Methane Production from the Anaerobic Co-digestion of Lignocellulosic Materials and Paper Plant Sludge Cakes

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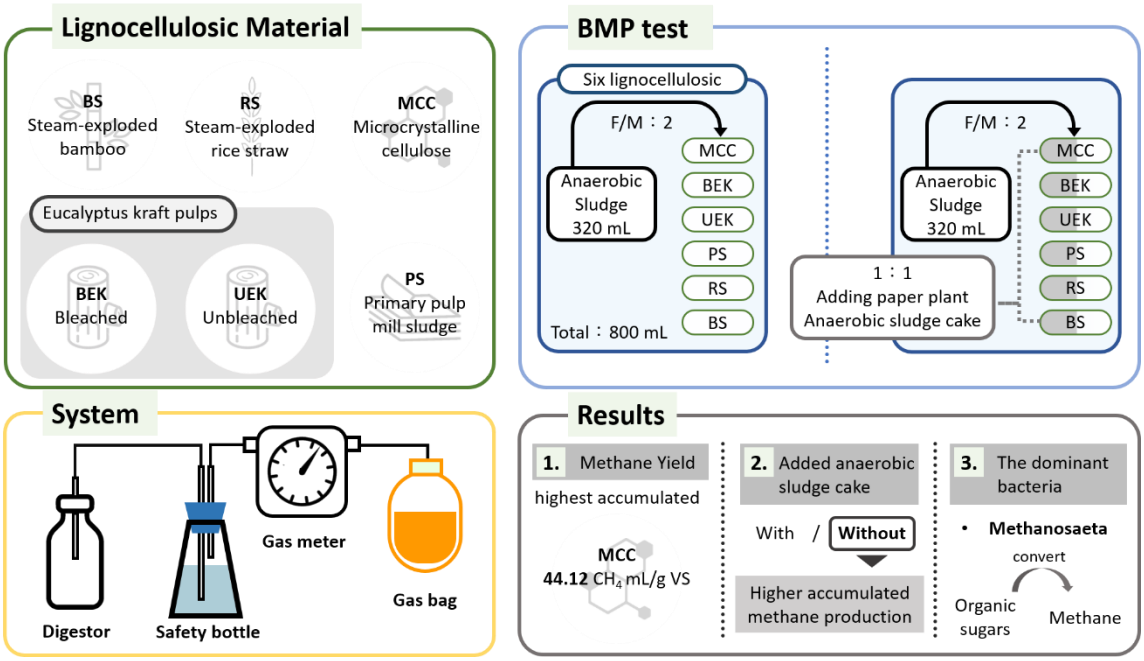
GRAPHICAL ABSTRACT



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Anaerobic co-digestion was evaluated for lignocellulosic materials and paper plant sludge cakes (PSL). The methane production, crystallinity, residual cellulose, and next-generation sequencing (NGS) were analyzed and compared. It was found that microcrystalline cellulose (MCC) had the highest accumulated methane production among the different materials in the anaerobic digestion system. The residual content and crystallinity of cellulose both decreased to a much larger extent, and the accumulated methane production was higher than that of the anaerobic digestion system with the added anaerobic sludge cake. NGS showed that the domain bacteria in the anaerobic digestion system with the added anaerobic sludge cake were *Methanosaeta*, which can convert organic sugars into methane. This substantially reduced the number of bacteria that can degrade cellulose. As the ability to degrade cellulose decreased, the residual cellulose content and crystallinity of cellulose became higher than those of the anaerobic digestion system without adding anaerobic sludge cake.

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Keywords: Methane production; Lignocellulosic materials; Next generation sequencing; Anaerobic co-digestion; Paper plant sludge cakes

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INTRODUCTION

The amount of agricultural waste produced daily from farms, poultry houses, slaughterhouses, rice straws, and wheat straws make biomass the most promising renewable energy resource. Using agricultural waste to produce energy reduces not only these wastes but also greenhouse gases because anaerobic digestion produces methane and carbon dioxide from agricultural waste (Zhang *et al.* 2022). The use of lignocellulosic biomass to generate energy produces zero net carbon emissions because its growth requires the absorption of carbon in the environment. Although the burning of biomass generates carbon dioxide, this system could be regarded as a closed carbon recycling system. This method is preferable to using fossil fuels, which extract carbon stored underground and then generate more atmospheric carbon. Therefore, using lignocellulose biomass to generate power is environmentally preferable (Nahak *et al.* 2022).

In this study, lignocellulose biomass was anaerobically digested to generate biogas. As the main ingredient in the fermentation of anaerobic digestion, the cellulose and hemicellulose of lignocellulose biomass decompose into monosaccharides (Malik *et al.*

2021). Lignocellulose biomass can be decomposed in several ways, such as acid- or enzyme-catalyzed hydrolysis. Acid-catalyzed hydrolysis requires a higher temperature, generates toxic byproducts, and causes corrosive conditions, whereas enzyme-catalyzed hydrolysis uses less energy and has a higher conversion efficiency (Ballesteros 2010). Thus, enzyme-catalyzed hydrolysis is more preferable than acid-catalyzed hydrolysis. The natural structure of lignocellulose biomass makes it difficult for an enzyme to decompose it; therefore, pretreatment of lignocellulose biomass is necessary for efficient decomposition. This decomposes the lignin structure, breaks down crystalline cellulose, and increases the accessibility of the enzyme (Mosier *et al.* 2005).

Because each step in the process can substantially affect all subsequent stages, selecting appropriate pretreatment methods determines the requirements of the process configuration for hydrolysis and fermentation. Physical, thermal, chemical, and thermochemical techniques have been widely used to pretreat lignocellulosic materials, destroying their structures during anaerobic digestion (Khan *et al*. 2022). The pretreatment and cellulase enzymatic hydrolysis of lignocellulosic biomass are among the major technical and economic impediments to the overall success of biorefineries (Reis *et al.* 2023). Pretreatment also affects the cost of the subsequent operating process, and the downstream cost is affected by the toxicity of fermentation and rates of enzyme hydrolysis. Furthermore, the enzyme loading varies depending on the fermentation process. Thus, the effects of pretreated biomass, minimization of cellulase dosage, and enzyme recycling to reduce the cost of lignocellulosic materials are critical parameters for lignocellulose biorefineries (Reis *et al.* 2023).

Steam explosion is an effective method to pretreat various lignocellulosic biomass, such as hardwood and softwood, and agricultural residues, such as corn stover, sugarcane bagasse, and wheat straw. It also significantly increases the digestibility of cellulose, enzymatic hydrolysis yield, and soluble portion of the hemicellulosic component (Yu *et al.* 2022). Lizasoain *et al.* (2016) demonstrated the positive impact of steam explosions on the production of biogas from reed biomass, achieving a biogas yield of up to 700 L/g volatile solids (VS). However, the major drawback of steam explosion is that it produces toxic components from the sugars degraded during pretreatment. The major inhibitors are furan derivatives, mild acid, and the phenol component (Guo *et al.* 2022; Yu *et al.* 2022).

Organic materials are degraded and stabilized (the relatively high resistance of humic acids to further biodegradation) by microbial organisms during anaerobic digestion. In this process, microbial organisms use organic materials to produce microbial biomass and biogas, which is a mixture of carbon dioxide and methane and is a renewable energy source to replace fossil fuels (Ameen *et al.* 2021). Anaerobic digestion can also reduce pollution from the great quantities of agricultural and industrial sources. For energy recovery and waste treatment, the anaerobic co-digestion of sewage sludge, food waste, or sugarcane filter cake is the optimal method for methane production (Karki *et al.* 2021; Wongarmat *et al.* 2022). Steam explosion methods can enhance the hydrocarbons in methane during anaerobic digestion while biological methods can increase process stability by completely degrading biomass to methane. The environmental and economic impacts of methane production and its pretreatment methods are important, necessitating anaerobic biomethane production systems (Stanley *et al.* 2022).

Additionally, the paper mill wastewater system seems likely to be a reliable source of bacteria and possibly fungi to inoculate the mixture, thereby having a suitably diverse and acclimated population of biota. Therefore, the aims of this study were to verify the feasibility of co-digestion (for methane production) of different biomass substrates with

paper plant sludge cakes and to investigate the anaerobic conversion efficiency and microbial functional analysis during the co-digestion process. The methane production, crystallinity, residual cellulose, and next generation sequencing (NGS) was analyzed and compared.

EXPERIMENTAL

Materials

Anaerobic sludge was collected from a pig farm in Taoyuan, Taiwan. The pH, total solids, and volatile solids were 7.16, 7.18%, and 3.57%, respectively. The accumulated methane production was about 100 mL.

Microcrystalline cellulose (MCC, FLUKA Avicel® PH-101, powder form, approximately 50 μm particle size) was purchased from Sigma Aldrich (St. Louis, MO, USA). Bleached eucalyptus kraft pulps (BEK) and unbleached eucalyptus kraft pulps (UEK) were obtained from the Chung-Hwa Pulp Corporation (Hualien, Taiwan) laboratory. In the kraft pulping process, a mixed alkaline solution of sodium hydroxide and sodium sulfide is used to decomposed the chemical structure of lignin and dissolved in digesting liquid.

After processing the wastewater from the pulp factory, a large amount of suspended matter was used to generate the primary pulp mill sludge (PS). The cellulose residue found in this sludge makes this method a more beneficial and eco-friendly approach for handling pulp sludge. The pulp sludge for this experiment was also provided by the Chung-Hwa Pulp Corporation laboratory.

The steam explosion pretreatment of rice straw (steam-exploded rice straw, RS) and bamboo (steam-exploded bamboo, BS) were at 180 to 190 °C with a 10 min soak time. All samples were oven-dried, crushed using a grinder, and screened through a 40- to 60-mesh before the chemical composition analysis. The chemical components of biomass were determined according to the method described by Van Soest *et al.* (1991).

Research Method

Six different lignocellulosic materials (BEK, UEK, MCC, PS, RS, and BS) were used as substrates for anaerobic digestion. The volume of anaerobic sludge for all six substrates was 320 mL. A fixed substrate-to-anaerobic sludge F/M ratio of 2 was prepared by adjusting the concentrations of the six different materials using VS. The total volume of the six experiments was 800 mL. A serum bottle containing 320 mL of anaerobic sludge was used as a blank. The differences among the six lignocellulosic materials after anaerobic digestion were observed.

Anaerobic sludge cake from the paper plant was used to replace half of the six different lignocellulosic materials (VS basis) in the first experiment. The volume of the anaerobic pig farm sludge for the six substrates with the added anaerobic paper plant sludge cake was 320 mL. A fixed F/M ratio of 2 was maintained using VS to adjust the concentrations of the six materials and the anaerobic sludge cake. The total volume for each of the six experiments was 800 mL. In addition, one experiment of only anaerobic paper plant sludge cake with 320 mL of anaerobic sludge from a pig farm was made to evaluate how well the anaerobic sludge cake from the paper plant functioned as a substrate for anaerobic digestion. A serum bottle containing 320 mL of anaerobic sludge was used as a blank. Experiments were conducted to evaluate the effects of adding anaerobic paper

plant sludge cake to an anaerobic digestion system to generate biogas. The addition of an anaerobic paper plant sludge cake was also evaluated.

Analysis

The biogas production yield was measured using a wet gas meter (W-NK-0.5, Shinagawa Co., Tokyo, Japan). The pH before and after fermentation was measured using a pH meter (pH Microcomputer pH-vision 6071; Jenco, Taipei, Taiwan). Total solids (TS) and VS were measured before and after fermentation using standard methods (APHA 1992). The methane content was measured using a sampling probe to aspirate the gas from the gas bag and analyse the ratio of methane to carbon dioxide in the gas bag. The analysis was performed using a gas chromatograph (8700T, China Chromatography Co., Ltd., Taiwan), and a thermal conductivity detector was used with a Porapaq Q (Supelco, Inc., MO, USA, 6 ft × 1/8 in) analysis column to analyse the gas components.

The reducing sugar content before and after fermentation was measured using the DNSA method (following König *et al.* 2002). Enzyme activity was measured using a filter paper activity (filter-paper-ase, FPase) assay to test the cellulase activity in the anaerobic systems. While there is no enzyme that is identified a "filter-paper-ase," there are various natural enzymes that happen to be good at biodegrading filter paper, which is not a natural material. The crystallinity was measured using X-ray diffraction (XRD MiniFlex 600, Rigaku, Japan) provided by the Department of Agricultural Chemistry in National Taiwan University. To identify bacteria in the anaerobic digestion system, the 16S rDNA of the PCR amplicon was used because highly reserved and variable sequences in the regions of 16S and 18S rDNA can serve as tags for bacteria identification. Appropriate primers were chosen and NGS was used to obtain information on the sequences. The sequences were then separated using operational taxonomic unit similarity analysis.

RESULTS AND DISCUSSION

Chemical Composition of Lignocellulosic Materials

Table 1 lists the compositions of different lignocellulosic materials. The moisture content of the substrates with steam explosion pretreatment are higher than those of un-pretreated ones, and the TS content was lower than that of the un-pretreated substrates. The VS content of the primary sludge was only 53.8%, which was much lower than that of the other substrates (>95%).

Table 1. Chemical Compositions of Six Lignocellulosic Materials

Lignocellulosic materials	Moisture (%)	Ash (%)	Extractive (%)	Lignin (%)	Holocellulose (%)	α cellulose (%)
PS	9.23	41.94	1.84	1.86	44.47	21.14
BS	65.65	0.26	15.51	5.11	14.13	4.35
RS	75.43	2.58	9.36	4.44	8.56	2.33
MCC	1.18	1.51	0	0.09	82.10	85.30
BEK	11.72	0.70	2.28	0.16	83.45	74.56
UEK	7.28	3.02	2.06	3.20	82.21	71.64

*PS: Primary pulp mill sludge, BS: steam-exploded bamboo, RS: Steam-exploded rice straw, MCC: Microcrystalline cellulose, BEK: Bleached eucalyptus kraft pulps , UEK: Unbleached eucalyptus kraft pulps

The cellulose contents of the steam explosion substrates were lower than those of the other substrates because cellulose was broken down into small molecules such as reducing sugars during the steam explosion process. The cellulose content of the primary sludge was only 44.5% based on dry weight, whereas the cellulose contents of the other substrates were all over 75%. This indicated that a large amount of cellulose was composed of glucose in the substrates that could be utilised by anaerobic bacteria. The bonds in cellulose have extensive intermolecular and intramolecular hydrogen bonding, which make it difficult to break down the material. The alcohol benzene extract content of the steam explosion substrates was higher than that of the other substrates because the extracted ingredient was dissolved from the substrates using acetic acid.

Lignocellulosic Materials in Anaerobic Digestion

Figure 1 shows the accumulated methane content of six different lignocellulosic materials. MCC achieved the highest accumulated methane production, followed by BEK, primary sludge, UEK, steam exploded rice straw, and steam exploded bamboo. Table 2 shows the sequence of the methane yields (CH_4 mL/g cellulose). Steam explosion of rice straw produced the highest methane yield of 62.6 mL/g cellulose. It had the highest methane yield because during the steam explosion process, cellulose was broken down, thereby lowering the cellulose content.

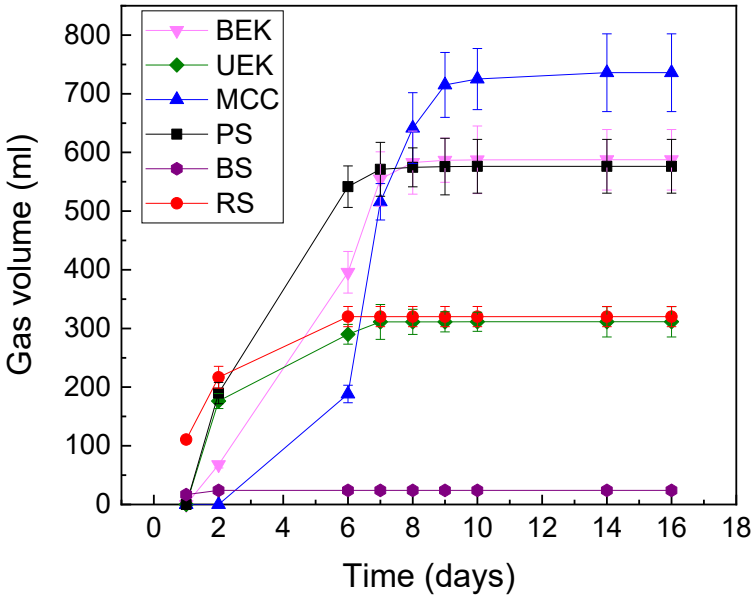

Fig. 1. Accumulated methane yield of six biomass substrates

Table 2. Methane Yield of Six Lignocellulosic Materials

Lignocellulosic materials	F / M	Cellulose /Microorganism VS	pH before	pH after	CH_4 mL/g cellulose	CH_4 mL/g VS
PS	2.00	1.82	7.37	7.28	37.94	34.55
BS	2.00	0.83	7.37	7.08	3.52	1.86
RS	2.00	0.78	7.39	7.24	62.55	24.34
MCC	2.00	1.89	7.24	6.87	46.61	44.12
BEK	2.00	1.90	7.19	6.99	36.97	35.23
UEK	2.00	1.83	7.21	5.25	20.37	18.67

When the accumulated methane content was divided by the gram of cellulose, the methane yield increased. The methane yields from the primary sludge and BEK were similar, but the cellulose content of the primary sludge was significantly less than BEK, and the methane yield was higher than that of BEK.

Figure 2 shows that with the high reducing sugar content of the substrates with steam explosion pretreatment, increased methane was produced rapidly at the beginning of the experiment, with the highest methane yield. The other substrates, which did not undergo steam explosion pretreatment, did not initially produce this large amount of methane. Over time, as the reducing sugar was gradually generated by the bacteria, methane production increased; therefore, more time was needed to reach the highest methane production.

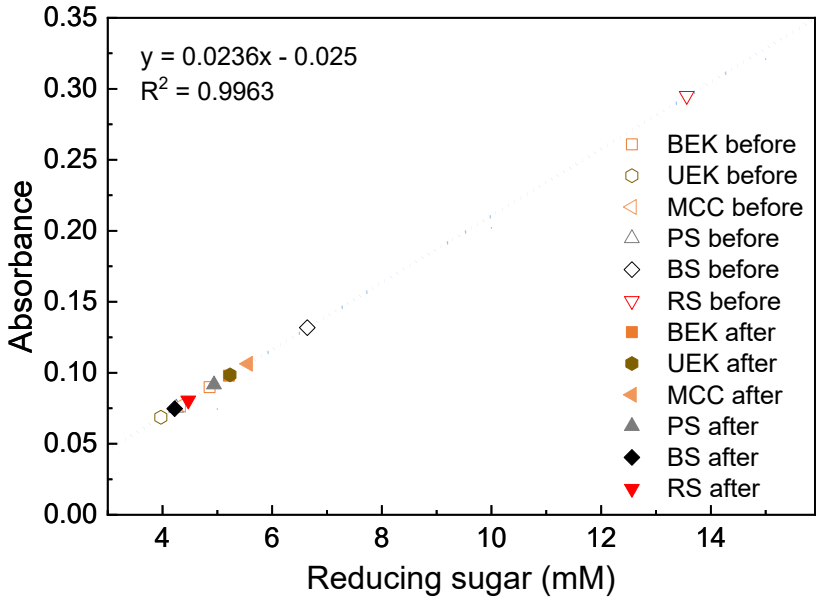

Fig. 2. Reducing sugar content of six different substrates

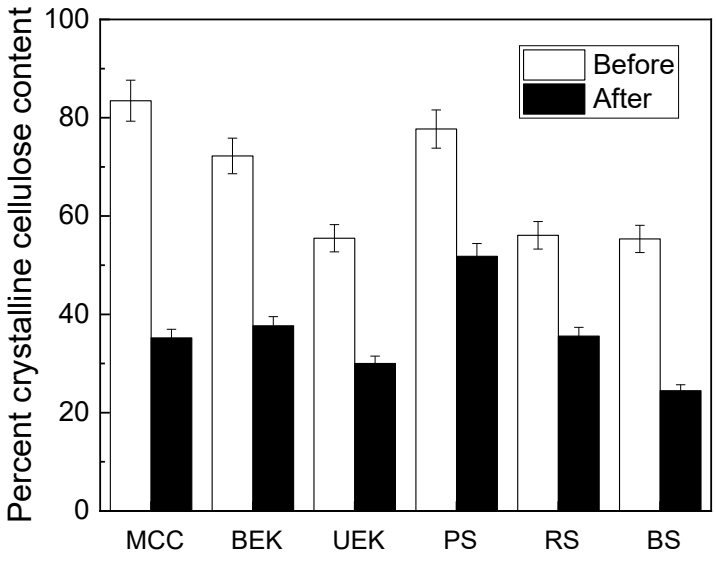

Fig. 3. Crystallinity of six substrates before and after fermentation

Figure 3 shows that crystallinity substantially decreased after fermentation, indicating that the bacteria truly degraded the structure of cellulose, producing smaller molecules and reducing sugars for use by anaerobic bacteria. The reduction of crystallinities of the substrates were ranked as follows: MCC, BEK, PS, UEK, RS, and BS. Comparing the decreasing amount with that shown in Fig. 1, the decreasing amount of crystallinity was proportional to accumulated methane production. The decrease in crystallinity was proportional to the decrease in residual cellulose content, which represents the cellulose content, excluding the primary sludge. This is because anaerobic bacteria degrade cellulose and reduce the residual cellulose content. The degradation of cellulose reduces its crystallinity. Consequently, the increased production of reducing sugars and smaller molecules by bacteria degrading cellulose led to higher methane production.

Figure 4 shows the phylum level distribution of the seven anaerobic systems with six lignocellulosic materials and a blank. These results indicated that Proteobacteria were the dominant bacteria in the system. Proteobacteria are all Gram-negative, and the bacterial membrane is composed of lipopolysaccharides. Much of the Proteobacteria use flagella to move, while the others glide. Many bacteria in this phylum of Proteobacteria are obligate or facultative anaerobes, and it is reasonable to find these bacteria in an anaerobic system. The second most common bacteria were *Firmicutes*, which are mainly Gram-positive bacteria. *Firmicutes* are typically divided into clostridia, which are anaerobic, and bacilli, which are obligate or facultatively aerobic. Therefore, *Firmicutes* can reasonably occur in anaerobic systems. More *Firmicutes* were found in the anaerobic systems without added lignocellulosic materials than in the systems with added lignocellulosic materials. This may have been due to an environmental adaptation of the bacteria. The red area at the bottom of the column (Fig. 4) represents Euryarchaeota, a phylum of the Archaea. Euryarchaeota include methanogens, which produce methane and are often found in the intestines; halobacteria, which survive extreme saline concentrations; and some extremely thermophilic aerobes and anaerobes. They are distinguished from other archaea mainly based on rRNA sequences and their unique DNA polymerase. Although Euryarchaeota are not major components of the bacterial community, they are important for methane production in anaerobic digestion systems.

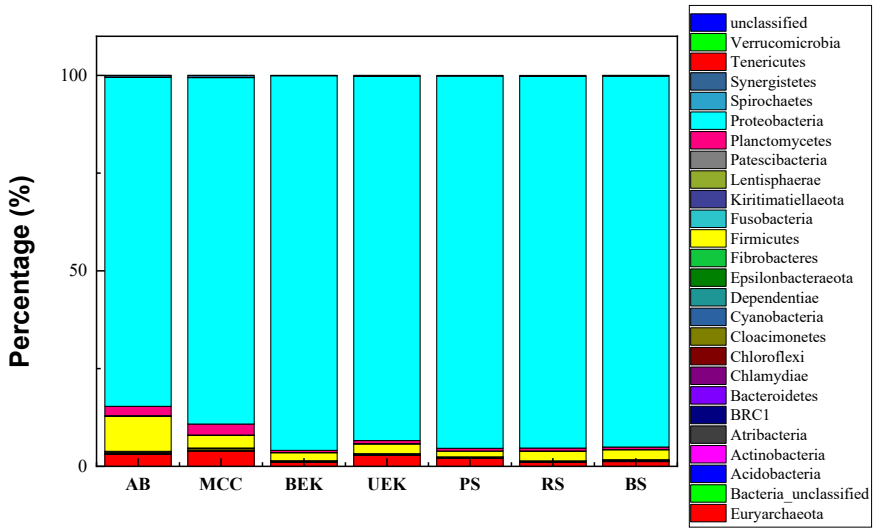

Fig. 4. Phylum level distribution of six anaerobic systems for the six lignocellulosic materials and blank (AB) in the absence of anaerobic sludge cake

Lignocellulosic Materials with Added Anaerobic Paper Plant Sludge

Figure 5 shows the cumulative methane production of the six substrates with added anaerobic sludge cake. The methane produced with MCC, BEK, UEK, primary sludge, and steam explosion of rice straw decreased, especially for BEK. In contrast, for UEK and steam explosion bamboo, the accumulated methane production was similar with that without the addition of anaerobic paper plant sludge cake, which did not help anaerobic digestion to produce methane. Compared with lignocellulosic materials, anaerobic sludge cake might be more difficult to use with anaerobic pig farm sludge because additives in the anaerobic sludge cake might inhibit digestion by anaerobic bacteria. The ranking of methane yield efficiency was as follows: MCC, UEK, PS, RS, BS, and BEK. The methane yields from BEK and RS decreased substantially. The added anaerobic sludge cake may reduce the ability of bacteria to break down cellulose into smaller molecules for utilisation by anaerobic bacteria. This is due to the anaerobic sludge from the pulp mill could contain a substantial amount of lignin-related compounds. These area prone to the non-productive binding (strong adsorption) of enzymes (cellulase enzymes).

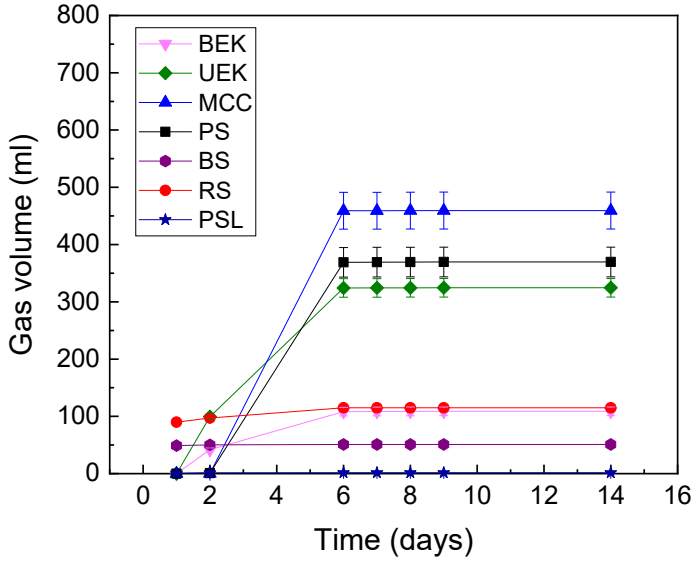

Fig. 5. Accumulated methane yield of six substrates with added anaerobic paper plant sludge cake

Figure 6 compares the crystallinities of the original lignocellulosic materials, lignocellulosic materials after anaerobic digestion without the added anaerobic sludge cake, and lignocellulosic materials after anaerobic digestion with the added anaerobic sludge cake. The decrease in the crystallinity of lignocellulosic materials after anaerobic digestion without added anaerobic sludge cake was higher than that of lignocellulosic materials after anaerobic digestion with added anaerobic sludge cake. This might be because the bacteria in the anaerobic digestion system without added anaerobic sludge cake were better able to degrade lignocellulosic materials. This can be demonstrated by comparing the decreased residual cellulose content of the systems with and without the added anaerobic sludge cake. The decrease in the residual cellulose content of the anaerobic digestion system without the added anaerobic sludge cake was twice that of the decreased residual cellulose content of the anaerobic digestion system with the added anaerobic sludge cake. This demonstrates that cellulose in the anaerobic digestion system without added anaerobic sludge cake was degraded more by bacteria than cellulose in the anaerobic digestion system with added anaerobic sludge cake.

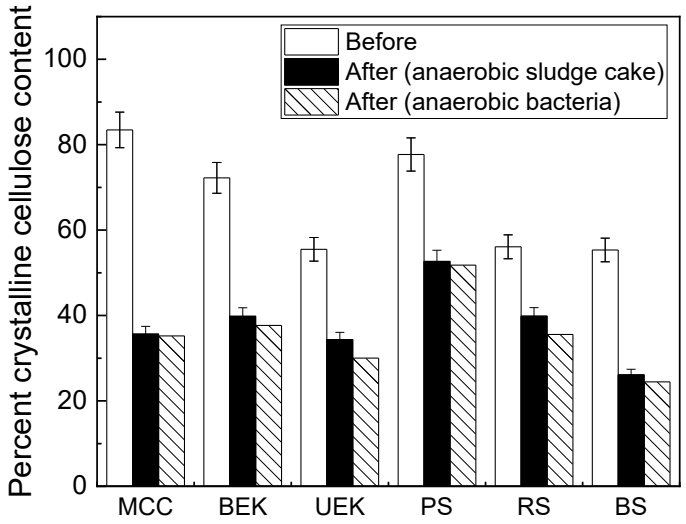

Fig. 6. Crystallinity of original lignocellulosic materials, and the lignocellulosic materials after anaerobic digestion with and without adding anaerobic sludge cake.

Figure 7 shows the phylum distribution during anaerobic digestion of the seven materials. With the addition of the anaerobic paper plant sludge cake, Euryarchaeota and *Firmicutes* became the dominant bacteria, and the original dominant bacteria, Proteobacteria, were much less abundant. The abundances of *Planctomycetes* and *Epsilonbacteraeota* also increased substantially.

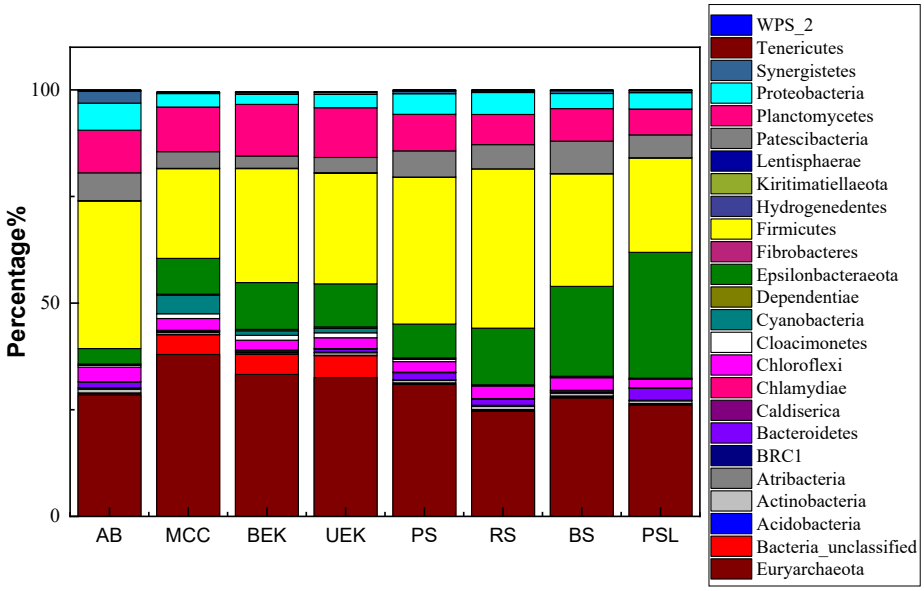

Fig. 7. Phylum level distribution of anaerobic systems with added anaerobic sludge cake and blank (AB)

Planctomycetes is a phylum of aquatic bacteria found in brackish, marine, and freshwater environments. Euryachaeota, which comprised 25 to 40%, was the main methanogen. Although Euryarchaeota showed a substantial increase, the accumulated methane production decreased significantly, indicating that the amount of Euryarchaeota was not proportional to the accumulated methane produced.

The dominant bacteria in the experiment without the addition of the anaerobic sludge cake were *Enterobacterales* and *Clostridiales*, which can metabolise sugar into short carbonate chains. Some *Pseudomonadales*, which could metabolise fatty acids, were also found in the anaerobic system without anaerobic sludge cake. The bacteria in the anaerobic system without anaerobic sludge cake produced a large number of short carbonate chains, which could be used by *Methanosaeta*. In contrast, the dominant bacteria in the anaerobic system with the added anaerobic sludge cake were *Methanosaeta*, but the bacteria producing short carbonate chains for *Methanosaeta* to use were much less abundant. Only some *Clostridiales* species can metabolise sugars into short carbonate chains. The decrease in accumulated methane production in the anaerobic digestion system with added anaerobic sludge cake was due to insufficient bacteria to produce short carbonate chains for use by *Methanosaeta*. Comparing the residual cellulose content with and without the addition of anaerobic sludge cake, the decrease in the cellulose content of the anaerobic digestion without the anaerobic sludge cake was higher than that of the anaerobic digestion system with the added anaerobic sludge cake. This shows that the ability to degrade cellulose in an anaerobic digestion system without added anaerobic sludge cake was better than that in an anaerobic digestion system with added anaerobic sludge cake.

Methane Yield and Microbial Community Comparison

A comparison with other anaerobic fermentation methods is shown in Table 3. Compared with Pore *et al.* (2016), the NGS results showed a large difference in bacterial flora content. The methane content was similar to that of without paper mill bacteria. However, it is the most important flora in terms of bacteria, and *Firmicutes* is the second most abundant, followed by Proteobacteria (Xu *et al.* 2019). The source of the differences in the flora may be the difference in lignocellulose, strain source, or whether they have been domesticated. Compared with Xu *et al.* (2019), the fermented methane production of maize stalks was much higher, which may be because the bacteria used in the present work were not domesticated. As far as crystallinity is concerned, the drop rate of our crystallinity was much higher than that of theirs, and the difference in crystallinity may be caused by differences in the bacterial species and lignocellulose components. In the analysis of the NGS bacterial flora results, the flora was roughly similar, but their proportions were quite different. Moreover, *Bacteroides* was the main flora, whereas the present results showed that Euryarchaeota was the main flora and *Firmicutes* was the supplementary flora. Adding paper mill bacteria, Proteobacteria is the largest group, and Euryarchaeota is the second largest group. The source of the difference in the bacterial groups may be the difference in lignocellulose and the source of the bacteria or it may have been caused by domestication. Compared with Latifi *et al.* (2019), the total methane production rate was higher after removing the methane gas produced by the strain; however, the VS used for methane gas production was slightly better than that used in this study. The F/M ratio was also similar to that used in this study. Additionally, the greater the amount of biomass added, the higher the gas production.

Table 3. Comparison of the Methane Yield and Microbial Community of Different Materials

Materials	Methods	Results	References
Cow dung slurry, rice straw	Anaerobic digestion Sodium hydroxide	23-450 ml/g VS NGS domain: Bacteria 95.2% Archaea 4.2% NGS phylum: Bacteroidetes 36.8%, Proteobacteria 13.4%, *Firmicutes* 28%,	Pore *et al.* (2016)
Corn stover stem bark	Anaerobic digestion	194 ml/g VS Crystallinity 47.8-42.2% NGS phylum: Bacteroidetes 23%	Xu *et al.* 2019
Poultry slaughterhouse wastes with sewage sludge	Anaerobic co-digestion	218 ml/g VS 54 ml/g VS Better F/M ratio: 1, 2, and 4.4	Latifi *et al.* (2019)
MCC, BEK, UEK PS, BS, RS	Anaerobic co-digestion Steam explosion	MCC: 44.72 ml/g VS RS: 55.76 ml/g VS Crystallinity: 83.47% to 35.21% NGS (with PSL) phylum: *Firmicutes* 21.03%, Euryarchaeota 37.95% NGS (without PSL) phylum: Proteobacteria 88.61 %,	This study

CONCLUSIONS

1. The anaerobic digestion of different biomass substrates with paper plant sludge cakes was evaluated and compared in this study. Microcrystalline cellulose (MCC) showed the highest accumulated methane production among the different materials in the anaerobic digestion system.
2. Crystallinity and enzyme activity were not proportional to accumulated methane production. The steam explosion materials had a higher reducing sugar content before fermentation because the cellulose was broken down into small molecules and reducing sugars during the steam explosion process; thus, the remaining cellulose was difficult to break down.
3. Comparing the results of anaerobic digestion with and without added anaerobic sludge cake, the anaerobic digestion system without anaerobic sludge cake had a higher accumulated methane production.
4. Next-generation sequencing (NGS) revealed that the dominant bacteria in the anaerobic digestion system without added anaerobic sludge cake could convert sugars into organic acids and degrade cellulose. This substantially reduced the residual cellulose content and cellulose crystallinity; therefore, the yield of accumulated methane was higher than that of the anaerobic digestion system with the added anaerobic sludge cake.

5. The dominant bacteria in the anaerobic digestion system with anaerobic sludge cake were *Methanosaeta*, which can convert organic sugars into methane. This substantially reduced the number of bacteria that can degrade cellulose.

ACKNOWLEDGMENTS

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