รายงานวิจัยฉบับสมบูรณ์

โครงการ: การคัดเลือกแบคทีเรียที่มีความสามารถในการผลิตก๊าซไฮโดรเจน และการศึกษาสภาวะที่เหมาะสมในการผลิตก๊าซไฮโดรเจนของแบคทีเรีย

โดย: ดร. สังวาลย์ แก่นโส

มีนาคม 2555

สัญญาเลขที่ MRG4880163

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มหาวิทยาลัยอุบลราชธานี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และสำนักงานคณะกรรมการการอุดมศึกษา

กิตติกรรมประกาศ

ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และ สำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) ผู้อุดหนุนทุนวิจัย “ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่” ที่ทำให้งานวิจัยนี้เป็นไปได้ และที่ให้โอกาสอาจารย์ที่พึ่งจบการศึกษาได้ฝึกฝนในการทำงานวิจัยด้วยตนเองอย่างอิสระ นอกจากนั้นยังให้กำลังใจ คำแนะนำ และแนวทางในการทำงานและการพัฒนาตนเอง ผ่านทางการจัดอบรมต่างๆ ผู้วิจัยเห็นคุณค่าและซาบซึ้งในความปรารถนาดีของเจ้าหน้าที่ในสกว.ที่ได้ทุ่มเท อดทน อดกลั้น ในการติดตาม ส่งเสริม สนับสนุนและผลักดันให้การวิจัยไทยก้าวไปข้างหน้าอย่างเข้มแข็ง

ขอขอบพระคุณ ภาควิชาวิทยาศาสตร์ชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยอุบลราชธานี ที่ให้สถานที่ทำวิจัยและเครื่องมือในการวิจัย

ขอกราบขอบพระคุณ รศ.ดร.รมิดา วัฒนโภคาสิน อาจารย์ที่ปรึกษาพี่เลี้ยง ที่ได้กรุณาให้ความรู้ คำแนะนำและให้กำลังใจ ตลอดการทำงานที่ผ่านมา

สุดท้ายนี้ ขอกราบขอบพระคุณ คุณพ่อวิบูลย์ และคุณแม่กองศรี แก่นโส บุพการีผู้ให้ทุกสิ่งทุกอย่างกับผู้วิจัย และขอขอบคุณสมาชิกในครอบครัวที่เป็นแรงบันดาลใจให้มุ่งมั่นในการทำงานวิจัย ความดีอันเกิดจากการศึกษาค้นคว้าครั้งนี้ ผู้วิจัยขอมอบแด่บิดา มารดา ครู อาจารย์ และผู้มีพระคุณทุกท่าน

ดร. สังวาลย์ แก่นโส

บทคัดย่อ

รหัสโครงการ : MRG4880163

ชื่อโครงการ : การคัดเลือกแบคทีเรียที่สามารถผลิตก๊าซไฮโดรเจน
และการศึกษาสภาวะที่เหมาะสมในการผลิตก๊าซไฮโดรเจนของแบคทีเรีย

ชื่อนักวิจัย : ดร. สังวาลย์ แก่นโส มหาวิทยาลัยอุบลราชธานี

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ระยะเวลาโครงการ : 2548-2550

โครงการวิจัยนี้มีวัตถุประสงค์เพื่อคัดแยกเชื้อแบคทีเรียที่สามารถผลิต H_2 ได้ในปริมาณมาก และหาสภาวะที่เหมาะสมในการผลิต H_2 โดยเชื้อที่แยกได้ รวมทั้งศึกษารายละเอียดของแบคทีเรียนั้นเพื่อการจัดจำแนกเชื้อ และเพื่อการตีพิมพ์ ซึ่งดำเนินการโดยคัดแยกแบคทีเรียทั้งในสภาวะปกติและสภาวะไร้ออกซิเจน ทดสอบความสามารถในการผลิต H_2 และศึกษาสภาวะที่เหมาะสมในการผลิตก๊าซไฮโดรเจนของแบคทีเรียสายพันธุ์ KRS4C-6 ที่ผลิต H_2 ได้ดีที่สุด พร้อมทั้งทำการศึกษาเพื่อระบุชนิดเชื้อ ผลการทดลองคัดแยกเชื้อได้ทั้งหมด 106 ไอโซเลต ที่ผลิตก๊าซได้มี 23 ไอโซเลต แต่มีเพียง 13 ไอโซเลตเท่านั้นที่ผลิต H_2 ได้ดี คือไอโซเลตดังต่อนี้ (ข้อมูลในวงเล็บแสดงเปอร์เซนความเหมือนเปรียบเทียบกับลำดับเบสของสปีชีส์ที่คล้ายคลึงมากที่สุดที่พบในฐานข้อมูล) WS-2-2 (98% *Paenibacillus polymyxa*), WS-4-2 (99% *Escherichia coli*), WS-7-11 and Lao-1-2 (99% *Pantoea agglomerans*), AS-1 (99% *Klebsiella pneumoniae*), AS-4 (99% *Klebsiella variicola*), AS-I-1 (98% *Paenibacillus polymyxa*), AS-I-2 (98% *Paenibacillus polymyxa*), CD-5 (99% *Enterobacter cloacae*), CD-6 (*Pantoea agglomerans*), KRS4B-5 (98% *Paenibacillus polymyxa*), KRS4C-6 (99% *Paenibacillus polymyxa*) และ Lao-1-11 (Sequencing failed)

การผลิต H_2 ของเชื้อ KRS4C-6 เกิดขึ้นควบคู่กับการเจริญและการใช้กลูโคส เชื้อ KRS4C-6 เจริญและผลิต H_2 ได้ในช่วง pH 5.0-8.5 แต่เจริญและผลิต H_2 ได้ดีที่สุดที่ pH 6 อุณหภูมิที่เชื้อสามารถเจริญได้คือระหว่าง 13-43°C โดยเจริญได้ดีที่สุดที่ 30-35°C แต่การผลิตไฮโดรเจนเกิดขึ้นในช่วงอุณหภูมิ 20-40°C ผลการศึกษาการผลิต H_2 ของเชื้อ KRS4C-6 ในสภาวะที่เหมาะสมที่สุด(H_2 production at optimal conditions) ในอาหาร NB ที่เติมน้ำตาลกลูโคส 1 % (10 mg/ml) ที่มีค่า pH เริ่มต้น 6.0 ปริมาณ 500 ml ในฟลาสค์ขนาด 1000 ml พ่นด้วยก๊าซไนโตรเจน เป็นเวลา 5 นาที บ่มที่อุณหภูมิ 35°C เป็นเวลา 144 ชั่วโมง (ที่มีการผลิตก๊าซไฮโดรเจน) พบว่าเชื้อ KRS4C-6 ผลิต H_2 ได้ 495 ml ในปริมาณก๊าซทั้งหมด 1146 ml คิดเป็นก๊าซ H_2 43.17% ประสิทธิภาพการผลิตไฮโดรเจนที่สภาวะดังกล่าว คิดเป็น 0.9692 mol H_2/mol Glucose โดยคำนวณจากกลูโคสที่ใช้ทั้งหมด 4.09 g (0.0227 mol)

คำหลัก : แบคทีเรียผลิตก๊าซไฮโดรเจน สภาวะที่เหมาะสมในการผลิตก๊าซไฮโดรเจน

Abstract

Project Code : MRG4880163

Project Title : Isolation of hydrogen-producing bacteria and optimization of hydrogen gas production by bacterial isolates.

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Project Period : 2005-2007

This project aimed to isolate hydrogen-producing bacteria and to find an optimal condition for H_2 production for a chosen isolate with good H_2 producing ability as well as to study a chosen isolate in details for the purpose of identification, description and publication. Bacteria isolation performed both aerobically and anaerobically using environmental samples as inoculum yielded 106 pure isolates with twenty three isolates capable of H_2 production under condition tested. However only thirteen of them with high amount of gas produced were studied further. These isolates were listed as followed with percentage similarity of 16S rRNA gene partial sequence in the parenthesis as WS-2-2 (98% *Paenibacillus polymyxa*), WS-4-2 (99% *Escherichia coli*), WS-7-11 and Lao-1-2 (99% *Pantoea agglomerans*), AS-1 (99% *Klebsiella pneumoniae*), AS-4 (99% *Klebsiella variicola*), AS-I-1 (98% *Paenibacillus polymyxa*), AS-I-2 (98% *Paenibacillus polymyxa*), CD-5 (99% *Enterobacter cloacae*), CD-6 (*Pantoea agglomerans*), KRS4B-5 (98% *Paenibacillus polymyxa*), KRS4C-6 (99% *Paenibacillus polymyxa*) and Lao-1-11 (Sequencing failed). KRS4C-6 was the best for H_2 production therefore chosen for further detailed study and optimization of H_2 production.

H2 production by KRS4C-6 occurred in couple to cell growth and glucose utilization. KRS4C-6 grew and produced H_2 at the pH 5.0-8.5 but the optimal pH for growth and H_2 production were found at pH 6. Growth temperatures were between 13-43°C while H_2 production occurred at 20-40°C. However optimal temperature for growth and H_2 production were between 30-35°C. KRS4C-6 produced H_2 at optimal condition (in 500 ml NB medium containing 1% glucose (10 mg/ml), pH 6.0) in a 1000 ml flask flushed with N_2 for 5 minutes incubated at 35 C for 144 hours) yielding 495 ml H_2 from the total biogas produced of 1146 ml calculating to be 43.17% H_2 which was equivalent to 0.9692 mol H_2/mol glucose on account of 4.09 g. (0.0226 mol) total glucose utilized.

Keywords : Hydrogen-producing bacteria, Optimization of hydrogen production

4. เนื้อหางานวิจัย (บทนำ วิธีการทดลอง ผลการทดลอง บทวิจารณ์ หนังสืออ้างอิง)

4.1 บทนำ (Introduction)

Environmental pollution is a global problem. Its impacts have been experienced more intensely than ever before everywhere on earth. Global warming, longstanding drought and dramatic seasonal changes are some obvious examples. But human consumption and demand for resources and energy have never stopped going up while the amount of wastes that pollutes the environment has ski-rocked. The two inter-related problems of increased energy demand and environmental pollution have been global concerns. In most countries including Thailand, the main sources of energy are fossil based, which include petroleum, coal and natural gas as examples. Burning of fossil-based fuel generates green house gas CO_2. Decomposition of waste that comes from living material results in another green house gas, CH_4, in addition to CO_2. In the current situation the more amount of waste generated the more greenhouse gasses are released to the atmosphere. In Bangkok alone the amount of waste generated is more than 9,521 tons daily (2545). More over the process of getting rid of these wastes consumes enormous amount of energy, which the world is already short on.

With global-scale impacts of environmental pollution and the foreseen shortness of fossil fuel, every country has realized the necessity of replacing finite fossil fuel with sustainable and environmentally friendly alternatives. Because waste is full of potential energy, the thought of combining the need to generate energy and the need to get rid of waste appears ideal. This combination can possibly be made practical with the invention of hydrogen fuel cells. The technology makes use of hydrogen gas to directly generate electricity without combustion. This means that there is no pollutant emitted. This method of electricity generation is therefore superior when compared to other methods that rely on burning process, which will eventually result in the generation of green house gasses and other pollutants (http://www.eere.energy.gov/hydrogenandfuelcells).

Hydrogen has been used in many applications including as fuel. However the amount of gas generated currently is still short for the use as fuel. The bottleneck of the use of hydrogen as fuel is the production of the gas in the large enough quantity in a sustainable way. Therefore there is a real need for research into sustainable energy production technology such as hydrogen in Thailand if we are going to be able to avoid the energy and environmental crisis.

4.2 วัตถุประสงค์ (Aims)

1. To survey various sources for hydrogen producing bacteria, particularly thermophiles.
2. To isolate bacteria, naturally capable of producing hydrogen in significant amount.
3. To find optimal conditions for H_2 production by chosen strain.
4. To carry out detail studies on a chosen strain for the purpose of description and identification.

4.3 ระเบียบวิธีวิจัย (Methodology)

4.3.1 Sample collection:

Environmental samples used in isolation included 1) soil from a rice paddy in Warinchamrab (WS), 2) cow dung (CD), 3) soil settlement and water samples from the Mae Khong River in an area in Phonpisai, Nongkhai (KRS), 4) sludge from an open pond of cassava-processing factory waste water at Chonjaren Factory, Chonburi (AS), 5) soil settlement and water samples from various thermally heated springs in northern Thailand (THS) and 6) soil from coffee plantation area in Jampasak, Laos (Lao). Samples 1-2 were collected in Warinchamrab area, Ubonratchathani, Thailand whereas sample 5 were collected from 4 hot springs (namely Phang, Huay-Maaklium, Pha-Sert and Huay-Sai-Khaow) in northern Thailand. Soil samples were collected from the depth of 1-10 cm below the surface. Sludge from an open pond of a cassava processing factory waste water was collected at three meters away from the bank and the depth was one meter below the surface. Cow dung sample was collected from the middle of the pile and about an hour after it had been dropped. Soil settlement and water samples 3 and 6 were collected from the bottom of the ponds. Temperature and pH were measured and recorded at the sampling sites. Then sample bottles were placed on ice, transported to the laboratory at Ubon Rajathanee University, Ubonratchathani and stored at 4 $^{\circ}$C until processed.

4.3.2 Enrichment and isolation of bacteria:

Bacteria, both thermophiles and mesophiles, were isolated aerobically on nutrient agar plate (NA, composted of yeast extract 3.0 g, peptone 5.0 g, agar 15.0 g. in 1 l, pH was adjusted to 6.8 $\pm$ 0.2) at various temperatures and anaerobically in an anaerobic chamber (Sheldon Manufacturing, USA). The isolates were simply screened for the generation of gas by an application of Durham tubes in nutrient broth (NB) medium, same ingredients as NA except no agar was added. Bacterial isolate in cultures that contain evolved gas seen in Durham tubes were purified under suitable culture conditions by the method of streaking or end point dilution accordingly.

4.3.3 Test for the ability of pure isolates to produce H_2:

Once pure isolates were obtained, the ability of individual isolates to produce gas (and H_2 gas) was examined in NB pH 6.8 supplemented with 1% glucose. Total amount of gas formed was easily measured using glass syringe to measure the gas volume. Hydrogen gas formed in headspace of a vessel was analyzed by a hydrogen detector (Hy-optima Model 700, H2SCAN, USA) following the manufacturer recommendation.

4.3.4 Phylogenetic study by analyzing 16S rRNA gene sequences

Genomic DNA were extracted from each hydrogen-producing isolate and 16S rRNA genes were amplified by PCR. After that PCR products were subjected to RFLP analysis. Isolates were grouped into OTUs according to their restriction cutting pattern. Then some representatives of the OTUs were sent for partially sequencing of their 16S rRNA genes. The gene sequences were analyzed and phylogenetic trees were drawn.

4.3.4.1 Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted using modified CTAB/NaCl method (Andrews and Patel 1996). The 16S rRNA gene was PCR amplified using Fd1 primer (5'-AGAGTTTGATCCTGGCAG-3') and Rd1 primer (5'-AAGGAGGTGATCCAGCC-3'). Each reaction which was prepared in the total volume of 25 µl. composted of 12.5 µl PCR Master Mix (2X), 2.5 µl Fd1 Primer (50 µM), 2.5 µl Rd1 Primer (50 µM) and 5.5 µl. de-ionized water mixed very well. To the mixture 2 µl genomic DNA was added and the tube was placed in a thermal cycler programmed as follow: preheat at 94^{O}C (1 min) followed by 30 cycles of denaturation at 94^{O}C (30 s.), annealing at 55^{O}C (30 s.) and extension at 72^{O}C (2 min). PRC products were visualized along with standard DNA size marker under UV after gel electrophoresis using 2% agarose gel containing 1 µg/ml ethidium bromide.

4.3.4.2 RFLP Analysis

PCR amplified 16S rRNA gene products of 1500 bp. in size obtained for each of all the isolates were subjected to two types of restriction enzyme cutting, *Hinf*I and *Taq*I. Each reaction which was prepared in the total volume of 20 µl. composted of 13.3 µl. de-ionized water, 0.2 µl. acetylated BSA (10 µg/µl), 2.0 µl. buffer (10x), 4 µl. PCR product. After mixing, 0.5 µl. of *Hinf*I (10 U/µl.) was added and incubated at 37 ^{O}C for 2 hrs. Another tube was set in the same way but the enzyme used was 0.5 µl. of *Taq*I (10 U/µl.) followed by incubation at 65 ^{O}C for 2 hrs. Restriction cutting profiles were analyzed by gel electrophoresis using 2% agarose gel containing 1 µg/ml ethidium bromide. Bacteria isolates with the same restriction cutting patterns were grouped into the same operational taxonomic units (OTUs).

4.3.4.3 16S rRNA Gene Sequencing and Analysis

PCR amplified 16S rRNA gene products obtained for each of all the isolates were also partially sequenced (ranging from 367 to 973 nucleotides at the 5') at the Genomic Institute, Thailand. Sequences generated were assembled and the consensus sequence corrected manually for errors using BioEdit (Hall, 1999). The most closely related sequences in GenBank and Ribosomal Database Project II were identified using BLAST (Altschul, *et al*., 1997) and the Sequence Match program (Maidak, *et al*., 2001); sequences were then extracted from the

databases, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Sequence uncertainties were omitted and phylogenetic reconstruction achieved using TreeCon (Van de Peer & De Wachter, 1994) in which pairwise evolutionary distances were computed from percentage similarities (Jukes & Cantor, 1969) and phylogenetic trees constructed from the evolutionary distances using the neighbour-joining method (Saitou & Nei, 1987). Tree topology was re-examined by using the bootstrap method of re-sampling (Felsenstein, 1985) using 1000 bootstraps.

4.3.5 Study of optimal conditions for growth and hydrogen production by a chosen strain, KRS4C-6:

Growth and production of hydrogen gas by strain KRS4C-6 at various temperatures (10-45°C) and pH (5-7.6) were examined. Optimal initial pH for growth and H_2 production by strain KRS4C-6 were determined in 500 ml NB medium containing 1% glucose adjusted the pH to 5.1, 6.0, 6.7, 7.3 and 7.6. The flasks were incubated at 35°C cell growth and gas production were monitored every 24 hr for 8 days. Cell growth were determined by cell count and dry weight. Total gas produced was measured by water replacement method while H_2 content of the gas was determine by a hydrogen detector (Hy-optima Model 700, H2SCAN, USA). Once optimal pH for H_2 production was determined to be at pH 6, optimal temperature for H_2 production by the strain KRS4C-6 was determined at pH 6 using the same medium and condition but incubated at various temperatures which were 10, 20, 25, 30, 35, 40 and 45°C. Cell growth and gas production were monitored every 24 hr for 8 days in the same way as described.

4.3.6 Detailed studies on strain KRS4C-6 with high potential for gas production for the purpose of characterization to the level of species:

Strain KRS4C-6 that exhibited a high potential for further applications was further studied in detailed for the purpose of characterization and classification to the level of species according to the system of polyphasic taxonomy (Vandamme *et al.*, 1996). The studies included both traditional methods for studying phenotypic characteristics such as cell and colony morphology, cell wall composition, fatty acid composition, biochemical characteristics, fermentation of sugars and fermentation product as well as modern methods used for studying genotypic characteristics such as 16S rRNA gene sequences analysis, %GC content and DNA:DNA hybridization. These analyses are widely regarded as a necessarily information for description and classification of novel bacteria if the information are to be published internationally in a journal with peer review.

5. ผลการทดลอง (Results)

5.1 Isolation:

Out of 106 isolates obtained, there were only 13 isolates capable of producing 2 ml of gas or more in 48 hours. Hydrogen-producing bacteria were successfully isolated from all sample sources used except from hot springs. However amount of biogas and hydrogen gas produced by each isolates varied as shown in table 1. Isolate KRS4C-6 gave the highest amount of 3.3 ml. of hydrogen gas from 16.1 ml. biogas calculated to be 20.5% v/v while isolate AS_I-2, Lao-1-2 and AS_I-1 gave 2.70 ml, 2.51 ml. and 2.12 ml. of hydrogen gas respectively. However it must be noted that these H_2 production had not been conducted under optimal condition. Therefore the percent H_2 obtained were rather low. Bacteria isolates from samples collected from 4 hot springs (namely Phang, Huay-Maaklium, Pha-Sert and Huay-Sai-Khaow) in northern Thailand all did not generate any gas in the media and condition tested.

Table 1 Biogas and hydrogen gas produced by the 13 isolates growing in NB supplemented with 1% glucose and incubated at 30^{O}C for 48 hrs.

No.	Isolate Name [a]	Total biogas (ml.)	Hydrogen gas (ml.)
1	AS-1	11.6	1.46
2	AS-4	13.0	1.83
3	AS_I-1	15.1	2.12
4	AS_I-2	15.7	2.70
5	CD-5	13.1	1.97
6	CD-6	13.9	1.92
7	WS-2-2	15.0	1.81
8	WS-4-2	2.4	0.35
9	WS-7-11	13.1	2.08
10	Lao-1-2	15.3	2.51
11	Lao-1-11	12.6	1.83
12	KRS4B-5	16.0	2.90
13	KRS4C-6	16.1	3.30

[a] Name of isolates and corresponding sources of sample were as follow: WS= Warinchamrab soils, CD= cow dung, KRS= Khong River settlement, AS= Sludge from cassava-processing factory waste water, THS= Thai hot springs in northern Thailand.

5.2 Basic Morphological study

Basic morphological studies of hydrogen-producing bacteria growing on NA plate at 30°C for 2 hrs. yielded 8 Gram-negative and 5 Gram-positive isolates with cell and colony morphology as shown in the table 2.

Table 2 Basic morphology of 13 hydrogen-producing isolates cultured in NA 24 hrs. at 30°C.

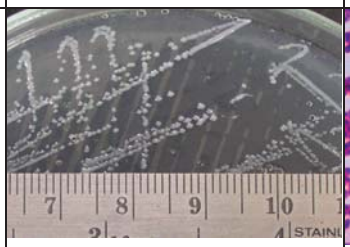
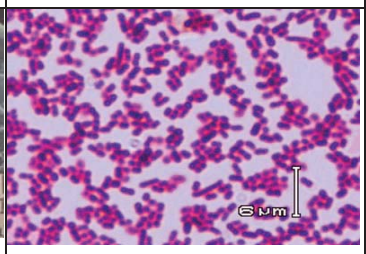
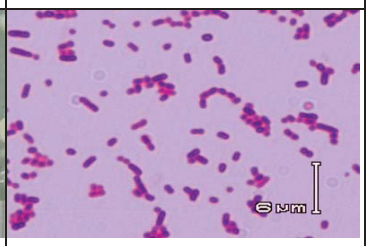
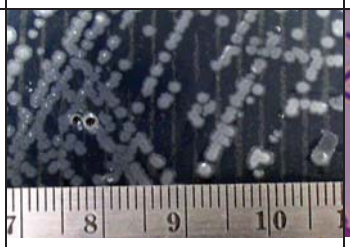
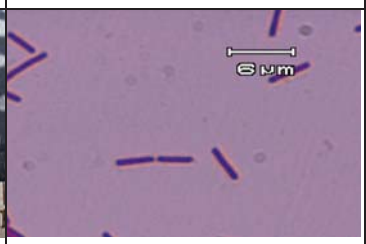
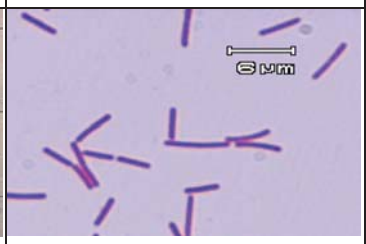
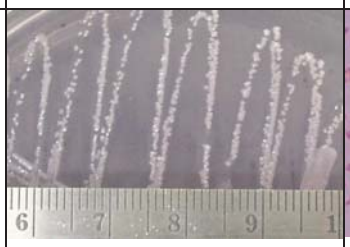
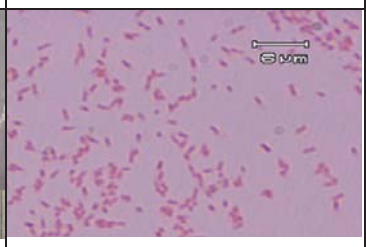
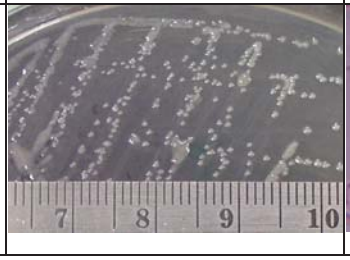
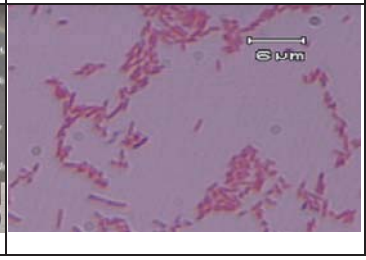
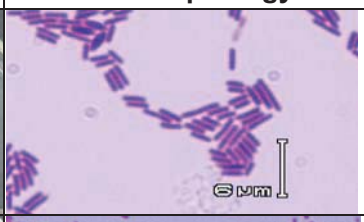
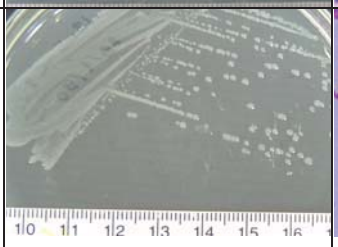
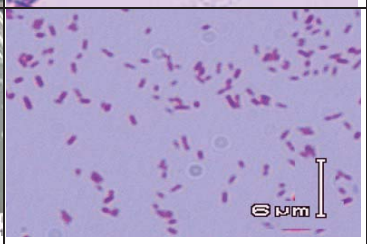
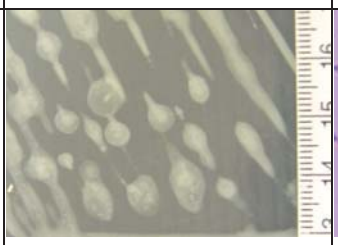
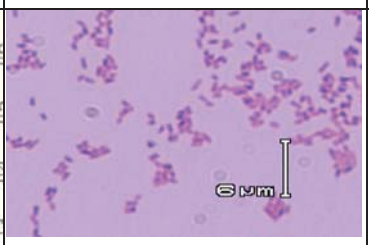
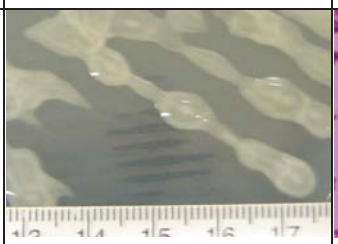
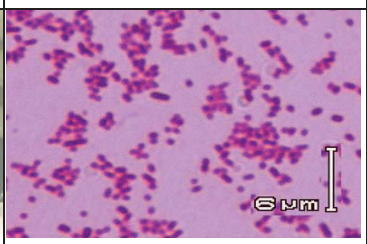
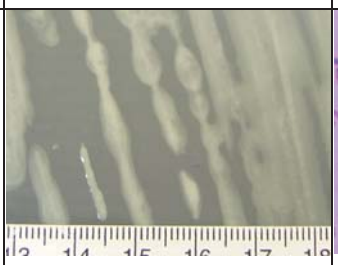
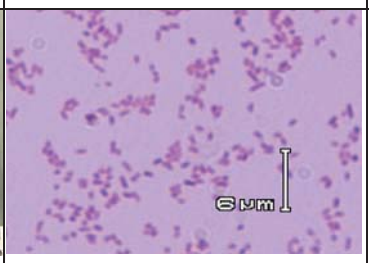
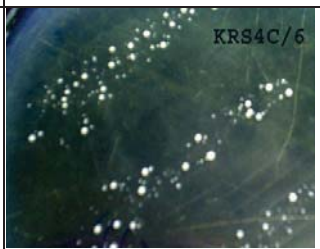
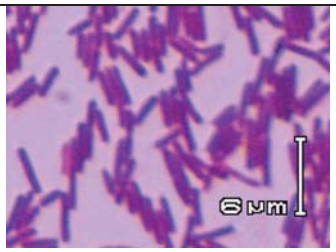
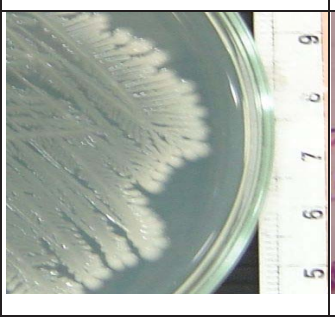
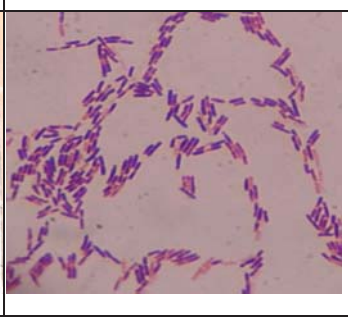
Isolate	Colony Morphology	Cell Morphology	Description
AS-1			Glistering white, 1-2 mm, Entire, convex colony, short rod, 0.5-1x1 μm, Gram-negative
AS-4			Glistering white, 2-4mm, Entire, convex colony, cocci or short rod, 0.5-1x1 μm, Gram-negative
AS_I-1			White colony with transparent edge, 1-2 mm, smooth, rhizoid, rod, 0.5x3-4 μm, gram-positive
AS_I-2			White, 1-3 mm, smooth, rhizoid, rod, 0.5x3-5 μm, gram-positive
CD-5			White, 0.5-1 mm. smooth, entire, short rod, 0.5x1 μm, Gram-negative
CD-6			White turbid, 0.5-1 mm. smooth, entire, short rod, 0.5x1-2 μm, Gram-negative

Table 2 (cont) Basis morphology of 13 hydrogen-producing isolates in NA for 24 hrs. at 30°C.

Isolate	Colony Morphology	Cell Morphology	Description
WS-2-2			White, 1-5 mm. rough, entire, rod, 0.5x1-2 µm, gram-positive
WS-4-2			White turbid, 1-2 mm. Convex, entire, short rod, 0.5x1-1.5 µm, Gram-negative
WS-7-11			White turbid, 3-6 mm, Convex, entire, short rod, 0.5x0.5-1 µm, Gram-negative
Lao-1-2			White turbid, 3-6 mm, Convex, entire, cocci to short rod, 0.5x0.5-1 µm, Gram-negative
Lao-1-11			White turbid, 3-6 mm, Convex, entire, cocci to short rod, 0.5x0.5-1 µm, Gram-negative
KRS4C-6			White turbid or transparent, 1-2 mm, convex, entire, 1-1.2x3-4 µm, spore-forming rod, single or pair, motile and Gram-positive
KRS4B-5			White with turbid edge, rhizoid, 1x3-4 µm, spore-forming rod, single or pair, motile and Gram-positive

5.3 Phylogenetic study by analyzing 16S rRNA gene sequences

5.3.1 PCR Amplification and RFLP Analysis

Genomic DNA from 11 isolates were successfully PCR amplified and products of about 1500-1600 bp in size were obtained for every isolates (Figure 1). Restriction enzyme cutting profile from digestion with *Hin*fI appeared as in figure 2 and by *Taq*I appeared as in Figure 3. The other 2 isolates (KRS4C-6 and KRS4B-5) had been phylogenetically analyzed by sequencing of 16S rRNA gene without employing the RFLP technique and results were presented in sections 5.3.2 and 5.5.3.

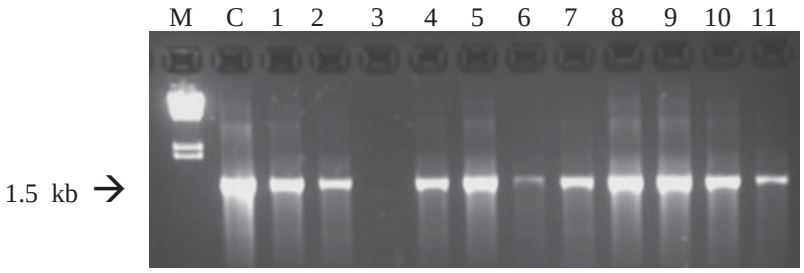


Figure 1 PCR amplified products of about 1500-1600 bp in size were obtained for every isolates. M=λ *Hin*dIII DNA Size Marker, C=Positive PCR control, 1=WS-7-11, 2=Lao-1-11, 3*=Lao-1-2 (Good PCR product was later obtained but not shown here), 4=WS-4-2, 5=WS-2-2, 6=CD6, 7=CD5, 8=AS-I-2, 9=AS-I-1, 10=AS-4, 11=AS-1

According to figure 2 there were 4 distinct restriction cutting profiles designated *a, b, c* and *d* generated from restriction cutting with *Hin*fI. However restriction cutting profiles generated from restriction cutting with *Taq*I showed 6 distinct profiles. Therefore the 11 isolates were put into 6 groups (OTUs). OTU *a* composted of 2 isolates which were WS-7-11 and Lao-1-2 and OTU *d* composted of 3 isolates which were WS-2-2, AS-I-2 and AS-I-1. These grouping results were in agreement for both restriction cutting enzymes. However OTU *b* which composted of Lao-1-11, CD6 and CD5 according to *Hin*fI restriction cutting, could be further separated into 2 groups, with an additional band of about 450 bp in size for Lao-1-11 (OTU *b1)* while restriction patterns were the same for CD6 and CD5 (OTU *b2)*. OTU c which composted of WS-4-2, AS-4 and AS-1 according to *Hin*fI restriction (similar pattern in figure 2 lane 5, 10 and 11) could also be further separated into 2 OTUs, *c1* (WS-4-2) and *c2* (AS-4 and AS-1). Restriction pattern of WS-4-2 cut with *Taq*I (figure 3 lane 4) was clearly different from those of AS-4 and AS-1 (figure 3 lane 10 and 11).

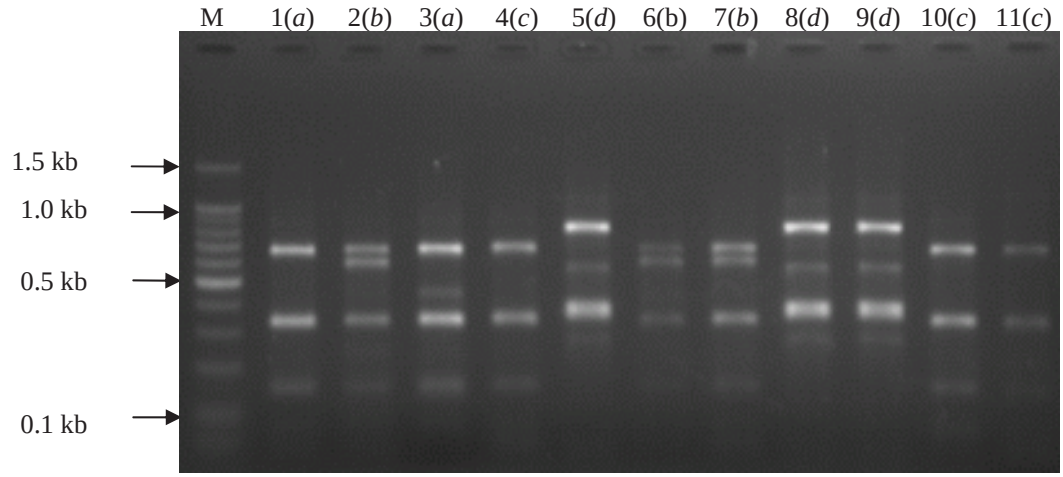


Figure 2 Restriction enzyme cutting profile from digestion with *Hin*fI. M=100 bp DNA ladder, 1=WS-7-11, 2=Lao-1-11, 3=Lao-1-2, 4=WS-4-2, 5=WS-2-2, 6=CD6, 7=CD5, 8=AS-I-2, 9=AS-I-1, 10=AS-4, 11=AS-1. Alphabets *a, b, c* and *d* represented 4 distinct restriction cutting profiles.

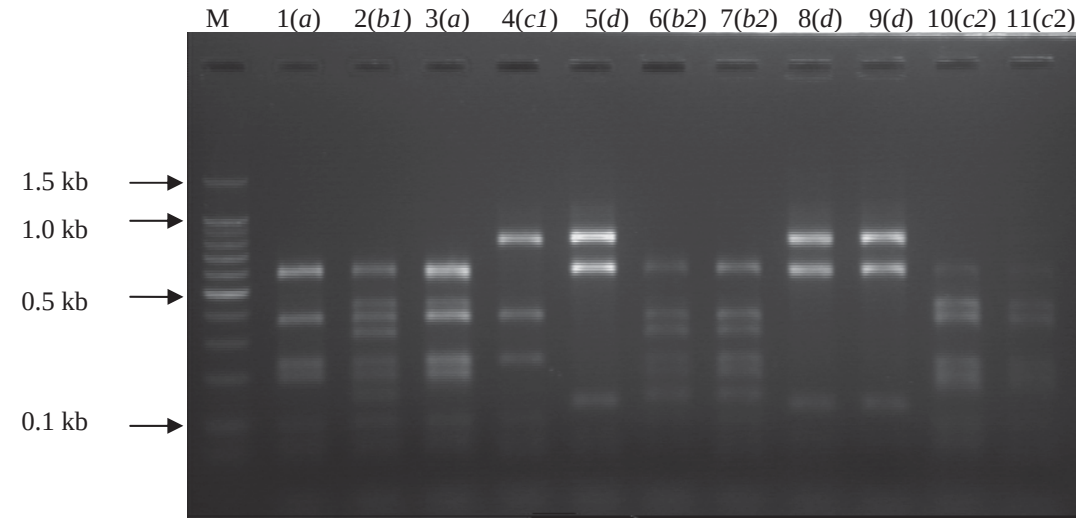


Figure 3 Restriction enzyme cutting profile from digestion with *Taq*I. M=100 bp DNA ladder, 1=WS-7-11, 2=Lao-1-11, 3=Lao-1-2, 4=WS-4-2, 5=WS-2-2, 6=CD6, 7=CD5, 8=AS-I-2, 9=AS-I-1, 10=AS-4, 11=AS-1. Alphabets *a*, *b1*, *b2*, *c1*, *c2* and *d* represented 6 distinct restriction cutting profiles hence OTUs.

To summarize, the 11 isolates were put into 6 distinct OTUs according to restriction pattern of 16S rRNA gene PCR amplified products designated OTU *a* (WS-7-11 and Lao-1-2), *b1* (Lao-1-11), *b2* (CD6 and CD5), *c1* (WS-4-2), *c2* (AS-4 and AS-1) and OTU *d* (WS-2-2, AS-I-2 and AS-I-1). The 6 OTUs designated were evaluated against results from 16S rRNA gene sequence analysis in the following section.

5.3.2 16S rRNA Gene Sequence Analysis and Phylogeny

Table 3 BLASTn analysis of 16S rRNA gene of hydrogen-producing isolates against GenBank database.

No.	Isolate Name	Description	Accession Number	Maximum Identity	No. of Nucleotide
1	WS-2-2	*Paenibacillus polymyxa* str. GBRR-465	AY359623.1	98%	963/973
2	WS-4-2	*Escherichia coli* C2	AF403733.1	99%	891/894
3	WS-7-11	*Pantoea agglomerans* str. WAB1969	AM184307.1	99%	640/642
4	AS-1	*Klebsiella pneumoniae* ATCC13884T	Y17657.1	99%	882/884
5	AS-4	*Klebsiella variicola*	AJ783916.1	99%	859/865
6	AS_I-1	*Paenibacillus polymyxa* str. KCTC3627	AY359636.1	98%	360/367
7	AS_I-2	*Paenibacillus polymyxa* str. KCTC3627	AY359636.1	98%	529/537
8	CD-5	*Enterobacter cloacae* subsp. Dissolvens	DQ988523.1	99%	641/642
9	CD-6	*Pantoea agglomerans* str. WAB1927	AM184266.1	99%	687/689
10	Lao-1-2	*Pantoea agglomerans* str. WAB1872	AM184214.1	98%	849/860
11	Lao-1-11	Sequencing failed	-	-	-
12	KRS4C-6	*Paenibacillus kribbensis* str. AM49	NR_025169.1	99%	1416/1428
13	KRS4B-5	*Paenibacillus polymyxa* str. A10	AM882681.1	98%	362/371

From the analysis of partial 16S rRNA gene sequence (367-973 nucleotides on the 5' end of the gene) by BLASTn, it was found that all the 13 isolates could be affiliated with bacteria in 5 different genera which were *Paenibacillus*, *Escherrichia*, *Pantoea*, *Klebsiella* and genus *Enterobacter* with very high similarity between 98-99% in all the case as shown in table 3. These high levels of similarity were in the range which could be assigned in the same species when the entire genes were analyzed (Stackebrant & Goebel, 1994). Analysis of 16S rRNA gene has been generally accepted for its use in bacterial classification and taxonomy with accuracy (Weisburg, *et al*., 1991; Petti, *et al*., 2005). Furthermore partial sequence analysis on the 5' of the gene had also been shown to give acceptably good results in bacteria identification due to the fact that these regions were highly variable (Byers, *et al*., 2005; Okhravi, *et al*., 2000). The 16S rRNA gene of isolate KRS4C-6 which produced the greatest amount of H_2 (3.3 ml) was phylogenetically analyzed fully in a separate section (5.5.3) while for Lao-1-11 the gene was unable to be sequenced.

AS_I-2 which produced 2.71 ml hydrogen gas along with AS-I-1 (giving 2.12 ml H_2) and WS-2-2 (1.8 ml H_2) were grouped in OTU *d* and were affiliated with *Paenibacillus polymixa* with 98-99 % similarity. *P. polymixa* was a member of the genus *Paenibacillus*, family *Paenibacillaceae,* order *Bacillales* and class *Bacilli.* In addition to being put in the same OTU *(d)* basic morphology and biochemical test results of these three isolates were in agreement with the characteristics of the genus *Paenibacillus.* Furthermore phylogenetic inferring confirmed grouping resulted from restriction cutting with a phylogenetic tree showing no distance between them (fig.4).

Lao-1-2 which produced 2.51 ml. hydrogen gas and isolate WS-7-11 were grouped together in OTU *a*. The two isolates were affiliated with members of the genus *Pantoea* with similarity of 98-99% to *Pantoea agglomerans.* Phylogenetic inferring also showed the two isolates related to one another with 88% bootstrap value (figure 5).

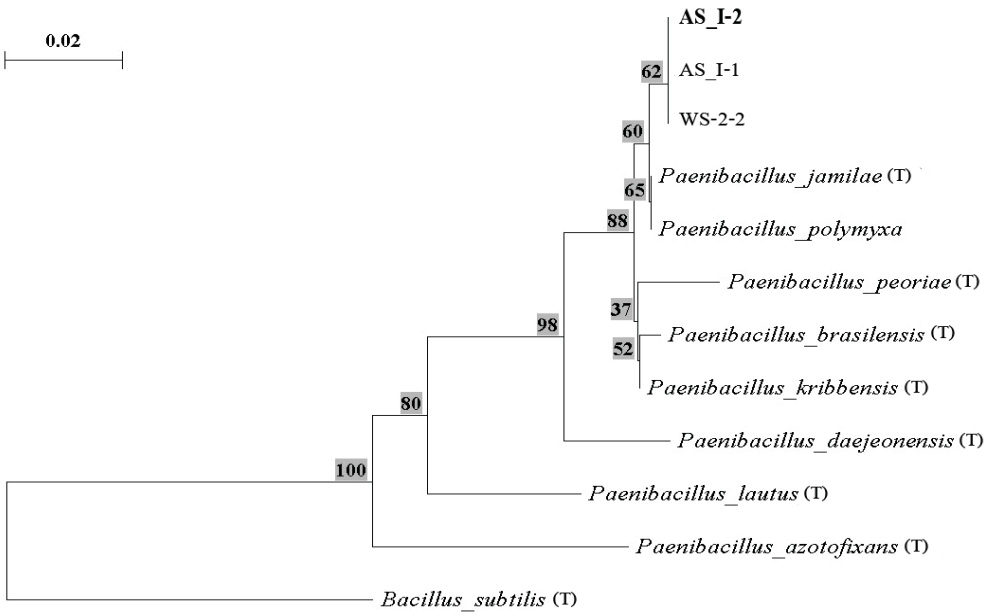


Figure 4 Phylogenetic tree drawn for hydrogen-producing bacterial isolates affiliated with members of genus *Paenibacillaceae. Bacillus subtilis* $^{(T)}$ was an out group species. Bar=2 nucleotide difference per 100 nucleotides.

In the cases of OTU *b2,* isolates CD6 and CD5 showed different BLASTn results. CD6 had 99% similarity to *Pantoea agglomerans* strain while CD5 showed 99% similarity to *Enterobacter cloacae* strain. These did not indicate a disagreement of results from two different methods because the two genera were very closely related and their current taxonomies were super imposed. OTU *b1* could not be confirmed by 16S rRNA sequence analysis because sequencing failed. For OTU *c2* (AS-4 and AS-1), 16S rRNA analysis clearly confirmed the grouping as the two isolates showed similarity of 99% to member of the genus *Klebsiella*, although maximum

similarities were with different *Klebsiella* species. WS-4-2, the only member of OTU *c1* was also the only isolate that was affiliated with the genus *Escherichia* (99% similarity to *E. coli* C2). This very well explained its distinct restriction digestion pattern.

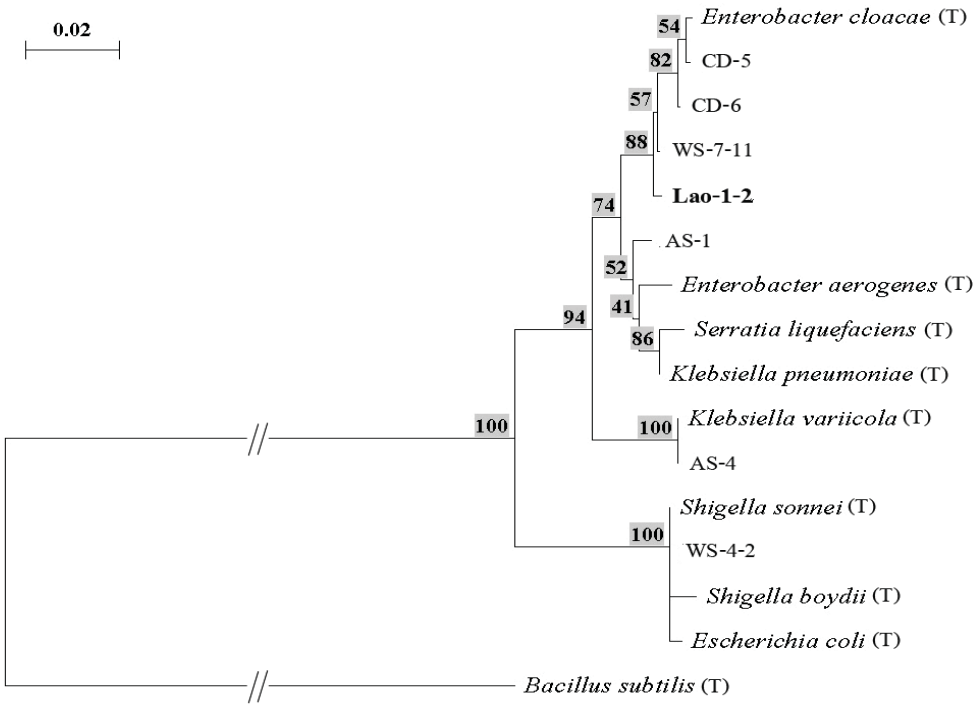


Figure 5 Phylogenetic tree drawn for hydrogen-producing bacterial isolates affiliated with members of family *Enterobacteriaceae*. *Bacillus subtilis*$^{(T)}$ was an out group species. Bar=2 nucleotide difference per 100 nucleotides.

Considering results from species affiliation of the 11 hydrogen-producing isolates, it was found that all the genera that the isolates were affiliated with had been reported to have members capable of producing hydrogen gas which were as anticipated. For example *Paenibacillus polymyxa, a member of the genus Paenibacillus* in which AS_I-2, AS_I-1 and WS-2-2 were closely related to, was reported to be one of the two species responsible for hydrogen production from basic-treated-waste (Cai, *et al*., 2004). In addition Sakka and others (2005) concluded that the explosion of a silo storing Refuse-Derived Fuel (RDP) pellets in Mie Prefecture, Japan in 2003 was likely to be connected with accumulated hydrogen gas in the silo. His study also revealed the presence of *Paenibacillus* sp. in the RDP pellets stored.

Other four genera the other 7 isolates were affiliated with were *Escherrichia*, *Pantoea, Klebsiella* and *Enterobacter members of the family Enterobacteraceae.* Members of this family were bacteria associated with digestion tract of worm blooded animal and also generally found in soil and water. It was well known that these group of bacteria produced acid and gas from glucose fermentation. The gas produce composted of carbon dioxide and hydrogen. Member of

the genera *Escherichia* and *Pantoea* were capable of fermentation of glucose and other carbohydrate producing lactic acid, acetic acid as well as formic acid. Some formic acid was further digested by enzyme hydrogenlyase generating carbon dioxide and hydrogen gas. Members of the genus *Klebsiella* were also known to produce carbon dioxide and hydrogen with the greater amount of the former gas. *Enterobacter* sp. produced carbon dioxide and hydrogen in the ratio of 2:1 at 37 $^{\circ}$C but ceased to produce gas from glucose at 44.5 $^{\circ}$C (Rachman, *et al*., 1998; Minnan, *et al*., 2005; Nath, *et al*., 2005).

In conclusion, cultivable hydrogen-producing bacteria in the northeastern Thailand and western Laos were not as diverse as firstly anticipated. Two possibilities were that no other bacteria apart from the two main groups found, were capable of growth under isolation condition used or there were no other bacteria capable to produce hydrogen in those sample sources.

5.4 Optimal conditions for growth and hydrogen production by strain KRS4C-6

5.4.1 Determination of optimal pH for H_2 production by strain KRS4C-6

From a preliminary test (results not shown) KRS4C-6 was able to grow at initial pH between 5-8.5. But the optimal pH for H_2 production (tested at pH 5-7.6) was found to be at pH 6.0 in which 442.65 ml of H_2 was produced (table 4) with the highest H_2 content obtained of 33 %. This was slightly more than 431.53 ml produced at pH 7.3. However according to figure 6, during the fermentation H_2 was produced in the greatest amount in the first 24 hr of fermentation at every initial pH except at pH 5.1 and the amount produced at initial pH of 6-7.6 were not very different. At initial pH 5.1 H_2-production appeared to have a longer lag phase of 48 hr and showed peaked H_2 after 72 hr.

Table 4 Production of H_2 by KRS4C-6 at various initial pH 5–7.6 in 500 ml NB medium supplemented with 1 % glucose at 35 $^{\circ}$C.

Volume of H_2 gas produced at 24 hr interval (ml).										
Time (hr) / **pH**	**0**	**24**	**48**	**72**	**96**	**120**	**144**	**168**	**192**	Total H_2 (ml)
pH 5.1	0.00	29.06	21.08	90.99	65.32	60.39	0.00	0.00	0.00	266.84
pH 6.0	0.00	132.54	103.29	83.14	52.78	45.19	18.90	6.81	0.00	**442.65**
pH 6.7	0.00	130.48	56.50	73.80	57.69	28.59	0.00	0.00	0.00	347.06
pH 7.3	0.00	166.32	106.65	63.44	37.90	54.20	3.02	0.00	0.00	431.53
pH 7.6	0.00	137.12	98.99	52.24	48.90	21.81	0.00	0.00	0.00	359.06

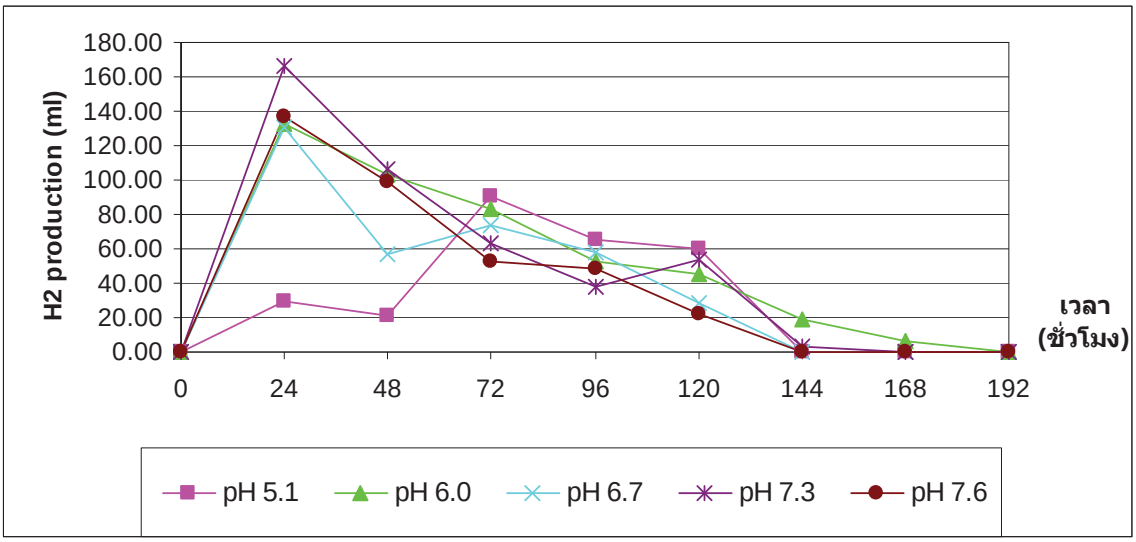


Figure 6 Production of H_2 by KRS4C-6 at various initial pH 5-7.6 in 500 ml NB medium supplemented with 1 % glucose at 35 °C.

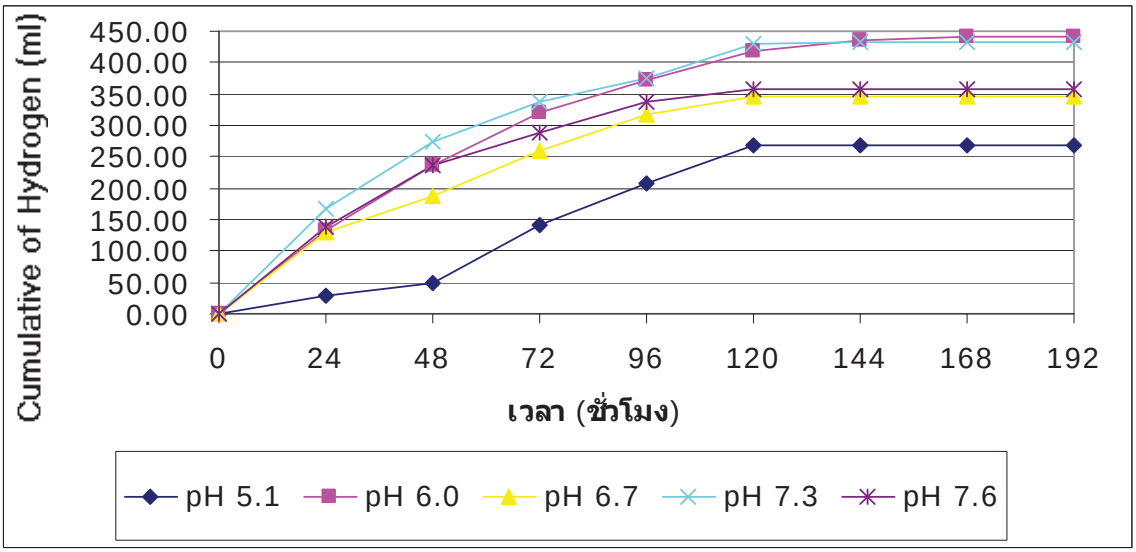


Figure 7 Accumulative H_2 produced by KRS4C-6 at various initial pH 5–7.6 in 500 ml NB medium supplemented with 1 % glucose at 35 °C.

5.4.2 Determination of optimal temperature for H_2 production by strain KRS4C-6

Abilities of isolate KRS4C-6 to grow and produce H_2 at various temperatures were tested between 10-45 °C at optimal initial pH 6. KRS4C-6 was able to grow at temperatures between 13-43 °C but H_2 production was observed only at temperatures between 20-40 °C (table 5). The highest H_2 volume and content were obtained at 35 °C and were equal to 494.82 ml and 32.5 % respectively. Figure 8 and 9 also showed that H_2 production increased with the increase in temperature between 20-35 °C but further increase in temperature to 40 °C and 45°C stopped the gas production.

Table 5 Production of H_2 by KRS4C-6 at various temperatures between 10-45 °C at optimal initial pH 6 in 500 ml NB medium supplemented with 1 % glucose.

Volume of H_2 gas produced at 24 hr interval (ml).									
Time (hr) / Temperature(°C)	0	24	48	72	96	120	144	168	Total H_2 (ml)
10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20	0.00	14.31	57.76	58.32	73.10	61.17	48.83	26.73	340.22
25	0.00	56.42	118.43	96.59	89.04	64.45	14.96	0.00	439.89
30	0.00	92.04	124.06	119.44	96.33	39.77	0.00	0.00	471.64
35	0.00	125.94	**146.79**	99.63	71.09	37.57	13.81	0.00	**494.82**
40	0.00	0.00	0.00	0.00	0.00	0.00	199.63	0.00	199.63
45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

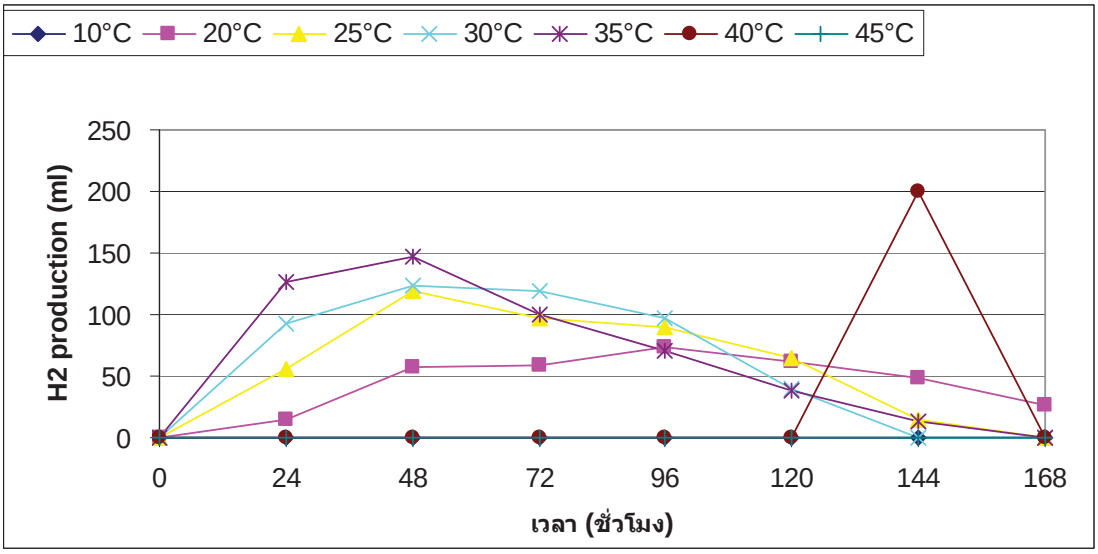


Figure 8 Production of H_2 by KRS4C-6 at various temperatures between 10-45°C in 500 ml NB medium supplemented with 1 % glucose at optimal initial pH 6.

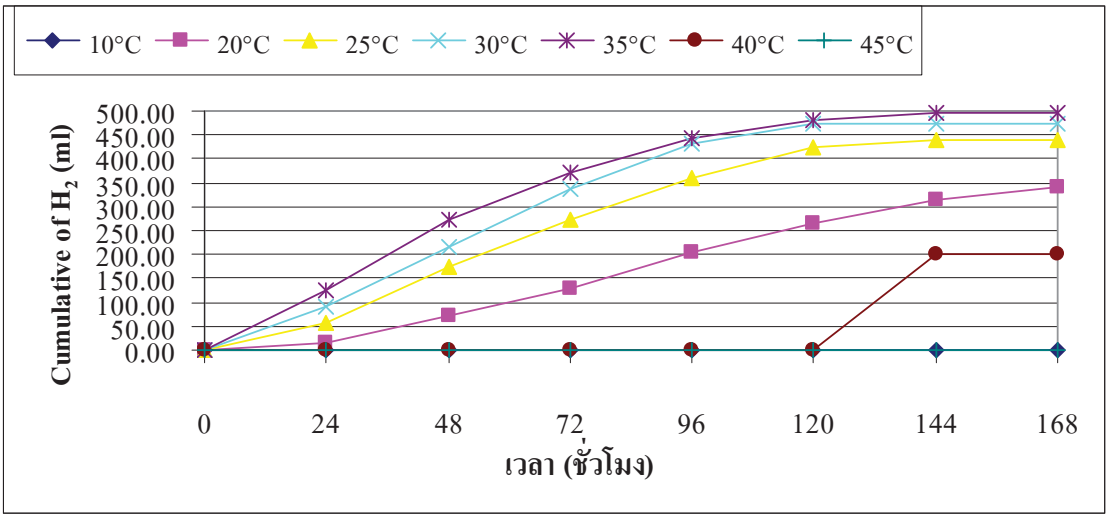


Figure 9 Accumulative H_2 produced by KRS4C-6 at various temperatures between 10-45°C in 500 ml NB medium supplemented with 1 % glucose at optimal initial pH 6.

5.4.3 H_2 production by KRS4C-6 at optimal conditions (initial pH 6, 35 °C)

H_2 production at optimal condition (pH 6, temperature 35 °C) was studied in 500 ml NB medium supplemented with 1% glucose in 1000 ml flask flashed with N_2 gas for 5 minutes. Cell growth, H_2 production, glucose utilization and pH changes were monitored for 144 hr, times with gas generation. H_2 gas production appeared to occur with very little lag period as a great amount of H_2 was measured after 24 hr and peaked at 48 hr which was in the log phase of cell growth. This indicated that H_2 production and growth occurred at the same time and in expense of glucose as could be seen by the sharp decrease of glucose simultaneously. Under optimal condition KRS4C-6 produced the greatest amount of highest H_2 of 495 ml from the total biogas of 1146 ml which was equal to 43.17 %. H_2 yield was calculated to be 0.9692 mol H_2/mol glucose from the total glucose utilized of 4.09 g (0.0227 mol).

Table 6 H_2-production by KRS4C-6 at optimal condition (pH 6, temperature 35 °C) in 500 ml NB medium supplemented with 1% glucose in 1000 ml flask flashed with N_2 gas for 5 minutes.

Volume of H_2 gas produced at 24 hr interval (ml).								
Time (hr) / **Parameter measured**	**0**	**24**	**48**	**72**	**96**	**120**	**144**	**168**
H_2 (ml)	0.00	125.94	146.79	99.63	71.09	37.57	13.81	0.00
H_2 accumulation (ml)	0.00	125.94	272.73	372.36	443.45	481.02	494.82	494.82
Glucose (mg/ml)	8.67	6.70	3.72	2.25	0.44	0.39	0.49	0.43
pH	6.0	5.2	5.1	5.2	5.3	5.8	5.8	5.9
OD_{600}	0.02	0.40	0.52	0.58	0.74	0.65	0.58	0.56

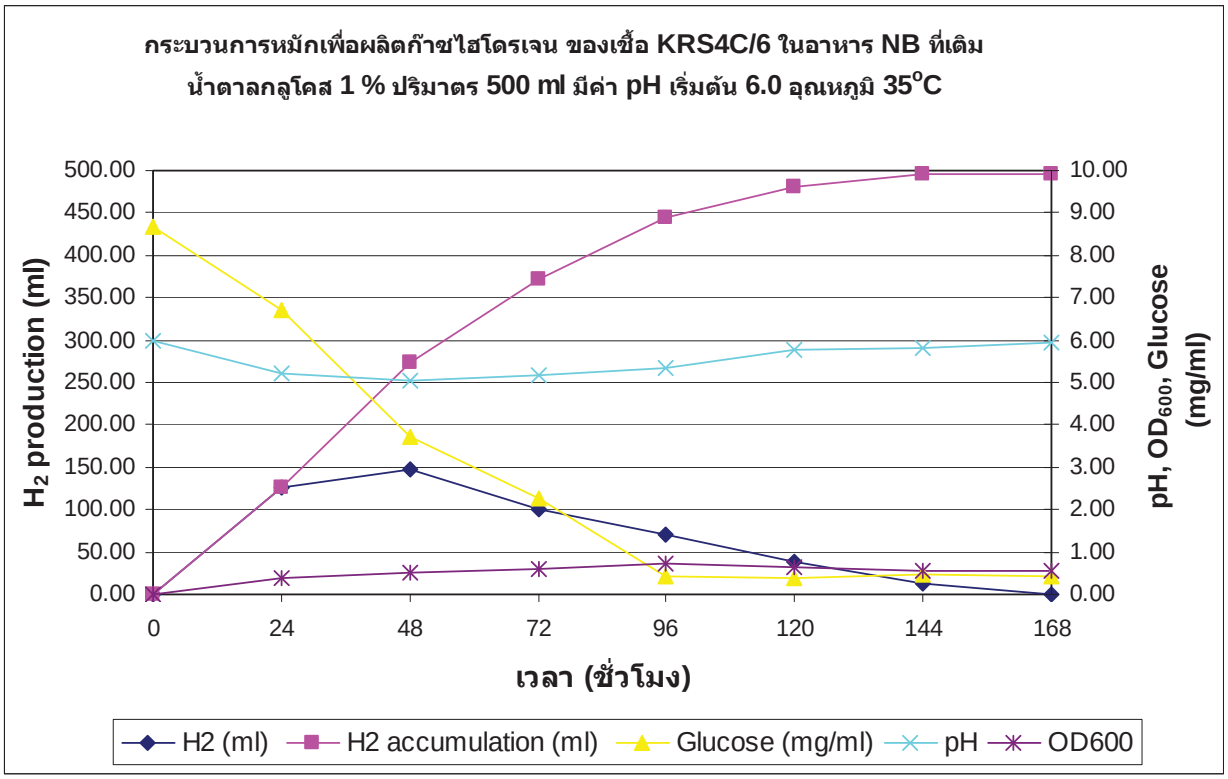


Figure 10 H_2-production by KRS4C-6 at optimal condition (pH 6, temperature 35 °C) in 500 ml NB medium supplemented with 1% glucose in 1000 ml flask flashed with N_2 gas for 5 minutes.

5.5 Detailed study on KRS4C-6, the isolate producing the greatest amount of H_2.

5.5.1 Morphological, biochemical studies and fermentation of sugars

The colonies of strain KRS4C-6 were 1-2 mm in size, white in color, circular and convex with smooth surface and entire edges. Cellular characterization and sporulation tests performed as described by Andrews and Patel (1996) showed that the cells of strain KRS4C-6 stained Gram-positive, occurred singly or in pairs. The cells were usually rods to slightly curved rods (1-1.2 x 3-4 μm.) Strain KRS4C-6 was motile and ellipsoidal spores were observed. Strain KRS4C-6 was a facultative anaerobe as it also grew in both NB prepared aerobically and anaerobically (boiled and flushed with N_2 gas for 30 minutes before dispensed under a stream of oxygen-free N_2 into 10 ml aliquot in Hungate tubes sealed with air-proof butyl septum. Morphological characteristics and biochemical test results in comparison with related strains were present in the table 7.

Peptidoglycan was isolated from the strain KRS4C-6 and its structure was determined. The total hydrolysate (4N NCl, 100 °C, 16h) of the peptidoglycan contained the amino acid meso-diaminopimelic acid, alanine and glutamic acid in a quantitative ratio of ca. 1.0 : 2.0 : 1.6, respectively. The strain KRS4C-6 showed the peptidoglycan type A1γ. Analysis of respiratory quinones carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschewig, Germany, showed that the Strain KRS4C-6 possessed the Menaquinone 7.

As analyzed by gas chromatography (Shimadzu GC-7AG), fermentation products from glucose were neither acetate (Rt=1.63 min.), proprionate (Rt=3.53 min.) nor butyrate (Rt=7.60 min.). Two major peaks of fermentation products from glucose shown in analytical trace had retention times of 0.86 minutes and 10.14 minutes but their identities were not known but likely to be some other types of acid and alcohol.

Table 7 Comparison of morphological characteristic and biochemical test results of the isolate KRS4C-6 and its most related species, modified from Yoon, J.H. *et. al*., 2003.

Characteristic	*P.polymyxa*	*P.azotofixans*	*P.peoriae*	***P.kribbensis***	**KRS4C-6**
Spore shape and position*	Oval	Oval	Oval	Ellipsoidal	Ellipsoidal
Nitrate reduction	+	-	+	+	-
Hydrolysis of:					
Casein	+	-	+	+	+
Gelatin	+	-	+	+	-
Starch *	+	-	+	+	+
Urea	-	NT	-	-	-
Tween80	NT	NT	NT	+	+
Utilization of:					
L-Arabinose	NT	NT	NT	+	NT
D-Fructose	NT	NT	NT	+	+
D-Ribose	NT	NT	NT	+	NT
D-Xylose	NT	NT	NT	+	+
Citrate	-	-	+	W	-
Succinate	-	NT	+	W	NT
Acid production from:					
Adonitol	-	-	-	-	NT
L-Arabinose	+	-	+	+	NT
D-Arabinose	-	-	-	-	+
D-Xylose	+	-	+	+	+
Growth in presence of 2% NaCl	NT	V	NT	+	+
Optimum growth temperature (°C)	30	30–37	30	30–37	30-35
DNA G+C content (mol%)	43–46	48–53	45–47	48	46.8

NT= not test; +=positive reaction; -= negative reaction; W= weak reaction; V= variable reaction

5.5.2 Whole cell fatty acid composition

Strain KRS4C-6 had whole cell fatty acid profile similar to that of *P. kribbensis* AM49^T. The values of each type of fatty acids were very close to those values obtained from *P. kribbensis* AM49^T especially for anteiso C15:0, the major fatty acid component, in which the values were 52.4 for both strains.

Table 8 Comparison of whole cell fatty acid profile of the isolate KRS4C-6 and those of its most related species: 1=*P.polymyxa* DSM36^T; 2=*P.azotofixans* DSM5976^T; 3=*P.peoriae* DSM8320^T; 4=*P.kribbensis*^{T}; 5=KRS4C-6; 6=*P.terrae*^{T}

Fatty acid	1	2	3	4	5	6
Saturated fatty acid:						
C14:0	0.7	5.0	1.1	1.9	2.1	1.3
C15:0	0.5	2.2	2.6	1.7	1.21	2.2
C16:0	9.1	15.5	6.3	10.2	5.83	9.1
C17:0	ND	ND	0.4	0.5	0.2	0.3
Branched fatty acid:						
iso-C13:0	ND	1.3	0.4	ND	0.25	0.1
iso-C14:0	0.6	4.7	2.2	1.2	2.04	1.1
iso-C15:0	5.5	8.7	8.7	10.4	12.03	6.5
anteiso-C15:0	49.9	45.4	56.4	52.4	52.43	62.3
iso-C16:0	7.7	5.3	7.4	6.4	7.57	4.5
iso-C17:0	7.0	1.1	5.9	6.3	5.64	3.8
anteiso-C17:0	16.7	2.1	7.9	8.8	7.72	8.3

5.5.3 Phylogenetic tree analyses

16S rRNA gene sequence of strain KRS4C-6, accession number FJ937757, showed the greatest similarity to members of the genus *Paenibacillus*, family *Paenibacillaceae, order Bacillales* and *class Bacilli.* The closest relatives were *Paenibacillus kribbensis* AM49^T (similarity value of 99.3%). The high level of similarity between strain KRS4C-6 and *Paenibacillus kribbensis* AM49^T indicated that they were very closely related.

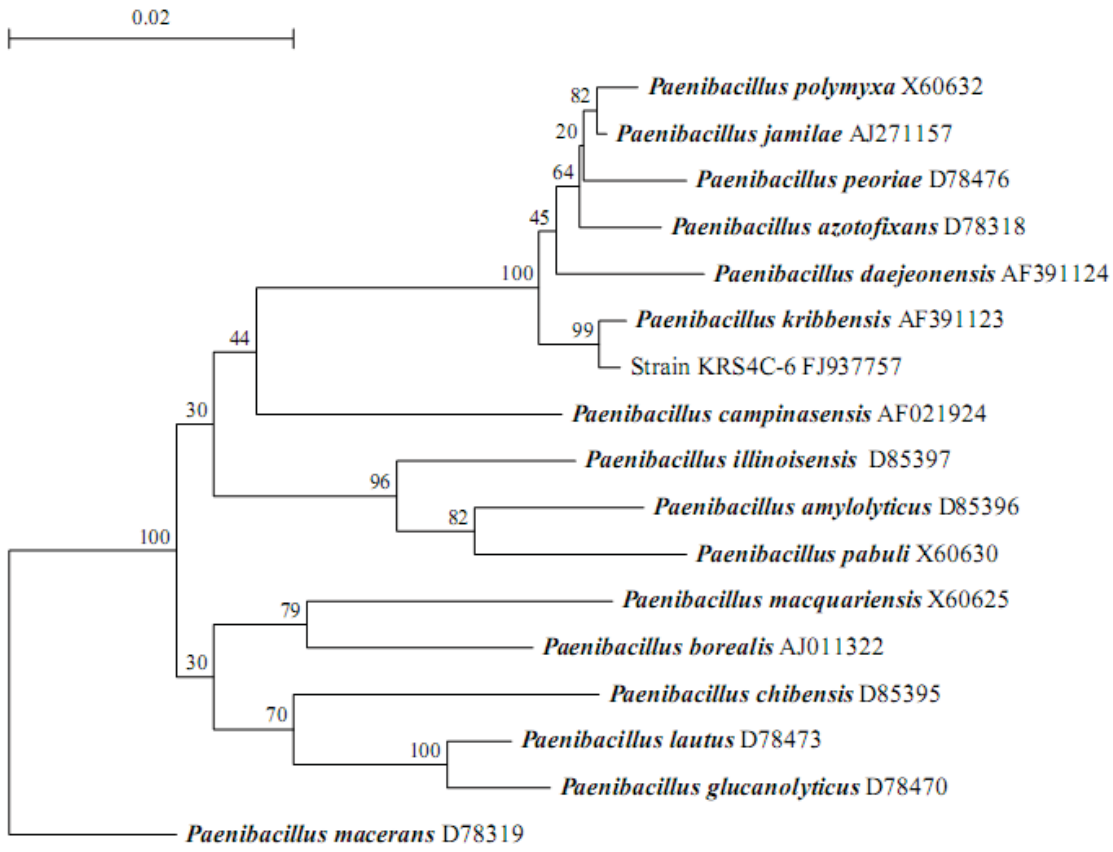


Figure 11 Dendrogram showing the phylogenetic position of str. KRS4C-6 (=DSMZ 19575 =TISTR 1857) as a member of the genus *Paenibacillus* and belong to the *Paenibacillus kribbensis* species. The dendrogram was constructed by using the neighbour-joining method and Jukes & Cantor evolutionary distance matrix of data obtained from 17 unambiguous aligned nucleotides. The sequences were extracted from the Ribosomal Database Project (RDP) version 8.0 and GenBank database, release 121. Bootstrap values shown in percentage are indicated at the nodes. GenBank/EMBL/DDBJ accession numbers for the sequences are shown after species names. Scale bar indicates 2 substitutions per 100 nucleotides.

5.5.4 %GC content and DNA:DNA hybridization

The DNA base composition of the strain KRS4C-6 was determined by HPLC to be equivalent to 46.8 mol% G+C.

DNA:DNA hybridization of the strain KRS4C-6 was performed against *P. kribbensis* in 2xSSC at 69 $^{\circ}$C. The average similarity between the two strains was measured to be 89.25 % (with the two actual values of 85.9 % and 92.6 %). As the recommendations of a threshold value of 70% DNA:DNA hybridization similarity for the definition of bacterial species by the *ad hoc* committee are considered, the strain KRS4C-6 appeared to belong to the species *Paenibacillus kribbensis*.

6. บทวิจารณ์ (Discussion)

Cultivable H_2-producing bacteria isolated were members of the genus *Paenibacillus* and the family *Enterobacteriaceae*. They were not as diverse as firstly anticipated. Possibilities were that no other bacteria capable of growth under isolation condition used, apart from the two main groups found. However the 13 isolated bacteria were affiliated with species reported to have capability of producing H_2. Although H_2 produced by these species has not been among maximum value reported, members of the genus *Paenibacillus* and the family *Enterobacteriaceae* have been reported to have an interesting property of bio-flocculant formation (You *et. al*., 2008) which could be advantages in wastewater treatment. More over these strains have been reported to be isolated from effluent of H_2 production reactor. This meant that the strains were indigenous to H_2 production system and hence must be well adapted to such environment making them likely to be very suitable as bio-flocculant maximizing floc formation in treatment of effluent from H_2 production reactor which normally required further treatment before it could be disposed off.

No *Clostridium* strain was isolated in this search for H_2-producing bacteria. This could have been because of a few reasons. The isolating media used and a short incubation time of 72 hours could lead to an overgrowth and subsequent isolation of facultative anaerobic and fast growing strains leaving out slow growing and fastidious strains like strict anaerobic *Clostridia* which would take longer time to develop into visible colony. Direct isolation from the environmental sample without enrichment could also contribute to the absence of *Clostridium*. If one aimed to specifically isolate *Clostridia* species, it would be better to set up an enrichment culture in a specific media suitable for *Clostridia* strain prior to isolation rather than isolation directly from an environmental sample. The incubation time should also be longer. The minimum of five to seven days should be more suitable for slow growing strains to develop. Diverse type of media and additives should also need to be tried in exploring for a novel strain. Finally to ensure that strict anaerobe were not excluded because of possible unsuitable degree of anaerobic condition, it is necessary to set up a positive control culturing of strict anaerobe a long during isolation.

Although cultivable H_2-producing bacteria found in this search were not as diverse as firstly anticipated, isolation and identification of H_2-producing bacteria should still be a desirable practice in parallel to H_2 production experiments since the production relying on mixed and often unidentified community of microbe such as sludge from the field as seed has a drawback that it can be non-reproducible. This is so because by its nature the microbial community is highly dynamic. It changes all the time according to various environmental factors and the changes are far too great and beyond one to predict. Therefore it is unlikely to always obtain seeds with the same microbial properties and content from such fields. In addition having a suitable effective H_2 producing strains or mixed culture of known strains available for re-inoculation following adjustment of some parameters could be one good way which would save the resource and effort already put into setting up the fermentation system, which can be vital in real practices.

7. References

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You Y, Ren N, Wang A, Ma F, Gao L, Peng Y. (2008) Use of waste fermenting liquor to produce bioflocculants with isolated strains. International Journal of Hydrogen Energy. 33: 3295-3301.

5. output จากโครงการวิจัยที่ได้รับทุนจาก สกว. (ตามรายละเอียดในเอกสารแนบหมายเลข 3)

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ** (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

    1. **Kanso. S.,** Dasri. K., Tingthong. S., Watanapokasin. R.Y. (2011), Diversity of cultivable hydrogen-producing bacteria isolated from agricultural soils, waste water sludge and cow dung. International Journal of Hydrogen Energy. 36 (14), 8735-8742. (Impact factor in 2011 = 3.945)
    2. Watanapokasin. R. Y., Boonyakamol. A., Sukseree. S., Krajarng. A., Sophonnithiprasert, T., **Kanso, S**., Imai, T. (2009). Hydrogen production and anaerobic decolorization of wastewater containing Reactive Blue 4 by a bacterial consortium of *Salmonella subterranea* and *Paenibacillus polymyxa*. Biodegradation. 20 (3), 411-418.
    (Impact factor in 2009 = 1.873)
    3. **Kanso. S.** and Dasri. K. *Paenibacillus kribbensis* strain KRS4C-6 16S ribosomal RNA gene, partial sequence deposition. GenBank Accession Number FJ937757.1
    4. **Kanso. S.** and Dasri. K. Characterization of H_2-producing bacteria *Paenibacillus kribbensis* strain KRS4C-6 isolated from the Mae Khong River and its bio-hydrogen production. Research in Microbiology. In preparation.

2. **การนำผลงานวิจัยไปใช้ประโยชน์**

    - **เชิงพาณิชย์ -**
    - **เชิงนโยบาย -**
    - **เชิงสาธารณะ** (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

    - สร้างเครือข่ายความร่วมมือกับ Green Energy Development Center, Feng Chia University, Taiwan (ซึ่งเป็นศูนย์ความเป็นเลิศด้านพลังงานไฮโดรเจนจากของเสียที่สร้างสถิติการผลิตไฮโดรเจนในระดับโลก) โดยทางศูนย์ได้ให้การตอบรับนักศึกษาระดับ ป. โท-เอก ไปทำวิจัยที่ศูนย์ได้เริ่มจากปี 2554

    - ได้รับทุนให้เข้าร่วมทำวิจัยใน Green Energy Development Center, Feng Chia University, Taiwan เป็นเวลา 3 เดือน โดยได้ทำหน้าที่

    1.ร่วมทำวิจัยเรื่องการผลิตไฮโดรเจนจากเซลลูโลส

    2.สอนบรรยายและปฏิบัติการแก่นักศึกษาระดับปริญญาโทและเอกในการเพาะเลี้ยงและแยกเชื้อแบคทีเรียและการประยุกต์ใช้เทคนิค PCR และ DGGE ในการศึกษาความหลากหลายของเชื้อแบคทีเรียในถังหมักไฮโดรเจน

3.เป็นผู้บรรยายให้ความรู้เรื่องแบคทีเรียกับการผลิตไฮโดรเจนจากของเสียโดยวิธีชีวภาพให้กับกลุ่มนักวิทยาศาสตร์รุ่นเยาว์จากประเทศไต้หวัน ฮ่องกง และออสเตรเลีย ที่เข้าเยี่ยมชมศูนย์ Green Energy Development Center.

- **เชิงวิชาการ** (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
  - **สร้างนักวิจัยใหม่ 2 คน คือ** นายขจรพงศ์ ดาศรี และ นายสุริยา ติ่งทอง
  - **พัฒนาการเรียนการสอน คือ** จัดให้มีปฏิบัติการแยกเชื้อกลุ่ม Strict anaerobe โดยใช้เทคนิค Roll tube technique และ end point dilution เป็นครั้งแรกในวิชานิเวศวิทยาจุลินทรีย์

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร

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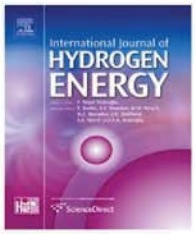

Diversity of cultivable hydrogen-producing bacteria isolated from agricultural soils, waste water sludge and cow dung

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ABSTRACT

This project aimed to study the diversity of cultivable hydrogen-producing bacteria, isolated from agricultural soils, waste water sludge and cow dung by analyzing 16S rRNA gene. Isolation performed anaerobically on nutrient agar using environmental samples as inoculum yielded 106 pure isolates. These isolates were tested for their capability to produce hydrogen. Then 16S rRNA gene of the 11 isolates having such ability were PCR amplified and subjected to restriction fragment length polymorphism (RFLP) analysis in order to group them into operational taxonomic units (OTUs). RFLP was evaluated for its ability to group the 1500–1600 bp PCR products into OTUs. Isolates were presumptively identified by analysis of partial 16S rRNA gene sequence data. Each OTU was found to be phylogenetically cohesive. The 11 isolates were put into 2 main groups. Group 1 composted of WS-2-2, AS_I-1 and AS_I-2 whose partial 16S rRNA genes showed similarity of 98–99% to members of the genus *Paenibacillus*. Group 2 composted of WS-4-2, Lao-1-2, WS-7-11, AS-1, AS-4, CD-5 and CD-6 having highest similarity of 98–99% to members of four genera in the family Enterobacteriacea. AS_I-2 which produced the highest amount of hydrogen gas of 2.70 ml had the highest 16S rRNA gene similarity of 99% to *Paenibacillus polymixa*.

1. Introduction

In an energy crisis era as the world is currently encountered, hydrogen gas as a clean renewable energy is what many countries pay attention to. Hydrogen can be generated in many ways but hydrogen production is still an energy consuming and very expensive process. Bio-hydrogen production by microorganism is one of the most attractive ways. There have been many reports on using bacteria for producing hydrogen gas with various results.

Many research groups have reported the isolation of bacteria capable of producing hydrogen gas and the production of the gas from organic substrate by various groups of bacteria. Van Ooteghem et al. [1] isolated bacteria from soil sample pre-boiled for 15 min to kill heat labile bacteria strain and found bacteria member of the genus *Thermotoga* which could use carbohydrate and protein for growth and hydrogen production. *Thermotoga neapolitana* could produce gas containing 80% hydrogen by using ammonia as substrate under aerobic condition. It was believed that the hydrogen gas was likely to be generated by an unknown metabolic pathway. Rachman et al. [2] studied a continuous production of hydrogen gas by self-flocculated cells of *Enterobacter aerogenes* strain AY-2. He found that hydrogen production rate of the strain increased with the

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increase of dilution rate. Furthermore the cell was still capable of producing floc at very high dilution rate of HRT as low as 0.9 h which was a very good characteristic for hydrogen production in liquid media. Minnan et al. [3] isolated *Klebsialla oxytoca* HP1 from a hot spring and found that it could produce hydrogen gas very well. Hydrogen production by mixed culture has also been reported. Koskinen et al. [4] studied the production of hydrogen from carbohydrate by a mixed culture of thermophilic bacteria yielding carbon dioxide gas and acetic acid at 58 °C. He found that 3.2 mol of hydrogen gas per mole glucose was produced. Examination of the fermentation vessel for bacteria species identification showed that in a closed fermentation system *Thermobrachium celere* dominated the culture. In contrast, in a continuous culture with constant mixing, the dominating species was *Thermoanaerobacterium oatearoense* whereas *T. celere* was not detected. An interesting hydrogen production system performed by Nath et al. [5] employed dark fermentation by *Enterobacter cloacae* strain DM11 coupling with photo-fermentation by *Rhodobacter sphaeroides* O.U.001. Fermentation product, acetic acid, produced as the result of dark fermentation was utilized as substrate for photo-fermentation. This resulted in the increase of total hydrogen yield which was much greater than using each of the two systems alone. Another bacterium which had been widely experimented in hydrogen production was *Clostridium butyricum*. Yokoi et al. [6] set a continuous hydrogen production system by filling a column with *C. butyricum* cells fixed in glass beads. The system gave out 1150 ml hydrogen gas per liter per hour calculated to be 1.9 mol hydrogen per mole glucose in the medium containing glucose at 0.5 g/l.

It appeared many different groups of bacteria were capable of producing hydrogen gas. This research aimed to explore the diversity of cultivable hydrogen-producing bacteria in agricultural soils, waste water sludge and cow dung. Hydrogen-producing bacteria isolated from such sources would be of very much interest and a potential use in further hydrogen production research.

2. Materials and methods

2.1. Environmental samples and culturing media

Environmental samples used in isolation included a) soil from a rice paddy (28 °C, pH 7.02, designated WS), b) sludge from a cassava processing factory waste water (open pond, 26 °C, pH 7.23, designated AS), c) cow dung (37 °C, pH 7.45, designated CD) and d) soil from coffee plantation (27 °C, pH 7.40, designated Lao). Samples a, b and c were collected in Warinchamrab area, Ubon Ratchathani, Thailand whereas sample d was collected from an area in Jumpasak, Laos. Soil samples were collected from the depth of 1–10 cm below the surface. Sludge from an open pond of a cassava processing factory waste water was collected at 3 m away from the bank and the depth was 1 m below the surface. Cow dung sample was collected from the middle of the pile and about an hour after it had been dropped. Temperature and pH were measured and recorded at the sampling sites. Then sample bottles were placed on ice, transported to the laboratory at Ubon Rajathanee University, Ubonratchathani and stored at 4 °C until processed. Nutrient Agar (NA) used in isolation of bacteria composted of yeast extract 3.0 g, peptone 5.0 g, agar 15.0 g in 1 l. The pH was adjusted to 6.8 ± 0.2. Nutrient broth (NB) pH 6.8, which contained the same ingredients except no agar was added, was used to maintain the bacteria culture. H_2-producing activity of isolates was tested in NB pH 6.8 supplemented with 1% glucose.

2.2. Screening, isolation and Cultivation

Samples were serially diluted using oxygen depleted water (pre-boiled and flushed with N_2 for 30 min before aliquot to Hungate tubes and sterilized) and spread on anaerobically prepared nutrient agar plates in anaerobic chamber (Sheldon Manufacturing, USA) and the plates were incubated at room temperature (30–37 °C) for 72 h in an anaerobic chamber filled with N_2 gas. Pure isolates were cultured in nutrient broth (NB) prepared anaerobically by boiling and purging with nitrogen gas prior to being aliquot into Hungate tubes and autoclaved. Isolates were tested for an ability to produce hydrogen gas at 37 °C in anaerobic NB medium supplemented with 1% glucose using Hungate tubes. Excess pressure was released after 1 h incubation at a 37 °C. After 48 h incubation, gas formed inside Hungate tubes were examined by measuring the volume and hydrogen composition every 12 h followed by releasing all the gas formed. A needle fit with an air proof 2 or 5 ml syringe was punched into a butyl septum of Hungate tube and the gas volume showed in the syringe was recorded. After that 1 ml of the gas was transferred to inject into a hydrogen detector device Hy-optima model 700 (H2scan, USA) and hydrogen content was recorded in percent. The volume of hydrogen gas produced and reported was calculated from the percent of hydrogen gas measured divided by one hundred and the result was multiplied by the total volumes of biogas formed. All strains were also found to be capable of aerobic growth therefore their cultures were later maintained aerobically in common nutrient broth.

2.3. Morphological and biochemical studies

Isolates were capable of aerobic growth therefore morphology study was done with culture growing on NA agar plates. Colony and cell characteristics were observed and recorded and cells were Gram stained and observed under microscope.

2.4. Genomic DNA Extraction and PCR amplification

Genomic DNA was extracted using modified CTAB/NaCl method previously described [7]. The 16S rRNA gene was PCR amplified using Fd1 primer (5′-AGAGTTTGATCCTGGCAG-3′) and Rd1 primer (5′-AAGGAGGTGATCCAGCC-3′). Each reaction which was prepared in the total volume of 25 μl composted of 12.5 μl PCR Master Mix (2×), 2.5 μl Fd1 Primer (50 μM), 2.5 μl Rd1 Primer (50 μM) and 5.5 μl de-ionized water mixed very well. To the mixture 2 μl genomic DNA was added and the tube was placed in a thermal cycler programmed as follow: preheat at 94 °C (1 min) followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and extension at 72 °C (2 min). PCR products were visualized along with standard DNA size marker under UV after gel electrophoresis using 2% agarose gel containing 1 μg/ml ethidium bromide.

2.5. RFLP analysis

PCR amplified 16S rRNA gene products of 1500 bp in size obtained for each of all the isolates were subjected to two types of restriction enzyme cutting, *Hin*fI and *Taq*I. Each reaction which was prepared in the total volume of 20 μl composed of 13.3 μl de-ionized water, 0.2 μl acetylated BSA (10 μg/μl), 2.0 μl buffer (10×), 4 μl PCR product. After mixing, 0.5 μl of *Hin*fI (10 U/μl) was added and incubated at 37 °C for 2 h. Another tube was set in the same way but the enzyme used was 0.5 μl of *Taq*I (10 U/μl) followed by incubation at 65 °C for 2 h. Restriction cutting profiles were analyzed by gel electrophoresis using 2% agarose gel containing 1 μg/ml ethidium bromide.

2.6. 16S rRNA gene sequencing and analysis

PCR amplified 16S rRNA gene products obtained for each of all the isolates were also partially sequenced (ranging from 367 to 973 nucleotides at the 5′) at the Genomic Institute, Thailand. Sequences generated were assembled and the consensus sequence corrected manually for errors using BioEdit [8]. The most closely related sequences in GenBank and Ribosomal Database Project II were identified using BLAST [9] and the Sequence Match program [10]; sequences were then extracted from the databases, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Sequence uncertainties were omitted and phylogenetic reconstruction achieved using TreeCon [11] in which pairwise evolutionary distances were computed from percentage similarities [12] and phylogenetic trees constructed from the evolutionary distances using the neighbour-joining method [13]. Tree topology was re-examined by using the bootstrap method of re-sampling using 1000 bootstraps [14].

3. Results and discussion

3.1. Isolation

Out of 106 isolates obtained, there were only 11 isolates capable of producing 2 ml of gas or more in 48 h. Hydrogen-producing bacteria were successfully isolated from four sample sources used. However amount of biogas and hydrogen gas produced by each isolates varied as shown in Table 1. Isolate AS_I-2 gave the highest amount of 2.70 ml of hydrogen gas from 15.70 ml biogas calculated to be 17.2% v/v while isolate Lao-1-2 and AS_I-1 gave 2.51 and 2.12 ml of hydrogen gas respectively.

3.2. Basic morphology

Colony and cell morphology are the most elemental characteristics traditionally used by microbiologist to distinguish bacterial species. In general, in a given medium, a certain group of bacteria shares a certain set of characteristics which differ from other groups. Basic morphological studies of hydrogen-producing bacteria growing on NA plate at 37 °C for 24 h yielded 3 Gram-positive and 8 Gram-negative isolates with colony and cell morphology as shown in Fig. 1. Colony and cell morphology appeared in Fig. 1 were in agreement with phylogenetic affiliation results. Isolates AS-1 and AS-4 had morphological appearances of typical members of the genus *Klebsiella* which were facultative anaerobic, stained Gram-negative, rod in shape with polysaccharide-based capsule giving them the glistering wet appearance. Isolates AS-I-1, AS_I-2 and WS-2-2 grew both in the presence and absence of oxygen showing typical large white colony with rod shape cells (0.5–0.8 × 3–6 μm) stained Gram-positive fitting with general characteristics of the genus *Paenibacillus*. Other isolates CD-5, CD-6, WS-4-2, WS-7-11, Lao-1-2, Lao-1-11 were facultative anaerobic bacteria having both respiratory and fermentative type of metabolism and capable of growing very well aerobically and anaerobically at 30–37 °C. Cells were straight short rods (0.5–0.8 × 3–6 μm), motile, stained Gram-negative. These were general characteristics of member of the family Enterobacteriaceae which were widely distributes in soil, water, fruits, vegetable, grain, flowering plant, trees, animals and human [15].

Table 1 – Biogas and hydrogen gas produced by the 11 isolates growing in NB supplemented with 1% glucose and incubated at 37 °C for 48 h.

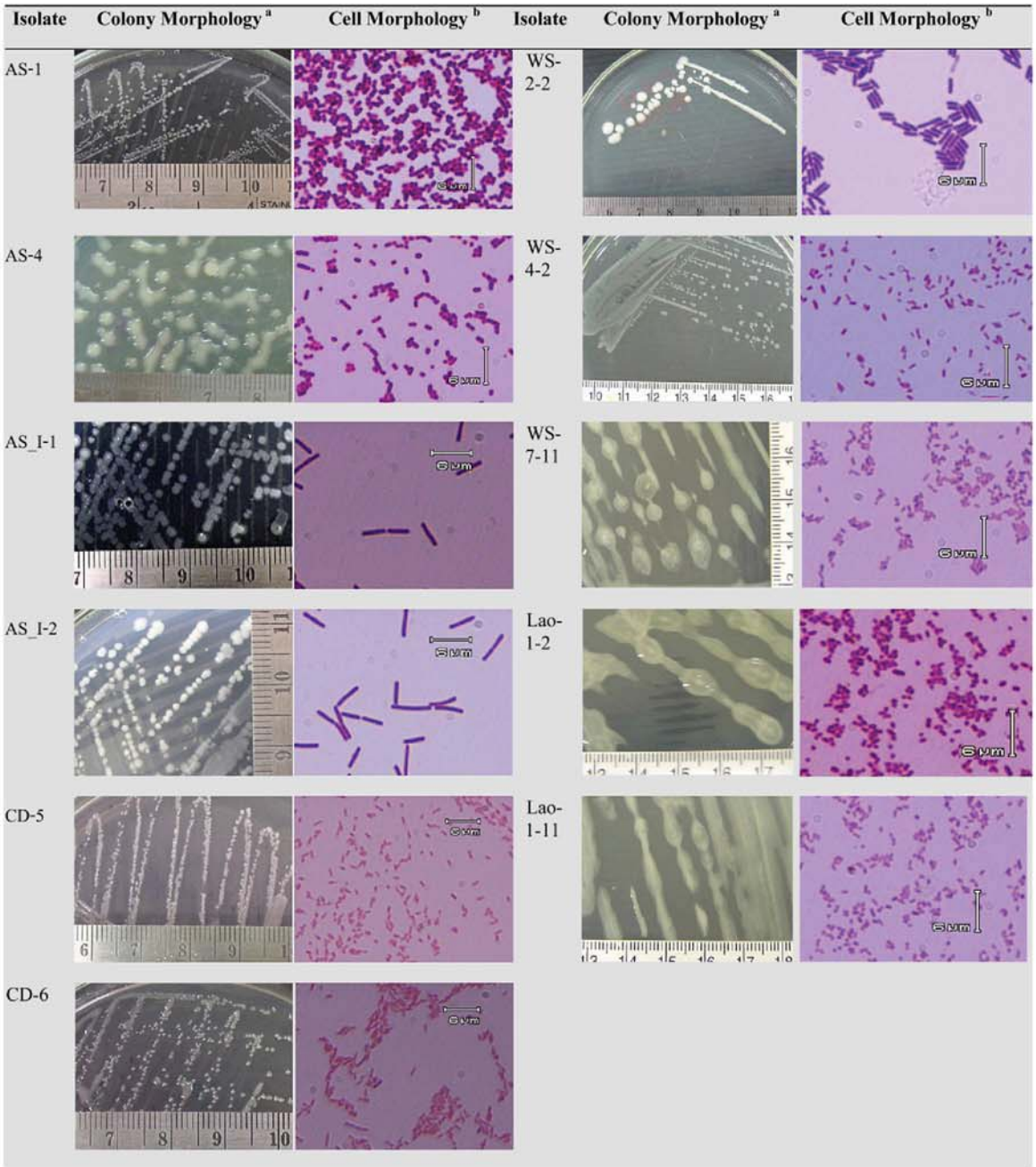
No.	Isolate[a] Name	Total Biogas Volume (ml.)	Hydrogen Gas Volume (ml.)
1	AS-1	11.6	1.46
2	AS-4	13.0	1.83
3	AS_I-1	15.1	2.12
4	AS_I-2	15.7	2.70
5	CD-5	13.1	1.97
6	CD-6	13.9	1.92
7	WS-2-2	15.0	1.81
8	WS-4-2	2.4	0.35
9	WS-7-11	13.1	2.08
10	Lao-1-2	15.3	2.51
11	Lao-1-11	12.6	1.83

a Origin of the isolates: AS; Sludge from cassava processing factory waste (open pond), CD; Cow dung, WS; Warinchumrab soil, Lao; Coffee plantation area in Jumpasak, Laos.

3.3. Amplified PCR products and RFLP analysis

Genomic DNA from the 11 isolates were successfully PCR amplified and products of about 1500–1600 bp in size were obtained for every isolates as shown in Fig. 2. Restriction enzyme cutting profile from digestion with *Hin*fI appeared as in Fig. 3 and by *Taq*I appeared as in Fig. 4.

According to Fig. 3 there were 4 distinct restriction cutting profiles designated *a*, *b*, *c* and *d* generated from restriction cutting with *Hin*fI. However restriction cutting profiles generated from restriction cutting with *Taq*I showed 6 distinct profiles. Therefore the 11 isolates were put into 6 groups (OTUs). OTU *a* composted of 2 isolates which were WS-7-11 and Lao-1-2 and OTU *d* composted of 3 isolates which were WS-2-2, AS-I-2 and AS-I-1. These grouping results were in agreement for both restriction cutting enzymes. However OTU *b* which composted of Lao-1-11, CD6 and CD5 according to *Hin*fI restriction cutting, could be further separated into 2 groups, with an additional band of about 450 bp in size for Lao-1-11 (OTU *b1*) while restriction patterns were the same for CD6 and CD5 (OTU *b2*). OTU *c* which composted of WS-4-2, AS-4 and AS-1 according to



a, b Colony and cell description of isolates were as follow:

AS-1: Glistering white, 1-2 mm, entire, convex colony, short rod, 0.5-1x1μm, non-motile, Gram-negative.

AS-4: Glistering white, 2-4mm, entire, convex colony, cocci or short rod, 0.5-1x1μm, non-motile, Gram-negative.

AS_I-1: White colony with transparent edge, 1-2 mm, smooth, rhizoid, rod, 0.5x3-5 μm, Gram-positive.

AS_I-2: White, 1-3 mm, smooth, rhizoid, rod, 0.5x3-6 μm, Gram-positive.

CD-5: White, 0.5-1 mm, smooth, entire, short rod, 0.5x1 μm, motile, Gram-negative.

CD-6: White turbid, 0.5-1 mm, smooth, entire, short rod, 0.5x1-2 μm, motile, Gram-negative.

WS-2-2: White, 1-5 mm. rough, entire, rod, 0.5x1-5 μm, Gram-positive.

WS-4-2: White turbid, 1-2 mm, convex, entire, short rod, 0.5x1-1.5 μm, motile, Gram-negative.

WS-7-11: White turbid, 3-6 mm, convex, entire, short rod, 0.5x0.5-1 μm, motile, Gram-negative.

Lao-1-2: White turbid, 3-6 mm, convex, entire, cocci to short rod, 0.5x0.5-1 μm, motile, Gram-negative.

Lao-1-11: White turbid, 3-6 mm, convex, entire, cocci to short rod, 0.5x0.5-1 μm, motile, Gram-negative.

Fig. 1 – Basic morphology of 11 hydrogen-producing isolates cultured in NA for 24 h at 37 °C.

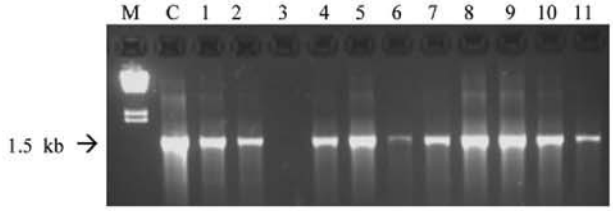


Fig. 2 – PCR amplified products of about 1500–1600 bp in size were obtained for every isolates. M = λ *Hin*dIII DNA Size Marker, C = Positive PCR control, 1 = WS-7-11, 2 = Lao-1-11, 3* = Lao-1-2 (Good PCR product was later obtained but not shown here), 4 = WS-4-2, 5 = WS-2-2, 6 = CD6, 7 = CD5, 8 = AS-I-2, 9 = AS-I-1, 10 = AS-4, 11 = AS-1.

*Hin*fI restriction (similar pattern in Fig. 2 lane 5, 10 and 11) could also be further separated into 2 OTUs, *c1* (WS-4-2) and *c2* (AS-4 and AS-1). Restriction pattern of WS-4-2 cut with *Taq*I (Fig. 4, lane 4) was clearly different from those of AS-4 and AS-1 (Fig. 4, lane 10, 11).

To summarize, the 11 isolates were put into 6 distinct OTUs according to restriction pattern of 16S rRNA gene PCR amplified products designated OTU *a* (WS-7-11 and Lao-1-2), *b1* (Lao-1-11), *b2* (CD6 and CD5), *c1*(WS-4-2), *c2* (AS-4 and AS-1) and OTU *d* (WS-2-2, AS-I-2 and AS-I-1). The 6 OTUs designated were evaluated against results from 16S rRNA gene sequence analysis in the following section.

3.4. 16S rRNA gene sequence analysis and phylogeny

From the analysis of partial 16S rRNA gene sequence (367–973 nucleotides on the 5′ end of the gene) by BLASTn, it was found that all the 10 isolates could be affiliated with bacteria in 5 different genera which were *Paenibacillus*, *Escherrichia*, *Pantoea*, *Klebsiella* and genus *Enterobacter* with very high similarity between 98 and 99% in all the cases as shown in Table 2. These high levels of similarity were in the range which could be assigned in the same species when the entire genes were analyzed [16]. Analysis of 16S rRNA gene has been generally accepted for its use in bacterial classification and taxonomy with accuracy [17,18]. Furthermore partial sequence analysis on the 5′

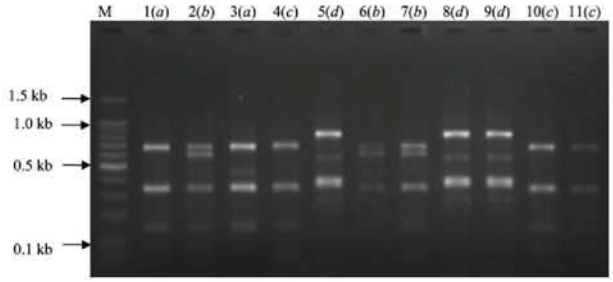


Fig. 3 – Restriction enzyme cutting profile from digestion with *Hin*fI. M = 100 bp DNA ladder, 1 = WS-7-11, 2 = Lao-1-11, 3 = Lao-1-2, 4 = WS-4-2, 5 = WS-2-2, 6 = CD6, 7 = CD5, 8 = AS-I-2, 9 = AS-I-1, 10 = AS-4, 11 = AS-1. Alphabets *a*, *b*, *c* and *d* represented 4 distinct restriction cutting profiles.

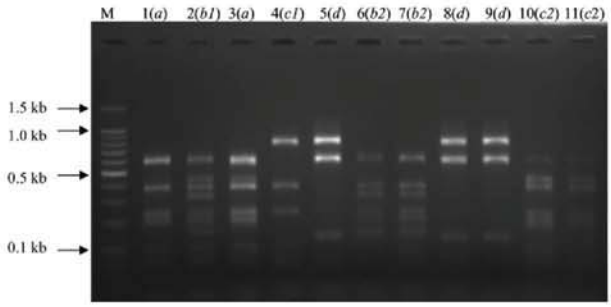


Fig. 4 – Restriction enzyme cutting profile from digestion with *Taq*I. M = 100 bp DNA ladder, 1 = WS-7-11, 2 = Lao-1-11, 3 = Lao-1-2, 4 = WS-4-2, 5 = WS-2-2, 6 = CD6, 7 = CD5, 8 = AS-I-2, 9 = AS-I-1, 10 = AS-4, 11 = AS-1. Alphabets *a*, *b1*, *b2*, *c1*, *c2* and *d* represented 6 distinct restriction cutting profiles hence OTUs.

of the gene had also been shown to give acceptably good results in bacteria identification due to the fact that these regions were highly variable [19,20].

AS_I-2 which produced the greatest amount of hydrogen gas along with WS-2-2 and AS-I-1 which were grouped in OTU *d* were affiliated with *Paenibacillus polymyxa* with 99% similarity. *P. polymyxa* was a member of the genus *Paenibacillus*, family Paenibacillaceae, order Bacillales, class Bacilli. AS_I-1 and WS-2-2 which gave 2.12 and 1.8 ml hydrogen respectively were also affiliated with genus *Paenibacillus* with 98% similarity with *P. polymyxa*. In addition to being put in the same OTU (*d*) basic morphology and biochemical test results of these three isolates were in agreement with the characteristics of the genus *Paenibacillus*. Furthermore phylogenetic inferation confirmed grouping results from restriction cutting with a phylogenetic tree showing no distance between them (Fig. 5).

Lao-1-2 which produced 2.51 ml hydrogen gas and isolate WS-7-11 were grouped together in OTU *a*. The two isolates were affiliated with members of the genus *Pantoea* with similarity of 98–99% to *Pantoea agglomerans*. Phylogenetic inferation also showed the two isolates related to one another with 88% bootstrap value (Fig. 6).

In the cases of OTU *b2*, isolates CD6 and CD5 showed different BLASTn results. CD6 had 99% similarity to *P. agglomerans* strain while CD5 showed 99% similarity to *E. cloacae* strain. These did not indicate a disagreement of results from two different methods because the two genera were very closely related and their current taxonomies were super imposed. OTU *b1* could not be confirmed by 16S rRNA sequence analysis because sequencing failed. For OTU *c2* (AS-4 and AS-1), 16S rRNA analysis clearly confirmed the grouping as the two isolates showed similarity of 99% to member of the genus *Klebsiella*, although maximum similarities were with different *Klebsiella* species. WS-4-2, the only member of OTU *c1* was also the only isolate that was affiliated with the genus *Escherrichia* (99% similarity to *Escherrichia coli* C2). This very well explained its distinct restriction digestion pattern.

Considering results from species affiliation of the 11 hydrogen-producing isolates, it was found that all the genera that the isolates were affiliated with had been reported to have members capable of producing hydrogen gas which were as

Table 2 – BLASTn analysis of 16S rRNA gene of hydrogen-producing isolates against GenBank database.

No.	Isolate Name	Description	Accession Number	Maximum Identity	No. of Nucleotide
1	AS-1	*Klebsiella pneumoniae* ATCC13884T	Y17657.1	99%	882/884
2	AS-4	*Klebsiella variicola*	AJ783916.1	99%	859/865
3	AS_I-1	*Paenibacillus polymyxa* strain KCTC3627	AY359636.1	98%	360/367
4	AS_I-2	*Paenibacillus polymyxa* strain KCTC3627	AY359636.1	98%	529/537
5	CD-5	*Enterobacter cloacae* subsp. Dissolvens	DQ988523.1	99%	641/642
6	CD-6	*Pantoea agglomerans* strain WAB1927	AM184266.1	99%	687/689
7	WS-2-2	*Paenibacillus polymyxa* strain GBRR-465	AY359623.1	98%	963/973
8	WS-4-2	*Escherrichia coli* C2	AF403733.1	99%	891/894
9	WS-7-11	*Pantoea agglomerans* strain WAB1969	AM184307.1	99%	640/642
10	Lao-1-2	*Pantoea agglomerans* strain WAB1872	AM184214.1	98%	849/860
11	Lao-1-11	Sequencing failed	–	–	–

anticipated. For example *P. polymyxa*, a member of the genus *Paenibacillus* in which AS_I-2, AS_I-1 and WS-2-2 were closely related, was reported to be one of the two species responsible for hydrogen production from basic-treated-waste [21]. In addition Sakka et al. [22] concluded that the explosion of a silo storing Refuse-Derived Fuel (RDP) pellets in Mie Prefecture, Japan in 2003 was likely to be connected with accumulated hydrogen gas in the silo. His study also revealed the presence of *Paenibacillus* sp. in the RDP pellets stored.

Other four genera the other 7 isolates were affiliated with were *Escherrichia*, *Pantoea*, *Klebsiella* and *Enterobacter* members of the family Enterobacteriaceae. Members of this family were bacteria associated with digestion tract of warm-blooded animal and also generally found in soil and water. It was well known that these group of bacteria produced acid and gas from glucose fermentation. The gas produced composted of carbon dioxide and hydrogen. Member of the genera *Escherrichia* and *Pantoea* were capable of fermentation of glucose and other carbohydrate producing lactic acid, acetic acid as well as formic acid. Some formic acid was further digested by enzyme hydrogenlyase generating carbon dioxide and hydrogen gas. Members of the genus *Klebsiella* were also known to produce carbon dioxide and hydrogen with the greater amount of the former gas. *Enterobacter* sp. produced carbon dioxide and hydrogen in the ratio of 2:1 at 37 °C but ceased to produce gas from glucose at 44.5 °C [2,3,5].

Hydrogen produced by these species has not been among maximum value reported. However members of genus *Paenibacillus* and the family Enterobacteriaceae isolated in this study have been reported to have an interesting property of bio-flocculant formation [23], which could be advantages in waste water treatment. More over these strains have been reported to be isolated from effluent of hydrogen production reactor. This meant that the strains were indigenous to hydrogen production system and hence must be well adapted to such environment making them likely to be very suitable as bio-flocculant maximizing floc formation in treatment of effluent from hydrogen production reactor which normally required further treatment before it could be disposed off.

No *Clostridium* strain was isolated in this search for hydrogen-producing bacteria. This could have been because of a few reasons. The isolating media used and a short incubation time of 72 h could lead to an overgrowth and subsequent isolation of facultative anaerobic and fast growing strains leaving out slow growing and fastidious strains like strict

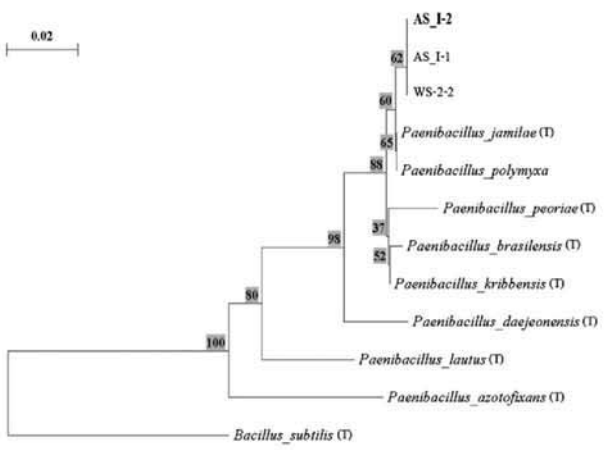


Fig. 5 – Phylogenetic tree drawn for hydrogen-producing bacterial isolates affiliated with members of genus *Paenibacillus*. *Bacillus subtilis* (T) was an out group species. Bar = 2 nucleotide difference per 100 nucleotides.

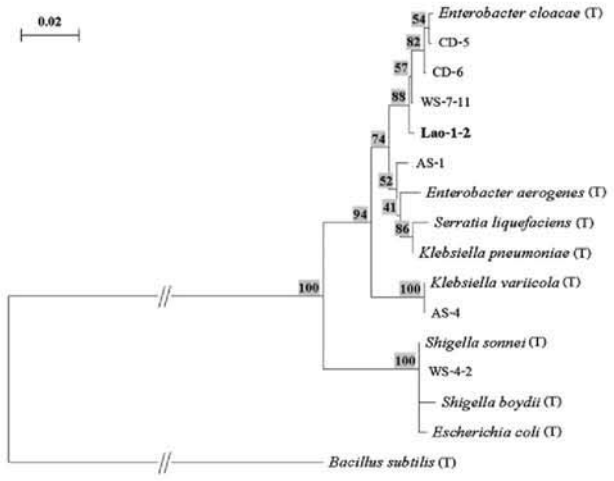


Fig. 6 – Phylogenetic tree drawn for hydrogen-producing bacterial isolates affiliated with members of family Enterobacteriaceae. *Bacillus subtilis*(T) was an out group species. Bar = 2 nucleotide difference per 100 nucleotides.

anaerobic *Clostridia* which would take longer time to develop into visible colony. Direct isolation from the environmental sample without enrichment could also contribute to the absent of *Clostridium*. If one aimed to specifically isolate Clostridia species, it would be better to set up an enrichment culture in a specific media suitable for *Clostridium* strain prior to isolation rather than isolation directly from an environmental sample. The incubation time should also be longer. The minimum of five to seven days should be more suitable for slow growing strains to develop. Diverse type of media and additives should also need to be tried in exploring for a novel strain. Finally to ensure that strict anaerobe were not excluded because of possible unsuitable degree of anaerobic condition, it is necessary to set up a positive control culturing of strict anaerobe along in the incubation chamber.

Although cultivable hydrogen-producing bacteria found in this search were not as diverse as firstly anticipated, isolation and identification of hydrogen-producing bacteria should still be a desirable practice in parallel to hydrogen production experiments since the production relying on mixed and often unidentified community of microbe such as sludge from the field as seed has a drawback that it can be non-reproducible. This is so because by its nature the microbial community is highly dynamic. It changes all the time according to various environmental factors and the changes are far too great and beyond one to predict. Therefore it is unlikely to always obtain seeds with the same microbial properties and content from such fields. In addition having a suitable effective hydrogen-producing strains or mixed culture of known strains available for re-inoculation following adjustment of some parameters could be one good way which would save the resource and effort already put into setting up the fermentation system, which can be vital in real practices.

4. Conclusion

Isolation of bacteria capable of producing hydrogen directly from environmental samples yielded 11 bacterial isolates. Among them 3 strains were identified to be members of the genus *Paenibacillus* and 8 strains belong in the family Enterobacteriaceae. The ability to produce hydrogen of the strains isolated has been known and reported in literatures but their hydrogen production performances have not been among maximum value reported. However the ability to produce bioflocculant of these strains has gained a lot of attention from researcher in the hope of applying them in waste water treatment especially in treatment of fermentation liquor from hydrogen production bioreactor which normally required further treatment before it could be disposed off.

Acknowledgements

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ORIGINAL PAPER

Hydrogen production and anaerobic decolorization of wastewater containing Reactive Blue 4 by a bacterial consortium of *Salmonella subterranea* and *Paenibacillus polymyxa*

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Abstract Anaerobic biodegradability of wastewater (3,000 mg CODcr/l) containing 300 mg/l Reactive Blue 4, with different co-substrates, glucose, butyrate and propionate by a bacterial consortium of *Salmonella subterranea* and *Paenibacillus polymyxa*, concomitantly with hydrogen production was investigated at 35°C. The accumulative hydrogen production at 3,067 mg CODcr/l was obtained after 7 days of incubation with glucose, sludge, the bacterial consortium. The volatile fatty acids, residual glucose and the total organic carbon were correlated to hydrogen obtained. Interestingly, the bacterial consortium possess decolorization ability showing approximately 24% dye removal after 24 h incubation using glucose as a co-substrate, which was about two and eight times those of butyrate (10%), propionate (12%) and control (3%), respectively. RB4 decolorization occurred through acidogenesis, as high volatile fatty acids but low methane was detected. The bacterial consortium will be the bacterial strains of interest for further decolorization and hydrogen production of industrial waste water.

Keywords Hydrogen · Decolorization · RB4 · *Salmonella subterranea* · *Paenibacillus polymyxa* · Wastewater

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Introduction

Hydrogen is a clean and environmentally friendly fuel, when it burns, it only produces water as the by product. Hydrogen can be produced in many ways including; chemically (e.g., gasification of coal), electrochemically (e.g., electrolysis of water) or by the use of microorganisms (Takabatake et al. 2004; Wang et al. 2007; Bothe et al. 2008). There are two main systems of microbial hydrogen production, photochemical system using photosynthetic microorganisms such as algae and photosynthetic bacteria (Melis and Happe 2001) and fermentative system using facultative anaerobes and obligate anaerobes (Nandi and Sengupta 1998). The fermentation substrates most studied in the laboratory are glucose (Mu et al. 2006) and sucrose (Tao et al. 2007). In addition,

it is well known that carbohydrates are the main source of hydrogen during fermentative processes and therefore, renewable energy sources, wastes/wastewater or agricultural residues rich in carbohydrates can be considered as potential sources of hydrogen (Kapdan and Kargi 2006). Hydrogen could be converted into electricity via fuel cells or directly used in internal combustion engines. It can also be used for the syntheses of ammonia, alcohols and aldehydes, as well as for the hydrogenation of petroleum, coal, shale oil, and edible oil (Hart 1997). Many believe that hydrogen will replace fossil fuels as the next generation of energy supply. A hydrogen-based economy will impose no risk of global warming, and will improve significantly the urban air quality (Fang et al. 2004).

Dyes are annually produced and applied in textiles, cosmetics, pharmaceutical, photographic, plastics, paper, and food industry. They are classified as acidic, basic, azo, diazo, disperse, metal complex and anthraquinone-based dyes (Fu and Viraraghavan 2001; Aksu and Tezer 2005) according to their structural varieties. Due to the increased demand for textile products, the textile industry and its wastewater have been increasing proportionally, making it one of the main sources of severe environmental problems (Vandevivere et al. 1998). Reactive Blue 4 (RB4), an anthraquinone-based chlorotriazine dye, is very important in dyeing of cellulosic fabrics. Reactive dyes have environmental implications since up to 50% of the initial dye mass used in the dyeing process remains in the spent dyebath in its hydrolyzed form which no longer has an affinity for the fabric, and therefore cannot be reused in the dyeing process (Laszlo 1995). They are not readily removed by typical wastewater treatment processes due to their stability and resistance towards light or oxidizing agents (Lee et al. 2005). In addition, the high pH and high salt concentrations under typical reactive dyeing conditions further complicate the management of used reactive dyebaths (Rys and Zollinger 1989). Therefore, establishing dye removal technologies is an urgent problem. The most commonly used method for the treatment of textile wastewater is the combination of physicochemical treatment and biological oxidation (Manu and Chaudhari 2002; Singh et al. 2008; Omar 2008). However, these processes are quite ineffective in wastewater color removal since dyestuff, such as anthraquinone-based dye, are biorecalcitrant owing to their aromatic structure. RB4 was selected as a targeted dye for this research due to its relatively slow biodecolorization kinetics (Lee et al. 2005).

Therefore, this study aims to investigate hydrogen production and decolorization potential of waste water containing RB4 by facultative anaerobic bacterial consortium of, *Salmonella subterranea* and *Paenibacillus polymyxa*, which possess both hydrogen production and dye decolorization properties using different co-substrates, glucose, butyrate and propionate.

Materials and methods

Chemicals

Reactive Blue 4 (RB4, IC 61205), the anthraquinone dye class, was used as a targeted dye. Glucose, butyrate and propionate were used as co-substrates or electron donor substrates. All chemicals were purchased from Sigma–Aldrich and Wako, Japan.

Bacterial strains used were *Salmonella subterranea* and *Paenibacillus polymyxa* capable of hydrogen production and dye decolorization.

Preparation of culture medium containing wastewater and RB4

Salmonella subterranea and *Paenibacillus polymyxa*, capable of hydrogen production were obtained from Dr. Sungwan Kanso. The two bacterial strains were isolated from Mae Khong River, Ubolratchathani Province, Thailand. Screening, purification, morphological and biochemical characterization and phylogenetic analysis of 16SrRNA sequences were conducted and are currently under preparation for publication by Dr. Kanso's group. Interestingly, we later found that the bacterial strains also showed dye decolorization property using 1% molasses in agar plates and then also preliminary experiment with molasses wastewater decolorization (data not shown).

Anaerobic sludge obtained from municipal wastewater treatment plant, Western Purification Center, Ube City, Yamaguchi Prefecture, Japan was centrifuged to separate the wastewater and sludge cell. Sludge was concentrated to 3,000 mgVSS/l and separated wastewater was supplemented with co-substrate

and nutrient to make up the chemical oxygen demand (COD) concentration to 3,000 mg CODcr/l. The co-substrates used were glucose (9.4 g/l), propionate (6.6 g/l) and butyrate (5.5 g/l) and supplemented with yeast extract (0.1 g/l), and K_2HPO_4 (4 g/l). In order to understand the effect of RB4 on microbial activity, neutral pH (pH 7) was obtained by the addition of buffering chemical, $NaHCO_3$ (4 g/l). For bacterial consortium used, 1×10^{10} cell of each of *S. subterranea* and *P. polymyxa* were mixed with anaerobic sludge before use.

Before the experiment was conducted, the 500 ml serum bottle contained 50 ml bacterial consortium was sealed with butyl rubber stopper, capped with aluminium crimp cap and purged with Argon gas to deplete the oxygen accumulated in bottle, then incubated at 35°C overnight to reduce the nutrients remaining in the sludge solution before the addition of 200 ml wastewater (3,000 mg CODcr/l) and 300 mg/l RB4. The detailed experimental condition is shown in Table 1. The operational parameters used were optimized previously in our laboratory.

Analysis of samples

During the incubation period, a 5-ml sample was taken every 24 h from each bottle, and centrifuged at 12,000*g* for 10 min. The supernatant was collected for measuring of pH, accumulated fatty acids (VFAs), total organic carbon (TOC) and decolorizing efficiency. The gas evolved was measured volumetrically by water displacement in a burette and the volume was calculated using the mass balance equation (Zheng and Yu 2005). As from equation $H_2 + 1/2\ O_2 \rightarrow H_2O$, so 1 mole H_2 is equal to 16 g COD/l, the unit of H_2 was used as mg CODcr/l so that it will be the same unit used for VFAs and co-substrate, i.e., glucose. Gas samples were taken from the headspace of each bottle by a gas-tight syringe. The biogas composition was analyzed by a gas chromatograph (Shimadzu GC-8APT) equipped with a thermal conductivity detector (TCD) and 1.5 m stainless column packed with activated charcoal 60/80, Shinwa Co. Ltd, Japan.

Decolorization of the samples was determined using a spectrophotometer (Hitachi U-2001), pH of all samples were adjusted to 7.6 with NaOH or H_2SO_4, before measuring the absorbance at the maximum wavelength of 598 nm. Dye removal defined as a percentage of differences between the initial and final absorbance (% decolorization). The reduced form of treated dye was detected by monitoring the changes of spectral wavelengths between 200 and 800 nm. The appearance of a new peak indicated a reduced form of treated dye. Residual glucose was measured spectrophotometrically (Hitachi U-2001) using dinitrosalicylic acid (DNS) method. Chemical oxygen demand (COD) was measured as the corresponding oxygen consumption during oxidation with dichromate, defined as CODcr (Pitwell 1983).

The biogas composition was analyzed by a gas chromatography (Shimadzu GC-8APT) equipped with a thermal conductivity detector (TCD) and 1.5 m stainless column packed with activated charcoal 60/80, Shinwa Co. Ltd, Japan. The temperature of injector, column and detector were kept at 50, 60, and 50°C, respectively. Argon was used as carrier gas at a flow rate of 20 ml/min. Volatile fatty acids (VFAs) concentrations were detected using Shimadzu GC-8APF with Packed Column Unisol F-200 30/60. The temperature of injector, column and detector were kept at 250, 140, and 140°C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. Total organic carbon (TOC) were also determined using total organic carbon analyzer (Shimadzu TOC-5000).

Table 1 The experimental condition of controls and treatments for hydrogen production and RB4 decolorization at 35°C

	Bacteria consortium	Sludge	Co-substrate	300 mg/l RB4
Control 1	X	O	X	O
Control 2	O	O	X	O
Sample 1	O	O	Glucose	O
Sample 2	O	O	Propionate	O
Sample 3	O	O	Butyrate	O

Results and discussion

Hydrogen production

Hydrogen production by biological methods, determined from various renewable resources, are less energy intensive than chemical or electrochemical

Table 2 Decolorization (%), volatile fatty acid concentrations, gas production and TOC removal after 7 days of incubation at 35°C of RB4 and wastewater in the presence and absence of glucose, propionate and butyrate, and the bacterial consortium

Treatments	Decolorization (%)	VFAs (mg COD l^{-1})				Gas production (mg COD l^{-1})		TOC removal (%)
		Hac	HPr	i-Hbu	*n*-Hbu	CH_4	H_2	
Control 1	32.3	97.7	18.7	3.6	0	1,015.5	33.7	16.9
Control 2	64.8	270.8	215.8	9.1	41.5	1,065.5	436.3	3.04
Sample 1	74.3	1,522.8	1,190.3	39.9	330.4	395.7	3,067.5	41.8
Sample 2	82.5	199.8	143.0	0	2,702.6	987.7	711.9	20.4
Sample 3	79.7	392.7	4,263.1	0	202.9	468.6	1,374.0	15.4

ones since they are carried out at ambient temperature and pressure (Elam et al. 2003). Therefore, hydrogen production by the bacterial consortium, mixed culture of anaerobic sludge, *S. subterranea* and *P. polymyxa*, was determined. The accumulative H_2 production after 7 days was as high as 3,067.5 mg CODcr/l, in the presence of the bacterial consortium and glucose (sample 1), as compared to propionate (1,374.0 mg CODcr/l) (sample 2) and butyrate (711.9 mg CODcr/l) (sample 3), which are 3 times and 5 times less H_2 was detected (Table 2). The results were correlated with the VFAs produced (Table 2). In general, after glucose hydrolysis, VFAs were then produced by acidogenic activity of the bacterial consortium and hydrogen production occurred. Table 2 indicated that in the presence of glucose, H_2 and acetic acid (Hac) was increased. On the other hand, methane production using glucose and propionate as substrate was low, implying that methanogenic activities may be inhibited by color adsorption to the bacterial consortium. In general, methanogenic activity could be inhibited by low pH and high VFA, however, in this study pH was not less than 6 (Fig. 1) and VFAs are not so high to cause inhibition of methanogenic activities. Dye adsorption to the cells leading to methanogenic activity inhibition is a possible explanation. The total organic carbon (TOC) was shown in Table 2, supporting carbon degradation by the anaerobic bacterial consortium.

The higher efficiency of glucose as a co-substrate for H_2 production after 7 days (Table 2) in the presence of the bacterial consortium as compared to butyrate and propionate was supported by many studies on hydrogen production using *Clostridia* (Taguchi et al. 1993) and *Enterobacteria* (Kumar and Das 2000). In this study, during the experimental period of 7 days, pH was relatively constant for each

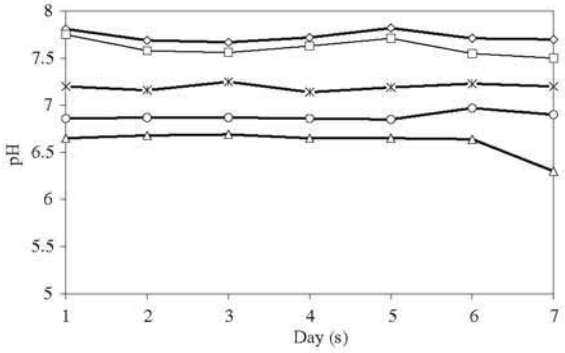

Fig. 1 pH changes during anaerobic decolorization at 35°C of wastewater with sludge and 300 mg/l reactive blue4 in the absence (◇) and presence (□) of the bacterial consortium. Different substrates were added; glucose (Δ), butyrate (*) and propionate (○)

sample (Fig. 1), therefore, hydrogen production was not affected by pH variation. Since fermentative hydrogen production is affected by pH, temperature as well as the nature of the microorganisms. pH is crucial due to its effects on hydrogenase activity, metabolism pathways (Lay 2000; Jun et al. 2008; Liu et al. 2008) and microbial communities (Fang and Liu 2002). In general, the dominant metabolism in a mixed acidogenic culture depends strongly on pH of the microbial culture and hydrogen production is suppressed by both low and high pH (Chen et al. 2002). Thus, it is important to control the pH in order to maintain satisfactory hydrogen production.

In general, degradation of sugars is accompanied by the production of hydrogen and different metabolic products, mainly VFAs (acetic acid, Hac; propionic acid, HPr; *n*-butyric acid, *n*-Hbu; isobutyric acid; i-Hbu), lactic acid and ethanol, during the fermentation process. Different organic matter conversion pathways under mesophlilic condition, of which glucose degradation by acidogens and

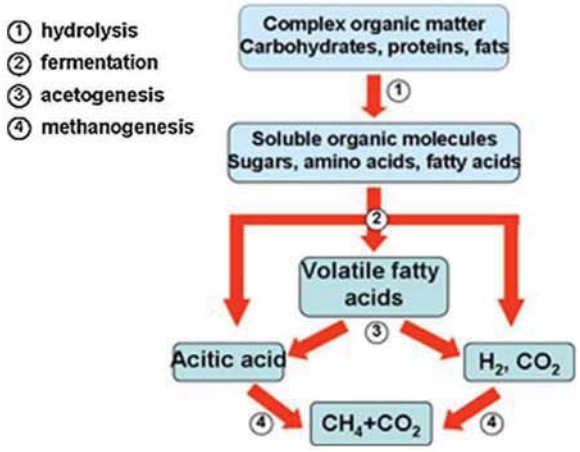

Fig. 2 Suggestion of the organic matter conversion pathways at 35°C (mesophilic condition)

acetogens to intermediate products (acetate, butyrate, propionate, etc.), which further be used as substrate for methane production (Fig. 2). Since, the hydrogen yield varies proportionally to the final metabolic products, acetic and butyric acid production favors hydrogen production (Nandi and Sengupta 1998; Hawkes et al. 2002) according to Eqs. (1) and (2) with the fermentation to acetic acid giving the highest theoretical yield of 4 mol H_2/mol hexose. The final products of fermentation (acetate, hydrogen and CO_2) are the precursors of methane formation (methanogenesis) (McCarty and Smith 1986). In addition, lowering the pH to 4.5 or below may shift the VFA-producing pathway to an alcohol-producing pathway. As the study by Khanal et al. 2004, showing that if there is a shift of pH to be more acidic, this will affect VFA and metabolic alteration, which was not occurred in our experiment.

Therefore, our results obtained in Table 2 showed correlation between hydrogen production and the VFAs produced, showing increased acetic acid production accompanied by increased hydrogen production as of sample 1 (glucose, the bacterial consortium).

Acetic acid production

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

Butyric acid production

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

Decolorization of RB4

Apart from H_2 production, the bacterial consortium also possesses decolization ability. The spectra of RB4 after 24 h treatment with the bacterial consortium scanned at 400–800 nm, with maximum absorption at 598 nm revealed the evident reduction of absorbance in the presence of glucose as compared to controls (Fig. 3a). It is readily apparent from Fig. 3b that in the presence of glucose, the bacterial consortium, at day 1, 24% dye removal was achieved, which is approximately 2 times and 8 times higher than those of butyrate (10%), propionate (12%) and control (3%), respectively, implying that the presence of glucose in the decolorizing system was needed for the fast decolorization efficiency. This result was supported by the TOC removal and decreased residual glucose

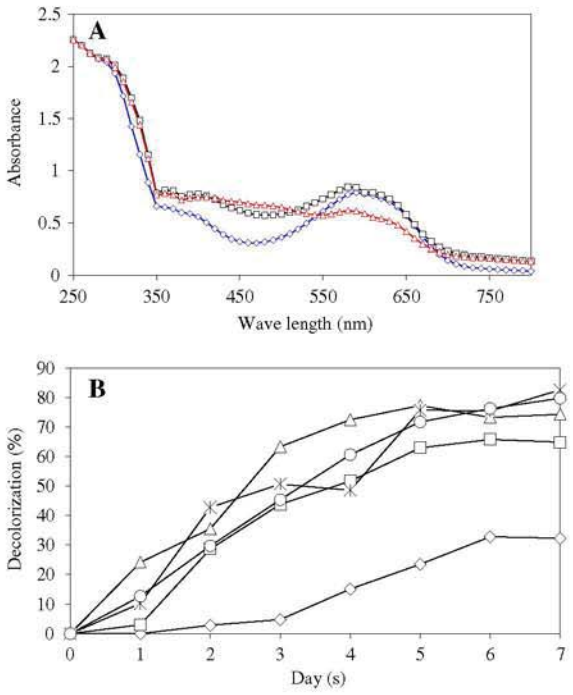

Fig. 3 **a** Wastewater spectra after 24 h of incubation with RB4, sludge and the bacterial consortium at 35°C in the presence of glucose, sample 1 (Δ) as compared to control 1, no bacterial consortium (◇) and control 2, with the bacterial consortium (□). **b** Percantage of anaerobic decolorization of waste water with sludge and 300 mg/l RB4 in the absence (◇) and presence (□) of the bacterial consortium. Different substrates were added; glucose (Δ), butyrate (*) and propionate (○), measured at 598 nm

concentration (Table 2, Fig. 4). The dye removal was increased continuously until day 7, however, the increase in dye decolorization of control was not so evident as in the presence of glucose, regardless of the bacterial consortium addition. Approximately 32.3–82.5% total color removal was obtained after 7 days of decolorization in different types of substrates used as demonstrated in Table 2.

The slow RB4 decolorization of the control at 35°C, showing blue cells, may be due to the dye adsorption to the cells during experimental period. Dye adsorption to the cells leading to methanogenic activity inhibition is a possible explanation. The result showed that the TOC removal was decreased from 90% (start-up condition) to 30%, approximately, during the decolorization (Table 2), which corresponded with other researches. The inhibition of decolorizing microorganisms also occurred by textile dye and its intermediates, the VFAs accumulation was mainly in the form of acetate and propionate with traces of iso-butyric, *n*-butyric and iso-valeric acid when RB4 or RB19 was amended in culture (Lee et al. 2004). In addition, the methanogenic culture amended with 250–300 mg/l of Brilliant Red Resolin (BLS) showed 78.9% inhibition of specific methane yield and 59.6% production via aceticlastic methanogenesis (Melpei et al. 1998).

The adsorbed dye may also block the substrate transportation pathway involved in decolorizing mechanism. The increased dye removal in the presence of glucose may be due to glucose utilization, as glucose is the best electron donor for the dye decolorizing. This result was correlated with our previous study that

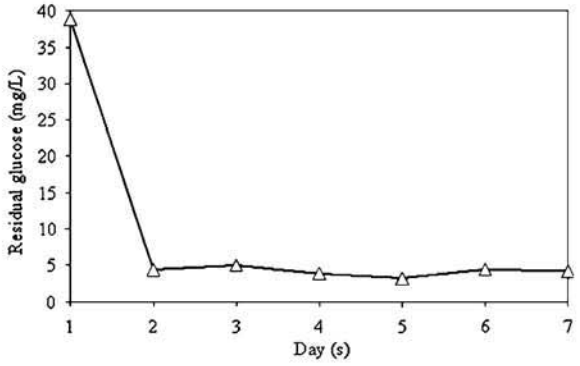

Fig. 4 Residual glucose (mg/l) of anaerobic decolorization at 35°C of sample 1 containing waste water, sludge and 300 mg/l RB 4 in the presence of glucose (300 mg CODcr/l) and the bacterial consortium

increasing the glucose concentrations showed an increase in decolorizing efficiency, at 35°C (data not shown). In general, anthraquinone dye reduction occurs by the mechanism of reversible quinone reduction to hydroquinone in two steps: benzoquinone ↔ semiquinone ↔ hydroquinone (Zollinger 1991). Thus, in the reduction process, transformation of RB4 in terms of the anthraquinone to hydroquinone was related to H^+ generated from glucose degradation and reductive transformation of the anthraquinone nucleus (Revenga et al. 1994). For RB4 decolorization mechanism, the results indicated evidently that acidogenesis was involved, as high decolorization efficiency, high VFAs but low methane were obtained when using glucose as compared to control and other co-substrates. This can be explained by Fig. 2, under mesophilic condition, degradation of glucose by acidogens and acetogens produced acetate, butyrate and propionate etc., which then will be used as substrates for methane production. These indicated that the reduction of RB4, anthraqionone form to hydroquinone, was related with H^+, generated from organic matter conversion process, and reductive transformation of anthraquinone nucleus (Revenga et al. 1994). Reducing byproduct of RB4 did not show any auto-oxidizing reaction, as the treated wastewater containing RB4 showed light yellow due to the unsubstituted antraquinone. The slower dye removal found when using other co-substrate, may be due to dye adsorption or accumulation to the cells.

Anthraquinone and phthalocyanine dyes are shown to be rather recalcitrant (Lee et al. 2005; Dos Santos et al. 2005). Therefore, the bacterial consortium is beneficial for enhanced the RB4 removal efficiency.

In conclusion, the isolated microbial cultures of the bacterial consortium shown in this study, deserve attention as a new biomass media, which can be utilized with combined anaerobic sludge treatment in the decolorization of wastewater effluents containing dyes as well as hydrogen production.

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