Original Article

Microbial diversity analysis of thermophilic hydrogen-producing consortia from hot spring in the south of Thailand and evaluate cashew apple juice as a substrate

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Abstract

This study aims to develop thermophilic hydrogen-producing consortia from ten sediment samples of hot springs in southern Thailand by repeated batch cultivation at 60°C with fructose, glucose and combination of fructose and glucose. The promising hydrogen production potentials consortia were obtained from thermophilic enriched cultures and encoded as NST1 consortia. NST1 consortia exhibited the maximum hydrogen yields of 259 ml/g sugar consumed when fructose-glucose mixed was used as a substrate. PCR amplified 16S rDNA product was separated by using denaturing gradient gel electrophoresis (DGGE) to identify the diversity of bacteria in NST1 consortia. The phyologenetic profile of the NST1 consortia showed a significant diversity in the microbial community, where major nucleotide sequences were affiliated to class Clostridia, followed by Bacteroidetes, Deltaproteobacteria, and Flavobacteria. Time course of hydrogen production revealed that NST1 consortia gave the maximum hydrogen yield of 540 ml H_2/g sugar consumed after 96 hrs of cultivation when 60% of cashew apple juice was used as a substrate.

Keyword: biohydrogen, cashew apple juice, hot springs, microbial community analysis, thermophilic consortia

1. Introduction

Hydrogen is an ideal fuel source and clean energy carrier for the future because of its high conversion, absence of greenhouse gases, recyclability and non-polluting nature (Morimoto *et al.,* 2004). Many believe that using hydrogen as an alternative energy source may replace fossil fuels (Mariakakis *et al.,* 2011). Hydrogen production using microorganisms or biohydrogen is an exciting new area of feasible technology (Hasyim *et al.,* 2011). Either thermophilic or mesophilic microorganisms can produce biohydrogen by fermentative process from several substrates (Hniman *et al.,*

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2011). Although many previous investigations have been conducted, thermophilic bacteria have more promising properties for hydrogen production. Thermophiles show a wide spectrum of substrate and produce less variety of fermentation end-products compared to mesophilic bacteria (Hasyim *et al.,* 2011). Moreover, by increasing acetate production while decreasing butyrate, ethanol and lactate levels during fermentation, thermophilic bacteria can produce higher amounts of biohydrogen (O-Thong *et al.,* 2007).

Mixed consortia enriched from natural environments have been widely used for biohydrogen production. One of the difficulties associated with biohydrogen production using mixed cultures is the utilization of hydrogen produced by hydrogen-consuming microorganisms, such as metha-nogens, homoacetogens, and sulfate-reducing bacteria. Hydrogen-consuming bacteria must be eliminated using pre-treatment methods. Several pretreatments of mixed cultures have been used such as heat, chemical, acid and base treatments (Chang & Lin, 2004; Gadhe *et al.,* 2014; Hasyim *et al.,* 2011; Hniman *et al.,* 2011; Mariakakis *et al.,* 2011; O-Thong *et al.,* 2007; Si *et al.,* 2015; Sydney *et al.,* 2014; Zheng *et al.,* 2008). Hot springs are a potential source for mixed culture of thermophilic hydrogen-producing micro-organisms (Fraj *et al.,* 2013). In this study, thermophilic hydrogen-producing consortia were enriched from hot spring samples by enrichment culture techniques using fructose,

glucose, and fructose-glucose mixed substrates. The most effective consortia were selected for microbial community studies and tested for hydrogen production from cashew apple juice, which is an important by-product of the cashew nut industry. The use of low cost substrate will push these technologies towards large-scale industrial implementation.

2. Materials and Methods

2.1 Sample collection

Sediment samples were collected from 10 hot springs in southern Thailand, namely in Lamae, Chumphon Province (CP); Ban Nam Ron, Krabi Province (KB); Huy Pring, Nakhon Si Thammarat Province (NST1); Kung Ching, Nakhon Si Thammarat Province (NST2); Bo Dan, Phang Nga Province (PN); Lam Sin, Phatthalung Province (PT); Thung Ya Porn Rang, Ranong Province (RN1); Ban Had Yai, Ranong Province (RN2); Ta Sa Torn, Surat Thani Province (SRT) and Su So, Trang Province (TR). The physico-chemical properties of the water from each sampling site were measured of each visit, and the physico-chemical ranges for each hot spring are summarized in Table 1. At each hot spring, the site was surveyed to characterize the temperature profile range, and water temperature clines were established in the range of 40-49°C using 5°C intervals.

Table 1. Physical and chemical properties of the hot spring samples.

Hot spring sample	pH	Air temp. (^{o}C)	Water temp. (^{o}C)	Total alkalinity (mg/l)	Hardness (mg/l CaCO$_3$)	Conductivity (Us/cm)	Ca (mg/l)	Iron (µg/l)	K (mg/l)
CP	6.81±0.21	32.42±0.85	45.72±0.38	258.81±9.58	65.14±3.25	857.21±23.58	12.25±2.07	5.78±1.24	189.00±15.75
KB	7.22±0.07	33.17±0.99	49.14±0.60	145.42±5.76	112.54±10.54	532.78±27.25	857.57±57.56	4.57±1.04	147.34±26.20
NST1	7.30±1.21	30.58±0.64	43.09±0.49	214.55±10.02	124.36±12.85	478.65±11.45	29.58±7.54	3.58±0.89	125±10.10
NST2	6.53±0.94	34.53±0.93	48.24±0.46	217.42±6.54	58.78±5.78	1210.75±19.87	45.85±9.58	1.87±0.54	172.19±24.70
PN	8.20±0.12	33.05±0.25	40.33±0.37	164.67±9.47	190.14±6.14	752.85±28.74	38.57±6.78	2.58±0.47	152.41±27.85
PT	8.77±0.87	28.01±0.16	45.62±0.56	254.16±5.80	178.85±6.89	892.14±31.89	1358.21±45.80	4.98±1.24	198.58±25.84
RN1	7.68±0.65	32.20±0.28	48.00±0.33	267.78±7.42	125.61±9.28	562.78±40.54	33.75±3.25	1.87±0.24	128.75±24.78
RN2	6.82±0.85	30.79±0.87	49.15±0.73	210.53±5.23	120.14±14.58	1025.78±29.87	19.58±1.58	2.58±0.56	139.08±34.54
SRT1	7.40±0.47	29.16±0.35	40.00±0.35	135.21±12.06	134.02±12.78	687.25±58.74	15.78±5.74	3.28±0.24	152.44±18.93
TR	8.31±0.34	28.30±0.36	47.71±0.68	157.40±10.42	98.74±8.54	985.74±25.78	61.58±8.58	1.04±0.08	148.52±15.78

	Mg (mg/l)	NH$_4^+$ (mg/l)	NO$_3^-$ (mg/l)	Na (mg/l)	SO$_4^{2-}$ (mg/l)	S^{2-} (mg/l)	Cl^{-1} (mg/l)	SRP* (mg/l)
CP	25.85±2.57	1.14±0.05	2.87±0.85	78.25±12.58	58.25±5.82	0.12±0.04	8.25±1.25	0.58±0.08
KB	140.58±34.12	1.35±0.87	68.90±5.40	149.08±12.50	236.80±46.80	0.05±0.01	3058.45±106.58	1.25±0.14
NST1	10.25±4.56	1.07±0.47	1.24±0.47	102.58±21.85	121.35±14.25	0.08±0.02	25.78±5.47	0.85±0.11
NST2	9.58±2.25	1.25±0.85	2.58±0.65	58.23±8.59	58.74±6.58	2.45±0.47	42.89±6.41	0.47±0.05
PN	154.17±5.50	0.09±0.02	52.84±7.4t5	2417.84±54.50	215.80±25.48	1.25±0.24	3557.54±148.70	0.28±0.03
PT	6.78±2.85	2.04±0.87	15.57±2.54	125.25±13.45	35.62±5.28	0.58±0.07	40.28±9.74	0.98±0.14
RN1	4.24±3.75	0.89±0.25	5.65±1.87	60.25±10.85	28.85±3.24	0.04±0.01	18.45±5.21	0.85±0.10
RN2	16.31±2.89	1.54±0.41	3.58±0.78	47.25±8.57	36.47±6.25	1.21±0.28	12.87±3.25	0.64±0.04
SRT1	34.8±8.57	1.89±0.25	4.85±0.05	36.85±13.25	42.57±5.47	0.58±0.08	45.87±5.47	0.27±0.08
TR	32.14±6.72	1.51±0.34	3.45±0.54	65.89±9.64	58.56±4.21	0.96±0.10	25.78±6.21	0.32±0.06

*soluble reactive phosphorus

2.2 Enrichment of thermophilic hydrogen-producing consortia

Enriched hydrogen-producing consortia were performed in 150 ml serum bottles with a working volume of 100 ml. Each bottle was filled with 1% (w/v) of fructose or glucose or fructose-glucose mixed at the ratio of 1:1, sediment (1 g), nutrient stock (1 ml), and phosphate buffer (0.07M, pH 6.5). The nutrient stock solution was diluted to a liter can containing the following (g/l): NH_4HCO_3, 2; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.001; NaCl, 0.001; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.0015; $FeCl_2$, 0.00278 (O-Thong *et al.,* 2007). Bottles were wrapped with aluminum foil to eliminate substrate photolysis, and nitrogen gas was flushed for 10 min to remove oxygen within the headspace. The operational condition was 60°C for five days under strictly anaerobic condition. During the enrichment, H_2, H_2S, CO_2 and CH_4 and soluble metabolites were monitored. The selected thermophilic hydrogen-producing consortia were enriched in five successive batch cycles (10% inoculum in the medium with 5 days cultivation per cycle). The final cultures (5th batch cycle) were used for analysis of hydrogen production, soluble metabolite production and responsible microbial community. Thermophilic enriched consortia with the highest hydrogen production were selected for further study.

2.3 DNA extraction

The 16S rDNA sequence analysis was performed to determine the composition of the bacterial community in the samples. Template genomic DNA was extracted and purified according to the manual provided with the bacterial genomic DNA prep kit (Sol-Gent. SGD62-S120, South Korea). Briefly, the sludge and fermented culture broth were centrifuged at 10,000 ×g for 10 min to harvest the bacterial cells. Pellets were resuspended with 300 µl of the cell resuspension solution after the super-natant was discarded. Two µl lysozyme (100 mg/ml, Sigma-Aldrich, USA) was added and incubated at 37°C for 1 hour. After centrifugation (10,000 ×g, 1min), the pellet was resuspended with 300 µl of cell lysis solution. Protein precipitation solution (100 µl) was added and vigorously vortexed for 20-30 seconds. After centrifugation (10,000 ×g, 5 min) the supernatant was transferred to a new 1.5 ml micro tube containing 300 µl of 100% isopropanol. The solution was mixed by gently inverting 50 times followed by centrifugation (10,000 ×g, 1 min). After discarding the supernatant, 500 µl of the washing buffer was added and the tube containing the solution was inverted several times to wash the DNA pellet. After centrifugation (10,000 ×g, 1 min), the DNA pellet was air dried and 50 µl of elution buffer was added. This genomic DNA was used for PCR.

2.4 PCR amplification

The variable region (V3) of 16S rDNA was amplified by PCR with primers to conserved regions of the 16S rDNA

genes. The nucleotide sequences of the primers were as follows: primer 341F, 5'-AGGCCTAACACATGCAAGTC-3'; primer 517R, 5'-ATTACCGCGGCTGCTGG-3'; GC clamp was added to primer 63FGC, 5'-CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGGGAGGCCTAACACATGCAAGTC-3'. The GC rich sequence attached to the 5'-end of forward primer prevents the PCR products from complete melting during separation via DGGE (Mohan *et al.,* 2010). Both primers have shown to anneal with a majority of bacterial sequences in the ribosomal database. All the PCR amplifications were conducted in 50 µl volume containing 2 µl of total DNA having 100 ng/ml concentrations, 200 mM each of the four deoxynucleotide triphosphates, 15 mM $MgCl_2$, 0.1 mM of individual primers and 1 unit of Taq polymerase. An automated thermal cycler (Eppendorf, Germany) was used for PCR amplification, which was programmed for an initial denaturation at 96°C for 5 min, 35 cycles of denaturation (40 s at 94°C), annealing (50 s at 52.6°C) and extension (1 min at 72°C) and a final extension (72°C for 8 min). Finally, the amplified PCR product was stored at 4°C. The samples were verified on 1% agarose gel.

2.5 Denaturing gradient gel electrophoresis screening

Denaturing gradient gel electrophoresis (DGGE) was performed using the DCodeUniversal Mutation Detection System (Bio-Rad, USA). Samples containing approximately equal amounts of PCR amplicons (35 ml) were loaded onto 1 mm thick vertical gels containing 7% (w/v) polyacrylamide with a linear gradient of denaturants (urea and formamide). A denaturing gradient of 35-75% was applied to separate 16S rDNA fragments (where 100% denaturant is defined as 7 M urea and 40% (v/v) formamide). Gels were prepared in 1 TBE buffer (90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0), which was also used as the electrophoresis buffer. Electrophoresis was run at 60°C, initially for 1 hr under 80 V and then for 12 hrs at a constant voltage of 100 V. After electrophoresis, the gels were stained with ethidium bromide (0.5 mg/l) for 15 min followed by destaining in distilled water for 20 min. Images were captured using Molecular Imager (G:BOXEF System; Syngene, UK).

2.6 DNA sequence and phylogenetic analysis

The middle portion of the selected DGGE band was excised with a sterile pipette tip. The excised gel was incubated overnight at 4°C in 50 µl of sterile distilled water. A 10 µl of eluted DNA was used as the template for PCR performed under the conditions described above, except that the forward primer lacked the GC clamp. A 5 µl sample of each PCR product was subjected to agarose gel electrophoresis to confirm product recovery and to estimate product concentration. Five microlitres of each reaction mixture was again subjected to DGGE analysis as described above to check the purity and to confirm the melting behavior of the band recovered. Some DNA samples still contained mixed products,

as shown by multiple DGGE bands. In each of these cases, the target band was excised from the recovered pattern and reanalyzed with DGGE to obtain single bands.

For sequencing analysis, amplified PCR products were sent to MWG Biotech. All the 16S rDNA partial sequences were aligned with those of the reference microorganisms in same region of the closest relative strains available in the GenBank database by using the BLASTN facility (http://www. ncbi.nlm.nih.gov/BLAST/) and were also tested for possible chimera formation with the CHECK CHIMERA program (http://www.35.8.164.52/cgis/ chimera.cgi?su¼ SSU). These sequences were further aligned with the closest matches found in the GenBank Database with the CLUSTALW function of MEGA version 4.0. Neighbor-joining phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 4.0) (Tamura *et al.,* 2004). A bootstrap analysis with 500 replicates was carried out to check the robustness of the tree. Bootstrap re-sampling analysis for the replicates was performed to estimate the confidence of the tree topologies.

2.7 Nucleotide sequence accession numbers

Nucleotide sequence was submitted to the Nucleotide Sequence Database to the GeneBank public database under the accession numbers from AB919092-AB919105.

2.8 Batch test of the NST1 enrichment consortia for hydrogen production from cashew apple juice

The cashew apple juice was obtained through mechanical process. This juice, which contains high levels of tannins, was clarified by adding gelatin to remove tannins and suspended solids. The clarified cashew apple juice was physico-chemically characterized (Table 2); then filtered and stored frozen (-20°C) prior to use. In order to select thermophilic enriched consortia to use cashew apple juice as a substrate for biohydrogen production, batch cultivations were carried out by adding cashew apple juice at different concentrations. The thermophilic enriched culture inoculum (obtained at the exponential growth phase) was added at 10% (v/v) into 100 ml of cultivation medium in 150 ml glass serum bottles. Control vials contained only cultural medium and inoculum, in order to account for possible background hydrogen production from these additions. All experiments were conducted in triplicate and the hydrogen in gas phase was monitored periodically.

2.9 Analytical methods

The composition of biogas was analyzed using gas chromatography (Hewlett Packard, HP 6850, USA) equipped with thermal conductivity detectors (TCD). Hydrogen gas was analyzed by GC-TCD fitted with a 1.5 m stainless steel column SS350A packed with a molecular sieve (80/100 mesh).

Table 2. Physico-chemically characteristic of cashew apple juice.

Parameter	Content[*]
pH	4.21±0.17
Brix	7.42±1.57
Reducing sugars (g/l)	92.84±5.47
Fructose (g/l)	37.08±1.84
Glucose (g/l)	43.63±2.35
Soluble solids (g/l)	78.31±4.87
Starch (g/l)	43.78±2.45
Non reducing sugar (g/l)	36.96±3.17
Potassium (g/l)	13.74±2.57
Zinc (g/l)	12.97±1.89
Manganese (g/l)	6.43±0.71
Nitrogen as total protein (g/l)	2.64±0.91
Phosphorous (g/l)	1.37±0.34
Magnesium (g/l)	1.20±0.58
Carbohydrates (g/l)	0.95±0.14
Sulfur (g/l)	0.87±0.05
Sodium (g/l)	0.12±0.04
Ferrous (g/l)	$7.24±0.68 \times 10^{-3}$
Titratable acidity (%)	0.31±0.07

[*] Values are given as means±SD from triplicate determinations.

Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. The temperatures of the injection port, oven and detector were 100, 50 and 100°C, respectively (Morimoto *et al.,* 2004). Methane and carbon dioxide were analyzed by GC-TCD fitted with 1.0 m stainless steel column packed with Porapak T (60/ 80 mesh). Helium was used as a carrier gas at a flow rate of 35 ml/min. The temperatures of the injection port, oven and detector were 150, 50 and 100°C, respectively (Chang and Lin, 2004). The gas samples (100 ml for methane and 500 ml for hydrogen) were injected in duplicate.

Fermentation end products (volatile fatty acids (VFA) and ethanol) in the supernatant were determined by gas chromatography (Hewlett Packard, HP 6850, USA) equipped with a flame ionization detector (FID). A column capillary packed with nitroterephthalic acid-modified polyethylene-glycol (DB-FFAP) and with a length of 30 m was used. The temperature of the injection port was 250°C. The chromatography was performed using the following program: 100°C for 5 min, 100-250°C with a ramping of 10°C/min, 250°C for 12 min, and the detector temperature was 300°C. Chemical oxygen demand (COD), pH, suspended solid (SS), total suspended solid (TSS), oil concentration, total phosphorus and total Kjeldahl nitrogen (TKN) were determined in accordance with the procedures described in O-Tong *et al.* (2007). Ammonium-nitrogen and phosphate concentrations were analyzed using commercial test kits from Spectroquant (Merck Co., Ltd., Germany). The total carbohydrate content was analyzed by the method of Morris (1948).

3. Results and Discussion

3.1 Enrichment of thermophilic biohydrogen-producing consortia

The site of each of the ten hot springs from eight provinces in southern Thailand, was surveyed to characterize the temperature profile range, and water temperature clines were established in the range of 53.1-70.4°C using 5°C intervals with the pH range of 6.53-8.77 (Table 1). Thermophilic biohydrogen-producing microorganisms from these sources were successfully enriched with fructose, glucose and fructose-glucose mixed substrate under batch cultivation at pH 7.0 and 60°C. Different types of microbial communities were obtained from different hot spring samples, after preliminary screening for hydrogen production capacity and soluble metabolites produced from 10 g/l of fructose, glucose and the mixed fructose-glucose (Table 3). During incubation, all samples produced gas after 24 hrs and stopped after 120 hrs of incubation which consisted of a mixture of CO_2 (40-62%) and H_2 (27-58%), with traces of N_2 but no trace of CH_4 or H_2S. The hydrogen production from enriched bacterial consortia using fructose and glucose as a substrate ranged from 97.78 to 1962.12 ml H_2/l medium and 50.27 to 1825.87 ml H_2/l medium, respectively. However, the highest hydrogen production of 2362.25 ml/l was from NST1 sample when fructose-glucose mixed was used as a substrate. These data are in accordance with Hniman *et al.* (2011) who reported that mixed substrate of glucose and xylose yielded higher biohydrogen production by bacterial culture isolated from geothermal springs in the south of Thailand when compared to each sugar separately. The highest gas production was obtained from the hot spring sample collected from Noppitam, Nakhon Si Thammarat Province named NST1 (1825.87-2362.25 ml H_2/l medium) followed by the sample from Lam Sin, Phatthalung Province named PT (1057.52-1704.25 ml H_2/l medium), whereas the lowest quantity was observed from the hot spring sample CP (97.78-187.85 ml H_2/l medium) collected from Lamae, Chumphon Province. The explanation for this result may be the difference in physical and chemical properties of the hot spring samples from each site (Table 1). The physical and chemical properties of the hot spring samples including temperature, total alkalinity, conductivity and some minerals (Fe, Mg, NH_4^+, NO_3^- and sulphides) of NST1 samples were lower than those of the CP samples, whereas pH and hardness of NST1 samples were higher than in the CP samples. Temperature, pH and presence of mineral elements in environment have a strong effect on the bacterial community (Crevecoeur *et al.*, 2015; Liu *et al.*, 2015). For instance, Kumar *et al.* (2004) reported that soil pH, temperature and mineral were the best predictors of bacterial diversity in two hot springs located in the Garhwal region of Uttaranchal Himalaya. In addition, Mathur *et al.* (2007) reported a strong correlation between phylogenetic diversity of sediment bacterial communities and pH, temperature, as well as mineral chemistry among six sulfur springs.

The thermophilic enriched cultures NST1 and PT exhibited satisfaction hydrogen production yields indicating the potential role of microbial consortia in utilization of fructose and glucose without much variation in overall metabolism. The observed higher cumulative hydrogen production of the enriched culture NST1 from fructose, glucose and combination of fructose and glucose were 1962.12, 1825.87 and 2362.25 ml H_2/l medium, respectively. The amount of fructose, glucose and fructose-glucose mixed consumed after fermentation by NST1 was 95.60, 93.34 and 90.95%, respectively (Table 3). These data gave the maximum hydrogen yields of 205.25, 195.62, and 259.74 ml H_2/g sugar consumed, respectively. It was noted that hydrogen production from fructose was higher than that from glucose, suggesting that mixed fructose and glucose-utilizing microorganism were dominant in the consortia. The cumulative hydrogen production of the enriched culture PT from fructose, glucose and fructose-glucose mixed was 1057.52, 1425.87, and 1704.25 ml H_2/l medium, respectively. The amount of fructose, glucose and mixed fructose-glucose consumed after fermentation by PT was 92.16, 95.97, and 96.13%, respectively (Table 3), giving the hydrogen yields of 114.75, 128.58, and 157.29 ml ml/g sugar consumed, respectively. The hydrogen yields of the enriched cultures NST1 and PT were comparable to that obtained from geothermal hot spring using sago starch as a substrate (422 ml H_2/g sugar consumed) at 60°C (Hasyim *et al.*, 2011). The yield was higher than that obtained with inoculum from geothermal hot spring isolated from southern of Thailand using glucose-xylose mixture as a substrate (241.4 ml H_2/g sugar consumed) at 60°C (Hniman *et al.*, 2011). The high hydrogen production occurred together with the production of acetic acid and butyric acid (Table 3) with small amount of ethanol and propionic acid. On the other hand, low hydrogen production occurred with the presence of butanol and lactic acid. Lactic acid is not common as an end product but it was intermediate product during fermentation under thermophilic condition (Zheng *et al.*, 2008). The reason for lactic acid accumulation was not clear; however it was likely to be related to environmental change during repeated cycles or substrate overloading (Hniman *et al.*, 2011). It was suggested that when there is environmental change, bacteria would try to optimize the carbon flow rate through the cell by using lactate as an electron sink and reduce the acetate production, which would give a high energy yield. As a result, high lactate was found in all enriched cultures.

3.2 Bacterial communities of thermophilic enriched consortia

The thermophilic enriched consortia coded NST1 gave the highest biohydrogen production when fructose was used as a substrate, and was selected for study of the microbial community by DGGE. DGGE profiles generated from the universal bacterial primers revealed the structural composition of communities of NST1 consortia based on the V3

Table 3. Biohydrogen production and soluble metabolites of thermophilic enriched consortia from various sources of hot spring in southern of Thailand using fructose, glucose and fructose-glucose mixed as a substrate.

Sample name	Enrichment substrates	H_2 production (ml/l)[*]	H_2 yield (ml/g sugar consumed)[*]	Acetic acid (mM)[*]	Butanol (mM)[*]	Butyric acid (mM)[*]	Ethanol[*] (mM)	Lactic acid (mM)[*]	Substrate[*] consumed (%)
CP	Fructose	97.78±42.25	13.51±2.25	6.32±0.21	15.85±1.87	2.21±0.85	1.21±0.05	12.25±2.84	72.38±2.58
	Glucose	50.27±39.25	8.92±0.57	5.81±1.47	17.02±3.25	3.58±0.98	0.58±0.07	0.52±0.04	56.36±3.24
	Fructose-glucose mixed	187.85±10.85	39.84±3.25	4.32±0.89	18.95±4.25	2.54±0.41	1.23±0.12	4.58±1.24	47.15±4.89
KB	Fructose	784.85±12.14	117.86±14.32	8.97±2.65	8.54±2.87	4.96±1.21	0.87±0.21	5.21±0.89	66.59±5.46
	Glucose	428.56±8.56	87.58±5.79	6.25±1.21	9.58±3.25	8.32±2.58	0.56±0.08	2.89±0.64	48.93±6.84
	Fructose-glucose mixed	527.89±9.54	94.21±12.34	7.89±2.45	10.85±2.14	3.25±1.96	0.21±0.05	6.25±1.84	56.03±5.41
NST1	Fructose	1962.12±89.25	205.25±32.65	12.57±2.31	4.23±2.68	15.64±2.54	2.14±0.24	3.85±0.87	95.60±5.46
	Glucose	1825.87±47.25	195.62±25.46	7.54±1.24	6.74±1.54	18.75±3.14	1.25±0.14	7.25±1.54	93.34±6.21
	Fructose-glucose mixed	2362.25±85.55	259.74±34.14	10.25±2.87	4.52±0.87	20.54±3.25	0.32±0.11	10.32±2.84	90.95±5.89
NST2	Fructose	254.87±15.25	48.58±10.54	5.96±0.87	18.52±3.24	3.87±0.87	2.00±0.34	4.18±1.64	52.46±5.12
	Glucose	537.48±9.24	98.67±6.72	6.35±0.74	12.65±2.56	4.56±0.31	1.25±0.54	7.21±2.74	54.47±3.25
	Fructose-glucose mixed	652.87±35.25	118.79±20.89	9.87±1.24	10.76±3.20	6.54±1.52	0.89±0.14	6.25±1.35	54.96±2.56
PN	Fructose	321.45±12.25	68.25±14.25	5.24±0.87	17.68±0.57	2.71±0.87	0.54±0.01	2.07±0.45	47.10±3.47
	Glucose	124.85±14.25	28.25±3.25	6.32±1.25	18.85±1.41	1.87±0.45	0.62±0.02	10.58±3.21	44.19±3.98
	Fructose-glucose mixed	458.14±20.25	89.54±10.25	4.41±0.78	8.63±0.57	2.65±0.21	0.74±0.08	6.58±1.54	51.17±3.52
PT	Fructose	1057.5±25.25	114.75±20.75	15.84±3.25	7.85±0.96	14.58±3.25	1.21±0.14	4.15±0.58	92.16±2.45
	Glucose	1425.87±23.25	148.58±5.34	16.74±4.25	6.25±1.74	15.64±2.14	1.03±0.23	3.64±0.94	95.97±3.25
	Fructose-glucose mixed	1704.25±14.25	177.29±30.25	8.57±2.57	4.25±0.74	17.89±3.52	0.54±0.11	4.19±0.47	96.13±5.46
RN1	Fructose	632.25±23.25	110.79±16.85	6.89±2.45	15.65±2.14	3.25±0.54	0.98±0.08	8.14±3.84	57.07±4.87
	Glucose	542.25±30.25	98.54±9.25	5.64±0.75	13.25±3.25	5.45±1.25	0.65±0.06	3.76±1.25	55.03±6.25
	Fructose-glucose mixed	458.27±45.21	88.63±7.43	6.78±1.25	12.74±2.85	3.74±0.47	0.35±0.10	4.37±1.04	51.71±3.25
RN2	Fructose	1124.89±55.25	148.94±2.54	9.45±0.85	4.56±0.56	19.21±3.25	0.87±0.24	3.72±0.36	75.53±4.87
	Glucose	1225.35±60.25	173.25±6.25	6.92±0.64	5.24±0.87	20.78±2.25	0.25±0.07	6.43±0.87	70.73±5.98
	Fructose-glucose mixed	1527.28±12.25	195.87±3.74	5.34±1.24	4.65±0.66	18.95±5.41	1.25±0.51	11.69±2.34	91.31±5.21
SRT	Fructose	1084.75±23.25	110.65±18.58	10.78±2.32	2.54±0.40	19.85±6.25	1.56±0.14	6.21±2.14	92.77±6.35
	Glucose	1214.57±17.85	168.57±26.32	14.56±3.45	5.32±2.01	15.21±2.35	1.87±0.21	1.85±0.45	87.05±3.41
	Fructose-glucose mixed	1045.58±20.25	217.25±40.25	17.89±2.58	3.65±0.74	20.85±3.21	0.85±0.05	3.74±1.01	88.13±4.05
TR	Fructose	425.25±12.25	88.74±25.34	8.57±3.21	11.74±2.11	12.78±0.56	0.47±0.03	1.95±0.54	47.92±4.29
	Glucose	465.25±32.25	90.32±16.24	4.56±0.54	15.89±3.25	15.62±4.25	0.56±0.10	3.25±1.21	51.51±3.54
	Fructose-glucose mixed	587.58±25.25	108.64±11.97	6.32±0.78	18.75±4.20	13.25±3.24	0.25±0.07	4.61±0.78	54.09±5.14

[*] Values are given as means ± SD from triplicate determinations.

region of 16S rDNA. Different banding patterns of microbial community structure as well as species diversity were observed (Figure 1). Each of the distinguishable bands in the separation pattern represents an individual bacterial genus. The phylogenetic distribution was established with a boot-strap neighbour-joining method as shown in Figure 2. Dominant operational taxonomic units (OTUs) observed in NST1 consortia could be divided into four groups, viz., Aquificae, Bacilli, Clostridia and Gammaproteobacteria. Major bands were phylogenetically related to class Clostridia
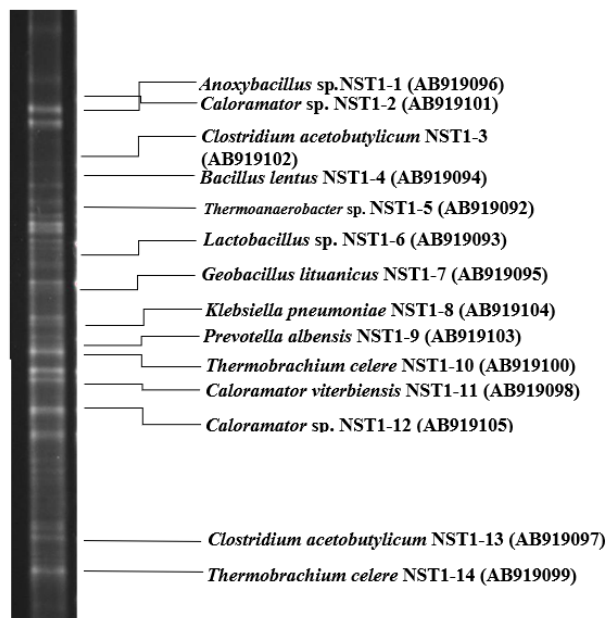
Figure 1. DGGE profiles generated from the universal bacterial primers revealed the structural composition of communities of NST1 consortia based on the V3 region of 16S rDNA.
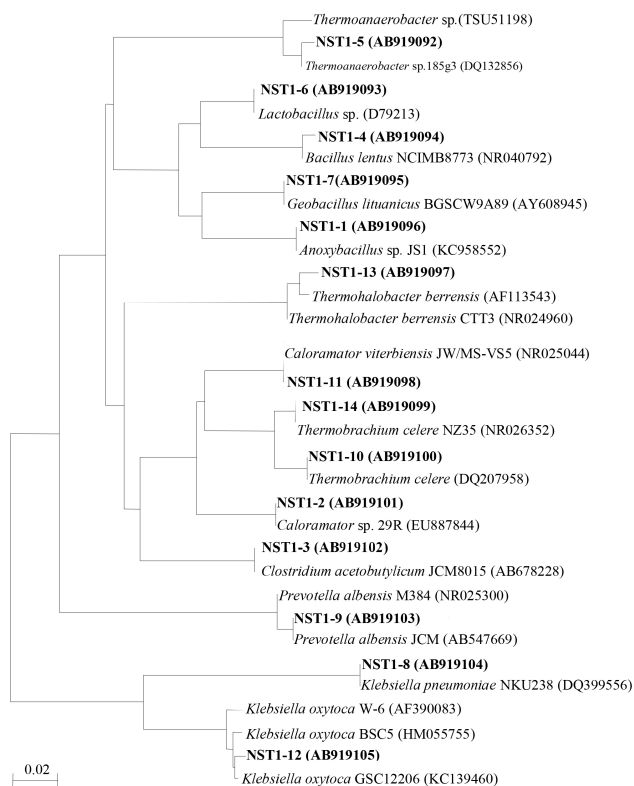

Figure 2. Phylogenetic tree showing the relationship between DGGE bands detected in this study and reference sequences based on a comparison of 16S rDNA (V3) sequences. The numbers shown next to the nodes indicate percent bootstrap values from 1,000 iterations.

(7 out of 14; NST1-2, NST1-3, NST1-5, NST1-10, NST1-11, NST1-13 and NST1-14) followed by Bacilli (4 out of 14; NST1-1, NST1-4, NST1-6 and NST1-7), Gammaproteobacteria (2 out of 14; NST1-8 and NST1-12) and Aquificae (1 out of 14; NST1-9).

Bacteria in the class Clostridia were the majority group of DGGE bands presented in this study including NST1-2 (*Caloramator* sp. 29R; EU8878441, 98%); NST1-3 (*Clostridium acetobutylicum* JCM8015; AB678228, 97%); NST1-5 (*Thermoanaerobacter* sp. 185g3; DQ132856, 97%); NST1-10, (*Thermobrachium celere*; DQ207958, 97%); NST1-11 (*Caloramator viterbiensis* JW/MS-VS5; NR025044, 99%); NST1-13 (*Thermohalobacter berrensis*; AF113543, 97%) and NST1-14 (*Thermobrachium celere* NZ35; NR026352, 97%). Within the domain bacteria, many thermophiles and hyper-thermophiles belonging to the class Clostridia have been isolated from hot springs (Mohan *et al.,* 2010). Several *Clostridium* spp. are described as biohydrogen-producing bacteria, including *C. thermocellum* 27405 (Levin *et al.,* 2006), *C. acidisoli* (Sun *et al.,* 2010), *C. beijerinckii* RZF-1108 (Zhao *et al.,* 2012), *C. butyricum* CGS5 (Liu *et al.,* 2012) and *C. hydrogeniproducens* HR-1 (Tang *et al.,* 2013). Isolates affiliated with these bacteria have been isolated from hot springs and used for thermophilic H_2 production in dark fermentation (Makinen *et al.,* 2012). Next to the class Clostridia, Bacilli were dominant in the thermophilic bio-hydrogen enrichment consortia [NST1-1 (*Anoxybacillus* sp. JS1 (KC958552, 98%); NST1-4 (*Bacillus lentus* NCIMB8773 (NR040792, 97%); NST1-6, (*Lactobacillus* sp. (D79213, 98%) and NST1-7, (*Geobacillus lituanicus* BGSCW9A89 (AY608945, 98%). *Clostridium* and *Bacillus* were found to be dominate in NST1, and there previously been reported as prominent biohydrogen producers from the phylum Firmicutes (Chang *et al.,* 2008; Kannaiah *et al.,* 2012).

OTUs NST1-8 and NST1-12 have shown 98 and 97% similarity to *Klebsiella pneumoniae* NKU238 (DQ399556) and *Klebsiella oxytoca* GSC12206 (KC139460), respectively. Some Gamma-proteobacteria (Gram-negative) are of anaerobic genera and most of them are known as putative nitrate reducers (Bomberg *et al.,* 2011). *K. pneumoniae* and *K. oxytoca* HP1, hydrogen-producing bacterial strains, were newly isolated from a hot spring, and were reported to be biohydrogen producers (Minnan *et al.,* 2005; Noparat *et al.,* 2012). OTUs NST1-9 has shown 100% similarity to *Prevotella albensis* JCM (AB547669, 97%). *Prevotella* spp. are a genus in Bacteroidetes, the Gram-negative anaerobes, able to grow on a variety of carbohydrates. *Prevotella* spp. are generally pathogenic in nature producing biohydrogen under anaero-bic conditions (Nandi & Sengupta, 1998). *Prevotella buccae* isolated from upflow anaerobic sludge blanket was characterized as a biohydrogen producing strain (Mohan *et al.,* 2010). *Prevotella* spp. have been also detected in other biohydrogen-producing systems (Mariakakis *et al.,* 2011).

3.3 Hydrogen production by thermophilic enriched consortia NST1 with cashew apple juice

Thermophilic enriched culture NST1 was selected to test for hydrogen production from cashew apple juice. In the first set of trials, we used initial cashew apple juice concentration ranging from 10 to 80 g/l, in increments of 10 g/l (Table 4). Hydrogen production increased with the increasing cashew apple juice concentration from 10 to 60% (v/v) and decreased thereafter when cashew apple juice concentration was higher than 60% (v/v). The maximum hydrogen production of 2998 ml/l medium was obtained at cashew apple juice concentration of 60% (v/v). The thermophilic enriched consortia NST1 degraded completely reducing sugars in the cashew apple juice to hydrogen, acetate, butyrate, lactic acid and butanol. Another component in cashew apple juice such as amino acid, starch, mineral and vitamin are also provide the growth and fermentation products from bacteria consortia (Deenanath

et al., 2015; Lowor & Agyente-Badu, 2009; Rocha *et al.*, 2006). The enriched consortium NST1 was noted to effectively assimilate reducing sugars and showed promise for application for biohydrogen production from cashew apple juice.

Figure 3 demonstrates the time-course profiles and characteristics of hydrogen fermentation of NST1 consortia under optimized conditions. Hydrogen production began immediately after a short lag phase of 12 hrs and the hydrogen production yield maintained a high level at 96-120 hrs, where cell growth had entered latter-stage of exponential growth. Exponential cell growth was observed from the beginning and the final dry cell weight was estimated to be 1.85 g/l. The hydrogen content in the biogas increased sharply after the onset of hydrogen production and reached a maximum of 540 ml/g sugar consumed after cultivation for 96 hrs. The major by-products of hydrogen fermentation were acetic, butyric and propionic acids, with lesser amounts of ethanol and butanol. The concentration of total volatile fatty acids

Table 4. Characteristics of biohydrogen production and soluble metabolites of thermophilic enriched consortia NST1 during batch experiment at different cashew apple juice concentration.

Cashew apple juice (%, v/v)	Reducing sugar content (g/l)[*]	H_2 production (ml/l medium)[*]	H_2 yield (ml/g sugar consumed)[*]	Acetic acid (mM)[*]	Butanol (mM)[*]	Butyric acid (mM)[*]	Ethanol (mM)[*]	Lactic acid (mM)[*]
10	0.92±0.04	528.85±32.58	574.21±15.25	7.20±0.14	1.21±0.54	5.89±2.14	0.89±0.14	5.28±1.25
20	1.85±0.24	974.54±40.87	526.85±10.54	5.14±1.25	1.34±0.78	7.85±3.21	0.95±0.21	4.56±2.54
30	2.78±0.16	1547.58±14.89	556.47±21.52	11.57±3.75	3.52±0.87	11.42±3.50	0.52±0.11	6.32±2.01
40	3.71±0.74	1958.74±32.85	527.62±19.87	14.36±2.89	4.25±2.47	15.89±2.58	1.21±0.89	5.47±2.14
50	4.64±0.32	2471.25±25.87	532.35±13.25	15.74±3.45	3.89±1.85	18.28±3.14	0.72±0.21	6.32±3.25
60	5.57±1.57	2998.64±34.57	538.14±16.74	18.25±4.10	3.75±1.21	16.78±2.56	0.91±0.10	4.78±1.25
70	6.49±1.85	2985.31±28.25	459.56±16.85	20.14±3.26	5.28±0.89	15.21±3.14	1.54±0.78	5.69±2.47
80	7.42±1.64	2987.78±36.78	403.87±20.25	18.21±2.85	4.58±0.25	14.78±2.78	1.25±0.89	6.17±2.36

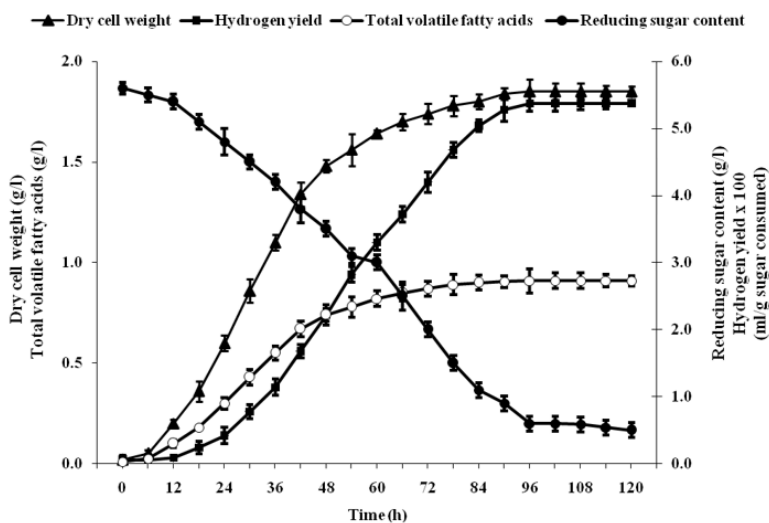[*] Values are given as means ± SD from triplicate determinations.

Figure 3. Time course for biohydrogen production by NST1 consortia using 60% (v/v) of cashew apple juice concentration as a substrate.

increased sharply with hydrogen production and reached a top value of 0.89 g/l at the late stationary phase. The residual reducing sugar declined gradually as fermentation proceeded and the pH of the culture dropped significantly from 6.50 to 4.87 in the first 24 hrs and then remained steady at about 4.60 correspondingly with H_2 production and formation of acidic metabolites (data not shown).

4. Conclusions

Potential thermophilic microorganisms for hydrogen production were enriched from Southern Thailand hot springs possessing a wide temperature range (53.1-70.4°C) and pH range (6.53-8.77). The enriched consortium NST1 produced a high hydrogen yield either from glucose, fructose or mixture of both sugars at 60°C with the maximum value of 375 ml of H_2/g sugars consumed. Enriched consortium NST1 was dominated by bacteria closely affiliated with Bacteroidetes, Firmicutes and Proteobacteria. Promising bacterial enrichments were obtained from Southern Thailand hot spring samples with a high potential for hydrogen production under thermophilic conditions from cashew apple juice.

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