รายงานวิจัยฉบับสมบูรณ์

ศักยภาพทางชีวภาพของจุลินทรีย์ชอบร้อนจากบ่อน้ำพุร้อนในเขตภาคใต้สำหรับการผลิตไบโอฟูเอล

ดร.สมพงศ์ โอทอง

13 กรกฎาคม 2554

สัญญาเลขที่MRG5280236

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ดร.สมพงศ์ โอทอง มหาวิทยาลัยทักษิณ

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณสำนักงานคณะกรรมการอุดมศึกษา (สกอ.) และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ได้สนับสนุนงบประมาณในการวิจัยและทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่

ผู้วิจัยขอขอบพระคุณ รองศาสตราจารย์ ดร.พูนสุข ประเสริฐสรรพ์ นักวิจัยที่ปรึกษา ซึ่งกรุณาสละเวลา ให้ความรู้และคำแนะนำตลอดการทำงานวิจัยเรื่องนี้ และ Prof.Dr.Tsuyoshi Imai ซึ่งให้การสนับสนุนทุนทำวิจัยต่างประเทศระยะเวลา 1 เดือน ณ Yamaguchi University, Japan

ผู้วิจัยขอขอบพระคุณ สาขาวิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยทักษิณ และ Yamaguchi University, Japan ที่เอื้อเฟื้อสถานที่ วัสดุอุปกรณ์ต่าง ๆ สำหรับการทำวิจัย

ผู้วิจัยขอขอบคุณ นายอดิลัน หนิมัน และ นายพงษ์ศักดิ์ นพรัตน์ นิสิตปริญญาเอก สาขาเทคโนโลยีชีวภาพ มหาวิทยาลัยสงขลานครินทร์ ที่ได้ให้ความช่วยเหลือในการทำงานวิจัยชิ้นนี้ ขอขอบคุณ Rafiani Hasyim ที่ได้ช่วยเหลือการประยุกต์ใช้กลุ่มเชื้อจุลินทรีย์ชอบร้อนในการผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งสาคู

สมพงศ์ โอทอง

บทคัดย่อ

รหัสโครงการ : **MRG5280236**

ชื่อโครงการ : **ศักยภาพทางชีวภาพของจุลินทรีย์ชอบร้อนจากบ่อน้ำพุร้อนในเขตภาคใต้สำหรับการผลิตไบโอฟูเอล**

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งานวิจัยนี้ศึกษาโครงสร้างชุมชนของจุลินทรีย์ชอบร้อนในตะกอนดินและน้ำจากบ่อน้ำพุร้อนในเขตภาคใต้ของประเทศไทย โดยใช้เทคนิค Denaturing gradient gel electrophoresis (DGGE) และเพิ่มจำนวนประชากรจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนและเอทานอลจากน้ำตาลไซโลส (C5) กลูโคส (C6) และแป้งจากตะกอนดินน้ำพุร้อน คัดเลือกแหล่งของเชื้อจุลินทรีย์ชอบร้อนที่มีความสามารถในการผลิตไฮโดรเจนและเอทานอลได้สูง นำกลุ่มเชื้อจุลินทรีย์ชอบร้อนที่คัดเลือกได้ไปคัดแยกให้บริสุทธิ์และศึกษาคุณลักษาณะทางสรีรวิทยา ชีวเคมี และ เอ็นไซม์ ศึกษาศักยาพทางชีวภาพสำหรับการผลิตการผลิตไฮโดรเจนและเอทานอลจากเฮมิเซลลูโลสไฮโดรไลเสตลำต้นปาล์มซึ่งประกอบด้วยน้ำตาลไซโลสและกลูโลส น้ำทิ้งโรงงานแป้งมันสำปะหลัง และน้ำทิ้งโรงงานแป้งสาคู ตะกอนดินและน้ำจากน้ำพุร้อนในเขตภาคใต้ของประเทศไทยจำนวน 8 แหล่ง มีอุณหภูมิ อยู่ในช่วงระหว่าง 53-75 องศาเซลเซียส และมีพีเอชระหว่าง 6.5-8.3 กลุ่มประชากรแบคทีเรียที่พบเป็นจำนวนมากในตะกอนดินและน้ำจากบ่อน้ำพุร้อนประกอบด้วย *Clostridium Bacillus* และ *Pseudomonas* ส่วนแบคทีเรียชนิดอื่นๆที่ตรวจพบได้แก่ *Flavobacterium Thermodesulfovibrio Thermotoga Brevundimonas* และ *Micrococcus* ส่วนกลุ่มอาเคียประกอบด้วย *Thermoprotei Methanothermobacter* และสมาชิกในสกุล *Thermoproteaceae* และ *Methanosaeta* เมื่อนำตะกอนดินและน้ำจากบ่อน้ำพุร้อนมาเพิ่มจำนวนประชาการแบคทีเรียที่สามารถผลิตก๊าซไฮโดรเจนพบว่าตะกอนดินและน้ำจากบ่อน้ำพุร้อนรหัส PGR และ YLT สามารถผลิตก๊าซไฮโดรเจนได้ปริมาตรสูงจากอาหารเลี้ยงเชื้อที่เติมกลูโคสผสมไซโลส (1506 มิลลิลิตรไฮโดรเจนต่อลิตรอาหาร หรือ 301.3 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป) และเติมไซโลส (1487.3 มิลลิลิตรไฮโดรเจนต่อลิตรอาหาร หรือ 297.4 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป) การผลิตไฮโดรเจนของกลุ่มจุลินทรีย์ชอบร้อน รหัส PGR และ YLT เกิดพร้อมกับการผลิตกรดอะซิติกและกรดบิวทิริก ผลการศึกษาโครงสร้างชุมชนกลุ่มจุลินทรีย์ชอบร้อนรหัส PGR และ YLT ประกอบด้วยกลุ่มเชื้อที่สามารถผลิตไฮโดรเจนได้สูง *Thermoanaerobacterium* sp. และ *Caldicellulosiruptor* sp. จุลินทรีย์กลุ่มนี้มีศักยภาพที่จะพัฒนาขึ้นมาใช้สำหรับการผลิตไฮโดรเจนจากไฮโดรไลเสตซึ่งมีทั้งไซโลสและกลูโคสเป็น

ส่วนประกอบ กลุ่มเชื้อจุลินทรีย์รหัส PGR และ YLT ผลิตไฮโดรเจนได้สูงใกล้เคียงกัน ถูกนำมาทดสอบความสเถียรในการผลิตไฮโดรเจนจากไซโลสผสมกลูโคสในระบบแบบสลับเป็นกะ พบว่ากลุ่มเชื้อจุลินทรีย์รหัส PGR สามรถผลิตไฮโดรเจนในระบบสลับเป็นกะได้ดีและมีผลผลิตสูงกว่ากลุ่มเชื้อจุลินทรีย์รหัส YLT ร้อยละ 30 กลุ่มเชื้อจุลินทรีย์รหัส PGR ผลิตไฮโดรเจนในระบบสลับเป็นกะได้ 375 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป ส่วนกลุ่มเชื้อจุลินทรีย์รหัส YLT ผลิตได้ 287 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป การผลิตไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมันด้วยกลุ่มเชื้อจุลินทรีย์รหัส PGR สามารถผลิตไฮโดรเจนได้สูงสุด 301 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไปที่ความเข้มข้นของไฮโดรไลเสตร้อยละ 40 ในขณะที่การผลิตไฮโดรเจนจะถูกยับยั้งเมื่อใช้ความเข้มข้นของไฮโดรไลเสตมากกว่าร้อยละ 80 กลุ่มเชื้อจุลินทรีย์รหัส PGR มีประสิทธิภาพในการผลิตไฮโดรเจนและมีศักยภาพที่ดีสำหรับการผลิตจากไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมัน

ทำการแยกเชื้อแบคทีเรียผลิตไฮโดรเจนที่อุณหภูมิสูงจากกลุ่มเชื้อจุลินทรีย์รหัส PGR และ YLT สามารถแยกแบคทีเรียได้จำนวน 171 สายพันธุ์ คัดเลือกจากความเหมื่อนของยีน 16S rRNA ด้วยเทคนิค DGGE ได้ 6 สายพันธุ์ รหัส AH1-AH6 ผลิตไฮโดรเจนได้จากทั้งน้ำตาลไซโลสและกลูโคส มีผลผลิตไฮโดรเจนเท่ากับ 278 292 280 310 150 และ 225 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาล ตามลำดับ การจัดจำแนกโดยการเทียบเคียงยีน 16S rRNA พบว่าสายพันธุ์ AH1 AH2 AH3 และ AH4 อยู่ในสกุล *Thermoanaerobacterium* ในขณะที่ AH5 อยู่ในสกุล *Caloramator* และ AH6 อยู่ในสกุล *Clostridium*

การเพิ่มจำนวนประชากรจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจน โดยใช้แป้งมันสำปะหลังเป็นเป็นแหล่งคาร์บอน พบว่ากลุ่มเชื้อจุลินทรีย์ชอบร้อนที่สามารถผลิตไฮโดรเจนได้ดีคือ รหัส PGK SRW และ PGR สามารถในการผลิตไฮโดรเจนจากแป้งได้ 249.3 180 และ 124.9 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งดิบตามลำดับ กลุ่มจุลินทรีย์ชอบร้อน PGK SRW และ PGR ผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งมันสำปะหลังได้สูงสุดคือ 287 264 และ 232 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งดิบในน้ำทิ้ง โครงสร้างประชากรแบคทีเรียของกลุ่มจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนพร้อมกับย่อยแป้งดิบในน้ำทิ้งโรงงานแป้งมันสำปะหลังเด่นด้วยแบคทีเรียในกลุ่ม *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum*, *Anoxybacillus* sp., *Geobacillus* sp. และ *Clostridium* sp. กลุ่มเชื้อจุลินทรีย์ PGK ได้นำไปทดสอบความสามารถในการผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งสาคู พบว่ากลุ่มเชื้อจุลินทรีย์ PGK สามารถผลิตไฮโดรเจนได้ความเข้มข้นร้อยละ 55 สภาวะที่ให้ผลผลิตสูงสุดคือ ที่พีเอช 6.5 ความเข้มข้นของแป้งสาคู 2.5 ให้ผลผลิตไฮโดรเจน 422 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งสาคู

คำหลัก : น้ำพุร้อนในเขตุภาคใต้ จุลินทรีย์ชอบร้อน การผลิตไบโอฟูเอล

Abstract

Project Code : MRG5280236

Project Title : Bioprospecting thermophilic microorganisms from Southern Thailand geothermal spring for biofuel production

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This research studied the microbial community structure in sediment rich water from southern Thailand geothermal spring by using denaturing gradient gel electrophoresis techniques (DGGE). Natural microbial consortia from geothermal spring samples were used to developed thermophilic mixed cultures for biohydrogen production from xylose (C_5) glucose (C_6) and starch. The best thermophilic mixed culture that has the ability to produce hydrogen and ethanol was selected for isolation of thermophilic pure culture. Hydrogen and ethanol producing isolated was purified and tested for morphology, physiology, biochemistry and enzyme properties. The potential for the hydrogen production from the hemicellulose hydrolyzate of oil palm trunk, cassava starch processing wastewater and sago starch processing wastewater by thermophilic mixed was investigated. The temperature and pH of geothermal spring ranged between 53-75°C and 6.5-8.3, respectively. All hot springs contains plant biomass-rich sediment that is a carbon source of microorganisms in hot springs. Bacteria community developing on plant biomass-rich sediment was comprised of *Clostridium* spp., *Bacillus* spp. and *Pseudomonas* spp. constituted the major groups in these communities. Other bacteria detected in the biomass-rich sediment were *Flavobacterium* spp., *Thermotoga* spp., *Thermodesulfovibrio* spp., *Brevundimonas* spp. and *Micrococcus* spp. Archaea comprised of *Thermoprotei* spp., *Methanothermobacter* spp., members in family *Thermoproteaceae* and *Methanosaeta* spp. Enriched cultures encoded as PGR and YLT showed high cumulative hydrogen production in xylose glucose mixed and xylose with hydrogen evolution of 1506 and 1487 mL H_2/L medium and hydrogen yields of 301.3 and 297.4 mL H_2/g $sugar_{comsumed}$, respectively. The enriched cultures coded PGR and YLT were produced acetate and butyrate as main soluble metabolites with high hydrogen production. Thermophilic mixed culture comprised of efficient hydrogen-producing species relating to *Thermoanaerobacterium* sp. and *Caldicellulosiruptor* sp. Significant hydrogen production potentials were obtained from thermophilic enriched

cultures PGR and YLT, after repeated batch cultivation, the hydrogen yield from xylose-glucose mixed substrate of PGR increased to 375 mL H_2/g $sugar_{consumed}$, which was 30% higher than that of YLT (287 mL H_2/g $sugar_{consumed}$). Cultivation of the enriched culture PGR in oil palm trunk hydrolysate, the maximum hydrogen yield of 301 mL H_2/g $sugar_{consumed}$ was achieved at hydrolysate concentration of 40% (v/v). At higher concentration to 80% (v/v), the hydrogen fermentation process was inhibited. Thermophilic hydrogen producing bacteria were isolated from enriched cultures code PGR and YLT. A total of 171 isolates were screened by DGGE of V_3 region of 16S rRNA and 6 candidates were selected for screening test on hydrogen production. Six isolates, namely AH1-AH6 were selected as they successfully fulfilled the criteria defined for the screening test. Analysis of 16S rRNA gene revealed that the strains AH1, AH2, AH3 and AH4 belong to the genus *Thermoanaerobacterium*, whereas the strain AH5 belong to genus *Caloramator* and the isolate AH6 belong to various clusters of *Clostridium*. They degraded both xylose and glucose into hydrogen with the production yield of 278, 292, 280, 310, 150 and 225 mL H_2/g sugar, respectively.

Natural microbial consortia from geothermal spring samples were used to developed thermophilic mixed cultures for biohydrogen production from cassava starch processing wastewater (CSPW). Significant hydrogen production potentials were obtained from three thermophilic mixed cultures namely PGK, SRW and PGR with maximum hydrogen production yields of 249.3, 180 and 124.9 mL H_2/g-starch, respectively. The thermophilic mixed cultures PGK, SRW and PGR exhibited the maximum hydrogen yield of 287, 264 and 232 mL H_2/g-starch in CSPW, respectively. Phylogenetic analysis of thermophilic mixed cultures revealed that members involved cassava starch degrading bacteria and hydrogen producers were phylogenetically related to the *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum*, *Anoxybacillus* sp., *Geobacillus* sp. and *Clostridium* sp. We investigated the feasibility of producing biohydrogen from sago starch in wastewater using a thermophilic mixed culture enriched PGK. The methane-free biogas evolved contained up to 55% hydrogen, with the remainder comprising carbon dioxide. Gelatinized dry starch at an initial pH of 6.5 and an initial starch concentration of 2.5 g/l gave the maximum hydrogen yield of 422 ml-H_2/g-$starch_{added}$ (80% of the theoretical limit).

Keywords : Southern Thailand geothermal spring, thermophilic bacteria, biofuel production

บทนำ

วิกฤตการณ์พลังงานเป็นปัญหาเร่งด่วนที่ต้องแก้ไข และหามาตรการป้องกัน ทั้งนี้เนื่องจากประเทศไทย มีทรัพยากรพลังงาน เช่น น้ำมัน และก๊าซธรรมชาติน้อย จึงจำเป็นต้องพึงพาประเทศอื่นๆ ที่สามารถส่งออกพลังงานมาจำหน่ายได้ ส่งผลให้ขาดความมั่นคงทางด้านพลังงาน และทรัพยากรพลังงานเป็นแหล่งพลังงานที่ใช้แล้วหมดไป จึงมีความสนใจที่จะหาแหล่งพลังงานทดแทนแหล่งใหม่ ซึ่งก๊าซไฮโดรเจน และ เอทานอลจากมวลชีวภาพประเภทเซลลูโลสเป็นอีกทางเลือกของแหล่งพลังงานในอนาคตที่คาดว่าจะนำมาทดแทนพลังงานเชื้อเพลิงจากซากดึกดำบรรพ์ (Levin et al., 2004; Kapdan and Kargi, 2006) กระบวนการหมักโดยจุลินทรีย์สามารถผลิตพลังงานทดแทนจากชีวมวลได้ กระบวนการหมักโดยจุลินทรีย์ที่อุณหภูมิสูง (60-70 องศาเซลเซียส) มีข้อดีกว่าการหมักที่อุณหภูมิห้อง (25-30 องศาเซลเซียส) แต่การศึกษาการหมักที่อุณหภูมิสูงยังมีน้อย การหมักที่อุณภูมิสูงสามารถเพิ่มอัตราการเกิดปฏิกิริยาทางเคมีและเอ็นไซม์ เพิ่มค่า thermodynamic favorability ของปฏิกิริยา ลดการละลายของ H_2 และ CO_2 ในน้ำหมัก ลดความหลากหลายของผลผลิตจากการหมักทำให้ง่ายต่อการทำให้บริสุทธิ์ เชื้อจุลินทรีย์ชอบร้อนส่วนใหญ่ผลิตเอ็นไซม์ขับออกมานอกเซลล์ในการย่อยสลายไบโอพอลิเมอร์ต่างๆ และ เหมาะสมที่จะใช้จุลินทรีย์ชอบร้อนในการผลิตพลังงานชีวมวลจากน้ำเสียโรงงานอุตสากรรมการเกษตรที่มีอุณหภูมิสูง การหมักที่อุณหภูมิสูงทำให้น้ำเสียที่ผ่านการหมักปราศจากเชื้อก่อโรค (van Groenestijn et al., 2002; van Niel et al., 2003; O-Thong et al., 2008) จุลินทรีย์ชอบร้อนเป็นจุลินทรีย์ที่สามารถใช้น้ำตาล เฮกโซส และ เพนโตส ได้ดี ซึ่งน้ำตาลสองชนิดนี้เป็นองค์ประกอบหลักของมวลชีวภาพประเภทลิกโนเซลลูโลส จุลินทรีย์ชอบร้อนสามารถผลิตเอทานอลจากมวลชีวภาพประเภทเซลลูโลสได้ดี ซึ่งกระบวนการผลิตเอทนอลในปัจจุบันโดยยีสต์ไม่สามาถผลิตได้ (Dien et al., 2003) การใช้มวลชีวภาพประเภทเซลลูโลส อย่างเช่น วัสดุเศษเหลือจากป่าไม้ เศษเหลือจากการเกษตร หญ้า ชีวมวลต่างๆ มาผลิตเอทานอล สามารถลดต้นทุนในการผลิตเอทานอลได้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับการหมักเอทานอลด้วยยีสต์จากน้ำตาลหรือกากน้ำตาล (Lin et al., 2006) จากคุณสมบัติที่กล่าวมาข้างต้น ทำให้การประยุกต์ใช้เชื้อจุลินทรีย์กลุ่มชอบร้อนในการผลิตไฮโดรเจนและเอทานอลมีความคุ้มค่าทางด้านเศรษฐศาสตร์ และเป็นไปได้ในทางปฏิบัติ อย่างไรก็ตามจำนวนเชื้อจุลินทรีย์ชอบร้อนที่สามารถเปลี่ยนมวลชีวภาพประเภทเซลลูโลสไปเป็น ไฮโดรเจน และเอทานอลยังมีจำนวนน้อยที่มีผลผลิตและอัตราการผลิตที่ยอมรับได้

ดังนั้นงานวิจัยชิ้นจึงมุ่งเน้นศึกษากลุ่มจุลินทรีย์ชอบร้อน ซึ่งแหล่งของเชื้อจุลินทรีย์ชอบร้อนส่วนใหญ่มาจากน้ำพุร้อน ตัวอย่างน้ำพุร้อนจากแหล่งต่างๆในเขตภาคใต้ของประเทศไทยซึ่งส่วนใหญ่ยังไม่มีการศึกษา เก็บตะกอนดินและน้ำมาศึกษาความหลากหลายทางชีวภาพโดยเทคนิคทางอณูวิทยา (Denaturing gradient gel electrophoresis; DGGE) และศึกษาศักยาพทางชีวภาพสำหรับการผลิตการผลิตเอทานอล และไฮโดรเจน โดยใช้ข้อมูลที่ได้ไปออกแบบอาหาร

เลี้ยงเชื้อเพื่อให้สามารถแยกจุลินทรีย์ที่ต้องการออกมาได้ ตะกอนดินถูกนำมาเพิ่มจำนวนจุลินทรีย์โดยใช้น้ำตาลเฮกโซส เพนโตส และแป้งเป็นสารตั้งต้น และคัดเลือกแหล่งของเชื้อที่มีความสามารถในการผลิตเอทานอลและไฮโดรเจน นำกลุ่มเชื้อที่มีความสามารถในการผลิตไฮโดรเจนและเอทานอลไปคัดแยกและศึกษาคุณลักษาณะทางสริวิทยา ชีวเคมี และ เอ็นไซม์ ข้อดีของการศึกษาทางด้านอณูวิทยาก่อนการคักแยกจุลิทรีย์ ทำให้เราเลือกใช้อาหารเลี้ยงเชื้อที่เหมาะสมกับจุลินทรีย์ที่เราต้องการและคักแยกจุลินทรีย์ใหม่ที่ไม่เคยศึกษามาก่อนได้

องค์ความรู้ใหม่และผลกระทบขององค์ความรู้ต่อความก้าวหน้าในเชิงวิชาการของสาขาที่ทำวิจัย การผลิตพลังงานจากชีวมวลซึ่งองค์ประกอบส่วนใหญ่เป็นน้ำตาลเฮกโซสและเพนโตส แต่วิธีการในการผลิตปัจจุบันไม่สามารถใช้ประโยชน์จากน้ำตาลเพนโตสได้ และต้องมีกระบวนการย่อยชีวมวลด้วยกระบวนการเคมีหรือกายภาพเพื่อให้ได้น้ำตาลเฮกโซส ซึ่งทำให้ต้นทุนในการผลิตสูง และทำให้การผลิตพลังงานจากชีวมวลไม่มีความคุ้มค่าทางด้านเศษฐศาสตร์ เชื้อจุลินทรีย์ชอบร้อนจะลดข้อจำกัดของการผลิตเอทานอลด้วยยีสต์และทำให้การผลิตเอทานอลจากชีวมวลมีความคุ้มค่าทางด้านเศษฐศาสตร์ และทำให้ทราบความหลากหลายของจุลินทรีย์จากน้ำพุร้อนในเขตภาคใต้ที่ยังไม่มีการศึกษา และการค้นพบจุลินทรีย์สายพันธุ์ใหม่จากแหล่งน้ำพุร้อน

วัตถุประสงค์

1.เพื่อศึกษาความหลากหลายของจุลินทรีย์ชอบร้อนจากน้ำพุร้อนในเขตภาตใต้และแนวโน้มในการน้ำมาใช้ประโยชน์ในการผลิตพลังงานจากมวลชีวภาพ

2. เพื่อแยกเชื้อจุลินทรีย์ชอบร้อนที่สามารถเปลี่ยนน้ำตาล เอกโซส เพนโตส และแป้ง เป็นเอทานอลหรือไฮโดรเจนจากแหล่งน้ำพุร้อนในเขตภาคใต้

3. คัดเลือกจุลินทรีย์ที่มีความสามารถในการผลิตพลังงานจากมวลชีวภาพ จากเชื้อจุลินทรีย์ที่แยกได้และหาสภาวะที่เหมาะสมในการผลิต

4. การผลิต ไฮโดรเจน และ เอทานอล ด้วยเชื้อจุลินทรีย์ชอบร้อนจากมวลชีวภาพ เซลลูโลส เฮมิเซลลูโลสไฮโดรไลเสต และน้ำทิ้งโรงงานแป้งมันสำปะหลัง และศึกษาความเป็นไปได้ทางเศษฐศาสตร์ในการผลิต

ระเบียบวิธีดำเนินการวิจัย

1. ศึกษาความหลากหลายของจุลินทรีย์ชอบร้อนจากน้ำพุร้อนในเขตภาคใต้และแนวโน้มการนำมาใช้ประโยชน์

เก็บตัวอย่างน้ำและตะกอนดินจากบ่อน้ำพุร้อนต่าง ๆ ในเขตภาคใต้ของประเทศไทย นำไปศึกษาความหลากหลายของ bacteria และ archaea โดยเทคนิค DGGE และนำข้อมูลที่ได้ไปประเมินศักยภาพทางชีวภาพของจุลินทรีย์ชอบร้อนในการผลิตเอทานอลและไฮโดรเจน โดยการเปรียบเทียบกับฐานข้อมูล genome ของจุลินทรีย์ โดยนำตัวอย่างน้ำและ ตะกอนดินตัวอย่างจากน้ำพุร้อนแต่ละแหล่งมาปั่นเหวี่ยงที่ 14,000g เป็นเวลา 10 วินาที เพื่อเก็บเกี่ยวเซลล์จุลินทรีย์ จากนั้นล้างด้วยฟอสเฟตบัฟเฟอร์ pH 7.4 แล้วปั่นเหวี่ยงที่สภาวะเดิมอีกครั้งเพื่อแยกบัฟเฟอร์ออก นำกากตะกอนที่ได้ไปทำการสกัด DNA จากกลุ่มจุลินทรีย์ในกากตะกอนทั้งหมดด้วยวิธี beading method โดยใช้ชุดสกัดสำเร็จรูป (Fast DNA Spin kit of soil, Bio 101 (Biospec Products, Bartlesville, สหรัฐอเมริกา) ทำการเพิ่มจำนวนชิ้น DNA ที่สกัดได้โดยใช้เทคนิค PCR (automated thermal cycle (Biorad, USA)) การเพิ่มจำนวนดีเอ็นเอของแบคทีเรียใช้ชุดไพรเมอร์ forward primer 16Sf (5’gagtttgatcctggctcag -3’) และ reverse primer 16Sr (5’-gaaaggaggtgatccagcc -3’) สำหรับเพิ่มจำนวนชิ้นยีน 16S ribosomal DNA และนำ PCR product ที่ได้ไปทำ PCR อีกครั้งโดยใช้ชุดไพรเมอร์ forward primer 357f-CG (5’GC clamp-cctacgggaggcagcag-3’) และ reverse primer 517r (5’-attaccgcgctgctgg-3’) สำหรับการเพิ่มจำนวนดีเอ็นเอในกลุ่ม archaea ใช้ชุดไพรเมอร์ forward primer Arch21f (5’ttccggttgatcc(c/t)gccgga-3’) และ reverse primer Arch958r (5’-(c/t)ccggcgttga(a/c)tccaatt-3’) สำหรับเพิ่มจำนวนชิ้นยีน 16S ribosomal DNA และนำ PCR product ที่ได้ไปทำ PCR อีกครั้งโดยใช้ชุดไพรเมอร์ forward primer PARCH340f-CG (5’GC clamp-cctacgggg(c/t)gca(g/c)cag -3’) และ reverse primer PARCH519r (5’-ttaccgcggc(g/t)gctg-3’) จากนั้นนำผลิตภัณฑ์ที่ได้จากการทำ PCR (PCR product) จาก primer ชุด CG-clamp ไปแยกชิ้น DNA ของจุลินทรีย์ที่แตกต่างกัน บนแผ่น gel โดยใช้เทคนิค DGGE ใช้สารละลาย acrylamide/Bis 40% ในการเท gel ใช้ denaturant gradients จาก 30% ถึง 70% ทำการแยกชิ้น DNA บนแผ่น gel ในสารละลาย 1xTAE บัฟเฟอร์ ที่ 130 V อุณหภูมิ 60 องศาเซลเซียสเป็นเวลา 5 ชั่วโมง แล้วย้อมแถบ (band) DNA บนแผ่น gel ด้วย ethidium bromide ตัดแถบ DNA ที่แยกได้แต่ละแถบมาทำ PCR ซ้ำ 5 รอบ เพื่อให้ได้ชิ้น DNA ที่บริสุทธิ์สำหรับจุลินทรีย์แต่ละชนิด จากนั้นนำ PCR product ส่งไปวิเคราะห์ลำดับเบสบนชิ้น DNA ที่บริษัท Macrogen ประเทศเกาหลี แล้วเปรียบเทียบกับลำดับเบสของจุลินทรีย์อ้างอิง (reference microorganisms) ที่มีอยู่ในฐานข้อมูลของ GenBank โดยใช้ BLAST search (www.NCBI.com) เพื่อทราบชนิดจุลินทรีย์ที่มีลำดับเบสใกล้เคียงกันมากที่สุด

แล้วสร้าง phylogenetic tree โดยใช้โปรแกรมจาก BioEdit (Hall, 2005) เพื่อจัดกลุ่มของจุลินทรีย์ที่มีความใกล้เคียงกัน

2. แยกเชื้อจุลินทรีย์ชอบร้อนที่สามารถเปลี่ยนน้ำตาล เฮกโซส เพนโตส แป้งไปเป็นไฮโดรเจนและเอทานอล

เก็บตัวอย่างน้ำพุร้อนและเพิ่มจำนวนจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนและเอทานอลโดยนำตัวอย่างน้ำและตะกอนดินจากน้ำพุร้อนปริมาณ 7 มิลลิลิตรมาถ่ายลงขวดอาหาร ซึ่งมีอาหารที่ใช้ในการเลี้ยงเชื้อมี 4 ชนิด ปริมาณชนิดละ 70 มิลลิลิตร อาหารเลี้ยงเชื้อประกอบด้วย peptone 5 กรัมต่อลิตร yeast extract 1 กรัมต่อลิตร $FeSO_4.7H_2O$ 0.2 กรัมต่อลิตร KH_2PO_4 1.5 กรัมต่อลิตร K_2HPO_4 3.0 กรัมต่อลิตร $NaHCO_3$ 2.5 กรัมต่อลิตร และใช้ Glucose Xylose Soluble starch และ Cellulose เป็นแหล่งคาร์บอน แหล่งคาร์บอนละ 10 กรัมต่อลิตรโดยบ่มไว้ที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 2 – 4 วัน เมื่อครบกำหนด ทำการวัดก๊าซ เพื่อตรวจสอบความสามารถในการสร้างไฮโดรเจน และนำของเหลวไปวัดปริมาณเอทานอลด้วย gas chromatography เก็บน้ำหมัก 2 มิลลิลิตรใส่หลอดเก็บในตู้เย็นเพื่อนำไปวิเคราะห์ปริมาณและชนิดของกรดอินทรีย์ระเหยง่ายที่กลุ่มจุลินทรีย์ผลิต เก็บกลุ่มชื้อที่เก็บไว้ในหลอด ปั่นแยกเอาส่วนใสไปวิเคราะห์องค์ประกอบของกรดและน้ำตาลที่เหลือ หาประสิทธิภาพในการผลิตเทียบต่อหน่วยสารตั้งต้น สำหรับส่วนที่เป็นตะกอนจะนำมาตรวจกลุ่มเชื้อโดยเทคนิค DGGE ตามอธิบายข้างบน

นำกลุ่มเชื้อจุลินทรีย์จากน้ำพุร้อนที่เพิ่มขยายจำนวนแล้วมาทำการคัดแยกจุลินทรีย์ด้วย Hungate techniques (Holt and Sneath, 1994; O-Thong et al., 2008) โดยเตรียมอาหารเลี้ยงเชื้อจุลินทรีย์ แล้วนำไปไล่ออกซิเจนออกโดยการพ่นด้วยก๊าซไนโตรเจนเป็นเวลา 15 นาที แล้วถ่ายอาหารลงขวด serum bottle ปริมาณ 4.5 มิลลิลิตร จากนั้นเติมตัวอย่างกลุ่มจุลินทรีย์ลงไปแล้วทำการเจื่อจางภายในขวด serum bottle โดยเจือจางตั้งแต่ 10^{-1} ถึง 10^{-10} หลังจากนั้นนำมาอุ่นในอ่างน้ำร้อนที่อุณหภูมิ 60 องศาเซลเซียส และเติม gellen gum ลงไปเพื่อทำให้อาหารแข็ง และนำไปบ่ม ที่ 60 องศาเซลเซียส และเก็บเกี่ยว colony ที่โตใน gellan gum นำโคโลนีที่แยกได้มาทำซ้ำอย่างน้อยสามครั้งเพื่อให้แน่ใจว่าได้สายพันธ์จุลินทรีย์บริสุทธิ์ และ ตรวจสอบอีกครั้งด้วยเทคนิค DGGE เปรียบเทียบเชื้อที่แยกได้กับข้อมูลเชื้อที่ได้จากเทคนิค DGGE ของกลุ่มเชื้อ ทำการจัดจำแนก และ ศึกษาลักษณะทางสรีรวิทยา ชีวเคมี และ เอ็นไซม์ที่จุลินทรีย์ผลิต ศึกษาในเชิงลึกของเชื้อจุลินทรีย์ที่แยกได้และคาดว่าจะเป็นสายพันธุ์ใหม่เพื่อการเสนอชื่อใหม่ของจุลินทรีย์ที่พบ อย่างเช่น การทำ DNA hybridization การศึกษา GC contain การศึกษาโครงสร้างผนังเซลล์ และ การถ่ายจุลทรรศ์อิเล็กตรอนเพื่อศึกษาโครงสร้างต่างๆ ภายในเซลล์

3. คัดเลือกจุลินทรีย์ที่มีความสามารถในการผลิตเอทานอลและไฮโดรเจนได้สูง

นำเชื้อจุลินทรีย์ที่คัดแยกได้มาคัดเลือกจุลินทรีย์ที่มีความสามารถในการผลิต ไฮโดรเจน และ เอทานอล และหาสภาวะที่เหมาะสม แบบกะโดย เติมหัวเชื้อ 5 มิลลิลิตร (10 เปอร์เซ็นต์ ลงในขวด Serum bottle ที่มีอาหารเลี้ยงเชื้อ 50 มิลลิลิตร เลี้ยงที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง เก็บข้อมูลสุ่มตัวอย่างจากขวด เพื่อตรวจหาปริมาณเซลล์แบคทีเรีย ปริมาณน้ำตาล ปริมาณไฮโดรเจน และปริมาณเอทานอลที่ระยะเวลาสิ้นสุดการหมัก และหาสภาวะที่เหมาะสมในการผลิตไฮโดรเจนและเอทานอลโดยศึกษาปัจจัยของ pH ความเข้มข้นของสารตั้งต้น แหล่งไนโตรเจน ความเข้มข้นของเหล็ก

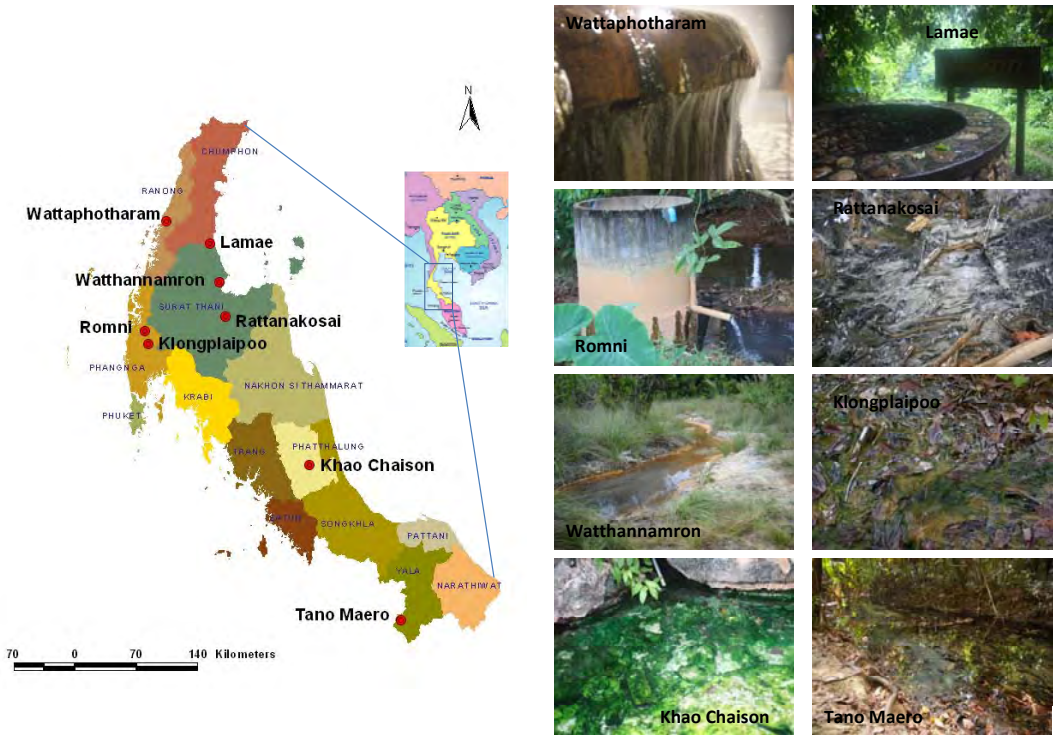
4. การผลิตเอทานอลและไฮโดจรเจนจากมวลชีวภาพเซลลูโลสและศึกษาความเป็นไปได้ทางเศษฐศาสตร์

นำเชื้อจุลินทรีย์ชอบร้อนที่คัดเลือได้มาผลิตไฮโดรเจนและเอทานอลจากเฮมิเซลลูโลสลำต้นปาล์ม โดยนำลำต้นปาล์มมาย่อยด้วยเตรียมด้วยกรดและด่างแบบสองขั้นตอน นำส่วนของ Cellulose ไปย่อยด้วยกรดและนำไปผลิตเอทานอล ส่วนของไฮโดรไลเสจากกระบวนการเตรียมด้วยกรดและด่างแบบสองขั้นตอนนำไปผลิต ไฮโดรเจน นำเชื้อจุลินทรีย์ชอบร้อนที่คัดเลือได้มาผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งมันสำปะหลัง และ โรงงานแป้งสาคู และศึกษาความเป็นไปได้ทางเศษศาสตร์ในการใช้จุลินทรีย์กลุ่มชอบร้อนในการผลิตพลังงานจากวัตถุดิบมวลชีวภาพ

ผลการวิจัย

1. ความหลากหลายของจุลินทรีย์ชอบร้อนจากบ่อน้ำพุร้อนในเขตภาคใต้

น้ำพุร้อนในเขตภาตใต้ของประเทศไทย มีอุณหภูมิอยู่ในช่วง 53-75 องศาเซลเซียส พีเอชอยู่ในช่วง 6.5-8.3 และตะกอนดินในบ่อน้ำพุร้อนประกอบด้วยใบของพืชจำนวนมาก ซึ่งเป็นแหล่งคาร์บอนสำหรับการเจริญเติบโตของจุลินทรีย์ (ภาพที่ 1) ดังนั้นจึงมีโอกาศที่จะพบแบคทีเรียชอบร้อนที่ย่อยสลาย เซลลูโลส ไซโลส กลูโคส และแป้งได้ บ่อน้ำพุร้อนจากจังหวัดระนองรหัส RNW ในน้ำมีซัลไฟด์ปริมาณเล็กน้อย (0.6 มิลลิกรัมต่อลิตร) และซัลเฟส (1.3 มิลลิกรัมต่อลิตร) มีพีเอชเป็นด่าง (8.3) และมีสารอินทรีย์ละลายน้ำ (DOC) สูง ซึ่งเป็นตัวบงชี้ถึงกิจกรรมของจุลินทรีย์ในบ่อน้ำพุร้อน บ่อน้ำพุร้อนจังหวัดสุราษฎร์ธานี รหัส SRW และ บ่อน้ำพุร้อนจังหวัดพังงา รหัส PGK และ PGR ในน้ำมีปริมาณซัลไฟด์ต่ำอยู่ในช่วง 0.7-1.5 มิลลิกรัมต่อลิตร มีพีเอชเป็นกลาง 6.5-7.0 ในขณะที่บ่อน้ำพุร้อนจังหวัดพัทลุง รหัส PTK และ บ่อน้ำพุร้อนจังหวัดสุราษฎร์ธานี รหัส SRR มีปริมาณซัลไฟด์อยู่ในน้ำสูง (10-12 มิลลิกรัมต่อลิตร) บ่อน้ำพุร้อนจังหวัดยะลารหัส YLT และ บ่อน้ำพุร้อนจังหวัดชุมพร รหัส CHL ในน้ำมีสารอินทรีย์ละลายน้ำ (DOC) สูง และไม่พบซัลไฟด์และซัลเฟสในน้ำ (ตารางที่ 1)



ภาพที่ 1 แสดงลักษณะตะกอนดินจากน้ำพุร้อนในเขตภาคใต้

ตารางที่ 1 คุณภาพทางเคมีและกายภาพของน้ำจากบ่อน้ำพุร้อนในเขตภาคใต้

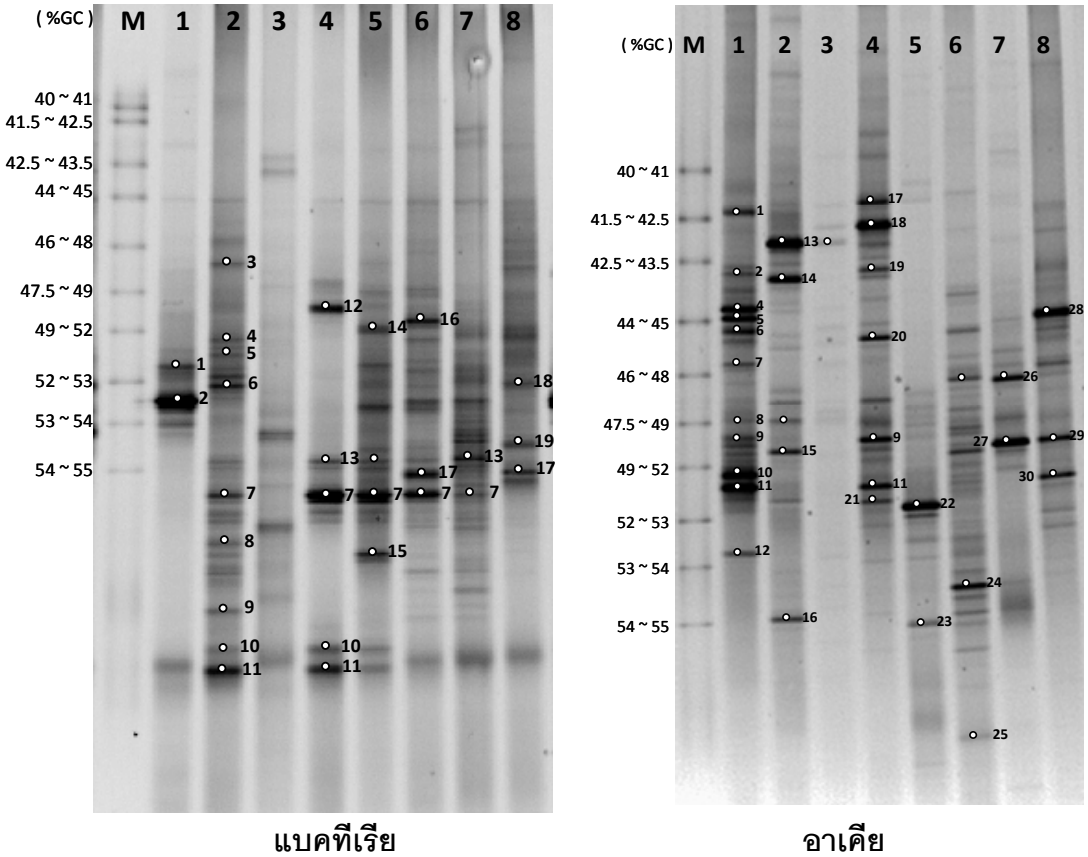
Geothermal spring	Code	Temp (°C)	pH	Sulfide (mg/L)	Sulfate (mg/L)	DOC (mg/L	DIC (mg/L)	NH^{+4}-N (mg/L)
Ranong province								
Wattaphotharam	RNW	65	8.3	0.6	1.3	523	121	12
Suratthani province								
Wat Than Nam Ron	SRW	63	7.0	1.5	25	12	128	0
Rattanakosai	SRR	67	7.9	10	70	34	135	0.6
Chumporn Province								
Lamae	CHL	53	7.8	0	0.2	342	78	1.2
Phatthalung Province								
Khao Chai Son	PTK	60	7.9	12	30	54	98	0.2
Phang Nga province								
Khong Pay Pao	PGK	60	6.5	0.7	1.5	44	112	0.5
Romanee	PGR	63	6.8	1.1	4	123	124	0.8
Yala province								
Ta Na Ma Rao	YLT	80	7.8	0	0.3	287	88	1.1

Temp = temperature, DOC = dissolved organic carbon, DIC = dissolve inorganic carbon

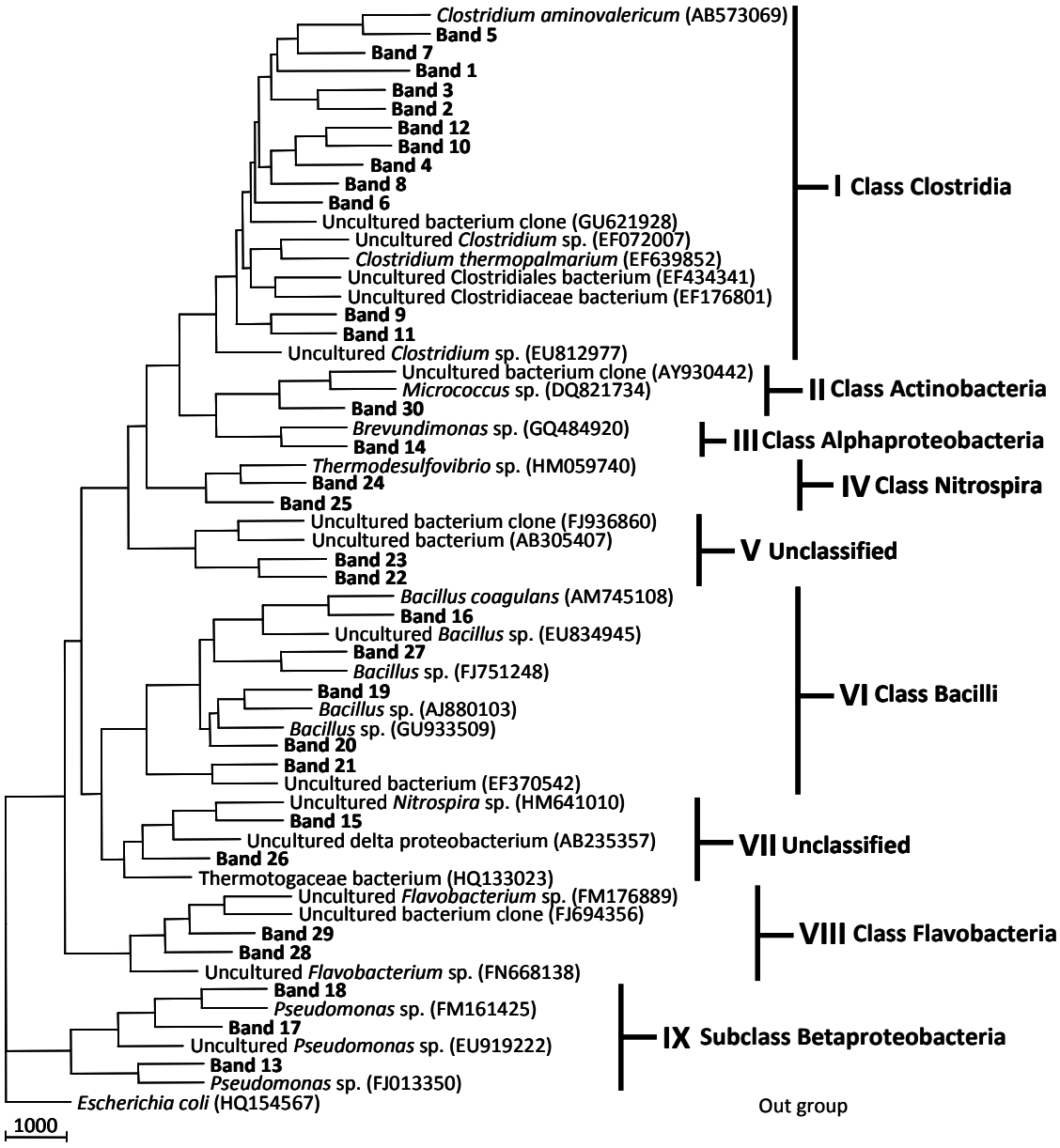
ตะกอนดินและน้ำจากน้ำบ่อพุร้อนในเขตภาคใต้ของประเทศไทยจำนวน 8 แหล่ง ศึกษาโครงสร้างชุมชนของจุลินทรีย์ทั้งแบคทีเรียและอาเคียด้วยเทคนิค DGGE ที่มีความจำเพาะต่อยีน 16S rRNA พบว่าทั้งแบคทีเรียและอาเคียมีการเจริญเติบโตในตะกอนดินและน้ำจากบ่อน้ำพุร้อน ยีน 16S rRNA มีความหลากหลายมากกว่า 30 ชนิดทั้งในกลุ่มของแบคทีเรียและอาเคีย (ภาพที่ 2) กลุ่มประชากรแบคทีเรียที่พบเป็นจำนวนมากในตะกอนดินและน้ำจากบ่อน้ำพุร้อนประกอบด้วย *Clostridium Bacillus* และ *Pseudomonas* ส่วนแบคทีเรียชนิดอื่นๆที่ตรวจพบได้แก่ *Flavobacterium Thermodesulfovibrio Thermotoga Brevundimonas* และ *Micrococcus* (ภาพที่ 3) ส่วนกลุ่มอาเคียประกอบด้วย *Thermoprotei Methanothermobacter* และสมาชิกในสกุล *Thermoproteaceae* และ *Methanosaeta* (ภาพที่ 4) จากผลการศึกษานี้แสดงให้เห็นว่าตะกอนดินและน้ำจากบ่อน้ำพุร้อนมีจุลินทรีย์หลากหลายชนิดที่สามารถใช้เป็นแหล่งของเชื้อแบคทีเรียและอาเคียที่ชอบอุณหภูมิสูงและสามารถย่อยสลายมวลชีวภาพจากพืชได้

เมื่อนำตะกอนดินและน้ำจากบ่อน้ำพุร้อนทั้ง 8 แหล่งมาเพิ่มจำนวนประชาการแบคทีเรียที่สามารถผลิตก๊าซไฮโดรเจนในอาหารเลี้ยงเชื้อที่เติมกลูโคส ไซโลส และกลูโคสผสมไซโลสเป็นแหล่งคาร์บอน พบว่าตะกอนดินและน้ำจากบ่อน้ำพุร้อนรหัส PGR และ YLT สามารถผลิตก๊าซไฮโดรเจนได้ปริมาตรสูงจากอาหารเลี้ยงเชื้อเติมกลูโคสผสมไซโลส (1506 มิลลิลิตรไฮโดรเจนต่อ

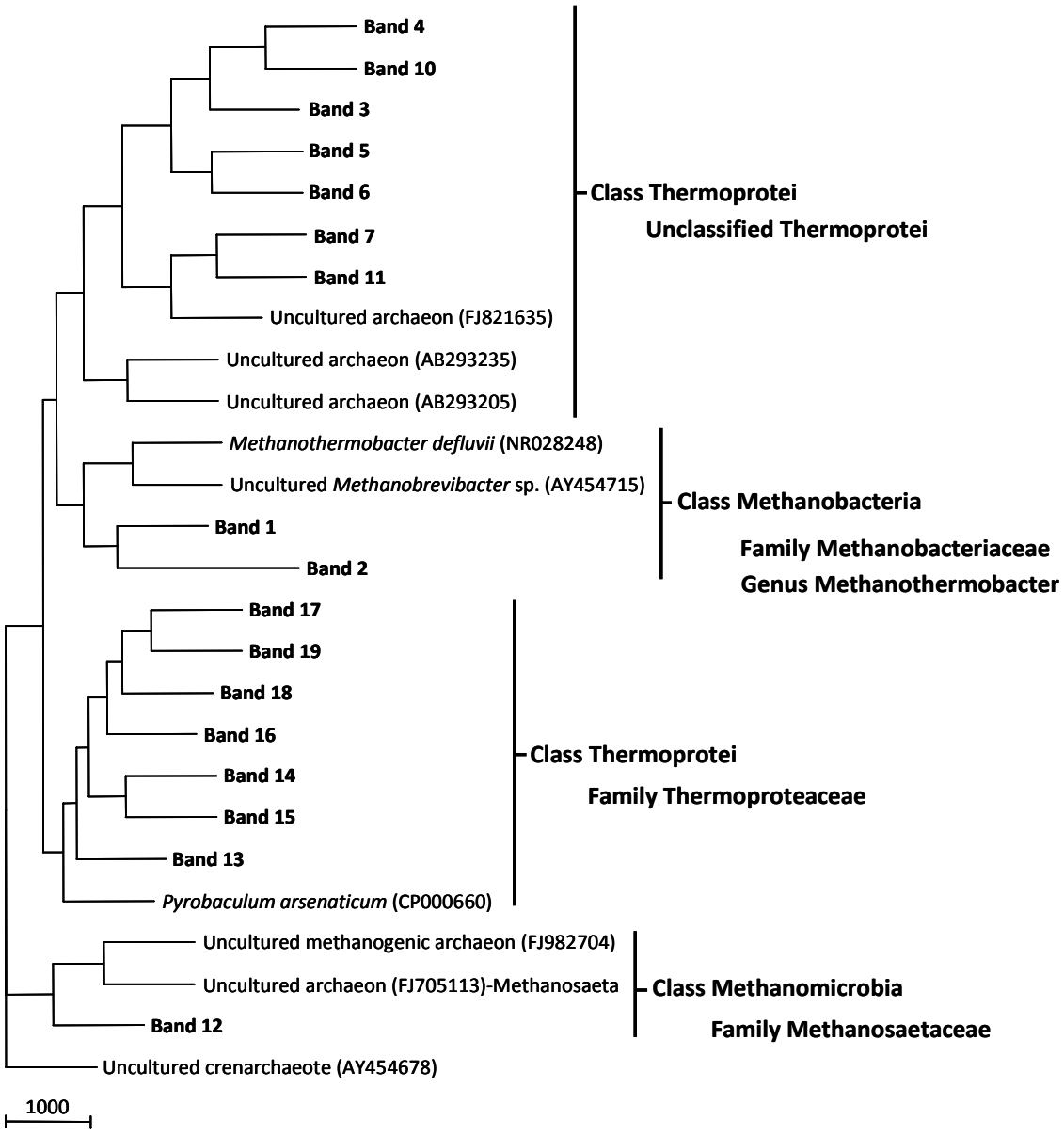
ลิตรอาหาร หรือ 301.3 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป) และไซโลส (1487.3 มิลลิลิตรไฮโดรเจนต่อลิตรอาหาร หรือ 297.4 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป) เป็นแหล่งอาหาร ตามลำดับ การผลิตไฮโดรเจนของกลุ่มจุลินทรีย์ชอบร้อน รหัส PGR และ YLT เกิดพร้อมกับการผลิตกรดอะซิติกและกรดบิวทิริก ผลการศึกษาโครงสร้างชุมชนกลุ่มจุลินทรีย์ชอบร้อนรหัส PGR และ YLT สามารถจำแนกจุลินทรีย์ได้ 3 กลุ่มคือ กลุ่ม A มีความสามารถในการผลิตไฮโดรเจนต่ำประกอบด้วย *Staphylococcus* sp. *Bacillus* sp. *Anoxybacillus* sp. และ uncultured Firmicutes bacterium กลุ่ม B มีความสามารถในการผลิตไฮโดรเจนปานกลางประกอบด้วย *Caloramator* sp. และ *Clostridium* sp. และกลุ่ม C ซึ่งเป็นกลุ่มเชื้อที่สามารถผลิตไฮโดรเจนได้สูงประกอบด้วย *Thermoanaerobacterium* sp.และ *Caldicellulosiruptor* sp. (ภาพที่ 5) จุลินทรีย์ที่ตรวจพบเหล่านี้แสดงถึงความเป็นไปได้ที่จะพัฒนาขึ้นมาใช้สำหรับการผลิตไฮโดรเจนจากไฮโดรไลเสต ที่มีทั้งไซโลสและกลูโคสเป็นส่วนประกอบ



ภาพที่ 2 โครงสร้างประชากรแบคทีเรียและอาเคียจากบ่อน้ำพุร้อนแหล่งต่าง ๆในเขตภาคใต้ของประเทศไทย วิเคราะห์ด้วยเทคนิค denaturing gradient gel electrophoresis (DGGE) โดยเลน 1=RNW, 2=SRW, 3=SRR, 4=CHL, 5=PTK, 6=PGK, 7=PGR and 8=YLT



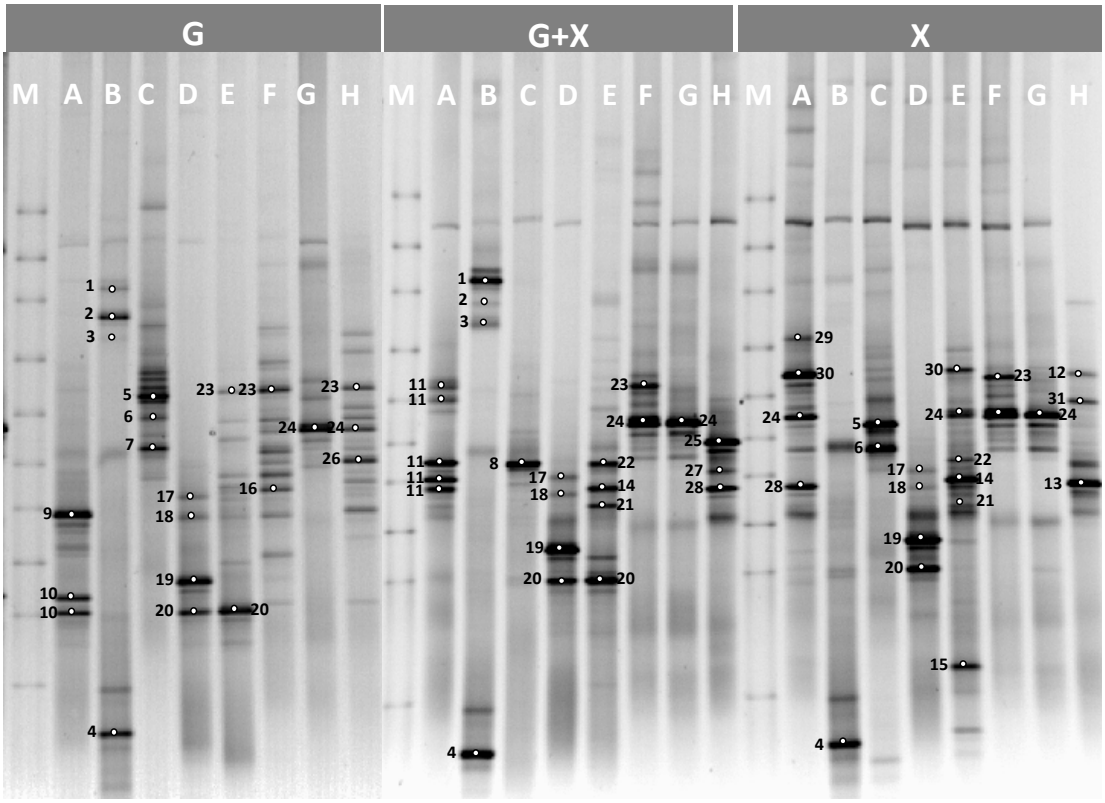
ภาพที่ 3 แสดงความสัมพันธ์ทางพันธุกรรมระหว่างยีน 16S rRNA ของเชื้อแบคทีเรียจากบ่อน้ำพุร้อนในเขตภาคใต้ของประเทศไทย กับเชื้อแบคทีเรียอ้างอิงจากฐานข้อมูล Ribosomal Database Project (RDP) โดยใช้เทคนิค Neighbor joining ทำซ้ำ 1000 ครั้ง และใช้ *E. coli* เป็น out group (แบนในภาพสอดคล้องกับภาพที่ 2)



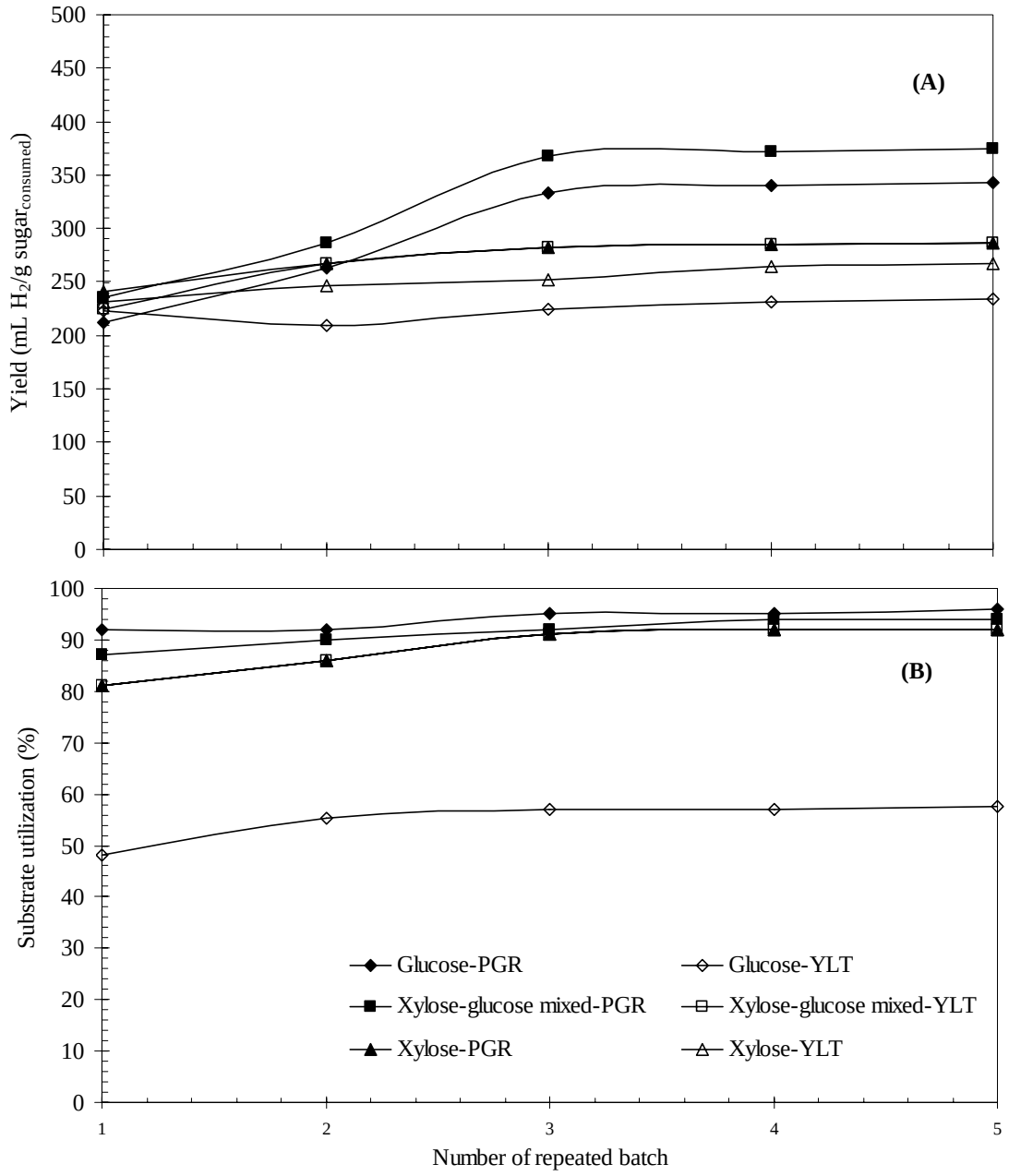
ภาพที่ 4 แสดงความสัมพันธ์ทางพันธุกรรมระหว่างยีน 16S rRNA ของเชื้ออาเคียยจากบ่อน้ำพุร้อนในเขตภาคใต้ของประเทศไทย กับเชื้ออาเคียอ้างอิงจากฐานข้อมูล Ribosomal Database Project (RDP) โดยใช้เทคนิค Neighbor joining ทำซ้ำ 1000 ครั้ง และใช้ *E. coli* เป็น out group (แบนในภาพสอดคล้องกับภาพที่ 2)

กลุ่มเชื้อจุลินทรีย์รหัส PGR และ YLT ผลิตไฮโดรเจนได้สูงใกล้เคียงกัน ถูกนำมาทดสอบความสเถียรในการผลิตไฮโดรเจนจากไซโลสผสมกลูโคสในระบบแบบสลับเป็นกะ พบว่ากลุ่มเชื้อจุลินทรีย์รหัส PGR สามรถผลิตไฮโดรเจนในระบบสลับเป็นกะได้ดีและมีผลผลิตสูงกว่ากลุ่มเชื้อจุลินทรีย์รหัส YLT ร้อยละ 30 กลุ่มเชื้อจุลินทรีย์รหัส PGR ผลิตไฮโดรเจนในระบบสลับเป็นกะได้ 375 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป ส่วนกลุ่มเชื้อจุลินทรีย์รหัส YLT ผลิตได้

287 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป (ภาพที่ 6) น้ำหมักไฮโดรเจนประกอบด้วยกรดบิวทิริก (20.6-21.8 มิลลิโมลาร์) กรดอะซิติก (7.2-13.5 มิลลิโมลาร์) กรดแลคติก (8.2-11.7 มิลลิโมลาร์) และบิวทานอล (4.4-13.0 มิลลิโมลาร์) โครงสร้างชุมชนแบคทีเรียที่พบในกลุ่มเชื้อจุลินทรีย์รหัส PGR ที่มีการเติมกลูโคส ไซโลส และกลูโคสผสมไซโลส เป็นแหล่งคาร์บอนในอาหารเลี้ยงเชื้อมีจุลินทรีย์ที่เด่นและพบจำนวนมาก ได้แก่ *Thermoanaerobacterium* sp. *Thermoanaerobacter* sp. *Caloramater* sp. และ *Anoxybacillus* sp. การผลิตไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมันด้วยกลุ่มเชื้อจุลินทรีย์รหัส PGR เชื้อจุลินทรีย์สามารถผลิตไฮโดรเจนได้สูงสุด 301 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไปที่ความเข้มข้นของไฮโดรไลเสตร้อยละ 40 ในขณะที่การผลิตไฮโดรเจนจะถูกยับยั้งเมื่อใช้ความเข้มข้นของไฮโดรไลเสต มากกว่าร้อยละ 80 กลุ่มเชื้อจุลินทรีย์รหัส PGRมีประสิทธิภาพในการผลิตไฮโดรเจนและมีศักยภาพที่ดีสำหรับการผลิตจากไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมัน



ภาพที่ 5 โครงสร้างประชากรแบคทีเรียจากกลุ่มเชื้อจุลินทรีย์ชอบร้อนเพิ่มจำนวนจากน้ำพุร้อนแหล่งต่าง ๆในเขตภาคใต้ของประเทศไทยด้วยน้ำตาลกลูโคส (G) ด้วยน้ำตาลไซโลส (X) ด้วยน้ำตาลไซโลสและกลูโคส (G+X) วิเคราะห์ด้วยเทคนิค denaturing gradient gel electrophoresis (DGGE) โดย A=RNW, B=SRW, C=SRR, D=CHL, E=PTK, F=PGK, G=PGR and H=YLT

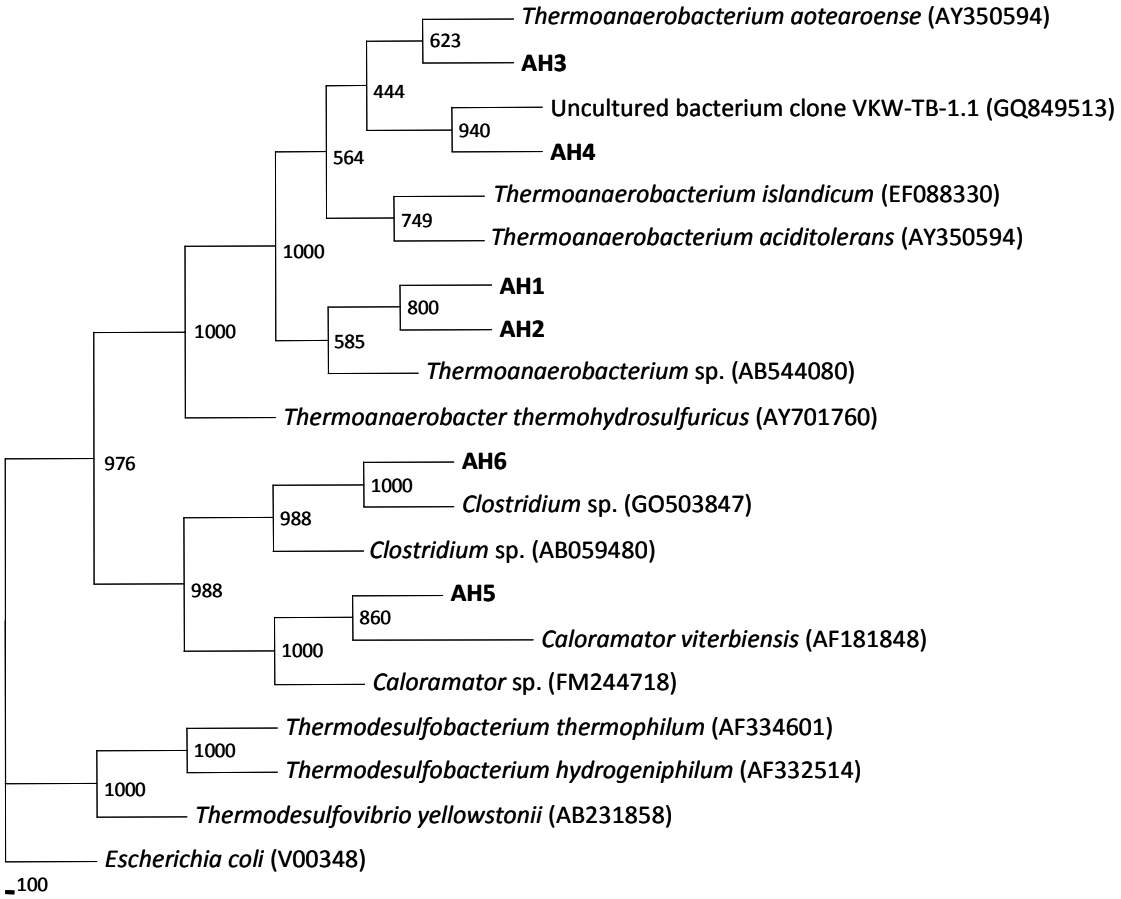


ภาพที่ 6 แสดงผลผลิตไฮโดรเจน (A) การใช้สับสเตรทโดยกลุ่มจุลินทรีย์ชอบร้อน PGR และ YLT ภายใต้การหมักแบบสลับเป็นกะ

2. แยกเชื้อจุลินทรีย์ชอบร้อนที่สามารถเปลี่ยนน้ำตาล เฮกโซส เพนโตส แป้งไปเป็นไฮโดรเจนและเอทานอล

ทำการแยกเชื้อแบคทีเรียผลิตไฮโดรเจนที่อุณหภูมิสูงจากกลุ่มเชื้อจุลินทรีย์รหัส PGR และ YLT สามารถแยกแบคทีเรียได้จำนวน 171 สายพันธุ์ คัดเลือกจากความเหมื่อนของยีน 16S rRNA ด้วยเทคนิค DGGE ได้ 6 สายพันธุ์ นำจุลินทรีย์ที่คัดเลือกได้ทั้ง 6 พันธุ์มาทดสอบ

ความสามารถในการผลิตไฮโดรเจน ประกอบด้วย 3 ขั้นตอน คือ ทดสอบการใช้ไซโลสเพื่อผลิตไฮโดรเจน ทดสอบการใช้ไซโลสผสมกลูโคสเพื่อการผลิตไฮโดรเจน และทดสอบการใช้ไฮโดรไลเสตจากลำต้นปาล์มเพื่อการผลิตไฮโดรเจน จุลินทรีย์ 6 สายพันธุ์ รหัส AH1-AH6 ผ่านการทดสอบดังกล่าวทั้งสามขั้นตอนเชื้อจุลินทรีย์ที่แยกได้ทั้ง 6 สายพันธุ์ ผลิตไฮโดรเจนได้จากทั้งน้ำตาลไซโลสและกลูโคส มีผลผลิตไฮโดรเจนเท่ากับ 278 292 280 310 150 และ 225 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาล ตามลำดับ การจัดจำแนกโดยการเทียบเคียงยีน 16S rRNA พบว่าสายพันธุ์ AH1 AH2 AH3 และ AH4 อยู่ในสกุล *Thermoanaerobacterium* ในขณะที่ AH5 อยู่ในสกุล *Caloramator* และ AH6 อยู่ในสกุล *Clostridium* (ภาพที่ 7) จุลินทรีย์ชอบร้อนทั้ง 6 สายพันธุ์สามารถผลิตไฮดรเจนจากน้ำตาลไซโลส และไฮโดรไลเสตจากลำต้นปาล์มที่ความเข้มข้นร้อยละ 50 ได้ทุกสายพันธุ์ สายพันธุ์ Thermoanaerobacterium AH4 สามารถผลิตไฮโดรเจนจากไฮโดรไลเสตจากลำต้นปาล์มที่ความเข้มข้นร้อยละ 50 ได้สูงที่สุดคือ 310 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาล (ตารางที่ 2)



ภาพที่ 7 แสดงความสัมพันธ์ทางพันธุกรรมระหว่างยีน 16S rRNA ของเชื้อจุลินทรีย์อ้างอิงจากฐานข้อมูล Ribosomal Database Project (RDP) กับเชื้อจุลินทรีย์ที่แยกได้ โดยใช้เทคนิค Neighbor joining ทำซ้ำ 1000 ครั้ง และใช้ *E. coli* เป็น out group

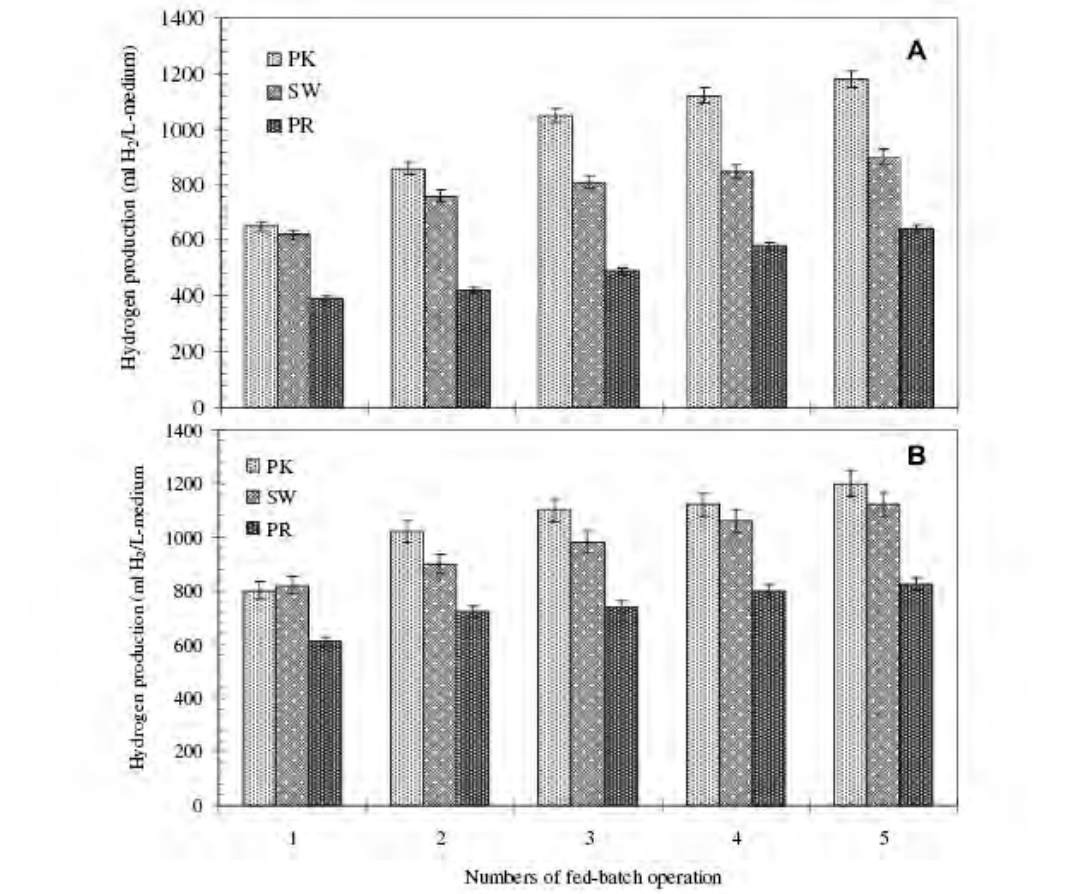
ตารางที่ 2 แสดงผลผลิตไฮโดรเจนของจุลินทรีย์ชอบร้อนทั้ง 6 สายพันธุ์ที่คัดเลือกได้ในอาหารเลี้ยงเชื้อที่เติมไซโลส และ เฮมิเซลลูโลสจากลำต้นปาล์มน้ำมัน

Strain	Classification	Pure xylose		Oil palm trunk hydrolysate	
		H_2 yield (mL H_2/g sugar)	Substrate utilization (%)	H_2 yield (mL H_2/g sugar)	Substrate utilization (%)
AH1	Thermoanaerobacterium sp.	260	100	278	91
AH2	Thermoanaerobacterium sp.	298	100	292	88
AH3	Thermoanaerobacterium sp.	310	100	280	92
AH4	Thermoanaerobacterium sp.	327	100	310	94
AH5	Caloramator sp.	180	100	150	68
AH6	Clostridium sp.	240	100	225	91

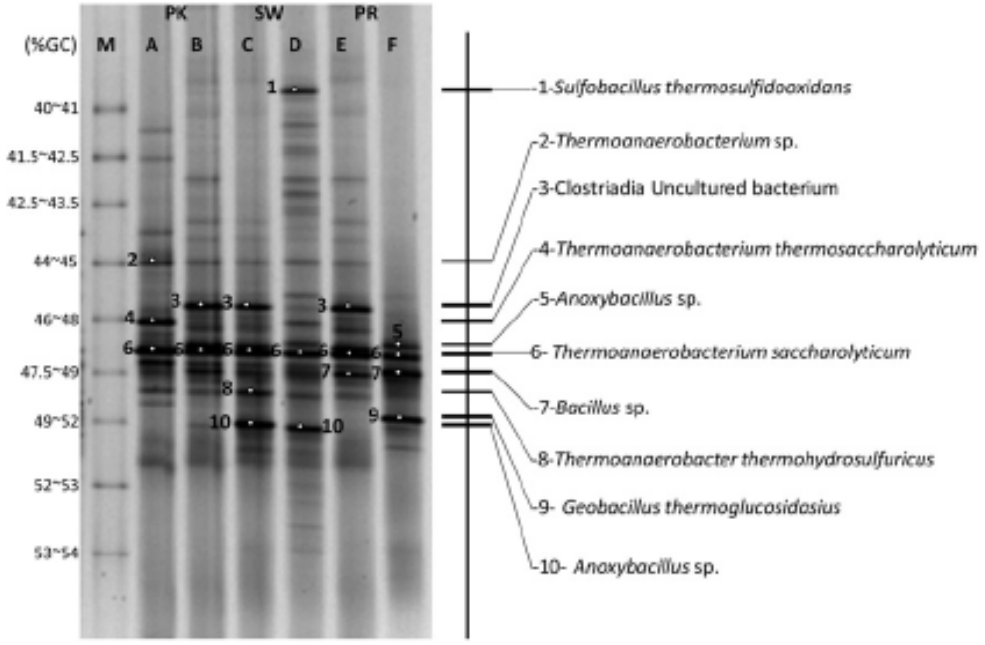
3. การผลิตไฮโดรเจนจากแป้งด้วยจุลินทรีย์ชอบร้อน

การเพิ่มจำนวนประชากรจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจน จากน้ำพุร้อนทั้ง 8 แหล่งโดยใช้แป้งมันสำปะหลังเป็นเป็นแหล่งคาร์บอน พบว่ากลุ่มเชื้อจุลินทรีย์ชอบร้อนที่สามารถผลิตไฮโดรเจนได้ดีคือ รหัส PGK SRW และ PGR สามารถในการผลิตไฮโดรเจนจากแป้งได้ 249.3 180 และ 124.9 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งดิบตามลำดับ และให้ผลผลิต 252.4 224.4 และ 165.4 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งสุกตามลำดับ (ภาพที่ 8) กลุ่มจุลินทรีย์ทั้งสามผลิตไฮโดรเจนพร้อมกับผลิตกรดอะซิตริก และเอทานอล หรือ กรดอะซิตริก และกรดแลกติก กลุ่มจุลินทรีย์ชอบร้อน PGK SRW และ PGR ผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งมันสำปะหลังได้สูงสุดคือ 287 264 และ 232 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งดิบในน้ำทิ้ง และสามารถผลิตได้เทียบค่าทางทฤษฎีเท่ากับ 53% 48.7% และ 42.8% ของค่าทางทฤษฎีการผลิตไฮโดรเจนจากแป้ง โครงสร้างประชากรแบคทีเรียของกลุ่มจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนพร้อมกับย่อยแป้งดิบในน้ำทิ้งโรงงานแป้งมันสำปะหลังเด่นด้วยแบคทีเรียในกลุ่ม *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum*, *Anoxybacillus* sp., *Geobacillus* sp. และ *Clostridium* sp. (ภาพที่ 9)

กลุ่มเชื้อจุลินทรีย์ PGK ได้นำไปทดสอบความสามารถในการผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งสาคู โดยศึกษาผลของพีเอช (5-8) ผลของความเข้มข้นของสับสเตรท (2.5-60 กรัมต่อลิตร) พบว่ากลุ่มเชื้อจุลินทรีย์ PGK สามารถผลิตไฮโดรเจนได้ความเข้มข้นร้อยละ 55 และไม่มีมีเทนในก๊าซชีวภาพ สภาวะที่ให้ผลผลิตสูงสุดคือ ที่พีเอช 6.5 ความเข้มข้นของแป้งสาคู 2.5 ให้ผลผลิตไฮโดรเจน 422 มิลลิลิตรไฮโดรเจนต่อกรัมแป้งสาคู โครงสร้างประชากรแบคทีเรียของกลุ่มจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งสาคูเด่นด้วยแบคทีเรียในกลุ่ม *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosulfurigenes*, และ uncultured *Thermoanaerobacterium* sp (ภาคผนวก 7)



ภาพที่ 8 แสดงผลผลิตไฮโดรเจนจากแป้งโดยกลุ่มเชื้อจุลินทรีย์ชอบร้อน PGK SRW PGR ในการเลี้ยงแบบสลับเป็นกะ



ภาพที่ 9 แสดงโครงสร้างประชากรแบคทีเรียของกลุ่มจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจนพร้อมกับย่อยแป้งดิบในน้ำทิ้งโรงงานแป้งมันสำปะหลัง

4. ความเป็นไปได้ทางเทคนิคในการขยายกำลังผลิตสู่ระดับเชิงพาณิชย์

จากการศึกษาความเป็นไปได้ทางเทคนิคในการขยายกำลังการผลิตสู่ระดับอุตสาหกรรม พบว่าจุลินทรีย์ที่แยกได้ Thermoanaerobacterium AH4 มีความสามารถในการผลิตไฮโดรเจนสูง โดยให้ผลผลิต 310 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาล จากการศึกษาสมดุลมวลของต้นปาล์ม พบว่าจากลำต้นปาล์มหนัก 1,680 กิโลกรัม สามารถคั้นน้ำได้ 252 ลิตร มีความเข้มข้นของน้ำตาล 82 กรัมต่อลิตร ผลิตเอทนอลด้วยเชื้อยีสต์ได้ 8.5 กิโลกรัมเอทานอลต่อต้น หรือ 10 ลิตรเอทานอลต่อต้น และได้นำกากของแข็งเหลือจากการคั้นไประเบิดด้วยไอน้ำเพื่อเตรียมเซลลูโลสได้เซลลูโลสปริมาณ 980 กิโลกรัมต่อต้น และได้เฮมิเซลลูโลสไฮโดรไลเสตซึ่งมีองค์ประกอบส่วนใหญ่เป็นน้ำตาลคาร์บอน 5 อะตอม อย่าง ไซโลส และ อะราบิโนสปริมาตร 700 ลิตรต่อต้น นำเฮมิเซลลูโลสไฮโดรไลเสตไปผลิตไฮโดรเจนด้วยกลุ่มเชื้อ Thermoanaerobacterium AH4 ได้ผลผลิตไฮโดรเจน 4650 มิลลิลิตรไฮโดรเจนต่อลิตร คิดเป็นผลผลิตไ ฮโดรเจนจากไฮโดรไลเสตทั้งหมดได้ 3255 ลิตรไฮโดรเจน ส่วนเซลลูโลสนำไปผลิตเอทานอลด้วยแบคทีเรียชอบร้อนได้ผลผลิต 0.3 กรัมเอทานอลต่อกรัมเซลลูโลส คิดเป็นผลผลิตเอทานอลจากเซลลูโลสทั้งหมดได้ 109 ลิตรเอทานอล จากการผลิตไฮโดรเจนและเอทานอลได้ไฮโดรเจน 3255 ลิตรไฮโดรเจนต่อต้น เอทานอล 119 ลิตรเอทานอลต่อต้น สามารถเก็บเกี่ยวพลังงานจากต้นปาล์มได้ ประมาณร้อยละ 30 ยังมีพลังงานเหลืออยู่ในน้ำทิ้งจากกระบวนการผลิตไฮโดรเจน กระบวนการผลิตเอทานอล และ เศษของแข็ง อีกร้อยละ 70 ซึ่งมีแนวโน้มที่จะนำไปผลิตมีเทนได้ กระบวนการผลิตไฮโดรเจนและเอทานอลได้พลังงานที่มีความคุ้มค่าแต่ต้องเพิ่มประสิทธิภาพของกระบวนการผลิตจึงจะสามารถนำไปผลิตเชิงพานิชย์ได้ และจะมีความคุ้มค่าทางด้านเศษฐศาสตร์สูงสุดเมื่อได้นำน้ำทิ้งจากกระบวนการผลิตไฮโดรเจน กระบวนการผลิตเอทานอล และ เศษของแข็ง อีกร้อยละ 70 ไปผลิตมีเทน

สรุปผลการวิจัย

1. กลุ่มประชากรแบคทีเรียที่พบเป็นจำนวนมากในตะกอนดินและน้ำจากบ่อน้ำพุร้อนประกอบด้วย *Clostridium* *Bacillus* และ *Pseudomonas* ส่วนแบคทีเรียชนิดอื่นๆที่ตรวจพบได้แก่ *Flavobacterium* *Thermodesulfovibrio* *Thermotoga* *Brevundimonas* และ *Micrococcus* ส่วนกลุ่มอาเคียประกอบด้วย *Thermoprotei* *Methanothermobacter* และสมาชิกในสกุล *Thermoproteaceae* และ *Methanosaeta*

2. ตะกอนดินและน้ำจากบ่อน้ำพุร้อนสามารถนำมาเพิ่มจำนวนประชาการแบคทีเรียที่สามารถผลิตก๊าซไฮโดรเจนได้ และตะกอนจากบ่อน้ำพุร้อนรหัส PGR และ YLT สามารถผลิตก๊าซไฮโดรเจนได้ปริมาตรสูงจากอาหารเลี้ยงเชื้อที่เติมกลูโคสผสมไซโลส (1506 มิลลิลิตรไฮโดรเจนต่อลิตรอาหาร หรือ 301.3 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป) และเติมไซโลส (1487.3 มิลลิลิตรไฮโดรเจนต่อลิตรอาหาร หรือ 297.4 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไป)

3. จุลินทรีย์ชอบร้อนที่สามารถผลิตไฮโดรเจนได้สูงพบในกลุ่มจุลินทรีย์ที่เพิ่มจำนวนจากตะกอนดินบ่อน้ำพุร้อนคือ*Thermoanaerobacterium* sp. และ *Caldicellulosiruptor* sp.

4. กลุ่มเชื้อจุลินทรีย์รหัส PGR สามารถผลิตไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมันได้สูงสุด 301 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาลที่ถูกใช้ไปที่ความเข้มข้นของไฮโดรไลเสตร้อยละ 40 ในขณะที่การผลิตไฮโดรเจนจะถูกยับยั้งเมื่อใช้ความเข้มข้นของไฮโดรไลเสตมากกว่าร้อยละ 80 กลุ่มเชื้อจุลินทรีย์รหัส PGR มีประสิทธิภาพในการผลิตไฮโดรเจนและมีศักยภาพที่ดีสำหรับการผลิตจากไฮโดรเจนจากไฮโดรไลเสตลำต้นปาล์มน้ำมัน

5. ได้จุลินทรีย์ชอบร้อน 6 สายพันธุ์ รหัส AH1-AH6 สามารถผลิตไฮโดรเจนได้จากทั้งน้ำตาลไซโลสและกลูโคส มีผลผลิตไฮโดรเจนเท่ากับ 278 292 280 310 150 และ 225 มิลลิลิตรไฮโดรเจนต่อกรัมน้ำตาล ตามลำดับ การจัดจำแนกโดยการเทียบเคียงยีน 16S rRNA พบว่าสายพันธุ์ AH1 AH2 AH3 และ AH4 อยู่ในสกุล *Thermoanaerobacterium* ในขณะที่ AH5 อยู่ในสกุล *Caloramator* และ AH6 อยู่ในสกุล *Clostridium*

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ภาคผนวก 1

Diversity and abundance of *Bacteria* and *Archaea* of plant biomass-rich sediment from hot spring in Southern Thailand

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Abstract

A sequence based survey of bacterial and archaeal diversity from eight hot spring locations in Southern Thailand was investigated. The temperature and pH of hot spring ranged between 53-75°C and 6.5-8.3, respectively. All hot springs contains plant biomass-rich sediment that is a carbon source of microorganisms in hot springs. Microbial community structure was investigated by polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences from DNA extracted from sediments of each hot spring, followed by PCR-DGGE and sequencing. Both bacterial and archaeal DNA was retrieved from all samples and several bacteria and archaea were growing in situ on plant biomass-rich sediment. Diversity and abundance of occurrence for 60 unique 16S rRNA gene phylotypes were obtained and most of bacteria and archaea particularly affiliating with deep-branching, but uncultivated organisms. Bacteria community developing on plant biomass-rich sediment was comprised of *Clostridium* spp. and *Pseudomonas* spp. constituted the major groups in these communities. Other bacteria detected in the biomass-rich sediment were *Flavobacterium* spp., *Thermotoga* spp., *Bacillus* spp., *Thermodesulfovibrio* spp., *Brevundimonas* spp. and *Micrococcus* spp. *Archaea* comprised of *Thermoprotei* spp., *Methanothermobacter* spp., members in family *Thermoproteaceae* and *Methanosaeta* spp.

Keywords: Geothermal spring, microbial diversity, molecular survey

Introduction

Thailand has about 90 hot springs with surface temperatures ranging from 40 to 100°C distributed across the country mainly in the northern and southern part (Chuaviroj, 1988). Southern part have at least 33 hot spring with surface temperature ranging from 40-80°C and pH from 6.5-8.5 (Hniman *et al*., 2010). These hot spring areas are evidently associated with forest and a lot of biomass fall into hot spring. The investigation of these hot springs has considerable biotechnological potential on the discovery of novel thermophiles coupled with the utilization of thermophiles and their enzymes for thermophilic hydrogen fermentation and other industrial purpose (Tirawongsaroj *et al*., 2008; Blumer-Schuette *et al*., 2008). Microbial communities associated with forest hot springs have attracted broad interest because of the unique thermophilic properties of the constituent organisms. Up to date, only a few published studies describe diversity of bacteria in Thailand's hot springs but no archaea communities were reported. Portillo *et al*. (2009) have characterized the microbial communities developing in different mat layers at Boekleung Hot Spring, Western Thailand. Bacterial communities were showing *Cyanobacteria* and *Chloroflexi* as the dominant species of the community and other significant members were Candidate Division *OP10*, *Bacterodetes*, *Planctomycetes* and *Actinobacteria*. Sompong *et al*. (2006) have characterized molecular diversity of cyanobacterial mats in different temperature regions from Ranong Hot Spring. *Mastigocladus*-like cyanobacteria were found at every temperature rang from 40-60°C and others species were found in most temperature ranges related to *Synechococcus*-like and *Oscillatoria*-like cyanobacteria.

However, little attention has been given to analysis hot spring communities as whole microbial ecosystems. The application of molecular phylogenetic techniques based

on 16S rRNA gene to study natural microbiotas in hot springs without the traditional requirement for cultivation has resulted in the discovery of abundance unique and numerous unexpected of prokaryotes (Ghosh *et al*., 2003**;** Kanokratana *et al*., 2004). 16S rRNA based techniques provided a valid approach to the assessment of true microbial community and the vast majority of this microbial community has proved obstinate to cultivation (Jeanthon, 2000**;** Zengler *et al*., 2002).

The most common and powerful 16S rRNA based techniques are denaturing gradient gel electrophoresis (DGGE) and clone library methods. Muyzer *et al*. (1993) were a developed DGGE method that has potential to study the microbial flora quickly. DGGE method has been applied to characterize bacterial community from many habitats such as hot spring (Hniman *et al*., 2010; Bakera *et al*., 2001**;** Aminin *et al*., 2008; Malkawi and Al-Omari, 2010; Löhr *et al*., 2006**)** and hot compost (Takaku *et al*., 2006; Blanc *et al*., 1999). Sequencing of the clone library generated from environmental DNA has advantages over DGGE, as it provides precise identification and quantification of the phylotypes present in samples. However, clone library approach can be laborious in producing a number of sequences large enough to cover a whole community and also limited by the difficulty to compare libraries and in determining if they are significantly different (Hur and Chun, 2004). Finally, based on results obtained from DGGE and clone library were applied to design a specific culture medium for isolation interested bacterial stains**.** This approach has allowed isolation of the novel species (Hetzer *et al*., 2008).

In these study report on a culture-independent survey of the prokaryotic community present in a hot spring in Southern Thailand. To our knowledge these hot spring has not been explore. The purpose of this study was to obtain information on the presence of prokaryotes valuable for biotechnology and to map the complexity of the prokaryotes population in hot spring. Bacteria and archaea diversity from eight hot spring

locations in Southern Thailand was analyzed by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE).

Materials and methods

Field locations and sampling

Nine sediments sampling hot spring sites located in Southern Thailand (**Fig. 1**) were sampled in April 2008. At all localities, the hot springs were associated with forest and a lot of biomass material such plant leaf, fronds, fruit faults in side hot spring. During collection and transportation, samples were stored on ice; upon arriving at the laboratory, the samples were stored at -20°C. Physico-chemical parameters known to have the greatest influence on thermophilic diversity (temperature, pH and hydrogen sulphide) were tested in the field when selecting sampling sites. In order to ensure, all samples were taken from within the known optimum temperature and pH range for thermophilic prokaryotic mat diversity using previously described protocols (Lau *et al*. 2006). Temperature and pH at each sampling location was recorded using a combined temperature/pH electrode (Orion, Boston, MA, USA) and the values obtained were shown in Table 1. Hydrogen methane and carbon dioxide were measured by gas chromatography (GC-8A Shimadzu) equipped with thermal conductivity detector (TCD) with a protocol according to Hniman *et al*. (2010) with a gas samples 100 µl for methane and 500 µl for hydrogen were injected in duplicate. The sediment rich samples were centrifuged at 10,000g for 10 min. Hydrogen sulphide was below the detection limit of 0.1 mg/l (as determined by methylene blue titration, HSWR, Hach, Loveland, CA, USA) at each location. Volatile fatty acids and ethanol in the supernatant were determined by gas chromatograph (HP6850) equipped with a flame ionization detector (FID) and Stabilwax-DA column (dimensions 30m x 0.32mm x 0.25um). The temperature of the injection port

was 230°C. The chromatography was performed using the following program: 70°C for 1 min, 70-180°C with a ramping of 20°C/min, 180°C for 6 min. The detector temperature was 250°C. Lactic acid, xylose and glucose were analyzed with a high performance liquid chromatograph (HPLC; Agilent 1200 series), equipped with Aminex® HPX-87H ion exclusion column.

DNA Isolation

Total genomic DNA was extracted from sediment-rich samples by using modified standard bacterial genomic DNA isolation method (Burrell *et al*., 1998). Pellet cells in enrichment culture were harvested in a micro-centrifuge tube by centrifugation at 10,000g for 5 min. The pellet cells were suspended in 500 µl of TENS buffer pH 8.0 (containing; 100 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl and 100 mM Sodium phosphate). After that, 40 µl of lysozyme (final conc. 3.7 mg/ml) was added and incubated for 1.5 hrs at 37°C with gentle manual mixing every 10 min. The mixtures were subjected to four cycle of freezing and thawing at 0°C (ice bath) and 65°C respectively, each step for 3 min. A 200 µl of 10% SDS and 50 µl of proteinase K (final conc. 1.2 mg/ml) were added and mixed by inversion tube by hand following incubated for 1.5 hrs at 60°C with gentle manual mixing every 10 min. Then, the DNA was recovered from the tube by phenol-chloroform extraction. The mixtures were added equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and mix gentle. The aqueous layer was selected by centrifugation at 10,000g for 10 min. The aqueous phase was transferred to new sterile tube and repeated extract with chloroform/isoamyl alcohol (24:1). After centrifugation at 10,000g for 10 min the crude DNA extract was precipitated with absolute ethanol for 2 hrs or overnight at -20°C followed by centrifugation at 12,000g for 10 min. The genomic DNA pellet was re-suspended in 30-50 µl of TE buffer and stored at

-20°C until needed. The genomic DNA was visualized by electrophoresis on 0.8% agarose gel stained with 0.5 μg/ml ethidium bromide.

PCR-DGGE analysis

The microbial community structure of enrichment culture was studied using PCR-DGGE as described by Prasertsan *et al*. (2009) with primers sequence shown in Table 2. Briefly, the bacterial 16S rDNA (1,400 bp) was amplified by the first polymerase chain reaction (PCR) with universal primer 1492r and 27f. Amplification mixtures were used TopTaq^{TM} Master Mix Kit (Qiagen) with a final volume of 25 μl. The mixtures contained 25 pmol of each primer, under conditions of an initial denaturation at 95.5°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 40 sec, extension at 72°C for 1 min, with final extension at 72°C for 10 min. The reactions were subsequently cooled to 4°C. The PCR product was analyzed on 1.0% agarose gels electrophoresis. In the second PCR, primer 518r and 357f with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from first PCR (Zhao *et al*., 2009). The PCR program were corresponded to an initial denaturation 95°C for 3 min follow by 30 cycles of three steps: 95°C for 1 min, 55°C for 30 sec, and 72°C for 1 min and final extension at 72°C for 10 min. PCR products were stored at 4°C and analyzed on 1.0% agarose before DGGE.

Amplification of Archaea 16SrDNA sequences was carried out using Archaea-specific primers (Table 2). The majority of the Archaea 16S rDNA fragment was amplified using Arch958r and Arch21f primers. The amplification mixture was carried out using the same mix as for bacteria. PCR began with a 94°C denaturation for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, extension at 72°C for 1 min, with final extension at 72°C for 10 min. The 16SrDNA PCR

product was used as template for next PCR. The V3 region fragment was amplified with the PARCH519r and PARCH340f-GC primers and carried out with the same mixture as above. The amplification condition started with an initial denaturation 94°C for 3 min follow by 34 cycles of three steps: 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min and final extension at 72°C for 10 min. PCR products were stored at 4°C and analyzed on 1.0% agarose before DGGE.

The DGGE analysis of PCR products obtained from the second PCR were performed using the DGGE unit, V20-HCDC (Scie-Plas limited, UK) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30-60% and 40-80% for bacteria and archaea respectively. DGGE gels were stained with Sybr-Gold for 60 min and photographed on Gel DocXR system (Bio-Rad Laboratories) and the bands in the gel were excised. The DNA in the excised gel slices were incubated in 20 µl of distilled water at 4°C for 24 hrs and re-amplified by PCR with the second PCR primers. The PCR products were separated again by DGGE, and then the bands were excised again. Most of the bands were excised from the gel and re-amplified with primer 357f (without a GC clamp) and the reverse primer. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer 518r (bacteria) and PARCH519r (archaea) by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

Phylogenetic analysis and statistics

16S rRNA gene sequences were identified and determined their approximate phylogeny by ribosomal database project (http://rdp.cme.msu.edu/) with SeqMatch program and NCBI web interface (http://www.ncbi.nlm.nih.gov).

All DGGE band sequences were aligned with closely related sequences identified from the SeqMatch search using ClustalX version 2.0 (Larkin *et al*., 2007). Phylogenetic

tree were constructed with bootstrap N-J tree with 1000 bootstrap replicates to determine the robustness of resulting tree topologies and opened with TreeViewX program.

Results and Discussion

Chemistry of Southern Thailand hot spring

To provide a chemical context for interpretation of the results of microbiological studies, we conducted chemical analyses of selected hot springs in geologically distinct areas of Southern Thailand with evidence of significant plant biomass-rich sediment (**Fig. 1)**. The results of these analyses and other available data are presented in **Tables 1.** Collectively, the sites analyzed are representative of geothermal springs worldwide. The temperature and pH of hot spring ranged between 53-75°C and 6.5-8.3, respectively. All hot springs contains plant biomass-rich sediment that is a carbon source of microorganisms in hot springs. Hot springs in Ranong Province (RNW), for instance, contain little sulfide and tend toward alkalinity (pH 8.3), with high concentrations of dissolve organic carbon (DOC). Hot springs in Suratthani Province (SRW and SRR), Phang Nga Province (PGK and PGR) and Phatthalung Province (PTK) contain relatively high concentrations of sulfides and neutral pH (6.5-7). Hot springs in Yala Province (YLT) and Chumpron Province (CHL) contain relatively high concentrations of dissolve organic carbon and low concentration of sulfides and neutral pH (7.8). Concentrations of potential carbon sources (DOC) are highly variable in all hot springs

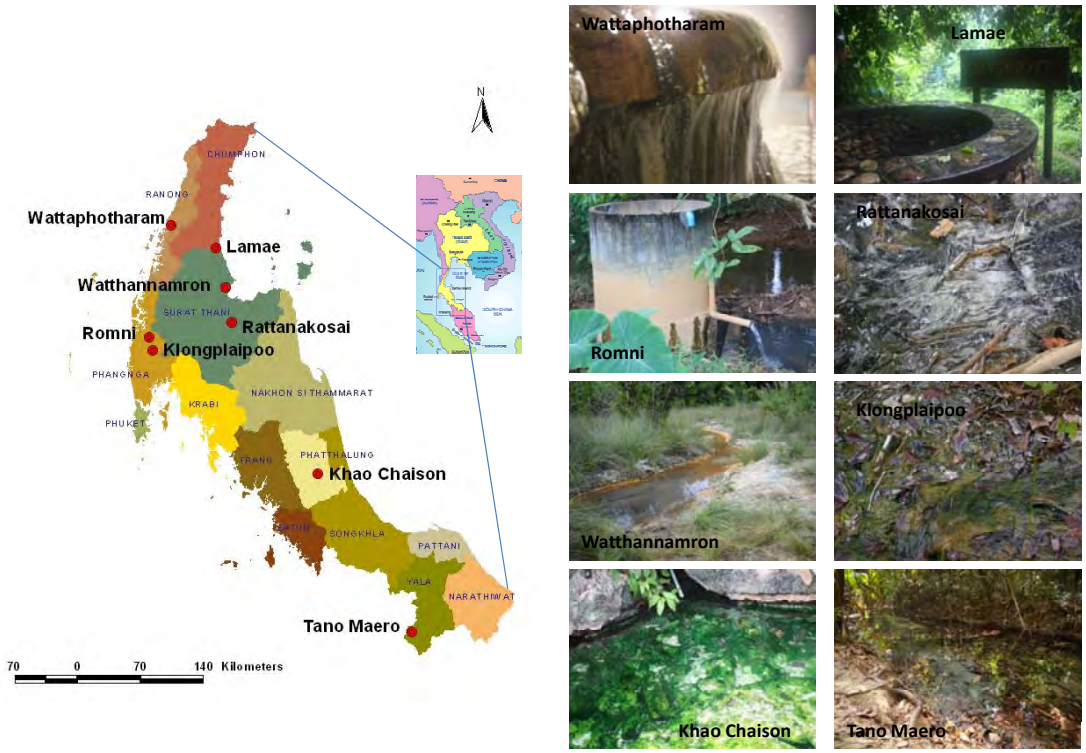

Fig. 1. Site locations map of Southern Thailand hot spring of sediment rich samples site indicated by red circle

Bacterial community

A sequence based survey of bacterial diversity from eight hot spring locations in Southern Thailand was investigated. Microbial community structure was investigated by polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences from DNA extracted from sediments of each hot spring, followed by PCR-DGGE and sequencing. Several bacteria were growing in situ on plant biomass-rich sediment. Diversity and abundance of occurrence for 60 unique 16S rRNA gene phylotypes were obtained and most of bacteria particularly affiliating with deep-branching, but uncultivated organisms. Bacteria community developing on plant biomass-rich sediment was comprised of *Clostridium* spp. *Bacillus* spp., and *Pseudomonas* spp. constituted the major groups in

these communities. Other bacteria detected in the biomass-rich sediment were *Flavobacterium* spp., *Thermotoga* spp., *Thermodesulfovibrio* spp., *Brevundimonas* spp. and *Micrococcus* spp.. PCR-DGGE analysis of 16S rRNA genes in plant biomass-rich sediment of Southern Thailand hot springs showed a diversity of bacteria (**Fig. 2 and Fig. 3**). Bacteria detect in plant biomass-rich sediment from RNW hot spring represented genus *Clostridium* spp., those from SRW and CHL hot spring represented genus *Pseudomonas* spp. and *Fervidobacterium* spp., those from SRB and PTK hot spring represented genus *Pseudomonas* spp. and and Bacillus spp., those from PGK hot spring represented genus *Clostridium* spp. and *Thermodesulfovibrio* spp., those from PGR hot spring represented genus *Clostridium* spp. and *Bacillus* spp., and those from YLT hot spring represented genus *Flavobacterium* spp. Bacteria of the genus *Fervidobacterium* are known to be able to degrade proteins (Kublanov *et al*., 2009) that found dominant in SRW hot spring.

To determine the phylogenetic types of organisms present, we compared the sequences to sequences of known organisms in public databases. We also compared the compositions of the communities. Although the detail of compositions varied, all of the communities contained sequences representative of the same phylogenetic groups. Samples obtained on artificial growth surfaces generally overlapped with the environmental sediment samples. **Fig. 3** summarizes the census results. The phylogenetic distribution of rRNA genes amplified with the universal PCR primers provides some perspective on the overall microbial composition of the Southern Thailand geothermal ecosystem. **Fig. 4** shows the main phylogenetic groups of bacteria identified in springs. Sequences representative of *Clostridium* spp. and *Pseudomonas* spp. were most abundant in the communities, and sequences representative of *Fervidobacterium* spp., *Thermodesulfovibrio* spp., *Bacillus* spp. and *Flavobacterium* spp. also were common.

Comparison of community compositions in hot spring with other reports (**Table 2**) indicates that organisms recognized for biomass degradation such as *Bacillus* and *Clostridium* were commonly found.

Archaea community

DGGE with archaeal primers revealed the presence of noncultivated archaea in all hot spring samples. Communities were dominated by bacterial rRNA genes. *Archaea* comprised of *Thermoprotei* spp. and *Methanothermobacter* spp., members in family *Thermoproteaceae* and *Methanosaeta* spp. Organisms present in RNW hot spring was belong to *Methanothermobacter* spp. to which many uncultured organisms from Yellowstone, Iceland, and Kamchatka hot springs were found to belong (Kublanov *et al*., 2009). Archaea are considered common in geothermal and other ‘‘extreme’’ environments, but these and all previous surveys indicate that such organisms are not more abundant than bacteria (Hugenholtz et al., 1998). Most of the archaeal sequences encountered were related to environmental crenarchaeote sequences previously observed in Bor Khlueng Hot spring (Kanokratana et al., 2004), none with a specific relationship to a cultured organism (**Fig. 5**). **Fig. 6** shows the main phylogenetic groups of archaea identified in springs with eight plant biomass-rich sediment. Although several hundred unique bacterial sequences were determined, these fell into only a few phylogenetic groups. Sequences representative of *Thermoprotei* spp. and *Methanobacteria* spp. were most abundant in the communities. Fig.7 shows the most of unclassified and uncultured archaeaon belong to class Thermoprotei whereas Methanobacteria were belonged to Methanobrevibater sp. and Methanothermobacter sp..

Acknowledgments

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Table 1. Water chemistry of Southern Thailand hot spring

Geothermal spring	Code	Temp (°C)	pH	Sulfide (mg/l)	Sulfate (mg/l)	DOC (mg/l)	DIC (mg/l)	NH^{+4}-N (mg/l)
Ranong province								
Wattaphotharam	RNW	65	8.3	0.6	1.3	523	121	12
Suratthani province								
Wat Than Nam Ron	SRW	63	7.0	1.5	25	12	128	0
Rattanakosai	SRR	67	7.9	10	70	34	135	0.6
Chumporn Province								
Lamae	CHL	53	7.8	0	0.2	342	78	1.2
Phatthalung Province								
Khao Chai Son	PTK	60	7.9	12	30	54	98	0.2
Phang Nga province								
Khong Pay Pao	PGK	60	6.5	0.7	1.5	44	112	0.5
Romanee	PGR	63	6.8	1.1	4	123	124	0.8
Yala province								
Ta Na Ma Rao	YLT	80	7.8	0	0.3	287	88	1.1

Table 2. Microbial community analysis of high temperature environmental samples based on 16S-rDNA approaches

Samples and sources	T (°C), pH	Dominant species	Techniques	References
Sediment-rich water, Domas, Hot springs, Indonesia	82-92°C, pH 2	Bacteria: *Flavobacterium*, *Preteobacteria γ sub-division*, *Bacillus*	Clone library	Baker *et al*., 2001
Microbail mats, Boekeung Hot spring, Ratchaburi Province, Thailand	50-57°C, pH 6.8	Bacteria: *Cyanobacteria*, *Chloroflexi*, Candidate Division *OP10*, *Bacteriodetes*, *Planctomycetes*, *Actinobacteria*	Clone library	Portillo *et al*., 2009
Microbial Mats, Hot spring , central Tibet	60-65°C, pH 7-7.4	Bacteria: *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Proteobacteria*	Clone library	Lau *et al*., 2009
Water, Acidic volcanic lake, Indonesia	19.7-24°C, pH 0.3-7.62	Bacteria: *Gammaproteobacteria*, *Firmicutes*, *Betaproteobacteria*, *Flavobacteria* Archaea: *Methanomicrobacteria*, *Methanococcales*, *Thermococcales*,	PCR-DGGE	Lohr *et al*., 2006
Water, Gedongsongo Hot spring, Indonesia	50°C, pH 4	Bacteria: *Betaproteobacteria*, *Firmicutes*	PCR-DGGE	Aminin *et al*., 2008
Volcanic environments of Canary Islands, Spain	85°C	Bacteria: *Deltaproteobacteria*, *Bacteroidetes*, *Propionibacterium*, *Pseudomonas*	PCR-DGGE	Portillo and Gonzalez, 2008
Water and Mats, Hot springs, Jordan	47-63°C	Bacteria: *Bacillus* sp.	Specific-PCR	Malkawi and Al-Omari, 2010
Water and sediment, Boiling Spring Lake, Lassen Volcanic National Park, northern California, USA	55°C, pH 2	Bacteria: *Thermoprotei*, *Clostridia*, *Thermoplasmata*, *Aquificae*, *Bacilli*, *Gammaproteobacteria*, *Betaproteobacteria*, *Thermotogales*, *Actinobacteria*	T-RFLP	Wilson *et al*., 2008

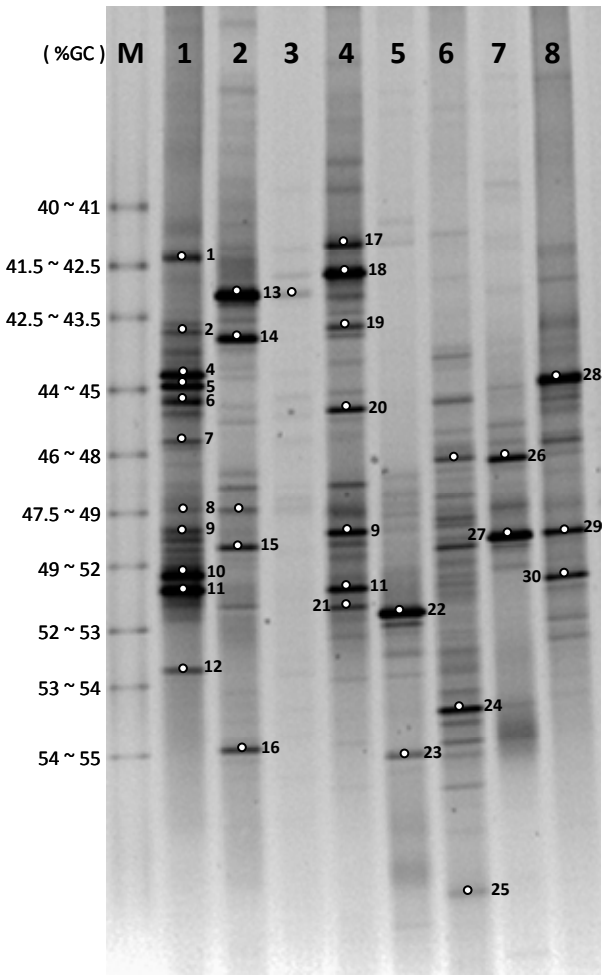

Fig. 2 Bacteria community profile determined with PCR-DGGE of partial 16S rRNA genes fragments from original hot spring. Lanes: M, DGGE marker II; 1, Wat Potharam ; 2, Wat Than Nam Ron; 3, Lamae; 4, Ban Tha Sathon; 5, Chao chaison; 6, Khog Pay Pao; 7, Romanee; 8, Ta Na Ma Rao.

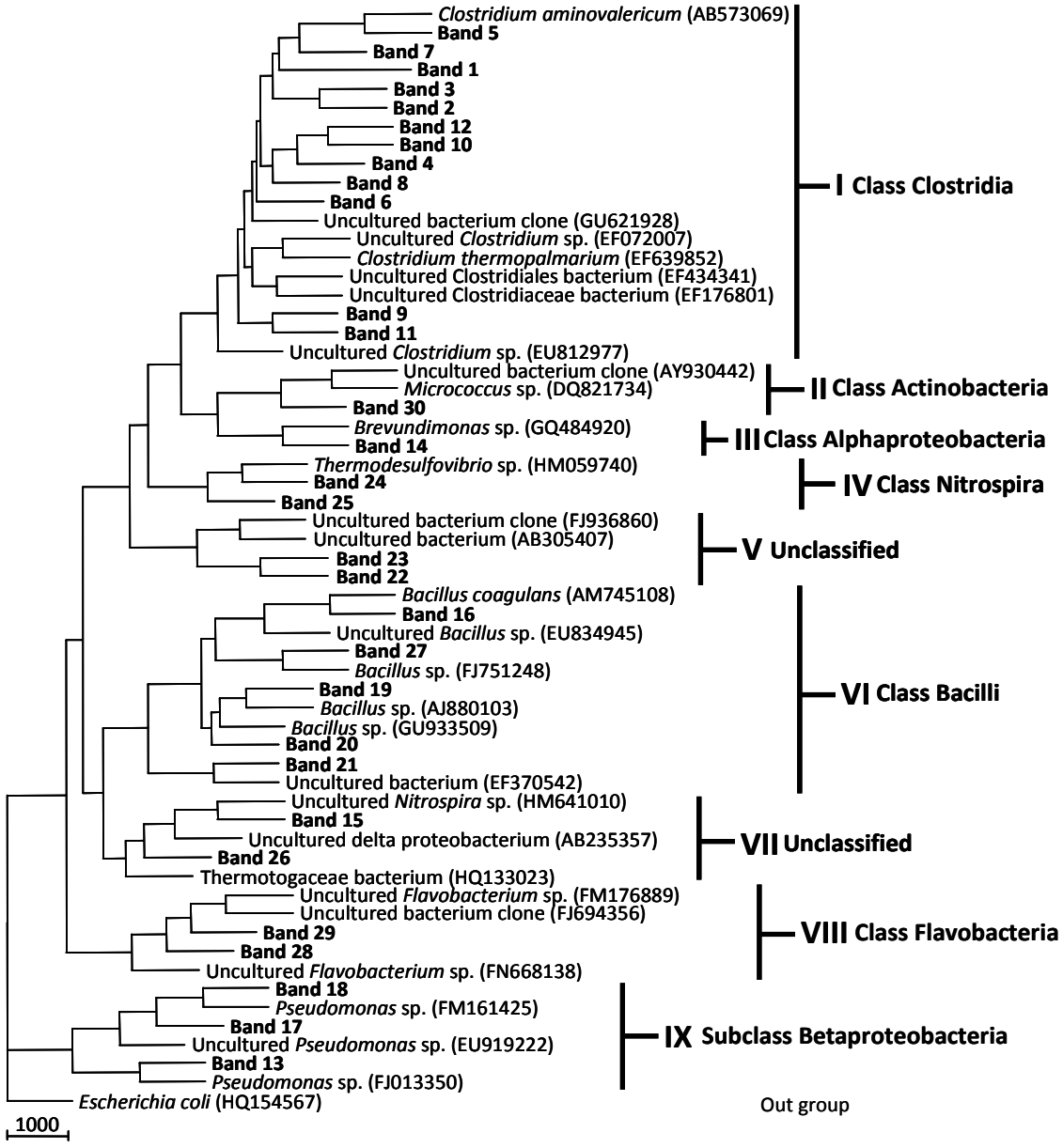

Fig. 3 Phylogenetic tree of bacteria consortium from original hot springs samples

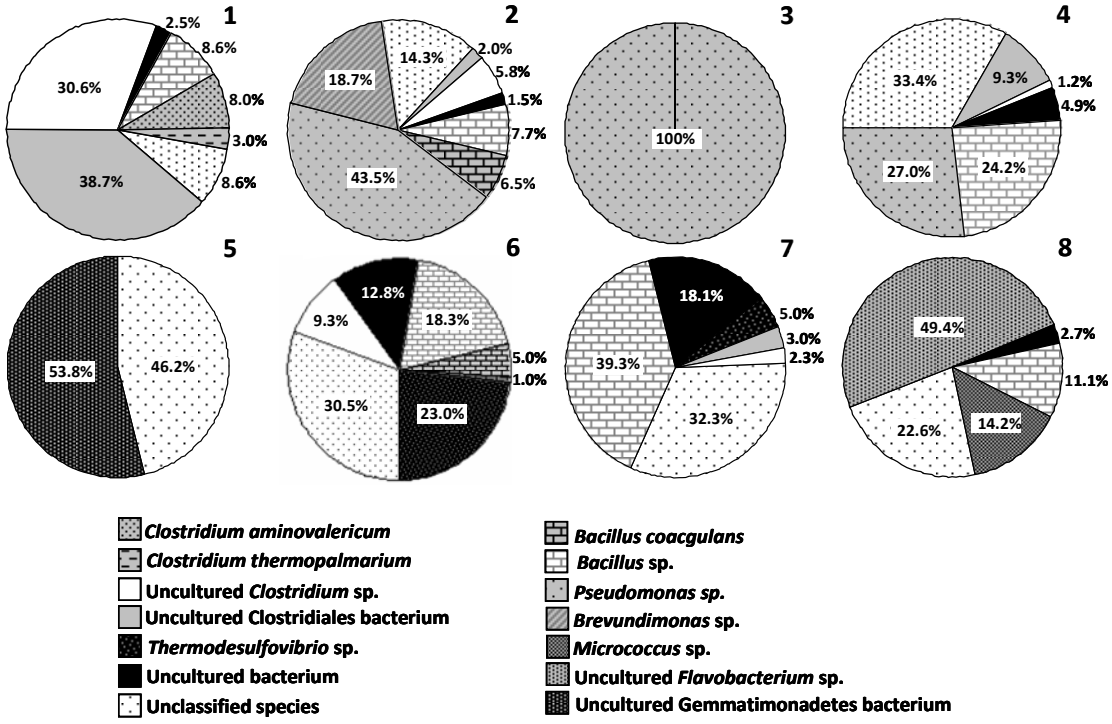

Fig. 4 Shows the main phylogenetic groups of bacteria identified in springs; 1, Wat Potharam ; 2, Wat Than Nam Ron; 3, Lamae; 4, Ban Tha Sathon ; 5, Chao chaison; 6, Khog Pay Pao; 7, Romanee; 8, Ta Na Ma Rao.

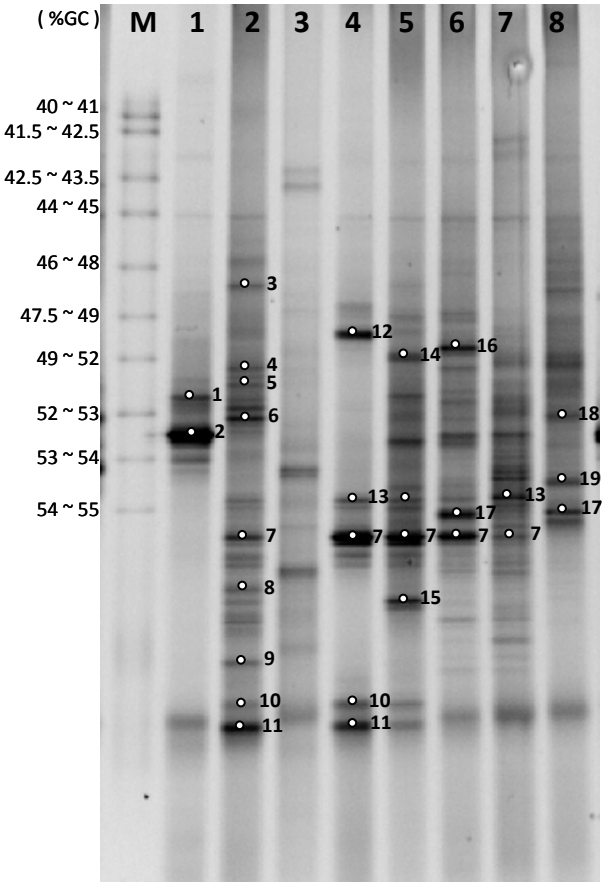

Fig. 5 Archaea community profile determined with PCR-DGGE of partial 16S rRNA genes fragments from original hot spring. Lanes: M, DGGE marker II; 1, Wat Potharam ; 2, Wat Than Nam Ron; 3, Lamae; 4, Ban Tha Sathon; 5, Chao chaison; 6, Khog Pay Pao; 7, Romanee; 8, Ta Na Ma Rao.

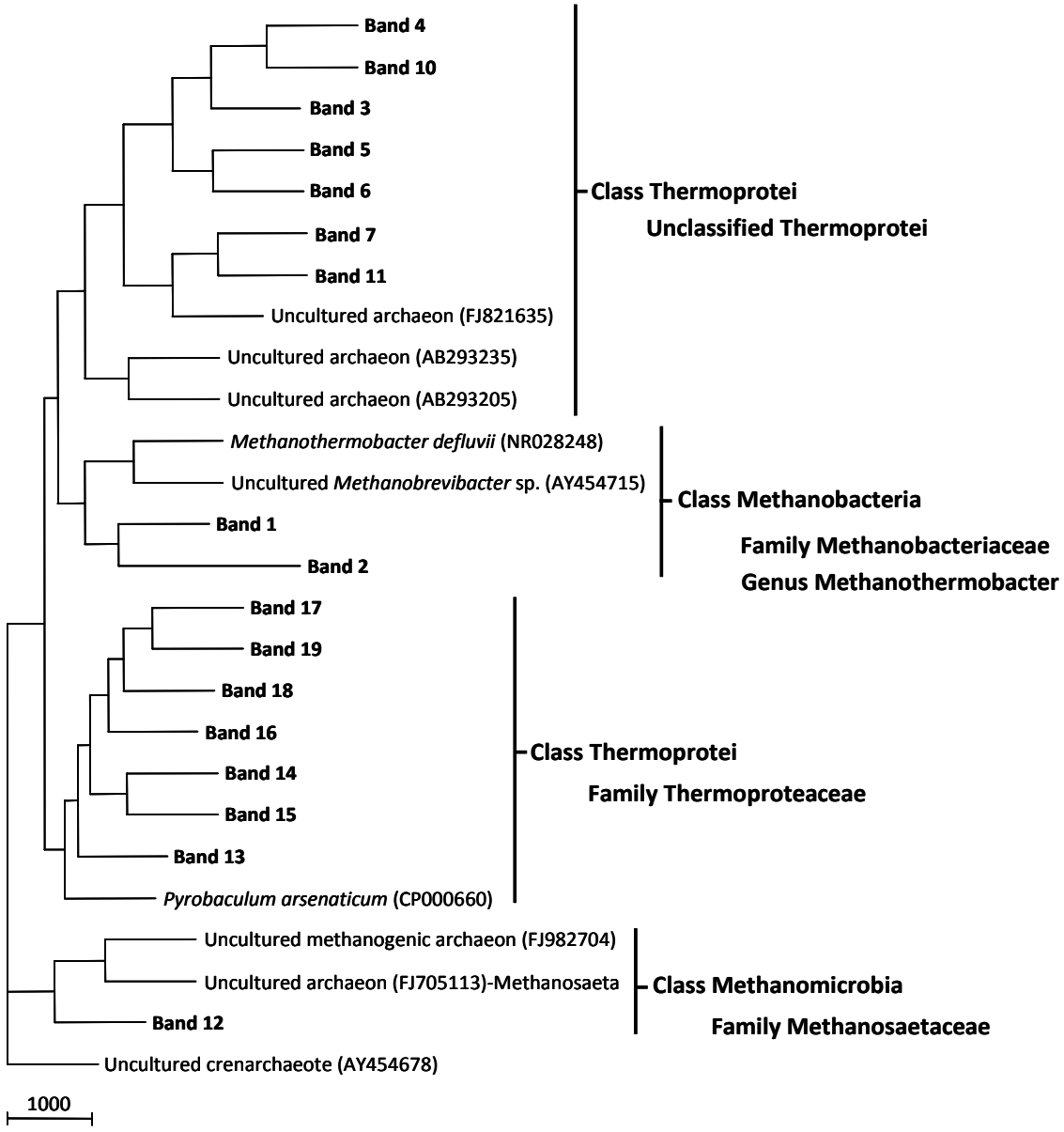

Fig. 6 Phylogenetic tree of archaea consortium from original hot springs samples

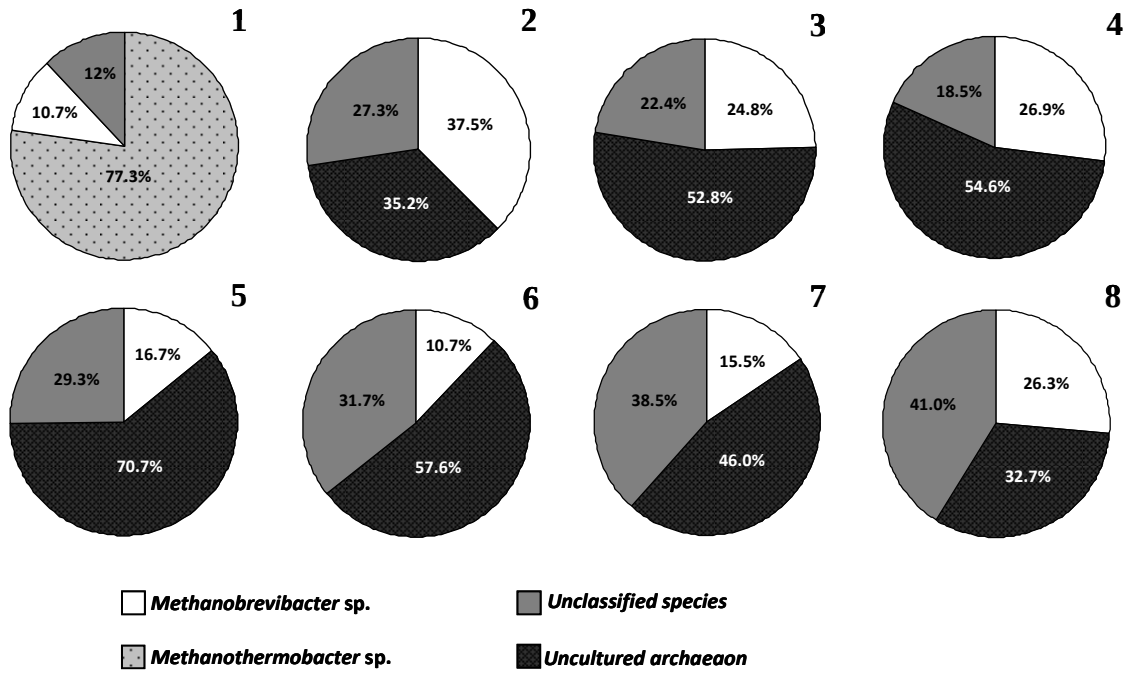

Fig. 7 Shows the main phylogenetic groups of archaea identified in hot springs; 1, Wat Potharam ; 2, Wat Than Nam Ron; 3, Lamae; 4, Ban Tha Sathon ; 5, Chao chaison; 6, Khog Pay Pao; 7, Romanee; 8, Ta Na Ma Rao.

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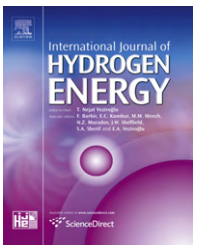

Highlights

► Bioprospecting thermophilic microorganisms from Thailand hot springs. ► It efficient to utilized xylose–glucose mixed substrate. ► Enriched cultures from hot spring yielded hydrogen of 301.3 mL H_2/g sugar. ► It dominated by *Thermoanaerobacterium* sp. and *Caldicellulosiruptor* sp. ► It shows promising to apply for hydrogen production from lignocellulosic hydrolysate.

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Community analysis of thermophilic hydrogen-producing consortia enriched from Thailand hot spring with mixed xylose and glucose

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ABSTRACT

Sediment samples from hot spring were enriched for hydrogen-producing bacteria with xylose, glucose and mixed of both sugars at high temperature (60 °C). Enriched cultures encoded as PGR and YLT showed higher cumulative hydrogen production in xylose–glucose mixed and xylose than glucose with hydrogen evolution of 1506 and 1487 mL H_2/L-medium and hydrogen yields of 301.3 and 297.4 mL H_2/g $sugar_{consumed}$, respectively. The enriched cultures coded SRR2, PGK, PGR and YLT were produced acetate and butyrate as main soluble metabolites with high hydrogen production. PCR-DGGE profiling showed that the enriched cultures gave best hydrogen production from xylose–glucose mixed comprised of three groups of hydrogen-producing species, (A) relating to genus *Bacillus* sp. and *Anoxybacillus* sp., (B) relating to genus *Caloramator* sp. and *Clostridium* sp. and (C) relating to efficient hydrogen-producing species related to *Thermoanaerobacterium* sp. and *Caldicellulosiruptor* sp. These microbial consortia developed show promising to apply for biohydrogen production from lignocellulosic hydrolysate containing xylose–glucose mixed.

1. Introduction

The challenge of biohydrogen process is the feasibility to produce at a commercial scale with a low cost to meet the need of sufficient and cost-effective energy supply. Consequently, the substrate used for fermentative hydrogen production must be abundant, easily available, and inexpensive. From these aspects, lignocellulosic materials obtained from crop or food industrial wastes could be a commercially viable biohydrogen feedstock [1]. Crop wastes such as straw, bagasse, oil palm trunks felled for replanting and palm empty fruit brunch are promising base substrates for cost-effective bioenergy production [2,3]. Basically, lignocellulose contains a large amount of cellulose (40–50%), hemicellulose (25–30%), and lignin (10–20%) [4]. Pre-treatment of lignocellulosic materials generates hemicellulose hydrolysate containing two main sugars of xylose and glucose. Pentose sugar xylose accounts for up to 35–45% of the total sugars in the lignocellulosic hydrolysate derived from wood, agricultural by-products or crops [5]. Microbial fermentation of xylose derived from such wastes is quite promising for combining energy extraction and waste reduction. However, bioconversion of pentoses (xylose) to bioethanol is not very effective in terms of ethanol yield and production rate [2]. Due to this limitation, an alternative

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utilization of xylose needs to be investigated to increase the economical feasibility of the biofuels production from lignocellulosic material. Therefore, utilization of the hydrolysate as a substrate for biohydrogen production is attractive.

It has been shown that xylose could be successfully converted to hydrogen by extreme thermophilic *Caldicellulosiruptor saccharolyticus* achieving a relatively high yield of 333.4 mL H_2/g xylose [6]. A thermophilic strain of *Thermoanaerobacterium thermosaccharolyticum* strain PSU-2, W16 and extreme thermopiles *C. saccharolyticus* have been reported to utilize mixed sugars (glucose and xylose) simultaneously to generate hydrogen with a hydrogen yield 315, 305–323 and 295 mL H_2/g sugar, respectively [7–9]. Natural dark fermentative inoculum using mixed consortia edges over pure inoculum in allowing process at non-sterile environment and capable of dealing with mixture of substrate due to a high microbial diversity [10,11], which significantly reduce the process operation cost with low biomass yield. Thermophilic mixed cultures have been reported to utilize mixed sugars (glucose and xylose) simultaneously to generate hydrogen with a hydrogen yield 218 mL H_2/g sugar [12]. Thermophilic mixed cultures that were enriched from sediment-rich samples of geothermal spring in Southern Thailand with mixed sugars under acidic conditions produced a significant amount of hydrogen gas [13]. High yield of hydrogen was observed in mixed sugars medium (375 mL H_2/g-sugar $_{consumed}$) and oil palm trunk hysrolysate (301 mL H_2/g-sugar $_{consumed}$) [13]. However, the problem of fermentation of lignocellulosic material-based substrate process is the inability of microorganisms to metabolize efficiently hemicellulose hydrolysate which contains mixed hexose, pentose and toxic compounds such as phenolics, furfurals and 5-hydroxymethyl-2-furaldehyde (HMF) into hydrogen with satisfied yield and productivity.

Although product formation by thermophilic mixed cultures is strongly affected by the specific characteristics of the feed substances and operational conditions such as pH, temperature, redox potential and available trace minerals [14]. Furthermore, it is also not clear whether a change in product formation by anaerobic microflora is caused by a metabolic change within a particular microbial population, by a change in the microflora population itself or a change in both. Knowledge of the microbial composition of the major hydrogen-producing microorganisms would result in efficient and optimal operation of fermentative hydrogen-producing systems (effective control of the start-up and operation) [15]. The comparative microbial community analysis will provides an accelerated approach to understanding community structure and function. The identification of unique or numerically dominant strains or groups under defined or controlled conditions is also possible. Therefore, tools for identification of the microbes present in the hydrogen production process are necessary. It was recommended that the step to overcome such instability and to provide high hydrogen-production efficiency, the insight into the hydrogen fermentation microbiology and factors involved in the stabilization/destabilization of the process should be further investigated [16,17].

Hot springs are a potential source for thermophilic hydrogen-producing microorganisms. In this study, changes in fermentation pattern of glucose and xylose mixed substrate as the model hemicellulose hydrolysate by thermophilic mixed cultures were investigated with respect to product formation and bacterial community structure during hydrogen production. Parallel to observations of fermentation behaviour, microbial community structure in mixed cultures was investigated using PCR-DGGE analysis targeted on bacterial 16S rDNA. The PCR-DGGE data were subjected to statistic analysis in order to evaluate hydrogen production performance relative to microbial community structure.

2. Materials and methods

2.1. Geothermal spring sampling

Sediment-rich water samples were collected from eight geothermal springs located in Southern Thailand (Table 1). Temperature and pH of samples were measured in the fields using thermometer and pH paper. The samples were kept in serum bottles for return to the laboratory.

2.2. Enrichment culture

Enriched hydrogen-producing cultures were developed as previously reported by O-Thong et al. [18]. At each site, 3 mL of sediment-rich water was transferred by sterile syringe into 27 mL basic anaerobic medium (BA medium, pH 5.5) supplemented with 5 g/L each of xylose, glucose and the mixture of these sugars in the ratio 1:1 [19] as a carbon source and incubated at 60 °C for 2 day at strict anaerobic condition. During the enrichment, hydrogen, carbon dioxide and methane gas and soluble metabolites were monitored. The cultures resulting from these initial cultivations were used in five successive batch cycles (10% inoculum in the medium, 2 days per cycle). The final cultures (5th batch cycle) were used for analysis hydrogen production, soluble metabolite production and responsible microbial community.

2.3. Product analysis

The evolved biogas was collected in a headspace of serum bottle. The total volumes at each time interval were measured at room temperature by syringe. Hydrogen, carbon dioxide and

Table 1 – The list of geothermal spring sources and characteristics used for enrichment culture.

Geothermal springx	Code	Temperature (°C)	pH
Ranong province			
Wat Potharam	RNW	65	8.3
Suratthani province			
Wat Than Nam Ron 1	SRW1	63	7.0
Wat Than Nam Ron 2	SRW2	53	7.8
Ban Tha Sathon 1	SRR1	67	7.9
Ban Tha Sathon 2	SRR2	60	7.9
Phangnga province			
Khong Pay Pao	PGK	60	6.5
Romanee	PGR	63	6.8
Yala province			
Ta Na Ma Rao	YLT	80	7.8

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methane in the biogas were measured. The biogas composition was confirmed by gas chromatography (GC-8A Shimadzu) equipped with thermal conductivity detector (TCD) and a 2.0-m packed column (ShinCarbon ST 100/120, Restek) and argon at a flow rate of 15 mL/min as the carrier gas. The culture broth was centrifuged at 10,000×*g* for 10 min. Fermentation end products (volatile fatty acids and ethanol) in the supernatant were determined by GC (HP6850, Hewlett Packard) equipped with a flame ionization detector (FID) and Stabilwax-DA column (dimensions 30 m × 0.32 mm × 0.25 μm). The temperature of the injection port was 230 °C. The chromatography was performed using the following program: 70 °C for 1 min, 70–180 °C with a ramping of 20 °C/min, 180 °C for 6 min. The detector temperature was 250 °C. Lactic acid, xylose and glucose were analyzed with a high performance liquid chromatograph (HPLC; Agilent 1200 series), equipped with Aminex® HPX-87H ion exclusion column.

2.4. *DNA extraction*

Total genomic DNA was extracted from enrichment culture samples by using a slightly modified standard bacterial genomic DNA isolation method [20]. Pellet cells in enrichment culture were harvested in micro-centrifuge tubes by centrifugation at 10,000*g* for 10 min. The pellet cells were suspended in 500 μl of TENS buffer pH 8.0 (containing; 100 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl and 100 mM Na_3PO_4). After that, 40 μl of lysozyme (final conc. 3.7 mg/mL) was added and incubated for 1.5 h at 37 °C with gentle manual mixing every 10 min. The mixtures were subjected to four cycles of freezing and thawing at 0 °C (ice bath) and 65 °C respectively, each step for 3 min. A 200 μl of 10% sodium dodecyl sulfate (SDS) and 50 μl of proteinase K (final conc. 1.2 mg/mL) were added and mixed by inverting the tubes by hand following incubation for 1.5 h at 60 °C with gentle manual mixing every 10 min. Then, the DNA was recovered from the tube by phenol–chloroform extraction. The mixtures were added equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and mix gently. The aqueous layer was selected by centrifugation at 10,000×*g* for 10 min. The aqueous phase was transferred to new sterile tube and repeated extraction with chloroform/isoamyl alcohol (24:1). After centrifugation, the crude DNA extract was precipitated with absolute ethanol for 2 h or overnight at −20 °C followed by centrifugation at 12,000×*g* for 10 min. The genomic DNA pellet was re-suspended in 30–50 μl of TE buffer and stored at −20 °C until needed. The genomic DNA was visualized by electrophoresis on 0.8% agarose gel stained with 0.5 μg/mL ethidium bromide.

2.5. *PCR-DGGE analysis*

The microbial community structure of the enriched culture was studied using PCR-DGGE as described by Prasertsan et al. [21] with primers sequence as shown in Table 2. Briefly, the bacterial 16S rDNA (~1400 base pair) was amplified by the first polymerase chain reaction (PCR) with universal primer 1492r and 27f. Amplification mixtures were used TopTaq™ Master Mix Kit (Qiagen) with a final volume of 25 μl and contained 25 pmol of each primers, under conditions of an initial denaturation at 95.5 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 40 s, extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. The reactions were subsequently cooled to 4 °C. The PCR product was analyzed on 1.0% agarose gels electrophoresis. In the second PCR, primer K517r and L340f with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR [22] with the program corresponded to an initial denaturation 95 °C for 3 min follow by 30 cycles of three steps: 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min and final extension at 72 °C for 10 min. PCR products were stored at 4 °C and analyzed on 1.0% agarose gel electrophoresis before DGGE.

Table 2 – Primers used for PCR-DGGE in this study.

Group	Primer name	Sequence (5′->3′)	Target
Bacteria	1492r	GAAAGGAGGTGATCCAGCC	16S rDNA
	27f	GAGTTTGATCCTTGGCTCAG	16S rDNA
	K517r	ATTACCGCGCTGCTGG	V3 region
	L340f	CCTACGGGAGGCAGCAG	V3 region
	L340f-GC	GC clamp-CCTACGGGAGGCAGCAG	V3 region
Archaea	Arch958r	YCCGGCGTTGAMTCCAATT	16S rDNA
	Arch21f	TTCCGGTTGATCCYGCCGGA	16S rDNA
	PARCH519r	TTACCGCGGCKGCTG	V3 region
	PARCH340f-GC	GC-clamp-CCTACGGGGYGCASCAG	V3 region
	GC clamp	CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGG CACGGGGGG	

Amplification of Archaea 16S rDNA sequences was carried out using archaea-specific primers (Table 2). The majority of the archaea 16S rDNA fragment was amplified using Arch958r and Arch21f primers. The amplification mixture was carried out using the same mixture for bacteria. PCR began with a 94 °C denaturation for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. The 16S rDNA PCR product was used as template for next PCR. The V3 region fragment was amplified with the PARCH519r and PARCH340f-GC primers and carried out with the same mixture as above. The amplification condition started with an initial denaturation 94 °C for 3 min follow by 34 cycles of three steps: 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min and final extension at 72 °C for 10 min. PCR products were stored at 4 °C and analyzed on 1.0% agarose gel electrophoresis before DGGE.

The DGGE analysis of PCR products obtained from the second PCR were performed using the DGGE unit, V20-HCDC (Scie-Plas limited, UK) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60% and 40–80% for bacteria and archaea respectively. DGGE gels were stained with Sybr-Gold for 60 min and photographed on Gel DocXR system (Bio-Rad Laboratories) and the bands in the gel were excised. The DNA in the excised gel slices were incubated in 20 μl of distilled

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water at 4 °C for 24 h and re-amplified by PCR with the second PCR primers. The PCR products were separated again by DGGE, and then the bands were excised again. Most of the bands were excised from the gel and re-amplified with primer 357f (without a GC clamp) and the reverse primer. After re-amplification, PCR products were purified and sequenced using primer 518r (bacteria) and PARCH519r (archaea) by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Raw data of DNA sequenced were analyzed with program Chromas and BioEdit before affiliated strains performed. Closest matches for partial 16S rRNA gene sequences were identified by ribosomal database project (http://rdp.cme.msu.edu/) with SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results and discussion

3.1. *Enrichment of thermophilic hydrogen-producing bacteria from geothermal spring samples*

Different types of microbial communities were obtained from different hot spring sediment-rich samples taken from 5 Provinces in Southern Thailand. During incubation in BA medium (pH 5.5) at 60 °C, all samples produced gas consisting of H_2 (34–61%) and CO_2 (38–60%), with traces of N_2. In some experiments, methane formation was also detected during the initial stages of consortium development but not found in the second generation. Gas production started after 1 day and stopped after 2–4 days incubation in all samples. Four sediment sources encoded with RNW, SRW1, SRW2 and SRR1 gave significantly low hydrogen production yield (<69.6 mL H_2/L-medium). In all carbon sources tested, except the source encoded with RNW enriched with xylose produced 566 mL H_2/L-medium indicated that it was xylose–utilizing thermophilic bacteria. Interestingly, the other group showed high cumulative hydrogen production. Among these, the two sediment sources encoded as PGR and YLT showed higher cumulative hydrogen production (1506.5 and 1487.3 mL H_2/L-medium, respectively) than the other sources encoded as SRR2 and PGK (326.1–967.6 mL H_2/L-medium, Table 3). The maximum hydrogen evolution (1506.5 mL H_2/L-medium) was achieved from consortium PGR enriched with xylose–glucose mixed sugars while the values from enrichment with xylose and glucose were 1319.8 and 1190.1 mL H_2/L-medium, respectively. The maximum hydrogen yields from the consortium PGR with mixed sugars, glucose and xylose were 301.3, 263.9 and 238.1 mL H_2/g $sugar_{consumed}$, respectively, whereas the values from the consortium YLT were 264.9, 156.5, and 297.4 mL H_2/g $sugar_{consumed}$, respectively. These results indicated the potential role of microbial consortia in utilization of xylose and glucose without much variation in overall metabolism. This data is in agreement with the report that biohydrogen production from xylose and glucose using pure culture of *T. themosaccharolyticum* PSU-2, *T. themosaccharolyticum* W16 and mixed culture of compost microfora [7–9]. However, hydrogen production values differed with type of carbon source. For the consortium PGR and YLT, xylose was a better carbon source than glucose as it exhibited higher cumulative hydrogen production (1319.8 and 1487.3 mL H_2/L-medium, respectively)

Table 3 – Hydrogen production, metabolic production concentration, and substrate utilization of enriched cultures from different geothermal spring source.

Samples	Enrichment substrates	H_2 production (mL/L)	H_2 yield (mL/g sugar consumed)	Substrate utilization (%)	Acetic acid (mM)	Butyric acid (mM)	Propionic Acid (mM)	Ethanol (mM)	Lactic acid (mM)	Butanol (mM)	Final pH
RNW	Glucose	36.2	7.2	57.6	1.60	0.52	0.33	0.11	2.45	6.80	5.0
	Glucose + xylose	20.6	4.1	51.8	1.80	0.92	0.19	0.16	1.87	7.30	4.9
	Xylose	566.0	113.2	76.0	2.52	9.20	0.24	0.13	0.00	8.00	4.6
SRW1	Glucose	11.9	2.4	52.0	1.15	0.52	0.32	0.15	4.65	6.00	4.6
	Glucose + xylose	12.6	2.5	60.6	0.81	1.08	0.26	0.18	4.55	7.80	4.5
	Xylose	5.2	1.1	61.6	0.81	1.72	0.23	0.28	1.37	8.80	6.9
SRW2	Glucose	27.1	5.4	63.6	1.02	4.52	0.26	0.12	3.02	6.70	5.2
	Glucose + xylose	26.2	5.2	56.0	0.81	5.12	0.19	0.16	1.18	8.80	7.0
	Xylose	8.9	1.8	32.0	1.44	1.72	0.19	0.22	6.10	0.50	7.0
SRR1	Glucose	32.5	6.5	48.8	2.74	0.52	0.26	0.15	0.00	7.80	5.6
	Glucose + xylose	69.6	13.9	56.0	2.28	1.16	0.23	0.21	1.72	8.80	6.3
	Xylose	8.5	1.7	40.0	1.05	1.92	0.19	0.28	6.60	1.20	6.8
SRR2	Glucose	326.1	65.2	54.0	4.39	8.52	0.26	0.14	3.63	1.20	4.9
	Glucose + xylose	538.9	107.7	66.0	5.91	9.24	0.19	0.21	2.00	4.40	4.8
	Xylose	657.2	131.4	40.0	4.72	7.56	0.23	0.29	1.40	0.00	4.6
PGK	Glucose	819.7	163.9	76.0	2.77	8.52	0.25	0.17	3.16	5.20	4.4
	Glucose + xylose	958.9	191.7	72.0	5.58	9.20	0.19	0.18	3.82	2.20	4.0
	Xylose	967.6	193.5	88.0	12.81	9.80	0.19	0.27	3.07	4.30	3.7
PGR	Glucose	1190.1	238.1	82.0	3.58	10.72	0.19	0.28	4.10	2.20	3.7
	Glucose + xylose	1506.5	301.3	96.0	6.73	10.32	0.19	0.15	5.24	3.40	3.8
	Xylose	1319.8	263.9	84.0	3.6	10.92	0.19	0.10	5.84	6.50	3.9
YLT	Glucose	782.9	156.5	48.0	8.23	2.12	0.26	0.12	3.61	0.00	3.8
	Glucose + xylose	1324.9	264.9	92.0	4.39	11.08	0.19	0.15	4.04	4.40	3.6
	Xylose	1487.3	297.4	92.0	8.19	8.92	0.19	0.18	5.66	3.20	3.6

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Table 4 – Percentage of COD distribution at the end of repeated batch enrichment of each hot spring samples and biomass was estimated as 15% according to the previous study [26].

Samples source	Enrichment substrates	Consumed sugars (%)	H_2 (%)	Butanol (%)	Acetic acid (%)	Butyric acid (%)	Propionic acid (%)	Ethanol (%)	Lactic acid (%)	Cell mass (%) ($C_5H_7O_2N$)	Balance (%)
RNW	Glucose	−100.0	0.8	42.5	3.3	1.6	1.2	0.3	12.8	15.0	−22.4
	Glucose + xylose	−100.0	0.5	50.7	4.3	3.2	0.8	0.6	10.8	15.0	−14.0
	Xylose	−100.0	10.0	37.9	4.0	21.8	0.7	0.3	0.0	15.0	−10.4
SRW1	Glucose	−100.0	0.3	41.5	2.7	1.8	1.3	0.5	26.8	15.0	−10.0
	Glucose + xylose	−100.0	0.3	46.3	1.6	3.2	0.9	0.5	22.5	15.0	−9.6
	Xylose	−100.0	0.1	51.4	1.6	5.0	0.8	0.8	6.7	15.0	−18.6
SRW2	Glucose	−100.0	0.6	37.9	1.9	12.8	0.9	0.3	14.2	15.0	−16.3
	Glucose + xylose	−100.0	0.6	56.6	1.7	16.5	0.7	0.5	6.3	15.0	−2.1
	Xylose	−100.0	0.4	5.6	5.4	9.7	1.2	1.2	57.2	15.0	−4.3
SRR1	Glucose	−100.0	0.9	72.3	6.7	1.9	1.1	0.6	0.0	15.0	−16.2
	Glucose + xylose	−100.0	1.7	56.6	4.9	3.7	0.9	0.7	9.2	15.0	−7.4
	Xylose	−100.0	0.3	10.8	3.2	8.6	1.0	1.3	49.5	15.0	−10.4
SRR2	Glucose	−100.0	8.1	8.0	9.8	28.4	1.0	0.5	20.2	15.0	−9.1
	Glucose + xylose	−100.0	10.9	24.0	10.7	25.2	0.6	0.6	9.1	15.0	−3.9
	Xylose	−100.0	22.0	0.0	14.2	34.0	1.2	1.3	10.5	15.0	−1.8
PGK	Glucose	−100.0	14.4	24.6	4.4	20.2	0.7	0.4	12.5	15.0	−7.8
	Glucose + xylose	−100.0	17.8	11.0	9.3	23.0	0.6	0.5	15.9	15.0	−6.9
	Xylose	−100.0	14.7	17.6	17.5	20.0	0.5	0.6	10.5	15.0	−3.7
PGR	Glucose	−100.0	19.4	9.7	5.2	23.5	0.5	0.6	15.0	15.0	−11.0
	Glucose + xylose	−100.0	21.0	12.8	8.4	19.4	0.4	0.3	16.4	15.0	−6.4
	Xylose	−100.0	17.0	22.5	4.2	18.9	0.4	0.2	16.8	15.0	−5.0
YLT	Glucose	−100.0	21.8	0.0	20.6	8.0	1.1	0.5	22.6	15.0	−10.5
	Glucose + xylose	−100.0	19.3	17.2	5.7	21.7	0.4	0.3	13.2	15.0	−7.2
	Xylose	−100.0	21.7	12.5	10.7	17.5	0.4	0.4	18.5	15.0	−3.4

than glucose (1190.1 and 782.9 mL H_2/L-medium, respectively). Furthermore, the maximum hydrogen yields of the thermophilic consortium PGR and YLT (301.3 and 297.4 mL H_2/g $sugar_{consumed}$, respectively) were comparable with other studies such as 274 mL H_2/g sugar at 60 °C using sludge inoculum taken from a municipal sewage treatment plant [23] and 2-fold higher than the earlier report (138 mL H_2/g sugar at 55 °C) using inoculum from a continuous reactor [24]. So far, the highest hydrogen production yields by thermophilic mixed cultures are 322 mL H_2/g sugar at 60 °C with anaerobic digested sludge

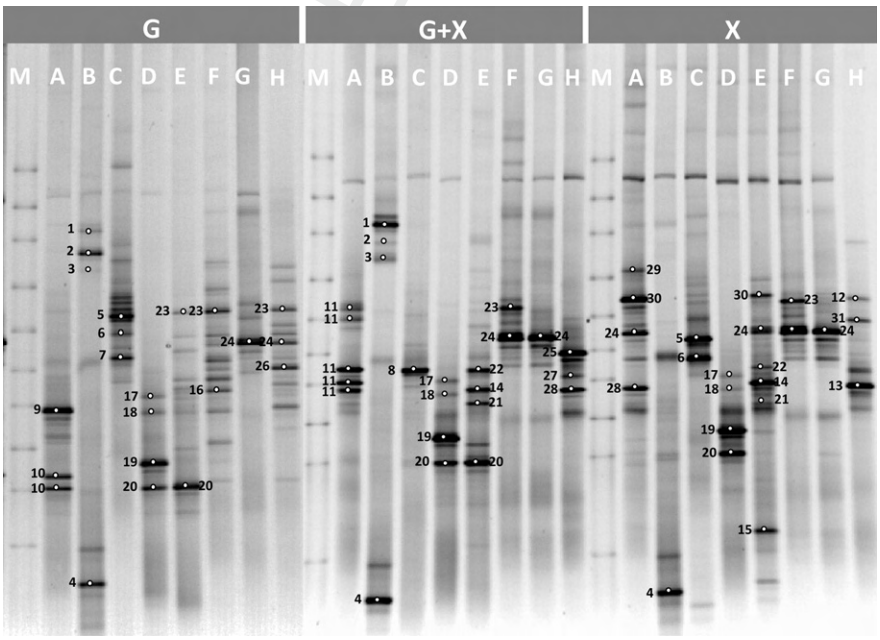

Fig. 1 – Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes fragments from enriched cultures at 5th cycles repeated batch cultivation with glucose (G), mixture of glucose and xylose (G + X) and xylose (X). Lanes: M, DGGE marker II; A, hot spring code RNW; B, hot spring code SRW1; C, hot spring code SRW2; D, hot spring code SRR1; E, hot spring code SRR2; F, hot spring code PGK; G, hot spring code PGR; H, hot spring code YT. Numbers indicate DNA bands were affiliated show in Table 5.

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[25] and 307 mL H_2/g sugar at 70 °C with inoculum from a bench-scale methanogenic manure-treating reactor [26]. Therefore, the hydrogen production of the enriched consortium PGR and YLT are comparable with the previous reports.

3.2. *Soluble metabolites of enrichment cultures*

The hydrogen production was going along with the production of volatile fatty acids and alcohols (Table 4). Main volatile fatty acids of the enriched cultures were acetic acid, butyric acid, lactic acid and butanol. Small amount of ethanol and propionic acid were observed throughout the enrichment culture fermentation. Among the metabolites, acetic acid and lactic acid accumulated were dominated which caused the acidic condition (low pH). The dominated metabolites correlated closely with high hydrogen producer encoded as SRR2, PGK, PR and YLT. The pH lower than 5.5 (initial pH) were observed in high hydrogen-producing group and the lowest pH (pH 3.8) was obtained from the highest hydrogen producer PGR in the xylose–glucose mixed. Most COD balances of soluble metabolites were under 10% error, indicating the measurement were accurate. Only COD balances of the enriched cultures with glucose were over 10%, it may cause from high cell mass conversion from this sugar. The soluble metabolites from high hydrogen producer were composed mostly butyric acid, butanol, lactic acid and acetic acid with a COD distribution of 18–23, 9–22, 15–16 and 4–8% on COD basis (Table 4). The enriched cultures (SRR2, PGK, PGR and YLT) had different metabolic patterns for hydrogen production mostly acetate and butyrate metabolic pathways. Some enriched cultures produced small amount of hydrogen but produced butanol and lactic acid as the main soluble metabolites (RNW, SRW1 and SRR1) or produced butanol and butyric acid as main soluble metabolites (SRW2). Lactic acid was found in all enriched cultures, while lactate is not common as end product but intermediate product during fermentation. The reason for lactic acid accumulation was not clear but likely related to environmental change during repeated cycle or substrate overloading [27]. It was suggested when environmental change, bacteria would try to optimize the carbon flow rate through the cell by using lactate as electron sink and lower down the acetate production or change in the microflora population itself, which gave high energy yield. As a result, high lactate was found in all enriched cultures. In the present study, fermentation patterns involving hydrogen fermentation of glucose–xylose mixed substrate was characterized relative to the soluble metabolites and microbial characteristics of the system. It was shown that the

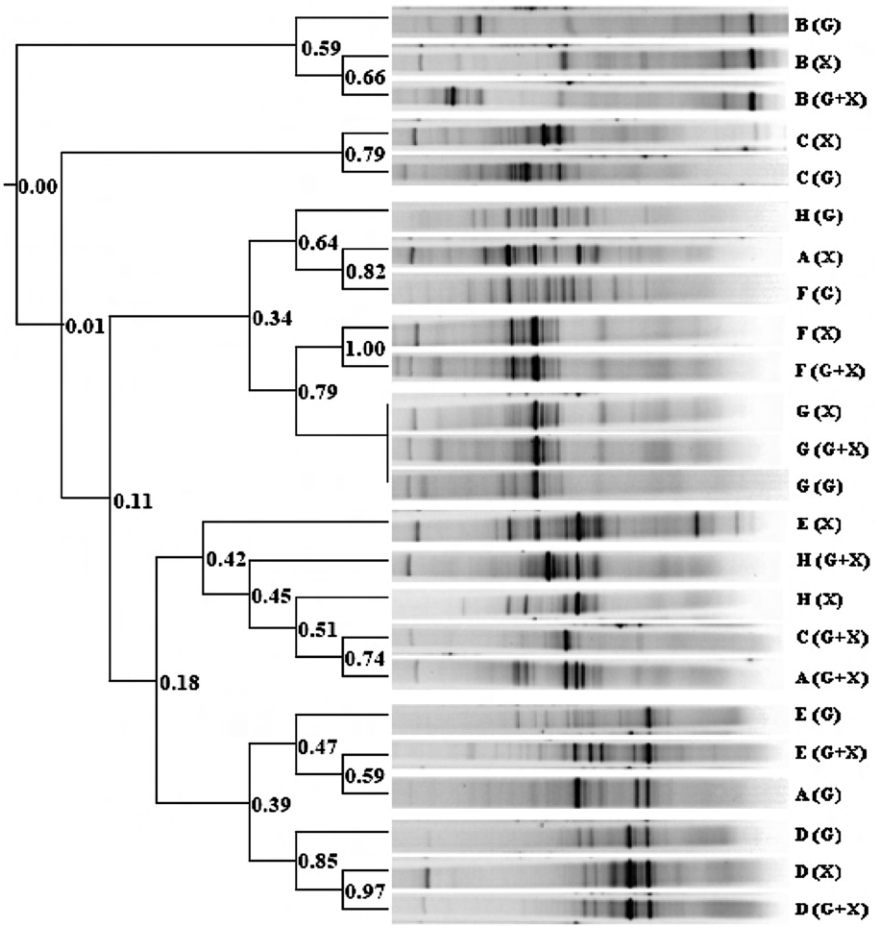

Fig. 2 – Phylogenetic tree showing the similarity of DGGE profiles of enrichment cultured. A, hot spring code RNW; B, hot spring code SRW1; C, hot spring code SRW2; D, hot spring code SRR1; E, hot spring code SRR2; F, hot spring code PGK; G, hot spring code PGR; H, hot spring code YT. Enriched carbon source are glucose (G), mixture of glucose and xylose (G + X) and xylose (X).

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distribution of fermentation products was dependent upon microorganisms that were dominant in the microflora under various inoculum sources.

3.3. *Microbial community analysis of enrichment cultures*

Bacterial community samples were taken from the last repeated batch cultures (5th cycle). The results demonstrated that numerous hydrogen-producing thermophilic bacteria could be obtained from liquid and sediment samples collected from hot springs in Southern Thailand. The community structures of the enriched cultures from hot spring were analyzed with PCR-DGGE, using the V3 region of 16S rRNA gene. The results showed that sample sources and enrichment carbon sources were affected to diversity of bacteria community (Fig. 1). The enriched culture consortium SRW1 (B), SRR1 (D) and PGR (G) showed 60–100% DGGE profiles similarity in all enrichment cultures with glucose, xylose–glucose mixed and xylose alone as carbon source. The consortium PGK (F) were

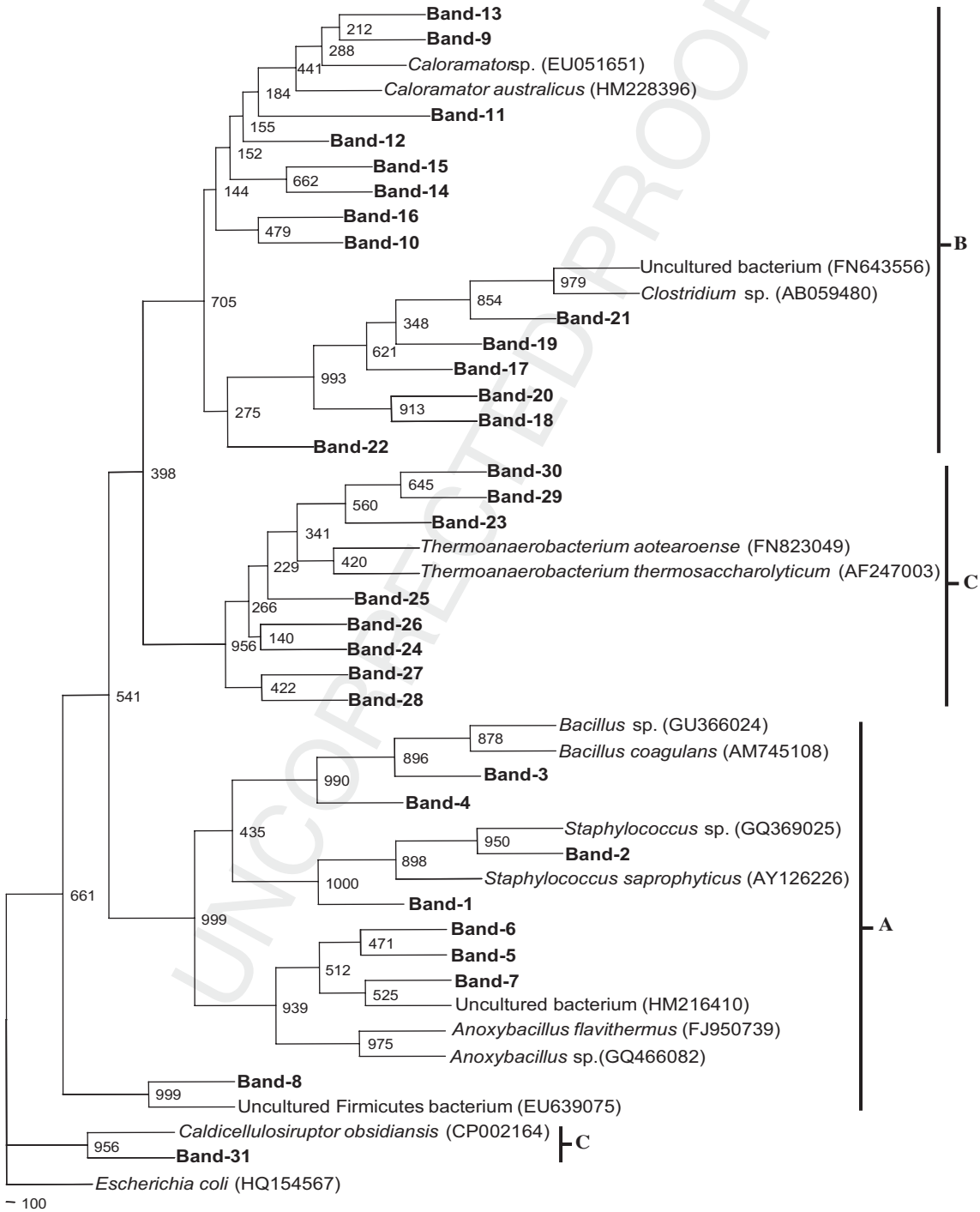

Fig. 3 – Phylogenetic tree showing the relationships between affiliation bands of DGGE profiled based on 16S rRNA gene sequences. The sequence of *Escherichia coli* served as an out-group sequence. Bar represents sequence distance.

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Table 5 – Affiliations and grouped of dominant bands excised from the DGGE gels (Fig. 1) of the enrichment cultured hydrogen-producing bac
springs.

Groups	Affiliations	Glucose	Xylose–glucose mixed
	Bands	A B C D E F G H	A B C D E F G H
A	*Staphylococcus* sp.		
	1	− + − − − − − −	− + − − − − − −
	2	− + − − − − − −	− + − − − − − −
	Bacillus sp.		
	3	− + − − − − − −	− + − − − − − −
	4	− + − − − − − −	− + − − − − − −
	Anoxybacillus sp.		
	5	− − + − − − − −	− − − − − − − −
	6	− − + − − − − −	− − − − − − − −
	7	− − + − − − − −	− − − − − − − −
	Uncultured Firmicutes bacterium		
	8	− − − − − − − −	− − + − − − − −
B	*Caloramator* sp.		
	9	+ − − − − − − −	− − − − − − − −
	10	+ − − − − − − −	− − − − − − − −
	11	− − − − − − − −	+ − − − − − − −
	12	− − − − − − − −	− − − − − − − −
	13	− − − − − − − −	− − − − − − − −
	14	− − − − − − − −	− − − − + − − −
	15	− − − − − − − −	− − − − − − − −
	16	− − − − − + − −	− − − − − − − −
	Clostridium sp.		
	17	− − − + − − − −	− − − + − − − −
	18	− − − + − − − −	− − − + − − − −
	19	− − − + − − − −	− − − + − − − −
	20	− − − + + − − −	− − − + + − − −
	21	− − − − − − − −	− − − − + − − −
	22	− − − − − − − −	− − − − + − − −
C	*Thermoanaerobacterium* sp.		
	23	− − − − + + − +	− − − − − + − −
	24	− − − − − − + +	− − − − − + + −
	25	− − − − − − − −	− − − − − − − +
	26	− − − − − − − +	− − − − − − − −
	27	− − − − − − − −	− − − − − − − +
	28	− − − − − − − −	− − − − − − − +
	29	− − − − − − − −	− − − − − − − −
	30	− − − − − − − −	− − − − − − − −
	Caldicellulosiruptor sp.		
	31	− − − − − − − −	− − − − − − − −

strongly dominant species presented 100% similarity between xylose–glucose mixed, and xylose alone as enrich carbon source, while the enriched cultures possessed more diverse dominant species in the presence of glucose. The bacterial community profiles of the other consortium; RNW (A), SRW2 (C), SRR2 (E) and YLT (F), also were different depending on the carbon sources enriched (Fig. 2).

Q2 Bacterial dominant species in the enrichment culture were grouped by phylogenetic tree. The dominant species were divided into three groups (Fig. 3, Table 5) including bacterial strains belong to Group A were related to *Staphylococcus* sp., *Bacillus* sp., *Anoxybacillus* sp. and uncultured Firmicutes bacterium. Bacterial species in this group were related to low hydrogen-producing species as the results showed presence dominant species in low hydrogen production enriched cultures (RNW, SRW1, SRW2 and SRR1). *Staphylococcus* sp. was dominated in the enriched cultures with glucose and xylose–glucose mixed substrate while xylose alone not found. It may be growth only glucose as carbon source. Two bands dominant were affiliated to different *Bacillus* sp. because dominant Band 3 seems to be preferred only glucose but Band 4 can used both of glucose and xylose as substrate. *Staphylococcus* sp. and *Bacillus* sp. were found only in the enriched culture SRW1 (B). *Bacillus coagulans* was found a sporogenic lactic acid bacterium and produces lactic acid as the primary fermentation product from both hexose and pentose sugars [28]. This result was corresponded to the presence lactic acid as main fermentation product in the enriched cultured with glucose and xylose–glucose mixed substrate of SRW1 (Table 3). For *Anoxybacillus* sp. was found dominant in enriched cultures SRW2 (C). This strain was utilized both of glucose and xylose substrate but in enriched culture with xylose–glucose mixed not clearly dominant because it seems can not competed with strongly dominated band affiliated to uncultured Firmicutes bacterium. Genus Anoxybacillus were reported as both glucose and xylose-utilizing and endospore-forming bacterium [29]. Bacterial dominant species belong to Group B were *Caloramator* sp. and *Clostridium* sp. with found mainly in the enriched cultures with glucose and xylose–glucose mixed of sample code RNW and all the enrichment cultured of samples coded SRR1 and SRR2, respectively. *Caloramator* sp. presented in the enriched cultures RNW seems to prefer both glucose and xylose but can not competed with other strains presented in the enriched culture with xylose as substrate. *Caloramator* sp. was also presented in the enriched culture with xylose of YLT. *Caloramator* sp. was found in enrichment culture for hydrogen-producing extreme thermophilic anaerobic microflora from cow manure enriched with glucose and xylose by repeated batch cultures at 75 °C [30] and in biohydrogen production from palm oil mill effluent with ASBR reactor tank system [21]. The bacterial strain related to *Caloramator indicus* were proposed as a new hydrogen-producing strain with optimum temperature 60–62 °C [31,32]. For *Clostridium* sp. were presented dominant in xylose–glucose mixed and xylose enrichment cultured. However, theses two genus were efficient utilized xylose–glucose mixed substrate and appeared moderate hydrogen-producing bacteria when compared with the last group dominant strains. The last group, Group C was related to *Thermoanaerobacterium* sp. and *Caldicellulosiruptor* sp. Genus Thermoanaerobacterium were presented dominant in the enriched cultures of samples code PGK, PGR and YLT. It showed efficient utilize xylose–glucose mixed substrate and gave the highest accumulation hydrogen production compared with other two groups. *T. thermosaccharolyticum* PSU-2, *T. thermosacchrolytium* W16, *T. saccharolyticum* and *T. aotearoense* were reported effectively hydrogen-producing species at thermophilic condition and utilized both hexose and pentose sugars [7,8,33]. One of the evidence clearly was the presence *Thermoanaerobacterium* sp. in the enriched culture with xylose of RNW gave hydrogen production higher significant than other two enrichment substrate. Genus Thermoanaerobacterium and Caloramator were frequency found to be dominant species in thermophilic hydrogen enriched cultures [30,34].

Caldicellulosiruptor sp. was also found in the enriched culture with xylose of YLT. The band was lower strong than other bands may be cause by not optimum cultured temperature. Finally this study, microbial consortia developed from Thailand hot springs were *Caloramator* sp., *Clostridium* sp., *Thermoanaerobacterium* sp., and *Caldicellulosiruptor* sp. as major groups of hydrogen-producing bacteria in glucose, xylose–glucose mixed and xylose substrate and show promising to apply for biohydrogen production from lignocellulosic hydrolysate with mostly contained xylose–glucose mixed.

Archaeal community of enrichment cultured found that no amplification products of archaeal 16S rRNA genes were obtained from the DNA extracts of all enriched cultures samples with a nested PCR procedure. This result indicated the absence of archaea in the enriched cultures.

4. Conclusion

Potential thermophilic microorganisms for hydrogen production were enriched from Southern Thailand hot springs over a wide temperature range (53–80 °C). The batch enrichments had different metabolic patterns, including high hydrogen or high butanol yields from glucose and xylose. Two enrichment cultures produced a high hydrogen yield from glucose, xylose and mixture of both sugars at 60 °C. The PGR and YLT enrichment had maximum hydrogen yields of 301.3 and 297.4 mL of H_2/g $sugars_{consumed}$. The consortium PGR and YLT, dominated by bacteria closely affiliated with *T. thermosaccharolyticum*. In summary, promising bacterial enrichments were obtained from Southern Thailand hot spring samples with a high potential for hydrogen production under thermophilic condition from lignocellulose hydrolysate. The community structures of enrichment with the xylose, glucose and the mixture composed of high hydrogen-producing bacterial strains affiliated with low-G+C-content Gram-positive bacteria, *Thermoanaerobacterium* sp. and *Caldicellulosiruptor* sp. based on the 16S rRNA gene. The moderate and low hydrogen-producing strains were comprised of genera Caloramator and Clostridium, Staphylococcus, Bacillus and Anoxybacillus, respectively.

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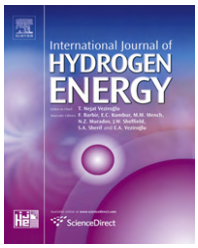

Developing a thermophilic hydrogen-producing microbial consortia from geothermal spring for efficient utilization of xylose and glucose mixed substrates and oil palm trunk hydrolysate

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ABSTRACT

Xylose and glucose are the major sugar components of lignocellulosic hydrolysate. This study aims to develop thermophilic hydrogen-producing consortia from eight sediments-rich samples of geothermal springs in Southern Thailand by repeated batch cultivation at 60 °C with glucose, xylose and xylose–glucose mixed substrates. Significant hydrogen production potentials were obtained from thermophilic enriched cultures encoded as PGR and YLT with the maximum hydrogen yields of 241.4 and 231.6 mL H_2/g $sugar_{consumed}$, respectively. After repeated batch cultivation the hydrogen yield from xylose–glucose mixed substrate of PGR increased to 375 mL H_2/g $sugar_{consumed}$ which was 30% higher than that of YLT (287 mL H_2/g $sugar_{consumed}$). Soluble metabolites from xylose–glucose mixed substrates were composed mostly of butyric acid (20.6–21.8 mM), acetic acid (7.2–13.5 mM), lactic acid (8.2–11.7 mM) and butanol (4.4–13.0 mM). Denaturing gradient gel electrophoresis (DGGE) profiles illustrated small difference in microbial patterns of PGR enriched with glucose, xylose–glucose mixed substrate and xylose. The dominant populations were affiliated with low G + C content Gram-positive bacteria, *Thermoanaerobacterium* sp., *Thermoanaerobacter* sp., *Caloramater* sp. and *Anoxybacillus* sp. based on the 16S rRNA gene. Cultivation of the enriched culture PGR in oil palm trunk hydrolysate, the maximum hydrogen yield of 301 mL H_2/g $sugar_{consumed}$ was achieved at hydrolysate concentration of 40% (v/v). At higher concentration to 80% (v/v), the hydrogen fermentation process was inhibited. Therefore, the efficient thermophilic hydrogen-producing consortia PGR has successfully developed and has great potential for production of biohydrogen from mixed sugars hydrolysate.

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1. Introduction

Lignocellulosic materials obtained from crop or food industrial wastes could be a commercially viable biohydrogen feedstock [1] as they are abundant, easily available, inexpensive and not compete with food and feed production [2]. Lignocellulosic agro-industrial wastes such as straw, bagasse, and palm empty fruit bunch are a promising base substrate for cost-effective biological hydrogen production as they contain a large amount of cellulose (40–50%), hemicellulose (25–30%), and lignin (10–20%) [3]. Direct fermentation of raw lignocellulosic feedstock is typically inefficient due to their complex structures. Hence, pre-treatments are commonly needed for saccharification process prior to biological conversion [4,5].

Natural dark fermentative inoculum (mixed cultures) gave advantages over pure inoculum in allowing process at non-sterile condition and capable of dealing with mixture of substrates due to a high microbial diversity [6,7], which significantly reduce the process operation cost. The advantage of using mixed cultures for biohydrogen production is high adaptive capacity owing to the microbial diversity and the possibility of obtaining a stable and continuous process. The most common bacteria used in dark fermentation to produce hydrogen are *Clostridium* [8] and *Thermoanaerobacterium* [9]. Normally, hydrogen production yield of 125–250 mL H_2/g $sugar_{consumed}$ are obtained with mesophiles, while thermophiles yield was higher than 250 mL H_2/g $sugar_{consumed}$ [10,11]. Hydrogen yields can be improved by increasing acetate end product formation, and decreasing or preventing butyrate formation by using a high-temperature fermentation process with thermophiles or extreme thermophiles, operating at temperatures higher than 60 °C [12]. Thermophilic mixed cultures have a high potential as hydrogen producers [11] and able to utilize a wide range of organic wastes [13]. High temperatures favor the stoichiometry of hydrogen production, resulting in higher hydrogen yields [14], have a narrower spectrum of end products as compared to mesophilic fermentation [10], and also reduce the contaminants. Nevertheless, thermophilic temperatures have main disadvantage of more heat energy input to maintain operation condition. This problem, however, can reduce by system design.

Some thermophilic bacteria are able to utilize mixed hexose and pentose into hydrogen [15]. *Thermoanaerobacterium thermosaccharolyticum* strain PSU-2, *T. thermosaccharolyticum* strain W16 and extreme thermophiles *Caldicellulosiruptor saccharolyticus* have been reported to simultaneously utilize mixed sugars to generate hydrogen with hydrogen yields of 281 and 290 mL H_2/g $sugar_{consumed}$, respectively [16–18]. However, the problem of lignocellulosic material-based substrate fermentation process is the inability of microorganisms to metabolize efficiently all mixed carbohydrates (hexose and pentose) into hydrogen with satisfied yield and productivity [12,15,19].

Hot springs are a potential source for thermophilic hydrogen-producing microorganisms. In this study, thermophilic hydrogen-producing microflora were enriched from geothermal spring samples by repeated batch cultivation at 60 °C with glucose, xylose, and xylose–glucose mixed substrates. The most effective consortia were selected for microbial community studies and tested for hydrogen production from oil palm trunk hydrolysate.

2. Materials and methods

2.1. Geothermal spring sampling

Sediment-rich samples were collected from eight geothermal springs located in Southern Thailand. Temperature and pH of all samples were measured on-site using thermometer and pH paper, respectively. The samples were kept in serum bottles for transportation to the laboratory.

2.2. Enrichment of thermophilic hydrogen-producing cultures

Enriched hydrogen-producing cultures were developed following the procedure as previously reported by O-Thong et al. [9]. In brief, a basic anaerobic medium (BA medium, pH 5.5) was prepared by using glucose, xylose and xylose–glucose mixed substrate as carbon source. At each site, 3 mL of sediment-rich water was transferred by sterile syringe into 27 mL BA medium supplemented with 10 g/L each of glucose, xylose and xylose–glucose mixed substrate at the ratio of 1:1 [20] and incubated at 60 °C for 4 day under strictly anaerobic condition. During the enrichment, H_2, H_2S, CO_2 and CH_4 and soluble metabolites were monitored. The selected thermophilic hydrogen-producing cultures were enriched in five successive batch cycles (10% inoculum in the medium with 2 days cultivation per cycle). The final cultures (5^{th} batch cycle) were used for analysis of hydrogen production, soluble metabolite production and responsible microbial community. Thermophilic enriched culture producing the highest hydrogen production was selected for further study.

2.3. Batch test of the selected thermophilic enriched cultures for hydrogen production from oil palm trunk hydrolysate

The oil palm trunk hydrolysate was prepared using the two-stage dilute acid and base hydrolysis. In the first stage, hydrolysis was carried out in 0.1% H_2SO_4 in an autoclave at 120 °C under 1 bar pressure for 25 min with 30% (w/w) of solid concentration. After pretreatment, the liquid hemicellulose-rich fraction (the hydrolysate) was separated from the solid cellulose-rich fraction of the oil palm trunk. In the second stage hydrolysis, the solid fraction was hydrolyzed in 0.1% NaOH under the same condition as described above. The two sources of hydrolysate were mixed and stored at 4 °C before use. After mixing, the pH of the hydrolysate was 6.2, containing 15.2 g/L of glucose and 9.6 g/L of xylose. In order to adapt the selected thermophilic enriched culture to the mixed hydrolysate, batch cultivations were carried out by adding hydrolysate at different concentrations; 10%, 20%, 40%, 60% and 80% (v/v). Hydrolysate media with 10–80% (v/v) hemicellulose-rich fraction were prepared and supplemented with the same concentrations of minerals, trace metals and yeast extract as described above for the BA medium. The

thermophilic enriched culture inoculum (obtained at the exponential growth phase) was added at 10% (v/v) into 50 mL hydrolysate in 125 mL glass serum bottles. Control vials containing only water, minerals, trace metals, yeast extract and inoculum were included, in order to account for possible background hydrogen production from these additions. The background hydrogen, if any, would be subtracted from hydrogen produced in vials with the mixed hydrolysate. All experiments were conducted in triplicate. The hydrogen in gas phase was monitored periodically.

2.4. Analytical methods

The evolved biogas was collected in a headspace of serum bottle. The total volumes at each time interval were measured at room temperature by syringe. Hydrogen, carbon dioxide, hydrogen sulfide and methane in the biogas were measured by gas meter (MX2100 OLDHAM, MULTIGAS Type MX2100). The biogas composition was confirmed by gas chromatography (Hewlett Packard GC 6850) equipped with thermal conductivity detector (TCD) every week to standardize gas meter. Hydrogen and methane gas were analyzed by GC-TCD with a protocol according to O-Thong et al. [21] with a gas samples 100 μl for methane and 500 μl for hydrogen injected in duplicate. The culture broth was centrifuged at 10,000×*g* for 10 min. Fermentation end products (volatile fatty acids (VFA) and ethanol) in the supernatant were determined by gas chromatograph (HP6850) equipped with a flame ionization detector (FID) and Stabilwax-DA column (dimensions 30 m × 0.32 mm × 0.25 μm). The temperature of the injection port was 230 °C. The chromatography was performed using the following program: 70 °C for 1 min, 70–180 °C with a ramping of 20 °C/min, 180 °C for 6 min. The detector temperature was 250 °C. Lactic acid, xylose and glucose were analyzed with a high performance liquid chromatograph (HPLC) (Agilent 1200 series), equipped with Aminex® HPX-87H ion exclusion column. The microbial community structure of the enrichment culture was studied using PCR–DGGE as described by Prasertsan et al. [22]. Briefly, the bacterial 16S rDNA (1400 bp) was amplified by the first polymerase chain reaction (PCR) with universal primer. In the second PCR, V3-specific reverse and forward primers with CG clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR. The DGGE analysis of PCR products obtained from the second PCR were performed using the DGGE unit, V20-HCDC (Scie-Plas limited, UK) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60%. DGGE gels were stained with Sybr-Gold for 60 min and photographed on Gel DocXR system (Bio-Rad Laboratories) and the bands in the gel were excised. The DNA in the excised gel slices were incubated in 20 μl of distilled water at 4 °C for 24 h and re-amplified by PCR with the second PCR primers. After re-amplification, PCR products were purified and sequenced using reverse primer by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by ribosomal database project (http://rdp.cme.msu.edu/) with SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results and discussion

3.1. Enrichment of thermophilic hydrogen-producing cultures

Eight geothermal springs from various places in Southern Thailand had the temperature in the range of 53–80 °C with the pH range of 6.5–8.3 (Table 1). Thermophilic hydrogen-producing bacteria from these sources were successfully enriched with glucose, xylose and xylose–glucose mixed substrate under batch cultivation at pH 5.5 and 60 °C. Different types of microbial communities were obtained from different hot spring samples. After preliminary screening for hydrogen production capacity and soluble metabolites produced from 10 g/L of glucose, xylose and the mixed xylose–glucose (Table 2), the most promising consortia PGR were further characterized. During incubation, all samples produced gas after 1 day and stopped after 2–4 days incubation which consisted of a mixture of H_2 (34–61%) and CO_2 (38–60%), with traces of N_2 but no trace of CH_4 or H_2S. The highest gas production was obtained from the hot spring sample coded as PGR (1785.2–2259.8 mL H_2/L medium) followed by YLT (1074.4–2130.0 mL H_2/L medium), whereas the lowest quantity was observed from the hot spring sample coded as SRW1 (7.8–18.9 mL H_2/L medium). The thermophilic enriched cultures PGR and YLT exhibited satisfied hydrogen production yields indicating the potential role of microbial consortia in utilization of xylose and glucose without much variation in overall metabolism. This data is in accordance with the reported biohydrogen production from xylose and glucose using mixed culture or pure culture [16,17]. The observed higher cumulative hydrogen production of the enriched culture PGR from glucose, xylose–glucose mixed and xylose were 1785.2, 2259.8 and 1979.7 mL H_2/L medium, respectively these data gave the maximum hydrogen yields of 212.5, 241.4 and 235.4 mL H_2/g $sugar_{consumed}$, respectively. It was noted that hydrogen production from xylose was higher than that from glucose, suggesting that xylose-utilizing microorganism were dominant in the consortia. A cumulative hydrogen production of the enriched culture YLT from glucose, xylose–glucose

Table 1 – The list of geothermal spring sources and characteristics used for enrichment cultures.

Geothermal spring	Code	Temperature (°C)	pH
Ranong province			
Wattaphotharam	RNW	65	8.3
Suratthani province			
Wat Than Nam Ron 1	SRW1	63	7.0
Wat Than Nam Ron 2	SRW2	53	7.8
Rattanakosai 1	SRR1	67	7.9
Rattanakosai 2	SRR2	60	7.9
Phang Nga province			
Khong Pay Pao	PGK	60	6.5
Romanee	PGR	63	6.8
Yala province			
Ta Na Ma Rao	YLT	80	7.8

Table 2 – Characteristics of hydrogen production and soluble metabolites of thermophilic enriched cultures from various sources of geothermal spring in Southern Thailand by using xylose, xylose–glucose mixed and glucose.

Samples code	Enrichment substrates	H_2 production (mL/L)	H_2 yield (mL/g sugar comsumed)	Acetic acid (mM)	Butyric acid (mM)	Ethanol (mM)	Lactic acid (mM)	Butanol (mM)	Substrate consumed (%)
RNW	Glucose	54.3	9.4	3.2	1.0	0.2	4.9	13.6	57.6
	Glucose–xylose mixed	30.9	6.0	3.6	1.8	0.3	3.7	14.6	51.8
	Xylose	849.0	111.7	5.0	18.4	0.3	0.0	16.0	76.0
SRW1	Glucose	17.9	3.5	2.3	1.0	0.3	9.3	12.0	52.0
	Glucose–xylose mixed	18.9	3.2	1.7	2.2	0.4	9.1	15.6	60.6
	Xylose	7.8	1.4	1.6	3.4	0.6	2.7	17.6	61.6
SRW2	Glucose	40.7	6.5	2.0	9.0	0.2	6.0	13.4	63.6
	Glucose–xylose mixed	39.3	7.2	1.6	10.2	0.3	2.4	17.6	56.0
	Xylose	13.4	4.4	2.9	3.4	0.4	12.2	1.0	32.0
SRR1	Glucose	48.8	10.2	5.5	1.0	0.3	0.0	15.6	48.8
	Glucose–xylose mixed	104.4	18.6	4.6	2.3	0.4	3.4	17.6	56.0
	Xylose	12.8	3.3	2.1	3.8	0.6	13.2	2.4	40.0
SRR2	Glucose	489.2	90.6	8.8	17.0	0.3	7.3	2.4	54.0
	Glucose–xylose mixed	808.4	122.5	11.8	18.5	0.4	4.0	8.8	66.0
	Xylose	985.8	246.4	9.4	15.1	0.6	2.8	0.0	40.0
PGK	Glucose	1229.6	161.7	5.5	17.0	0.3	6.3	10.4	76.0
	Glucose–xylose mixed	1438.4	199.8	11.2	18.4	0.4	7.6	4.4	72.0
	Xylose	1451.4	165.3	25.6	19.6	0.5	6.1	8.6	88.0
PGR	Glucose	1785.2	212.5	7.2	21.4	0.6	8.2	4.4	84.0
	Glucose–xylose mixed	2259.8	241.4	13.5	20.6	0.3	10.5	6.8	96.0
	Xylose	1979.7	235.4	7.2	21.8	0.2	11.7	13.0	82.0
YLT	Glucose	1074.4	223.8	16.5	14.2	0.2	3.2	2.0	48.0
	Glucose–xylose mixed	1887.4	205.2	14.8	22.2	0.3	4.1	4.8	92.0
	Xylose	2131.0	231.6	16.4	17.8	0.4	5.3	4.4	92.0

mixture and xylose were 1074.4, 1887.4 and 2131.0 mL H_2/L medium, respectively, giving the hydrogen yields of 223.8, 205.2 and 231.6 mL H_2/g $sugar_{consumed}$, respectively. The hydrogen yields of the enriched cultures PGR and YLT were comparable to that obtained with sludge inoculum from a municipal sewage treatment plant (244 mL H_2/g $sugar_{consumed}$) at 60 °C [23]. The yield was higher than that obtained with inoculum from a continuous, H_2-producing bench-scale reactor (138 mL H_2/g $sugar_{consumed}$) at 35 °C [24]. The high hydrogen production was going along with the production of acetic acid and butyric acid (as shown in Table 2) with small amount of ethanol and propionic acid. On the other hand, low hydrogen production occurred with the presence of butanol and lactic acid. Lactic acid is not common as end product but intermediate product during fermentation under thermophilic condition [25]. The reason for lactic acid accumulation was not clear but likely related to environmental change during repeated cycle or substrate overloading [26]. It was suggested when environmental change, bacteria would try to optimize the carbon flow rate through the cell by using lactate as electron sink and lower down the acetate production, which gave high energy yield. As a result, high lactate was found in all enriched cultures.

3.2. *Development of enriched cultures PGR and YLT by repeated batch fermentation*

Repeated batch cultivations were conducted by transferring the cultures from the previous batch to the new medium containing the same sugar concentration. The thermophilic enriched cultures encoded as PGR and YLT gave the maximum hydrogen yields of 241.4 and 231.6 mL H_2/g $sugar_{consumed}$, respectively, in the first generation which correlated to 46–48% of the theoretical value (495 mL H_2/g sugar). After repeated batch cultivation, the hydrogen yields of the enriched culture PGR in xylose, xylose–glucose mixed substrate and xylose increased from 212.5, 241.4 and 235.4 to 343.0, 375.0 and 287.0 mL H_2/g $sugar_{consumed}$, respectively. For the enriched culture YLT, the hydrogen yields from xylose, xylose–glucose mixed substrate and xylose increased from 223.8, 205.2 and 231.6 to 234.0, 287.0 and 267.0 mL H_2/g $sugar_{consumed}$, respectively. The increase of hydrogen yield from xylose–glucose mixed substrate of PGR to 375.0 mL H_2/g $sugar_{consumed}$ was 30% higher than that of YLT (287.0 mL H_2/g $sugar_{consumed}$). The hydrogen yields reached the maximum values (287.0–375.0 mL H_2/g $sugar_{consumed}$) in the third transfer and no further increase thereafter (Fig. 1). This result indicated that bacteria can adapt to new condition by repeated batch cultivation to achieve the increased hydrogen yield. Similar observation was reported by Imachi et al. [27] that successful bacteria cultivation with propionate was achieved by more than 10 times repeated transfers. Interestingly, the thermophilic enriched culture PGR produced very high hydrogen from xylose and xylose–glucose mixed substrate and prefers xylose over glucose. Apparently, they also exhibited efficient degradation of xylose for hydrogen production as glucose. The final yield of hydrogen in the xylose–glucose mixed substrate was higher than single sugar medium. In other words, thermophilic enriched culture PGR could utilize the mixture of xylose–glucose from lignocellulose hydrolysate for hydrogen production.

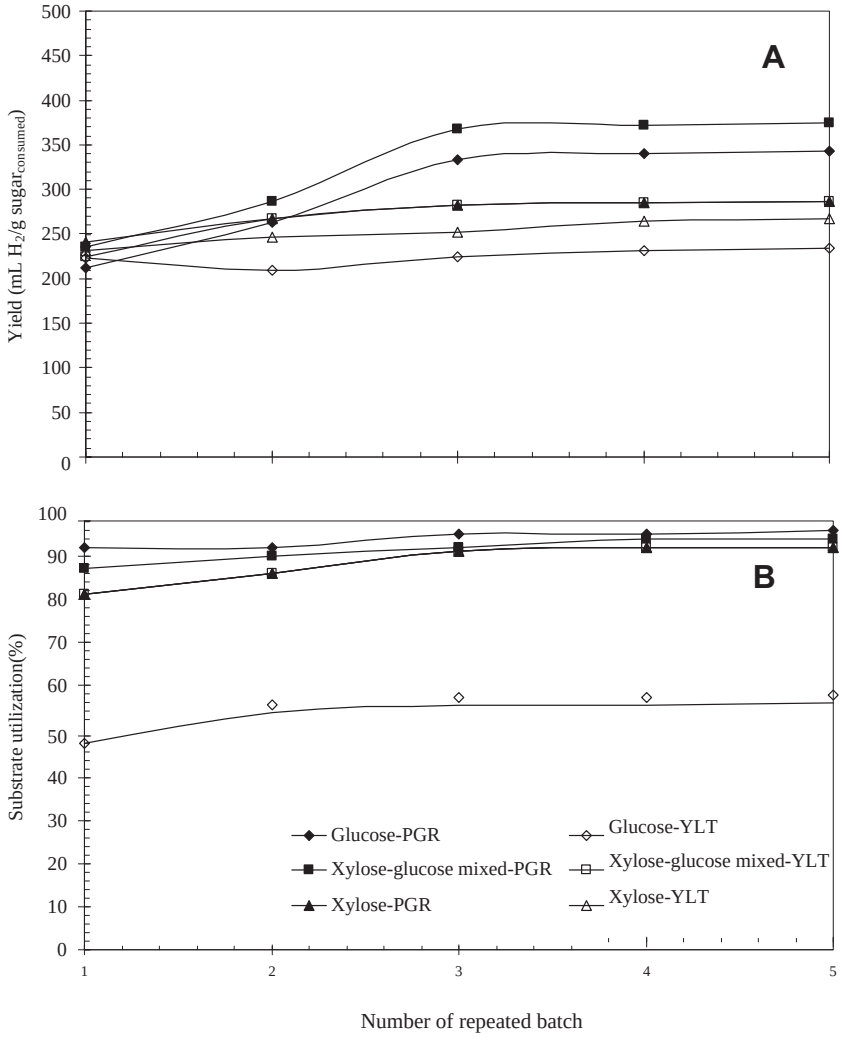

Fig. 1 – Hydrogen yield (A) and substrate utilization (B) at different transfer in the repeated batch cultivation of the enriched cultures PGR and YTL.

3.3. *Microbial community of thermophilic enriched culture PGR*

Bacterial community samples were taken from the first batch cultures and the final repeated batch cultures at 5th cycles. Diversity of bacterial community reduced when repeated batch cultures applied (Fig. 2). The results demonstrated that numerous thermophilic hydrogen-producing microorganisms in the enriched cultures could be obtained from liquid and sediment samples collected from hot springs in Southern Thailand. The DGGE profiles demonstrated that the community structures of the final repeated batch cultures had low microbial diversity and some was consisted of single band with high hydrogen production. The microbial community in the enriched culture PGR was affiliated with low G + C content Gram-positive bacteria, *Thermoanaerobacterium* sp. *Thermoanaerobacter* sp., *Caloramater* sp and *Anoxybacillus* sp. based on the 16S rRNA gene. The strong intensity of band related to *Themoanaerobacterium* sp., *T. thermosaccharolyticum*, *Thermoanaerobacter lactiethylicus* and *Caloramator indicus* indicated a very efficient hydrogen producer and corresponded with hydrogen production [21,22]. Genus *Caloramater* sp. was found in thermal water [28] and in enriched cow manure, which was capable to produce hydrogen from glucose and xylose [15]. *C. indicus* was proposed as a new hydrogen-producing strain with optimum temperature of 60–62 °C [29]. Phylogenetic analysis indicated that the dominant bacterial groups were low G + C Gram-positive bacteria, *Thermoanaerobacterium*, *Thermoanaerobacter*, *Caloramator* and *Anoxybacillus* sp. presented as major groups in the hydrogen production from the enriched cultures PGR with mixed sugars (xylose and glucose) (Fig. 3). The community structures of xylose enrichment revealed slightly difference in microbial community with the other carbon sources, which composed of high hydrogen-producing bacterial strains affiliated to *Caloramator*. Microbial consortia PGR from Thailand hot spring enriched with xylose–glucose mixed substrate showed that high hydrogen production was dominated by *Thermoanaerobacterium* species and *T. thermosaccharolyticum*. PGR showed promising to apply for biohydrogen production from lignocellulosic hydrolysate with

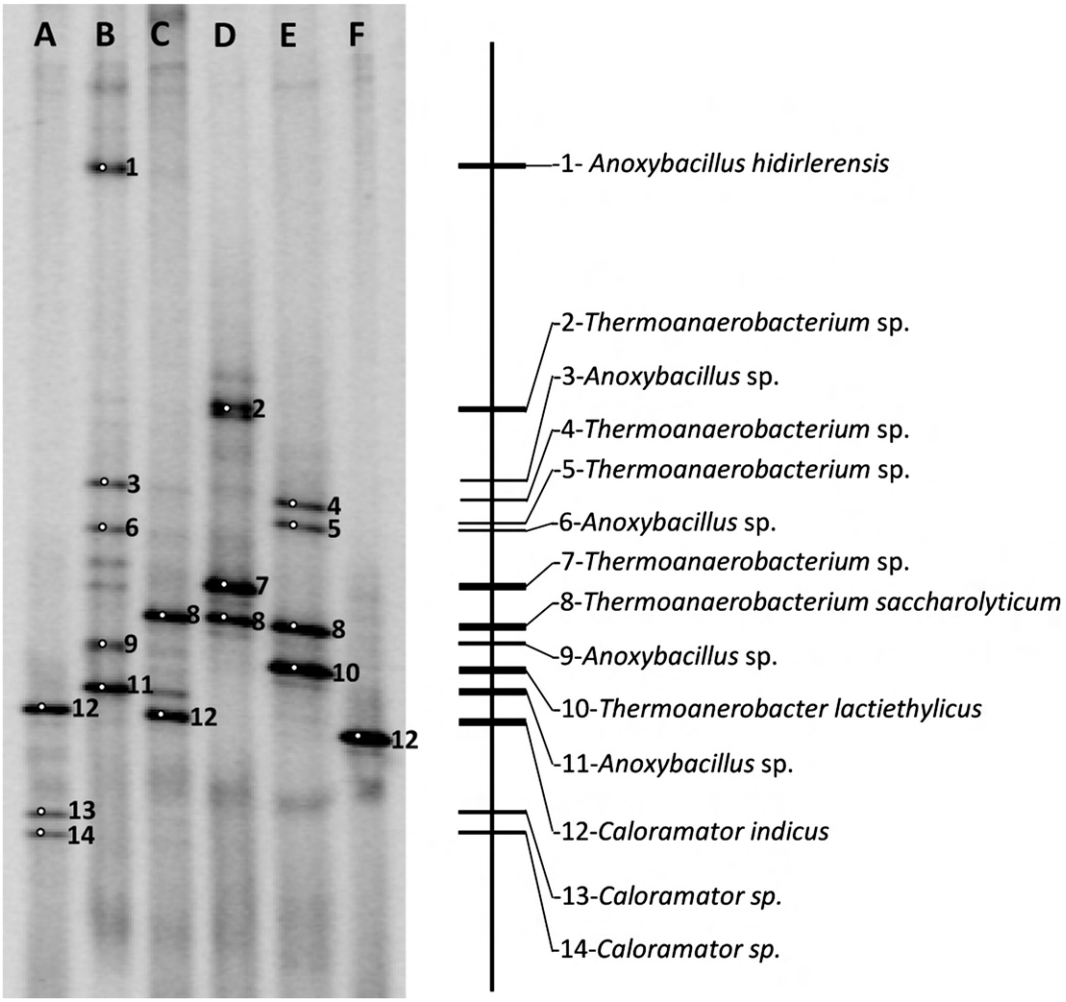

Fig. 2 – DGGE profiles of V3 region of 16S rDNA from the first batch of thermophilic enriched cultures PGR (A, B and C) and the final repeated batch cultures at 5th cycle (D, E and F). A and D enriched with glucose; B and E enriched with xylose–glucose mixed substrate; C and F enriched with xylose.

mostly contained mixed xylose and glucose. No amplification products of archaeal 16S rRNA genes were obtained from the DNA extracts of the enriched cultures samples with a nested PCR procedure, while the positive control (DNA from anaerobic digester sludge) had a high amplification yield (data not shown). This result indicated the absence of archaea in the enriched cultures.

3.4. *Hydrogen production by thermophilic enriched cultures PGR with hydrolysate of oil palm trunk*

Thermophilic enriched culture PGR was selected to test for hydrogen production from hydrolysate of oil palm trunk. Hydrogen production in the batch culture at different hydrolysate concentrations of 10%, 20%, 40%, 60% and 80% (v/v) was conducted (Table 3). Hydrogen production increased with the increase of hydrolysate concentration up to 40% and decreased thereafter with no hydrogen was produced at hydrolysate concentration of 80% (v/v). The results indicated that the efficient thermophilic hydrogen-producing microbial consortia PGR could be employed at higher hydrolysate loading of 40% (v/v) compared with that from the extreme thermophilic mixed culture (70 °C) operated at 30% (v/v) hydrolysate in both batch and continuously fed reactors [30]. Kongjan et al. [30] also found that the high hydrolysate concentration inhibited the hydrogen fermentation process because lignocellulosic pretreatment generated not only mixed sugars but also some toxic compounds such as phenolics, furfurals and 5-hydroxymethyl-2-furaldehyde (HMF) that inhibiting the fermentation process [30–32]. The maximum hydrogen yield of 301 mL H_2/g $sugar_{consumed}$ was achieved at hydrolysate concentration of 40% (v/v). The thermophilic enriched culture PGR degraded completely reducing sugars in the old oil palm trunk hydrolysate to hydrogen, acetate, butyrate, lactic acid and butanol. The tests with synthetic medium of the same reducing sugar levels also revealed a similar result. Specifically, the thermophilic enriched culture PGR produced biohydrogen from oil palm trunk hydrolysate and synthetic medium at 206–310 mL H_2/g $sugar_{consumed}$. Apparently, the components in hydrolysate of

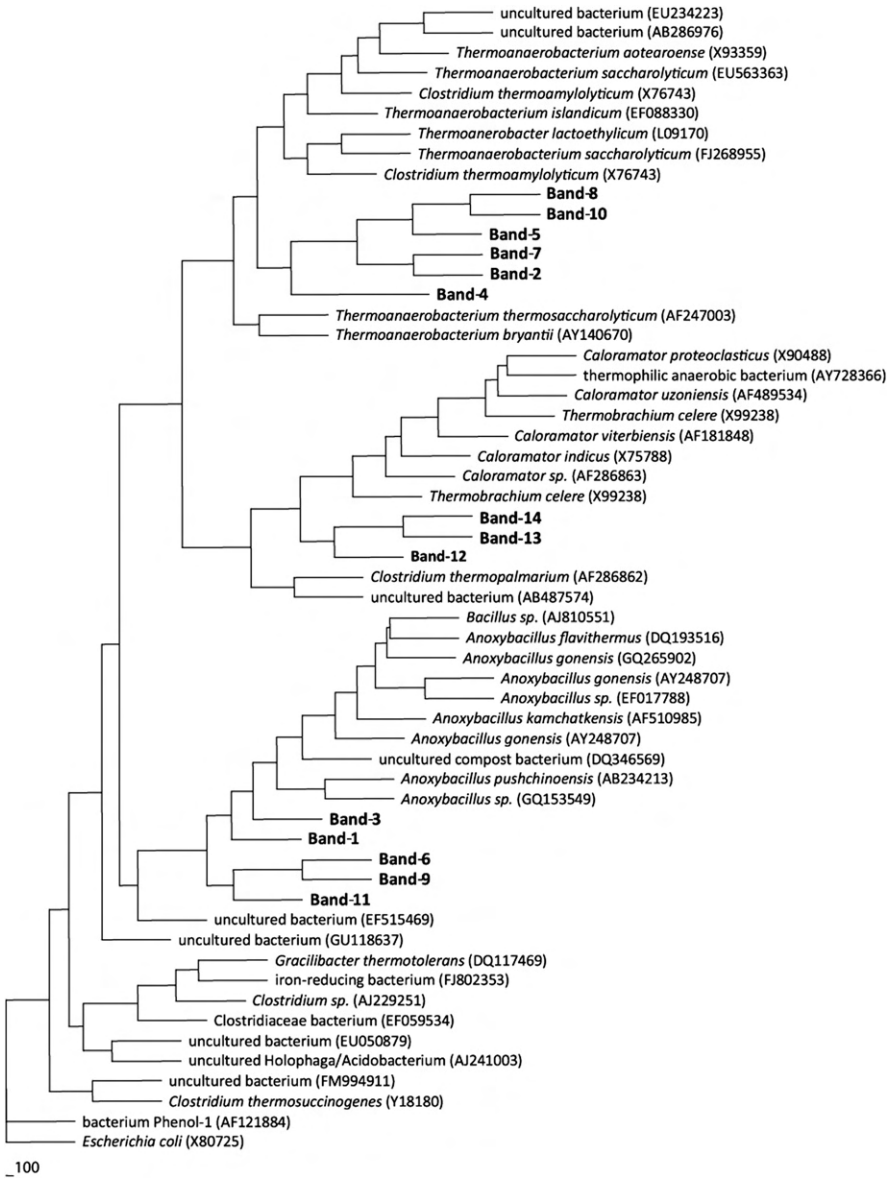

Fig. 3 – Phylogenetic tree of the dominated thermophilic enriched cultures PGR from Southern Thailand geothermal spring.

oil palm trunk inhibited the growth of thermophilic enriched cultures PGR at concentration higher than 40% (v/v). Additionally, the thermophilic enriched cultures PGR produced equally well the biohydrogen from old oil palm trunk hydrolysate and that from mixed pure glucose and xylose at 40% (v/v) corresponded to 9.92 g total sugar which produced 301 mL H_2/g $sugar_{consumed}$. The enriched culture PGR was noted to effectively assimilate reducing sugars and showed

Table 3 – Characteristics of hydrogen production and soluble metabolites of thermophilic enriched cultures PGR during batch experiment at different hydrolysate concentration.

Hydrolysate (% v/v)	Sugar concentration (g/L)	H_2 production (mL/L-medium)	H_2 yield (mL/g $sugar_{consumed}$)	Acetic acid (mM)	Butyric acid (mM)	Ethanol (mM)	Lactic acid (mM)	Butanol (mM)
10	2.48	768	310	5.4	6.3	0.5	2.3	1.2
20	4.92	1498	306	7	11	0.9	3.8	2.3
40	9.92	2981	301	22	23	1.2	5.3	5.4
60	14.88	3048	206	27	29	2.9	6.2	8.8
80	19.84	–	–	–	–	–	–	–

promising to apply for biohydrogen production from hemicellulose hydrolysate from old oil palm trunk.

4. Conclusion

Potential thermophilic microorganisms for hydrogen production were enriched from Southern Thailand hot springs possessing a wide temperature range (53–80 °C) and pH range (6.5–8.3). The enriched cultures PGR produced a high hydrogen yield from glucose, xylose and mixture of both sugars at 60 °C with the maximum value of 375 mL of H_2/g $sugars_{consumed}$. Enriched cultures PGR was dominated by bacteria closely affiliated with *Thermoanaerobacterium*, *Thermoanaerobacter* and *Caloramator*, promising bacterial enrichments were obtained from Southern Thailand hot spring samples with a high potential for hydrogen production under thermophilic conditions from lignocellulose hydrolysate.

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ภาคผนวก 4

Hydrogen production from oil palm trunk hemicellulose hydrolysate by thermophilic bacteria isolated from Southern Thailand hot spring

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Abstract

Thermophilic hydrogen producing bacteria were isolated from eight hot springs in Southern Thailand. Two enrichments consortia were used for isolation. A total 170 isolates were screened by denaturing gradient gel electrophoresis of V3 region of 16S rRNA gene and 6 candidates were selected for screening test on hydrogen production. The test was composed of three steps; (i) test for conversion of xylose to hydrogen; (ii) test for conversion of mixed sugars (xylose and glucose) to hydrogen; (iii) test for hydrogen production from pretreated oil palm trunk hemicellulose hydrolysate. Six isolates, namely AH1-AH6 were selected as they successfully fulfilled that the criteria defined for the screening test. Analysis of 16S rRNA gene revealed that the strains AH1, AH2, AH3 and AH4 belong to the genus *Thermoanaerobacterium*, whereas the strain AH5 belong to genus *Caloramator* and the isolate AH6 belong to various clusters of *Clostridium*. They degraded both xylose and glucose into hydrogen with the production yields of 278, 292, 280, 310, 150 and 225 mL H_2/g sugar, respectively and produce hydrogen in 50% palm oil trunk hydrolysate with glucose (7.5 g/L), mannose (0.7 g/L), xylose (4.8 g/L), galactose (1.05 g/L), furfural (2.15 g/L), 5-hydroxymethyl-2-furaldehyde (HMF) (1.15 g/L) and acetic acid (1.25 g/L) as substrate. All of the five effective strains showed promising application for biohydrogen production from hemicellulose hydrolysate of palm oil trunk.

Keywords: biohydrogen, thermophilic fermentation, hemicelluloses hydrolysate, xylose

1. Introduction

Dark fermentative hydrogen production has attracted increasing attention recently due to its high rate of hydrogen evolution and its applicability to different types of organic wastes and wastewaters from industrial processes [1]. Furthermore, production of hydrogen from organic wastes is not only cleans up the environment but also produces a clean and readily usable energy in a sustainable fashion [2]. Microbiological hydrogen production is considered one of the most promising alternatives for sustainable production. Biohydrogen processes are feasible for production hydrogen at a commercial scale with a low cost to meet the need of sufficient and cost effective energy supply. Consequently, the substrate used for fermentative hydrogen production must be abundant, easily available, and inexpensive. From these aspects, lignocellulosic materials obtained from crop or food industrial wastes could be a commercially viable as biohydrogen feedstock [3] including palm empty fruit branch (EFB). Lignocellulose contains a large amount of cellulose (40-50%), hemicellulose (25-30%), and lignin (10-20%) [4]. Pre-treatment of lignocellulose generates hemicellulose hydrolysate containing mixture of glucose and xylose. A few pentose-fermenting microorganisms have been identified compared to hexose-fermenting microorganisms and information on their conversion to hydrogen by defined cultures or mixed microflora is very limited [5].

Natural dark fermentative inoculum or mixed consortia have advantages over pure inoculum in allowing process at non-sterile environment and capable of dealing with mixture of substrate due to a high microbial diversity [6,7], which significantly reduce the process operation cost with low biomass yield. In addition, high temperatures favour the stoichiometry of hydrogen production, resulting in higher hydrogen yields

and [8] have a narrower spectrum of end products as compared to mesophilic fermentation [9] as well as reduce the contaminants. Furthermore, thermophilic bacteria are able to utilize mixed hexose and pentose for hydrogen production [10] with hydrogen yields of 2.32-2.37 mol-H_2/mol-substrate from *Thermoanaerobacterium thermosaccharolyticum* strain PSU-2, *T. thermosaccharolyticum* strain W16 and extreme thermophilic *Caldicellulosiruptor saccharolyticus* [11,12,13]. However, the problem of fermentation of lignocellulosic material-based substrate process is the inability of microorganisms to metabolize efficiently all mixed carbohydrates (hexose and pentose) into hydrogen with satisfied yield and productivity. This was because of the pre-treated hydrolysate contains various inhibitors of microbial fermentation such as weak organic acids like acetate, furan derivatives and phenolics monomers from sugar and lignin degradation respectively [14].

Oil palm trunk (OPT) was with over 25 years old need to be cut down for replantation. At present, they left unused in the oil palm plantation. These studies aimed to pretreat the old OPT and use the hemicellulose hydrolysate as feedstock for hydrogen production. Thermophilic anaerobic bacterial strains isolated from different natural geothermal springs and thermophilic anaerobic enriched cultures were tested for their capability to produce hydrogen from D-xylose and hemicellulose hydrolysate from oil palm trunk.

2. Materials and Methods

2.1 Hot spring samples and enrichment method

Sediment-rich water samples were collected from eight hot springs located in Southern Thailand [15]. Temperature and pH of samples were measured in the field

using thermometer and pH paper. The samples were kept in serum bottles for return to the laboratory.

Enrichment hydrogen-producing cultures were developed according to the procedure described previously [16]. In brief, A basic anaerobic medium (BA medium, pH 5.5) was prepared by using xylose, glucose and mixture of both sugars as a carbon source. For each site, 3 mL of sediment-rich water was transferred by sterile syringe into 27 mL BA medium supplemented with 5 g/L each of xylose, glucose and the mixture of these sugars in the ratio 1:1 [17] as a carbon source and incubated at 60°C for 2 day at strictly anaerobic conditions. During the enrichment, H_2, CO_2 and CH_4 and soluble metabolites were monitored. The hydrogen-producing enriched cultures were used in five successive batch cycles (10% inoculum in medium, 2 days cultivation per cycle). Two promising enriched cultures were used further for isolation and screening for their hydrogen production from xylose and hemicellulose hydrolysate.

2.2 Isolation of new thermophilic strains

Bacterial strains were isolated at 60°C and at neutral pH using the anaerobic roll-tube technique [18] with medium solidified by 1.2% (w/v) Gellan gum and using 5 g/L D-xylose as substrate supplemented with 1 g/L yeast extract. Colonies were picked up using sterile pasteur pipettes and the procedure was repeated until only one colony type was obtained.

2.3 Preparation of hemicellulose from palm oil trunk

The hydrolysate was prepared using the two stage diluted acid and base hydrolysis, the first stage of hydrolysis was carried out in 0.1% H_2SO_4 in autoclave for 25 min at 120°C and pressure at 1 bar with 30% w/w of solid concentration. After pretreatment, the liquid hemicellulose fraction (the hydrolysate) was separated from the

cellulose-rich fraction of the palm oil trunk by filtration. In second stage, hydrolysis of remaining solid was carried out in 0.1% NaOH in autoclave for 25 min at 120°C and pressure at 1 bar with 30% w/w of solid concentration. The second hydrolysate were mixed with the first stage hydrolysate and stored at 4°C before used. The composition of the hydrolysate is presented in Table 1. Hydrolysate medium with hemicellulose concentrations from 10 to 100% (v/v) were prepared and supplemented with the same concentrations of minerals, trace metals and yeast extract as described above for mineral anaerobic medium. Different concentrations of soluble hemicellulose fraction of two stage diluted acid and base oil palm trunk were prepared by addition of the respective volume of redistilled water given the desired concentrations (10-100% v/v).

2.4 Analytical methods

The evolved biogas was collected in a headspace of serum bottle. The total volumes at each time interval were measured at room temperature by syringe. Hydrogen and carbon dioxide in the biogas were measured by gas chromatography (Shimadzu GC-8A) equipped with thermal conductivity detector (TCD). Hydrogen and methane gas were analyzed by GC-TCD with a protocol according to O-Thong et al.[19] using gas sample volumes of 100 µL for methane and 500 µL for hydrogen injected in duplicate. The culture broth was centrifuged at 10000*xg* for 10 min. Fermentation end products (volatile fatty acids and ethanol) in the supernatant were determined by gas chromatograph (HP6850) equipped with a flame ionization detector (FID) and Stabilwax-DA column (dimensions 30 m x 0.32 mm x 0.25 µm). The temperature of the injection port was 230°C. The chromatography was performed using the following program: 70°C for 1 min, 70-180°C with a ramping of 20°C/ min, 180°C for 6 min. The detector temperature was 250°C. Lactic acid, xylose and glucose were analyzed with a

high performance liquid chromatograph (Agilent 1200 series), equipped with Aminex® HPX-87H ion exclusion column. The isolated strains from the enriched culture were grouped using PCR-DGGE as described by Prasertsan et al. [20].

2.5 Screening test

The screening program for testing the bacterial strains and the enriched cultures consisted of the following three steps. Fermentation of D-xylose to hydrogen: All strains were inoculated into the cultivation medium at neutral pH containing D-xylose in the range 5–15 g/L and 1 g/L yeast extract, incubated at 60°C for 4 days. Fermentation of hemicellulose hydrolysate from oil palm trunk to hydrogen: All strains were inoculated into serum vials containing the hydrolysate in concentrations of 10–100% (v/v) at neutral pH and incubated at 60°C for 7 days. For production of hydrogen from mixed sugars D-xylose and glucose at ratio 1:1 were added to the medium with neutral pH. The strains were incubated at 60°C for 4 days.

3. Results and discussion

3.1 Hydrogen production from xylose, xylose glucose mixed and glucose by the enriched cultures

Sediment-rich water samples from hot springs were used for establishing enriched cultures. A total of 8 enrichment cultures were tested for conversion of xylose, glucose and mixture of both sugars to hydrogen at neutral pH and 60°C. All the enriched cultures from the hot spring samples can produce hydrogen (Table 2) with two of them namely PGR and YLT gave the highest hydrogen yields of 301.3 and 297.4 mL H_2/g sugar from xylose glucose mixed and xylose as substrate, respectively. These correspond with the substrate utilization of 96% and 92% respectively. These samples

were therefore considered to be of interest for isolation of thermophilic hydrogen-producing bacteria.

3.2 Isolation of thermophilic hydrogen producing bacterial strains

The two selected enriched cultures (PGR and YLT) were selected for isolation. Colonies were visible after 2–4 days incubation time with single colony presented on 10^{-5} to 10^{-7} dilution rate cultures and a total of 171 single colonies were isolated from YLT (93 colonies) and PGR (78 colonies). Six of them were grouped by denaturing gradient gel electrophoresis (DGGE) profiles (Fig. 1 and Fig. 2). The hydrogen yields (180-327 mL H_2/g sugar) were achieved from the group strains tested for hydrogen production with corresponding to the original enriched cultures. 16S rRNA gene analysis revealed that the strains AH1, AH2, AH3 and AH4 gave the closest phylogenetic relative to genus *Thermoanaerobacterium*, the isolate AH5 belong to genus *Caloramator* and the strain AH6 belong to various clusters of genus *Clostridium* (Fig. 3). Several species of the genus *Thermoanaerobacterium* have been reported hydrogen-producing species including *T. islandicum*, *T. aciditolerans*, *T. aotearoense* and *T. thermosaccharolyticum* with glucose and xylose as substrate [21]. This strain is rod-shaped, Gram-positive, strictly anaerobic, and spore-forming [22]. *Caloramator* sp. was found in enrichment culture for hydrogen-producing extreme thermophilic anaerobic microflora from cow manure enriched with glucose and xylose by repeated batch cultures at 75°C [10] and in biohydrogen production from palm oil mill effluent with ASBR reactor tank system [20]. The bacterial strain related to *Caloramator indicus* were proposed as the new hydrogen-producing strain with optimum temperature 60-62^{o}C [23,24] and *Caloramator viteribiensis* has been isolated from sediment/water of a hot spring at Bagnaccio with growth optimum at 58°C and pH 6-6.5 [25]. The genus

Clostrium, C. thermohydrosulfuricum and *C. thermocellum* have been reported for hydrogen production with glucose and xylose fermentation [26,10].

3.3. Hydrogen production from xylose and hemicellulose hydrolysate of oil palm trunk by the selected isolates

The six thermophilic anaerobic bacterial strains (AH1-AH6) originating from hot springs in Southern Thailand were tested for hydrogen production by using 5 g/L xylose as substrate. Results (Table 3) indicated that they produced high hydrogen yields in the range of 180-327 mL H_2/g sugar with the highest H_2 yield of 327 mL H_2/g sugar was obtained from AH4 (*Thermoanaerobacterium* sp.). All strains completed xylose fermentation with acetic and butyric final end product. The isolated strain related with *Thermoanaerobacterium* sp. shown higher average hydrogen production than *Clostridium* sp. and *Caloramator* sp. and produced hydrogen yield in comparable with other reported such as *T. thermoanaerobacterium* PSU2 and *T. thermoanaerobacterium* W16 with hydrogen yield of 281 and 290 mL H_2/g sugar, respectively. For tested hydrogen production from palm oil trunk hydrolysate, the results shown that all isolated strains produce hydrogen in 50% hydrolysate medium and the highest hydrogen yield (310 mL H_2/g sugar) was achieved from the strain AH4. Hydrogen production yields were shown slightly lower than tested with pure xylose of all strains. *Caloramator* sp. (AH5) gave about 2-folds lower hydrogen yields (150 mL H_2/g sugar) than the strain AH4 (310 mL H_2/g sugar). The present of toxic compound in hydrolysate such as Furfural and 5-hydroxymethyl-2-furaldehyde (HMF) shown more affected to *Caloramator* sp. than *Thermoanaerobacterium* sp.. This mention could observe from studies community analysis of hydrogen production with hydrolysate usually found *Thermoanaerobacterium* sp. as dominant species whereas no *Caloramator* sp. [27,20].

The hydrogen yields of isolated strains were comparable with other thermophilic hydrogen-producing strain in previously report as showed in Table 4.

4. Conclusion

Among 171 isolates of thermophilic anaerobic hydrogen producing bacteria, 6 strains demonstrated high hydrogen producers. Four strains belong to the genus *Thermoanaerobacterium*, one isolate each belong to the genus *Caloramator* and *Clostridium*. They were able to utilize xylose and glucose as well as hemicellulose hydrolysate from oil palm trunk into hydrogen. Hence, all of the six effective strains showed promising application for biohydrogen production from hemicellulose hydrolysate of oil palm trunk.

Acknowledgements

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Figure captions

Fig. 1. DGGE profiles grouping of thermophilic hydrogen producing bacteria isolated from the enriched cultures YLT

Fig. 2. DGGE profiles grouping of thermophilic hydrogen producing bacteria isolated from the enriched cultures PGR

Fig. 3. Phylogenetic tree of the grouping isolated strains base on 16S rRNA gene sequences closet strains. The numbers at the nodes indicate the levels of bootstrap with 1000 replicates. Bar 100 distance of sequence and GenBank accession numbers are given in parentheses.

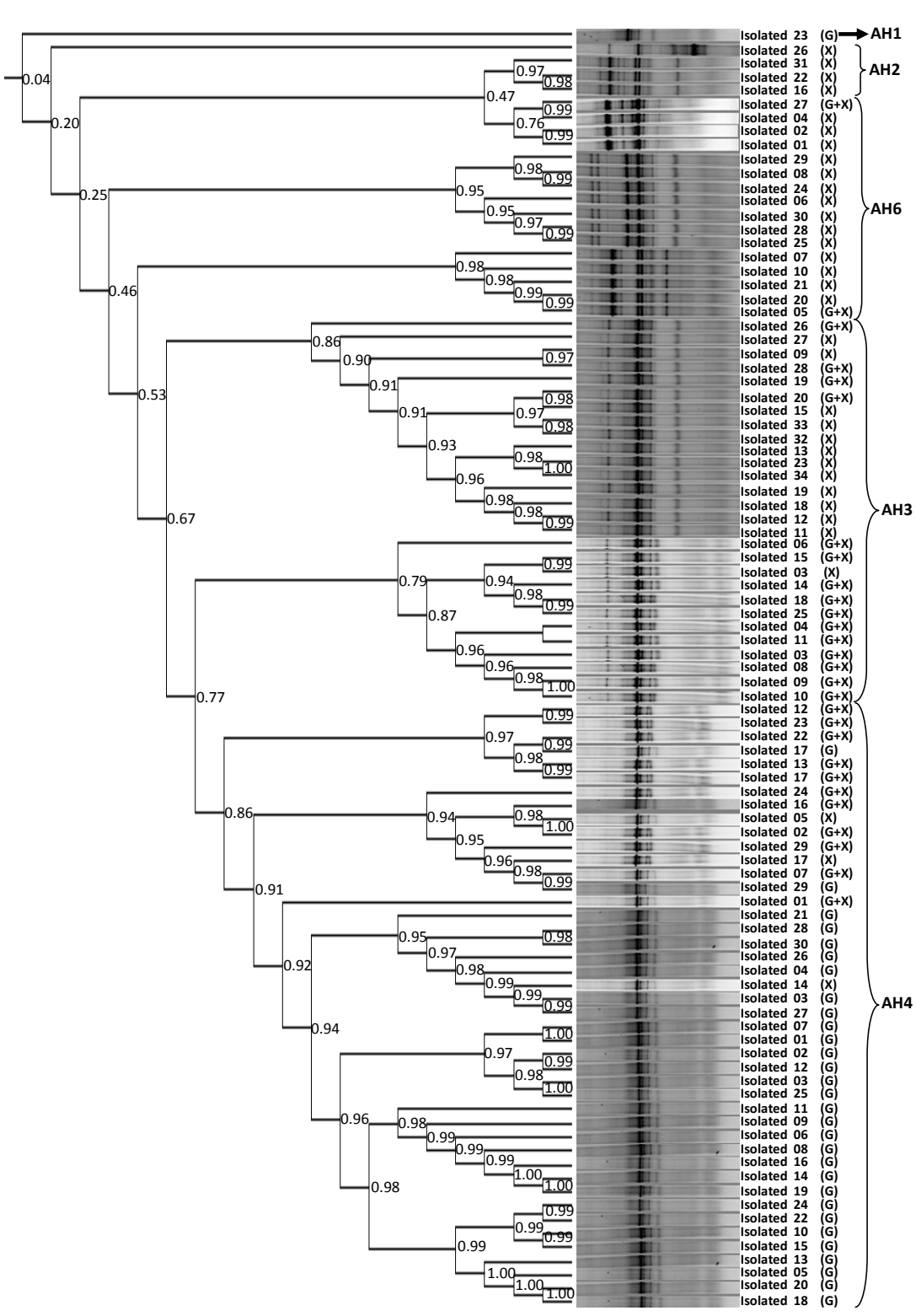

Fig. 1. DGGE profiles grouping of thermophilic hydrogen producing bacteria isolated from the enriched cultures YLT.

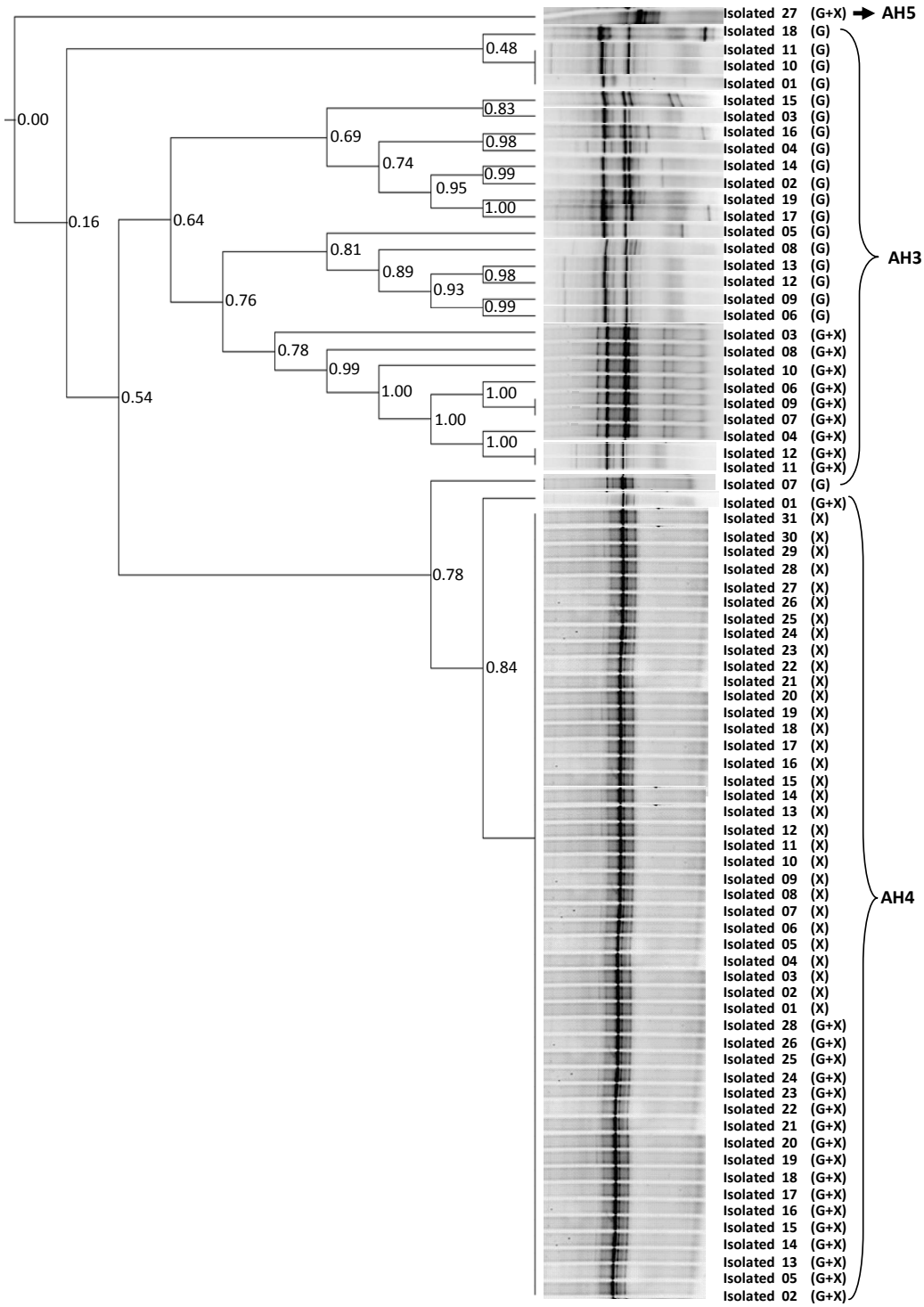

Fig. 2. DGGE profiles grouping of thermophilic hydrogen producing bacteria isolated from the enriched cultures PGR.

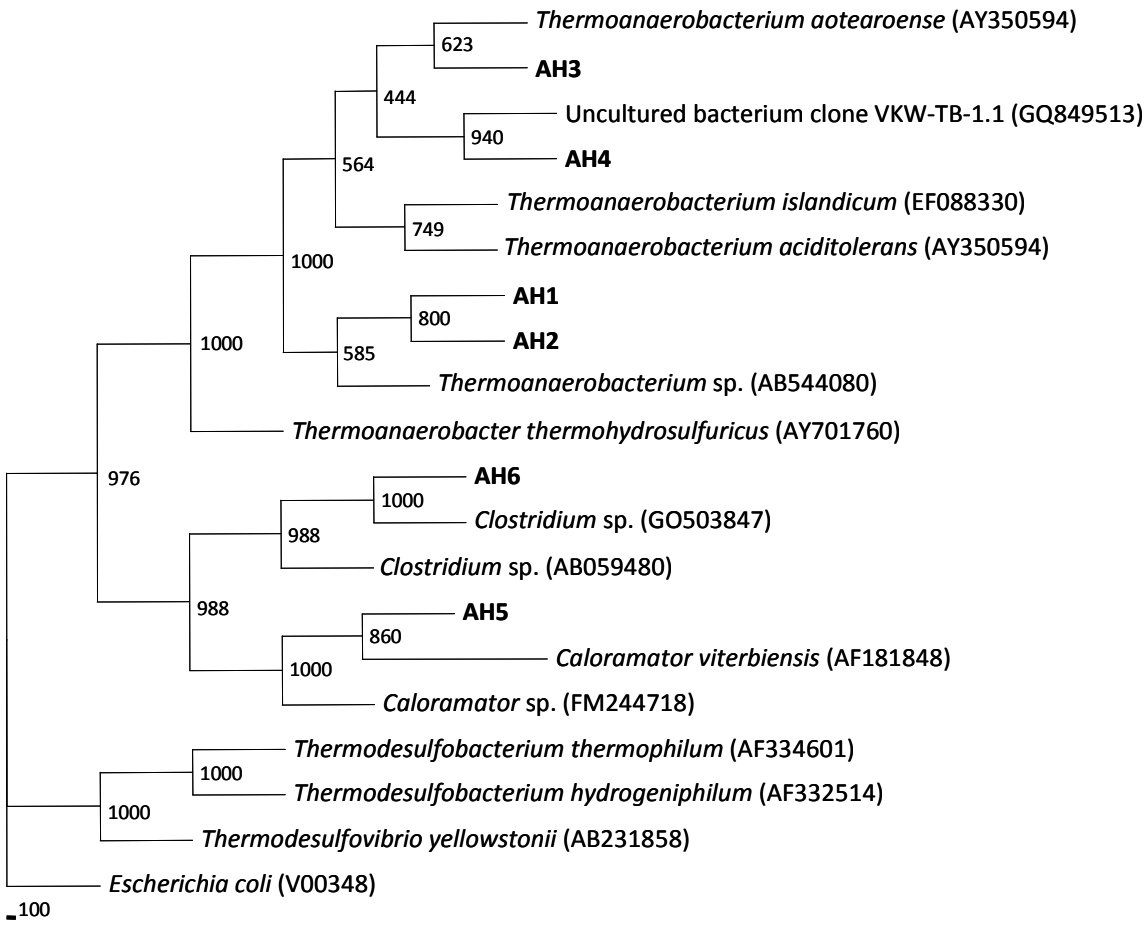

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Table 1.

Composition of two stage diluted acid and base hydrolysate of oil palm trunk

Component	Concentration (g/L)
Glucose	15
Mannose	1.4
Xylose	9.6
Galactose	2.1
Furfural	4.3
5-hydroxymethyl-2-furaldehyde (HMF)	2.3
Acetic acid	2.5

Table 2.

Hydrogen production by thermophilic enriched cultures from Thailand hot springs using glucose (G), xylose (X) and glucose xylose mixed (G+X) as substrates.

Samples	Substrates	H_2 yield (mL H_2/ g sugar)	Substrate utilization (%)
RNW	G	7.2	57.6
	G + X	4.1	51.8
	X	113.2	76.0
SRW1	G	2.4	52.0
	G + X	2.5	60.6
	X	1.1	61.6
SRW2	G	5.4	63.6
	G + X	5.2	56.0
	X	1.8	32.0
SRR1	G	6.5	48.8
	G + X	13.9	56.0
	X	1.7	40.0
SRR2	G	65.2	54.0
	G + X	107.7	66.0
	X	131.4	40.0
PGK	G	163.9	76.0
	G + X	191.7	72.0
	X	193.5	88.0
PGR	G	238.1	82.0
	G + X	301.3	96.0
	X	263.9	84.0
YLT	G	156.5	48.0
	G + X	264.9	92.0
	X	297.4	92.0

Table 3.

Performance of screening pure cultures by DGGE of the six selected strains and their capability to produce hydrogen from pure xylose and oil palm trunk hydrolysate (50% v/v dilution)

Strain	Classification	Pure xylose		Oil palm trunk hydrolysate	
		H_2 yield (mL H_2/g sugar)	**Substrate utilization (%)**	**H_2 yield (mL H_2/g sugar)**	**Substrate utilization (%)**
AH1	*Thermoanaerobacterium* sp.	260	100	278	91
AH2	*Thermoanaerobacterium* sp.	298	100	292	88
AH3	*Thermoanaerobacterium* sp.	310	100	280	92
AH4	*Thermoanaerobacterium* sp.	327	100	310	94
AH5	*Caloramator* sp.	180	100	150	68
AH6	*Clostridium* sp.	240	100	225	91

Table 4.

Effective comparison on hydrogen production of selective mesophilic, thermophilic, extreme thermophilic and hyper thermophilic hydrogen-producing strains

Microorganisms	Substrates	Condition			H_2 Yields	References
		Culture	T (°C)	pH	(mol/mol sugar)	
Thermotoga neapolitana	Glucose	Batch	75	6.9	2.90	[28]
Thermotoga elfii	Glucose	Batch	65		3.30	[29]
Clostridium thermolacticum	Lactose	Continuous	58	7.0	1.50	[30]
Clostridium thermobutyricum	Glucose	Batch	57	8.0	1.13	[31]
Clostridium thermosaccharolyticum	Glucose	Batch	55	5.4	1.72	[32]
*Clostridium sp.(*AH6)	Xylose	Batch	60	5.5	1.60	This study
Calidicellulosiruptor saccharolyticus	Glucose	Batch	72	6.9	3.40	[28]
Calidicellulosiruptor kristjanssonii	Glucose + xylose (1:1)	Batch	70	6.5	3.00	[13]
Calidicellulosiruptor owensensis	Glucose + xylose (1:1)	Batch	70	6.5	2.70	[13]
Thermoanaerobacterium thermosaccharolyticum W16	Xylose	Batch	60	6.5	2.19	[11]
Thermoanaerobacterium thermosaccharolyticum PSU2	Sucrose	Batch	60	6.3	2.53	[12]
Thermoanaerobacterium sp.-AH1	Xylose	Batch	60	5.5	1.73	This study
Thermoanaerobacterium sp.-AH2	Xylose	Batch	60	5.5	1.99	This study
Thermoanaerobacterium sp.-AH3	Xylose	Batch	60	5.5	2.07	This study
Thermoanaerobacterium sp.-AH4	Xylose	Batch	60	5.5	2.18	This study
*Caloramator sp.*AH5	Xylose	Batch	60	5.5	1.20	This study

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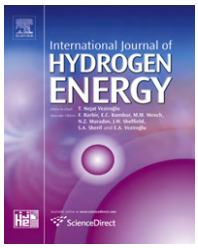

Biohydrogen production from cassava starch processing wastewater by thermophilic mixed cultures

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ABSTRACT

Natural microbial consortia from hot spring samples were used to developed thermophilic mixed cultures for biohydrogen production from cassava starch processing wastewater (CSPW). Significant hydrogen production potentials were obtained from three thermophilic mixed cultures namely PK, SW and PR with maximum hydrogen production yields of 249.3, 180 and 124.9 mL H_2/g starch, respectively from raw cassava starch and 252.4, 224.4 and 165.4 mL H_2/g starch, respectively from gelatinized cassava starch. Acetic acid-ethanol and acetic-lactic acid type fermentation were observed in cassava starch fermentation, based on three thermophilic mixed cultures performance. The thermophilic mixed cultures PK, SW and PR exhibited the maximum hydrogen yield of 287, 264 and 232 mL H_2/g starch in CSPW, respectively corresponding to 53%, 48.7% and 42.8% of the theoretical values. Phylogenetic analysis of thermophilic mixed cultures revealed that members involved cassava starch degrading bacteria and hydrogen producers in both raw cassava starch and CSPW were phylogenetically related to the *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum*, *Anoxybacillus* sp., *Geobacillus* sp. and *Clostridium* sp.

1. Introduction

Microbiological hydrogen production is considered to be one of the most promising alternative methods for renewable energy production. The challenge of biohydrogen processes is the feasibility of producing hydrogen at a commercial scale with a cost effective energy supply. Consequently, the substrate used for fermentative hydrogen production must be abundant, easily available and inexpensive. From those aspects, starch from crop or food industrial wastes could be a commercially viable biohydrogen feedstock [1]. Dark fermentative hydrogen production has attracted increasing attention recently due to its high rate of hydrogen evolution and its applicability to different types of organic wastes and wastewaters [2]. Furthermore, production of hydrogen from organic wastes is not only clean up the environment but also produces a clean and readily usable energy in a sustainable fashion [3]. Much of the waste is carried in water and is a product of food processing usually having high chemical oxygen demand (COD) values. Food processing wastewater mostly contained starch because starch is one of the major components in many agricultural products and biopolymer in foods [4] as well as the most abundant energy storage reserve carbohydrate in plants.

Thailand produces about 18 million tons of cassava starch per year. One kilogram of fresh roots yields 0.2 kg of starch,

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0.4–0.9 kg of cake and about 5–7 L of wastewater [5]. Cassava starch processing wastewater (CSPW) is a carbohydrate-rich waste. The wastewater contains high biochemical oxygen demand (BOD), COD and total solids (TS) as organic substances are extracted from the cassava roots. Starch can be hydrolyzed into glucose and maltose by acid or enzymatic hydrolysis followed by biological conversion of the carbohydrates into organic acids and then into hydrogen gas [1]. It is thus possible to treat these effluents under thermophilic condition, which presumably also favored the degradation kinetics and destruction of pathogens. Thermophilic bacteria utilize a wide range of organic wastes and produce less variety of fermentation end products compared to mesophiles [6]. Moreover, thermophiles can produce higher amounts of H_2 by increasing acetate production while decreasing butyric acid, ethanol and lactic acid during fermentation [7]. Thus, a higher temperature is more feasible for H_2 production due to favorable thermodynamics [8]. Although working at thermophilic temperatures may cause higher costs for heating, a smaller reactor volume due to short HRT as a consequence of higher hydrolysis and hydrogen production rate at thermophilic temperature could compensate the energy expenses from temperature increased. The costs for operation at higher temperatures will depend largely on the heat exchange efficiency of the plant, insulation of reactors, etc. It has been found that Danish biogas plants, operating at thermophilic temperature (55 °C), the energy cost for operating at 55 °C is approx. 10% of the energy produced at the plant. The extra energy cost for operating at thermophilic compared to mesophilic temperature is marginal (1–2%). Additionally, according to the regulations, biogas plants (and presumably also biohydrogen plants), have to include a sanitation step where biomass is heated up to 70 °C for 1 h, in order to secure sanitation of the effluents. Consequently, a combined sanitation and fermentation step will probably, reduce the total operation and construction expenses [9]. Thermophilic fermentative bacteria such as *Clostridium* and *Thermoanaerobacterium* can produce hydrogen from carbohydrate-rich waste at elevated temperatures [10]. However, the initial hydrolysis is the rate-limiting step in microbial conversion of starch as it dominates the efficiency of dark fermentative hydrogen production. Information on the conversion of starch to hydrogen by defined cultures or mixed microflora is very limited [11]. In addition, a few studies have been conducted to produce hydrogen from carbohydrate-rich wastewater under thermophilic condition [12].

This study aims to investigate the feasibility of using enriched cultures from a hot spring located in Southern Thailand for hydrogen production from cassava starch and cassava starch processing wastewater (CSPW). The microbial populations of the thermophilic hydrogen-producing mixed cultures were analyzed using DGGE techniques.

2. Materials and methods

2.1. Enrichment of thermophilic hydrogen-producing mixed cultures

The hydrogen-producing mixed cultures were enriched from sediment samples collecting from a geothermal spring located in Southern Thailand. Temperature of the sampling site ranged from 53 °C to 68 °C. Enriched thermophilic mixed cultures were developed according to O-Thong et al. [13]. Enrichments were conducted in 120 mL serum bottles with 50 mL working volume. One liter of cassava starch (CS) medium contained (g/L) cassava starch, 5.0; peptone, 1.0; yeast extract, 1.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.2; $FeCl_3.6H_2O$, 0.05; $CaCl_2$, 0.02. Five milliliter of sediment samples from the hot spring and 50 mL of CS medium were added to the serum bottles. The pH of the growth medium was adjusted to pH 5.5 to prevent growth of methanogens in series of batch cultures at 60 °C. Bottles were flushed with nitrogen gas for 5 min to provide anaerobic condition, capped with a rubber stopper and placed in a shaker (100 rpm) for 4 days. Gas production was measured according to Owen et al. [14]. During the enrichment, H_2, CO_2 and CH_4 and soluble metabolites were monitored.

2.2. Cassava starch processing wastewater (CSPW)

CSPW was obtained from VP Starch (2000) Factory (Nakornrachasima, Thailand). The main characteristics of CSPW were as follows: pH 5.52, total solids 38.2 g/L, volatile solids 33 g/L, total carbohydrate 9.6 g/L, total chemical oxygen demand (COD) 52.3 g/L, soluble COD 34.5 g/L, total nitrogen 0.87 g/L, total phosphate 0.43 g/L. After collected, the CSPW was stored at 4 °C before usage.

2.3. Experimental procedure

In order to adapt thermophilic mixed cultures, the cultures resulting from these initial enrichment cultivations with high hydrogen production performance were used in five successive batch cycles (30% inoculum in CS medium, 4 days per cycle). Subsequently, repeated batch cultures at 4 day intervals were performed with 250 mL serum bottles. In each batch culture, a volume representing about 50 mL of the content in the serum bottles was manually withdrawn, and an equal volume of fresh medium (50 mL) was added to each bottle. After the gas phase was replaced with nitrogen gas, the mixture was cultured at 60 °C for 2 days. Experiments to study different starch type (raw and gelatinized) were conducted with 5 g/L cassava starch. The starch was thermally gelatinized at 100 °C for 30 min prior to microbial fermentation. The final cultures (5th batch cycle) were further tested for their hydrogen production from CSPW supplemented with the same concentrations of minerals, trace metals and yeast extract as in CS medium. Control vials containing only water, minerals, trace metals, yeast extract and inoculum were included, in order to account for possible background hydrogen production from these supplements. The background hydrogen was subtracted from hydrogen produced in vials with CSPW. All experiments were conducted in triplicate. The hydrogen in gas phase was monitored periodically. Microbial sludge from the final cultures (5th batch cycle) and from CSPW was used for analysis of responsible microbial community. The promising thermophilic enrichment cultures were further tested for their hydrogen production from CSPW in CSTR by fed-batch operation.

Three identical 1 L glass continuous stirred tank (CSTR) reactors with 0.8 L working volume were used for continuous experiment operated at 5-day HRT. The inocula were taken

from the adapted thermophilic mixed cultures for CSPW fermentation in the repeated batch cultivation experiment. Experimental setup consists of feed bottle, feed pump, reactor, effluent bottle and gas meter. The CSPW feedstock was semi-continuously pumped into reactors six times a day at 4 h intervals, each time 44 mL CSPW with 9.2 g starch/L was added as feedstock. The temperature was controlled at 60 °C by circulating hot water inside the water jacket of the reactors. Mixing was provided by a magnetic stirrer located underneath the reactor.

2.4. Analytical methods

The volume of biogas was measured with a wet syringe and the composition was analyzed by gas chromatography GC-8APT with thermal conductivity detector (TCD), Shimadzu, Japan. Operating temperature was set at 50 °C for injector and detector. Argon as a carrier gas and the activated charcoal packed column were used for the analysis. Liquid samples were also taken from the culture at designated time intervals to analyze for the composition of soluble metabolites including pH, VFA, and total organic carbon (TOC). The VFA was determined by gas chromatography GC-8APF with flame ionization detector (FID), Shimadzu, Japan. Nitrogen as a carrier gas and Unisole F-200 30/60 mesh packed column were used. The operating temperature was 140 °C for the column, 250 °C for the injector and also 250 °C for the detector. Lactate and formate were analyzed by suppressed ion exclusion chromatography equipped with a high performance liquid chromatography (HPLC) pump L2100 Hitachi [9]. TOC was measured by TOC-5000, Shimadzu, Japan. Suspended solid (SS) and volatile suspended solid (VSS) were determined in accordance with the procedures described in the Standard Methods [16]. The total carbohydrate content was analyzed by the Anthrone method [17]. Reducing sugar was determined by using the dinitrosalicylic acid (DNS) method [18].

2.5. Community analysis by DGGE

Polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) was used to study microbial community structure in thermophilic mixed cultures. The sludge samples from each thermophilic mixed culture in cassava starch (CS) medium and cassava starch manufacturing wastewater (CSPW) were mechanically homogenized using a mini-bead beater (Biospec) followed by DNA extraction using Soil DNA Isolation Kit, ISOIL for beads beating, Nippon Gene Co. Ltd. Genomic DNA extractions as well as DGGE for bacteria were made as previously described by Prasertsan et al. [19]. Genomic DNA was used as a template for PCR reactions with a primer pair specific Arch21f and Arch958r [20] in order to detect archaea population in methanogenic reactor. Genomic DNA from *Caldicellulosiruptor saccharolyticus* and *Sulfolobus islandicus* were used as negative and positive control for archaea-PCR, respectively. First archaea-PCR products were used as a template for PCR reactions with a primer pair specific for *archaea* PARCH519R and PARCH340F with 40 bp GC clamp at the 5′ end [21]; were used to amplify the 200 bp fragment of the V3 region. The amplicons were used as DNA template to incorporate a GC clamp in the DNA fragment prior to DGGE [22]. The second archaea-PCR program corresponded to 20 cycles of three steps: 94 °C for 1 min, 65 °C for 0.75 min, and 72 °C for 1 min, 10 cycles of three steps: 94 °C for 1 min, 55 °C for 0.75 min, and 72 °C for 1 min followed by a final step at 72 °C for 10 min. Amplification was checked by agarose gel electrophoresis of PCR products before DGGE analysis. DGGE analysis of the amplicons obtained from second PCR was performed as previously described by Zoetendal et al. [23] and the DGGE Marker II set (Nippon Gene, Tokyo, Japan) was co-electrophoresed with the samples. DGGE profiles were compared using the Quantity One software package (version 4.6.0; Bio-Rad Laboratories). Most of the bands were excised from the gel and re-amplified. After re-amplification, PCR products were purified and sequenced by Macrogen (Macrogen; http://www.macrogen.com). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gene Bank using BLAST [24].

3. Results and discussion

3.1. Development of starch-degrading hydrogen producing bacteria

Different types of thermophilic microbial communities were obtained from different hot spring samples. During incubation all samples produced gas that consisted of a mixture of hydrogen (9–43%) and carbon dioxide (38–60%) with traces of nitrogen. In some experiments methane formation was also detected, especially during the initial stages of consortium development but not found in the second generation, indicating that methanogens were fully inhibited. The highest volume (633 mL H_2/L-medium) of gas produced obtained from thermophilic mixed culture namely PK that had been enriched from Klong Pai Poo hot spring, Phang Nga Province. On the other hand, the lowest quantity (70 mL H_2/L-medium) was obtained from thermophilic mixed culture namely PB enriched from Ban Thung Na Poh hot spring, Patthalung Province. Interestingly, all enriched thermophilic mixed cultures can degrade cassava starch with ability to produce hydrogen gas and acetic acid as main by-products. Hydrogen production and yields for individual mixed thermophilic cultures are summarized in Table 1. After preliminary screening for hydrogen production capacity, significant hydrogen production potentials were obtained from three thermophilic mixed cultures from Klong Pai Poo hot spring (PK), Romani hot spring (PR) Phang Nga Province and Wat Than Nam Ron hot spring (SW), Suratthani Province, Southern Thailand. Three thermophilic mixed cultures namely PK, SW and PR showed most promising for hydrogen production from cassava starch with hydrogen production of 633, 550 and 413 mL H2/L-medium, respectively, and hydrogen yield of 126.6, 110 and 82.6 mL H_2/g starch, respectively, corresponding to 23%, 20.3 and 15.3% of theoretical value. Such a yield was substantially higher than the 92 mL H_2/g starch at 60 °C by digested sludge [12]. The dominated soluble microbial products were acetic acid (28–88%), butyric acid (33–53%) and ethanol (3–23%). The concentration and component of soluble microbial products could reflect the metabolism of thermophilic hydrogen producing anaerobes, which has considerable effect on the hydrogen production. As presented in Table 1, the acetic acid (50–88%) and ethanol

Table 1 – Characteristics of starch degrading bacteria enriched from various Southern Thailand geothermal springs.

Inoculum sources	Samples code	Original temperature (°C)	H_2 content (%)	Final pH	H_2 production (mL/L)	H_2 yield (mL/g starch)	Soluble metabolite products (%)
Wattaphotharam hot spring, Ranong Province	RW	65	12	5.12	215	43	Acetic acid (28%) Butyric acid (53%)
Wat Than Nam Ron hot spring, Suratthani Province	SW	63	33	4.56	550	110	Acetic acid (100%)
Ban Nam Phuron hot spring, Suratthani Province	SB	67	15	5.1	285	57	Acetic acid (48%) Butyric acid (33%)
Rattanakosin hot spring, Suratthani Province	SR	61	12	5.3	237.5	47.5	Acetic acid (38%) Butyric acid (43%)
Klong Pai Poo hot spring, Phangnga Province	PK	60	43	4.39	633	126.6	Acetic acid (88%) Ethanol (11%)
Romani hot spring, PhangNga Province	PR	63	38	4.42	413	82.6	Acetic acid (94%) Ethanol (3%)
Ta Na Ma Rao hot spring, Yala Province	YT	80	22	4.7	310	62	Acetic acid (44%) Ethanol (33%)
Khao Chai Son hot spring, Patthalung Province	LK	53	18	4.8	245	49	Acetic acid (54%) Ethanol (23%)
Ban Thung Na Poh hot spring, Patthalung Province	PB	60	9	5.0	70	14	Acetic acid (48%) Butyric acid (43%)

(11–23%) as main soluble microbial products in enriched cultures (PK, PR and SW) obtained hydrogen production higher than acetic acid (28–48%) and butyric acid (33–43%) as main soluble microbial products. Among the soluble microbial products acetic acid was predominant and accounted for more than 88% of total volatile fatty acids in enriched cultures that shown high hydrogen production.

3.2. *Hydrogen production by repeated batch cultivation*

Three thermophilic mixed cultures (PK, PR and SW) were selected to further evaluate the long term influence on hydrogen production and microbial communities from raw and gelatinized cassava starch under thermophilic condition in repeated batch operation. Starch-degrading thermophilic bacteria (PK, PR and SW) were successfully produced hydrogen in long-term repeated batch operation using raw and gelatinized starch (Fig. 1). Long term operation of 20 days by repeated batch operation increased hydrogen production yield around 26–44%, when compared with batch enrichment. The maximum hydrogen production yields of thermophilic mixed cultures PK, SW and PR from raw cassava starch were 236, 180 and 128.4 mL H_2/g starch, respectively and from gelatinized cassava starch were 240, 224.4 and 165.2 mL H_2/g starch, respectively. An analysis of variation (ANOVA, $P > 0.05$) further demonstrated that using raw cassava starch or gelatinized cassava starch as substrate had no significant effects on hydrogen production. Performances of hydrogen production were in steady-state from the third fed-batch operation and also demonstrated that long term operation had no effect on hydrogen production from thermophilic enriched cultures. The compositions of VFA and ethanol at the end of fermentation were similar with the results obtained from batch tests that acetic acid, ethanol and lactic acid were the main soluble metabolites (Table 2). Two different cassava starch fermentation types from the three thermophilic mixed cultures were observed; acetic acid-ethanol and acetic acid-lactic acid type fermentation. Table 3 summarizes the COD mass balance, which described the effects of the thermophilic mixed cultures PK, SW and PR on the hydrogen production and fermentation patterns from the raw and gelatinized cassava starch. The volatile suspended solids (VSS) formula was assumed to be $C_5H_7NO_2$ [25]. Fermentation patterns depended on substrates types (raw and gelatinized cassava starch). All of the three thermophilic mixed cultures PK, SW and PR were acetic acid-ethanol type fermentation when cultivation on raw cassava starch, with an acetic acid yield of 26–44% (based on COD) and ethanol yield of 10–30% (based on COD). Acetic acid–lactic acid type fermentation occurred when cultivation on gelatinized starch, with an acetic acid yield of 36–49% (based on COD), ethanol yield of 10% (based on COD). Acetic acid–ethanol type fermentation trended to show higher hydrogen production potentials; by contrast, acetic acid–lactic acid type fermentation showed lower hydrogen production potentials. Lactic acid was found in some enriched cultures, while lactic acid was not accompanied with hydrogen. The reason for lactic acid accumulation was not clear but likely related to environmental change during repeated cycle or substrate overloading [26]. It was suggested when environment changes, bacteria would try to optimize the carbon flow rate through the cell by using lactate as electron sink and lower down the acetate production, which gave high energy yield. As a result, high lactate was found in all enriched cultures.

3.3. *Continuous hydrogen production from cassava starch processing wastewater*

Adaptation of thermophilic mixed cultures to cassava starch processing wastewater (CSPW) for long term stability on hydrogen production in CSTR was investigated. Fig. 2 shows the hydrogen production from three thermophilic mixed cultures with continuous operation of 30 days. The enriched cultures PK, SW and PR could completely degrade starch in CSPW to hydrogen, acetic acid, lactic acid and ethanol. It was

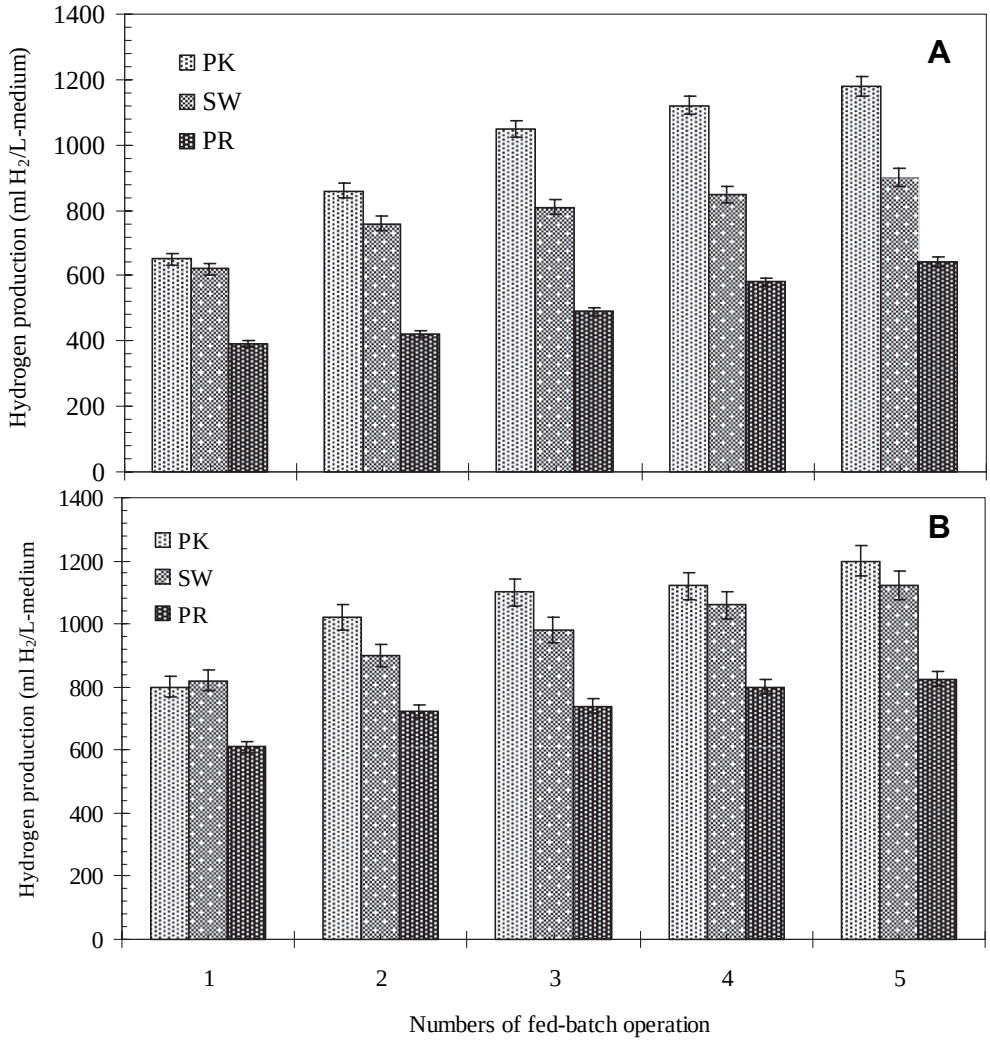

Fig. 1 – Time courses of fed-batch transfer hydrogen production by selected thermophilic mixed cultures. The thermophilic mixed cultures were enriched from hot spring with raw and gelatinized starch by repeated batch cultures at 4 day intervals.

found that repeated batch cultivation was a very useful method to adapt and enrich thermophilic mixed cultures that could ferment CSPW with high hydrogen yield and without significant lag phase. After adaptation, thermophilic mixed cultures PK, SW and PR could produce hydrogen directly in the CSPW feedstock, giving a maximum hydrogen yield of 287, 264 and 232 mL H_2/g starch, respectively. These hydrogen yields were close to that produced by sludge inoculum from a municipal sewage treatment plant (244 mL H_2/g sugar at 60 °C) [27]. Fermentation of CSPW resulted in comparable by-products, independent of the fact that it has been previously cultivated on cassava starch (CS) medium. When CSPW was added, the present populations of all thermophilic mixed cultures were able to use starch. Under condition with CSPW

Table 2 – Characteristics of gas production from starch and gelatinized starch for thermophilic mixed cultures enriched from hot spring.

Inoculum sources	Enrichment substrates	H_2 production (mL/L)	H_2 Yield (mL/g starch)	Substrate utilization (%)	Metabolite formation (mM)				Final pH	VSS (g/l)
					Acetic acid	Butyric acid	Lactic acid	Ethanol		
PK	Raw	1246	249.3	53.8	16.3	0	0	4.1	4.7	0.9
	Gelatinized	1266	252.4	69.6	21.0	0	0.89	6.3	4.4	0.8
SW	Raw	910	180.0	82.6	18.2	0	0	15.4	4.6	0.7
	Gelatinized	1122	224.4	73.2	24.7	0	1.5	6.7	4.4	0.9
PR	Raw	642	124.9	52.6	19.5	0	0	4.3	4.5	0.7
	Gelatinized	826	165.4	58.0	23.8	0	1.24	2.6	4.4	1.0

Table 3 – COD mass balance for each inoculum.

Inoculum sources	Enrichment substrates	H_2 (%)	Substrate utilization (%)	Metabolite formation (%)			Biomass ($C_5H_7O_2N$) (%)	Balance (%)
				Acetic acid	Lactic acid	Ethanol		
PK	Raw	31.0	−100	36.4	0	13.8	13.5	−5.2
	Gelatinized	24.4	−100	36.2	1.9	16.3	12.0	−9.2
SW	Raw	14.8	−100	26.4	0	33.6	10.5	−14.7
	Gelatinized	20.5	−100	40.4	3.1	16.6	13.5	−5.8
PR	Raw	16.3	−100	44.5	0	14.9	10.5	−13.8
	Gelatinized	19.1	−100	49.3	3.3	8.1	15.0	−5.3

feeding, acetic acid was the major soluble microbial product while lactic acid was the minor soluble microbial product.

3.4. *Microbial community analysis*

Microbial community structures of the three thermophilic mixed cultures (PK, PR and SW) cultivated in raw cassava starch (CS) medium and cassava starch processing wastewater (CSPW) were analyzed using DGGE analysis. Results indicated that hydrogen-producing *Thermoanaerobacterium*-related organisms in the inoculums were found in the hydrogen fermentation both substrates (CS and CSPW), although no aseptic operations were applied. The small different band patterns were illustrated for each thermophilic mixed culture (Fig. 3). The closest relative of the thickest band obtained in all thermophilic mixed cultures and both substrates was the *Thermoanaerobacterium saccharolyticum*. Thermophilic mixed culture PK gave the highest hydrogen production and its microbial community in CS medium was comprised of *T. saccharolyticum*, *Thermoanaerobacterium thermosaccharolyticum* and *Thermoanaerobacterium* sp., while in CSPW *T. saccharolyticum* and uncultured *Clostridium* sp. were dominant. Low microbial diversity was found in the thermophilic mixed culture PK, *T. saccharolyticum* and *T. thermosaccharolyticum* were considered to be the dominated thermophilic hydrogen-producing bacteria. Microbial community of thermophilic mixed culture SW in CS medium was comprised of *T. saccharolyticum*, uncultured *Clostridium* sp., *Thermoanaerobacterium thermohydrosulfuricus* and *Anoxybacillus* sp. while in CSPW comprised of *T. saccharolyticum* and *Anoxybacillus* sp. Microbial community of thermophilic mixed culture PR in CS medium was comprised of *T. saccharolyticum*, uncultured *Clostridium* sp. and *Geobacillus* sp. while in CSPW comprised of *T. saccharolyticum*, *Geobacillus* sp. and *Anoxybacillus* sp. No amplification products of archaeal 16S rRNA genes were obtained from the DNA extracts of the three enriched cultures samples with a nested PCR procedure, while the positive control (DNA from anaerobic digester sludge) had a high amplification yield (data not shown). This result indicated the absence of archaea in the enrichment cultures.

The presence of *T. saccharolyticum* and *T. thermosaccharolyticum* species was in good agreement with other studies that had identified *Thermoanaerobacterium* sp., as the main hydrogen producing bacteria, at thermophilic conditions [19,28]. *T. saccharolyticum* is a thermophilic anaerobic bacterium, grows in a temperature range of 45–65 °C and a pH range of 4.0–6.5 [29], able to directly ferment hemicellulose, starch and xylan polymers, as well as primary sugars found in cellulosic biomass, including cellobiose, glucose, xylose, mannose, galactose, and arabinose to acetic acid and ethanol as the end products. *T. thermosaccharolyticum* is a thermophile with optimal growth temperature at 60 °C and also able to convert carbohydrates to hydrogen with butyrate as the end soluble product [7,30]. Several species of the genus *Thermoanaerobacterium* are known for their hydrogen production characteristics, including *T. saccharolyticum*, *T. thermosaccharolyticum*, *Thermoanaerobacterium polysaccharolyticum*, *Thermoanaerobacterium zeae*,

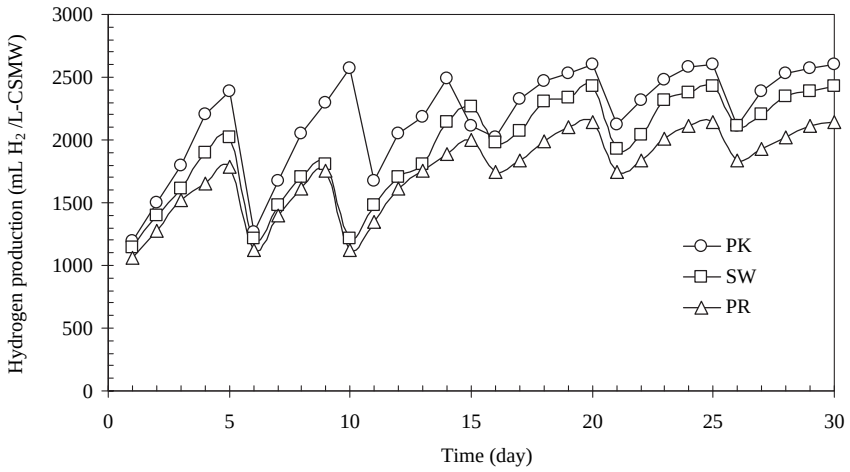

Fig. 2 – Hydrogen production from cassava starch manufacturing wastewater in CSTR with fed-batch mode of three thermophilic mixed cultures developed from hot spring.

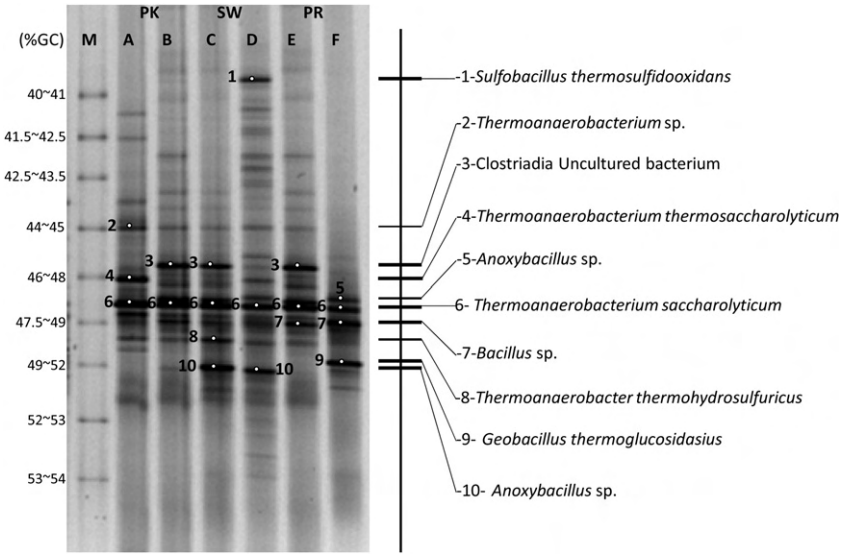

Fig. 3 – DGGE profile of 16S rRNA gene fragments. The fragments were PCR-amplified from total DNA extract of thermophilic mixed cultures for hydrogen production from raw cassava starch medium and cassava starch processing wastewater (CSPW) (M, DGGE maker; A, PK sludge sample from raw cassava starch medium; B, PK sludge sample CSPW; C, SW sludge sample from raw cassava starch medium; D, SW sludge sample CSPW; E, PR sludge sample from raw cassava starch medium; F, PR sludge sample CSPW).

Thermoanaerobacterium lactoethylicum, *T. thermohydrosulfuricus* and *Thermoanaerobacterium aotearoense*. All these organisms have optimal growth conditions at 55–70 °C and at pH 5.2–7.8 [15], which are accordance with operating conditions used in this investigation.

The second dominant bacterial group found during our experiments in the CS medium and CSPW was in the genera *Geobacillus*, *Clostridium* and *Anoxybacillus*. Both *Geobacillus* and *Anoxybacillus* are gram positive facultative anaerobic thermophiles, spore forming, and rod shape. *Anoxybacillus* had the optimal growth condition of 62 °C and pH 9.5–9.7 and able to produce hydrogen from monosaccharides [31]. *Geobacillus* species are facultative anaerobic by reduced nitrate to nitrogen gas, growth on various types of starch (such as potato, corn, and cassava) since they are good starch degrading enzyme producer including the alpha-amylase to hydrolyze raw starch [32,33]. Therefore, we proposed that *Geobacillus* species degrade starch into small molecules first and then these less complex compounds would be utilized by *Thermoanaerobacterium*, *Anoxybacillus* and *Clostridium* species for biohydrogen production. Third dominant bacterial group found in the CS medium and CSPW was in the genera *Sulfobacillus* which is able to degrade lactate to acetate and/or hydrogen [34]. DGGE profile of bacterial communities from sludges cultivation in CS and CSPW was not significant difference. Both microbial communities were dominated by *T. saccharolyticum*, *Geobacillus* sp. and *Clostridium* sp. These thermophilic mixed cultures show promising to apply for biohydrogen production from cassava starch processing wastewater.

4. Conclusions

High potential thermophilic mixed cultures were enriched from Southern Thailand hot springs over a wide temperature range (53–80 °C). Significant hydrogen production was obtained from three thermophilic mixed cultures PK, SW and PR in cassava starch (CS) medium and cassava starch processing wastewater (CSPW). The maximum hydrogen yield from CSPW by thermophilic mixed cultures PK, SW and PR were 287, 264 and 232 mL H_2/g starch, respectively, corresponding to 53%, 48.7% and 42.8% of the theoretical value. Both microbial communities in CS medium and CSPW were dominated by *T. saccharolyticum*, *T. thermosaccharolyticum*, *Anoxybacillus* sp., *Geobacillus* sp. and *Clostridium* sp.

Acknowledgments

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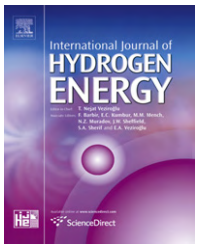

Biohydrogen production from sago starch in wastewater using an enriched thermophilic mixed culture from hot spring

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Hot spring

ABSTRACT

We investigated the feasibility of producing biohydrogen from sago starch in wastewater using a thermophilic mixed culture enriched from a hot spring in southern Thailand (PGK). The effects of repeated batch cultivations, the dependence of the initial pH values ranging from 5.0 to 8.0 and the initial sago starch concentrations ranging from 2.5 to 60 g/l as well as the utilization of 4 different starch types (gelatinized dry starch, non-gelatinized dry starch, gelatinized wet starch, and non-gelatinized wet starch) were examined in batch experiments at 60 °C. Microbial community structures were also investigated. The methane-free biogas evolved contained up to 55% hydrogen, with the remainder comprising carbon dioxide. Repeated batch cultivation under identical conditions improved the hydrogen yield. Gelatinized dry starch at an initial pH of 6.5 and an initial starch concentration of 2.5 g/l gave the maximum hydrogen yield of 422 ml-H_2/g-starch-added (80% of the theoretical limit). PCR-DGGE profiles of 16S rRNA gene fragments from the cultures showed that the predominant hydrogen producers were closely related to thermophilic anaerobic bacteria, including *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacterium thermosulfurigenes*, and uncultured *Thermoanaerobacterium* sp., all of which probably produced hydrogen with simultaneous generation of ethanol. The thermophilic bacilli capable of utilizing starch, including *Bacillus* sp., *Anoxybacillus* sp., *Bacillus lentus*, and *Geobacillus* sp., were also found in the starch cultures, but mainly in the non-gelatinized wet starch culture.

1. Introduction

Production of biohydrogen by fermentative processes is desirable because such methods generate high yields of hydrogen and high rates of bacterial growth with relatively low energy inputs, compared to the photobiological methods [1,2]. Not only is hydrogen a clean energy carrier, some biohydrogen production processes are environmentally sustainable. In particular, fermentation processes that utilize free carbon available in large-volume discharges of agro-industrial wastewater containing carbohydrates or lignocellulosic materials can recover available energy as well as purify the effluent [3–8]. In addition, high-temperature waste streams such as those from food processing plants are especially conducive to hydrogen-

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producing reactions, because of the thermodynamics of the reaction processes [9] and the resistance of these waste streams to contamination by pathogens [10–13]. O-Thong et al. [1] have reported that thermophilic bacteria can utilize a variety of carbon sources and generate high yields of hydrogen as well as tolerate acidic fermentation conditions. Thermophilic anaerobic fermentation processes hold tremendous promise for the forthcoming generations as well as for the commercial production of hydrogen fuel and concomitant purification of waste streams.

The capacity of fermentative microorganisms to produce hydrogen from carbohydrates is well known and has been studied and applied in a wide range of applications [14]. Researchers have utilized a variety of carbohydrates for fermentation, from simple sugars, including glucose [15] and sucrose [16], to more complex carbohydrates such as starch and carbohydrate-rich agricultural products such as rice [17,18]. Researchers have also investigated the production of hydrogen from carbohydrate-rich wastewaters to benefit the economy and to support the Clean Development Mechanism (CDM) [19]. Hydrogen has been produced by fermentation of the wastewater from a sugar factory [20], a sweet potato-starch-manufacturing plant [21], and a brewery [22]. In this study, we present data and observations on the production of hydrogen from sago starch in wastewater.

Starch from the sago palm (*Metroxylon sagu* Rottb.) has long been a staple food for populations in the Moluccas, West Papua (Indonesia), and Papua New Guinea, and is widely used as a dietary supplement in Southeast Asia. In food industries, sago starch is used as an ingredient while making noodles, vermicelli, biscuits, monosodium glutamate, glucose, caramel, fructose, syrups, and many other food products [23]. Sago palm is considered a potential commercial crop because of its numerous benefits compared to the other starch crops, including high starch yields (15–25 ton/ha of dry starch), relatively sustainable production, vigor, environmental friendliness [24], and low cost of production [25]. On Bengkalis Island and the surrounding areas of Riau Province in Indonesia, starch is already being produced by the local sago industries, and palm is being cultivated for large-scale commercial production [26]. Moreover, Bustaman [27] has documented the potential for sago-based bioethanol production in Moluccas, and several other studies have studied the production of bioethanol from this starch [28–30]. However, few studies have used sago starch or the wastewater from the sago starch-manufacturing units for biogas production [25] and no report for hydrogen production.

Sago starch factories are usually located near rivers or straits, and many of them discharge wastewaters containing starch and pith residue directly into the waterways without proper treatment. Production of wastewater in an average facility can reach 10^6 l/day, with a biological oxygen demand (BOD) and chemical oxygen demand (COD) of 3.4 g/l and 11.4 g/l, respectively [31]. BOD and COD are directly attributed to starch and pith residues. Pith residues or "hampas", containing about 60–70% dry weight starch, are a by-product of the rinsing procedures; approximately 1 ton/day of hampas is generated by an average factory [25,32].

To reduce environmental impacts and recover the energy lost in the waste stream, we investigated the feasibility of using sago starch in wastewater for fermentative hydrogen production by using a thermophilic mixed culture from a hot spring in Thailand. A mixed culture from a hot spring was selected because hot springs are recognized as a potential source of thermophilic microorganisms with fermentative abilities, such as hydrogen-producing bacteria [33,34]; also, mixed cultures have benefits in industrial applications because of their high microbial diversity, and because media sterilization is not required [6,35].

In the present study, we examined the effects of repeated batch cultivations, the use of different initial conditions of pH, sago starch concentration, and starch type on biohydrogen production in 4 serial batch experiments conducted at 60 °C. In addition, we investigated the microbial community of the thermophilic mixed culture in the sago starch media by using 16S rRNA-based molecular techniques, including polymerase chain reaction-based denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing.

2. Materials and methods

2.1. *Thermophilic enrichment of culture from a hot spring*

The enriched culture from Khong Pay Pao hot spring (PGK) used in this study was obtained from previous research conducted by Hniman et al. [36]. The culture was enriched from sediment-rich water sample collected from a geothermal hot spring located in Khong Pay Pao, Phang Nga, Southern Thailand. Temperature and pH of sample were 60 °C and 6.5, respectively. The enrichment of culture in basic anaerobic (BA) medium supplemented with 10 g/l each of glucose, xylose and xylose-glucose mixed substrate at the ratio of 1:1 under strictly anaerobic condition at 60 °C was performed by repeated batch cultivations following the procedure of O-Thong et al. [37] as described in detail by Hniman et al. [36]. This culture gave maximum hydrogen yield of 199.8 ml-H_2/g-$sugar_{consumed}$ using glucose-xylose mixed substrates. Subsequently, this culture was used in this study for producing hydrogen from sago starch in wastewater.

2.2. *Preparation of sago medium*

Bushnell–Haas medium (BHM) consisting of (g/l): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 1; KH_2PO_4, 1; NH_4NO_3, 1.0; $FeCl_3.6H_2O$, 0.05; $CaCl_2$, 0.02 supplemented with yeast extract (1.0 g/l), peptone (1.0 g/l) and sago starches were prepared for further batch hydrogen production. Sago starches, wet and dry types, from Bengkalis, Indonesia were supplied by local producer which were processed for commercial uses. Gelatinization of those starches was performed by autoclaving at 121 °C for 15 min to compare with the raw starches.

2.3. *Batch hydrogen production under thermophilic conditions*

In batch fermentation, we investigated the effects of repeated batch cultivations, the effects of different initial pHs, initial sago starch concentrations and starch types on biohydrogen

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production from sago starch in wastewater by enriched PGK culture in 4 serial experiments. For all experiments, 50 ml modified BHM supplemented with sago starch as substrate was distributed anaerobically in 125 ml serum bottles capped with rubber stopper and closed with aluminum caps. The 5 ml of enriched PGK culture was transferred into the medium and after the headspace was replaced with nitrogen gas, the mixture was cultivated at 60 °C for 3 days. The initial VSS concentration measured in each serum bottle was around 90 mg/l. The pH was set to 6.5 in all experiments, except for the second series. The initial concentration of sago starch was adjusted to 10 g/l except for the third series and gelatinized dry starch was used for all experiments except for the fourth series. All experiments were performed triplicate for verification purposes. The detail procedures for each serial experiment are explained below.

First, to determine the effects of repeated batch cultivations, 5 ml of enriched PGK culture was cultivated in 50 ml medium at 60 °C for 3 days. Then, 5 ml of inoculum from the first batch was taken for cultivating into the second batch. This procedure was repeated until 6 batch cultivations under identical conditions. Second, to determine the dependence of initial pH, the pH was set to various levels (in the range of 5.0–8.0, with increments of 0.5) in 7 serum bottles using 1 N HCl and 1 N NaOH. Third, to verify the dependence of initial sago starch concentration, the experiment was started with the initial concentrations ranging from 10 to 60 g/l at increments of 10 g/l in 6 serum bottles. Afterward, lower range concentration of sago starch (from 2.5 to 15 g/l with increments of 2.5) was set in another 6 serum bottles to confirm the result. In the fourth series, in order to observe the effects of sago starch type, we used 10 g/l of gelatinized and non-gelatinized of dry and wet starches in 4 serum bottles.

2.4. Analytical methods

Biogas volume was measured daily with a glass syringe and the composition was analyzed by gas chromatography. Liquid samples were also taken from the culture before and at the end of each experiment for analyzing the composition of soluble metabolites including pH, volatile fatty acids (VFA), total organic carbon (TOC), reducing sugar, and total carbohydrates. The measurement condition for biogas, VFA, and TOC analysis were similar with our previous report [38]. Concentration of reducing sugar and total carbohydrates were determined by DNS [39] and anthrone-sulfuric acid methods [40] using a spectrophotometer U-2001 (Hitachi, Japan) at 540 nm and 620 nm, respectively. Volatile suspended solid (VSS) was quantified according to the standard methods for the examination of water and wastewater [41].

2.5. Microbial community analysis

PCR-DGGE was used to study microbial community structure in two-stage process. Liquid samples were collected from serum bottles under steady state conditions. The microorganisms cells in 2 ml of sample were harvested in a tube by centrifuged at 5,000g for 5 min. The pelletized cells were washed three times with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) before being resuspended in 1 ml of TE buffer. Genomic DNA was extracted and purified using QIAamp DNA Stool Mini Kit (QIAgen, Hilden, Germany). Genomic DNA was used as a template for PCR reactions with a primer pair specific for *Eubacteria* (universal primer 1492r and primer 27f) as well as for *Archeae* (Arch21f and Arch958r) [42]. Each 50 μl (total volume) reaction mixture containing 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 200 mM, 1 μl of Taq polymerase (2U/ml; Sigma–Aldrich, St. Louis, MO), 10 pmol of each primer, and 1 μl of DNA extract solution. Sterile water was used as no template control, 1 μl genomic DNA from *Caldicellulosiruptor saccharolyticus* and *Sulfolobus islandicus* as positive and negative control, respectively. The thermal cycling program used for first amplification was as follows: predenaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s and elongation at 72 °C for 90 s. The reactions were subsequently post elongation at 72 °C for 30 min and cooled to 4 °C. Amplification was checked by agarose gel electrophoresis of PCR products using 1% agarose in 1×TAE buffer. First PCR products were used as a template for nested PCR reactions with a primer pair specific for *Eubacteria* (Primer 518r and 357f with 40 bp GC clamp at the 5′ end [43];) as well as for *Archeae* (PARCH519R and PARCH340F with 40 bp GC clamp at the 5′ end [44];) were used to amplify the 200 bp fragment of the V_3 region. The amplicons were used as DNA template to incorporate a GC clamp in the DNA fragment prior to DGGE [45]. The second PCR program corresponded to 20 cycles of three steps: 94 °C for 1 min, 65 °C for 0.75 min, and 72 °C for 1 min, 10 cycles of three steps: 94 °C for 1 min, 55 °C for 0.75 min, and 72 °C for 1 min followed by a final step at 72 °C for 10 min. Amplification was checked by agarose gel electrophoresis of PCR products before DGGE analysis.

DGGE analysis of the amplicons obtained from second PCR was performed as previously described by Zoetendal et al. [46] using the Dcode Universal Mutation Detection system (Bio-Rad, Hercules, CA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60%. A 100% denaturing solution was defined as 7 M urea and 40% formamide. Electrophoresis was performed for 16 h at 70 V in a 0.5× TAE buffer at 60 °C. The DGGE Marker II set (Nippon Gene, Tokyo, Japan) was co-electrophoresed with the samples. DGGE gels were stained with SYBR Green for 15 min and analyzed on GelDoc XR 1708170 system (Bio-Rad Laboratories, Hertfordshire, UK). DGGE profiles were compared using the Quantity One software package (version 4.6.0; Bio-Rad Laboratories). Most of the bands were excised from the gel and re-amplified with primer 357f without a GC clamp and the reverse primer 518r. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer 518r and were directly sequenced (Macrogen; http://www.macrogen.com). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gene Bank using BLAST [47].

3. Results and discussion

3.1. Biogas composition and the effects of repeated batch cultivation

The composition of biogas measured in all series of our experiments was approximately 40–55% hydrogen and 45–60%

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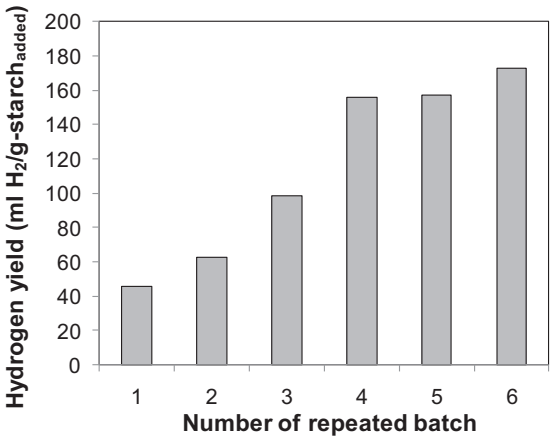

Fig. 1 – Hydrogen production at different batch cultivations with 10 g/l initial gelatinized dry sago starch concentration using enriched PGK culture at 60 °C.

carbon dioxide (excluding the residual nitrogen from the initial sparging). No methane was detected in any treatment, verifying the merit of an enriched PGK culture for biohydrogen production, as previously reported [36]. The utilization of this enriched culture for the fermentation of more complex carbohydrates, namely, sago starches, yielded promising results. We found that hydrogen yields were increased by increasing the number of repeated batch cultivations until a nearly stable level of hydrogen production was achieved, as shown in Fig. 1. The advantage of repeated batch cultivations is that it allows the microorganisms in a culture to adapt to their environment, resulting in increased hydrogen yields as compared to single batch cultivations described in previous reports [1,48].

3.2. *Dependence of biohydrogen production on initial pH*

Initial pH has been recognized as an important determinant of biohydrogen productivity. Table 1 summarizes the effect of initial pH levels on final pH, H_2 yields (ml/g-starch$_{added}$), cell yields (g-VSS/g-starch$_{added}$), VFA yields (g/l), reducing sugar content (g/l), and substrate removal (%) for 10 g/l gelatinized dry sago starch fermented for 3 days at 60 °C. The data indicate that hydrogen production is high for initial pH values of 6.5–8.0, and that maximum H_2 yields are obtained at pH 6.5. The final pH under conditions of high hydrogen yield decreased to approximately 4.5 because of the accumulation of VFAs. Acetic acid was the sole VFA product at all pH values, indicating that the enriched PGK culture produced H_2 via the acetic acid fermentation pathway, which is known to produce more hydrogen than other pathways. As summarized by Hasyim et al. [38], fermentation reactions producing hydrogen from glucose can occur by several pathways: acetic acid fermentation, butyric acid fermentation, ethanol fermentation, and a mixture of acetic acid and butyric acid fermentation. Only acetic acid fermentation can produce the maximum levels of hydrogen generation (4 mol H_2/mol glucose), whereas the other fermentation mechanisms can produce only 2 mol H_2/mol glucose.

The fermentation culture with an initial pH of 6.5 also attained the highest cell yield of all trials, confirming that this pH is preferable for the growth of starch-hydrolyzing and hydrogen-producing microorganisms in this culture. Interestingly, the maximum substrate utilization was attained at pH 7–8, not at pH 6.5, implying that at least one other important metabolite is also produced during fermentation. Ethanol fermentation is a possible route for producing H_2 within this pH range, because of the lower H_2 yields. Other metabolites that might be present in this pH range are alcohols and lactic acid, as shown by Hniman et al. [36] in their study using PGK cultures. Lee et al. [49] found that limiting the pH to 5.5–7.0 inhibits the production of ethanol in the H_2-producing fermentation of cassava starch. Therefore, adjustment of the initial pH to values of approximately 6.5 is considered important for the production of hydrogen from sago starch using the hot spring culture from Thailand.

At an initial pH of 5.0–6.0, hydrogen yields in our trials were very low compared to the yields at higher pH levels. Low pH fermentation was accompanied by lower substrate utilization and undetected VFAs (Table 1), indicating that H_2 production is inhibited at low pH. Similarly, Zhang et al. [17] reported low hydrogen yields at pH 5, and no hydrogen production at pH 4. On the other hand, Lin et al. [50] found that initial pH values of 5.0–5.5 were more favorable than higher pH levels for hydrogen production from the starch in a paper mill-wastewater treatment sludge digested by a natural mixed culture, suggesting that the microflora in this environment are preferentially adapted to low pH conditions. Thus, inconsistent results regarding the effects of initial pH on hydrogen production from starch are

Table 1 – Hydrogen production from sago starch using PGK culture at stationery phase of different initial pHs.

Initial pH	Final pH	H_2 yield (ml/g–starch $_{added}$)	Cell yield (g–VSS/g–starch $_{added}$)	VFA (g/l)			Reducing Sugar (g/l)	Substrate removal (%)
				HAc	HPr	HBu		
5	4.9	3.71	0.08	0	0	0	1.13	10.35
5.5	5.3	2.96	0.08	0	0	0	5.63	46.51
6	5.9	1.59	0.06	0.25	0	0	2.57	27.54
6.5	4.4	62.83	0.14	1.11	0	0	0.68	79.51
7	4.5	45.15	0.06	1.08	0	0	1.44	91.20
7.5	4.4	30.50	0.09	0.77	0	0	0.78	91.89
8	4.6	32.49	0.07	0.86	0	0	0.88	90.24

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probably because of the different characteristics of the microorganisms in the cultures and due to the variations in the substrate environment. Hence, it is necessary to study the effects of initial pH on hydrogen production from diverse environments and cultures before establishing large-scale hydrogen production systems.

At low pH values, especially at a pH of approximately 5.5, relatively high levels of reducing sugars were observed (5.63 g/l) in our trials (from initial reducing sugar concentrations of approximately 0.3 g/l). This indicates that the hydrogen-producing microbe in the culture is not active in these conditions (indicated by the low hydrogen yield), but that the starch-hydrolyzing microorganism in the culture still demonstrates high amylase activity. Konsula and Liakopoulou-Kyriakides [51] examined the effects of pH on the activities of the enzymes involved in starch hydrolysis, and found that the enzyme involved in amylase production displayed considerable activity in the pH range of 5–7.5, and optimal activity at pH 6.5. This might explain our observation that hydrogen production is highest at pH 6.5, but that reducing sugars are still produced at other pH values.

3.3. *Dependence of biohydrogen production on initial sago starch concentrations*

In addition to pH and substrate composition, substrate concentrations are recognized as one of the critical factors in the production of hydrogen. Biological metabolic processes, including specific metabolic pathways and the reaction kinetics of hydrogen production, are considered to be affected by substrate concentrations [49,52]. The measurements of hydrogen yield in response to the amount of available substrate (starch) provide valuable information about the potential of the substrate to generate hydrogen. Therefore, many research studies report the efficiency of hydrogen production as a function of the hydrogen produced per amount of substrate added [6,17].

In this study, we examined optimal hydrogen production efficiency as a function of the initial sago starch concentration. In the first set of trials, we used initial starch concentrations ranging from 10 to 60 g/l, in increments of 10 g/l Fig. 2 illustrates H_2 yield (ml/g-starch$_{added}$), substrate removal (%), reducing sugar content (g/l), and cell yield (g-VSS/g-starch$_{added}$) at different initial sago starch concentrations, digested for 3 days at 60 °C and an initial pH of 6.5. Fig. 2B shows that hydrogen yield and substrate removal tend to decrease at sago starch concentrations greater than 10 g/l.

Further investigations using lower initial starch concentrations (2.5–15 g/l) showed that higher hydrogen yields (maximum, 442.2 ml/g-starch$_{added}$) were achieved at initial sago starch concentrations of 2.5 g/l (Table 2). This hydrogen yield is nearly 80% of the theoretically derived maximum value of 553 ml of hydrogen per gram of starch [17]. To our knowledge, such efficiencies have never been reported in other studies on hydrogen production from starch. The high yield of hydrogen at low initial starch concentrations was concomitant with the consumption of nearly 100% of the starch, as shown in Table 2. Acetic acid was the sole VFA product under these conditions (data not shown), confirming that starch is digested via the acetate fermentation pathway.

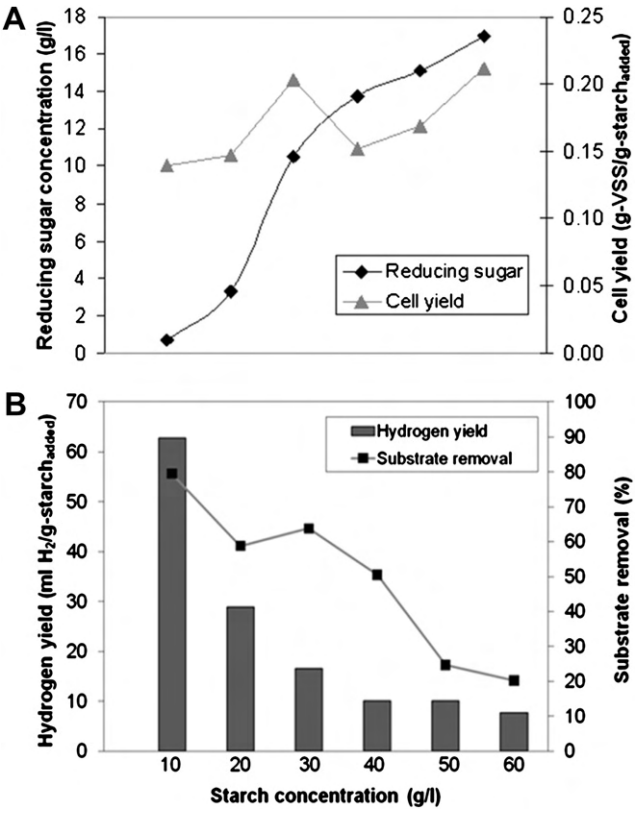

Fig. 2 – Hydrogen production from sago starch using PGK culture at initial starch concentration of 10–60 g/l after 3 days cultivation (A) Reducing sugar concentration and cell yield (B) H_2 yield and substrate removal.

Final pH levels in all conditions were approximately 4, due to the release of organic acids during the fermentation process. Lower hydrogen yield at higher initial starch concentrations have been observed by several researchers, and this is possibly caused by factors such as incomplete hydrolysis of starch, increase in the concentration of byproducts (such as VFAs and alcohols), accumulation of undissociated organic acids, or inhibition of the microorganisms involved in starch digestion or hydrogen production [6,8,17,52]. These factors might explain why lower initial concentrations of sago starch are favorable for hydrogen production, as observed in our study.

Fig. 2A shows that the reducing sugar concentration after 3 days of cultivation tends to increase with increasing sago starch concentrations, indicating that the starch-degrading capacity of this culture is quite high. Once again, this result may be possibly due to the high amylase activity of starch-degrading microorganisms in the culture, as well as the effects of temperature (60 °C). We also considered that a higher temperature of cultivation would facilitate the degradation of starch into simple sugars, as compared with lower temperatures, because starches start to swell at 55 °C or higher depending on the type of starch, pH, water content, and other factors [53]. Hence, cultivation under thermophilic conditions is recommended for hydrogen production from starch, including sago starch, because the starch gelatinization temperature is around 70 °C [24].

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Table 2 – Starch balance and hydrogen yield from sago starch using PGK culture at static state of different initial starch concentrations (2.5–15 g/l).

Initial conc.	Starch balance				H_2 yield	Final pH
(g/l)	input (mg)	remain (mg)	consumed (mg)	removal %	ml/g-starch$_{added}$	
2.5	125	0	125.00	100	442.2	4.69
5.0	250	67.53	182.47	72.99	276.4	4.02
7.5	375	177.53	197.47	52.66	236.2	3.88
10.0	500	350.19	149.81	29.96	156.1	3.93
12.5	625	354.37	270.63	43.30	111.6	4.39
15.0	750	269.43	480.57	64.08	91.5	4.36

Fig. 2A also shows that low reducing sugar contents accompany higher hydrogen yields that are produced from an initial starch concentration of 10 g/l (Fig. 2B), as compared to other initial starch concentrations, indicating that sugars in the medium are consumed by the hydrogen producers in the culture. The high hydrogen yields are also accompanied by the highest percentage of substrate removal. Cell yield at an initial starch concentration of 20 g/l were similar to the yield at 10 g/l, but with lower hydrogen yield, lower substrate utilization, and higher sugar concentration. Cell yield was substantially increased at an initial starch concentration of 30 g/l, paralleled by an increasing concentration of reducing sugars. In summary, these observations suggest that higher initial starch concentrations do not promote the growth of hydrogen-producing microbes in this culture; nevertheless, the results reveal the potency of starch degraders to grow.

3.4. *Comparison of biohydrogen production from different sago starch types*

Two types of starch are commercially produced in sago starch-manufacturing factories, namely, wet and dry. The main difference between wet and dry starch is the moisture content. In addition, the structure of starch may change because of spontaneous fermentation during long-term storage. Wet starch cannot be stored without proper storage methods because microbial activity may corrode the grain, thus diminishing the quality of the starch [24,54]. Regional variation is observed in the traditional methods of storing wet starch.

In this study, each type of starch was gelatinized at 121 °C for 15 min to examine the effects of starch gelatinization on biohydrogen production; results were compared with the data on raw starch. The cumulative hydrogen yields (ml H_2/g-starch$_{added}$) from the different types of sago starch for 4 days of cultivation with 10 g/l initial starch concentrations are depicted in Fig. 3. H_2 production ceased after 2 days of cultivation, indicating the short hydraulic retention time of the culture, probably caused by substrate- or product-induced inhibition. The highest hydrogen yields were achieved from gelatinized dry starch (157.3 ml H_2/g-starch$_{added}$); hydrogen productivity decreased when using gelatinized wet starch, non-gelatinized dry starch, and non-gelatinized wet starch (H_2 yields of 107.4, 52.2, and 8.5 ml H_2/g-starch$_{added}$, respectively). From these data, it appears that the highest hydrogen yields are obtained using enriched cultures and gelatinized dry starch.

Hydrogen yields obtained from both dry and wet gelatinized starch were higher than the yields obtained from non-

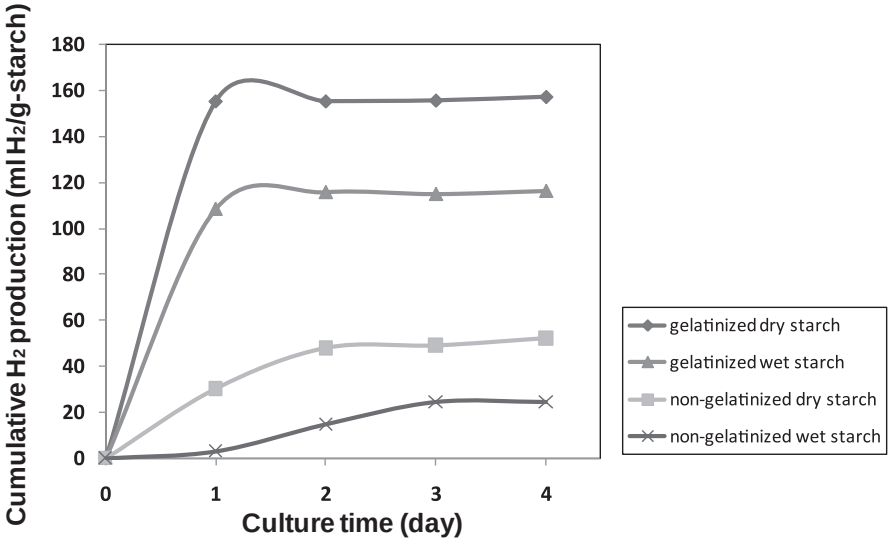

Fig. 3 – Hydrogen production from sago starch using PGK culture at different starch type with 10 g/l initial starch concentration for 4 days cultivation.

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gelatinized starch, clearly indicating that gelatinization favors hydrogen production. Starch gelatinization can cause irreversible changes in grain properties such as granular swelling, native crystallite melting, loss of birefringence, and starch solubilization [55,56]. Thus, gelatinization might create favorable conditions for microbial starch consumption in PGK cultures. Lower hydrogen yield from wet starch was possibly due to the properties of the starch. Further investigation is required to completely understand the chemical and physical properties of starch and to study the effects of gelatinization on hydrogen production.

3.5. *Comparison of hydrogen yield from starch, as determined in previous studies*

The utilization of starch as a substrate for hydrogen production has been described in many research studies. Starch contains approximately 20–25% linear and helical amylose and 75–80% branched amylopectin [57]. Sago starch generally contains 27% amylose and 73% amylopectin [58], with minor amounts of protein [55]. The structure and composition of the molecules constructed by these 2 major high-molecular-weight carbohydrate components are characteristic of each particular plant species [59,60]. Therefore, different sources of starch will result in different hydrogen yields; yields also depend on factors such as the structure and composition of the microbial community, composition of the medium, and temperature.

Table 3 compares the hydrogen yields obtained by anaerobic fermentation of soluble starch, cornstarch, and cassava starch from previous studies [8,17,61,62] and sago starch (this study). The data show that the highest hydrogen yields are obtained using sago starch with initial starch concentrations of 2.5 g/l. Hydrogen yields using initial concentrations of 5 g/l sago starch (276 ml H_2/g-starch$_{added}$) can be compared to the yields obtained using other starch sources. These results demonstrate the potency of the sago starch in wastewater for producing hydrogen by using an enriched PGK culture at moderate thermophilic conditions (60 °C) and an initial pH of 6.5.

3.6. *Microbial communities of sago starch thermophilic mixed cultures*

Bacterial cultures were collected from 4 serum bottles under steady state conditions, each containing a different type of sago starch, i.e., non-gelatinized dry starch, gelatinized dry starch, non-gelatinized wet starch, and gelatinized wet starch. Fig. 4 shows the DGGE profiles of 16S rRNA gene fragments for enriched PGK cultures from each bottle. A rapid comparison of the DGGE patterns of PCR products from each culture did not reveal any significant differences between the bacterial community structures in these samples, except for the communities from the non-gelatinized wet sago starch. Thermophilic anaerobic bacteria of the genus *Thermoanaerobacterium* were the dominant member in 3 of the 4 cultures (all except the non-gelatinized wet starch culture), with the strongest band intensity affiliated with the uncultured *Thermoanaerobacterium* sp.

Further observations of the DGGE profiles from the gelatinized and non-gelatinized sago starch cultures indicate that cultures from gelatinized starch (both dry and wet) possess similar microbial community structures, which are affiliated with Gram-positive bacteria, uncultured *Thermoanaerobacterium* sp., *Thermoanaerobacterium saccharolyticum*, and *Thermoanaerobacterium thermosulfurigenes*. *Thermoanaerobacterium* sp., an anaerobic spore-forming thermophilic microbe, has been known for its capacity to utilize various types of substrates for hydrogen production [1]. The saccharolytic fermentative bacterium *T. saccharolyticum* participates in interspecific hydrogen transfer in its natural environment, thereby producing hydrogen from carbohydrates. The species has been engineered to produce high yields of ethanol from a wide array of biomass-derived sugars [63]. *T. thermosulfurigenes*, also known as *Clostridium thermosulfurigenes*, produces an extracellular thermoactive thermostable β-amylase and a cell-bound glucoamylase as the major amylolytic enzymes responsible for starch degradation. It also produces ethanol, acetate, lactate, and hydrogen as the main end products [64].

In non-gelatinized dry starch cultures, *Geobacillus* sp. was observed along with *Thermoanaerobacterium* sp. *Geobacillus* sp. is widely distributed, and has been successfully isolated from geothermal environments. *Geobacillus* sp. has aroused interest in the industrial sector, on account of its potential applications in the biotechnological processes, for example, as sources of thermostable enzymes such as proteases, amylases, lipases, and pullanases [65]. In the non-gelatinized wet starch culture, bands related to the *Bacillus* sp., including *Bacillus lentus* and *Anoxybacillus* sp., were detected. These thermophilic bacilli are generally isolated from geothermal hot springs, and mainly produce amylolytic enzymes capable of utilizing carbohydrates, including starch; some can also produce hydrogen [1,66,67].

Table 3 – A comparison of hydrogen yields obtained by anaerobic fermentation of soluble starch, cornstarch, and cassava starch from previous studies and sago starch (this study).

Starch source	Initial Conc. (g/l)	Temp. (°C)	pH	Organism	Reactor	H_2 yield (ml/g starch)	Ref.
Starch (Farco Chemical, China) in wastewater	4.6	55	6	H_2-producing sludge	Batch	92	[17]
Cornstarch	2	35	8	Mixed bacteria	Batch	194	[61]
Cassava starch	10	35	7	Anaerobic activated sludge	Batch	240	[62]
Starch wastewater	5	37	6.5	Municipal WWTP sludge	Batch	186	[8]
Sago starch in wastewater	2.5	60	6.5	Enriched mixed culture (PGK)	Batch	442	This study

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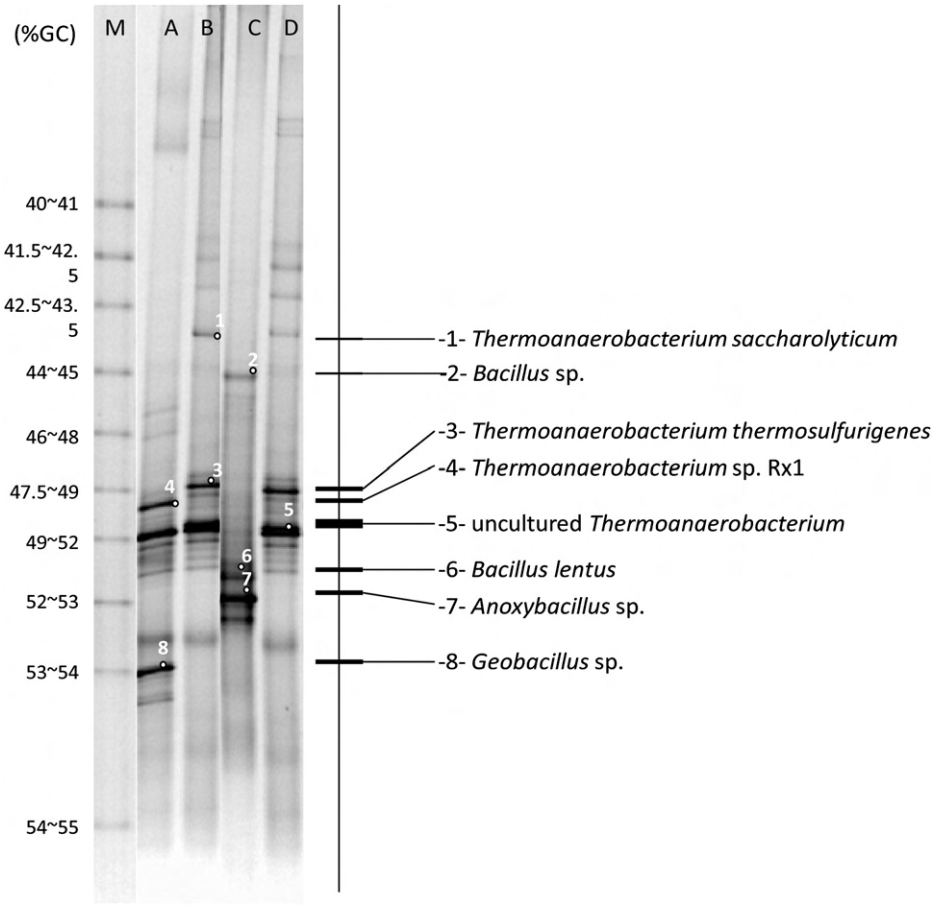

Fig. 4 – DGGE profile of 16S rRNA gene fragments. The fragments were PCR-amplified from total DNA extracted of enriched culture with sago starch used for hydrogen production (M, DGGE marker; A, bacteria community in non-gelatinized dry sago starch; B, gelatinized dry sago starch; C, non-gelatinized wet sago starch; and D, gelatinized wet sago starch).

Since lower hydrogen yields were observed in the non-gelatinized wet starch cultures (Fig. 3), we suggest that the bacteria in these cultures are associated with the starch-hydrolyzing processes, rather than with hydrogen production, as indicated by the high percentage of starch utilization (data not shown). Another possible contribution of the *Bacillus* sp. observed in this system is oxygen consumption, thereby generating the anaerobic conditions required by the hydrogen-producing microbes. The presence of some members of the aerobic *Bacillus* sp. in a hydrogen production system, in conjunction with some members of the anaerobic *Clostridium* sp., was observed in previous studies also, and is considered to positively contribute to the conditions essential for hydrogen production (an "added-value") by mixed culture methods [68–70].

The result of this microbial community analysis in combination with the higher hydrogen yields obtained from the gelatinized sago starch cultures (as depicted in Fig. 3) indicate that gelatinized cultures possess microorganisms capable of simultaneously degrading starch and efficiently producing hydrogen. In conclusion, we state that the enriched mixed culture from the hot spring in Southern Thailand (i.e., the PGK culture) shows promising potential for application in biohydrogen production from starch; the bacterial activity simultaneously degrades starch and produces hydrogen. Nested PCR of the DNA extracts of the enriched culture samples did not detect any amplification products of the archaeal 16S rRNA genes, thus indicating the absence of archaea in the enriched cultures.

4. Conclusions

The enriched PGK culture used in this study can produce a methane-free biogas with a hydrogen content of up to 55%. The optimal conditions for producing hydrogen from sago starch by using PGK culture at 60 °C were achieved when using gelatinized dry starch medium with an initial pH of 6.5 and an initial starch concentration of 2.5 g/l. The maximum hydrogen yield obtained under these conditions was 442.2 ml H_2/g-$starch_{added}$ (80% of the theoretical limit). Hydrogen yields decreased at the lower and higher initial pH values, at higher initial starch concentrations, and when using non-gelatinized starch.

PCR-DGGE profiles of the 16S rRNA gene fragments from the cultures showed that the predominant species associated with efficient hydrogen production were closely related to the thermophilic anaerobic bacteria, including *T. saccharolyticum*,

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T. thermosulfurigenes, and the uncultured *Thermoanaerobacterium* sp.; these microbes may simultaneously produce hydrogen and ethanol. Thermophilic bacilli capable of utilizing starch, including *Bacillus* sp., *Anoxybacillus* sp., *B. lentus*, and *Geobacillus* sp., were also found in the cultures, but mainly in the non-gelatinized wet starch culture.

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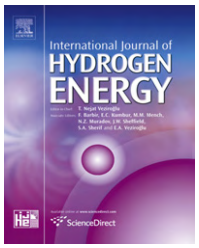

Isolation and characterization of high hydrogen-producing strain *Clostridium beijerinckii* PS-3 from fermented oil palm sap

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ABSTRACT

Felled oil palm trunk (OPT) (25 years old) is an abundant biomass in Southern Thailand. The OPT composition was 31.28–42.85% cellulose, 19.73–25.56% hemicellulose, 10.74–18.47% lignin, 1.63–2.25% protein, 1.60–1.83% fat, 1.12–1.35% ash and trace amount of minerals (0.01–0.40%). Oil palm sap extracted from OPT was found to contain 15.72 g/L glucose, 2.25 g/L xylose, and 0.086 g/L arabinose. A total of twenty samples from hot springs (45–75 °C and pH 6.5–8.4), oil palm sap and palm oil mill effluent were enriched for isolation of hydrogen-producing bacteria. The highest hydrogen-producing strain was isolated from oil palm sap and identified as *Clostridium beijerinckii* PS-3 using biochemical test and 16S rRNA gene analysis. Among various carbon sources tested, glucose, xylose, starch and cellulose were the preferred substrates for hydrogen production. The strain PS-3 could produce the maximum hydrogen yield of 140.9 ml H_2/g total sugar and the cumulative hydrogen production of 1973 ml/L-oil palm sap. Therefore, *C. beijerinckii* PS-3 is a potential candidate for fermentative hydrogen production from mixed sugars of the oil palm sap.

1. Introduction

Hydrogen is one of the major and most important biofuels which can be obtained by the fermentation of biomass using microorganisms [1]. It may not have a significant environmental impact as it did not involve any fossil fuel combustion and has less effect on air pollution [2] with its low atmospheric photochemical reactivity that further reduces its impact on ozone formation. The production of hydrogen from lignocellulosic biomass could improve energy security, reduce trade deficits, decrease urban air pollution and contribute little net CO_2 accumulation to the atmosphere [3].

Biomass is an important contributor to the world economy. Thailand is one of the largest food exporters in the world and is a major palm oil producer, producing 2.46–2.85% of world production [4]. Thailand therefore has abundant supplies of biomass resources with about 77 million oil palm trunks distributed over 20 provinces in the country but mainly in the south [4]. In 2008, there were 0.68 million tons of oil palm biomass including trunk, fronds, and empty fruit bunches [5]. The oil palm trunks appear to be waste on replanting and left unused in the plantation area. Hence, their economically utilization is urgently needed and should have been intensively studied.

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Various hydrogen-producing strains, including species of *Enterobacter* [6] and *Clostridium* [7], have been studied. Some *enterobacter* species can degrade soluble starch, while some *clostridium* species are able to degrade insoluble starch without any pretreatment [8]. Among the hydrogen-producers, *Clostridium butyricum* has been studied most extensively due to its fast growth with a high hydrogen yield (1.6–2.4 mol/mol-glucose). Numerous investigations of biohydrogen production using *Clostridium* sp. have been reported, which generally gives a hydrogen yield of 1.4–2.8 mol/mol-glucose [9–11]. *Clostridium acetobutylicum* [12], *Clostridium pasteurianum* CH4 [13] and *Clostridium paraputrificum* [14] exhibited hydrogen yields of 2.0 mol/mol-glucose, 2.07 mol/mol-hexose and 2.2 mol/mol-substrate, respectively.

This study aims to isolate mesophilic and thermophilic anaerobes from different natural and man-made systems such as hot springs, oil palm sap and oil palm mill effluent (POME) for production of hydrogen.

Table 1 – Composition analysis at various heights and positions of oil palm trunk (% dry weight).

Composition	Top (%)	Middle (%)	Bottom (%)
Cellulose	42.85 ± 2.5	40.65 ± 2.8	31.28 ± 3.5
Hemicellulose	25.56 ± 3.4	22.12 ± 1.5	19.73 ± 2.1
Lignin	10.74 ± 1.8	15.32 ± 2.2	18.47 ± 1.6
Protein	2.25 ± 0.05	1.84 ± 1.2	1.63 ± 0.4
Fat	1.83 ± 0.02	1.61 ± 0.02	1.60 ± 0.01
Ash	1.12 ± 0.03	1.17 ± 0.03	1.35 ± 0.02
N	0.26 ± 0.05	0.25 ± 0.01	0.23 ± 0.01
P	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
K	0.40 ± 0.02	0.40 ± 0.01	0.45 ± 0.01
S	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Ca	0.12 ± 0.01	0.12 ± 0.01	0.15 ± 0.01
Mg	0.13 ± 0.01	0.13 ± 0.01	0.11 ± 0.01
Fe	0.03 ± 0.01	–	–
Zn	0.01 ± 0.01	–	–

Results are means ± SD of three determinations.

2. Materials and methods

2.1. Culture medium

The synthetic medium used for bacterial growth contained 1.5 g/L KH_2PO_4, 2.9 g/L K_2HPO_4, 2.1 g/L urea, 4.5 g/L yeast extract, 0.05 g/L $MgCl_2.6H_2O$, 0.0075 g/L $CaCl_2.2H_2O$ and 0.015 g/L $FeSO_4.7H_2O$ [15] supplemented with mixed substrate; 5 g/L glucose, 5 g/L starch, 5 g/L xylose, 5 g/L cellulose. The initial pH was adjusted to 7.0.

The oil palm (OP) sap medium used for enrichment of the hydrogen-producing bacteria contained OP sap supplemented with 1% (w/v) pulp (residues after pressing oil palm trunk through a screw press) and 1 g/L yeast extract. The initial pH was adjusted to 7.0.

2.2. Oil palm trunk (OPT) and preparation of oil palm sap

Twenty-five year-old oil palm trunks were collected from an oil palm plantation in Phangnga Province, Southern Thailand. The length of felled OPT was in the range of 12 m–15 m, with a diameter of 35 cm–80 cm. The whole OPT was cut at a length of 3 m each at various heights and positions (top, middle, bottom). The OPT of each position were cut into small pieces, then stored in a −20 °C freezer until used. The OPT was determined for cellulose, hemicellulose and lignin [16], protein, fat, ash and mineral content [17]. The composition of OPT was 31.28–42.85% cellulose, 19.73–25.56% hemicellulose, 10.74–18.47% lignin, 1.63–2.25% protein, 1.60–1.83% fat, 1.12–1.35% ash and with trace amounts of mineral content (0.01–0.40%) (Table 1).

The top of the OPT was cut in cross section into three parts: part A (core); B (middle); and C (outer) (modified from Yutaka et al. [18]). Each part was soaked in water for 24 h, then pressed through a screw press to obtain oil palm sap (OP sap) and pulp. Concentrations of hexose (glucose) and pentose (xylose, arabinose) were determined using high performance liquid chromatography (HPLC) (Agilent 1200 series), equipped with Aminex HPX-87H ion exclusion column. The HPLC was operated with a refractive index detector under conditions of 0.005 M H_2SO_4 as effluent for 0.6 ml/min and at 65 °C [19]. The moisture content and free sugar content of each part are given in Table 2. The amount of fermentable sugars was highest (nearly 2%) in the sap (core) from the top of OPT. This OP sap was consisted of 15.72 g/L glucose, 2.25 g/L xylose, and 0.086 g/L arabinose. The OP sap was stored in a −20 °C freezer until used.

2.3. Samples from hot springs, oil palm sap and palm oil mill effluent (POME)

Water, sediment and soil samples were collected from four-tenn geothermal springs located in Southern Thailand. The temperature (45–75 °C) and pH (6.5–8.4) of all samples were measured using a thermometer (on-site) and pH meter, respectively. The samples were kept in serum bottles for transportation to the laboratory and incubated at room temperature (30 °C) for 24 h. The OP sap from the core section of top OPT (Section 2.2) was used for enrichment the cultures. Two sources of raw palm oil mill effluent (POME); sludge and decanter effluent, were collected from the receiving tank of Permkiat Palm Oil Co, Ltd. at Satun Province, Southern

Table 2 – Moisture content and free sugar content of each part in cross section of the top oil palm trunk.

Parameters	Parts		
	A (core)	B (middle)	C (outer)
Moisture content (%)	81.8 ± 1.8	74.4 ± 1.5	71.5 ± 3.5
Free sugars			
Glucose	15.72 ± 2.4	12.06 ± 1.2	7.71 ± 1.1
Xylose	2.25 ± 1.2	1.15 ± 0.8	0.75 ± 0.2
Arabinose	0.086 ± 0.01	0.045 ± 0.01	0.036 ± 0.01
Total sugar (g/L)	18.06 ± 1.6	13.25 ± 2.4	8.50 ± 1.8

Samples from different part of oil palm trunk were prepared according to the method described in Materials and methods. Results are means ± SD of three determinations.

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Thailand. Samples were collected in sterile plastic bags and kept at 4 °C until used.

2.4. Culture enrichment

The samples (1 mL each) from hot spring and POME were transferred into a 9 mL sterile OP sap medium. These were compared to the 10 mL non-sterile OP sap medium that did not contain any hot spring and palm oil mill effluent samples. They were incubated at 30 °C and 60 °C for 5 days. The cultures from the above three sources (hot spring, POME, non-sterile OP sap) were then sub-cultured into the OP sap medium and incubated again for hydrogen production. Samples were taken at time intervals to determine the cumulative hydrogen production. The consortia were kept in 50% glycerol at −20 °C. The enriched cultures with high cumulative hydrogen production were selected for isolation.

2.5. Isolation and characterization of the selected bacterial strains

A synthetic medium was used for isolation [15] and prepared in the OP sap (100% v/v) (as a diluent and a carbon source). The selected culture was transferred into a 50 ml synthetic medium and incubated at 30 °C for 24 h. The culture was serially diluted in fresh medium and incubated as strict anaerobes at the same temperature as that used for the primary enrichment. Colonies from each dilution were transferred to a fresh liquid medium and analyzed further for hydrogen production. The isolated strains were tested for hydrogen production from OP sap medium. Culture broth from the serum bottles was taken for measurement of final pH and analysis of substrate utilization, cumulative H_2 production, H_2 yield, H_2 content and soluble metabolites (ethanol, acetic cid, butyric acid and proionic acid).

The volumetric cumulative hydrogen production (ml/L) was calculated from the total gas production and the concentration of hydrogen in the headspace. The hydrogen production yield was calculated as ml H_2/g total sugar. The strain producing the highest cumulative hydrogen production was selected for further study.

2.6. Strain identification

The characterization and morphology of the isolated strains were determined according to Bergey's Manual of Determinative Bacteriology [20]. Morphological examinations were performed with a scanning electron microscope (FEI Quanta 400, SEM-Quanta). Gram staining was performed by the Hucker method [21]. Substrates were tested with the following concentrations: 10 g/L for arabinose, fructose, galactose, glucose, mannose, rhamnose, xylose, cellobiose, lactose, maltose, sucrose, starch, xylan, cellulose, and glycerol. The identification was confirmed using 16S rDNA technique.

The bacterial 16S rDNA (1400 bp) was amplified by the first polymerase chain reaction (PCR) with universal primer. In the second PCR, V3-specific reverse and forward primers with GC clamp were used to amplify the fragment of V3 region of 16S rDNA product from the first PCR. The DNA in the excised gel slices were incubated in 20 ml of distilled water at 4 °C for 24 h and re-amplified by PCR with the second PCR primers [22]. After re-amplification, PCR products were purified and sequenced using reverse primer by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

The closest matches for partial 16S rRNA gene sequences were identified by the ribosomal database project (http://rdp.cme.msu.edu/). This used the SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Analytical methods

The evolved biogas was collected in the headspace of a serum bottle. The total volumes at each time interval were measured at room temperature using a syringe. Hydrogen in the biogas was measured by a gas meter (MX2100 OLDHAM, MULTIGAS Type MX2100) [23]. The culture broth was centrifuged at 10,000 × g for 10 min. Fermentation end products (volatile fatty acids (VFA) and ethanol) in the supernatant were determined by gas chromatography (GC HP 6890 series) equipped with a flame ionization detector (FID) and Innowax column (dimensions 30 m × 320 um × 0.25 um). The temperature of the injection port was 230 °C. The chromatography was performed using the following program: 70 °C for 1 min, 70–180 °C with a ramping of 20 °C/min, 180 °C for 6 min. The detector temperature was 250 °C [21]. Data were the average of three determinations.

Table 3 – Hydrogen production using enrichment cultures grown on OP sap medium supplemented with pulp (1% w/v) at neural pH and incubated at 30 and 60 °C for 5 days.

Samples	Growth Temp. (30 °C)		Growth Temp. (60 °C)	
	H_2 (ml/L)	H_2 (%)	H_2 (ml/L)	H_2 (%)
OP sap				
A (core)	1008.5	29.8	37.4	5.2
B (middle)	134.6	11.1	15.1	3.4
C (outer)	107.3	10.1	13.5	3.2
Palm oil mill effluent (POME)				
Sludge	40.5	2.2	80.1	4.9
Decanter effluent	67.8	4.9	72.6	5.3
Hot spring				
81RN1	1085.4	29.4	35.4	4.2
83RN3	0.0	0.0	32.3	4.6
85RN5	993.5	28.7	37.2	4.4
86RN6	33.3	3.9	42.9	4.7
89SR3-1	37.4	3.8	59.2	4.4
89SR3-2	982.9	27.1	45.4	4.5
93SR7	35.2	3.3	48.4	4.3
94SR8	57.1	2.2	86.1	5.0
97PG1	65.1	3.1	65.2	4.0
98PG2	58.3	2.2	72.3	4.3
99PG3	36.5	2.6	77.1	4.5
105TR1	65.5	3.3	45.6	3.8
107PL1	46.8	3.9	36.4	4.4
109PL3	35.2	4.4	38.6	3.6
112YL1	778.2	27.5	87.8	4.3

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Table 4 – Screening of fermentative hydrogen-producing bacteria supplemented with mixed substrates (5 g/L xylose, 5 g/L glucose, 5 g/L starch and 5 g/L cellulose) in synthetic medium at neural pH and incubated at 30 and 60 °C for 5 days.

Mixed cultures	Code of pure culture	Cumulative H_2 production (ml/L)	H_2 yield (ml H_2/g total sugar)	H_2 content (%)	Final pH	Ethanol (mM)	Acetic acid (mM)	Butyric acid (mM)	Propionic acid (mM)
OP sap	PS-3	1873.0	130.6	27.5	4.5	13.4	30.5	41.9	5.3
85RN5	PS-4	1773.4	109.6	26.4	5.1	15.1	4.3	1.1	0.2
85RN5	PS-5	1739.6	104.8	25.2	5.2	4.7	1.8	0.4	4.7
89SR3	PS-7	1869.6	119.7	28.0	4.6	12.5	29.5	39.1	4.9
112YL1	PS-8	1834.4	109.6	25.5	4.4	13.6	11.6	39.4	2.4

3. Results and discussion

3.1. *Strain enrichment, isolation and screening*

Twenty samples from mesophilic and thermophilic natural and man-made environments were used for enrichment. A total of 20 enrichment cultures were tested for conversion of OP sap to hydrogen at neutral pH and incubated at 30 °C and 60 °C. The top for hydrogen production was obtained from the hot spring and OP sap incubated at 30 °C (778.2–1085.4 ml/L) (Table 3). Their hydrogen content (27.1–29.8%) was also higher than those from POME (2.2–4.9%). It was observed that the hot spring and OP sap samples, hydrogen production from these top mesophilic strains was much higher than those from the thermophilic strains (35.4–87.8 ml/L). However, this was not the case for the sludge and decanter effluent whereby hydrogen production from the mesophilic strains (40.5 and 67.8 ml/L, respectively) were lower than those of the thermophilic strain (80.1 and 72.6 ml/L, respectively).

The selected top 5 enriched cultures were subsequently enriched in the OP sap medium (with no addition of pulp) for screening. They exhibited higher hydrogen production (1757–1967 ml/L) and hydrogen content (27–28.6%) (data not shown). These five sources were employed for the isolation of high hydrogen-producing strains. Colonies were visible after 2–7 days incubation. Among 36 strains isolated, five of them demonstrated high cumulative hydrogen production (Table 4) and the top 5 hydrogen-producing strains encoded as the isolate PS-3, PS-4, PS-5, PS-7 and PS-8 were selected. They demonstrated great capacity in hydrogen production (1739–1873 ml/L) as well as hydrogen content (25.2–28.0%) closed to those original enriched mixed cultures. Their hydrogen yields were in the range of 104.8–130.6 ml H_2/g total sugar. The soluble metabolites were composed butyric acid (0.4–41.9 mM), acetic acid (1.8–30.5 mM), ethanol (4.7–15.1 mM) and propionic acid (0.2–5.3 mM).

The OP sap contained free monomeric sugars dominated by glucose and xylose and also had starch (2%) which could be a substrate for hydrogen production. For screening of the most efficient hydrogen-producing strain, the selected pure strains were cultivated in 100% (v/v) OP sap with the addition of 1% (w/v) pulp for another carbon source (in term of cellulose) and 1 g/L yeast extract for nitrogen source and vitamin. The results (Table 5) showed that they produced hydrogen as the major fermentation products (1069–1973 ml/L, 26.4–33.7% H_2 content and 71–141 ml H_2/g total sugar) and soluble metabolites were composed mostly of butyric acid (1.9–42.5 mM), acetic acid (4.5–31.8 mM), ethanol (11.2–19.3 mM) and propionic acid (0.3–5.4 mM). The highest hydrogen production of 1973 ml/L was obtained from the isolate PS-3. This was higher than those from *C. butyricum* CGS5 grown in rice husk hydrolyzate (88.1 ml/L) [13] and *Enterobacter asburiae* SNU-1 using glucose as a carbon source (350 ml/L) [24].

3.2. *Characterization of fermentative hydrogen-producing bacteria*

Five selected isolates designated as PS-3, PS-4, PS-5, PS-7 and PS-8; were identified. They were different in colony shape and identified using 16S rRNA gene sequence analysis. Among them, the isolate PS-3 from oil palm sap gave the highest hydrogen yield of 140.9 ml H_2/g total sugar which was higher than *C. butyricum* CGS5 (84 ml H_2/g total sugar) [9]. This is compared to those of 78.0, 71.3, 95.3 and 126.7 ml H_2/g total sugar from the isolates PS-4, PS-5, PS-7 and PS-8, respectively

Table 5 – Hydrogen production from OP sap (100% v/v) supplemented with 1 g/L yeast extract at neural pH and incubated at 30 and 60 °C for 5 days.

Pure culture	Cumulative H_2 production (ml/L)	H_2 yield (ml H_2/g total sugar)	H_2 content (%)	Final pH	Ethanol (mM)	Acetic acid (mM)	Butyric acid (mM)	Propionic acid (mM)
PS-3	1973.0	140.9	33.7	4.3	15.6	31.8	42.5	5.4
PS-4	1169.6	78.0	31.5	4.8	19.3	4.5	2.1	0.3
PS-5	1069.7	71.3	26.4	4.9	17.2	4.8	1.9	0.3
PS-7	1334.5	95.3	32.5	4.5	13.4	31.2	41.2	5.3
PS-8	1773.5	126.7	29.6	4.4	11.2	13.2	32.2	1.2

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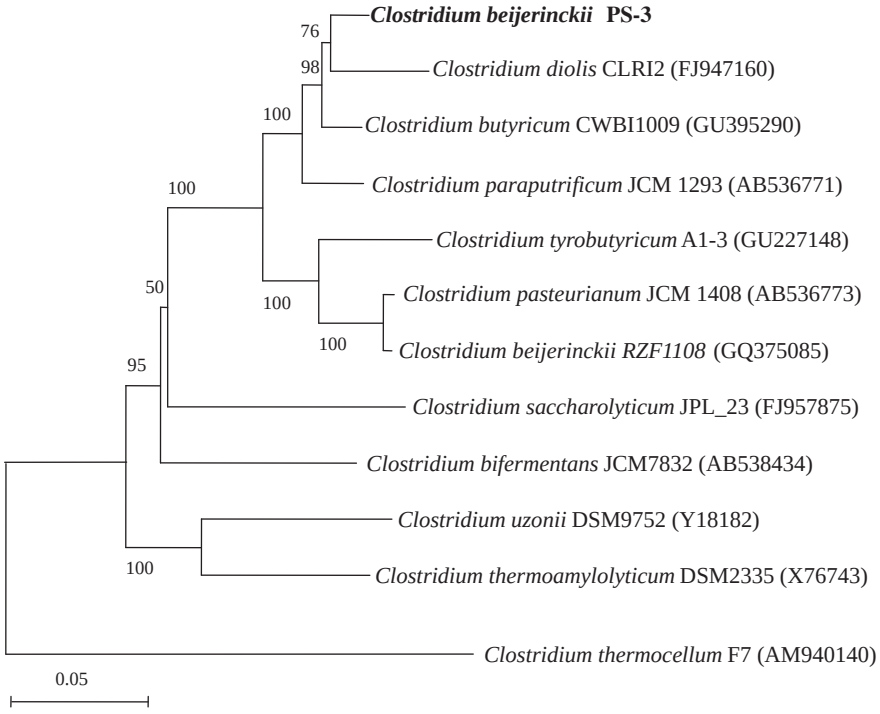

Fig. 1 – Neighbor-joining tree showing the phylogenetic position of isolated strain, *C. beijerinckii* PS-3, based on the 16S rRNA sequences of the genus *Clostridium*. The numbers at the nodes indicate the levels of bootstrap support percentages based on the neighbor-joining of 1000 replicates. Bar 0.05 nucleotide substitutions per site. GenBank accession numbers are given in parentheses.

(Table 5). 16S rRNA analysis revealed that the isolate PS-3 is a member of the genus *Clostridium*.

The closet phylogenetic relative was *Clostridium beijerinckii*, where a similarity was recorded to the 16S rRNA genes of 97%. A phylogenetic tree was constructed (Fig. 1). The phenotypic characteristics properties such as endospore-Forming, being Gram-positive, rod shape, motility, catalase positive and the strict anaerobes of the isolate PS-3 were consistent with those of the *Clostridium* species. However, most of them have been reported to produce hydrogen [25]. These include mesophilics (at 37 °C) of: *C. acetobutylicum* (1.8–2.0 mol/mol-glucose); *Clostridium bifermentans* (not determined); *C. beijerinckii* (2.0–2.8 mol/mol-glucose); *C. butyricum* (1.1–2.3 mol/mol-glucose); *Clostridium diolis* (not determined); *C. pasteurianum* (2.1–2.4 mol/mol-glucose); *C. paraputrificum* (1.0–2.2 mol/mol-glucose); and *Clostridium tyrobutyricum* (1.47 mol/mol-glucose). They also include thermophilics (at 55 °C) of: *Clostridium thermocellum* (0.8–1.6 mol/mol-glucose); *Clostridium thermolacticum* (1.1–1.5 mol/mol-glucose); *Clostridium uzonii* (0.55–0.67 mol/mol-glucose) and *Clostridium thermosaccharolyticum* (1.4–2.53 mol/mol-glucose) [26]. The isolated PS-3 can growth with various types of carbohydrates as carbon sources under mesophilic conditions. The substrate spectrum of the strain PS-3 has an ability to produce growth from arabinose, fructose, galactose, glucose, mannose, rhamnose, xylose, cellobiose, lactose, maltose, sucrose, starch, xylan, cellulose, and glycerol. Effective fermentative bacteria should use various type of substrates especially complex carbohydrate. Therefore, the level of hydrogen production of *C. beijerinckii* PS-3 from various types of carbohydrates was examined.

3.3. *Identification of the isolated bacteria*

Bacteria with the highest cumulative hydrogen production were then examined under a microscope (20 kV, 20,000×). This

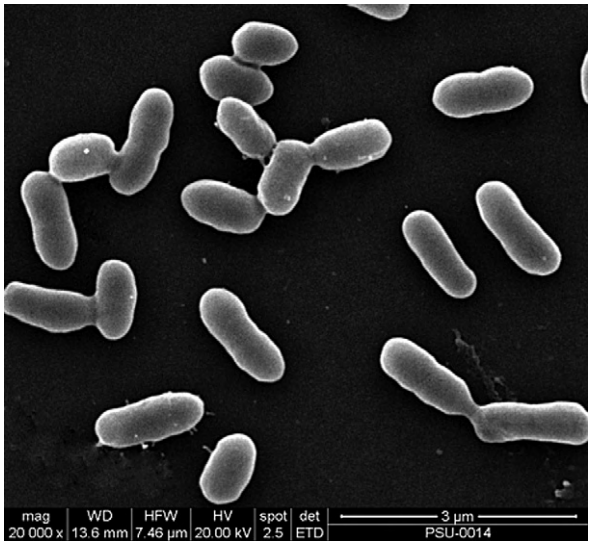

Fig. 2 – Scanning electron microscope (SEM) imaged of *Clostridium beijerinckii* PS-3.

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revealed that the bacteria have a rod shape (Fig. 2), with a diameter from 0.2 to 0.4 μm and a length from 0.9 to 1.5 μm. The cells had rounded ends. They occurred singly or in pairs. They exhibited Gram-positive staining in both the exponential and stationary growth phases. Spore was observed when nutrients were exhausted or other conditions become unfavorable for growth.

The nearly full-length sequences of 16S rDNA gene (1400 bp) were identified for the isolate. By aligning with the 16S rDNA gene sequences from GenBank releases, the strain PS-3 exhibited 97% sequence identity with *C. beijerinckii* JCM 7845. According to the multiple analysis results, this high hydrogen-producing strain was placed in the species *C. beijerinckii* PS-3. In addition, the 16S rDNA gene sequence of *C. beijerinckii* PS-3 had been deposited in the GenBank under accession number AB020190.

4. Conclusions

C. beijerinckii and *C. acetobutylicum* were found to be the dominant bacteria in the OP sap and hot spring samples (89SR3-2 and 112YL1). A mesophilic hydrogen-producing bacterial strain was isolated from oil palm sap (OP sap) and designated as *C. beijerinckii* PS-3. The bacterium can utilize different carbon sources including glucose and xylose to produce hydrogen. The maximal hydrogen yield and the cumulative hydrogen production were 140.9 ml H_2/g total sugar and 1973 ml/L-oil palm sap, respectively. It can be concluded that *C. beijerinckii* PS-3 is a potential candidate for fermentative hydrogen production from oil palm sap.

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