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Selection of the Non-methanogenic Microorganism for Biogas Production from Napier Grass Extract

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Abstract

This study selected outward methanotrophic bacteria for biogas potential experiments in Napier at the age of 60 and over 90 days using the Biochemical Methane Potential method (BMP) until steady state at mesophilic temperature. In this experiment, Clostridium beijerinckii and Cutibacterium acnes were chosen because they both have biogas potential in extracted Napier at the age of 60 and over 90 days of 59.98% maximal methane production. The accumulation of methane production was 4.84, 1.02, and 0.53 ml, respectively, as a result of Napier extract of 60 days with *Clostridium beijerinckii* ($N_{60}G$), 90 days with *Clostridium beijerinckii* ($N_{90}G$), and 90 days with *Cutibacterium acnes* ($N_{90}K$), and 0.28, 2.26, and 0.33 ml/TSadded of BMP.

Keywords : Biogas; Non-methanogenic; DNA sequence; Methane production; Napier grass extract

Introduction

Energy is the main instrument for driving economic growth, reducing poverty, and livelihood opportunities. Intense research has been conducted into new technological techniques for generating clean and sustainable energy from renewable sources [1] of energy because of the constantly rising global energy demand, the depletion of fossil fuels [2, 3], rising prices for oil, and the growing worries about environmental issues. However, there is a problem with energy sufficiency because there are not enough fossil resources. Consequently, there has been an increase in the utilization of various forms of renewable energy in recent years [4, 5].

The treatment and stabilization of organic component wastes using anaerobic technology are well recognized as being effective, and it also produces methane in the process [6] and anaerobic digestion is a popular approach for converting biomass into energy [7, 8]. Organic wastes and a variety of lignocellulose materials can be used in anaerobic treatment [9] and also used to prevent wastewater pollution [10], industrial waste [11], and municipal waste [12] and has proven to be a potential method of producing hydrogen and biogas, two renewable energy sources [13]. Moreover, A huge and diverse array of agricultural feedstocks are useable in Thailand for the manufacture of biogas. Numerous energy crops can be used to produce biogas, such as sugarcane, sorghum, and Napier grass. The net yield per hectare, high nutrient content, and cultivation ease are all key considerations when selecting energy crops for biogas production [14]. Feedstocks should be easy to cultivate, harvest, and store, drought-tolerant, and able to grow in low-nutrient soil. With a high organic content, including protein and carbohydrates, Napier grass (*Pennisetum purpureum*) is a substance that is used for energy crops (livestock) [15], Napier grass gives the highest methane production rate of approximately 11.46 ml/day compared with other feedstock in Thailand [16]. The harvest age of Napier grass was affecting the biogas and methane production because the composition in each harvesting age was different cellulose decomposition [17]. With the ability of Napier grass was easy to plant and grow [18] making it popular to be a feedstock for animals, and the unharvested Napier grass turn into agricultural waste. Thus, the researcher sees an opportunity to change Napier grass to alternative livestock into methane production for reducing gricultural waste by non-methanogenesis microbe. Many non-methanogenesis that can produce methane in oxygen-saturated aquatic and terrestrial ecosystems such as cyanobacteria, algae, fungi, purple non-sulfur bacteria, and cryptogamic covers, produce methane in oxygen-saturated aquatic and terrestrial ecosystems [19].

The potential of biogas production from the extraction of Napier grass that was harvested in 60 and over 90 days inoculated with the outward microbe group from various sites was described in this study.

Materials and Methods

Selection of methane-producing anaerobic microbe

Microbe selection and methane production ability

Samples were collected from two different locations. 3 samples from Klong Ha, Klong Luang, Pathum Thani province and 4 samples from an organic goat farm, which is located in Bang Sai, Phra Nakhon Si Ayutthaya province (Table 1). The inoculation was done by using an anaerobic jar. Anaero pack™ was incubated in 37ºC for 12-18 hrs and suspended solids at dilution 10^{-6} cell/ml, later were evacuated into RCM (Reinforce Clostridium Medium). Determination of colony was carried out by simple streak method, [20] then analysis of methane production by gas chromatography (Agilent Technologies GC 6890, USA).

Table 1 Sites and types of soil samples

Site	Samples
Klong Ha, Klong Luang, Pathum Thani	1. Soil at 10 cm deep 2. Soil under the garbage dump 3. Water-well sediment
Organic goat farm Bang Sai, Phra Nakhon Si Ayutthaya	4. Soil at 10 cm deep 5. Soil under the garbage dump 6. Water-well sediment point-1 7. Water-well sediment point-2

Biomolecular approach for morphological characterization and classification of microbe species

All samples were DNA extraction following by Zhou et al (1996) [21]. Pair primers of 27F (5'- GAGTTTGATCMTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3') were used for Polymerase Chain Reaction (PCR). Mixture PCR solution contained 5 pmol/µl each primer, 10 mM dNTP, 10X PCR buffer+$MgCl_2$ 5U/µl Taq DNA poly-merase, and adjusted DI water to total volume at 50 µl. The PCR condition with the thermal profile of denaturation step by 1 cycle, 94°C for 3 min, annealing step followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.1 mins, and final extension step at 72°C for 10 min. PCR products were kept under 4°C and then were checked targeting size by 1% agarose gel with 1X TBE buffer under electrode at 100 volts for 40 min and fragment DNA base was sent tosequencing and blasted result by NCBI's GenBank.

Napier grass extract preparation and Characteristic Analysis

Napier grass was obtained from the Faculty of Agricultural, Kasetsart University, Kamphaeng Sean campus, Nakorn Prathom province, Thailand. The Napier grass (*Pennisetum purpureum*) was harvested at 60 (Pak Chong 1 stain) and >90 days (mixed Pak Chong 1 stain at the age of 90 days with Taiwan stain at the age of 110 days), and, extracted by cold screw press shown in Figure 1(A). Napier grass of 60 days and over 90 days were extracted approximately 100 ml/kg and 50 ml/kg (Figure 1B), respectively.

(A) (B)

Figure 1 Cold screw press for produce extract Napier grass (A) and Napier grass extract age >90 days in serum vials (B)

The physical and chemical characteristics of Napier extract were determined in COD, SCOD, TKN, ammonia, TS, VS, alkalinity, VFA, and pH in triplicate according to the procedures in the APHA Standard Method [22] and analyzed in 3 replicates shown in Table 2.

Biochemical methane potential assay

The BMP method (biochemical methane potential) was used to determine the optimum day of Napier grass and hydraulic retention time (HRT) with the highest biogas yield in lab-scale [23], and the studies were carried out in serum vials in replicate, as shown in Table 2.

Table 2 Conditions of the experiments

Age (days)	Sample	Napier Grass extract (ml)	Microbe (mg)	
			G	K
60	$N_{60}G$	60	600	-
	$N_{60}K$	60	-	600
	$N_{60}KG$	60	300	300
	$N_{60}C$	60	-	-
>90	$N_{90}G$	60	600	-
	$N_{90}K$	60	-	600
	$N_{90}KG$	60	300	300
	$N_{90}C$	60	-	-

Remark: G: *Clostridium beijerinckii*, K: *Cutibacterium acnes*, C: without inoculation

All sample-experiment tests were incubated at 35°C with 120 RMP for 24 hrs, and the samples were collected on an alternate day until the reaction ended.

Analysis

COD, SCOD, TKN, ammonia, TS, VS, alkalinity, VFA, and pH were determined before and after the experiments. Gas samples were analyzed with gas chromatography in three replicates, and the biochemical methane potential (BMP) was calculated as follows:

$$\text{BMP} = \frac{(ml\ Methane)}{gTS\ removal} = \frac{Biogas\ (l) \times Methane\ (\%)}{(TS\ influence - TS\ effluence)(mg/l)}$$

Results and Discussion

The result of anaerobic microbe selection and biogas production

Anaerobic microbes from nine samples produced methane, including soil under garbage dumps (Isolates 3 and 5), water-well sediment (Isolates 6 and 7), and the organic goat farm Bang Sai in Phra Nakhon Si Ayutthaya. These samples included soil 10 cm deep (Isolate No. 9), soil under garbage dumps (Isolate No. 13), and water-well sediment (Point 1). However, Ananou et al. [24] found that 10 cm of the soil sample had the ability to produce biogas from fermented paper. 3,000 and 4,000 ml produced 6 and 9 ml/g of paper, which was consistent with this study (Figure 2).

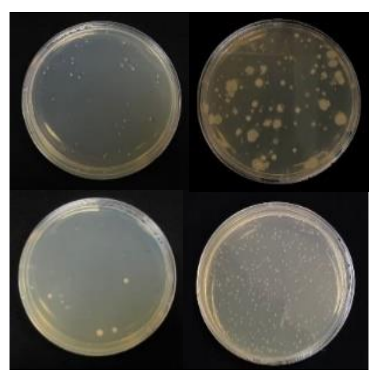

Figure 2 Colony characteristics of microorganisms at dilution 10^{-2}-10^{-8}

Result of methane production

The K5-1, G-5, and G-2 samples had the highest methane contents of 87.32%, 81.46%, and 48.13%, respectively (Table 3). The K5-1 and G-5 showed the highest methane content. And the production of methane in the extraction process was at Napier's age of 60, and over 90 days were tested in the experiments.

Morphology

Methane-producing microbes from nine isolates were gram-stained to determine their arrangement and staining. According to Table 4, nine isolates were gram-positive, seven isolates were bacilli, and group arrangement Cocci and group A were the other two isolates.

Results of biomolecular analysis

Classification of microbe species by biomolecular method. Furthermore, the DNA base content was compared to the NCBI's GenBank program. The identified microbe species resulting from K5-1 and G-5 were Clostridium beijerinckii (MN733990.1) and Cutibacterium acnes (MT613579.1), respectively, and were more similar than 99%.

Table 3 Characteristics of the growth, gas production, and methane content of microbe

Sample	Isolate (label)	Colony	Biogas Production	Methane content (%)
1. soil at 10 cm deep	1	circular, milky white, small	-	/
	2	circular, milky white, small	-	/
2. soil under the garbage dump	3 (K5-1)*	smooth-edged, cream	+	87.32
	4	circular, milky white, small	-	/
	5 (K5-4)	circular, milky white, small	+	19.78
3. Water-well sediment	6 (K5-2)	jagged-edge, clear white, small	+	/
	7 (K5-3)	smooth-edged, cream	+	/
4. soil at 10 cm deep	8	circular, white, smooth-edged	-	/
	9 (G-1)	circular, white, smooth-edged	+	21.74
	10	jagged edge, milky white	-	/
5. soil under the garbage dump	11	circular, cream, smooth-edged	-	/
	12	circular, clear, smooth-edged	undeveloped	/
	13 (G-2)	circular, milky white, small	+	48.13
6. Water-well sediment point1	14	smooth edge, milky white, small	-	/
	15 (G-3)	jagged-edge, milky white, jagged surface	+	21.13
	16 (G-4)	jagged-edge, milky white, jagged surface	+	9.09
	17	circular, milky white, small	-	/
7. Water-well sediment point2.	18	cocci, clear, small	undeveloped	/
	19 (G-5)**	cocci, white, smooth-edged	+	81.46

Remark * : *Clostridium beijerinckii*, ** : *Cutibacterium acnes*, **-** : No gas production, + : Generate gas, / : No methane content

Table 4 Morphology by gram's straining method

Isolate	Appearance	Arrangement
3 (K5-1)	bacilli	single
6 (K5-2)	bacilli	single
7 (K5-3)	cocci	group
5 (K5-4)	bacilli	single
9 (G-1)	cocci	group
13 (G-2)	bacilli	single
15 (G-3)	bacilli	single
16 (G-4)	bacilli	single
19 (G-5)	cocci	group

Biochemical methane potential (BMP)

Biogas producing potential

Table 5 shows the physical and chemical properties of the Napier extract at 60 and 90 days. The high organic content of Napier extract makes it suitable for anaerobic treatment, with methane produced as a byproduct of selected microbe activity. The C/N ratio was 286.40 and 262.34 after inoculating selected microbes into Napier extract age 60 and over 90 days [25] A high C/N ratio reduces nitrogen production and reduces biogas production. A C/N ratio of 20 to 30 is appropriate for anaerobic digestion, and biogas produces the highest yield [26]. However, if the C/N ratio is low, it will cause the system to accumulate a lot of ammonia as a result of the breakdown of nitrogen, which will result in alkalinity and the formation of biogas [27]. According to J. Dioha et al. [26], who studied the effect of the C/N ratio of the substrate on biogas methane inoculated with cow dung, poultry droppings, rice husks, neem tree leaves, and sugar cane bagasse, the C/N ratios of neem tree leaves and sugar cane bagasse were 82:1 and 47:1, respectively. While digesting, those substrates have little or no odor. However, the biogas yield of neem tree leaves and sugar cane bagasse was low (0.65 and 0.20 m^3/kgVS). Napier's extract VFA/Alkalinity Ratios at 60 and over 90 days were 1.64 and 3.50, respectively: a VFA/Alkalinity Ratio greater than 0.3-0.4 reduced stability in anaerobic digestion [28]. by accumulating in the system volatile fatty acids, causing the pH to become acidic [29].

Table 5 Characteristic of Napier grass extract at 60 and >90 days (mean±standard deviation)

Parameter	unit	Days	
		60	**>90**
COD	mg/l	133,653.33± 1,293.33	134,666.67± 4,664.99
sCOD	mg/l	123,946.67± 466.93	60,131.20± 4528.27
TKN	mg/l	466.67±80.83	513.33±80.83
NH4	mg/l	5.13±0.81	4.20±1.40
Alk	mg/l as $CaCO_3$	305.33±18.04	166.67±28.87
TS	mg/l	104.2083± 1.2925	103.9064± 28.9751
VS	%	52.37±23.69	77.20±30.00
VFA	mg/l as $CaCO_3$	500.00±0.00	583.33.00±72.17
pH	-	5.00±0.00	5.43±0.20

Complex molecules were broken down into simple molecules by the enzyme of hydrolytic bacteria, and those molecules were transformed into other organic substances such as organic acids and alcohol. process of anaerobic acetic acid, carbon dioxide, hydrogen, and methane Thus, methane production can indicate hydrolytic activity [30].

Archaea, a methanogenic microbe, produces methane as a metabolic byproduct in anoxic environments. It has an unusual metabolic system because bacteria use H_2, CO_2, methylated C_1 compounds, or acetic acid as a carbon energy source to grow [31]. Methane production occurs by two methods: splitting the acetic acid molecule to create carbon dioxide and methane or reducing carbon dioxide with hydrogen [32]. It was observed that during the first 24 hr. no lag phase occurred in all experiments, like the research of Amornpan et al. [33], who studied the activity relationships in anaerobic sludge anaerobic digestion systems from various commercial digesters, such as pig farms, palm oil mills, and concentrated rubber latex factories, and found that no lag phase appeared from any inoculate sets over the first 24 hr.

The biogas potential of anaerobic digestion systems of microbes selected for fermentation in Napier extract at 60 and over 90 days is shown in Figures 3 and 4, respectively, for Clostridium beijerinckii at 60 and 90 days. Napier extract had biogas accumulations of 86.07 and 134.40 ml, respectively. 59.98% and 1.22% of maximal methane, methane accumulation was 0.16 and 0.11 ml, respectively, and 0.28 and 0.26 ml/gTS_{added} of BMP, respectively.

Cutibacterium acnes accumulated 99.70 and 108.80 ml of biogas in Napier extract ages 60 and over 90 days, respectively. Methane accumulation was 0.10 and 0.15 ml, respectively. Maximal methane was 22.65 and 21.07%, respectively and 0.44 and 0.17 ml/gTD of BMP, respectively.

Clostridium beijerinckii mixed with *Cutibacterium acnes* in equal proportions has methane accumulations of 54.27 and 107.63 ml, respectively. 59.98% of maximal methane, 0.02 and 0.08 ml of accumulated methane, respectively, and 2.29 and 0.33 ml/gTS_{added} of BMP, respectively. *Clostridium beijerinckii* and *Cutibacterium acnes* were anaerobic bacteria, and when they were in the right condition, they would generate biogas. However, research on the biogas potential and methane production of *Clostridium beijerinckii* and *Cutibacterium acnes* is limited.

The control experiment at 60 (N60C) and 90 (N90C) days had biogas accumulation at 20.80 and 16.16 ml, respectively; maximal methane at 0.18 and 0.15%, respectively; and methane accumulation at 0.65 and 0.03 ml, respectively. The BMP concentrations were 0.06 and 0.03 ml/g T added, respectively. The experiment with the control (N60C and N90C) produced biogas with the microbe that could be contaminated by the microbes on the Napier grass extract without sterilized and under anaerobic conditions.

In all experiments with 60 and 90 days of extracted Napier grass, Napier had HRT at 11 and 21. The methanogen bacteria are pH-sensitive and do not cause degradation activity at pH below 6.2, causing less production of methane [34]. Most anaerobic bacteria, including methanogen bacteria, have an optimal pH in the range of 6.8-7.2 [35]. The generation of biogas is influenced by a variety of variables, including temperature, concentration, material characteristics, and the carbon-nitrogen ratio. The production of biogas depends on mixing, acidity, and material type [36].

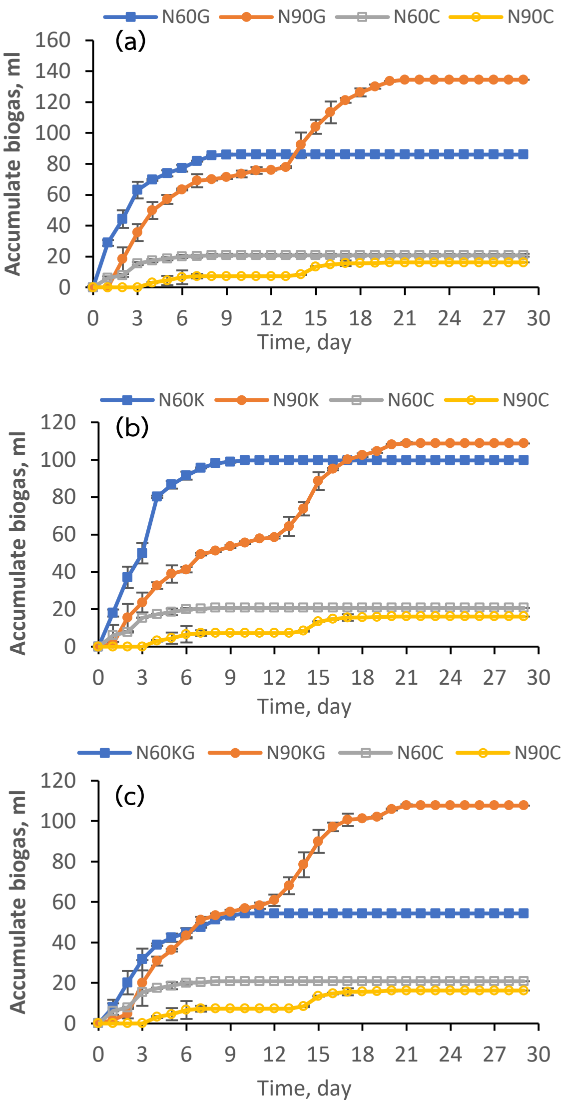

Figure 3 Biogas accumulation of extraction Napier age 60 days and >90 days inoculated with microbe (a) *Clostridium beijerinckii*, (b) *Cutibacterium acnes*, (c) *Clostridium beijerinckii* mix with *Cutibacterium acnes*

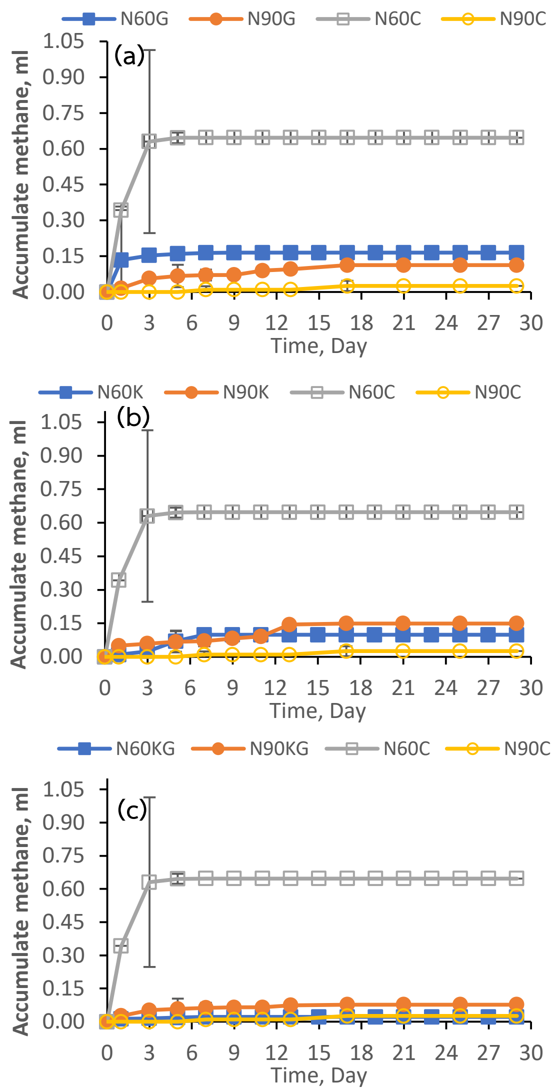

Figure 4 Methane accumulation of Napier extract age 60 days and >90 days inoculated with microbe (a) *Cutibacterium acnes*, (b) *Clostridium beijerinckii*, (c) *Clostridium* beijerinckii mix with *Cutibacterium acnes*

Conclusions

The study of biogas production potential from water Napier extract aged 60 and over 90 days by outward microbes found that N60G, N60GK, and N90GK had maximal methane of 59.98%, total methane accumulation of 4.84, 1.02, and 0.53 ml, respectively, and BMP of 0.28, 2.26, and 0.33 ml/TS_{added}, respectively.

The results showed that Clostridium beijerinckii and Cutibacterium acnes were the outward group of biogas-producing microbes that had the potential for biogas production by anaerobic digestion from extraction Napier at age 60 and over 90 days, with 11 days of HRT. Therefore, anaerobic digestion depends on the C/N ratio and the VFA/alkalinity ratio. The microbe will be encouraged to perform more effectively if it is in the optimal range, including in terms of producing methane and biogas.

The goal of this study was to experiment with outwardly visible microbes that are easily found and natural at those sites. This result demonstrated that the visible microbes have the potential to produce methane, but at a lower yield than the methanotroph. Because they are a carbon source for methanotrophs and have a low tolerance and slow rate of production in the anaerobic digestion system, these visible microbes can also produce CO_2. However, we expect the outwardly facing microbes would help to actuate the activity of methanotrophs in the sense of producing more carbon sources to improve methane production.

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