Process Development of Biohydrogen Production from Crude Glycerol of Biodiesel Plant by Thermotolerant Bacteria

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ชื่อวิทยานิพนธ์ การพัฒนากระบวนการผลิตไฮโดรเจนจากกลีเซอรอลโรงงานผลิตไบโอดีเซลโดยแบคทีเรียทนร้อน

ชื่อผู้แต่ง นาย ธีระ ชูแก้ว

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บทคัดย่อ

เมื่อนำตัวอย่างจากดินที่ปนเปื้อนกลีเซอรอลดิบและน้ำพุร้อนในภาคใต้ของประเทศไทยจำนวน 19 ตัวอย่างมากระตุ้นในอาหารที่มีกลีเซอรอลและบ่มที่อุณหภูมิ 45 องศาเซลเซียสเพื่อแยกเชื้อจุลินทรีย์ทนร้อนที่ผลิตไฮโดรเจนพบว่า ตัวอย่างดินที่ปนเปื้อนกลีเซอรอลดิบสามารถผลิตไฮโดรเจนได้สูงเป็น 2 เท่าเมื่อเทียบกับตัวอย่างจากน้ำพุร้อน จากแบคทีเรีย 38 สายพันธุ์ที่แยกได้พบว่า 10 สายพันธุ์สามารถผลิตไฮโดรเจนได้สูง เชื้อที่คัดเลือกไว้คือ สายพันธุ์ TR17, TR20 และ TR32 มีประสิทธิภาพในการผลิตไฮโดรเจนจากกลีเซอรอลดิบเมื่อเลี้ยงที่อุณหภูมิ 45 องศาเซลเซียส (17.17, 15.17 และ 15.97 มิลลิโมลไฮโดรเจนต่อลิตร ตามลำดับ) ได้สูงกว่าการเลี้ยงที่อุณหภูมิ 30 องศาเซลเซียส (9.07, 7.82 และ 13.48 มิลลิโมลไฮโดรเจนต่อลิตร ตามลำดับ) ดังนั้นเชื้อสามสายพันธุ์ดังกล่าวจึงจัดเป็นเชื้อทนร้อน โดยสายพันธุ์ TR17 ผลิตไฮโดรเจน (20.90 มิลลิโมลไฮโดรเจนต่อลิตร) และให้ผลผลิตไฮโดรเจนได้สูงสุด (0.21 โมลต่อโมลกลีเซอรอล) รวมทั้งผลิต 2,3-บิวเทนไดออล (17.15 มิลลิโมลต่อลิตร) และให้ผลผลิต 2,3-บิวเทนไดออลสูงสุด (0.16 โมลต่อโมลกลีเซอรอล) ส่วนสายพันธุ์ TR20 จัดเป็นสายพันธุ์ที่ผลิต 1,3-โพรเพนไดออล (54.32 มิลลิโมลต่อลิตร) และสายพันธุ์ TR32 จัดเป็นสายพันธุ์ที่ผลิตเอทานอล (73.32 มิลลิโมลต่อลิตร) โดยให้ค่าผลผลิต1,3-โพรเพนไดออลและเอทานอลจากกลีเซอรอลได้สูงสุด 0.62 และ 0.77 โมลต่อโมลกลีเซอรอล ตามลำดับ

เมื่อจำแนกสายพันธุ์ทนร้อน TR17 ที่ผลิตไฮโดรเจน โดยการศึกษาลักษณะทางสรีรวิทยาร่วมกับคุณสมบัติทางชีวเคมีและการเทียบเคียงลำดับยีน 16S rRNA พบว่าเป็น *Klebsiella pneumoniae* เมื่อศึกษาสภาวะที่เหมาะสมต่อการผลิตไฮโดรเจนโดย *Klebsiella pneumoniae* TR17 พบว่าเชื้อสามารถผลิตไฮโดรเจนในช่วงอุณหภูมิ 30-50 องศาเซลเซียส พีเอชเริ่มต้น 4.0-9.0 ความเข้มข้นของกลีเซอรอล 20-100 กรัมต่อลิตรและยีสต์สกัดเป็นแหล่งไนโตรเจน สภาวะที่เหมาะสมสำหรับการผลิตไฮโดรเจนแบบกะคือ อุณหภูมิ 40 องศาเซลเซียส พีเอชเริ่มต้น 8.0 ความเข้มข้นของ

กลีเซอรอล 20 กรัมต่อลิตรและยีสต์สกัด 2 กรัมต่อลิตร โดยให้ค่าสูงสุดในการผลิตไฮโดรเจน 27.7 มิลลิโมลไฮโดรเจนต่อลิตรและผลผลิตไฮโดรเจน 0.25 โมลต่อโมลกลีเซอรอล นอกจากนี้ยังได้ผลิตภัณฑ์พลอยได้อื่นๆได้แก่ 1,3-โพรเพนไดออล, 2,3-บิวเทนไดออล และเอทานอลในปริมาณเท่ากับ 3.52, 2.06 และ 3.95 กรัมต่อลิตร ตามลำดับ

จากการศึกษาผลขององค์ประกอบของอาหารเลี้ยงเชื้อต่อการผลิตไฮโดรเจนจากกลีเซอรอลดิบของ *Klebsiella pneumoniae* TR17 ด้วยวิธีทางสถิติแบบพื้นผิวตอบสนอง โดยใช้วิธี Plackett-Burman design พบว่าองค์ประกอบที่สำคัญของอาหารเลี้ยงเชื้อได้แก่ โพแทสเซียมไดไฮโดรเจนฟอสเฟต (KH_2PO_4) และแอมโมเนียมคลอไรด์ (NH_4Cl) เป็นปัจจัยที่มีความสำคัญทางสถิติ ($p<0.05$) จากนั้นนำปัจจัยที่มีความสำคัญดังกล่าวรวมทั้งกลีเซอรอลดิบซึ่งใช้เป็นแหล่งคาร์บอนมาศึกษาหาความเข้มข้นที่เหมาะสมเพื่อให้ได้ผลผลิตสูงของไฮโดรเจนและเอทานอลด้วยวิธีทางสถิติแบบพื้นผิวตอบสนอง โดยใช้วิธี Central Composite design พบว่า กลีเซอรอล โพแทสเซียมไดไฮโดรเจนฟอสเฟตและแอมโมเนียมคลอไรด์ มีอิทธิพลเฉพาะตัวต่อการผลิตไฮโดรเจนและเอทานอลอย่างมีนัยสำคัญทางสถิติ($p<0.05$) ขณะที่อิทธิพลร่วมระหว่างโพแทสเซียมไดไฮโดรเจนฟอสเฟตและแอมโมเนียมคลอไรด์ มีความสำคัญอย่างมีนัยสำคัญทางสถิติต่อการผลิตเอทานอล ($p<0.05$) สำหรับสภาวะที่เหมาะสมในการผลิตไฮโดรเจนและเอทานอลประกอบด้วย กลีเซอรอล 11.14 กรัมต่อลิตร โพแทสเซียมไดไฮโดรเจนฟอสเฟต 2.47 กรัมต่อลิตร และแอมโมเนียมคลอไรด์ 6.03 กรัมต่อลิตร สภาวะดังกล่าวให้ค่าสูงสุดที่ได้จากการทำนายสำหรับการผลิตไฮโดรเจน 0.27 โมลต่อโมลกลีเซอรอล และเอทานอล 0.63 โมลต่อโมลกลีเซอรอล ซึ่งค่าดังกล่าวใกล้เคียงกับค่าที่ได้จากการทดลองคือ ผลผลิตไฮโดรเจน 0.26 โมลต่อโมลกลีเซอรอลและผลผลิตเอทานอล 0.58 โมลต่อโมลกลีเซอรอล

เมื่อศึกษาการผลิตไฮโดรเจนจากกลีเซอรอลดิบโดย *Klebsiella pneumoniae* TR17 ด้วยระบบตรึงเซลล์ในถังหมักยูเอเอสบี (UASB) และระบบเซลล์อิสระในถังหมักยูเอ (UA) ภายใต้สภาวะไม่ฆ่าเชื้อและศึกษาผลของระยะเวลากักพักน้ำ (Hydraulic Retention Time, HRT) จาก 12 ถึง 2 ชั่วโมง และความเข้มข้นของกลีเซอรอลจาก 10 ถึง 30 กรัมต่อลิตร พบว่าในถังหมักยูเอเอสบีที่ความเข้มข้นของกลีเซอรอล 30 กรัมต่อลิตรและระยะเวลากักพักน้ำ 4 ชั่วโมงให้อัตราการผลิตไฮโดรเจนสูงสุด (242.15 มิลลิโมลไฮโดรเจนต่อลิตรต่อวัน) โดยสามารถลดค่าความเข้มข้นของสารอินทรีย์ (ในรูปของซีโอดี) ในช่วง 31.1-59.0% จากการวิเคราะห์โครงสร้างจุลินทรีย์ของระบบตรึงเซลล์ในถังหมักยูเอเอสบีและระบบเซลล์อิสระในถังหมักยูเอที่ใช้ความเข้มข้นของกลีเซอรอล 30 กรัมต่อลิตรโดยเทคนิค polymerase chain reaction-denaturing gradient gel electrophoresis

(PCR-DGGE) พบว่า *Klebsiella pneumoniae* TR17 เป็นจุลินทรีย์ที่เด่นซึ่งพบได้ในทุกระยะเวลากักพักน้ำของระบบตรึงเซลล์ในถังหมักยูเอเอสบี ขณะเดียวกันในระบบเซลล์อิสระ *Klebsiella pneumoniae* TR17 จะแข่งขันกับ *Escherichia coli* และ *Citrobacter freundii* ซึ่งเป็นจุลินทรีย์ที่เด่นในถังหมักยูเอ การทดลองแสดงให้เห็นว่าในสภาวะไม่ฆ่าเชื้อ ระบบตรึงเซลล์ช่วยให้ *Klebsiella pneumoniae* TR17 สามารถเจริญแข่งขันกับเชื้อจุลินทรีย์อื่นๆแม้ใช้ระยะเวลากักพักน้ำที่ต่ำ นอกจากนี้ภาพจากกล้องจุลทรรศน์อิเล็กตรอน (Scanning electron microscopy, SEM) แสดงให้เห็นว่า *Klebsiella pneumoniae* TR17 เป็นเชื้อจุลินทรีย์ที่เด่นบนพื้นผิวของวัสดุตรึงเซลล์

การผลิตไฮโดรเจนจากกลีเซอรอลดิบโดย *Klebsiella pneumoniae* TR17 ด้วยระบบตรึงเซลล์ในถังหมักยูเอเอสบีพบว่า เมื่อลดระยะเวลากักพักน้ำสามารถเพิ่มผลผลิตไฮโดรเจนจากกลีเซอรอล โดยที่ระยะเวลากักพักน้ำ 4 ชั่วโมงและความเข้มข้นของกลีเซอรอล 10 กรัมต่อลิตร ให้ผลผลิตไฮโดรเจนสูงสุด (44.27 มิลลิโมลไฮโดรเจนต่อลิตรต่อกรัมกลีเซอรอล) นอกจากนี้พบว่าผลิตภัณฑ์พลอยได้หลักในระบบคือ 1,3-โพรเพนไดออล ซึ่งบ่งชี้ว่า *Klebsiella* sp. เป็นจุลินทรีย์ที่เด่นระหว่างการผลิตไฮโดรเจนในสภาวะไม่ฆ่าเชื้อ เมื่อศึกษากลุ่มจุลินทรีย์โดยใช้เทคนิค Fluorescence *in situ* hybridization (FISH) พบว่าในถังหมักยูเอเอสบีประกอบด้วยจุลินทรีย์ในสกุล *Klebsiella* sp. 56.96, 59.45 และ 63.47% ของถังหมักที่มีความเข้มข้นกลีเซอรอล 10, 20 และ 30 กรัมต่อลิตร ตามลำดับ

เมื่อผลิตไฮโดรเจนจากกลีเซอรอลดิบแบบกะด้วยระบบสองขั้นตอนแบบไร้แสงและแบบให้แสงโดย *Klebsiella pneumoniae* TR17 และ *Rhodopseudomonas palustris* TN1 ตามลำดับ พบว่าในกระบวนการไร้แสงให้ค่าการผลิตไฮโดรเจนและผลผลิตไฮโดรเจนสูงสุด 64.24 มิลลิโมลไฮโดรเจนต่อลิตรและ 5.74 มิลลิโมลไฮโดรเจนต่อกรัมซีโอดีที่ใช้ไป ตามลำดับ โดยมีประสิทธิภาพในการใช้กลีเซอรอล 80.21% เมื่อศึกษาสภาวะที่เหมาะสมต่อการผลิตไฮโดรเจนในกระบวนการให้แสงที่มีการใช้ความเข้มข้นของน้ำทิ้งหลังการผลิตไฮโดรเจนแบบไร้แสง (dark fermentation effluent, DFE) (อัตราเจือจาง 0-5 เท่า) การเติมและไม่เติมยีสต์สกัด (yeast extract) (2.3 กรัมต่อลิตร) ร่วมกับโซเดียมไบคาร์บอเนต ($NaHCO_3$) (0.63 กรัมต่อลิตร) และการเติมกลูตาเมท (glutamate) (2-8 มิลลิโมลาร์) พบว่าการเจือจางน้ำทิ้งหลังการผลิตไฮโดรเจนแบบไร้แสง 5 เท่าโดยไม่เติมยีสต์สกัดร่วมกับโซเดียมไบคาร์บอเนตและเติมกลูตาเมท 2 มิลลิโมลาร์ให้ค่าสูงสุดในการผลิตไฮโดรเจน 3.12 มิลลิโมลไฮโดรเจนต่อลิตร โดยให้ผลผลิตไฮโดรเจนจากกลีเซอรอลสูงสุดในระบบสองขั้นตอนแบบไร้แสงและแบบให้แสงคิดเป็น 6.42 มิลลิโมลไฮโดรเจนต่อกรัมซีโอดีที่ใช้ไป คิดเป็น 10.4% ของค่าผลผลิตทางทฤษฎี

Thesis Title Process Development of Biohydrogen Production from Crude Glycerol of Biodiesel Plant by Thermotolerant Bacteria

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ABSTRACT

Nineteen samples from crude glycerol contaminated soil and hot springs in Southern Thailand was enriched in the medium containing glycerol and incubated at 45^0C for isolation of thermotolerant hydrogen-producing bacteria. Samples from crude glycerol contaminated soil produced hydrogen at least 2 folds higher than those from hot springs. Among 38 isolates obtained, 10 strains demonstrated great capacity in hydrogen production. The efficiency of hydrogen production by the selected isolates TR17, TR20, and TR32 at 45^0C (17.17, 15.17 and 15.97 mmol H_2/L, respectively) was superior to those observed at 30^0C (9.07, 7.82 and 13.48 mmol H_2/L, respectively). Thus, they were classified as thermotolerant strains. The isolate TR17 exhibited the maximum hydrogen production (20.90 mmol H_2/L) and yield (0.21 mol H_2/mol glycerol) with the highest 2,3-butanediol (17.15 mmol/L) and yield (0.16 mol/mol glycerol). The isolate TR20 was 1,3-propanediol producer (54.32 mmol/L) while the isolate TR32 was ethanol producer (73.32 mmol/L) with a yield of 0.62 and 0.77 mol/mol glycerol, respectively.

A thermotolerant fermentative hydrogen-producing isolate TR17 was identified as *Klebsiella pneumoniae* on the basis of the 16S rRNA gene analysis as well as physiological and biochemical characteristics. Optimization on production of hydrogen from *Klebsiella pneumoniae* TR17 was conducted. The strain produced hydrogen within a wide range of temperature ($30\text{-}50^0$C), initial pH (4.0-9.0) and glycerol concentration (20-100 g/L) with yeast extract as a favorable nitrogen source. In batch cultivation, the optimal conditions for hydrogen production were: cultivation temperature at 40^0C, initial pH at 8.0, 20 g/L glycerol and 2 g/L yeast extract. This resulted in the maximum cumulative hydrogen production of 27.7 mmol H_2/L and

hydrogen yield of 0.25 mol H_2/mol glycerol. In addition, the main soluble metabolites were 1,3-propanediol, 2,3-butanediol, and ethanol corresponding to the production of 3.52, 2.06, and 3.95 g/L, respectively.

Studies on the effect of medium components on simultaneous hydrogen and ethanol production from crude glycerol of *Klebsiella pneumoniae* TR17 by response surface methodology (RSM) using Plackett-Burman design were investigated. The significant variables ($p<0.05$), which influenced hydrogen production, were KH_2PO_4 and NH_4Cl. Subsequently, the two selected variables and glycerol as a carbon source were optimized by the Central Composite design (CCD) for achieving maximum hydrogen and ethanol yield. The concentration of glycerol, KH_2PO_4, and NH_4Cl had an individual effect on both hydrogen and ethanol yield ($p<0.05$), while KH_2PO_4 and NH_4Cl had an interactive effect on ethanol yield ($p<0.05$). The optimum medium components for production of hydrogen and ethanol were 11.14 g/L glycerol, 2.47 g/L KH_2PO_4, and 6.03 g/L NH_4Cl. The predicted maximum simultaneous hydrogen and ethanol yield were 0.27 mol H_2/mol glycerol and 0.63 mol EtOH/mol glycerol, respectively. Validation of the predicted optimal conditions exhibited similar values from the experiment. The hydrogen yield was 0.26 mol H_2/mol glycerol and ethanol yield was 0.58 mol EtOH/mol glycerol.

Biohydrogen production from crude glycerol using immobilized-cell and suspended-cell system of *Klebsiella pneumoniae* TR17 was investigated in up-flow anaerobic sludge blanket (UASB) and up-flow anaerobic (UA) reactors, respectively, under non-sterile condition. The reactors were operated under hydraulic retention time (HRT) from 12 to 2 h and glycerol concentration from 10 to 30 g/L. UASB performance at 30 g/L of glycerol and 4 h HRT gave the highest hydrogen production rate (242.15 mmol H_2/L d) with the chemical oxygen demand (COD) removal efficiency in the range of 31.1 to 59.0%. The microbial community of UASB and UA reactors at 30 g/L of glycerol was analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). In UASB reactor, *Klebsiella pneumoniae* TR17 was dominant in all HRT tested. In contrast, *K. pneumoniae* TR17 was competed with *Escherichia coli* and *Citrobacter freundii* in UA reactor. This indicated that the immobilized-cell system could compete with other microorganisms

in non-sterile condition even at low HRT. Scanning electron microscopy (SEM) illustrated that the morphological properties of *Klebsiella pneumoniae* TR17 were dominant on the immobilized materials.

Biohydrogen production from crude glycerol by immobilized *Klebsiella pneumoniae* TR17 in UASB reactor indicated that decreasing the HRT led to an increase in hydrogen yield (HY). The highest HY (44.27 mmol H_2/g glycerol consumed) were obtained at 4 h HRT with the glycerol concentration of 10 g/L. The main soluble metabolites were 1,3 propanediol which implied that *Klebsiella* sp. was dominant among other microorganisms during the operation. Fluorescence *in situ* hybridization (FISH) results revealed that the microbial community was dominated by *Klebsiella* sp. with 56.96, 59.45, and 63.47% of total DAPI binding cells, at the glycerol concentration of 10, 20, and 30 g/L, respectively.

Hydrogen production from crude glycerol by two stage process of dark fermentation using *Klebsiella pneumoniae* TR17 and photo fermentation using *Rhodopseudomonas palustris* TN1 was investigated in batch experiments. In dark fermentation, the cumulative hydrogen production and hydrogen yield were 64.24 mmol H_2/L and 5.74 mmol H_2/ g COD consumed, respectively with 80.21% glycerol conversion rate. The dark fermentation effluent (DFE) was employed for photo fermentation. Effect of DFE concentration (0 to 5 times dilution), with and without supplementation of yeast extract (2.3 g/L), $NaHCO_3$ (0.63 g/L), and glutamate (2-8 mM) were optimized. The optimal conditions for hydrogen production from *Rps. palustris* TN1 were 5 times dilution of DFE without supplementation of yeast extract, $NaHCO_3$, and 2 mM glutamate. Under the optimum conditions, the cumulative hydrogen production of 3.12 mmol H_2/L was obtained. The total hydrogen yield in the two stage process was estimated to be 6.42 mmol H_2/g COD which was 10.4% of the theoretical yield.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP	=	Adenosine triphosphate
NH_3-N	=	Ammonia nitrogen
CCD	=	Central composite design
COD	=	Chemical oxygen demand
CLSI	=	Clinical and Laboratory Standards Institute
CSTR	=	Continuous stirred-tank reactor
DAPI	=	Dye 4′,6′-diamidino-2-phenilindol
DFE	=	Dark fermentation effluent
^0C	=	Degree Celsius
DGGE	=	Denaturing gradient gel electrophoresis
ESBL	=	Extended-spectrum beta-lactamases
Fd	=	Ferredoxin
FID	=	Flame ionization detector
FISH	=	Fluorescence in situ hybridization
g/L	=	Gram per liter
HC	=	Hydrogen content
HPR	=	Hydrogen production rate
HRT	=	Hydraulic retention time
HY	=	Hydrogen yield
λ	=	Lag-phase time
η	=	Light conversion efficiency
mL	=	Milliliter
mmol H_2/L d	=	Millimole hydrogen per liter per day
mmol/L h	=	Millimole per liter per hour
N/C	=	Nitrogen to carbon source
NADH	=	Nicotinamide adenine dinucleotide
OD	=	Optical density
OLR	=	Organic loading rate

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

PBD	=	Plackett-Burman design
PBS	=	Phosphate buffer solution
PCR-DGGE	=	Polymerase chain reaction-denaturing gradient gel electrophoresis
KOH	=	Potassium hydroxide
PNSB	=	Purple non-sulfur bacteria
PS	=	Photosystem
PSB	=	Purple sulfur bacteria
PUFA	=	Polyunsaturated fatty acids
RFLP	=	Restriction fragment length polymorphism
RSM	=	Response surface methodology
SEM	=	Scanning electron microscope
SHPR	=	Specific hydrogen production rate
NaOH	=	Sodium hydroxide
SMP	=	Soluble metabolite products
SS	=	Suspended solid
Temp.	=	Temperature
TGGE	=	Temperature gradient gel electrophoresis
TN	=	Total nitrogen
T-RFLP	=	Terminal restriction fragment length polymorphism
TS	=	Total solid
UA	=	Up-flow anaerobic
UASB	=	Up-flow anaerobic sludge blanket
v/v	=	Volume per volume
VFA	=	Volatile fatty acid
VSS	=	Volatile suspended solid
W/m^2	=	Watts per square meter
w/v	=	Weight by volume

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The major global crisis is environmental problems and energy requirements that make the increasing demand for alternative environmentally-friendly energy (Baykara, 2005). Hydrogen is renewable energy carrier, considered to be a clean fuel for future, and has a high energy content (Das and Veziroglu, 2008; Das and Veziroglu, 2001). Among the methods for production of hydrogen, biological process of dark and photo fermentation generates high efficiency of hydrogen (Tao *et al*., 2007). Moreover, dark and photo fermentation can utilize the renewable resources and wastes as substrate for hydrogen production (Argun and Kargi, 2010).

Thai government's biodiesel development strategy is to replace 10% of petro-diesel in transport sector with biodiesel by 2012. The plan is to increase the use of biodiesel from 365 million liters in 2007 to 3,100 million liters by 2012 (Siriwardhana *et al*., 2009). The increase of biodiesel production will increase the quantity of crude glycerol. This was due to the fact that production of 100 kg of biodiesel would generate 10 kg of crude glycerol. The crude glycerol from biodiesel production usually presents 55-90% of purity. The rest of the crude glycerol consists of unconverted triglycerides, unconverted methanol, biodiesel, soaps and contamination (Meher *et al*., 2006). Therefore, this crude glycerol contains too many contaminants for a useful application in chemistry or pharmacy without treatment. Moreover, the high purification cost of crude glycerol makes the application in pharmaceutical and chemical applications limited (Amaral *et al*., 2009). In order to make biodiesel sustainable for using, bioconversion of crude glycerol to a variety of value added products have been studied. Since crude glycerol does not require additional pretreatment for the microorganisms like most wastes materials. It's abundant, easily available, and inexpensive for large scale production. (Sarma *et al*., 2012). From these aspects, crude glycerol has a potential used as a substrate for biohydrogen production. In addition, a metabolic pathways of dark fermentation

of crude glycerol exhibited that a high valuable products such as 1,3-propanediol, 2,3-butanediol, and ethanol, which used in many industrial applications, were also produced from this waste (Syu, 2001;Hu and Wood, 2010;Wu *et al*., 2008).

Hydrogen production from dark fermentation mostly conducted in three categories of temperature including mesophilic (30-39^{0}C), thermophilic (50-64^{0}C), and hyper-thermophilic (>65^{0}C) (Lee *et al*., 2011). The use of thermotolerant temperature (30-45^{0}C) is more attractive for hydrogen production. Since this temperature is lower risk of microbial contamination compared with mesophilic temperature. Also, it is lower costs for heating compared to thermophilic temperature (Hawkes *et al*., 2007; Eberly and Ely, 2008). Thus, it is possible to produce hydrogen under thermotolerant condition.

In dark fermentation, substrate is converted to hydrogen and organic acids, but the production of organic acids can inhibit the dark fermentative bacteria resulting in low hydrogen production obtained. In photo fermentation, organic acids from dark fermentation effluent can assimilate by photosynthetic bacteria (Ozgur *et al*., 2010a; Ozgur *et al*., 2010b). From this point, combination of two-stage process could achieve high hydrogen production and increase conversion efficiency (Su *et al*., 2009a). Recently, waste materials such as potato starch (Laurinavichene *et al*., 2010), beet molasses (Ozgur *et al*., 2010c), cheese whey wastewater (Azbar and Cetinkaya-Dokgoz, 2010), and corncob (Yang *et al*., 2010) can successfully be used as substrate for two stage process of hydrogen production as well as waste minimization.

Therefore, this research aims to investigate the conversion of crude glycerol from biodiesel plant for biohydrogen production using the thermotolerant bacteria isolate by dark fermentation and the dark fermentation effluent was further utilized by *Rhodopseudomonas palustris* TN1 in photo fermentation for hydrogen production with two-stage process.

Literature Review

1. Status of biodiesel in Thailand

An interest of using biofuel has been increased since the 1990s. Many countries are looking for renewable energy sources because of rising oil prices. The Thai government has come up with various strategies to reduce energy consumption as well as maximizing the utilization of available resources in energy supply (Pichalai, 2005; Pichalai, 2007). Thailand development plans are to increase the use of alternative fuels in transport sector. The plan is to develop raw materials and replace 5% of the total petro-diesel consumption with biodiesel by 2011 and increase it up to 10% by 2012. The biodiesel consumption of about 3,100 million liters in 2012 will result in savings of foreign exchange of about US $675 million per year (27 billion Baht per year) (Siriwardhana *et al*., 2009).

1.1 Biodiesel production and generation of crude glycerol

The methods for biodiesel production include chemical, biological, and supercritical method. Chemical method is mainly used in industry at present (Fig. 1). Vegetable oils or animal oil is substituted by short chain alcohols such as methanol or ethanol to produce fatty acid methyl esters or fatty acid ethyl esters via transesterification in the presence of acidic or basic catalysts, which sodium hydroxide (NaOH) or potassium hydroxide (KOH) are mainly used as catalyst (Fig. 2) (Ma and Hanna, 1999).

Glycerol or crude glycerol is a by-product of biodiesel production by the transesterification. The increase of biodiesel production will increase the quantity of crude glycerol. This was due to the fact that production of 100 kg of biodiesel would generate 10 kg of crude glycerol. The crude glycerol from biodiesel production usually presents 55-90% of purity. The rest of the crude glycerol consists of unconverted triglycerides, unconverted methanol, biodiesel, soaps and contamination (Meher *et al*., 2006). Therefore, this crude glycerol contains too many contaminants for a useful application in chemistry or pharmacy without treatment. Moreover, the

high purification cost of crude glycerol makes the application in pharmaceutical and chemical applications limited.

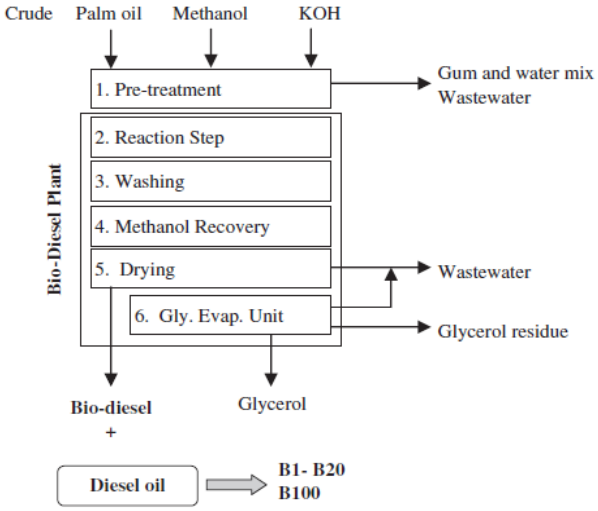

Figure 1. Biodiesel production process

Source: Siriwardhana *et al*. (2009)

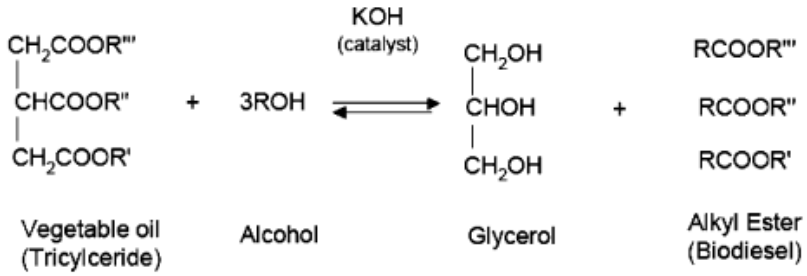

Figure 2. General equation for transesterification of triglycerides

Source: Siriwardhana *et al*. (2009)

2. Fermentation of glycerol to valuable products

There are many developments for conversion of glycerol by microbial fermentation to a valuable products, such as succinic acid from *Anaerobiospirillum*

succiniciproducens, *E.coli*, and *Basfia succiniciproducens* (Blankschien *et al*., 2010; Zhang *et al*., 2010b; Lee *et al*., 2001; Scholten *et al*., 2009). Production of propionic acid was obtained from *Propionibacterium* (Bories *et al*., 2004). Moreover, it has been reported that using *E. coli* could achieve several products such as ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi *et al*., 2006). The amino acids could be produced from glycerol using *Corynebacterium glutamicum* (Rittmann *et al*., 2008). The algal fermentation processes was also developed to convert glycerol into high value omega-3-polyunsaturated fatty acids (PUFA) (Pyle *et al*., 2008). Furthermore, *Yarrowia lipolytica* could use glycerol to produce citric acid (Papanikolaou *et al*., 2004). Ethanol was produced from glycerol by *Kluyvera cryocrescens* and *Klebsiella pneumoniae* (Choi *et al*., 2011; Oh *et al*., 2011). Moreover, it has been reported that *Klebsiella pneumonia* and *Clostridium butyricum* are the efficient strains for production of 1,3-propanediol (Sattayasamitsathit *et al*, 2011; Mu *et al*., 2006a; Gonzalez-Pajuelo *et al*., 2004).

The fermentation of glycerol to valuable products above clearly shows that glycerol has been successfully used for different bioconversion to different products. However, there is a need to find out another economically attractive and environmentally products from glycerol. Since biodiesel has a sudden increase in the production as a renewable energy, a rapid enlargement in glycerol is being observed. In order to make biodiesel sustainable for using, the conversion of glycerol to hydrogen may be a suitable option. Since hydrogen is environmentally-friendly energy and could be the potential alternative energy for the future. There are few bacteria that could convert glycerol into hydrogen such as *Klebsiella*, *Citrobacter*, *Enterobacter*, and *E. coli* (Ito *et al*., 2005; Yazdani *et al*., 2007). Fermentative metabolic pathways of glycerol utilization were studied intensively using *E. coli*. *E. coli* can further convert formate into hydrogen and carbon dioxide. *Enterobacter* could convert glycerol into hydrogen under anaerobic conditions with a minimal amount of other by-products such as lactate, acetate, 1,3-propanediol, and formate (Markov *et al*., 2011). *Klebsiella* was used to produce hydrogen from glycerol with other valuable soluble metabolites such as ethanol, 1,3-propanediol, and 2,3-butanediol (Wu *et al*., 2011). Moreover, mixed culture was used to produce hydrogen

from glycerol. A maximum hydrogen production rate of 1.37 mmol H_2/L h was obtained using mixed cultures from brewery wastewater (Sittijunda and Reungsang, 2012a). Hydrogen yields obtained during pure glycerol fermentation were 0.05-0.28 mol H_2/mol glycerol using mixed cultures (Temudo *et al*., 2008; Selembo *et al*., 2009), and 0.61-1.05 mol H_2/mol glycerol using pure cultures (Ito *et al*., 2005; Biebl *et al*., 1998). The waste glycerol from biodiesel produced hydrogen yields up to 0.31 mol H_2/mol glycerol using mixed cultures and 1.12 mol H_2/ mol glycerol using pure cultures (Ito *et al*., 2005; Selembo *et al*., 2009).

3. Hydrogen production

Hydrogen is an odorless, colorless, and non-poisonous gas. It generates only water as a combustion product. It has the higher energy content per unit weight (142 kJ/g) compared to methane (55.7 kJ/g) (Chong *et al*., 2009b). Hydrogen is now accepted as a renewable energy and expected to be the alternative energy for the future (Das and Veziroglu, 2008). Hydrogen can be generated through various processes such as steam reforming of natural gas, gasification of coal, and electrolysis of water (Nath and Das, 2004). However, these processes require huge amounts of energy intensive and release of large quantities of greenhouse gases. Thus, they are not ideal for sustainable hydrogen production (Khanal, 2008). From this point, biological hydrogen production seems to be the promising method for hydrogen production, since these are less energy intensive and more environmentally-friendly.

3.1 Biological hydrogen production

A biological process for hydrogen production used less energy input than chemical or electrochemical ways. This process can be mainly classified into two groups that is light dependent and light independent process. Light dependent processes include direct/indirect biophotolysis and photo fermentation whereas light independent process is dark fermentation (Kotay and Das, 2008). Among the biological hydrogen production, dark and photo fermentation could utilize renewable resource such as agricultural wastes and waste materials, which could eliminate the

pollution as well as waste minimization (Ozgur *et al*., 2010a). Moreover, a combination system of dark and photo fermentation by using a two-stage process is a promising method for effectively hydrogen production, in which the maximum conversion of substrates to hydrogen could be obtained. Since the dark fermentation effluent provides organic acids that could be further utilized to produce hydrogen by photosynthetic bacteria in the presence of light (Wu *et al*., 2010). The biological hydrogen production processes are presented in Table 1.

Table 1. Biological hydrogen production processes

Process	Organism
Direct photolysis $2H_2O + light \longrightarrow 2H_2 + O_2$	Green algae
Indirect photolysis $6H_2O + 6CO_2 + light \longrightarrow C_6H_{12}O_6 + 9O_2$ $C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2$	Cyanobacteria
Dark-fermentation $C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2$	Heterotrophic bacteria
Photo-fermentation $C_6H_{12}O_6 + 6H_2O + light \longrightarrow 12H_2 + 6CO_2$	Photoheterotrophic bacteria, Purple non sulfur bacteria
Two-stage fermentation (dark + photo) $C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2$ $2CH_3COOH + 4H_2O + light\ energy \longrightarrow 8H_2 + 4CO_2$	Heterotrophic bacteria, Photoheterotrophic bacteria, Purple non sulfur bacteria

Source: modified from Kotay and Das (2008)

4. Dark fermentation

4.1 Type of microorganisms

Two types of dark fermentative microorganisms for hydrogen production with high rate and yield called strictly anaerobic and facultative anaerobic bacteria. The strictly anaerobic bacteria belong to the family Clostridiaceae and facultative anaerobic

bacteria belong to the family Enterobactericeae (Chen *et al*., 2006b).

Clostridium species produce hydrogen during the exponential growth phase. Investigations on microbial diversity of a mesophilic hydrogen producing sludge indicated the presence of *Clostridium* species as 64.6% (Fang *et al*., 2002). The dominant culture of *Clostridium* can be easily obtained by heat treatment of biological sludge. The spores formed at high temperatures can be activated when required environmental conditions are provided for hydrogen production.

The species of the family Enterobactericeae have the ability to metabolize glucose by mixed acid fermentation. Carbon dioxide (CO_2) and hydrogen are produced from formic acid in addition to ethanol and the 2,3-butanediol (Podesta *et al*., 1997). Hydrogen production from anaerobic facultative bacteria of *Enterobacter aerogenes* has been widely studied (Palazzi *et al*., 2002; Nakashimada *et al*., 2002; Kapdan and Kargi, 2006).

4.2 Biochemical pathways for hydrogen production

A strictly anaerobic bacterium, *Clostridium* sp., is classical as the main groups for dark fermentation of carbohydrates to butyrate, acetate, CO_2 and hydrogen (Papanikolaou *et al*., 2004). As shown in the Fig. 3 that first pyruvate is produced which is then converted to different metabolites and hydrogen via different pathways. Pyruvate is broken down to acetyl-CoA via reduction of a ferredoxin (Fd) catalyzed by pyruvate ferredoxin oxidoreductase. Reduced ferredoxin (Fd) is then oxidized by a hydrogenase that reproduces oxidized Fd and hydrogen. Some additional amount of hydrogen can be produced during glycolysis when NADH is oxidized by Fd reduction and NADH-ferredoxin reductase (Juanita and Guangyi, 2009; Vardar-Schara *et al*., 2007). Theoretically a maximum of 4 mol hydrogen can be produced per mole glucose when acetate is the fermentation end product. However, only 2 mol hydrogen per mol glucose can be generated when butyrate is the fermentation end product (Levin *et al*., 2004; Sarma *et al*., 2012).

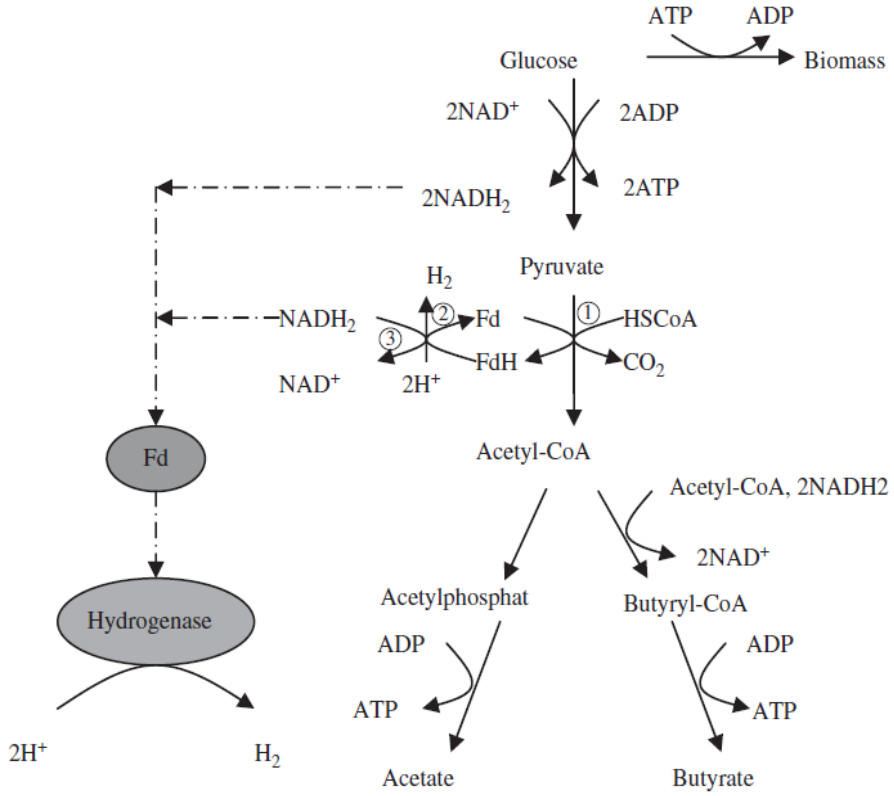

Figure 3. Metabolic pathway of glucose by *Clostridium butyricum* under anaerobic conditions. 1 Pyruvate: ferredoxin oxidoreductase; 2 Hydrogenase; 3 NADH: ferredoxin oxidoreductase

Source: Chen *et al*. (2006b)

4.3 Hydrogen production from glycerol by dark fermentation

4.3.1 Glycerol metabolism

The oxidative and reductive pathways of glycerol are known for species of *Klebsiella*, *Citrobacter*, *Clostridium*, and *Enterobacter* (Agnieszka *et al*., 2001). For oxidative pathway, glycerol is first converted to dihydroxyacetone using enzyme glycerol dehydrogenase. After that it changes to phosphorylated by the enzyme dihydroxyacetone kinase. Finally the phosphorylated product is metabolized through glycolysis (Daniel *et al*., 1995; Luers *et al*., 1997; Macis *et al*., 1998) (Fig. 4). For reductive pathway, glycerol is finally converted to 1,3-propanediol via production of the intermediate product 3-hydroxypropionaldehyde. Conversion of glycerol to 3-

hydroxypropionaldehyde is catalyzed by B12-dependent glycerol dehydratase and related diol dehydratases, which is then reduced to 1,3-propanediol by the $NADH^{+}H^{+}$-dependent enzyme 1,3-propanediol dehydrogenase (Ahrens *et al*., 1998; Nemeth *et al*., 2003).

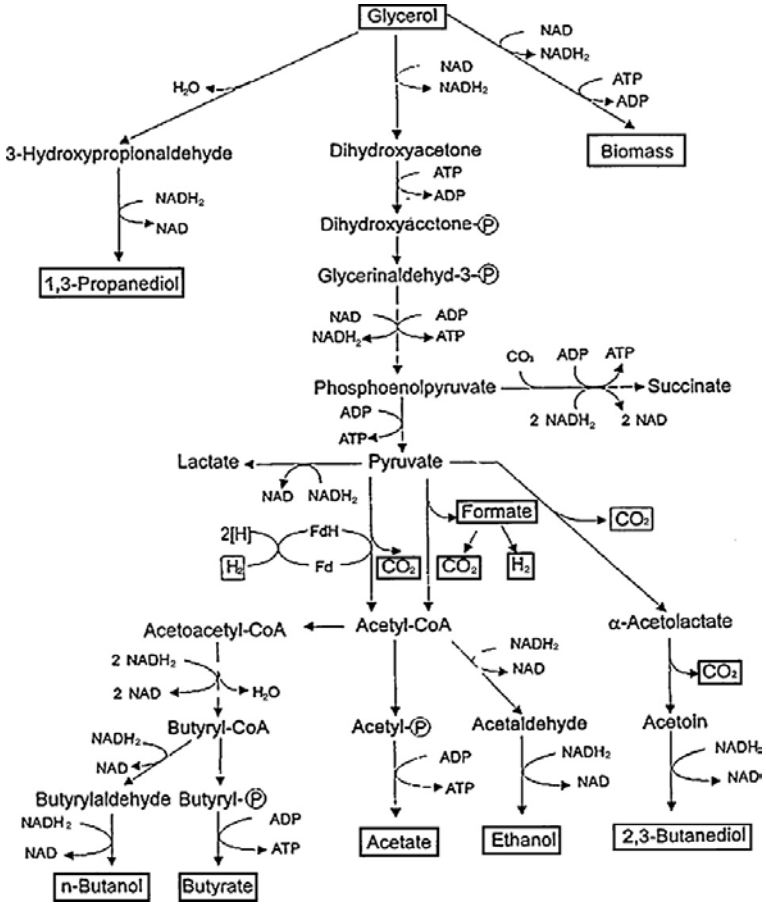

Figure 4. Biochemical pathways of glycerol fermentation
Source: Biebl *et al*. (1999)

During oxidative pathway of glycerol, pyruvate is formed as an intermediate and metabolized to different end products by different microorganisms. In most of the glycerol pathways, along with different metabolites hydrogen is produced during oxidative pathway as presented in equation 1-4. This reaction is catalyzed by some hydrogen-producing enzymes that are nitrogenases, [NiFe]-

hydrogenases, and [FeFe]- hydrogenases (Juanita and Guangyi, 2009; Vignais *et al*., 2001; Sarma *et al*., 2012). Hydrogenase is the main enzyme for hydrogen production. It has been reported that activity of different hydrogenase enzymes (Hyd) is dependent on pH. Hyd-1 activity is induced under anaerobic conditions at acidic pH whereas maximal activity of Hyd-2 is attained in alkaline medium (King and Przybyla, 1999). Hyd-3 with formate dehydrogenase H (Fdh-H), the component of the formate hydrogen lyase (FHL) complex, produces hydrogen mostly at acidic pH (Sauter *et al*., 1992). However, at alkaline pH, hydrogen production was found to be largely by Hyd-4 with Fdh-H forming the FHL pathway to produce hydrogen (Bagramyan *et al*., 2002).

$$C_3H_8O_3 + H_2O \longrightarrow CH_3COOH \text{ (Acetic acid)} + CO_2 + 3H_2 \quad (1)$$

$$2C_3H_8O_3 \longrightarrow C_4H_8O_2 \text{ (Butyric acid)} + 2CO_2 + 4H_2 \quad (2)$$

$$2C_3H_8O_3 \longrightarrow C_4H_{10}O \text{ (Butanol)} + 2CO_2 + H_2O + 2H_2 \quad (3)$$

$$C_3H_8O_3 \longrightarrow C_2H_6O \text{ (Ethanol)} + CO_2 + H_2 \quad (4)$$

4.4 Factors influencing fermentative hydrogen production from glycerol

4.4.1 Inoculum

Many microorganisms are able to metabolize glycerol (Table 2). Therefore, fermentative of glycerol has been studied with several bacteria, such as pure culture and mixed culture. Facultative anaerobes are the most studied organism for hydrogen production from glycerol. Mixed cultures from environmental sources is the other mostly used inoculum for glycerol fermentation.

Ito *et al*. (2005) evaluated hydrogen and ethanol production from pure glycerol using *E. aerogenes* HU-101 in a packed bed reactor using self-immobilized cells. The maximum hydrogen production rate was 80 mmol/L h and ethanol yield was 0.8 mol/mol glycerol. However, hydrogen production is decreased at high glycerol concentration and little ethanol is produced. Kivisto *et al*. (2010) demonstrated hydrogen production using pure glycerol by *Halanaerobium saccharolyticum* subspecies *saccharolyticum* and *senegalensis*. The main metabolites of both strains were CO_2 and acetate. Subspecies *saccharolyticum* also produced 1,3-

propanediol, butyrate, and ethanol. The maximum hydrogen yield of subsp. *saccharolyticum* and subsp. *senegalensis* was 0.6 and 1.6 mol H_2/mol glycerol, respectively. Wu *et al*. (2011) investigated the hydrogen production from glycerol by *Klebsiella* sp. HE1. The maximum hydrogen yield of 0.345 mol H_2/ mol glycerol was obtained at 35^0C, pH 6.0, and glycerol concentration of 50 g/L. These conditions are also preferable for producing ethanol, 1,3-propanediol, and 2,3-butanediol. Ngo *et al*. (2010) evaluated hydrogen production by *Thermotoga neapolitana* DSM 4359 using waste glycerol. It was found that pre-treated waste glycerol achieved higher hydrogen production than untreated waste glycerol. Moreover, nitrogen sparging could enhance the hydrogen yield of 2.22 fold to the maximum of 2.73 mol H_2/mol glycerol consumed, compared with the non nitrogen sparged. Ito *et al.* (2005) also investigated hydrogen and ethanol production from crude glycerol using *E. aerogenes* HU 101. The results found that the crude glycerol should be diluted with a synthetic medium to increase the rate of glycerol utilization, and the addition of yeast extract and tryptone in the medium could enhance the production of hydrogen and ethanol. Moreover, they also reported that using porous ceramics as a support material to fix cells in the reactor, the maximum hydrogen production rate reached 63 mmol/L h and ethanol yield of 0.85 mol/mol glycerol was obtained. Liu and Fang (2007) used *Klebsiella pneumoniae* DSM 2026 to produce hydrogen from crude glycerol. The hydrogen yield under the optimal medium conditions in a 5 L stirred tank bioreactor was 0.53 mol H_2/mol glycerol. Furthermore, 1,3-propanediol was also obtained from the liquid medium as a by-product.

4.4.2 Reactor type

As shown in Tables 2 most of the studies on fermentative hydrogen production from glycerol were studied in batch mode due to its simple operation and control. However, large scale operations would require continuous production processes for further improvement to increase the hydrogen yield and hydrogen production rate before being applied industrially. Glycerol has a high viscosity that might cause a decrease in the substrate transfer to the microorganisms, which is an important step for hydrogen production (Seifert *et al*., 2009; Ito *et al*.,

2005). Therefore, an up-flow system was conducted in order to eliminate this problem as well as to offer high treatment efficiency (Kumar and Das, 2001; O-Thong *et al*., 2008c). Ito *et al*. (2005) investigated a packed bed reactor of 60 mL working volume which was operated in continuous mode. Crude glycerol was fed from the bottom of reactor, and the effluent was discharged from the top of the reactor. Chen *et al*. (2008c) demonstrated that increasing HRT could increase the ability of hydrogen production, but HRT at much higher levels could decrease hydrogen production.

4.4.3 Temperature

Temperature is one of the most important factors that influence the activities of hydrogen-producing bacteria as well as hydrogen production. It has been demonstrated that in an appropriate range, increasing temperature could increase the ability of hydrogen-producing bacteria to produce hydrogen, but temperature at much higher levels could decrease hydrogen production (Wang and Wan, 2009). Wu *et al*. (2011) examined the effect of temperature (30, 35, and 40^0C) on hydrogen production from glycerol using *Klebsiella* sp. HE1. The result achieved maximum hydrogen yield at 35^0C. Temperatures from 25 to 40^0C were found favorable for hydrogen production from glycerol by *Klebsiella pneumoniae* DSM 2026, but the maximum hydrogen production was obtained at 37^0C (Liu and Fang, 2007). Seifert *et al*. (2009) also reported that 37^0C was ideal temperature for hydrogen production with an anaerobic digested sludge. Moreover, Ngo *et al*. (2010) studied hydrogen production from glycerol by using *T. neapolitana* DSM 4359 at 75^0C. It was found that the maximum hydrogen yield of 2.73 mol H_2/mol glycerol was obtained. Thus, as shown in Tables 2, it can indicate that the initial temperature with glycerol as a carbon source for hydrogen production was in the range of 30-37^0C.

4.4.4 pH

pH is another important factor that influences the activities of hydrogen producing bacteria, because it may affect the hydrogenase activity as well as the metabolism pathway. It has been demonstrated that increasing pH could increase the ability of hydrogen-producing bacteria to produce hydrogen, but pH at

much higher levels could decrease hydrogen production with increasing levels (Wang and Wan, 2009). Liu and Fang, 2007 reported that the optimal initial pH for converting glycerol to hydrogen by *Klebsiella pneumoniae* DSM 2026 was 6.5. Another study showed that an initial pH 6.0 favored hydrogen production from glycerol by *Klebsiella* sp. HE1 (Wu *et al*., 2011). Kivisto *et al*. (2010) reported hydrogen production from halophilic bacteria. The highest hydrogen yield of *Halanaerobium saccharolyticum* subspecies *saccharolyticum* was achieved at pH 7.4, while the highest hydrogen yield of *Halanaerobium saccharolyticum* subspecies *senegalensis* was achieved at pH 7.0. It can indicate that the initial pH for hydrogen production with glycerol was in the range of 6.0-7.5 (Table 2).

Table 2. Different microorganisms, bioreactor systems, and operation conditions used for hydrogen production from glycerol

Substrate	Microorganisms	Bioreactor type	Operation conditions			Maximum H_2 production	References
			Temp. (^{0}C)	pH	Mode of operation		
Pure glycerol	*Halanaerobium saccharolyticum* subspecies *saccharolyticum*	Small sealed glass tube	37	7.4	Batch	0.62 mol H_2/mol glycerol	Kivisto *et al*. (2010)
Pure glycerol	*Halanaerobium saccharolyticum* subspecies *senegalensis*	Small sealed glass tube	37	7.0	Batch	1.61 mol H_2/mol glycerol	Kivisto *et al*. (2010)
Pure glycerol	*Klebsiella* sp. HE1	2.5 L fermenter containing 1.5 L media	35	6.0	Batch	0.34 mol H_2/mol glycerol	Wu *et al*. (2011)
Pure glycerol	Mixed culture (anaerobic digested sludge)	Glass reactors (60 cc) with working capacity of 30 cc	37	6.0	Batch	0.41 mol H_2/mol glycerol	Seifert *et al*. (2009)
Pure glycerol	Mixed culture (wheat soil)	500 mL serum glass bottles filled with 250 mL media	30	6.2	Batch	0.28 mol H_2/mol glycerol	Selembo *et al*. (2009)
Pure glycerol	*E. aerogenes* HU-101	Packed-bed reactor of 60 mL working volume	37	6.8	Continuous	80 mmol H_2/L h	Ito *et al*. (2005)
Pure glycerol	*E. aerogenes* ATCC 13048	UASB reactor	37	5.5	Continuous	9 mmol H_2/L h	Reungsang *et al*. (2013a)

Table 2. Different microorganisms, bioreactor systems, and operation conditions used for hydrogen production from glycerol (cont.)

Substrate	Microorganisms	Bioreactor type	Operation conditions			Maximum H_2 production	References
			Temp. (^{0}C)	pH	Mode of operation		
Crude glycerol	*Thermotoga neapolitana* DSM 4359	120 mL serum bottles containing 40 mL media	75	7.5	Batch	2.73 mol H_2/mol glycerol	Ngo *et al*. (2011)
Crude glycerol	Mixed culture (wheat soil)	500 mL serum glass bottles filled with 250 mL of media	30	6.2	Batch	0.31 mol H_2/mol glycerol	Selembo *et al*. (2009)
Crude glycerol	*K. pneumoniae* DSM 2026	5 L stirred tank bioreactor	37	6.5	Batch	0.53 mol H_2/mol glycerol	Liu and Fang, (2007)
Crude glycerol	Mixed culture (hot spring sediment)	100 mL serum bottles containing 70 mL media	55	5.5	Batch	1502.84 mL H_2/L	Sittijunda and Reungsang, (2012b)
Crude glycerol	*E. aerogenes* KKU-S1	130 mLserumbottles containing 90 mL media	37	8.14	Batch	0.12 mol H_2/mol glycerol	Reungsang *et al*. (2013a)
Crude glycerol	*E. aerogenes* HU-101	Packed bed reactor of 60 mL working volume	37	6.8	Continuous	63 mmol H_2/L h	Ito *et al*. (2005)
Crude glycerol	*E. aerogenes* ATCC 13048	UASB reactor	37	5.5	Continuous	6.2 mmol H_2/L h	Reungsang *et al*. (2013b)

5. Photo fermentation

5.1 Type of microorganisms

Photoautotrophic organisms and photoheterotrophic bacteria have the ability to generate hydrogen. Photoautotrophic organisms such as microalgae and cyanobacteria are able to use sunlight to metabolize CO_2 with water as an additional substrate. Under anaerobic conditions, microalgae can produce hydrogen by water photolysis with light as the energy source and hydrogenase enzyme as a catalyst (Akkerman *et al*., 2002). Photoheterotrophic bacteria can convert organic acids to hydrogen under anaerobic conditions with light. Photoheterotrophic bacteria produce energy through photosynthesis and generate ATP through the cyclic functioning of the single photosystem. They depend upon reduced fixed carbon compounds, inorganic ions, and hydrogen as source of electrons for their metabolic activities. Photoheterotrophic bacteria have several subtypes; purple sulfur bacteria (PSB) and purple non sulfur bacteria (PNSB) (Bryant and Frigaard, 2006; Keskin *et al*., 2011).

The genus *Rhodobacter* of PNSB has been widely used for photo hydrogen production. All PNSB obtained their electrons and carbon from reduced fixed carbon compounds, some species can use S^{2-}, H_2 or Fe^{2+} as electron donors, and CO_2 as the sole carbon source. PNSB are able to use a wide variety of organic compounds such as pyruvate, acetate, and other organic acids, amino acids, alcohols, and carbohydrates. Some species can use one carbon atom compounds such as methanol and formate, while some other species can use aromatic organic compounds such as benzoate, chlorobenzoate, phenylacetate, and phenol (Keskin *et al*., 2011).

Nitrogenase plays an important role for hydrogen production by photosynthetic bacteria, and the particular requirements for its regulation, biosynthesis, and enzyme activity (Masepohl and Hallenbeck, 2010). Nitrogenase is an iron-sulfur molybdenum enzyme that is repressed by high concentrations of fixed nitrogen, especially ammonium, and oxygen. Active enzyme requires iron and molybdenum, which is also a regulatory factor (Masepohl and Hallenbeck, 2010). It has been reported that nitrogen for cell growth that is supplied in the form of proteins, glutamate or yeast extract could enhance hydrogen production much better than

ammonium (Oh *et al*., 2004; Takabatake *et al*., 2004). Metabolism shifts to the utilization of organic compounds for cell synthesis or storage of polyhydroxybutyrate rather than hydrogen production in the presence of high nitrogen concentrations resulting in excess biomass growth and reduction in light diffusion (Oh *et al*., 2004). The inhibitory action of ammonium is reversible, and hydrogen production activity can be recovered after ammonia is consumed (Takabatake *et al*., 2004; Masepohl and Hallenbeck, 2010; Keskin *et al*., 2011).

5.2 Metabolic pathways of photo fermentation

The photosynthetic of purple bacteria, as the main groups for photo fermentation, consists of one photosystem (PS) as shown in Fig. 5. This photosystem is fixed in the intracellular membrane. The photosystem itself is not powerful enough to split water. Under anaerobic condition, these bacteria are able to use simple organic acids or hydrogen disulfide (H_2S) as electron donor. The electrons that are released from the organic carbon or H_2S are pumped around through a large number of electron carriers (amongst which are Q and C_2 in Fig. 5). During the electron transport, protons are pumped through the membrane (e.g. in the cytochrome bc_1 protein complex). A proton gradient is developed (high and low H+), and this is used by the ATP synthase enzyme to generate ATP. The extra energy in the form of ATP can be used to transport the electrons further to the electron acceptor ferredoxin. Under nitrogen limited condition, these electrons can be used, using extra ATP energy by the nitrogenase enzyme to reduce molecular nitrogen into ammonium. When molecular nitrogen is not present, this enzyme can, again with the help of extra energy in the form of ATP, reduce protons into hydrogen, with the electrons derived from the ferredoxin. The nitrogenase enzyme is also sensitive to oxygen. In this case, it is not a problem because no oxygen is produced during the process (anoxygenic photosynthesis) (Akkerman *et al*., 2002).

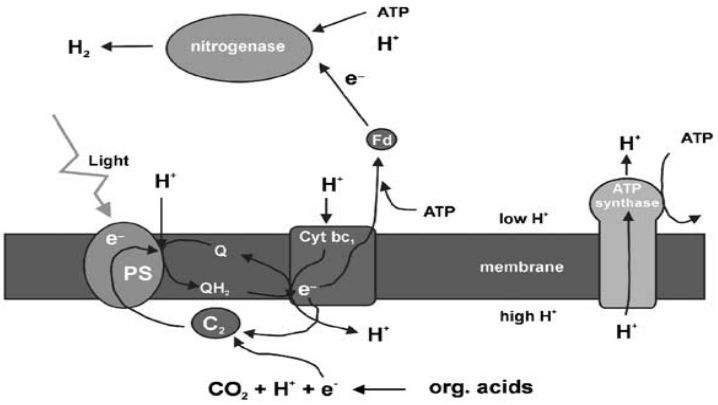

Figure 5. Photo fermentation by purple bacteria

Source: Akkerman *et al*. (2002)

5.3 Photo fermentative hydrogen production from wastes

A wide variety of wastes are potentially for hydrogen production by photosynthetic bacteria (Table 3). These include wastes from organic acids such as brewery wastewater, wastes containing simple sugars such as dairy wastewater, and carbohydrate (starch and cellulose) wastes such as wheat starch. Brewery wastewaters contain a variety of organic compounds including amino acids, proteins, organic acids, sugars, alcohols, and vitamin B. Many of these are useful for photo bacteria as growth factors. Brewery wastewater has been used for photo hydrogen production by *R. sphaeroides* after filtration and sterilization (Seifert *et al*., 2010b). Under an illumination of 116 W/m^2 and 10% v/v dilution, the maximum of hydrogen production of 2.24 L H_2/L medium was achieved with 1.7% light conversion efficiency (Seifert *et al*., 2010b). Dairy wastewater was studied by using *R. sphaeroides* O.U.001 with the optimum light intensity of 9,000 lux. However, the dairy wastewater needed to be diluted by at least 60% to prevent ammonium inhibition. Under these conditions, maximum hydrogen of 3.6 L H_2/L medium was obtained with 0.36 g dry weight/L (Seifert *et al*., 2010a). The starch in ground wheat was used for hydrogen production by photosynthetic bacteria. Ground wheat was pretreated by acid hydrolysis (pH 3) and autoclaved at 90^0C for 15 min using *R. sphaeroides* RV. The maximum hydrogen yield of 1.23 mol H_2/mol glucose was achieved (Kapdan *et al*., 2009).

Crude glycerol is available in large quantities as it is a waste obtained from biodiesel plant. A study from Sabourin-Provost and Hallenbeck (2009) has shown that crude glycerol is an effective substrate for photo fermentative hydrogen production with 6 mol H_2/mol glycerol obtained. Crude glycerol has a low nitrogen concentration, and for this reason supplement of nitrogen could improve the hydrogen yield. Several improvements such as reducing the lag phase, optimizing the light intensity, and improving the *R. palustris* strain with genetic engineering could be made to this system possibly.

Table 3. Hydrogen yield of photo fermentation obtained from wastes

Photosynthetic bacteria	Carbon source	Total H_2 yield	Reference
R. sphaeroides	Wheat starch	1.23 mol H_2/mol glucose	Kapdan *et al*. (2009)
R. sphaeroides	Brewery wastewater	2.24 L H_2/L medium	Seifert *et al*. (2010b)
R. sphaeroides	Olive mill effluent	0.25 L H_2/L medium	Eroglu *et al*. (2004)
R. sphaeroides	Dairy wastewater	3.62 L H_2/L medium	Seifert *et al*. (2010a)
R. palustris	Glycerol	6 mol H_2/mol glycerol	Sabourin-Provost and Hallenbeck (2009)

Source: modified from Keskin *et al*. (2011)

5.4 Factor for photo fermentation

5.4.1 Carbon sources

PNSB are able to use a wide range of substrates as carbon sources. The most carbon sources used for hydrogen production by photo fermentation are the acetate, butyrate, propionate, and lactate (Barbosa *et al*., 2001; Wang *et al*., 2010). Table 4 summarizes the performance of hydrogen production by photo fermentation on the type of carbon sources used. It shows that a wide variety of carbon sources were used to produce hydrogen by photo fermentation with different photosynthetic bacteria. It has been reported that *Rhodopseudomonas palustris* P4 could convert acetate effectively which over 70% theoretical yield was achieved (Oh *et al*., 2004; Chen *et al*., 2011).

Table 4. Comparison of photo hydrogen production performance using various carbon sources and photosynthetic bacteria

Photosynthetic bacteria	Carbon source	Carbon source concentration (mg/L)	H_2 production rate (mL/L h)	References
Rhodopseudomonas palustris P4	Acetate	720-3,300	NA	Oh *et al*. (2004)
Rhodopseudomonas faecalis	Acetate	1,000	NA	Liu *et al*. (2009)
Rhodobacter Capsulatus JP91	Lactate	4,020	38.5	He *et al*. (2006)
Rubrivivax gelatinosus L31	Lactate	2,700	2.9	Li and Fang (2008)
Rhodopseudomonas palustris WP 3-5	Acetate and butyrate	2,270 and 900	41.1	Chen *et al*. (2008a)
Rhodopseudomonas capsulate	Acetate, propionate and, butyrate	1,800, 200 and 1,000	NA	Shi and Yu (2005)

NA: not available.

Source: modified from Keskin *et al*. (2011)

5.4.2 Nitrogen sources

The type and concentration of nitrogen source is important for the efficiency of hydrogen production by photo fermentation. Since nitrogenase is strongly inhibited when an excess amount of NH^+_4 ions is present (Chen *et al*., 2008b; Koku *et al*., 2002). Lee *et al*. (2002) reported that wastewater that contained a small amount of ammonia was more suitable for photo hydrogen production than that with a higher ammonia concentration. Chen *et al*. (2008c) showed that ammonia is a favorable nitrogen source for the growth of PSB, as the addition of a small quantity of NH^+_4 ions could enhance photo hydrogen production. It has been reported that glutamate is widely used as the nitrogen source for photo fermentation, since it seems to have less of an inhibitory effect on nitrogenase (Chen and Chang, 2006; Koku *et al*., 2002). Shi and Yu (2005) also reported that glutamate was readily used by *R. capsulate* as a nitrogen source for improving photo hydrogen production from mixed volatile fatty acids (VFAs).

5.4.3 Light intensity

ATP synthesis in photosynthetic bacteria is a light-dependent and requiring light energy at the wavelengths of 522, 805, and 850 nm (Akkerman *et al*., 2002; Chen *et al*., 2006a). Since hydrogen production by photo fermentation requires ATP from the reducing power. Thus, sufficient supply of ATP becomes one of the major concerns for efficient photo hydrogen production (Miyake *et al*., 1999; Chen *et al*., 2011). Light intensity can become a growth limiting factor for photo hydrogen production, due to self-shading and absorption of light by the cells close to the illuminated surface (Barbosa *et al*., 2011). It has been reported that increased light intensity resulted in an increase in the hydrogen production. However, light conversion efficiency decreased by increasing light intensity. Miyake and Kawamura, (1987) and Nakada *et al*. (1995) demonstrated that a decrease in light intensity from 720 to 22 W/m^2 leads to an increase in light conversion efficiency from 0.5 to 1.8%. The difference between light conversion efficiencies when a low and high light intensity were supplied could be described that all light is not absorbed at high light intensities. Another reason could be the supply of energy in excess of the capability of

the nitrogenase when light intensity is high, leading to low light conversion efficiency (Nakada *et al*., 1995; Nath and Das, 2009).

5.4.4 Temperature

The operating temperature is an important factor influencing the performance of cell growth of photosynthetic bacteria as well as substrate conversion efficiency. The optimal growth temperature range for many photosynthetic bacteria is between 30 and 40^0C (Stevens *et al*., 1984). Wang *et al*. (2010) found that optimal growth temperature for the *Rhodopseudomonas palustris* CQK01 is between 27.5 and 32.5^0C, whereas a sharp decline in hydrogen production was observed when the temperature was above 35^0C. He *et al*. (2006) showed that the culture temperature significantly influenced the bacteria growth, hydrogen production rate, and substrate conversion efficiency. The optimal growth temperature was 30^0C for photo hydrogen production with *Rhodobacter capsulatus* JP91.

5.4.5 pH

The pH value of the culture medium normally affects the biochemical reaction characteristics of photosynthetic bacteria. Koku *et al*. (2002) reported that the optimum pH for the nitrogenase and hydrogenase was 7.1-7.3 and 6.5-7.5, respectively. Jamil *et al*. (2009) studied the optimum pH of *Rhodopseudomonas palustris* PUBM 001 using palm oil mill effluent in the range of 6.0-8.0. The results found that the optimum pH for the hydrogen production was 6.0 with the hydrogen yield of 0.66 mL/mL medium. Ren *et al*. (2009) also studied the optimum pH of *Rhodopseudomonas faecalis* RLD-53 using basal medium in the range of 4.0-9.0 and found that the optimum pH for the hydrogen production was 7.0 with the hydrogen yield of 2.71 mol H_2/mol acetate.

6. Two-stage process for hydrogen production

Two-stage process of dark and photo fermentation have been shown to be efficient process to produce hydrogen through biological methods and have a certain advantages over single stage dark or photo fermentation (Yang *et al*., 2010). In the first stage, dark fermentation, a relatively easily applied and cost-effective technology for hydrogen production which can operate continuously without light input, produces hydrogen along with the simultaneous production of short chain organic acids. In the second stage, photo fermentation with PNSB is used to efficiently convert the short chain organic acids to hydrogen. Therefore combining these two systems hold the promise of being a very useful and productive method for the economic production of hydrogen. The maximum hydrogen yield of dark fermentation is 4 mol hydrogen per mol glucose when acetic acid is the main liquid by-products (Eq. 5). In the case of photo fermentation, the acetic acid that is produced from dark fermentation could be used as a substrate for photo fermentation, resulting in production of 8 mol hydrogen per mol glucose (Eq. 6). In the two-stage process of dark and photo fermentation, the overall production of 12 mol hydrogen per mol glucose could obtain (Eq. 7) (12 mol hydrogen/mol glucose, are 61.7 mmol H_2/g COD) (Keskin *et al*., 2011). However, in practice, the maximum hydrogen yield per mol glucose via two-stage dark and photo fermentation systems is lower than theoretical since part of the substrate is used for microbial growth and metabolism (Manish and Banerjee, 2008).

Dark fermentation

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 4H_2 + 2CO_2 + 2CH_3COOH \quad (5)$$

Photo fermentation

$$2CH_3COOH + 4H_2O + \text{Light Energy} \longrightarrow 8H_2 + 4CO_2 \quad (6)$$

Complete oxidation

$$C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2 \quad (7)$$

6.1 Two-stage process for hydrogen production from wastes

Numerous studies on hydrogen production with two-stage of dark and photo fermentation from wastes have been carried out. These include wastes from organic acids, wastes containing simple sugars, and carbohydrate (starch and cellulose) wastes. Table 5 indicated that combining dark and photo fermentation can improve the hydrogen production from wastes materials and wastewaters. The success of using dark fermentation effluent depends on their compositions with the presence of elemental nutrients such as iron (Fe) and sodium molybdate (Mo) were beneficial, since they are required for the hydrogen producing enzymes. Moreover, the presence of NH^{+}_{4} was harmful, since it can inhibit hydrogen production. Therefore, the concentration of these compounds should be carefully adjusted before the photo fermentation operation (Keskin *et al*., 2011).

Table 5. Hydrogen yields obtained from two-stage process of dark and photo fermentation of wastes

Substrate	Microorganism in process		H_2 yield	Reference
	Dark fermentation	Photo fermentation		
Wheat starch	Mixed culture	*Rhodobacter* sp.	176 mL H_2/g starch	Argun *et al*. (2009)
Beet molasses	*Caldi-cellulusiruptor saccharolyticus*	*R. capsulatus*	13.7 mol H_2/mol sucrose	Ozgur *et al*. (2010c)
Potato starch	Mixed culture	*R. capsulatus*	5.6 mol/mol hexose	Laurinavichene *et al*. (2010)
Cheese whey	Mixed culture	*R. palustris*	10 mol H_2/mol lactose	Azbar and Cetinkaya-Dokgoz (2010)
Cassava starch	Mixed culture	*R. palustris*	840 mL H_2/g starch	Chen *et al*. (2010)
Cassava starch	Activated sludge	*R. palustris*	503 mL H_2/g starch	Su *et al*. (2009a,b)

Source: modified from Keskin *et al*. (2011)

7. Molecular techniques for microbial community structure analysis

The abundance of bacterial and diversity in anaerobic processes for hydrogen production have been studied using many microbiological approaches. Traditional methods such as counting techniques can only provide a precursory estimate of bacterial diversity without the majority of unculturable bacteria as its limitations inherent to the growth media. In contrast, molecular methods could detect nucleic acids in microbial samples that may not be culturable as well as achieve more complete assessment of microbial diversity (Khanal, 2008).

Molecular techniques such as fluorescence *in situ* hybridization (FISH) (Sanz and Kochling, 2007), denaturing gradient gel electrophoresis (DGGE) (O-Thong *et al*., 2007; Sanz and Kochling, 2007), Temperature gradient gel electrophoresis (TGGE) (Dahllof, 2002), Terminal restriction fragment length polymorphism (T-RFLP) (Dahllof, 2002), Cloning and sequencing (Dahllof , 2002; Gilbride *et al*., 2006; Sanz and Kochling, 2007), and Restriction fragment length polymorphism (RFLP) (Dahllof, 2002) are the techniques that have been used most frequently for microbial community structure analysis. Advantages and disadvantages of molecular techniques are summary in Table 6.

Table 6. Summary of advantages and disadvantages of molecular techniques for microbial diversity and community analysis

Molecular methods	Advantages	Disadvantages
Fluorescence *in situ* hybridization (FISH)	-Direct analysis and quantification -Suitable for targeting specific group/species	-Requires genes/RNA with high number of copies -Limit for total diversity mapping
Denaturing gradient gel electrophoresis (DGGE)	-Suitable for pattern analysis or microbial community diversity -Further sequencing not required, except using a non heterogeneous gene	-Can produce multiple bands from one template with bias PCR of heterogeneous genes (e.g., 16S) -Lacks resolution -Needs sequencing for species -May have PCR bias problems -Cannot actually indicate species richness
Temperature gradient gel electrophoresis (TGGE)	-Suitable for pattern analysis	-Lacks resolution -Needs further sequencing -May have PCR bias problems -Cannot actually indicate species richness

Table 6. Summary of advantages and disadvantages of molecular techniques for microbial diversity and community analysis (Cont.)

Molecular methods	Advantages	Disadvantages
Cloning and sequencing	-Contains larger sequences -Provides more positive ID	-A large number of clones must be sequenced for positive diversity -Bias from PCR -Sequences need to compare with each other and libraries -Laborious and time consuming
Restriction fragment length polymorphism (RFLP)	-Suitable for screening clone libraries or isolates before sequencing	-Not suitable for pattern analysis -Produces multiple bands for one species
Terminal restriction fragment length polymorphism (T-RFLP)	-Suitable for pattern analysis -One band per species -High resolution -Sensitive detection	-Restriction enzyme selection with unknown sequences -May have PCR bias problems -Cannot actually indicate species richness -Some species may show the same length of fragments

Objectives

1. To screen and isolate thermotolerant fermentative hydrogen-producing bacteria with high hydrogen production from crude glycerol.

2. To identify the selected thermotolerant fermentative hydrogen-producing bacteria and optimize the condition for maximum hydrogen production from crude glycerol.

3. To optimize the medium composition affecting hydrogen production from crude glycerol by the selected thermotolerant bacteria using response surface methodology.

4. To compare hydrogen production and microbial community structure of the immobilized-cell and suspended-cell system by the selected thermotolerant bacteria using crude glycerol as substrate under non sterile condition.

5. To investigate the hydrogen production and bacterial quantification in UASB reactor using crude glycerol by the selected thermotolerant bacteria under non sterile condition.

6. To evaluate the potential use of crude glycerol as a substrate for hydrogen production by the two-stage process of dark and photo fermentation using the selected thermotolerant bacteria and *Rhodopseudomonas palustris* TN1, respectively.

CHAPTER 2

BIOCONVERSION OF CRUDE GLYCEROL FROM BIODIESEL PLANT TO HYDROGEN WITH SIMULTANEOUS DIOLS AND ETHANOL PRODUCTION BY THERMOTOLERANT BACTERIAL ISOLATES

2.1 Abstract

Nineteen samples from crude glycerol contaminated soil and hot springs in Southern Thailand was enriched in the medium containing glycerol and incubated at 45^0C for isolation of thermotolerant hydrogen-producing bacteria. Samples from crude glycerol contaminated soil produced hydrogen at least 2 folds higher than those from hot springs. Among 38 isolates obtained, 10 strains demonstrated great capacity in hydrogen production. The efficiency of hydrogen production by the selected isolates TR17, TR20, and TR32 at 45^0C (17.17, 15.17, and 15.97 mmol H_2/L, respectively) was superior to those observed at 30^0C (9.07, 7.82, and 13.48 mmol H_2/L, respectively). Thus, they were classified as thermotolerant strains. The isolate TR17 exhibited the maximum hydrogen production (20.90 mmol H_2/L) and yield (0.21 mol H_2/mol glycerol) with the highest 2,3-butanediol (17.15 mmol/L) and yield (0.16 mol/mol glycerol). The isolate TR20 was 1,3-propanediol producer (54.32 mmol/L) while the isolate TR32 was ethanol producer (73.32 mmol/L) with a yield of 0.62 and 0.77 mol/mol glycerol, respectively.

2.2 Introduction

Due to the reduction of petroleum supply and the concern about environmental pollution from the hydrocarbon fuels, biodiesel have been consider as the most feasible alternative energy (Fan *et al*., 2010). Crude glycerol is generated as a major by-product of biodiesel production and has been widely used in many industries such as cosmetic, paint, automotive, and food (Temudo *et al*., 2008). However, rapidly increasing in biodiesel production led to generate exceeding in crude glycerol, which rising of disposal for treating of this waste (Yang *et al*., 2008). In order to decrease costs for treating and utilization of crude glycerol to obtain more valuable products, a practical processes for converting crude glycerol should be developed.

Hydrogen is considered as an ideal and efficient energy for the future due to its environmentally-friendly and higher energy yield (Das and Veziroglu, 2001). It has been reported that crude glycerol has a potential for using as substrate to produce hydrogen by many methods such as pyrolysis and electrolytic process. However, pyrolysis of crude glycerol requires high temperature and electrolytic process needs more energy to generate hydrogen (Valliyappan *et al*., 2008). Among these methods, biological process by dark fermentation is more attractive. Since high hydrogen production rate and yield were obtained, low energy input and various waste materials can be used as substrate (Schara *et al*., 2008). In addition, a metabolic pathways of dark fermentation of crude glycerol exhibited that a high valuable products such as 1,3-propanediol, 2,3-butanediol, and ethanol, which used in many industrial applications, were also produced from this waste (Syu, 2001;Hu and Wood, 2010;Wu *et al*., 2008).

As hydrogen was produced in the gas phase and the valuable products were produced in liquid phase. Therefore, it is technically and economically favorable for the downstream operation (Wu *et al*., 2011). However, high efficiency of microorganisms used for conversion of crude glycerol into higher valuable products should be applied into this process. It has been reported that using *Enterobacter aerogenes* for crude glycerol fermentation, a maximum hydrogen yield was obtained but low yield of 1,3-propanediol and also less ethanol production (Ito *et al*., 2005). Conversely, a maximum yield of 1,3-propanediol was obtained with *Clostridium butyricum*, but there was no hydrogen production (Saint-Amans *et al*.,1994;

Gonzalez-Pajuelo *et al*., 2004). Thus, the screening of simultaneous high hydrogen and valuable products strains is needed for development of the commercial process for conversion of crude glycerol (Niu *et al*., 2010).

Dark fermentation of hydrogen production mostly conducted in three categories of temperature including mesophilic (30-39^{0}C), thermophilic (50-64 ^{0}C), and hyper-thermophilic (>65^{0}C) (Lee *et al*., 2011). Using thermotolerant temperature (30-45^{0}C) is more attractive for hydrogen production. Since this temperature required low temperature for operation compared with thermophilic and hyper-thermophilic. Moreover, thermotolerant temperature is superior to mesophilic for the lower risk of microbial contamination (Hawkes *et al*., 2007; Eberly and Ely, 2008). However, the use of thermotolerant temperature for hydrogen and valuable metabolite products from crude glycerol has not been reported yet.

Thus, the objective of this work is to isolate the effective thermotolerant anaerobic bacteria for converting the crude glycerol to mainly hydrogen with simultaneous production of 1,3-propanediol, 2,3-butanediol, and ethanol.

2.3 Materials and methods

2.3.1 Crude glycerol

Crude glycerol without pretreatment was obtained from Prince of Songkla University (PSU) Biodiesel Pilot Plant at Faculty of Engineering, PSU, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification using methanol as a reactant and sodium hydroxide as a catalyst. The purity of the crude glycerol was calculated base on its glycerol content and found to be 50%. In addition, the impurities of crude glycerol were mainly potassium and sodium salts (4-5%), methanol (1-3%), non-glycerol organic matter (1.6-7.5%), and water (36%) (Sattayasamitsathit *et al*., 2011).

2.3.2 Inocula source for isolation and cultivation

Water and soil samples were used as a source for screening of thermotolerant bacteria. Water samples were collected from ten hot springs located in Southern Thailand. The temperature (45-75^{0}C) and pH (6.5-8.4) of all samples were measured using a thermometer (on-site) and pH meter, respectively (Table 7). The

samples were kept at 4^0C before isolation. Four different areas of crude glycerol contaminated soil, designated as SL1-SL4, were collected from PSU Biodiesel Pilot Plant. Samples were collected in sterile plastic bags and kept at 4^0C until used.

Table 7. The list of hot spring sources and its characteristics

Hot springs	Code	Temperature (^{0}C)	pH
Ranong province			
Wattaphotharam	81RN1	65	8.3
Ban Pon Rung	83RN3	45	8.4
Ratchagrud	85RN5	46	7.5
Surat Thani province			
Wat Than Num Ron	89SR3-1	63	7.0
	89SR3-2	53	7.8
	89SR3-3	67	7.6
	89SR3-4	66	7.8
Rattanakosai	93SR7	67	7.9
Ban Khao Plu	94SR8	56	7.8
	94SR9	57	7.9
	94SR10	56	7.8
Phang Nga province			
Khong Pay Pao	97PG1	60	6.5
Romanee	98PG2	63	6.8
Ban Bor Dan	99PG3	45	7.1
Yala province			
Ta Na Ma Rao	112YL1	80	7.8

2.3.3 Culture medium for isolation and cultivation

The culture medium contained: 10.0 g/L glycerol (glycerol concentration contained in crude glycerol), 3.4 g/L K_2HPO_4, 1.3 g/L KH_2PO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution. Trace element

solution contained 0.07 g/L $ZnCl_2$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $H_3BO_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2$; 0.02 g/L $CuCl_2 \cdot 2H_2O$, 0.025 g/L $NiCl_2 \cdot 6H_2O$, 0.035 g/L $Na_2MoO_4 \cdot 2H_2O$ (Liu and Fang, 2007). The initial pH of the medium was adjusted to 7.0 using 5 N NaOH or HCI before sterilization at 121^{o}C for 20 min.

2.3.4 Cultures enrichment

The sample of 1 mL water or 1 g soil was inoculated into a 9 mL of the culture medium for dark fermentation and incubated at 45^{0}C for 48 h. The cultures from each sample were then sub-cultured into 60 mL serum bottles with 36 mL working volume under anaerobic condition (flushing with nitrogen gas) and incubated at 45^{0}C for 48 h. After 3 times enrichment, samples were determined for the cumulative hydrogen production and hydrogen yield. The enriched cultures with high cumulative hydrogen production were selected for isolation.

2.3.5 Isolation and selection of thermotolerant fermentative hydrogen producing bacteria

The selected cultures were then serially diluted. Aliquots (0.1 mL) from 10^{-6}-10^{-7} dilution were spread on the glycerol medium agar plates and incubated under anaerobic condition at 45^{0}C for 48 h. Colonies obtained from agar plates were restreak to ensure the purity of the strains then transferred to a fresh liquid medium and analyzed further for hydrogen production. The strains producing high cumulative hydrogen production were selected and tested for their thermotolerant properties. They were transferred into a 60 mL serum bottles with 36 mL working volume and incubated at 30^{0}C and 45^{0}C for 24 h under anaerobic condition. The isolates that could grow and produce cumulative hydrogen at two levels of temperature but produce higher cumulative hydrogen at 45^{0}C were considered as thermotolerant isolates (Saeki *et al*., 1997).

2.3.6 Analytical methods

The biogas production in the headspace of a serum bottle was measured by a syringe technique described by Owen *et al*. (1979). Hydrogen content of the biogas was determined using an Oldham MX 2100 gas detector (Cambridge

Sensotec Ltd., England). Cell growth was determined as dry cell weight (gram dry weight per liter) by centrifugation the culture broth at 10,000 *g* for 10 min, washed cell sample twice with sterile distilled water, dried at 105ºC overnight and then weighed (Barbirato *et al*., 1998). Glycerol and organic acids concentration were determined by High Performance Liquid Chromatography (HPLC) (Agilent 1200). The culture medium was centrifuged at 10,000 *g* for 10 min then, the supernatant was filtered through 0.22 μm Nylon membrane filter (Sartorius, German) before injection. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostat column compartment; an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA); and ChemStation Software. Operation conditions were: 20 μl sample volume; 5 mM H_2SO_4 as a mobile phase; flow rate of 0.7 mL/min and a column temperature of 65^0C (Rujananon *et al*., 2011). Liquid samples from the serum bottles were taken for determination of pH, substrate utilization and metabolite products. The hydrogen production was calculated by measuring the total volume of hydrogen produced (mmol H_2/L). The hydrogen yield (mol H_2/mol glycerol) was calculated by measuring amount of hydrogen produced (mol) divided by amount of glycerol consumed (mol) (Chen *et al*., 2007). The glycerol conversion rate was calculated by using the equation: [(I-F)/I] x 100%, in which I and F are the initial and final glycerol concentrations (g/L), respectively (Markov *et al*., 2011).

2.3.7 Kinetic analysis

A modified Gompertz Eq. (8) was used to fit the experimental data to determine the cumulative hydrogen production in the batch experiment.

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t)+1\right]\right\} \quad (8)$$

Where, H is the cumulative hydrogen production (mL), P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL/h), λ is the lag-phase time (h), t is the incubation time (h), and e is the irrational constant (2.718).

The P, R_m and λ values for each batch were estimated by fitting the hydrogen production data using Microsoft Office Excel version 2007 (Suwansaard *et al*., 2009).

2.4. Results and discussion

2.4.1 Culture enrichment for production of hydrogen from crude glycerol

A total of 19 samples were used for enrichment and tested for conversion of glycerol to hydrogen in the dark fermentation medium containing 10 g/L glycerol as a major carbon source at pH 7.0 and incubated at 45^0C for 48 h. Among these samples, the top ten hydrogen-producing sources were the samples code as SL2, SL1, SL3, 81RN1, 99PG3, SL4, 93SR7, 94SR8, 98PG2, and 112YL1 that produced the cumulative hydrogen of 1.68-7.47 mmol H_2/L with hydrogen yield of 0.03-0.07 mol H_2/mol glycerol (Fig. 6). Results clearly indicated that the samples from the crude glycerol contaminated soil (code SL) except the sample SL4 produced cumulative hydrogen (6.54-7.47 mmol H_2/L) at least 2 folds higher than those from the hot spring samples (0.02-3.41 mmol H_2/L). Thus, the top ten samples were selected for isolation. Crude glycerol contaminated soil was a good source to obtain the best inoculum for hydrogen production. This may be due to the natural acclimatization of bacteria in the crude glycerol contaminated soil that ready for degradation of glycerol to hydrogen (Selembo *et al*., 2009). Moreover, hot springs are a potential source for thermotolerant bacteria as they could tolerant high temperature (of the hot spring) and survive in this environment (Abou-Shanab, 2007). However, lower cumulative hydrogen production obtained from the hot spring inoculum might be due to the inability to utilize glycerol for hydrogen production.

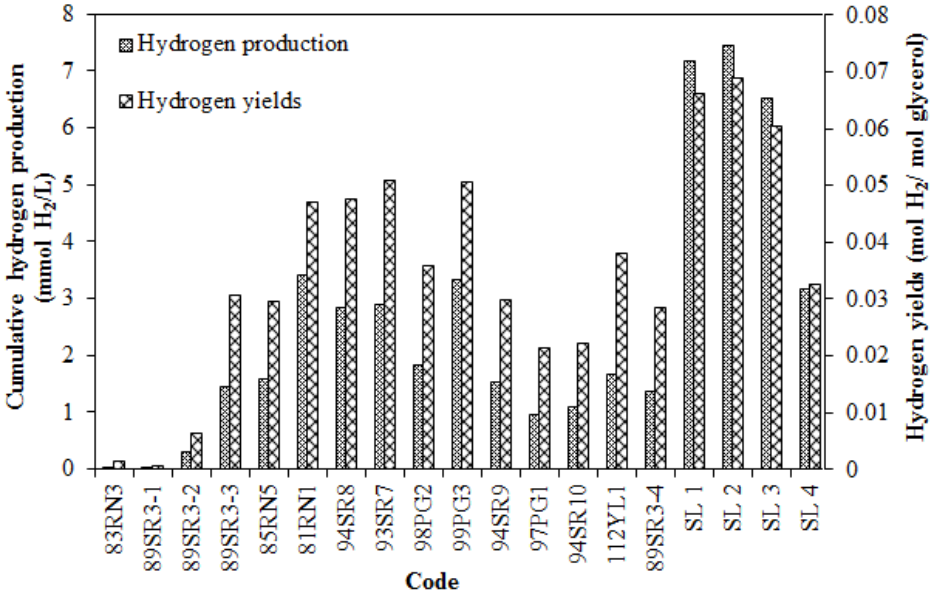

Figure 6. Hydrogen production from 19 samples of crude glycerol contaminated soil and hot spring sources in Southern Thailand cultivated in the fermentation medium with 10g/L glycerol, initial pH 7.0, and incubated at 45^0C for 48 h

2.4.2 Isolation of hydrogen-producing bacteria from the selected enrichment cultures

The selected top 10 enriched cultures were employed for isolation of hydrogen-producing strains. Colonies were visible after 24 h incubation. From these 10 enriched cultures, a total of 38 strains were isolated and coded as isolates TR1 to TR38. Twenty-four strains were isolated from the crude glycerol contaminated soil, while 14 strains were isolated from the hot spring samples taken from Ranong, Surat Thani, Phang Nga, and Yala Provinces. Among 38 isolates, 20 isolates exhibited hydrogen producing capability from glycerol. The top 10 isolates (Table 8) that demonstrated high cumulative hydrogen production were TR17, TR20, TR32, TR11, TR18, TR33, TR3, TR7, TR34, and TR8, respectively. They exhibited superior capacity in cumulative hydrogen production (4.95-15.03 mmol H_2/L) and hydrogen yield (0.19-0.47 mol H_2/mol glycerol). Glycerol conversion rate were in the range of 15.07 - 35.47%.

Table 8. Number of isolate strains from top 10 enriched cultures of 19 samples from crude glycerol contaminated soil and hot spring sources in Southern Thailand cultivated in the fermentation medium with 10 g/L glycerol, initial pH 7.0, and incubated at 45^0C for 24 h

Code (top10 enriched cultures)	Sources	Number of isolates	Isolated strains	Selected top 10 strains
SL2	soil2	7	TR20- TR26	TR20
SL1	soil1	5	TR15- TR19	TR17,TR18
SL3	Soil3	5	TR27- TR31	
81RN1	Wat Potharam- Ranong	2	TR1, TR2	
99PG3	Ban Bor Dan-Phangnga	2	TR9, TR10	
SL4	Soil4	7	TR32- TR38	TR32,TR33,TR34
93SR7	Ratanakosai-Suratthani	2	TR5, TR6	
94SR8	Ban Khao Plu-Suratthani	2	TR3, TR4	TR3
98PG2	Romanee-Phangnga	2	TR7, TR8	TR7,TR8
112YL1	Ta Na Ma Rao-Yala	4	TR11- TR14	TR11

2.4.3 Selection of thermotolerant hydrogen producing bacterial isolates

The definition of thermotolerant bacteria from Saeki *et al*. (1997) is that thermotolerant strains worked rapidly with a higher fermentation rate at higher temperature (40^0C), which the mesophilic strains at 30^0C were unable to do. Moreover, Moonmangmee *et al*. (2000) used two difference temperatures at 30^0C and 37^0C to screen thermotolerant *Gluconobacter* to produce L-sorbose. The result found that the production of L-sorbose by the strain CHM54 at 37^0C was superior to that obtained at 30^0C and this strain was classified as thermotolerant *Gluconobacter*. Saichana *et al*. (2009) also used two difference temperatures at 30^0C and 37^0C to screen thermotolerant *Gluconobacter* to produce L-sorbose 5-Keto-d-Gluconic Acid. The result found that thermotolerant *Gluconobacter* strains is able to produce 5-keto-d-gluconic acid at 37°C, a temperature at which regular mesophilic 5-keto-d-gluconic acid-producing strains showed much less growth and 5-keto-d-gluconic acid

production. In addition, Pechsuth *et al*. (2001) used thermotolerant polymer-producing fungal strains at 45ºC to remove the organic matter, oil, and grease of palm oil mill effluent. The result found that thermotolerant polymer-producing fungal strains at 45ºC can remove COD more than 50%. From these aspects, the strains that can grow and produce hydrogen both at 30^0C and 45^0C, but exhibited a higher hydrogen production at 45^0C were considered to be the thermotolerant strain. At 45^0C, the isolates TR7, TR17, TR18, TR20, and TR32 achieved higher cumulative hydrogen production (13.86-17.17 mmol H_2/L), cell growth (0.22-0.65 g dry cell weight/L), and hydrogen yield (0.22-0.33 mol H_2/mol glycerol) than those at 30^0C. However, this was not the case for the isolates TR3, TR8, TR11, TR33, and TR34 whereby cumulative hydrogen production (11.54-15.03 mmol H_2/L), and cell growth (0.52-0.70 g dry cell weight/L) at 30^0C were higher than those at 45^0C (Table 9). Thus, the isolates TR7, TR17, TR18, TR20, and TR32 were classified as thermotolerant bacteria. The efficiency of high hydrogen production at 45^0C by the top three isolates (TR17, TR20, and TR32) were selected for the next study.

Table 9. Comparison of hydrogen production performance and cell growth by selected isolates at 30^0C and 45^0C

Isolates	Cumulative hydrogen production (mmol H_2/L)		Hydrogen yield (mol H_2/mol glycerol)		Cell growth (g dry cell weight/L)		Final pH	
	30^0C	45^0C	30^0C	45^0C	30^0C	45^0C	30^0C	45^0C
TR3	14.66	13.78	0.14	0.27	0.55±0.001	0.37±0.002	5.66	5.60
TR7	9.99	14.01	0.09	0.25	0.57±0.03	0.65±0.21	5.39	5.75
TR8	13.38	12.77	0.13	0.25	0.70±0.007	0.47±0.10	5.59	5.82
TR11	13.21	10.19	0.12	0.21	0.57±0.17	0.57±0.10	5.80	5.04
TR17	9.07	17.17	0.08	0.33	0.35±0.07	0.45±0.07	5.50	5.46
TR18	7.90	13.86	0.09	0.26	0.50±0.14	0.65±0.07	5.37	5.55
TR20	7.82	15.17	0.09	0.27	0.40±0.14	0.50±0.008	5.57	5.50
TR32	13.48	15.97	0.13	0.22	0.15±0.07	0.22±0.10	5.71	5.72
TR33	15.03	14.03	0.15	0.21	0.20±0.007	0.45±0.14	5.65	5.41
TR34	11.54	9.69	0.10	0.22	0.52±0.03	0.32±0.03	5.51	5.77

2.4.4 Simultaneous production of hydrogen, 1,3-propanediol, 2,3-butanediol, and ethanol by the selected thermotolerant isolates

The hydrogen production from crude glycerol by three thermotolerant isolates (TR17, TR20, and TR32) exhibited the ability to produce high 1,3-propanediol, 2,3-butanediol, and ethanol in the culture medium. From this point, the feasibility to produce both fuels energy and biochemical products from these bacterial isolates could be possible. The advantage of this process could maximize the utilization of crude glycerol while reducing the waste material and also obtained high valuable products. The selected thermotolerant isolates were evaluated with respect to these advantages. The included hydrogen production with simultaneous 1,3-propanediol, 2,3-butanediol, and ethanol production were performed in the medium containing 10 g/L glycerol at initial pH 7.0 and incubated at 45^0C. The results found that glycerol was depleted within 20 h (Fig. 7) with a concomitant cumulative hydrogen production and cell growth. The specific growth rates of the selected three thermotolerant isolates were variable with the highest rate was 0.057 h^{-1} for the isolate TR20 and the lowest rate was 0.032 h^{-1} for the isolate TR32. The isolate TR17 had a cumulative hydrogen production, hydrogen yield and glycerol conversion rate of 20.90 mmol H_2/L, 0.21 mol H_2/mol glycerol, and 96.27%, respectively, which was the highest of the three isolates (Table 10). The shortest lag times were observed with the isolate TR17 (3.69 h), followed by the isolate TR20 (3.85 h), and the isolate TR32 (4.16 h). Although the isolate TR20 had the highest specific growth rate, it had the lowest cumulative hydrogen production. In contrast, the isolate TR17 had a high specific growth rate and also the highest hydrogen production performance. Thus, the isolate TR17 is the suitable strain for hydrogen production. The results demonstrated great capacity in 1,3-propanediol production (17.02-54.14 mmol/L) as well as 1,3-propanediol yield (0.17-0.62 mol/mol glycerol). They produced 2,3-butanediol in the range of 12.99-17.17 mmol/L with the yield of 0.14-0.16 mol/mol glycerol. The ethanol production and yield were 18.32-73.23 mmol/L and 0.21-0.77 mol/mol glycerol, respectively (Table 11). The isolate TR32 gave the highest ethanol production (73.32 mmol/L) and yield (0.77 mol/mol glycerol). The highest 1,3-propanediol production (54.36 mmol/L) and yield (0.62 mol/mol glycerol) were obtained from the isolate TR20, whereas the highest 2,3-butanediol production (17.15

mmol/L) and yield (0.16 mol/mol glycerol) were obtained from the isolate TR17. There are oxidative and reductive pathways for glycerol degradation. In the reductive pathway, glycerol is reduced to 3-hydroxypropionaldehyde and converted to 1,3-propanediol. In the oxidative pathway, glycerol converts to pyruvate and further cleavage into lactate, acetyl-CoA, α acetolactate, and formate. Formate further converted to hydrogen. Acetyl-CoA is further converted to ethanol and acetate. α Acetolactate is further converted to 2,3-butanediol (Biebl *et al*., 1999). In general, hydrogen is also produced along with different metabolites during oxidative metabolism of glycerol (Sarma *et al*., 2012). The dominant in difference products of the strain TR17, TR20, and TR32 might be due to the different microorganisms let to different consume NADH which used as the reductant for 1,3-propanediol, 2,3-butanediol, and ethanol production as well as different in metabolized end products (Sarma *et al*., 2012; Reungsang *et al*., 2013a).

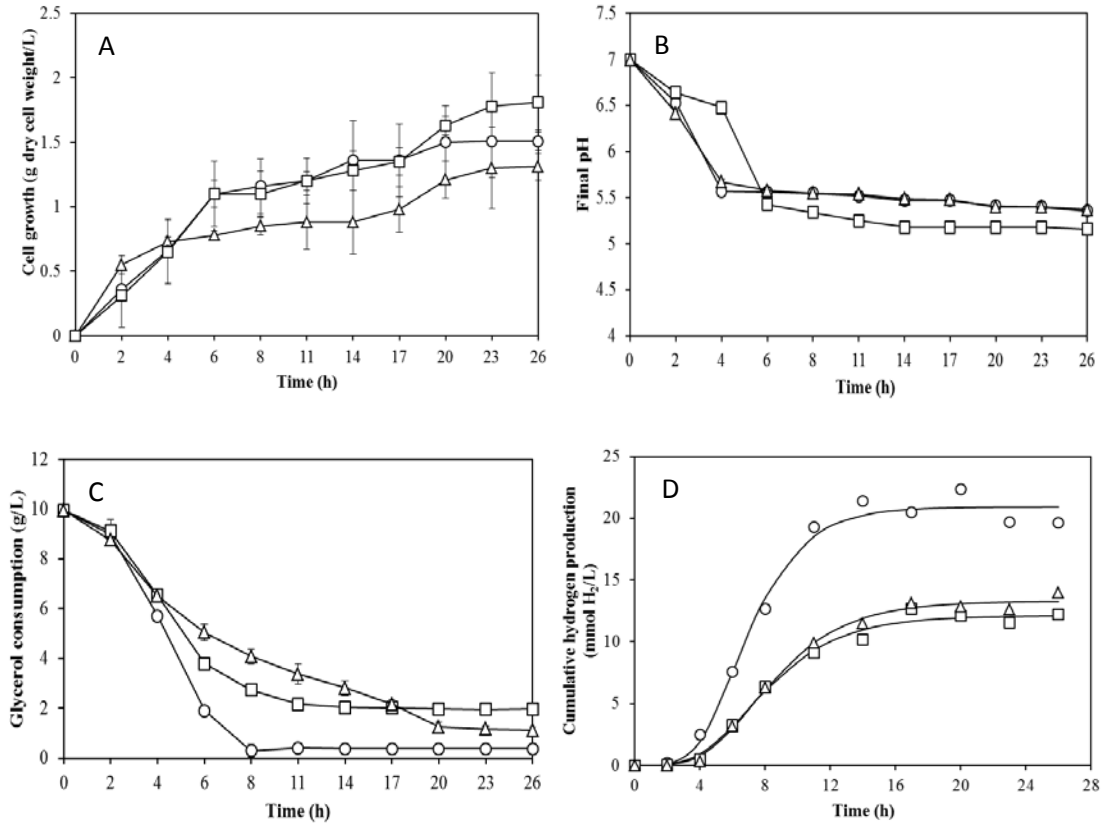

Figure 7. Time course cultivation of the top three isolates in the medium with 10 g/L glycerol as sole carbon source (pH 7.0) and incubated at 45^0C for 26 h. Cell growth (A), pH change (B), glycerol consumption (C), and cumulative hydrogen production (D). In each panel, symbols are ○ for TR17, □ for TR20, and Δ for TR32

Table 10. Characteristics of the top three thermotolerant hydrogen-producing isolates

Isolates	Growth yield ($Y_{x/s}$)	Specific growth rate (h^{-1})	Hydrogen yield (mol/mol glycerol)	Glycerol conversion (%)	P ($mmolH_2/L$)	λ (h)	R^2
TR17	0.151	0.051	0.21	96.27	20.90	3.69	0.975
TR20	0.223	0.057	0.14	80.25	12.10	3.85	0.982
TR32	0.147	0.032	0.14	88.44	13.27	4.16	0.991

P (the hydrogen production potential), λ (lag time of hydrogen production), and R^2 (goodness of fit) values were determined by best-fitting the cumulative hydrogen production data to a modified Gompertz equation.

Table 11. Production performance of 1,3-propanediol (1,3-PD), 2,3-butanediol (2,3-BD), and ethanol (EtOH) by top three isolates

Isolates	1,3-PD production (mmol/L)	1,3-PD yield (mol/mol glycerol)	2,3-BD production (mmol/L)	2,3-BD yield (mol/mol glycerol)	EtOH production (mmol/L)	EtOH yield (mol/mol glycerol)
TR17	54.14±4.65	0.52±0.04	17.15±3.12	0.16±0.03	32.94±3.54	0.32±0.05
TR20	54.36±5.73	0.62±0.06	13.51±2.77	0.15±0.01	18.32±1.84	0.21±0.02
TR32	17.02±2.44	0.17±0.04	12.99±2.37	0.14±0.02	73.23±5.75	0.77±0.1

2.4.5 Comparison of hydrogen yield from dark fermentation of crude glycerol with different microorganisms

This study compared the hydrogen yield from anaerobic fermentation of glycerol (10 g/L) at initial pH 7.0 and incubated at thermotolerant condition (45^0C) (Table 12). The three selected isolates TR17, TR20, and TR32, achieved the hydrogen yield of 0.21, 0.14, and 0.14 mol H_2/mol glycerol, respectively. These results demonstrated that the thermotolerant bacterial isolates could produce higher hydrogen yield than the other reports under mesophilic condition (Table 12). However, the

lower hydrogen yield was observed when compared to those of Seifert *et al*. (2009) and Selembo *et al*. (2009). In fact, hydrogen yield depend on many factors such as inoculum sources and condition for cultivation (Wang and Wan, 2007). However, the presence of thermotolerant bacterial isolates in this study could produce simultaneous hydrogen, diols, and ethanol which were superior to individual production of these products.

2.5 Conclusion

Enrichments of hydrogen producing bacteria at 45^0C from crude glycerol contaminated soil were found to be the potential sources for isolation of thermotolerant bacteria. Among the 38 isolates obtained, 20 isolates exhibited high hydrogen production from glycerol. The isolate TR17, TR20, and TR32 was selected based on thermotolerant performance with high cumulative hydrogen production. The isolate TR17 gave the highest cumulative hydrogen production with high 2,3-butanediol production. The isolate TR20 was 1,3-propanediol producer whereby the isolate TR32 was ethanol producer.

Table 12. A comparison yield (mol/mol glycerol) of hydrogen, 1,3-propanediol (1,3-PD), 2,3-butanediol (2,3-BD), and ethanol (EtOH) by anaerobic fermentation of crude glycerol

Inoculum	Initial glycerol Conc. (g/L)	Temp. (^{0}C)	Initial pH	Yield (mol/mol glycerol)				References
				H_2	1,3-PD	2,3-BD	EtOH	
Anaerobic digested sludge	10	37	6.0	0.41	-	-	-	Seifert *et al*. (2009)
Heat-treated mixed cultures	3	30	7.0	0.31	0.59	-	-	Selembo *et al*. (2009)
Heat treated soil	3	30	5.5	0.18	0.46	-	-	Sharma *et al*. (2011)
Klebsiella pneumoniae DSM 2026	20	37	7.0	0.09	-	-	-	Liu and Fang (2007)
Klebsiella sp. HE1	10	35	6.0	0.04	0.16	0.00	0.80	Wu *et al*. (2011)
Isolate TR17	10	45	7.0	0.21	0.52	0.16	0.32	This study
Isolate TR20	10	45	7.0	0.14	0.62	0.15	0.21	This study
Isolate TR32	10	45	7.0	0.14	0.17	0.14	0.77	This study

- : Not reported

CHAPTER 3

FERMENTATIVE PRODUCTION OF HYDROGEN AND SOLUBLE METABOLITES FROM CRUDE GLYCEROL OF BIODIESEL PLANT BY THE NEWLY ISOLATED THERMOTOLERANT *KLEBSIELLA PNEUMONIAE* TR17

3.1 Abstract

A thermotolerant fermentative hydrogen-producing isolate TR17 was identified as *Klebsiella pneumoniae* on the basis of the 16S rRNA gene analysis as well as physiological and biochemical characteristics. Optimization on production of hydrogen from *Klebsiella pneumoniae* TR17 was conducted. The strain produced hydrogen within a wide range of temperature (30-50^{0}C), initial pH (4.0-9.0) and glycerol concentration (20-100 g/L) with yeast extract as a favorable nitrogen source. In batch cultivation, the optimal conditions for hydrogen production were: cultivation temperature at 40^{0}C, initial pH at 8.0, 20 g/L glycerol and 2 g/L yeast extract. This resulted in the maximum cumulative hydrogen production of 27.7 mmol H_2/L and hydrogen yield of 0.25 mol H_2/mol glycerol. In addition, the main soluble metabolites were 1,3-propanediol, 2,3-butanediol, and ethanol corresponding to the production of 3.52, 2.06, and 3.95 g/L, respectively.

3.2 Introduction

Hydrogen is an environmentally-friendly fuel since its complete combustion discharges only water (Dunn, 2002). The considerable promise in replacing fossil fuels with biohydrogen for generating energy depends upon obtaining hydrogen from renewable and economical sources (Suwansaard *et al*., 2010). Consequently, the substrate used for fermentative hydrogen production must be abundant, easily available and inexpensive. From these aspects, waste materials from various agro-industries have been used as renewable feedstock for biohydrogen production (Chong *et al*., 2009b).

Thai government's biodiesel development strategy is to replace 10% of petro-diesel in transport sector with biodiesel by 2012. The plan is to increase the use of biodiesel from 365 million liters in 2007 to 3,100 million liters by 2012 (Siriwardhana *et al*., 2009). The dramatically increase of biodiesel production will inevitably increase the quantity of crude glycerol. This was due to the fact that production of 100 kg of biodiesel would generate 10 kg of crude glycerol. While pure glycerol has many applications for the use in food, cosmetics, and drugs (Fan *et al*., 2010), the crude glycerol has very low value because of its impurities. Furthermore, the purification process is costly. Therefore, alternative approach by using crude glycerol as a carbon source for fermentative microorganisms has been proposed. The production of biohydrogen from crude glycerol has received much attention. Not only gaseous products, soluble metabolites such as 1,3-propanediol (1,3-PD), 2,3 butanediol (2,3-BD) (Sattayasamitsathit *et al*., 2011), succinic acid, dihydroxyacetone and ethanol (EtOH) were also produced (da Silva *et al*., 2009; Yazdani and Gonzales, 2007).

Crude glycerol fermentation to produce hydrogen has been reported in *Enterobacter aerogenes* (Ito *et al*., 2005; Sakai and Yagishita, 2007), mixed cultures (Selembo *et al*., 2009), *Klebsiella* sp. (Niu *et al*., 2010), and thermophilic *Thermotoga neapolitana* (Ngo *et al*., 2011). However, hydrogen production is repressed at high glycerol concentrations and little EtOH is produced using *E. aerogenes* (Ito *et al*., 2005). Furthermore, mixed culture fermentation is very complex and difficult to optimize due to the variable compositions of the microbial communities (Hallenbeck and Ghosh, 2009). *Klebsiella* sp. has been investigated for production of 1,3-PD and other valuable by-products based on its rapid growth rate and simple growth condition

(Niu *et al*., 2010; Jhonson and Taconi, 2007). Nevertheless, only few studies have focused on biohydrogen production from crude glycerol using *Klebsiella* sp. (Liu and Fang, 2007; Wu *et al*., 2011).

A higher temperature is not only more feasible due to favorable stoichiometry but also due to lower risk of contamination (O-Thong *et al*., 2011). However, working at thermophilic temperatures has higher costs for heating, compared to mesophilic temperature (Hniman *et al*., 2011). It is thus possible to produce hydrogen under thermotolerant condition, which requires less heat energy input to maintain operation condition.

The aim of this study is to identify a thermotolerant hydrogen-producing strain from crude glycerol contaminated soil. The effects of some factors on hydrogen production were investigated in order to determine the optimal conditions for the higher hydrogen production.

3.3 Materials and methods

3.3.1 Crude glycerol

Crude glycerol without pretreatment was obtained from Prince of Songkla University (PSU) Biodiesel Pilot Plant at Faculty of Engineering, PSU, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification using methanol as a reactant and sodium hydroxide as a catalyst. The purity of the crude glycerol was calculated base on its glycerol content and found to be 50%. In addition, the impurities of crude glycerol were mainly potassium and sodium salts (4-5%), methanol (1-3%), non-glycerol organic matter (1.6-7.5%), and water (36%) (Sattayasamitsathit *et al*., 2011).

3.3.2 Culture medium for hydrogen production

The culture medium contained: 10.0 g/L glycerol (glycerol concentration contained in crude glycerol), 3.4 g/L K_2HPO_4, 1.3 g/L KH_2PO_4, 2.0 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution. Trace element solution contained 0.07 g/L $ZnCl_2$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $H_3BO_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2$; 0.02 g/L $CuCl_2 \cdot 2H_2O$, 0.025 g/L $NiCl_2 \cdot 6H_2O$, 0.035 g/L $Na_2MoO_4 \cdot 2H_2O$

(Liu and Fang, 2007). The initial pH of the medium was adjusted to 7.0 using 5 N NaOH or HCI before sterilization at 121^{o}C for 20 min.

3.3.3 Microscopic examination and biochemical tests

Gram staining was performed as previously described by Noparat *et al*. (2011). Morphological examinations were performed with a scanning electron microscope (FEI Quanta 400, SEM-Quanta). The different biochemical characterization was performed and substrate utilization was tested with the following sources: lactose, citrate, glucose, mannitol, *meso*-inositol, D-sorbitol, L- rhamnose, sucrose, xylose, fructose, galactose, D-mannose, maltose, dulcitol and arabinose. The reactions were compared with the reference strain of *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884^{T} (Li *et al*., 2004; Xu *et al*., 2010a; Drancourt *et al*., 2001).

3.3.4 16S rRNA sequencing and phylogenetic analysis

Total genomic DNA was extracted from the selected strain using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA). The 16S rRNA gene was amplified as previously described by O-Thong *et al*. (2008b). The PCR product was purified using the QIAquick PCR purification Kit (QIAGEN, USA). The 16S rRNA gene sequence was aligned and identified against existing sequences in the GenBank database using the BLAST program (Altschul *et al*., 1990). The closely related sequences of reference sequences were retrieved from the database aligned and checked using the BioEdit version 7.0.5.3 (Chong *et al*., 2009a). Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA 3.1 (Kumar *et al*., 1993). Confidence in the tree topologies was evaluated by re-sampling 1000 bootstrap trees (Felsenstein, 1995).

3.3.5 Detection of extended spectrum β-lactamases (ESBL) by combination disk method

The combined disk method for phenotypic detection was evaluated using ceftazidime (30 µg) and cefotaxime (30 µg) disks, alone and in combination with clavulanic acid (10 µL). The tests were carried out in Mueller-Hinton agar and

interpreted according to the standards established by the Clinical and Laboratory Standards Institute (CLSI). In this test, selected bacteria were preincubated in nutrients broth at 37^0C to an optical density equal to that of a 0.5 McFarland turbidity standard. This suspension was then used to inoculate Mueller Hinton agar plates by swabbing them with a sterile cotton swab. Disks containing cefotaxime/clavulanic acid 30 µg/10 µL and cefotaxime 30 µg, and ceftazidime/clavulanic acid 30 µg/10 µL and ceftazidime 30 µg were then placed prior to incubation. After overnight incubation at 37^0C, an increase of more than 5 mm in the diameter of the inhibition zone around disks containing clavulanic acid as compared to the diameters of around disks free of this inhibitor indicated ESBL activity (Jeong *et al*., 2004).

3.3.6 Optimization of factors affecting hydrogen production from crude glycerol by conventional method

All batch-fermentation studies were conducted anaerobically in 60 mL serum bottles with 36 mL working volume using the culture medium as described above. Prior to testing, the medium was flushed with nitrogen gas, capped with rubber stopper, closed with aluminum caps and autoclaved at 121^0C for 15 min. Ten percent of starter culture of the selected isolate was inoculated. The effects of operating parameters on hydrogen production were studied by varying the incubation temperature (30 to 60^0C), initial pH (pH 4.0 to 9.0) adjusted using 5N HCl or 5N NaOH and glycerol concentrations (20 to 100 g/L). The effect of nitrogen source and concentration was varied from 2 to 8 g/L with 2 g/L increment of each source including peptone, yeast extract and urea.

3.3.7 Analytical methods

The biogas production in the headspace of a serum bottle was measured by a syringe technique described by Owen *et al*. (1979). Hydrogen content of the biogas was determined using an Oldham MX 2100 gas detector (Cambridge Sensotec Ltd., England). Cell growth was determined as dry cell weight (gram dry weight per liter) by centrifugation the culture broth at 10,000 *g* for 10 min, washed cell sample twice with sterile distilled water, dried at 105ºC overnight and then weighed (Barbirato *et al*., 1998). Glycerol and organic acids concentration were

determined by High Performance Liquid Chromatography (HPLC) (Agilent 1200). The culture medium was centrifuged at 10,000 *g* for 10 min then, the supernatant was filtered through 0.22 μm Nylon membrane filter (Sartorius, German) before injection. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostat column compartment; an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA); and ChemStation Software. Operation conditions were: 20 μl sample volume; 5 mM H_2SO_4 as a mobile phase; flow rate of 0.7 mL/min and a column temperature of 65^0C (Rujananon *et al*., 2011). Liquid samples from the serum bottles were taken for determination of pH, substrate utilization and metabolite products. The hydrogen production was calculated by measuring the total volume of hydrogen produced (mmol H_2/L). The hydrogen yield (mol H_2/mol glycerol) was calculated by measuring amount of hydrogen produced (mol) divided by amount of glycerol consumed (mol) (Chen *et al*., 2007). The glycerol conversion rate was calculated by using the equation: [(I-F)/I] x 100%, in which I and F are the initial and final glycerol concentrations (g/L), respectively (Markov *et al*., 2011).

3.4 Results and discussion

3.4.1 Identification of thermotolerant hydrogen-producing bacteria isolated from crude glycerol contaminated soil

Among the isolated hydrogen-producing strains, the isolate TR17 showed the highest cumulative hydrogen production from glycerol with thermotolerant property. The isolate TR17 is a facultative anaerobic bacterium, Gram negative, rod shape, with the diameter from 0.3-1.0 μm and the length from 0.6-3.0 μm (Fig.8). Results of standard tests for physiological and biochemical identification indicated that the strain TR17 was belonged to the genus *Klebsiella*. The sequences (1450 bp) of the 16S rRNA gene from the strain TR17 were determined and aligned with available sequences from GenBank releases. The 16S rRNA sequence analysis indicated that the strain TR17 had the 97% similarity with the reference strains AF129444, AF129443, U78182, Y17658 and AF129440; 98% similarity with the reference strain AB0004750; 99% similarity with the reference strains X87286, AJ783916, AF250285, AF010251 and AF130982. The strain TR17 had the highest

similarity of 99.52% to *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884^T (Y17657). Phylogenetic trees, constructed on the basis of 16S rRNA, also revealed that the strain TR17 was grouped together with the reference strain of *Klebsiella pneumoniae* as shown in Fig. 9. The 16S rRNA gene sequence of *Klebsiella pneumoniae* TR17 was deposited in the GenBank under accession number AB647144. Standard biochemical analyses (Table 13) showed that the strain TR17 was positive for the production of indole, Voges-Proskauer reaction and lysine decarboxylase; negative for the production of H_2S, methyl red test, urease and motility. The strain was also positive for the utilization (sole carbon source) of lactose, citrate, glucose, mannitol, *meso*-inositol, D-sorbitol, L- rhamnose, sucrose, xylose, fructose, galactose, D-mannose, maltose, dulcitol and arabinose. Comparison with those of the reference strain (ATCC 13884^T), characters of methyl red test and utilization of dulcitol were different from *Klebsiella pneumoniae* TR17. According to the results of 16S rRNA gene analysis and physio-biochemical examinations, the *Klebsiella* sp. isolated in this study was identified as a new strain.

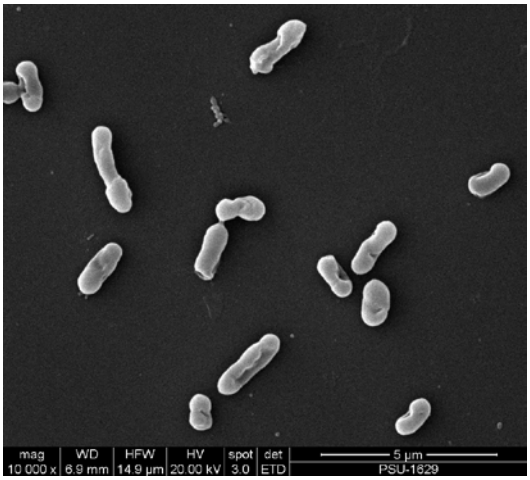

Figure 8. Scanning electron microscope (SEM) image of *Klebsiella pneumoniae* TR17

3.4.2 Detection of extended spectrum β-lactamases producing in *Klebsiella pneumoniae* TR17 by combination disk method

Klebsiella pneumoniae belongs to the family of Enterobacteriaceae that are classified as opportunistic pathogens in animals and humans (Zhang *et al*., 2010a).

The production of β-lactamase is the single most prevalent mechanism responsible for resistance to β-lactams among clinical isolates of the family Enterobacteriaceae. Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to penicillins, cephalosporins of the first, second and third generations and aztreonam via hydrolysis of the antibiotics (Oliveira *et al*., 2010). Thus, the test of ESBL producing in isolated bacteria, *Klebsiella pneumoniae* TR17, was evaluated. The National Committee for Clinical Laboratory Standards (NCCLS) recommends performing phenotypic confirmation of potential ESBL-producing isolates by testing both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. Testing can be performed by the broth micro dilution method or by disk diffusion. For disk diffusion testing, a $\geq$ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism (Carter *et al*., 2000). In this study, the combination disk method was used for detected ESBL producing in *Klebsiella pneumoniae* TR17. Pairs of disks contained a cefotaxime 30 µg and ceftazidime 30 µg, alone with and without clavulanic acid 10 µL. The tests were carried out in Mueller-Hinton agar. Zones of inhibition are measured following overnight incubation at 37^0C. The result in Fig. 10 found that *Klebsiella pneumoniae* TR17 showed decreases in zones of inhibition of $\leq$ 5mm for combination cefotaxime/clavulanic acid discs and ceftazidime/clavulanic acid discs compared with the plain cefotaxime and ceftazidime discs. This implies that *Klebsiella pneumoniae* TR17 could not produce ESBL enzymes as well as could not be a pathogen for resistance to β-lactam antibiotics.

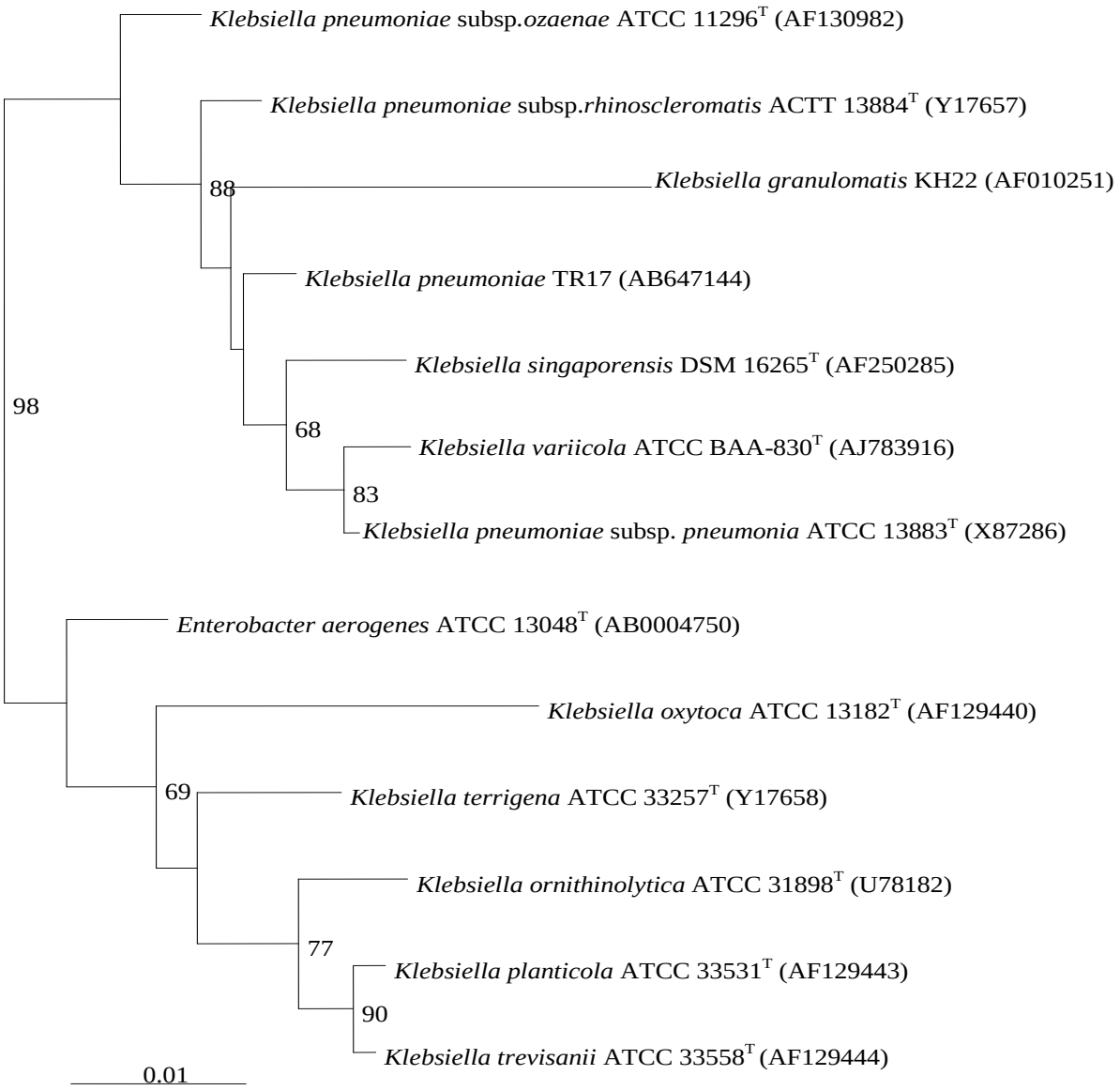

Figure 9. Phylogenetic relationships of the newly isolate *Klebsiella pneumoniae* TR17 recognized *Klebseilla* species and related taxonomic based on 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbor-joining method. Bootstrap values (expressed as percentages of 1,000 replications) greater than 60% are shown at the branch points. Bar 0.01 substitutions per nucleotide position

Table 13. The characteristics of the isolate TR17 in comparison with the reference strain *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884^T

Character	Strain ATCC 13884^T (Xu *et al*., 2010a)	Strain TR17 (This study)
Indole	-	+
Methyl red test	+	-
Voges-Proskauer reaction	-	+
Lysine decarboxylase	-	+
H_2S	-	-
Urease	-	-
Motility	-	-
Utilization of:		
Lactose	+	+
Citrate	+	+
Glucose	+	+
Mannitol	+	+
meso-Inositol	+	+
D-sorbitol	+	+
L- Rhamnose	+	+
Sucrose	+	+
Xylose	+	+
Fructose	ND	+
Galactose	ND	+
D-Mannose	+	+
Maltose	+	+
Dulcitol	-	+
Arabinose	+	+

Symbol: +, positive; -, negative

ND= not determined.

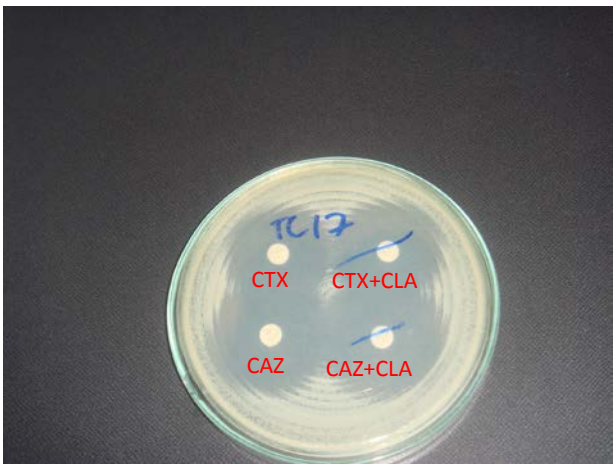

Figure 10. Non extended spectrum β-lactamases producer of *Klebsiella pneumoniae* TR17 showed ≤ 5mm in the diameter of the inhibition zone around disks containing a cefotaxime (CTX) and ceftazidime (CAZ), alone and in combination with clavulanic acid (CTX+CLA, CTX+CAZ) by combination disk method in Mueller-Hinton agar after 24 h of incubation at 37^0C

3.4.3 Effects of temperature on hydrogen production

Temperature is an important factor for production of hydrogen by microorganisms (Mu *et al*., 2006b). Hydrogen production by *Klebsiella* sp. was usually reported within the temperature range of 30-37^0C (Wu *et al*., 2011; Liu and Fang, 2007; Niu *et al*., 2010) but has not been examined at higher temperature. Therefore, the effect of incubation temperature in the range of 30^0C to 60^0C was tested for hydrogen production by *Klebsiella pneumoniae* TR17 in the medium with 10 g/L glycerol as a carbon source, initial pH at 7.0 and incubated for 24 h. The cumulative hydrogen production increased with increasing temperature from 30^0C to 40^0C and decreased thereafter. The maximum cumulative hydrogen production of 22.5 mmol H_2/L was achieved at 40^0C (Fig. 11A). The optimal temperature for cell growth (0.55 g dry cell weight/L) was at 30^0C. The final pH values were obviously at two levels; pH approximately 5.8 at the incubation temperature of 30^0C to 45^0C and pH approximately 6.6 at 50^0C to 60^0C. The residual glycerol increased with the increase of incubation temperature especially from 50^0C to 60^0C. No hydrogen production at the high temperature (50^0C to 60^0C) was observed due to the influence

of temperature on the activity of some essential enzymes such as hydrogenases that was affected along with the metabolic pathway of the microorganisms (Wang and Wan, 2008; Akutsu *et al*., 2009b).

The hydrogen yield (0.07-0.36 mol H_2/mol glycerol) increased with increasing temperature from 30^0C to 45^0C and dropped sharply thereafter. The maximum hydrogen yield (0.36 mol H_2/mol glycerol) was obtained at 45^0C. However, the maximum cumulative hydrogen production (22.5 mmol H_2/L) was obtained at 40^0C. These results were slightly different from the work on *Klebsiella* sp. that previously reported on the optimum cumulative hydrogen production at 35 ^{0}C (Wu *et al*., 2011) and 37^0C (Liu and Fang, 2007; Niu *et al*., 2010). The soluble metabolites were composed mostly of 1,3-propanediol (1,3-PD) (0.13-4.13 g/L), 2,3-butanediol (2,3-BD) (1.28-1.85 g/L), ethanol (EtOH) (0.79-2.58 g/L), acetic acid (0.08-0.53 g/L) and succinic acid (0.10-0.28 g/L) (Table 14). It was noted that the optimum temperature for production of 1,3-PD, 2,3-BD was at 30^0C, while that of acetic acid and succinic acid was at 35^0C. The EtOH production was highest at 40^0C.

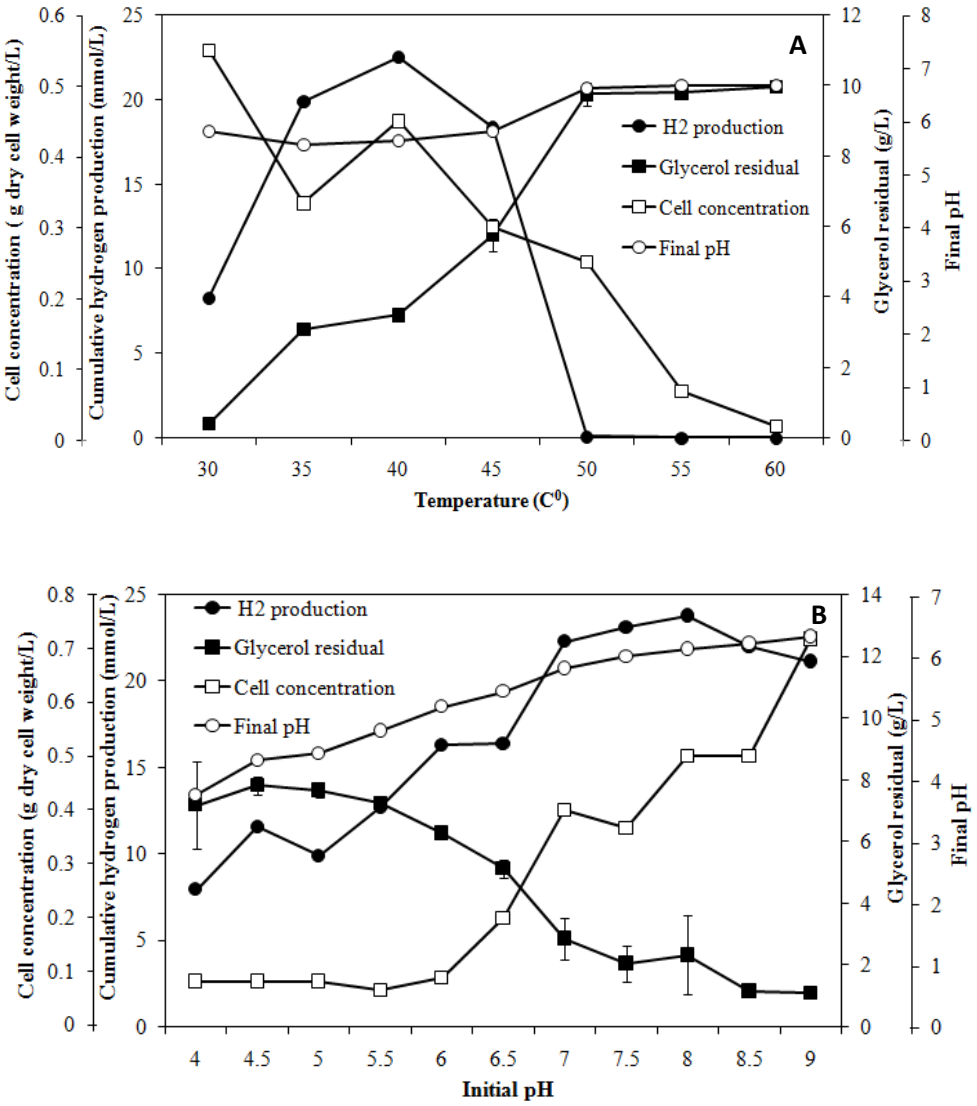

Figure 11. Effect of temperature (A) (at a fixed initial pH 7.0) and initial pH (B) (at 40^0C) from cultivation of *Klebsiella pneumoniae* TR17 in the medium with 10 g/L glycerol as a carbon sources and incubated for 24 h on cumulative hydrogen production, glycerol residual, cell concentration, and final pH

Table 14. Soluble metabolite products from fermentation of glycerol for hydrogen production from *Klebsiella pneumoniae* TR17

Conditions		Soluble metabolite products (g/L)					
		HAc	HSu	1,3-PD	2,3-BD	EtOH	SMP
Temp. (^{0}C)	30	0.23±0.03	0.33±0.05	4.13±0.05	1.85±0.02	1.65±0.01	8.18±1.57
	35	0.53±0.00	0.38±0.02	2.55±0.06	1.35±0.05	2.16±0.15	6.99±0.95
	40	0.27±0.04	0.32±0.01	1.85±0.07	1.33±0.04	2.58±0.08	6.36±0.99
	45	0.24±0.00	0.24±0.02	0.99±0.07	1.28±0.00	2.13±0.20	4.90±0.80
	50	0.08±0.00	0.10±0.01	0.13±0.00	1.28±0.03	0.79±0.00	2.40±0.54
	55	0	0	0	0	0	0
	60	0	0	0	0	0	0
Initial pH	4.0	0.18±0.00	0.15±0.00	0.55±0.01	1.05±0.01	1.16±0.01	3.12±0.47
	4.5	0.15±0.00	0.11±0.00	0.58±0.08	1.08±0.02	1.29±0.07	3.23±0.53
	5.0	0.16±0.03	0.12±0.01	0.64±0.07	1.06±0.01	1.30±0.07	3.29±0.53
	5.5	0.16±0.03	0.14±0.02	0.81±0.08	1.06±0.03	1.23±0.13	3.47±0.52
	6.0	0.27±0.00	0.21±0.01	1.29±0.02	1.11±0.09	1.42±0.00	4.32±0.57
	6.5	0.35±0.14	0.21±0.10	1.72±0.20	0.95±0.14	1.37±0.27	4.56±0.65
	7.0	0.49±0.06	0.33±0.03	2.40±0.23	1.07±0.02	1.71±0.15	6.01±0.86
	7.5	0.56±0.07	0.37±0.01	2.53±0.33	0.99±0.01	1.82±0.06	6.27±0.90
	8.0	0.60±0.11	0.38±0.07	2.58±0.38	1.06±0.08	1.92±0.28	6.55±0.92
	8.5	0.69±0.01	0.39±0.02	2.82±0.11	1.03±0.02	1.95±0.08	6.89±0.99
	9.0	0.33±0.02	0.38±0.01	1.75±0.13	1.04±0.02	3.12±0.28	6.62±1.15
Glycerol conc. (g/L)	20	0.74±0.2	0.94±0.1	7.73±1.6	2.70±0.1	5.47±0.1	17.6±3.0
	40	1.40±0.2	1.06±0.1	8.83±0.8	4.56±0.1	4.70±0.1	20.6±3.1
	60	2.91±0.7	1.45±0.1	13.2±1.8	10.5±3.2	5.44±0.1	33.6±5.0
	80	3.53±0.1	2.18±0.1	17.0±0.2	12.3±0.6	6.79±0.1	41.8±6.2
	100	3.65±0.1	2.09±0.1	18.3±0.5	13.3±0.2	7.36±0.1	44.7±6.8

HAc: acetic acid; HSu: succinic acid; 1,3-PD: 1,3-propanediol; 2,3-BD: 2,3-butanediol; EtOH: ethanol. SMP: soluble metabolite products (SMP= HAc + Hsu + 1,3-PD + 2,3-BD + EtOH). The second number in each entry is the standard deviation of triplicate experimental results

3.4.4 Effects of initial pH on hydrogen production

In order to determine the optimum initial pH for hydrogen production by *Klebsiella pneumoniae* TR17, various initial pHs (4.0 to 9.0) were investigated at a fixed initial glycerol concentration of 10 g/L and incubated at 40^0C for 24 h. The optimal initial pH for cumulative hydrogen production (23.8 mmol H_2/L) was at pH 8.0 while pH 9.0 was the optimal pH for cell growth (0.71 g dry cell weight/L) (Fig. 11B). Glycerol consumption was in the range of 70-88% at pH 7.0-9.0. The final pH dropped during the cultivation to the final values of 3.7-6.2. An increase in initial pH led to the increase in glycerol conversion rate and soluble metabolites, but decreased in the hydrogen yield. The maximum glycerol conversion rate (88.6%) and hydrogen yield (0.48 mol H_2/mol glycerol) was achieved at the initial pH of 9.0 and 5.5, respectively. The soluble metabolites were composed of 1,3-PD (0.55-2.82 g/L), 2,3-BD (0.95-1.11 g/L), EtOH (1.16-3.12 g/L), acetic acid (0.15-0.69 g/L) and succinic acid (0.11-0.39 g/L) (Table 14). The decrease in cumulative hydrogen production as the pH lower than 7.0 (pH 3.7-6.2) was the result of growth inhibition caused by penetration of proton from acid through the cell membrane into cytoplasms (Fangkum and Reungsang, 2010; Pessoa *et al*., 1996). In addition, the low pH (<4.5) can also affect the hydrogenase activity as well as the metabolic pathway of the microorganisms (Chong *et al*., 2009a; Fangkum and Reungsang, 2010; Wang and Wan, 2009). The optimum initial pH at 8.0 of *Klebsiella pneumoniae* TR17 was higher than those previously reported for cumulative hydrogen production from glycerol such as pH 6.0 from *Klebsiella* sp. HE1 (Wu *et al*., 2011), pH 6.3 from *Escherichia coli* (Hu and Wood, 2010), pH 6.5 from *Klebsiella pneumoniae* DSM 2026 (Liu and Fang, 2007), and pH 7.4 from *Halanaerobium saccharolyticum* subspecies *saccharolyticum* (Kivisto *et al*., 2010). Hydrogenase is the main enzyme for hydrogen production. It has been reported that activity of different hydrogenase enzymes (Hyd) is dependent on pH. Hyd-1 activity is induced under anaerobic conditions at acidic pH whereas maximal activity of Hyd-2 is attained in alkaline medium (King and Przybyla, 1999). Hyd-3 with formate dehydrogenase H (Fdh-H), the component of the formate hydrogen lyase (FHL) complex, produces hydrogen mostly at acidic pH (Sauter *et al*., 1992). However, at alkaline pH, hydrogen production was found to be largely by Hyd-4 with Fdh-H forming the FHL pathway

to produce hydrogen (Bagramyan *et al*., 2002). Moreover, Trchounian *et al*. (2011) study Hyd activity and hydrogen production by *Escherichia coli* at a low pH under glycerol fermentation. The results on hydrogen production by mutants with defects in different Hyd-enzymes suggest that under glycerol fermentation at a low pH, Hyd-4 were operating in a reversed with non-hydrogen producing mode. Thus, the maximum hydrogen production at pH 8.0 in this study might be due to the role of Hyd-4 as the main responsible for hydrogen production.

3.4.5 Effect of glycerol concentration on hydrogen production

The effect of initial glycerol concentration (20, 40, 60, 80, and 100 g/L) on hydrogen production from *Klebsiella pneumoniae* TR17 was tested under the optimum initial pH at 8.0 and incubated at 40^0C for 36 h. The optimal value was found to be 20 g/L glycerol (Fig. 12A) where the strain exhibited the maximum cumulative hydrogen production of 26.9 mmol H_2/L (23.8 mmol H_2/L obtained from 10 g/L glycerol) (data not shown) and cell growth of 2.85 g dry cell weight/L (Fig. 12B). An increase in glycerol concentration led to the decrease in glycerol conversion rate but increased in hydrogen yield up to 60 g/L glycerol. The maximum glycerol conversion rate (81.3%) and hydrogen yield (0.20 mol H_2/mol glycerol) was obtained at the initial glycerol concentration of 20 and 60 g/L, respectively. The soluble metabolites were composed mostly of 1,3-PD (7.73-18.3 g/L), 2,3-BD (2.70-13.3 g/L), EtOH (5.47-7.36 g/L), acetic acid (0.74-3.65 g/L) and succinic acid (0.94-2.09 g/L) (Table 14). Experimental results showed that the hydrogen production tended to decrease with the increase of glycerol concentration. It was also observed that glycerol limitation could lead to higher hydrogen production, while excessive glycerol favors production of 1,3-PD (Biebl *et al*., 1998; Deckwer,1995; Zeng *et al*., 1996). Glycerol limitation condition enhanced hydrogen production as it favored the conversion of pyruvate to acetyl CoA. When glycerol was in excess, more $NADH_2$ was used for the formation of 1,3-PD than hydrogen product (Deckwer,1995).

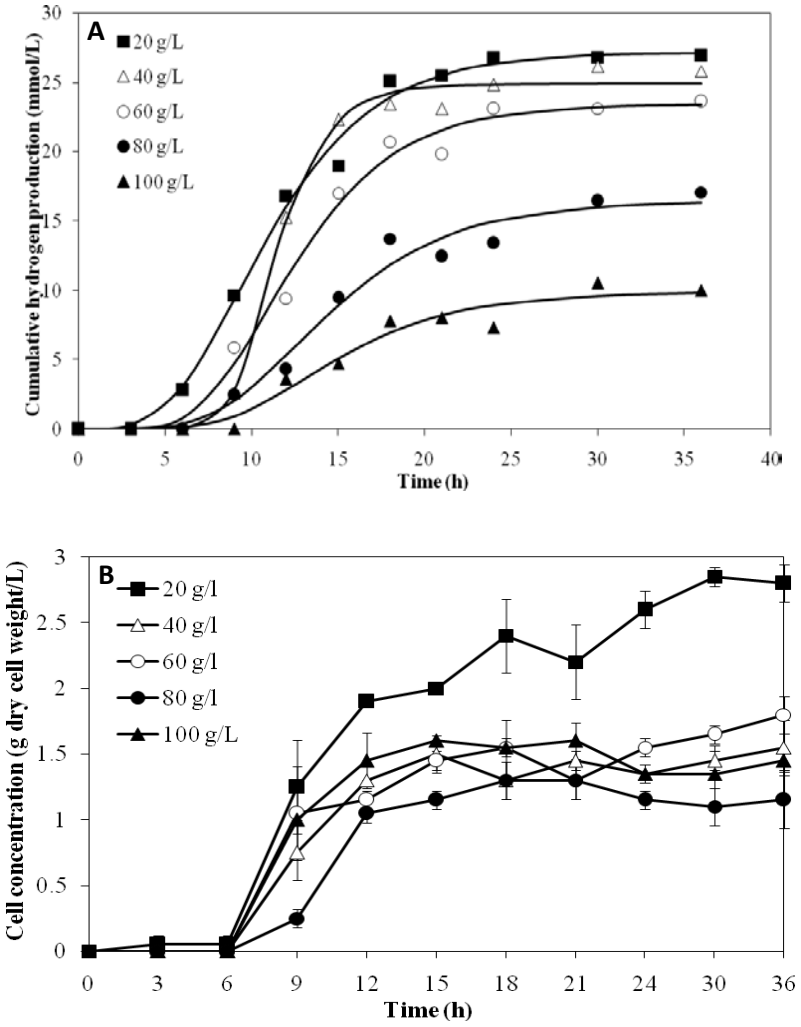

Figure 12. Effect of initial glycerol concentration on cumulative hydrogen production (A) and cell concentration (B) of *Klebsiella pneumoniae* TR17 during fermentation at the optimal initial pH 8.0 and incubation at 40^0C for 36 h

3.4.6 Effect of nitrogen sources on hydrogen production

Nitrogen source affects the synthesis of enzymes involved in both primary and secondary metabolisms (Pan *et al*., 2008b). The effect of various nitrogen sources were tested at the optimal initial pH of 8.0, glycerol concentration of 20 g/L and incubation at 40^0C for 24 h. The maximal cumulative hydrogen production (27.0 mmol H_2/L) and cell growth (1.12 g dry cell weight/L) were observed at 2 and 8 g/L yeast extract, respectively (Fig. 13). The maximum hydrogen yield (0.38 mol H_2/mol glycerol) was obtained at the yeast extract concentration of 6 g/L. The soluble

metabolites were composed of 1,3-PD (1.90-8.34 g/L), 2,3-BD (1.60-2.97 g/L), EtOH (1.34-5.09 g/L), acetic acid (0.42-2.77 g/L), and succinic acid (1.01-1.49 g/L). As a result, addition of peptone or yeast extract gave higher hydrogen production and cell growth than using urea. This was due to the fact that nitrogen of yeast extract and peptone exists in the form of protein and amino acids which can be used better by bacteria for growth as well as hydrogen production (Xu *et al*., 2010b).

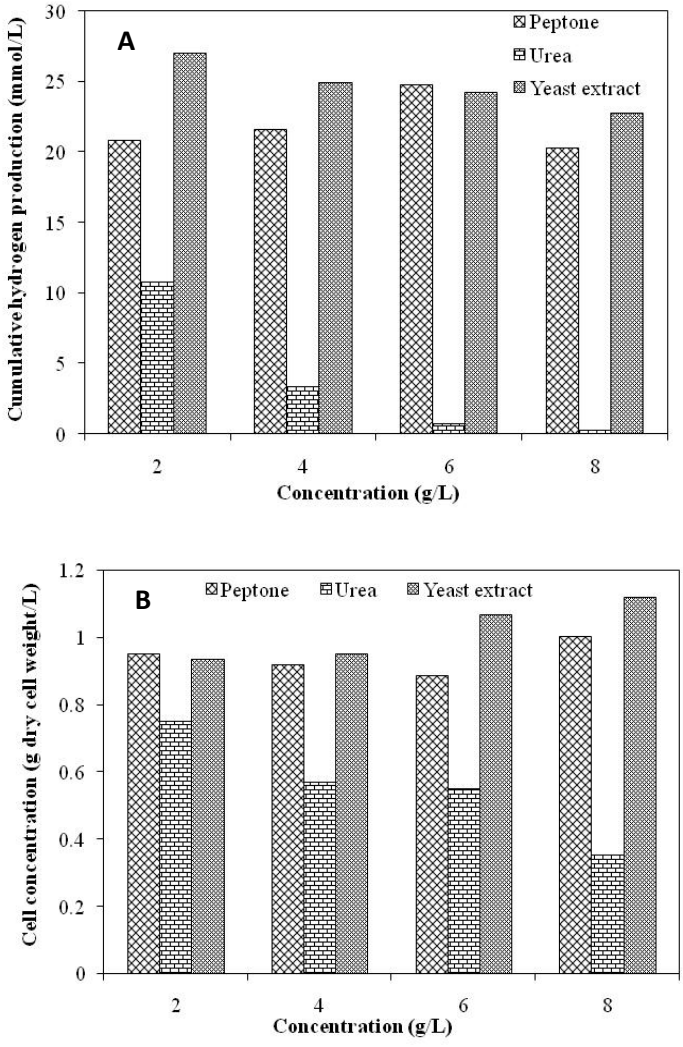

Figure 13. Effect of organic nitrogen sources concentration on cumulative hydrogen production (A) and cell concentration (B) of *Klebsiella pneumoniae* TR17 during fermentation at the optimal temperature of 40^0C, initial pH 8.0, 20 g/L glycerol and incubation for 24 h

3.4.7 Fermentation characteristic under the optimum condition

The time course profile and characteristics of hydrogen fermentation of *Klebsiella pneumoniae* TR17 under the optimum condition (40^0C, initial pH 8.0, 20 g/L glycerol and 2 g/L yeast extract) were illustrated in Fig. 14A. The maximum cumulative hydrogen production (27.7 mmol H_2/L) and hydrogen yield (0.25 mol H_2/mol glycerol) was achieved. The cell growth and final pH were 0.78 g dry cell weight/L and 6.1, respectively. The evolution of hydrogen in dark fermentation process is always accompanied by formation of soluble metabolites mainly 1,3-PD, 2,3-BD and EtOH with the highest values of 3.52, 2.06 and 3.95 g/L, respectively, as well as smaller quantity of acetic acid and succinic acid (Fig.14B). The theoretical maximum yield for hydrogen production was depended on the end products. If acetate is the main end metabolic product, the theoretical hydrogen yield is 3 mol H_2/mol glycerol (Selembo *et al.*, 2009). If the main end metabolic product is 1,3-propanediol, the theoretical hydrogen yield is 1 mol H_2/mol glycerol (Kivisto *et al*., 2010). In this study, the main metabolite was 1,3-propanediol. Thus, the theoretical maximum hydrogen yields of glycerol from the groups of *Klebsiella* sp. was 1 mol H_2/mol glycerol. The lower hydrogen yield mainly was due to the production of soluble metabolites which are competing with the cumulative hydrogen production. There are two approaches; one is to optimize the medium components affecting simultaneous fermentative hydrogen and ethanol production in order to obtain two biofuels from glycerol. The other way is to improve the hydrogen production using the immobilized systems on larger scale reactor.

The thermotolerant *Klebsiella pneumoniae* TR17 had the optimum temperature for cumulative hydrogen production at 40^0C. The use of thermotolerant bacteria has potential applications in bioreactor operation because high temperatures would lower the risk of contamination by competing mesophiles and require less energy than thermophiles. These make thermotolerant bacteria more likely to be the technically and economically favored option (Eberly and Ely, 2008). The optimum initial pH at 8.0 was near the neutral pH of crude glycerol generated from biodiesel production process used in this study. This seems to be important in term of economic feasibility for conversion of alkaline renewable waste like crude glycerol to hydrogen by *Klebsiella pneumoniae* TR17.

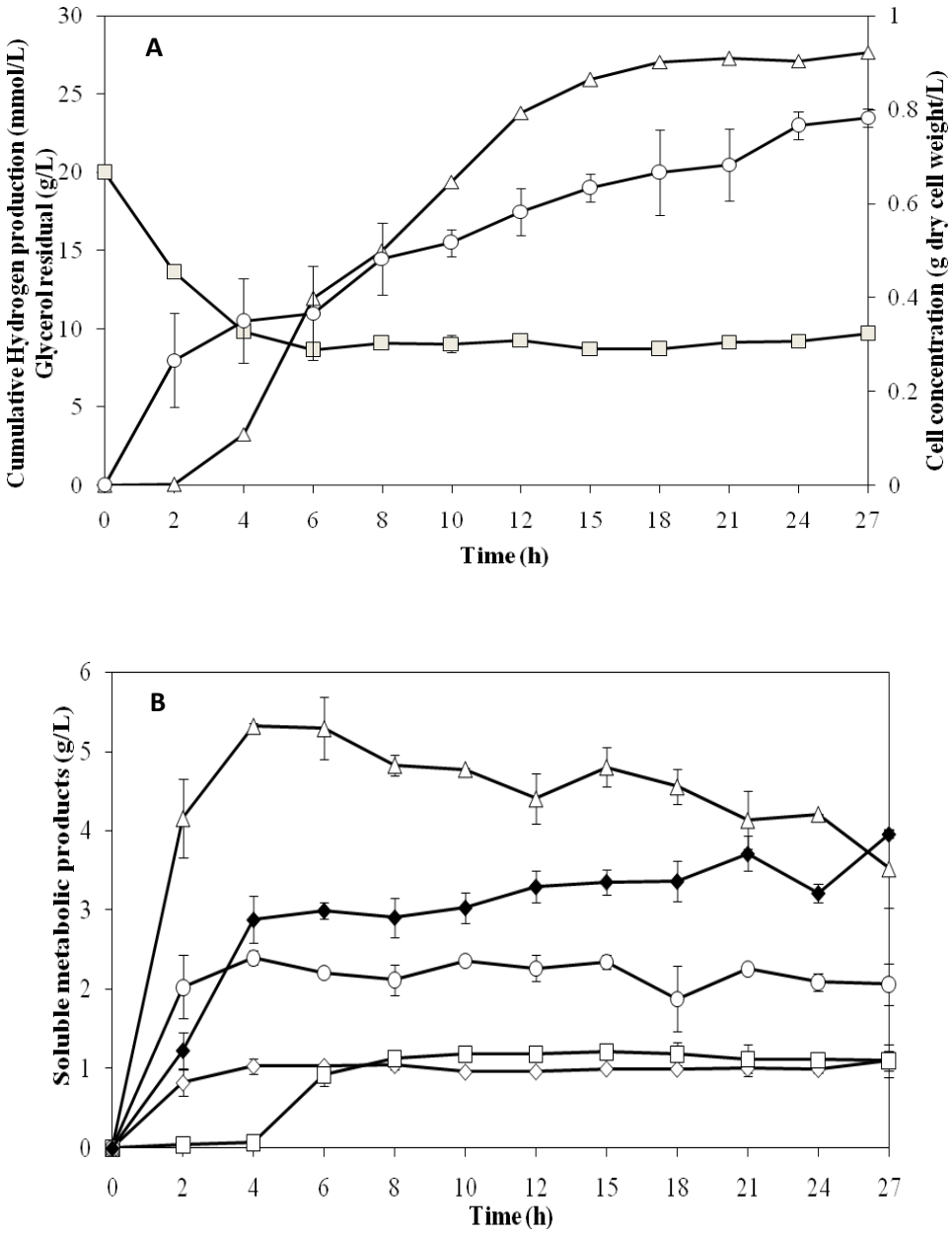

Figure 14. Time course during fermentation by *Klebsiella pneumoniae* TR17 under the optimum condition: cumulative hydrogen production (A), in each panel, symbols ○ for cell growth, □ for glycerol residual, and Δ for cumulative hydrogen production: soluble metabolite products (B), in each panel, symbols are □ for acetic acid, ◊ for succinic acid, Δ for 1,3- PD, ○ for 2,3-BD, and ♦ for EtOH

3.5 Conclusion

A thermotolerant fermentative hydrogen-producing strain was identified as *Klebsiella pneumoniae* TR17 on the basis of the 16S rRNA gene analysis as well as physiological and biochemical characteristics. The maximum cumulative hydrogen production of 27.7 mmol H_2/L and hydrogen yield of 0.25 mol H_2/mol glycerol were achieved from fermentation of crude glycerol under the optimal conditions: 40^0C, initial pH 8.0, 20 g/L glycerol and 2 g/L yeast extract. Besides hydrogen, the strain also gave valuable soluble metabolites especially 1,3-propanediol, 2,3-butanediol, and ethanol.

CHAPTER 4

STATISTICAL OPTIMIZATION OF MEDIUM COMPONENTS AFFECTING SIMULTANEOUS FERMENTATIVE HYDROGEN AND ETHANOL PRODUCTION FROM CRUDE GLYCEROL BY THERMOTOLERANT *KLEBSIELLA PNEUMONIAE* TR17

4.1 Abstract

Studies on the effect of medium components on simultaneous hydrogen and ethanol production from glycerol of *Klebsiella pneumoniae* TR17 by response surface methodology (RSM) using Plackett-Burman design were investigated. The significant variables ($p<0.05$), which influenced hydrogen production, were KH_2PO_4 and NH_4Cl. Subsequently, the two selected variables and glycerol as a carbon source were optimized by the Central Composite design (CCD) for achieving maximum hydrogen and ethanol yield. The concentration of glycerol, KH_2PO_4, and NH_4Cl had an individual effect on both hydrogen and ethanol yield ($p<0.05$), while KH_2PO_4 and NH_4Cl had an interactive effect on ethanol yield ($p<0.05$). The optimum medium components for production of hydrogen and ethanol were 11.14 g/L glycerol, 2.47 g/L KH_2PO_4, and 6.03 g/L NH_4Cl. The predicted maximum simultaneous hydrogen and ethanol yield were 0.27 mol H_2/mol glycerol and 0.63 mol EtOH/mol glycerol, respectively. Validation of the predicted optimal conditions exhibited similar values from the experiment. The hydrogen yield was 0.26 mol H_2/mol glycerol and ethanol yield was 0.58 mol EtOH/mol glycerol.

4.2 Introduction

The problems of oil prices and environmental pollution are convincing the Thai government to come up with the national energy policy to focuse on renewable energy for the energy security of the country (Papong *et al*., 2010). According to the national energy policy, the biodiesel and bioethanol were set. The targets of biodiesel production in Thailand were proposed by the years 2011, 2016, and 2022 at 3.0, 3.64, and 4.5 million liters/day, respectively (Silalertruksa *et al*., 2012). The increasing of biodiesel plants for supporting the Thai government's national energy policy will generate large amount of crude glycerol as a by-product, with about 1 liters of crude glycerol created from every 10 liters of biodiesel production. The more biodiesel produce, the more crude glycerol will be generated. Thus, finding the way to use crude glycerol is required, since it can eliminate the accumulation of crude glycerol as well as waste minimization in the future (Selembo *et al*., 2009).

The biological production of chemical industries or biofuels from crude glycerol could be an attractive way. Recently, crude glycerol was focused on the production of 1,3-propanediol, which used for monomer of polyester and polytrimethylene terephthalate (PTT) (Sattayasamitsathit *et al*., 2011). However, conversion of crude glycerol into biofuels such as biohydrogen and bioethanol are more attractive compared with pyrolysis, electrolytic, and reforming processes. Biohydrogen and bioethanol can be produced from renewable sources and waste materials (Zhao *et al*., 2009). Hydrogen has 2.75 times higher energy content than hydrocarbon fuels and was considered as a clean energy for the future (Sreela-or *et al*., 2011). In addition, ethanol can substitute gasoline for transportation, or replace methanol for biodiesel production (Han *et al*., 2011).

Hydrogen and ethanol production from glycerol by fermentation has been studied (Liu and Fang 2007; Markov *et al*., 2010; Oh *et al*., 2011). A conversion of glycerol into hydrogen, CO_2, and ethanol by fermentation is shown in Eq. (9).

$$C_3H_5(OH)_3 \longrightarrow H_2 + CO_2 + C_2H_5OH \qquad (9)$$

Based on Eq. (13), theoretical maximum yield of both hydrogen and ethanol are 1.0 mol/mol glycerol (Markov *et al*., 2010; Sakai and Yagishita, 2007). Glycerol can be

used for production of biofuels by fermentation which could competitive with glucose or xylose. When the fermentation of glycerol passes though pyruvate, it generates twice the amount of reducing equivalents as well as metabolism would be higher compared with those sugars (Yazdani and Gonzales, 2007). Thus, from Eq. (13) the combination of hydrogen and ethanol production is more advantage when compared with either hydrogen or ethanol production alone. In addition, valuable metabolite products were also obtained from dark fermentation of glycerol. Moreover, the production of hydrogen and ethanol is exhibited in separate phases, which are favorable for downstream separation (Zhao *et al*., 2010). However, combination of two biofuel products from crude glycerol has scarcely been investigated.

Hydrogen and ethanol production by dark fermentation is focused on mesophilic temperature (Ito *et al*., 2005; Hu and Wood, 2010; Wu *et al*., 2011). In fact, a higher temperature favored the stoichiometry of hydrogen production, resulting in higher hydrogen yields. However, thermophilic temperature requires heat energy input (Hniman *et al*., 2011). Thus, thermotolerant temperature (30-45^{0}C) presumably favored for the stoichiometry of hydrogen production and decreased heating cost. This makes thermotolerant operation more reasonable for the technically and economically option (Hawkes *et al*., 2007; Eberly and Ely, 2008).

Many factors are focused on the individual effects on hydrogen (Ngo *et al*., 2011; Kivisto *et al*., 2010) or ethanol production (Oh *et al*., 2011). Glycerol concentration exhibited strong effect with the decrease of hydrogen and ethanol production at higher concentration of glycerol (Hu and Wood, 2010). Tryptone and yeast extract were found to be essential for enhancing hydrogen and ethanol production (Ito *et al*., 2005). Nevertheless, the amount of compounds to be added into the medium must be cost minimized.

Optimization of the critical factors such as glycerol concentration, nutrient sources and concentration are needed. The response surface methodology (RSM) is the statistical experimental design that can achieve the optimum conditions from the experiments. Since RSM can identify critical factors which eliminates time consuming to perform the optimization conditions compared with a conventional method. Hence, using a statistical experimental design shows the interactive effects of the variables investigated (Saraphirom and Reungsang, 2010). Although many studies

have already done on the effect of nutritional and environmental conditions for improving on individual hydrogen or ethanol production from various substrates and waste materials, but the information on the using of statistical optimization of medium compositions on simultaneous hydrogen and ethanol production from crude glycerol by thermotolerant microorganism are still lacking.

Therefore, the objectives of this study are to optimize the medium composition affecting simultaneous hydrogen and ethanol production from crude glycerol by thermotolerant *Klebsiella pneumoniae* TR17. The Plackett-Burman design is used to screen the significant factors, then the Central Composite design is used to find the optimum levels of the selected factors for maximizing simultaneous hydrogen and ethanol production. The results on optimization of this study could be applied to actual hydrogen/ethanol production process, same as the other fermentation products. These two products are generated in different phase, so it is not difficult to separate hydrogen (in the gas phase) from ethanol (in the liquid phase).

4.3 Materials and methods

4.3.1 Crude glycerol

Crude glycerol without pretreatment was obtained from Prince of Songkla University (PSU) Biodiesel Pilot Plant at Faculty of Engineering, PSU, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification using methanol as a reactant and sodium hydroxide as a catalyst. The purity of the crude glycerol was calculated base on its glycerol content and found to be 50%. In addition, the impurities of crude glycerol were mainly potassium and sodium salts (4-5%), methanol (1-3%), non-glycerol organic matter (1.6-7.5%), and water (36%) (Sattayasamitsathit *et al*., 2011).

4.3.2 Microorganism and culture medium

Klebsiella pneumoniae TR17 (AB647144) was isolated from crude glycerol contaminated soil (Chookaew *et al*., 2012). It was grown in culture medium containing: 20.0 g/L glycerol, 3.4 g/L K_2HPO_4, 1.3 g/L KH_2PO_4, 4.0 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution. Trace element solution contained: 0.07

g/L $ZnCl_2$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $H_3BO_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2$; 0.02 g/L $CuCl_2 \cdot 2H_2O$, 0.025 g/L $NiCl_2 \cdot 6H_2O$, 0.035 g/L $Na_2MoO_4 \cdot 2H_2O$ (Chookaew *et al*., 2012). All experiments were carried out in 60 mL serum bottles with 36 mL of the culture medium as described above. The initial pH was adjusted to 8.0 with 5 N NaOH or 5 N HCl. The anaerobic condition was created by flushing the medium with nitrogen gas, capped with rubber stopper, then autoclaved at 121^0C 15 min. The strain at its exponential-growth phase (OD_{660} = 0.5) was used as the inoculums. The serum bottles contained 10% inoculum (v/v) with different concentration of variables according to the design. All treatments were incubated at 45^0C and carried out in three replications.

4.3.3 Experimental design and optimization

4.3.3.1 Plackett-Burman design (PBD)

This study used the PBD for screening the significant variables for hydrogen production by the *Klebsiella pneumoniae* TR17. The technique is based on the first order polynomial model (Eq. (10)) as follows:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (10)$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. Table 15 shows the experimental design with eight medium components (at a fixed 20 g/L of glycerol). The medium components were investigated to find the key significant factors influencing the hydrogen production. Based on PBD, each factor was prepared in two levels: -1 for low level and +1 for high level.

Table 15. Levels of the variables and statistical analysis of Plackett-Burman design

Code	Variables (g/L)	Low level (-1)	High level (+)
X_1	K_2HPO_4	1.00	7.00
X_2	KH_2PO_4	1.00	3.00
X_3	NH_4Cl	2.00	6.00
X_4	$MgSO_4 \cdot 7H_2O$	0.10	0.40
X_5	Yeast extract	1.00	4.00
X_6	$FeSO_4$	0.10	0.40
X_7	$CaCl_2$	1.00	3.00
X_8	Trace element	1.00	4.00

4.3.3.2 Central Composite design (CCD)

The selected variables from PBD were further studied for enhanced hydrogen together with ethanol production based on CCD. A 2^3 factorial CCD and six replicates at the center point with the 20 experiments were applied for this study. For statistical calculation, the relation between the coded values and actual values are described by equation (Eq. (11)) as follow:

$$X_i = (A_i - A_0)/\Delta A_i \quad (11)$$

where X_i is the coded value of the variable, A_i is the actual value of variable, A_0 is the actual value of the A_i at the center point, and ΔAi is the step change of variable. The experiments were performed in 60 mL serum bottles containing with 36 mL of working volume. The two responses of the design experiments were the hydrogen yield (mol H_2/mol glycerol) and ethanol yield (mol EtOH/mol glycerol).

In order to predict the optimal point, a second-order polynomial model was fitted to correlate relationship between variables and response (Box *et al*., 1976). Quadratic equation for the variables was shown below in the following equation (Eq. (12)):

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (12)$$

where Y_i is the predicted response; β_0 is the interception coefficient; β_i, β_{ii}, and β_{ij} is the linear, quadratic, and interactive coefficients, respectively. X_i is the independent variables. The statistical software package Design-Expert 7.0.0 (trial version), Stat-Ease. Inc., Minneapolis, MN, USA was used for the regression and graphical analysis of the experimental data obtained.

4.3.4 Analytical methods

The biogas production in the headspace of a serum bottle was measured by a syringe technique described by Owen *et al*. (1979). Hydrogen content of the biogas was determined using an Oldham MX 2100 gas detector (Cambridge Sensotec Ltd., England). Glycerol and organic acids concentration were determined by High Performance Liquid Chromatography (HPLC) (Agilent 1200). The culture medium was centrifuged at 10,000 *g* for 10 min then, the supernatant was filtered through 0.22 μm Nylon membrane filter (Sartorius, German) before injection. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostat column compartment; an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA); and ChemStation Software. Operation conditions were: 20 μl sample volume; 5 mM H_2SO_4 as a mobile phase; flow rate of 0.7 mL/min and a column temperature of 65^0C (Rujananon *et al*., 2011). The hydrogen yield (mol H_2/mol glycerol) was calculated by measuring amount of hydrogen produced (mol) divided by amount of glycerol consumed (mol). The ethanol yield (mol EtOH/mol glycerol) was calculated by measuring amount of ethanol produced (mol) divided by amount of glycerol consumed (mol).

4.4 Results and discussion

4.4.1 Screening of significant factors for hydrogen production

The environmental factors including temperature and pH were previously found to have directly an impact on growth and hydrogen production from

Klebsiella pneumoniae TR17 (Chookaew *et al*., 2012). In this study, medium components influencing fermentative hydrogen production were optimized. Table 16 illustrates the main effect of each variable upon cumulative hydrogen production (*Ps*). KH_2PO_4, NH_4Cl, $MgSO_4 \cdot 7H_2O$, yeast extract, $CaCl_2$, and trace element showed a positive effect for hydrogen production, whereas K_2HPO_4 and $FeSO_4$ had a negative effect on hydrogen production. The positive variables with confidence levels at ≥95% ($P<0.05$) were considered as significant. Among them, KH_2PO_4 and NH_4Cl were significant while $MgSO_4 \cdot 7H_2O$, yeast extract, $CaCl_2$, and trace element were insignificant with positive coefficients for hydrogen production. KH_2PO_4 and NH_4Cl had high impact on hydrogen production (Table 17). The reason may be that KH_2PO_4 act as a role of buffer capacity (Pan *et al*., 2008a), changing concentration of phosphate can strongly affect cell growth as well as hydrogen production (Pan *et al*., 2008b). NH_4Cl act as a nitrogen source for growth (Pan *et al*., 2008b). This result was similar to Liu and Fang, (2007) that NH_4Cl was found to be one of the most important components for hydrogen production from *Klebsiella* sp. Thus, KH_2PO_4 and NH_4Cl were selected for further optimization by CCD.

Table 16. Plackett-Burman design matrix for evaluating factors influencing hydrogen production (P_s) by *Klebsiella pneumoniae* TR17

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	P_s[a] (mLH_2/L)
1	+1	-1	+1	-1	-1	-1	+1	+1	493.57
2	+1	+1	-1	+1	-1	-1	-1	+1	513.04
3	-1	+1	+1	-1	+1	-1	-1	-1	601.50
4	+1	-1	+1	+1	-1	+1	-1	-1	513.04
5	+1	+1	-1	+1	+1	-1	+1	-1	547.25
6	+1	+1	+1	-1	+1	+1	-1	+1	608.97
7	-1	+1	+1	+1	-1	+1	+1	-1	597.76
8	-1	-1	+1	+1	+1	-1	+1	+1	560.40
9	-1	-1	-1	+1	+1	+1	-1	+1	449.89
10	+1	-1	-1	-1	+1	+1	+1	-1	430.95
11	-1	+1	-1	-1	-1	+1	+1	+1	547.25
12	-1	-1	-1	-1	-1	-1	-1	-1	449.37

[a] P_s represented for average cumulative hydrogen production of triplicate experiments

Table 17. Levels of the variables and statistical analysis of Plackett-Burman design

Code	Variables (g/L)	Low level (-1)	High level (+)	Effect (E_{Xi})	*t*-values	Prob > |t|
X_1	K_2HPO_4	1.00	7.00	-8.28	-1.22	0.3098
X_2	KH_2PO_4	1.00	3.00	43.21	6.36	0.0079[a]
X_3	NH_4Cl	2.00	6.00	36.46	5.37	0.0126[a]
X_4	$MgSO_4 \cdot 7H_2O$	0.10	0.40	4.15	0.61	0.5844
X_5	Yeast extract	1.00	4.00	7.08	1.04	0.3738
X_6	$FeSO_4$	0.10	0.40	-1.44	-0.21	0.8458
X_7	$CaCl_2$	1.00	3.00	3.45	0.51	0.6467
X_8	Trace element	1.00	4.00	2.77	0.41	0.7105

R^2 (predict) = 96.04%; R^2 (adjust) = 85.48%, [a]5% significance level

4.4.2 Effect of glycerol, KH_2PO_4, and NH_4Cl concentration on hydrogen yield by *Klebsiella pneumoniae* TR17

In order to evaluate the significant factors from PBD and the combination of the selected factors, the concentrations of glycerol (X_1), KH_2PO_4 (X_2), and NH_4Cl (X_3) were further explored by the CCD. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was obtained to describe the hydrogen yield as a function of the significant variables (Eq. (13)) as follow:

$$Y = 0.019 + 7.696E\text{-}03X_1 + 0.056X_2 + 0.044X_3 + 6.250E\text{-}05X_1X_2 - 4.167E\text{-}05X_1X_3 - 2.083E\text{-}04X_2X_3 - 3.404E\text{-}04X_1^2 - 9.760E\text{-}03X_2^2 - 3.782E\text{-}03X_3^2 \quad (13)$$

Where, Y is the hydrogen yield (mol H_2/mol glycerol), and X_1, X_2, and X_3 are the actual values of glycerol (g/L), KH_2PO_4 (g/L), and NH_4Cl (g/L), respectively. ANOVA of the fitting model (Table 18) showed that the model term was significant ($p<0.01$). The lack of fit was not significant ($p>0.05$). Non-significant lack of fit is indicating that the model equation was sufficient for predicting the hydrogen yield under combination of the variables. The R^2 value of 0.97 indicates the accuracy of the

model and also can explain how much variability of the response variables and their interactions. The R^2 close to 1 indicated the better of the model predicts the response (Acikel *et al*., 2010). All these indicated that Eq. (13) could illustrate the effect of response variables on the hydrogen yield of this study.

ANOVA of the model (Table 19) exhibited the linear effect of glycerol concentration, NH_4Cl, and the quadratic effect of glycerol concentration, KH_2PO_4 and NH_4Cl ($p<0.01$) were significant indicated that these terms had impact on hydrogen yield. In contrast, the linear effect of KH_2PO_4 and the interactive effect between three response variable were not significant ($p>0.05$). Subsequently, the maximum yield of 0.27 was predicted from Eq. (13) at the glycerol, KH_2PO_4, and NH_4Cl concentration of 11.21, 2.84, and 5.66 g/L, respectively. The response surface plots and corresponding contour plots are shown in Fig. 15, which leaving the two variables within the experimental range and keeping one variable constant at its optimum level. The response surface of hydrogen yield displayed a clear peak, indicating that the optimum conditions were fell inside the boundary range. Hydrogen yield increased with increasing concentration of KH_2PO_4 and NH_4Cl to the optimum conditions and decreased thereafter. The results in Fig. 15 indicated that KH_2PO_4 and NH_4Cl concentration had higher profound effect on hydrogen yield than the glycerol concentration. The optimum concentration of NH_4Cl for hydrogen production by *Klebsiella pneumoniae* TR17 (5.66 g/L) was lower than that of *Klebsiella pneumoniae* DSM 2026 (13.80 g/L) (Liu and Fang, 2007). Changing concentration of glycerol did not affect cell growth as well as hydrogen yield. This indicated that methanol and the other impurities in the crude glycerol did not affect *Klebsiella pneumoniae* TR17 for conversion of crude glycerol to hydrogen. In addition, the impurities in the crude glycerol were also reported to have no effect on production of 1,3-propanediol (Sattayasamitsathit *et al*., 2011; Chatzifragkou *et al*., 2010).

Table 18. The Central Composite experimental design with three independent variables

Run	Glycerol (g/L)		KH_2PO_4 (g/L)		NH_4Cl (g/L)		Hydrogen yield (mol H_2/mol glycerol)	Ethanol yield (mol EtOH/ mol glycerol)
	X_1	Code X_1	X_2	Code X_2	X_3	Code X_3		
1	10	-1	1	-1	2	-1	0.18	0.49
2	30	+1	1	+1	2	-1	0.06	0.36
3	10	-1	5	+1	2	-1	0.16	0.54
4	30	+1	5	+1	2	-1	0.07	0.33
5	10	-1	1	-1	8	+1	0.20	0.65
6	30	+1	1	-1	8	+1	0.10	0.39
7	10	-1	5	+1	8	+1	0.20	0.54
8	30	+1	5	+1	8	+1	0.08	0.33
9	5.86	-1.414	3	0	5	0	0.27	0.61
10	34.14	+1.414	3	0	5	0	0.07	0.32
11	20	0	0.172	-1.414	5	0	0.17	0.61
12	20	0	5.828	+1.414	5	0	0.15	0.48
13	20	0	3	0	0.758	-1.414	0.15	0.48
14	20	0	3	0	9.242	+1.414	0.19	0.52
15	20	0	3	0	5	0	0.24	0.56
16	20	0	3	0	5	0	0.24	0.54
17	20	0	3	0	5	0	0.26	0.62
18	20	0	3	0	5	0	0.24	0.57
19	20	0	3	0	5	0	0.23	0.60
20	20	0	3	0	5	0	0.22	0.57

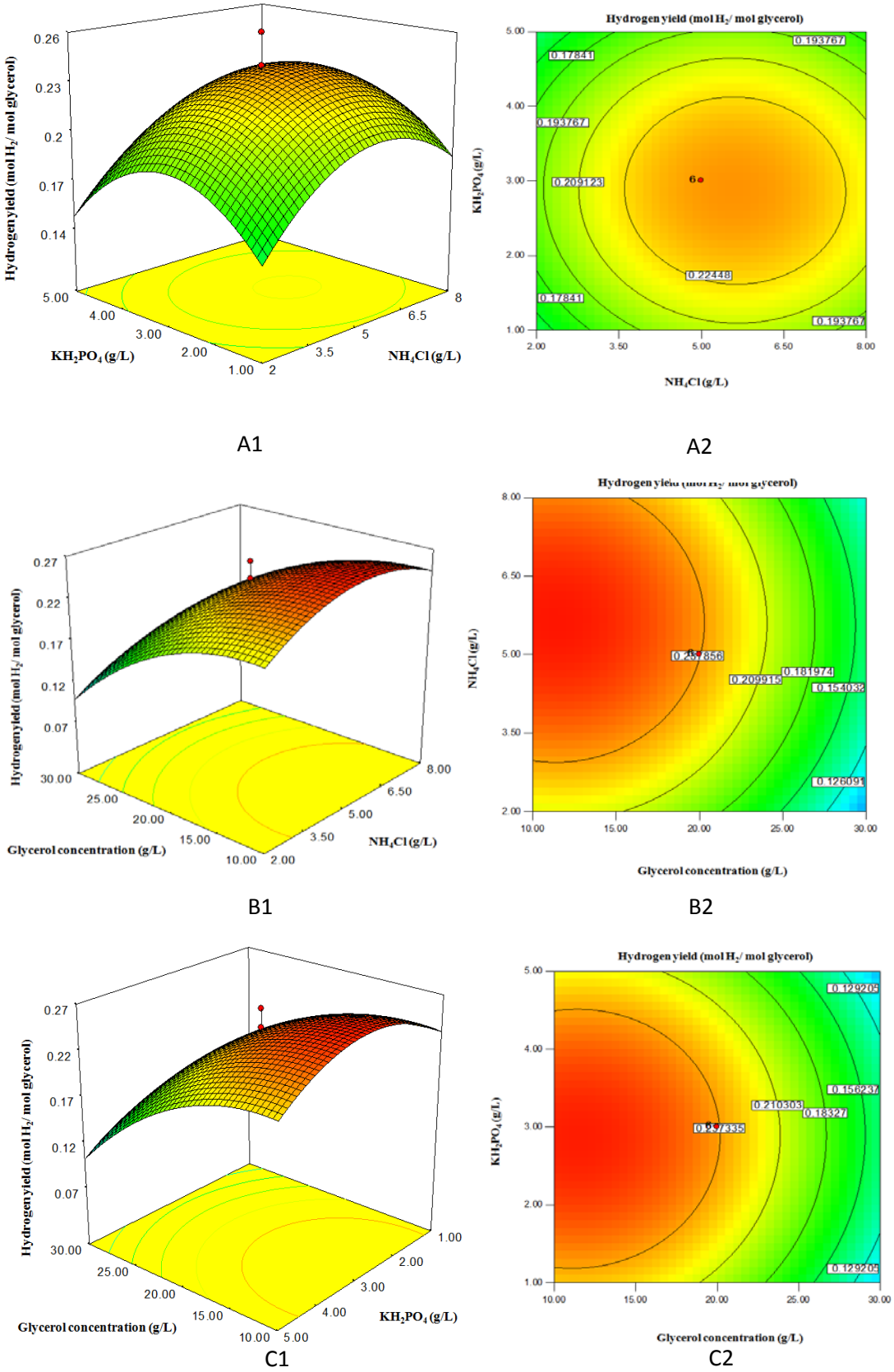

Figure 15. Response surface plot and corresponding contour plot for hydrogen yield

4.4.3 Effect of glycerol, KH_2PO_4, and NH_4Cl concentration on ethanol yield by *Klebsiella pneumoniae* TR17

The influences of key factors from PBD including glycerol (X_1), KH_2PO_4 (X_2) and NH_4Cl (X_3) on ethanol yield were investigated using a CCD. Regression analysis on the experimental data from Table 18 resulted in the second-order polynomial equation (Eq. (14)) as follow:

$$Y = 0.26 + 0.017X_1 + 0.039X_2 + 0.077X_3 - 1.875E\text{-}04X_1X_2 - 5.417E\text{-}04X_1X_3 - 3.958E\text{-}03X_2X_3 - 5.982E\text{-}04X_1^2 - 4.951E\text{-}03X_2^2 - 4.701E\text{-}03X_3^2 \quad (14)$$

where Y is the ethanol yield (mol EtOH/mol glycerol). X_1, X_2, and X_3 are the actual values of glycerol (g/L), KH_2PO_4 (g/L), and NH_4Cl (g/L), respectively. ANOVA of the fitting model (Table 19) showed that the model is significant ($p<0.01$). The lack of fit was not significant ($p>0.05$). The model demonstrated a high determination coefficient ($R^2 = 0.95$), which can explain a statistically significant model. The results of an ANOVA test (Table 19) exhibited that the linear effects of glycerol and KH_2PO_4 concentration, the quadratic effect of glycerol and NH_4Cl concentration on the ethanol yield were significant ($p<0.01$). Hence, the p values of linear effect of NH_4Cl ($p = 0.0372$) and interactive effect between KH_2PO_4 and NH_4Cl ($p = 0.0468$) were also significant ($p<0.05$). The maximum ethanol yield of 0.64 mol EtOH/mol glycerol was obtained from Eq. (14) at the concentration of glycerol, KH_2PO_4, and NH_4Cl were 10.86, 1.00, and of 7.10 g/L, respectively.

The response surface plots and corresponding contour plots described by the regression is shown in Fig. 16, which changing the two variables within the experimental range while keeping one variable constant at its optimum level. Ethanol yield increased with increasing KH_2PO_4 and NH_4Cl to their optimum concentrations and decreased thereafter. Moreover, increasing concentration of KH_2PO_4 and NH_4Cl was more effective than changing glycerol concentration, as shown in Fig. 16.

Table 19. Analysis of variance (ANOVA) for the model regression represents hydrogen yield and ethanol yield

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Model of hydrogen yield (mol H_2/mol glycerol)	0.085	9	9.432E-03	47.30	< 0.0001
X_1	-0.059	1	4.077E-03	-14.57	< 0.0001
X_2	-4.857E-03	1	4.077E-03	-1.19	0.2610
X_3	0.014	1	4.077E-03	3.41	0.0067
X_1X_2	1.250E-03	1	4.992E-03	0.25	0.8074
X_1X_3	-1.250E-03	1	4.992E-03	-0.25	0.8074
X_2X_3	-1.250E-03	1	4.992E-03	-0.25	0.8074
$X1^2$	-0.034	1	4.798E-03	-7.09	< 0.0001
$X2^2$	-0.039	1	4.798E-03	-8.14	< 0.0001
$X3^2$	-0.034	1	4.798E-03	-7.09	< 0.0001
Residual	1.994E-03	10	1.994E-04	-	-
Lack of Fit	1.111E-03	5	2.221E-04	1.26	0.4039
Pure Error	8.833E-04	5	1.767E-04	-	-
Cor Total	0.087	19	-	-	-
Coefficient of determination (R^2) = 0.97					
Model of ethanol yield (mol EtOH/mol glycerol)	0.20	9	0.023	25.71	< 0.0001
X_1	-0.10	1	8.553E-03	-11.89	< 0.0001
X_2	-0.028	1	8.553E-03	-3.25	0.0087
X_3	0.021	1	8.553E-03	2.40	0.0372
X_1X_2	-3.750E-03	1	0.01	-0.36	0.7278
X_1X_3	-0.016	1	0.01	-1.55	0.1519
X_2X_3	-0.024	1	0.01	-2.27	0.0468
$X1^2$	-0.060	1	0.01	-5.94	0.0001
$X2^2$	-0.020	1	0.01	-1.97	0.0775
$X3^2$	-0.042	1	0.01	-4.20	0.0018
Residual	8.778E-03	10	8.778E-04	-	-
Lack of Fit	4.644E-03	5	9.289E-04	1.12	0.4507
Pure Error	4.133E-03	5	8.267E-04	-	-
Cor Total	0.21	19	-	-	-
Coefficient of determination (R^2) = 0.95					

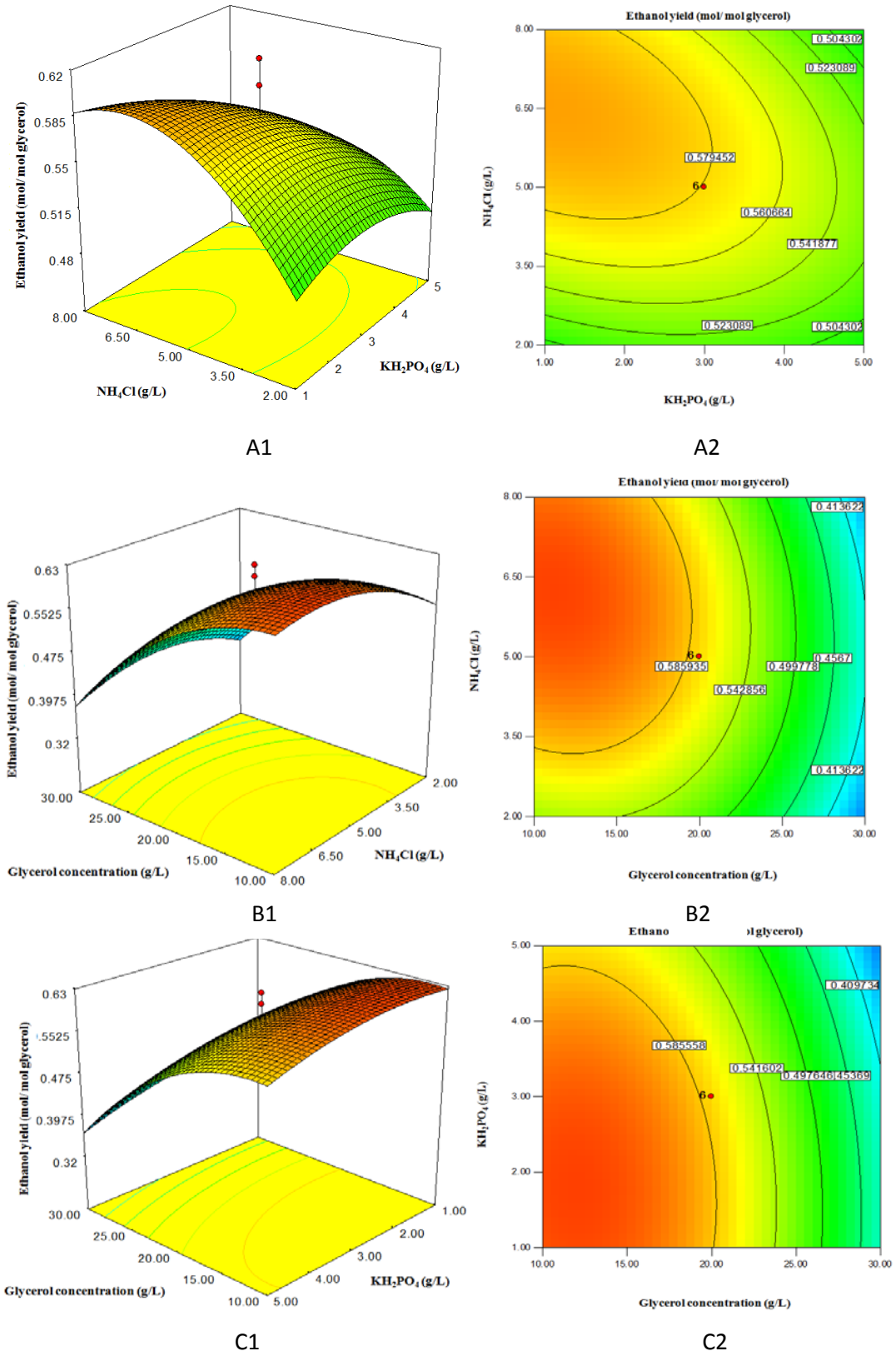

Figure 16. Response surface plot and corresponding contour plot for ethanol yield

4.4.4 Validation of the model

In order to validate the predicted optimal conditions from the models, verification tests were done in batch experiments. The maximum hydrogen yield (run 1) and ethanol yield (run 2) were obtained (Table 20) under the predicted optimal concentration of glycerol, KH_2PO_4, and NH_4Cl from the models. Under the optimal conditions, the model predicted hydrogen yield of 0.27 mol H_2/ mol glycerol and ethanol yield of 0.64 mol EtOH/ mol glycerol. These predicted values were close to the actual values with the hydrogen yield of 0.26 mol H_2/ mol glycerol and ethanol yield of 0.63 mol EtOH/ mol glycerol (Table 20).

The maximum simultaneous hydrogen and ethanol yield was obtained at the optimal conditions of 11.14 g/L glycerol, 2.47 g/L KH_2PO_4, and 6.03 g/L NH_4Cl (run 3). Under the optimal conditions, the predicted hydrogen and ethanol yield were 0.27 mol H_2/ mol glycerol and 0.63 mol EtOH/ mol glycerol, respectively. The actual results obtained were 0.26 mol H_2/ mol glycerol and 0.58 mol EtOH/ mol glycerol respectively, which were closed to the predicted values (Table 20). This confirmed that the CCD was an efficient tool for predicting conditions for a combination of hydrogen and ethanol production from *Klebsiella pneumoniae* TR17.

4.4.5 Comparison of the optimal conditions by RSM obtained in this study and those in other studies

Recently, only one study has done for using RSM to produce hydrogen from crude glycerol by *Klebsiella* sp. (Liu and Fang, 2007). However, there was no report on simultaneous hydrogen and ethanol production from crude glycerol by *Klebsiella* sp. using RSM. Although, Reungsang *et al.* (2013a) reported on simultaneous hydrogen and ethanol production from glycerol by *E. aerogenes* KKU-S1, the hydrogen yield was 0.12 mol H_2/mol glycerol. Moreover, Sittijunda and Reungsang. (2012b) used mixed cultures (hot spring sediment) for hydrogen production from glycerol at thermophilic condition. The hydrogen yield obtained was 0.30 mol H_2/mol glycerol with 1,3-propanediol, ethanol, and acetic acid as the main metabolite products. Zhang *et al*. (2008) reported that the metabolic flux of *Klebsiella* sp. for production of hydrogen and ethanol decreased when increasing the glycerol concentration. Nonetheless, the thermotolerant *Klebsiella pneumoniae* TR17 in this

study can produce simultaneous hydrogen and ethanol from crude glycerol which were superior to the other individual or combination of those two products under mesophilic condition (Hu and Wood, 2010; Wu *et al*., 2011). Moreover, thermotolerant *Klebsiella pneumoniae* TR17 exhibited the ability to produce other high valuable products such as 1,3-propanediol and 2,3-butanediol, compared to the other hydrogen/ethanol producing bacteria such as *Klebsiella pneumoniae* and *Enterobacter aerogenes* (Liu and Fang, 2007; Markov *et al*., 2010; Ito *et al*., 2005). The maximum yield of hydrogen from glycerol by RSM was reported at 0.53 mol H_2/mol glycerol, but no ethanol yield (Liu and Fang, 2007). The combination of hydrogen and ethanol yield obtained in this study was 0.26 mol H_2/mol glycerol and 0.58 mol EtOH/mol glycerol, respectively. Comparison to some hydrogen yields from various substrates (Table 21), the hydrogen yield from crude glycerol (this study) was lower than those from glucose and xylose. However, using crude glycerol as a carbon source gave the advantage of its low cost substrate for hydrogen production.

4.5 Conclusion

Two significant variables affecting hydrogen production including KH_2PO_4 and NH_4CL were selected by the PBD experiments. These selected factors and crude glycerol were optimized by the CCD to maximize simultaneous hydrogen and ethanol yield. The results exhibited that the optimum conditions for maximizing hydrogen yield and ethanol yield were 11.14 g/L glycerol, 2.47 g/L KH_2PO_4, and 6.03 g/L NH_4Cl which exhibited the predicted hydrogen yield of 0.27 mol H_2/mol glycerol and ethanol yield of 0.63 mol EtOH/mol glycerol. The hydrogen yield of 0.26 mol H_2/mol glycerol and ethanol yield of 0.58 mol EtOH/mol glycerol was obtained from verification experiments.

Table 20. Experimental evaluation of the optimal conditions predicted by RSM

Run	Conditions	Response variable					
		Hydrogen yield (mol H_2/ mol glycerol)			Ethanol yield (mol EtOH/ mol glycerol)		
		Predicted	Experimental	Deviation (%)	Predicted	Experimental	Deviation (%)
1. Hydrogen production	11.21 g/L glycerol, 2.84 g/L KH_2PO_4, 5.66 g/L NH_4Cl	0.27	0.26	3.70	-	-	-
2. Ethanol production	10.86 g/L glycerol, 1.00 g/L KH_2PO_4, 7.10 g/L NH_4Cl	-	-	-	0.64	0.63	1.56
3. Simultaneous hydrogen and ethanol production	11.14 g/L glycerol, 2.47 g/L KH_2PO_4, 6.03 g/L NH_4Cl	0.27	0.26	3.70	0.63	0.58	7.93

Table 21. Comparison of hydrogen yield at the optimum conditions by RSM with various substrates and microorganisms

Substrate	Microorganism	Optimum conditions	H_2 yield (mol H_2/mol substrate)	Major valuable byproduct	Reference
Glucose	*Clostridium* sp. Fanp2	23.75 g/L glucose, 0.159 M phosphate buffer and 13.3 mL/L vitamin solution	2.33 mol H_2/mol hexose	acetic, butyric, and propionic acids	Pan *et al*. (2008a)
Glucose	*Ethanoligenens harbinense* B49	14.5 g/L glucose, 180 mg/L Fe^{2+} and 690 mg/L Mg^{2+}	2.20 mol H_2/mol glucose	ethanol and acetate acids	Guo *et al*. (2009)
Xylose	*Enterobacter* sp. CN1	16.15 g/L xylose, 250.17 mg/L $FeSO_4$ and 2.54g/L peptone	2.0 mol H_2/mol xylose	-	Long *et al*. (2010)
Xylose	*T.thermosaccharolyticum* W16	12.24 g/L xylose, 0.170 M phosphate buffer and 4.11 g/L yeast extract	2.37 mol H_2/mol xylose	acetate and butyrate acids	Cao *et al*. (2010)
Glycerol	*K. pneumoniae* DSM 2026	20.4 g/L glycerol, 5.7 g/L KCl, 13.8 g/L NH_4Cl, 1.5 g/L $CaCl_2$ and 3.0 g/L yeast extract	0.53 mol H_2/mol glycerol	1,3-propanediol	Liu and Fang. (2007)
Glycerol	Mixed cultures (hot spring sediment)	20.33 g/L glycerol, 0.16 g/L urea, 3.97 g/L Na2HPO4, and 0.20 mL/L Endo-nutrient	0.30 mol H_2/mol glycerol	1,3-propanediol, ethanol, and acetic acid	Sittijunda and Reungsang. (2012b)
Glycerol	*E. aerogenes* KKU-S1	1.00 g/L yeast extract, 31 g/L glycerol, initial pH 8.13, temp. 37^0C	0.12 mol H_2/mol glycerol	ethanol	Reungsang *et al.* (2013a)
Glycerol	*K. pneumoniae* TR17	11.14 g/L glycerol, 2.47 g/L KH_2PO_4 and 6.03 g/L NH_4Cl	0.26 mol H_2/mol glycerol	1,3-propanediol, 2,3-butanediol, and ethanol	This study

CHAPTER 5

BIOHYDROGEN PRODUCTION AND MICROBIAL COMMUNITY STRUCTURE OF CRUDE GLYCEROL-FEEDING REACTOR USING IMMOBILIZED AND SUSPENDED SYSTEMS BY *KLEBSIELLA PNEUMONIAE* TR17

5.1 Abstract

Biohydrogen production from crude glycerol under non-sterile condition using immobilized-cell and suspended-cell system of *Klebsiella pneumoniae* TR17 was investigated in up-flow anaerobic sludge blanket (UASB) and up-flow anaerobic (UA) reactors, respectively. The reactors were operated under hydraulic retention time (HRT) from 12 to 2 h and glycerol concentration from 10 to 30 g/L. UASB performance at 30 g/L of glycerol and 4 h HRT gave the highest hydrogen production rate of 242.15 mmol H_2/L d with the chemical oxygen demand (COD) removal efficiency in the range of 31.1 to 59.0%. The microbial community analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) indicated that *Klebsiella pneumoniae* TR17 was dominant in all HRT tested in UASB reactor. In contrast, *Klebsiella pneumoniae* TR17 was competed with *Escherichia coli* and *Citrobacter freundii* in UA reactor. Scanning electron microscopy (SEM) illustrated that the morphological properties of *Klebsiella pneumoniae* TR17 were dominated on the immobilized materials.

5.2 Introduction

Hydrogen is one of the potential alternative energy for the future as it is environmentally friendly, clean and sustainable (Cheng *et al*., 2008; Lin and Lay, 2005). Biological hydrogen production by dark fermentation has become interest among the processes for hydrogen production as it can utilize various waste materials, pollution free, and used less energy intensive (Hung *et al*., 2007). Recently, crude glycerol obtained from biodiesel by-products has gained attention as a low-cost feedstock for hydrogen production (Han *et al*., 2012) since fermentation of crude glycerol simultaneous achieved high valuable products such as 1,3-propanediol, 2,3-butanediol, and ethanol, which used in many industrial applications (Hu and Wood, 2010; Wu *et al*., 2008).

The hydrogen production by dark fermentation was investigated using suspended-cell and immobilized-cell system (Zhang *et al*., 2007b). The immobilized-cell systems have been considered as a promising process for efficient hydrogen production (Akutsu *et al*., 2009a). Since this system is more capable of maintaining biomass concentration at low HRT (O-Thong *et al*., 2008b), and handling high organic loading rates (Shida *et al*., 2009). The immobilization systems can be categorized into 3 groups including surface attachment, self-flocculation, and gel entrapment (Lin *et al*., 2009). In particular, surface attachment system is extensively applied for hydrogen production. Recently, various surface attachment systems constructed and successfully conducted to achieve high hydrogen production such as packed-bed reactor (Wu *et al*., 2007), fluidized-bed reactor (Shida *et al*., 2009), fixed-bed reactor (Chang *et al*., 2002), and upflow anaerobic sludge blanket (UASB) reactor (O-Thong *et al*., 2008b). Among these systems, UASB reactor was the most promising for hydrogen production because of their high treatment efficiency and capable to maintain high levels of biomass at a short HRT (Chang and Lin, 2004).

Previous investigation reported that *Klebsiella pneumoniae* TR17 in suspended-cell system can convert crude glycerol into hydrogen with high hydrogen production (27.7 mmol H_2/L) and high valuable metabolite products such as 1,3-propanediol, 2,3-butanediol, and ethanol (Chookaew *et al*., 2012). However, hydrogen production from crude glycerol by *Klebsiella pneumoniae* TR17 in immobilized-cell system under non-sterile condition has not been reported yet. Furthermore, the direct

comparison of suspended-cell and immobilized-cell systems of *Klebsiella* sp. from crude glycerol in term of bacterial community has not been reported either. Analysis of microbial community could provide an accelerated approach for better understanding of bacterial diversity and function (Hniman *et al*., 2011).

In this study, the immobilized-cell system with *Klebsiella pneumoniae* TR17 was examined for hydrogen production in UASB reactor using crude glycerol as substrate in comparison to suspended-cell system in UA reactor. The effects of HRT (12-2 h) and glycerol concentration (10-30 g/L) were investigated. The microbial community structure of immobilized-cell and suspended-cell systems under non sterile condition were analyzed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Moreover, the performance of immobilized *Klebsiella pneumoniae* TR17 was evaluated by scanning electron microscopy (SEM).

5.3 Materials and methods

5.3.1 Crude glycerol

Crude glycerol without pretreatment was obtained from Prince of Songkla University (PSU) Biodiesel Pilot Plant at Faculty of Engineering, PSU, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification using methanol as a reactant and sodium hydroxide as a catalyst. The purity of the crude glycerol was calculated base on its glycerol content and found to be 50%. In addition, the impurities of crude glycerol were mainly potassium and sodium salts (4-5%), methanol (1-3%), non-glycerol organic matter (1.6-7.5%), and water (36%) (Sattayasamitsathit *et al*., 2011).

5.3.2 Microorganisms and culture medium

Klebsiella pneumoniae TR17 (accession number in Genbank AB647144) is a glycerol fermenting microorganism isolated by Chookaew *et al*. (2012). It was grown in a medium containing: 11.14 g/L glycerol, 3.4 g/L K_2HPO_4, 2.47 g/L KH_2PO_4, 6.03 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution.

Trace element solution contained: 0.07 g/L $ZnCl_2$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L $H_3BO_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2$; 0.02 g/L $CuCl_2 \cdot 2H_2O$, 0.025 g/L $NiCl_2 \cdot 6H_2O$, 0.035 g/L $Na_2MoO_4 \cdot 2H_2O$ (Chookaew *et al*., 2014).

5.3.3 Seed sludge

The methanogenic granular sludge were taken from a UASB reactor of seafood wastewater treatment system (Chotiwat Manufacturing Co., Ltd., Songkhla Province, Thailand). The methanogenic granular were autoclaved at 121^0C for 30 min to kill methanogenic activity before used as carriers.

5.3.4 Experimental set-up and operation of UASB and UA reactors

UASB and UA reactors (1.3 L each) with a working volume of 1.0 L were used (Fig. 17 and Fig. 18). The reactors were operated with immobilized-cell system (UASB) by using heat treated methanogenic granular as an immobilized materials and suspended-cell system (UA) as a control. All reactors were placed in a 40^0C water bath with the internal jacket recirculation. For set-up, 440 mL of treated methanogenic granules as carriers were transferred to UASB reactors. The inoculum (OD_{660} = 0.5) of 540 mL of *Klebsiella pneumonaie* TR17 was added into UASB systems (O-Thong *et al*., 2008c). After inoculation, the reactors were operated in batch mode for 24 h and fed with 10 g/L pure glycerol, then re-circulated medium for one week with the HRT of 12 h (flow rate of 1.38 mL/min), in order to enhance bacterial immobilization on the granules before changing to crude glycerol. When the reactors began to reach steady state, the reactors were operated at the HRT of 12, 10, 8, 6, 4, and 2 h, respectively. The steady state of each HRT was established when the value of hydrogen production rate was lower than 5% difference, and the final pH in the effluent was constant (Chang and Lin, 2004). The medium containing glycerol concentration of 10, 20, and 30 g/L with initial pH 8.0 were used in the three UASB and three UA reactors. The reactors were monitored by sampling the effluent every three days for COD and volatile suspended solids (VSS), twice a day for soluble metabolic products and residual glycerol. Gas production and pH were measured daily.

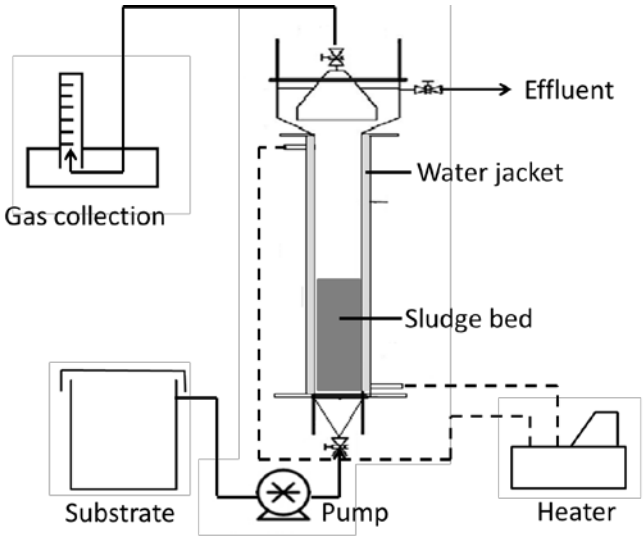

Figure 17. Schematic diagram of a UASB reactor (1.3 L) in dark fermentation for hydrogen production

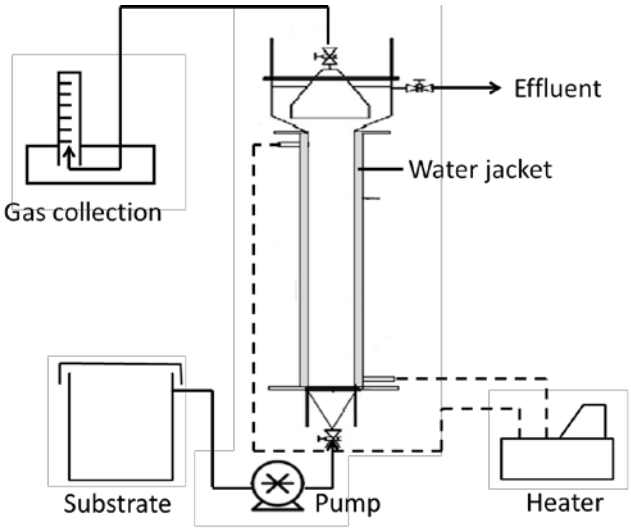

Figure 18. Schematic diagram of a UA reactor (1.3 L) in dark fermentation for hydrogen production

5.3.5 Scanning electron microscopy (SEM)

The granule samples for SEM observation were fixed in a solution of gluteraldehyde for 2.5 h and washed with phosphate buffer solution (PBS) (three times, five minutes each), followed by washing with deionized water. After fixation, each sample was dehydrated through ethanol series solutions: 50%, 60%, 70%, 80%, 90%, and 100% ethanol (two times for each concentration) and then dried to critical

point. Finally, the samples were coated with gold to enhance the electrical conductivity. The granule samples were performed with a SEM (FEI Quanta 400, SEM-Quanta).

5.3.6 Community analysis by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

PCR-DGGE was used to study the microbial community in the UASB and UA reactors. Total genomic DNA of samples were collected at steady state of each HRT and extracted by using a standard bacterial genomic DNA isolation method as described by Hniman *et al*., (2011). The bacterial 16S rDNA was amplified by the first PCR with primer set of 27f (5'-GAGTTTGATCCTTGGCTCAG-3') and 1492r (5'-GAAAGGAGGTGATCCAGCC-3') (Table 22). The amplification condition started with an initial denaturation at 95.5^0C for 5 min followed by 30 cycles of denaturation at 95^0C for 1 min, annealing at 54^0C for 40s, extension at 72^0C for 1 min, with final extension at 72^0C for 10 min. The reactions were subsequently cooled to 4^0C. DGGE analysis was amplified for the second PCR using the primer set of L340f with a GC-clamp at the 50 end (5'-CCTACGGGAGGCAGCAG-3') and K517r (5'-ATTACCGCGGCTGCTGG-3') under conditions of initial denaturation 95^0C for 3 min followed by 30 cycles of three steps: 95^0C for 1 min, 55^0C for 30 s, and 72^0C for 1 min and final extension at 72^0C for 10 min. PCR products were stored at 4^0C 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%. 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 mL of distilled water at 4^0C for 24 h and re-amplified by PCR with the second primer without GC-clamp. After re-amplification, PCR products were purified and sequenced by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were carried out with the BLAST server of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 22. Primers used for PCR-DGGE technique

Group	Primer name	Sequence (5' to 3')	Target
Bacteria	1492r	GAAAGGAGGTGATCCAG CC	16S rDNA
	27f	GAGTTTGATCCTTGGCTCAG	16S rDNA
	K517r	ATTACCGCGGCTGCTGG	V3 region
	L340f-GC	GC clamp-CCTACGGGAGGCAGCAG	V3 region

Source: Hniman *et al*. (2011)

5.3.7 Analytical methods

The volume of gas production was measured using a gas meter with water displacement method (O-Thong *et al*., 2008b). Hydrogen content of the biogas was measured by gas meter (Oldham MX 2100, Cambridge Sensotec Ltd., England) (Noparat *et al*., 2012). Soluble metabolites were analyzed by HPLC. The samples were centrifuged at 10,000 g for 10 min and the supernatants were filtered through a 0.22 µm Nylon membrane filter (Sartorius, German). The HPLC analyses were performed using an Agilent 1200 equipped with an Aminex HPX-87H column, 300 mm x 7.8 mm (Bio-Rad, USA) and a refractive index detector (RID). The conditions for HPLC were as follows: column temperature at 65ºC, 5 mM sulfuric acid as mobile phase at a flow rate of 0.7 mL/min, and an injection volume of 20 µL (Chookaew *et a*l., 2012). VSS, COD, and pH were determined in accordance with the procedures described in the Standard Methods (APHA, 1995). The hydrogen production rate (mmol H_2/L d) was calculated by measuring the total volume of hydrogen produced (mmol H_2/L) divided by the incubation time (d). The glycerol conversion rate was calculated by using the equation: [(I-F)/I] x 100%, in which I and F are the initial and final glycerol concentrations (g/L), respectively (Markov *et al*., 2011).

5.4. Results and discussion

5.4.1 Effects of HRT and glycerol concentration on hydrogen production performance of immobilized-cell and suspended-cell system

Hydrogen production rate (HPR) of *Klebsiella pneumoniae* TR17 in UASB and UA reactors (Fig. 19A and Fig.20A) increased with decreasing HRT from

12 to 2 h. In contrast, HPR increased with increasing glycerol concentration from 10 g/L to 30 g/L, this was occurred only in UASB reactor. The maximum HPR of 242.15 mmol H_2/L d was obtained by UASB reactors at 30 g/L glycerol and 4 h HRT, with a 5 times higher than that obtained from UA reactor (46.35 mmol H_2/L d) at the same condition. However, the maximum HPR (68.98 mmol H_2/L d) of UA reactor was achieved at 10 g/L glycerol with 6 h HRT (Table 23 and Table 24). The result exhibited that the immobilized-cell system could remove higher substrate concentration resulting in higher HPR. Fig. 19B and Fig. 20B showed the specific hydrogen production rate (SHPR) profiles of the UASB and UA reactors. SHPR of the UASB reactor was higher than those obtained from UA reactor as decreasing HRT from 12 h to 2 h. At a fixed glycerol concentration, the SHPR decreased with the increase of glycerol concentration from 10 g/L to 30 g/L. The maximum SHPR (47.74 mmol H_2/g VSS d) was obtained from UASB reactor at 20 g/L glycerol with 4 h HRT, but the SHPR obtained from UA reactor decreased to 6.67 mmol/g VSS d at the same condition (Table 23 and Table 24). It was noticed that SHPR obtained from UASB reactor was higher than that from UA reactor. These results implied that the microorganisms in UASB reactor were more stable under immobilized system and did not washout under low HRT tested. During the operation, the COD removal efficiency of UASB and UA reactors decreased with decreasing HRT from 12 to 2 h (Fig. 19C and Fig. 20C). The maximum COD removal efficiency (60.0%) was obtained from UASB reactor at 20 g/L glycerol with 10 h HRT, but the COD removal efficiency obtained from UA reactor was 33.3% at the same condition. The COD removal efficiency of UASB reactor was higher than that obtained from UA reactor. The reason might be that the immobilized-cell system was superior to suspended-cell system and had higher substrate consumption as well as decreased in COD. At 30 g/L glycerol, COD removal efficiency of UASB reactor decreased from 59.0 to 31.1% as HRT decreased from 12 to 2 h. In contrast, COD removal efficiency of UA reactor decreased from 25.0 to 20.0% at decreasing HRT from 12 h to 2 h (Table 23 and Table 24). This indicated that the immobilized-cell system was capable of assimilating substrate even at high concentration of glycerol (Yang *et al*., 2008).

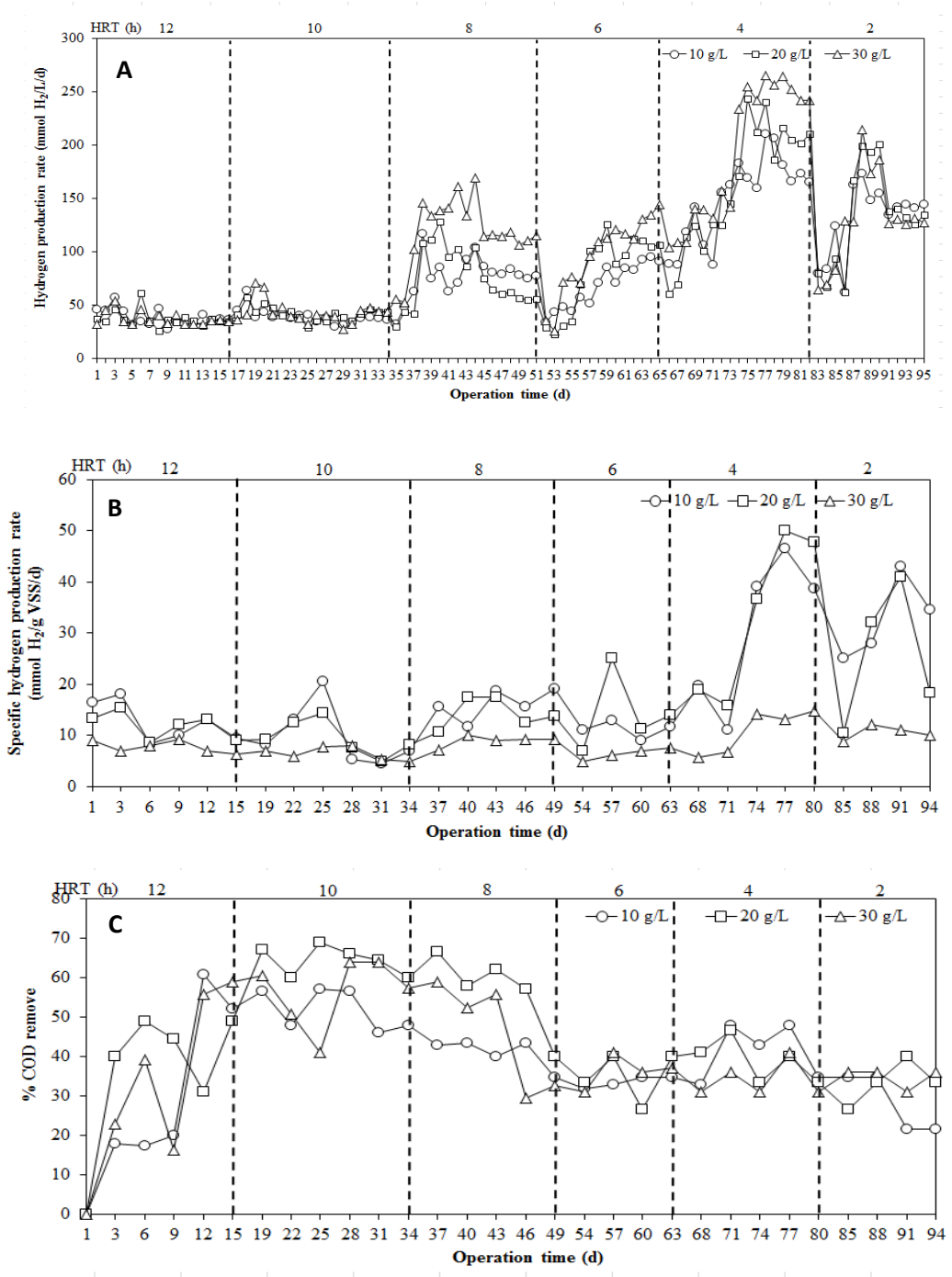

Figure 19. Performance of UASB reactor (A) hydrogen production rate, (B) specific hydrogen production rate, and (C) COD removal efficiency

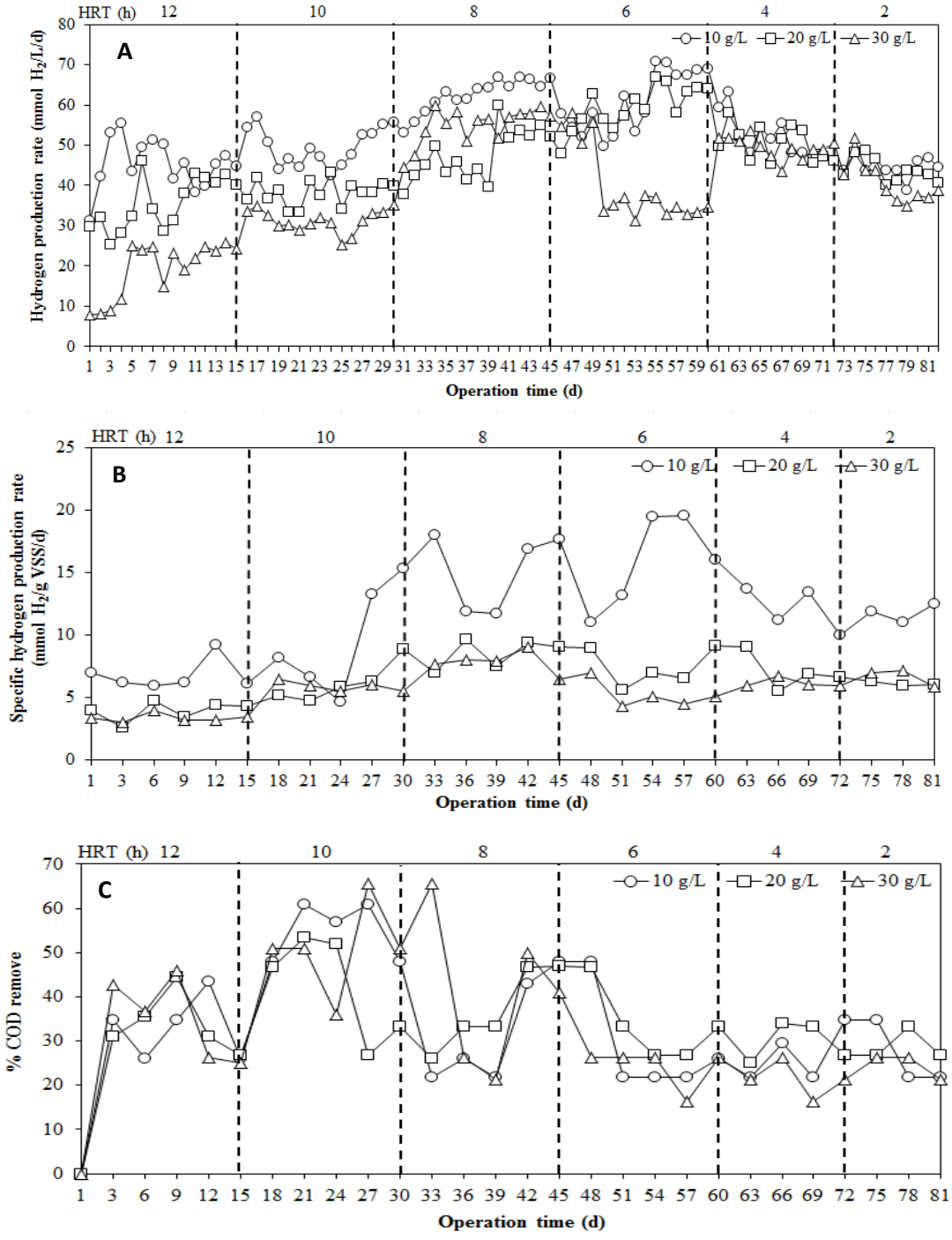

Figure 20. Performance of UA reactor (A) hydrogen production rate, (B) specific hydrogen production rate, and (C) COD removal efficiency

Table 23. Effect of HRT and glycerol concentration on process performance of UASB reactor for hydrogen production

HRT (h)	Hydrogen production rate (mmol H_2/L d)			Specific hydrogen production rate (mmol H_2/g VSS d)			COD removal efficiency (%)		
	Glycerol concentration (g/L)			Glycerol concentration (g/L)			Glycerol concentration (g/L)		
	10	20	30	10	20	30	10	20	30
12	36.5±0.7	35.1±1.2	34.6±0.9	9.4±2.5	8.9±2.9	6.2±0.5	52.2±6.1	48.9±2.5	59.0±2.3
10	36.4±1.3	43.3±1.4	43.4±2.0	6.9±1.8	8.2±2.4	4.9±0.2	47.8±1.2	60.0±3.1	57.4±4.6
8	77.2±1.8	55.6±0.7	115.1±4.5	19.1±2.5	13.7±0.8	9.1±0.1	34.8±6.2	40.0±5.6	32.8±2.3
6	90.8±2.0	106.3±2.7	144.5±7.0	11.7±1.9	13.9±1.9	7.5±0.4	33.0±1.0	26.7±9.4	37.0±6.9
4	165.2±4.3	210.4±4.5	242.2±5.8	38.7±5.6	47.7±1.6	14.8±1.1	35.8±3.2	33.3±4.7	31.1±6.9
2	144.2±1.6	134.9±4.7	127.1±3.2	34.6±5.9	18.3±6.5	10.0±0.7	21.7±1.5	33.0±4.7	36.1±3.5

Table 24. Effect of HRT and glycerol concentration on process performance of UA reactor for hydrogen production

HRT (h)	Hydrogen production rate (mmol H_2/L d)			Specific hydrogen production rate (mmol H_2/g VSS d)			COD removal efficiency (%)		
	Glycerol concentration (g/L)			Glycerol concentration (g/L)			Glycerol concentration (g/L)		
	10	20	30	10	20	30	10	20	30
12	44.9±1.4	40.2±1.4	24.2±1.1	6.1±2.2	4.3±0.1	3.5±0.2	26.1±6.3	26.6±3.1	25.0±6.9
10	55.8±1.5	39.7±1.1	35.1±1.1	15.3±1.5	8.8±1.8	5.5±0.4	47.8±9.3	33.3±4.5	50.8±9.4
8	66.5±1.1	51.9±1.6	57.1±1.4	17.7±0.5	9.0±0.3	6.5±1.7	47.0±9.2	47.0±8.9	40.9±9.9
6	68.9±0.9	64.1±0.5	34.5±0.8	16.0±2.5	9.1±1.8	5.1±0.4	26.0±8.2	33.0±4.7	26.2±6.5
4	46.1±0.8	46.1±0.8	46.4±0.9	11.9±1.3	6.7±0.2	5.9±0.1	34.8±9.1	25.7±4.1	21.3±3.5
2	44.6±1.2	40.6±1.5	38.8±0.9	12.5±1.1	6.0±0.1	5.8±0.9	21.7±2.1	26.0±4.4	20.0±3.7

5.4.2 Microbial community analysis during operation in UASB and UA reactors

Bacterial cultures were collected from each HRT in UA and UASB reactors under steady state conditions at 30 g/L glycerol where the maximum hydrogen production rate was obtained. The PCR-DGGE profiles of microbial community (Fig. 21 and Table 25) were revealed that the sequence affiliation in band on PCR-DGGE (18 bands) could be divided into five groups by family. These were: Enterococcus (band 1, 2, and 3); Enterobacteriaceae (band 4, 5, 6, 8, 10, 12, 13, 14, 16, 17, and 18); Clostridiaceae (band 11 and 15); Veillonellaceae (band 7), and Thermoanaerobacteriaceae (band 9). The PCR-DGGE band of 1, 2, and 3 were related to lactic acid bacteria which could decrease hydrogen production in the system because it released bacteriocins and had inhibitory effect on hydrogen-producing bacteria (Saraphirom and Reungsang, 2011). This was coincided with Reungsang *et al*. (2013c) who reported that *Enterococcus* sp. was capable of producing acetic acid via acetogenesis pathway which contributed to a low hydrogen production.

Further observations of the PCR-DGGE profiles indicated that band number 12 and 13 related to *Citrobacter freundii*. *Citrobacter* sp. is a facultative anaerobic, Gram-negative bacilli, grows in a temperature range of 25-40^{0}C, pH range of 4-9 (Oh *et al*., 2003). *Citrobacter* sp. has been reported for production of 1,3-propanediol from glycerol. Metsoviti *et al*. (2013) reported that *Citrobacter freundii* FMCC-B294 can produce 1,3-propanediol in fed-batch fermentation and achieved 1,3-propanediol production of 68.1 g/L with a volumetric productivity of 0.79 g/L/h. Moreover, this strain was tested for production of 1,3-propanediol under non-sterile conditions. The results found that 1,3-propanediol of 66.3 g/L was obtained with glycerol concentration of 176 g/L. In addition, Maru *et al*. (2013) reported that *Citrobacter werkmanii* DSM 17579 was able to use glycerol as substrate for production of 1,3-propanediol. The 1,3-propanediol yield of 0.62 mol/mol glycerol was achieved in fed-batch fermentation. Recently, *Citrobacter freundii* H3 was reported to produce hydrogen from glycerol with a yield of 0.94 mol/mol glycerol. However, this *Citrobacter* sp. have not been reported as hydrogen and 1,3-propanediol producer from glycerol at 40^{0}C.

Among all species from the PCR-DGGE profile, *Clostridium* sp. (band

11 and 15) could be strongly detected mostly in UASB reactor. *Clostridium* sp. seems to prefer the immobilized-cell system and could not compete with other strains presented in the UA reactor. In spite of the presence of uncultured *Clostridium* sp., the specific dominant strain of *Clostridium* could not be determined. Papanikolaou *et al*. (2004) reported that *Clostridium pasteurianum* and *Clostridium butyricum* were able to use glycerol as substrate for production of 1,3-propanediol. However, *Clostridium pasteurianum* and *Clostridium butyricum* are mesophilic bacteria and have not been reported as a hydrogen producer from crude glycerol (Selembo *et al*., 2009). According to Yossan *et al*. (2012) *Clostridium paraputrificum* has been reported as a hydrogen producer at widely temperature from 25 to 55^{o}C. Evvyernie *et al*. (2000) reported that *Clostridium paraputrificum* M-21 was capable of fermenting chitinous material and starch wastes to hydrogen but not for glycerol. Bowman *et al*. (2010) reported that *Clostridium hydrogeniformans* can grow at 15-40^{0}C, with a pH in the range 5.0-10.0, and capable of fermenting cellobiose, fructose, galactose (weak), glucose, maltose and salicin but not for glycerol. Thus, *Clostridium* sp. was not the dominant for hydrogen producer in UASB reactor.

Thermoanaerobacterium thermosaccharolyticum (band 9) was found only in UASB reactor at 10 h and 12 h HRT. This bacterial strain was considered to be thermophilic hydrogen producer (O-Thong *et al*., 2008a,c). In this case, *Thermoanaerobacterium* sp. was not dominant species for hydrogen production since *Thermoanaerobacterium* sp. has not been reported to consume glycerol for hydrogen production at 40^{0}C. Nonetheless, this strain was not observed when decreasing HRT lower than 10 h. However, Sittijunda and Reungsang (2012b) reported that *Thermoanaerobacterium thermosacharolyticum* was the main hydrogen producer present in the fermentation system using glycerol as substrate at thermophilic condition (55^{0}C).

Escherichia coli (band 8) were present at all HRT of UA reactors and seem to prefer in the suspended-cell system. It might not be able to compete with other strains presented in the UASB reactor. Glycerol fermentation performed by *Escherichia coli* can produce ethanol and 1,3-propanediol. Tang *et al*. (2009) reported that *Escherichia coli* can produce 1,3-propanediol and achieved 1,3-propanediol yield of 1.09 mol/mol glycerol. Moreover, Yazdani and Gonzalez, (2008) used engineered

Escherichia coli SY03 for production of ethanol and achieved ethanol yield of 1.0 mol/mol glycerol. In addition, *Escherichia coli* was reported to produce hydrogen. However, it has a very low specific growth rate in glycerol (Hu and Wu, 2010).

Veillonella parvula (band 7) were detected at all HRT of UASB reactors and up to 8 h HRT of UA reactor. *Veillonella parvula* is a gram negative, strict anaerobic, non spore forming bacterium. *Veillonella parvula* was not reported to utilize carbohydrates, but can use organic acids as a substrate (Luppens *et al*., 2008). It has been reported that *Veillonella parvula* can form biofilm and able to aggregate with other organisms (Huder and Dimroth, 1993). Thus, this aggregation by biofilm from *Veillonella parvula* might help the *Klebsiella pneumoniae* TR17 would not be washed out at lower HRTs as well as presence throughout the UASB reactor at all HRT tested. However, this strain has not been reported as a hydrogen producer, although it could grow at 40^0C (Fisher and Denison, 1996).

Klebsiella sp. (band 4, 5, 6, and 10) was the dominant species at all HRT in UA and UASB reactors. The affiliates of *Klebsiella* sp. was identified as the major species for hydrogen production especially at low HRT of UASB reactor (2-4 h HRT). The bands intensity of *Klebsiella pneumoniae* TR17 (band 14, 16, and 17) were present at all HRT of UASB reactor but showed stronger intensity at high HRT (8-12 h) than at lower HRT for UA reactor. In addition, decreasing HRT from 12 h to 2 h resulted in higher hydrogen production rate in UASB reactor. The results demonstrated the effect of augmented *Klebsiella pneumoniae* TR17 to compete with other microorganisms under non-sterile condition both in UASB and UA reactors. Liu and Fang (2007) reported that *Klebsiella pneumoniae* DSM 2026 was able to use glycerol as substrate for production of hydrogen. The hydrogen yield under the optimal medium conditions in a 5 L stirred tank bioreactor was 0.53 mol H_2/mol glycerol. Furthermore, 1,3-propanediol was also obtained from the liquid medium as a by-product. In addition, Wu *et al*. (2011) reported that *Klebsiella* sp. HE1 can produce hydrogen from glycerol and achieved hydrogen yield of 0.34 mol/mol glycerol with valuable metabolite products such as ethanol, 1,3-propanediol, and 2,3-butanediol. This result was corresponded to the production of 1,3-propanediol, 2,3-butanediol, and ethanol which were the main soluble metabolites produced from *Klebsiella pneumoniae* TR17 (data shown in section 6.3).

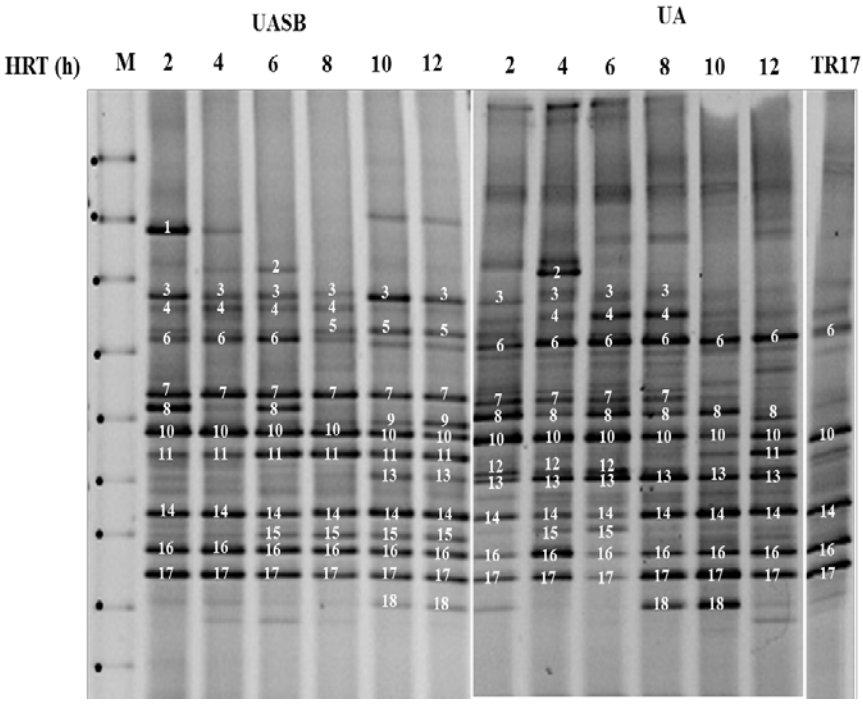

Figure 21. Bacterial community profile determined with PCR-DGGE of partial 16S rRNA genes fragments from enriched cultures of 30 g/L glycerol in UASB and UA reactor operated under different HRT. Lanes: M, PCR-DGGE marker; TR17, *Klebsiella pneumoniae* TR17. Numbers indicated DNA bands were affiliated to microorganisms given in Table 25

Table 25. PCR-DGGE analysis results for microbial community from hydrogen production in UASB and UA reactor

Band No.	Closest relatives	Identity (%)	Accession No.
1	*Enterococcus casseliflavus*	93	GU427867.1
2	*Enterococcus faecium*	97	AB627844.1
3	*Enterococcus faecalis*	90	GU417202.1
4	Uncultured *Klebsiella* sp.	93	GQ471864.1
5	Uncultured *Klebsiella* sp.	82	JX178708.1
6	Uncultured *Klebsiella* sp.	93	GQ471879.1
7	*Veillonella parvula*	90	GU406800.1
8	*Escherichia coli*	88	JF919875.1
9	*Thermoanaerobacterium thermosaccharolyticum*	91	FR870449.1
10	*Klebsiella pneumoniae*	97	JF919926.1
11	Uncultured *Clostridium* sp.	97	JN650257.1
12	*Citrobacter freundii*	99	HM629465.1
13	*Citrobacter freundii*	99	JF935132.1
14	*Klebsiella pneumoniae*	100	JF919904.1
15	Uncultured *Clostridium* sp.	94	JN650257.1
16	*Klebsiella pneumoniae*	98	JF919926.1
17	*Klebsiella pneumoniae*	100	JF919926.1
18	Uncultured *Raoultella* sp.	97	EU919218.1

5.4.3 Morphology of hydrogen producing granules

Fig. 22 shows the SEM image of (A) a methanogenic granule before use as a carrier and (B) an enriched granules with hydrogen producing microorganisms obtained from 30 g/L of glycerol concentration in UASB reactor after the end of operation time (130 days). The granules had the diameters between 0.5-4.0 mm and their surfaces were covered mainly by rod shaped microorganisms similar to the shape of *Klebsiella pneumoniae* TR17 that was dominant. *Klebsiella pneumoniae* TR17 had good agglutinating into granules as well as allowing efficient and stable

hydrogen production even at low HRT of UASB reactor compared to UA reactor. Therefore, using heat pretreated methanogenic granules as a carrier was an effective for enhancing hydrogen production and the immobilized cells were able to consume more glycerol concentration compared to suspended-cell system.

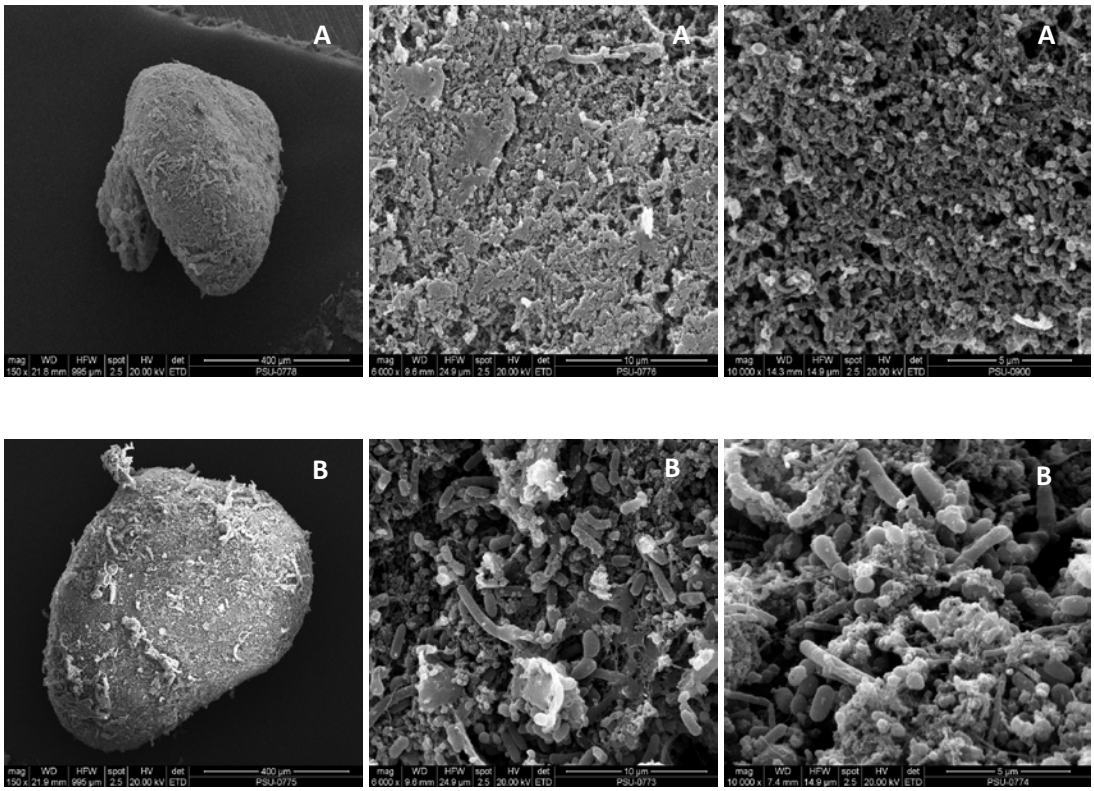

Figure 22. SEM images of (A) a methanogenic granule before use as a carrier, (B) a hydrogen-producing granule obtained from 30 g/L of glycerol concentration in UASB reactor after the end of experimental period

5.5 Conclusion

Klebsiella pneumoniae TR17 in the immobilized-cell was superior to suspended-cell system for effective hydrogen production from crude glycerol under non-sterile condition. The highest HPR (242.15 mmol H_2/L d) was obtained at 30 g/L of glycerol at 4 h HRT in UASB reactor with 5 folds higher than that obtained from UA reactor under the same conditions. The microbial community determined by PCR-DGGE analysis indicated that *Klebsiella pneumoniae* TR17 was dominant in all HRT tested in UASB reactor. However, *Klebsiella pneumoniae* TR17 present in UA reactor had to compete with *Escherichia coli* and *Citrobacter freundii*. Results proved that *Klebsiella pneumoniae* TR17 remained dominant in the immobilized-cell system even at low HRT compared with suspended-cell system. SEM showed that the surfaces of the immobilized materials were surrounded by the rod shape of *Klebsiella pneumoniae* TR17 as the strain was inoculated for hydrogen production.

CHAPTER 6

BIOHYDROGEN PRODUCTION AND BACTERIAL QUANTIFICATION FROM CRUDE GLYCEROL BY IMMOBILIZED *KLEBSIELLA PNEUMONIAE* TR17 IN UP-FLOW ANAEROBIC SLUDGE BLANKET (UASB) REACTOR UNDER NON STERILE CONDITION

6.1 Abstract

Biohydrogen production from glycerol by immobilized *Klebsiella pneumoniae* TR17 in UASB reactor indicated that decreasing the HRT led to an increase in hydrogen yield (HY). The highest HY (44.27 mmol H_2/g glycerol consumed) were obtained at 4 h HRT with the glycerol concentration of 10 g/L. The main soluble metabolites were 1,3 propanediol which implied that *Klebsiella* sp. was dominant among other microorganisms during the operation. Fluorescence *in situ* hybridization (FISH) results revealed that the microbial community was dominated by *Klebsiella* sp. with 56.96, 59.45, and 63.47% of total DAPI binding cells, at the glycerol concentration of 10, 20, and 30 g/L, respectively.

6.2 Introduction

Hydrogen has a potential as fuel for the future because it is clean and has high energy yield compared with hydrocarbon fuels (Jung *et al*., 2010). Among the biological method of hydrogen production, dark fermentation has various advantages such as it can use a wide range of substrates and no light requirement for operation. Thus, this method is relatively energy saving process and environmentally-friendly (Yossan *et al*., 2012; Chairattanamanokorn *et al*., 2009).

Crude glycerol is a by-product obtained from biodiesel. Since biodiesel has a sudden increase in the production as a renewable energy, a rapid enlargement in crude glycerol is being observed (Amaral *et al*., 2009). Crude glycerol has high impurities as well as too costly and energy intensive to be disposed (Saenge *et al*., 2011). In order to make biodiesel sustainable for using, the conversion of crude glycerol to a variety of value added products have been studied such as hydrogen (Markov *et al*., 2011), 2,3-butanediol (Petrov and Petrova, 2009), ethanol (Choi *et al*., 2011), and 1,3-propanediol (Mu *et al*., 2006a). One attractive approach for conversion of crude glycerol could be production of hydrogen as an alternative energy for the future.

Studies on hydrogen production from dark fermentation have been focused on pure culture (Lee *et al*., 2011), in which the genus Clostridium has been most studied from various waste materials such as food waste (Kim *et al*., 2008), palm oil mill effluent (Chong *et al*., 2009a), and molasses (Wang and Jin, 2009). However, the Clostridium is obligate anaerobe, requires strictly anaerobic condition as well as difficult for industrial production (Marone *et al*., 2012). Thus, the use of facultative bacteria to convert crude glycerol to hydrogen by dark fermentation is more appropriate for hydrogen production.

Klebsiella sp. was reported to convert crude glycerol to hydrogen at a high rate and yield (Liu and Fang, 2007; Chen *et al*., 2006b), easy to grow and also achieved various valuable by-products such as 1,3-propanediol, 2,3-butanediol (Sattayasamitsathit *et al*., 2011; Niu *et al*., 2010), and ethanol (Chookaew *et al*., 2014). Hydrogen production under non sterile condition was more attractive to minimize the production cost as well as possible for industrial application. The microorganisms in the reactor during operation under non sterile condition could be quantified in order to determine the role of the microorganisms present in the system.

UASB is an effective anaerobic process in wastewater treatment system and also achieved high organic removal efficiency (Buzzini *et al*., 2006; Khemkhao *et al*., 2012; Lopes *et al*., 2008). It was used to produce hydrogen from various substrates such as starch-wastewater (Akutsu *et al*., 2009a), desugared molasses (Kongjan *et al*., 2011), coffee drink manufacturing wastewater (Jung *et al*., 2010), and cheese whey (Castello *et al*., 2009). However, there has not been reported of hydrogen production from crude glycerol under UASB operation.

The objective of this work is to investigate the hydrogen production in a UASB reactor using crude glycerol as a substrate by *Klebsiella pneumoniae* TR17 immobilized on heat-pretreated methanogenic granules under non sterile condition. Consequently, the microbial communities in UASB reactor were analyzed by FISH in order to evaluate the role of immobilized *Klebsiella* sp. in the fermentation system.

6.3 Materials and methods

6.3.1 Crude glycerol

Crude glycerol without pretreatment was obtained from Prince of Songkla University (PSU) Biodiesel Pilot Plant at Faculty of Engineering, PSU, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification using methanol as a reactant and sodium hydroxide as a catalyst. The purity of the crude glycerol was calculated base on its glycerol content and found to be 50%. In addition, the impurities of crude glycerol were mainly potassium and sodium salts (4-5%), methanol (1-3%), non-glycerol organic matter (1.6-7.5%), and water (36%) (Sattayasamitsathit *et al*., 2011).

6.3.2 Microorganisms and culture medium

Klebsiella pneumoniae TR17 (accession number in Genbank AB647144) is a glycerol fermenting microorganism isolated by Chookaew *et al*. (2012). It was grown in a medium containing: 11.14 g/L glycerol, 3.4 g/L K_2HPO_4, 2.47 g/L KH_2PO_4, 6.03 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution. Trace element solution contained: 0.07 g/L $ZnCl_2$, 0.1 g/L $MnCl_2 \cdot 4H_2O$, 0.06 g/L

$H_3BO_3 \cdot 6H_2O$, 0.2 g/L $CoCl_2$; 0.02 g/L $CuCl_2 \cdot 2H_2O$, 0.025 g/L $NiCl_2 \cdot 6H_2O$, 0.035 g/L $Na_2MoO_4 \cdot 2H_2O$ (Chookaew *et al*., 2014).

6.3.3 Seed sludge

The methanogenic granular sludge were taken from a UASB reactor of seafood wastewater treatment system (Chotiwat Manufacturing Co., Ltd., Songkhla Province, Thailand). The methanogenic granular were autoclaved at 121^0C for 30 min to kill methanogenic activity before used as carriers.

6.3.4 Experimental set-up and operation of UASB reactors

UASB reactors (1.3 L each) with a working volume of 1.0 L were used (as shown in section 5.3.4). The reactors were operated with immobilized-cell system (UASB) by using heat treated methanogenic granular as an immobilized material. All reactors were placed in a 40^0C water bath with the internal jacket recirculation. For set-up, 440 mL of treated methanogenic granules as carriers were transferred to UASB reactors. The inoculum (OD_{660} = 0.5) of 540 mL of *Klebsiella pneumonaie* TR17 was added into UASB system (O-Thong *et al*., 2008c). After inoculation, the reactors were operated in batch mode for 24 h and fed with 10 g/L pure glycerol, then re-circulated medium for one week with the HRT of 12 h (flow rate of 1.38 mL/min), in order to enhance bacterial immobilization on the granules before changing to crude glycerol. When the reactors began to reach steady state, the reactors were operated at the HRT of 12, 10, 8, 6, 4, and 2 h, respectively. The steady state of each HRT was established when the value of hydrogen production rate was lower than 5%, and the final pH in the effluent was constant (Chang and Lin, 2004). The medium containing glycerol concentration of 10, 20, and 30 g/L with initial pH 8.0 were used in the three UASB. The reactors were monitored by sampling the effluent every three days for COD and volatile suspended solids (VSS), twice a day for soluble metabolic products and residual glycerol. Gas production and pH were measured daily.

6.3.5 Fluorescence *in situ* hybridization (FISH)

FISH technique was selected for detection and quantification of the selected thermotolerant bacteria immobilized on heat-pretreated methanogenic

granules. Table 26 shows the list of the specific oligonucleotide probes and hybridization conditions used in this study. Probes labeled with the sulfoindocyanine dyes Cy3, EUB338 (Tsuneda *et al*., 2003) and Enterbact D (Ootsubo *et al*., 2002), was used for the hybridization to target Eubacteria and *Klebsiella* sp., respectively. Fixation of samples started by adding 375 mL of sludge samples in 1,125 mL of 4% (v/v) paraformaldehyde (pH 7.2). Then, the samples were mixed and kept at 4^0C for 4 h before centrifuged at 10,000 g for 5 min, the supernatants were expelled and the cells were washed twice in phosphate buffer saline (PBS). The cell pellets were re-suspended in 150 mL of filter sterilized PBS, then 150 mL of filter sterilized 96% ethanol was added. The samples were mixed carefully and stored at -20^0C (Ogue-Bon *et al*., 2010). The fixed samples were further processed for FISH following the procedure as described by Amann *et al.* (1995). Quantitative determination was analyzed by counting 25 microscopic fields of view per sample. Dye 4′,6′-diamidino-2-phenilindol (DAPI) stain was used to count the total cell quantification (total DAPI binding cells). The quantitative of each bacteria group was counted as the ratio of area covered by samples stained of probes and DAPI to the area covered by DAPI stained samples alone. Slides were viewed under a microscope (Nikon Corporation, Japan) (Kongjan *et al*., 2011).

Table 26. Oligonucleotide probes used for FISH technique

Probe	Specificity	Sequence (5' to 3')	FA (%)[a]	NaCl (M)[b]	Reference
EUB338	Eubacteria	GCTGCCTCCCGTAGGAGT	35	0.08	Tsuneda *et al*. (2003)
Enterbact D	*Klebsiella* sp.	TGCTCTCGCGAGGTCGCTTCTCTT	0	0.90	Ootsubo *et al*. (2002)

[a]Formamide concentration in the hybridization buffer

[b]Sodium chloride concentration in the washing buffer

6.3.6 Analytical methods

The volume of gas production was measured using a gas meter with water displacement method (O-Thong *et al*., 2008b). Hydrogen content of the biogas was measured by gas meter (Oldham MX 2100, Cambridge Sensotec Ltd., England) (Noparat *et al*., 2012). Soluble metabolites were analyzed by HPLC. The samples were centrifuged at 10,000 g for 10 min and the supernatants were filtered through a 0.22 µm Nylon membrane filter (Sartorius, German). The HPLC analyses were performed using an Agilent 1200 equipped with an Aminex HPX-87H column, 300 mm x 7.8 mm (Bio-Rad, USA) and a refractive index detector (RID). The conditions for HPLC were as follows: column temperature at 65ºC, 5 mM sulfuric acid as mobile phase at a flow rate of 0.7 mL/min, and an injection volume of 20 µL (Chookaew *et a*l., 2012). VSS, COD, and pH were determined in accordance with the procedures described in the Standard Methods (APHA, 1995). The hydrogen yield (mmol H_2/g glycerol consumed) was calculated by measuring the total volume of hydrogen produced (mmol H_2/L) divided by the glycerol consumed (g/L) and hydrogen yield in mol H_2/mol glycerol was calculated by measuring amount of hydrogen produced (mol) divided by amount of glycerol consumed (mol) (Chen *et al*., 2007). The glycerol conversion rate was calculated by using the equation: [(I-F)/I] x 100%, in which I and F are the initial and final glycerol concentrations (g/L), respectively (Markov *et al*., 2011).

6.4 Results and discussion

6.4.1 Effect of HRT and glycerol concentration on hydrogen yield (HY) and hydrogen content (HC) of immobilized *Klebsiella pneumoniae* TR17 in UASB reactors

The variation of HRT and glycerol concentration led to the variation in HY and HC (Fig. 23). Increasing of glycerol concentrations (10, 20, and 30 g/L) resulted in the decrease of HY (44.27, 29.85, and 29.00 mmol H_2/g glycerol consumed, respectively) at 4 h HRT. It should be noted that the decline of HY at 2 h HRT can possibly be attributed to the too low mixing and poor contact of glycerol with the microorganisms (Liu *et al*., 2011). The HC increased when decreased the HRT from 12 to 2 h. However, increasing in glycerol concentration (10-30 g/L) had

no effect on HC (42-46%) at 4 h HRT. The result of HC in this study was similar to that of Zhang *et al*. (2007b) and Lin *et al*. (2011).

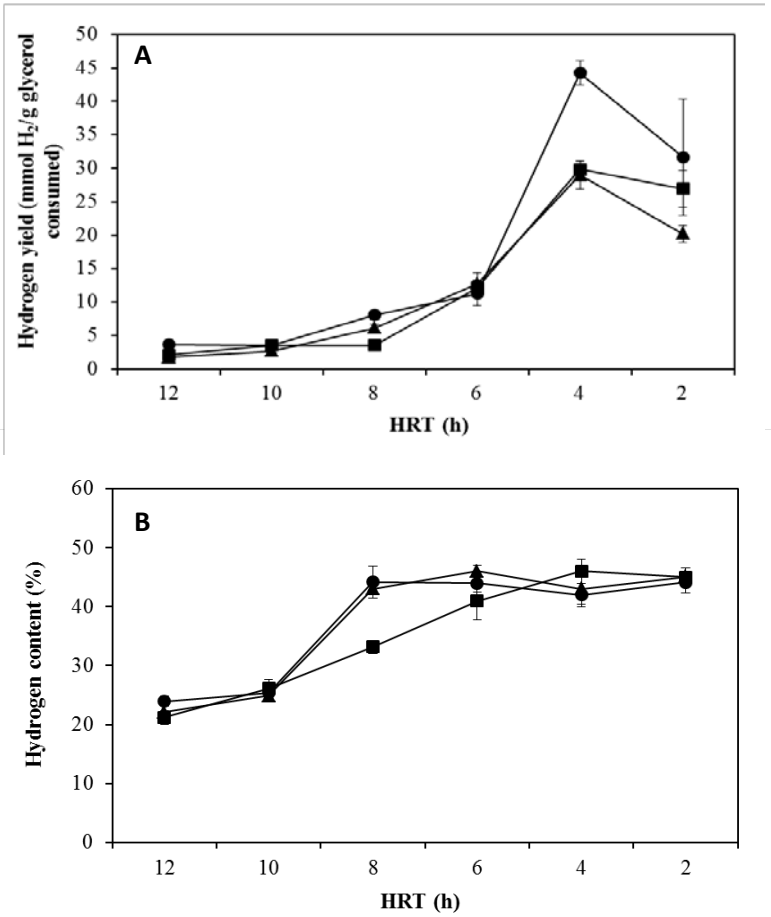

Figure 23. Variations in (A) HY and (B) HC with respect to different combination of HRT and glycerol concentration in the UASB reactors. In each panel, symbols are ● for 10 g/L, ■ for 20 g/L, and ▲ for 30 g/L

6.4.2 Effect of HRT and glycerol concentration on biomass concentration (VSS) and glycerol conversion rate of immobilized *Klebsiella pneumoniae* TR17 in UASB reactors

The optimum HRT for growth was at 6 h HRT for glycerol concentrations of 10 and 30 g/L while it was at 4 h HRT for 20 g/L of glycerol concentration (Fig. 24A). The maximum growth increased with the increase of glycerol concentration, giving the biomass concentrations of 7.89, 9.15, and 17.47 g VSS/L at 10, 20, and 30 g/L glycerol concentrations, respectively. Glycerol

conversion rate tend to decrease with the decrease of HRT. Therefore, the maximum glycerol conversion rate was obtained at 12 h HRT with the values of 97.34, 79.88, and 64.65% at 10, 20, and 30 g/L glycerol concentrations, respectively. On the contrary, increase the glycerol concentration from 10 to 30 g/L caused the decrease in the glycerol conversion rate from 46.94 to 32.09%, at 4 h HRT (Fig. 24B). It is likely that a shorter HRT led to a faster substrate conversion rate, resulting in the decrease of substrate utilization efficiency (Hung *et al*., 2007). During fermentation of glycerol to hydrogen, the increase of final pH with the decrease of HRT was observed (Fig. 24C). *Klebsiella* sp. TR17 utilizes glycerol and produce alcohol and organic acids. The oxidative pathway of glycerol provides energy and reducing equivalents (NADH). The most energy-advantageous metabolite products of this pathway are acetic acid, as its formation is connected with NAD+ regeneration and coenzymeA recycling. However, the high acetic acids secretion leads to the pH drop and the cell growth inhibition by the accumulation of its undissociated form (Petrov and Petrova, 2009).

6.4.3 Effect of HRT and glycerol concentration on soluble metabolic products of immobilized *Klebsiella pneumoniae* TR17 in UASB reactors

During UASB operation, *Klebsiella* sp. TR17 not only produced hydrogen but also succinic acid, acetic acid, 1,3-propanediol, 2,3-butanediol, and ethanol (Fig. 25). The maximum 1,3-propanediol, as the main soluble metabolic products, was achieved at 12 h HRT for all glycerol concentrations tested. The maximum 1,3-propanediol decreased with the decrease of HRT, and gave the values of 4.37, 9.00, and 7.52 g/L at 10, 20, and 30 g/L glycerol concentrations, respectively. 1,3-Propanediol is considered to be favorable metabolites for *Klebsiella* sp. TR17 (Chookaew *et al*, 2012). Thus, the presence of high concentration of 1,3-propanediol in this study could imply the dominance of *Klebsiella* sp. TR17 that successfully immobilized on heat treated anaerobic sludge granules in the UASB reactor and played important role for hydrogen production from glycerol. Decreasing HRT also led to a low ethanol concentration as the hydrogen yield increased in all glycerol concentrations tested. Ethanol may cause imbalances of NAD/NADH and alter movement of protons in and out of the cell membrane as well as their abilities to produce hydrogen (Wu *et al*., 2006). This result coincided with Zhang *et al*. (2007a)

who reported that the concentration of ethanol decreased when the HRT decreased from 2 to 0.5 h whereas the hydrogen production increased.

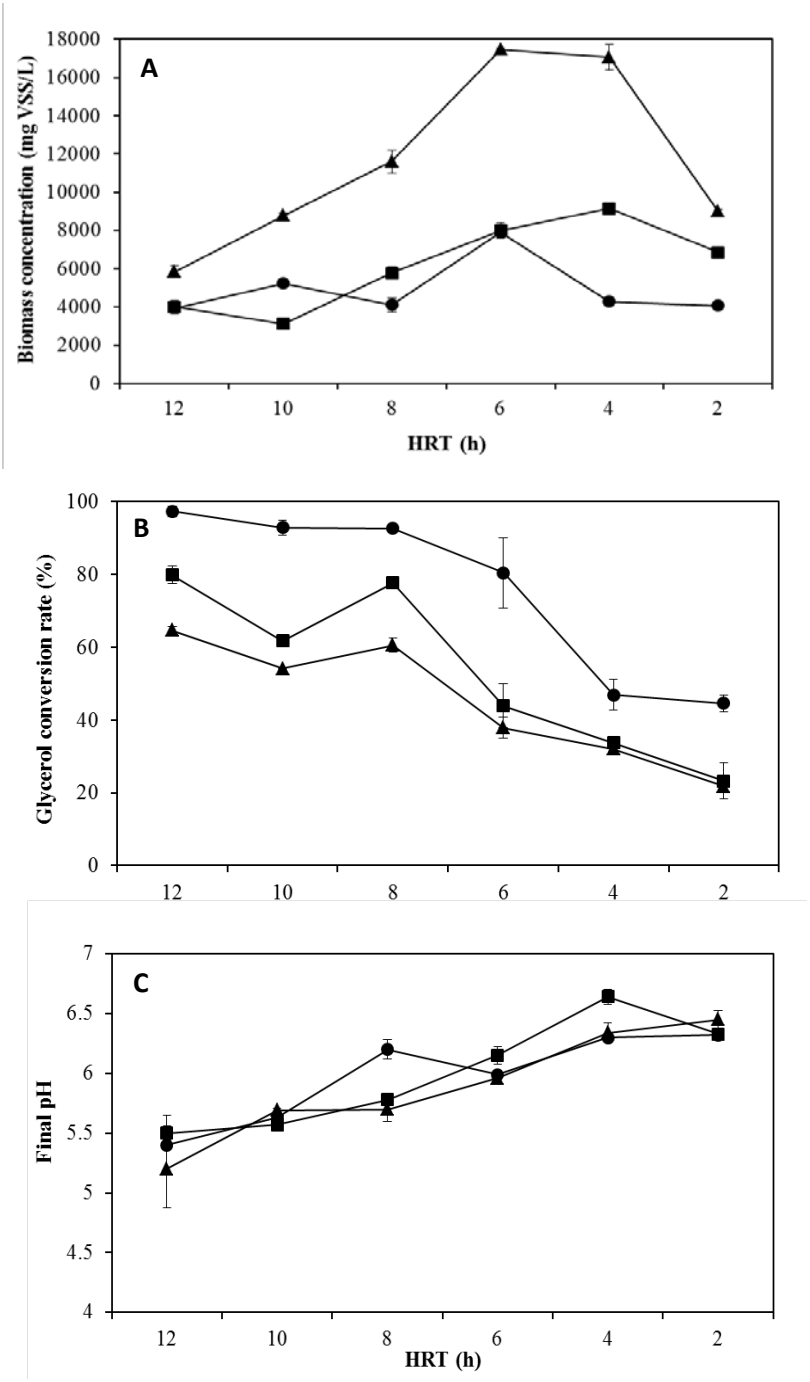

Figure 24. Variations in (A) biomass concentration, (B) glycerol conversion rate, and (C) final pH with respect to different combination of HRT and glycerol concentration in the UASB reactors. In each panel, symbols are ● for 10 g/L, ■ for 20 g/L, and ▲for 30 g/L

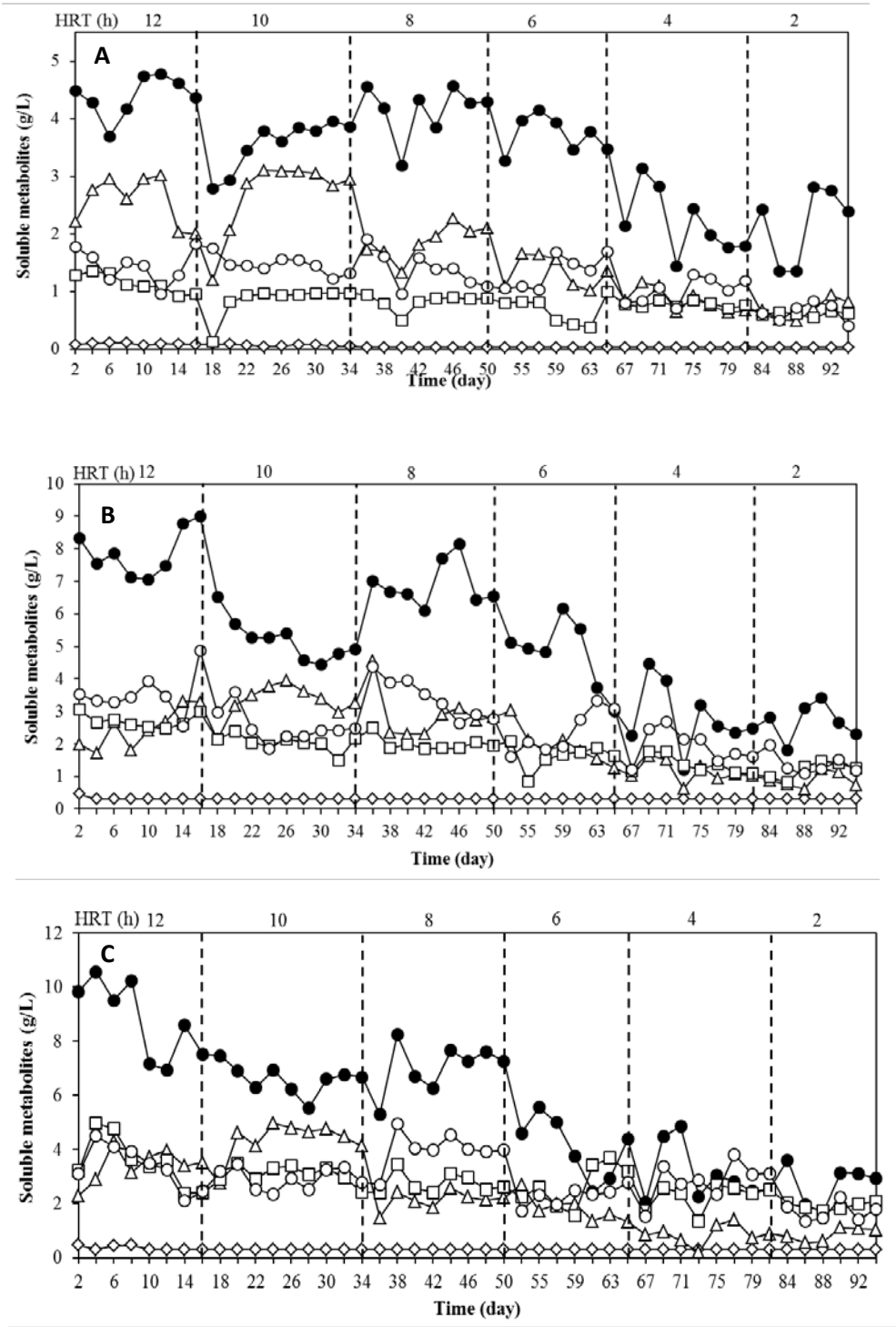

Figure 25. Time course profile of soluble metabolic products during the operation of UASB reactors: (A) 10 g/L glycerol concentration, (B) 20 g/L glycerol concentration, and (C) 30 g/L glycerol concentration. In each panel, symbols ◊ succinic acid, Δ acetic acid, □ 2,3-butanediol, o ethanol, and ● 1,3-propanediol

6.4.4 Analysis of the microbial community by FISH

The FISH technique was used to monitor the contribution of various microorganisms and quantification of the selected species under study in the three UASB reactors with different glycerol concentrations. Microbial composition of the sludge samples from the granules in UASB reactors after the end of experiment operation was shown in Fig. 26. The microbial community of UASB reactors fed with glycerol concentration of 10, 20, and 30 g/L was found to contain Eubacteria with 75.13%, 77.05%, and 80.8% of total DAPI binding cells, respectively. Among Eubacteria, *Klebsiella* sp. was accounted for 56.96%, 59.45%, and 63.47% of total DAPI binding cells, respectively. The FISH images (Fig. 27) showed that *Klebsiella* sp. accounted for more than 56% of total DAPI binding cells with the glycerol concentrations tested (10-30 g/L). The main soluble metabolic product in this study was 1,3-propanediol which confirmed that *Klebsiella pneumoniae* TR17 was dominant in the UASB reactors.

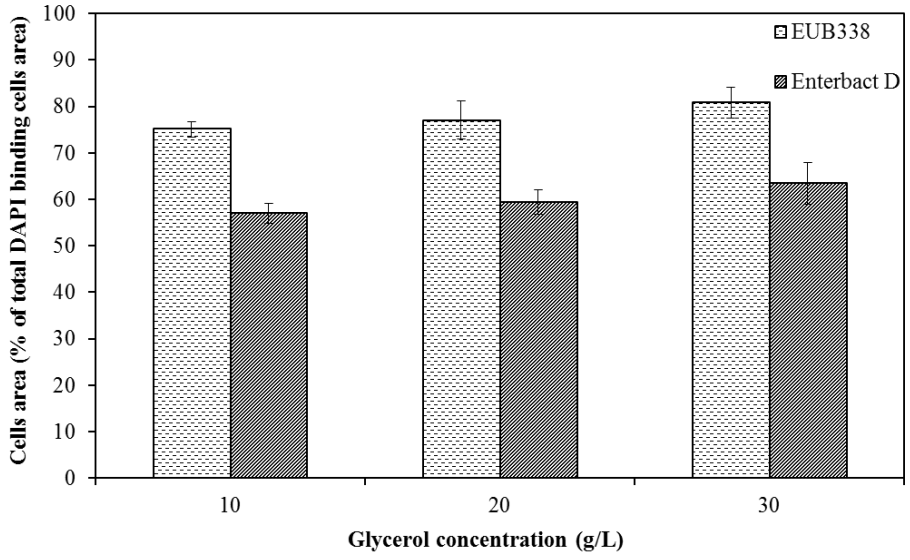

Figure 26. Microbial compositions of sludge samples obtained from granules in hydrogen producing UASB reactors. The error bars indicate the standard deviations from a triplicate sampling analysis

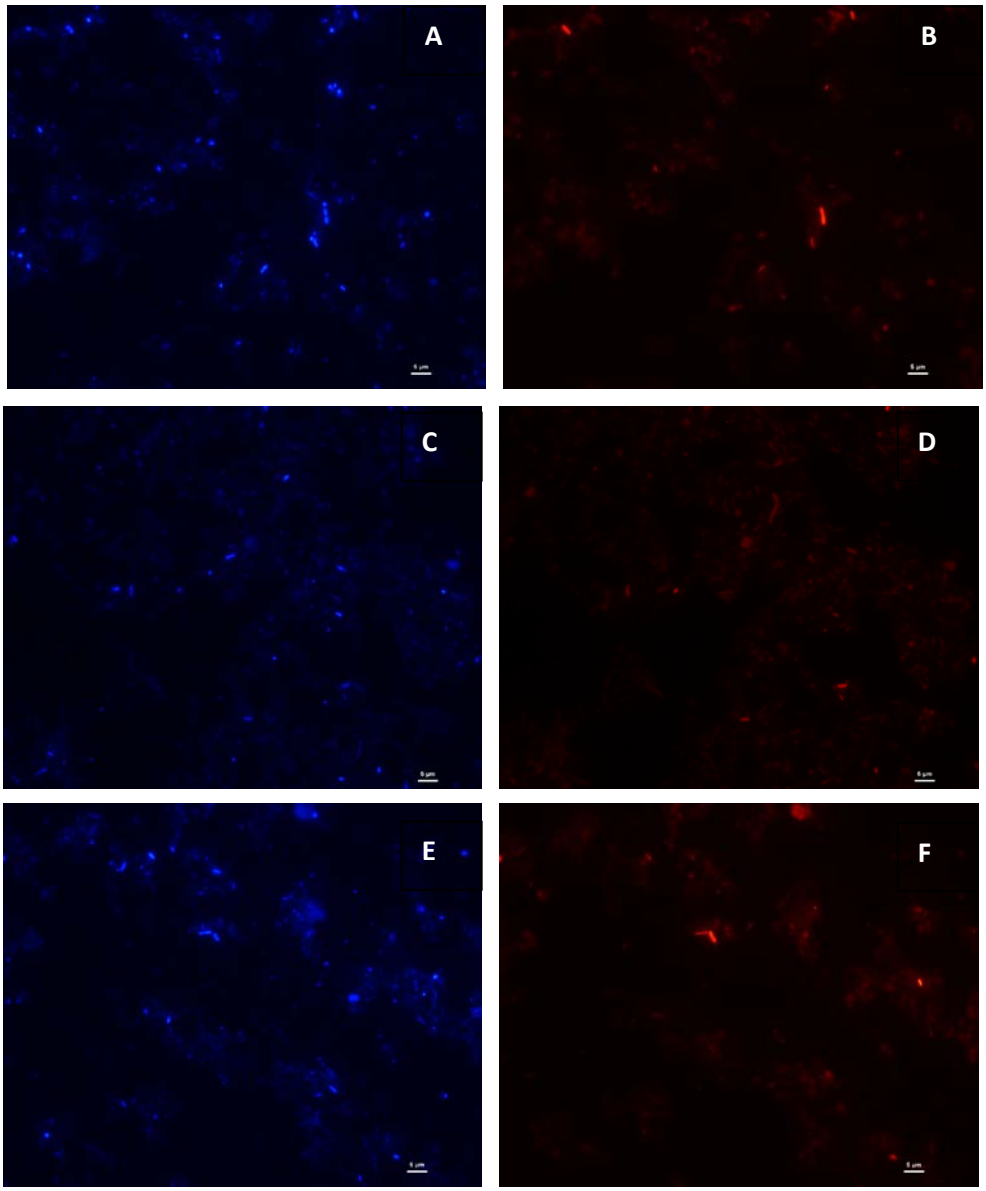

Figure 27. Image of the hydrogen producing sludge (A), (C), and (E) are sample from UASB reactors with 10, 20, and 30 g/L of glycerol, respectively, stained with DAPI for total cell. (B), (D), and (F) are samples from UASB reactors with 10, 20, and 30 g/L of glycerol, respectively, probe Enterbact D hybridization and labeled with Cy3 for detected *Klebsiella* sp.

6.5 Conclusion

The hydrogen yield of the immobilized *Klebsiella pneumoniae* TR17 increased with the decrease of HRT under non sterile condition in UASB reactors which the maximum values of 44.27 mmol H_2/g glycerol consumed at 10 g/L glycerol. However, the glycerol conversion rate tended to decrease as the HRT decreased from 12 to 2 h. Decreasing in HRT and glycerol concentration resulted in the decrease of soluble metabolites in which 1,3-propanediol was the main products. From FISH technique, the highest ratio of *Klebsiella* sp. with 63.47% of total DAPI binding cells and Eubacteria with 80.8% of total DAPI binding cells, were obtained at 30 g/L glycerol.

CHAPTER 7

BIOHYDROGEN PRODUCTION FROM CRUDE GLYCEROL BY TWO-STAGE OF DARK AND PHOTO FERMENTATION

7.1 Abstract

Hydrogen production from crude glycerol by two-stage process of dark fermentation using *Klebsiella pneumoniae* TR17 and photo fermentation using *Rhodopseudomonas palustris* TN1 was investigated in batch experiments. In dark fermentation, the cumulative hydrogen production and hydrogen yield were 64.24 mmol H_2/L and 5.74 mmol H_2/ g COD consumed, respectively with 80.21% glycerol conversion rate. The dark fermentation effluent (DFE) was employed for photo fermentation. Effect of DFE concentration (0 to 5 times dilution), with and without supplementation of yeast extract (2.3 g/L), $NaHCO_3$ (0.63 g/L), and glutamate (2-8 mM) were optimized. The optimal conditions for hydrogen production from *Rps. palustris* TN1 were 5 times dilution of DFE without supplementation of yeast extract, $NaHCO_3$, and 2 mM glutamate. Under the optimum conditions, the cumulative hydrogen production of 3.12 mmol H_2/L was obtained. The total hydrogen yield in the two-stage process was estimated to be 6.42 mmol H_2/g COD which was 10.4% of the theoretical yield.

7.2 Introduction

The major global crisis is environmental problem and energy requirement that make the increasing demand for alternative environmentally-friendly energy (Baykara, 2005). Hydrogen is high energy content, renewable energy carrier (Das and Veziroglu, 2008), and considered to be a clean fuel for future (Das and Veziroglu, 2001). Among the methods for production of hydrogen, biological process of dark and photo fermentation generates high efficiency of hydrogen (Tao *et al*., 2007). Moreover, dark and photo fermentation have potential to use renewable resources and wastes as substrate for production of hydrogen (Argun and Kargi, 2010).

In dark fermentation, substrate is converted to hydrogen and organic acids, but the production of organic acids can inhibit the dark fermentative bacteria resulting in low hydrogen production. In photo fermentation, organic acids from dark fermentation effluent (DFE) can assimilate by photosynthetic bacteria (Ozgur *et al*., 2010a,b). From this point, combination of two-stage process could achieve high hydrogen production and increase conversion efficiency (Su *et al*., 2009a). Recently, waste materials such as potato starch (Laurinavichene *et al*., 2010), beet molasses (Ozgur *et al*., 2010c), cheese whey wastewater (Azbar and Cetinkaya-Dokgoz, 2010), and corncob (Yang *et al*., 2010) can successfully be used as substrate for two-stage process of hydrogen production as well as waste minimization.

Crude glycerol is a by-product generated from biodiesel production in the amount of 1 kg per 10 kg of biodiesel (10% w/w) (Fountoulakis and Manios, 2009). Various investigations of single stage of dark or photo fermentation for hydrogen from crude glycerol have been reported (Selembo *et al*., 2009; Wu *et al*., 2011; Sabourin-Provost and Hallenbeck, 2009). However, to the best of our knowledge, there was no report on two-stage process of dark and photo fermentation of crude glycerol from biodiesel plant.

The objectives of this study were to evaluate the potential use of crude glycerol as a substrate for hydrogen production by the two-stage of dark and photo fermentation using *Klebsiella pneumoniae* TR17 and *Rhodopseudomonas palustris* TN1, respectively. The effects of dilution of dark fermentation effluent and nutrients supplementation were investigated.

7.3 Materials and methods

7.3.1 Bacterial strains and medium for dark fermentation

Klebsiella pneumoniae TR17 was used for the dark fermentative hydrogen producing bacteria. The hydrogen production medium for *Klebsiella pneumoniae* TR17 contained 11.14 g/L glycerol, 3.4 g/L K_2HPO_4, 2.47 g/L KH_2PO_4, 6.03 g/L NH_4Cl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, 2.0 mL/L trace element solution as described by Chookaew *et al*. (2014). The pH was adjusted to 8.0. The crude glycerol containing 50% of purity was used as carbon source in the culture medium.

7.3.2 Photosynthetic bacteria and medium for photo fermentation

Rhodopseudomonas palustris TN1 used for hydrogen production in photo fermentation of two-stage process was isolated from Songkhla Lake by Suwansaard *et al.* (2009). The culture medium (modified glutamate-acetate (GA) medium) contained: 0.5 g/L KH_2PO_4, 0.6 g/L K_2HPO_4, 0.4 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 1.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L H_3BO_3, 2.0 mg/L EDTA-2Na, 1.0 mg/L thiamine·HCl, 0.5 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg/L $ZnCl_2$, 0.01 mg/L $CoCl_2 \cdot 6H_2O$, 0.01 mg/L $CuCl_2$, 1.5 µg/L biotin, 0.935 g/L glutamic acid, 7.076 g/L acetic acid, 0.096 g/L propionic acid, 2.753 g/L butyric acid, 2.3 g/L yeast extract, 0.63 g/L $NaHCO_3$. The pH was adjusted to 7.0 using 5 N NaOH or HCI (Suwansaard *et al*., 2009).

7.3.3 Hydrogen production in dark fermentation by *Klebsiella pneumoniae* TR17

Dark fermentation was performed in 60 mL serum bottle containing 36 mL of the culture medium that was flushed with nitrogen gas to create anaerobic condition, closed with rubber stoppers and aluminum cap, and then sterilized at 121^0C for 20 min. Ten percent of starter culture of *Klebsiella pneumoniae* TR17 (OD_{660} = 0.5) was added into the medium and incubated at 40^0C. The cumulative hydrogen production and pH were determined every 4 h.

7.3.4 Hydrogen production in photo fermentation by *Rhodopseudomonas palustris* TN1

Dark fermentation effluent (DFE) sample was taken from the serum bottle in provious experiment and centrifuged (10,000 *g* for 10 min), then diluted in the range 1-5 times by mixing with deionized water. Effect of nutrients supplementation with and without yeast extract (2.3 g/L) + $NaHCO_3$ (0.63 g/L), were performed. The pH was adjusted to 7.0, flushed with argon to obtain an anaerobic condition and autoclaved. The inoculum of *Rps. palustris* TN1 (OD_{660} = 0.5) was added into serum bottles. Photo fermentation was conducted in batch mode using 60 mL of serum bottle with 36 mL working volume. The effect of glutamate concentration on hydrogen production at 0, 2, 4, 6, and 8 mM was tested. All photo fermentation experiments were conducted at 30^0C under anaerobic-light (3,000 lux) condition.

7.3.5 Analytical methods

The biogas production in the headspace of a serum bottle was measured by a syringe technique described by Owen *et al*. (1979). Hydrogen content of the biogas was determined using an Oldham MX 2100 gas detector (Cambridge Sensotec Ltd., England). Cell growth was determined as dry cell weight (gram dry weight per liter) by centrifugation the culture broth at 10,000 *g* for 10 min, washed cell sample twice with sterile distilled water, dried at 105ºC overnight and then weighed (Barbirato *et al*., 1998). Glycerol, succinic acid, 1,3 propanediol, and 2,3 butanediol concentration were determined by High Performance Liquid Chromatography (HPLC) (Agilent 1200). The culture medium was centrifuged at 10,000 *g* for 10 min then, the supernatant was filtered through 0.22 μm Nylon membrane filter (Sartorius, German) before injection. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostat column compartment; an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA); and ChemStation Software. Operation conditions were: 20 μl sample volume; 5 mM H_2SO_4 as a mobile phase; flow rate of 0.7 mL/min and a column temperature of 65^0C (Rujananon *et al*., 2011). Volatile fatty acids were determined using a gas chromatography (Hewlett Packard, HP 6890)

equipped with a flame ionization detector (FID) and Innowax column (dimensions 30 m x 320 μm x 0.25 μm). The temperature of the injection and detector were 240^0C and 280^0C, respectively. The chromatography was conducted using the following program: (i) 70^0C for 2.5 min, (ii) 70-100^0C with a ramping of 40^0C/min and hold at 100^0C for 0.2 min, (iii) 100-200^0C with a ramping of 10^0C/min and hold at 200^0C for 2 min. Helium was used for carrier gas at a flow rate of 1.8 mL/min (Yossan *et al*., 2012). Chemical oxygen demand (COD) and total nitrogen (TN) concentrations were analyzed using commercial test kits from Spectroquant (Merck Co., Ltd., Germany). Total solid (TS), suspended solid (SS), volatile suspended solid (VSS), pH, and ammonia nitrogen (NH_3-N) were determined with the procedures described in the Standard Methods (APHA, 1995). The light conversion efficiency was calculated using the following equation (15).

$$\eta(\%) = (33.6 \text{ x } p_{H2} \text{ x } V_{H2}) \text{ x } 100/(I \text{ x } A \text{ x } t) \quad (15)$$

where p_{H2} is the density of hydrogen production (g/L), V_{H2} is the volume of hydrogen production in L, I is the light intensity (W/m^2), A is the irradiated area in m^2 and t is the duration of produced hydrogen (h). The hydrogen yield (mmol H_2/g glycerol consumed) was calculated by measuring the total volume of hydrogen produced (mmol H_2/L) divided by the glycerol consumed (g/L). The glycerol conversion rate was calculated by using the equation: [(I-F)/I] x 100%, in which I and F are the initial and final glycerol concentrations (g/L), respectively (Markov *et al*., 2011).

7.4 Results and discussion

7.4.1 Hydrogen production in dark fermentation stage

The time course profiles of cumulative hydrogen production and pH during dark fermentation of crude glycerol by *Klebsiella pneumoniae* TR17 were illustrated in Fig. 28. Hydrogen production was generated after a lag phase of 6 h. The maximum cumulative hydrogen production (64.24 mmol H_2/L) and hydrogen yield (5.74 mmol H_2/ g COD consumed) was achieved with 80.21% of glycerol conversion rate. The initial COD of the culture medium (before process) was 36,800 mg/L, and the dark fermentation effluent (DFE) had a COD of 25,600 mg/L, indicating a COD

removal of 30.43%. The pH of the culture broth dropped from 8.0 to 5.94. The characteristics and concentration of organic acids in the DFE of crude glycerol were summarized in Table 27.

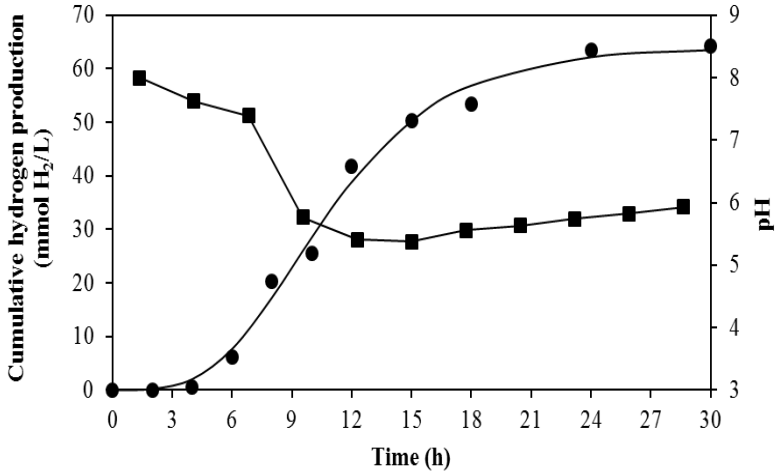

Figure 28. Time course of cumulative hydrogen production and pH during dark fermentation by *Klebsiella pneumoniae* TR17 under the condition: temperature 40^0C, initial pH 8.0, and 11.14 g/L glycerol. In each panel, symbols ● for hydrogen production, and ■ for pH

Table 27. Characteristics of dark fermentation effluent

Characteristics	Concentration
TN (mg/L)	148±11.31
NH_3-N (mg/L)	0.02
COD (mg/L)	25,600±4,525
TS (mg/L)	20.99±2.17
SS (mg/L)	3.16±0.02
Succinic acid (mM)	4.92±0.04
Glycerol (mM)	21.49±0.24
1,3 Propanediol (mM)	47.06±0.44
2,3 Butanediol (mM)	8.76±0.03
Ethanol (mM)	47.29±0.14
Acetic acid (mM)	11.95±0.29
Propionic acid (mM)	0.40±0.06
Iso-butyric acid (mM)	1.41±0.15
Butyric acid (mM)	0.36±0.07
Iso-valeric acid (mM)	0.23±0.01
n-Valeric acid (mM)	0.26±0.01
Iso-caproic acid (mM)	8.54±0.73
n-Caproic acid (mM)	0.16±0.002
Heptanoic acid (mM)	0.13±0.006

7.4.2 Effect of dilution and nutrients supplementation

The organic acids in the DFE can be used as substrate for production of hydrogen by photosynthetic bacteria. However, the organic acids obtained in this study were not suitable for *Rps. palustris* TN1 because the concentrations may be too high that may cause a decrease in productivity and yield (Ozgur *et al*., 2010a). So, evasion from the inhibitory effect of substrate concentration could be obtained by diluting the DFE to determine the optimum substrate concentration before reutilization in photo fermentation. It was reported by Suwansaard *et al*. (2010) that

yeast extract and $NaHCO_3$ are the significant factor for hydrogen production by *Rps. palustris* TN1 with the optimum concentrations of 2.30 g/L and 0.63 g/L, respectively. Thus, hydrogen production on diluted DFE with and without supplementation of yeast extract and $NaHCO_3$ were monitored.

The cumulative hydrogen production and hydrogen yield by *Rps. palustris* TN1 increased with the increase of the dilution of DFE from 1x (undiluted sample) to 5x (5 times diluted) both of with and without nutrients supplementation (Fig. 29). The maximum cumulative hydrogen production and hydrogen yield of 1.37 mmol H_2/L and 0.43 mmol H_2/ g COD consumed, respectively, were achieved at 5 times diluted DFE without nutrients supplementation. The final pH, light conversion efficiency, and COD removal efficiency both of with and without nutrients supplementation tend to increase with the increase of dilution from 1x to 5x (Table 28). At this optimum condition, the maximum light conversion efficiency was 0.72% with the total volatile fatty acid (VFA) consumption of 37.49% and COD removal efficiency of 33.3%. The highest hydrogen production rate (0.021 mmol H_2/L h) was observed at 24 h of 5x diluted DFE without nutrients supplementation. While nutrients supplementation at 24 h of 5x diluted DFE had the hydrogen production rate of 0.014 mmol H_2/L h. However, the maximum cell concentration (1.90 g dry cell weight/L) was obtained at 3x diluted DFE with nutrients supplementation. Therefore, a nutrients supplementation gave higher cell concentration than those without supplementation even though both DFE with and without supplements achieved the same level of hydrogen production of 1.31 and 1.37 mmol H_2/L, respectively, at 5x diluted DFE.

Supplementation with $NaHCO_3$ could increase the microbial growth resulting in a significant hydrogen production (Takabatake *et al*., 2004). Moreover, yeast extract was a growth factor which could affect the activity of enzyme in metabolism of bacteria as well as increasing in cell concentration (Xu *et al*., 2008). Results in this study showed that supplementation of yeast extract and $NaHCO_3$ could increase cell concentration but not hydrogen production. A similar result was also reported by Xu *et al*. (2008). It seems like the lighter color of DFE from the higher dilution resulted in the higher hydrogen production (Table 28). This might be due to light could easily pass through the medium in the higher dilution of DFE compared to

the no dilution which had dark yellow color. These results were similar to the result using olive mill wastewater in which the lighter color from higher dilution could increase hydrogen production (Eroglu *et al*., 2004).

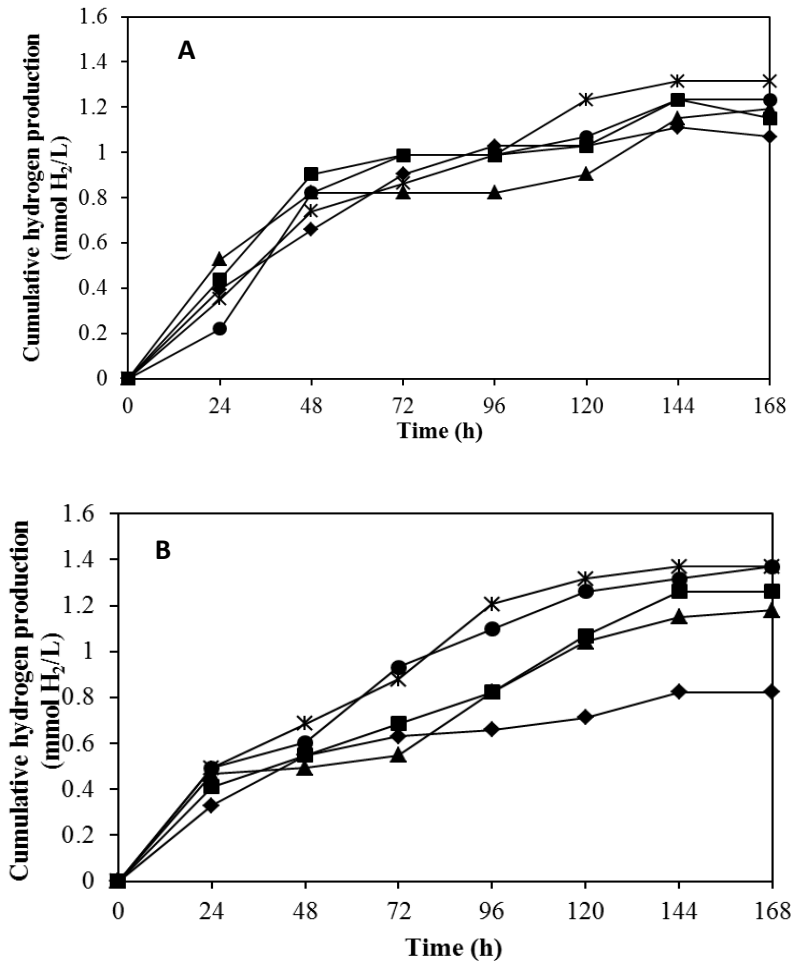

Figure 29. Effect of dilution with supplementation of yeast extract + $NaHCO_3$ (A), and without supplementation (B) on cumulative hydrogen production of DFE by *Rps. palustris* TN1 under anaerobic-low light condition (3,000 lux) at 30^0C. In each panel, symbols are ♦ for 1x, ■ for 2x, ▲for 3x, ● for 4x, and * for 5x; 1X means undiluted DFE, 2X means 50% diluted DFE.

Table 28. Effect of dilution of dark fermentation effluent (DFE) with and without nutrients supplementation (suppl.) by *Rps. Palustris* TN1 under anaerobic-low light condition (3,000 lux) after 168 h of cultivation

Dilution DFE	Final pH		Cell concentration (g DCW/L)		Total VFA consumption (%)		Light conversion efficiency (%)		COD removal efficiency (%)		H_2 yields (mmol H_2/ g COD consumed)	
	Suppl.	Without suppl.	Suppl.	Without suppl.	Suppl.	Without suppl.	Suppl.	Without suppl.	Suppl.	Without suppl.	Suppl.	Without suppl.
1x	7.15±0.02	7.08±0.03	0.90±0.14	0.28±0.03	68.12	31.38	0.56	0.56	25.0	12.5	0.17	0.26
2x	7.78±0.02	7.80±0.02	1.80±0.14	1.35±0.49	65.21	38.39	0.60	0.66	28.5	14.2	0.18	0.40
3x	8.15±0.07	8.10±0.01	1.90±0.49	1.65±0.07	54.64	39.56	0.62	0.61	40.0	20.0	0.19	0.37
4x	8.10±0.14	8.05±0.02	1.60±0.28	0.62±0.04	48.85	39..90	0.64	0.72	25.0	25.0	0.39	0.43
5x	8.05±0.07	7.82±0.04	0.73±0.03	0.60±0.07	66.96	37.49	0.69	0.72	33.3	33.3	0.41	0.43

7.4.3 Effect of glutamate concentration on hydrogen production

Glutamate is one of the efficient nitrogen sources for hydrogen production from photosynthetic bacteria (Koku *et al*., 2002; Shi and Yu, 2005). *Rps. palustris* TN1 was employed to study the effect of glutamate concentration (0-8 mM) on hydrogen production from DFE using the optimum condition obtained from the previous studies (5x dilution without supplementation of yeast extract + $NaHCO_3$). The optimum glutamate concentration was found to be 2 mM, giving the highest hydrogen production (3.12 mmol H_2/L) and cell concentration (0.88 g dry cell weight/L) (Fig. 30). The final pH was 8.15. The total VFA consumption was 53.31% and COD removal efficiency was 41.81% (Table 29). Light conversion efficiency decreased from 1.44 to 0.53% with increasing glutamate concentration from 2 to 8 mM. This result was similar to Shi and Yu, (2005) that hydrogen production was high at low concentration of glutamate. Moreover, glutamate concentration above 11 mM could decrease hydrogen production (Sasikala *et al*., 1995).

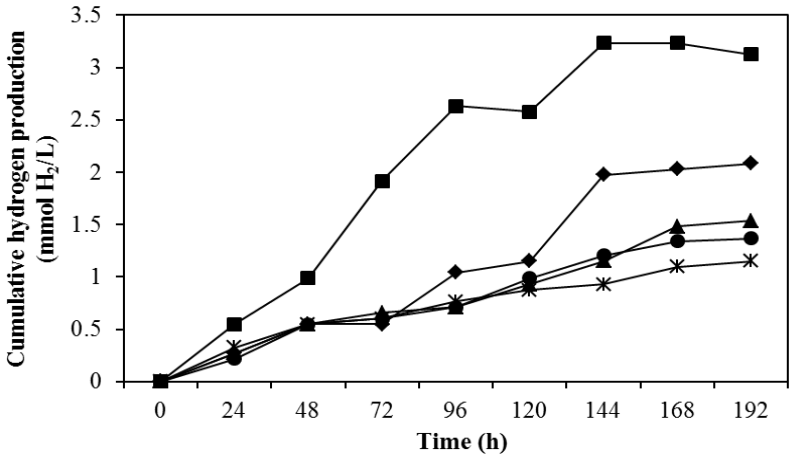

Figure 30. Effect of glutamate concentration on cumulative hydrogen production of DFE by *Rps. palustris* TN1 under anaerobic-low light conditions (3,000 lux) at 30^0C. In each panel, symbols are ♦ for 0 mM (control), ■ for 2 mM, ▲ for 4 mM, ● for 6 mM, and ∗ for 8 mM

Table 29. Effect of glutamate concentration on hydrogen production by *Rps. palustris* TN1 under anaerobic-low light condition (3,000 lux) after 192 h of cultivation

Glutamate concentration (mM)	Final pH	Cell concentration (g DCW/L)	Total VFA consumption (%)	Light conversion efficiency (%)	COD removal efficiency (%)	H_2 yield (mmol H_2/ g COD consumed)
0 (Control)	7.73±0.10	0.80±0.14	58.52	0.56	24.36	0.78
2	8.15±0.11	0.88±0.10	53.31	1.44	41.81	0.68
4	7.99±0.06	0.63±0.17	50.71	0.70	24.36	0.57
6	8.02±0.03	0.73±0.03	50.23	0.63	24.33	0.51
8	7.91±0.14	0.67±0.03	52.45	0.53	12.72	0.82

7.4.4 Overall yield

The hydrogen yield obtained by two-stage process of dark and photo fermentation was 6.42 mmol H_2/ g COD which was 10.4% of the theoretical yield. Many studies have reported on biohydrogen production by two-stage process with the difference in various types of wastewater, photosynthetic bacterial strains, and operating conditions. Therefore, it is rather difficult to make a comparison. The total hydrogen yield obtained in this study (6.42 mmol H_2/ g COD) was slightly lower than those from the previous reports using wastes such as ground wheat waste (6.9 mmol H_2/ g COD) (Argun *et al*., 2009), corncob (28.1 mmol H_2/ g COD) (Yang et al., 2010), and cheese whey wastewater (26.2 mmol H_2/ g COD) (Azbar and Cetinkaya-Dokgoz, 2010).

7.5 Conclusion

A two-stage of dark and photo hydrogen production could be used for conversion of crude glycerol to hydrogen. In a batch experiments, the optimum conditions for photo hydrogen production from dark fermentation effluent (DFE) were 5 times diluted DFE without supplementation of yeast extract + $NaHCO_3$, and 2 mM glutamate. The total hydrogen yield in the two-stage process of dark and photo fermentation was estimated to be 6.42 mmol H_2/g COD.

CHAPTER 8

SUMMARY AND SUGGESTIONS

8.1 Summary

1. Enrichments of hydrogen producing bacteria at 45^0C from crude glycerol contaminated soil were found to be the potential sources for isolation of thermotolerant bacteria. The isolate TR17, TR20, and TR32 was selected based on thermotolerant performance with high cumulative hydrogen production. The isolate TR17 gave the highest cumulative hydrogen production with high 2,3-butanediol production. The isolate TR20 was 1,3-propanediol producer whereby the isolate TR32 was ethanol producer.

2. A thermotolerant fermentative hydrogen-producing strain was identified as *Klebsiella pneumoniae* TR17 on the basis of the 16S rRNA gene analysis as well as physiological and biochemical characteristics. The maximum cumulative hydrogen production of 27.7 mmol H_2/L and hydrogen yield of 0.25 mol H_2/mol glycerol were achieved from fermentation of crude glycerol under the optimal conditions: 40^0C, initial pH 8.0, 20 g/L glycerol and 2 g/L yeast extract.

3. The optimum conditions for maximizing hydrogen yield and ethanol yield were 11.14 g/L glycerol, 2.47 g/L KH_2PO_4, and 6.03 g/L NH_4Cl. The predicted hydrogen yield was 0.27 mol H_2/mol glycerol with the ethanol yield of 0.63 mol EtOH/mol glycerol. The verification experiments revealed hydrogen yield of 0.26 mol H_2/mol glycerol and ethanol yield of 0.58 mol EtOH/mol glycerol.

4. The highest hydrogen production rate (242.15 mmol H_2/L d) was obtained at 30 g/L of glycerol at 4 h HRT in UASB reactor. This was 5 folds higher than that obtained from UA reactor under the same conditions. The microbial community determined by PCR-DGGE analysis indicated that *Klebsiella pneumoniae* TR17 was dominant in all HRT tested in UASB reactor. However, *Klebsiella pneumoniae* TR17

present in UA reactor had to compete with *Escherichia coli* and *Citrobacter freundii*. The hydrogen yield of the immobilized *Klebsiella pneumoniae* TR17 increased with the decrease of HRT under non sterile condition in UASB reactors. The maximum value was 44.27 mmol H_2/g glycerol consumed at 10 g/L glycerol. From FISH technique, the highest ratio of *Klebsiella* sp. was 63.47% of total DAPI binding cells and Eubacteria with 80.8% of total DAPI binding cells, were obtained at 30 g/L glycerol.

5. In a batch experiments, the optimum conditions for photo hydrogen production from dark fermentation effluent (DFE) were 5 times diluted DFE without supplementation of yeast extract + $NaHCO_3$, and 2 mM glutamate. The total hydrogen yield in the two-stage process of dark and photo fermentation was estimated to be 6.42 mmol H_2/g COD.

8.2 Suggestions

1. 1,3-Propanediol, 2,3-butanediol, and ethanol are major valuable by-products and have a potential for many applications such as monomer for manufacture of polyurethanes, polytrimethylene terephthalate (PTT), and fuel additives. Therefore, the fermentation condition for production of each product is necessary to be optimized.

2. Decreasing of pH in UASB reactor led to the decreasing of hydrogen production rate. Therefore, control of pH in the reactor could improve the hydrogen production.

3. Dark fermentation effluent of crude glycerol had dark yellow color as well as light could not easily penetrate through the medium. Thus, photobioreactor design such as plate-type photobioreactor for increase hydrogen production could be studied.

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APPENDICES

1. Chemical oxygen demand (COD) analysis

Using COD cell test from Spectroquant (Merck Co., Ltd., Germany), Cat. No. 14555

1.1 Procedure

1.1.1 Dilute the sample in the range of 500-10,000 mg/L COD.

1.1.2 Suspend the bottom sediment in the cell by swirling.

1.1.3 Carefully pipette 1.0 mL of the sample into a reaction cell, close tightly with the screw cap, and mix vigorously. (Caution, the cell becomes hot)

1.1.4 Heat the reaction cell in the thermoreactor at 148°C for 2 hours.

1.1.5 Remove the cell from the thermoreactor and place in a test-tube rack to cool.

1.1.6 Swirl the cell after 10 minutes.

1.1.7 Replace the cell in the rack for complete cooling to room temperature.

1.1.8 Place the cell into the cell compartment. Align the mark on the cell with that on the photometer.

2. Total Nitrogen analysis

Using Nitrogen cell test from Spectroquant (Merck Co., Ltd., Germany), Cat. No. 14763

2.1 Procedure

2.1.1 Dilute the sample in the range of 10-150 mg/L N

2.1.2 Pipette 1.0 mL of the sample into an empty round cell.

2.1.3 Add 9.0 mL of distilled water with pipette.

2.1.4 Add 1 level blue microspoon of N-1K.

2.1.5 Add 6 drops of N-2K, close the cell with the screw cap, and mix.

2.1.6 Heat the cell in the thermoreactor at 120°C (100°C) for 1 hour.

2.1.7 Remove the cell from the thermoreactor and place in a test-tube rack to cool to room temperature (pretreated sample).

2.1.8 Swirl the cell after 10 minutes.

2.1.9 Pipette 1.0 mL of the pretreated sample into a reaction cell (do not mix).

2.1.10 Add 1.0 mL of N-3K with pipette, close the cell with the screw

cap, and mix. (Caution, cell becomes hot)

2.1.11 Reaction time: 10 minutes

2.1.12 Place the cell into the cell compartment. Align the mark on the cell with that on the photometer.

3. Hydrogen detection

3.1 Standard curve of hydrogen

3.1.1 Inject pure hydrogen (99%) to MX2100 OLDMAN gas detector at different volume.

1.1.2 Plot the standard curve by the relationship of hydrogen volume and hydrogen concentration.

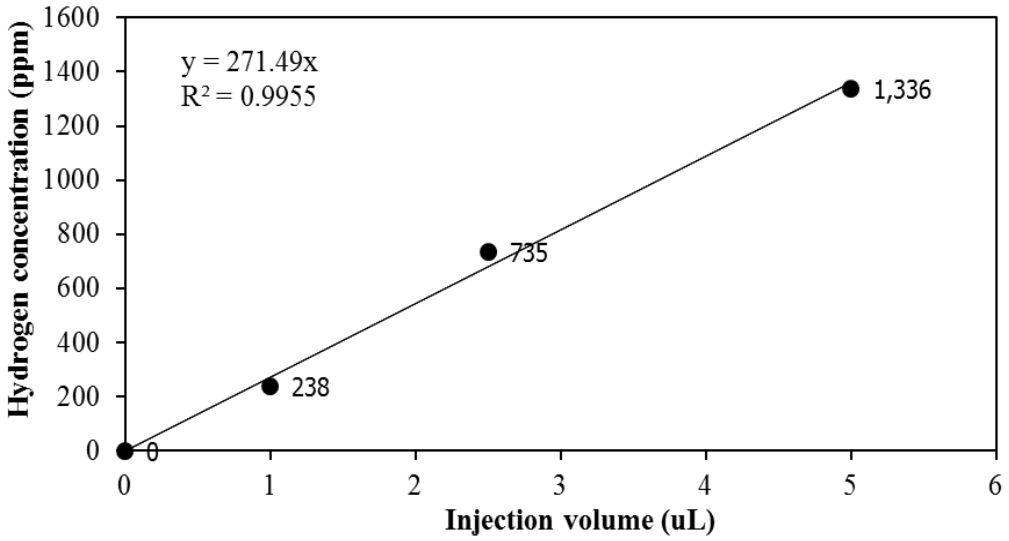

Figure 31. Standard curve of hydrogen concentration (ppm) detected by MX2100 OLDMAN gas detector

3.2 Hydrogen calculation corrected to standard curve

Bacterial cell produce total biogas of 200 mL. The biogas (1 mL) was withdrawn to determine hydrogen concentration (ppm) by injection into gas detector. The 170 ppm hydrogen concentration was detected. Calculation of hydrogen volume correlated to standard curve.

Y (ppm) = 271.49X (μL)

170 ppm = 271.49X

X = 1.597 μL

Therefore 1 mL of injection contains hydrogen 1.597 μL

200 mL of total biogas contains hydrogen = 1.579 x 200 = 319.4

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Reviewer for journals

International Journal of Hydrogen Energy

List of Publications and Proceeding

Publications

Chookaew, T., O-Thong, S. and Prasertsan, P. Bioconversion of crude glycerol from biodiesel plant to hydrogen with simultaneous diols and ethanol production by thermotolerant bacteria isolates. (Submitted)

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