars* with the standard errors are shown with the confidence interval of 95 % for the $\delta^{13}C$ values and 65 % for the $\delta^{2}H$ values

Only on days 0 and 9 in co-digestion reactors inoculated with FCM, the predominance of one methanogenic pathway was observed. On day 0, the αC value indicated the predominance of hydrogenotrophic methanogenesis in accordance with a 68 % predominance of the strictly hydrogenotrophic genera *Methanospirillum* (ca. 50 %) and *Methanobacterium* (18 %). In addition, the versatile genus *Methanosarcina*, which contributed 32 % to the community profile, most probably produced methane via the hydrogenotrophic pathway during this period. On day 9, the methanogenic pathway was shifted drastically to the dominance of aceticlastic methanogenesis. The αC value was slightly lower than 1.025 and the predominance of the versatile *Methanosarcina* (ca. 75 %) suggested that members of this genus became not just numerically abundant, but switched to methane production from acetate. On day 21, again a sudden change was observed from the αC dynamics. At this time, *Methanosarcina* was still the most abundant taxon, which, however, probably used both methanogenic pathways.

The methanogenic pathway dynamics during the experiment is possibly also observed in plots combining $\delta^{13}C$ and $\delta^{2}H$ isotopic variations of methane during start-up (Fig. 8b) and steady state (Fig. 8c). Nikolausz et al. [7] had used this combination of $\delta^{13}C_{CH4}$ and $\delta^{2}H_{CH4}$ to identify the dominant methanogenic pathway in laboratory-scale biogas reactors. Accordingly, the suggested predominance of the hydrogenotrophic methanogenesis at day 0 (marked in the graphic with the hull) for FCM-inoculated reactors is confirmed as suggested by Sugimoto and Wada [51]. Although their classification did not correspond to the other sampling points in terms of predominant methanogenic pathway defined by αC and the molecular biological results, the differences between the inocula and the substrate were still observed in the combined $\delta^{13}C_{CH4}$ and $\delta^{2}H_{CH4}$ graphics (hulls). A general trend of less negative $\delta^{2}H_{CH4}$ values observed in case of later samples in all reactors is probably due to the influence of the isotopic composition of the supplemented process water.

Conclusion

Our results confirmed that FCM is a reliable and efficient inoculum for the co-digestion of filter cake and bagasse, since very similar methane and biogas yields were obtained under the steady-state phase, independent of the inoculation strategy. The bacterial and methanogenic communities were also very similar at the end of the experiment, regardless of whether the reactors had been inoculated with FCM or MIX. Bacterial composition and succession showed that the major phyla involved in the anaerobic degradation of the waste products from the Brazilian bioethanol/sugar industry were *Bacteroidetes*, *Firmicutes* and *Synergistetes*. The co-digestion of filter cake and bagasse in both inocula setups led to the development of polysaccharide-degrading specialists affiliated to the genera *Prevotella* and *Bacteroides* (both comprised around 50 % relative abundance of the bacterial community at steady phase). Methanogenic communities varied mainly in the first 3 weeks of operation for co-digestion reactors and in the first 5 weeks for mono-digestion. The

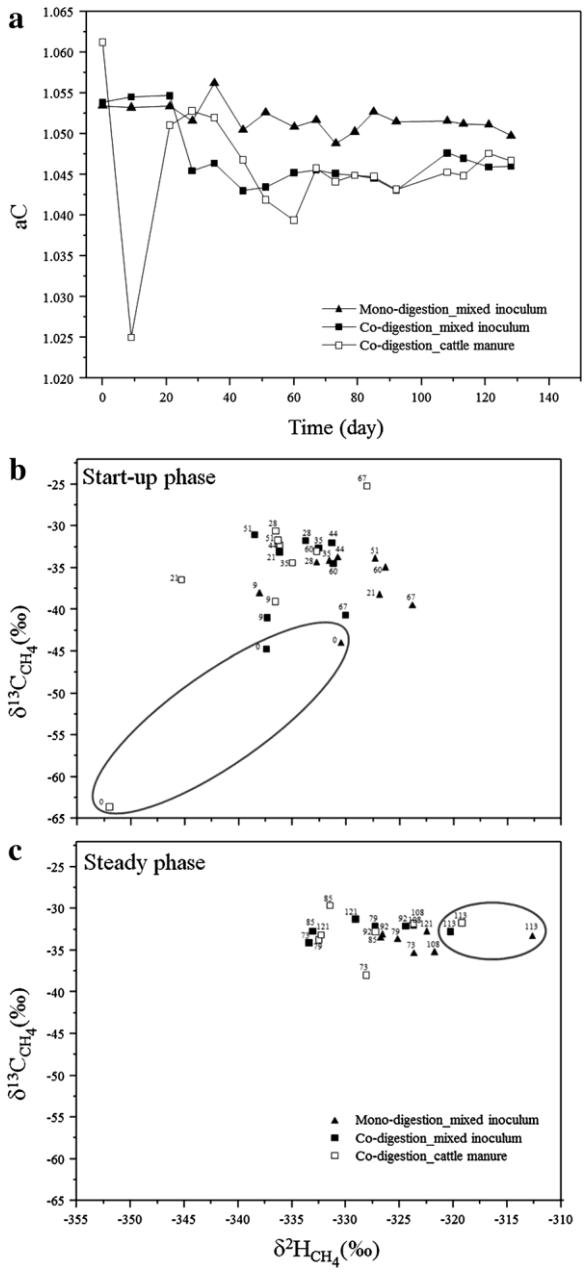


Fig. 8 Determination of the potential predominant methanogenic pathway. The estimate dynamics shifts were based on the αC calculation (**a**) and on the correlation of δ^2H and $\delta^{13}C$ of methane. The *hulls* indicate the effect of the different inoculation strategies (**b**) and digestion setups (**c**) on the methanogenic pathways. The sampling days are plotted together with the representative shape point

key methanogens were affiliated with *Methanosarcina*, *Methanobacterium*, *Methanoregulaceae* and *Methanomassiliicoccus* in co-digestion reactors, as opposed to *Methanosarcina*, *Methanobacterium*, *Methanoregulaceae* and a non-identified taxon in mono-digestion reactors.

The most important reactor parameter correlating with the methanogenic community structure in both digestion setups was the pH. Stable isotope fingerprinting showed clearly, especially in case of carbon dioxide, the influence of different inoculum strategies and substrate feeding on the methanogenic activity. Based on the isotope analysis, in agreement with the molecular approach, both aceticlastic and hydrogenotrophic methanogenesis pathways contributed importantly to methane production throughout the experiment.

Additional files

Additional file 1: Figure S1. Duplicate T-RFLP profiles of the methanogenic community dynamics for each reactor in order to show the reproducibility of the T-RFLP approach. **Figure S2.** Rarefaction curves of the pyrosequencing data of the 16S ribosomal RNA genes from the four co-digestion reactors (R3.5, R3.6, R3.7 and R3.8) at three different sampling points along the experiment. **Table S1.** Beta diversity index showing the community similarities between samples. **Figure S3.** 3D PCA diagram of the beta diversity. **Figure S4.** N-MDS plot showing the Bray–Curtis similarity of the methanogenic communities in parallel reactors.

Additional file 2: Table S2. List of obtained OTUs for the co-digestion reactors at three different sampling points.

Abbreviations

AD: anaerobic digestion; FCM: fresh cattle manure; GC: gas chromatograph; HRT: hydraulic retention time; NH_4–N: ammonium–nitrogen; NMDS: non-metric multidimensional scaling; MIX: engineered mixed inoculum; OLR: organic loading rate; OTUs: operational taxonomic units; T-RFLP: terminal restriction fragment length polymorphism; VOA: volatile organic acid; VOA/TIC: volatile organic acids per total inorganic carbonate buffer.

Authors' contributions

AFL designed the experiment, performed the molecular and isotope analyses, evaluated the data and prepared the manuscript. LJ designed the experiment, contributed to the data interpretation and reviewed the manuscript. HH supervised the study and thoroughly revised the manuscript. HHR contributed to the evaluation and interpretation of the isotope data and revised the manuscript. MN designed the experiment, contributed to the data interpretation, supervised the study and thoroughly revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

De-multiplexed sequences from the pyrosequencing analysis were deposited under the EMBL-EBI accession number PRJEB12073 (http://www.ebi.ac.uk/ena/data/view/PRJEB12073).

Further supporting data related to this study were included as additional supporting materials.

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References

1. Leite AF, Janke L, Harms H, Zang JW, Fonseca-Zang WA, Stinner W, Nikolausz M. Assessment of the variations in characteristics and methane potential of major waste products from the Brazilian bioethanol industry along an operating season. Energy Fuels. 2015;29(7):4022–9.
2. Janke L, Leite A, Nikolausz M, Schmidt T, Liebetrau J, Nelles M, Stinner W. Biogas production from sugarcane waste: assessment on kinetic challenges for process designing. Int J Mol Sci. 2015;16:20685–703.
3. Janke L, Leite A, Wedwitschka H, Schmidt T, Nikolausz M, Stinner W. Biomethane Production Integrated to the Brazilian Sugarcane Industry: The Case Study of São Paulo State. In: Proceedings of the 22nd European biomass conference and exhibition. 2014. p. 1295–9.
4. Janke L, Leite AF, Nikolausz M, Radetski CM, Nelles M, Stinner W. Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. Waste Manag. 2016;48:199–208.
5. Demirel B. Major pathway of methane formation from energy crops in agricultural biogas digesters. Crit Rev Environ Sci Technol. 2014;44:199–222.
6. Oh ST, Martin AD. Long chain fatty acids degradation in anaerobic digester: thermodynamic equilibrium consideration. Process Biochem. 2010;45:335–45.
7. Nikolausz M, Walter RF, Strauber H, Liebetrau J, Schmidt T, Kleinsteuber S, Bratfisch F, Gunther U, Richnow HH. Evaluation of stable isotope fingerprinting techniques for the assessment of the predominant methanogenic pathways in anaerobic digesters. Appl Microbiol Biotechnol. 2013;97:2251–62.
8. Gehring T, Klang J, Niedermayr A, Berzio S, Immenhauser A, Klocke M, Wichern M, Lubken M. Determination of methanogenic pathways through carbon isotope (delta13C) analysis for the two-stage anaerobic digestion of high-solids substrates. Environ Sci Technol. 2015;49:4705–14.
9. Lv Z, Hu M, Harms H, Richnow HH, Liebetrau J, Nikolausz M. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. Bioresour Technol. 2014;167:251–9.
10. Ferry JG. Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. Curr Opin Biotechnol. 2011;22:351–7.
11. Zinder S. Physiological ecology of Methanogens. In: Ferry J, editor. *Methanogenesis.* Chapman & hall microbiology series. Berlin: Springer; 1993. p. 128–206.
12. Abendroth C, Vilanova C, Gunther T, Luschnig O, Porcar M. Eubacteria and archaea communities in seven mesophile anaerobic digester plants in Germany. Biotechnol Biofuels. 2015;8:87.
13. Nettmann E, Bergmann I, Pramschufer S, Mundt K, Plogsties V, Herrmann C, Klocke M. Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. Appl Environ Microbiol. 2010;76:2540–8.
14. Sun L, Pope PB, Eijsink VG, Schnurer A. Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure. Microb Biotechnol. 2015:8:815–27.
15. Sun L, Müller B, Westerholm M, Schnürer A. Syntrophic acetate oxidation in industrial CSTR biogas digesters. J Biotechnol. 2014;171:39–44.
16. Batstone D, Picioreanu C, Van Loosdrecht M. Multidimensional modelling to investigate interspecies hydrogen transfer in anaerobic biofilms. Water Res. 2006;40:3099–108.
17. Westerholm M, Muller B, Isaksson S, Schnurer A. Trace element and temperature effects on microbial communities and links to biogas digester performance at high ammonia levels. Biotechnol Biofuels. 2015;8:154.
18. Polag D, Krapf LC, Heuwinkel H, Laukenmann S, Lelieveld J, Keppler F. Stable carbon isotopes of methane for real-time process monitoring in anaerobic digesters. Eng Life Sci. 2014;14:153–60.
19. Karakashev D, Batstone DJ, Angelidaki I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl Environ Microbiol. 2005;71:331–8.
20. Conrad R. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. Org Geochem. 2005;36:739–52.
21. Lv Z, Leite AF, Harms H, Richnow HH, Liebetrau J, Nikolausz M. Influences of the substrate feeding regime on methanogenic activity in biogas reactors approached by molecular and stable isotope methods. Anaerobe. 2014;29:91–9.
22. Polag D, May T, Muller L, Konig H, Jacobi F, Laukenmann S, Keppler F. Online monitoring of stable carbon isotopes of methane in anaerobic digestion as a new tool for early warning of process instability. Bioresour Technol. 2015;197:161–70.
23. Steinberg LM, Regan JM. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. Appl Environ Microbiol. 2008;74:6663–71.
24. Lucas R, Kuchenbuch A, Fetzer I, Harms H, Kleinsteuber S. Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. FEMS Microbiol Ecol. 2015. doi:10.1093/femsec/fiv004.
25. Popp D, Schrader S, Kleinsteuber S, Harms H, Strauber H. Biogas production from coumarin-rich plants-inhibition by coumarin and recovery by adaptation of the bacterial community. FEMS Microbiol Ecol. 2015;91:fiv103.
26. Leite A, Janke L, Lv Z, Harms H, Richnow H-H, Nikolausz M. Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: effects of increasing organic loading rate on methanogenic community dynamics. Int J Mol Sci. 2015;16:23210.
27. Buhligen F, Lucas R, Nikolausz M, Kleinsteuber S. A T-RFLP database for the rapid profiling of methanogenic communities in anaerobic digesters. Anaerobe. 2016;39:114–6.
28. Ziganshin AM, Liebetrau J, Proter J, Kleinsteuber S. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. Appl Microbiol Biotechnol. 2013;97:5161–74.
29. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.
30. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–1.
31. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 2012;6:610–8.
32. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261–7.
33. Nawrocki EP, Kolbe DL, Eddy SR. Infernal 1.0: inference of RNA alignments. Bioinformatics. 2009;25:1335–7.
34. Magurran AE. Measuring biological diversity. Hoboken: Wiley; 2004.
35. Hill TCJ, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol. 2003;43:1–11.
36. Strauber H, Lucas R, Kleinsteuber S. Metabolic and microbial community dynamics during the anaerobic digestion of maize silage in a two-phase process. Appl Microbiol Biotechnol. 2016;100(1):479–91.

37. Krober M, Bekel T, Diaz NN, Goesmann A, Jaenicke S, Krause L, Miller D, Runte KJ, Viehover P, Puhler A, Schluter A. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J Biotechnol. 2009;142:38–49.
38. Klocke M, Mahnert P, Mundt K, Souidi K, Linke B. Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate. Syst Appl Microbiol. 2007;30:139–51.
39. Bayer EA, Belaich JP, Shoham Y, Lamed R. The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol. 2004;58:521–54.
40. Latham M, Wolin M. Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of Methanobacterium ruminantium. Appl Environ Microbiol. 1977;34:297–301.
41. Schwarz WH. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biotechnol. 2001;56:634–49.
42. Ueki A, Akasaka H, Satoh A, Suzuki D, Ueki K. *Prevotella paludivivens* sp. nov., a novel strictly anaerobic, gram-negative, hemicellulose-decomposing bacterium isolated from plant residue and rice roots in irrigated rice-field soil. Int J Syst Evol Microbiol. 2007;57:1803–9.
43. Chouari R, Le Paslier D, Dauga C, Daegelen P, Weissenbach J, Sghir A. Novel major bacterial candidate division within a municipal anaerobic sludge digester. Appl Environ Microbiol. 2005;71:2145–53.
44. Pelletier E, Kreimeyer A, Bocs S, Rouy Z, Gyapay G, Chouari R, Riviere D, Ganesan A, Daegelen P, Sghir A, et al. "Candidatus Cloacamonas acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division. J Bacteriol. 2008;190:2572–9.
45. Blume F, Bergmann I, Nettmann E, Schelle H, Rehde G, Mundt K, Klocke M. Methanogenic population dynamics during semi-continuous biogas fermentation and acidification by overloading. J Appl Microbiol. 2010;109:441–50.
46. Griffin ME, McMahon KD, Mackie RI, Raskin L. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol Bioeng. 1998;57:342–55.
47. Chen S, He Q. Persistence of Methanosaeta populations in anaerobic digestion during process instability. J Ind Microbiol Biotechnol. 2015;42(8):1129–37.
48. De Vrieze J, Hennebel T, Boon N, Verstraete W. Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. Bioresour Technol. 2012;112:1–9.
49. Whiticar MJ, Faber E, Schoell M. Biogenic methane formation in marine and freshwater environments: CO_2 reduction vs. acetate fermentation—isotope evidence. Geochim Cosmochim Acta. 1986;50:693–709.
50. Galand PE, Yrjälä K, Conrad R. Stable carbon isotope fractionation during methanogenesis in three boreal peatland ecosystems. Biogeosciences. 2010;7:3893–900.
51. Sugimoto A, Wada E. Hydrogen isotopic composition of bacterial methane: CO_2/H_2 reduction and acetate fermentation. Geochim Cosmochim Acta. 1995;59:1329–37.
52. Oliveros JC. VENNY. An interactive tool for comparing lists with Venn diagrams. 2007. http://bioinfogp.cnb.csic.es/tools/venny/index.html. Accessed Nov 2015.

3.4. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment

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Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment

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HIGHLIGHTS

- A dual-pool two-step model was fitted to the batch experiment data.
- 6 g NaOH/100 g FC_{FM} achieved the highest methane potential in batch experiment.
- Urea addition did not enhance VFA yield during semi-continuous experiment.
- Optimum pH for VFA production during semi-continuous experiment was between 5 and 5.5.
- NaOH pretreatment increased the VFA yield by 37% during semi-continuous experiment.

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ABSTRACT

Different methods for optimization the anaerobic digestion (AD) of sugarcane filter cake (FC) with a special focus on volatile fatty acids (VFA) production were studied. Sodium hydroxide (NaOH) pretreatment at different concentrations was investigated in batch experiments and the cumulative methane yields fitted to a dual-pool two-step model to provide an initial assessment on AD. The effects of nitrogen supplementation in form of urea and NaOH pretreatment for improved VFA production were evaluated in a semi-continuously operated reactor as well. The results indicated that higher NaOH concentrations during pretreatment accelerated the AD process and increased methane production in batch experiments. Nitrogen supplementation resulted in a VFA loss due to methane formation by buffering the pH value at nearly neutral conditions (~6.7). However, the alkaline pretreatment with 6 g NaOH/100 g FC_{FM} improved both the COD solubilization and the VFA yield by 37%, mainly consisted by *n*-butyric and acetic acids.

1. Introduction

Sugarcane is the most produced agricultural commodity (fresh mass basis) in the world (FAO, 2013). It is mainly used for sugar, first-generation bioethanol and bioelectricity production. Nowadays, new developments aim to add value to the underused biomass fractions derived from the cane processing with the intention to diversify the product portfolio of the sugarcane plants in a biorefinery concept (Mariano et al., 2013).

Filter cake (FC), also known as press mud or filter mud, is a solid waste generated during the clarification (physical–chemical process) of sugarcane juice before been used for sugar and first-generation bioethanol production. It is mainly composed of water, inorganic soil particles, residual sugars and small pieces of sugarcane bagasse, which are often intentionally added to improve the permeability during the recovery of sucrose at the rotary vacuum-drum filter. In most cases, FC is applied as organic fertilizer on the sugarcane fields without any previous recovery of value-added products (Elsayed et al., 2008).

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Anaerobic digestion (AD) is a promising strategy to treat such type of waste, since methane and/or platform chemicals for value-added products could be produced as a result of the different syntrophic biochemical phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Deublein and Steinhauser, 2008; Merklein et al., 2014). These cascade reactions are performed by various microorganisms, thereby the composition and dynamics of the microbial community is influenced by different process parameters such as organic loading rate (OLR), hydraulic retention time (HRT), pH value, balance of macronutrients and trace elements as well as presence of inhibitors (FNR, 2010; Kayhanian and Rich, 1995).

For optimization of AD, hydrolysis/acidogenesis and acetogenesis/methanogenesis may be improved independently by physical separation of these phases in two reactors (Cysneiros et al., 2012a). It is well known that hydrolysis is often the rate-limiting step during AD when fibrous material, such as filter cake is used as feedstock due to the recalcitrant presence of lignin, which prevents the action of microorganisms and enzymes by its hydrophobic nature (Montgomery and Bochmann, 2014; Yu et al., 2014). One option to improve the hydrolysis of lignocellulosic substrates is to perform a physical (comminution, hydrothermolysis), chemical (acid, alkali, solvents, ozone), physico-chemical (steam explosion, ammonia fiber explosion) or biological (enzymes, fungi) pretreatment process to increase the accessible surface area, to modify the crystalline structure or partially depolymerize cellulose, to solubilize hemicellulose and/or lignin, or to modify lignin structure (Hendriks and Zeeman, 2009; Silverstein et al., 2007).

Only few studies have assessed the effects of different pretreatment methods on methane production from FC in batch systems (López González et al., 2013, 2014). To our knowledge, pretreatment of FC for VFA production in a semi-continuous stirred-tank reactor (SCSTR) has not been studied yet. Only by applying a similar feeding system used during large-scale applications (semi-continuous) it is possible to have a broader understanding of the reactor's behavior in terms of effect of inhibitors, nutrient deficiencies and optimum pH value for a specific AD phase.

Previous studies from our group (Leite et al., 2015) assessed the main characteristics of sugarcane waste along an operating season. Apparently, FC presented a proper C:N ratio for AD, varying from 24 to 41, which is according to several authors (FNR, 2010; Fricke et al., 2007; Weiland, 2010) into the optimum range of around 20–40. However, it is noteworthy that in case of low nitrogen release during fermentation, it could negatively influence the microbial community functioning due to nitrogen deficiency. In the meantime, the sodium concentration of FC was found to be very low (up to maximum 3.7 mg L^{-1}) much lower than recommended values (100–200 mg L^{-1}) by early studies (Mccarty, 1964), which might also negatively affect the microbial growth during the AD process.

The use of sodium hydroxide (NaOH) for substrate pretreatment could be a potential strategy to overcome these possible drawbacks. At the same time cellulose and hemicellulose would become more accessible to hydrolytic enzymes by breaking down the lignocellulosic structure of FC, and sodium as an important macroelement for microbial growth would be provided (Sambusiti et al., 2013a). However, particular attention should be paid to the NaOH dosage, since the ion Na^+ in high concentrations could negatively affect the activity of non-halotolerant microorganisms and interfere with their metabolism (Chen et al., 2008).

Therefore, in this study the effect of alkaline FC pretreatment was previously investigated in batch experiments evaluating the influence of different NaOH concentrations on the short-term AD, followed by fitting the cumulative methane yields to a dual-pool two-step model to provide a preliminary estimation of VFA production. Moreover, a SCSTR was operated as an acidogenic reactor at high OLRs and low HRTs to investigate how VFA production could be optimized providing urea as a NH_4-N source and NaOH pretreatment as a measure to improve the microbial hydrolysis.

2. Methods

2.1. Substrate and inoculum

Sugarcane FC was obtained from a distillery plant in the State of Goiás (Brazil) during the 2013/2014 season, transported to Germany in sealed plastic bags and stored at 4 °C until its use. A large-scale biogas plant that uses maize silage and cattle manure as substrate provided fresh digestate, which was used as inoculum for the batch experiments and the start-up of the semi-continuous reactor. To avoid inlet and outlet pipes from clogging, the digestate was sieved prior to inoculation in the reactors. During the operation of the semi-continuous experiment tap water was utilized to keep the total solids (TS) of the feed in below 15% for wet fermentation process.

2.2. Alkaline pretreatment

For batch experiment, alkaline pretreatment was carried out in 500 mL glass flasks applying different NaOH concentrations (0, 1.5, 3 and 6 g NaOH/100 g FC_{FM}), hereafter referred as to untreated, low, mild and high NaOH concentrations. The substrate TS concentration was 68 g TS L^{-1}. FC and NaOH mixture was stirred for 30 min (100 rpm) at 45 °C using a magnetic stirrer (Heidolph Instruments). After pretreatment, the FC was neutralized with hydrochloric acid and immediately used for subsequent AD trials. The same pretreatment procedure was used prior semi-continuous digestion in a concentration of 6 g NaOH/100 g FC_{FM}.

2.3. PCA analysis

A principal component analysis (PCA) was run on VFA and chemical oxygen demand (COD) concentrations obtained after pretreatment to evaluate the relationship between the effects of the different NaOH concentrations on solubilization of the main substrate components. The analysis was run with the software Statistica 6.0 (Statsoft, Tulsa, OK, USA).

2.4. Batch experiment

The methane yield of the FC pretreated with different NaOH concentrations was obtained through biochemical methane potential (BMP) assays according to VDI (2006) using an Automatic Methane Potential Test System II (AMPTS II, Bioprocess Control, Sweden) under mesophilic temperature (38 °C) during 10 days. Based on the different model derivations presented by Brulé et al. (2014) an exponential dual-pool two-step model (Model D) was used to evaluate the methane production kinetics of the anaerobic batch experiment. This modeling approach differentiates between rapidly and slowly degradable fractions (dual-pool) of the available substrate. Furthermore, it includes the acidification of the two fractions to VFA as well as the degradation of the resulting intermediate VFA concentration to methane (two-step).

Thus, five model parameters and constants needed to be adjusted to depict the respective measurement results: the total methane potential S ($mL_N\,gVS^{-1}$), the ratio of rapidly degradable substrate to total degradable substrate a (–) and the three first-order reaction constant for the degradation of rapidly degradable substrate k_F (d^{-1}), slowly degradable substrate k_L (d^{-1}) and the degradation of VFA k_{VFA} (d^{-1}). The model implementation as well as the numeric parameter identification (Levenberg–Marquard

algorithm) were realized in the software environment Matlab as described by Brulé et al. (2014).

2.5 Semi-continuous experiment

A lab-scale SCSTR with 5 L total volume (3 L working volume) was used for this experiment. The reactor was continuously stirred (100 rpm) using a central stirrer with helix shaped blades located in the lower part of the reactor. The operation temperature was kept under mesophilic conditions (38 ± 1 °C) by recirculating hot water through the double-walled reactor.

The experiment was carried out over nearly 100 consecutive days with the same feeding frequency (once per day). For the reactor start-up the HRT was set to 2.5 days and gradually increased to 4.5 days in order to wash-out methanogens from the inoculum and overload the reactor with organic acids. After achieving a steady phase for VFA production at a HRT of 5.0 days, 3 g of urea was supplemented at day 30. During a second urea supplementation period (days 54–71), 5 mL of nitric acid (10 mL L^{-1}) was added for pH control. From day 72 until the end of the experiment, NaOH pretreatment was applied to the FC prior to feeding as described above. Detailed information about different feeding rates, OLR and HRT are listed in Table 1.

2.6 Analytical methods

TS and volatile solids (VS) were determined by drying the samples for 24 h at 105 °C in a drying oven (Binder, Germany) and further reducing the organic content to ashes for 2 h at 550 °C in a high temperature oven (Carbolite, UK). The COD of non-centrifuged aliquots of the untreated and pretreated FC was analyzed using LCK 014 COD kit (Hach-Lange, Germany) according to the manufacturer's protocol. VFA (acetic, propionic, *n*-butyric, *iso*-butyric, *n*-valeric, *iso*-valeric and hexanoic acid) were determined using a 5890 series II gas chromatograph (Hewlett Packard, USA) equipped with an HS40 automatic headspace sampler (Perkin Elmer, USA) and an Agilent HP-FFAP column (30 m × 0.32 mm × 0.25 µm) according to a previously described method (Sträuber et al., 2015).

The daily biogas production in the semi-continuous experiment was measured by a drum-type gas meter TG 05 (Ritter, Bochum, Germany), and corrected to standard temperature and pressure conditions. Specific biogas production (SBP) was presented in norm milliliters per g of VS ($mL_N\,gVS^{-1}$). The composition of the biogas (CH_4, CO_2, O_2 and H_2S) in the headspace of the reactor was measure three times a week using a GA2000 Landfill Gas Analyzer (Geotechnical Instruments Ltd., UK).

Every day the pH value of fresh digestate was measured immediately after sampling of the semi-continuous reactor with a pH-electrode (WTP type pH 3310 Sentix 41, Germany). Three times a week fresh digestate samples were centrifuged for 10 min at 10,000 rpm and 10 °C. The supernatant was used after filtration for subsequent analysis, including the measurement of volatile organic acids (VOA) by using a Titration Excellence T90 titrator (Mettler-Toledo GmbH, Switzerland) and soluble chemical oxygen demand (SCOD) using the equipment as previously described. Ammonium-nitrogen concentration (NH_4-N g L^{-1}) was determined from 500 µL filtered supernatant diluted with distilled water (1:500) with the Nessler method using a benchtop spectrophotometer (Hach-Lange DR 3900, Loveland, US).

To reveal the hydrolytic potential of the acidification step in the semi-continuous experiment the partial, and unintentional, gas production was converted into a theoretically corresponding amount of acetate in the liquid phase. Based on the stoichiometric pathways of the acetoclastic methanogenesis presented by Angelidaki et al. (1993) 3.96 g of acetic acid are needed for the production of 1 g of methane. Using the ideal gas law at standard conditions (1.01325 bar und 273.15 K) the specific weight of methane equals 1.40 $L_N\,g^{-1}$, which results into 2.83 g of acetic acid per L_N of methane. Finally, the theoretical amount of VOA (g) needed for the respective methane production is divided by the liquid volume (3L), added to the measured VOA concentration and presented as calculated volatile organic acids (CVOA).

3. Results and discussion

3.1 Substrate composition

3.1.1 Raw FC

The main characteristics of the sugarcane FC are presented in Table 2. Having an appearance similar to sludge, the substrate presented a TS value of 27.03% and relatively low VS concentration of 61.75% of TS, since inorganic soil particles could be attached during the sugarcane juice treatment process where FC is generated. Our group has already assessed the characteristics of sugarcane FC in details (Leite et al., 2015). The most relevant observations are the high lignin content found, which is equivalent to about 23% of the total organic matter of the substrate, and the low sodium concentration (1.27 mg L^{-1}), which is below the recommended values for AD (Mccarty, 1964).

3.1.2 Pretreated FC

The COD and VFA content after FC pretreatment with different NaOH concentrations are presented in Fig. 1. The mild and high NaOH concentrations were responsible for increasing the COD

Table 1
Overview of the semi-continuous experiment.

Phases	Period (d)	FC Input ($g\,d^{-1}$)	Water Input ($mL\,d^{-1}$)	HRT (d)	OLR ($gVS\,L^{-1}\,d^{-1}$)
Start-up	0–3	300	900	2.5	16.7
	4–7	250	750	3.0	13.9
	8–10	220	650	3.4	12.1
	11–14	190	565	4.0	10.5
	15–17	165	490	4.6	9.1
Steady	18–30	150	450	5.0	8.3
Urea supplementation	31–48	150	450	5.0	8.3
	49–53	220	650	3.4	12.1
Urea + nitric acid supplementation	54–56	220	650	3.4	12.1
	57–71	150	450	5.0	8.3
Sodium hydroxide pretreatment	72–99	150	450	5.0	8.3

Table 2
Main characteristics of the sugarcane FC.

Parameters		FC	Units
Total solids (TS)*		27.03 ± 0.13	%FM[a]
Volatile solids (VS)*		61.75 ± 0.13	%TS
Carbon (C)		42.52 ± 3.63	%TS
Nitrogen (N)		1.38 ± 0.36	%TS
Sodium (Na)		1.27 ± 2.10	$mg\,L^{-1}$
Raw protein		107.9 ± 15.9	$g\,kg^{-1}$ TS
Raw fat		39.31 ± 5.83	$g\,kg^{-1}$ TS
Carbohydrates	NFC[b]	56.78 ± 19.8	$g\,kg^{-1}$ TS
	Cellulose	184.6 ± 35.2	$g\,kg^{-1}$ TS
	Hemi-cellulose	170.9 ± 32.5	$g\,kg^{-1}$ TS
Lignin		169.6 ± 13.8	$g\,kg^{-1}$ TS
C:N ratio		31 ± 10	–

[a] Fresh matter.
[b] Non-fiber carbohydrate.

* All values, except TS and VS, were previously published by our group (Leite et al., 2015).

value by 12% and 17%, respectively, in comparison to the untreated FC. In the meantime, the lower NaOH concentration did not show any improvement for the same parameter, which is in accordance to previous studies conducted by Sambusiti et al. (2013b), where a pretreatment with a low NaOH concentration (1 g NaOH/100 g TS) at 40 °C did not improve COD release from wheat straw and ensiled sorghum forage.

For all pretreatment conditions the VFA were manly composed by *n*-butyric and acetic acids, whereas it was observed a reduction on their concentration along the increase of NaOH dosage. Fact that can be explained by a possible dilution of such major VFA by the additional COD released during mild and high alkaline dosages. On the other hand, the same effect was not observed on concentration of the minor VFAs, which were mainly composed by phenylacetic, hexanoic and formic acids. Moreover, it is important to note that furfural and 5-methylfurfural, which could potentially inhibit the AD process (Barakat et al., 2012), where not detected for any pretreatment condition.

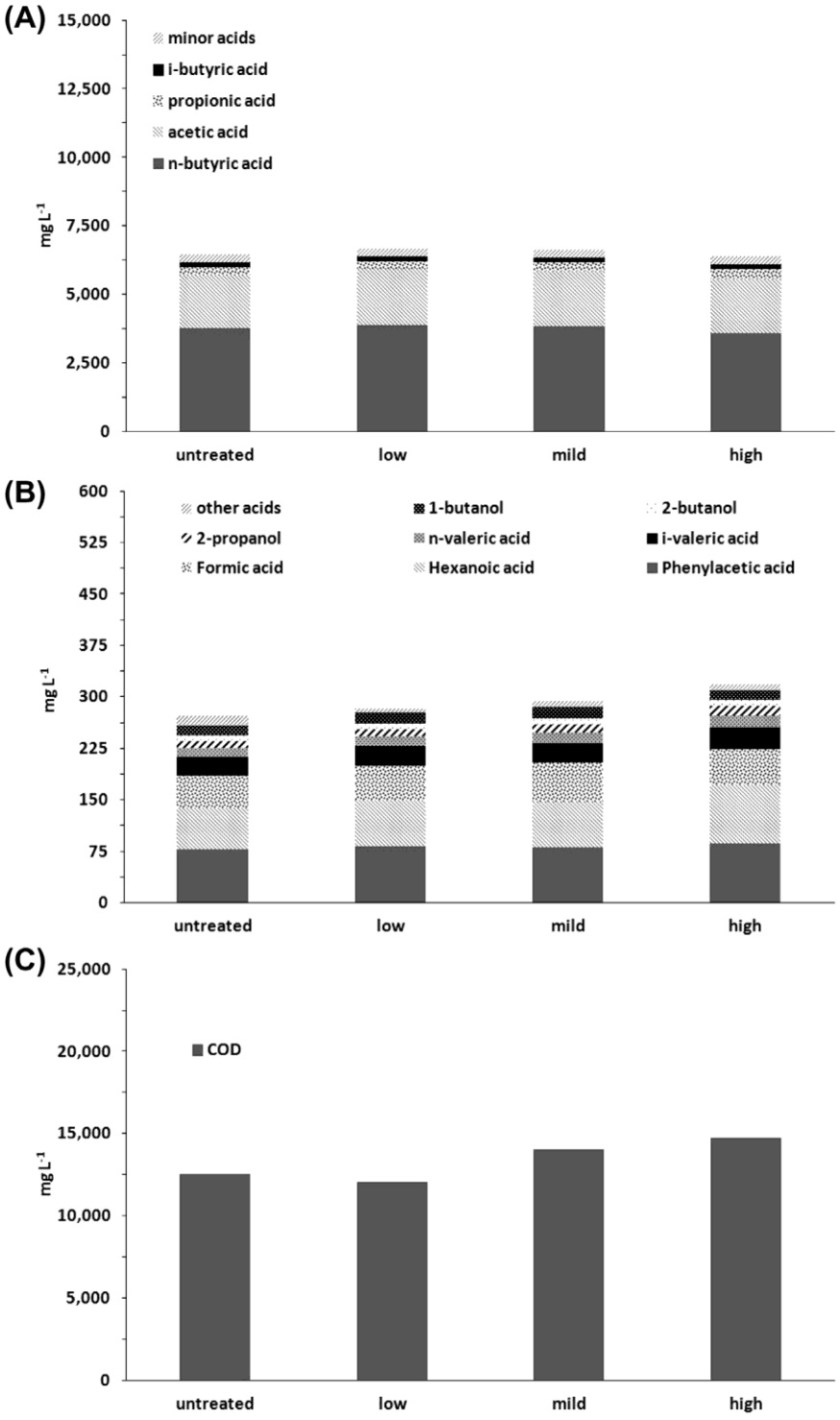

Fig. 1. Characteristics of the FC after alkaline pretreatment. (A) major VFA, (B) minor VFA, and (C) COD of the pretreated substrate, untreated (0 g NaOH/100 g FC_{FM}), low (1.5 g NaOH/100 g FC_{FM}), mild (3 g NaOH/100 g FC_{FM}) and high (6 g NaOH/100 g FC_{FM}).

The PCA between the main solubilized components and the different pretreatment conditions recovered 69.8% of the total variance. As can be seen in Fig. 2, PCA was effective in grouping the solubilized components according to the different pretreatment conditions. It is clearly observed that a deviation occurred after alkaline pretreatment, and the untreated FC emerged as an isolated group from the other components. PC1 (53.4%) is related with high NaOH concentration and evidences the relationship between this pretreatment condition and most of the analyzed components, including the increase on COD release, which is considered the primary effect of an efficient pretreatment process. On the other hand, the mild NaOH concentration is related to PC2 (16.4%) and describes the influence of this pretreatment in the concentration of acetic and *n*-butyric acids, which were the main VFA observed for all pretreatment conditions. In this case, increasing the NaOH dosage from low to mild or high did not result in a higher concentration of *n*-butyric acid. Similarly, the high pretreatment did not display higher acetic acid concentration than mild dosage.

3.2. Batch experiment

The methane yields obtained from the batch experiment and fitted to the dual-pool two-step model are shown in Figs. 3 and 4 and Table 3. The untreated FC presented a total methane potential of 259 $mL_N\ gVS^{-1}$, which is similar to values previously reported (Janke et al., 2014; Leite et al., 2015). The lower NaOH concentration (1.5 g NaOH/100 g FC_{FM}) was not able to significantly improve the total methane potential (262 $mL_N\ gVS^{-1}$) in comparison to the untreated FC. This is explained by the lower COD release by the low NaOH concentration during the substrate pretreatment.

The mild NaOH concentration (3 g NaOH/100 g FC_{FM}) was not only able to achieve 14.7% higher methane potential (297 $mL_N\ gVS^{-1}$), but also accelerate the AD process, since the ratio of rapidly degradable substrate to total degradable substrate (a value) increased from 0.36 (untreated) to 0.59 (mild NaOH concentration). Whereas, the additional 12% of COD released as an effect of the mild NaOH pretreatment seems to be the reason of such higher performance.

In the meantime, by applying the highest sodium hydroxide dosage (6 g NaOH/100 g FC_{FM}) in this experiment, the saponification effect provided by the alkali reagent was even higher, responsible for solubilizing additional 17% of COD, which has resulted in the highest a value (0.67) of all tested pretreatment conditions and 22.4% higher methane potential (317 $mL_N\ gVS^{-1}$) in comparison to the untreated EFC.

From the remaining model constants it is possible to observe that in all cases the hydrolysis was the rate-limiting step, since the k_F and k_L values are lower than k_{VFA}, which is in accordance to previous studies on lignocellulosic substrates conducted by Weinrich and Nelles (2015). Additionally, if the k_{VFA} of the different pretreatment conditions is considered for the short-term AD, the high NaOH concentration was responsible for increasing the VFA peak by 38.6% in compassion to the untreated FC. Fact that could give an advantage if the high NaOH concentration would be used in a semi-continuous process aiming the production of VFA, since low HRT should be maintained to wash-out the slow growing methanogens.

Another aspect that should be taken into consideration during semi-continuous AD process is the possibility of reactor's salinization by high Na^+ concentrations. Previous studies (Chen et al., 2008) reported that depending on the adaptation period, antagonistic/synergistic effects, substrate and reactor configuration, methanogens could be inhibited with sodium concentrations between 5.6 and 53 $g\ L^{-1}$. Thus, if methane is further intended to be produced in a two-stage reactor system, special care should be taken with the highest NaOH concentration (6 g NaOH/100 g FC_{FM}), since in this case the Na^+ concentration would achieve around 8 gL^{-1}.

3.3. Semi-continuous experiment

3.3.1. Start-up phase

For the reactor's start-up phase (days 0–17) the initial OLR was set to 16.7 g VS $L^{-1}\ d^{-1}$ and gradually decreased to 9.1 g VS $L^{-1}\ d^{-1}$, while the HRT was increased from 2.5 days to 4.6 days. Such start-up strategy was responsible for overloading the AD process (between days 8 and 17) by increasing the initial VOA concentration from 1.0 g L^{-1} to an average value of 6.9 g L^{-1}, followed by a decrease of the pH value from 7.7 to nearly 5.2 at the same period (Fig. 5).

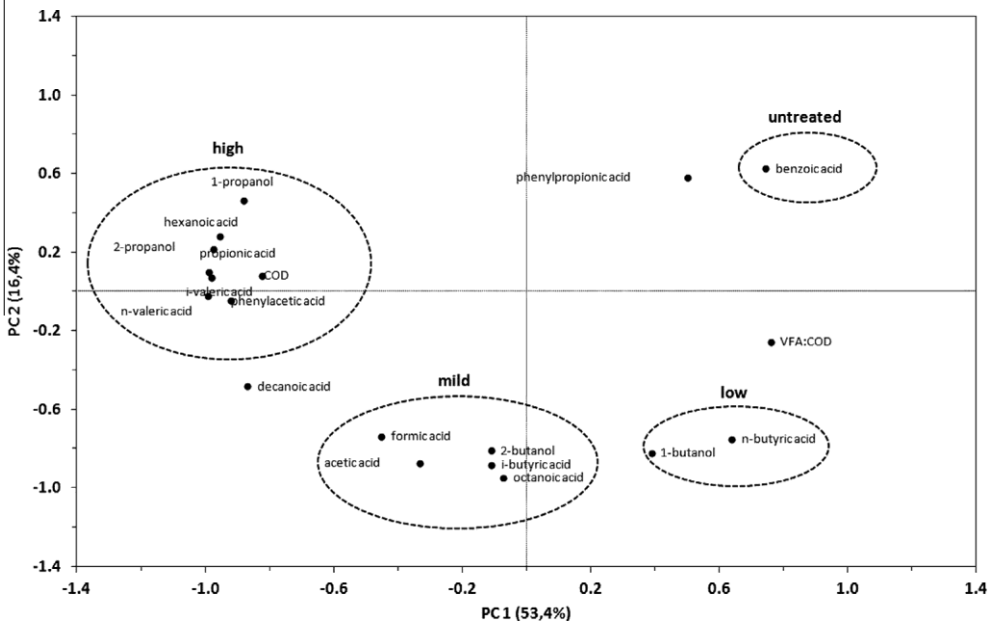

Fig. 2. PCA of the main characteristics of the FC pretreated with different sodium hydroxide concentrations applied for the batch experiment.

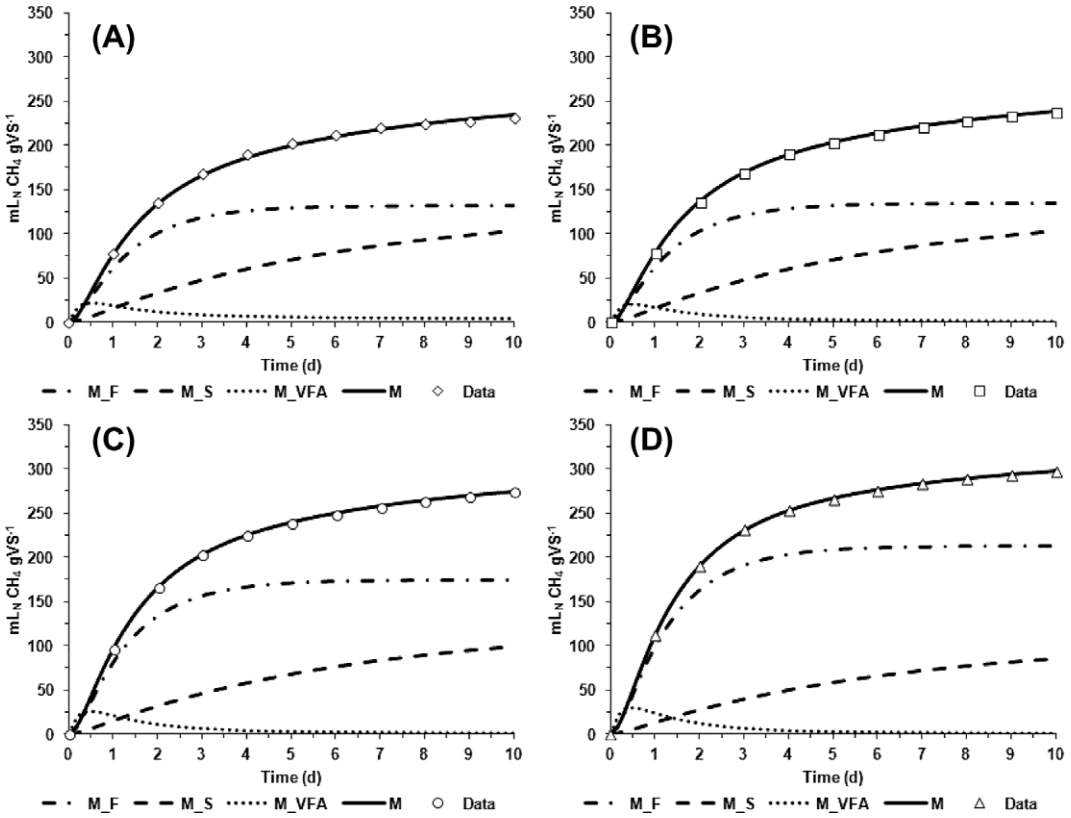

Fig. 3. Individual cumulative methane yields obtained from the batch experiment and fitted to the dual-pool two-step model. (A) untreated (0 g NaOH/100 g FC_{FM}); (B) low (1.5 g NaOH/100 g FC_{FM}); (C) mild pretreatment (3 g NaOH/100 g FC_{FM}) and (D) high (6 g NaOH/100 g FC_{FM}). Abbreviations refer to (M_F) methane from rapidly degradable fraction, (M_S) methane from slowly degradable fraction, (M_VFA) intermediate VFA production, (M) total methane potential and (Data) experimental measurements.

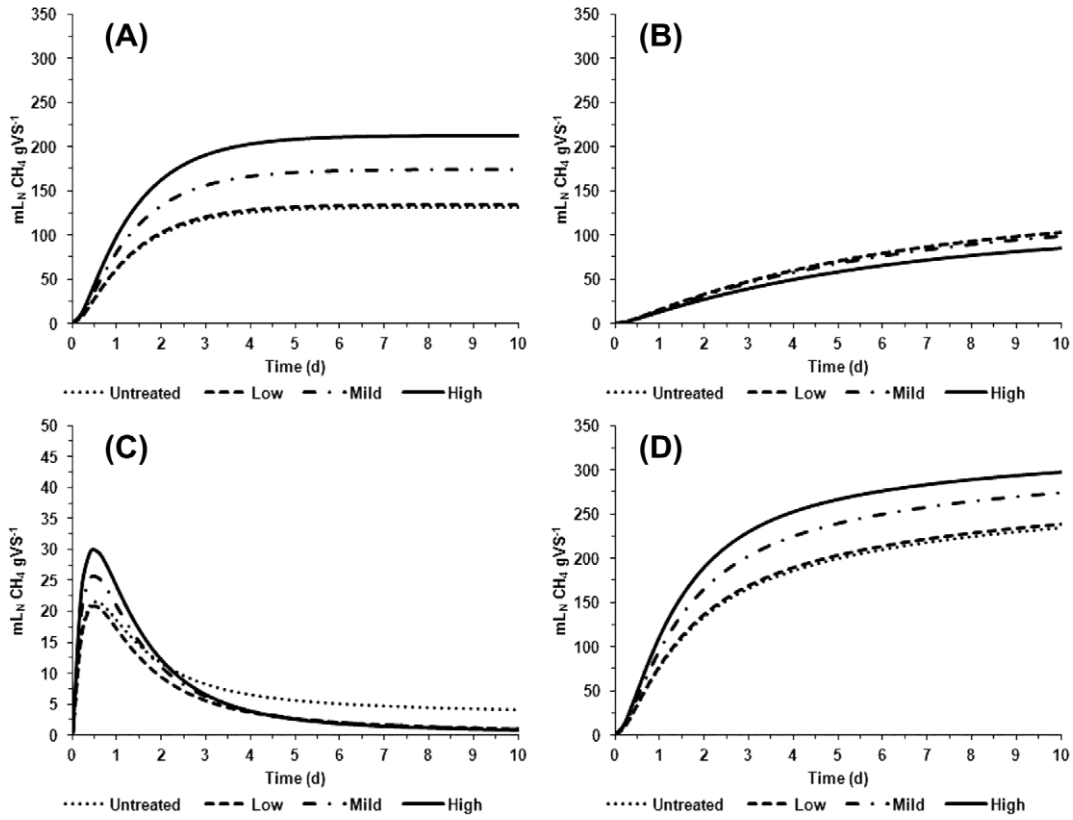

Fig. 4. Grouped cumulative methane yields obtained from the batch experiment and fitted to the dual-pool two-step model. (A) methane from rapidly degradable fraction; (B) methane from slowly degradable fraction; (C) intermediate VFA production and (D) total methane potential of untreated, low, mild and high FC pretreatment conditions.

Table 3
Results of the batch experiment fitted to the dual-pool two-step model.

Condition	S ($mL_N\ CH_4\ gVS^{-1}$)	α (–)	k_F (d^{-1})	k_L (d^{-1})	k_{VFA} (d^{-1})	S^* ($mL_N\ CH_4\ gVS^{-1}$)	α^* (–)	Increase S^* (%)
Untreated	238	0.36	0.83	0.12	4.40	259	0.51**	–
Low	267	0.58	0.71	0.10	4.95	262	0.51	1.24
Mild	325	0.61	0.77	0.14	4.59	297	0.59	14.7
High	333	0.67	0.83	0.32	4.09	317	0.67	22.4

* Optimized values (curve fitting) for constant values of k_F = 0.83 (d^{-1}), k_L = 0.17 (d^{-1}) and k_{VFA} = 4.51 (d^{-1}), respectively.
** Manually adjusted (see Figs. 3 and 4).

Although the SBP achieved a maximum value of 97 $mL_N\ gVS^{-1}$ (methane 53.8% v/v) at days 2–5 when the average pH value and the VOA concentrations were 7.0 and 3.4 g L^{-1}, respectively. The fast increase of the VOA concentration and decrease of the pH value observed during the start-up phase caused a strong inhibition of the methane production, depicted by a SBP of nearly zero between days 6 and 17.

Additionally, the process of inoculum washing-out that usually occurs immediately after the start-up of a SCSTR also seems to have contributed to the fast pH decrease, since the initial NH_4-N concentration in the inoculum (1.7 g L^{-1}) was rapidly decreased to lower than 0.1 g L^{-1} at day10, reducing the capacity of NH_4^+ buffer the system.

3.3.2. Steady phase

In order to provide stable conditions for VFA production during steady phase (days 18–30) the OLR and HRT were kept at 8.3 g VS L^{-1} d^{-1} and 5 days, respectively. The VOA concentration achieved a constant value of 6.6 g L^{-1} at an average pH value of 5.2, conditions in which nearly no methane production was observed.

The NH_4-N concentration displayed the same value as observed at the end of the start-up phase (0.1 g L^{-1}), indicating a possible nitrogen deficiency of the substrate. According to a previous study (Zhang et al., 2005) the optimum concentration of NH_4-N for VFAs production is about 1.0–1.2 g L^{-1}, which is 10–12 times higher than found during the steady phase.

The Fig. 6 shows the VFA profile of the semi-continuous experiment. Under the above mentioned operation conditions, 3.8 g L^{-1} of *n*-butyric acid, 2.7 g L^{-1} of acetic acid and 0.5 g L^{-1} of propionic acid were produced (total VFA of 8.0 g L^{-1}), resulting in a VFA yield of 0.32 g VFA gVS^{-1}. Based on the average SCOD concentration observed during steady conditions (14.6 g L^{-1}), a conversion of approximately 54% of the soluble compounds produced by hydrolysis into VFAs were assumed.

3.3.3. Urea supplementation

As mentioned previously, the NH_4-N concentration indicated a potential nitrogen deficiency of the substrate. Additionally, previous studies (Cysneiros et al., 2012b) on AD of maize silage using leach bed reactors reported an increase of 50% in VFA production by controlling the pH at around 6.5. Therefore, 3 g of urea was supplemented at day 30 to buffer the reactor's pH, and at the same time to balance the C:N ratio of the substrate in case of low nitrogen solubilization during hydrolysis/acidogenesis.

After supplementation, the NH_4-N concentration increased to 0.45 g L^{-1} at day 36, also resulting in an increase of the pH value from 5.2 to 6.7. However, instead of optimizing the acidogenic process, a decrease in the VOA concentration from 6.6 g L^{-1} to a minimum level of 1.7 g L^{-1} at day 47 was observed, followed by an increase of the SBP from 10 $mL_N\ gVS^{-1}$ to a maximum production of 275 $mL_N\ gVS^{-1}$ (methane 49.3% v/v) at day 44. This fact suggests that the slow-growing methanogens were able to recover and convert the accumulated VOA into biogas even at a low HRT (5 days) setup, possibly due to the pH increase, which is close to the optimum range for the methanogenesis phase (Schmidt et al., 2014; Solera et al., 2002).

Moreover, it is assumed that acetogenesis also benefited from the pH increase, since the *n*-butyric acid concentration decreased from around 3.8–0.01 g L^{-1}. Interestingly, during the same period the propionic acid concentration increased from 0.5 g L^{-1} to 1.1 g L^{-1}. In fact, the urea supplementation strategy was responsible for reducing the VFA yield by 75% (0.08 g VFA gVS^{-1}), which is the lowest level observed during the entire experimental period.

At day 49 the OLR was increased to 13.1 g VS L^{-1} d^{-1} while the HRT decreased to 3.4 days in order to recover the VFA production. Such procedure allowed a faster NH_4-N wash-out provided by the urea supplementation, followed by the reduction of the pH value to around the same value (5.5) found at steady phase.

3.3.4. Urea with nitric acid supplementation

At day 57 the OLR and HRT were set to the same levels as during steady and urea supplementation phases, and kept stable until the end of the semi-continuous experiment at day 99 to allow a reliable comparison among the different experiment phases.

Between the days 54 and 71 a second urea supplementation was performed, however at this time the pH value was also controlled to prevent VFA conversion to biogas. Therefore, at day 57 urea was daily supplemented (1.2 g) and HNO_3 added every two days (5 mL of 10 mL L^{-1} solution) to keep the pH close to the same level observed during steady phase (5–5.5).

Along the increasing on NH_4-N concentration (0.8 g L^{-1} at day 64), the pH varied between 5.1 and 5.9, and the VOA concentration reached a maximum value of 6.1 g L^{-1} (11% lower than in steady phase). Apparently, the urea supplementation and pH control by HNO_3 addition did not result in keeping the SBP at the same low level as observed during steady phase, i.e., nearly zero. Occasionally, the SBP achieved 100 $mL_N\ gVS^{-1}$ (methane 45.0% v/v) when the pH increased above 5.5. Such results are in the contrary to other studies (Veeken et al., 2000; Wang et al., 2014) that reported improvements of hydrolysis and acidogenesis by controlling the pH above 6, possibly due to their different reactor configuration (i.e., leach bed) and/or feeding regime (i.e., batch system).

The produced VFA spectrum consisted mainly of acetic acid 2.8 g L^{-1}, *n*-butyric acid 1.7 g L^{-1} and propionic acid 0.7 g L^{-1} (total VFA concentration of 6.3 g L^{-1}). Although the VFA:SCOD ratio demonstrated that 57% of the soluble compounds were converted into VFAs, which is 3% higher than during steady phase, the VFA yield (0.24 g VFA gVS^{-1}) was 25% lower. The main reason for that is the relatively high pH value where it was difficult to counteract the buffering capacity provided by NH_4-N with HNO_3 additions to keep the pH relatively constant between 5 and 5.5. At a higher pH value acetogenesis and methanogenesis are recovered resulting in VFA loss through biogas production.

3.3.5. Sodium hydroxide pretreatment

The NaOH concentration for substrate pretreatment that resulted in the highest methane yield during batch AD has been chosen for substrate pretreatment between days 72 and 99 of the semi-continuous experiment. Mostly during this phase the

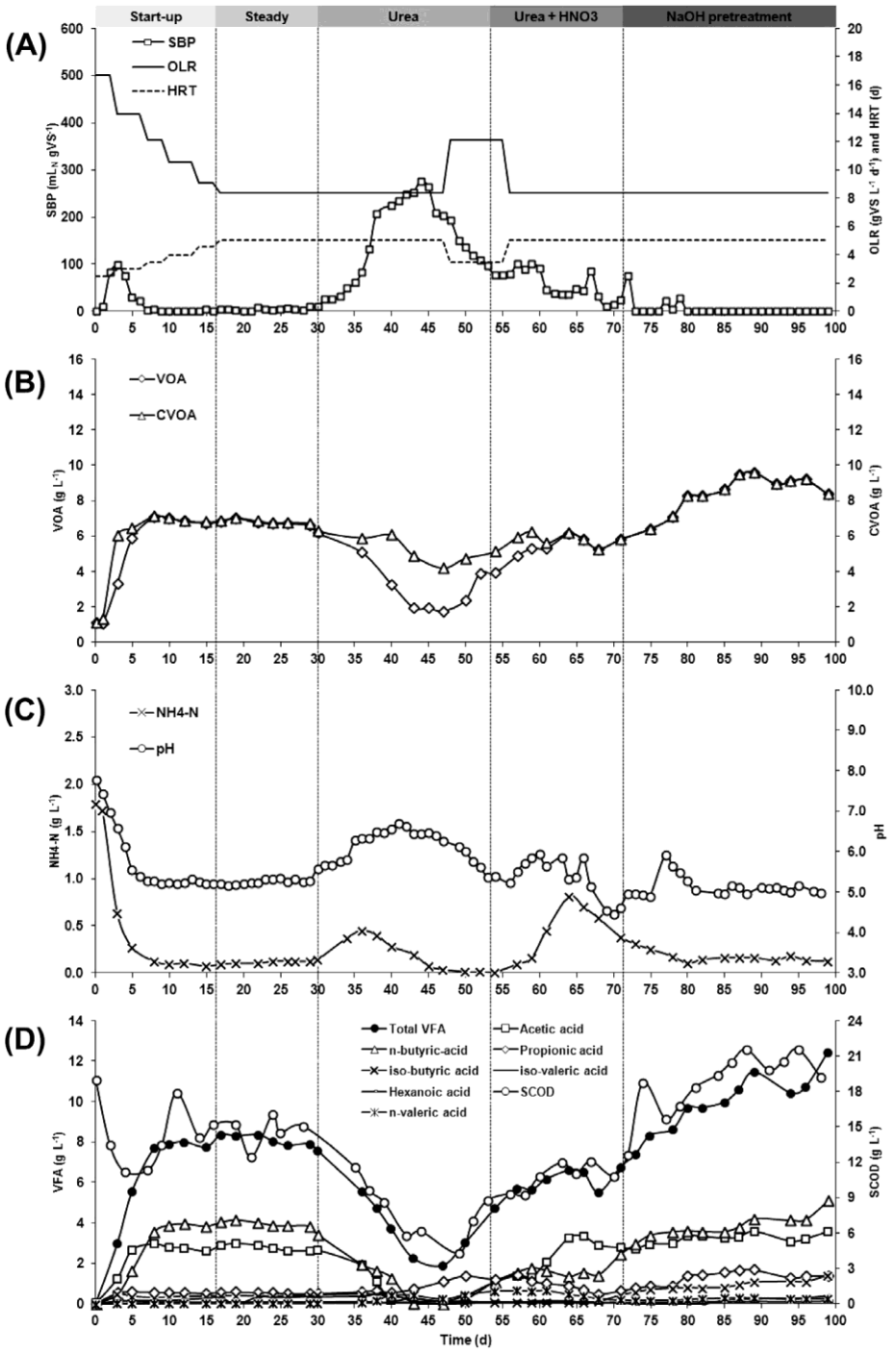

Fig. 5. Main parameters monitored during the semi-continuous experiment. (A) specific biogas production (SBP), organic loading rate (OLR) and hydraulic retention time (HRT); (B) volatile organic acids (VOA) and calculated volatile organic acids (CVOA); (C) ammonium-nitrogen (NH_4-N) and pH value; (D) volatile fatty acid (VFA) and soluble chemical oxygen demand (SCOD).

pH value was kept around 5.0, resulting in an average VOA concentration of 8.8 g L^{-1}. The NH_4-N concentration returned to the same low level (0.1–0.15 g L^{-1}) as found during steady phase. Under these conditions it was possible to prevent any losses of VFA through methanogenesis, since the observed SBP was nearly zero.

The average VFA yield reached its highest value during this phase (0.44 g VFA gVS^{-1}), representing an increase of 37% in comparison to the steady phase. Meanwhile, the VFA:SCOD ratio (0.54) did not show any improvement, suggesting that NaOH pretreatment enhanced primarily the hydrolysis phase, which reinforces the hydrolysis as the rate-limiting step during AD of FC. The total VFA reached its highest average production (10.9 g L^{-1}), which is 36% higher than the average value found during the steady phase.

Finally, it is possible to assume that the syntrophic relationship between acetogenesis and methanogenesis was inhibited by the low pH observed during the sodium hydroxide pretreatment phase, since the *n*-butyric acid production recovered from 0.01 g L^{-1} during urea supplementation phase to 4.0 g L^{-1} and propionic acid increased from 0.5 g L^{-1} during steady phase to 1.4 g L^{-1}.

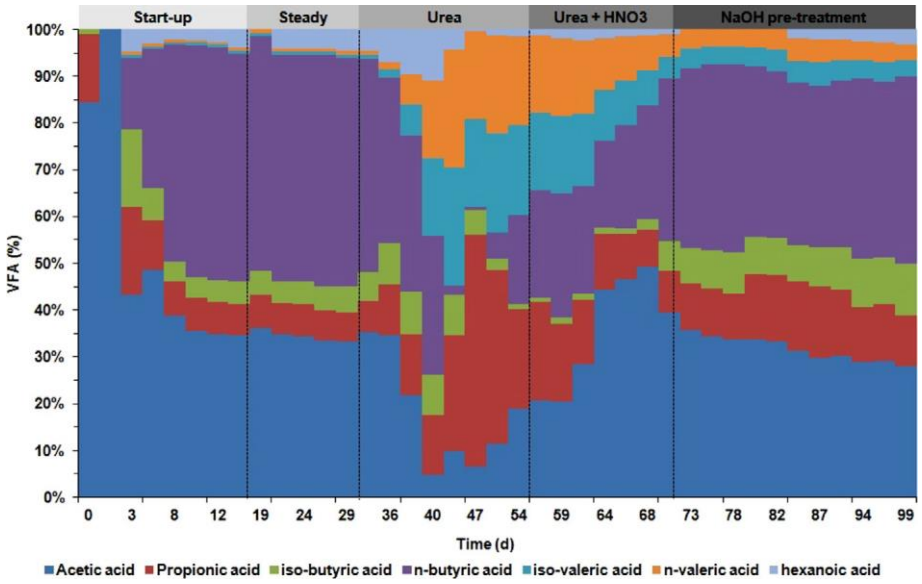

Fig. 6. Relative VFA concentrations measures during semi-continuous experiment.

4. Conclusions

The present study showed the effects of urea addition and NaOH pretreatment on AD of FC. By increasing NH_4-N provided by urea, the VFA accumulation was drastically reduced by 75% during semi-continuous experiment. NaOH pretreatment provided positive effects during batch and semi-continuous experiment. During batch experiment methane potential was increased by 22.4%, while during semi-continuous experiment VFA yield achieved maximum value of 0.44 g VFA gVS^{-1} (37% higher than found at steady phase). Such results can enhance the development of a bio-based economy, since the optimized VFA production could substitute fossil based raw materials in several industrial applications.

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References

Angelidaki, I., Ellegaard, L., Ahring, B.K., 1993. A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnol. Bioeng. 42, 159–166. http://dx.doi.org/10.1002/bit.260420203.

Barakat, A., Monlau, F., Steyer, J.P., Carrere, H., 2012. Effect of lignin-derived and furan compounds found in lignocellulosic hydrolysates on biomethane production. Bioresour. Technol. 104, 90–99. http://dx.doi.org/10.1016/j.biortech.2011.10.060.

Brulé, M., Oechsner, H., Jungbluth, T., 2014. Exponential model describing methane production kinetics in batch anaerobic digestion: a tool for evaluation of biochemical methane potential assays. Bioprocess Biosyst. Eng. 1–12.http://dx.doi.org/10.1007/s00449-014-1150-4.

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99, 4044–4064. http://dx.doi.org/10.1016/j.biortech.2007.01.057.

Cysneiros, D., Banks, C.J., Heaven, S., Karatzas, K.A.G., 2012a. The role of phase separation and feed cycle length in leach beds coupled to methanogenic reactors for digestion of a solid substrate (Part 1): Optimisation of reactors' performance. Bioresour. Technol. 103, 56–63. http://dx.doi.org/10.1016/j.biortech.2011.09.094.

Cysneiros, D., Banks, C.J., Heaven, S., Karatzas, K.A.G., 2012b. The effect of pH control and ''hydraulic flush" on hydrolysis and Volatile Fatty Acids (VFA) production and profile in anaerobic leach bed reactors digesting a high solids content substrate. Bioresour. Technol. 123, 263–271. http://dx.doi.org/10.1016/j.biortech.2012.06.060.

Deublein, D., Steinhauser, A. (Eds.), 2008. Biogas from Waste and Renewable Resources: An Introduction. WILEY-VHC Verlag GmbH & Co. KGaA, Weinheim.

Elsayed, M.T., Babiker, M.H., Abdelmalik, M.E., Mukhtar, O.N., Montange, D., 2008. Impact of filter mud applications on the germination of sugarcane and small-seeded plants and on soil and sugarcane nitrogen contents. Bioresour. Technol. 99, 4164–4168. http://dx.doi.org/10.1016/j.biortech.2007.08.079.

FAO, 2013. Food and agricultural commodities production [WWW Document]. FAOSTAT. URL: faostat.fao.org (accessed 5.25.15).

FNR, 2010. Guide to Biogas From production to use.

Fricke, K., Santen, H., Wallmann, R., Hüttner, A., Dichtl, N., 2007. Operating problems in anaerobic digestion plants resulting from nitrogen in MSW. Waste Manag. 27, 30–43. http://dx.doi.org/10.1016/j.wasman.2006.03.003.

Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18. http://dx.doi.org/10.1016/j.biortech.2008.05.027.

Janke, L., Leite, A.F., Wedwitschka, H., Schmidt, T., Nikolausz, M., Stinner, W., 2014. Biomethane production integrated to the Brazilian sugarcane industry: the case study of São Paulo state. pp. 23–26.

Kayhanian, M., Rich, D., 1995. Pilot-scale high solids thermophilic anaerobic digestion of municipal solid waste with an emphasis on nutrient requirements. Biomass Bioenergy 8, 433–444. http://dx.doi.org/10.1016/0961-9534(95)00043-7.

Leite, A.F., Janke, L., Harms, H., Zang, J.W., Fonseca-Zang, W.A., Stinner, W., Nikolausz, M., 2015. Assessment of the variations in characteristics and methane potential of major waste products from the Brazilian bioethanol industry along an operating season. Energy Fuels. http://dx.doi.org/10.1021/ef502807s 150318103543009.

López González, L.M., Pereda Reyes, I., Dewulf, J., Budde, J., Heiermann, M., Vervaeren, H., 2014. Effect of liquid hot water pre-treatment on sugarcane press mud methane yield. Bioresour. Technol. 169, 284–290. http://dx.doi.org/10.1016/j.biortech.2014.06.107.

López González, L.M., Vervaeren, H., Pereda Reyes, I., Dumoulin, A., Romero Romero, O., Dewulf, J., 2013. Thermo-chemical pre-treatment to solubilize and improve anaerobic biodegradability of press mud. Bioresour. Technol. 131, 250–257. http://dx.doi.org/10.1016/j.biortech.2012.12.167.

Mariano, A.P., Dias, M.O.S., Junqueira, T.L., Cunha, M.P., Bonomi, A., Filho, R.M., 2013. Butanol production in a first-generation Brazilian sugarcane biorefinery: technical aspects and economics of greenfield projects. Bioresour. Technol. 135, 316–323. http://dx.doi.org/10.1016/j.biortech.2012.09.109.

Mccarty, P.L., 1964. Anaerobic waste treatment fundamentals. Public Work 95, 107–112.

Merklein, K., Fong, S.S., Deng, Y., 2014. Production of butyric acid by a cellulolytic actinobacterium *Thermobifida fusca* on cellulose. Biochem. Eng. J. 90, 239–244. http://dx.doi.org/10.1016/j.bej.2014.06.012.

Montgomery, L.F.R., Bochmann, G., 2014. Pretreatment of feedstock for enhanced biogas production. IEA Bioenergy.

Sambusiti, C., Ficara, E., Malpei, F., Steyer, J.P., Carrère, H., 2013a. Benefit of sodium hydroxide pretreatment of ensiled sorghum forage on the anaerobic reactor

stability and methane production. Bioresour. Technol. 144, 149–155. http://dx.doi.org/10.1016/j.biortech.2013.06.095.

Sambusiti, C., Monlau, F., Ficara, E., Carrère, H., Malpei, F., 2013b. A comparison of different pre-treatments to increase methane production from two agricultural substrates. Appl. Energy 104, 62–70. http://dx.doi.org/10.1016/j.apenergy.2012.10.060.

Schmidt, T., Ziganshin, A.M., Nikolausz, M., Scholwin, F., Nelles, M., Kleinsteuber, S., Pröter, J., 2014. Effects of the reduction of the hydraulic retention time to 1.5 days at constant organic loading in CSTR, ASBR, and fixed-bed reactors – Performance and methanogenic community composition. Biomass and Bioenergy 69, 241–248. http://dx.doi.org/10.1016/j.biombioe.2014.07.021.

Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J., 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour. Technol. 98, 3000–3011. http://dx.doi.org/10.1016/j.biortech.2006.10.022.

Solera, R., Romero, L.I., Sales, D., 2002. The evolution of Biomass in a two-phase anaerobic treatment process during start-up. Chem. Biochem. Eng. Q 16, 25–29.

Sträuber, H., Bühligen, F., Kleinsteuber, S., Nikolausz, M., Porsch, K., 2015. Improved anaerobic fermentation of wheat straw by alkaline pre-treatment and addition of alkali-tolerant microorganisms. Bioengineering 2, 66–93. http://dx.doi.org/10.3390/bioengineering2020066.

VDI, 2006. VDI 4630 – Fermentation of organic materials: Characterisation of the substrate, sampling, collection of material data, fermentation tests. VDI-Gesselschaft Energietechnik.

Veeken, A., Kalyuzhnyi, S., Scharff, H., Hamelers, B., 2000. Effect of pH on hydrolysis of organic solid waste. J. Environ. Eng. 126, 1076–1081.

Wang, K., Yin, J., Shen, D., Li, N., 2014. Anaerobic digestion of food waste for volatile fatty acids (VFAs) production with different types of inoculum: effect of pH. Bioresour. Technol. 161, 395–401. http://dx.doi.org/10.1016/j.biortech.2014.03.088.

Weiland, P., 2010. Biogas production: current state and perspectives. Appl. Microbiol. Biotechnol. 85, 849–860. http://dx.doi.org/10.1007/s00253-009-2246-7.

Weinrich, S., Nelles, M., 2015. Critical comparison of different model structures for the applied simulation of the anaerobic digestion of agricultural energy crops. Bioresour. Technol. 178, 306–312. http://dx.doi.org/10.1016/j.biortech.2014.10.138.

Yu, L., Bule, M., Ma, J., Zhao, Q., Frear, C., Chen, S., 2014. Enhancing volatile fatty acid (VFA) and bio-methane production from lawn grass with pretreatment. Bioresour. Technol. 162, 243–249. http://dx.doi.org/10.1016/j.biortech.2014.03.089.

Zhang, B., Zhang, L.-L., Zhang, S.-C., Shi, H.-Z., Cai, W.-M., 2005. The influence of pH on hydrolysis and acidogenesis of kitchen waste in two-phase anaerobic digestion. Environ. Technol. 26, 329–339. http://dx.doi.org/10.1080/09593332608618563.

4. Discussion and conclusion

4.1. Challenges for novel substrates in bioenergy production

Based on recent alarming figures regarding population growth and climate change (Thornton et al., 2014; Wheeler & von Braun, 2013), the assessment of potential novel substrates for biogas and biofuel production has to be reconsidered, since it involves, more than ever, complex issues related to land use and waste management (Rey, 2013). Many substrates already used for biogas and biofuel production are cultivated in agricultural regions, resulting in potential competition with food production in regard to land use. For example, the cost-efficiency of biogas and biofuels in Germany, Brazil and USA mainly relies on the use of agricultural crops. Germany, as the greatest biogas producer worldwide (Hijazi et al., 2016; Weiland, 2010) and as an extensive biodiesel producer (De Oliveira & Coelho, 2016), uses mostly maize for biogas production and vegetable oils for biodiesel. Brazil and USA, leading countries in bioethanol (Marin, 2016) and biodiesel production (Martins & de Andrade Júnior, 2016; Tolmac et al., 2014), use sugarcane and maize, respectively, for bioethanol production and vegetable oils for biodiesel (Fischer et al., 2009; Herrmann, 2013; Lewandowski, 2015).

Significant efforts and extensive research have been devoted to produce biofuels and biogas from alternative substrates with low or no requirement of agricultural areas, such as algae (Dębowski et al., 2013; Mussgnug et al., 2010), cassava (Moshi et al., 2014) and crops grown on marginal lands (Popp et al., 2015; Scordia et al., 2010). Waste products have also been intensively investigated for their potential in biofuel and biogas production, e.g. apple waste with swine manure (Kafle & Kim, 2013), coffee grounds (Kim et al., 2016), and fruit and vegetable waste (Bouallagui et al., 2003), among others. Hence, considering the advances in research in recent years regarding process monitoring and controlling, and efficient pre-treatment options, the decision to use novel substrates is oriented not towards agricultural crops but rather towards alternative substrates and waste products.

Applying pre-treatments to novel substrates as a strategic part of the biogas process design is common practice. Pre-treatment strategies for exploiting higher methane yields are predominately performed firstly in batch tests in order to quickly estimate the conversion ratio of the novel substrate. However, batch tests may show only the energy potential and disregard for instance the toxicity of most inhibitors present in the treated substrate. The negative effects are masked due to the larger content of inoculum compared to substrate used in batch tests. Also, the deficiency of micro- and macro-elements of the substrate is normally disguised by the balanced elemental composition of the inoculum. Furthermore, the adaptation of microorganisms to inhibitory substances is not considered in batch tests due to the short running time. For example, Lv et al. (2014a) and Nie et al. (2015) have reported long-term microbial adaptation under high ammonia concentration in a continuous biogas process.

Therefore, combining the pre-treatment strategy together with a (semi-) continuous feeding system is crucial in a feasibility and cost-efficiency assessment of the whole concept.

Many of the potential substrates considered for biogas production lack essential elements which ensure an efficient microbial process. In mono-digestion processes, these substrates would not be implemented extensively in the biogas sector due to eventual further costs of adding chemicals with micro- and macro-elements. For this reason, the co-digestion of substrates which complement each other in relation to the elemental composition is considered. For instance, Li et al. (2015) showed that the optimal feeding ratio of rice straw to cow manure during anaerobic mesophilic co-digestion was 1:1 based on volatile solids. Wan et al. (2011) reported that the co-digestion of thickened waste-activated sludge with fat, oil and grease at 64% volatile solids, increased biogas production by 137%. During the assessment of potential co-digestion options, the local available resources are also taken into consideration. Otherwise, the transportation costs of the co-substrate may circumvent profitability.

To ensure continuous biogas production from novel substrates, storage options are evaluated through the different weather conditions throughout the year. In Europe, in order to guarantee an effective storage of substrates for biogas production, ensilaging is commonly applied. In this case, a process under low pH values and high concentration of organic acids, and cold temperature during long storage period prevents further microbial degradation of the substrate. Still, due to energy losses during undesirable aerobic degradation in the ensiling process, Weiland (2010) suggested compacting the substrate in silos and covering it with plastic wrap. Later, after opening the bunker silo, Weiland (2010) further recommends the application of heterofermentative starter cultures. In tropical countries, where temperatures are much higher and rains are more intense, storage options, including previous ensilage, are assessed according to each specific condition, e.g. dry and rainy seasons, hot days and cold nights, and available local infrastructure.

The ensiling of substrates for biogas production is also considered as a pre-treatment process, because part of the structural polysaccharides of the plant material is degraded. In order to further increase the degradation ratio, some researchers, considering the long storage period, have investigated additional biological pre-treatment with fungus to enhance methane production in the biogas process (Liu et al., 2014; Zhong et al., 2011). Altogether, the great advantage of storing substrates is the flexibilization of bioenergy production. In contrast to solar and wind energy, the stored substrates in the biogas sector can be used to produce energy on demand (Lv et al., 2014b; Mulat et al., 2016). Hence, this contributes to the consolidation of biogas as a reliable energy source.

4.2. Implementing the biogas process in the sugarcane industry

Biogas production from sugarcane wastes has been described in a number of biorefinery concepts in which Brazil, with its strong sugarcane industry, was presented as a case study (Rabelo et al., 2011; Renó et al., 2014). Furthermore, in life cycle assessments performed on other sugarcane producing countries such as Cuba (Contreras et al., 2009), India (Chauhan et al., 2011) and Columbia (Pabon Pereira et al., 2006), biogas production from sugarcane wastes was considered as a sustainable option for energy recovery. In this context, the application of biogas technology in the sugarcane industry follows the current global trend towards sustainable development. This PhD thesis is part of the early stage development of practical biogas research on sugarcane wastes with laboratory-scale reactors simulating large biogas plants.

Few other scientists, who have contributed to the development of concepts for AD process in the sugarcane industry, have studied basically the theory of a possible sustainable application of the biogas production from sugarcane wastes (Chandel et al., 2012; Lettinga & Haandel, 1993; Moraes et al., 2014; Moraes et al., 2015; Pabon Pereira et al., 2006). Only few other studies used laboratory-scale reactors investigating continuous biogas production from sugarcane wastes (Costa et al., 1986; Lakshmanan et al., 1990; Souza et al., 1992; Thangamuthu, 2010). However, they were using very simple monitoring techniques and vague approaches. Publications arising from this PhD thesis were based on clear monitoring of parameters with modern techniques to undoubtedly show the effects of controlled variations on the process.

In view of the evaluation of the experiments performed in this work, it was observed that despite promising biochemical methane potential (BMP) results, bagasse in mono-digestion process for continuous biogas production is not recommended due to its very high C:N ratio and low concentrations of important trace elements. Moreover, potential competition with the thermal energy recovery (incineration) and with the production of second generation bioethanol was consider as risk for the mono-digestion of bagasse (Bezerra & Ragauskas, 2016). The other lignocellulosic waste, straw, was evaluated in a similar fashion to bagasse with respect to high carbohydrate and lignin content, and low trace elements concentration. However, straw can still be used for energy recovery purposes, because it is simply left on the fields or burned without energy recovery to facilitate harvesting. Although a few other studies have reported the pre-treatment of filter cake (Lopez Gonzalez et al., 2014; Lopez Gonzalez et al., 2013), they conducted experiments only in batch tests. Vinasse has shown satisfactory potential for biogas production in the batch essays in comparison to other industrial wastewaters (Maya-Altamira et al., 2008).

Considering the final products from AD process, Brazil has a great potential to implement biogas technology successfully in the sugarcane industry, since the initial infrastructure for the use of biogas commodities already exists. Electricity demand is continuously increasing in emerging economies and developing countries. Heat can be used in the industry itself during the distillation process. Biomethane can serve as a biofuel for vehicles used in the agriculture and industry, from the sugarcane fields to bioethanol/sugar distribution. Additionally, it can also replace the use of imported natural gas. In this case, the pipes coming from Bolivia and going to São Paulo could also be used for transportation of upgraded biogas (Janke et al., 2014). Digestate as a well-balanced fertilizer can easily stop the direct disposal of sugarcane wastes into the environment in the sugarcane fields. Furthermore, it can prevent the use of mineral fertilizer. In addition to all these benefits in supporting the implementation of biogas technology in the sugarcane industry, Brazil may provide additional conditions for biogas development after winning the long fight in regulating the laws on waste management (Jabbour et al., 2014; Janke et al., 2014).

Based on the sugarcane production of the 2015/2016 season (UNICA, 2016) and on the BMP of raw straw, bagasse, filter cake and vinasse found in this PhD thesis, the biogas process in the Brazilian sugarcane industry is able to provide a total electricity production around 56 TWh (203 PJ) per year (as much as 42% conversion in the combined heat and power system). In this case, only 50% of the straw and 12% of the bagasse were considered available for the biogas process, since the burning of straw prior to manual harvesting and the thermo-conversion of bagasse into heat and electricity are currently applied (De Paoli et al., 2011). Filter cake and vinasse were considered to be 100% available for the biogas process. By selling biogas electricity, the Brazilian sugarcane industry could earn, in local currency, close to R$ 16 billion (considering an electricity price of 0.28 R$/kWh). In Figure 8, the potential electricity capacity of biogas power plants is shown for each single sugarcane waste. Considering only the potential for biogas production from the four sugarcane wastes in Brazil, the electricity capacity in the sugarcane industry would be approximately 41% larger than the current biogas-installed capacity in Germany. Furthermore, the sugarcane industry could cover about 12% of the total electricity consumption of Brazil in 2016 simply by using its wastes for biogas production. If Brazil substituted natural gas with biogas as a fuel source for electricity generation, approximately 23 Tg CO_2 emissions could be saved. Despite this, GHG emissions from sugarcane fields and vinasse lagoons could be prevented by using sugarcane wastes in biogas processes.

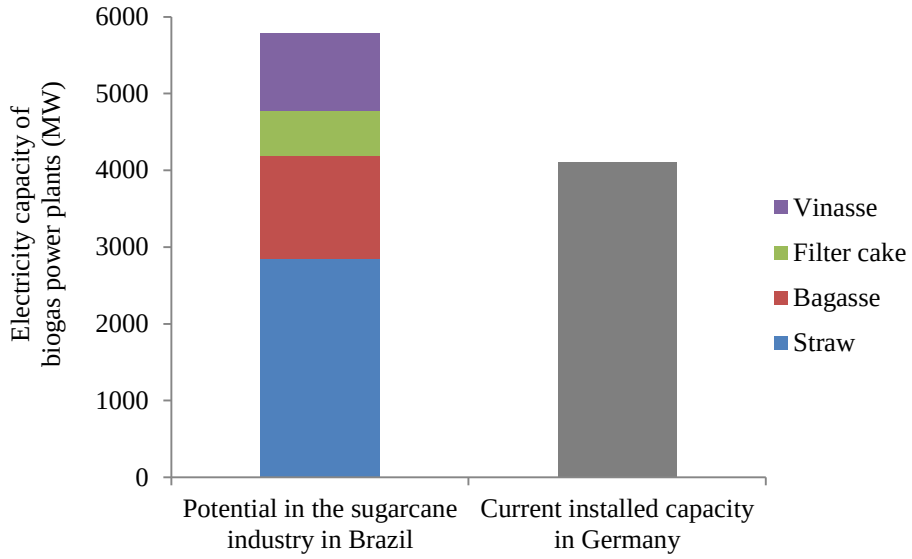

Figure 8. Potential electricity capacity of biogas power plants in the Brazilian sugarcane industry compared to the total biogas-installed capacity in Germany. By estimating the potential values, a capacity factor of 90% was considered for the biogas combined heat and power system.

4.3. Microbial ecology reveals opportunities for an efficient process

Very often the biotic component of engineered ecosystems, such as in biogas processes, is ignored by operators and researchers, who in recent decades have mostly focused on the abiotic component (e.g. pH, VOA and VFA concentration) in order to solve certain process imbalances and/or enhance productivity (e.g. application of pre-treatment to the substrates) (Koch et al., 2014). However, the relation between abiotic and biotic components is crucial for the stability of the biogas process, especially for novel substrates such as sugarcane wastes. The combination of microbial community dynamics and physico-chemical process parameters forms the basis for the development of predictive process models for monitoring biogas production. Additionally, information regarding microbial community structure at high performance provides insights into microbial resource management in order to avoid process disturbances and thereby profit disruptions due to lower biogas production (Koch et al., 2013; Merlin Christy et al., 2014).

The development of new concepts based on microbial resources management relies on a deep understanding of the microbial interactions and their dependency on technical process set-ups. Nevertheless, current knowledge on this matter is limited, which hinders further development (Walter et al., 2012). Although changing the process conditions and/or managing the microbes seems to be much easier in laboratory-scale reactors compared to full plants, this should not be a limiting factor for the development of microbial resource management at the large scale. As an example, a biogas process

with a number of disturbances and low production could be managed with robust specific microbes which provide fast process recovery. In this context, in the experiment with the gradual OLR increase (subsection 3.1.), the bioaugmentation of the versatile genus *Methanosarcina* in the reactor ecosystem under acidification could have potentially recovered the process in the sense that it would have reduced the large accumulation of VFA by consuming acetate. In this case, *Methanosarcina* was the most promising methanogen, because this genus has been shown to predominate in the mono-digestion of filter cake and its co-digestion with bagasse under stable process conditions also in the experiments with different inoculation strategies (subsection 3.3.).

With the advance of microbial fingerprinting technologies applied to the optimisation of consolidated industrial process, new questions have been raised regarding the Baas-Becking hypothesis, in which the following assumption is formulated '*Everything is everywhere, but, the environments selects*' (Becking, 1934; De Wit & Bouvier, 2006). The reason for that refers to the limitations in detecting many microbial species that are only latently present, which implies a distorted observation of the microbial biodiversity found in a certain environment (De Wit & Bouvier, 2006; O'Malley, 2008). Considering the Baas-Becking hypothesis, microbial management in a bioprocessing system such as the biogas process should focus on the communities environmentally selected by previously determined conditions, which are based on an effective biodegradation ratio and/or process recovery. In this way, a microbial community with stability, resistance and resilience might improve the process yield and/or prevent drawbacks in biogas production when eventually the environmental parameters change. Further reference to the Baas-Becking hypothesis is applied to the inoculation of a bioprocessing system. The experiments with different inoculation strategies performed in this work show that, at steady-state, the microbial community in all reactors with the same environmental conditions was very similar, independently of whether cattle manure or mixed inoculum were used to inoculate the reactors. Although the research developed in this PhD thesis was performed in Germany and not in Brazil, where the biogas process should be implemented in the sugarcane industry, the same microbial community detected in the experiments presented here is expected to be found in Brazil under the same process conditions, even when using fresh manure from Brazilian cattle.

4.4. Stable isotope fingerprinting as a monitoring tool for biogas process

The microbial community dynamics assessed through molecular biology techniques was followed by stable isotope fingerprinting, as shown in experiments with different inoculation strategies and gradual increase in OLR. This revealed that the isotope technique is a very promising monitoring tool for biogas process. Its effectiveness in detecting rapid changes in microbial activity provides crucial

information about process disturbances at an earlier stage. The development of stable isotope fingerprinting as a monitoring tool for the biogas process is still at an initial phase and requires, therefore, specific ecological models that combine results from microbial ecology and isotope composition. Inconveniently, in the up-to-date analysis of stable isotope composition of methane, the diagram combining the carbon and hydrogen isotope composition of methane ($\delta^{13}C_{CH_4}$ and $\delta^{2}H_{CH_4}$) and the apparent fractionation factor αC proposed for evaluating the predominant methanogenic pathway in environmental samples (Conrad, 2005; Sugimoto & Wada, 1995; Whiticar, 1999; Whiticar et al., 1986) do not completely agree with the results from the biogas process.

In the experiment performed in this work, it was observed that more frequent sampling with an automated measurement procedure is strongly recommended in order to achieve greater reproducibility and reliability, especially regarding the ^{2}H isotope signature of methane, which was further influenced by the isotope composition of the process water. Blaser and Conrad (2016) have suggested monitoring the performance of several experiments under identical conditions to ensure the variability of the isotopic signatures. They also recommend the investigation of distinctive effectors influencing the range of fractionation factors in microbial cultures in order to better understand the biogeochemical pathway. Unfortunately, applicability of stable isotope fingerprinting in the context of biogas production in the sugarcane industry is currently speculative due to the fact that only very few laboratories in the country have this so-far expensive technology. New developments of optical methods based on laser absorption spectroscopy enable the continuous measurement of stable carbon isotopes with significant precision (Polag et al., 2015). The more widespread utilization of this approach, also outside the biogas sector, is expected in future. This might influence the price of the technology in a way to be affordable for the biogas industry.

4.5. Final remarks and future perspectives

During continuous biogas production of sugarcane wastes, it was observed that the nitrogen bioavailability of filter cake was very low despite its suitable C:N ratio for the AD process. In this case, nitrogen supplementation is recommended in order to ensure a stable process for biogas production. Special monitoring of nitrogen addition should be carried out during the production of VFA under low pH values (around 5.5). This is due to fact that the increase in nitrogen in the process contributes to the buffer capacity, tending thus to elevate the pH to neutral values.

The pre-treatment of filter cake with sodium hydroxide provided an increase in chemical oxygen demand (COD) solubilization and VFA yield. However, in order to implement such pre-treatment on a larger scale, further research should be conducted to evaluate its cost-efficiency under different

conditions related to the location of the sugarcane industry (e.g. transportation and nitrogen supply costs). Other pre-treatment strategies applied to continuous biogas production of sugarcane wastes (e.g. steam explosion, liquid hot water, enzymes and acid/biological/alkaline pre-treatments) should also be further investigated, taking into consideration their cost-effectiveness to the process.

Bagasse, straw and filter cake as solid waste products have the potential to be used as in CSTRs, which is the most common biogas reactor configuration applied nowadays in Germany (Weiland, 2010). In order to lower the total solid concentration of the biogas process using these waste products as feeding substrates, vinasse could be used as a co-substrate to keep the process under wet conditions. In the case of the mono-digestion of vinasse, the best suitable options regarding reactor type are considered to be those with biomass retention, such as fixed bed reactors, UASB reactors or fluidised bed reactors (Pant & Adholeya, 2007; Rajeshwari et al., 2000).

Process inhibition under a surplus of certain chemical elements such as sulphur and potassium present in the sugarcane wastes was not observed in the semi-continuous feeding experiments. However, foaming and acidification in the mono-and co-digestion set-ups limited the application of higher OLR and lower HRT. Since this may be related to certain elements, further investigation into this matter is required. In this context, microbial resource management could provide microbes associated with the degradation of specific foam generating compounds, or microbes that may contribute to a faster conversion of acetate into methane, avoiding thus VFA accumulation.

Further experiments on the microbial ecology of AD processes from sugarcane wastes would provide further knowledge about the functional organisation of microbial communities that yield greater productivity, or even single species that are able to perform the desired functions. Based on the current infrastructure situation of the sugarcane industry, on the characteristics of the waste products and on the already performed experiments, research on the following topics is strongly recommended: (a) restart-up of the biogas process after storage of the digestate under environmental temperatures during the sugarcane industry offseason; (b) gradual change of the feeding substrate using silage of sugarcane wastes in order to maintain biogas production throughout the entire year; (c) application of distinct reactor types, focusing on biomass retention during the AD process of vinasse; (d) addition of macro- and microelements in the biogas process of sugarcane wastes.

Considering the research conducted in this PhD thesis, the future of the Brazilian sugarcane industry would appear promising in terms of efficient biogas production from its different wastes. Despite the recent drawbacks in Brazil’s economy and therefore limited financial resources, the country still has an opportunity to develop its waste management along with economic growth, environmental protection

and social health. Brazil currently has a cultural tendency to search for new opportunities during economic recession. Now may be the right time for promoting innovation through biogas production from sugarcane wastes, and thus reactivating the economy and ensuring sustainable development. The benefits of biogas processes to the sugarcane industry shown in this PhD thesis provide the insurance to develop a reliable investment plan of biogas projects. Considering the present situation in Brazil, a pilot biogas plant is strongly recommended based on its lower investment costs. By increasing the number of installed biogas plants in the sugarcane industry, more companies would then have the courage to invest further and the market would consequently develop by itself. However, this optimistic and reasonable pathway should be carried out together with governmental incentives regarding subsides for biogas commodities. Moreover, the government should provide stricter environmental regulations and monitoring of the sugarcane industry in order to promote sustainable waste management.

References

Balat, M., Balat, H. 2009. Recent trends in global production and utilization of bio-ethanol fuel. *Applied Energy*, **86**(11), 2273-2282.

Batstone, D.J., Jensen, P.D. 2011. 4.17 - Anaerobic Processes. in: *Treatise on Water Science*, (Ed.) P. Wilderer, Elsevier. Oxford, pp. 615-639.

Becking, L.G.M.B. 1934. *Geobiologie of inleiding tot de milieukunde*. WP Van Stockum & Zoon.

Bergman, P.C.A., Boersma, A.R., Kiel, J.H.A., Prins, M.J., Ptasinski, K.J., Janssen, F. 2005. Torrefaction for entrained-flow gasification of biomass, Contributions of the Energy Research Centre of the Netherlands to "The 2nd World Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection".

Bezerra, T.L., Ragauskas, A.J. 2016. A review of sugarcane bagasse for second-generation bioethanol and biopower production. *Biofuels, Bioproducts and Biorefining*, **10**(5), 634-647.

Blaser, M., Conrad, R. 2016. Stable carbon isotope fractionation as tracer of carbon cycling in anoxic soil ecosystems. *Curr Opin Biotechnol*, **41**, 122-129.

Bouallagui, H., Ben Cheikh, R., Marouani, L., Hamdi, M. 2003. Mesophilic biogas production from fruit and vegetable waste in a tubular digester. *Bioresource Technology*, **86**(1), 85-89.

Bundesgesetzblatt. 2014. Gesetz zur grundlegenden Reform des Erneuerbare-Energien-Gesetzes und zur Änderung weiterer Bestimmungen des Energiewirtschaftsrechts. in: Teil I, (Ed.) Bundesgesetzblatt, Vol. 33.

Carvalho, J.L.N., Otto, R., Franco, H.C.J., Trivelin, P.C.O. 2013. Input of sugarcane post-harvest residues into the soil. *Scientia Agricola*, **70**, 336-344.

Cavalett, O., Junqueira, T.L., Dias, M.O.S., Jesus, C.D.F., Mantelatto, P.E., Cunha, M.P., Franco, H.C.J., Cardoso, T.F., Maciel Filho, R., Rossell, C.E.V., Bonomi, A. 2011. Environmental and economic assessment of sugarcane first generation biorefineries in Brazil. *Clean Technologies and Environmental Policy*, **14**(3), 399-410.

Cerqueira Leite, R.C.d., Verde Leal, M.R.L., Barbosa Cortez, L.A., Griffin, W.M., Gaya Scandiffio, M.I. 2009. Can Brazil replace 5% of the 2025 gasoline world demand with ethanol? *Energy*, **34**(5), 655-661.

Chandel, A.K., da Silva, S.S., Carvalho, W., Singh, O.V. 2012. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. *Journal of Chemical Technology & Biotechnology*, **87**(1), 11-20.

Chauhan, M.K., Varun, Chaudhary, S., Kumar, S., Samar. 2011. Life cycle assessment of sugar industry: A review. *Renewable and Sustainable Energy Reviews*, **15**(7), 3445-3453.

Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: a review. *Bioresour Technol*, **99**(10), 4044-64.

Christy, P.M., Gopinath, L.R., Divya, D. 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Reviews*, **34**, 167-173.

Conrad, R. 2005. Quantification of methanogenic pathways using stable carbon isotopic signatures: a review and a proposal. *Organic Geochemistry*, **36**(5), 739-752.

Contreras, A.M., Rosa, E., Pérez, M., Van Langenhove, H., Dewulf, J. 2009. Comparative Life Cycle Assessment of four alternatives for using by-products of cane sugar production. *Journal of Cleaner Production*, **17**(8), 772-779.

Costa, F.J.C.B., Rocha, B.B.M., Viana, C.E., Toledo, A.C. 1986. Utilization of Vinasse Effluents from an Anaerobic Reactor. *Water Science and Technology*, **18**(12), 135.

Damaso, M., Machado, C., Rodrigues, D., Belem, S., Salum, T. 2014. Bioprocesses for biofuels: an overview of the Brazilian case. *Chemical and Biological Technologies in Agriculture*, **1**(1), 6.

Dawson, T.E., Brooks, P.D. 2001. Fundamentals of stable isotope chemistry and measurement. in: *Stable isotope techniques in the study of biological processes and functioning of ecosystems*, Springer, pp. 1-18.

de Campos, F.d.S., Alves, M.C., de Souza, M., Torres, J.L.R. 2015. Cropping systems evaluation, fertilization, and effects on technological quality and sugarcane productivity. *African Journal of Agricultural Research*, **10**(34), 3387-3393.

De Oliveira, F.C., Coelho, S.T. 2016. History, evolution, and environmental impact of biodiesel in Brazil: A review. *Renewable and Sustainable Energy Reviews*, http://dx.doi.org/10.1016/j.rser.2016.10.060.

De Paoli, F., Bauer, A., Leonhartsberger, C., Amon, B., Amon, T. 2011. Utilization of by-products from ethanol production as substrate for biogas production. *Bioresource Technology*, **102**(11), 6621-6624.

De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W. 2012. Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. *Bioresour Technol*, **112**, 1-9.

De Wit, R., Bouvier, T. 2006. 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? *Environmental Microbiology*, **8**(4), 755-758.

Dębowski, M., Zieliński, M., Grala, A., Dudek, M. 2013. Algae biomass as an alternative substrate in biogas production technologies—Review. *Renewable and Sustainable Energy Reviews*, **27**, 596-604.

Demirbas, M.F., Balat, M., Balat, H. 2009. Potential contribution of biomass to the sustainable energy development. *Energy Conversion and Management*, **50**(7), 1746-1760.

Demirel, B. 2014. Major Pathway of Methane Formation From Energy Crops in Agricultural Biogas Digesters. *Critical Reviews in Environmental Science and Technology*, **44**(3), 199-222.

EurObserv'ER. 2014. Biogas Barometer.

Fachverband Biogas. 2016. Branchenzahlen 2015 und Prognose der Branchenentwicklung 2016.

FAO. 2016. Food and Agriculture Organization of the United Nations. FAOSTAT.

Finnveden, G., Ekvall, T., Arushanyan, Y., Bisaillon, M., Henriksson, G., Gunnarsson Östling, U., Söderman, L.M., Sahlin, J., Stenmarck, Å., Sundberg, J., Sundqvist, J.-O., Svenfelt, Å., Söderholm, P., Björklund, A., Eriksson, O., Forsfält, T., Guath, M. 2013. Policy Instruments towards a Sustainable Waste Management. *Sustainability*, **5**(3).

Fischer, G., Hizsnyik, E., Prieler, S., Shah, M., van Velthuizen, H.T. 2009. Biofuels and Food Security, Final Report to Sponsor: The OPEC Fund for International Development (OFID). Vienna, Austria

Franke-Whittle, I.H., Walter, A., Ebner, C., Insam, H. 2014. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste Manag*, **34**(11), 2080-9.

Fuess, L.T., Garcia, M.L. 2014. Implications of stillage land disposal: a critical review on the impacts of fertigation. *J Environ Manage*, **145**, 210-29.

Giusti, L. 2009. A review of waste management practices and their impact on human health. *Waste Management*, **29**(8), 2227-2239.

Goes, T., Marra, R., Araújo, M.d., Alves, E., Souza, M.O.d. 2011. Sugarcane in Brazil: Current technologic stage and perspectives. *Revista de Politica Agrícola - MAPA*, **Ano XX, N° 1**.

Grabowski, A., Tindall, B.J., Bardin, V., Blanchet, D., Jeanthon, C. 2005. Petrimonas sulfuriphila gen. nov., sp. nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. *Int J Syst Evol Microbiol*, **55**(Pt 3), 1113-21.

Halvorsen, B. 2012. Effects of norms and policy incentives on household recycling: An international comparison. *Resources, Conservation and Recycling*, **67**, 18-26.

Hendriks, A., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology*, **100**(1), 10-18.

Herrmann, A. 2013. Biogas Production from Maize: Current State, Challenges and Prospects. 2. Agronomic and Environmental Aspects. *BioEnergy Research*, **6**(1), 372-387.

Hijazi, O., Munro, S., Zerhusen, B., Effenberger, M. 2016. Review of life cycle assessment for biogas production in Europe. *Renewable and Sustainable Energy Reviews*, **54**, 1291-1300.

Hunkeler, D., Meckenstock, R.U., Sherwood Lollar, B., Schmidt, T.C., Wilson, J.T. 2008. *A guide for assessing biodegradation and source identification of organic ground water contaminants using compound specific isotope analysis (CSIA)*. Office of Research and Development, National Risk Management Research Laboratory, US Environmental Protection Agency.

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R.J., Sicheritz-Ponten, T., De Bellis, G., Visca, P., Cassone, A., Carattoli, A. 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant Acinetobacter baumannii strain belonging to the European clone II group. *Antimicrob Agents Chemother*, **52**(7), 2616-25.

Isa, Z., Grusenmeyer, S., Verstraete, W. 1986. Sulfate Reduction Relative to Methane Production in High-Rate Anaerobic Digestion: Technical Aspects. *Applied and Environmental Microbiology*, **51**(3), 572-579.

Jabbour, A.B.L.d.S., Jabbour, C.J.C., Sarkis, J., Govindan, K. 2014. Brazil's new national policy on solid waste: challenges and opportunities. *Clean Technologies and Environmental Policy*, **16**(1), 7-9.

Janke, L., Leite, A., Wedwitschka, H., Schmidt, T., Nikolausz, M., Stinner, W. 2014. Biomethane Production Integrated to the Brazilian Sugarcane Industry: The Case Study of São Paulo State.

Kafle, G.K., Kim, S.H. 2013. Anaerobic treatment of apple waste with swine manure for biogas production: Batch and continuous operation. *Applied Energy*, **103**, 61-72.

Karakashev, D., Batstone, D.J., Angelidaki, I. 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Appl Environ Microbiol*, **71**(1), 331-8.

Keppler, F., Laukenmann, S., Rinne, J., Heuwinkel, H., Greule, M., Whiticar, M., Lelieveld, J. 2010. Measurements of 13C/12C methane from anaerobic digesters: Comparison of optical spectrometry with continuous-flow isotope ratio mass spectrometry. *Environmental science & technology*, **44**(13), 5067-5073.

Khalili, N.R., Duecker, S., Ashton, W., Chavez, F. 2015. From cleaner production to sustainable development: the role of academia. *Journal of Cleaner Production*, **96**, 30-43.

Kim, J., Kim, H., Baek, G., Lee, C. 2016. Anaerobic co-digestion of spent coffee grounds with different waste feedstocks for biogas production. *Waste Management*, http://dx.doi.org/10.1016/j.wasman.2016.10.015.

Koch, C., Fetzer, I., Schmidt, T., Harms, H., Muller, S. 2013. Monitoring functions in managed microbial systems by cytometric bar coding. *Environ Sci Technol*, **47**(3), 1753-60.

Koch, C., Muller, S., Harms, H., Harnisch, F. 2014. Microbiomes in bioenergy production: from analysis to management. *Curr Opin Biotechnol*, **27**, 65-72.

Krober, M., Bekel, T., Diaz, N.N., Goesmann, A., Jaenicke, S., Krause, L., Miller, D., Runte, K.J., Viehover, P., Puhler, A., Schluter, A. 2009. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. *J Biotechnol*, **142**(1), 38-49.

Lakshmanan, A.R., Kuppuswamy, G., Jeyabal, A. 1990. Biogas generation from sugarcane filtercake-laboratory scale and pilot plant studies. *Urja*, **27**(2), 25-28.

Lantz, M. 2013. *Biogas in Sweden-Opportunities and challenges from a systems perspective*.

Laukenmann, S., Polag, D., Heuwinkel, H., Greule, M., Gronauer, A., Lelieveld, J., Keppler, F. 2010. Identification of methanogenic pathways in anaerobic digesters using stable carbon isotopes. *Engineering in Life Sciences*, **10**(6), 509-514.

Laurent, A., Bakas, I., Clavreul, J., Bernstad, A., Niero, M., Gentil, E., Hauschild, M.Z., Christensen, T.H. 2014. Review of LCA studies of solid waste management systems–Part I: Lessons learned and perspectives. *Waste management*, **34**(3), 573-588.

Leal, M.R.L.V., Galdos, M.V., Scarpare, F.V., Seabra, J.E.A., Walter, A., Oliveira, C.O.F. 2013. Sugarcane straw availability, quality, recovery and energy use: A literature review. *Biomass and Bioenergy*, **53**, 11-19.

Lebuhn, M., Munk, B., Effenberger, M. 2014. Agricultural biogas production in Germany - from practice to microbiology basics. *Energy, Sustainability and Society*, **4**(1), 1-21.

Leschine, S.B. 1995. Cellulose degradation in anaerobic environments. *Annual Reviews in Microbiology*, **49**(1), 399-426.

Lettinga, G., Haandel, A.C.v. 1993. Anaerobic digestion for energy production and environmental protection, Earthscan. London, pp. 817-839.

Levén, L., Nyberg, K., Schnürer, A. 2012. Conversion of phenols during anaerobic digestion of organic solid waste–a review of important microorganisms and impact of temperature. *Journal of environmental management*, **95**, S99-S103.

Lewandowski, I. 2015. Securing a sustainable biomass supply in a growing bioeconomy. *Global Food Security*, **6**, 34-42.

Li, D., Liu, S., Mi, L., Li, Z., Yuan, Y., Yan, Z., Liu, X. 2015. Effects of feedstock ratio and organic loading rate on the anaerobic mesophilic co-digestion of rice straw and cow manure. *Bioresource Technology*, **189**, 319-326.

Liu, S., Li, X., Wu, S., He, J., Pang, C., Deng, Y., Dong, R. 2014. Fungal Pretreatment by Phanerochaete chrysosporium for Enhancement of Biogas Production from Corn Stover Silage. *Applied Biochemistry and Biotechnology*, **174**(5), 1907-1918.

Lopez Gonzalez, L.M., Pereda Reyes, I., Dewulf, J., Budde, J., Heiermann, M., Vervaeren, H. 2014. Effect of liquid hot water pre-treatment on sugarcane press mud methane yield. *Bioresour Technol*, **169**, 284-90.

Lopez Gonzalez, L.M., Vervaeren, H., Pereda Reyes, I., Dumoulin, A., Romero Romero, O., Dewulf, J. 2013. Thermo-chemical pre-treatment to solubilize and improve anaerobic biodegradability of press mud. *Bioresour Technol*, **131**, 250-7.

Lucas, R., Kuchenbuch, A., Fetzer, I., Harms, H., Kleinsteuber, S. 2015. Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. *FEMS Microbiol Ecol*, **91**(3).

Lv, Z., Hu, M., Harms, H., Richnow, H.H., Liebetrau, J., Nikolausz, M. 2014a. Stable isotope composition of biogas allows early warning of complete process failure as a result of ammonia inhibition in anaerobic digesters. *Bioresource technology*, **167**, 251-259.

Lv, Z., Leite, A.F., Harms, H., Richnow, H.H., Liebetrau, J., Nikolausz, M. 2014b. Influences of the substrate feeding regime on methanogenic activity in biogas reactors approached by molecular and stable isotope methods. *Anaerobe*, **29**, 91-9.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*, **66**(3), 506-577.

Madigan, M., Martinko, J., Stahl, D., Clark, D. 2010. *Brock Biology of Microorganisms (13th Edition)*. Benjamin Cummings.

Marin, F.R. 2016. Understanding sugarcane production, biofuels, and market volatility in Brazil--A research perspective. *Outlook on Agriculture*, **45**(2), 75-77.

Marshall, R.E., Farahbakhsh, K. 2013. Systems approaches to integrated solid waste management in developing countries. *Waste Management*, **33**(4), 988-1003.

Martinelli, L.A., Filoso, S. 2008. Expansion of Sugarcane Ethanol Production in Brazil: Environmental and Social Challenges. *Ecological Applications*, **18**(4), 885-898.

Martins, C.A., de Andrade Júnior, P.P. 2016. Production of biodiesel: Source strategies and efficiency in the Brazilian energy matrix. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, **38**(2), 277-285.

Maya-Altamira, L., Baun, A., Angelidaki, I., Schmidt, J.E. 2008. Influence of wastewater characteristics on methane potential in food-processing industry wastewaters. *Water Research*, **42**(8–9), 2195-2203.

Merlin Christy, P., Gopinath, L.R., Divya, D. 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Reviews*, **34**, 167-173.

Michener, R., Lajtha, K. 2008. *Stable isotopes in ecology and environmental science*. John Wiley & Sons.

Modesto, M., Aoki, A.C., Lodi, A., Pina, E.A. 2016. Assessment of the Potential to Increase Electricity Generation from Sugarcane Straw in Brazilian Sugarcane Cogeneration Plants. *Chemical Engineering Transactions*, **50**, 193-198.

Monlau, F., Sambusiti, C., Barakat, A., Quéméneur, M., Trably, E., Steyer, J.-P., Carrère, H. 2014. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. *Biotechnology advances*, **32**(5), 934-951.

Moraes, B.S., Junqueira, T.L., Pavanello, L.G., Cavalett, O., Mantelatto, P.E., Bonomi, A., Zaiat, M. 2014. Anaerobic digestion of vinasse from sugarcane biorefineries in Brazil from energy, environmental, and economic perspectives: Profit or expense? *Applied Energy*, **113**, 825-835.

Moraes, B.S., Zaiat, M., Bonomi, A. 2015. Anaerobic digestion of vinasse from sugarcane ethanol production in Brazil: Challenges and perspectives. *Renewable and Sustainable Energy Reviews*, **44**, 888-903.

Moshi, A.P., Crespo, C.F., Badshah, M., Hosea, K.M.M., Mshandete, A.M., Elisante, E., Mattiasson, B. 2014. Characterisation and evaluation of a novel feedstock, Manihot glaziovii, Muell. Arg, for production of bioenergy carriers: Bioethanol and biogas. *Bioresource Technology*, **172**, 58-67.

Mulat, D.G., Jacobi, H.F., Feilberg, A., Adamsen, A.P.S., Richnow, H.-H., Nikolausz, M. 2016. Changing Feeding Regimes To Demonstrate Flexible Biogas Production: Effects on Process Performance, Microbial Community Structure, and Methanogenesis Pathways. *Applied and Environmental Microbiology*, **82**(2), 438-449.

Müller, N., Worm, P., Schink, B., Stams, A.J.M., Plugge, C.M. 2010. Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. *Environmental Microbiology Reports*, **2**(4), 489-499.

Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O. 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of Biotechnology*, **150**(1), 51-56.

Nelles, M., Grünes, J., Morscheck, G. 2016. Waste Management in Germany – Development to a Sustainable Circular Economy? *Procedia Environmental Sciences*, **35**, 6-14.

Nie, H., Jacobi, H.F., Strach, K., Xu, C., Zhou, H., Liebetrau, J. 2015. Mono-fermentation of chicken manure: ammonia inhibition and recirculation of the digestate. *Bioresour Technol*, **178**, 238-46.

Nikolausz, M., Walter, R.F., Strauber, H., Liebetrau, J., Schmidt, T., Kleinsteuber, S., Bratfisch, F., Gunther, U., Richnow, H.H. 2013. Evaluation of stable isotope fingerprinting techniques for the assessment of the predominant methanogenic pathways in anaerobic digesters. *Appl Microbiol Biotechnol*, **97**(5), 2251-2262.

O'Malley, M.A. 2008. 'Everything is everywhere: but the environment selects': ubiquitous distribution and ecological determinism in microbial biogeography. *Studies in History and Philosophy of Science Part C: Studies in History and Philosophy of Biological and Biomedical Sciences*, **39**(3), 314-325.

Pabon Pereira, C.P., Lier, J.B.v., Sanders, W.T.M., Slingerland, M.A., Rabbinge, R. 2006. The Role of Anaerobic Digestion in Sugarcane Chains in Colombia. http://agris.fao.org/agris-search/search.do?recordID=NL2012058038.

Pant, D., Adholeya, A. 2007. Biological approaches for treatment of distillery wastewater: A review. *Bioresource Technology*, **98**(12), 2321-2334.

Petrosino, J.F., Highlander, S., Luna, R.A., Gibbs, R.A., Versalovic, J. 2009. Metagenomic pyrosequencing and microbial identification. *Clin Chem*, **55**(5), 856-66.

Pippo, W., Luengo, C. 2013. Sugarcane energy use: accounting of feedstock energy considering current agro-industrial trends and their feasibility. *International Journal of Energy and Environmental Engineering*, **4**(1), 1-13.

Polag, D., Krapf , L.C., Heuwinkel, H., Laukenmann, S., Lelieveld, J., Keppler, F. 2014. Stable carbon isotopes of methane for real-time process monitoring in anaerobic digesters. *Engineering in Life Sciences*, **14**(2), 153-160.

Polag, D., May, T., Muller, L., Konig, H., Jacobi, F., Laukenmann, S., Keppler, F. 2015. Online monitoring of stable carbon isotopes of methane in anaerobic digestion as a new tool for early warning of process instability. *Bioresour Technol*, **197**, 161-70.

Popp, D., Schrader, S., Kleinsteuber, S., Harms, H., Sträuber, H. 2015. Biogas production from coumarin-rich plants—inhibition by coumarin and recovery by adaptation of the bacterial community. *FEMS Microbiology Ecology*, **91**(9).

Rabelo, S.C., Carrere, H., Maciel Filho, R., Costa, A.C. 2011. Production of bioethanol, methane and heat from sugarcane bagasse in a biorefinery concept. *Bioresour Technol*, **102**(17), 7887-7895.

Rajeshwari, K.V., Balakrishnan, M., Kansal, A., Lata, K., Kishore, V.V.N. 2000. State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. *Renewable and Sustainable Energy Reviews*, **4**(2), 135-156.

Renó, M.L.G., Olmo, O.A.d., Palacio, J.C.E., Lora, E.E.S., Venturini, O.J. 2014. Sugarcane biorefineries: Case studies applied to the Brazilian sugar–alcohol industry. *Energy Conversion and Management*, **86**, 981-991.

Rey, J.M.M. 2013. Biofuels and food security. *Cuadernos de estrategia*(161), 196-224.

Ronaghi, M. 2001. Pyrosequencing sheds light on DNA sequencing. *Genome Res*, **11**(1), 3-11.

Schink, B., Stams, A.M. 2013. Syntrophism Among Prokaryotes. in: *The Prokaryotes*, (Eds.) E. Rosenberg, E. DeLong, S. Lory, E. Stackebrandt, F. Thompson, Springer Berlin Heidelberg, pp. 471-493.

Schmidt, T., Ziganshin, A.M., Nikolausz, M., Scholwin, F., Nelles, M., Kleinsteuber, S., Pröter, J. 2014. Effects of the reduction of the hydraulic retention time to 1.5 days at constant organic loading in CSTR, ASBR, and fixed-bed reactors – Performance and methanogenic community composition. *Biomass and Bioenergy*, **69**, 241-248.

Schnürer, A., Nordberg, A. 2008. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. *Water Sci Technol*, **57**(5), 735-40.

Scordia, D., Cosentino, S.L., Jeffries, T.W. 2010. Second generation bioethanol production from Saccharum spontaneum L. ssp. aegyptiacum (Willd.) Hack. *Bioresource Technology*, **101**(14), 5358-5365.

Souza, M.E., Fuzaro, G., Polegato, A.R. 1992. Thermophilic Anaerobic Digestion of Vinasse in Pilot Plant UASB Reactor. *Water Science and Technology*, **25**(7), 213.

Strauber, H., Lucas, R., Kleinsteuber, S. 2015. Metabolic and microbial community dynamics during the anaerobic digestion of maize silage in a two-phase process. *Appl Microbiol Biotechnol*.

Sträuber, H., Schröder, M., Kleinsteuber, S. 2012. Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. *Energy, Sustainability and Society*, **2**(1), 13.

Sugimoto, A., Wada, E. 1995. Hydrogen isotopic composition of bacterial methane: CO2/H2 reduction and acetate fermentation. *Geochimica et Cosmochimica Acta*, **59**(7), 1329-1337.

Sun, L. 2015. Biogas production from lignocellulosic materials. Swedish University of Agricultural Sciences, Uppsala. Doctoral Thesis. 2015:083. ISBN (electronic version) 978-91-576-8365-6.

Sun, L., Müller, B., Westerholm, M., Schnürer, A. 2014. Syntrophic acetate oxidation in industrial CSTR biogas digesters. *J Biotechnol*, **171**, 39-44.

Thangamuthu, P. 2010. Eco-friendly cooking gas from sugar mill waste. *Proc. Int. Soc. Sugar Cane Technol*, **27**.

Thornton, P.K., Ericksen, P.J., Herrero, M., Challinor, A.J. 2014. Climate variability and vulnerability to climate change: a review. *Global Change Biology*, **20**(11), 3313-3328.

Tolmac, D., Prulovic, S., Lambic, M., Radovanovic, L., Tolmac, J. 2014. Global Trends on Production and Utilization of Biodiesel. *Energy Sources, Part B: Economics, Planning, and Policy*, **9**(2), 130-139.

Ueki, A., Akasaka, H., Suzuki, D., Ueki, K. 2006. Paludibacter propionicigenes gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. *Int J Syst Evol Microbiol*, **56**(Pt 1), 39-44.

UNICA. 2016. Unicadata: Production data 2015/2016 season. *UNICA (Brazilian Sugarcane Industry Association).* (Available online: http://www.unicadata.com.br), Accessed on Dezember 2016.

USDA. November, 2015. Sugar: World Markets and Trade. Foreign Agricultural Service, United States Department of Agriculture (USDA).

Valdes, C. 2011. Brazil's Ethanol Industry: Looking Forward. United States Department of Agriculture (USDA). .

van den Wall Bake, J.D., Junginger, M., Faaij, A., Poot, T., Walter, A. 2009. Explaining the experience curve: Cost reductions of Brazilian ethanol from sugarcane. *Biomass and Bioenergy*, **33**(4), 644-658.

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W. 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Current Opinion in Biotechnology*, **27**, 55-64.

Veeken, A., Kalyuzhnyi, S., Scharff, H., Hamelers, B. 2000. Effect of pH and VFA on hydrolysis of organic solid waste. *Journal of environmental engineering*, **126**(12), 1076-1081.

Volmer, J., Schmid, A., Bühler, B. 2015. Guiding bioprocess design by microbial ecology. *Current Opinion in Microbiology*, **25**, 25-32.

Walter, A., Galdos, M.V., Scarpare, F.V., Leal, M.R.L.V., Seabra, J.E.A., da Cunha, M.P., Picoli, M.C.A., de Oliveira, C.O.F. 2014. Brazilian sugarcane ethanol: developments so far and challenges for the future. *Wiley Interdisciplinary Reviews: Energy and Environment*, **3**(1), 70-92.

Walter, A., Knapp, B.A., Farbmacher, T., Ebner, C., Insam, H., Franke-Whittle, I.H. 2012. Searching for links in the biotic characteristics and abiotic parameters of nine different biogas plants. *Microb Biotechnol*, **5**(6), 717-30.

Wan, C., Zhou, Q., Fu, G., Li, Y. 2011. Semi-continuous anaerobic co-digestion of thickened waste activated sludge and fat, oil and grease. *Waste Management*, **31**(8), 1752-1758.

Weiland, P. 2010. Biogas production: current state and perspectives. *Appl Microbiol Biotechnol*, **85**(4), 849-860.

Westerholm, M., Müller, B., Arthurson, V., Schnürer, A. 2011. Changes in the Acetogenic Population in a Mesophilic Anaerobic Digester in Response to Increasing Ammonia Concentration. *Microbes and Environments*, **26**(4), 347-353.

Wheeler, T., von Braun, J. 2013. Climate Change Impacts on Global Food Security. *Science*, **341**(6145), 508.

Whiticar, M.J. 1999. Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chemical Geology*, **161**(1), 291-314.

Whiticar, M.J., Faber, E., Schoell, M. 1986. Biogenic methane formation in marine and freshwater environments: CO2 reduction vs. acetate fermentation—Isotope evidence. *Geochimica et Cosmochimica Acta*, **50**(5), 693-709.

Zaman, A.U., Lehmann, S. 2011. Challenges and Opportunities in Transforming a City into a "Zero Waste City". *Challenges*, **2**(4).

Zancaner, M.G., de Souza Santos, T.B. 2013. Cogeração: Ampliação da Oferta de Energia Elétrica com a Biomassa (Bagaço da Cana-de-Açúcar). *Diálogos Interdisciplinares*, **2**(2), 47-65.

Zhang, D.Q., Tan, S.K., Gersberg, R.M. 2010. Municipal solid waste management in China: Status, problems and challenges. *Journal of Environmental Management*, **91**(8), 1623-1633.

Zhong, W., Zhang, Z., Luo, Y., Sun, S., Qiao, W., Xiao, M. 2011. Effect of biological pretreatments in enhancing corn straw biogas production. *Bioresource Technology*, **102**(24), 11177-11182.

Ziganshin, A.M., Liebetrau, J., Proter, J., Kleinsteuber, S. 2013. Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Appl Microbiol Biotechnol*, **97**(11), 5161-74.

Zinder, S., Koch, M. 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Archives of Microbiology*, **138**(3), 263-272.

Declaration of authorship

I hereby declare that

- I have written this thesis independently including my own ideas and judgment. All external resources used were properly referred.
- all authors listed in the publications have their respective contribution stated under ‘Author contributions of published articles’.
- no other people were involved on the elaboration and preparation of this thesis. Neither PhD consultancy nor third party that could have financial interest on goods or services contributed to the work presented in this thesis.
- This thesis has not been submitted earlier at any other academic institution and it has not been published or under other doctoral examination process.

Athaydes Francisco Leite Junior
Leipzig, March 9th 2017

Author contributions of published articles

Title: **Assessment of the Variations in Characteristics and Methane Potential of Major Waste Products from the Brazilian Bioethanol Industry along an Operating Season**

Journal: Energy & Fuels

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Journal: International Journal of Molecular Sciences

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Journal: International Journal of Molecular Sciences

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Title: **Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse**

Journal: Waste Management

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Title: **Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment**

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10/2010 – 07/2012 Master of Science in International Material Flow Management at the Environmental Campus Birkenfeld (UCB), FH Trier, Germany.

Master thesis developed at UFZ in cooperation with the DBFZ: Effect of the substrate feeding on the activity of the methanogens in biogas reactors.

08/2008 – 07/2010 Bachelor of Science in Pharmacy (minor) at the Federal University of Goiás, Goiânia, Brazil.

01/2007 – 07/2010 Bachelor of Science in Agro-Industrial Chemistry (major) at the Federal Institute of Education, Science and Technology of Goiás, Goiânia, Brazil.

Bachelor thesis developed at Institute for Applied Material Flow Management at UCB: Material flow management applied in bioethanol production in the State of Goiás.

Work experience

09/2011 – 03/2017 Scientific member at UFZ, Department Environmental Microbiology, Research Group Microbiology of Anaerobic Systems. Major activity: Assessment of microbial communities in engineered ecosystem.

09/2011 – 03/2017 Scientific partner at DBFZ, Department Biochemical Conversion. Major activities: Process engineering and diversification of inputs for energy production.

Skills and qualifications

Methods	Stable isotope fingerprinting Molecular biology techniques: DNA fingerprinting Laser scanning microscopy
Biostatistics	R and Python: basics of programming Microsoft excel
Soft skills	Scientific project management Grant proposal writing Writing of scientific publications Optimizing published science research reading Patent writing Academic presenting in English Time and self-management Intercultural communication Communication skills Professionalization of supervision
Additional training	Industrial biotechnology for lignocellulose based processes Microbial services in times of global change

Languages

Full professional proficiency: English, German, Portuguese

List of publication

Leite AF, Janke L, Harms H, Richnow H-H, Nikolausz M. Lessons learned from the microbial ecology resulting from different inoculation strategies for biogas production from waste products of the bioethanol/sugar industry. *Biotechnology for Biofuels*. 2016; 9:1-16.

Leite A, Janke L, Lv Z, Harms H, Richnow H-H, Nikolausz M. Improved Monitoring of Semi-Continuous Anaerobic Digestion of Sugarcane Waste: Effects of Increasing Organic Loading Rate on Methanogenic Community Dynamics. *International Journal of Molecular Sciences*. 2015; 16:23210.

Leite AF, Janke L, Harms H, Zang JW, Fonseca-Zang WA, Stinner W, Nikolausz M. Assessment of the Variations in Characteristics and Methane Potential of Major Waste Products from the Brazilian Bioethanol Industry along an Operating Season. *Energy & Fuels*. 2015; 29:4022-4029.

Leite AF, Janke L, Guimaraes E, Harms H, Richnow H-H, Walter S, Nikolausz M. Developing a toolbox for microbial resource management addressed to abrupt ammonia inhibition in nonacclimatized biogas reactors. *In preparation for speedy submission.*

Lv Z, Leite AF, Harms H, Richnow HH, Liebetrau J, Nikolausz M. Influences of the substrate feeding regime on methanogenic activity in biogas reactors approached by molecular and stable isotope methods. *Anaerobe*. 2014; 29:91-99.

Lv Z, Leite AF, Harms H, Glaser K, Liebetrau J, Kleinsteuber S, Nikolausz M. Ecological succession reveals microbial driving factors for partially and completely ammonia-inhibited biogas processes. *In preparation for speedy submission*.

Janke, Leite AF, Weinrich S, Schüch A, Nikolausz M, Nelles M, Stinner W. Optimization of semi-continuous anaerobic digestion of sugarcane straw co-digested with filter cake: Effects of macronutrients supplementation on conversion kinetics. *Proceedings of Asia-Pacific Conference on Biotechnology for Waste Conversion*. 2016; ISBN 978-988-19988-3-5.

Janke L, Leite A, Nikolausz M, Schmidt T, Liebetrau J, Nelles M, Stinner W. Biogas Production from Sugarcane Waste: Assessment on Kinetic Challenges for Process Designing. *International Journal of Molecular Sciences*. 2015; 16:20685-20703.

Janke L, Leite A, Wedwitschka H, Schmidt T, Nikolausz M, Stinner W. Biomethane Production Integrated to the Brazilian Sugarcane Industry: The Case Study of São Paulo State. *Proceedings of the 22nd European Biomass Conference and Exhibition*; 2014:1295-1299.

Janke L, Leite A, Batista K, Weinrich S, Sträuber H, Nikolausz M, Nelles M, Stinner W. Optimization of hydrolysis and volatile fatty acids production from sugarcane filter cake: Effects of urea supplementation and sodium hydroxide pretreatment. *Bioresource Technology*. 2016; 199:235-244.

Janke L, Leite AF, Nikolausz M, Radetski CM, Nelles M, Stinner W. Comparison of start-up strategies and process performance during semi-continuous anaerobic digestion of sugarcane filter cake co-digested with bagasse. *Waste Management*. 2016; 48:199-208.

Janke L, Leite AF, Batista K, Silva W, Nikolausz M, Nelles M, Stinner W. Enhancing biogas production from vinasse in sugarcane biorefineries: Effects of urea and trace elements supplementation on process performance and stability. *Bioresource Technology*. 2016; 217:10-20.

Janke L, Weinrich S, Leite AF, Terzariol FK, Nikolausz M, Nelles M, Stinner W. Improving anaerobic digestion of sugarcane straw for methane production: Combined benefits of mechanical and sodium hydroxide pretreatment for process designing. *Energy Conversion and Management*. 2016; In press.

Conference contributions

Oral presentations

1. Sommerakademie IX Rot International, Rot an der Rot, 12th – 24th August 2013.
   Leite, A. F.; Nikolausz, M. Biotech und Biofuels in dem Thema Ernährungssicherheit.

2. 2nd International Conference on Anaerobic Digestion, Vienna, 26th – 30th October 2014.
   Leite, A. F.; Janke, L.; Schmidt, T.; Harms, H.; Fonseca-Zang, W. A.; Zang, J. W.; Nikolausz, M. Assessment of the characteristics and potential of major waste products of the Brazilian bioethanol industry to produce biogas.

3. Workshop "Research Into Use" (RIU), Goiânia, 20th – 22nd January 2015.
   Leite, A. F.; Nikolausz, M. Molecular and isotope techniques applied for process optimization of the biogas production from bioethanol waste products.

4. International Conference on Anaerobic Digestion: AD Technology and Microbial Ecology for Sustainable Development, Chiang Mai, 3rd – 6th February 2015.
   Leite, A. F.; Janke, L.; Nikolausz, M. Start-up phase and the influence of inoculum characteristics in bioreactors fed with waste products from the Brazilian bioethanol industry.

5. Waste-to-Resources 2015, 6th International Symposium MBT, MRF & Recycling: Energy and resources from MSW and organic waste, Hanover, 5th – 8th May 2015.
   Janke, L.; **Leite, A. F.**; Nikolausz, M.; Stinner, W. Biogas production from sugarcane filter cake: Start-up strategies, co-digestion with bagasse and plant design.

6. 9th International Symposium on Anaerobic Microbiology, Portorož, 25th – 27th June 2015.
   Leite, A. F.; Janke, L.; Harms, H.; Nikolausz, M. Methanogenic community dynamics during reactor acidification by overloading.

7. 6th Energy.Juniors Doc Colloquium: EnergyLandUse, Leipzig, 27th October 2016.
   Leite, A. F.; Janke, L.; Harms, H.; Nikolausz, M. Improving the energy conversion efficiency in the Brazilian bioethanol industry: biogas from waste products.

Poster presentations

8. 13th World Congress on Anaer. Digestion, Santiago de Compostela, 25th – 28th June 2013.
   Lv, Z.; **Leite, A. F.**; Harms, H.; Richnow, H.; Nikolausz, M. Investigation of the effect of the substrate feeding on methanogenic activity in biogas reactors by a combined molecular and stable isotope approach.

9. 2nd International Conference on Biogas Microbiology, Uppsala, 10th – 12th June 2014.
   Leite, A. F.; Lv, Z.; Janke, L.; Schmidt, T.; Harms, H.; Nikolausz, M. Effect of the gradual increase of organic loading rate on the activity of methanogens in biogas reactors fed with residues of bioethanol industry.

10. 22nd European Biomass Conference and Exhibition: Setting the course for a biobased economy, Hamburg 23rd – 26th June 2014.
    Janke, L.; **Leite, A. F.**; Nikolausz, M.; Stinner, W. Biogas production from sugarcane filter cake: Start-up strategies, co-digestion with bagasse and plant design.

11. Conference on Tropical and Subtropical Agricultural and Natural Resource Management, Berlin, 16th – 18th September 2015.
    Zang, J. W.; Fonseca-Zang, W. A.; Leandro, W. M.; Santos C.; Jablonoviski, N.; Schuch, A.; **Leite, A.;** Oliveira, S. B.; Muniz, M. P. Plant phenotyping tests using anaerobic digested vinasse and filter cake from the Brazilian sugar cane industry.

12. HIGRADE Fall Conference 2015, Leipzig, 17th November 2015.
    Leite, A. F.; Janke, L.; Harms, H.; Nikolausz, M. Bioenergy conversion in the bioethanol industry: let´s open the black box!

13. 16th International Symposium on Microbial Ecology, Montreal, 21st – 26th August 2016.
    Leite, A.; Janke, L.; Harms, H.; Nikolausz, M. Comparative microbial community analyses of different inoculation strategies applied for biogas production from bioethanol waste products.

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Supplementary materials

Supplementary material for the subsection 3.1.

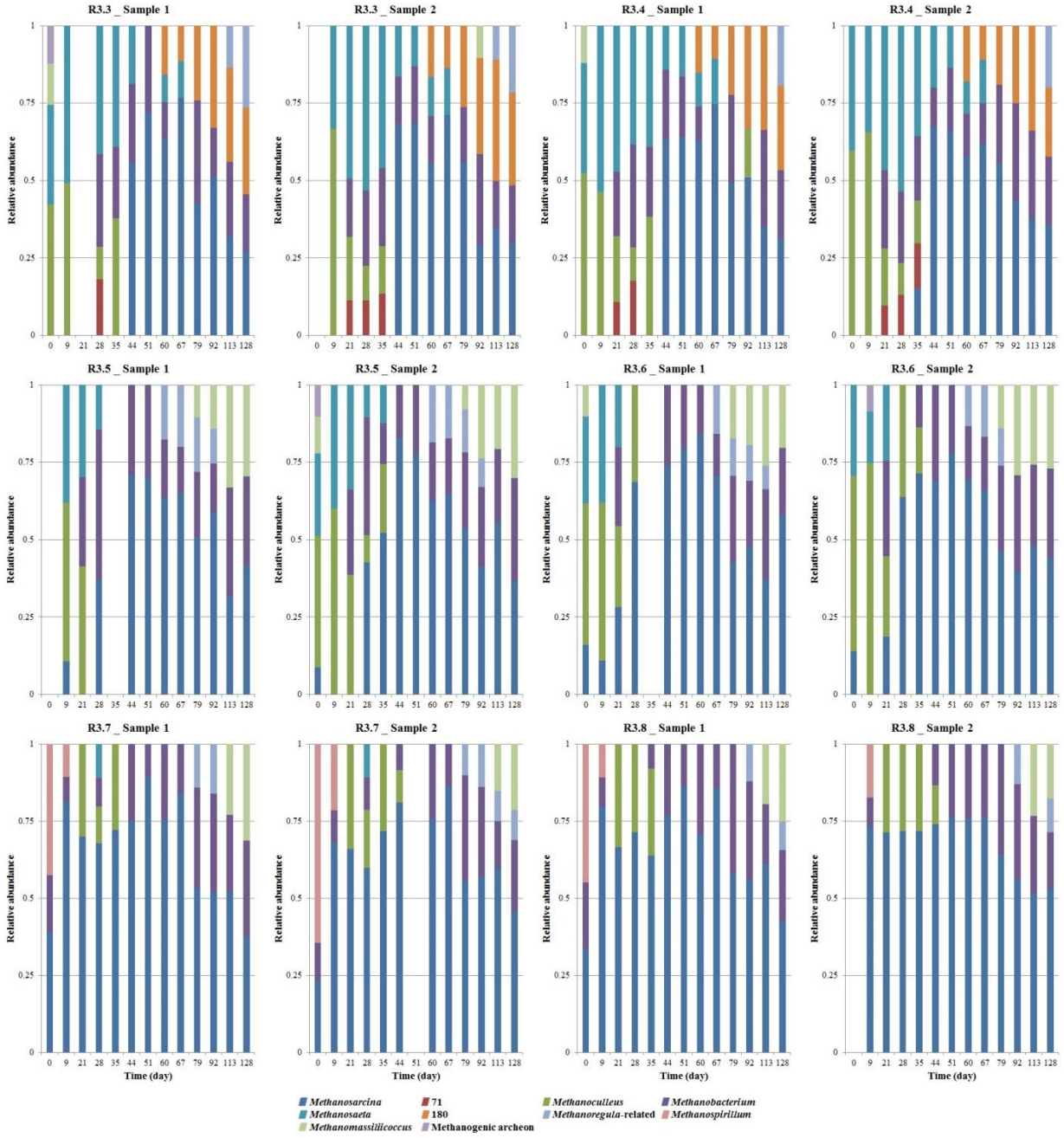
Table S1. Sequence analyses of representative *mcrA/mrtA* gene clones.

Clone (Acc. No.)	Sequences	Closest cultivable relative (Acc. No.) with sequence identity	BstNI T-RF (bp)
Methanobacterium	-	-	-
E10-8H2 (LN847074)	*4*	*Methanobacterium congolense* strain NBRC 105227 (AB542748.1) 73%	469
E2-8H1 (LN847075)	*6*	*Methanobacterium formicicum* (LN515531.1) 86%	470
C9-8C1 (LN847076)	*6*	*Methanobacterium formicicum* strain BRM9 (CP006933.1) 86%	463
C1-8H2 (LN847077)	*8*	*Methanobacterium kanagiense* (AB551869.1) 90%	469
E8-8H1 (LN847078)	*3*	*Methanobacterium sp.* MB1 (HG425166.1) 99%	332
Methanoculleus	-	-	-
A1-8C1(LN847079)	*6*	*Methanoculleus bourgensis* MS2T (HE964772.2) 99%	93
H8-8C2 (LN847080)	*1*	*Methanoculleus bourgensis* strain MAB1 (KJ708788.1) 93%	93
C6-8C2 (LN847081)	*9*	*Methanoculleus chikugoensis* (AB288270.1) 93%	91
D10-8C2 (LN847082)	*1*	*Methanoculleus chikugoensis* strain NBRC 101202 (AB703634.1) 90%	92
A12-8C1 (LN847083)	*6*	*Methanoculleus sp.* M07 (AB288284.1) 93%	92
Methanomassiliicoccus	-	-	-
C10-8C1 (LN847084)	*2*	*Methanomassiliicoccus luminyensis* strain B10 (HQ896500.1) 93%	408
Methanoregula	-	-	
H3-8H2 (LN847085)	*6*	*Methanoregula formicicum* SMSP (CP003167.1) related 81%	338
Methanosaeta	-	-	-

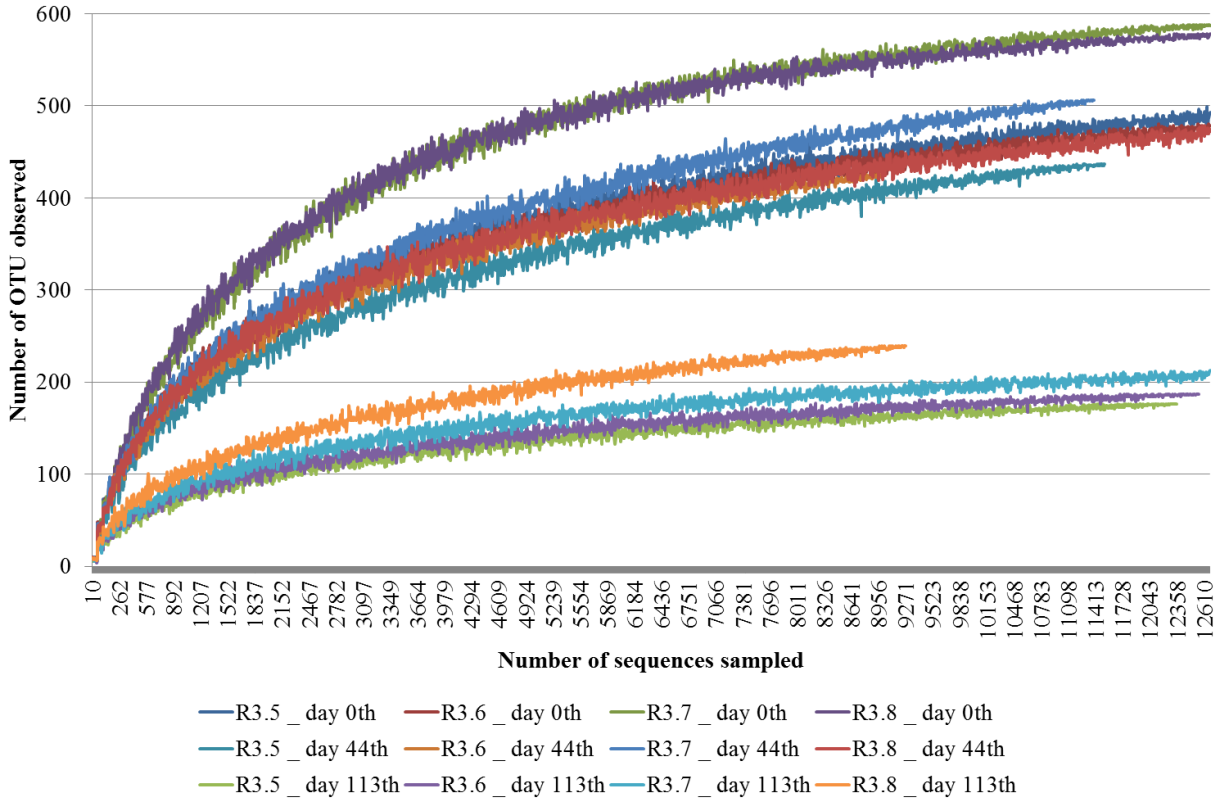
A10-8C1(LN847086)	*15*	*Methanosaeta concilii* strain NBRC 103675 (AB679170.1) 93%	127
Methanosarcina	-	-	-
A4-8C1 (LN847087)	*9*	*Methanosarcina mazei* Tuc01 (CP004144.1) 93%	55
B3-8C1 (LN847088)	*29*	*Methanosarcina thermophila* TM-1 (AB353225.1) 96%	56
B1-8XH1 (LN847089)	*1*	*Methanosarcina mazei* strain KOR-4 (KC292223.1) 99%	55
B9-8XH1 (LN847090)	*1*	*Methanosarcina mazei* strain NBRC 101201 (AB703645.1) 99%	487
Methanospirillum	-	-	-
B11-8C1 (LN847091)	*3*	*Methanospirillum stamsii* strain Pt1 (KC951357.1) 95%	344

The *mcrA*/*mrtA* gene sequences detected in the study under subsection 3.1 were deposited in the European Bioinformatics Institute (EMBL-EBI) database under the accession numbers LN847074-LN847091.

Supplementary materials for the subsection 3.3.



Supplementary Figure S1. Duplicate T-RFLP profiles of the methanogenic community dynamics for each reactor in order to show the reproducibility of the T-RFLP approach.

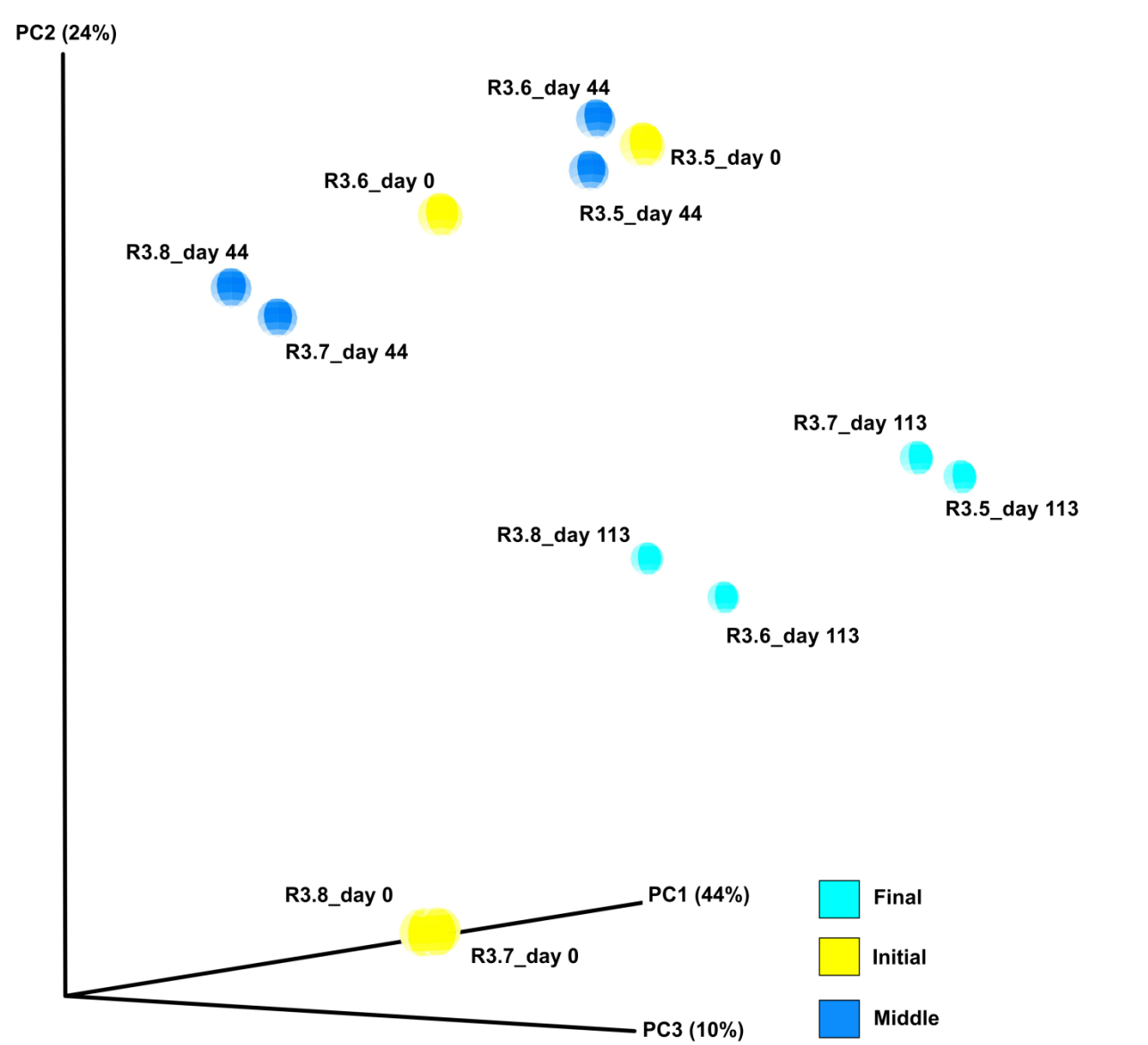


Supplementary Figure S2. Rarefaction curves of the pyrosequencing data of the 16S ribosomal RNA genes from the four co-digestion reactors (R3.5, R3.6, R3.7 and R3.8) at three different sampling points along the experiment.

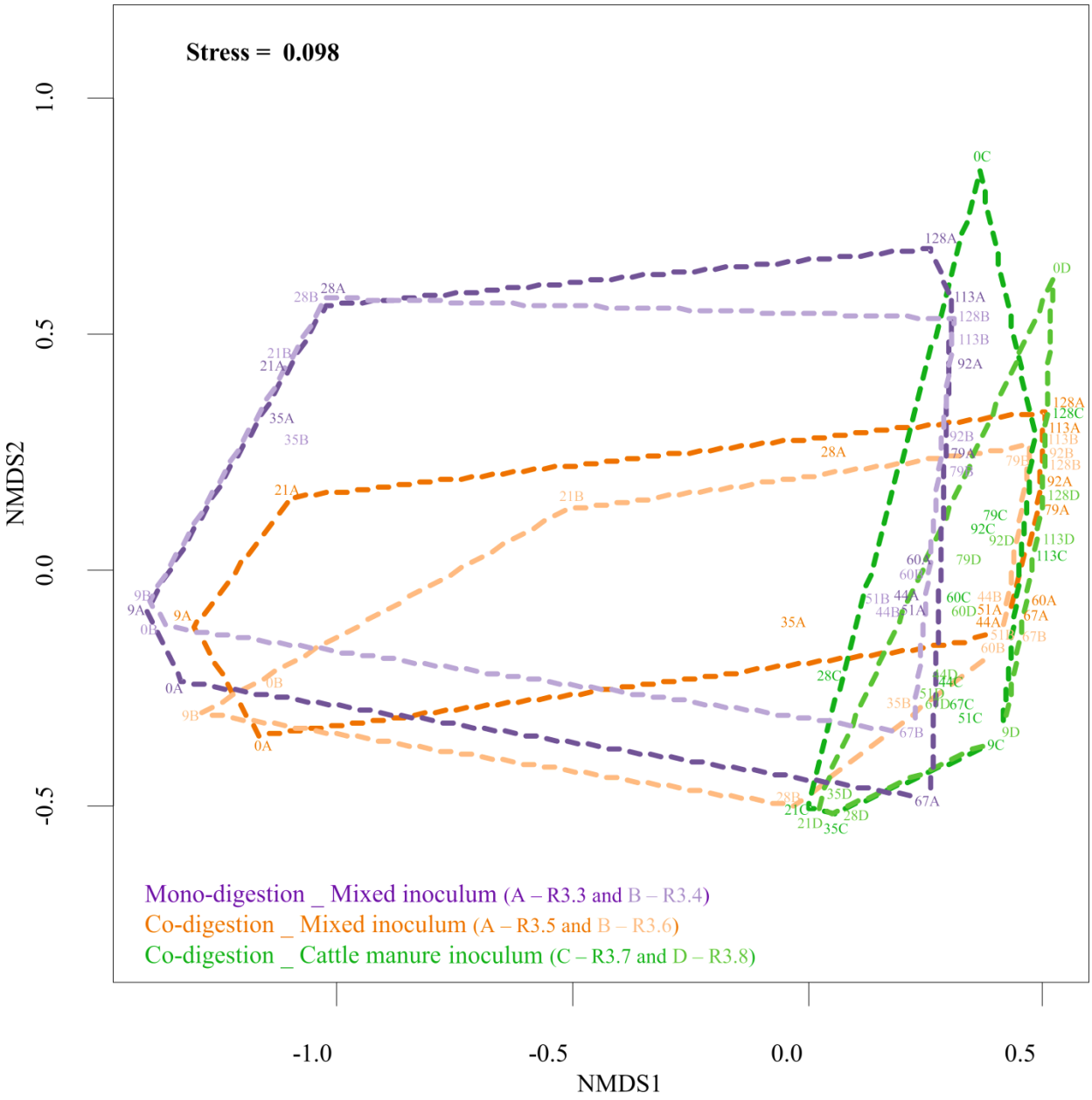
Supplementary Table S1. Beta diversity indices based on 16S rRNA gene amplicon sequences showing the community similarities between samples.

		Day 0				Day 44				Day 113			
		R3.5	R3.6	R3.7	R3.8	R3.5	R3.6	R3.7	R3.8	R3.5	R3.6	R3.7	R3.8
Day 0	R3.5	-0.0087	0.0265	0.0110	-0.0067	-0.2098	0.1676	0.1964	0.1496	-0.0073	-0.0659	0.0386	-2.70E-09
	R3.6	-0.0121	0.0147	0.0080	-0.0033	-0.2560	0.1325	0.0481	0.2478	0.0082	0.0607	-0.0391	-2.70E-09
	R3.7	0.0143	-0.0193	-0.0118	-0.0246	-0.2830	-0.3782	0.0503	-0.0446	-0.0227	0.0491	0.0503	-2.70E-09
	R3.8	0.0083	-0.0041	0.0041	0.0367	-0.2858	-0.3787	0.0403	-0.0580	0.0181	-0.0460	-0.0510	-2.70E-09
Day 44	R3.5	0.0963	-0.1116	0.0610	-0.0733	-0.0533	0.2056	0.0437	-0.1497	0.0293	0.0014	-0.0108	-2.70E-09
	R3.6	0.0500	-0.0626	-0.0760	0.0951	-0.0440	0.2502	0.0461	-0.1116	-0.0385	0.0125	0.0008	-2.70E-09
	R3.7	-0.0964	0.0405	0.1058	0.0492	-0.1455	0.1062	-0.2145	-0.0675	-0.0193	0.0024	0.0135	-2.70E-09
	R3.8	-0.0552	0.0630	-0.0974	-0.0642	-0.2067	0.1219	-0.2087	-0.0591	0.0260	-0.0147	-0.0012	-2.70E-09
Day 113	R3.5	-0.1344	0.0070	-0.0065	0.0237	0.4046	-0.0215	0.1460	-0.0582	0.0864	0.0176	0.0093	-2.70E-09
	R3.6	-0.0786	-0.1341	-0.0132	-0.0232	0.3983	-0.1198	-0.1162	0.1384	-0.0492	-0.0195	-0.0049	-2.70E-09
	R3.7	0.0213	0.1308	0.0102	-0.0381	0.3669	-0.0039	0.1201	-0.0932	-0.0730	0.0064	-0.0216	-2.70E-09
	R3.8	0.1950	0.0493	0.0048	0.0288	0.3142	-0.0818	-0.1515	0.1060	0.0420	-0.0041	0.0161	-2.70E-09
eigvals		0.0873	0.0609	0.0309	0.0263	0.8956	0.4846	0.2108	0.1745	0.0213	0.0137	0.0093	8.73E-17
% variation explained		4.3334	3.0200	1.5319	1.3069	44.4419	24.0463	10.4624	8.6594	1.0586	0.6785	0.4606	4.33E-15

Legend: The values were obtained using unweighted UniFrac.



Supplementary Figure S3. Two dimensional version of 3D principal component analysis diagram based on the beta diversity of bacterial communities in various reactor samples taken at different time points.



Supplementary Figure S4. N-MDS plot showing the similarity of the methanogenic community compositions in parallel reactors based on the Bray-Curtis dissimilarity index.

The Supplementary Table S2 containing the list of obtained OTUs for the co-digestion reactors at three different sampling points can be found under the following link <https://static-content.springer.com/esm/art%3A10.1186%2Fs13068-016-0548-4/MediaObjects/13068_2016_548_MOESM2_ESM.xlsx>.

Furthermore, the de-multiplexed sequences from the pyrosequencing analysis obtained in the study under subsection 3.3. are deposited under the EMBL-EBI accession number PRJEB12073 <http://www.ebi.ac.uk/ena/data/view/PRJEB12073>.