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Biochemical hydrogen and methane potential of sugarcane syrup using a two-stage anaerobic fermentation process

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ABSTRACT

The potential of using a two-stage hydrogen and methane fermentation of sugarcane juice was studied. The effects of pure and mixed culture as inocula on hydrogen production were compared. Additionally, the effects of pure culture inocula form, i.e., free cells or immobilized cells of *Clostridium butyricum* TISTR1032 was compared to different forms of mixed cultures, i.e., granules or suspended cells of heat-treated upflow anaerobic sludge blanket (UASB) granules. The hydrogenogenic effluents from all treatments were used as substrates to evaluate the potential of methane production by non-pretreated UASB granules. Results showed that a pure culture gave a higher hydrogen production potential and a shorter lag time in comparison to mixed cultures. Immobilized cells of *C. butyricum* TISTR1032 gave a hydrogen production potential that was 1.2 times higher than that of free cells. However, there was no significant difference in hydrogen production potential of granules and suspended cells. Moreover, hydrogenogenic effluent from the first stage fermentation showed a high efficiency in methane production by non-pretreated UASB granules. Although fermentation of mixed cultures resulted in lower hydrogen yield, its hydrogenogenic effluent yielded a higher methane production than that of pure culture. Therefore, overall energy yield when using mixed cultures in hydrogen production stage was higher. The two-stage hydrogen and methane production process removed 94–95% of chemical oxygen demand (COD) resulting in energy recovery in the range of 12–13.4 kJ/gCOD_{added}. Results indicated that the COD removal efficiency and the energy recovery from two-stage hydrogen and methane production was improved 6–7 fold when compared to hydrogen production alone.

1. Introduction

Due to energy shortages and environmental crises, renewable energy has become promising energy to substitute fossil fuels. Renewable energy like hydrogen has been widely recognized as a promising energy carrier since it has many advantages including high energy density, clean combustion and they can be produced from various biomass or wastes (Das and Veziroglu, 2001). Several technologies are available for producing hydrogen, such as electrolysis of water, partial oxidation of methane, and steam reforming of hydrocarbon substances (Levin et al., 2004). However, these technologies require fossil fuel or electricity to operate, which can aggravate energy shortages and hydrocarbon emission problems. Alternatively, biological hydrogen production through dark or photo fermentation is more suitable due to its unique characteristics, including environmental friendliness and being less energy intensive than other processes. The advantages of bio-hydrogen production through dark fermentation include a high production rate, no light energy requirement, and it is a simple and economical process (Argun and Kargi, 2011). However, the major drawbacks in dark fermentation are low yields and the production of volatile fatty acids (VFAs) causing effluents with high COD values necessitating treatment before discharge to the environment.

Anaerobic digestion is a biological process that can be used to treat waste/wastewater and recover methane from waste/wastewater. It is divided into two main stages. In the first stage, acidogenic bacteria digest organic compounds to hydrogen, carbon dioxide and VFAs, which are converted to acetic acid by acetogenic bacteria and subsequently converted to methane by methanogenic bacteria. The traditional one-stage methane production processes encounter problems such as substrate degradation and low energy conversion efficiency due to the different growth rates and optimum pHs of acidogenic and methanogenic bacteria (Liu et al., 2004). Therefore, two-stage fermentations have been proposed to improve substrate degradation and energy conversion

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(Hallenbeck, 2009; Hallenbeck and Ghosh, 2009). By separating the fermentation process into two stages, the process can give both hydrogen and methane with easier control of first and second stage conditions. This yields higher overall energy recovery and substrate degradation than single-stage fermentation processes.

Previous researchers reported a successful hydrogen and methane production by two-stage fermentation process from various kinds of biomass. Mamimin et al. (2015) conducted a two-stage fermentation process of palm oil mill effluent (POME) and found that the hydrogen and methane yields of 210 and 315 L/kgCOD, respectively, were achieved with a total energy yield of 15.34 MJ/kgCOD. Kumari and Das (2015) investigated the two-stage fermentation process of pretreated sugarcane bagasse and obtained the maximum hydrogen and methane yields of 93.4 and 221.8 mL/gVS, respectively. The energy conversion efficiency of 44.8% by the two-stage process was higher than a single stage hydrogen production process of 5.4%. Wang et al. (2013a) used a continuous two-stage fermentation to produce the hydrogen and methane from sugary wastewater and achieved the hydrogen and methane yields of 106 and 340 mL/$gCOD_{added}$, respectively. In this study, sugarcane juice was evaluated for the potential of hydrogen and methane production using a two-stage fermentation process. Sugarcane juice was chosen as the substrate because of its abundant supply in Thailand (Office of The Cane and Sugar Board, 2014). The main component of sugarcane juice is sucrose, which is a highly suitable carbon source for microbial growth; thus it can be directly used for hydrogen or methane production without any pretreatment or saccharification. Therefore, using a sugar-rich biomass like sugarcane juice and sweet sorghum juice can result in a high energy yield in comparison to lignocellulosic materials.

Bio-hydrogen production can be conducted by mixed and pure cultures. Hydrogen production using anaerobic mixed cultures is known for its operational simplicity, no requirement for sterile conditions and its ability to use a variety of organic wastes. However, the major disadvantage of using mixed cultures is low hydrogen yield because hydrogen may be removed by hydrogen consuming bacteria during hydrogen production. Anaerobic sludge from wastewater treatment plants can be used as an inoculum for hydrogen production after undergoing pretreatment to inhibit methanogenic bacteria. In this study, upflow anaerobic sludge blanket (UASB) granules from a brewery wastewater treatment plant were chosen as an inoculum of a mixed culture to produce hydrogen from sugarcane juice. The hydrogen production efficiency of UASB granules in free and suspended forms were compared. We hypothesized that a reduction of granule size, i.e., the suspended form, might increase the contact surface as well as the mass transfer rate and lead to increased hydrogen production.

Clostridium is a well-known pure culture capable of producing hydrogen. These bacteria include *Clostridium butyricum* DSM10702 (Ortigueira et al., 2015), *C. butyricum* TISTR1032 (Plangklang et al., 2012), *C. butyricum* CGS5 (Liu et al., 2012), *Clostridium tyrobutyricum* ATCC 25755 (Jiang et al., 2013), and *Clostridium acetobutylicum* ATCC824 (Cappelletti et al., 2011). Hydrogen production using pure cultures has high hydrogen production rates and hydrogen yields, but sterile conditions are needed for these processes. To improve the hydrogen production rate and yield, immobilization techniques have been used for hydrogen fermentation since hydrogen producing bacteria can be protected from harsh conditions e.g., substrate or product inhibition (Plangklang et al., 2012) by supporting materials. Therefore, the effects of immobilizing *C. butyricum* TISTR1032 on hydrogen production were studied and compared to hydrogen production by free cells.

The aim of this study was to explore the hydrogen and methane production potential of sugarcane juice in a two-stage fermentation. A simple bench-scale batch fermentation was used to evaluate the hydrogen and methane potential based on the COD equivalent of hydrogen and methane in gCOD unit. This method is a rapid and easily applicable method to estimate the two-stage hydrogen and methane potential and can be used for a clear comparison between different organic matters from various origins (Giordano et al., 2011). For two-stage fermentation, hydrogen was produced in the first stage and the differences in the abilities of mixed cultures in different forms (granules and suspended forms) and pure culture (free cell and immobilized cell) were compared. After the hydrogen production ceased, the hydrogenogenic effluent of the first stage was used as a substrate for methane production in a second stage with non-pretreated UASB granules as an inoculum. The hydrogen and methane production of the two-stage fermentation process was then evaluated on the basis of COD equivalents and energy recovery of biogas in kilojoule (kJ) units. Furthermore, glucose, sucrose and xylose were also used as model substrates in order to compare two-stage hydrogen and methane production on pure sugar with that on sugarcane juice.

2. Materials and methods

2.1. Feedstock preparation and fermentation media

Sugarcane stalks (*Saccharum offinarum* Linn.) was obtained from a local sugarcane field in Chaiyaphum Province, Thailand. To prepare sugarcane juice, sugarcane stalks were crushed using a sugarcane press and filtered through a thin layer of cloth. Sugarcane juice was boiled to concentrate the sugarcane syrup, cooled and kept in refrigerator at 4 °C before use. Sugarcane syrup had a total sugar concentration of 800 g/L (equivalent to 900 gCOD/L).

Sugarcane syrup was diluted with distilled water to a concentration of 25 gCOD/L before use as a carbon source. Additionally, glucose, sucrose and xylose (Sigma–Aldrich, Germany) at 25 gCOD/L were used as model substrates for comparison to sugarcane syrup.

Fermentation media was used to produce hydrogen. It consisted of (all in mg/L): K_2HPO_4 125; $MgCl_2{\cdot}6H_2O$ 15; $FeSO_4{\cdot}7H_2O$ 25; $CuSO_4{\cdot}5H_2O$ 5; $CoCl_2{\cdot}5H_2O$ 0.125; NH_4HCO_3 5240 and $NaHCO_3$ 6720 (modified from Endo et al., 1982).

2.2. Inoculum preparation

UASB granules were obtained from the UASB wastewater treatment plant of a local brewery in Khon Kaen, Thailand. The granules had the following characteristics (all in g/kg): total solids (TS) 115.5; volatile solids (VS) 102.2; total suspended solids (TSS) 109.7; and volatile suspended solids (VSS) 98.2. The granules were heat-treated in a hot air oven (Lab Tech Model LDO-100E, Korea) at 105 °C for 4 h to inhibit methanogenic bacteria. For an inoculum in granule form, the heat-treated granules were directly used as an inoculum for hydrogen production. For the suspended form, the heat-treated UASB granules were grinded by blender (Philips HR2118, Indonesia) and sieved (approximately 1 mm) before use as an inoculum. UASB granules with no pretreatment were used as inocula in methane production studies.

The pure culture used for bio-hydrogen production was *C. butyricum* TISTR1032 purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand. *C. butyricum* cells were kept in 50% glycerol at −20 °C as stock culture. Prior to use, they were activated and enriched in Tryptone Sucrose Yeast Extract medium with sucrose as a carbon source following the method of Pattra et al. (2011).

2.3. Immobilization of C. butyricum TISTR1032

Ca-alginate was used as a support material. Cell immobilization followed the method of Cheng et al. (2011). Briefly, enriched

C. butyricum TISTR1032 at a volume of 10% inoculum (v/v) (cell concentration of 10^7 cells/mL) was mixed with 3% (w/v) sterile Na-alginate at a volumetric ratio of 1:1. The mixed solution was filled into a 20 mL syringe and formed into Ca-alginate beads by gradually injected this material into a 1.5% sterile $CaCl_2$ solution. The beads had a diameter of approximately 2.0–2.5 mm and were allowed to harden for 1 h in a $CaCl_2$ solution prior to use. The immobilized *C. butyricum* TISTR1032 in Ca-alginate were visualized by scanning electron microscopy (JEOL, Model JSM-5410LV, Japan) (Rachman et al., 1998) at 1000×–10000× magnifications.

2.4. Two-stage hydrogen and methane fermentation and one-stage methane production

Batch fermentations to assay two-stage hydrogen and methane production were conducted in 120 mL serum bottles with a working volume of 70 mL. For first stage hydrogen production by mixed cultures, the fermentation media with 25 gCOD/L of sugarcane syrup, glucose, sucrose, or xylose as a carbon source was inoculated with 0.83 gTS (1% VSS, w/v) of heat-treated UASB granules or heat-treated UASB granules in suspended form. For hydrogen production by a pure culture, 7 mL of enriched *C. butyricum* TISTR1032 (10^7 cells/mL) as free cells or equivalent amount of immobilized *C. butyricum* in Ca-alginate beads (10% inoculum, v/v) were added into the fermentation media. Initial pH was adjusted to 6.5 with 5 M HCl or 5 M NaOH. After capping with rubber stoppers and aluminum caps, the headspace in serum bottles was purged with nitrogen gas for 10 min to ensure anaerobic conditions. All of the serum bottles were incubated at room temperature (30 ± 3 °C) and shaken at 150 rpm on an orbital shaker. Fermentation broth was collected at the beginning and end of fermentation to check COD and VFAs concentrations. All treatments were conducted in triplicate. Biogas was measured during fermentation using a wetted glass syringe method (Owen et al., 1979).

After the first stage hydrogen fermentation ended, the serum bottles were uncapped and drained of 50 mL of the hydrogenogenic effluent. The effluent was placed in a new serum bottle and 7.13 g wet weight (1% VSS w/v) of non-pretreated UASB granules were added to produce methane. For one-stage methane production, the fermentation media were mixed with 7.13 g wet weight (1% VSS w/v) of non-pretreated UASB granules. The pH of the fermentation broth was adjusted to 7.0 by addition of either 5 M HCl or 5 M NaOH. Each serum bottle was capped again and further incubated under the same conditions.

2.5. Reusability of the immobilized cells

After hydrogen fermentation from sugarcane syrup by immobilized *C. butyricum* TISTR1032 finished, fermentation broth in the serum bottle was discarded and then replaced by 63 mL of the fresh fermentation medium with 25 gCOD/L of sugarcane syrup as a carbon source. The bottles were incubated under the same conditions as described above. The immobilized *C. butyricum* was repeatedly used five times to determine its reusability in hydrogen production. Ca-alginate beads with immobilized *C. butyricum* were collected at the end of each reuse. Bead surfaces were microscopically examined (Olympus Model CH-2) at 100× magnification.

2.6. Analytical methods

pH was measured using a pH meter (pH 500Clean, USA). TS, VS, TSS, VSS and COD were analyzed according to standard methods (APHA et al., 1995). Total sugar concentration was measured using a phenol sulfuric acid method with glucose as a standard (Dubois et al., 1956).

Hydrogen, methane, and carbon dioxide contents of the biogas were determined using gas chromatography (GC) (GC-2014, Shimadzu Co., Ltd.) and expressed as percent biogas content. The GC was equipped with a thermal conductivity detector (TCD) and a 2-m stainless steel column packed with Shin carbon (50/80 mesh). The GC conditions followed the method of Pattra et al. (2008). The hydrogen and methane volume in biogas were calculated by mass balance (Zheng and Yu, 2005). The volumes of hydrogen and methane were expressed at standard temperature and pressure (STP, 0 °C and 760 mmHg). Hydrogen and methane yield were expressed in mLH_2 or $CH_4/gCOD_{added}$, respectively. A modified Gompertz equation (Eq. (1)) was used to fit the cumulative hydrogen and methane yield curves (Khanal et al., 2004).

$$H = P\exp\left\{-\exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where H is the cumulative hydrogen and methane production (mL), λ is the lag phase time (h), P is the hydrogen and methane production potential (ml), R_m is the maximum hydrogen or methane production rate (mL/h), t is the incubation time (h), e is the exp(1) = 2.718. Parameters (P, R_m, λ) were estimated using non-linear curve fitting in SigmaPlot 11.0 Trial Version (Systat Software Inc., USA). The specific maximum hydrogen or methane production rate (R_s) (mL/$gCOD_{added}$ × h) were obtained by dividing R_m of hydrogen or methane by COD_{added}.

VFA concentration in fermentation broth was analyzed by sampling the liquid in the serum bottles, centrifuging at 10,000 rpm for 5 min, acidifying with 0.2 M oxalic acid, filtered through 0.45 mm cellulose acetate membrane and kept at −20 °C prior to analysis by high performance liquid chromatography (HPLC) (Shimadzu LC-10AD). The HPLC was equipped with an Aminex HPX-87H column and a reflective index detector (RID). The temperature of the column was 45 °C. 5 mM of H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL/min.

2.7. Gas recovery estimation, COD balance, and energy yield

Hydrogen and methane recovery from one- and two-stage fermentation processes was estimated on a COD basis, i.e., the amount of the initial substrate that was converted to hydrogen and methane (Giordano et al., 2011). Recovery of hydrogen and methane in% (COD/COD) were calculated from Eq. (2).

$$\mathrm{Ri} = \frac{\mathrm{Pi} \times \mathrm{fi}}{\mathrm{CODs}} \quad (2)$$

where Ri is the hydrogen or methane recovery (% COD/COD), Pi is the maximum cumulative hydrogen or methane (mol), fi is the COD equivalent of hydrogen or methane (gCOD/mol), i.e., 16 $gCOD/molH_2$ and 64 $gCOD/molCH_4$, and CODs is the amount of substrate added (gCOD) at the beginning of hydrogen production.

To confirm whether the experimental data were consistent with the substrate distribution in the one- and two-stage processes, a COD balance was conducted according to the COD values of the products formed (i.e., hydrogen and methane). The COD balance was done by using Eq. (3).

$$\mathrm{CODbalance}\,(\%) = \Sigma\left[\frac{\mathrm{COD_{distribution}}}{\mathrm{COD_{initial}}} \times 100\right] - 100 \quad (3)$$

where $COD_{distribution}$ is a COD concentration of hydrogen or methane or the effluent. $COD_{initial}$ is the COD of substrate added to the hydrogen production process.

For the energy yield calculation, hydrogen and methane yield from one- and two-stage processes in $L/gCOD_{added}$ units were converted to energy yield in units of $kJ/gCOD_{added}$ by multiplying gas yield (hydrogen or methane in $L/gCOD_{added}$ units) and hydrogen with an energy content of 10.8 kJ/L (STP) (equivalent to 121 kJ/gH_2)

Table 1
Two-stage hydrogen and methane potential (mean ± standard deviation) of sugarcane syrup, glucose, sucrose and xylose.

Substrates	Inoculums	Hydrogen					Methane					Total recovery% (COD/COD)	COD removal (%)
		H_2 yield ($mL/gCOD_{added}$)	H_2 recovery (%COD/COD)	R_s[a] ($mL/gCOD_{added}$ h)	λ[a] (h)	R^{2a}	CH_4 yield ($mL/gCOD_{added}$)	CH_4 recovery (% COD/COD)	R_s[a] ($mL/gCOD_{added}$ h)	λ[a] (h)	R^{2a}		
Sugarcane syrup	Granules	91.7 ± 1.5	6.55	7.0	11.4	0.998	298.3 ± 14.0	85.22	0.8	170.4	0.992	91.77	94.72
	Suspended cells	88.8 ± 7.1	6.35	9.9	13.8	0.996	309.6 ± 0.6	88.46	0.9	178.6	0.991	94.81	95.17
	Immobilized cells	173.7 ± 2.4	12.41	6.4	7.0	0.994	272.8 ± 2.6	77.95	0.7	389.0	0.991	90.35	94.83
	Free cells	140.4 ± 1.2	10.03	14.0	10.2	0.999	258.5 ± 5.4	73.92	0.5	463.3	0.987	83.95	94.77
Glucose	Granules	143.9 ± 3.4	10.28	9.0	13.4	0.997	302.4 ± 10.6	86.41	0.8	151.9	0.991	96.69	95.98
	Suspended cells	140.8 ± 2.0	10.05	20.2	14.2	0.998	307.7 ± 22.3	87.92	0.9	163.2	0.992	97.97	96.68
	Immobilized cells	207.5 ± 1.9	14.75	13.1	5.9	0.999	273.3 ± 7.0	78.08	0.6	153.3	0.999	92.83	96.78
	Free cells	180.4 ± 6.1	12.90	11.1	9.0	0.997	263.4 ± 4.9	75.32	0.6	205.8	0.994	88.22	95.05
Sucrose	Granules	102.4 ± 1.4	7.31	8.3	13.4	0.998	287.2 ± 9.0	82.07	0.8	164.9	0.993	89.38	95.07
	Suspended cells	117.9 ± 2.4	8.42	15.9	13.7	0.997	313.6 ± 10.3	85.78	1.0	194.0	0.991	94.20	96.01
	Immobilized cells	165.9 ± 19.5	11.85	8.5	6.2	0.999	281.7 ± 7.8	80.50	0.5	220.7	0.992	92.35	98.74
	Free cells	150.2 ± 4.5	10.75	11.5	8.8	0.998	261.7 ± 5.8	74.77	0.5	440.1	0.982	85.52	98.69
Xylose	Granules	65.8 ± 4.6	4.70	2.5	18.6	0.991	277.1 ± 2.0	79.16	0.8	139.2	0.995	83.86	95.51
	Suspended cells	67.3 ± 0.4	4.81	2.0	18.3	0.994	290.1 ± 0.4	82.90	1.1	178.0	0.989	87.71	94.46
	Immobilized cells	129.8 ± 2.7	9.25	2.6	12.7	0.997	262.7 ± 4.2	75.06	0.5	334.8	0.994	84.32	89.61
	Free cells	125.1 ± 5.3	8.94	4.5	11.2	0.999	223.8 ± 2.5	64.02	0.4	449.6	0.986	72.96	83.15

[a]Parameters were obtained from modified Gompertz equation.

or methane with an energy content of 36 kJ/L (STP) (equivalent to 50 kJ/gCH_4).

2.8. Microbial community analysis

The effluent was collected at the end of hydrogen fermentation and centrifuged at 10,000 rpm for 5 min to separate solids and supernatant. The solids were kept in 50% sterile glycerol at −20 °C prior to microbial community analysis using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) following the method of Kongjan et al. (2010). Most of the bands were excised from the gel and re-amplified with primer 357f without a GC clamp and the reverse primer 518r. After re-amplification, PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by database searches in GenBank using BLAST (Altschul et al., 1997).

3. Results and discussion

3.1. Two-stage hydrogen and methane potential of UASB granules

Hydrogen production by both granules and suspended cells started after 12 h (Fig. 1a and b). No methane was detected during the fermentation, indicating that heat pretreatment was effective in inhibiting methanogenic bacteria. Hydrogen production from sugarcane syrup by UASB granules gave a hydrogen yield of 91.7 ± 1.5 mL/$gCOD_{added}$ or 6.55% H_2 recovery (Table 1). This was close to the hydrogen yield by suspended cells of 88.8 ± 7.1 mL/$gCOD_{added}$ or 6.35% H_2 recovery. Lag times of hydrogen fermentation by UASB granules and suspended cells were similarly (Table 1). A maximum hydrogen yield of 143.9 ± 3.4 mL/$gCOD_{added}$ was obtained when using glucose as a substrate and heat-treated granules as an inoculum. Hydrogen yield was lowest when xylose was used as substrate i.e., 65.8 ± 4.6 mL/$gCOD_{added}$ by granules and 67.3 ± 0.4 mL/$gCOD_{added}$ by suspended cells. The results suggested that the microorganisms present in UASB granules consumed both hexose and pentose sugars but preferred hexose over pentose sugars to produce hydrogen.

The hydrogen yields obtained from sugarcane syrup by both granules and suspended cells were lower, 36–37%, than that obtained from glucose. However, the hydrogen yield of sugarcane syrup by granules and suspended cells were close to that of sucrose. This is not surprising since the main component of sugarcane syrup is sucrose. Moreover, hydrogen yield from glucose, sucrose and xylose by granules and suspended cells were not significantly different. This may be due to the porous nature of UASB granules facilitating mass transfer.

The primary metabolite in the hydrogenogenic effluent from granules and suspended cells was butyric acid with concentrations in all treatments higher than 60% (Table 2), suggesting a butyrate type fermentation. Generally, hydrogen production via dark fermentation produces acetic and butyric acids as by-products. The theoretical yield of hydrogen production from glucose while acetic or butyric acid is only by-product is shown in Eqs. (4) and (5).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 4H_2 + 2CO_2 \quad (4)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_4H_8O_2 + 2H_2 + 2CO_2 \quad (5)$$

From Eq. (4), the maximum hydrogen yield by dark fermentation is 4 molH_2/mol hexose or 33.33% H_2 was recovered from the substrate when acetic acid was the only by-product. If butyric acid was the only fermentation by-product, the maximum hydrogen yield was only 2 molH_2/mol hexose or 16.67% H_2 was recovered from the substrate. However, the actual hydrogen yield is commonly lower than the theoretical yield since the substrate is often converted to other

Table 2
Soluble metabolite products (SMPs) in hydrogenogenic effluent from hydrogen production process (mean ± standard deviation).

Substrate	Inoculum	SMPs (gCOD/L)								Total VFAs (gCOD/L)	Total SMPs (gCOD/L)
		Acetic	Butyric	Propionic	Formic	Lactic	Citric	Succinic	Residual sugar		
Sugarcane syrup	Granules	4.64 ± 0.01	10.81 ± 3.42	–	0.19 ± 0.02	6.19 ± 2.01	–	–	1.60 ± 0.45	15.64 ± 3.45	23.43 ± 5.91
	Suspended cells	5.30 ± 0.33	10.80 ± 1.39	–	0.31 ± 0.17	5.63 ± 0.59	–	0.30 ± 0.10	1.74 ± 0.72	16.41 ± 1.89	24.08 ± 3.30
	Immobilized cells	1.87 ± 0.14	11.47 ± 0.24	–	0.01 ± 0.00	–	–	0.03 ± 0.01	2.85 ± 0.20	13.35 ± 0.38	16.23 ± 0.59
	Free cells	2.61 ± 0.26	9.77 ± 0.31	–	0.01 ± 0.00	–	0.24 ± 0.06	0.04 ± 0.01	5.30 ± 0.19	12.39 ± 0.57	17.97 ± 0.83
Glucose	Granules	3.50 ± 0.27	10.37 ± 1.36	–	0.40 ± 0.10	6.31 ± 1.66	–	0.10 ± 0.00	1.24 ± 0.02	14.27 ± 1.73	21.92 ± 3.41
	Suspended cells	4.53 ± 1.11	11.36 ± 0.49	–	0.39 ± 0.05	2.97 ± 0.00	–	0.08 ± 0.03	0.76 ± 0.19	16.28 ± 1.65	20.09 ± 1.87
	Immobilized cells	2.12 ± 0.33	11.48 ± 0.10	–	0.02 ± 0.00	–	–	0.07 ± 0.02	1.16 ± 0.30	13.62 ± 0.43	14.85 ± 0.75
	Free cells	2.55 ± 0.09	10.85 ± 1.08	–	0.04 ± 0.00	–	1.89 ± 0.12	0.19 ± 0.01	3.93 ± 0.29	13.44 ± 1.17	19.45 ± 1.59
Sucrose	Granules	4.90 ± 0.22	13.86 ± 0.39	–	0.26 ± 0.04	1.30 ± 0.07	–	0.19 ± 0.01	2.07 ± 0.79	19.02 ± 0.65	22.58 ± 1.52
	Suspended cells	4.49 ± 0.34	11.78 ± 0.97	–	0.34 ± 0.04	4.58 ± 1.46	–	0.07 ± 0.01	0.67 ± 0.14	16.61 ± 1.35	21.93 ± 2.96
	Immobilized cells	2.68 ± 0.37	10.80 ± 0.29	–	0.08 ± 0.04	–	0.94 ± 0.27	0.06 ± 0.03	4.65 ± 0.41	13.56 ± 0.70	19.21 ± 1.41
	Free cells	2.45 ± 0.05	9.38 ± 0.26	–	0.03 ± 0.02	–	2.63 ± 0.14	0.02 ± 0.00	7.38 ± 0.34	11.86 ± 0.33	21.89 ± 0.81
Xylose	Granules	7.00 ± 0.11	13.82 ± 0.41	0.55 ± 0.00	–	–	–	2.08 ± 1.14	0.90 ± 0.37	21.37 ± 0.52	24.35 ± 2.03
	Suspended cells	7.81 ± 1.16	13.95 ± 0.70	0.08 ± 0.00	–	–	–	0.03 ± 0.02	0.97 ± 0.20	21.84 ± 1.86	22.84 ± 2.08
	Immobilized cells	1.28 ± 0.28	8.98 ± 0.11	–	–	–	0.21 ± 0.01	0.24 ± 0.04	4.77 ± 0.19	10.26 ± 0.39	15.48 ± 0.63
	Free cells	1.93 ± 0.26	9.84 ± 0.54	–	0.01 ± 0.00	–	0.28 ± 0.02	0.13 ± 0.01	5.43 ± 0.20	11.78 ± 0.80	17.62 ± 1.03

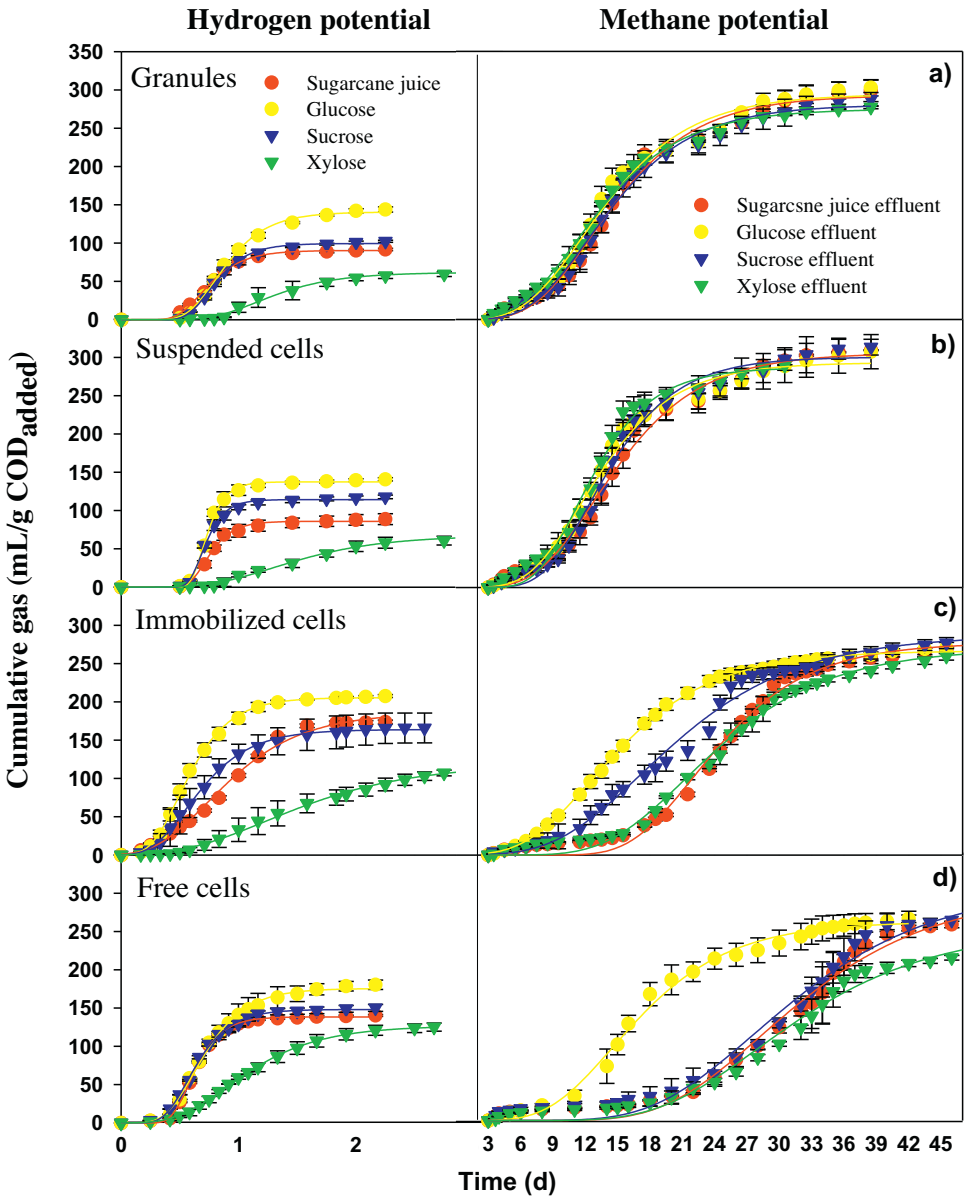

Fig. 1. Hydrogen and methane yield during the evaluation of two-stage hydrogen and methane potential from different carbon sources by using different forms of inoculum: (a) granules; (b) suspended cells; (c) immobilized cells; and (d) free cells. Error bars represent standard deviations ($n = 3$).

metabolite products and biomass (Hallenbeck and Ghosh, 2009). Thus, the hydrogen recovery from the substrate by dark fermentation process is usually below 20% (Antonopoulou et al., 2008; Das and Veziroglu, 2001). The results in Table 2 showed that the effluent after the end of hydrogen fermentation consisted of various kinds of VFAs including acetic, butyric, formic, succinic and lactic acids. This could explain why hydrogen recovery in this experiment was relatively low, in the range of 4.70–10.28%. Moreover, the presence of lactic acid as a metabolic product is another contribution to the low hydrogen yield in this experiment. This is because even though hydrogen is neither consumed nor produced in the lactic acid production pathway, substrate is consumed for lactic acid production as shown in Eq. (6). Thus, carbon sources were converted to lactic acid instead of hydrogen.

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 \quad (6)$$

Hydrogenogenic effluent of all treatments were further used as substrates for methane production by non-pretreated UASB granules in a second stage. Methane production started within the first day and continued for 36 days. Approximately 70% of the total methane was produced during the first 15 days of fermentation (Fig. 1a and b). Methane yield from the hydrogenogenic effluent of sugarcane syrup fermented using granules and suspended cells were not significantly different (Table 1). Additionally, the methane yield obtained from the hydrogenogenic effluent fermented from different carbon sources in the first stage by granules and suspended cells were similar (Table 1). This could have resulted from the relatively similar VFA concentrations and compositions for all the treatments (Table 2).

3.2. *Two-stage hydrogen and methane production by free and immobilized cells of* C. butyricum *TISTR1032 followed by non-pretreated UASB granules*

Hydrogen production by immobilized cells showed a higher hydrogen yield and a shorter lag time compared to free cells (Table 1). Immobilized cells gave a hydrogen yield from sugarcane syrup of 173.7 ± 2.4 mL/gCOD$_{added}$ or 12.41% H_2 recovery.

The highest hydrogen yield of 207.5 ± 1.9 mL/gCOD$_{added}$ or 14.75% H_2 recovery was obtained from glucose while xylose showed the lowest hydrogen yield of 129.8 ± 2.7 mL/gCOD$_{added}$ or 9.25% H_2 recovery. These results suggest that *C. butyricum* TISTR1032 could better utilize hexose sugars than pentose sugars to produce hydrogen. It produced hydrogen from sugarcane syrup as well as from sucrose.

The primary soluble metabolite product of free and immobilized cell fermentation from sugarcane syrup was butyric acid with concentrations of 79 and 86%, respectively, suggesting a butyrate type fermentation. The butyrate type fermentation was found in all experiments where glucose, sucrose or xylose was used as a carbon source (Table 2). This finding was consistent with the previous research reports that a hydrogen production from various types of sugars by *C. butyricum* was a butyrate type fermentation (Plangklang et al., 2012; Pattra et al., 2008; Lo et al., 2008). Moreover, we found that residual sugar in the hydrogenogenic effluent of immobilized cells in all treatments was lower than that of free cells (Table 2), indicating that the immobilized cells utilized the substrates better than the free cells. This revealed a more efficient substrate utilization by immobilized cells, even with high accumulation of VFAs. VFA accumulation caused a decrease in pH and thereby affected microbial activity. Previous studies suggested that cell immobilization can improve hydrogen yield and increased tolerance to inhibitory compounds and harsh conditions (i.e., pH and substrate or product inhibitions) (Anjana and Kaushik, 2014; Plangklang et al., 2012; Zhao et al., 2012).

Hydrogenogenic effluents from all treatments were subsequently used as substrates for methane production by non-pretreated UASB granules in a second stage fermentation. The results show that the methane yield from hydrogenogenic effluents with different carbon sources by immobilized cells was higher than that of free cells (Fig. 1c and d). Methane production from hydrogenogenic effluent by immobilized cells showed a shorter lag time in comparison to free cells (Table 1). Residual sugars in the hydrogenogenic effluent of the free cell fermentation were higher than those of immobilized cell fermentations (Table 2). Therefore, it might take a longer time for the residual sugars of the hydrogenogenic effluent of free cell fermentation to be degraded in the hydrolysis and acidogenesis steps before methanogenesis.

Our results indicated that hydrogen and methane fermentation by immobilized *C. butyricum* TISTR1032 results in a higher two-stage hydrogen and methane production in terms of a higher biogas yields, a shorter lag time, and a higher substrate utilization efficiencies over that of free cells.

3.3. Reusability of the immobilized cells

The reusability of the immobilized *C. butyricum* TISTR1032 was studied. The results suggested that hydrogen production slightly decreased after the first and the second reusability tests (Fig. 2). The outer surface of Ca-alginate bead before fermentation is depicted in Fig. 3a and the presence of *C. butyricum* TISTR1032 entrapped in Ca-alginate bead is depicted in Fig. 3a-1. After the first and the second reuse, it was found that the outer surface of Ca-alginate beads were not broken (Fig. 3b and c). However, after the third reuse, hydrogen production decreased dramatically and continued decreased until the fifth reuse (Fig. 2). This corresponded to breakage of the outer surface of the beads (Fig. 3d–f). The breakage of the beads could be the result of escaping hydrogen gas produced by entrapped *C. butyricum* TISTR1032 in the matrix. Consequently, the bacterial cells leaked from the beads resulting in a decline in hydrogen production in later reuse (Fig. 2). Seol et al. (2011) reported breakage of Ca-alginate beads during a hydrogen fermentation in batch mode and a lower stability of the Ca-alginate beads in comparison to agar or agarose as an immobilization matrix. Therefore, Ca-alginate may not be a suitable immobilization material for hydrogen production over a long term use.

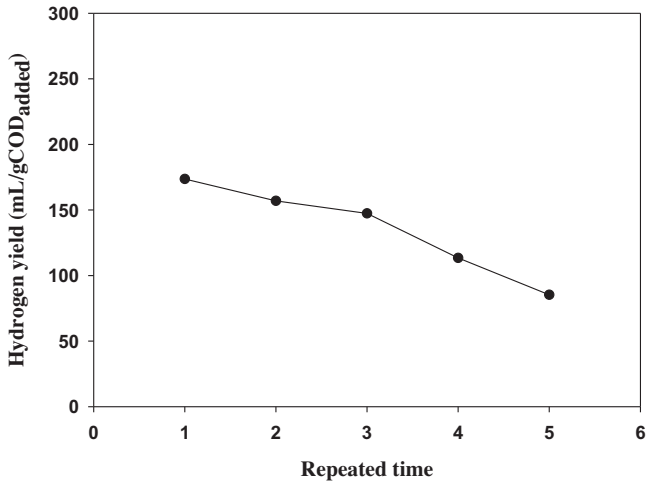

Fig. 2. Hydrogen yield from sugarcane syrup by immobilized *C. butyricum* TISTR1032 at each reusability run.

3.4. One-stage methane production by non-pretreated UASB granules

Biogas production from all treatments started within 24 h and then stopped after 72 h. The biogas consisted of hydrogen and methane in a similar proportion of approximately 20% each (data not shown). The pH of the effluent from all treatments dropped to 4.5–5.0 revealing acidic conditions in the fermentation broth. Substrates in this experiment were sugars that could be rapidly consumed by acidogenic bacteria and converted to a large amount of VFAs, such as acetic and butyric acids. Accumulation of VFAs in the fermentation broth caused the pH of fermentation broth to drop to 5.0, indicating the activity of acidogenic bacteria was greater than the activity of methanogenic bacteria (Mao et al., 2015). Moreover, methanogenic activity was significantly inhibited at pH values lower than 6.6 (Mosey and Fernandes, 1989), thus the methane production stopped. Generally, acidogenic bacteria prefer acidic conditions in the pH range of 5–7 with an optimum pH of 5.5 (Fang and Liu, 2002; Khanal et al., 2004). This could explain why hydrogen was found in the biogas and the pH sharply decreased at the early stage of the fermentation. This result was similar to the findings of Mosey and Fernandes (1989). They found that hydrogen present in biogas is a useful indicator for monitoring the failures in an anaerobic digestion process of rapidly fermenting materials such as milk and sugars. Xu et al. (2014) reported that the decrease in methane production can be found at high organic loads due to rapid accumulation of VFAs. This problem can be remedied by maintaining the pH of the fermentation broth at 7.0, which is the optimum pH of methanogenic bacteria (Ward et al., 2008).

3.5. COD balance

The COD balance of the two-stage hydrogen and methane production had a relatively small error, in the range of +1.29 to −13.17% (Table 3). This error might result from unmeasured biomass formation and errors arising from the measurements of metabolites. For an anaerobic fermentation, approximately 10% of the biodegradable organic matter is utilized for bacterial growth (Khanal, 2008). The results indicate that the experimental data of the current study were quite accurate and consistent with the COD distribution in the two-stage process.

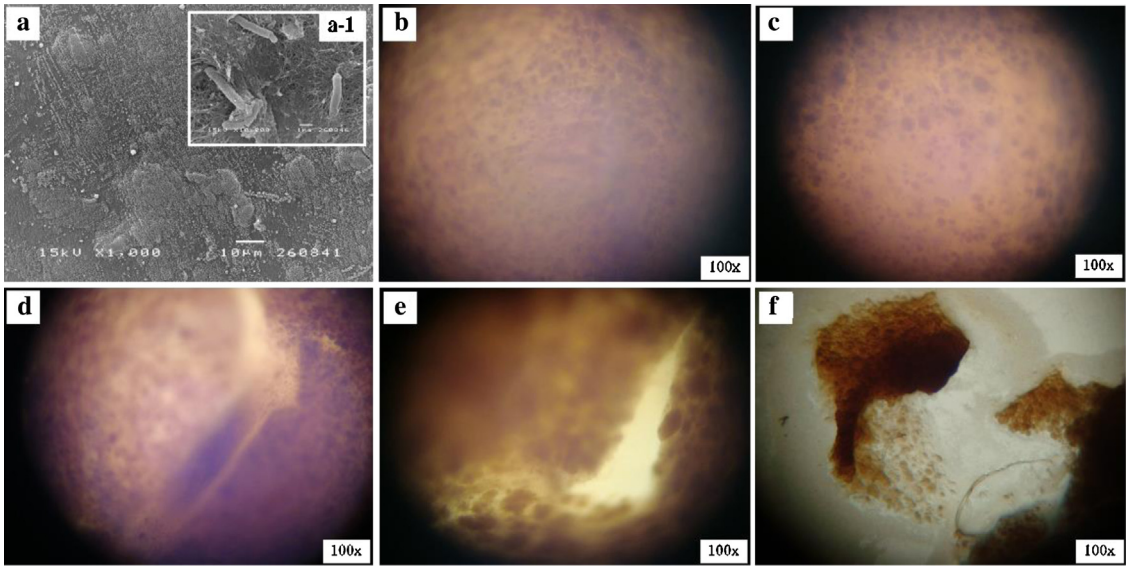

Fig. 3. Morphological observations on the immobilized *C. butyricum* TISTR1032 in Ca-alginate beads during the reusability tests ((a) Scanning electron microscope of outer surface of the Ca-alginate bead before fermentation (1000×); (a-1) inner surface of the Ca-alginate bead before fermentation (10000×); (b) microscope of outer surface of the Ca-alginate bead (100×) after the first batch; (c) after the second batch; (d) after the third batch; (e) after the fourth batch; and (f) after the fifth batch).

Table 3
COD balance for two-stage hydrogen and methane production process.

Substrate	Inoculum	Initial substrate (%)	H_2 (%)	CH_4 (%)	Final effluent (%)	Balance (%)
Sugarcane syrup	Granules	100	6.55	85.22	5.28	−2.95
	Suspended cells	100	6.35	88.46	4.83	−0.36
	Immobilized cells	100	12.41	77.95	5.17	−4.47
	Free cells	100	10.03	73.92	5.23	−10.82
Glucose	Granules	100	10.28	86.41	4.02	+0.71
	Suspended cells	100	10.05	87.92	3.32	+1.29
	Immobilized cells	100	14.75	78.08	3.22	−3.95
	Free cells	100	12.90	75.32	4.95	−6.83
Sucrose	Granules	100	7.31	82.07	4.93	−5.69
	Suspended cells	100	8.42	85.78	3.99	−1.81
	Immobilized cells	100	11.85	80.50	1.26	−6.39
	Free cells	100	10.75	74.77	1.31	−13.17
Xylose	Granules	100	4.70	79.16	4.49	−11.65
	Suspended cells	100	4.81	82.90	5.54	−6.75
	Immobilized cells	100	9.25	75.06	10.39	−5.30
	Free cells	100	8.94	64.02	16.85	−10.19

3.6. Energy yield and economic evaluation

Overall energy yield from all experiments is shown in Fig. 4. Pure culture showed a higher hydrogen energy yield in the first stage fermentation than that of mixed cultures (Fig. 4a). This could be due to the substrate in hydrogen production process being more effectively converted to hydrogen by a pure culture than by a mixed culture. However, hydrogenogenic effluent from the pure culture fermentation gave a lower methane energy yield from the second stage fermentation in comparison to mixed cultures (Fig. 4b).

To obtain the overall energy yield from the two-stage fermentation process, hydrogen and methane energy yield from each treatment were combined. The results suggested that using mixed cultures in the first stage can give slightly higher overall energy yield than using a pure culture (Fig. 4c). Although hydrogen yield recovered from mixed cultures was lower than that obtained from pure culture, the methane yield from hydrogenogenic effluent by mixed cultures was higher. This resulted in higher overall energy yield when using mixed cultures in a two-stage energy production.

Sugarcane syrup gave a maximum overall energy yield of 13.44 kJ/gCOD_{added} or 94.81% recovery from the initial substrate when using heat-treated suspended cells in the first stage followed by non-pretreated UASB granules in the second stage. Glucose showed the highest overall energy yield in a two-stage process whereas xylose was the lowest (Fig. 4c).

Table 4 shows a comparison of hydrogen and methane yields from carbohydrate-rich substrates using two-stage fermentation. Hydrogen and methane obtained in our study are relatively high compared with other studies. However, it should be noted that most studies were conducted using complex substrates such as durum wheat (Giordano et al., 2011), potato wastes, bean curd wastes, kitchen garbage (Chu et al., 2012), and cassava wastewater (Intanoo et al., 2014). These complex substrates have to be hydrolyzed into soluble organic substances before being converted to hydrogen in an acidogenesis step and then to methane. The results implied that the types of substrate significantly influence hydrogen yield in the first stage fermentation. Therefore, substrate type affects the total recovery of hydrogen and methane in a two-stage process.

The energy yield of a two-stage hydrogen and methane production from sugarcane juice in this study was compared with the literature search on ethanol production from sugar-rich substrates

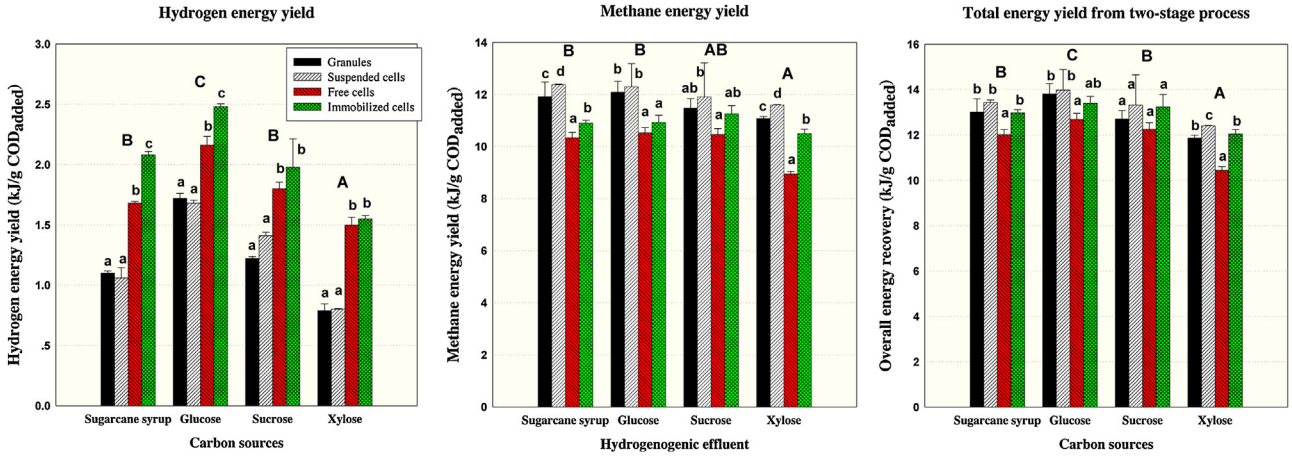

Fig. 4. Hydrogen, methane and overall energy yield of two-stage hydrogen and methane production process. (Bar groups with different capital letters indicate a significant difference ($P < 0.05$) between the different carbon source, bars with different lowercase letters indicate a significant difference ($P < 0.05$) among the different types of inoculum in different forms.).

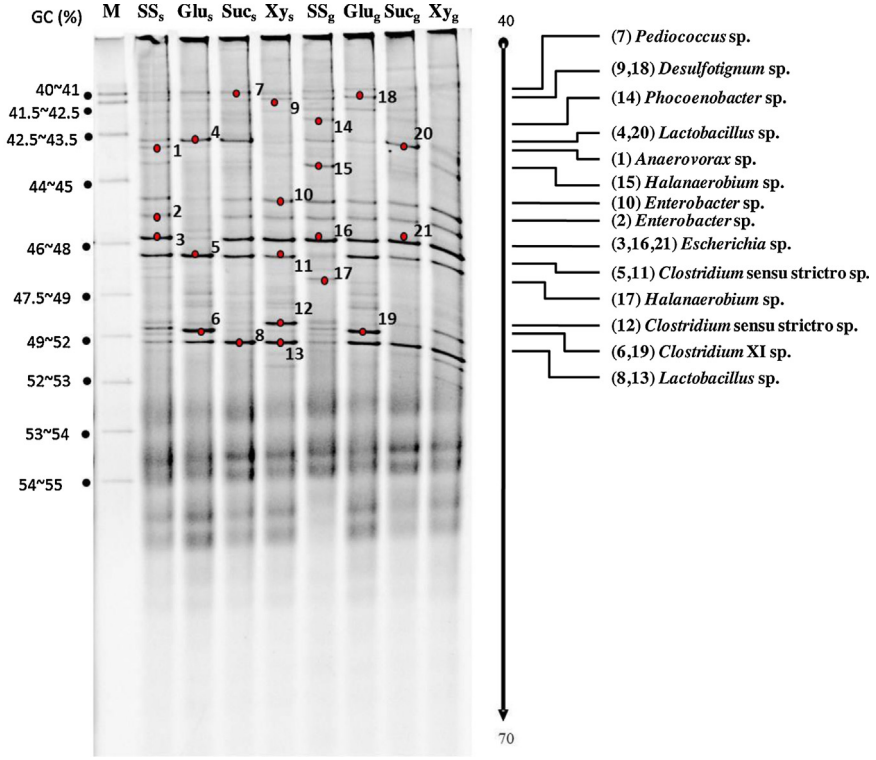

Fig. 5. DGGE profile of 16S rRNA gene fragments. The fragments were PCR-amplified from total DNA extracted of heat-treated UASB granules as inoculum in hydrogen production process (M—DGGE marker; SS_s—sugarcane syrup + suspended cells; Glu_s—Glucose + suspended cells; Suc_s—sucrose + suspended cells; Xy_s—xylose + suspended cells; SS_g—sugarcane syrup + granules; Glu_g—glucose + granules; Suc_g—sucrose + granules; Xy_g—xylose + granules).

(Table 5). We found that the two-stage fermentation process gave a higher energy yield than ethanol fermentation process suggesting that the two-stage fermentation process can be an alternative way to recover a high energy yield from a valuable biomass like sugarcane juice.

To provide a cursory economic evaluation of two-stage fermentation process, the economic profit of hydrogen and methane produced by two-stage fermentation as well as ethanol were calculated. For economic profit of ethanol, the ethanol yield from the literature search was used for a calculation. Economic profit is calculated by multiplying the hydrogen, methane or ethanol yield by energy price. Energy price for hydrogen, methane and ethanol were 0.50 USD/m^3 of hydrogen (USEPA, 2015), 0.42 USD/m^3 of methane (USEIA, 2015), and 0.4 USD/L ethanol (USEIA, 2015), respectively. The economic profit of ethanol production from sugarcane juice, sweet sorghum juice and sucrose were in the ranges of 0.20–0.26 USD/kg hexose while from molasses were quite low (0.14 USD/kg hexose) (Table 5). Results suggested that apart from ethanol production, sugarcane juice can be used to produce other valuable renewable energy like hydrogen and methane.

It is important to note that the economic profit of hydrogen, methane and ethanol is varied depending on the deviations in the market prices of hydrogen, methane and ethanol. In addition, this evaluation does not consider the important factors such as construction and operation costs due to lack of information. Since this study used the mixed cultures as inoculum and the fermentation

Table 4
Comparison of hydrogen and methane yield from two-stage fermentation process in this study to some other studies.

Substrate	Hydrogen			Methane			Total recovery (% COD/COD)	References
	H_2 noculum	H_2 yield ($mL/gCOD_{added}$)	H_2 recovery (% COD/COD)	CH_4 Inoculum	CH_4 yield ($mL/gCOD_{added}$)	CH_4 recovery (% COD/COD)		
Glucose	UASB granules	185	13.30	UASB granules	267	75.50	88.80	Giordano et al. (2011)
Glucose	UASB granules	144	10.28	UASB granules	302	86.41	96.69	This study
Glucose	*C. butyricum* TISTR1032 (imobilized cells)	207	14.75	UASB granules	273	78.08	92.83	This study
Waste lactose	AS	100	7.14[a]	AS	303	86.57[a]	93.71	Banks et al. (2010)
Durum wheat	UASB granules	76	5.50	UASB granules	243	69.50	75.00	Giordano et al. (2011)
Potatoes wastes	AS	59	4.21[a]	AS	247	70.57[a]	74.78	Chu et al. (2012)
Bean curd wastes	AS	17	1.21[a]	AS	276	78.86[a]	80.07	Chu et al. (2012)
Kitchen garbage	AS	48	3.43[a]	AS	266	76.00[a]	79.43	Chu et al. (2012)
Cassava wastewater	UASB granules	54	3.86[a]	UASB granules	165	47.14[a]	51.00	Intanoo et al. (2014)
Synthetic wastewater	UASB granules	33	2.36[a]	UASB granules	320	91.43[a]	93.79	DiStefano and Palomar (2010)
Sugarcane syrup	UASB granules	92	6.55	UASB granules	298	85.22	91.77	This study
Sugarcane syrup	*C. butyricum* TISTR1032 (imobilized cells)	174	12.41	UASB granules	273	77.95	90.35	This study

AS: Anaerobic digester sludge.
[a] Calculated from the hydrogen or methane yield.

Table 5
Comparison on ethanol production from sugar-rich substrates to two-stage hydrogen and methane production from sugarcane juice.

Substrate	Process	Yield			Energy yield[c] (kJ/gCOD)	Economic profit of energy products (USD/kg_{hexose}[d])				References
		Ethanol[a] (g/gCOD, L/kg_{hexose})	Hydrogen (mL/gCOD, L/kg_{hexose}[b])	Methane (mL/gCOD, L/kg_{hexose}[b])		Ethanol	Hydrogen	Methane	Sum	
Sugarcane juice	Ethanol fermentation	0.40, 0.54	–	–	10.72	0.21	–	–	0.21	Dhaliwal et al. (2011)
Sugarcane juice	Ethanol fermentation	0.37, 0.50	–	–	9.92	0.20	–	–	0.20	Limtong et al. (2007)
Sweet sorghum juice	Ethanol fermentation	0.43, 0.58	–	–	11.52	0.23	–	–	0.23	Sasaki et al. (2014)
Sweet sorghum juice	Ethanol fermentation	0.48, 0.65	–	–	12.86	0.26	–	–	0.26	Laopaiboon et al. (2009)
Sucrose	Ethanol fermentation	0.43, 0.58	–	–	11.52	0.23	–	–	0.23	Wang et al. (2013b)
Molasses	Ethanol fermentation	0.26, 0.35	–	–	6.97	0.14	–	–	0.14	Cazetta et al. (2007)
Sugarcane syrup	Two-stage fermentation	–	88.80, 94.75	309.60, 330.34	13.44	–	0.05	0.14	0.19	This study

[a] Ethanol yields were calculated from the original data and expressed in $g/gCOD_{hexoseequivalent}$ and L/kg_{hexose} units (1 g of hexose = 1.067 gCOD; ethanol density = 0.789 g/L).
[b] Hydrogen and methane yield from experimental data ($mL/gCOD_{added}$) were converted and expressed in L/kg_{hexose} unit (1 g of hexose = 1.067 gCOD).
[c] Energy yields were calculated from ethanol energy content of 26.8 kJ/gEtOH, hydrogen energy content of 10.8 kJ/LH_2, and methane energy content of 36 kJ/LCH_4.
[d] Energy price (Nov. 8, 2015): 0.50 USD/m^3 of hydrogen (USEPA, 2015), 0.42 USD/m^3 of methane (USEIA, 2015), 1.51 USD/gallon of ethanol (USEIA, 2015).

was conducted under non-sterile condition; therefore the operation costs of two-stage fermentation might be lower than that of ethanol production.

3.7. Microbial community analysis

The microbial community analysis of the hydrogen fermentation indicated that *Escherichia* sp. (bands 3, 16 and 21), *Clostridium sensu stricto* sp. (bands 5, 11 and 12), *Lactobacillus* sp. (bands 4, 8, 13 and 20) and *Clostridium* XI sp. (bands 6 and 19) (Fig. 5) were the predominant species. *Escherichia* sp. (Morsy, 2014; Seppala et al., 2011) and *Clostridium* sp. (Ortigueira et al., 2015; Plangklang et al., 2012; Pattra et al., 2011) are well known hydrogen producers.

Results in Fig. 5 indicated that other bacterial species responsible for hydrogen production in UASB granules include *Enterobacter* sp. (bands 2 and 10) and *Halanaerobium* sp. (bands 15 and 17). The low intensity of the bands suggested that these bacteria were present at low levels in the UASB granules. Zhang and Fang (2000) reported that the staining intensity of each band represents the relative concentration of that microbial species. *Enterobacter* sp. is a facultative hydrogen producing bacteria that has been used in many studies of hydrogen production (Reungsang et al., 2013; Sun et al., 2015; Patel et al., 2014). *Halanaerobium* sp. is a halophilic hydrogen producing bacteria that has been used for wastewater treatment and produce hydrogen under hypersaline conditions (Kivisto et al., 2010). This indicated that *Enterobacter* sp. and *Halanaerobium* sp. might have a role in hydrogen production even though they are in low concentration. In contrast, *Anaerovorax* sp., *Phocoenobacter* sp., and *Desulfotignum* sp. found in this study are not hydrogen producers. They might compete to consume the substrates for growth.

Lactic acid bacteria (LAB) i.e., *Lactobacillus* sp. (bands 4, 8, 13 and 20) and *Pediococcus* sp. (band 7) were found in the UASB granules. LAB can cause low hydrogen yield in hydrogen fermentations by mixed cultures due to excreted bacteriocins which inhibit other bacteria including hydrogen producers (Noike et al., 2002).

4. Conclusions

The analysis of hydrogen production and metabolic products indicated that size reduction of heat-treated UASB granules was found unnecessary for hydrogen production by mixed cultures. The immobilization of *C. butyricum* TISTR1032 could improve hydrogen yield and substrate utilization over that of free cells. The hydrogen yield by mixed cultures fermentation was lower than that of the pure culture. This was due to the presence of LAB in the UASB granules. Hydrogenogenic effluent from the fermentation by mixed cultures had a high COD. It could be converted to methane with a higher yield in the second stage fermentation than the effluent obtained from pure culture. Hence, the overall energy recovery from two-stage fermentation process when using mixed cultures in hydrogen production was superior than using pure culture. Methane could not be produced from sugar-rich substrates at high concentration by a one-stage fermentation without pH control. This was due to rapid accumulation of VFAs. The results implied the two-stage fermentation process is more appropriate in terms of better process stability and energy recovery. The two-stage fermentation process had an overall energy yield from sugarcane syrup of up to 13.44 kJ/gCOD_{added}.

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