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Pretreatment of lignocelluloses for enhanced biogas production: A review on influencing mechanisms and the importance of microbial diversity

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ABSTRACT

As one of the most efficient methods for waste management and sustainable energy production, anaerobic digestion (AD) countenances difficulties in the hydrolysis of lignocelluloses biomass. Different pretreatment methods have been applied to make lignocelluloses readily biodegradable by microorganisms. These pretreatments can affect biogas yield by different mechanisms at molecular scale, including changes in chemical composition, cellulose crystallinity, degree of polymerization, enzyme adsorption/desorption, nutrient accessibility, deacetylation, and through the formation of inhibitors. The present article aims at critically reviewing the reported molecular mechanisms affecting biogas yield from lignocelluloses *via* different types of pretreatments. Then, a new hypothesis concerning the impact of pretreatment on the microbial community developed (throughout the AD process from an identical inoculum) was also put forth and was experimentally examined through a case study. Four different leading pretreatments, including sulfuric acid, sodium hydroxide, aqueous ammonia, and sodium carbonate, were performed on rice straw as model lignocellulosic feedstock. The results obtained revealed that the choice of pretreatment method also plays a pivotally positive or negative role on biogas yield obtained from lignocelluloses through alteration of the microbial community involved in the AD. Considerable changes were observed in the archaeal and bacterial communities developed in response to the pretreatment used. Sodium hydroxide, with the highest methane yield (338 mL/g volatile solid), led to a partial switch from acetoclastic to the hydrogenotrophic methane production pathway. The findings reported herein

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undermine the default hypothesis accepted by thousands of previously published papers, which is changes in substrate characteristics by pretreatments are the only mechanisms affecting biogas yield. Moreover, the results obtained could assist with the development of more efficient biogas production systems at industrial scale by offering more in-depth understanding of the interactions between microbial community structure, and process parameters and performance.

List of abbreviations, units, and nomenclatures

°C	Temperature unit
AA	Aqueous ammonia
AD	Anaerobic digestion
AIL	Acid insoluble lignin
ASL	Acid soluble lignin
atm	Atmosphere (pressure unit)
bar	Pressure unit
d	Day (time unit)
DP	Degree of polymerization
GC	Gas chromatograph
GHG	Greenhouse gas
g	Gram (mass unit)
h	Hour (time unit)
HMF	5-Hydroxymethylfurfural
kGy	kilogray (ionizing radiation dose unit)
L	Liter (volume unit)
m	Meter (length unit)
M	Molar (concentration unit)
mg	Milligram (mass unit)
min	Minute (time unit)
mL	Milliliter (volume unit)
mm	Millimeter (length unit)
NMMO	N-methylmorpholine-N-oxide
PCA	Principal components analysis
PCR	Polymerase chain reaction
Prin	Principal component
s	Second (time unit)
S_0	Severity parameter
SA	Sulfuric acid
SC	Sodium carbonate
SH	Sodium hydroxide
VFAs	Volatile fatty acids
VS	Volatile solid

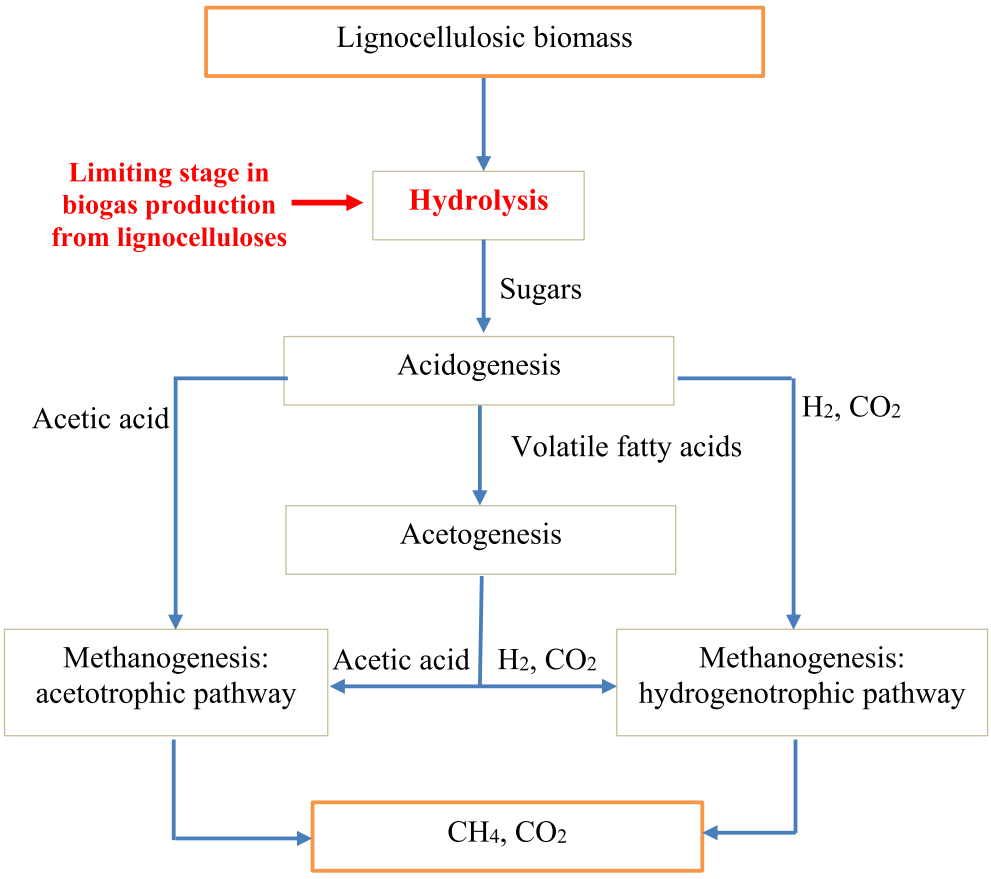

Fig. 1. Schematic representation of biogas production from organic materials including lignocelluloses; Cellulose and hemicellulose are the only digestible components available in lignocelluloses and given their recalcitrant nature, their "hydrolysis" into fermentable sugars in the limiting stage throughout the whole process.

1. Introduction

Biogas production has attracted an increasing deal of interest in the agricultural sector. The ability of biogas plants in using many substrates and their flexibility in terms of scale are considered among the main advantages of this process for universal applications [1]. Anaerobic digestion (AD) refers to a series of biological processes in which a microbial consortium synergistically decomposes organic matters in an oxygen-depleted environment [2]. AD provides not only an alternative source of energy but also an alternative option to divert organic wastes from landfills and reduce greenhouse gas (GHG) emissions [3]. AD, as a part of an integrated solid waste management system, is a promising technology to increase the efficiency of municipal solid waste management [4]. AD of organic materials including lignocellulosic biomass is performed in four sequential stages, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 1) [5]. During the process, organic materials are converted into biogas, consisting of methane (45–70%), carbon dioxide (24–40%), and small amounts of other components (nitrogen, oxygen, hydrogen, hydrogen sulfide) [6]. Biogas is a promising renewable source of energy that can be used for different applications including as vehicular fuel and for heat and electricity production [6]. Utilization of biogas-based electricity (of biowaste origin), instead of fossil-based electricity in existing sugar plants was shown to decrease the environmental impacts in all the investigated categories [7]. Moreover, apart from biogas, other value-added products, i.e., both liquid and solid biofertilizers, could be generated throughout the AD process. These value-added products are associated with less environmental burdens compared to the bioelectricity produced from biogas [8]. Various biological techniques, including upstream, mainstream, and downstream strategies, aimed at boosting biogas production have been recently reviewed [9,10]. It should also be noted that AD could also reduce the natural emissions of methane through the self-degradation of biomass [11]. This is significantly important given the fact that the greenhouse effect of methane is 21 times higher than that of carbon dioxide. The sustainability aspects of the whole process could be enhanced if various processes would be integrated through multi-products biorefineries [12]. Biorefineries and the implementation of circular economy could also compensate for the generally low return on investment of biogas production plants (5% in 2017) and enhance their economic feasibility [13].

Biogas production from lignocelluloses, including agricultural and forestry wastes, municipal solid wastes, and energy crops, has remarkable potentials in terms of environmental and social sustainability. However, the main challenge faced for AD of such feedstocks is their recalcitrant structure limiting their hydrolysis to sugars. The large gap between the actual and potential biogas production values could in fact be ascribed to the mentioned challenge [14]. Various types of pretreatments are performed to overcome this obstacle and to enhance methane yield from lignocelluloses [15].

To date, the attempts aimed at investigating the effects of different pretreatment processes on AD were only focused on substrate characteristics [16]. In other words, so far the impacts of pretreatments on AD of lignocelluloses has only been investigated by taking into consideration the changes in main substrate properties, including composition, surface properties, crystallinity, the degree of polymerization (DP), enzymes adsorption/desorption, and accessibility [17,18].

In light of the above, the present research-review article is aimed at first reviewing the molecular mechanisms reportedly affecting biogas yield from lignocelluloses through different types of pretreatment. Then, a case study was performed to investigate if pretreatments could also exert their influence through the alteration of the microbial consortium developed throughout the AD process leading to consequent improvements/deterioration in biogas production. To examine this mechanism experimentally, four different outstanding pretreatments, including sulfuric acid (SA), sodium hydroxide (SH), aqueous ammonia (AA), and sodium carbonate (SC), were selected and performed on rice straw. It should be noted that these pretreatment methods were not meant to represent all available methods but rather, they were solely selected to serve the purpose of the mentioned case study. More specifically, the new hypothesis argues that in addition to substrate physicochemical characteristics, the pretreatment of lignocelluloses also affects the microbial community involved in the AD (developed from an identical inoculum). Moreover, it also asserts that such pretreatment-induced alterations in the developed microbiome, under similar inoculum conditions, could positively or negatively impact biogas production. To the best of our knowledge, there is no study reporting on the changes in microbial communities during the AD in response to the application of different pretreatments for a given lignocellulosic substrate, which is the focus of the case study reported herein. These findings could be of substantial assistance to explain the controversial results reported in the literature.

2. Lignocelluloses as AD feedstocks

Agricultural wastes, mainly lignocellulosic materials, have attracted a wide interest as AD feedstock because of their abundance and renewability [19]. Lignocellulosic materials have a complex structure consisting of a high amount of cross-linked polysaccharide networks, glycosylated proteins, and lignin [20]. From the structural point of view, long cellulose microfibrils are surrounded and interconnected by sheaves of hemicellulose polysaccharides while pectins and lignins fill the remaining spaces in the structure [21]. Microorganisms can ferment the lignocelluloses carbohydrates such as cellulose and hemicellulose during the AD process. Cellulose, the main constituent of lignocelluloses, is a linear homopolysaccharide of glucose with the strong linkages of β-1,4-glycosidic [22]. The individual molecules of cellulose are microfibrils containing hydroxylic groups, forming hydrogen bonds inside and between microfibrils. Cellulose has amorphous and crystalline regions, depending on the different orientations of cellulose molecules [23]. In addition to hydrogen bonds, cellulose microfibrils are linked to each other by hemicellulose and pectin and are also covered by lignin [21,23]. Therefore, this complex structure makes cellulose resistant to chemical and biological degradation.

Unlike cellulose, hemicellulose is an amorphous and branched heteropolysaccharide composed of different hexoses (glucose, mannose, galactose, and rhamnose), pentoses (xylose and arabinose), and acids (methyl glucuronic acid, glucuronic acid, and galacturonic acid). The amorphous structure of hemicellulose forms a rigid matrix throughout lignocellulosic materials. Nevertheless, hemicellulose itself is greatly susceptible to anaerobic degradation [24]. Lignin is a hydrophobic heteropolymer reinforcing the strength of cellulose. It is the most recalcitrant component of lignocelluloses that consists of phenylpropane units. Lignin is the main barrier in the use of lignocelluloses for biofuel purposes. Softwoods generally have more recalcitrant structures to bioconversion than hardwoods because of their higher lignin contents [25].

Cellulose, hemicellulose, and lignin constitute over 80% of lignocelluloses. The rest is extraneous materials, including numerous materials divided into extractives and nonextractives [26]. Extractives are mainly fats, terpenes, waxes, and phenols, while nonextractives are mostly proteins, starches, silica, pectins, alkali earth carbonates, and oxalates [27]. Several review papers have well summarized and discussed the typical composition of common lignocelluloses [28,29]. It should be noted that the composition of lignocelluloses differs under different growth conditions and maturation level, even in the case of similar species [29]. Generally, lignocelluloses have a high C/N ratio that results in a low biogas yield. Therefore, the direct utilization of these organic materials in the AD process is difficult due to the nutritional imbalance and complex structure of lignocellulosic [30]. Anaerobic co-digestion is an efficient method for biogas production from lignocelluloses. A comprehensive review on the achievements and perspectives of anaerobic co-digestion has been recently published [31].

3. Pretreatment, an essential step prior to AD

In principle, cellulose and hemicellulose are digestible by the anaerobic microorganisms, among different components available in lignocelluloses [32]. However, untreated lignocellulosic feedstocks are bulky and difficult to feed into conventional biogas digesters. Even when fed, these substrates float and can only be partly degraded during the process [24]. Also, the hydrolysis stage is the limiting stage throughout lignocelluloses digestion [33], as hydrolytic microorganisms, which are responsible for initiating the AD process, cannot effectively degrade these compounds [34]. Therefore, increasing the biodegradability of lignocelluloses by a pretreatment is an essential element for biogas plants running on agro-wastes to be economically feasible. From the technical point of view, the pretreatment process is a preliminary key stage included in biogas production processes to overcome this problem [35].

Different pretreatments are used for improving biogas yield from lignocelluloses. In this context, various pretreatment methods have been recently reviewed by Tabatabaei et al. [12]. For instance, dilute sulfuric acid pretreatment was applied for biogas production from garden wastes in co-digestion with biomass of the fungi: *Mucor indicus* [36]. Sodium hydroxide successfully improved anaerobic biogas production from corn stover [37], birch, spruce [38], and maize stalk [39]. Besides, a few research works have been studied ionic liquid pretreatments prior to biogas production from lignocelluloses [40,41]. The main obstacles to applying ionic liquids for pretreatment are their high costs and their high viscosity values, rendering their industrial application impractical. An increase in methane yield was reported from pinewood using concentrated phosphoric acid [42]. A combined hydrothermal alkaline pretreatment at 175 °C with 8% NaOH was reported to enhance biogas yield by 57% [43]. Lignin removal was introduced as the main mechanism responsible for enhancing methane yield in this work. In another study, 122% increase in methane yield was recorded in reposnse to the incorporation of an alkaline-photocatalytic pretreatment (with 1.5% w/v NaOH, 0.25 g/L TiO_2 at 37 °C for 3 h) [44]. The authors cited delignification and an increase in cellulose content as major reasons behind the observed improvements [44]. Sulfuric acid pretreatment of water hyacinth increased biogas yield by 131% at the optimum conditions (121 °C, 2 atm, 60 min, 5% v/v H_2SO_4) [45]. These are just examples of many studies portraying the favorable impacts of pretraetment methods.

On the contrary, some studies also claim decreases in biogas yields due to the implementation of pretreatment processes [39,42]. As an example, urea pretreatment was reported to decrease biogas production yield from corn stalk [39]. Concentrated phosphoric acid (85%) at 60 °C for 45 min also decreased methane yield from poplar and berry woods by up to 43% [42]. Acid pretreatments (with 2–20 g H_2SO_4, H_3PO_3, and HCl/100 g total solids at 121 °C for 1 h) of grass lawn waste were also reported to decrease the total methane potential (from the separated fractions of liquid and solid) by up to 23% [46]. Therefore, based on these controversial findings, it could be concluded that the choice of pretreatment method prior to the biogas production from lignocelluloses may increase or decrease methane yield through different mechanism as discussed in the subsequent section.

4. Pretreatment of lignocelluloses to improve biogas: mechanisms

Various mechanisms can lead to changes in methane yield, considering the complicated nature of the AD process. There are over a

Table 1
A summary of the studies attributing the improvements in biogas yield from lignocelluloses to the impact of pretreatments on physicochemical characteristics of feedstocks.

Biomass	Pretreatment	Pretreatment conditions	Justification of biogas improvement	Reference
Softwood spruce, rice straw, and triticale straw	NMMO*	130 °C, 1–15 h, 85% solution	Breakdown of the crystalline structure	[47]
Sunflower residues	NaOH	55 °C, 24 h, 4 g NAOH/g total solid	Delignification	[48]
Rice straw, corn stalk	Banana peel ash + CaOH	Room temperature for 7 d or 60–90 °C, 2–10 h	Delignification, decease in crystallinity	[49]
Corn stover	NaOH	20 °C, 24 h, 50% solid loading, 1–7.5% solution	Lignin degradation and lignocellulose depolymerization	[50]
Fallen leaves	Simultaneous NaOH treatment with AD	2–5% NaOH loading	Delignification, cellulose and hemicellulose degradation	[51]
Rice and triticale straw	NMMO*	130 °C, 1–15 h, 7.5% solid loading, 85% solution	Increases in the accessible surface area and decreases in crystallinity	[52]
High-crystalline cellulose	NMMO*	90–120 °C, 0.5–15 h, 3% solid loading, 73–85% solution	Changes in cellulose structure and water swelling capacity	[53]
Wheat plant	NaOH	0–100 °C, 60 min, 5% solid loading, 8% solution	Changes in crystallinity and removal of surface layers of lignin and hemicellulose	[54]
Pinewood	NaOH	0–100 °C, 10–60 min, 5% solid loading, 8% solution	Changes in cellulose crystallinity and disruption of recalcitrant structure	[55]
Oil palm empty fruit bunches	NaOH	100 °C, 10–60 min, 1:20 solid:liquid ratio, 8% w/v solution	Lignin removal and reduction in crystallinity	[56]
Oil palm empty fruit bunches	H_3PO_4	50 °C, 30 min, 1:8 solid:liquid ratio, 85.7% solution	Structure modification	[56]
Pine tree wastes	NaOH	0–100 °C, 10–60 min, 5% solid loading, 8% solution	Crystallinity reduction and lignin removal	[57]
Water hyacinth	[Bmim]Cl/DMSO**	100–140 °C, 1–4 h, 5% solid loading	Changes in composition and structure crystallinity	[58]
Corncob waste	Organosolv	175 °C, 2 h, ethanol:acetic acid ration of 1:10,	Lignin removal	[59]
Elm, pine, and rice straw	Organosolv	150–180 °C, 30–60 min, 1:8 solid:liquid ratio, 75% ethanol solution with 1% H_2SO_4 as the catalyst	Changes in lignin content	[60]
Water hyacinth, rice straw, mango leaves, and spruce	[C4mim]Cl***	120 °C, 2 h, 5% solid loading	Changes in lignin content and crystallinity	[61]
Birch	Steam explosion	170–230 °C, 5–15 min	Xylan degradation and formation of pseudo-lignin	[62]
The straw fraction of manure	NMMO*	120 °C, 5–15 h, 85% solution	Changes in crystallinity	[63]
Wheat straw	Ammonia	20–80 °C, 6–48 h, 10% solid loading, 0–30.8% solution	Changes in lignin content	[64]

* N-methylmorpholine-N-oxide.
** 1-N-butyl-3-methyimidazolium chloride/dimethyl sulfoxide.
*** 1-butyl-3-methylimidazolium chloride.

thousand scientific articles highlighting feedstock changes as the main reason affecting biogas yield. A summary of these studies is presented in Table 1. Moreover, these studies are discussed categorically in this section.

As mentioned in Section 3, pretreatment is a necessary step in the AD of lignocelluloses to disrupt their recalcitrant structure [35]. Nevertheless, the pretreatment process usually changes several parameters simultaneously [65]. Therefore, it is difficult to investigate the effect of a single parameter on the AD yield. However, it is generally believed that the most influencing parameters in bioconversion of lignocelluloses are their chemical composition, cellulose crystallinity, cellulose DP, accessible surface area, enzyme adsorption and desorption, the degree of hemicellulose acetylation, and water swelling capacity.

4.1. Changes in chemical composition

The chemical composition of lignocelluloses is known as an essential parameter affecting their degradability due to the shielding effect of lignin. Alkali pretreatments mostly solubilize lignin and acid pretreatments mostly solubilize hemicellulose and cellulose, while thermal pretreatments are capable of solubilize all [66]. He et al. [67] investigated the effects of changes in lignocelluloses compositions on biogas production from rice straw pretreated with sodium hydroxide [67]. The authors reported that the changes in major components considerably contributed to the enhancement of biogas yield. Similarly, the effects of compositional changes caused by phosphoric acid pretreatment on biogas yield were highlighted by a different study [42]. Lignin content higher than 100 g/kg volatile solid was reported as a critical value in AD, leading to remarkably low methane yield [68]. In light of that, lignin removal has been proposed as one of the most influencing factors on biogas production yield [69]. A decrease of 3.2–38.6% in lignin content by NaOH pretreatment (at the initial pH of 8–13 for 24 h) was reported as to enhance biogas yield from organic fraction of municipal solid wastes by 19.6–34.8% [70]. Lignin content has been reported as the most important factor hindering methane production [71] as compared with the other lignocelluloses' characteristics, even more strongly than cellulose crystallinity [72]. A combined metal oxide (CuO 4%) and UV-based photocatalytic (180 min) pretreatment that led to the maximum delignification was reported to cause the maximum increase in methane yield (by 57%) from wheat straw [73]. Lignin removal (by up to 50.5%) from a mixture of cotton straw and cow manure by potassium ferrate and peroxymonosulfate pretreatment was also found instrumental in boosting methane yield [74]. White-rot fungi such as *Ceriporiopsis subvermispora*, *Phellinus pini*, etc., as delignifying microorganisms, can improve biogas yield. However, the fungal strain should be carefully chosen, and the biological pretreatment conditions should be well optimized to avoid hemicellulose loss (degradation) [75].

Hemicellulose removal can also improve biogas yield by breaking the physical structure of lignocellulose and promoting the accessibility of microorganisms [76]. Lignin and hemicellulose removal by thermal pretreatment (120–180 °C for 60 min) was reported to contribute to increasing biogas yield (by up to 53%) [77]. Increasing the pretreatment temperature from 120 to 180 °C led to a continuous decrease in hemicellulose and lignin contents and increased biogas yields [77]. It can be concluded that increments in temperature, and consequently in pressure, act *via* hemicellulose and lignin removal mechanism to improve methane yield. Steinbach et al. [78] reported that hemicellulose degradation by too severe steam explosion (with the severity parameter of $S_0 > 4.3$ min) caused drops in methane yield, whereas altering hemicellulose structure and increasing its porosity under moderate conditions ($S_0 = 4.1$ min) enhanced methane yield.

An increase in water extractive value can also lead to improvements in biodegradability and biogas production. Water extractives are typically simple compounds with low molecular weights and are the most biodegradable materials in lignocelluloses. Pretreatments and resultant degradation of hemicellulose, cellulose, and lignin could lead to increases in water extractives and consequently in biogas yields [79]. Ethanol and benzene extractives (usually consisting of resins, waxes, fattiness, and tannins) are not biodegradable during the AD [79], and more importantly, could also play an inhibitory role in the process. Hence, decreases in ethanol and benzene extractives during the pretreatment of lignocelluloses are considered favorable to the AD process.

At the first glance, it may seem that mechanical pretreatments are solely aimed at size reduction and do not exert compositional changes. This perception was questioned by Dahunsia [80] who attempted to predict methane yields from the structural components of lignocelluloses after mechanical pretreatments. He showed that the contents of lignin, cellulose, hemicellulose, and arabinan were considerably decreased, and the protein content was increased during comminution.

4.2. Changes in cellulose crystallinity

Cellulose crystallinity is one of the main parameters affecting the biodegradability of lignocelluloses. As evidence, the low digestibility of natural cotton that is almost pure cellulose is attributed to the high crystallinity of its cellulose [81]. Cellulose, with a polymorphic structure, consists of crystalline and amorphous regions. The amorphous region, with a high accessible surface area, can readily adsorb water, chemicals, and enzymes, and consequently, its hydrolysis rate is 2–25 times faster than that of the crystalline region [82]. The crystalline cellulose can adopt different forms by changing the location of hydrogen bonds. The natural cellulose is described by cellulose I model, suggesting that it is composed of cellulose I_α (triclinic unit with one cellulose chain) and cellulose I_β (monoclinic unit with two cellulose chains). These two polymorphs of cellulose are found in different proportions in lignocelluloses. Cellulose I_α is dominant in lower plants since it is synthesized simultaneously with the microfibrils. Whereas, cellulose I_β has more proportions in higher plants, and it is deposited within the secondary wall [83]. The more stable structure of I_β compared with I_α is because of the higher number of intramolecular hydrogen bonds in the structure. Another crystalline form of cellulose is described by cellulose II model that typically exists in pretreated lignocelluloses. Cellulose II, called regenerated cellulose, has a nonparallel arrangement of molecules, and it is produced by cellulose dissolution in a solvent followed by a precipitation process [84].

The crystallinity of cellulose is defined as the ratio of the amount of crystalline region to the total amount of cellulose, including both amorphous and crystalline regions [85]. It has been reported that reductions in crystallinity index increased biogas yields [38,57]. Patowary and Baruah [49] reported decreases in the crystallinity index of rice straw from 0.97 to 0.85 and corn stalk from 0.96 to 0.87 by increasing the pretreatment severity through applying a combination of chemical and thermal pretreatments (using banana peel ash and CaOH at 60–90 °C for 2–10 h). The decreases in crystallinity index were simultaneous with increases in biogas production in all cases except that of the most severe pretreatment conditions (the highest temperature of 90 °C for the highest duration time of 10 h). This finding could be attributed to the formation of inhibitors as a result of harsh pretreatment conditions. Similarly, 24.5–56.0% decrease in crystallinity index of wheat straw by liquid digestate pretreatment for 3–7 d led to up to 39.8% increase in biogas production [86]. However, some contradictory results have also been reported by several researchers [79]. For instance, by using a combined H_2SO_4 with steam explosion pretreatment, crystallinity index of rape straw was increased by 41–49%, which also coincided 14–53% increase in methane yield [87]. The authors attributed the increased crystallinity index to the degradation of hemicellulose, amorphous cellulose, and lignin. Nevertheless, the complicated nature of the AD process make it difficult to explain such conflict. Overall, the crystallinity is undoubtedly an influencing factor but its magnitude of impact seems proportional to the other factors involved.

4.3. Decreases in degree of polymerization (DP)

The DP of cellulose, which determines the relative abundance of interior and terminal β-glucosidic bonds, is the primary parameter affecting the digestibility rate of lignocelluloses [35]. The DP of cellulose contained in lignocellulosic substrates is in a range of 1510–5500, depending on the substrate source and the applied pretreatment [88]. For instance, the cellulose DP of softwoods and hardwoods is 4–5.5 times more than that of agricultural wastes (about 1000). Hemicellulose has very low DP values (50–300) [88] explaining its higher digestibility compared to cellulose. Higher DP values of cellulose indicate longer cellulosic chains, i.e., more hydrogen bonds, stronger network, and less accessibility of enzymes and microorganisms [88]. In contrast, the lower DP values indicate higher numbers of cellulose ends available for exo-enzymes and higher reactivity of cellulose to these enzymes [88]. In spite of its significance, no study has directly investigated the effects of cellulose DP on AD yield.

The reduction of cellulose DP has been mentioned as one of the main objectives of lignocelluloses comminution that is a necessary pretreatment for AD [89]. Chemical pretreatments can also decrease the DP of cellulose, up to the point that some cellulose chains would be solubilized in the pretreatment solution. The cellulose chains with DP < 6 are soluble and those with $6 <$ DP < 12 are slightly soluble [90]. This could be a drawback of the chemical pretreatments of lignocelluloses if the use of soluble cellulose available in the supernatant would not be considered.

In an interesting study by Wyman et al. [91], Fe (1000 mg/L) was added as a trace element to the AD of model lignocellulosic compounds [91]. However, the authors found that the supplement acted as a chemical pretreatment on lignin structure and reduced its DP, which led to a 28% improvement in methane yield coefficient from the model lignin. In a recent investigation, electron beam irradiation pretreatment at 900 kGy could also decrease the DP from 2160 to 245 and promote enzymatic hydrolysis [92].

4.4. Changes in enzyme adsorption/desorption

Lignocellulose degradation occurs through the function of different enzyme systems, including enzymes cocktails (free enzymes with one or more catalytic domains per enzyme) and cellulosomes (large multi-enzyme complexes, with several catalytic units per complex) [93]. Resch et al. [94] suggested that cellulosomes provide access to the deep lamella layers by peeling up pockets of cell wall lamellas. Synergistically, the accessible microfibrils are hydrolyzed by free enzymes that penetrate into the accessible walls. Mesophilic anaerobic bacteria from *Ruminococcus Clostridium*, *Bacteroides*, and *Acetivibrio* genera produce cellulosomes [95].

Cellulose degradation starts with enzymatic hydrolysis, a heterogeneous catalysis stage, which is preceded by exoenzymes adsorption. Adsorption is the attachment of exoenzymes to the surface of lignocellulose and is initiated with the physical forces between lignocellulose and exoenzymes, such as van der Waals forces, Brownian motion, and gravitational forces and ends with chemical interactions [96]. The established substrate-enzyme complex must be detached, and the active sites of exoenzymes must be emptied to initiate the adsorption-desorption cycle again. Therefore, not only enzyme adsorption but also the adsorption reversibility and the process dynamic equilibrium are among the main characteristics affecting the lignocellulose degradation rate. The irreversibility of enzyme adsorption can be due to the strong binding of carbohydrate-binding modules or changes in the enzyme conformation [97]. Furthermore, nonspecific and non-productive binding of enzymes to the modified lignin and not to the native lignin, has inhibitory effect on enzymatic hydrolysis [98]. Lignin is in fact known as the main obstacle to the specific and productive adsorption of exoenzymes onto cellulose [99]. Cellulosomes, compared to free enzymes, cause fewer off-rates because of having multiple binding specificities [100]. Whole cell biocatalysts have also been shown to be effective in enhancing enzyme adsorption. For example, modification of rice straw by *Pleurotus ostreatus* for 25 d was reported to increase the cellulase adsorption by 18.8% and xylanase adsorption by 58.1% that improved methane yield by 26.9% [101]. Overall, enzyme adsorption/desorption is an essential attribute affecting the hydrolysis stage, as it could overshadow some other important properties, including particle size, hemicellulose and lignin contents, crystallinity, and accessible surface area [18]. In line with that, it was experimentally shown that cellulase adsorption on cellulose was the primary parameter controlling the enzymatic hydrolysis [102]. However, there is little knowledge available on the relationship between enzyme adsorption/-desorption and microbial digestion and biogas production yield from lignocelluloses.

4.5. Increases in nutrient accessibility (accessible surface area)

The surface area of lignocelluloses defines the accessibility of nutrients to microorganisms and their exoenzymes, and it can influence the rate and yield of AD. The accessible surface area includes internal surface area and external surface area. The internal surface area corresponds to the porosity and capillary structure of the lignocelluloses, while the external surface area corresponds to the size and shape of the particles [65]. Water swelling capacity and porosity are two parameters that can determine the enzymatic accessibility of biomass [103,104].

As a matter of fact, most pretreatment methods are performed on lignocelluloses to enhance their accessible surface area. Biomass comminution, a mechanical pretreatment, increases the external surface area and improves the digestibility of the carbohydrates. Therefore, it is a necessary step for biomass conversion into fermentable sugars, even though it contributes to a major proportion of the expenses of the whole process [89]. Lignin removal considerably increases cellulose accessibility [35]. For instance, concentrated phosphoric acid can greatly enhance cellulose accessibility by disrupting the linkages between cellulose, hemicellulose, and lignin through biomass dissolution [105]. In spite of the significance of high nutrient accessibility for a successful AD process, it is worth stating that increasing nutrient accessibility requires careful considerations, especially at high solid loadings rates. In other words, high nutrient accessibilities may also cause imbalances within the microbial communities involved in the organic matters degradation and methane production, leading to the accumulation of volatile fatty acids (VFAs) and ultimately AD failure. This has been experimentally shown when hardwoods were reportedly pretreated by concentrated phosphoric acid, and as a result of high nutrient accessibility, very low methane yields were obtained [42].

4.6. Deacetylation

The hydrolysis of cellulose and hemicellulose is hampered by the acetyl groups of xylans and mannans through creating a steric hindrance for binding of hydrolytic enzymes [106]. In other words, acetyl groups exert inhibitory effects on the formation of hydrogen bonds between enzymes and cellulose molecules [107]. Increases in cellulose diameter and changes in enzymes' hydrophobicity have been highlighted as the reasons to the hampering effect of acetyl groups on the degradation process [108]. Hemicelluloses deacetylation is, therefore, vital for enhanced exposure of the cellulose surface to the enzymes and for improving its digestibility [109]. The acetyl groups can be removed by acid, alkaline, hydrothermal, and N-Methylmorpholine-N-oxide (NMMO) pretreatments [25]. Thermal pretreatment of wheat straw has been reported to break acetyl bonds (existing between hemicellulose and lignin), and that higher treatment temperatures led to higher degrees of deacetylation and higher methane yields [77]. In spite of the favorable outcomes associated with deacetylation, it should also be noted that lignocellulose deacetylation can also lead to the formation of acetate in the pretreatment solution. These acetate ions can interfere with the AD process if the pretreated substrate is used without

performing prior washing processes. The interference of acetate, as a VFA, in the AD process is further discussed in Section 5.

5. Pretreatment of lignocelluloses: formation of inhibitors

Physicochemical pretreatments of lignocelluloses might also result in the release of some byproducts, in addition to the applied chemicals, which can inhibit the enzyme activity and the growth and metabolism of the microbial community involved in the AD process (Table 2) [110]. The potential byproducts with inhibitory effects are aliphatic (acids such as formic, acetic, and levulinic acids), furaldehydes (such as 5-hydroxymethylfurfural (HMF) and 2-furaldehyde (furfural)), uronic acid, cinnamaldehyde, vanillin, 4-hydroxybenzoic acid, formaldehyde, and phenol. Weak acids can diffuse through the lipoprotein plasma membrane of the microbes and change the cytosol to an acidic nature. Cells should maintain a neutral cellular environment by excreting protons through the plasma ATPase, and this could finally lead to cell lysis and death [111]. Some compounds like *p*-hydroxybenzoic acid and salicylic acid are amphiphilic molecules that cause the disruption of eukaryotic cells. These compounds dissolve the inner mitochondrial membrane, compromise the ability of mitochondria to produce ATP from ADP, and lead to starvation and death [112]. Furan derivatives can also interfere with the activity of some enzymes including alcohol dehydrogenase, aldolase, hexokinase, phosphofructokinase, and triosephosphate dehydrogenase [113].

The formation of inhibitors largely depends on the properties and composition of the feedstock. The chemical composition of lignin and hemicelluloses differs between lignocelluloses, while cellulose has a uniform composition in most of them [114]. In addition, the nature and the amount of the inhibitors generated also depend on the pretreatment method used and the conditions employed. For instance, acidic pretreatments lead to the formation of furfural and HMF, mainly formed during the hydrolysis of hemicelluloses, resulting in the dehydration of pentose and hexose sugars, respectively [113]. HMF, under severe conditions, can be degraded into formic acid and levulinic acid [115]. Also, furfural can be further degraded into formic acid and formed resins [116]. Acetic acid is another typical compound formed during acidic pretreatments, resulting from the hydrolysis of the acetyl groups available in hemicelluloses. As mentioned earlier, acetic acid is an intermediate product of AD and its accumulation beyond certain limits could jeopardize the efficiency of the process. Phenolic compounds, i.e., vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, dehydroconiferyl alcohol, syringic acid, *p*-coumaric acid, ferulic acid, pyrogallol acid, and gallic acid, are other products formed through acidic pretreatments, which are originated from lignin macromolecules and extractives [117,118]. Hydrothermal pretreatment could also generally lead to acidification because of the release of acetic acid and uronic acid [119]. Therefore, most of the products of acidic pretreatments can also be formed during hydrothermal processes, but in lower concentrations.

Alkaline pretreatments are accompanied with more favorable preservation of carbohydrates compared with their acidic counterparts. However, their low degradation rates may lead to the formation of carboxylic acids. Peeling reactions can occur during alkaline pretreatments resulting in the production of saccharinic, lactic, formic, dihydroxy, and dicarboxylic acids [115]. Other typical compounds formed by alkaline pretreatments are acetic acid and phenols, produced from acetyl groups and lignins, respectively. Using agricultural wastes and hardwoods as substrates as well as high solid loading processes could bring about inhibition by aliphatic carboxylic acids, like acetic acid. This is ascribed to the higher amounts of acetyl groups in agricultural wastes and hardwoods compared with softwoods [120,121].

VFAs, aliphatic monocarboxylic acids with 2–8 carbon atoms in a molecule, e.g., acetic, butyric, and propionic acids, are the AD intermediates and also the potential inhibitors to the process. These components at high concentrations could inhibit the methanogens' activity and yield, jeopardizing the whole process [122]. The threshold concentration of undissociated acetic and butyric acids reportedly stands at 19 mM [123].

Table 2
Inhibitors formed during the pretreatment of lignocelluloses.

Inhibitor	Original compound	pretreatment	Reference
Aliphatic carboxylic acids:	Cellulose, Hemicellulose	Acidic, Hydrothermal,	[124]
Acetic acid, Formic acid, Levulinic acid		Alkaline,	[123]
Phenolic compounds	Lignin, Extractives	Alkaline	[124]
Furans	Cellulose, Hemicellulose	Acidic	[124]

6. Case study

As mentioned earlier, a case study was also performed to prove if the microbial consortium developed throughout the AD process (from an identical inoculum) and the consequent biogas production can be influenced by various pretreatments. To investigate that, four different leading pretreatments, including SA, SH, AA, and SC, were performed on rice straw and the microbial population profiles along with methane productions were assessed.

6.1. Materials and methods

6.1.1. Substrate

Rice straw was obtained from an agricultural field in Lenjan area (Isfahan, Iran). It was milled and screened, and the straw particles which passed through a 35-mesh screen (0.50 mm) but were too large to pass through a 60-mesh (0.25 mm) screen were used.

6.1.2. Pretreatments

Four different chemical pretreatment methods, including a dilute acid (SA), two basic (SH and AA), and an inorganic salt (SC), were applied on rice straw. Pretreatments were conducted under the optimum conditions reported in the literature (Table 3). High-temperature pretreatments were carried out in a 500 mL high-pressure stainless steel reactor [125].

- Dilute sulfuric acid pretreatment

SA pretreatment was conducted on rice straw according to the method described previously [126]. Briefly, 20 g (dry weight) of rice straw was mixed with 380 g sulfuric acid solution (1% w/w) to obtain a solid loading of 5%. A high-pressure reactor, containing rice straw and the acidic solution mixture, was transferred to an oil bath at 160 °C and at 6.1 bar, heated at an average rate of 1.3 °C/min to the desired

Table 3
Pretreatment conditions performed on rice straw.

Pretreatment	Chemical	Chemical loading (g chemical/g rice straw)	Temperature (°C)	Time (min)	Ref.
Sulfuric acid (SA)	H_2SO_4	0.19	160	5	[126]
Sodium hydroxide (SH)	NaOH	2.28	0	180	[127]
Aqueous ammonia (AA)	NH_3	3.99	69	600	[128]
Sodium carbonate (SC)	Na_2CO_3	1.01	100	180	[129]

temperature. After holding the temperature for 5 min, the reactor was immediately cooled in an ice bath. Then, the pretreated materials were removed and left to swell in water for 24 h. During this time, water was replaced three times by fresh distilled water to reach a neutral pH. The pretreated rice straw was then freeze-dried (Christ, Alpha 1–2 LDplus Model, Germany) for 48 h and finally stored in airtight plastic bags at room temperature until use.

- Sodium hydroxide pretreatment

SH pretreatment was performed using 12% w/v NaOH with a 5% w/w solid loading [127]. Dry straw (10 g, dry weight) was mixed with 190 g of the NaOH solution, and the mixture was held at 0 °C for 3 h at atmospheric pressure using an ice bath. After the pretreatment, the mixture was washed with distilled water until pH 7 was obtained. The solids were then separated using a filtration cloth, freeze-dried for 48 h, and stored in airtight plastic bags until use.

- Aqueous ammonia pretreatment

Rice straw (10 g, dry weight) was pretreated in 190 g aqueous ammonia solution (21% w/w) in glass bottles at 69 °C for 10 h at atmospheric pressure [128]. The glass bottles were covered with aluminum foils to prevent the high rate of evaporation during the pretreatment. After being soaked in the aqueous-ammonia solution, the solids were filtered and washed using distilled water until the pH of the liquid reached 7. The pretreated rice straw was then freeze-dried for 48 h and stored in airtight plastic bags until use.

- Sodium carbonate pretreatment

Sodium carbonate solution (0.5 M) at 100 °C for 3 h at atmospheric pressure was used for SC pretreatment of rice straw [129]. Rice straw (10 g) was added to 190 g of the sodium carbonate solution to obtain a solid loading of 5%, and the desired temperature was applied using an oil bath. The solids were then separated and washed with distilled water to achieve a neutral pH. Similar to the other pretreatment procedures, the SC-pretreated sample was freeze-dried for 48 h and stored in airtight plastic bags until use.

6.1.3. Anaerobic digestion (AD)

Anaerobic digestion of the untreated and treated samples was performed in triplicate according to a method developed by Hansen et al. [130]. The inoculum was taken from a 3000 m^3 mesophilic AD bioreactor (Isfahan Municipal Sewage Treatment, Isfahan, Iran) and was kept at 37 °C for 1 week for stabilization. The inoculum had a total solid content of 8.4 ± 0.4% and a volatile solid content of 4.3 ± 0.2%. Media containing 0.25 g substrate (either pretreated or untreated straw, based on dry weight), 5 g water, and 20 mL of the inoculum were prepared in 118 mL black serum bottles. The serum bottles were sealed with rubber septa and aluminum caps, purged with nitrogen for 2 min, and incubated in a convection oven under mesophilic conditions (37 ± 1 °C) for 60 d. The inoculum was also digested alone as a reference to consider the methane production from the inoculum. Gas samples were periodically taken and analyzed for produced biogas every 3 d during the first 9 d of the experimental period and then every 4 or 5 d until 60 d. Afterward, liquid samples were taken from the bottles and transferred to centrifuge tubes. The tubes were sealed with Parafilm M and shipped on ice packs to another laboratory for microbial community analysis.

6.1.4. Analytical techniques

Total solids and volatile solids contents of the untreated and pretreated rice straw, as well as the inoculum, were determined by weighting the samples before and after drying at 105 °C [131] and also after the ignition of the dried samples at 575 °C [132]. Structural carbohydrates and lignin contents of untreated and pretreated straw were analyzed using a two-stage acid hydrolysis procedure developed by the National Renewable Energy Laboratory [133]. Briefly, the samples (300 mg) were first hydrolyzed using 72% sulfuric acid solution (3 mL) at 30 °C for 60 min and then using 4% sulfuric acid (86.73 mL) at 121 °C for 60 min. The hydrolysate was filtrated to obtain acid insoluble lignin, which was determined using a gravimetric method. Acid soluble lignin was determined by a UV–Vis spectrophotometer (Rayleigh UV-1601, BRAIC, Beijing, China) at a wavelength of 320 nm. Sugar concentrations in the filtrate were analyzed by a high-performance liquid chromatograph equipped with a refractive index detector (Agilent 1100, Agilent Technologies, Palo Alto, CA) and an Aminex HPX-87 P column (Bio-Rad, Richmond, CA, USA). The column temperature was set at 80 °C, and the mobile phase was deionized water at a flow rate of 0.6 mL/min. All compositional analyses were performed in duplicates, and the average values were presented.

The volume and composition of the biogas produced during the AD were analyzed [134] by a gas chromatograph (GC) with a thermal conductivity detector (Sp-3420 A, Beijing Beifen Ruili Analytical Instrument Co., China) equipped with a packed column (3 m length and 3 mm internal diameter, stainless steel, Porapak Q column, Chrompack, Germany). Nitrogen was used as a carrier gas at a flow rate of 45 mL/min. The column, injector, and detector temperatures were at 40, 100, and 150 °C, respectively. A pressure-tight syringe (SGE analytical science, Australia) with a volume of 0.250 mL was used for gas sampling and injection to GC with the ability to take gas samples at the fermenters' real pressure. The excess gas was released after each gas sampling to avoid overpressure built-up in the fermenters, and a new gas analysis was performed to determine the gas composition in the headspace after discharging. The method presented by Hansen et al. [130] was applied to determine the methane production volume, which is based on measuring the methane content by GC at the real fermenter pressure and then converting it to the standard conditions.

The pH values and VFAs to alkalinity ratios of the digested substrates were determined by a two-step titration method as described by Lossei and Pütz [135]. In this method, 4 g of the samples was suspended in 40 mL of distilled water and then separated by centrifugation at 4500 rpm for 20 min. The obtained supernatant was tittered with a 0.1 N H_2SO_4 solution from the initial pH to pH 5 and then, from pH 5 to pH 4.4, corresponding to the alkalinity and total VFA, respectively [135].

6.1.5. Microbial community analysis

- DNA extraction

DNA extraction from the samples was performed in a harsh manner by combining several lysis methods. The physical lysis, including liquid N_2 treatment and bead beating, was accompanied with the lysis buffer treatment to yield better extraction results. The lysis buffer was formulated according to Siddhapura et al. [136] with minor modifications. Two grams of sludge samples were added to the enzymatic buffer (Tris-HCl pH 8: 20 mM, EDTA pH 8: 10 mM and Triton X-100 1.2%) and 20 mg/mL lysozyme and were incubated under shaking overnight. Before the addition of the lysis buffer, the sludge sample was vigorously vortexed with 1 g glass beads followed by intermittent freezing-thawing in liquid N_2. The subsequent steps, used for chemical lysis and purification, were conducted according to Siddhapura et al. [136]. The extracted metagenomic DNA samples were quantitated using the Quant-iT dsDNA Assay Kit and the Qubit fluorometer (Invitrogen, USA).

- Bacterial and archaeal 16S rRNA Tag-encoded amplicon pyrosequencing

The composition and abundance of both bacterial and archaeal communities were analyzed through pyrotag sequencing of the 520 bp of bacterial and 457 bp of archaeal 16S rRNA gene amplicons. Polymerase chain reaction (PCR) for amplification of 10 bp-barcoded

amplicons of each sample was carried out using the bacterial 27F: 5′-AGAGTTTGATCCTGGCTCAG -3′ and 534R: 5′-ATTACCGCGGCTGCTGG -3′ as well as the archaeal 349 F: 5′-GYGCASCAGKCGMGAAW -3′ and 806 R: 5′- GGACTACVSGGGTATCTAAT -3′ primers. The reaction mixture of both bacterial and archaeal PCR was prepared by addition of 0.5 of each primer, 1.5 mM $MgCl_2$, 1 U of pfu polymerase enzyme and 1 × pfu buffer (Fermentas, Lithuania) and 0.2 mM dNTPs to 2 μL of the diluted DNA sample.

The PCR program included 95 °C for 2 min; 30 cycles of 95 °C for 20 s; 56 °C for 30 s; 72 °C for 1 min followed by a final extension stage at 72 °C for 5 min for the bacterial 16S rRNA. For the archaeal 16-S rRNA amplicon libraries preparation, the PCR program included 94 °C for 3 min; 35 cycles of 94 °C for 45 s; 50 °C for 60 s; 72 °C for 90 s; and a final extension stage at 72 °C for 10 min. Equimolar multiplexing on purified PCR product was performed using the Qubit fluorometer (Invitorgen, USA) and pooled libraries were pyrosequenced on a GS Junior platform (454 Life Sciences, Roche, Macrogen).

- Bioinformatics and data analysis

Raw reads were first filtered according to the 454 amplicon processing pipeline. To quantitatively analyze the filtered sequences, QIIME 1.6.0 pipeline [137] was used for OUT picking, taxonomic assignment, and for obtaining the relative abundance of each OTU using the Greengenes 16S rRNA gene database.

6.1.6. Data analysis

Principal components analysis (PCA) was performed on compositions and methane yields by using the SAS 9.1.3 software.

6.2. Results and discussion

6.2.1. Effects of pretreatments on the composition of rice straw

Solid recoveries and composition of untreated and pretreated rice straw are shown in Table 4. The maximum and minimum solid recoveries were 59.6 and 41.6% that were obtained with the AA and SA pretreatments, respectively. The rice straw lost 25, 58, 61, and 68% of its acid-insoluble lignin content through the SA, SH, SC, and AA pretreatments, respectively, considering the recovery of the solids. However, the weight percentage of the acid-insoluble lignin fraction increased after the SA pretreatment due to the further reduction of the other fractions. On the other hand, acid soluble lignin was decreased by all the pretreatments. Among the pretreated samples, the SA-pretreated sample had the lowest amount of acid soluble lignin (0.9%), while the AA-pretreated sample had the highest amount (1.2%).

As shown in Table 4, all the pretreatments resulted in an increase in glucan content by 16.2–128.9%. The SA-pretreated sample contained the highest amount of glucan followed by SH-, AA-, and SC-pretreated samples in a descending order. The SA pretreatment completely removed xylan while the SH pretreatment partly removed it. Xylan content was increased from 10.7% in the untreated sample to 17.1 and 16.7% through the AA and SC pretreatments, respectively. Arabinan was increased from 4.7% in the untreated sample to 6.5, 7.9, and 7.8 in response to the SH, AA, and SC pretreatments, respectively, but it was eliminated in the SA-pretreated sample.

Arabinan content of the SH-pretreated sample was increased from 4.7% in the untreated sample to 6.5%, unlike the trend observed for xylan content. Nevertheless, considering their standard deviations, both changes in xylan and arabinan contents of the SH-pretreated sample in comparison with the untreated sample were not significant. Considering the recovery of the solids, rice straw lost 54–70% of its ash content through the pretreatments. The SC and SH pretreatments resulted in the highest and lowest amounts of ash removal, respectively.

The used pretreatments could be divided into two main groups of acidic and basic pretreatments. Therefore, it could be concluded that the acidic group in general resulted in the removal of dominant hemicellulosic carbohydrates such as xylan and arabinan, whereas the basic pretreatments mainly resulted in lignin removal.

6.2.2. Methane production

As shown in Fig. 2a, the AD of the pretreated rice straw resulted in the production of different amounts of methane depending on the pretreatment method applied. Increased methane yields were observed using SH-, AA-, and SC-pretreated samples, while SA-pretreated sample led to decreased methane yield, in comparison with the UT straw. The possibility of remaining the acidic agent in the acid-pretreated sample was rejected because the pH was analyzed for the SA-pretreated sample after the pretreatment. On the other hand, the pH of the digesters was also measured at the end of the AD process (Table 5). The obtained results demonstrated that all digesters had pH values above 8. Hence, the decrease in methane yield could not be attributed to the retention of acidic agents (H^+) in the pretreated rice straw.

Discarding the pretreatment liquor, which contained considerable amounts of sugars and VFAs released from hemicellulose degradation, could be one of the probable reasons to the very low methane yield obtained from the SA-pretreated straw in the present study. Kim et al. [138] similarly reported a decrease in methane production after SA pretreatment (with 0.01–2% H_2SO_4, at 121 °C for 1 h) even without discarding the pretreatment liquor. They stated that the inhibitory effect of sulfate ion, added by sulfuric acid pretreatment, was responsible for the reduced methane yield. The presence of sulfate triggers a competition between methanogens and sulfur reducing bacteria over hydrogen and limits hydrogenotrophic methane production.

The decrease in methane yield by SA pretreatment can also be due to the application of high temperature (160 °C) and, consequently, high pressure (6.1 bar) in the SA pretreatment, unlike the other treatments which were performed at atmospheric pressure and temperatures below 100 °C. Wang et al. [76] similarly reported 30% decrease in methane

Table 4
Solids recovery and composition of untreated and pretreated rice straw samples.

Sample	Recovery (%)	Composition (%)					
		AIL*	ASL**	Glucan	Xylan	Arabinan	Ash
UT[a]	–	14.0 ± 1.2	2.7 ± 0.0	37.7 ± 5.6	10.7 ± 3.5	4.7 ± 1.3	16.8 ± 0.0
SA-pretreated[b]	41.6 ± 1.5	25.1 ± 0.2	0.9 ± 0.1	86.4 ± 6.0	0.0 ± 0.0	0.0 ± 0.0	15.6 ± 1.0
SH-pretreated[c]	54.5 ± 0.9	10.9 ± 1.7	1.1 ± 0.0	52.6 ± 7.9	7.5 ± 1.1	6.5 ± 0.9	14.1 ± 0.9
AA-pretreated[d]	59.6 ± 3.7	7.5 ± 1.2	1.2 ± 0.0	50.4 ± 3.9	17.1 ± 1.5	7.9 ± 0.4	12.2 ± 1.2
SC-pretreated[e]	55.6 ± 2.2	9.8 ± 2.9	1.1 ± 0.1	43.8 ± 3.3	16.7 ± 0.6	7.8 ± 1.2	9.0 ± 0.5

[a] UT: Untreated.
[b] SA: Sulfuric acid.
[c] SH: Sodium hydroxide.
[d] AA: Aqueous ammonia. SC: Sodium carbonate.
[e] SC: Sodium carbonate.
* AIL: Acid insoluble lignin.
** ASL: Acid soluble lignin.

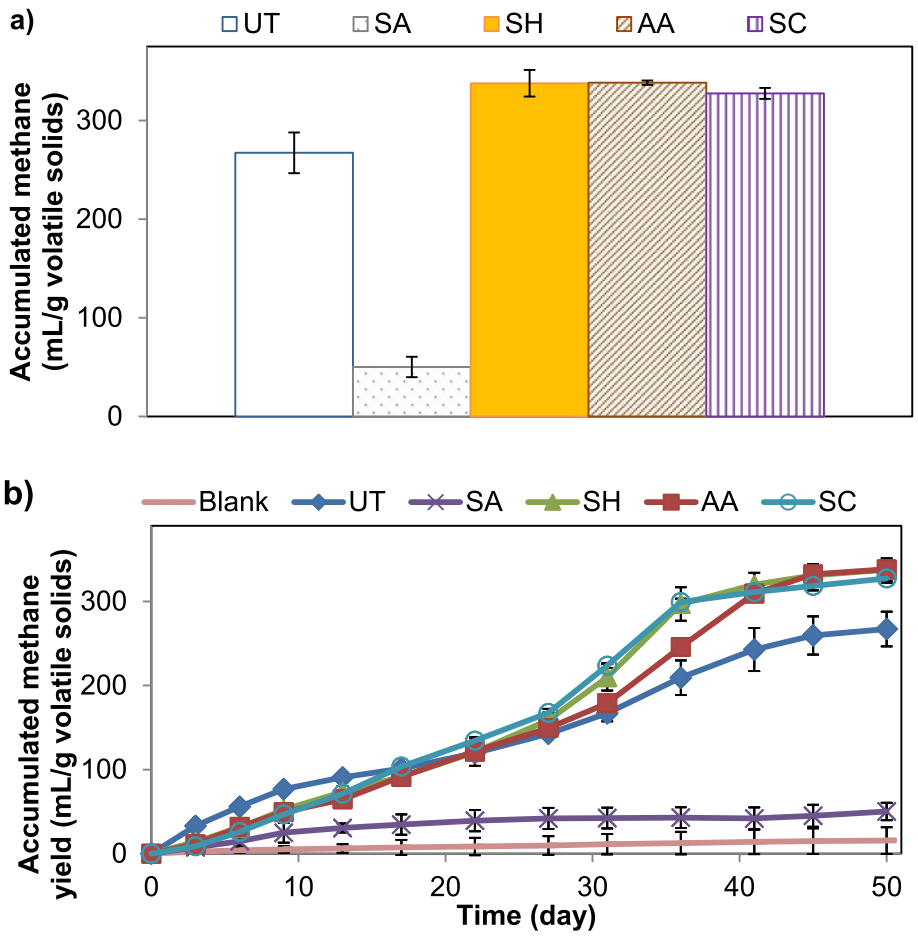

Fig. 2. Total accumulated methane yield (a) and its time profile (b) for different samples used.

Table 5
Final pH and VFA/alkalinity of the digesters fed with rice straw pretreated by different pretreatment methods.

Substrate	Final pH	Final VFA/alkalinity
UT-pretreated rice straw	8.53	0.11
SA-pretreated rice straw	8.45	0.21
SH-pretreated rice straw	8.64	0.05
AA-pretreated rice straw	8.58	0.35
SC-pretreated rice straw	8.47	0.22

yield from rice straw due to applying hydrothermal pretreatment at high temperature (210 °C for 15 min). Because of the presence of H_3O^+ formed through the deionization of water molecules in the solution, the hydrothermal pretreatment in fact must have acted similar to a dilute acid pretreatment and led to hemicellulose removal. Hemicellulose was easily hydrolyzed into xylan, and xylan was subsequently degraded into inhibitors such as formic acid and furfural jeopardizing methane yield [76]. The findings reported by Wang et al. [76] were later justified by those of Syaichurrozi et al. [139]. They employed SA pretreatment (with 4% H_2SO_4, at 30 °C for 2 d) on *Salvinia molesta* and could reportedly increase methane production by 81.7% [139]. In their experiment, the pretreated solids were washed and the liquor was discarded. The observed increase in methane yield could be ascribed to the fact that the authors used low pretreatment temperatures which did not result in the introduction of sulfate into the structure and therefore, enabled its easy wash-out.

The time profiles of the cumulative methane yield from rice straw pretreated with different methods are presented in Fig. 2b. Biogas yields were significantly affected by different pretreatments performed on the feedstock. The highest cumulative biogas yields for the 60-d digestion was obtained using the SH, AA, and SC pretreatments with minor differences. More rapid initial biogas production from the UT straw in comparison with the pretreated samples was due to readily biodegradable organic matters found in the UT substrate, which were removed through the pretreatments. Digesters require a VFA/alkalinity ratio of 0.4–0.6 for stable operation [135]. Final VFA/alkalinity ratios of all digesters were below 0.4, showing the lack of biomass for microorganisms (Table 6). The lack of biomass in the batch digesters after 60 d was expectable.

The low methane yields obtained in the present work for all the pretreatments could be explained by the fact the pretreatment liquor (containing sugars and VFAs with considerable biomethane potential) were discarded. This was done to prevent the adverse effects of the inhibitors and chemicals present in the liquor on the microbial consortium. It should also be noted that the differences between the methane yields recorded in the present study and the values reported in the literature could also be attributed to differences in the rice straw and the inoculum used. For instance, Du et al., also discarded the pretreatment liquor following the alkaline pretreatment of rice straw at optimum conditions (with 5% g CaOH/g straw, at 80 °C for 6 h) but obtained a higher methane yield (411.1 mL/g volatile solids) [140] than the present study.

The results obtained for the straw components, including glucan, xylan, arabinan, acid-soluble lignin (ASL), acid insoluble lignin (AIL), and ash, were subjected to PCA to determine the relationship between straw composition and methane yield. The results of PCA, including loading map and score map, with glucan, xylan, arabinan, ASL, AIL, and ash contents as well as methane yield as variables are shown in Fig. 3a

Table 6
Eigenvectors, eigenvalues, and proportions from a principal component analysis of straw compositions and methane yields.

	Glucan	Xylan	Arabinan	ASL	AIL	Ash	Methane yield	Eigenvalue	Proportion
Prin 1	−0.36	0.43	0.45	0.08	−0.45	−0.29	0.44	4.79	0.68
Prin 2	−0.44	−0.05	−0.10	0.72	0.03	0.52	0.03	1.82	0.26

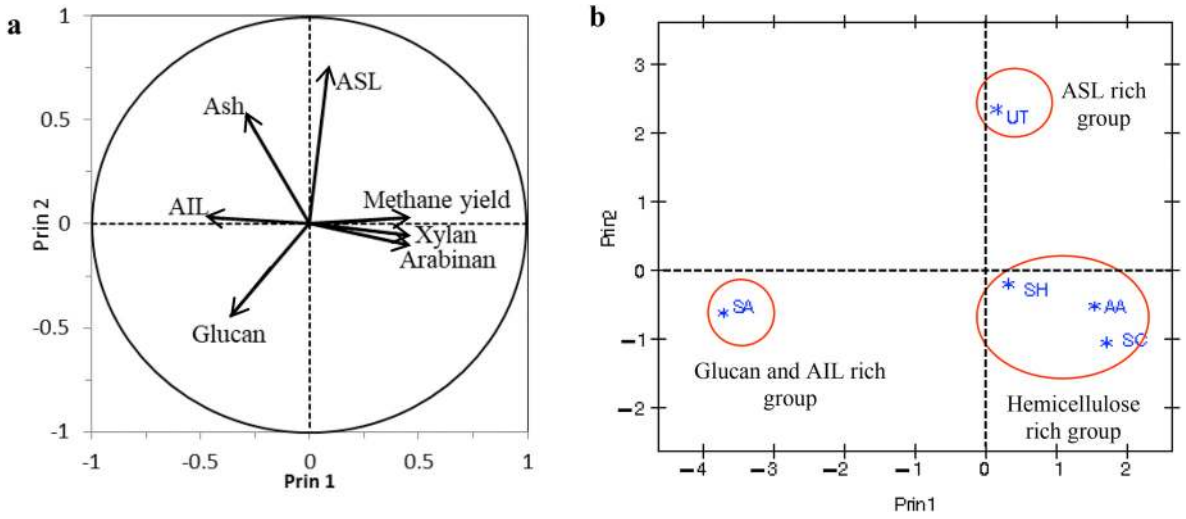

Fig. 3. The plot of (a) loading map and (b) the score map obtained from a principal component analysis of methane production from rice straw pretreated with different pretreatment methods.

and b. It was found that only the first two components were meaningful (eigenvalue > 1) and hence, only these components are presented. Combined, components 1 and 2 accounted for 94% of the cumulative contribution ratio in the PCA. The variables, corresponding factor loadings (eigenvectors), eigenvalues, and proportions are presented in Table 6. In interpreting the rotated factor pattern, a variable was said to load on a given component if the factor loading was 0.40 or greater for that component and was less than 0.40 for the other. Using these criteria and the loading map, four variables were found to load on the first principal component (Prin 1). It was revealed that xylan and arabinan contents and methane yield were highly correlated and had a considerable positive influence on the principal component 1. ASL content had a minor influence on component 1. Glucan had a slight influence on both components. Glucan, AIL, and ash contents were located on the opposite side of methane yield. AIL had a considerable negative effect on component 1. Overall, the main predictor component for methane yield was the component 1, which was formed from a combination of the concentrations of xylan and arabinan (i.e., hemicellulose) with a positive portion and AIL with a negative potion. The component 1 seems to measure the preponderance of hemicellulose (xylan and arabinan) over glucan. Two variables, i.e., ASL and ash, have positive influence on component 2, which seems to measure non-digestible parts of lignocellulose and has a negligible effect on methane yield. The interesting point revealed is that methane yield is highly correlated with hemicellulose content, even more than cellulose content. Therefore, applying the pretreatments which are aimed at hemicellulose removal would not be appropriate for biogas production.

The SA treatment is at the extreme left of the plot, with a low ratio of hemicellulose to lignin contents. Untreated sample tends to be in the upper part of the plot, with a greater than average content of non-digestible materials. The SH-, AA-, and SC-pretreated samples are on the extreme right with a high ratio of hemicellulose to lignin contents. Overall, it is possible to identify three clear groups on the score map, UT, basic treatments, and acidic treatment, according to the components responsible for methane yield. This PCA analysis provides the ability to choose the most suitable pretreatment in terms of methane production.

6.2.3. Phylogenetic analysis

A systematic analysis of the changes in microbial communities in response to the application of different pretreatments on the lignocellulosic substrate was performed. As mentioned earlier, different pretreated rice straw samples, including SA-, SH-, AA-, and SC-pretreated samples, in addition to the UT straw and the inoculum were anaerobically digested for 60 d. Sixty days seems likely enough for the adaptation of the initial microbial community with the pretreated substrates. The amplicon sequencing of the five biogas-producing microbial communities (developed on rice straw pretreated by SH, AA, and SC, with highest methane yields; on SA-pretreated sample, with lowest methane yield; on UT sample, as a reference; and on the starting inoculum, as initial reference) revealed considerable variations in the composition and abundance of the archaeal and bacterial communities.

- Archaeal communities

The main archaea genera presented at a relative abundance of more than 1% in at least one of the digesters, their taxonomy, and metabolism are tabulated in Table 7. Shifts in archaeal communities were observed because of using different pretreatments on the feedstock. Compared with the reactor running on UT rice straw, the methanogenic population in the reactor fed with the SH-pretreated sample (leading to the highest methane yield) shifted from *Methanosaeta* species, i.e., acetoclastic methanogenesis [141], towards *Methanobacterium* genus, i.e., hydrogenotrophic methanogenesis. In other words, these results demonstrated that using the SH treatment resulted in changing the methanogenesis pathway from the acetoclastic pathway to hydrogenotrophic. Furthermore, archaeal communities shifted from *Methanosaeta* to *Candidatus nitrososphaera*, an ammonia-oxidizing genus, in the reactors fed with AA-, SA-, and SC-pretreated samples.

The relative abundance of bacterial groups in the anaerobic reactors utilizing different pretreated rice straw samples was analyzed at the phylum, class, order, family and genus levels, including unclassified sequences (Fig. 4). The main detected bacterial families presented at a relative abundance of more than 1% in at least one of the digesters are

Table 7
The main archaea genera present at a relative abundance>1% in at least one of the digesters as well as their taxonomies.
- Bacterial communities

Phylum	Class	Order	Family	Genus	Metabolism
Crenarchaeota	Thaumarchaeota	Nitrososphaerales	*Nitrososphaeraceae*	*Candidatus nitrososphaera*	Ammonia-oxidizing
Euryarchaeota	Halobacteria	Halobacteriales	*Halobacteriaceae*		
Euryarchaeota	Halobacteria	Halobacteriales	*Halobacteriaceae*	*Haloterrigena*	
Euryarchaeota	Methanobacteria	Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium*	Methanogenesis using H_2 and CO_2 as a substrate, strictly anaerobic, acid tolerant, Gram-negative
Euryarchaeota	Methanomicrobia	Methanosarcinales	*Methanosaetaceae*	*Methanosaeta*	Methanogenesis from acetate only, Gram-negative, ability to grow on hydrophobic surfaces
Euryarchaeota	Methanomicrobia	Methanosarcinales	*Methanosarcinaceae*	*Methanosarcina*	Produce methane using all three known metabolic pathways

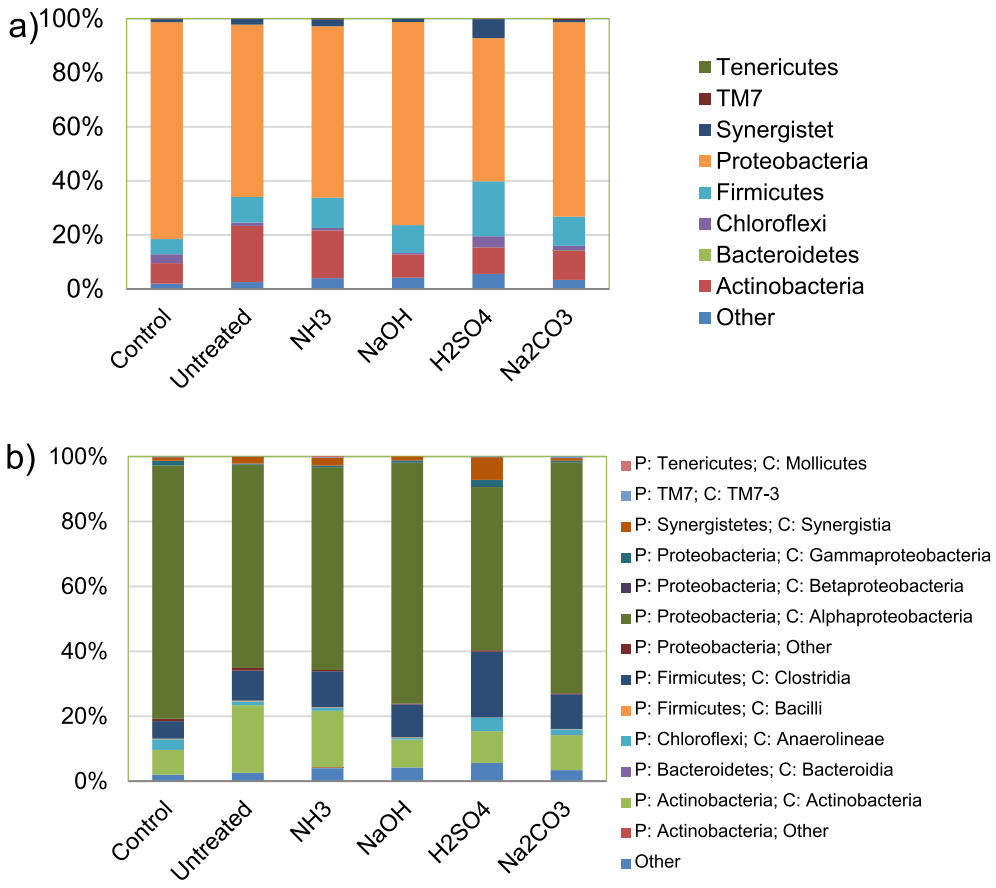

Fig. 4. Bacterial community distribution in different digesters fed with untreated, NH_3-pretreated, NaOH-pretreated, H_2SO_4-pretreated, Na_2CO_3-pretreated rice straw at (a) phylum, (b) class, (c) order, and (d) family levels.

tabulated in Table 8. Only 2–5.7% of the bacterial sequences in different reactors were designated as unclassified bacteria at the phyla level. However, the unclassified bacteria were considerably high (71–80.2%) at the genus level.

All the reactors were dominated by sequences from the *Hyphomicrobiaceae* family (up to 26.3% of total bacterial sequences), affiliated with the *Rhizobiales* order from *Alphaproteobacteria* class and *Proteobacteria* phylum. *Proteobacteria* and *Firmicutes* are typical phyla in the AD process, which can hydrolyze carbohydrates and proteins and have an important role in the degradation of VFAs. *Proteobacteria* are mostly in the liquid fraction of digestion media, whereas *Firmicutes* are firmly attached to the lignocelluloses surface. A few representatives of the *Hyphomicrobiaceae* family can grow anaerobically by mixed-acid fermentation [142]. The relative abundance values of this family in reactors fed with SH-, AA-, and SC-pretreated samples were the highest (23.5, 26.3, and 24.8%, respectively) and very close to each other.

Similarly, these reactors demonstrated the highest methane yields with close values. The community fed with the UT sample contained few species of *Hyphomicrobiaceae* family (19%) compared with the reactors fed with SH-, AA-, and SC-pretreated samples, even *vs.* the reactor fed with the initial inoculum (with a 22.4% abundance). Noticeably, the abundance of this family in the reactor utilizing SA-pretreated sample (7.7%), with the least methane yield, was massively less than those in the other reactors.

Clostridium genus, which is an efficient genus and appears in all phases of the fermentation, had a relative abundance of 1.9–2.7% in all reactors except in the reactor fed by the SA-pretreated sample. The reactor with minimum methane yield, fed with SA-pretreated sample, had the minimum amount (0.5%) of *Clostridium* genus. *Clostridium* genera are generally responsible for cellulose degradation, which are

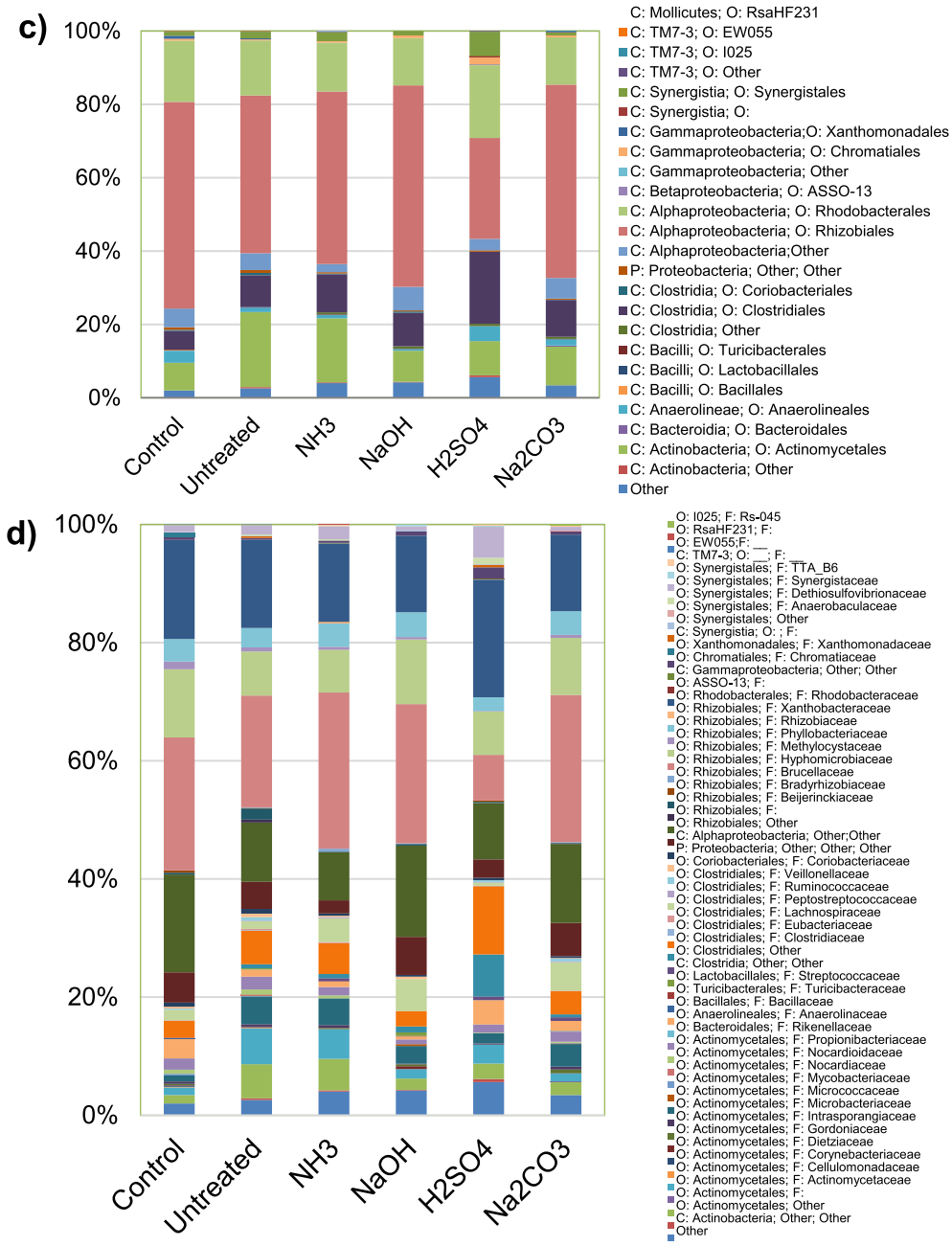

Fig. 4. (*continued*).

firmly attached to the insoluble substrate by the mediation of cellulosomes (multi-enzyme complexes), and degrade cellulose into soluble sugars [143]. *Chromatiaceae*, the main family of purple sulfur bacteria, demonstrated the highest relative abundance (1.9%) in the reactor fed with SA-pretreated sample among the other reactors (with 0.3–0.7%). Purple sulfur bacteria use hydrogen sulfide, which was subsequently oxidized to produce granules of elemental sulfur. In other words, these bacteria produce sulfur globules and store them inside their cells.

Cellulolytic bacteria such as members of the families *Microbacteriaceae* and *Clostridiaceae* increased in all the digesters, as compared to the initial inoculum. This showed the progress of the inoculum towards adopting itself to the cellulosic substrate. These families can colonize complex plant materials and degrade recalcitrant polymers, such as cellulose and hemicellulose. Hence, these families play a common role as active plant degraders [144]. Interestingly, the bacterial communities recorded in different digesters were related to the pretreatment employed and their respective methane yields. The bacterial population in the reactor with the least methane yield (fed with SA-pretreated sample) was dominated by the family *Rhodobacteraceae* (19.8%) and *Clostridiaceae* (11.5%). While, in the other digesters, *Hyphomicrobiaceae* was the prevalent family.

The minimum or maximum relative abundances of some families were observed in the reactor fed with SA-pretreated sample, among other reactors. The relative abundances of families like *Anaerolinaceae, Clostridiaceae, Rhodobacteraceae, Chromatiaceae, Dethiosulfovibrionaceae, Synergistaceae* were maximal in the microbial community fed with the SA-pretreated sample, among the other microbial communities. *Chromatiaceae* and *Rhodobacteraceae* are two families of purple sulfur bacteria, which use hydrogen sulfide and oxidize it to elemental sulfur. Increasing the population of these bacteria was indicative of increased concentrations of hydrogen sulfide. This could be attributed to the sulfate remaining in the rice straw structure after pretreatment or more

Table 8
The main bacterial genera present at a relative abundance >1% in at least one of the digesters.

Family	Genus	Description
Microbacteriaceae	–	Gram-positive
Microbacteriaceae	*Leucobacter*	Aerobic heterotroph
Propionibacteriaceae	Other	Gram-positive, anaerobic to aerotolerantrodsor filaments; ferment carbohydrates, with propionic acid as the principal product.
Anaerolinaceae	*T78*	–
Clostridiaceae	Other	–
Clostridiaceae	*Clostridium*	The genus is well known to accomplish the first steps of anaerobic digestion, appear in all phases of the fermentation process but are dominant in the acidogenic phase
Peptostreptococcaceae		Gram-positive, anaerobe
Other	Other	Metabolizing C1-compounds
Other	Other	Gram-negative, rhizobia fix nitrogen
Beijerinckiaceae	–	Free-living nitrogen-fixing bacteria, the *alphaproteobacterial* family *Beijerinckiaceae* has generalist species that thrive on a wide variety of feedstocks, and specialist species that thrive only on methanol and methane
Hyphomicrobiaceae	–	Many species are *Oligocarbophilic*, thriving in the presence of low concentrations of suitable carbon source. The oligocarbophilic bacteria can satisfy their requirement with traces of organic substances from the air.
Methylocystaceae	–	They are only capable of obtaining carbon and energy from methane and methanol, type II Methanotroph
Methylocystaceae	*Pleomorphomonas*	Nitrogen-fixing bacterial strain
Phyllobacteriaceae		Rod-shaped, ovoid, or reniform cells, Gram-negative, Aerobic, Grow well on complex solid media
Rhizobiaceae	Other	Gram-negative, aerobic, a rod shape, many species of them can fix nitrogen
Rhodobacteraceae	Other	They are deeply involved in sulfur and carbon biogeochemical cycling
Rhodobacteraceae	*Paracoccus*	They can gain energy from both inorganic, such as sulfur and hydrogen, and organic compounds, such as methanol and methylamine. A feature of this bacterium is its ability to convert nitrate to dinitrogen in a process called denitrification single-handedly. gram-negative, in both aerobic or anaerobic environments
Rhodobacteraceae	*Rhodobacter*	A kind of purple bacteria
Chromatiaceae		The main family of purple sulfur bacteria, which consume hydrogen sulfide, and elemental sulfur with granular form is produced. This sulfur can also be oxidized and produce sulfuric acid. Sulfur globules can be produced and be stored inside their cells.
Synergistaceae	*VadinCA02*	–

specifically after discarding the liquor and washing the biomass. On the other hand, among microbial communities fed with different pretreated samples, the minimum relative abundances of *Peptostreptococcaceae*, *Hyphomicrobiaceae*, *Microbacteriaceae*, *Phyllobacteriaceae*, *Rhizobiaceae* families were also observed in the reactor fed with the SA-pretreated sample. The relative abundance of *Microbacteriaceae* in the reactor fed with the SA-pretreated was less than half of its relative abundance in the other reactors.

The high abundance of purple sulfur bacteria in the digester fed with the SA-pretreated straw revealed the retention of the sulfate ion in the substrate structure even when exposed to the washing process. This finding suggests that discarding the liquor and washing the pretreated biomass failed to totally remove all the inhibitors and chemicals. Consequently, it is recommended that future research works focused on underlying mechanisms corresponding to microbial community, also consider the impact of cooking or hydrothermal pretreatments. These pretreatments are promising techniques that do not use chemicals, thus eliminating the risk of chemical adsorption into the structure of lignocelluloses. Moreover, these methods offer other advantageous compared with thermo-chemical pretreatments, such as being environmentally friendly, the possibility of heat recovery, and lower costs. Hydrothermal pretreatment is typically performed at the range of 90–260 °C. The efficiency of hydrothermal pretreatments depends on a number of parameters including substrate type, pressure, temperature, pretreatment time, and solid loading [145].

For the pretreated samples with high biogas production rates such as SH- and AA-pretreated samples, the abundance of *Methanobacterium* genus was observed to be much higher. The growth and metabolism of *Methanobacterium* were non-linearly proportional with xylan and arabinan concentrations. This was very significant for the SA pretreatment, where the Xylan + Arabinan was 0.0% resulting in the lowest *Methanobacterium* population of 0.4%. On the other hand, by comparing SH- and SC-pretreated samples with AA-pretreated sample separately, it was found that both AA- and SC- pretreated samples with similar Xylan + Arabinan percentages led to similar *Methanobacterium* abundance too. However, both of them had lower *Methanobacterium* abundance compared with the SH-pretreated sample containing a moderate composition of Xylan + Arabinan (Table 9). On the contrary, it was revealed that with the high abundance of the other methanogenic archaea, i.e., *Methanosaeta* genus, the production of methane did not increase.

Also, according to the hypothesis presented by Yamamoto et al., ammonia-oxidizing archaea species like *Candidatus nitrosphaera* can play a methanogenic role in the archaeal communities [146]. However, based on the findings of the present study, it seems that the sole presence of this genus could not guarantee a high volume of methane production such as that observed in the SA-pretreated sample, but when accompanied with *Methanobacterium* in high abundance, this genus can also exert positive effects leading to synergism and increased methane production.

Surprisingly, when analyzing the influence of bacterial groups on gas production, *Hyphomicrobiaceae*, a methylotroph bacterium [147], can play the primary role in methane production by converting the methyl group of methylated compounds such as methanol and methyl-amine into methane *via* the methylotrophic pathway [148]. The correlation between the methane production value and the abundance of *Hyphomicrobiaceae* was significant in all the investigated samples.

To date, the universally accepted hypothesis is that variations in biogas yield following the application of different pretreatment methods on a given substrate lie in changes in substrate composition. For instance, it could be articulated that using SA pretreatment in this study resulted in the removal of easily digestible carbohydrates (xylan or hemicellulose); hence, it resulted in a decrease in biogas yield [148]. Surprisingly, the phylogenetic analysis in the present study showed that alteration of the microbial community was equally important in the yields of biogas obtained from the different pretreated samples. Thus, it could be concluded that changes in methane yield should not be attributed to changes in substrate composition caused by different pretreatments only and that the impacts of various pretreatment methods could go beyond. Overall, to further enhance biogas yields from lignocelluloses, the key is to obtain more in-depth understanding about the signature microorganisms and their functions and to engineer more favorable interactions between microbial community structure, and process parameters and performance.

7. Conclusions and future prospects

Biogas production can improve the sustainability attributes of

Table 9
Main groups of archaeal and bacterial methanogens and their abundance in digesters fed with differently pretreated rice straw samples.

Sample	Accumulated methane (mL/g VS*)	Saccharide composition % (Xylan + Arabinan)	Main groups of methanogenic archaea and their abundances			The main group of methanogenic bacteria and its abundance
			Methanobacterium	Candidatus nitrososphaera	Methanosaeta	Hyphomicrobiaceae
Inoculum	–	–	0.8	0.2	98.7	22.3
UT	267.22	15.4 ± 4.8	39.6	11.2	44.8	19.0
AA-pretreated	338.31	25 ± 1.9	27.9	51.0	3.4	26.2
SH-pretreated	337.76	14 ± 2	73.0	2.7	23.4	23.5
SA-pretreated	50.16	0.0 ± 0.0	0.4	59.9	36.3	7.7
SC-pretreated	327.46	24.5 ± 1.8	15.0	71.8	9.3	24.8

* Volatile solids

lignocellulosic waste management. However, the impacts of arguably the most essential stage of the whole process, i.e., pretreatment, on the AD of lignocelluloses is controversial. The changes in substrate characteristics, including chemical composition, cellulose crystallinity, cellulose degree of polymerization, enzyme adsorption/desorption, nutrient accessibility, deacetylation, and inhibitors formation, by pretreatments, have been considered as the major molecular mechanisms governing biogas production yield from lignocelluloses. These characteristics and their effects on methane yield were reviewed herein. In addition to that, a new hypothesis concerning the impact of pretreatment on the microbial community developed (throughout the AD process from an identical inoculum) was also put forth and was experimentally examined through a case study. It was revealed that in addition to substrate physicochemical characteristics, the choice of pretreatment method also plays a pivotally positive or negative role on biogas yield obtained from lignocelluloses through alteration of the microbial community involved in the AD. Interestingly, both bacterial and archaeal communities were influenced by the choice of pretreatment used.

The obtained results showed that acidic pretreatments (causing hemicellulose removal) are not appropriate for biogas production from solid residues, as hemicellulose content was found to have a higher correlation with methane yield than cellulose content. NaOH pretreatment associated with the highest methane yield almost changed the methanogenic pathway from acetoclastic to hydrogenotrophic pathway. Therefore, the findings of the present study challenge the default hypothesis accepted by thousands of previously published papers, which is changes in substrate characteristics, caused by different pretreatments, are the only mechanisms affecting biogas yield from lignocelluloses. The results obtained herein can explain the common antithetical observations made on the effects of different pretreatment methods of AD of lignocelluloses. Moreover, they can assist with obtaining a more in-depth understanding of the AD process, leading to the development of more efficient biogas production systems at industrial scale.

Finally, it should be noted that the present study employed thermochemical pretreatments to verify the hypothesis laid forth. However, this could also introduce a level of uncertainty and therefore, future investigations should also include non-chemical pretreatments such as cooking or hydrothermal pretreatments to further verify the findings presented here.

CRediT authorship contribution statement

Safoora Mirmohamadsadeghi: Investigation, Writing - original draft. **Keikhosro Karimi:** Conceptualization, Supervision. **Reza Azarbaijani:** Software, Data curation. **Laleh Parsa Yeganeh:** Investigation, Formal analysis. **Irini Angelidaki:** Resources. **Abdul-Sattar Nizami:** Methodology. **Rajeev Bhat:** Data curation, Formal analysis. **Kavya Dashora:** Validation, Visualization. **Virendra Kumar Vijay:** Validation, Visualization. **Mortaza Aghbashlo:** Conceptualization, Supervision, Writing - review & editing. **Vijai Kumar Gupta:** Conceptualization, Supervision, Writing - review & editing. **Meisam Tabatabaei:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

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