Anaerobic Biogas Production Using Microalgae *Chlorella* sp. as Biomass Co-digested by Cow Manure and Cow Rumen Fluid as Inoculum.

To cite this article: H Ambarsari *et al* 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **209** 012053

Anaerobic Biogas Production Using Microalgae *Chlorella* sp. as Biomass Co-digested by Cow Manure and Cow Rumen Fluid as Inoculum.

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World's demand for energy is steadily increasing following population increase, on the other hand reserves for fossil and coal energy are limited. Therefore research for renewable sources of energy is important. Biogas produced from renewable source could be one of the answer for future energy production. Researches regarding biogas production utilizing microalgae biomass such as *Chlorella* sp. have received much attention. In this study, co-digestion of *Chlorella* sp. "CD01" isolated from Cideng/Krukut River using cow rumen fluid and cow manure as inoculum were used to enhance biogas production. Our experiment used a simple non-stirred batch anaerobic bioreactor that was carried out for 29 days. Biogas volume, COD value, and microorganism were the parameters in this study. Our result showed that *Chlorella* sp. "CD01" and rumen fluid alone could not produce biogas. Co-digestion of *Chlorella* sp. "CD01" substrate and rumen fluid inoculum produced 314.5 mL biogas with yield of 43.23 mL/gTS. Co-digestion of *Chlorella* sp. "CD01" substrate and manure inoculum produced 1758 mL biogas with yield of 98.96 mL/gTS. The best substrate inoculum ratio (S/X) in this study was 0.33 based on cumulative biogas production. Attempts for isolating anaerobic culture from each reactor had been done using methanogenic selective media. Two methanogens isolates were morphologically characterized.

Keywords: biogas, anaerobic digestion, *Chlorella*, co-digestion, rumen fluid, cow manure

1. Introduction

The world today is in need for more energy than ever. Population growth is the key aspect of a raise in energy demand. It is predicted that the human population will reach up to 10 billion people in 2050. Thus, a new, cleaner, and more sustainable energy is required. Indonesia has also experienced the same problem with population growth and more energy demand each year. Indonesia energy requirement has increased over 1.3% each year from 2010 to 2015. Coal and oil are mainly used as the sources of energy [1]. Even though there is still none regulated law from the government, attempts to use other sources of energy have been done, one of those is biomass utilization.

Biomass utilization for energy producing has attract many increasing attentions in the last few decades. Biomass can be both clean and sustainable as the source of energy. Biomass can be converted into biofuel. Biogas is one type of biofuel that utilizes gas produced by decomposition of organic material as burnable fuel [2]. Biogas utilization and attempts have been done in Indonesia, such as "Kampung Biogas" in Malang city and "Pertanian Terpadu Berkelanjutan" by [3]. Other organic waste such as animal manure [4], crop leftover [5], and vegetables [6] have been studied and used as biomass to produce biogas. The other biomass potential that has been overlooked is microalgae.

Microalgae is considered as the future biomass for energy production because it has high carbohydrate and lipid content, fast growing [7], and a sustainable biomass source [8]. One of the popular species of microalgae is *Chlorella*. It is ubiquitous in most water bodies, fast growing, and has a relatively high tolerance against waste. This species can use waste water as nutrient for its growth [9]. Biomass from *Chlorella* can be converted to biofuel, especially biogas using anaerobic digestion [10]. Recently a study regarding a more efficient, more yielding, and a better production process is being researched intensively. One method to produce more and better yielding biogas is by using co-digestion.

Co-digestion is when two or more biomass are digested in the reactor. The aim of co-digestion is to make the carbon nitrogen ratio inside the reactor about 20 to 30, as it will increase the production of a more desirable biogas like methane rather than ammonia and other nitrogen containing gasses [11]. Thus by combining crop waste and animal waste will achieve the desired carbon nitrogen ratio [12]. Other studies showed a different point of view in combining different biomass sources inside a reactor. Substrate (S) and inoculum (X) interactions are also observed inside the anaerobic reactor. The substrate inoculum ratio (S/X) is an important factor in co-digestion. Just as carbon nitrogen ratio, it has a specific value that is different for every substrate and inoculum used.

Chlorella in anaerobic digestion plays the role of the substrate as its only purpose is to be degraded and converted to biogas. Several studies have shown that *Chlorella* alone can be degraded to biogas using anaerobic digestion, unfortunately it required more time compared to other substrate like manure. Co-digestion of *Chlorella* using inoculums from waste water sludge has shown an increase of biogas production [13]. However, the inoculum from waste water sludge is not commonly found in Indonesia for biogas production, thus an alternative inoculum is required. Cow manure is one of the common source of inoculum used in several biogas studies. It has both the substrate and microorganism to produce biogas [4]. There have been research studying the effect of co-digestion between *Chlorella vulgaris* and cow manure, and the result was an increase in biogas production [13]. Another common inoculum source that is rich in biogas producing microorganism that have yet to be fully utilized is the cow's rumen fluid.

Rumen fluid has been studied and observed to house diverse microorganism specialized in anaerobic digestion such as hydrolytic bacteria that cleaves long poly/oligosaccharides, acidogenic and acetogenic bacteria that utilize simple acids and fats to produce gases, and methanogenic microorganisms that utilize gases and other simple acids to form methane. Previous research have studied the effect of rumen fluid addition for co-digestion with microalgae *Scenedesmus* [14] and *Ulva lactuca* [15]. Both researches showed an increase in the biogas production. Co-digestion between *Chlorella* and rumen fluid have not been studied before. It is compelling to study both *Chlorella* and rumen fluid for biogas production because both are obtainable bio-source in Indonesia.

Therefore, this research is aimed to study the effect of co-digestion between *Chlorella* sp. "CD01" biomass isolated from domestic river in Indonesia as substrate (S) and cow's rumen fluid as inoculum (X) for biogas production. Co-digestion between the same *Chlorella* biomass and cow manure will also be studied and compared to the amount of biogas produced by *Chlorella* biomass and rumen fluid inoculum.

2. Material and Methods

Source and preparation of *Chlorella*

Chlorella was isolated from Cideng/Krukut River in Jakarta, Indonesia. The species has yet to be identified genetically, thus it will be referred as *Chlorella* sp. "CD01". It was cultivated using Bold's Basal media. There were 2 stage of cultivation, first stage using a 1-L Erlenmeyer for 1 week and second stage using a plastic container with 10-L working volume for 4 weeks. Extra illumination using 100 W white neon lamps and air from aerator for 24 hours were provided. *Chlorella* sp. "CD01" was harvested by dewatering, 3-L of dense dark green liquid was recovered. Cultivation was done 3 times to meet the required amount. The final concentration was 2 g/L measured by Total Solids (TS) analysis [13]. It was re-characterized and thermally pretreated by autoclaving 121°C for 20 minutes [16]. After pretreatment, *Chlorella* sp. "CD01" were stored inside a plastic jerrycan in room temperature.

Source of rumen fluid and manure
Cow rumen fluid and manure were collected from a slaughterhouse in Tangerang city, Banten province. The rumen fluid was obtained from the rumen stomach of a fresh slaughtered cow, whilst the manure was collected from the cage. The rumen fluid and manure were stored inside a black plastic trash bag and stored in a plastic box container. Both materials were used the day after. The rumen fluid was diluted 1:1 (v/v) with water and filtered using unbleached cloth [17]. The cow manure was also diluted with water 1:1 (w/v).

Experimental set up
This experiment set up consisted of a simple anaerobic bioreactor using HDPE plastic bottles with 1 L working volume. Bottle body were dark and possible leakage were sealed using hot glue gun. The bioreactor worked in a batch mode without stirring. The temperature was controlled at 35°C using water bath. The biogas produced was collected from the top of the bioreactor and measured using water displacement system (WDS). A sampling port was included at the top of the bioreactor. Both ports were equipped with plastic stoppers. Figure 1 demonstrates the bioreactor used in this experiment. The reactor design was based on a previous research [4] [10].

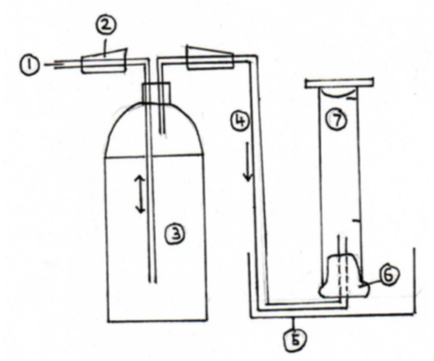

Figure 1. Bioreactor setup. Descriptions by number as shown by the figure are as follows; 1: sampling port; 2: stopper; 3: bioreactor body; 4: biogas hose; 5: water container (part of WDS); 6: Styrofoam plug (WDS); 7: graduated measuring cylinder 100 mL (WDS).

Experimental procedure
This study have 12 bioreactor variations that were divided into 4 groups. The content of bioreactor was indicated by 2 letters, whilst the (S/X) ratio variation was indicated by the numbers. K was manure, R was rumen fluid, and C was *Chlorella* sp. "CD01". There are 3 (S/X) ratio variations which are ratio of 3, 1, and 0.33. This ratio was obtained by dividing the content volume of substrate to inoculum. The positive control and treatment groups have (S/X) ratio variations. Negative control groups were the Chlorella bioreactor, the manure bioreactor, and the rumen bioreactor consisting as the name suggest. Negative control groups were all added water until reaching 1 L working volume. The positive control group which was the KR bioreactor contained rumen fluid and cow manure based on a previous study [17]. The function of KR bioreactor in this study was to be compared to the treatment groups using *Chlorella* sp. "CD01" as the substrate.

Table 1. Components of each bioreactor

Bioreactor	(S/X) ratio	*Chlorella* sp. "CD01" (mL)	Rumen (mL)	Manure (mL)
Chlorella	0	500	0	0
Rumen	0	0	500	0
Manure	0	0	0	500
KR3	3	0	250	750
KR1	1	0	500	500
KR033	0,33	0	750	250
CK3	3	750	0	250
CK1	1	500	0	500
CK033	0,33	250	0	750
CR3	3	750	250	0
CR1	1	500	500	0
CR033	0,33	250	750	0

The treatment groups were divided into two groups based on the inoculum inside. Group CK treatments were using *Chlorella* sp. "CD01" as the biomass and cow manure as the inoculum. Group CR treatments were using *Chlorella* sp. "CD01" as the substrate and rumen fluid as the inoculum. Control group did not have any replication, treatment groups have 2 replication each. Detailed composition can be seen in Table 1. *Chlorella* sp. "CD01" was always considered as the substrate. Rumen fluid was always considered as the inoculum. Manure can be both the substrate and the inoculum. This experiment was carried out for 29 days.

Analytical methods

Total solids (TS) of each bioreactor was measured initially, later to be calculated as yield. Biogas volume was observed daily and measured using a 100-mL graduated cylinder. Samples were taken once each week to measure the COD value. The COD value were measured using the standard closed-reflux colorimetric method. Samples for COD value determination were taken each week. Samples for microbial analysis were taken after the last day of observation and brought to the lab stored inside a sealed plastic jar. Isolation and characterization were ensured using selective media and anaerobic condition, thus had excluded most obligate aerobes present in the bioreactor. Sample were streaked onto the anaerobic methanogenic media as described previously. Petri dishes were then stored inside an anaerobic jar flushed using pure CO_2 gas for 5 minutes. Positive pressure was created inside the jar using the same gas. Previous study [18] used a combination of H_2:CO_2 with the ratio of 80:20 for flushing and creating the anaerobic condition, which have to be modified in this study because that specific gas was unavailable. L-cysteine hydrochloride$_2$O and $Na_2S.9H_2O$ are not added to the media because it was unavailable. Microorganism are characterized by Gram staining.

Statistical analysis

Regression analysis was used to determine whether a cumulative biogas volume had any relation to *Chlorella* sp. "CD01" volume, rumen fluid volume, or cow manure volume used in this study. Such a cumulative biogas volume was the dependent variable, whereas the other volume were the independent variables. Data presented in the graph for CK and CR group will be the averaged value of two bioreactor replicate.

3. Results and Discussion

The influence of (S/X) ratio to cumulative biogas production

The aim of this study was to investigate how different substrate inoculum (S/X) ratio using *Chlorella* sp. "CD01" as the substrate would affect biogas production. Two different inoculums, cow rumen fluid and cow manure, were used to digest *Chlorella* sp. "CD01" in a simple anaerobic bioreactor. The cumulative biogas production of each bioreactor grouped by 4 is given at Figure 2.

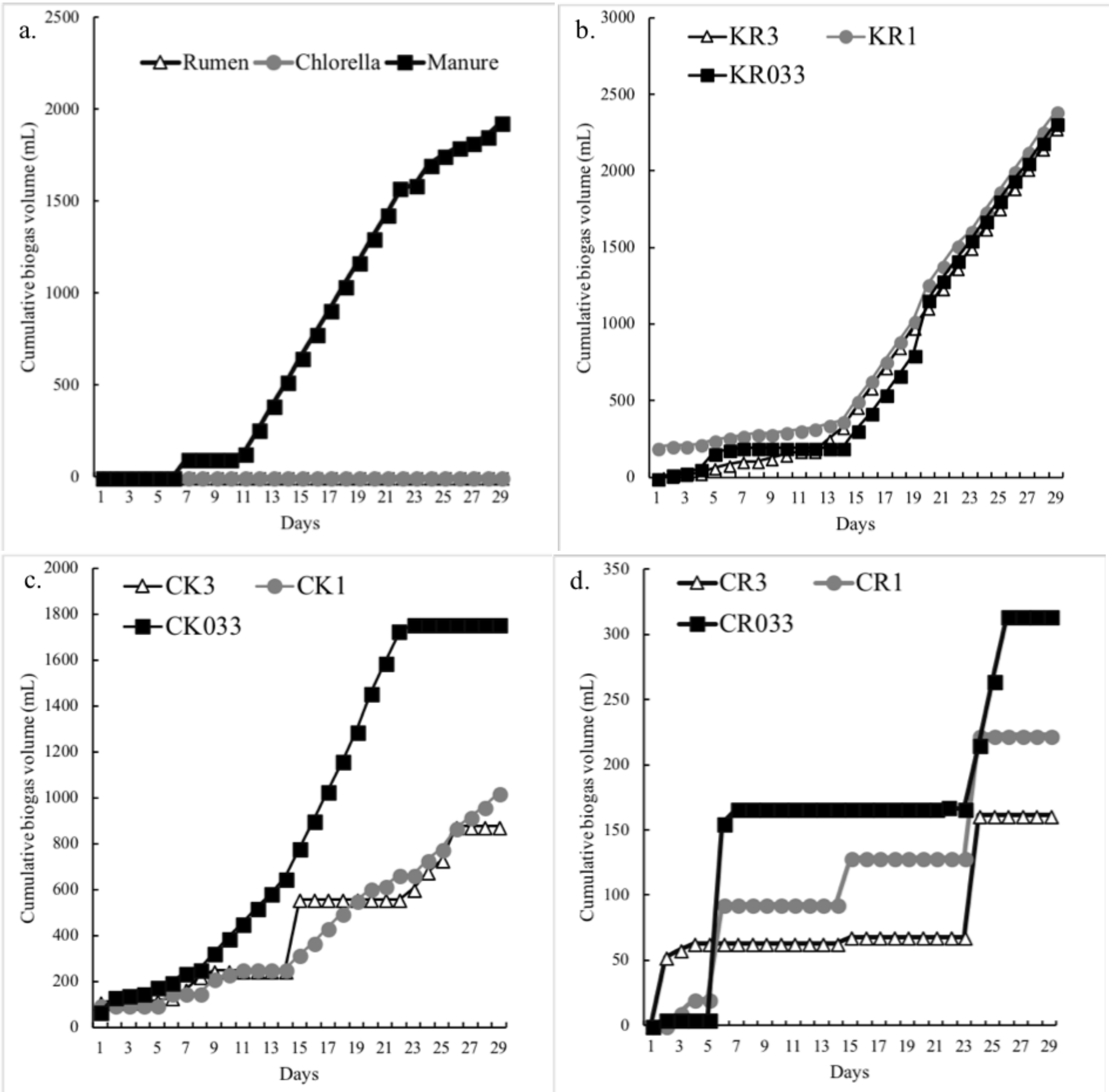

Figure 2. Cumulative biogas production of each bioreactor. a. Negative control group of Rumen, Chlorella, and Manure bioreactor; b. Positive control group of KR3, KR1, and KR033; c. Treatment group CK3, CK1, and CK033; d. Treatment group CR3, CR1, and CR033. Positive control and treatment group were named using two letters and numbers. Letters indicated the content of the bioreactor: K-Manure; R-Rumen fluid; C-Chlorella sp. “CD01”. Numbers indicated (S/X) ratio of 3, 1, and 0.33. Data for graph c. and d. is the average value of two replication bioreactor.

Figure 2.a. showed that rumen bioreactor did not produce any biogas after 29 days of observation. This showed that filtered rumen fluid did not contain any degradable organic material for the microorganism to digest, thus no biogas was produced. Previous study [17] stated that filtered rumen fluid of the cow could not produce biogas on its own. Microalgae bioreactor which contained *Chlorella* sp. “CD01” showed the same result as rumen bioreactor.

Chlorella should have been able to produce biogas even without any additional organic material. *Chlorella* used in this study had also been pretreated to enhance degradation which lead to biogas production. Previous study [13] had shown that untreated 2 g/L *Chlorella vulgaris* was able to produce about 200 mL of biogas after 30 days of experiment. It was deduced that the amount of biomass presented by total solid of Chlorella bioreactor were too low to produce a measurable amount of biogas. Even though *Chlorella* sp. “CD01” stock in this study had reached 2 g/L, it didn’t seem enough. *Chlorella* sp. “CD01” might have higher resistance toward degradation as it was isolated from a polluted river.

Manure bioreactor had produced 1930 mL biogas. Manure itself had the capability to produce biogas without any additional organic material as stated by previous studies [19] [20]. Manure had both the substrate and microorganism to undergo anaerobic digestion and biogas production, even in the presence of some complex hydrocarbon such as lignocellulosic material that were deemed to be un-optimal for anaerobic digestion [4]. Biogas accumulated by this bioreactor was burnable, resulting a blueish flame.

The KR bioreactor produced the most biogas in this study as seen on Figure 2.b. The KR1 produced the most which was 2391 mL biogas. The KR1 had (S/X) ratio of 1 between rumen fluid and cow manure. Previous studies [4] [17] reported the same result that the most biogas was produced using 1:1 rumen fluid and cow manure. All the 3 ratio variations of the KR bioreactors had not shown any difference in the cumulative biogas production. This was to be expected, as previous study [4] had reported that differences in the cumulative biogas production with various ratio of manure and rumen fluid could be seen after 40 days of digestion. This is not a concern because the KR bioreactor was not the main focus of this study, it was only to be used as a comparison to the treatment groups, which were the CK and the CR bioreactors.

The CK bioreactor had *Chlorella* sp. “CD01” as the substrate and cow manure as the inoculum. Figure 2.c. showed that the cumulative biogas production increased as value of (S/X) ratio went down. The CK3 with (S/X) ratio of 3 produced 876 mL biogas, the CK1 with (S/X) ratio of 1 produced 1022.5 mL, and the CK033 with 0.33 (S/X) ratio produced 1758 mL biogas. The K3 bioreactor began producing biogas rapidly on the 8th day of observation. CK3 and CK1 bioreactors began their biogas production on the 15th day. The cumulative biogas production from the CK3 bioreactor was not as steady as the CK1 and the CK033 bioreactor. This could be seen by the linear line indicating non-growing cumulative volume of biogas on the 15th to 21th day. This stagnant period can also be seen on the 26th day onwards, thus indicating that (S/X) ratio of 3 was less efficient compared to the other ratio.

The CK1 bioreactor showed a steady biogas production until the end of experiment. Unlike the CK033 bioreactor, a stagnant period was not present in the CK1 bioreactor. The (S/X) ratio of 1 showed by the CK1 bioreactor might potentially produce more cumulative biogas than the CK033 bioreactor if given more time, alas its production was not as fast as the CK033 in this study. Daily biogas production of CK033 and CK1 bioreactor was 62.62 mL and 35.25 mL, respectively. This result indicated that lower (S/X) ratio produce faster daily biogas production when using *Chlorella* sp. “CD01” and cow manure as the substrate and the inoculum.

Result from the CK bioreactor differed from a previous research [13] that the cumulative biogas production increased with the addition of *Chlorella* using dairy manure waste as the inoculum. But another study had reported that the low substrate inoculum ratio was preferred for producing biogas using mix culture of microalgae as the substrate and activated anaerobic sludge as the inoculum [16]. These two contradicting studies showed that different substrates and inoculums would have different optimum ratios. Perhaps dilution of the inoculum was the factor affecting the results between our study and the previous studies. The concentrated manure perhaps affected the number of microorganism inside the inoculum that could interact with the *Chlorella* biomass. This findings were also supported by the fact that the biogas production increased by increasing the volume of inoculum. Thus the (S/X) ratio of 0.33 produced the best result in our study, even though the amount were less than what the KR group had produced.

The CR bioreactor had the same trend of the cumulative biogas production as the CK bioreactor. Smaller (S/X) ratio had increased the cumulative biogas production. It was likely that the smaller (S/X) ratio contained more microorganism, thus increasing the productivity. The highest cumulative biogas in the CR group was 314.5 mL, produced by the CR033 with (S/X) ratio of 0.33. It was 48% more than what the CR3 had produced and 41% more than the CR1.

Co-digestion of *Chlorella* sp. "CD01" and rumen fluid had enhanced the biogas productivity by 150% compared to *Chlorella* alone in the previous study [13]. Addition of rumen fluid inoculum percentage inside a working bioreactor had increased the biogas production for microalgae degradation [16]. Numerous studies have also reported that microalgae co-digestion gave better result compared to using microalgae as a sole feed. Although the co-digestion using *Chlorella* sp. "CD01" and rumen fluid was able to produce biogas, it was not optimal compare to previous study. Other experiments [21] in biogas production using microalgae biomass was capable in producing twice the volume compared to our study.

Biogas yield

Biogas yield is obtained by dividing the cumulative volume of biogas by TS of respected bioreactor. Yield for Chlorella and rumen bioreactor were 0 because there were no biogas. Yield of manure bioreactor was 190.28 mL/gTS. Positive control group KR have an average yield of 163.44 mL/gTS. CK3 has the most yield in CK group, which was 179.84 mL/gTS. CK1 and CK033 have similar yield, which were 95.6 and 98.96 mL/gTS respectively. CR3 has the most yield in CR group, which was 68.99 mL/gTS. CR1 has the lowest yield of all, at 39.58 mL/gTS. Yield of CR033 was 43.23 mL/gTS.

It is not safe to assume that the highest yield was the best in this study because there was limitation. The amount of *Chlorella* sp. "CD01" substrate were not constant throughout the bioreactor. Ratio, as in volume was the main variable, thus did not focus on the consistency of biomass. Yield stated in this study were available for future reference only.

Statistical analysis of biogas production

Regression analysis is valid because significance F value from the data was significant ($\alpha = 0.05$). Our analysis showed that manure was the only independent variable that had a significant effect (p-value < 0.05) toward the cumulative biogas volume. There was a positive relation between the manure and the biogas volume. *Chlorella* sp. "CD01" and rumen fluid did not showed any correlation toward the cumulative biogas volume because their relation were not significant.

Correlation of COD value and biogas production

The COD removal indicated that more organic material were broken down to gases such as H_2, CO_2, and CH_4. Most COD values of manure containing bioreactors like the KR and the CK decreased at the end of our experiment. The COD value of all CR bioreactor increased by 80%. This showed that biomaterials inside CR bioreactor were just starting to be broken down as the COD value went up. It meant that the biogas production stage had not been fully achieved for all the CR bioreactors. This was why the cumulative biogas production in our study was lower compared to other previous study [21].

Aanaerobic microorganism in the bioreactor

Several microorganism had been isolated and characterized morphologically using Gram staining method. All isolated microorganism were around 6 to 10 μm in size. There were both Gram positive and Gram negative bacteria. Specific methanogenic media and anaerobic culturing technique [18] were used, thus raises the probability that isolates were either methanogens or anaerobic microorganism.

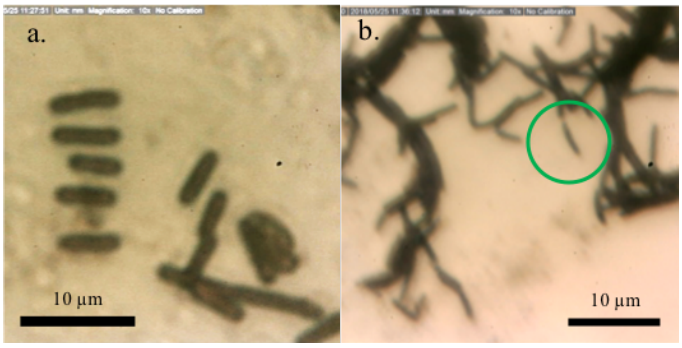

Figure 3. Isolated microorganisms from bioreactor. Observed under light microscope with 1000 X magnification. a. isolate "IM01"obtained from manure bioreactor; b. isolate "ICR01"obtained from CR3 bioreactor.

The microorganism isolated from the manure bioreactor was 6-8 μm in size. It was a Gram positive indicated by the blue stain, long basil bacterium with a rounded edge and did not form chains between cells. Based on the selective media and culturing technique used, microorganism "IM01" is presumed to be methanogens genus *Methanobacterium*. Figure 3.a. showed the observation photograph of the manure bioreactor isolate. The microorganism isolated from CR3 bioreactor was about 10 μm in size. It was a Gram positive with an elongated basil form. It was often found in groups forming a long structure. This microorganism named "ICR01" was presumed to also be methanogens from the same genus as before. Figure 3.b. showed photograph of the microorganism "ICR01". The green circle in Figure 3.b. pointed out a single cell.

Some common methanogens that were previously isolated in other studies from cow's rumen fluid and manure were *Methanobacterium bryantii* and *Methanobacterium formicium*. *Methanobacterium bryantii* is a common methanogenic bacterium that could be found in waste and sludge, including barns and cattle manure [22]. *Methanobacterium formicium* were reported present inside the rumen of cow [24]. Isolate "IM01" have similar cell shape and gram stained to *Methanobacterium bryantii,* and isolate "ICR01" have similar cell shape and gram to *Methanobacterium formicium*. The presence of anaerobic and methanogenic bacteria showed that anaerobic condition was achieved inside the simple bioreactor.

4. Conclusion

Several ratios of substrate and inoculum (S/X) were studied using a simple anaerobic digestion system. The biomass of *Chlorella* sp. "CD01" was the main substrate while cow manure and rumen fluid were the inoculum. The highest cumulative biogas production could be achieved by the (S/X) ratio of 0.33; which were the CK033 and the CR033 bioreactor. The CK033 produced as much as 1758 mL, and the CR033 produced 314.5 mL biogas within 29 days. There were two microorganisms; "IM01" and "ICR01"; presumed to be methanogens were isolated from different bioreactors. Morphological analysis were not enough to fully identify the corresponding microorganism as it sometimes can be elusive. Unfortunately molecular analysis was unavailable for these isolates. The evidences of anaerobic microorganism present inside the simple digester proved that the digester was able to achieve anaerobic condition for biogas production. The biogas production using *Chlorella* sp. "CD01" biomass as the substrate using rumen fluid and cow manure inoculum was proven to be plausible, however more focused studies are required to further enhance biogas production and understand the interaction between *Chlorella* sp. "CD01" and each inoculum. The amount of total solid using this specific substrate and inoculum also needs further researches. Further study of cow manure and rumen fluid inoculum enrichment and acclimatization to enhance substrate degradation may also be explored.

Acknowledgements

This work was funded by the project of "Insentif Riset Sistem Inovasi Nasional 2018" (INSINAS

2018) from Indonesian Ministry of Research Technology and Higher Education (KemenRistekDikti). Authors would also express their gratitude to all staff members of Laboratorium Pusat Teknologi Lingkungan (PTL) - BPPT and the Biotechnology Laboratory of Surya University for the supports and guidance.

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