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Effects of Nutrient Supplement and Chitosan on Microbial Population Change in Up-Flow-Anaerobic-Sludge-Blanket Reactor during Biogas Production

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Abstract

The objective of this research was to study the effects of nutrient supplement and chitosan on microbial change in an up-flow-anaerobic-sludge-blanket (UASB) reactor during biogas production. Three UASB reactors were operational in this study. All reactors were operated by feeding dilute stillage with chemical oxygen demand (COD) concentration at 10,000 mg/L and 9 days of hydraulic retention time (HRT) under anaerobic conditions. Reactor 2 and reactor 3 were supplemented with nutrient supplement and chitosan, respectively. The results of the environment and pH values of all UASB reactors showed similar conditions with total volatile acid/alkalinity (TVA/Alk) values of 0.27-0.31. The COD removal efficiency of reactor 1 (stillage), 2 (stillage and nutrient supplement) and 3 (stillage and chitosan) showed about 79%, 84% and 87%, respectively. In addition, it was found that the UASB reactors supplemented with nutrient supplement or chitosan produced higher levels of biogas than those without additives. The 16S rRNA technique by PCR reaction showed that the dominant archaea in the final fermentation of all UASB reactors and in inoculum sample were hydrogenotrophic (genus *Methanobacterium*) and acetotrophic methanogens (genus *Methanosaeta*). The methanogens population in the reactor supplemented with chitosan (18.11%) produced more biogas than the ones in the reactor supplemented with nutrient supplement (14.44%) and in the control reactor (15.95%).

Introduction

Currently, Thailand is a country considered as one which has many factories, especially agricultural-industrial factories. These agricultural processing plants discharge a large amount of wastewater which will affect the environment in the future, if not managed well. According to the study of Owusu-Agyeman et al.

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(2020), one of the most suitable and popular wastewater treatment systems for these wastewaters was the UASB system. It is effective for treating high organic wastewater, less costly to operate, and the system can produce high volumes of biogas, making it economically cost-effective (Udomsinrot, 2000; Verbyla et al., 2013), but the efficiency of the UASB system depends on the granular sludge in the system. Granular sludge consists of several groups of microorganisms such as hydrolytic, methanogens and acetogens (Xu et al., 2018). Granular sludge provides a high treatment efficiency due to its active anaerobic microorganisms as well as a good sedimentation process, thereby reducing the loss of sludge outside the system (Kiran et al., 2016). Moreover, granular sludge is resistant to various changing environmental conditions in the system, such as pH fluctuation, flow rate fluctuation and change in organic matter (Kobayashi et al., 2018).

That is why several researchers had tried to increase the efficiency of granular sludge regarding the performance of UASB reactors. Lertsitthichai (2006) studied the addition of nutrient supplement to affect the efficiency of the UASB system, and it was found that the biogas production rate was higher and the microbial pellet size was larger compared to the system without supplementation. It was shown that the supplement added resulted in a huge pellet size, which affects system efficiency of biogas production and organic removal. Jijai et al. (2015) found that the particle size of granular sludge affects system efficiency. On the other hand, many researchers tried to use chitosan to increase the granular sludge size for higher efficiency of the UASB reactor (Jijai et al., 2015). Tiwari (2005) used chitosan to improve the granular sludge for wastewater treatment in a UASB system. The result showed that 95-98% of the COD efficiency was removed and it also increased significantly the granular sludge in the UASB system. Torres (2018) used chitosan to start up their UASB system. The result showed that COD was removed about 92% within 11 days. Moreover, Khemkhao et al. (2011) have studied the effect of chitosan on UASB treating palm oil mill effluent during a transition from mesophilic to thermophilic conditions. It was found that chitosan addition showed a higher biogas production rate, decreased the washout of biomass, and increased the granular size.

As mentioned above, both nutrient supplement and chitosan led to a higher efficiency of the UASB system and also increased the pellet size of granular sludge, especially in adding chitosan. Nutrient supplement and chitosan may affect the microbial changes in the system. However, the study on the change of the microbial population and particularly the comparison between adding chitosan and nutrient supplement in an UASB reactor was limited. Therefore, this study aimed to find detailed effects of nutrient supplement and chitosan on microbial population change in UASB reactors. In addition, the organic matter removal as well as biogas production were studied.

Materials and methods

1. Cassava stillage

The effluent from a cassava ethanol production plant was used in this experiment. It was collected from the water outlet of a reservoir before entering the UASB system. Samples were preserved at a temperature below 4°C to prevent bacterial activity. The water samples were analyzed for various properties including pH, biochemical oxygen demand (BOD), COD, total solids (TS), total volatile solids (TVS), total dissolved solids (TDS), suspended solids (SS), and total Kjeldahl nitrogen (TKN). These parameters were analyzed according to the standard method of APHA (2012).

2. Nutrient supplement and chitosan

For testing nutritional supplement and chitosan regarding optimizing the removal of organic matter and biogas production the nutrient supplement was mixed with cassava stillage according to the application of Speece's formula (1996) as shown in Table 1.

Table 1 Composition of nutrient supplement

Ingredient	Concentration (mg/L)
Major	
NH_4Cl	400
KCl	400
$MgSO_4 7H_2O$	400
$Na_2S\ 9H_2O$	300
$(NH_4)_2\ HPO_4$	80
$CaCl_2\ 2H_2O$	50
Minor	
$FeCl_2\ 4H_2O$	40
$CoCl_2\ 6H_2O$	10
$NiCl_2\ 6H_2O$	0.5

The major ingredients of the supplements were ammonium, potassium, magnesium, sodium and calcium.

Minor other ingredients were ferrous, cobalt and nickel. The stillage that had been mixed with nutrient supplement was fed into the UASB every day.

Chitosan original flakes from aquacultural Yannamei shrimp. Shell with a molecular weight of 2,100 kilodalton (kDa) was used in this experiment. The long-chain of chitosan was prepared at a concentration of 2% by dissolving 100 g of chitosan powder in concentrated 150 mL acetic acid. Then, water was filled up to a net volume of 5 L. On every cycle of hydraulic retention time (HRT), 1.6 L of cassava stillage mixed with 50 mL of prepared chitosan was fed to the UASB.

3. Granular sludge and specific methanogenic activity (SMA)

Granular sludge from the same plant as the stillage sample was used in this study. Granular sludges were collected from the UASB system of a factory and stored by soaking in the effluent of the UASB system of the factory for use as a nutrient for the sludge prior to further testing. Granular sludges were analyzed by pH, moisture, total solids and total volatile solids following the APHA standard method. In the testing step, the concentrated granular sludge that was fed into the UASB reactor was approximately at 20,000 mg/L. One third of the reactor volume was fed into the reactor before the operation testing step.

The SMA test was performed in a 120 mL serum vial with a food to microorganism ratio (F/M ratio) of 0.5 using glucose as food, whereas the amount of sludge was calculated from the volatile suspended solids (VSS) (Smolder et al., 1995). The biogas was measured at least 20 days or until the biogas was at its balanced level. The biogas gas composition was then analyzed on a weekly basis using a gas chromatography-flame ionization detector (GC-FID) model SHIMADZU GC-14. The SMA was calculated from the slope of the methane production curve, divided by gram of VSS and expressed as $gCOD.gVSS^{-1}.day^{-1}$. Theoretically, 350 mL of methane was produced from 1 g of COD.

4. UASB reactor

In the experiment, all three UASB reactors used in this study were made of acrylic material. Each had a width of 15 cm., length of 15 cm, and height of 80 cm. The total volume of each reactor was 18 liters, with a working volume of 15 liters. Inside the reactor, there were 2 separated sections. The lower part was the reacting section whereas the upper part was the gas separating section (GSS). Cassava stillage was fed from the bottom of the reaction tank through 3 taps equipped with a pump. Effluent from the reactor was collected at the height of 67 cm and the biogas sampling point was located at the top of the reactor as showed in Fig 1.

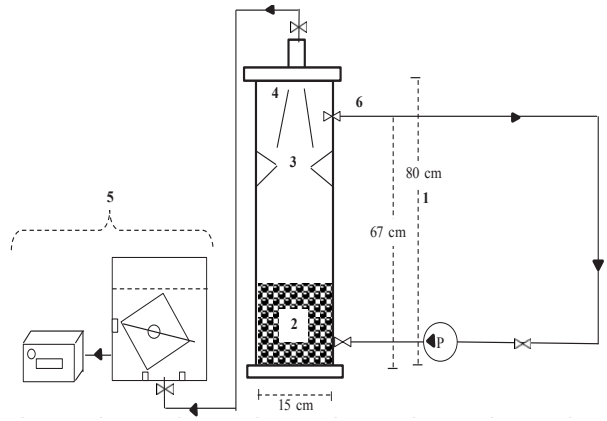

Fig. 1 Up-flow anaerobic sludge blanket reactor

5. UASB Operation

UASB reactors: reactor 1 was operated with pure cassava stillage, reactor 2 was operated with cassava stillage including nutrient supplement, and reactor 3 was operated with cassava stillage including chitosan. All UASB reactors were operated by feeding stillage diluted with COD concentration at 10,000 mg/L. They were fed at a flow rate of 1.6 L/d approximately 9 days of drolysisdraulic retention time (HRT) under anaerobic conditions. The recirculation rate of all three UASB reactors were 5L/min by a Mirano Water Pump ID8 (220 V A.C. 50 Hz 0.37 kW).

6. Analysis

The effluent and biogas production of the three UASB reactors were measured and analyzed. The effluent of each reactor was analyzed for pH, COD, Alk and TVA to assess the optimal environment and system performance. It was analyzed according to the APHA standard method (Ableling & Seyfired, 1992). The biogas production of each reactor was measured by a gas collector and counter for comparing the efficiency of each reactor.

7. Study of microbial population in UASB

Granular sludge samples from the UASB system before (inoculum source) and after adding wastewater from the cassava ethanol production plant (reactor 1),

nutritional supplements (reactor 2) and chitosan (reactor 3) were studied to extract the DNA for microbial populations with the following E.Z.N.ATM Mag-Bind Soil DNA Kit (Omega, USA). The 16S rRNA gene was increased by PCR reaction using 341F (5/TCGTCGG CAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG-3/) and 805R(5/GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGGACTACH VGGGTATCTAACC-3/) primers with specific V3-V4 variable regions using sparQ HiFi PCR master mix (Quanta bio, USA) imported to a Perkin-Elmer GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, USA) by programming the DNA amplification as denaturation cycle (94°C for 3 min), 25 cycles (98°C for 20 sec, 55°C for 30 sec and 72°C for 30 sec) and final extension (72°C for 5 min). The nuclear ribosomal DNA was increased by PCR reaction by using ITS-1F and ITS-2R primers and imported to the Perkin-Elmer GeneAmp PCR system 2400 thermal cycler by programming the DNA enrichment as denaturation cycle (94°C for 3 min), 25 cycles (98°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec) and a final extension (72°C for 5 min). The newly created DNA was then purified with AMPure XP beads and the specimens were classified by using 5 µl of each Nextera XT index kit. The attachment of DNA strands to classified samples was carried out by using 50 µl of PCR reaction followed by 8-10 cycles of PCR states, the final PCR products were then cleaned and collected. After the completion, the size of the DNA fragment was examined using agarose gel technique electrophoresis; 0.8% agarose gel was applied and was cut in the DNA band area of 16S rRNA at an approximate target DNA size of i.e., 550 bp and it was then diluted to the final concentration of 6 pM performing a sequencing analysis with the Illumina MiSeq genome analyzer by Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand).

FASTQC software was used to analyze the sequences' evolutionary relationships (Phylogenetic analysis) by comparing the genetic sequence using a PEAR program (Zhang et al., 2017). The FASTX-Tool kit read less than 90 % of non-quality data and would not be less than 300 bp when comparing the similarity of nucleotide sequences obtained using the UCHIME method (Edgar et al., 2011) in vsearch 1.1.1 (Rognes et al., 2016). Uchime_ref, group classification was carried out using operational taxonomic units (OTU) using pick_open_reference_otus.py command in QIIME 1.9.0. The specificity of the sequences used SortMeRNA for reference. The taxonomy was generated at 97% of Greengenes database. The error information was further read using SUMACLUST. The sequence used was 30,000 for each sample to analyze the bacterial population. Alpha diversity data was performed with the Chao1 index (Chao, 1984) and Shannon index (Shannon, 1984). Beta diversity data was estimated by calculating the weighted and unweighted UniFrac distances matrix which used principal coordinate analysis (PCoA) technique (Lozupone et al., 2011). The archaeal and bacterial 16S rRNA gene sequences obtained from inoculum samples of reactor 1, reactor 2 and reactor 3 were deposited in GenBank under Bioproject accession number PRJNA750574.

Results and discussion

1. Cassava stillage properties

The inspection and observation of the physical appearance of cassava stillage revealed that the water appeared in a light brown color with rancid odor and unclear sediment. As for the examination of the biochemical properties, relatively high values in COD and TS were found, with the average value at 42,777 ± 2,525 and 29,820 ± 1,220 mg/L, respectively (Table 2). With the consideration of the proportion of COD: N: P at 100: 1.69: 0.004, the result did not seem suitable for bacteria growth in an UASB reactor. Ableling & Seyfired (1992) found that for an anaerobic process, the appropriate COD: N: P in the wastewater is 100:0.625: 0.125. Moreover, it was found that some of the nutrients contained in the wastewater cannot be used for the growth of the bacteria. Therefore, additives that affect the growth of microorganisms under anaerobic conditions should be considered to further increase the efficiency of the treatment system. Regarding the proportion of BOD to COD in this study, a value of 0.57 was found. This means that the stillage used in this study had more than 50% of organic substances that were biodegradable, considering that any values greater than 0.5 are suitable for biodegradation in the anaerobic fermentation process (Lee & Nikraz, 2015).

2. Granular sludge and SMA

The UASB granules were characterized before they were used as inoculum in an UASB reactor (Table 3). The pH value showed a result of 7.0. The moisture content showed a high value at about 94% of the UASB wet sludge. Total volatile solid was 81% as a dry basis. From the 30 days of SMA test, it was found that the

Table 2 Characterization of cassava stillage

Property indexes	Values	Average ± S.D.
pH	4.04-4.21	4.12 ± 0.09
Alkalinity (mg/L)	42.5-112	83.17 ± 36.23
Total volatile acid (mg/L)	3,932-4,745	4,308 ± 410
BOD (mg/L)	21,500-27,180	24,428 ± 2,844
COD (mg/L)	40,250-45,300	42,777 ± 2,525
Total solids (mg/L)	28,750-31,150	29,820 ± 1,220
Total volatile solid (mg/L)	24,400-29,750	27,633 ± 2,844
Suspended solids (mg/L)	2,033-2,500	2,248 ± 236
Total nitrogen (mg/L)	678-785	724 ± 54
Phosphorus (mg/L)	1.12-1.92	1.5 ± 0.4

granular sludge could eliminate the average organic matter for about 0.28 gCOD.gVSS^{-1}.day^{-1}. These values showed that the sludge contains microorganisms capable of removing organic matter in the system. In addition, these values were used to prepare the inoculum to be suitable for the organic content in the next step of the experiment.

Table 3 Characterization of granular sludge

Property indexes	Average ± S.D.
pH	7.0 ± 0.03
Moisture (% wet basis)	94 ± 0.56
Total solids (% wet basis)	6 ± 0.065
Total volatile solid ((% dry basis)	81 ± 0.27
SMA (gCOD.gVSS^{-1}.day^{-1})	0.28 ± 0.01

3. Performance of the UASB reactors

UASB reactors: reactor 1 was operated with cassava stillage, reactor 2 was operated with cassava stillage with additional nutrient supplement and reactor 3 was operated with cassava stillage with additional chitosan.

3.1 Stability of UASB

The stability of the three UASB systems was determined by measuring Alk, TVA and pH in effluent during 60 days of operation (Fig. 2). It was found that the pH value of reactors 1, 2 and 3 were similar (6.5 ± 0.2, 6.7 ± 0.2 and 6.6 ± 0.3), showing neutral conditions suitable for microorganisms. The Alk of the reactors 1, 2 and 3 were 886 ± 151, 1,086 ± 161 and 929 ± 129 mg/L, respectively, not exceeding the reference value (range between 1,000-3,000 mg/L).

The average TVA of the three reactors also did not exceed the reference value as shown in Table 4 (Udomsinrot, 2000). The TVA/Alk ratio of the three reactors were 0.27 ± 0.02, 0.22 ± 0.05 and 0.31 ± 0.05, indicating a high buffering capability of the system. Normally, with a TVA/Alk ratio greater than 0.8, it would show an accumulation of acid, especially propionic and

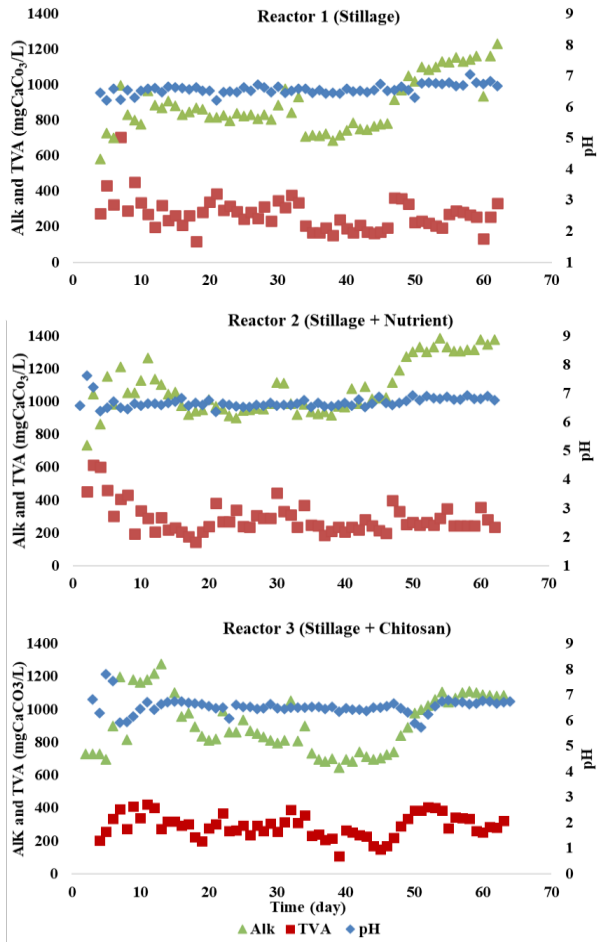

Fig. 2 Alkalinity, total volatile acid, and pH of UASB reactor

butyric, which affects the inhibition of microbial groups in anaerobic treatment (Dogan, et al., 2005). It was indicated in the study of Pooltawee regarding the inhibition of the methanogenic bacteria group that a high propionic acid level resulted in an increased removal of organic matter (Dogan et al., 2005; Pooltawee, 1994).

3.2 COD removal and biogas production

The influent and effluent of all three UASB reactors were determined by COD and SS for evaluating removal efficiency of the UASB system. It was found that the average COD concentration of effluent of the reactors 1, 2 and 3 were 2,382, 1,935 and 1,350 mg/L, respectively. Thus, the COD removal efficiency of the reactor 1, 2 and 3 showed about 79 ± 8.8%, 84 ± 6.4% and 87 ± 6.6%, respectively (Table 4 and Fig. 3). The results of the efficiency of COD removal indicated that adding nutrients to the UASB reactor causes an increase

Table 4 Stability and performance indexes of UASB

Indexes	Reactor 1	Reactor 2	Reactor 3	Reference value (Udomsinrot, 2000)
Stability				
pH	6.5 ± 0.2	6.7 ± 0.2	6.6 ± 0.3	6.6-7.6
TVA/Alk	0.27 ± 0.02	0.22 ± 0.05	0.31 ± 0.05	< 0.4
Alkalinity (mg/L)	886 ± 151.1	1,086 ± 161.4	929 ± 129.5	1,000-3,000
Total volatile solid (mg/L)	271 ± 33.4	288 ± 32.7	291 ± 30.4	< 2,000
Performance				
COD removal (%)	79 ± 8.8	84 ± 6.4	87 ± 6.6	
SS removal (%)	60 ± 7.3	61 ± 10.1	74 ± 10.6	

of the efficiency of COD removal. This may be due to the addition of nutrients that contribute to the growth of microorganisms in the system, resulting in a higher system efficiency in removing organic matter. Furthermore, adding chitosan to the UASB reactor showed a higher COD removal efficiency similar to adding a nutrient supplement to UASB. Chitosan as a coagulant helps to increase the efficiency of sludge coagulation and the flocculation process. Thus, the system UASB had a high efficiency of COD removal and biogas production including SS removal efficiency. Similar to the study of Zou et al. (2021), it was found that chitosan was beneficial in accelerating sludge granulation and reducing sludge loss in UASB systems.

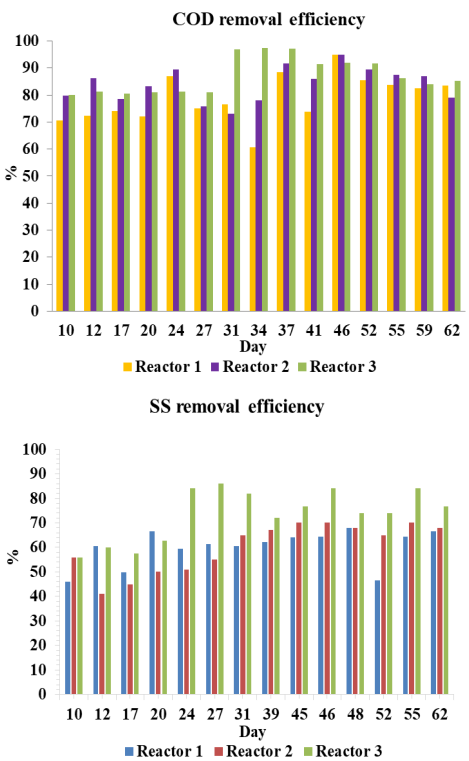

Fig. 3 COD and SS removal efficiency of UASB reactors

Considering the efficiency of SS removal of the three UASB reactors, it was found that to be consistent with the efficiency of removing COD as shown in Fig. 3. The reactor 3 had a higher SS removal efficiency (74 ± 10.6%) than the other reactors (Table 4 and Fig. 3). Chitosan filled in a reactor acted as a coagulant. The granular sludges were formed from binding of a cationic polymer of chitosan and negatively charged cell surfaces of bacteria, which leads to a higher density of sludge retained in the reactor (Khemkhao et al., 2011), as evidenced by an increase of SS removal efficiency (Table 4). Reducing the amount of SS in the system could affect the removal of organic matter, resulting in higher organic removal efficiency and higher biogas production (Fig. 4).

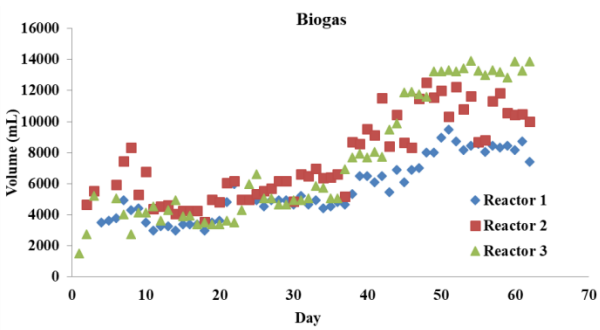

Fig. 4 Biogas production of UASB reactors

Biogas production was determined during 60 days of experimenting as shown in Fig. 4. Reactors 2 and 3 had a high average biogas production which was about 7,565 mL/day and 7,762 mL/day, respectively. While the reactor 1 produced biogas less than the two other reactors: about 5,645 mL/day. Reactor 2, which was filled with nutrient additives, had a ratio of COD:N:P, and was thus suitable for the growth of microorganisms and as a result caused a higher biogas production. Reactor 3, which was filled with chitosan, performed its biogas production at

an average level, which was not different from reactor 2. This was to be expected from chitosan, since it is a flocculant. It can reduce the loss of granular sludge from UASB reactors, which may result in a growing number of bacteria in the system or may reduce the SS out of the system (Zou et al., 2021). It was consistent with the SS removal efficiency in Fig. 3. Consequently, the result of the treatment of COD and the biogas production were higher than from the reactors 1 and 2.

4. Microbial communities

As shown in Fig. 5, a total of 78 phylotypes, which were affiliated with two phyla, Euryarchaeota and Crenachaeota, were detected in archaeal communities, and Euryarchaeota remained predominant throughout the end-up stage. These results were similar to previous findings of the archaeal communities regarding agricultural anaerobic digesters, which were dominated by members of the phylum Euryarchaeota (Langer et al., 2019).

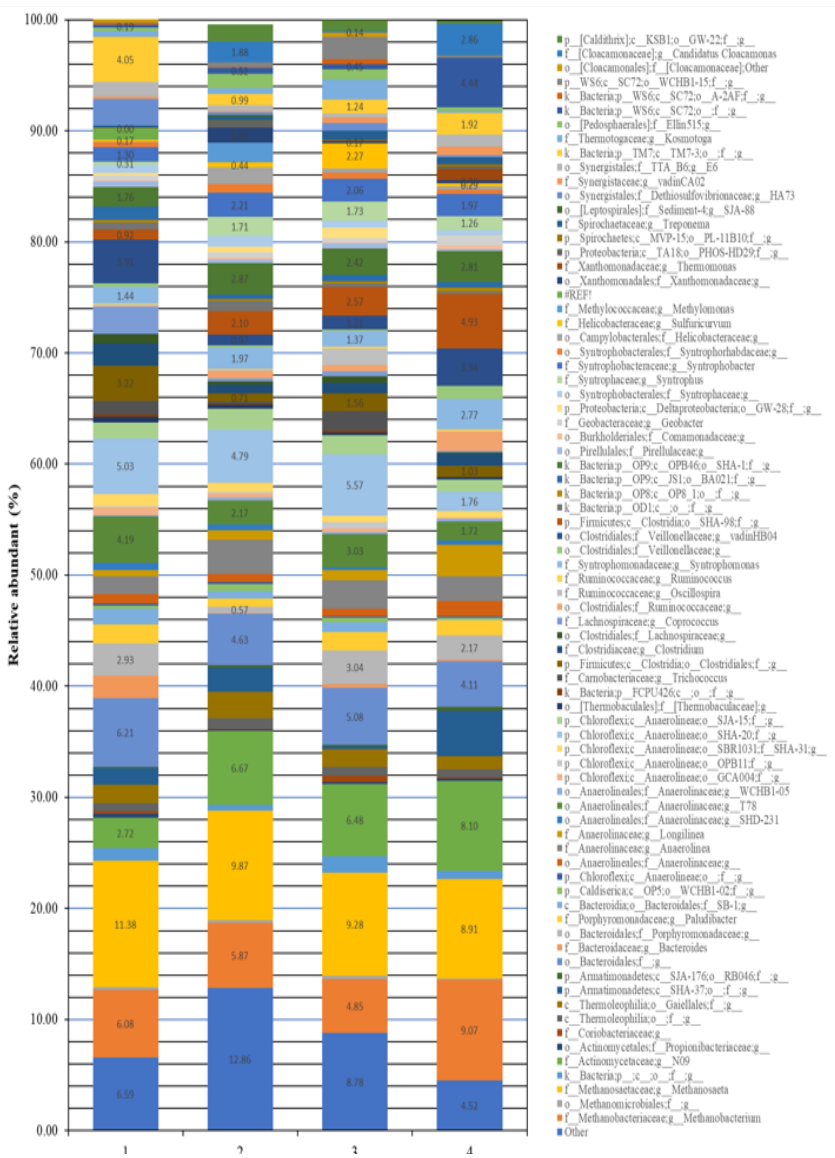

Fig. 5 Correlation of microbial content from 16S rRNA in UASB treatment tanks from factory wastewater. Cassava ethanol: (1) Inoculum sample, (2) reactor 1 with stillage, (3) reactor 2 with stillage and nutrient supplement, (4) reactor 3 with stillage and chitosan

In the methanogen populations, the acetotrophic *Methanosaeta* were highly abundant in inoculum sample (11.38%), in reactor 1 with stillage (9.87%) and reactor 2 with stillage and nutrient supplement (9.28%), while in the reactor 3 with stillage and chitosan it was the hydrogenotrophic *Methanobacterium* (9.07 %). In these findings, the hydrogenotrophic genera *Methanoculleus* and *Methanobacterium* dominated in agricultural anaerobic digesters (Langer et al., 2019). Furthermore, the acetotrophic *Methanosaeta* was the key methanogen in the digestion chamber for biogas production from a pig farm (Pampillón-González et al., 2017) and the sewage sludge from a wastewater treatment plant (Pyzik et al., 2018). The genus *Methanosaeta* acted as an acetotrophic methanogens, converted acetate into CH_4 and CO_2 and led to a higher methane production in the reactors (Pampillón-González et al., 2017). It was reported that genus *Methanoseata* favors low levels of acetic acid (Wu et al., 2016).

The predominant archaeal genus *Methanosaeta* (11.38%) and *Methanobacterium* (6.21%) and bacterial order Bacteroidales (6.21%), class Anaerolineae (5.30%), famies Anaerolinaceae (4.19%) and Veillonellaceae (3.91%) were predominant in the inoculum sample. These results indicate that the inoculum sample was highly methanogenic. Normally, the content of the inoculum sample is important for starting up biogas reactors. However, it does not seem to be the main factor for both the process performance and the overall microbial community, being dependent on an optimal organic material composition and on operating parameters.

In particular, the tendency towards an increase in the genus *Methanobacterium* (9.07%), family Actinomycetaceae (8.10%), class Clostridia (4.93%), kingdom Bacteria (4.44%), family Veillonellaceae (3.34%), kingdom Armatimonadetes (4.12%), genus *Candidatus Cloacamonas* (2.86%) and genus *Syntrophomonas* (2.77%) in reactor 3 with stillage and chitosan was higher than in reactor 1 with stillage and in reactor 2 with stillage and nutrients supplement. The bacterial populations play an important role in anaerobic digesters, such as: methane production (*Methanobacterium*); lactate, acetate and succinate production (family Actinomycetaceae); protein, cellulose, and other polysaccharides degradation (genus *Clostridium* and *Syntrophomonas*); polysaccharide degradation (family Veillonellaceae); pectin and xylan degradation (phylum Armatimonadetes) and the acidogenesis process (*Candidatus Cloacamonas*) (Langer et al., 2019; Pyzik et al., 2018; Wu et al., 2016; Ren et al., 2014; Lee et al., 2014; Esquivel-Elizondo et al., 2017). According to the report of Pachiega et al. (2019) there is a predominance of Veillonellaceae and Streptococcaceae families in UASB reactors treating brewery wastewater for hydrogen production. The Veillonellaceae family can be tolerated in high organic material and can be stabilized in the process of biogas production. The results indicated that in the reactor with chitosan there was a higher rate of archeal and bacterial communities than in the reactor with nutrient supplement and the control reactor. The granular sludges were formed from binding of a cationic polymer of chitosan and the negatively charged cell surfaces of bacteria leading to a higher density of the sludge retaining in the reactor. The chitosan addition showed a higher biogas production rate and decrease washout of biomass and increased granular size (Khemkhao et al., 2011).

Class Anaerolineae (5.57%) Order Bacteroidales (5.08%), family Porphyromonadaceae (3.40%), family Anaerolinaceae (3.03%), genus *Sulfuricurvum* (2.27%) Order Clostridiales (1.56%) and family Veillonellaceae (1.21%) were higher in reactor 2 with stillage and nutrient supplement than in the control reactor. Some bacterial members associated with the acetate production at the end of the start-up stage were Class Anaerolineae (genus *Anaerolinea*), including genera *Ruminococceae, Proteiniphillum, Syntrophomonas,* and *Coprothermobacter* (Wu et al., 2016). These bacterial populations play an important role in carbon compound degradation (order Bacteroidales and family Porphyromonadaceae) (Burns et al., 2012) and H_2S oxidation (genus *Sulfuricurvum*). Previous research has demonstrated that the bacterial families Porphyromonadaceae, Tissierellaceae, and archaeal family Methanobacteriaceae were most abundant in the slaughterhouse industry of pigs and poultry and produced high amounts of biogas/methane (Granada et al., 2018). The order Clostridiales (family Veillonellaceae) is a symbiotic gut bacteria (Pampillón-González et al., 2017) and plays an important role in the polysaccharide-degrading (Pyzik et al., 2018). Pampillón-González et al. (2017) reported that the family Clostridiaceae dominated in a sedimentation pond for biogas production at a pig farm. The microorganisms used ammonia as a nitrogen source for their metabolisms (Nsair et al., 2020). Light metals such as potassium (K), magnesium (Mg), sodium (Na) and aluminum (Al) were added in order to promote the microbial growth and enhancement of the bacterial cell immobilization (Ca).

Ca^{2+} helps genus *Sulfuricurvum* (2.27%) Order Clostridiales to degrade cellulose (Karlsson et al., 2014). Trace metals such as Fe, Ni, Co and Mo play an important role in enhancing the catalytic efficiency of enzymes in the growth of methanogenic bacteria, which leads to an increase in methane production (Feng et al., 2010).

In the comparison of biogas production in reactor 2 with stillage and nutrient supplement and reactor 3 with stillage and chitosan there were no significant differences, but the output was higher compared to the control reactor. However, the reactor with chitosan addition (18.11%) showed a higher growth of methanogen populations of genera *Methanosaeta* and *Methanobacterium* than the reactor with nutrient supplement (14.44%) or the control reactor (15.95%). This corresponds to the efficiency of SS removal. It was found that reactor 3 with chitosan had a higher efficiency than the other ones (Table 4). In connection, chitosan is a biopolymer that can be used to enhance the sludge granulation (Kaseamchochoung et al., 2006) and to decrease biomass washout from the UASB (Khemkhao et al., 2011). The abundance and diversity of the microbial community was relatively high in organic material composition. The several factors affecting the biogas production during the anaerobic digestion were temperature, pH, and organic material composition (Karakashev et al., 2006; Pampillón-González et al., 2017). These results indicate that the sludge granulation from chitosan in the reactor with added chitosan protected the methanogen cells inside an acidogenic layer and decreased washing out of the methanogen cells from the reactor better than other reactors.

Furthermore, the population of the Porphyromonadaceae family and Clostridiales order found in reactor 2 with stillage and nutrient supplement of 3.40% and 1.56%, respectively, showed a higher percentage than the control reactor which accounted for 0.57% and 0.71%, respectively. As a result, the biogas production in reactor 2 with stillage and nutrient supplement showed better performance than the control reactor. According to Su et al. (2018), order Clostridiales has been reported as a hydrogen producer whereas family Porphyromonadaceae not only hydrolyzed complex carbohydrates and proteinaceous compounds, but it also generates volatile fatty acids such as acetic, isobutyric, propionic, and isovaleric acids during the acidogenesis phase. Therefore, it promotes biogas production related to methanogens (Zhang et al., 2017; Granada et al., 2018; Kurade et al., 2020)

Conclusion

Cassava stillage in this experiment showed a high level of organic matter. However, the addition of chitosan and nutrient supplement had improved the efficiency of organic removal and biogas production of the UASB reactor. Nutrient additives in reactor 2 made the ratio of COD:N:P suitable for the growth of microorganisms and resulted in a high biogas production. The chitosan additive in reactor 3 is a substance that flocculates and thereupon reduces the loss of granular sludge from the UASB reactor, which may it result as well in a growing number of bacteria in the system or reduce the SS in the system.

The dominant archaea in the final fermentation of all reactors and in the inoculum, sample were hydrogenotrophic (genus *Methanobacterium*) and acetotrophic methanogens (genus *Methanosaeta*). The acetotrophic *Methanosaeta* and hydrogenotrophic *Methanobacterium* were the key methanogens in the reactor for biogas production from stillage of the cassava ethanol production plant with added nutrients supplement.

Among these methanogenic populations, the populations of genera *Methanosaeta* and *Methanobacterium* in the reactor with chitosan addition (18.11%) showed up in higher amounts than in those reactors with added nutrient supplement (14.44%) and also than in the control reactor (15.95%), resulting in a higher biogas production.

In addition, the results showed that there was a high abundance of other bacterial groups in all three reactors, such as the family of Actinomycetaceae, orders Bacteroidales and Anaerolinea. The functions of these bacterial groups associate with the production of methanogen, lactate, acetate, and succinate, and degrade cellulose, xylan and polysaccharide. Furthermore, other bacterial groups in the reactor with added chitosan were also highly abundant. One hydrolytic group involved polysaccharides, lipids, proteins degradation (class Clostridia, family Veillonellaceae and genera *Clostridium* and *Syntrophomonas*). Another hydrolytic group involved pectin, xylan and cellulose degradation (phylum Armatimonadetes and genus *Clostridium*) and the acidogenesis group (family Actinomycetaceae and genera *Clostridium, Syntrophomonas, Candidatus* and Cloacamonas acidaminovorans) acetogenesis group (class Clostridia, order Clostridiales and genus *Syntrophomonas*).

Chitosan, which was added to reactor 3, is a substance that helps to increase the sludge concentration and the SS removal efficiency (74%), thereby increasing the efficiency of the system. Although reactor 2 with added nutrient supplement was quite effective, it was found that the SS removal efficiency was as low as 61%, which may affect the performance of the system in the future. Moreover, compared to the nutrient supplementation option, the preparation and use of chitosan were less complicated and cheaper. Therefore, chitosan could be used to control microbial growth, to enhance the operating lifetime, the stability and the survival of the microbial cells in the anaerobic digestion process for biogas production from wastewater of cassava ethanol production plants.

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