การผลิตไฮโดรเจนชีวภาพโดยไซยาโนแบคทีเรียที่ตรึงไนโตรเจน *Anabaena siamensis* TISTR 8012

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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BIOHYDROGEN PRODUCTION BY NITROGEN FIXING CYANOBACTERIUM *Anabaena siamensis* TISTR 8012

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By	Miss Wanthanee Khetkorn
Field of Study	Biotechnology
Thesis Advisor	Professor Aran Incharoensakdi, Ph.D.
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วันทนีย์ เขตต์กรณ์: การผลิตไฮโดรเจนชีวภาพโดยไซยาโนแบคทีเรียที่ตรึงไนโตรเจน *Anabaena siamensis* TISTR 8012. (BIOHYDROGEN PRODUCTION BY NITROGEN FIXING CYANOBACTERIUM *Anabaena siamensis* TISTR 8012) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร.อรัญ อินเจริญศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ ร่วม: Prof. Peter Lindblad, Ph.D., 166 หน้า

ในวิทยานิพนธ์นี้ ผู้วิจัยได้มุ่งเน้นที่จะศึกษาการผลิตไฮโดรเจนทางชีวภาพโดยไซยาโนแบคทีเรีย *Anabaena siamensis* TISTR 8012 สายพันธุ์ที่แยกได้จากทุ่งนาข้าวในประเทศไทย จากการวิเคราะห์สายวิวัฒนาการโดยติดตามลำดับยีน *16S* rDNA พบว่า *A. siamensis* TISTR 8012 ถูกจัดออกเป็นกลุ่มเดี่ยว และมีความสามารถเปลี่ยนแปลงโครงสร้างของเซลล์ได้หลากหลายเมื่อเจริญในสภาพวะที่ขาดแคลนแหล่งไนโตรเจน เช่น เซลล์ akinete และ heterocyst ปัจจัยทางกายภาพที่เหมาะสมต่อการเพิ่มอัตราการเจริญเติบโตของ *A. siamensis* TISTR 8012 ภายใต้สภาวะตรึงไนโตเจน คือ อุณหภูมิ 30 องศาเซลเซียส, ความเข้มแสงระหว่าง 40-50 ไมโครไอน์สไตน์ต่อตารางเมตรต่อวินาที และพีเอชเท่ากับ 7.5 เป็นต้น

ประสิทธิภาพในการผลิตก๊าซไฮโดรเจน ขึ้นอยู่กับปัจจัยหลายประการ โดยพบว่า *A. siamensis* TISTR 8012 จะมีการผลิตไฮโดรเจนน้อยลงทันที เมื่ออาหารมีสารประกอบไนโตรเจน หรือ เซลล์ได้รับความเครียดสูงจากเกลือต่างๆ แต่เมื่อควบคุมปัจจัยหลักที่มีผลต่อการผลิตก๊าซไฮโดรเจน เช่น อายุของเซลล์ ความเข้มแสง ช่วงเวลาการให้แสง และแหล่งคาร์บอน ส่งผลให้เซลล์มีการผลิตก๊าซไฮโดรเจนสูงขึ้น โดยพบว่า อัตราการผลิตก๊าซไฮโดรเจนสูงสุดประมาณ 32 ไมโครโมลไฮโดรเจนต่อมิลลิกรัมคลอโรฟิลล์เอต่อชั่วโมง เมื่อเลี้ยงเซลล์ที่อยู่ในระยะเจริญเติบโตแบบทวีคูณ (log phase) ในอาหารสูตร $BG11_0$ ที่ขาดแหล่งไนโตรเจน โดยเติมน้ำตาลฟรุกโตสที่ความเข้มข้น 0.5 เปอร์เซ็นต์ เป็นแหล่งคาร์บอน และบ่มภายใต้ความเข้มแสงที่ 200 ไมโครไอน์สไตน์ต่อตารางเมตรต่อวินาที เป็นเวลา 12 ชั่วโมงในสภาวะไร้อากาศ ซึ่งอัตราการผลิตนี้ เป็นอัตราที่สูงกว่าการผลิตในไซยาโนแบคทีเรียที่เป็นต้นแบบ เช่น *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133 และ *Synechocystis* PCC 6803 เป็นต้น การผลิตที่สูงขึ้นนี้เป็นผลมาจากกิจกรรมของเอ็นไซม์ไนโตรจีเนส และไบไดเรกชันนอลไฮโดรจีเนสที่สูงขึ้น โดยพบว่าแสง และน้ำตาลฟรุกโตส สามารถชักนำให้กิจกรรมของเอ็นไซไนโตรจีเนสเพิ่มขึ้นได้อย่างชัดเจน และจากการศึกษาการนำเข้าน้ำตาลไปใช้ในเซลล์ พบว่า เซลล์สามารถนำเข้าน้ำตาลฟรุกโตสได้ดีกว่าน้ำตาลชนิดอื่นๆ แต่อย่างไรก็ตาม การผลิตไฮโดรเจนไม่ได้เพิ่มขึ้นเมื่อบ่มเซลล์ในสภาวะที่มีแสงนานกว่า 12 ชั่วโมง ทั้งนี้อาจเป็นเพราะกิจกรรมของเอ็นไซม์อัพเทคไฮโดรจีเนสที่สูงขึ้น จากผลการทดลอง แสดงให้เห็นว่า การเลือกใช้ความเข้มแสง และระยะเวลาในการให้แสงที่เหมาะสม เป็นสิ่งสำคัญ เพื่อลดอันตรายที่เกิดจากแสง (photodamage) และการทำงานของเอ็นไซม์อัพเทคไฮโดรจีเนส

ถึงอย่างไรก็ตาม อุปสรรคสำคัญสำหรับการผลิตก๊าซไฮโดรเจนโดย *A. siamensis* คือ การบริโภคก๊าซไฮโดรเจนโดยกิจกรรมของเอ็นไซม์อัพเทคไฮโดรจีเนส ดังนั้น เพื่อจะเอาชนะอุปสรรคนี้ ผู้วิจัยได้สร้างสายพันธ์กลาย ที่ขาดยีนอัพเทคไฮโดรจีเนส (Δ*hupS*) โดยการแทรกยีนต้านยาปฏิชีวนะในยีน *hupS* พบว่า สายพันธุ์กลายสามารถผลิตก๊าซไฮโดรเจนได้มากว่าสายพันธ์ดั้งเดิมประมาณสามเท่า นอกจากนี้ สายพันธ์กลายสามารถรักษาการผลิตก๊าซไฮโดรเจน ภายใต้การเปิดรับแสงเป็นเวลานาน อีกทั้งมีกิจกรรมของเอ็นไซม์ไนโตรจีเนสเพิ่มขึ้นเป็นสองเท่า เมื่อเปรียบเทียบกับสายพันธุ์ดั้งเดิม จากการวิเคราะห์การแสดงออกของยีน โดยรีเวิร์สทรานสคริปเทสโพลีเมอเรสเชนรีแอคชัน (RT-PCR) แสดงให้เห็นว่า อิเล็กตรอนและพลังงานจำนวนมาก ที่จำเป็นสำหรับการผลิตก๊าซไฮโดรเจนในสายพันธ์กลาย อาจได้รับมาจากการขนส่งอิเล็กตรอน ที่ได้รับจากการเกิดออกซิเดชันโมเลกุลของน้ำผ่านระบบแสงที่สอง (PSII) ในเซลล์ปกติ จากผลงานวิจัยนี้ ชี้ให้เห็นว่า ไซยาโนแบคที่เรียที่ตรึงไนโตรเจน *A. siamensis* TISTR 8012 มีศักยภาพสูงในการผลิตก๊าซไฮโดรเจน และสามารถนำไปสู่การพัฒนา การผลิตไฮโดรเจนโดยกระบวนการทางชีวภาพ ในระดับใหญ่ขึ้น เพื่อเป็นแหล่งพลังงานทดแทนที่สะอาดในอนาคต

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2554..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

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WANTHANEE KHETKORN: BIOHYDROGEN PRODUCTION BY NITROGEN FIXING CYANOBACTERIUM *Anabaena siamensis* TISTR 8012. ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., CO ADVISOR: PROF. PETER LINDBLAD, Ph.D., 166 pp.

In this thesis, we focused to study biohydrogen production by using the cyanobacterium *Anabaena siamensis* TISTR 8012, a strain isolated from rice paddy field in Thailand. The phylogenetic analysis using *16S* rDNA gene sequence revealed that *A. siamensis* TISTR 8012 is classified into a single group which possesses akinete and heterocyst differentiations when grown under N-deprivation. Optimum physical parameters to enhance growth rate of *A. siamensis* TISTR 8012 under N_2 fixing condition were temperature at 30 ºC, light intensity between 40-50 μE m^{-2} s^{-1} and pH 7.5.

The efficiency of H_2 production depends on several factors, nitrogen compound available in medium and high salt stress could immediately decrease H_2 production in *A. siamensis* TISTR 8012. Optimizing key factors affecting H_2 production such as cell age, light intensity, time of light incubation and source of carbon resulted in enhanced H_2 production. The maximum H_2 production was about 32 μmolH_2 mg chl $a^{-1}h^{-1}$ when cells at log phase were adapted in $BG11_0$ medium without N-source supplemented with 0.5% fructose and incubated under continuous illumination of 200 μ$Em^{-2}s^{-1}$for 12 h under anaerobic condition. This rate was higher than that observed in the model organisms *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133 and *Synechocystis* PCC 6803. This higher production was likely caused by higher nitrogenase and bidirectional hydrogenase activities. Light and fructose were found to induce activity of nitrogenase. A study of sugar uptake by *A. siamensis* TISTR 8012 showed that fructose was taken up by cells more than other sugars tested. However, The production of H_2 did not increase after 12 h of light incubation which was probably due to an increased uptake hydrogenase activity, indicating that a proper adjustment of light conditions such as intensity and duration is important to minimize both the photodamage of the cells and the uptake hydrogenase activity.

Nevertheless, the main obstacle for high H_2 production by *A. siamensis* is H_2 consumption by uptake hydrogenase under light condition. To overcome this, we created the hydrogen uptake deficient mutant by interrupting the *hupS* gene with antibiotic resistance cassette. We could demonstrate that the mutant strain produced about 3-folds more H_2 than wild type strain. Moreover, The Δ*hupS* mutant could sustain H_2 production under light exposure for long period with about 2-folds higher activity of nitrogenase when compared to wild type. Gene expression analysis by RT-PCR showed that more electrons and ATP molecules required for H_2 production in Δ*hupS* mutant may be obtained from the electron transport chain associated with the photosynthetic oxidation of water in PSII in vegetative cells. The results obtained from this study indicated that *A. siamensis* TISTR 8012 has a high potential for H_2 production leading to further development for the production of biohydrogen in an expanded scale, thus serving as a renewable and clean energy source for the future.

Field of Study :….Biotechnology……Student's Signature……..…

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Co-advisor's Signature……..……..……..……..…

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LIST OF ABBREVIATIONS

H_2	Hydrogen
O_2	Oxygen
N_2	Nitrogen
N_2ase	Nitrogenase
Hox	Hydrogen oxidation
Hup	Hydrogen uptake
PSI	Photosystem I
PSII	Photosystem II
Chl *a*	Chlorophyll *a*
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
HEPES	Hydroxyethyl piperazineethanesulfonic acid
$\mu E\ m^{-2}s^{-1}$	Micromole einsteins per second per square meter
kb	Kilo base
h	hour
min	Minute
mM	Milimolar
µg	Microgram
µL	Microliter
nm	nanometer
OD	Optical density
DT	Doubling time
RT-PCR	Reverse transcriptase polymerase chain reaction

CHAPTER I

INTRODUCTION

1.1 The energy challenge

The worldwide energy demand has been growing at an alarming rate. The European “World Energy Technology and Climate Policy Outlook” (WETO) predicts that an average growth rate per annum for energy demand will be 1.8% in the period 2000-2030. And 80% of world energy demand is being met largely as fossil fuel reservation. Unfortunately, the limited fossil energy resources are likely to be depleted in the near future. As a consequence, the fossil fuel resources become increasingly expensive. However, the utilization of fossil fuels is cause of global warming because the combustion of fossil fuel emits both greenhouse gases and other pollutants. The National Oceanic and Atmospheric Administration (NOAA) reported the average annual concentration of CO_2 in the atmosphere in 2011 is 391.57 ppm which is higher than the upper safety limit for atmospheric CO_2, 350 ppm which mainly come from the combustion of fossil fuel. From these crises, many researchers have been working on the exploration of new sustainable energy resources that can substitute fossil fuels and are environmental friendly. H_2 energy is a clean renewable energy which has been suggested as the energy carrier of the future. (Michael *et al*., 1989 : online).

1.2 H_2 energy

H_2 is one of the potential future sustainable energy carriers that may substitute the use of fossil fuels because it has a high energy yield when compared to traditional hydrocarbon fuels, a unit weight of H_2 gas can generate heating value as 141.65

MJ kg^{-1} (Perry, 1963). H_2 is considered as clean energy since water is the main product from combustion; this is the greatest advantage of H_2 because it is environmental friendly and it can reduce global greenhouse gas emissions. Nowadays, H_2 has been used in various applications; it can generate electricity using fuel cells and it can be used for fuel in rocket engines including application in transportation (Veziroglu, 1974; Ramachandran and Menon, 1998). Moreover, H_2 gas is an important industrial gas and raw material in many industries. The major problem in utilization of H_2 gas as a fuel is the high cost of the process for H_2 production which requires other energy sources. At the present, H_2 gas can be prepared by many conventional ways such as steam reforming, electrolysis and biohydrogen production processes. The steam reforming is a method using high temperature steam to produce H_2 from fossil fuel or natural gas. The electrolysis is an electrolytic process to decompose water molecule into O_2 and H_2. However, these two methods are energy intensive and generate CO_2 and pollutants during process as by products, which is not always environment friendly. On the other hand, biohydrogen production processes are found to be more environmental friendly and less energy intensive as compared to thermochemical and electrochemical processes.

1.3 Biohydrogen production

Biohydrogen production is the method for producing of H_2 gas by using microorganisms. There are several microorganisms that can produce H_2 gas namely, green algae, cyanobacteria, photosynthetic bacteria and dark fermentative bacteria. These microorganisms have very diverse physiology and metabolism that allow them to generate H_2 by different pathways. The different biological H_2 production

processes of various microorganisms including the advantages and disadvantages of their processes are summarized in Table 1.

Table 1 Different biological H_2 production processes in various microorganisms

Organisms	**Different biological H_2 production processes**	**Advantages and disadvantages**
Green algae	<u>Direct biophotolysis</u> $2H_2O$+ light = $2H_2$+O_2 $2H^+ + e^- \longrightarrow H_2$ <u>Photo-fermentation</u> CH_3COOH+$2H_2O$+light = $4H_2$+$2CO_2$	<u>Advantages:</u> - Can produce H_2 from water and sunlight - Solar conversion energy increased by 10 folds as compared to trees, crops - Can reduce CO_2 in environment <u>Disadvantages:</u> - Require N-source for growth - O_2 can be dangerous for the system - Difficult to genetic engineering
Cyanobacteria	<u>Direct biophotolysis</u> $2H_2O$+ light = $2H_2$+O_2 N_2+$8H^+$+ $8e^-$+16ATP $\rightarrow$ $2NH_3$+H_2+16ADP+$16P_i$ $2H^+ + e^- \longleftrightarrow H_2$ <u>Indirect biophotolysis</u> $6H_2O$+ $6CO_2$+light = $C_6H_{12}O_6$+ $6O_2$ $C_6H_{12}O_6$+$2H_2O$ = $4H_2$+$2CH_3COOH$+$2CO_2$	<u>Advantages:</u> - Can produce H_2 from water and sunlight - Can grow in simple nutrient - Can reduce CO_2 in environment - Can fix N_2 from the atmosphere <u>Disadvantages:</u> - Consumption of H_2 by uptake hydrogenase - O_2 has inhibitory effect on nitrogenase

Table 1 (Continued)

Organisms	Different biological H_2 production processes	Advantages and disadvantages
Photosynthetic bacteria	Photo-fermentation $CH_3COOH+2H_2O+light = 4H_2+2CO_2$ $N_2+8H^+ + 8e^- +16ATP \rightarrow 2NH_3+H_2+16ADP+16P_i$	Advantages: - Can use different waste materials like, whey, distillery, etc. - Can use wide spectrum of light Disadvantages: - Require light for the H_2 Production - Fermented broth will cause water pollution problem
Fermentative bacteria	Dark fermentation $C_6H_{12}O_6+6H_2O = 12H_2+6CO_2$ Pyruvate + CoA $\rightarrow$ acetyl-CoA + formate Pyruvate + CoA + 2Fd(ox) $\longrightarrow$ Acetyl-CoA + CO_2 +2Fd(red)	Advantages: - Can produce H_2 all day long without light - Can utilize different carbon source like, starch, cellobiose, sucrose. - Produces valuable metabolites such as butyric acid, lactic acid - Anaerobic process Disadvantages: - Require C and N-sources for growth - Fermented broth will cause water pollution problem - CO_2 present in the gas

Photobiological production of H_2 by microorganisms has attracted public interest due to its potential as a renewable energy carrier which can be produced using nature's plentiful resources, solar energy and water. Cyanobacteria are one of the groups of microorganisms suitable for the photobiological production of H_2 (Hansel and Lindblad, 1998). They can grow in the simple nutrient, with the ability to fix CO_2

from atmosphere as carbon source, many strains are able to reduce atmospheric dinitrogen to ammonia and utilize sunlight as only source of energy to produce H_2. In this study, we focused on biohydrogen production by using cyanobacteria.

1.4 Cyanobacteria

Cyanobacteria (also called blue-green bacteria or blue-green algae) comprise a large and diverse group of oxygenic photoautotrophic prokaryotes. The name "cyanobacteria" comes from the color of the cell (Greek word: kyanós which means blue). They were classified in the phylum Cyanophyta. The fossil traces of cyanobacteria were found in sedimentary rocks formed 3.5 billion years ago, and ancestors of cyanobacteria most probably played a key role in the contributing oxygen to the atmosphere (Schopf, 2000). Some cyanobacteria can form symbiosis with a wide diversity of hosts; it is believed that ancestors of cyanobacteria evolved to become plastids after a long period of endosymbiosis. In biochemical and structural detail, cyanobacteria are especially similar to the chloroplasts of red algae (Douglas, 1994; Meeks, 1988; Rai *et al*., 2000).

Cyanobacteria constitute an extremely diverse group of prokaryotes which have colonized a wide range of habitats, in almost all environments, including freshwater, seawater, hot spring and deserts. Sometime, cyanobacteria may display a variety of colors due to different combinations of photosynthetic pigments chlorophyll *a*, carotenoids, and phycobiliproteins. However, cyanobacteria have no chloroplasts then the photosynthetic pigments are arranged on thylakoid membranes in cytoplasm.

The classifiable distinguishing of different species of cyanobacteria depends on morphological differentiation and biochemical characteristic (Whitton and Potts, 2000). The external morphology of cyanobacteria shows various forms such as

unicellular, filamentous, and colonial forms. Some filamentous colonies show the ability to differentiate into four different cell types; 1) Vegetative cell, is the normal cell contains the most essential metabolites, carboxysome aggregated the Calvin-cycle enzyme, ribulose biphosphate carboxylase (Rubisco) for CO_2 fixation and photosynthetic pigments which located in thylakoids inside the cell (Castenholz and Waterbury, 1989). 2) Homogonia, which involved in mobility and symbiosis. Some cyanobacteria such as species of the non heterocystous order Oscillatoriales can produce homogonia, which these mobile filaments formed in response to different environmental stresses. 3) Akinete, is spore-like resting cell found in some heterocystous filamentous cyanobacteria, such as *Anabaena cylindrica* but not found in *Anabaena* sp. PCC 7120 it is usually larger size than vegetative cell and has a thicker cell wall. Germination of akinete occurs when the environmental stress (light intensity or phosphate availability) (Meeks *et al.*, 2002). 4) Heterocyst, is specialized cell that differentiated from vegetative cell when cell was grown under nitrogen limitation. The heterocysts are basically anaerobic cells due to (1) they lack PSII resulting no activity of O_2-evolution, (2) heterocyst has a thick envelope cell wall consisting of an inner layer composed of glycolipids and an outer layer composed of polysaccharides protecting the rate of oxygen diffusion, and (3) they have an increased level of respiration (Meeks *et al*., 2002; Meeks and Elhai, 2002; Tamagnini *et al*., 2007). Heterocyst-forming strains such as *Anabaena* and *Nostoc s*pecies have the ability to fix N_2 gas into ammonia (NH_3), nitrites (NO_2^-) or nitrates (NO_3^-) which can be absorbed by plants and converted to protein and nucleic acids because atmospheric N_2 cannot be used by plants directly.

Rippka *et al* (1979) divide the cyanobacteria into the five sections coincide broadly with orders of other classifications: Chroococcales, Pleurocapsales,

Oscillatoriales, Nostocales, and Stigonematales. However, the data available for cyanobacteria are still scarce. Analysis of *16S* rRNA and different molecular methods are being used and developed to study phylogenetic relationships and genotypic characterizations of cyanobacteria. A milestone in the study of cyanobacteria was the publication of the entire genome (3,573,470 bp) of the non-nitrogen-fixing unicellular cyanobacterium *Synechocystis* strain PCC 6803 (Kaneko *et al*., 1996). Until now, a number of other cyanobacterial genome projects are being completed for example, *Anabaena* strain PCC 7120, *Nostoc puntiforme* (ATCC 29133, PCC 73102) etc.

1.4.1 N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012

The model organism was used to study the ability of biohydrogen production in this research, *Anabaena siamensis* TISTR 8012, is a filamentous heterocystous cyanobacterial strain belonging to the class Cyanophyceae, order Nostocales. The *A. siamensis* originally was isolated from a rice paddy field in Thailand (Antarikanonda, 1982a). Nowadays this strain is used in the Thai agricultural market as an algal bio-fertilizer for rice fields because it has a high capacity to fix atmospheric N_2 with subsequent increase of the rice growth rate. This strain also adapts well to temperature fluctuations (25 to 42°C) prevailing in rice fields due to the fact that Thailand is the tropical country with warm temperature and high intensity of sunlight in all year round. Thomas *et al* (1990) created mutant strain of *A. siamensis* by ethyl methanesulfonate mutagenesis to enhance ammonium excretion in *A. siamensis* for algal bio-fertilizer. In addition, *A. siamensis* has been reported to have a high selectivity for metals (Cd) adsorption in hard water when using NaOH-treated cells (Nagase *et al*., 1997). Moreover, this strain is considered to be one of good candidates for the bioreactor of CELSS (Controlled Ecological Life Support Systems)

because it is very nutritious, easy to grow and resistant to change in environmental condition. Furthermore, its physiological characteristics are useful in the food production and air recycle in space station to create a regenerative environment that can support and maintain human life via agricultural means and can solve the problem of extreme habitats on ground (Wang *et al*., 2006). Although, the H_2 yield of *A. siamensis* has not been reported, from the reviews above *A. siamensis* is a good model organism to study the efficiency of H_2 production because it has a potential for N_2-fixing by using sunlight as energy source.

1.5 Photosynthesis and H_2 production in N_2-fixing cyanobacteria

Cyanobacteria are photoautotrophic organism that can use sunlight as the energy source to synthesize food directly from CO_2 and water through oxygenic photosynthesis process. The thylakoid membrane in cyanoplasm of cyanobacteria contains pigment molecules such as chlorophyll *a*, phycocyanin and allophycocyanin which are used to absorb light energy (i.e. photons) for photosynthesis. The oxygenic photosynthesis involves the use of water as an electron donor, leading to the generation of O_2 molecule. Figure 1 shows the schematic mechanisms of oxygenic photosynthesis and H_2 metabolism in heterocystous filamentous cyanobacterial strain. The oxygenic photosynthesis is found in normal vegetative cell. The chlorophyll *a* molecule within reaction center of photosystem II (PSII) absorbs light energy (photons) at 680 nm, an electron within these molecules is promoted to a higher energy level and is shuttled through a linear electron transport chain to the terminal electron acceptor of ferredoxin (Fd) via plastoquinone pool (PQ/PQH_2), cytochrome b_6f complex (Cyt b_6f), plastocyanin (PC), and photosystem I (PSI). The pigment in PSI can absorb the light at 700 nm which further raises the electron to reduce

ferredoxin (Fd). PSII receives electron from the splitting of water which generates O_2 as by product. The proton gradient formed across the thylakoid membrane drives adenosine triphosphate (ATP) production using ATP synthase. Therefore, reduced Fd acts as a central hub of the electron flow, donating electrons to biochemical pathways in vegetative cell including Fd-NAD(P)$^+$ oxidoreductase (FNR) to generate NAD(P)H which is subsequently consumed in the Calvin–Benson cycle for CO_2 fixation. The electrons can be siphoned to respiratory terminal oxidase such as cytochrome c oxidase (Cyt ox) for generation of water, and to generate strong reductant NAD(P)H for H_2 production via bidirectional hydrogenase (Hox). For N_2 fixing cyanobacteria, heterocyst lacks PSII, electrons can be shuttled back to the PQ pool via PSI (i.e. cyclic electron transfer). The electrons and ATP molecules are obtained from either cyclic electron transfer or the catabolism of carbohydrates. These electrons can be transferred to nitrogenase for H_2 production in heterocyst cell (Madamwar *et al*., 2000; Lubitz *et al*., 2008; Manish and Banerjee, 2008; Ghirardi *et al*., 2009). Interestingly, N_2-fixing cyanobacteria are suitable for photobiological H_2 production. They can use solar energy to split water and shuttle electrons through the electron transport chain to the terminal electron acceptor ferredoxin (Fd) via plastoquinone pool. This will generate strong reductants such as NADPH and reduced ferredoxin, which can be utilized as substrates for H_2 production by either nitrogenase or hydrogenase.

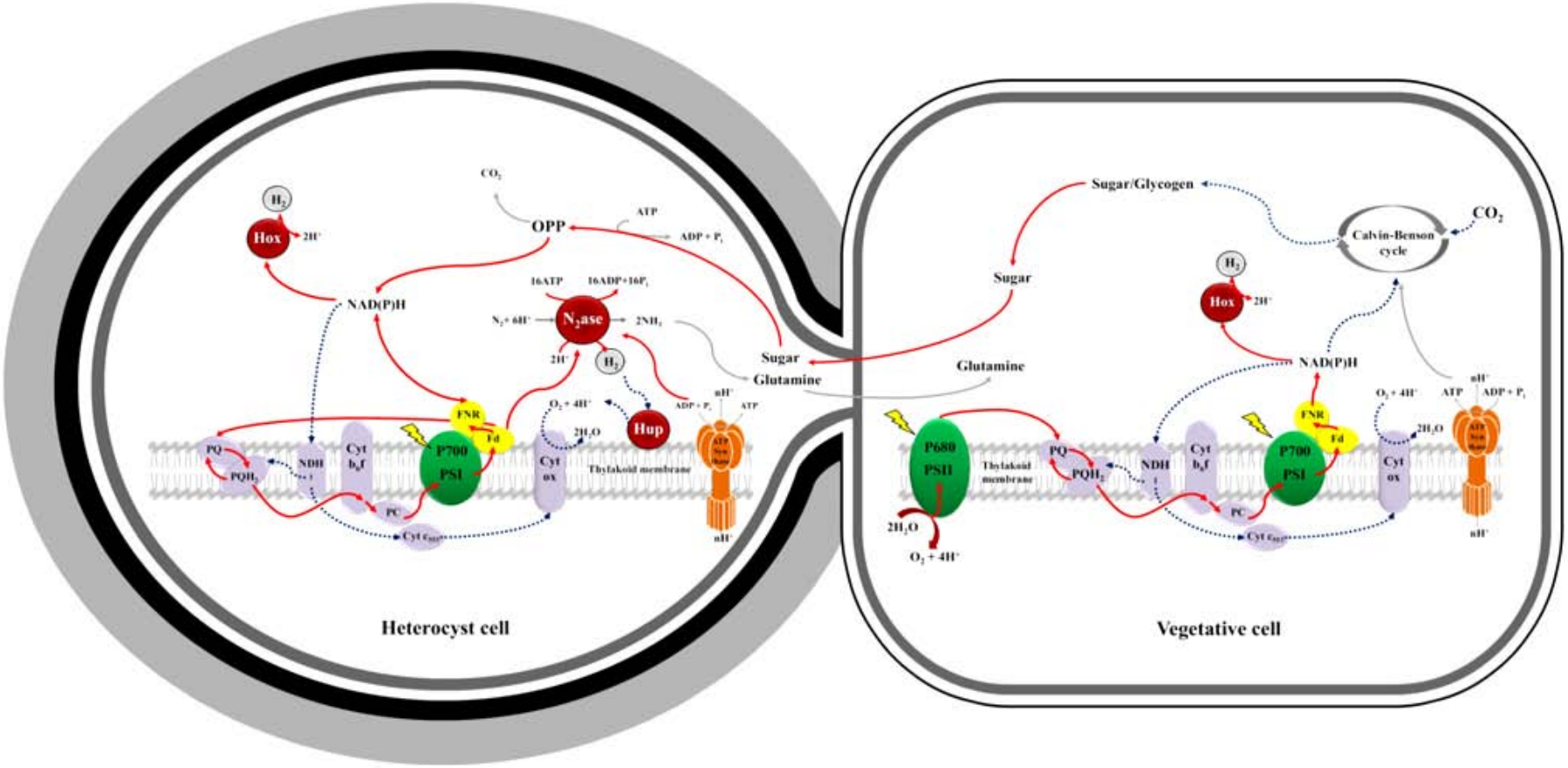

Figure 1 The schematic mechanisms of photosynthesis and H_2 metabolism in heterocystous filamentous cyanobacterial strain. Heterocyst imports carbohydrates from vegetative cell to generate energy and supply reducing power for N_2-fixation. In turn, it exports glutamine to the vegetative cell. The electrons and ATP required for H_2 production via nitrogenase (N_2ase) and bidirectional hydrogenase (Hox) are gained by either the photosyntic oxidation of water or carbohydrate catabolism (red line).

1.6 N_2- fixation in heterocystous filamentous cyanobacteria

The heterocystous filamentous cyanobacteria in the order of Nostocales such as *Anabaena* PCC 7120, *Nostoc punctiforme* and *A. siamensis*, for example, have the ability to fix dinitrogen (N_2) from atmospheric to produce ammonia when grown under nitrogen limitation. The N_2-fixing reaction is catalyzed by nitrogenase in which the reaction requires metabolic energy in the form of ATP and source of reducing power. The overall reaction can be written as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Moreover, in the absence of N_2 (e.g. under anaerobic condition), all the electrons are allocated to reduce proton ($H^{+)}$ into H_2 as follows:

$$2H^+ + 2e^- + 4ATP \longrightarrow H_2 + 4ADP + 4P_i$$

However, nitrogenase is irreversibly inactivated by O_2. To protect the nitrogenase, many heterocystous filamentous cyanobacteria have evolved mechanisms by differentiation of vegetative cell into specialized cell for N_2 fixation, the heterocyst cell. The heterocyst provides a microanaerobic environment suitable for the function of nitrogenase since "(i) it lacks PSII activity, therefore does not produce O_2 as a by-product, (ii) it has a higher rate of respiratory O_2 consumption, and (iii) it is surrounded by a thick envelope that limits the diffusion of O_2 through the cell wall" (Fay, 1992; Wolk *et al.*, 1994). Some filamentous cyanobacteria are able to differentiate 5-10% of their vegetative cells into heterocysts depended on species. The connection between the vegetative cell and heterocyst is narrow pore and occurs through microplasmodesmata. Under aerobic growth condition, their vegetative cells perform photosynthetic O_2 evolution and CO_2 fixation, whereas in heterocyst CO_2

fixation is absent because it lacks the key enzyme, ribulose bisphosphate carboxylase (Rubisco) (Stainier, 1988). Therefore, the heterocysts have to import carbohydrates or many sugars from vegetative cells using oxidative pentose phosphate (OPP) pathway for carbohydrate degradation to generate energy and supply reducing power for N_2-fixation. In turn, heterocysts export glutamine as N-source to the vegetative cells (Thomas *et al*., 1977; Currati *et al*., 2002) as shown in Figure 1. The heterocyst differentiation process in filamentous cyanobacteria is still not complete by understood; most studies have been performed in *Anabaena* PCC 7120. One of key genes involved in heterocyst differentiation, *ntcA*, encodes the transcription factor NtcA which is a global nitrogen regulator in cyanobacteria. NtcA is required for the development and function of mature hetercocysts. NtcA can activate the expression of *hetR*, which encodes a serine-type protease with DNA-binding activity. HetR is expressed early during heterocyst differentiation and is crucial to the differentiation process. Mutation in the *hetR* gene inhibits early steps in the differentiation process while over expression of the gene gives rise to multiple heterocysts (Buikena and Haselkorn, 2001).

1.7 Enzymes involved in H_2 metabolism in cyanobacteria

In cyanobacteria, there may be three enzymes directly involved in H_2 metabolism: nitrogenase catalyzes the N_2-fixation from atmospheric N_2 to produce ammonia and concomitantly produces H_2 as a by-product, uptake hydrogenase catalyzes the consumption of the H_2 produced by the nitrogenase and bidirectional hydrogenase catalyzes both consumption and production of H_2 as shown in Figure 2.

1.7.1 Nitrogenase

The heterocystous filamentous cyanobacteria such as *Anabaen*a species and some unicellular cyanobacteria, *Synechococcus* sp., *Cyanothece* sp. *Gloeothece* sp. contain nitrogenase (Mitsui, *et al*, 1986; Maryan, *et al*., 1986; Reddy, *et al*., 1993). Nitrogenase is a multiprotein emzyme complex consisting of two proteins: the dinitrogenase (MoFe protein or protein I) and dinitrogenase reductase (Fe protein or protein II). The dinitrogenase is a heterotetramer $\alpha_2\beta_2$ with a molecular weight of about 220 to 240 kDa comprising α and β subunits encoded by *nifD* and *nifK*, respectively. The function of dinitrogenase is reduction of N_2 bonds leading to the formation of ammonia (NH_3). The dinitrogenase reductase is a homodimer with a molecular weight of about 60 to 70 kDa and encoded by *nifH.* It has an important role for transferring electrons from the external electron donor to the dinitrogenase protein (Orme-Johnson, 1992). There are different types of nitrogenase depending on the metal cofactor in the catalytic site of the nitrogenase complex (Wall, 2004), as follows:

Mo-nitrogenase : $N_2 + 8H^+ + 8e^- \longrightarrow 2NH_3 + H_2$ (1)

Fe-nitrogenase : $N_2 + 21H^+ + 21e^- \longrightarrow 2NH_3 + 7.5H_2$ (2)

V-nitrogenase : $N_2 + 12H^+ + 12e^- \longrightarrow 2NH_3 + 3H_2$ (3)

This enzyme catalyzes the reduction of atmospheric N_2 to ammonia and is also responsible for the reduction of protons (H^+) into H_2 (Eqs. (1)-(3)). In the absence of N_2 substrate, nitrogenase may exclusively catalyze H_2 production and high potential-energy electrons. However, H_2 production by nitrogenase requires considerable amount of electrons, reductants and a mimimum of 16ATP molecules provided from photosynthesis or by carbohydrate degradation in the cell.

1.7.2 Uptake hydrogenase

An uptake hydrogenase has been found in all N_2-fixng cyanobacteria (Tamagnini *et al*., 2000). It is a heterdomeric enzyme consisting of at least two subunits, HupS (small subunit) and HupL (large subunit). The large subunit of about 60 kDa, encoded by *hupL*, contains the active site, consisting of four conserved cystein residues that are involved in the coordination of the metallic NiFe at center of the active site. The small subunit of about 35 kDa, encoded by *hupS*, contains three FeS clusters, which has a function in transferring electrons from active site on to electron acceptor connected to electron transport chain (Houchins, 1981; Lindblad and Sellstedt, 1990). The function of the uptake hydrogenase is recycling of H_2 produced by nitrogenase which has been suggested to have at least three advantages for organism by Bothe *et al* (1982) and Smith (1990); "(1) it provides additional reducing equivalents to PSI and various cell functions, (2) it provides ATP from oxyhydrogen reaction and, (3) it protects inactivation of hydrogenase by removing O_2 from nitrogenase"

The structural *hupS* and *hupL* genes have been cloned and sequenced in many cyanobacteria such as *Anabaena* PCC 7120, *Nostoc* sp. PCC 73102, *Anabaena variabilis* ATCC 29413 and *Gloeothece* sp. ATCC 27152 (Carrasco *et al*., 1995; Oxelfelt *et al*., 1998; Happe *et al*., 2000; Oliveira *et al*., 2004). The *hupS* is normally located upstream of the *hupL*. The RT-PCR analysis revealed that *hupS* and *hupL* are co-transcription and the enhanced transcription level was found when cells were grown under N_2-fixing condition or addition of external Nickel (Ni) in culture medium. In 2006, Phunpruch and co-worker have successfully cloned and sequenced *hupSL* of N_2-fixing cyanobacterium *A. siamensis*. The predicted gene products for *hupS* and *hupL* consist of 320 and 531 amino acids, respectively. Their deduced

amino acid sequence showed higher than 90% and 80% similarity for HupS and HupL, respectively when compared to other cyanobacteria.

1.7.3 Bidirectional hydrogenase

The bidirectional hydrogenase is a heteropentameric, NAD^+-reducing enzyme, encoded by *hoxEFUYH*. It consists of two protein complexs; hydrogenase complex (HoxY and HoxH) and a diaphorase complex (HoxE, HoxF and HoxU). The large subunit, HoxH contains the active metal NiFe center which is similar to the uptake hydrogenase. The bidirectional hydrogenase is commonly found in both N_2-fixing and non-N_2-fixing cyanobacteria (Kentamich *et al*., 1991; Ghirardi *et al*., 2007) which catalyzes both consumption and production of molecular H_2. The physiological function of this enzyme is not totally clear. Appel *et al* (2000) suggested that the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 acts as an electron valve during photosynthesis since inactivated HoxH resulted in oxidation of PSI and caused a higher fluorescence of PSII when compared to wild type. Moreover, this enzyme has been proposed to be a mediator in the release of excess reducing power under anaerobic condition (Troshina *et al*., 2002). Studies in *Synechocystis* PCC 6803 found that the enzyme was insensitive to light, reversibly inactivated by O_2 and could be quickly reactivated by NADH or NADPH (Cournac *et at*., 2004).

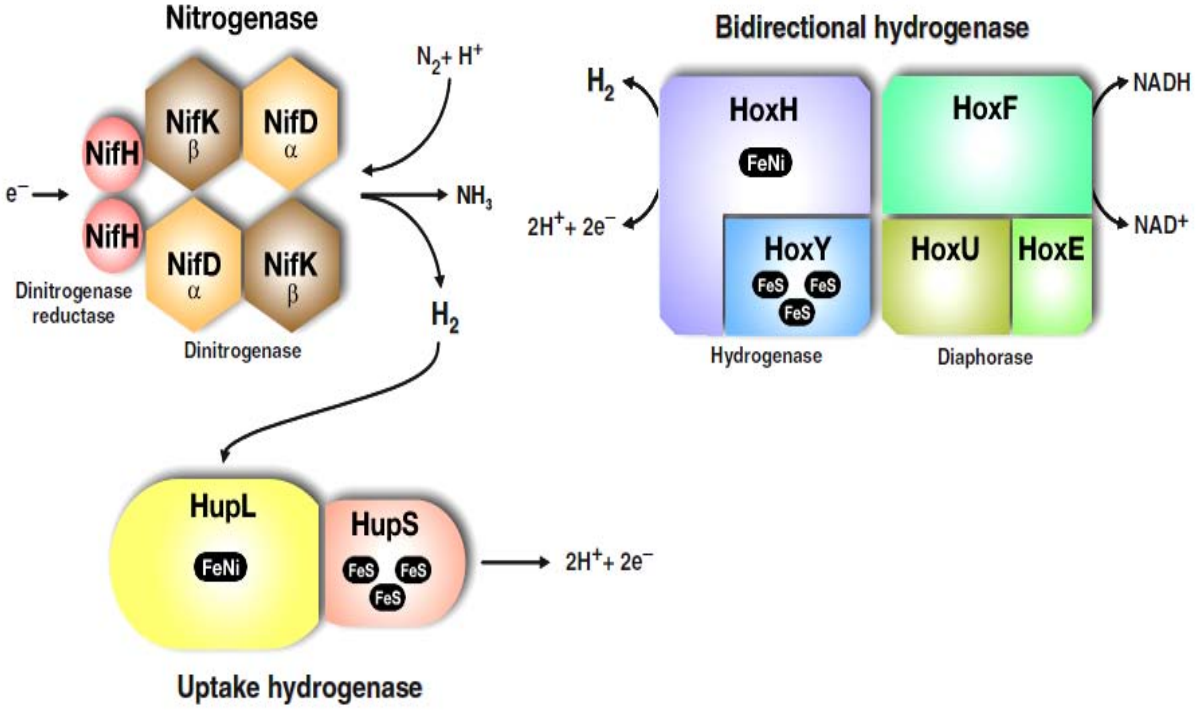

Figure 2 Schematic picture of the enzymes involved in H_2 metabolism in cyanobacteria. Nitrogenase catalyzes N_2-fixing from atmospheric to produce ammonia and H_2 as a by-product. The produced H_2 is consumed by the uptake hydrogenase. The bidirectional hydrogenase can either consume or produce molecule of H_2 depending on the redox potential. (Modified from Tamagnini *et al*., 2002 and Ghirardi *et al*., 2007)

1.8 Reviews of biohydrogen production in cyanobacteria

Most of the researches on biohydrogen production in cyanobacteria have been performed in heterocystous filamentous cyanobacteria through the activities of nitrognase and bidirectional hydrogenase, with a maximum H_2 production rates ranging from 0.17 to 4.2 μmol H_2 mg chl a^{-1} h^{-1} in wild type strain under anaerobic condition (Masukawa *et al*., 2001) as shown in Table 2. Interestingly, N_2-fixing cyanobacterium *A. variabilis* SPU 003 has a capacity to produce H_2 in darkness (Shah *et al*., 2001).

In addition, several marine cyanobacterial strians have a potential for hydrogen production such as *Oscillatoria brevis*, *Calothrix scopulorum*, and *Calothrix membranacea* (Lambert and Smith, 1977). The tested strains showed maximum H_2 production between 0.108 and 0.168 μmol H_2 mg dry $wt^{-1}h^{-1}$ under Ar and CO_2 (3%) atmosphere as shown in Table 3.

H_2 production has also been performed in unicellular non N_2-fixing cyanobacteria as shown in Table 4; these strains can produce H_2 gas via activity of bidirectional hydrogenase and the highest H_2 production was found in *Gloebacter* PCC 7421 with a rate of 1.38 μmol H_2 mg chl a^{-1} $h^{-1}$when incubated under low light (20-30 μE $m^{-2}s^{-1}$) intensity with CO and C_2H_2 (Hawarth and Codd, 1985). According to the review above, it is suggested that the capacity to H_2 production in non-N_2-fixing cyanobacteria is lower than N_2-fixing cyanobacterial strain.

Table 2 Comparison of H_2 production in some heterocystous filamentous cyanobacteria

Organism	**Maximum H_2- production ($\mu molH_2$ mg chl $a^{-1}h^{-1}$)**	**H_2 production condition**
Anabaena sp. PCC 7120	2.6	Ar; 60 μE $m^{-2}s^{-1}$
Anabaena cylindrica IAM M-1	2.1	Ar; 60 μE $m^{-2}s^{-1}$
Anabaena variabilis IAM M-58	4.2	Ar; 60 μE $m^{-2}s^{-1}$
Anabaenopsis circularis IAM M-4	0.31	Ar; 60 μE $m^{-2}s^{-1}$
Nostoc muscorum IAM M-14	0.6	Ar; 60 μE $m^{-2}s^{-1}$
Nostoc linckia IAM M-30	0.17	Ar; 60 μE $m^{-2}s^{-1}$
Nostoc commune IAM M-13	0.25	Ar; 60 μE $m^{-2}s^{-1}$
Anabaena cylindrica UTEX B 629	0.91	Ar; 60 μE $m^{-2}s^{-1}$
Anabaena fos-aquae UTEX 1444	1.7	Ar; 60 μE $m^{-2}s^{-1}$
Anabaena fos-aquae UTEX LB 2558	3.2	Ar; 60 μE $m^{-2}s^{-1}$

Table 3 Comparison of H_2 production in some marine cyanobacteria

Organism	**Maximum H_2 production ($\mu mol\ H_2$ mg dry $wt^{-1}h^{-1}$)**	**H_2 production condition**
Oscillatoria brevis B-1567	0.168	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels
Calothrix scopulorum 1410/5	0.128	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels
Calothrix membranacea B-379	0.108	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels

Table 4 Comparison of H_2 production in some non N_2-fixing cyanobacteria

Organism	Maximum H_2 production ($\mu molH_2$ mg chl $a^{-1}h^{-1}$)	H_2 production condition
Synechococcus PCC 6830	0.26	Ar + CO(13.4 μmol) + C_2H_2 (1.34 mmol); darkness
Synechococcus PCC 602	0.66	Ar + CO(13.4 μmol); 20-30 $\mu Em^{-2}s^{-1}$
Synechoccus PCC 6301	0.09	Ar + C_2H_2 (1.34 mmol); 20-30 $\mu Em^{-2}s^{-1}$
Synechococcus PCC 6307	0.02	Ar + C_2H_2 (1.34 mmol); 20-30 $\mu Em^{-2}s^{-1}$
Microcystis PCC 7820	0.16	Ar(100%); 20-30 μE $m^{-2}s^{-1}$
Gloebacter PCC 7421	1.38	Ar + CO(13.4 μmol) + C_2H_2 (1.34 mmol); 20-30 μE $m^{-2}s^{-1}$
Synechocystis PCC 6308	0.13	Ar + CO(13.4 μmol) + C_2H_2 (1.34 mmol); 20-30 μE $m^{-2}s^{-1}$
Synechocystis PCC 6714	0.07	Ar + CO(13.4 μmol); 20-30 $\mu Em^{-2}s^{-1}$
Aphanocapsa montana	0.40	Ar(100%); 20-30 μE $m^{-2}s^{-1}$

1.9 Strategies for enhanced H_2 production in N_2-fixing cyanobacteria

1.9.1 Optimization of physical parameters for growth and H_2 production

The efficiency of H_2 production depends on different cyanobacterial species and several parameters used for cultivation, such as light, temperature, sources of carbon and nitrogen or even micronutrients. Therefore, the study of optimum

condition for cultivation is necessary for efficient production of H_2 by N_2-fixing cyanobacteria in a large scale.

1.9.2 Improve cyanobacterial strain by using molecular biology technique

The major obstacle for enhanced H_2 production in N_2-fixing cyanobacteria is the consumption of H_2 produced from nitrogenase by uptake hydrogenase activity. Then, the mutant strains created by using genetic engineering techniques to inactivate uptake hydrogenase activity are needed. The previous studies have reported that H_2 uptake-deficient mutants of *A. variabilis* strain AVM13 (Δ*hupSL*) (Happe *et al*., 2000), *N. punctiforme* strain NHM5 (Δ*hupL*) (Lindberg *et al*., 2002), *Anabaena* sp. strain AMC 414 (Δ*xisC*) (Carrasco *et al*., 2005), *Anabaena* PCC 7120 (Δ*hupL* and Δ*hupL*/ Δ*hoxH*) (Masukawa *et al*., 2002) and *Nostoc* sp. PCC 7422 (Δ*hupL*) (Yoshino *et al*., 2007) have an ability to produce H_2 with higher rate than the respective wild types.

The aim of this project is to investigate various factors affecting bio-energy production, namely hydrogen, using N_2-fixing cyanobacterium *A. siamensis*. TISTR 8012. This cyanobacterium has high potential for hydrogen production which is attributed to the activities of two enzymes, nitrogenase and bidirectional hydrogenase. The project consists of two main steps. The first step deals with the optimum conditions for growth of the cyanobacterium yielding highest rates of H_2 production via the increase in the amounts of the two enzymes. The other step focuses on treatments leading to enhanced activities of the two enzymes. In addition, factors causing the reduction of the activity of another enzyme, uptake hydrogenase, will also be studied in order to maximize H_2 production. It is expected that the results obtained from this project will lead to further development for the production of biohydrogen

in an expanded scale, thus serving as a renewable and clean energy source for the future.

Objectives of this research are:

1. To investigate the optimum conditions for growth and hydrogen production by N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012
2. To monitor the activitiy of enzymes directly involved in H_2 metabolism
3. To study the competency of hydrogen production by uptake hydrogenase deficient mutant strain

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave	Model HA-30, Hirayama Manufactering Cooperation, Japan
Bead Beater	Hamilton Beach**,** Model 1G 918, USA
Balances	Model AB204-S, Mettler Toledo, Switzerland
Centrifuge	Jouan MR 1812, USA SORVALL® MC 12V DUPONT, USA HERMLE Z233 MK, USA
Fluorescence spectrophotometer	Perkin-Elmer luminescence spectrometer LS 55, England
Electrophoresis Unit	BIO-RAD PROTEIN® II xi Cell, USA
Gas Chromatography	Model GC-15A, Shimadzu, Japan, Clarus 500, USA, Perichrom PR2100, France
Geldoc® enabled	DNA visualization UV light, USA
O_2/H_2-electrode	Hansatech, Norfolk, UK
Laminar flow	BVT-124 International Scientific Supply, Thailand
Microcentrifuge	Model 5417C, Eppendorf, Germany

PCR apparatus	PERKIN ELMER DNA Thermal Cycler, Japan
pH meter	METTLER TOLEDO, Switzerland
Photometer	LI-COR Model LI-185B, USA
Power supply	BIO-RAD POWER PAC 1000, USA BIO-RAD Model 1000/500
Shaker Innova™	2100 PLATFORM SHAKER, USA
Spectrophotometer	SPECTRONIC® GENESYS™2, USA Jenway UV/VIS 6400, USA
Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.1.2 Chemicals

Acetic acid	BDH, England
Agarose	Promega Corporation, USA
β-mercaptoethanol	Sigma, USA
Brilliant blue	Sigma, USA
Bromophenol blue	Sigma, USA
Chloroform	Merck, Germany
EDTA	Sigma, USA
Ethanol	Scharlau Chemie S.A., Spain
Ethidium bromide	Sigma, USA
Ferredoxin	Sigma, USA
Glucose	Sigma, USA

Glycerol	Scharlau Chemie S.A., Spain
Isopropanol	Sigma, USA
Methanol	Scharlau Chemie S.A., Spain
Methyl viologen	Sigma, USA
N-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)	Sigma, USA
Phenol	Merck, Germany
Sodium bicarbonate	BDH, England
Sodium chloride	APS, Australia
Sodium citrate	Sigma, USA
Sodium dithionite	Sigma, USA
Sodium dodecyl sulfate	Sigma, USA
Sodium fluoride	Sigma, USA
Sodium thiosulfate	Sigma, USA
Sorbitol	Sigma, USA
Sucrose	Sigma, USA
TES sodium salt	Bio basic inc, Canada
Tris base	USB Corporation, USA
Tris HCl	Sigma, USA
Triton X-100	Packard, USA
Trizol® reagent	Invitrogen, USA

2.1.3 Enzymes

Klenow polymerase	Invitrogen, USA
Lysosyme	Sigma, USA
Restriction enzymes	Fermentas, Canada
Shrimp Alkaline Phosphatase (SAP)	Fermentas, Canada
Taq DNA polymerase	Invitrogen, USA

2.1.4 Antibiotics

Ampicillin	Sigma, USA
Chloramphenicol	Sigma, USA
Kanamycin	Bio basic inc, Canada
Neomycin sulfate	Bio basic inc, Canada

2.1.5 Kits and suppliers

λ DNA/*Hind*III Marker	Fermentas, Canada
GeneRuler™ 1 kb DNA Ladder	Fermentas, Canada
GeneRuler™ 100 bp DNA Ladder	Fermentas, Canada
Mixed cellulose ester filter	Merck, Germany
Nylon membrane filter 0.45 and 0.22 μm	Sartorius, Germany
PCR purification kit	Machery-Nagel, USA
NucleoSpin® Extract II	
Plasmid extraction Kit	Fermentas, Canada
Quick ligation™ Kit	BioLabs
iScript™ cDNA synthesis Kit	Bio-RAD, USA

2.1.6 Organisms

2.1.6.1 Cyanobacterium strains: The filamentous cyanobacterium *Anabaena siamensis* TISTR 8012, originally isolated from a rice paddy field in Thailand, was obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand and the other cyanobacterial strains, *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133 and *Synechocystis* sp. PCC 6803 were obtained from Department of Photochemistry and Molecular Science, The Ångström Laboratory, Uppsala University, Uppsala, Sweden.

2.1.6.2 *Escherichia coli*: The two strains of *E. coli* were obtained from Department of Photochemistry and Molecular Science, The Ångström Laboratory, Uppsala University, Uppsala, Sweden.

Strain DH5α (F- φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-, mk^+), *pho*A *sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1) was used for DNA manipulation.

Strain HB101 (F^-, *hsdS2*0(rB-, mB-), *xyl*5, λ^-, *rec*A13, *gal*K2, *ara*14, *sup*E44, *lac*Y1, *rps*L20(str^r), *leu*B6, *mtl*-1, *thi*-1 was used for DNA manipulation.

2.1.7 Plasmids

The plasmids used in this study are shown in Table 5. Some circle maps are shown in APPENDIX.

Table 5 Plasmids used in this study

Plasmids	**Relevant characteristic(s)**	**Sources or references**
pGEM-T easy	Cloning vector, Apr *lacZ', mcs*	Promega
pRL271	Cloning vector carrying *sacB*, Em and Cm	GenBank accession no L05081
pUC4K	Source of Nm cassette	Amersham
pRL632	Helper plasmid carrying metylates *AvaI*, *AvaII* and *AvaIII* sites	Elhai and Wolk (1988) personal communication
pRL443	Conjugative plasmid, Km spontaneous mutant of RK2	Elhai and Wolk (1988)
pGhupS	pGem-T easy vector contained *hupS* gene	This study
pGhupSNm	Nm cassette inserted into *Eco*RV site within *hupS* gene of pGhupS plasmid	This study
pRLhupSNm	pRL271 containing 2.2 kb hupSNm fragment of pGhupSNm	This study

2.1.8 Oligonucleotides

Table 6 Oligonucleotides used in this study

Primers	**Sequence 5′ to 3′**	**Purpose of primer**	**PCR product size (bp)**
16SF2	atgcttaacacatgcaagtcgaa	PCR for *16S* rDNA	1407
16SR2	cccagtcaccagtcctgcctt		
HupSF2	gcatgcatgactaacgtactctggct	PCR for *hupS*	992
HupSR2	gcatgcgtctccattcccattaccta		
pUC4KF	acgcgttgaggtctgcctcgtgaagaa	PCR for Neomycin cassette	1221
pUC4KR	acgcgtaaagccacgttgtgtctcaaa		
ASnifDF1	tcgtattcggtggtgacaaa	RT-PCR for *nifD*	204
ASnifDR1	gagacacaccacggaaacct		
AShoxHF1	gaatccgtctgcgtcaattt	RT-PCR for *hoxH*	284
AShoxHR1	gcaaatgtccgtcgtaggtt		
AShupLF1	ggcgatttagatgtccgtgt	RT-PCR for *hupL*	240
AShupLR1	taaattccgcgccaaaatag		
23F	gctaagcgatgtaccgaagc	RT-PCR for *23S* rDNA	200
23R	taacccagagtggacgaacc		
PsaAF1	ctgttgaaaggtgtattgtt	RT-PCR for photosystem I,	489
PsaAR1	aggagctaccttcagtttat	core protein A1	
PsbAF1	gcacattcaactttatgatt	RT-PCR for photosystem II,	390
PsbAR1	ccaaaattgagttattgaag	D1 protein	

Table 6 Oligonucleotides used in this study (continued)

Primers	Sequence 5′ to 3′	Purpose of primer	PCR product size (bp)
FdxHF1	atggctagctaccaagttag	RT-PCR for heterocyst	299
FdxHR1	ttaagcaaggtacggttctt	ferredoxin	
CoxAF1	gcgagattacttcagtttta	RT-PCR for cytochrome c	426
CoxAR1	atccaaataccttctcctac	oxidase	
NtcAF1	cgagtctactttcttttgaa	RT-PCR for nitrogen	354
NtcAR1	aaaatcacgacagagaatta	regulator, *NtcA*	
HetRF	ggatgaccggacatttgcac	RT-PCR for primary	321
HetRR	ccataagcgatcgcaagagg	activator heterocyst	

2.2 Methods for cyanobacterial strains

2.2.1 Growth condition

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 cells were grown in 50 mL of different media namely, BG11 (Rippka, 1979), $BG11_0$ without N-source, Allen–Arnon and N-free, respectively. The composition of nutrients in each medium is shown in Table 7. The other cyanobacterial strains, *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133 and *Synechocystis* PCC 6803 cells were cultured in 50 mL of $BG11_0$ medium (without N-source) and BG11 medium containing 18 mM $NaNO_3$ as N-source, both media were buffered with 20 mM HEPES-NaOH (pH 7.5). For Δ*hupS* mutant strain, cell was grown in either BG11 or $BG11_0$ media containing 25

mg mL^{-1} neomycin antibiotic. The initial cell concentration was adjusted to an OD_{730} of 0.1 and cultures were incubated aerobically under continuous illumination of 40 $\mu Em^{-2}s^{-1}$ with cool white fluorescent lamps from two sides on a rotatory shaker at 160 rpm and 30 °C. The growth rate was monitored by measuring the optical density of the culture at 730 nm with a spectrophotometer. The doubling time (DT) value was calculated using the equation formula below where $OD_{730(f)}$ is the final optical density and $OD_{730(i)}$ is the initial optical density.

$$DT = \text{time of incubation} \times \log 2 / \log (OD_{730(f)} / OD_{730(i)})$$

2.2.2 Cell adaptation under various conditions

The cells were pre-cultivated in $BG11_0$ medium at 160 rpm, 30 °C under continuous illumination of 40 $\mu E\ m^{-2}\ s^{-1}$ until reaching log phase. After that, cells were centrifuged and ressuspended twice with 50 mL of fresh $BG11_0$ medium and adapted in various conditions for 24 h before determining H_2 production.

2.2.2.1 Various pH: Cells at log phase were adapted in $BG11_0$ medium and adjusted pH with Universal buffer (Britton and Robinson, 1931) ranging from 3.5 to 12.0 and at the pH 7.5 which was adjusted with different four buffer types, TES, HEPES, K-phosphate and Tris-HCl at concentration of 10 mM to study effect of pH on H_2 production.

2.2.2.2 Various temperature: Cells at log phase were adapted in $BG11_0$ medium and incubated at various temperature ranging from 25 to 45 ºC to investigate effect of temperature on H_2 production.

2.2.2.3 Variation of N-source and concentrations: Cells at log phase were adapted in $BG11_0$ medium containing various N-sources, $NaNO_3$, $NaNO_2$ and NH_4Cl at the same concentration of 17.6 mM to study effect of N-source on H_2 production. The various concentrations of $NaNO_3$ ranging from 0 to 22.5 mM were also tested.

2.2.2.4 Various carbon sources supplementation: Cells at log phase were adapted in $BG11_0$ medium supplemented with various types of sugars such as glucose, galactose, lactose, fructose and sucrose at 0.5 % (w/v) to investigate the effect of various carbon sources supplementation on H_2 production compared with $BG11_0$ and $BG11_0$ without C-source.

2.2.2.5 Various concentrations of fructose: Cells at log phase were adapted in $BG11_0$ medium supplemented with various concentrations of fructose supplemented ranging from 0 to 2.0 % (w/v) were tested.

2.2.2.6 Light intensity and time of light exposure: Cells at log phase were adapted in fresh $BG11_0$ medium incubated under various light intensities at 0, 40, 70, 150, 200, 250 and 300 $\mu E\ m^{-2}\ s^{-1}$, respectively. Times of light exposure for 12, 24 and 48 h were observed at 0, 40 and 200 $\mu E\ m^{-2}\ s^{-1}$ of light intensity.

2.2.2.7 Salt and osmotic stress: Cells at log phase were adapted in $BG11_0$ medium by either supplementation with various salt such as NaCl, KCl, $CaCl_2$ and LiCl at the same concentration of 25 mM or with various concentrations of D-sorbitol at 0, 50, 100, 250 and 500 mM to investigate the effect of salt stress and osmotic stress on H_2 production.

2.2.2.8 Microelement supplementation: Cells at log phase were adapted in $BG11_0$ medium supplemented with different microelements such as cobalt (Co) from $Co(NO_3)_2.6H_20$, molybdenum (Mo) from $Na_2MoO_4.2H_2O$, vanadium (V) from NH_4VO_3, nickel (Ni) from $NiCl_2.6H_20$ and iron (Fe) from $FeSO_4$ to investigate the effect of microelement supplementation on H_2 production. The microelements were added by varying the final concentration in the range 0-6 μM for Mo, V and Ni and 0-100 μM for $FeSO_4$, respectively.

2.2.2.9 Reductant supplementation: β-mercaptoethanol was used to study the effect of physiological reductant on H_2 production under dark and light conditions at concentrations ranging from 0 to 1000 μM.

2.2.2.10 Sulfur derivation: Cells at log phase were adapted in $BG11_0$ medium without sulfur (S) by using $BG11_0$ medium with the following modifications: $MgSO_4.7H_2O$ was replaced with $MgCl_2.6H_2O$, $CuSO_4.5H_2O$ was replaced with $CuCl_2$ and $ZnSO_4.7H_2O$ was replaced with $ZnCl_2.2H_2O$, all in equal molarity.

2.2.2.11 Aerobic and anaerobic conditions: Cells were cultured in 600 mL of $BG11_0$ medium, incubated under growth condition at different times of air limitation. Cell culture was bubbled continuously with either (i) air or (ii) argon gas for 24 h and (iii) with air for 12 h followed by argon for 12 h.

Table 7 Nutrients composition of different culture media for growing cells of *A. siamensis* TISTR 8012

Concentration of nutrients in the four different culture media				
Nutrients	**Type of medium**			
	BG11	**$BG11_0$**	**Allen and Arnon (AA)**	**N-free**
KH_2PO_4	0.29 mM	0.29 mM	19.43 mM	3.40 mM
$MgSO_4.7H_2O$	0.30 mM	0.30 mM	10 mM	1.50 mM
$CaCl_2.2H_2O$	0.24 mM	0.24 mM	5.08 mM	0.54 mM
$Na_2EDTA.2H_2O$	2.70 μM	2.70 μM	0.80 mM	3.40 mM
$FeSO_4.7H_2O$	21 μM	21 μM	0.69 mM	0.035 mM
$NaCO_3$	0.19 μM	0.19 μM	1.90 mM	-
Citric acid	31 μM	31 μM	-	-
H_3BO_3	27 μM	27 μM	27 μM	48 μM
$MnCl_2.4H_2O$	9.10 μM	9.10 μM	9.10 μM	10 μM
$ZnSO_4.7H_2O$	0.76 μM	0.76 μM	0.76 μM	1 μM
$Co(NO_3)_2.6H_2O$	0.17 μM	0.17 μM	0.17 μM	0.07 μM
$Na_2MoO_4.2H_2O$	1.60 μM	1.60 μM	1.60 μM	32 μM
$CuSO_4.5H_2O$	0.30 μM	0.30 μM	0.30 μM	0.32 μM
$NaNO_3$	17.6 mM	-	-	-
NaCl	-	-	-	1.2 mM

2.2.3 Chlorophyll *a* measurement

The total amount of chlorophyll *a* (Chl *a*) was extracted from cyanobacterial cells by using 90% (v/v) methanol (Mackinney, 1941), centrifuged at 14,000 rpm for 5 min and removed the green supernatant to measure the absorbance at 665 nm using a

blank made of 90% (v/v) methanol and 10% (v/v) of BG11. The chlorophyll *a* content was calculated by following the formula below where value of 12.7 is based on the extinction coefficient of 78.74 L g^{-1} cm^{-1}.

$$\text{Chl } a \text{ content } (\mu g\ mL^{-1}) = 12.7 \times A_{665} \times \text{Dilution factor}$$

2.2.4 H_2 production and O_2 accumulation determination

After adapting cells under various conditions, cells were harvested by centrifugation at 5,000 rpm for 10 min at room temperature and resuspended in 2 mL desired medium in a glass vial, sealed with a rubber septum and a proper screw lid. The vial was bubbled with argon gas for 15 min for anaerobic condition and incubated under different conditions before determining H_2 gas. The H_2 and O_2 amount in 100 µL of head space gas sample, withdrawn after incubation time from the vial with a gas tight syringe, were analyzed by a Gas Chromatograph with a Molecular Sieve 5A 60/80 mesh column equipped with a thermal conductivity detector (Perkin-Elmer, USA). The Gas Chromatography condition and calculation of H_2 and O_2 using standard graph were described in APPENDIX K and L. The H_2 production rate was expressed is µmol H_2 mg chl a^{-1} h^{-1} and the O_2 accumulation rate was expressed as nmol O_2 mL^{-1}, respectively.

2.2.5 Bidirectional hydrogenase activity assay

In vivo bidirectional hydrogenase activity was analyzed by measuring the H_2 production in the presence of methyl viologen, which was reduced by sodium dithionite, using a Gas Chromatography. The 8 mL rubber septum sealed vial without O_2 gas by bubbling with argon gas, was injected with 1 mL of 5 mM methyl viologen and 20 mM sodium dithionite in 50 mM K-phosphate buffer (pH 7.5) under anaerobic

condition, the color of reaction mixture was changed from white to blue solution and then injected 1 mL of cells suspension into vial before determining H_2 gas. Activity was expressed as nmol H_2 mg chl a^{-1} h^{-1}.

2.2.6 Uptake hydrogenase activity assay

In vivo uptake hydrogenase activity was measured using a Hansatech DW1 O_2/H_2 electrode (Hansatech Ltd., Norfolk, UK) connected to a LKB 2210 2-channel recorder (LKB-Produkter, Bromma, Sweden) as described previously (Oxelfelt *el al*., 1995). The uptake hydrogenase activity was measured by mixing 0.9 mL of cyanobacterial cells suspension and 0.1 mL of H_2-saturated distilled water in the reaction vial and a magnetic stirrer was used to obtain homogeneous cells suspension. The uptake hydrogenase activity was calculated from the amount of consumed H_2 using known amounts of H_2 as standards as shown in APPENDIX M. Activity was expressed as μmol H_2 mg chl a^{-1} h^{-1}.

2.2.7 Nitrogenase activity assay

In vivo Nitrogenase activity was measured using the acetylene-reduction assay. It can catalyze the conversion of acetylene (C_2H_2) to ethylene (C_2H_4) gas. Reaction was carried out in a glass vial by incubation of the cells suspension (2 mL) with 1 mL of 10% (v/v) acetylene (C_2H_2) balanced in argon. The ethylene (C_2H_4) production was detected by using a Gas Chromatograph with a Porapak Q, 50/80 mesh column equipped with a Flame ionization detector (Shimadzu, Japan). Enzyme activity was expressed as μmol C_2H_4 mg Chl a^{-1} h^{-1}.

2.2.8 Sugar uptake determination

The content of remaining sugar in the growth medium after incubating cells in different sugar sources was determined using the phenol-sulfuric acid assay (Dubois *et al*., 1951). The 200 μL of a clear solution, obtained by filtering 5 mL of samples through a 0.22 μm membrane filter, was mixed with 200 μL of 5% (w/v) phenol reagent followed by the addition of 1 mL of concentrated sulfuric acid. After 10 min of incubation, the mixture was shaken vigorously and left standing for 30 min before measuring the absorbance at 490 nm using a spectrophotometer. Calculate the sugar uptake (in percentage) by comparing with control. The percentage of sugar utilized by the cells represented sugar uptake.

2.2.9 Fluorescence measurement

Fluorescence emission spectra were measured at room temperature in a Perkin-Elmer luminescence spectrometer LS 55 (England). The cells were adjusted to 10 mg chlorophyll *a* per mL by dilution with BG11 medium. Fluorescence was excited at 440 nm and fluorescence emission was recorded in the range 610-750 nm at a speed of 200 nm per min. The spectra were analyzed with FL WinLab program.

2.2.10 Physiological morphology determination

2.2.10.1 Phenotype determination: The morphology of *A. siamensis* TISTR 8012 cells were observed by using a Carl Zeiss Light Microscopy equipped with a digital camera. Image processing was performed using Adobe Photoshop CS4.

2.2.10.2 Cell surface and ultrastructure observation: *A. siamensis* TISTR 8012 cells were prepared for the analysis of cell surface and ultrasructure by

using Scaning Electron Microscope, SEM (JEOL model JSM-5410LV, Japan) and Transmission Electron Microscopy, TEM (JEM-2100, 200KV, Japan).

2.3 Molecular biology methods for cyanobacterial strain

2.3.1 Genomic DNA extraction

The 100 mL of *A. siamensis* TISTR 8012 cells culture at exponential growth phase were harvested by centrifugation at 6,000 rpm, 4 ºC for 10 min. The pellet was resuspended in 10 mL of TES buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA and 10 mM NaCl) and stored at room temperature for 10 min. Sarkosyl (SLS) was added to a final concentration of 0.1%, and kept the sample at 4 ºC for 30-90 min. The filaments were collected by centrifugation at 12,000 rpm for 10 min (the supernatant looked bluish), washed with 20 mL TES buffer, pH 8.0 and resuspended in 2.5 mL of TES; the filaments are often surrounded by a gelatinous sheath (polysaccharide), which may interfere with the enzymatic digestion of the cell wall, the use of 0.1% sarkosyl to clean lipid polysaccharide. After that, the lysozyme, SDS and proteinase K were added to the final concentration of 0.5 mg mL^{-1}, 1% and 50 mg mL^{-1}, respectively for cell lysis. The sample was stirred thoroughly and incubated at 37 ºC for 2 h; the solution should appear sticky when cells are completely lysed. The lysate was added with equal volume of phenol for protein precipitation and kept the sample on ice immediately, then centrifuged at 12,000 rpm at 4 ºC for 10 min and transferred upper aqueous phase into a new micro-centrifuge tube before addition of equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and then centrifuged at 12,000 rpm, 4 ºC for 10 min to separate of the interface and facilitate the recovery of the aqueous phase. The clear supernatant (containing total DNA) was transferred to a new micro-centrifuge tube. After that, equal volume of chloroform: isoamyl alcohol (24:1) was

mixed and centrifuged at 12,000 rpm, 4 ºC for 10 min to remove contaminated protein and transferred upper aqueous phase into new tube, then added 0.1 volume of 3 M sodium acetate and chilled 99% ethanol for DNA precipitation, kept sample at -20 C overnight to increase yields. The pellet of DNA was centrifuged and discarded supernatant. The pellet DNA was washed with chilled 70% ethanol and gentle mixing by inversing and then centrifuged at 12,000 rpm, 4^{o}C for 2 min. The pellet DNA was dried at room temperature and then resuspended with 50 µl of TE-Buffer (10 mM Tris-HCl, 1 mM EDTA), pH 8.0 and checked by monitoring concentration at optical density 260 nm (OD 1.0 = 50 µg mL^{-1}), while the purity was checked by the absorbance ratio A260/A280. DNA sample was run in 0.8% agarose gel electrophoresis with 0.5xTBE buffer pH 8.0.

2.3.2 RNA extraction

The 50 mL of *A. siamensis* TISTR 8012 cells culture in each condition were harvested by centrifugation at 6,000 rpm, 4 ºC for 10 min. The pellet was resuspended in 1 mL of Trizol® reagent in eppendorf tube and added 0.2 g of acid washed glass beads, then disrupted the cells using a bead beater for 2-5 times of repeatation to break the cell. The solution was centrifuged at 12,000 rpm, 4 ºC for 10 min and transferred the cleared solution to a new eppendorf tube and incubated at room temperature for 5 min. The 0.2 mL of chloroform was added, shaked vigorously by hand for 15 sec and incubated at room temperature of 3 min before centrifugation at 12,000 rpm, 4 ºC for 15 min. RNA remaining in the upper aqueous phase was transferred to a new eppendoft tube and added 0.25 mL of isopropanol followed by 0.25 mL of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl). Mixed the solution and incubated the sample at room temperature for 10 min and

centrifuged at 12,000 rpm, 4 ºC for 10 min. RNA precipitate forms a gel-like pellet on the side and bottom of the tube was washed with 2 mL of 75% ethanol. Mixed the sample by vortexing and centrifuged at 12,000 rpm, 4 ºC for 10 min. Remove the ethanol and dissolved the RNA in RNase-free water and incubated at 55 ºC for 5 min. The RNA concentration was calculated using the formula below:

RNA concentration ($\mu g\ \mu L^{-1}$) = A260 x 0.04 $\mu g\ \mu L^{-1}$ of RNA x Dilution factor

2.3.3 Primers design

Primers for amplification were designed by using the program primer3 (v.0.4.0) (Rozen and Skaletsky, 2000).

2.3.4 Phylogenetic tree analysis

PCR primers of 16S rDNA gene were designed by using consensus nucleotide sequences registered in GenBank database. The 16S rDNA partial- sequence of *A. siamensis* TISTR 8012 and other cyanobaterial strain (obtained from GenBank database) were aligned with ClustalX alignment program prior to Bootstrap NJ phylogenetic tree construction, the result was visualize as a radial tree with the interaction phylogenic tree plotting program Treeview (R, 1996).

2.3.5 Polymerase Chain Reaction

The 1X PCR mix (20 µL of reaction) containing 1X Taq buffer $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.5 pmol of each forward and reverse primer and 0.05 U of *Taq* polymerase using 0.1-1 µg of template DNA (or picked colony for colony PCR) and adjusting the volume with sterile DI water, The PCR condition as shown below:

Step 1: Innitial denature	94	ºC	3	min
Step 2: Denature	94	ºC	30	sec
Step 3: Anealing	50-65	ºC	1	min
Step 4: Extension	72	ºC	1	min
Step 5: Go to step 2	29	cycles		
Step 6: Final extension	72	ºC	10	min
Step 7: Hold	10	ºC		

The PCR product was analyzed by electrophoresis.

2.3.6 Transcription analysis

Extracted total RNA from cells in each condition by using the TRI Reagent® was treated with DNase (Fermentas) for DNA digestion. The treated RNA (1µg) was converted to single stranded cDNA with the iScript™ cDNA Synthesis Kit (Bio-RAD), according to the manufacturer's instruction. RT-PCR amplifications using cDNAs of the respective genes were performed using corresponding primers (Table 6) and RT-PCR on DNaseI treated RNA without RT-enzyme as negative controls for the RT-reaction. Negative controls for the PCR reactions were amplified by without cDNA added and positive controls were performed with genomic DNA. The PCR conditions consisted of 95 °C for 3 min, followed by 30 cycles of 95 °C for 15 sec, 50 °C for 20 sec and 72 °C for 20 sec, and then a final extension at 72 °C for 3 min. The PCR product was analyzed by 1.0% (w/v) agarose gel electrophoresis with 1X SB buffer.

2.3.7 Conjugative method for gene transfer into *A. siamensis* cell

The conjugative method can be divided in three steps, first step is cell preparation; 50 mL of *A. siamensis* cell culture was centrifuged at 6,000 rpm, 4 ºC for 10 min. The pellet of cells were resuspended in 2 mL of fresh BG11 medium in sterile falcon tube and kept on ice. Cells suspension were sonicated with ultrasonicatore using amplitude 45 (around 7 W on display), pulse at 1 s, sonicated for 15 sec and kept on ice for 15 sec per times, repeated for 4 times to separate the long filament to a single cell or very short filament (filament integrity was checked by microscopy). After ultrasonicating, cells were incubated under growth condition for at least 4 h before used in next step. Second step is triparental mating; 10 mL of helper strain (*E. coli* strain HB101 containing helper plasmid (pRL 623)) and 10 mL of cargo strain (*E coli* strain HB101 containing both of target gene transfer plasmid (pRLhupSNm) and conjugative plasmid (pRL 443)) were centrifuged at 3000 rpm for 10 min and resuspended each in 10 mL of LB without antibiotic. Mixed the helper and conjugative strains by hand and stored at room temperature for 10 min, then centrifuged and resuspended in 200 μL of LB in micro-centrifuge tube before adding 100 μL of *A. siamensis* cells (13 μg mL^{-1} of chlorophyll *a*) from first step and incubated at 30 ºC under light condition for 1 h before plating. The 300 mL mixture was spreaded on filter membrane BG11 medium without antibiotic and incubated at 30 ºC under light condition for 3 days. Third step is selection of recombinant colony; transferred filter membrane from second step to BG11 plate medium containing neomycin antibiotic at concentration of 25 μg mL^{-1} and incubated under light condition at 30 ºC until the recombinant colonies were appeared. To ensure the complete segregation, obtained gene knockout was analysed by colony PCRs.

2.4 Methods for *E. coli*

2.4.1 Growth condition

E. coli strain DH5α was grown in LB medium at 37 ºC with shaking at 250 rpm using a rotary shaker. The helper strain was grown in LB medium containing 30 μg mL^{-1} of chloramphenicol antibiotic and cargo strain was grown in LB medium containing 25 μg $mL^{-1}$of kanamycin and neomycin antibiotics.

2.4.2 Preparation of competent cells

A single colony of either *E. coli* strain DH5α or HB101 was cultured into 2 mL of LB medium and incubated at 37^{o}C overnight with shaking at 250 rpm on a rotary shaker. A 2 mL of cells was cultured in 125 mL of fresh LB medium, incubated at 37 ºC for 2 h with shaking at 250 rpm until OD_{600} reached to 0.3-0.5. Then, cell culture was centrifuged at 5000 rpm, 4 ºC for 10 min, the cell pellet was gently resuspended in 40 mL of cold CCMB80 buffer and incubated on ice for 20 min before centrifugation at 5000 rpm, 4 ºC for 10 min, removed supernatant and resuspended cell pellet with 10 mL of cold CCMB80 buffer again. After that, 100 mL of cells suspension were aliquoted into pre-chilled sterile micro-centrifuge tube and stored immediately at -80 ^{o}C.

2.4.3 Heat-shock transformation

An aliquot of competent *E. coli* DH5α or HB101 was gently thawed on ice for 5-10 min. A 2 μl of ligation reaction was mixed with the competent cells and kept on ice for 5 min. The cells were transformed by heat-shock at 42 ºC for 1 min, then placed on ice for 2 min followed by adding 900 μl of LB medium and incubated at 37 ºC with shaking at 250 rpm for 1 h. The mixture was spreaded on the LB agar plates containing appropriate antibiotic and incubated overnight at 37 ºC.

CHAPTER III

RESULTS

3.1 Physiological morphology and phylogenetic analysis of N_2-fixing cyanobacterium *A. siamensis* TISTR 8012

3.1.1 Phenotype examination of *A. siamensis* TISTR 8012

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012, a heterocystous filamentous cyanobacterium, was obtained from the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The cells can utilize many nitrogen sources in order to support growth such as nitrate, ammonia and are also able to fix dinitrogen from atmosphere to produce ammonia. Some filamentous cyanobacteria show the ability to differentiate into different cell types when cells are grown in nutrient limitation. The *A. siamensis* TISTR 8012 exhibited the morphological changes when the cells were grown in either BG11 medium containing nitrate as N-source or $BG11_0$ medium without N-source. Both treatments were incubated at 30 °C under continuous illumination of 40 μE $m^{-2}s^{-1}$. The micrographs were taken at 0, 2, 4, 7 and 9 days of cultivation.

After two days of transfer to medium without N-source, cells changed from blue-green to yellow-green, while cells in the medium with N-source stayed blue-green (Figure 3A). During 4-6 days of cultivation, all of cells were still organized in long filaments of vegetative cells until the depletion of nitrate from the medium induced the presence of heterocysts (Figure 3B). For cells grown without N-source, there were various changes. Akinetes were found after two days and some vegetative cells increased several folds in volume. The cell wall became thick and the pigment

composition was changed (Figure 3C). The heterocyst differentiation was first observed after four days and its percentage gradually increased with time (Figure 3D). The heterocyst cells were of various sizes with wide shorter filaments. A filament generally consists of 8-10 vegetative cells between two terminal heterocysts.

3.1.2 Cell surface and ultrastructure observation

Scanning Electron Microscopy (SEM) was used to observe cell surface of *A. siamensis* TISTR 8012 under different conditions, with and without N-source. The cell comprises filaments that divide exclusively by binary fission in one plane was found. A diameter of vegetative cell is about 1-1.5 μm. (Figure 4A-C). The heterocyst cells were found in either the end of filament or the middle of filament, with a diameter of heterocyst cell is about 2.5-3.5 μm. (Figure 4D-F).

Figure 5 shows the ultrastructure of *A. siamensis* cells under Transmission Electron Microscopy (TEM). The vegetative cell shows the three different layers, the cytoplasmic membrane layer (CM), peptidoglycan layer (PG) and outer membrane (OM). The obvious convoluted thylakoids (Th) and some carboxysomes (C) are present in vegetative cell. Heterocyst cell contains CM, PG and OM layers with same layer as vegetative cell. Moreover, the heterocyst cell shows the presence of thick cell wall consisting of heterocyst glycolipid layer (HGL) and heterocyst polysaccharide layer (HPL) in the envelope of heterocyst. The thickness is almost 0.5 μm which is thicker than those previously reported. This characteristic has the advantage in restriction of O_2 diffusion into the cells which is suitable for nitrogenase function.

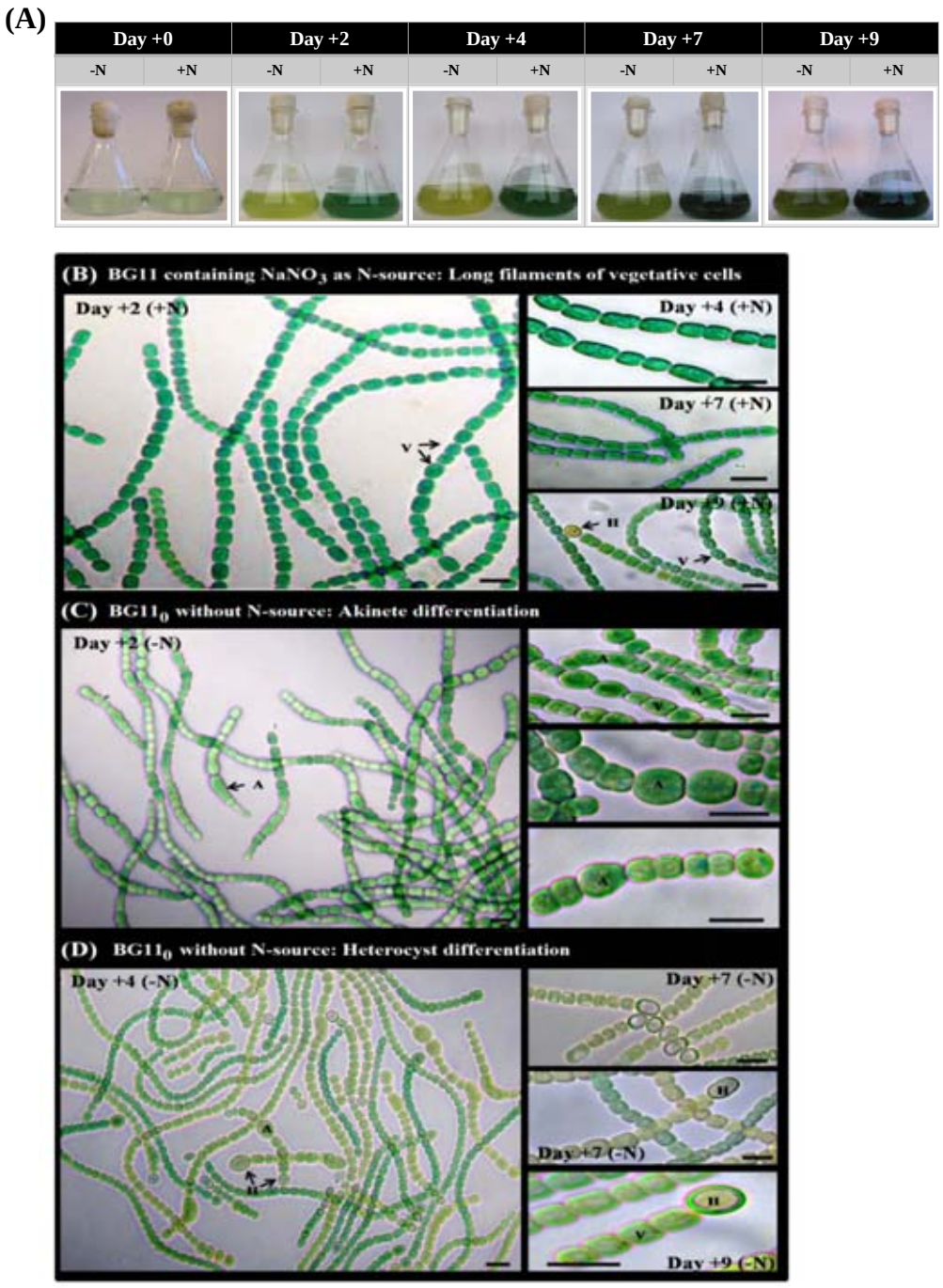

Figure 3 Phenotype examinations of cells of *A. siamensis* TISTR 8012 when grown under different media, BG11 medium containing nitrate as N-source and $BG11_0$ medium without N-source. (A) Photographic examination of the cell cultures. (B-D) Microscopic examination showing the important features of cell such as vegetative (V), akinete (A) and heterocyst (H), scale bars as 3 μm.

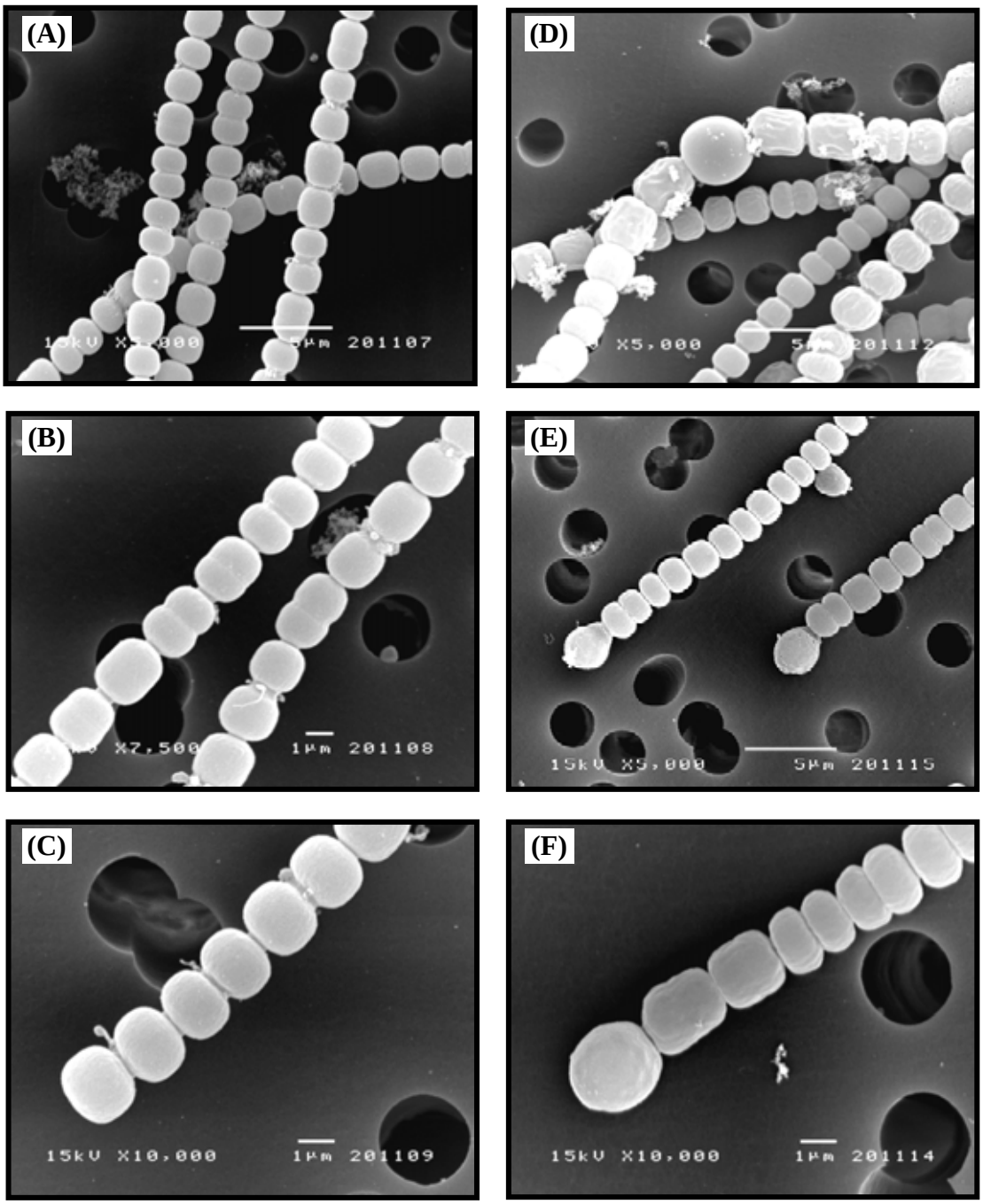

Figure 4 Image of *A. siamensis* TISTR 8012 cells under Scanning Electron Microscopy (SEM) when cells were grown in BG11 medium containing nitrate as N-source (A-C) and $BG11_0$ medium without N-source (D-F) with different magnifications.

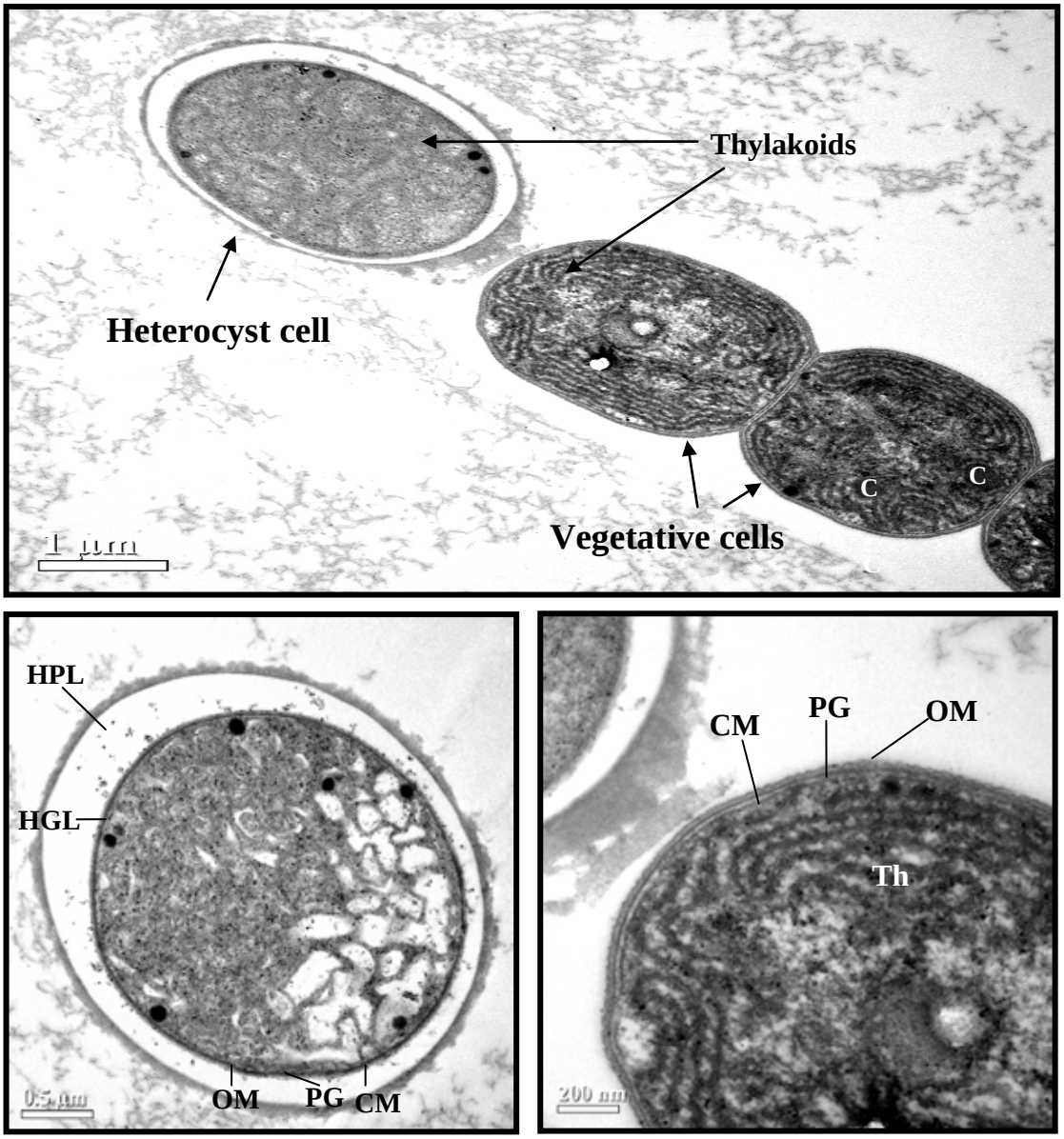

Figure 5 Image of *A. siamensis* TISTR 8012 cells under Transmission Electron Microscopy (TEM) when cells were grown in $BG11_0$ medium without N-source. Electron micrographs show the cytoplasmic membrane layer (CM), peptidoglycan layer (PG), outer membrane (OM), heterocyst glycolipid layer (HGL) and heterocyst polysaccharide layer (HPL), convoluted of thilakoid (Th) and the presence of carboxysomes (C) in only vegetative cell, respectively.

3.1.3 Phylogenetic analysis of *A. siamensis* TISTR 8012 using*16S* rDNA sequence

Phylogenetic analysis of cyanobacteria was performed using gene encoding the small subunit of ribosomal RNA (*16S* rDNA). The 1,407 bp of partial-sequence of *A. siamensis* TISTR 8012 was amplified by using primers of *16S* rDNA gene designed from conserved nucleotide sequences registered in GenBank database. This sequence has been submitted to the GenBank under accession number JQ657825.

The *16S* rDNA sequences obtained from 20 different cyanobacterial strains including *A. siamensis* TISTR 8012 were aligned with ClustalX alignment program prior to Bootstrap NJ phylogenetic tree construction as shown in Figure 6. The phylogenetic tree exhibits different cyanobacterial groups, separated with different colors. Interestingly, *A. siamensis* TISTR 8012 is classified into a single group separated from other *Anabaena* species in green group even though the physical morphology is quite similar. The diversity of strains for survival in different geography and environmental stresses may have some influence. However, the distance between *A. siamensis* TISTR 8012 and the potential H_2 production strains such as *Anabaena* sp. PCC 7120 and *Anabaena variabilis* ATCC 29413 is still very close. Therefore, *A. siamensis* TISTR 8012 originally isolated from rice paddy field in Thailand was an interesting model organism to study the ability of H_2 production.

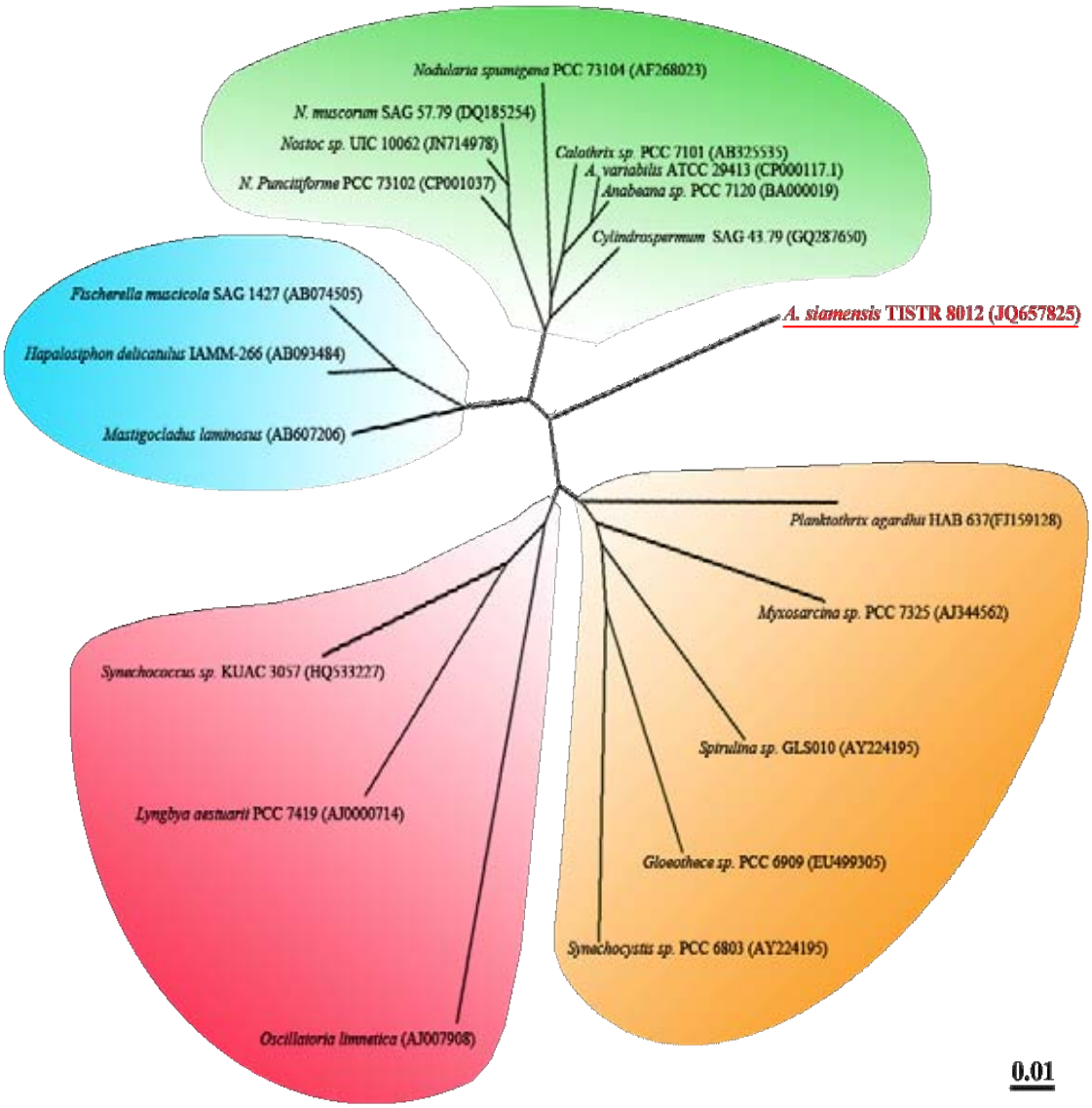

Figure 6 Phylogenetic tree based on *16S* rDNA sequences from 20 cyanobacterial strains including *A. siamensis* TISTR 8012 (underlined). The accession number of each strain was inserted at the end of name referred to sequences are available in GenBank database.

3.2 Optimization of growth condition for H_2 production by N_2-fixing cyanobacterium *A. siamensis* TISTR 8012

3.2.1 Growth rate under different culture media

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 cells were grown in different media namely, BG11, $BG11_0$, Allen–Arnon and N-free, respectively. The composition of nutrients in each medium was shown in Table 7. Only BG11 medium contained nitrate whereas the other media were used to grow *A. siamensis* TISTR 8012 under N_2-fixing condition. Cells were cultured at 160 rpm, 30 °C under continuous illumination of 40 $\mu E\ m^{-2}\ s^{-1}$. Samples were taken from a culture flask every three days of cultivation to determine growth rate by measuring the optical density of the culture at 730 nm and chlorophyll *a* content at 665 nm with spectrophotometer. The growth rate of *A. siamensis* TISTR 8012 (shown in Figure 7), could be divided into three different growth phases, log phase (3-6 days of cultivation), late log phase (6-9 days of cultivation) and stationary phase (more than 9 days of cultivation), respectively. In BG11 medium containing nitrate as N-source, cells could grow fast and both optical density (OD_{730}) and chlorophyll *a* content increased almost linearly with the time of cultivation while cells in medium without N-source, $BG11_0$, Allen–Arnon and N-free had slower growth rates than cells in BG11 medium (Figure 7A, B).

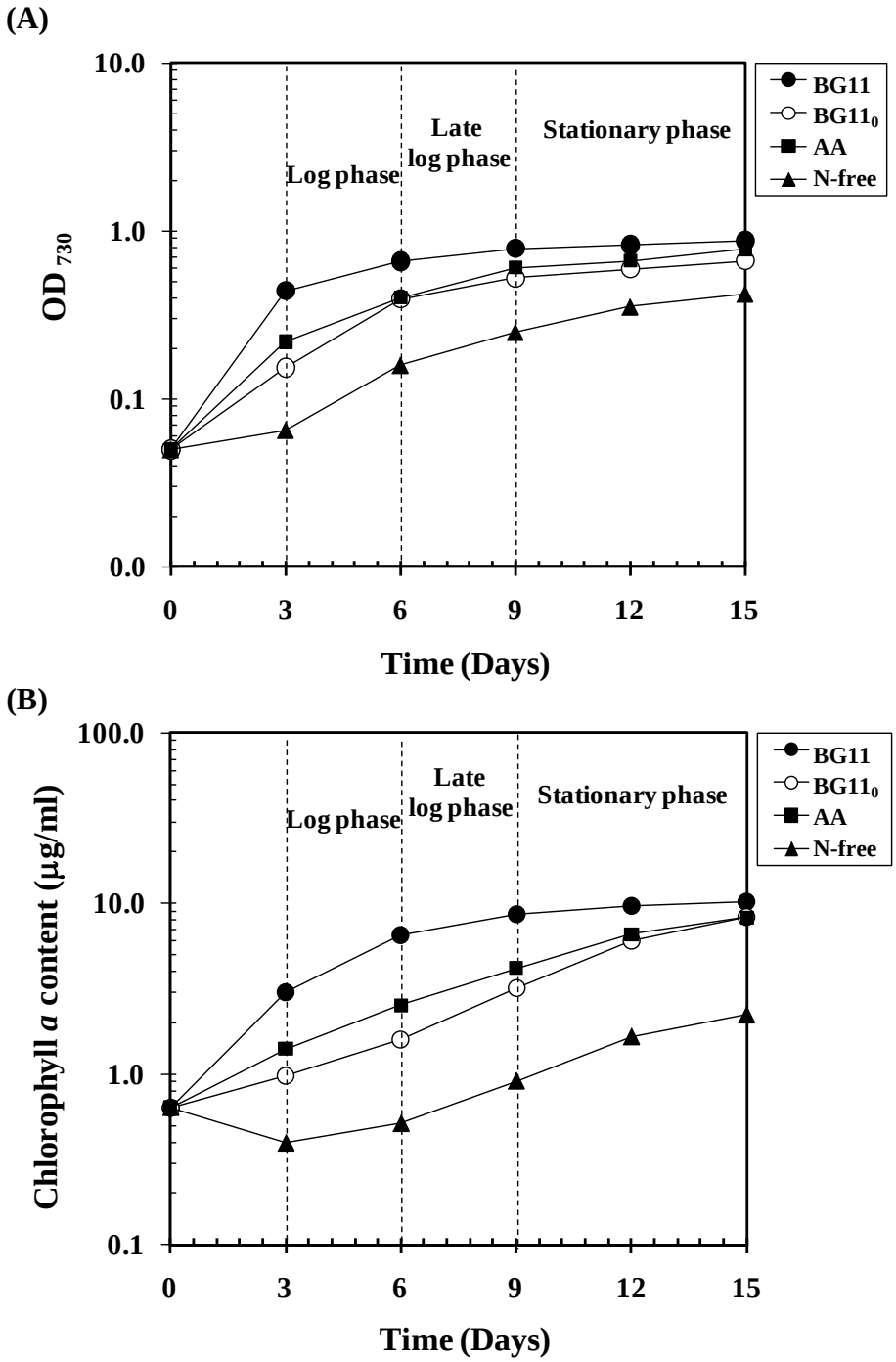

Figure 7 Effect of different culture media on growth rate of *A. siamensis* TISTR 8012 by measuring the optical density of cells at 730 nm (A) and chlorophyll *a* content (B). Cells were grown in different culture media namely, (●) BG11, (○) $BG11_0$, (■) Allen–Arnon and (▲) N-free media, respectively.

3.2.2 Time course of H_2 production

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 cells were grown in different culture media under growth condition until the optical density of cells culture at 730 nm shows different growth phases such as log phase, late log phase and stationary phase. After that, cells at different growth phases were harvested and resuspended in fresh medium in glass vial, incubated in darkness under anaerobic condition at room temperature before determining H_2 production at indicated times for 30 h to initially study effect of different culture media , cell age and time of incubation on H_2 production. Figure 8 shows time course of H_2 production by using *A. siamensis* in different culture media, BG11 (Figure 8A), $BG11_0$ (Figure 8B), Allen–Arnon (Figure 8C) and N-free (Figure 8D), respectively.

The results indicated that cells at log phase had better ability to produce H_2 than cell at other phases in every culture medium and time course experiments revealed that maximum H_2 production was obtained after 12 h. In addition, the comparison of H_2 production rate under time point at 12 h of anaerobic incubation showed that the cell at log phase grown in $BG11_0$ without N-source has significantly higher H_2 production rate than cell grown in other culture media, with a production rate was 5.23 ± 0.034 μmol H_2 mg chl a^{-1} h^{-1}. This rate is about 13-15 folds higher H_2 production rate when compared with cells in BG11 medium (Figure 9).

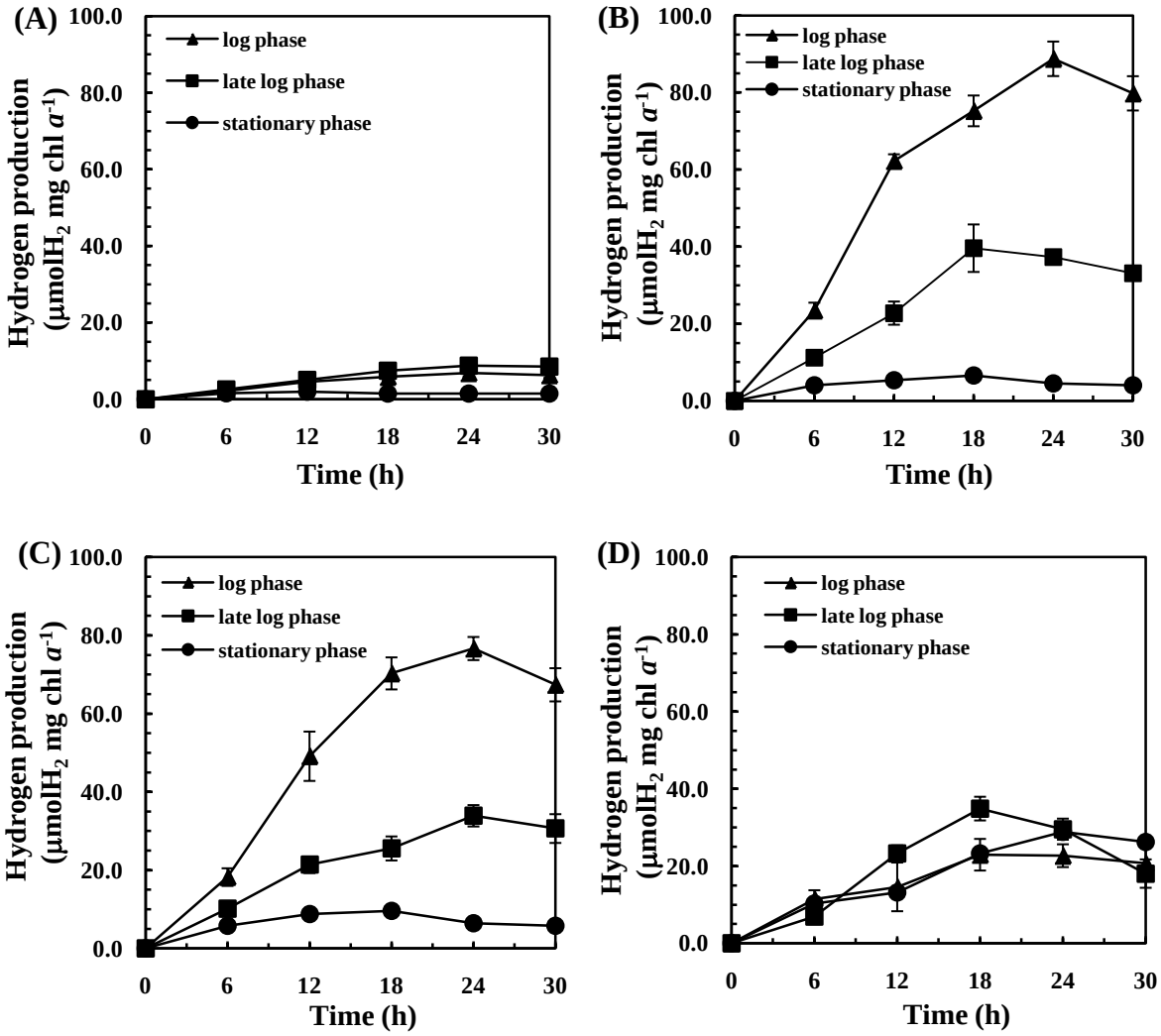

Figure 8 Time course of H_2 production by *A. siamensis* TISTR 8012 using cell were grown in different culture media namely, BG11 (A), $BG11_0$ (B), Allen-Arnon (C) and N-free media (D). The H_2 amount was determined at indicated times. Means ± S.D. (n=3).

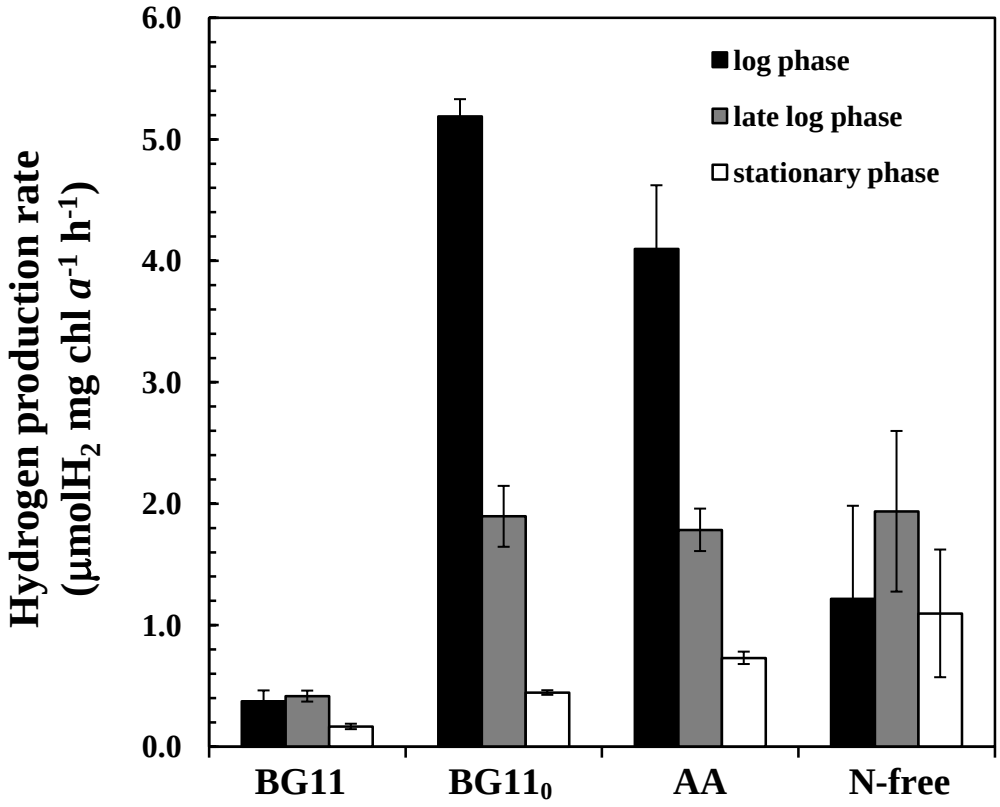

Figure 9 Effect of different culture media and cell age on H_2 production of *A. siamensis* TISTR 8012. The H_2 production rate was derived from Figure 8 by comparing of H_2 production rate under time point at 12 h of anaerobic incubation in each culture medium. Means ± S.D. (n=3).

3.2.2 Optimum physical parameters for growth in $BG11_0$ medium

The suitable culture medium for growing *A. siamensis* cell prior to produce H_2 was $BG11_0$ medium without N-source, in which the production was almost 15 fold higher H_2 production rate than BG11 medium containing nitrate as N-source. However, a lower growth rate of cell under N-deprivation was observed. Therefore, the screening of optimum physical parameters to enhance growth rate in $BG11_0$ medium were investigated by comparing the doubling time (DT) value. The physical parameters such as light intensity, pH and temperature were used to study doubling time value by varying light intensity ranging from 40 to 300 μE m^{-2} s^{-1}, temperature ranging from 30 to 50 ºC and pH ranging from 5.5 to 9.5 with Universal buffer as shown in Figure 10A-C, respectively. The results revealed that the shortest doubling time was achieved when cells were grown at 30 ºC under light intensity of 40-50 μE m^{-2} s^{-1} and pH 7.5, indicating that the N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 took less than one day to adjust themselves under N-deprivation. Therefore, the optimum physical parameters to enhance growth rate of *A. siamensis* TISTR 8012 in $BG11_0$ medium were temperature at 30 ºC, light intensity between 40-50 μE m^{-2} s^{-1} and pH 7.5.

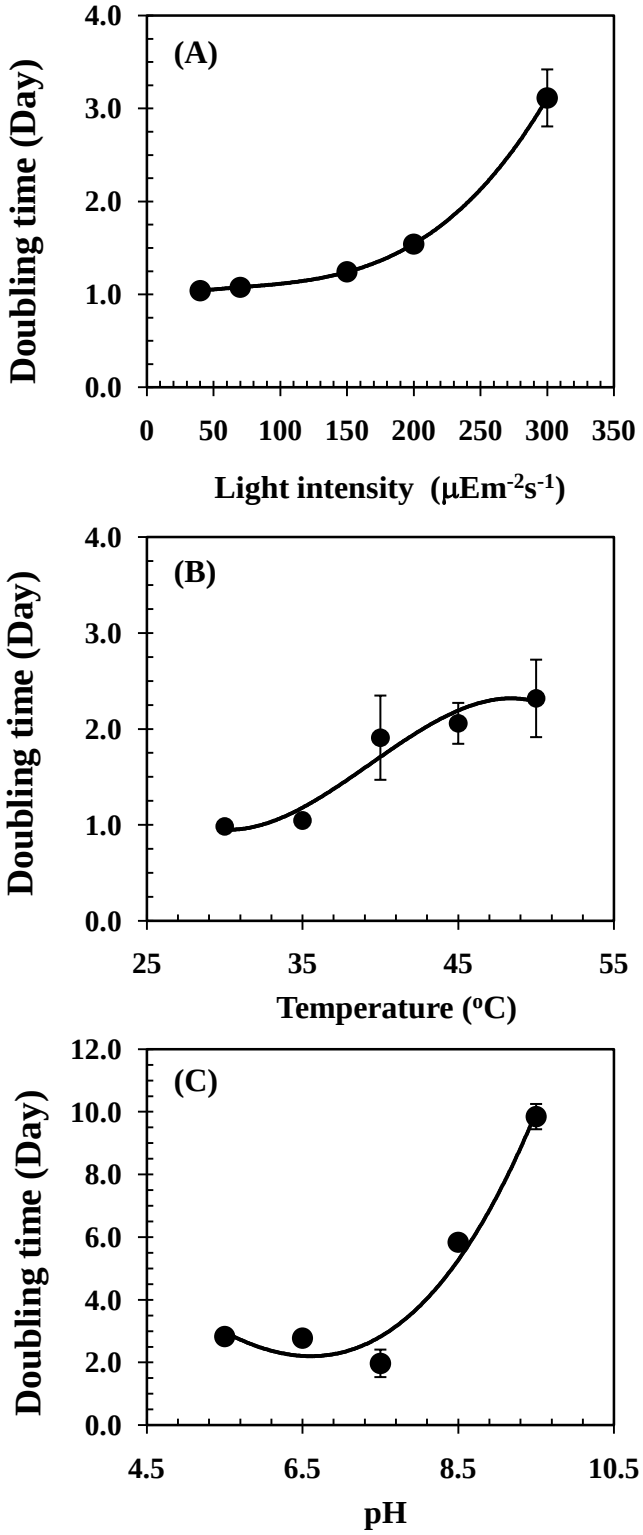

Figure 10 The doubling time of N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 when grown under different physical parameters namely, light intensity (A), temperature (B) and pH (C), respectively.

3.3 Characterization of H_2 production under various conditions

The *A. siamensis* TISTR 8012 cells were pre-cultured under optimum growth condition, in $BG11_0$ medium buffered with 20 mM HEPES (pH 7.5) and incubated at 30 ºC under continuous illumination of 40 μE m^{-2} s^{-1} until reaching log phase before cells were adapted in various conditions for 24 to investigate optimum condition for H_2 production. The various conditions were tested.

3.3.1 Effect of pH on H_2 production

The cells were grown in $BG11_0$ medium in which the pH was adjusted with Universal buffer ranging from 3.5-12.0 to study effect of pH on cell growth and H_2 production. The results showed that cells could not grow in medium with a lower pH at 3.5 and 4.5 whereas cells grew well with the increase of pH from 5.5 (Figure 11). This suggested that N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 preferred base condition than acid condition.

For H_2 production, the cells were pre-cultivated in $BG11_0$ medium at 160 rpm, 30 °C under continuous illumination of 40 μE m^{-2} s^{-1} until cells reached log phase. After that, cells were centrifuged and resuspended twice with fresh $BG11_0$ medium and adapted in $BG11_0$ medium adjusted pH with Universal buffer as 3.5-12.0 for 24 h before detecting H_2 production in darkness under 12 h anaerobic condition. The results in Figure 12A showed that the optimal pH for H_2 production was 7.5 for cells cultivated in $BG11_0$ medium. The lower pH of 5.5 and higher pH of 9.5 significantly reduced H_2 production. However, there are several buffer types for controlling pH at 7.5. Four buffer types such as TES, HEPES, K-phosphate and Tris-HCl buffers at concentration of 10 mM were investigated. The proper buffer type at pH 7.5 achieving highest H_2 production was 10 mM HEPES buffer (Figure 12B).

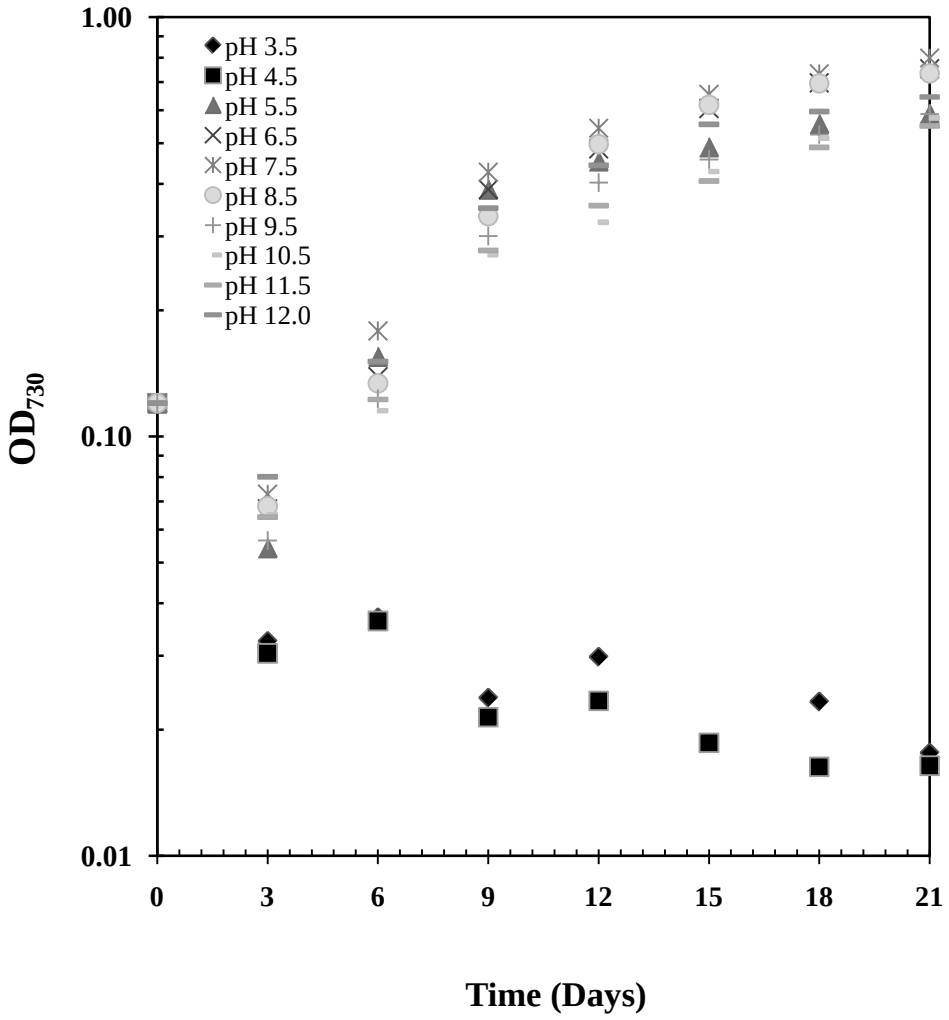

Figure 11 The growth rate of *A. siamensis* TISTR 8012 when cells were grown in $BG11_0$ medium with Universal buffer pH ranging from 3.5-12.0. The optical density of cell culture at 730 nm was detected every three days of cultivation. Means ± S.D. (n=3).

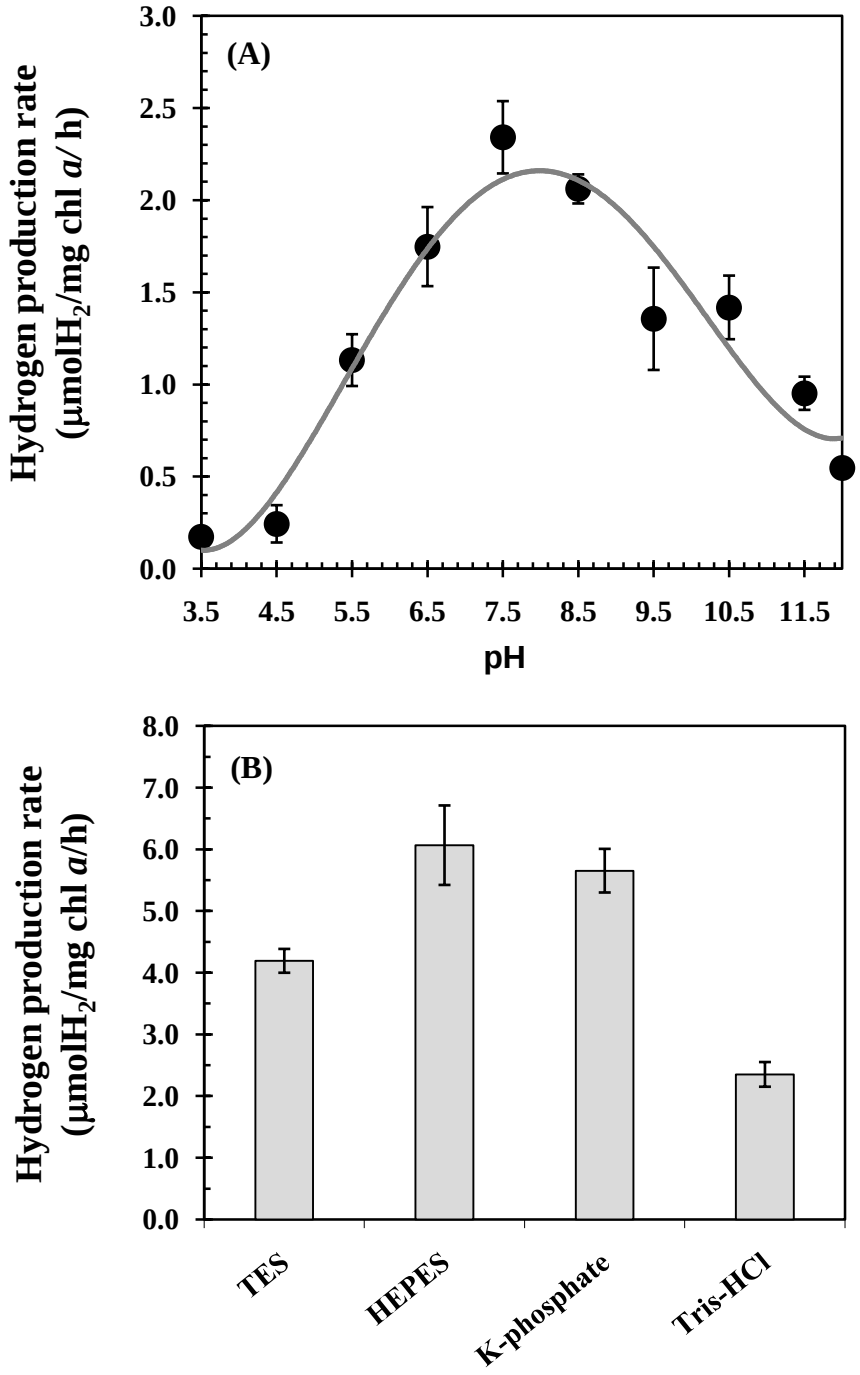

Figure 12 The H_2 production rate of *A. siamensis* TISTR 8012 when cells were adapted in $BG11_0$ medium with either Universal buffer pH ranging from 3.5-12.0 (B) or different buffer types at pH 7.5 (C) for 24 h before detecting H_2 production under darkness. Means ± S.D. (n=3).

3.3.2 Effect of temperature on H_2 production

The cells at log phase were adapted for 24 h in $BG11_0$ medium by varying temperatures at 25, 30, 35, 40 and 45 ºC, respectively before detecting H_2 production in darkness under 12 h of anaerobic condition. The results indicated that the H_2 production rate of *A. siamensis* TISTR 8012 was clearly highest at 30 ºC and gradually decreased when temperature was over 30 ºC (Figure 13).

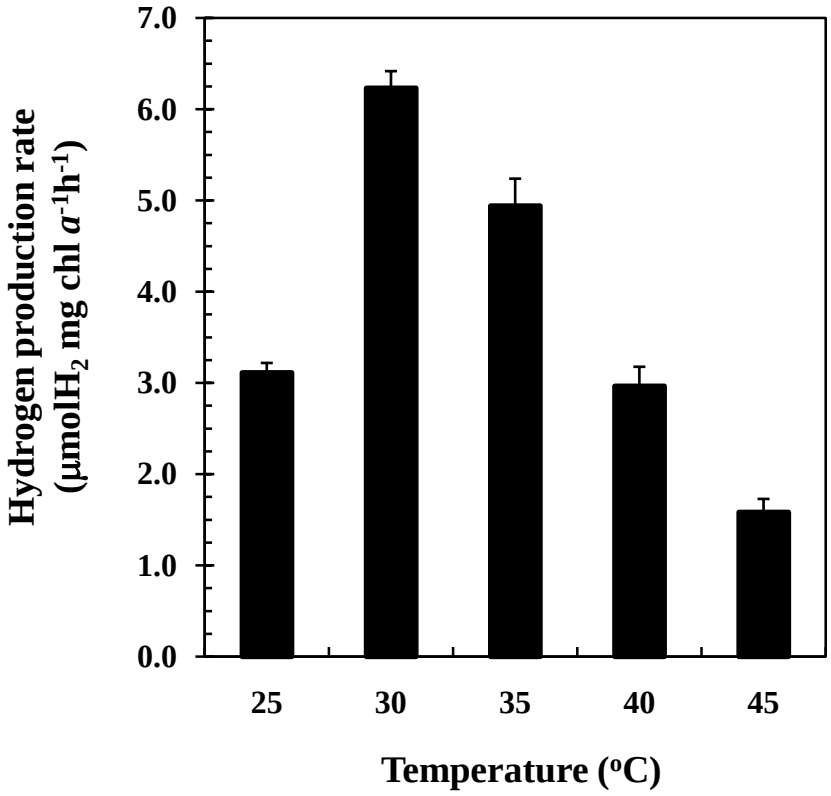

Figure 13 The H_2 production rate of *A. siamensis* TISTR 8012 when cells were adapted in $BG11_0$ medium incubated at different temperatures, 25, 30, 35, 40 and 45 ºC for 24 h before detecting H_2 production under darkness. Means ± S.D. (n=3).

3.3.3 Effect of N-sources and concentrations on H_2 production

The cells at log phase were adapted in BG11 medium containing different N-sources, namely $NaNO_3$, $NaNO_2$ and NH_4Cl at the same concentration of 17.6 mM to study effect of various N-sources on H_2 production by using $BG11_0$ medium without N-source as control. After 24 h of adaptation, cell in each condition was analyzed for H_2 production in darkness under anaerobic condition for 12 h. The results showed that all of added N-sources, $NaNO_3$, $NaNO_2$ and NH_4Cl could inhibit H_2 production in *A. siamensis* cell and the H_2 production rate was 0.208 ± 0.036, 0.640 ± 0.061, and 0.104 ± 0.026 μmol H_2 mg chl a^{-1} h^{-1} with $NaNO_3$, $NaNO_2$ and NH_4Cl as N-source, respectively (Figure 14A). Furthermore, various concentrations of $NaNO_3$ were examined to know the lowest concentration of $NaNO_3$ that can affect H_2 production in *A. siamensis* TISTR 8012. The experiment in Figure 14B showed that the H_2 production was clearly inhibited even at 1 mM of $NaNO_3$.

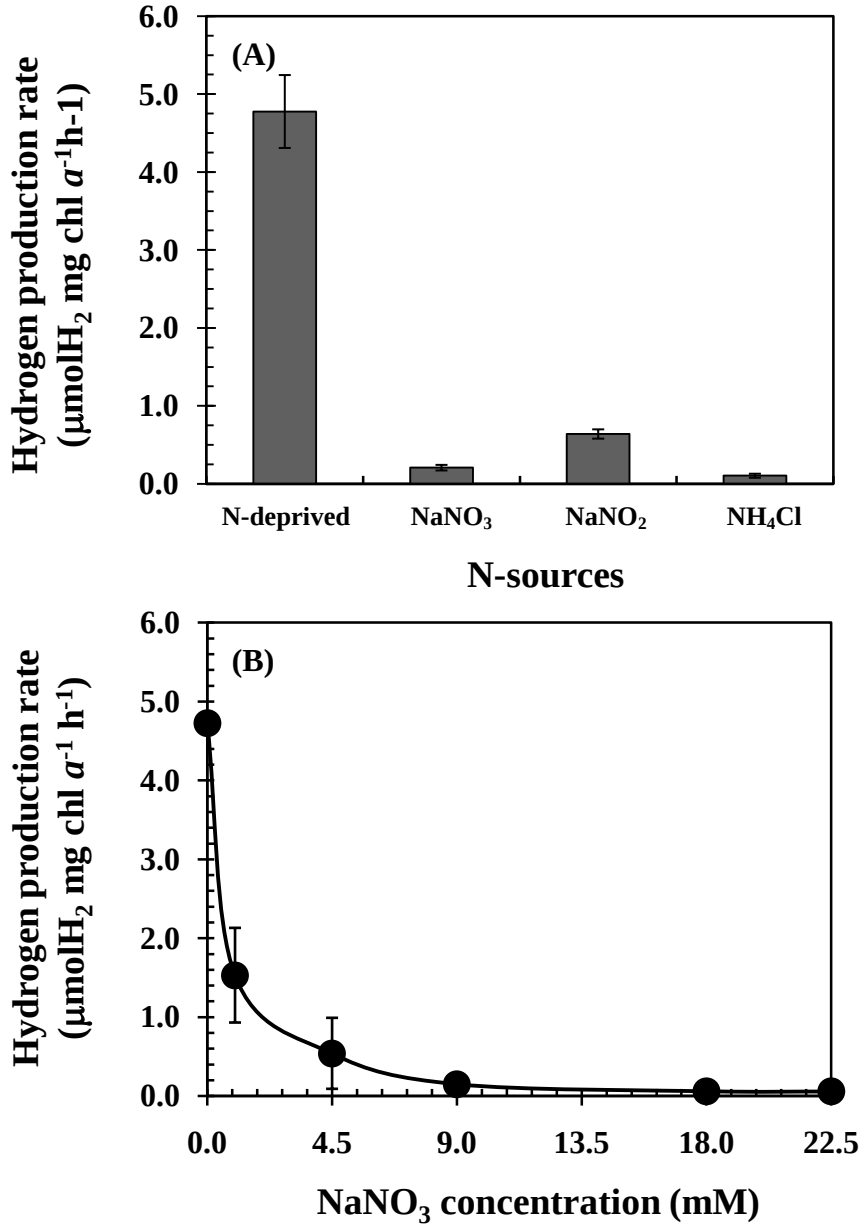

Figure 14 (A) Effect of various N-sources on H_2 production of *A. siamensis* TISTR 8012 when cells were adapted in BG11 medium containing different N sources, $NaNO_3$, $NaNO_2$ and NH_4Cl at the same concentration of 17.6 mM using $BG11_0$ medium without N-source as control. (B) Effect of various concentrations of $NaNO_3$ on hydrogen production. The H_2 production rate in each condition was measured under darkness. Means ± S.D. (n=3).

3.3.4 Effect of various supplemented carbon sources on H_2 production

Cyanobacteria can utilize organic substrates as a source of carbon. Therefore, cells were grown in $BG11_0$ medium supplemented with various sugars such as glucose, lactose, galactose, fructose and sucrose at the same concentration of 0.5 % (w/v) to investigate the effect of various carbon sources on growth and H_2 production. Normally, *A. siamensis* TISTR 8012 can utilize Na_2CO_3 and citric acid contained in $BG11_0$ as carbon source or can fix CO_2 from atmospheric when cells were grown in C-deprived medium. The growth rate of *A. siamensis* TISTR 8012 grown in various carbon sources as shown in Figure 15A, revealed that cells could grow with the same growth pattern in all C-sources studied.

Figure 15B shows the H_2 production of *A. siamensis* TISTR 8012 adapted in $BG11_0$ medium supplemented with various carbon sources for 24 h under growth condition before H_2 detection. The results demonstrated that the most preferred substrate for H_2 production by *A.siamnesis* TISTR 8012 was fructose, with a production rate of 11.034 ± 0.133 μmol H_2 mg chl a^{-1} h^{-1} followed by sucrose yielding 9.957 ± 0.624 μmol H_2 mg chl a^{-1} h^{-1}. Other sugars, glucose, galactose and lactose had no effect on H_2 production when compared with control normal $BG11_0$.

In addition, the percentage of sugar uptake in *A. siamensis* TISTR 8012 using the phenol-sulfuric acid assay was determined. The results suggested that *A. siamensis* TISTR 8012 rapidly took up the sugar within 12 h of cell adaptation. After that, the uptake rate was constant. Results showed that 0.5 % (w/v) of fructose was preferentially utilized by cells rather than other sugars studied (Figure 16).

3.3.5 Effect of fructose concentration on H_2 production

Various concentrations of fructose supplemented in $BG11_0$ medium ranging from 0 to 2.0 % (w/v) were tested. Cells had the higher H_2 production when increasing the concentration of fructose between 0.4 - 0.6 % (w/v), the H_2 production rate was approximately 11.0 - 12.0 µmol H_2 mg chl a^{-1} h^{-1}. Moreover, a continuous decrease of H_2 production rate was found when fructose concentration was higher than 0.6 % (Figure 17).

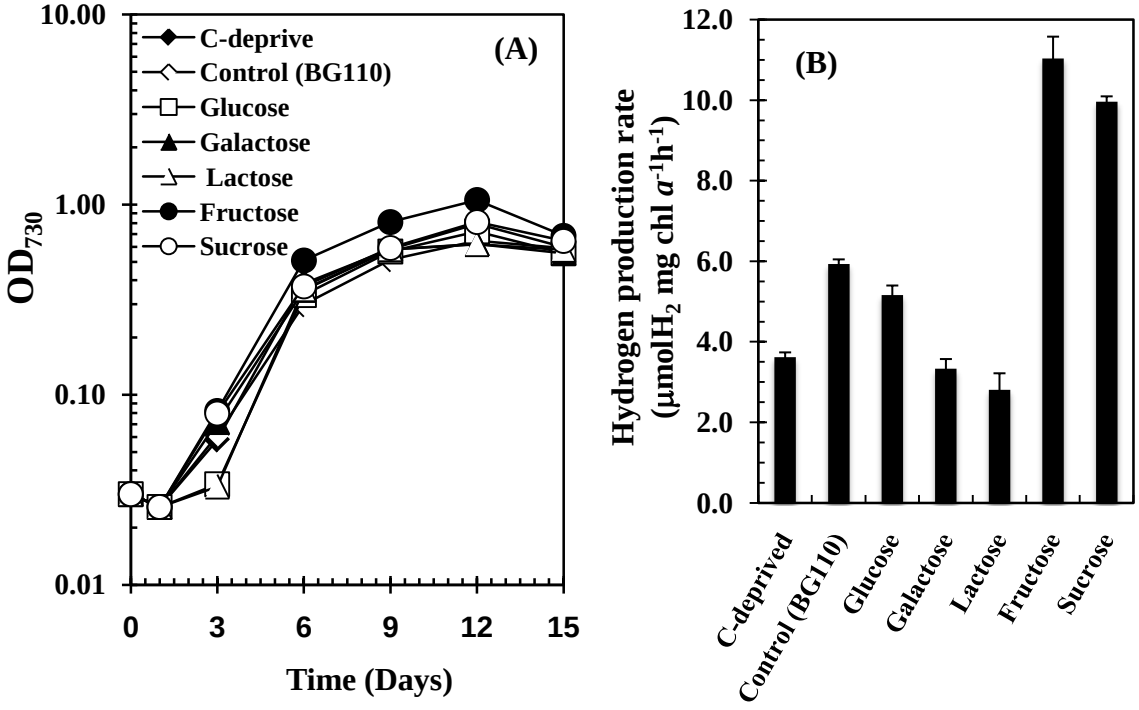

Figure 15 Effect of various supplemented carbon sources on growth and H_2 produciton. Growth rate of *A. siamensis* TISTR 8012 (A) and H_2 production rate ((B) in $BG11_0$ medium without carbon source (C-deprived) and $BG11_0$ medium supplemented with various types of sugars, glucose, galactose, lactose, fructose and sucrose at the same concentration of 0.5 % (w/v). $BG11_0$ medium (containing $NaCO_3$ and citric acid as carbon source) was used as a control. Means ± S.D. (n=3).

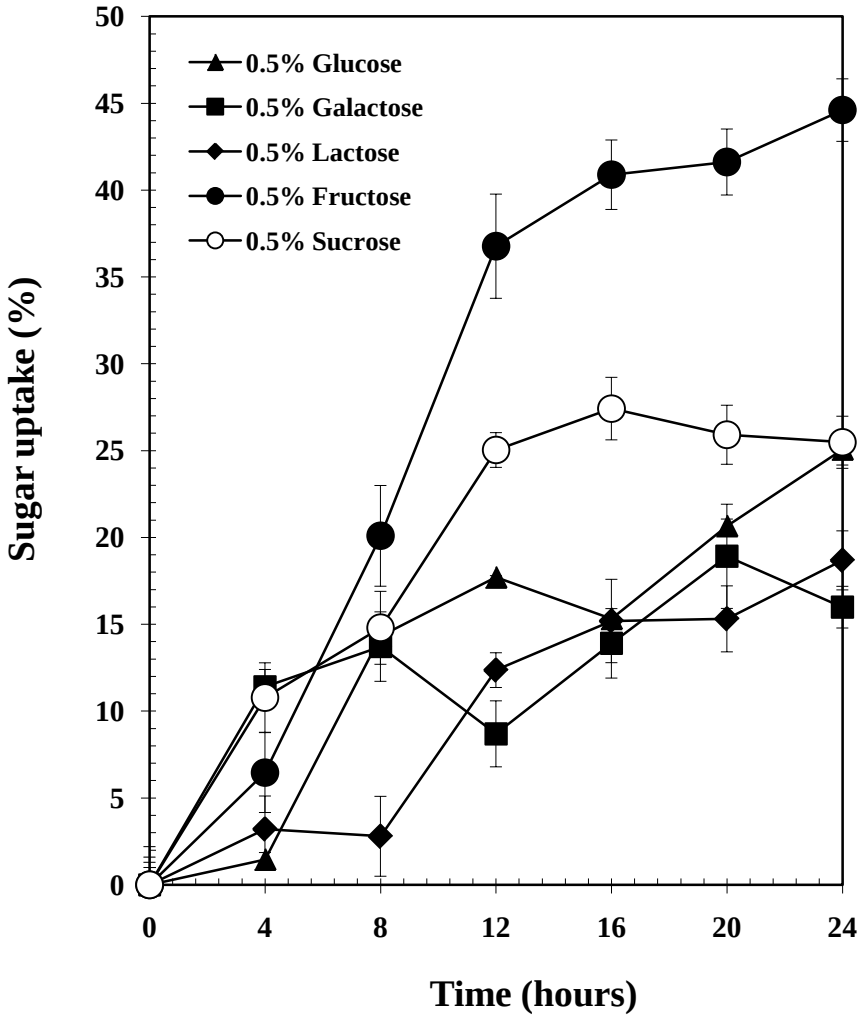

Figure 16 Uptake of various types of sugar by *A. siamensis* TISTR 8012. The remaining sugar in the medium after incubating cells in various types of sugar under N_2-fixing condition was determined every 4 h to calculate the percentage of sugar utilized by the cells when compared with the initial sugar concentration. Sugar uptake was calculated using a standard calibration curve. Means ± S.D. (n=3).

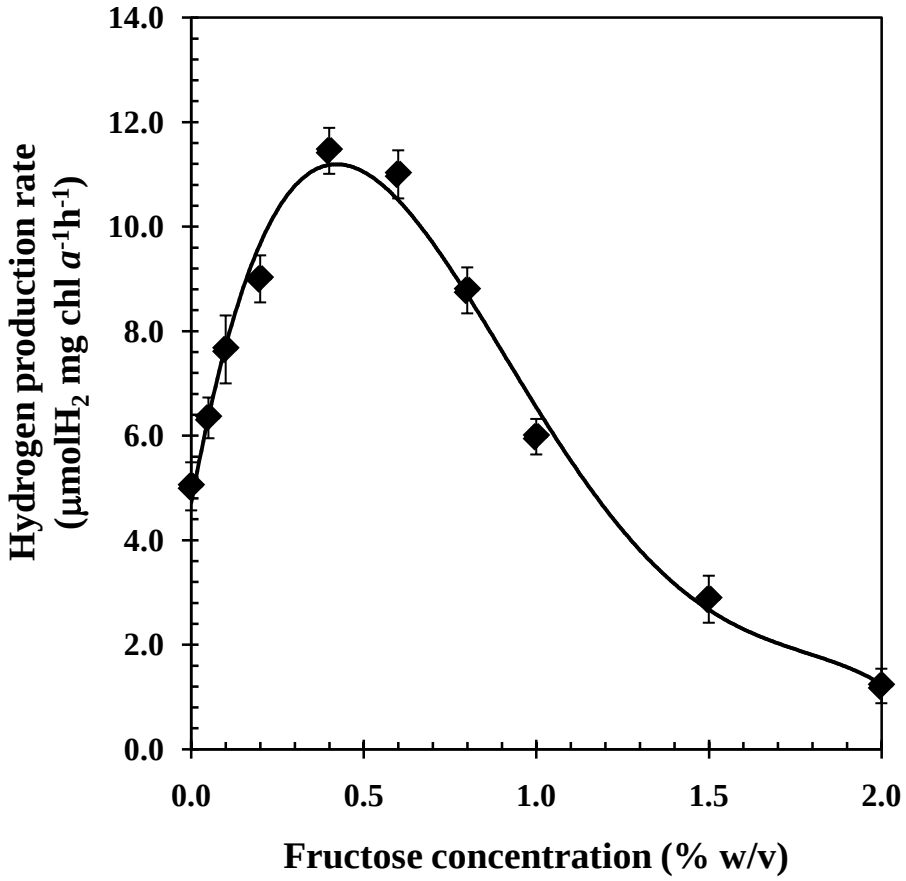

Figure 17 The H_2 production rate of *A. siamensis* TISTR 8012 when adapted in $BG11_0$ supplemented with various concentrations of fructose ranging from 0 to 2.0 % (w/v) for 24 h. The H_2 production was determined under dark and anaerobic condition for 12 h in each condition. Means ± S.D. (n=3).

3.3.6 Effect of light intensity and duration of light exposure on H_2 production

Cells at log phase were adapted in fresh $BG11_0$ medium incubated under different light intensities at 0, 40, 70, 150, 200, 250 and 300 μE m^{-2} s^{-1}, respectively compared with darkness for 24 h. After 24 h, cells were transferred to vials and incubated continuously in the same light intensity under anaerobic condition for 12 h before detecting H_2 production as shown in Figure 18A. The results indicated that increased light intensity could significantly enhance H_2 production in *A. siamensis* TISTR 8012 and the maximum H_2 production rate was found when cells were adapted under 200 μE m^{-2} s^{-1} of light intensity. However, light intensity higher than 200 μE m^{-2} s^{-1} reduced H_2 production. Moreover, cell lysis under high light intensity was also observed (Figure 18B).

Even though the 200 μE m^{-2} s^{-1} of light intensity was the optimum light incubation for H_2 production in *A. siamensis* TISTR 8012, the duration of light exposure may also be important to increase H_2 production. Then, time of light exposure for 12, 24 and 36 h was investigated by using cells at darkness and 40 μE m^{-2} s^{-1} (light intensity of growth condition) as control compared with light intensity at 200 μE m^{-2} s^{-1}. The results revealed that at 12 h of continuous light exposure the cells could produce the highest H_2 production (Table 8). The maximum H_2 production rate was 15.2 ± 1.1 μmol H_2 mg chl a^{-1} h^{-1}. This rate was about 2 and 3-fold of those at 40 μE m^{-2} $s^{-1}$and in darkness, respectively. However, light exposure over 12 h did not increase H_2 production, but rather caused increased O_2 accumulation and chlorophyll *a* content.

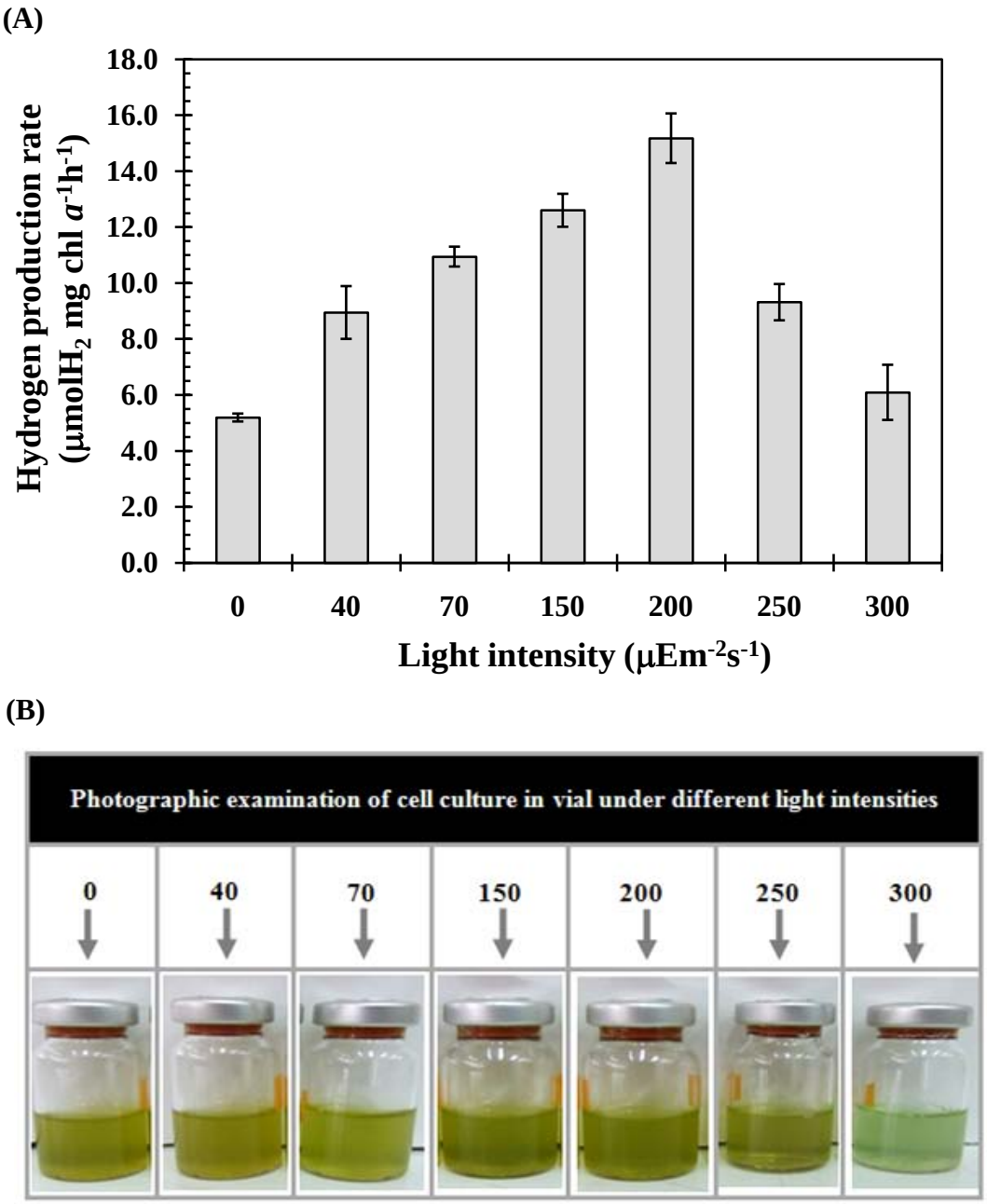

Figure 18 (A) Effect of light intensity on H_2 production rate of *A. siamensis* TISTR 8012. Cells were adapted in fresh $BG11_0$ incubated in different light intensities for 24 h before detecting H_2 production under anaerobic condition for 12 h of each sample. Means ± S.D. (n=3). (B) Photographic examination of cells culture adapted under different light intensities for 24 h.

Table 8 Effect of light intensity and time of light incubation on H_2 production, O_2 accumulation of N_2-fixing cyanobacterium *A. siamensis* TISTR 8012

Light intensity ($\mu Em^{-2}s^{-1}$)	**Time of light incubation (h)**	**Chl *a* content ($\mu g\ mL^{-1}$)**	**H_2 production rate ($\mu molH_2$ mg chl a^{-1} h^{-1})**	**Oxygen accumulation ($nmolO_2\ mL^{-1}$)**
Darkness	12	86.3 ± 1.8	5.5 ± 0.1	0.018 ± 0.004
	24	80.7 ± 2.2	4.9 ± 0.5	0.019 ± 0.004
	36	75.6 ± 1.5	3.7 ± 0.5	0.018 ± 0.004
40	12	86.8 ± 1.3	8.9 ± 0.3	0.406 ± 0.040
	24	93.4 ± 1.3	3.1 ± 0.1	0.874 ± 0.144
	36	101.0 ± 1.3	0.8 ± 0.1	1.508 ± 0.034
200	12	85.1 ± 1.6	15.2 ± 1.1	0.478 ± 0.032
	24	93.4 ± 1.3	7.4 ± 1.5	0.982 ± 0.071
	36	78.2 ± 3.2	6.4 ± 0.8	1.274 ± 0.054

Each sample was adapted in $BG11_0$ medium in various light intensities for 24 h and before transferring to vials, incubated at the same light intensity for 12, 24, 36 h under anaerobic condition before determining H_2 was quantified. Means ±S.D. (n=3).

3.3.7 Effect of salt and osmotic stress on H_2 production

Cells at log phase were adapted in $BG11_0$ medium by either supplementing with various salts such as NaCl, KCl, $CaCl_2$ and LiCl at 25 mM or supplementing with various concentrations of D-sorbitol for investigating the effect of salt stress and osmotic stress on H_2 production in *A. siamensis* TISTR 8012. Cells were adapted in each condition for 24 h before detecting H_2 production under darkness and anaerobic condition for 12 h. Figure 19A showed that all of salts tested decreased H_2 production.

In contrast, an increased H_2 production was observed when cells were adapted in $BG11_0$ supplemented with D-sorbitol at 50 mM (Figure 19B). Various concentrations of D-sorbitol were also tested, and the best concentration was the presence of 50 mM of D-sorbitol in medium. D-sorbitol higher than 50 mM did not increase H_2 production in *A. siamensis* TISTR 8012.

Furthermore, *A. siamensis* TISTR 8012 cells could not grow normally in medium containing either salt or D-sorbitol. The morphology changes were observed under light microscope and Scanning Electron Microscope (SEM) as shown in Figure 20A-D. The clear plasmolysis occurred where cells lost water because of hypertonic condition leading to shrinking of cell, when cells were grown in medium containing either salt or D-sorbitol. The cell damage under salt stress was also observed (Figure 20 A, B).

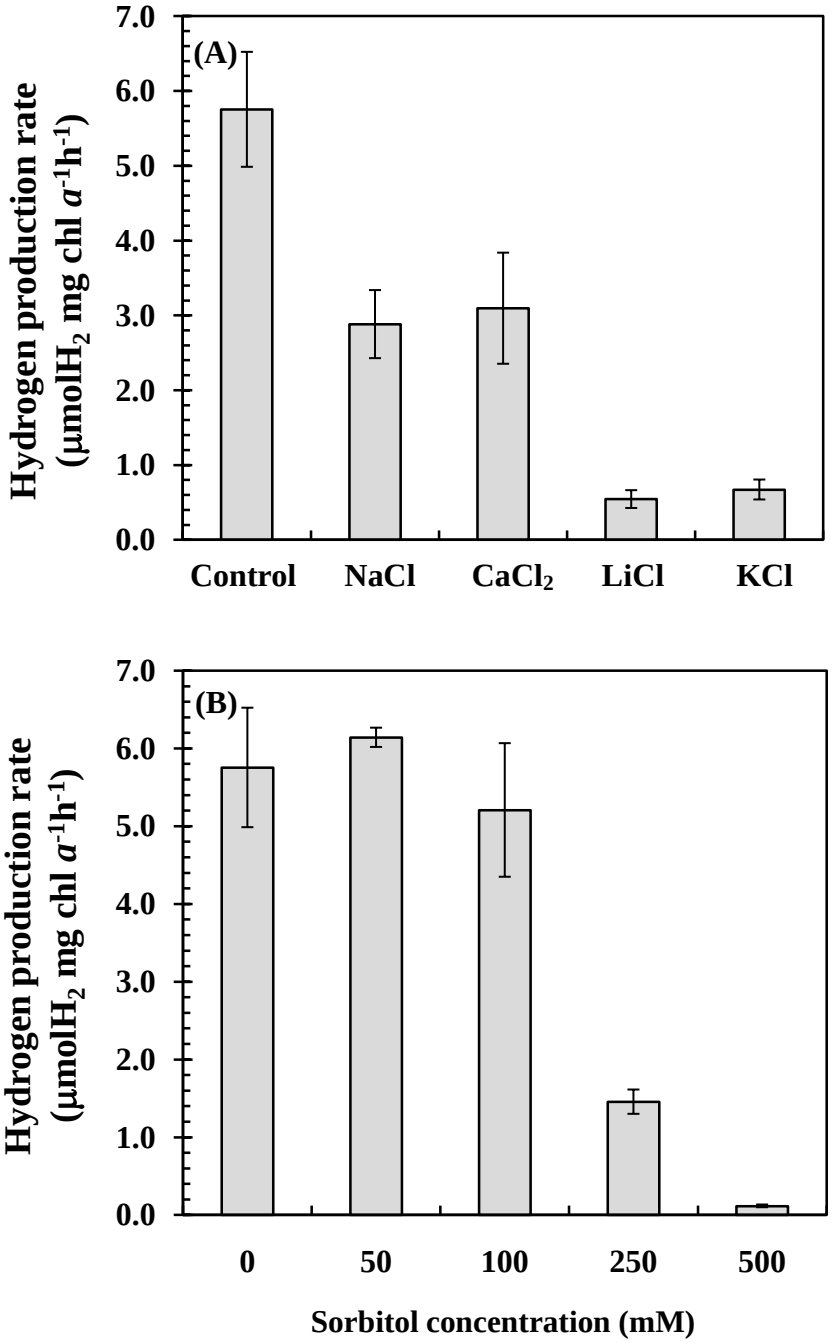

Figure 19 Effect of salt and osmotic stress on H_2 production rate of *A. siamensis* TISTR 8012. (A) H_2 production rate of cells was adapted in $BG11_0$ supplemented with 25 mM of different salts including, NaCl, $CaCl_2$, LiCl and KCl. (B) H_2 production rate of cells was adapted in $BG11_0$ supplemented with various concentrations of D-sorbitol. Means ± S.D. (n=3)

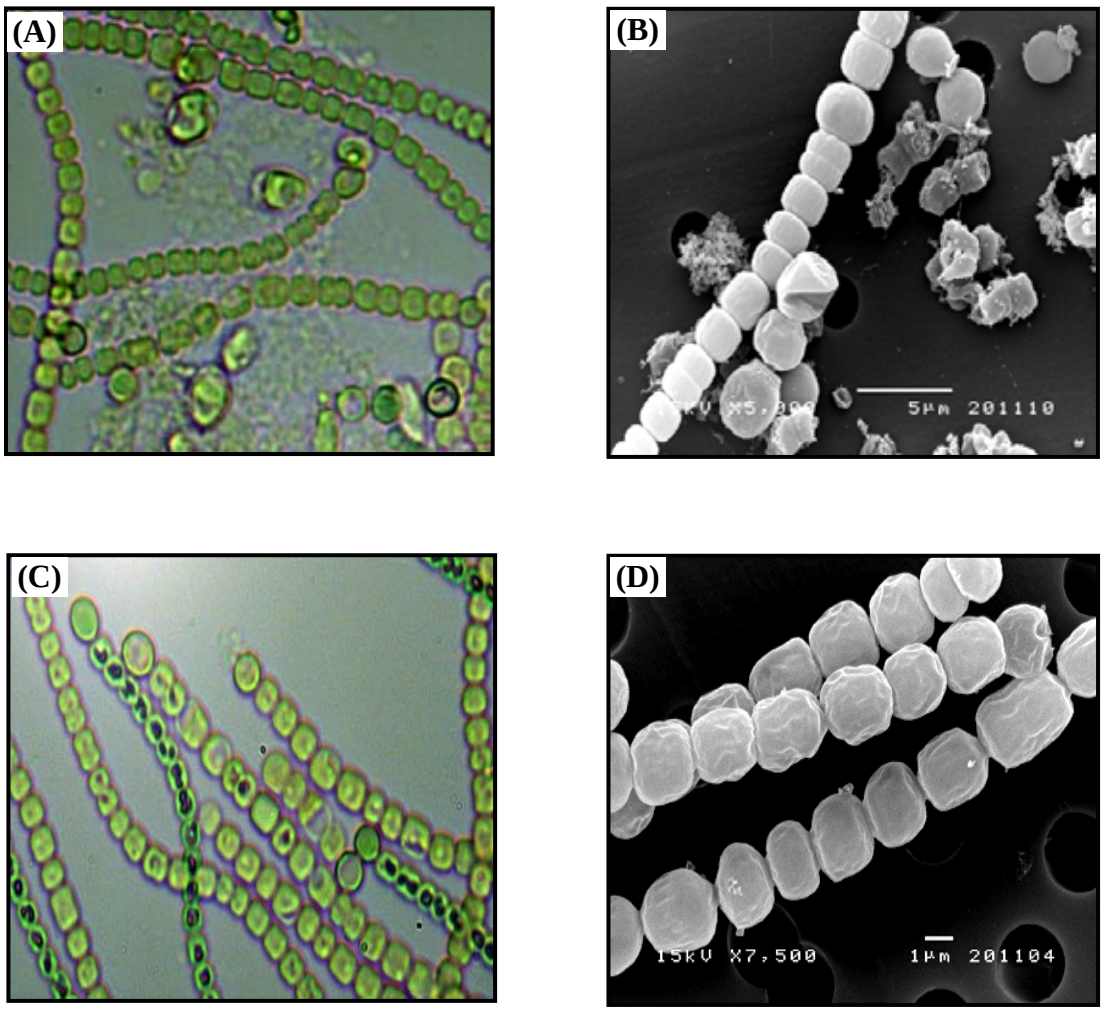

Figure 20 Microscopic image of *A. siamensis* TISTR 8012 under light microscope and Scanning Electron Microscope (SEM) when cells were adapted in $BG11_0$ supplemented with 25 mM NaCl (A-B) and 100 mM D-sorbitol (C-D) for 24 h.

3.3.8 Effect of supplementation of microelement on H_2 production

Microelements such as cobalt (Co), copper (Cu), molybdemum (Mo), vanadium (V), nickel (Ni) and iron (Fe) affect H_2 production in cyanobacteria because their metals are required as co-factors of enzymes involved in H_2 metabolism. Therefore, effect of supplemented microelements in $BG11_0$ medium on H_2 production in *A. siamensis* TISTR 8012 was investigated.

For Fe^{2+} ion, cells were adapted in $BG11_0$ medium by varying concentration of $FeSO_4$ ranging from 0 to 105 μM for 24 h before detecting H_2 production under darkness and anaerobic condition. Figure 21A revealed that increasing concentration of $FeSO_4$ obviously increased H_2 production. However, increasing the concentration of $FeSO_4$ higher than 21 mM resulted in no further increase in H_2 production. In the presence of 21 μM $FeSO_4$ in $BG11_0$, H_2 production rate was 10.5 ± 0.742 μmol H_2 mg chl a^{-1} h^{-1}, which was about 2-fold of cells in normal $BG11_0$ medium containing 2.1 mM $FeSO_4$.

For trace elements, the effect of Mo^{2+}, V^{2+} and Ni^{2+} ions in $BG11_0$ medium on H_2 production was investigated (Figure 21B-D). The results showed that increasing the concentration of Mo^{2+} in medium resulted in the increase in H_2 production while in the presence of V^{2+} and Ni^{+2} H_2 production was repressed in *A. siamensis* TISTR 8012. The maximum H_2 production was found upon addition of 4 mM Mo^{2+}, giving a 2.5-fold higher H_2 production than that without Mo^{2+} (Figure 21B). Moreover, too high concentrations of metal ions affected growth and cell survival.

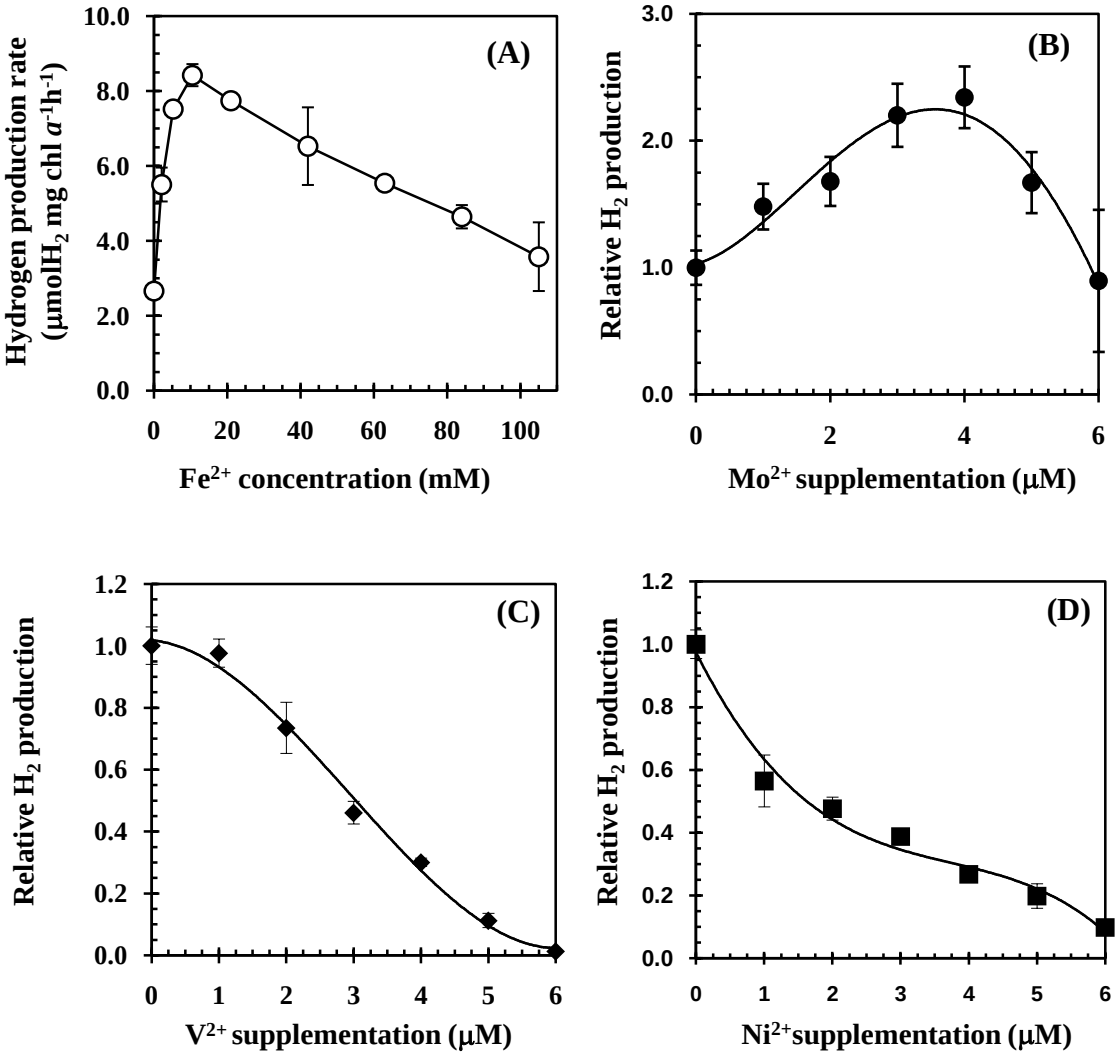

Figure 21 Effect of microelements on H_2 production by *A. siamensis* TISTR 8012, (A) H_2 production rate of cells adapted in $BG11_0$ medium supplemented with various concentrations of Fe^{2+} ion. (B-C) The relative H_2 production of cells adapted in $BG11_0$ medium supplemented with different concentrations of Mo^{2+}, V^{2+} and Ni^{2+} ions, respectively. H_2 production was measured under darkness and anaerobic condition and the relative H_2 production was calculated relative to the production observed in the control $BG11_0$. Means ± S.D. (n=3).

3.3.9 Effect of reductant on H_2 production

β-mercaptoethanol was investigated for the effect of physiological reductant on H_2 production in *A. siamensis* TISTR 8012 by using cells at log phase adapted in $BG11_0$ medium supplemented with various concentrations of β-mercaptoethanol ranging from 0 to 1000 μM for 24 h before detecting H_2 production. No apparent stimulation of H_2 production was observed when increasing the concentration of β-mercaptoethanol under darkness (Figure 22). Surprisingly, the presence of β-mercaptoethanol at concentration of 750 μM could induce higher H_2 production in *A. siamensis* TISTR 8012 when incubation cell under light condition (40 μE $m^{-2}s^{-1}$).

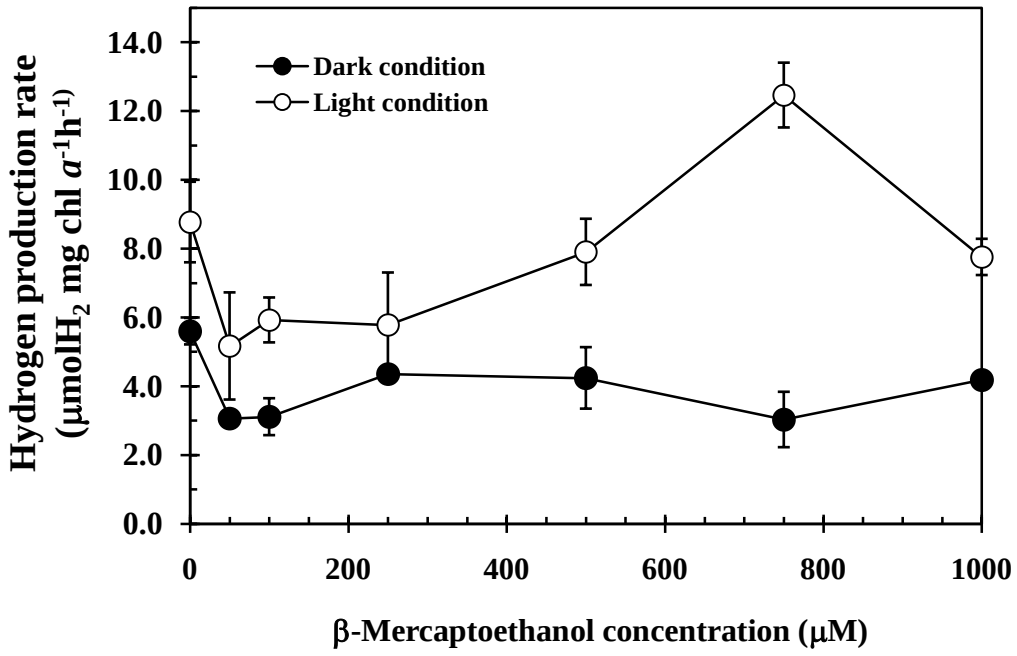

Figure 22 Effect of reductants on H_2 production. The H_2 production was detected under dark and light (40 μE $m^{-2}s^{-1}$) conditions using cell adapted in $BG11_0$ supplemented with various concentrations of β-mercaptoethanol for 24 h. Means ± S.D. (n=3).

3.3.10 Effect of sulfur deprivation on H_2 production

There have been reports that limited sulfur condition can induce H_2 production in some cyanobacteria. The *A. siamensis* TISTR 8012 cells were grown in BG11 and $BG11_0$ media. Log-phase cells were washed and transferred to either BG11 or $BG11_0$-S-deprived for 24 h before detecting H_2 production under light condition (40 $\mu E\ m^{-2}s^{-1}$). The results showed that S-deprived condition could increase H_2 production in *A. siamensis* TISTR 8012 in both media, with the maximum H_2 production of 10.1 ± 0.033 μmol H_2 mg chl a^{-1} h^{-1} when cells were adapted in $BG11_0$-S-deprived, representing about 2-folds of cells grown in normal $BG11_0$ medium (Figure 23).

The chlorophyll fluorescence analysis was used to study the effect of sulfur limitation on PSII activity of *A. siamensis* TISTR 8012. In general, the light energy absorbed by chlorophylls associated with PSII can be used to drive photochemistry in which an electron is transferred from the reaction center chlorophyll (P680), to acceptor of PSII (PQ). Alternatively, absorbed light energy can be lost from PSII as chlorophyll fluorescence or heat as shown in Figure 24A. In addition, PSII activity can reffect O_2 evolution activity since the reaction of photosynthesis in cyanobacteria utilizes water molecule (H_2O) as final electron donor and generates O_2 as a by-product. The loss of PSII activity indicated the reduced O_2 evolution in cells.

Cells in four media, BG11, BG11-S-deprived, $BG11_0$ and $BG11_0$-S-deprived were analyzed for chlorophyll fluorescence using fluorescence spectrometer. The results revealed that cells under S-deprived media showed higher level of fluorescence intensity at 680 nm (mostly PSII fluorescence) of emission wavelength than cells in normal media either BG11 or $BG11_0$ (Figure 24B).

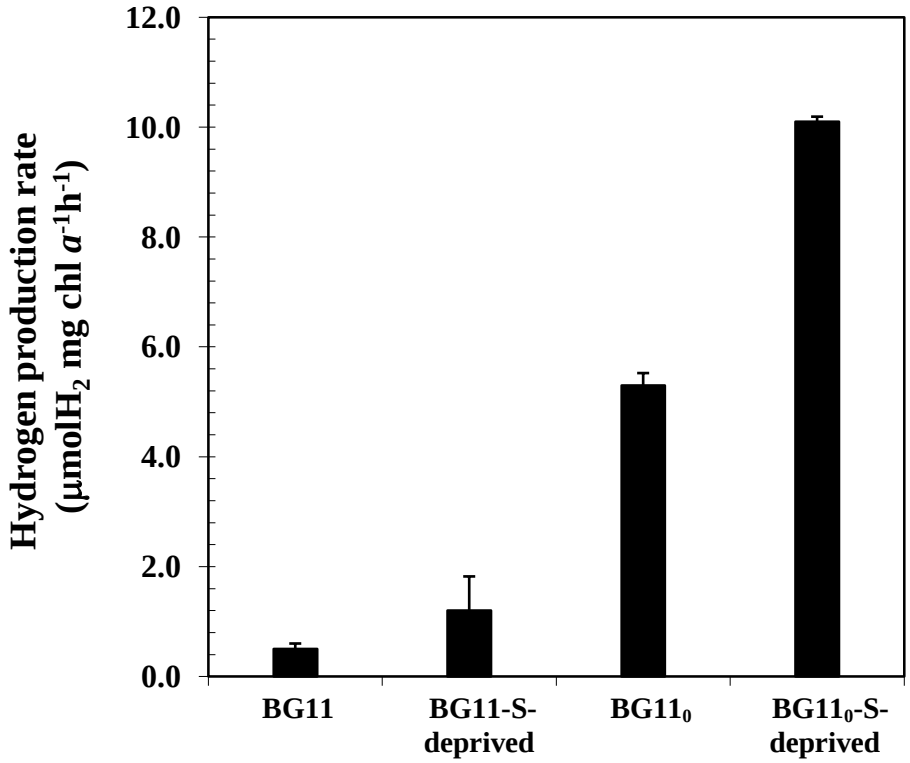

Figure 23 H_2 production rate by *A. siamensis* TISTR 8012 under sulfur and nitrogen deprivation. Cells were adapted in BG11, BG11-S-deprived, $BG11_0$ and $BG11_0$-S-deprived media, and incubated under growth condition for 24 h before detecting H_2 production. The H_2 production rate was detected in light (40 $\mu Em^{-2}s^{-1}$) under anaerobic condition for 12 h. Means ± S.D. (n=3).

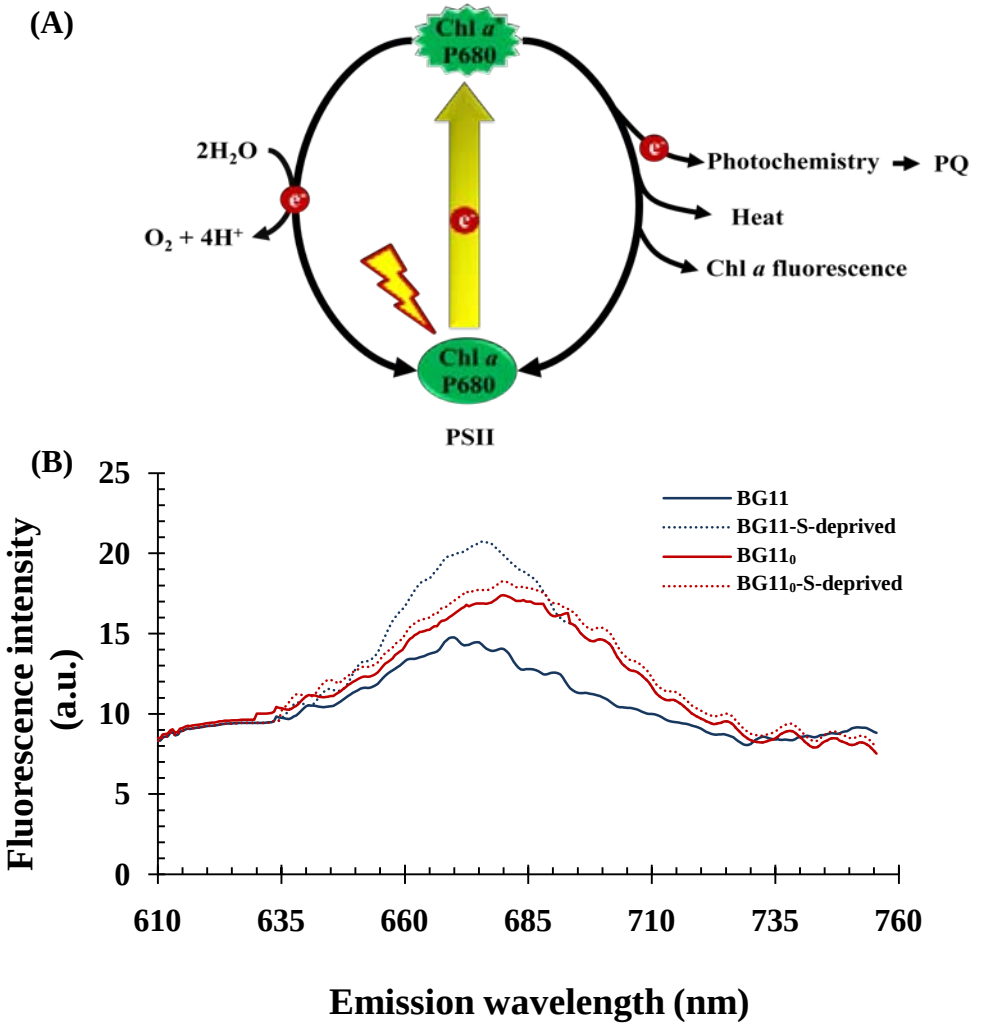

Figure 24 (A) light energy absorbed by chlorophylls associated with PSII, an electron is transferred from the reaction center chlorophyll (P680) to PQ pool. Alternatively, absorbed light energy can be lost from PSII as chlorophyll fluorescence or heat. PSII activity can be referred to O_2 evolution due to the reaction of photosynthesis in cyanobacteria utilizes water molecule (H_2O) as electron donor and generated O_2 as by product. The loss of PSII activity led to the reduction of O_2 evolution in cells. (B) Emission spectra of fluorescence in 298 K (room temperature) of *A. siamensis* cells adapted in BG11, BG11-S-deprived, $BG11_0$ and $BG11_0$-S-deprived media, wavelength of excitation was at 440 nm.

3.3.11 Effect of aerobic and anaerobic conditions on H_2 production

The enzymes involved in H_2 metabolism in N_2-fixing cyanobacterium are strongly inhibited in the presence of O_2. This experiment was done to test the competency of N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 for H_2 production under aerobic condition. Cells were cultured in 600 mL of $BG11_0$ medium, incubated under growth condition by bubbling with different gases as shown in Figure 25A. Cell culture was bubbled continuously with either air (i) or with argon gas (ii) for 24 h and with air for 12 h followed by argon for 12 h (iii), respectively. After 24 h, cells in each flask were transferred into a vial before determining H_2 gas under light condition for every 4 h. The results showed that H_2 production of *A. siamensis* TISTR 8012 was strongly inhibited when using cells were adapted under continuous aerobic condition. Interestingly, *A. siamensis* TISTR 8012 under temporal aerobic condition could produce H_2 even though the production rate was one half of that grown under anaerobic condition (Figure 25B).

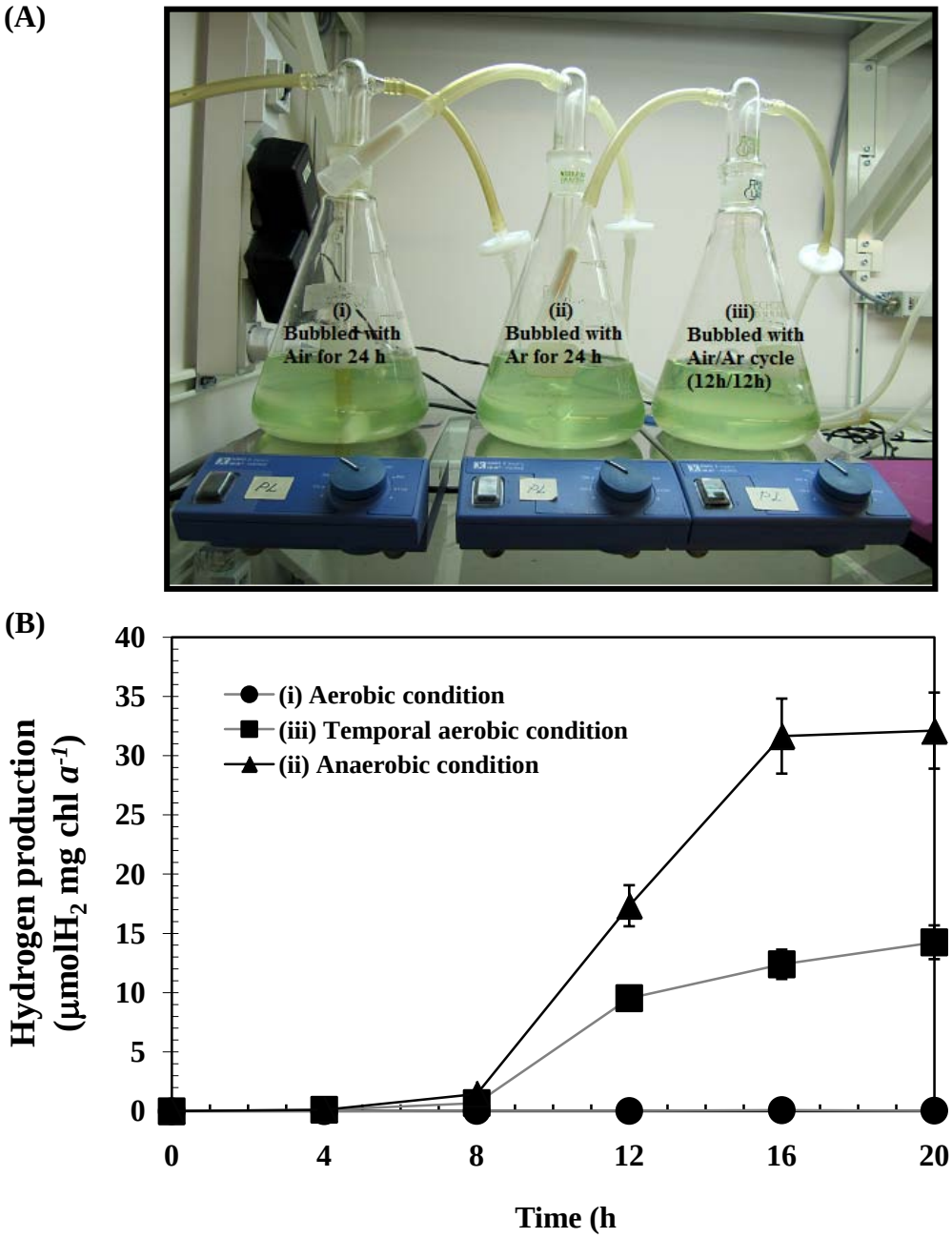

Figure 25 Effect of aerobic and anaerobic conditions on H_2 production. (A) Cells were grown in flask, incubated under continuous aerobic condition; (i) bubbled with air for 24 h, anaerobic condition; (ii) bubbled with argon gas for 24 h and temporal aerobic condition; (iii) bubbled with Air/Ar cycle (12h/12 h), respectively. (B) Time course of H_2 production in vial under light condition. (Means ± S.D. (n=3).

3.4 Enhanced H_2 production in *A. siamensis* TISTR 8012 by controlling key factors

The characterization of H_2 production by N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 under various conditions indicated that several factors could increase H_2 production such as cell age, carbon source, light intensity and time of light exposure. To enhance H_2 production in *A. siamensis* TISTR 8012, a combination effect of cell age, carbon source, light intensity and time of light exposure was investigated for H_2 production. The results showed an enhanced H_2 production when cells, at log phase, were adapted under N_2-fixing condition using 0.5% fructose as carbon source and a continuous illumination of 200 μE m^{-2} s^{-1} at 30 ºC for 12 h under anaerobic condition, in which the highest H_2 production rate was 31.79 ± 0.54 μmol H_2 mg chl a^{-1} h^{-1} (Figure 26).

3.5 Comparison of H_2 production in *A. siamensis* TISTR 8012 with other cyanobacterial strains

The model organisms *Anabaena* PCC 7120, *Nostoc punctiforme* ATCC 29133 and *Synechocystis* PCC 6803 were used to compare H_2 production with *A. siamensis* TISTR 8012. The results revealed that *A. siamensis* TISTR 8012 had the capacity to produce 4-and 400-folds more H_2 than *Anabaena* PCC 7120 and *Synechocystis* PCC 6803, respectively but less than *Nostoc punctiforme* ATCC 29133 under the same growth condition ($BG11_0$, 30 °C, under 40 μE m^{-2} s^{-1} of light intensity) (Table 9). However, higher hydrogen production rate was obtained by *A. siamensis* TISTR 8012 under controlled key factors, i.e. high light intensity (200 μE $m^{-2}s^{-1}$) and using fructose as the source of carbon yielding about 32 μmol H_2 mg chl a^{-1} h^{-1}.

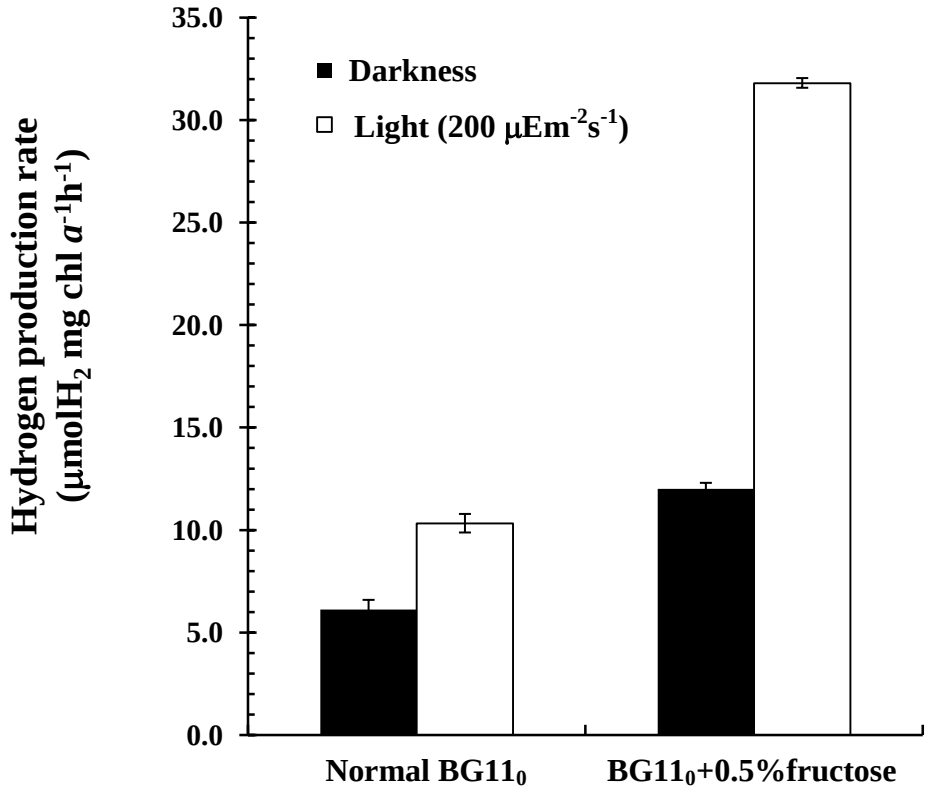

Figure 26 H_2 production rate of *A. siamensis* TISTR 8012 by controlling key factors using cells at log phase were adapted under normal $BG11_0$ and $BG11_0$ supplemented with 0.5% fructose either under darkness (■) or under continuous illumination of 200 μE m^{-2} s^{-1} (□) at 30 ºC for 24 h before detecting H_2 production. The H_2 gas was detected under the same conditions for 12 h in anaerobic condition. Means ± S.D. (n=3).

Table 9 Comparison of the ability of H_2 production of *A. siamensis* TISTR 8012 with other cyanobacterial strains.

Organisms	**Morphology**	**Maximum H_2-production rate ($\mu molH_2$ mg chl $a^{-1}h^{-1}$)**	**Growth condition**	**H_2-production condition**
Anabaena siamensis TISTR [a] 8012	Heterocystous filamentous	8.68 ± 0.31	Air, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$	Ar, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$
		31.79 ± 0.54	Air, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$	Ar, $BG11_0$ added 0.5% fructose, 30 °C, 200 $\mu Em^{-2}s^{-1}$
Anabaena PCC [b] 7120	Heterocystous filamentous	2.17 ± 0.07	Air, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$	Ar, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$
Nostoc punctiforme ATCC [c] 29133	Heterocystous filamentous	20.7 ± 0.72	Air, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$	Ar, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$
Synechocystis PCC [b] 6803	Unicellular	0.02 ± 0.02	Air, BG11, 30 °C, 40 $\mu Em^{-2}s^{-1}$	Ar, $BG11_0$, 30 °C, 40 $\mu Em^{-2}s^{-1}$

[a] TISTR = Thailand Institute of Scientific and Technological Research, (Bangkok, Thailand)

[b] PCC = Pasteur Culture Collection (Paris, France)

[c] ATCC = American Type Culture Collection (Rockville, MD, USA)

3.6 Transcription analysis of genes involved in H_2 metabolism under various conditions

3.6.1 Effect of different growth media and cell age on transcription level of *nifD, hoxH* and *hupL* genes

Transcription analysis of genes involved in H_2 metabolism of *A. siamensis* was investigated to gain more understanding on the function of nitrogenase, uptake hydrogenase and reversible hydrogenase affected by various conditions. Figure 27 showed RT-PCR product using RNA isolated from cells grown in either $BG11_0$ medium and BG11 medium for 0, 2, 4, 7 and 9 days. The cultivation was conducted under anaerobic condition, and incubated at 30 ºC under continuous illumination of 40 $\mu Em^{-2}s^{-1}$ and anaerobic condition. Faint bands for both *nifD* and *hupL* transcripts level were detected in BG11-grown cells whereas intensed bands were observed in those of $BG11_0$-grown cells. On the other hand, under both N_2-fixing and non N_2-fixing conditions, there were no significant changes on the transcript level of *hoxH* in *A. siamensis* TISTR 8012 with higher transcript levels under anaerobic conditions in all cell age periods.

3.6.2 Effect of light intensity and duration of light exposure on transcription level of *nifD, hoxH* and *hupL* genes

The expression levels of genes encoding enzymes involved in H_2 metabolism *nifD, hoxH* and *hupL* were examined by reverse transcription-PCR (RT-PCR). Total RNA was isolated from *A. siamensis* cells adapted in $BG11_0$ medium under different light intensities namely, 0 (darkness), 40 and 200 $\mu Em^{-2}s^{-1}$, respectively and incubated for 12, 24, 36 h under anaerobic condition at 30 °C. An increased light intensity resulted in an increased *nifD* transcription level whereas no increase in *hoxH*

transcription level was observed. Interestingly, strong signal of *hupL* transcription level was observed when cells were incubated under longer light exposure (Figure 28).

3.6.3 Transcription level of *nifD, hoxH* and *hupL* genes under controlled key factors

Total RNA of *A. siamensis* TISTR 8012 was isolated from cells adapted anaerobically in $BG11_0$ containing Na_2CO_3 as carbon source and in $BG11_0$ supplemented with 0.5% fructose, incubated under darkness and light (200 $\mu Em^{-2}s^{-1}$) condition for 12 h at 30 ºC. RT-PCR as shown in Figure 29 indicated that the presence of 0.5% fructose in $BG11_0$ medium could significantly enhance transcript level of *nifD* in either dark or light condition compared with other carbon sources. The stronger signal of *nifD* transcription level was observed under 200 $\mu Em^{-2}s^{-1}$ of light intensity than under dark condition. However, the transcript levels of *hoxH* and *hupL* showed little or no induction.

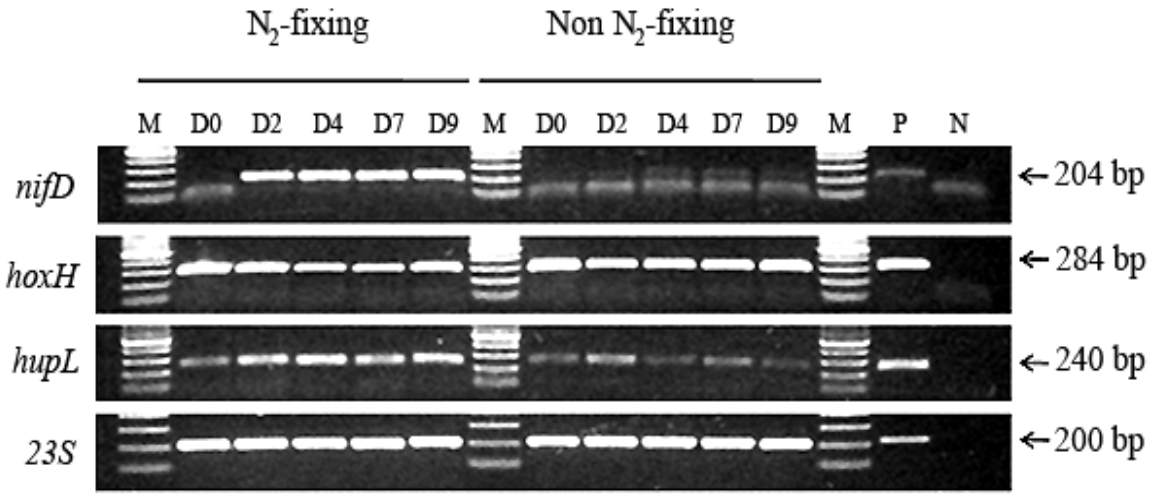

Figure 27 Effect of different growth conditions and cell age on transcript levels of genes encoding enzymes involved in hydrogen metabolism of *A.siamensis* TISTR 8012. Transcription levels of *nifD*, *hoxH*,and *hupL* genes by RT-PCR using total RNA isolated from cells grown in N_2-fixing ($BG11_0$ medium) and non N_2-fixing condition (BG11 medium) for 0, 2, 4, 7 and 9 days and incubated at 30 °C under continuous illumination of 40 $\mu Em^{-2}s^{-1}$ under anaerobic condition. PCR amplifications using cDNAs of respective gens were performed using specific primers. 23S rRNA was used as an internal reference gene, genomic DNA and no template added were used as positive control (P) and negative control (N), respectively for the PCR reaction. These experiments were repeated at least three times. Negative controls without reverse transcriptase in the RT reaction prior to the PCR were performed for all samples. M 100 bp DNA Ladder (Amersham Biosciences) was used as standard marker.

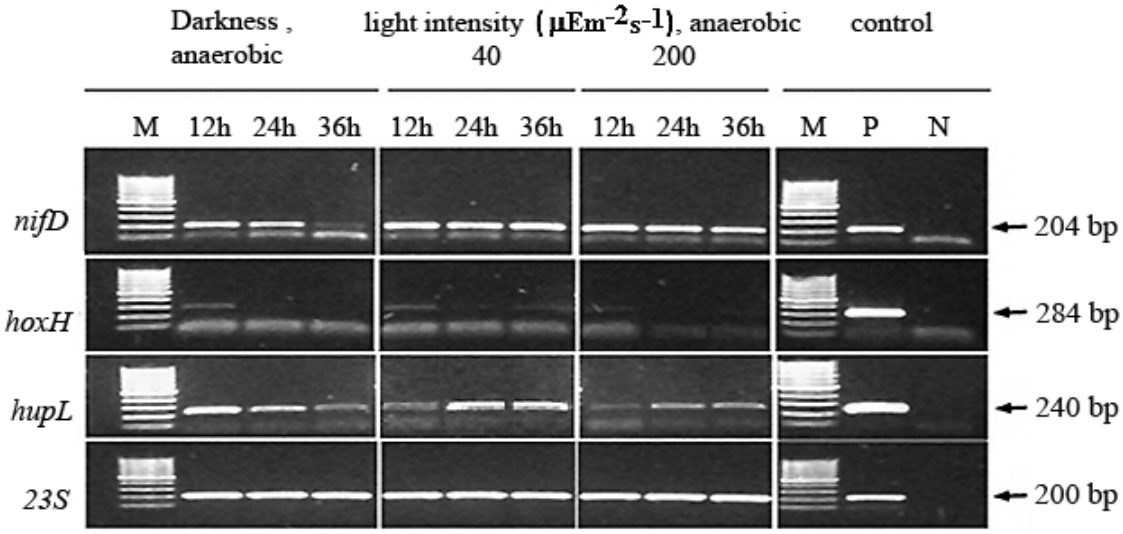

Figure 28 RT-PCR analysis of *nifD, hoxH and hupL* transcript levels in *A. siamensis* TISTR 8012 grown under different light intensities and durations. Total RNA was isolated from cells adapted in $BG11_0$ medium under different light intensities and incubated for 12, 24, 36 h under anaerobic condition at 30 °C. Other details were as described in the legend to Figure 27.

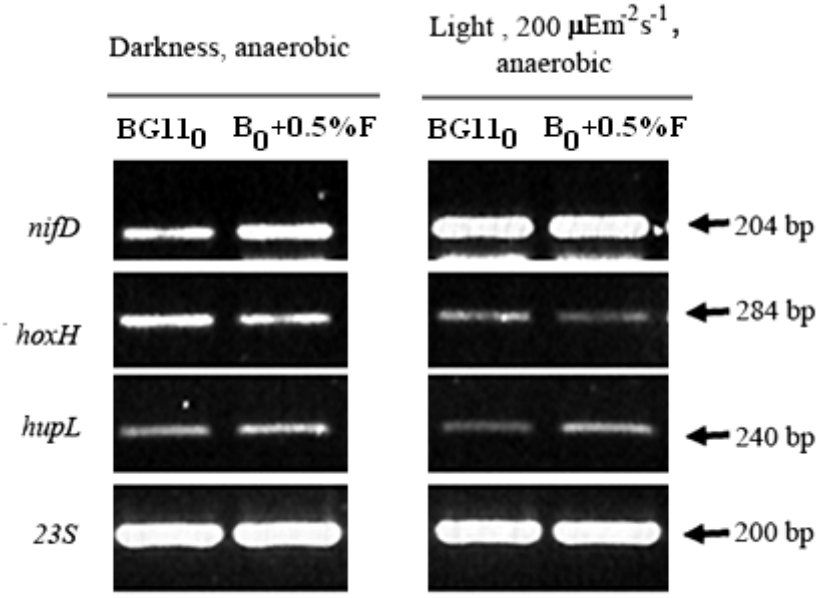

Figure 29 RT-PCR analysis of *nifD, hoxH and hupL* transcript levels in *A. siamensis* TISTR 8012 grown under optimum parameter for H_2 production. Total RNA was isolated from cells adapted anaerobically in $BG11_0$ containing Na_2CO_3 as carbon source and in $BG11_0$ supplemented with 0.5% fructose and incubated under darkness and 200 $\mu Em^{-2}s^{-1}$ of light intensity for 12 h at 30 °C. Other details were as described in the legend to Figure 27.

3.7 Study of activities of enzyme involved in H_2 metabolism under various conditions

3.7.1 Time course of nitrogenase activity by *A. siamensis* TISTR 8012

In vivo nitrogenase activity was analyzed using acetylene reduction assay, where nitrogenase can also catalyze the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). The *A. siamensis* TISTR 8012 cells grown in $BG11_0$ medium under N_2-fixing condition until reaching at log phase were transferred to a vial. After that, 1 mL of 20 % C_2H_2 was injected into the vial and incubated under anaerobic condition before detecting C_2H_4 production at indicated times as shown in Figure 30. Time course of nitrogenase activity under anaerobic condition revealed that nitrogease activity of *A. siamensis* TISTR 8012 was increased linearly with the time within the first 2 h of incubation. After that, the activity was decreased due to the depletion of substrate for activity of enzyme. From this result, 12 h of incubation time was used to detect activity of nitrogenase under various conditions in next experiment.

3.7.2 Effect of various conditions on nitrogenase activity

The nitrogenase activity in filamentous cyanobacteria has been reported to depend on several factors. It is very sensitive to O_2 and require more energy and reluctant for activity of enzyme. The *A. siamensis* TISTR 8012 cells were adapted under various conditions for 24 h before analyzing the activity of nitrogenase as shown in Table 10 to study optimum condition for enhanced nitrogenase function leading to increased H_2 production. The various conditions were investigated as followings:

3.7.2.1 Temperature: Optimum temperature for increasing of nitrogenases activity in *A. siamensis* TISTR 8012 was 30 °C. The lower or higher

than 30 ºC were not suitable for nitrogenase activity since there were no activities detected.

3.7.2.2 Light intensity: *A. siamensis* TISTR 8012 cells were adapted under different light intensities ranging from 0 40 and 200 μE $m^{-2}s^{-1}$ before determining nitrogenase activity. The result indicated that increased light intensity affected nitrogenase activity in *A. siamensis* TISTR 8012. Under 200 μE$m^{-2}s^{-1}$ of light intensity, cells showed highest nitrogenase activity and the maximum activity was approximately 36.81 ± 0.5 μmol C_2H_4 mg Chl a^{-1} h^{-1}. Increased time of light exposure showed no effect on the nitrogenase activity.

3.7.2.3 Combination effects: cells were adapted under controlled key factors such as source of carbon and light intensity to analyze nitrogenase activity. The results as shown in Table 10 indicated that *A. siamensis* TISTR 8012 had a high nitrogenase activity when supplemented with 0.5% fructose compared with normal $BG11_0$ medium. The maximum nitrogenase activity was approximately 48.31 ± 0.2 μmol C_2H_4 mg Chl a^{-1} h^{-1} when cells were adapted in $BG11_0$ supplemented with 0.5% fructose and incubated under light intensity of 200 μE$m^{-2}s^{-1}$.

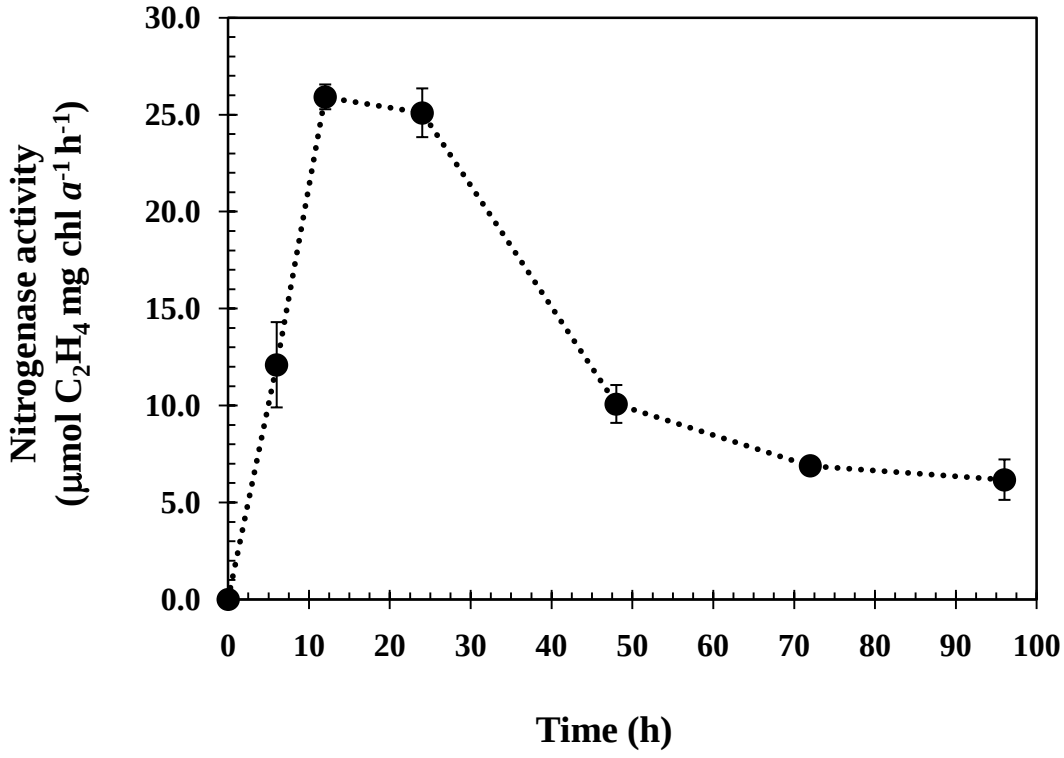

Figure 30 Time course of the nitrogenase activity by *A. siamensis* TISTR 8012. Cells at log phase grown in $BG11_0$ medium under growth condition were taken to determine activity of nitrognase using acetylene reduction assay under anaerobic condition. Means ± S.D. (n=3).

Table 10 Effect of various conditions on nitrogenase activity by *A. siamensis* TISTR 8012

Various conditions		**Nitorgenase activity (μmol C_2H_4 mg chl a^{-1} h^{-1})**
Temperature (ºC)	25	ND
	30	22.27 ± 4.2
	35	7.46 ± 0.1
	40	ND
	50	ND
Light intensity ($\mu Em^{-2}s^{-1}$)	Time of light exposure (h)	
0	12	17.27 ± 0.4
	24	12.72 ± 0.4
	36	18.34 ± 0.6
40	12	27.84 ± 0.2
	24	25.31 ± 0.5
	36	26.53 ± 0.3
200	12	36.81 ± 0.5
	24	34.22 ± 0.7
	36	35.61 ± 0.8
Darkness	$BG11_0$ contained Na_2CO_3	17.56 ± 0.4
	$BG11_0$ supplemented with 0.5% fructose	39.50 ± 0.8
200 $\mu Em^{-2}s^{-1}$	$BG11_0$ contained Na_2CO_3	34.68 ± 0.3
	$BG11_0$ supplemented with 0.5% fructose	48.31 ± 0.2

Each sample was incubated in each condition under anaerobic condition before measuring C_2H_4 production using gas chromatography. Means ± S.D. (n=3). ND= Not detectable.

3.7.3 Time course of bidirectional hydrogenase activity by *A. siamensis* TISTR 8012

In vivo bidirectional hydrogenase activity was analyzed by measuring the H_2 production in the presence of metyl viologen, which was reduced by sodium dithionite, using a gas chromatograph as described in materials and methods. The *A. siamensis* TISTR 8012 cells grown in different culture media, BG11 and $BG11_0$, were used to study time course of bidirectional hydrogenase activity. Linearly increased bidirectional hydrogenase activity of *A. siamensis* was observed in the first 30 min of incubation time under anaerobic dark condition in both media (Figure 31). Interestingly, cells in $BG11_0$ showed higher bidirectional hydrogenase activity than cells in BG11 medium. From the result, 10 min of incubation time was used to assay activity of bidirectional hydrogenate in the next experiment.

3.7.4 Effect of various conditions on bidirectional hydrogenase activity

Efficiency of bidirectional hydrogenase depends on several factors. In this experiment, *A. siamensis* cells were grown in $BG11_0$ medium, adapted under various conditions for 24 h before determination of activity as shown in Table 11, to study optimum condition for enhanced bidirectional hydrogenase activity leading to an increased of H_2 prodcution. The various conditions were observed as followings:

3.7.4.1 Temperature: Optimum temperature for increasing of bidirectional hydrogenase in *A. siamensis* TISTR 8012 was 40 °C. Temperature higher than 40 ºC could inhibit bidirectional hydrogenase activity.

3.7.4.2 Light intensity: *A. siamensis* cells grown in $BG11_0$ medium was analyzed the bidirectional hydrogenase activity under different light intensities ranging from 0, 40 and 200 $\mu Em^{-2}s^{-1}$. The results showed that activity of bidirectional

hydrgenase could be induced in the presence of low light intensities (40 μE $m^{-2}s^{-1}$). However, there were no significant changes of activity when cells were exposed to more intensity higher than 40 μE $m^{-2}s^{-1}$.

3.7.4.3 Combination effects: The bidirectional hydrogenase activity under controlled key factors as shown in Table 11 indicated that combination effects of 0.5% fructose-supplemented $BG11_0$ medium and incubation under darkness could significantly increase the activity of bidirectional hydrogenase higher than other conditions, with maximum bidirectional hydrogenase activity of 5.6 ± 0.2 nmol H_2 mg chl a^{-1} min^{-1} However, there were no significant changes of bidirectional hydrogenase activity under 200 μE$m^{-2}s^{-1}$ of light intensity either in the presence of fructose or in the absence of carbon source.

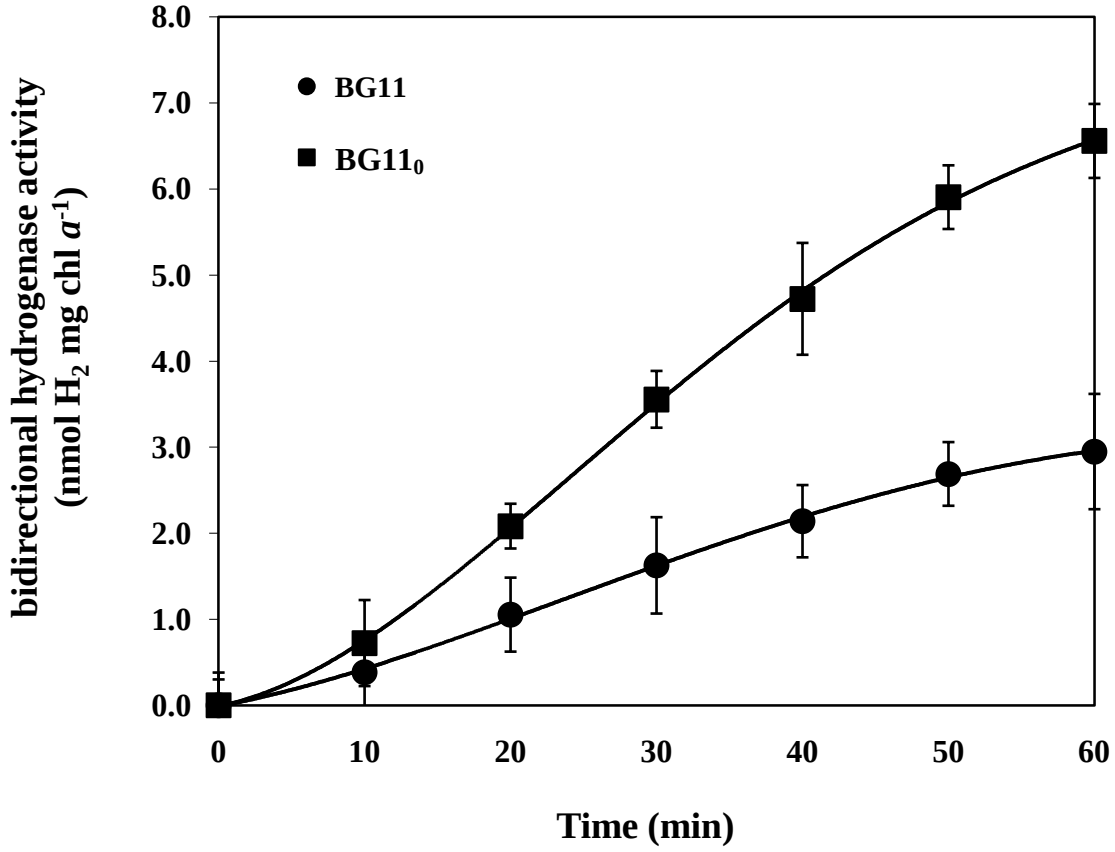

Figure 31 Time course of the bidirectional hydrogenase activity by *A. siamensis* TISTR 8012. Cells were grown in different media, BG11 (●) and $BG11_0$ (■) until cells at log phase, were used to determine activity of bidirectional hydrogenas under anaerobic condition by measuring H_2 production using methyl viologen (MV) as electron donor for bidirectional hydrogenase. Means ± S.D. (n=3).

Table 11 Effect of various conditions on bidirectional hydrogenase activity by *A. siamensis* TISTR 8012

Various conditions		**Bidirectional hydrogenase activity (nmol H_2 mg chl *a*$^{-1}$ h^{-1})**
Temperature (ºC)	25	2.4 ± 0.3
	30	3.8 ± 0.4
	35	4.1 ± 0.4
	40	4.7 ± 0.6
	45	3.6 ± 0.4
	50	2.4 ± 0.4
Light intensity ($\mu Em^{-2}s^{-1}$)	Time of light exposure (h)	
0	12	3.7 ± 0.1
	24	2.9 ± 0.2
	36	3.0 ± 0.6
40	12	5.1 ± 0.4
	24	4.3 ± 0.5
	36	4.0 ± 0.3
200	12	4.9 ± 0.5
	24	3.2 ± 0.1
	36	3.6 ± 0.5
Darkness	$BG11_0$ contained Na_2CO_3	4.4 ± 0.1
	$BG11_0$ supplemented with 0.5% fructose	5.6 ± 0.2
200 $\mu Em^{-2}s^{-1}$	$BG11_0$ contained Na_2CO_3	2.9 ± 0.3
	$BG11_0$ supplemented with 0.5% fructose	2.5 ± 0.8

Each sample was incubated in each condition under anaerobic condition for 10 min before measuring H_2 production using methyl viologen (MV) as electron donor for bidirectional hydrogenase. Means ± S.D. (n=3).

3.7.5 Effect of various conditions on uptake hydrogenase activity

The uptake hydrogenase catalyzes the consumption of the H_2 produced by the nitrogenase. This enzyme is a major obstacle to achieve an increase of H_2 production in the N_2-fixing cyanobacterium *A. siamensis* TISTR 8012. Therefore, in this experiment we focused on factors causing the reduction of uptake hydrogenase activity in order to maximize H_2 production such as:

3.7.5.1 Light intensity and time of light exposure: cells were adapted in $BG11_0$ medium under different light intensities namely, 0, 40 and 200 $\mu Em^{-2}s^{-1}$ and incubated in each condition for 12, 24 and 36 h before measuring uptake hydrogenase activity. The results from Table 12 revealed that the uptake hydrogenase activity of *A. siamensis* TISTR 8012 was not significantly different when cells were incubated under darkness for longer time. In contrast, when cells were incubated under both light intensities and longer light exposure, an induced H_2 uptake by *A. siamensis* TISTR 8012 was observed.

3.7.5.2 Combination effects: The uptake hydrogenase activity of cells adapted under combination effects of carbon source and light intensity was investigated. The results suggested that supplemented fructose in medium had a major effect on increasing uptake hydrogenase activity in either darkness or light condition (Table 12)

Table 12 Effect of various conditions on uptake hydrogenase activity by *A. siamensis* TISTR 8012

Various conditions		**Uptake hydrogenase activity ($\mu molH_2$ mg chl a^{-1} h^{-1})**
Light intensity ($\mu Em^{-2}s^{-1}$)	Time of light exposure (h)	
0	12	2.9 ± 0.7
	24	2.7 ± 0.5
	36	2.8 ± 0.6
40	12	3.3 ± 0.1
	24	5.3 ± 0.3
	36	6.5 ± 0.3
200	12	3.3 ± 0.5
	24	4.2 ± 0.3
	36	5.6 ± 0.8
Darkness	$BG11_0$ contained Na_2CO_3	5.2 ± 0.5
	$BG11_0$ supplemented with 0.5% fructose	6.1 ± 0.5
200 $\mu Em^{-2}s^{-1}$	$BG11_0$ contained Na_2CO_3	7.6 ± 0.5
	$BG11_0$ supplemented with 0.5% fructose	9.3 ± 0.2

Cell in each condition was measured for the reduction of H_2-saturated distilled water in the reaction vial and H_2 uptake was calculated using H_2 standard as described in APPENDIC M. Means ± S.D. (n=3).

3.8 Study of the competency of H_2 production by uptake hydrogenase deficient mutant strain

3.8.1 Creation of uptake hydrogenase deficient mutant by gene interruption mutagenesis

Although our results indicated that the *A. siamensis* TISTR 8012 has a high potential for H_2 production when cell were adapted under N_2-fixing condition using 0.5% fructose as carbon source, as well as growing anaerobically under continuous illumination of 200 μE $m^{-2}s^{-1}$ for 12 h. The enhanced H_2 production was mainly achieved from nitrogenase activity. However the net H_2 yield was lost due to the activity of the uptake hydrogenase. Therefore, knocking out the gene encoding uptake hydrogenase was conducted in order to maximize and sustain H_2 production by *A. siamensis* TISTR 8012. The uptake hydrogenase consists of at least two subunits encoded by *hupS* (small subunit) and *hupL* (large subunit) genes. In *A. siamensis* TISTR 8012, *hupS* and *hupL* contained 963 bp and 1596 bp, respectively, and the intergenic 195-bp sequence was found between *hupS* and *hupL* as shown in Figure 32A. A mutant of *A. siamensis* TISTR 8012 lacking the uptake hydrogenase (Δ*hupS*) was created by gene interruption mutagenesis where the *hupS* was inactivated by inserting neomycin antibiotic resistant cassette gene (Figure 32B).

3.8.2 Construction of recombinant plasmid to be transferred into *A. siamensis* TISTR 8012 cell

The strategy for construction of recombinant plasmid containing target gene interruption could be divided into three steps as shown in Figure 33. The first step, the *hupS* gene sequence information in *A. siamensis* TISTR 8012 was obtained from NCBI database, the accession number is AY152844. The *hupS* gene was amplified

from extracted genomic DNA of *A. siamensis* TISTR 8012 cells by using specific primers. The PCR condition was performed as described in materials and methods. An approximate size of 1.0 kb of *hupS* PCR product was purified and ligated into the pGEM-T easy vector (Promega), creating pGhupS plasmid. The pGhupS construct was digested with *Eco*RI restriction enzyme to prove the corrected size of pGhupS plasmid by using 0.8% agarose gel electrophoresis. The result showed two bands of *hupS* gene, an approximate size of 1.0 kb and pGEM-T easy vector, an approximate size of 3.0 kb, respectively (Figure 34) and this was then confirmed by sequencing.

The second step, the *Mlu*I fragment containing a neomycin (*NmR*) resistant cassette gene from pUC4K vector was modified blunt-ending and then inserted into *Eco*RV site within the *hupS* gene of the pGhupS plasmid to produce pGhupSNm plasmid. An approximate size of 2.2 kb of hupSNm fragment consists of hupS gene (1.0 kb) and neomycin resistant cassette gene (1.2 kb) this fragment was proved by digestion with *Eco*RI, analyzed by 0.8% agarose gel electrophoresis (Figure 35) and confirmed by sequencing.

The last step, a hupSNm fragment from pGhupSNm plasmid was amplified by using specific primers and then cloned into pRL271 vector to produce pRLhupSNm plasmid. The pRLhupSNm plasmid was called cargo plasmid suitable to be transferred into filamentous cyanobacterial strain. The pRLhupSNm plasmid was correctly checked by digesting with *Spe*I and *Pst*I restriction enzymes and analyzed by 0.8% agarose gel electrophoresis as shown in Figure 36. Two bands of pRL271 vector (6.3 kb) and hupSNm frangment (2.2 kb) were observed. After that, pRLhupSNm was analyzed by sequencing to ascertain 100 % correction.

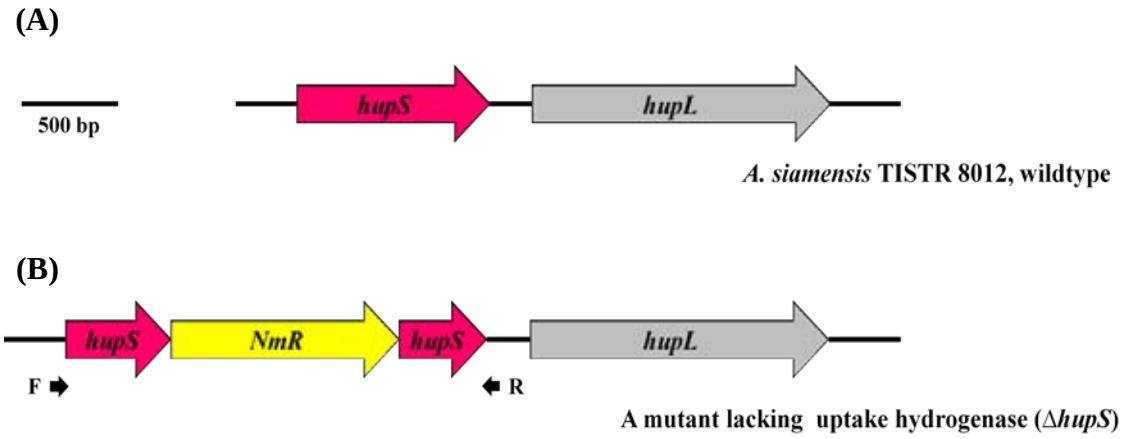

Figure 32 Physical map of *hupSL* gene of wild type *A. siamensis* TISTR 8012 (A) and a mutant strain lacking the uptake hydrogenase (Δ*hupS*) created by *hupS* gene interrupted with neomycin antibiotic resistant cassette gene (B). *hupS* and *hupL* contained 963 bp and 1596 bp, respectively and the intergenic 195 bp sequence was found between *hupS* and *hupL*. F and R are primers used for PCR analysis and mutant confirmation.

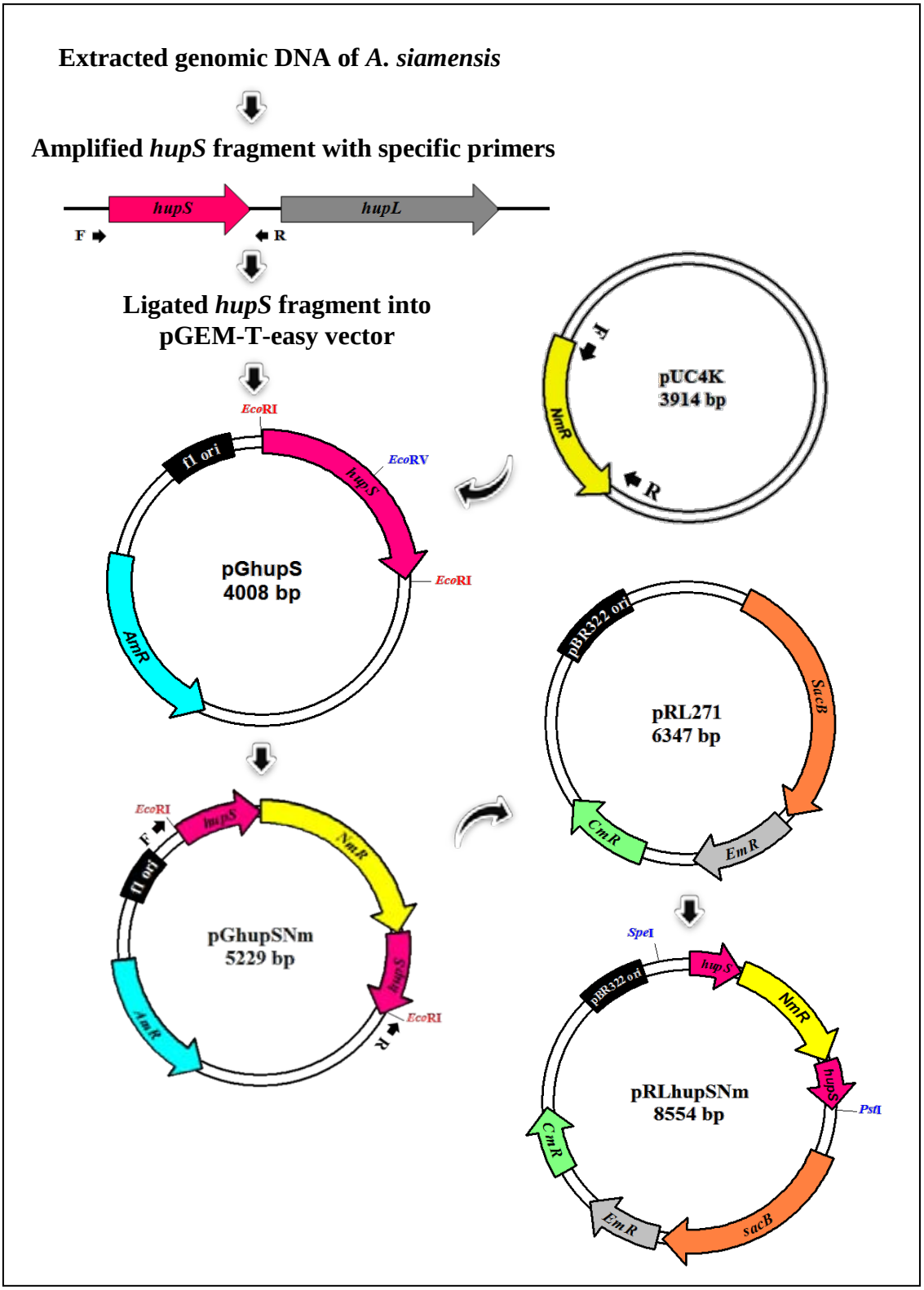

Figure 33 The strategy for construction of recombinant plasmid contained *hupS* gene interruption.

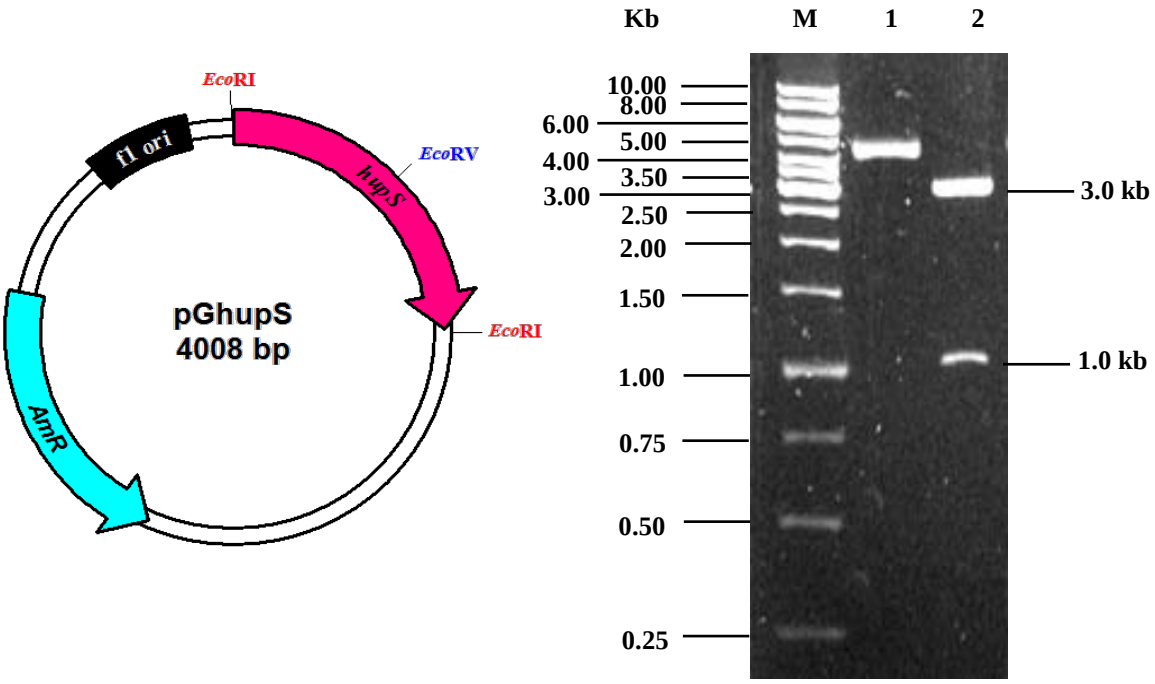

Figure 34 The physical map of pGhupS plasmid and 0.8% agarose gel electrophoresis analyzed pGhupS plasmid by digested with *Eco*RI restriction enzyme.

Lane M : 1 kb standard ladder marker

Lane 1: uncut pGhupS plasmid

Lane 2: pGupS plasmid was cut with *Eco*RI

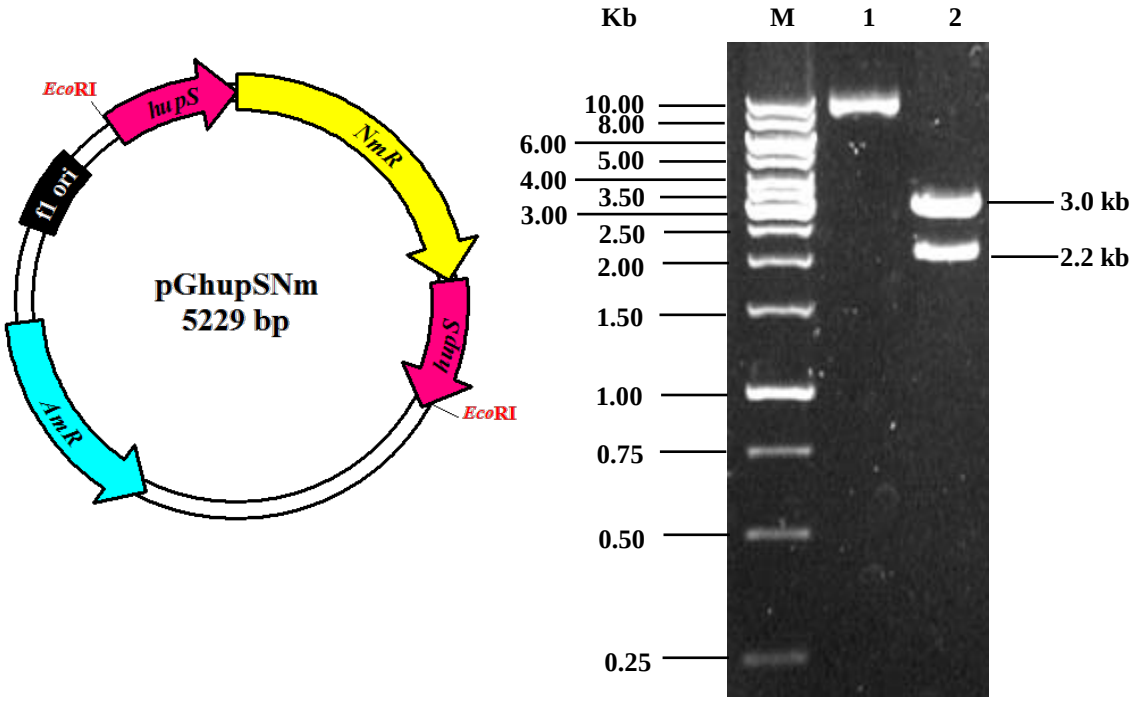

Figure 35 The physical map of pGhupSNm plasmid and 0.8% agarose gel electrophoresis analyzed pGhupSNm plasmid by digested with *Eco*RI restriction enzyme.

Lane M : 1 kb standard ladder marker

Lane 1: uncut pGhupSNm plasmid

Lane 2: pGupSNm plasmid was cut with *Eco*RI

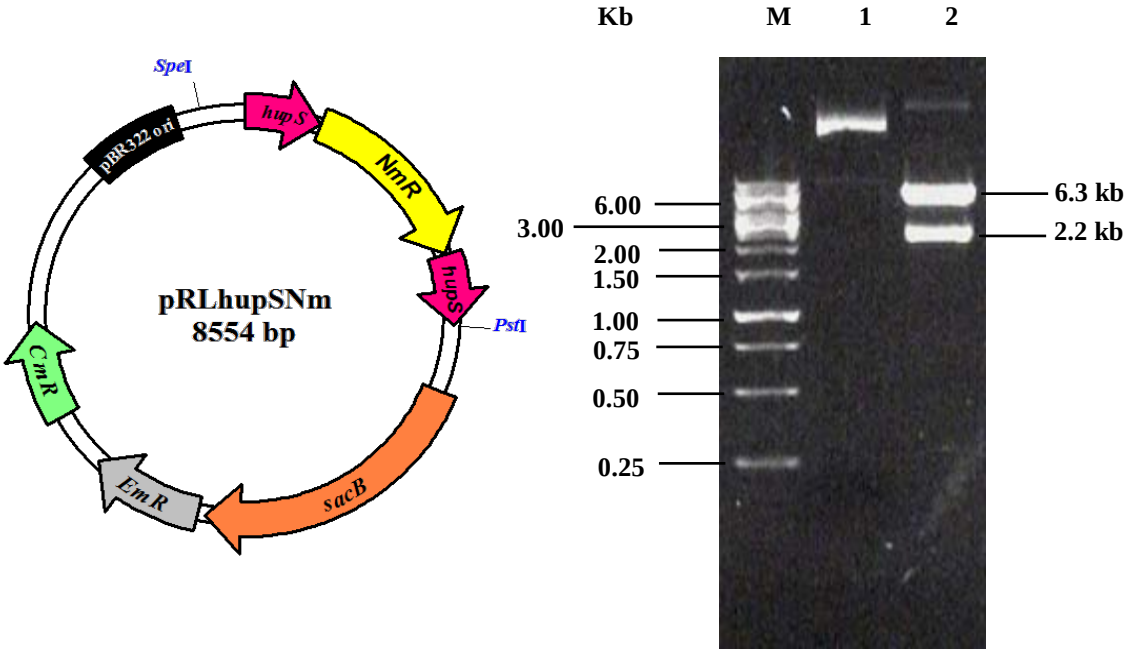

Figure 36 The physical map of pRLhupSNm plasmid and 0.8% agarose gel electrophoresis analyzed pRLhupSNm plasmid by digested with *Spe*I and *Pst*I restriction enzymes.

Lane M : 1 kb standard ladder marker

Lane 1: uncut pRLhupSNm plasmid

Lane 2: pRLhupSNm plasmid cut with *Spe*I and *Pst*I

3.8.3 Gene transfer by using conjugative method

Three plasmids used for transferring *hupS* target gene interruption into filamentous cyanobacterium *A. siamensis* TISTR 8012 were: (1) cargo plasmid (pRLhupSNm) containing *hupS* gene interruption, (2) helper plasmid (pRL623) containing methylase gene to protect degradation of cargo plasmid from restriction enzyme in filamentous cyanobacterial cell. This is due to some filamentous cyanobacteria have a specific restriction enzyme to destroy the foreign DNA into the cell, and (3) conjugative plasmid (pRL443) containing transfer gene to mobilize cargo plasmid to be transferred into *A. siamensis* cell.

Conjugative method is one of the methods for gene transfer into filamentous cyanobacteria by using cell to cell contact. It is based on the mobilization of DNA from bacteria (usually *Escherichia coli* strain HB101). The pRLhupSNm (cargo plamid) was transformed into *E. coli* HB101 carrying pRL623 (helper plasmid) and the clone was selected on LB agar plate supplemented with 30 μg mL^{-1} chloramphenicol and 25 μg mL^{-1} neomycin antibiotics. The clone was called cargo strain. The pRL443 (conjugative plasmid) was transformed into *E. coli* HB101 and the clone was selected on LB agar plate supplemented with 100 μg mL^{-1} amplicillin. The clone was called conjugative strain. After mixing suspension of cargo strain, conjugative strain and *A. siamnesis* cell, the pRLhupSNm would be transferred into *A. siamensis* TISTR 8012 cell by the conjugative plasmid helping.

3.8.4 Selection of recombinant colonies of *A. siamensis* TISTR 8012

Three weeks after transformation of *hupS* gene interruption into *A.siamensis* cell, the recombinant colonies were grown in BG11 medium supplemented with 25 μg mL^{-1} neomycin antibiotic as shown in Figure 37 by using cells grown in BG11

medium without antibiotic as positive control. Cells without target gene transformation grown in BG11 medium supplemented with 25 μg mL^{-1} neomycin antibiotic were used as negative control, respectively. The appeared recombinant colonies were cultured on BG11 plate containing neomycin antibiotic for at least three generations and then transferred to BG11 broth contained neomycin antibiotic for one week before checking the complete segregation using colony PCRs. To ensure the complete segregation of obtained *hupS* gene knockouts, colony PCR was performed by using primer pair specific to *hupS* gene. PCR products were analyzed by 0.8% agarose gel electrophoresis as shown in Figure 38. The PCR result showed that the DNA bands of Δ*hupS* mutant strain were up-shifted (*hupS* gene + neomycin resistant antibiotic cassette gene) compared to that of wild type strain of *A. siamensis* TISTR 8012.

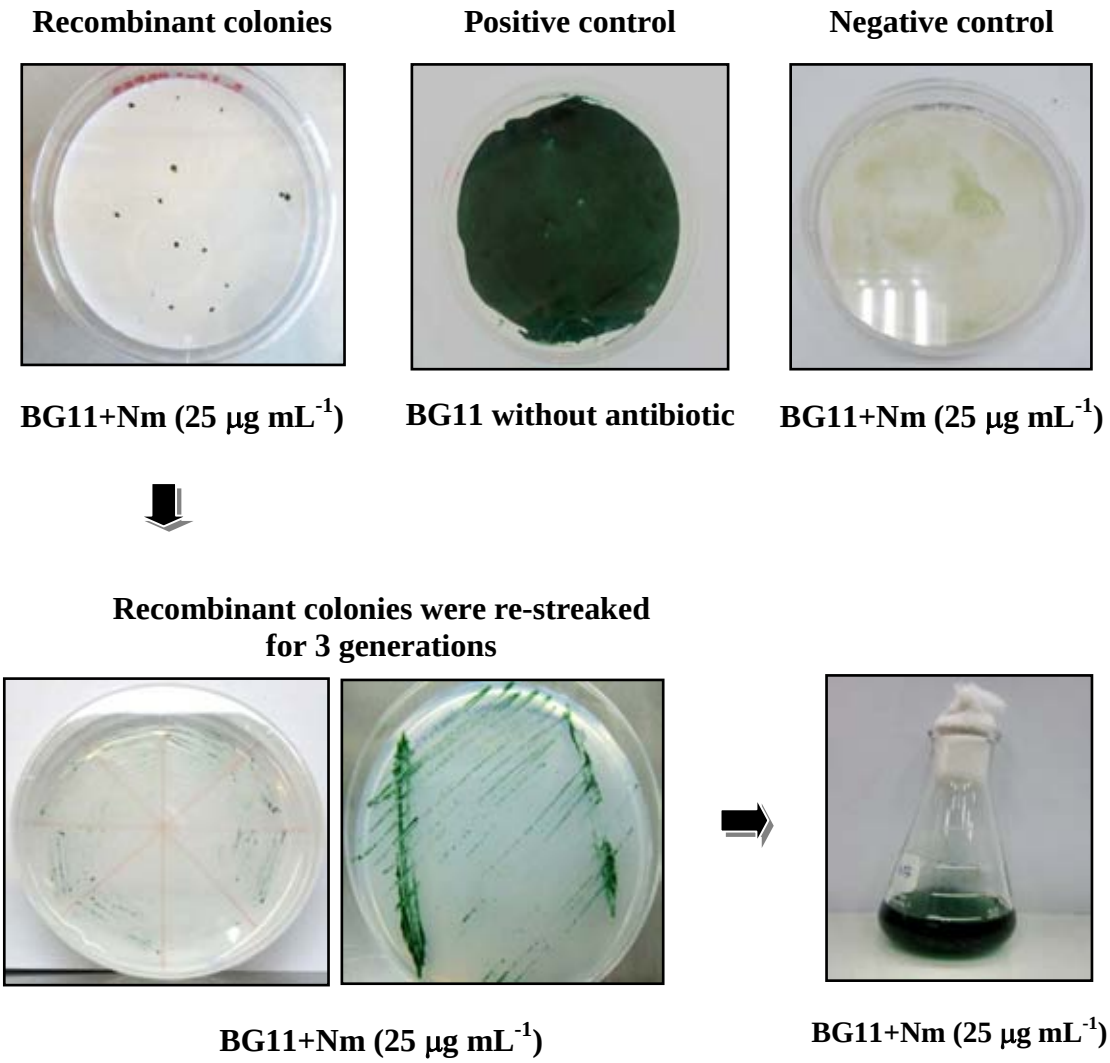

Figure 37 The recombinant colonies were grown in BG11 medium supplemented with 25 μg mL^{-1} neomycin antibiotic on agar and both medium. Positive control: cells grown in BG11 medium without antibiotic. Negative control: cells without gene transformation grown in BG11 medium supplemented with 25 μg mL^{-1} neomycin antibiotic.

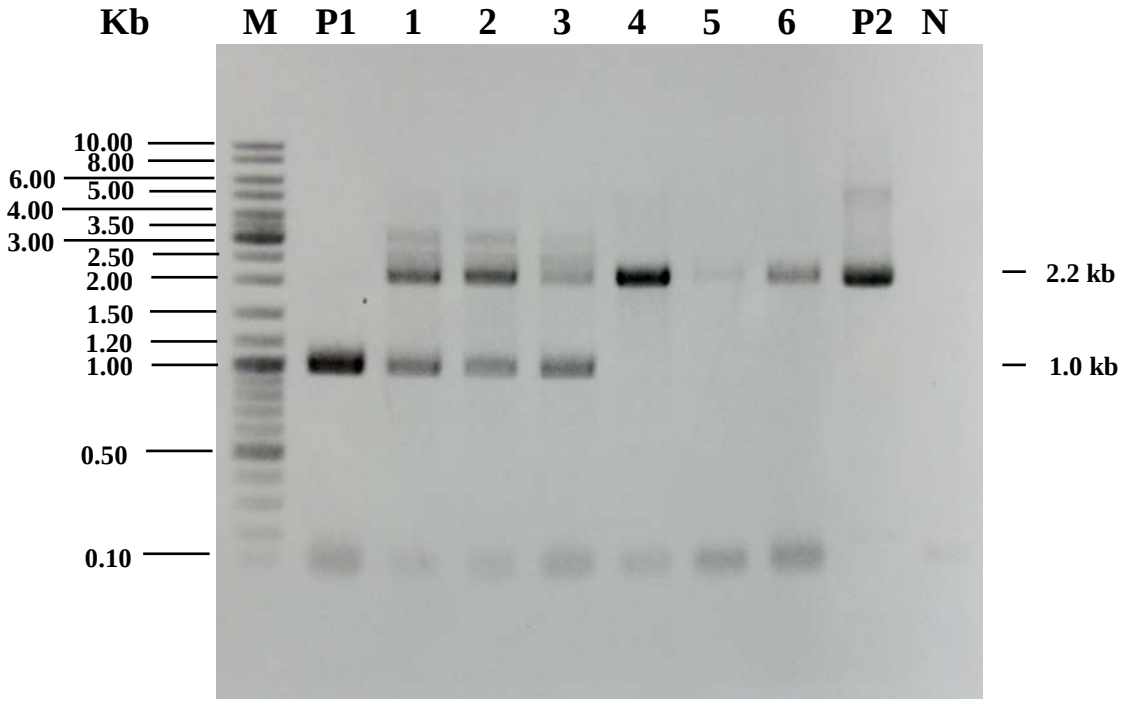

Figure 38 The confirmation of complete segregation of a mutant strain lacking the uptake hydrogenase (Δ*hupS*) using colony PCRs and analyzed by 0.8% agarose gel electrophoresis. Primer pair specific to *hupS* gene was used.

Lane M: GeneRuler^{TM} DNA ladder (Fermentas)

Lane P1: Positive control (PCR product of *hupS* gene using genomic DNA of wild type as template)

Lane1-3: PCR products of recombinant colonies cultured in BG11 medium plus antibiotic for 2 weeks show not completed segregation

Lane4-6: PCR products of recombinant colonies cultured in BG11 medium plus antibiotic for 4 weeks show completed segregation

LaneP2: Positive control (PCR product of hupSNm using pRLhupSNm plasmid as template)

Lane N: Negative control using H_2O as template

3.9 Comparison of growth rate and H_2 production between Δ*hupS* mutant and wild type of *A. siamensis* TISTR 8012

The *A. siamensis* TISTR 8012 wild type and Δ*hupS* mutant strain were grown in BG11 medium containing 18 mM of $NaNO_3$ as N-source and $BG11_0$ without N-source under growth condition. Samples were taken to measure the optical density of cell culture and chlorophyll *a* content every three days of cultivation. The results showed that the growth rate of wide type and Δ*hupS* mutant had a similar pattern in both media whereas Δ*hupS* mutant cells grew at a slightly slower rate than wild type cells (Figure 39A, B).

Analyzing H_2 production, wide type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 cells were grown under N_2-fixing condition in $BG11_0$ medium for 0, 12, 24, 48, and 72 h, respectively. H_2 production was then determined under continuous illumination of 40 $\mu Em^{-2}s^{-1}$ and anaerobic condition for 12 h. Interestingly, Δ*hupS* mutant substantially produced H_2 at a higher rate than that of wild type. The maximum H_2 production rate of Δ*hupS* mutant was about 29.7 $\mu molH_2$ mg chl $a^{-1}h^{-1}$ when grown in $BG11_0$ medium for 72 h, which is almost 4-folds higher H_2 production rate than wild type (Figure 40) under normal growth condition. This indicated that inactivation of *hupS* gene results in enhanced H_2 production in *A. siamensis* TISTR 8012.

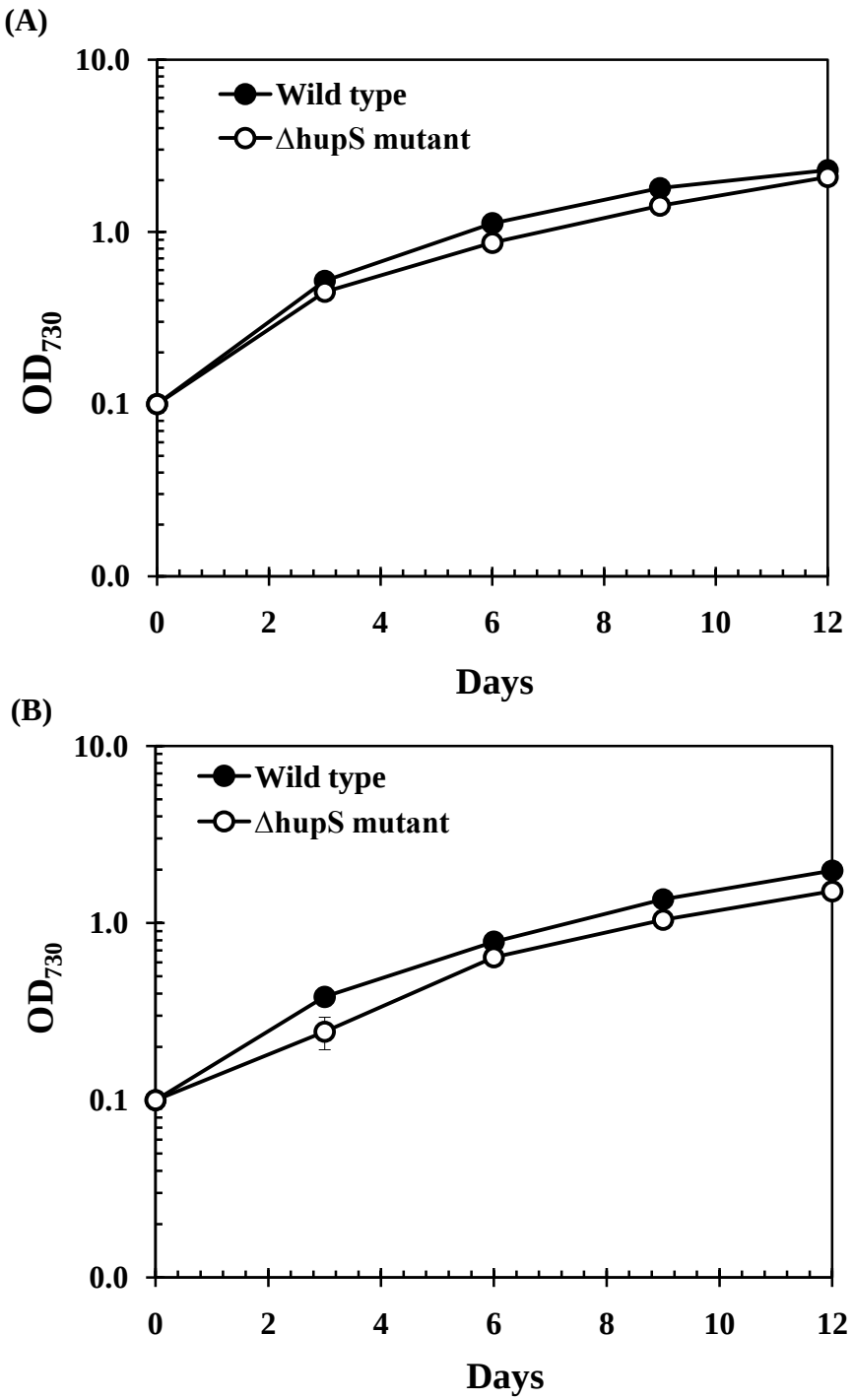

Figure 39 Comparison of growth rate between wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 when the cells were grown in BG11 (A) and $BG11_0$ (B) media, respectively, means ± S.D. (n=3).

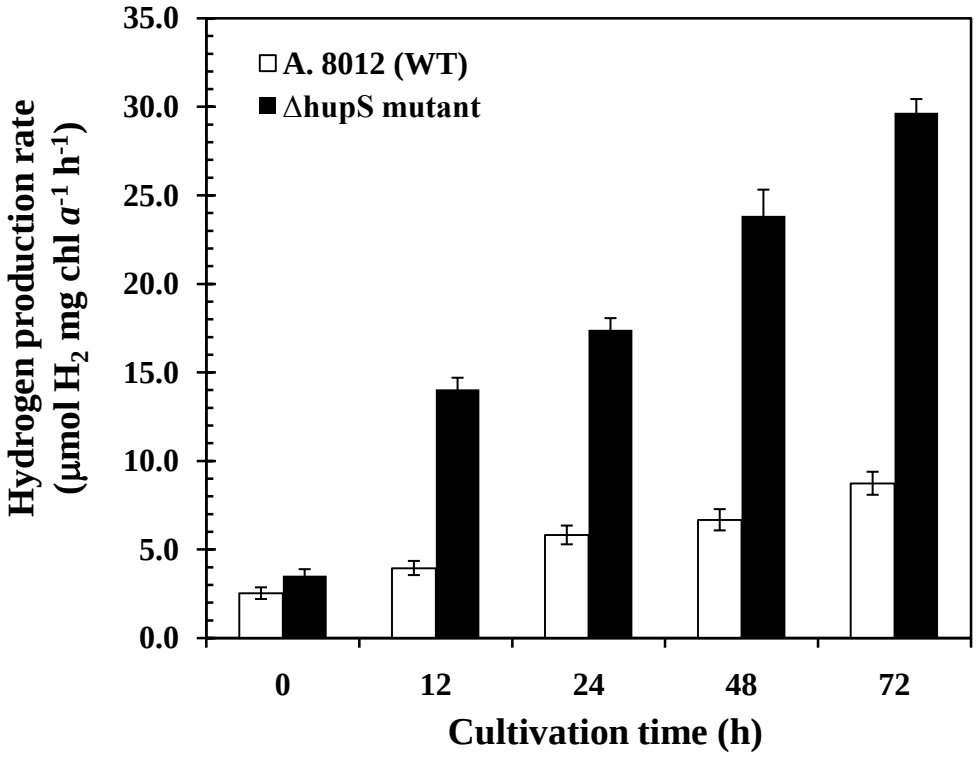

Figure 40 Comparison of H_2 production between wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 when the cells were grown in $BG11_0$ medium for 0, 12, 24, 48, and 72 h of cultivation time. H_2 production rate was determined under continuous illumination of 40 $\mu Em^{-2}s^{-1}$ and anaerobic condition for 12 h. Means ± S.D. (n=3).

3.10 Study of H_2 production and nitrogenase activity in Δ*hupS* mutant under long term of light exposure

The previous result showed that H_2 production of wild type *A. siamensis* TISTR 8012 could drop when cells were incubated under light condition for longer time because of a higher uptake hydrogenase activity. In this experiment, the Δ*hupS* mutant with inactivated uptake hydrogenase activity was grown in N_2-fixing condition under growth condition before determining H_2 production every 12 h under continuous illumination of 200 μE $m^{-2}s^{-1}$ and anaerobic condition compared with wild type strain. Interestingly, the results indicated that Δ*hupS* mutant had more efficiency for H_2 production under long term of light exposure than wild type strain. The H_2 production rate in the mutant was not decreased even after exposure to light longer than 12 h. (Figure 41A).

In addition, nitrogenase activity of Δ*hupS* mutant was also investigated under the same condition at above. The result revealed that nitrogenase activity of the mutant was increased when cells were incubated for longer time under light condition, with a 2-3 folds higher C_2H_4 production than wild type strain (Figure 41B).

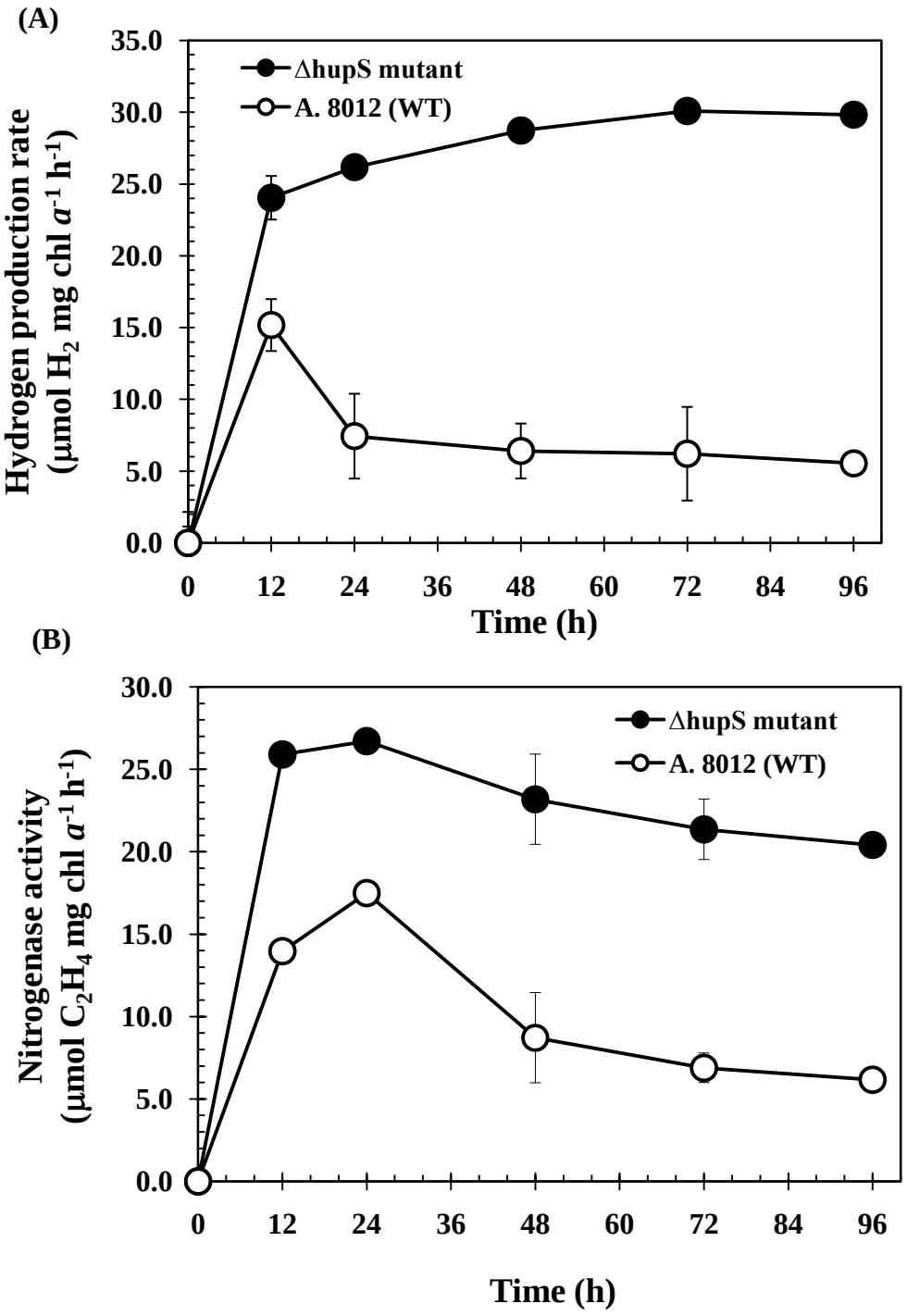

Figure 41 Comparison of H_2 production (A) and nitrogenase activity (B) between wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 when cells were grown in $BG11_0$ medium before measuring H_2 production and nitrogenase activity in every 12 h under continuous illumination of 200 μE $m^{-2}s^{-1}$ and anaerobic condition. Means ± S.D. (n=3).

3.11 Gene expression analysis of *A. siamensis* TISTR 8012 with inactivated HupS

The Δ*hupS* mutant had more efficiency for H_2 production than that of wild type strain because uptake hydrogenase acitivity was inactivated. However, the beneficial functions of uptake hydrogenase is known to supply the recycled reducing energy back to N_2-fixation and other processes and provide ATP from oxyhydrogen reaction. Importantly, how the cell can improve their metabolism for enhancing H_2 production through nitrogenae and bidirectional hydrogenase with the loss of uptake hydrogenase activity. In this experiment, the expression of genes was analyzed associating with H_2 metabolism in *A. siamensis* TISTR 8012 when knocking out *hupS* gene compared with that of wild type strain under N_2-fixing condition. The different genes analyzed in this experiment were genes involved in H_2 metabolism, such as *nifD*, *hupL*, and *hoxH*; gene associated with photosynthesis in either heterocyst or vegetative cell, *fdxH*, *psaA*, *psbA* and *coxA*, and genes involved in heterocyst differentiation, *hetR* and *ntcA*, respectively. Figure 42 showed RT-PCR products using RNA isolated from both wild type and Δ*hupS* mutant strains when grown in $BG11_0$ medium for 12, 24, 48, 72 h and incubated anaerobically at 30 ºC under continuous illumination of 40 μE $m^{-2}s^{-1}$.

The relative transcript of each respective gene was calculated by using GeenTools programme as shown in Figure 43, under nitrogen limitation, nitrogenase will be active for maintenance of the nitrogen cycle. Wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 showed higher expression of *nifD* when lacking N-source for longer time. Interestingly, *nifD* was found to be highly expressed in Δ*hupS* mutant compared to wild type strain at the first day of N-deprivation. Unexpectedly, *hupL* transcription level of Δ*hupS* mutant also gave stronger signal than wild type despite

inactivation of small subunit HupS. Another hydrogenase, *hoxH* transcription levels were not significantly changed in both wild type and Δ*hupS* mutant. Interestingly, *FdxH* gene, encoding heterocyst-spectific ferredoxin (FdxH) and considered to be electron donor to nitrogenase, was slightly increased in Δ*hupS* mutant when compared with wild type.

Moreover, genes involved in both photosynthesis and respiratory electron transport were also studied. In Δ*hupS* mutant, the expression of *psabA* and *coxA* encoding D1 protein of photosystem II and cytochrome c oxydase complex I, respectively, were more enhanced than wild type strain. In contrast, there were no changes in the expression of *psaA* gene encoding core protein of photosystem I in both the mutant and wild type.

In addition, unchanged transcription levels of key genes involved in heterocyst differentiation, *hetR* encoding primary activator protein and *ntcA* encoding the transcription factor NtcA were observed in Δ*hupS* mutant when compared with wild type strain.

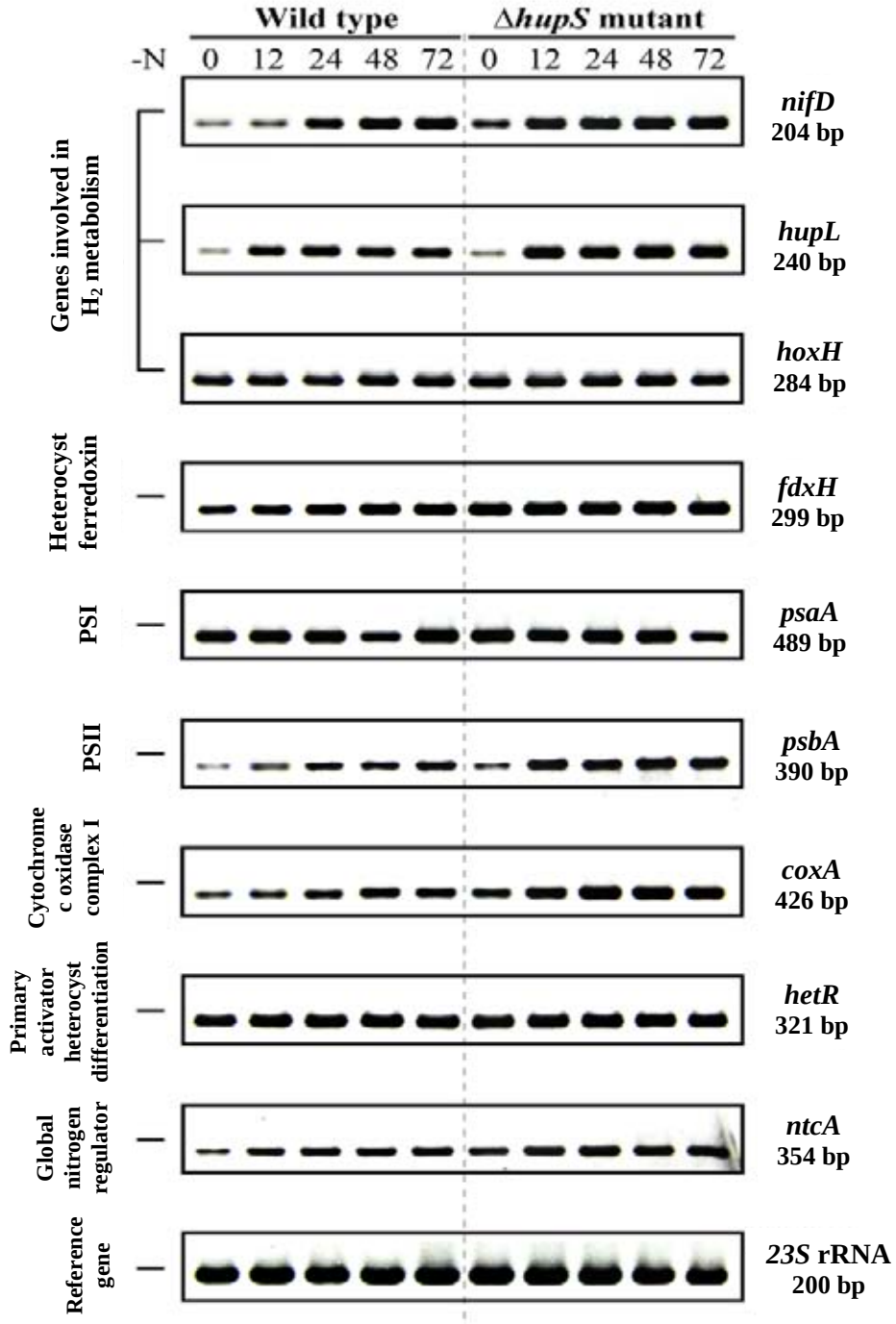

Figure 42 Genes expression analysis by RT-PCR using total RNA isolated from wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 grown in $BG11_0$ without N-source for 0, 12, 24, 48, 72 h under growth condition. PCR amplification using cDNAs of respective genes were performed using specific primers. *23S* rRNA was used as an internal reference gene.

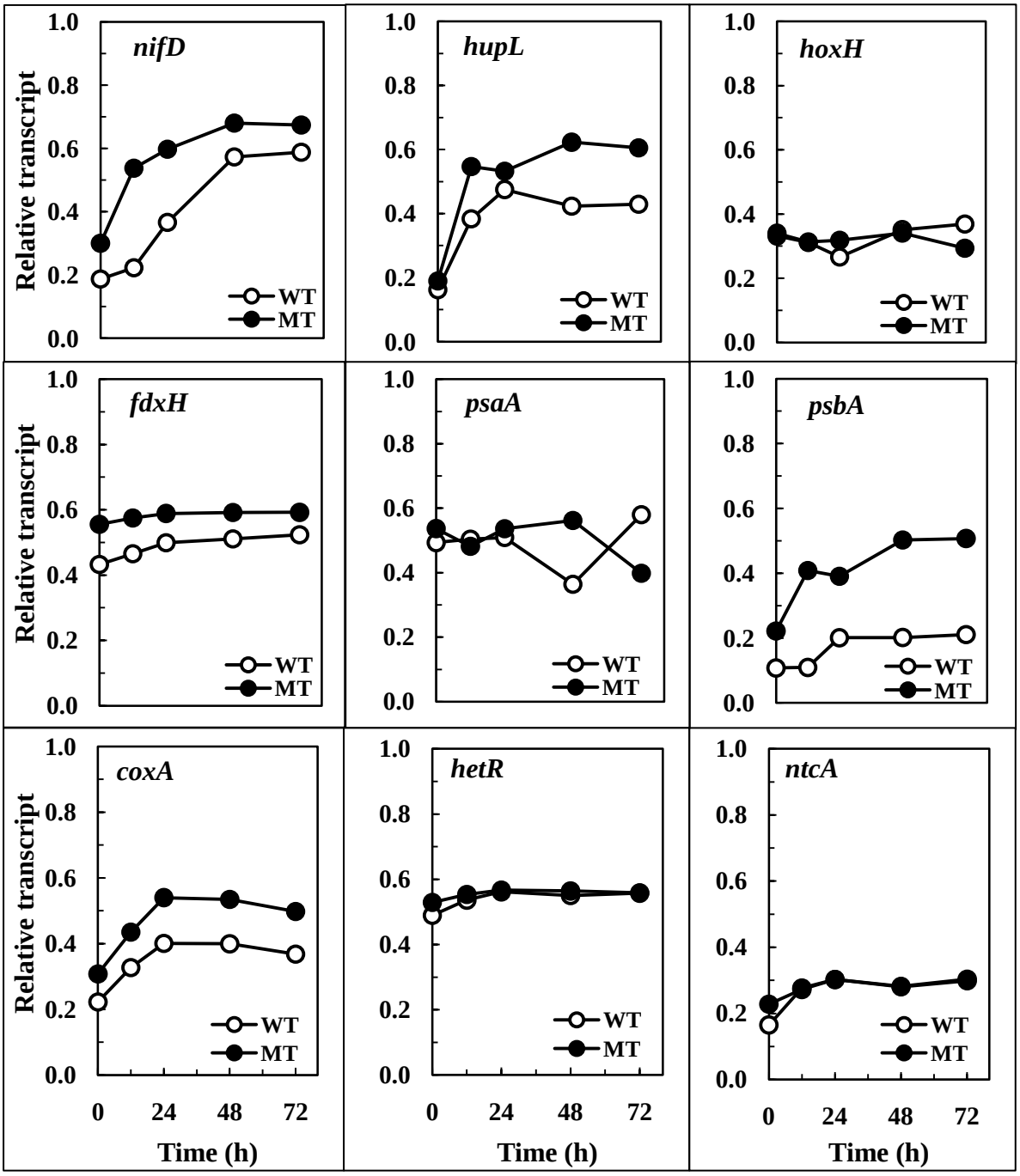

Figure 43 Comparison of genes expression in wild type and Δ*hupS* mutant of *A. siamensis* TISTR 8012 by using the relative transcript level. The transcript of each gene as shown in figure 42 was calculated by comparing to transcription of the reference gene (*23S* rRNA) and shown in the form of relative transcript.

CHAPTER IV

DISCUSSION

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 is the model organism in this study on the efficiency of biohydrogen production. The *A. siamensis* TISTR 8012 can utilize many nitrogen sources in order to support growth and also able to fix dinitrogen from atmosphere to produce ammonia. Nitrate did not have any effect on cell differentiation in *A. siamensis* TISTR 8012 whereas under N_2-fixing condition there were various morphology changes. Akinete and heterocyst differentiation were found under N-deprivation and heterocyst frequency gradually increased with time. In addition, *A. siamensis* TISTR 8012 cells show chlorosis when grown in N-deprived medium. This change of color was in line with a general response to nutrient deprivation (chlorosis) - nitrogen limitation likely induced a well-characterized set of cellular responses, visible chlorosis, alteration of the ratio of phycocyanin to allophycocyanin and decreasing amount of chlorophyll (Stevens *et al*., 1981). The cell surface observed under Scanning Electron Microscopy (SEM) revealed that the filaments of *A. siamensis* TISTR 8012 divide exclusively by binary fission in one plane and heterocyst cells appear in either the end of filament or the middle of filament. The ultrastructure of *A. siamensis* TISTR 8012 observed under Transmission Electron Microscopy (TEM) shows component similar to other heterocystous filamentous cyanobacteria *Anabaena* PCC 7120 and *N. punctiforme* PCC 73102 (Seabra *et al*., 2009). The vegetative cell exhibits obvious convoluted thylakoids and some carboxysomes. Interestingly, the thickness of heterocyst's envelope of *A. siamensis* TISTR 8012 is almost 0.5 μm which is thicker than those

previously reported. This characteristic has the advantage in restriction of O_2 diffusion into the cells and suitable for nitrogenase function.

The phylogenetic analysis of *A. siamensis* TISTR 8012 using *16S* rDNA gene was performed to study the phylogenetic relationship between different cyanobacterial strains. Surprisingly, *A. siamensis* TISTR 8012 is classified into a single group with different *Anabaena* species branching as shown in Figure 6. The causes of biodiversity, habitat and symbiotic relationship may have a major effect on unique group of *A. siamensis* TISTR 8012. However, the distance of *A. siamensis* TISTR 8012 shows still close to the potential H_2 production strains such as *Anabaena* PCC 7120 and *A. variabilis* ATCC 29413. Therefore, *A. siamensis* TISTR 8012 originally isolated from rice paddy field in Thailand is an interesting model organism to study the capacity of H_2 production.

In order to promote a high potential H_2 production, we divided the process for biohydrogen production by *A. siamensis* TISTR 8012 into three stage; (1) growth stage, (2) cell adaptation stage and (3) H_2 production stage. During the first stage, cells were grown under optimum condition yielding higher amount of cells before adapting cells under various conditions for 24 h to enhance activities of nitrogenase and bidirectional hydrogenase in the second stage. In the last stage, cells were placed H_2 under anaerobic condition to produce H_2.

For optimization of growth, the *A. siamensis* TISTR 8012 were grown in different culture media namely, BG11, $BG11_0$, Allen-Arnon and N-free, respectively. The growth rate pattern can be divided into three different phases, log phase, late log phase and stationary phase, respectively. After that cells in each phase were initially investigated for their ability of H_2 production. The results indicated that cells at log phase had better ability to produce H_2 than cell at other phases and cells under

N_2-fixing conditions ($BG11_0$ medium) showed a 15-fold higher H_2 production rate when compared with cells under non N_2-fixing conditions (BG11 medium). In *Synechocystis* PCC 6803, it was found that log phase was the best growth phase for H_2 production (Baebprasert *et al*., 2010). In contrast, Chen *et al* (2008) demonstrated that cells of the cyanobacterium *Anabaena* sp. strain CH3 in late-log phase had a better ability to produce hydrogen than at log phase, supporting that the H_2 production is strongly dependent on growth phase with differences depending on strain used. This may be explained by the fact that cells in the later growth phases need to maintain cellular metabolism rather than to release an excess of reducing power as H_2.

Moreover, the expression of genes encoding enzymes involved in H_2 metabolism, *nifD*, *hoxH* and *hupL* were examined under different growth conditions and cell ages by reverse transcription-PCR (RT-PCR) as shown in Figure 27. Strong expression of *nifD* was observed in N_2-fixing condition ($BG11_0$-grown medium) with gradually higher transcript levels in every cell age period. In addition, under both N_2-fixing and non N_2-fixing conditions there were no significant changes in the transcript level of *hoxH* in *A. siamensis* TISTR 8012 since its function is independent of N_2 fixation (Serebryakova *et al*., 1996). These results supported that a higher H_2 production by *A. siamensis* TISTR 8012 depended on a higher nitrogenase activity.

However, a lower growth rate of cell under N_2-fixing condition was observed. Therefore, the screening of optimum physical parameters to enhance growth rate in $BG11_0$ medium were investigated and found that cells were grown at 30 ºC under a light intensity between 40-50 μE m^{-2} s^{-1} and pH 7.5 indicated *A. siamensis* TISTR 8012 used times less than one day to adjust themselves in N-deprivation.

The characterization of H_2 production under various conditions by *A. siamensis* TISTR 8012 demonstrates the following:

(1) Effect of pH: In general, acidic pH of culture medium was not favorable for the growth of any cyanobacterial strains and the optimum pH for growth and H_2 production by *A. siamensis* TISTR was 7.5 using 10 mM HEPES buffer controlling pH. The lower pH of 5.5 and higher pH of 9.5 significantly reduced H_2 production. The reduction in H_2 production under higher pH might be due to the activation of uptake hydrogenase that functions optimally at pH 9 (Markov *et al*., 1995).

(2) Effect of temperature: The optimum temperature for most cyanobacterial H_2 production is between 30-40 ºC varying in different cyanobacterial species. The maximum H_2 production in *A. siamensis* TISTR 8012 was 30 ºC similar to *Anabaena variabilis* SPU 003 (Shah *et al*., 2001), whereas *Nostoc muscorum* SPU 004 demonstrated maximum H_2 production rate at 40 ºC (Shah *et al*., 2003).

(3) Effect of variation of N-sources and concentrations: Although most cyanobacterial strains can utilize various nitrogen sources to support their growth. However, all exogenously supplemented N-source strongly inhibit nitrogenase synthesis (Rawson, 1985). In *A. siamensis* TISTR 8012, the H_2 production was drastically depressed when grown in medium containing nitrate, nitrite, ammonium or even a low concentration of nitrate. The supplemented N-sources have also been reported to inhibit nitrogenase activity in *Anabaena variabilis* SPU 003 and *Anabaena cylindrical* (Datta *et al*., 2000), suggesting that H_2 production in N_2-fixing cyanobacteria depends greatly on nitrogenase activity.

(4) Effect of various supplemented carbon sources: Cyanobacteria can utilize both CO_2 and bicarbonate ions (CO_3^{2-}) as a source of carbon in photosynthesis, with bicarbonate being converted to CO_2 by an active carbonic anhydrase activity. In addition, they are able to use various sugars as source of carbon for heterotrophic growth (Smith, 1983). The presence of some sugars strongly induces a higher H_2

production in *A. siamensis* TISTR 8012 (Figure 15B), probably due to the organic substrates as sources of energy (ATP) and reductants for nitrogenase (Neuer and Bothe, 1985). The most preferred substrate for H_2 production in *A. siamensis* TISTR 8012 was fructose. This result is in agreement with previous study that the N_2-fixing cyanobacteria *A. variabilis* and *Anabaena* sp. CH3 also are able to use fructose as a substrate for increased H_2 production (Chen *et al*., 2008). Results of sugar uptake in Figure 16 indicated that *A. siamensis* TISTR 8012 was more efficient to take up fructose into the cells than other sugars, suggesting the use of fructose for heterotrophic growth by *A. siamensis* TISTR 8012. The ability of *A. siamensis* TISTR 8012 to utilize sugars as substrate to produce hydrogen may be beneficial for its potential usage in waste water treatment in the future. The previous studies have reported that the oxidative pentose phosphate pathway is the major pathway for fructose metabolism. Fructose has important role involved in N_2-fixation in heterocyst because its presence resulted in increased respiration leading to O_2 reduction, a condition that supports nitrogenase activity (Haury and Spiller, 1981). Nevertheless, higher fructose concentration could inhibit metabolism of cells and led to a decrease of H_2 production.

(5) Effect of light intensity and time of light exposure: Previous studies have reported that light is a key factor affecting H_2 production in cyanobacteria and that the light effect varies among different species and strains. The heterocystous cyanobacteria *A. variabilis* ATCC 29413, *N. muscorum*, *A. cylindrica* and *Anabaena* sp. PCC 7120 were found to produce H_2 under light conditions since the nitrogenase activity is strictly dependent on the energy supplied by the light (Hallenbeck *et al*., 1978; Tsygankov *et al*., 1999; Lindblad *et al*., 2002; Shah *et al*., 2003). However, the unicellular cyanobacteria *Synechococus* PCC 7942 and *Synechocystis* PCC 6803

could produce H_2 in complete anaerobic and dark condition (Asada and Miyake, 1999; Baebprasert *et al*., 2010). As expected, an increased light intensity resulted in an increased H_2 production in *A. siamensis* TISTR 8012 with saturation at 200 μE m^{-2} s^{-1} of light intensity (Figure 18A). Above 200 μE m^{-2} s^{-1} the cells produced less H_2 with a reduced level of chlorophyll *a* content and induced cell lysis. Under continuous illumination of 200 μE m^{-2} s^{-1} for 12 h, H_2 production rate of 15.2 μmolH_2 mg chl $a^{-1}h^{-1}$ was obtained which was about 2 and 3-folds of that at 40 μE m^{-2} s^{-1} and in darkness, respectively (Table 8). This higher production may be caused by higher nitrogenase activity since we observed an upregulation of *nifD* but no increase in the expression of *hoxH* (Figure 28). However, the production did not increase after 12 h of light exposure. This was caused by an efficient uptake hydrogenase as evidenced by an increase in *hupL* transcript level.

Therefore, the controlling of light intensity and time of light exposure to minimize the effect of photo damage of the cells and the uptake hydrogenase activity is necessary in order to increase H_2 production in *A. siamensis* TISTR 8012.

(6) Effect of salt and osmotic stress: Salt stress could not stimulate an enhanced H_2 production in N_2-fixing cyanobacterium *A. siamensis* TISTR 8012. This may be due to the diversion of energy and reductants needed for nitrogenase activity towards extrusion of ion from within the cells or the prevention of ion influx leading to a decrease of H_2 production (Rai and Abraham, 1995). Salt stress also causes of oxidative stress as reactive oxygen species (ROS) is produced as a result of cell damage (Zhu, 2001). Interestingly, the Ca^{2+} ion had less effect on reducing H_2 production than other ions. Smith *et al* (1987) reported that the Ca^{2+} may be involved in a very early step of the heterocyst differentiation.

In contrast, the presence of 50 mM of D-sorbitol showed higher H_2 production when compared with the control in normal $BG11_0$. The previous study has reported that the *Synechocystis* PCC 6803 can synthesize the natural solute glucosylglycerol (GG) to balance the osmotic potential and to protect proteins and membrane (Mikkat *et al*., 1996). Nevertheless, high concentration of either external salt or D-sorbitol had a negative effect on growth causing of cell shrinkage and cell damage.

(7) Effect of supplementation of microelement: Microelements have an effect on H_2 production in cyanobacteria because metals are co-factors of enzymes involved in H_2 metabolism. Metal ions namely, zinc (Zn), nickel (Ni), manganese (Mn), magnesium (Mg), cobalt (Co), iron (Fe), molybdenum (Mo) and vanadium (V) are involved in the stimulation of hydrogenase and nitrogenase activity (Asada *et al*., 1987). In *A. siamensis* TISTR 8012, a 2-fold enhanced H_2 production was found upon addition of 21 mM of $FeSO_4$ in medium without N-source. Similar result was observed in *Anabaena cylindrica* (Jeffries *et al*., 1978).

For trace elements, the presence of Mo^{2+} had a major effect on increasing H_2 production whereas in the presence of V^{2+} and Ni^{2+} H_2 production was inhibited in *A. siamensis* TISTR 8012. Different types of nitrogenases depend on different metal co-factors in the catalytic site of nitrogenase complex as described by Wall (2004). As a consequence, an enhanced H_2 production was observed in the presence of Mo^{2+} while the negative effect was found in the presence of V^{2+}, indicating that nitrogenase complex of *A. siamensis* TISTR 8012 may contain Mo^{2+} at the catalytic site. Negative effect of Ni^{2+} supplementation might be caused by a higher uptake hydrogenase activity as evidenced by an increase in *hupL* transpript level upon addition of Ni^{2+} in medium (Phunpruch *et al*., 2006). Morovere, the previous studies have reported that addition of external nickel to the growth medium could increase the uptake

hydrogenase activity in several cyanobacteria (Kumar *et al*., 1991; Daday *et al*., 1985; Oxelfelt *et al*., 1995).

(8) Effect of reductant: β-mercaptoethanol was used to study effect of addition of external reductant on H_2 production in *A. siamensis* TISTR due to its powerful reducing capacity. Although small amount of H_2 production was detected when added with β-mercaptoethanol under darkness, at concentration of 750 M β-mercaptoethanol H_2 production could be induced under light condition. This suggested that β-mercaptoethanol is a good source of electrons involved in redox state. However, the suitable concentration for use should be considered.

(9) Effect of sulfur limitation: Previous studies have reported that the cyanobacteria *Gloeocapsa alpicola* and *Synechocystis* sp.PCC 6803 showed high production of H_2 when grown in S-deprived medium (Antal and Lindblad, 2005). Sulfur is an important component in D1 protein essential for photosystem II (PSII). During sulfur deprivation, photosynthesis and respiration will be decreased and lead to higher H_2 production because less O_2 is generated (Melis *et al*., 2000). As expected, sulfur deprivation could significantly stimulate H_2 production in *A. siamensis* TISTR 8012 under light condition in either medium with or without N-source. In addition, PSII activity of *A. siamensis* TISTR 8012 was clearly reduced under sulfur deprivation. As a consequence, a decrease of PSII activity might lead to a reduction of O_2 evolution in *A. siamensis* TISTR cells as described above.

(10) Effect of oxygen: Enzymes involved in H_2 metabolism, nitrogenase and bidirectional hydrogenase are very sensitive to O_2 (Fay, 1992). The challenges to find cyanobactial strain that can produce H_2 even in aerobic condition has attracted attention for a long time. Our studies indicated that *A. siamensis* TISTR 8012 is a

promising candidate strain that could produce H_2 under temporal aerobic condition even though a decrease by 50% of produced H_2 was found.

The controlling key factors were used to increase the efficiency of H_2 production by *A. siamensis* TISTR 8012. Our results demonstrated that an enhanced H_2 production was obtained when cells at log phase, were adapted under N_2-fixing condition using 0.5% fructose as carbon source and a continuous illumination of 200 $\mu Em^{-2}s^{-1}$for 12 h under anaerobic condition. The maximum H_2 production rate was about 32 $\mu molH_2$ mg chl $a^{-1}h^{-1}$. In addition, using fructose as the carbon source instead of CO_2 under light condition strongly enhanced the *nifD* transcript level. It is therefore reasonable to observe a higher H_2 production under this condition (Figure 29).

Moreover, the ability of this particular strain for H_2 production was compared with other model cyanobacterial strains, *Anabaena* PCC 7120, *N. punctiforme* ATCC 29133 and *Synechocystis* PCC 6803. Table 9 revealed that *A. siamensis* TISTR 8012 has the capacity to produce 4-and 400-times more H_2 than *Anabaena* PCC 7120 and *Synechocystis* PCC 6803, respectively but less than *N. punctiforme* ATCC 29133 under the same growth condition ($BG11_0$, 30 °C, under 40 $\mu E\ m^{-2}\ s^{-1}$ of light intensity). However, higher H_2 production rate was obtained when the cells were adapted to optimal condition. The maximum H_2 production rates in heterocystous filamentous cyanobacteria was reported in the range from 0.17 to 4.2 $\mu molH_2$ mg chl $a^{-1}h^{-1}$ in wild type strains under anaerobic conditions (Masukawa *et al*., 2001). The maximum H_2 production by *A. siamensis* TISTR 8012 in the present study was at least 8-folds higher than those previously reported.

The N_2-fixing cyanobacterium *A. siamensis* TISTR 8012 has high potential for H_2 production which is attributed to the activities of two enzymes, nitorgenae and bidirectional hydrogenase. We demonstrated that nitrogenase of *A. siamensis* TISTR 8012 was activated in heterocyst cell under N_2-fixing condition. The bidirectional hydrogenase was found to be active in both vegetative cells and heterocysts but cells under N_2-fixing condition showed higher activity than cell under non-N_2-fixing condition. Similar results were observed in *A. variabilis* (Sheremetieva *et al*., 1996) and in *Nostoc* sp. PCC 7120 (Houchins and Burris, 1981). In addition, *A. siamensis* TISTR 8012 cells grown under controlling of light intensity at or lower than 200 $\mu Em^{-2}s^{-1}$ resulted in enhanced activities of both enzymes, nitrogenase and bidirectional hydrogenase. The optimum temperature of two enzymes was 30 ºC and 40 ºC for nitrogenase and bidirectional hydrogenase, respectively. Moreover, the nitrogenase activity was very active in the presence of fructose under light condition. This might be because of the heterocystous filamentous cyanobacteria provided more electron and reductant for nitrogenase through degradation of storage glycogen or additional organic substances as suggested by Smith (1983). However, incubation of cells under light condition and longer light exposure also led to an induction of H_2 uptake by *A. siamensis* TISTR 8012 which is a major problem to sustain H_2 production under light condition by using *A. siamensis* TISTR 8012.

The main obstacle for sustainable H_2 production by *A. siamensis* TISTR 8012 is H_2 consumption by uptake hydrogenase activity. To overcome this, we created the hydrogen uptake deficient mutant by interrupting *hupS* gene with neomycin resistance cassette gene. The previous studies have reported that many heterocystous filamentous cyanobacteria such as *N. punciforme*, *Anabaena* PCC 7120, *A. variabilis* and *Nostoc* sp. PCC 7942 with inactivated uptake hydrogenase show an ability to

produce H_2 at higher rate when compared to their wild type strains (Happe *et al*., 2000; Lindberg *et al*., 2002; Masukawa *et al*., 2002; Carrasco *et al*., 2005; Yoshino *et al*., 2007). Interestingly, many previous reports mainly focused on HupL inactivation because the active site of uptake hydrogenase is located in the large subunit. Therefore, we also would like to know the capacity of *A. siamensis* TISTR 8012 in H_2 production if HupS is not functional. The *hupS* gene encodes a small subunit of uptake hydrogenase. It has an important role in transferring electrons from active site to electron acceptor connected to the electron transport chain. Bock *et al* (2006) reported that HupS and HupL need to go through a maturation process before uptake hydrogenase enzyme will be fully functional. As a consequence the uptake hydrogenae might not function if *hupS* gene was interrupted. As expeted, the *A. siamensis* TISTR 8012 in which *hupS* gene was knocked out (Δ*hupS* mutant) showed higher capacity to produce H_2 than wild type strain. Moreover, the inactivation of HupS had no effect on growth, the Δ*hupS* mutant strain showed similar growth rate pattern to wild type strain. Surprisingly, Δ*hupS* mutant was more efficient for H_2 production under long term of light exposure than wild type strain and the production could be prolonged more than 72 h under light condition. This might be due to higher nitrogenase activity in Δ*hupS* mutant, with about 2 times higher activity than wild type strain under light exposure. In contrast, *A. variablilis* mutant strain AVM13 where *hupSL* was interrupted showed no increase of nitrogenase activity when compared with wild type strain and the higher H_2 production under N_2-fixing condition was not stable, the rate was decreased dramatically in the next 30 h of incubation (Happe *et al*., 2000). Our results indicate that Δ*hupS* mutant of *A. siamensis* TISTR 8012 has a high potential for H_2 production with the ability to sustain H_2 production under light exposure for a long period.

In addition, the Δ*hupS* mutant strain was also examined for the genes transcription level compared to wild type strain in order to provide important information for understanding their metabolism for enhanced H_2 production. The results demonstrated that the increased of H_2 production in Δ*hupS* mutant may be due to higher nitrogenase activity. This was in agreement with significantly enhanced *nifD* transcription level under N_2-fixing condition although one of the key enzymes to supply electrons to nitrogenase, uptake hydrogenase was inactivated. Therefore, the nitrogenase would be able to receive more ATP and reducing equivalents from another pathway. Ekman *et al* (2011) have reported that proteins encoded by an oxidative pentose phosphate (OPP) pathway were more abundant in heterocyst of hydrogen uptake deficient mutant *N. punciforme*. Interestingly, when analyzing genes expression, we found that the *psbA* encoding D1 protein of PSII and *fdxH* encoding heterocyst-specific ferredoxin meadiating electrons transport to nitrogenase in heterocysts, were significantly up-regulated in Δ*hupS* mutant. This suggested that the electrons and ATP molecules required for H_2 production in Δ*hupS* mutant were obtained from either the electron transport chain associated with the photosynthetic oxidation of water or the catabolism of carbohydrate, despite no changes in the expression of *psaA* gene encoding core protein of photosystem I.

Moreover, inactivated HupS of *A. siamensis* TISTR 8012 had a major effect on up-regulation of *coxA* gene that encodes cytochrome c oxidase subunit I found only in vegetative cells. One possible explanation for the increased expression of *coxA* in Δ*hupS* mutant was because of its requirement to maintain the high O_2 consumption rate in cells to protect inhibition of enzyme needed for H_2 production.

Interestingly, the key genes involved in heterocyst differentiation, *hetR* encoding primary activator protein and *ntcA* encoding the transcription factor NtcA

were not changed in Δ*hupS* mutant when compared with wild type strain. This supported that HupS inactivation had no major effect on growth of *A. siamensis* TISTR 8012. Unexpectedly, higher *hupL* transcription level was found in Δ*hupS* mutant when compared to wild type. This might be because a positive effect of higher intracellular H_2 concentrations could induce co-transcription of *hupSL* as described by Ekman *et al* (2011).

CHAPTER V

CONCLUSIONS

The knowledge gained from this study of biohydrogen production by N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012 can be summarized as following:

1. Nitrate was not have any effect on cell differentiation in *A. siamensis* TISTR 8012, akinete and heterocyst differentiations were found under N-deprivation.
2. The filaments of *A. siamensis* TISTR 8012 divide exclusively by binary fission in one plane and heterocyst cells appear in either the end of filament or the middle of filament.
3. *A. siamensis* TISTR 8012 contains the thick heterocyst envelope which provided the advantage in restriction of O_2 diffusion into the cells supporting the function of nitrogenase.
4. The phylogenetic analysis using *16S* rDNA gene revealed that *A. siamensis* TISTR 8012 is classified into a single group.
5. Cells at log phase had ability to produce H_2 more than cells at other phases and cells under N_2-fixing condition show higher H_2 production rate than cells under non N_2-fixing condition.
6. The H_2 production is strongly dependent on growth phase with differences depending on strains used.
7. Optimum physical parameters to enhance growth rate of *A. siamensis* TISTR 8012 in $BG11_0$ medium was temperature at 30 ºC under a light intensity between 40-50 $\mu E\ m^{-2}\ s^{-1}$ and pH 7.5.

8. Many sources of nitrogen could support growth but strongly inhibit H_2 production of *A. siamensis* TISTR 8012.
9. Fructose is the most preferred substrate for H_2 production in *A. siamensis* TISTR 8012 which shows an ability to use fructose for heterotrophic growth.
10. To increase H_2 production in *A. siamensis* TISTR 8012, it is necessary to control the light intensity and time of light exposure to minimize the effect of photodamage of the cells and the uptake hydrogenase activity.
11. Under salt and osmotic stress, H_2 production was inhibited in *A. siamensis* TISTR 8012. High concentration of either external salt or D-sorbitol had a negative effect on growth and could result in cell shrinkage and cell damage.
12. Enhanced H_2 production was observed in the presence of molybdenum while the negative effect was found in the presence of vanadium, indicating that nitrogenase complex of *A. siamensis* TISTR 8012 may contain molybdenum at the catalytic site.
13. The β-mercaptoethanol is a good source of electrons involved in redox state for H_2 production and sulfur deprived condition led to an increase of H_2 production in *A. siamensis* TISTR 8012.
14. The *A. siamensis* TISTR 8012 is a promising candidate strain that could produce H_2 under temporal aerobic condition.
15. The maximum H_2 production rate was about 32 $\mu molH_2$ mg chl $a^{-1}h^{-1}$ when *A. siamensis* TISTR 8012 at log phase was adapted under N_2-fixing condition using 0.5% fructose as carbon source and a continuous illumination of 200 $\mu Em^{-2}s^{-1}$for 12 h under anaerobic condition.

16. The maximum H_2 production by *A. siamensis* TISTR 8012 in the present study was at least 8-fold higher than those previously reported.
17. The bidirectional hydrogenase in cells under medium without N-source showed higher activity than that in cells under medium contain N-source.
18. The optimum temperature of nitrogenase and bidirectional hydrogenase activities of *A. siamensis* TISTR 8012 were 30 ºC and 40 ºC, respectively
19. Light intensity at or lower than 200 μE $m^{-2}s^{-1}$ resulted in enhanced activities of nitrogenase and bidirectional hydrogenase.
20. Long duration of light exposure could induce H_2 uptake in *A. siamensis*.
21. The *hupS* gene was successfully inactivated by interrupting the gene with antibiotic resistance cassette in *A. siamensis* TISTR 8012.
22. The Δ*hupS* mutant of *A. siamensis* TISTR 8012 showed higher capacity to produce H_2 than wild type strain, however, both strains had a similar growth rate pattern.
23. The Δ*hupS* mutant of *A. siamensis* had a high potential for H_2 production with the ability to sustain H_2 production under light exposure for a long period.
24. The increase of H_2 production in Δ*hupS* mutant might a result of higher nitrogenase activity.
25. The electrons and ATP molecules required for H_2 production in Δ*hupS* mutant might be obtained from the electron transport chain associated with the photosynthetic oxidation of water in vegetative cells.
26. The efficiency of H_2 production depends on cyanobacterial strains and various environmental conditions. When screening new potentially high H_2 producing strains, the N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012 was identified as a promising candidate

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APPENDICES

APPENDIX A

pGEM-T Easy Vector (Promaga)

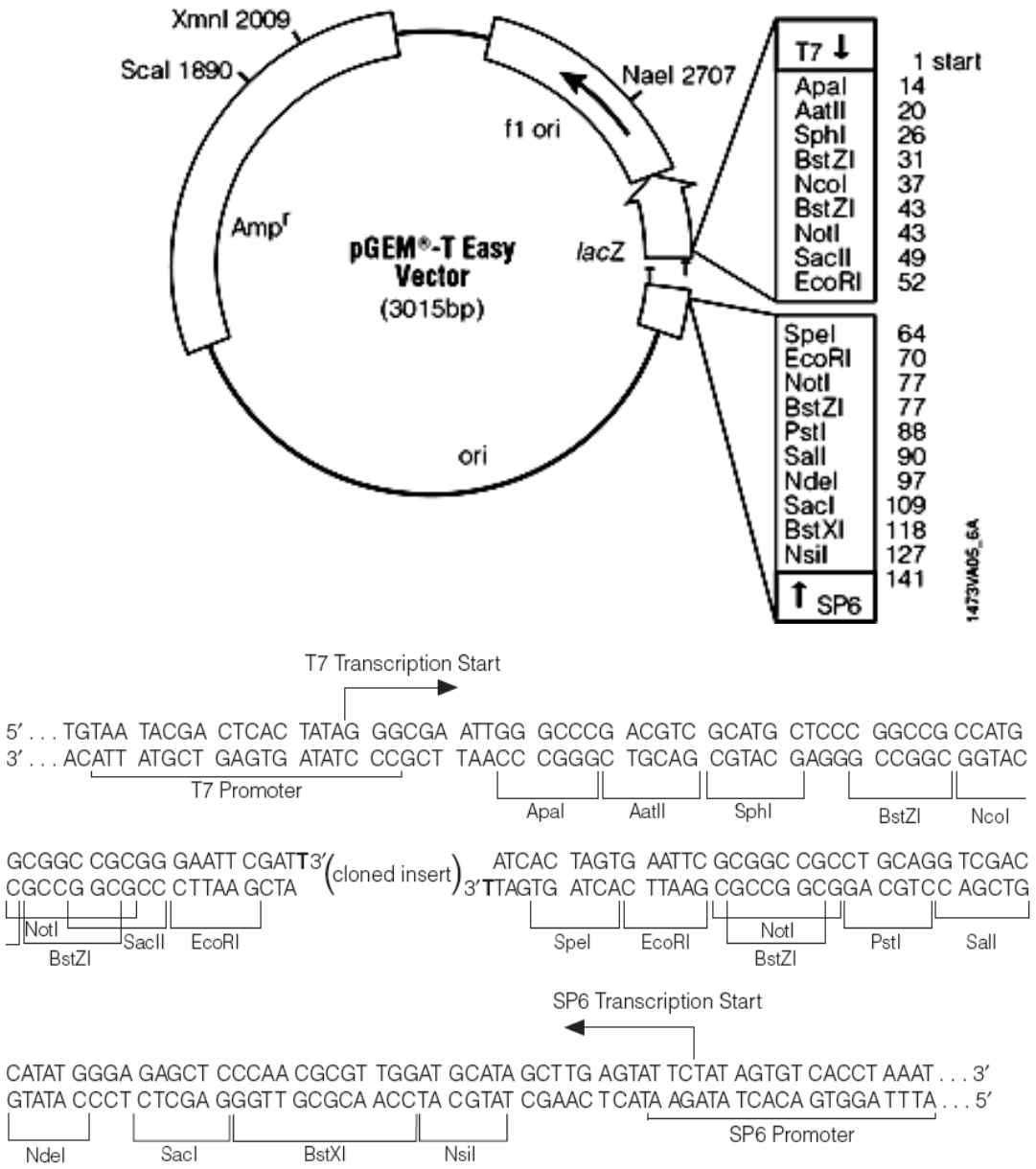

Figure A.1 Map of pGEM®-T Easy vector and cloning/expression region

APPENDIX B

pUC4K vector (Lablife)

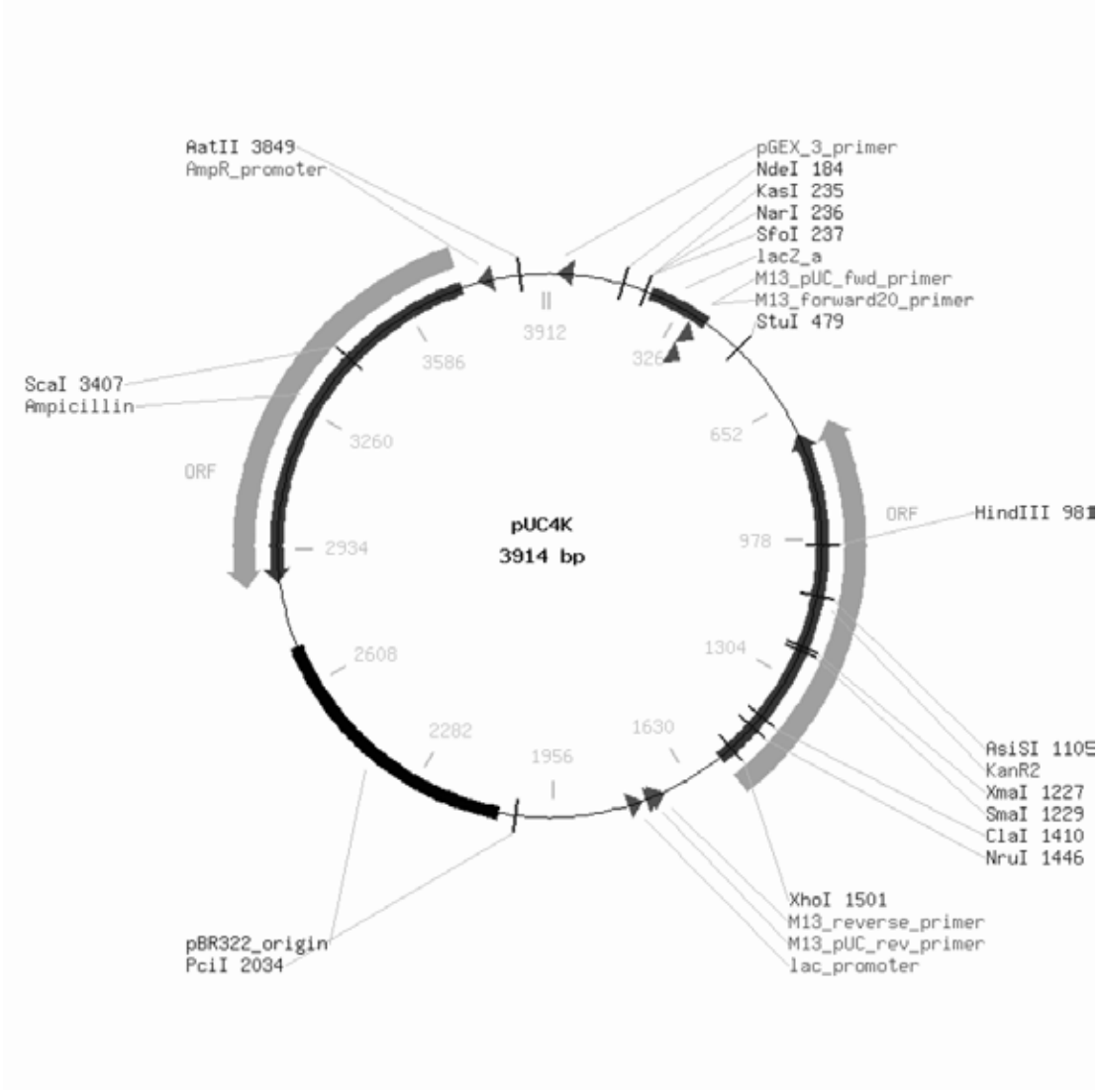

Figure A.2 Map of pUC4K vector

APPENDIX C

pRL 271 vector (Lablife)

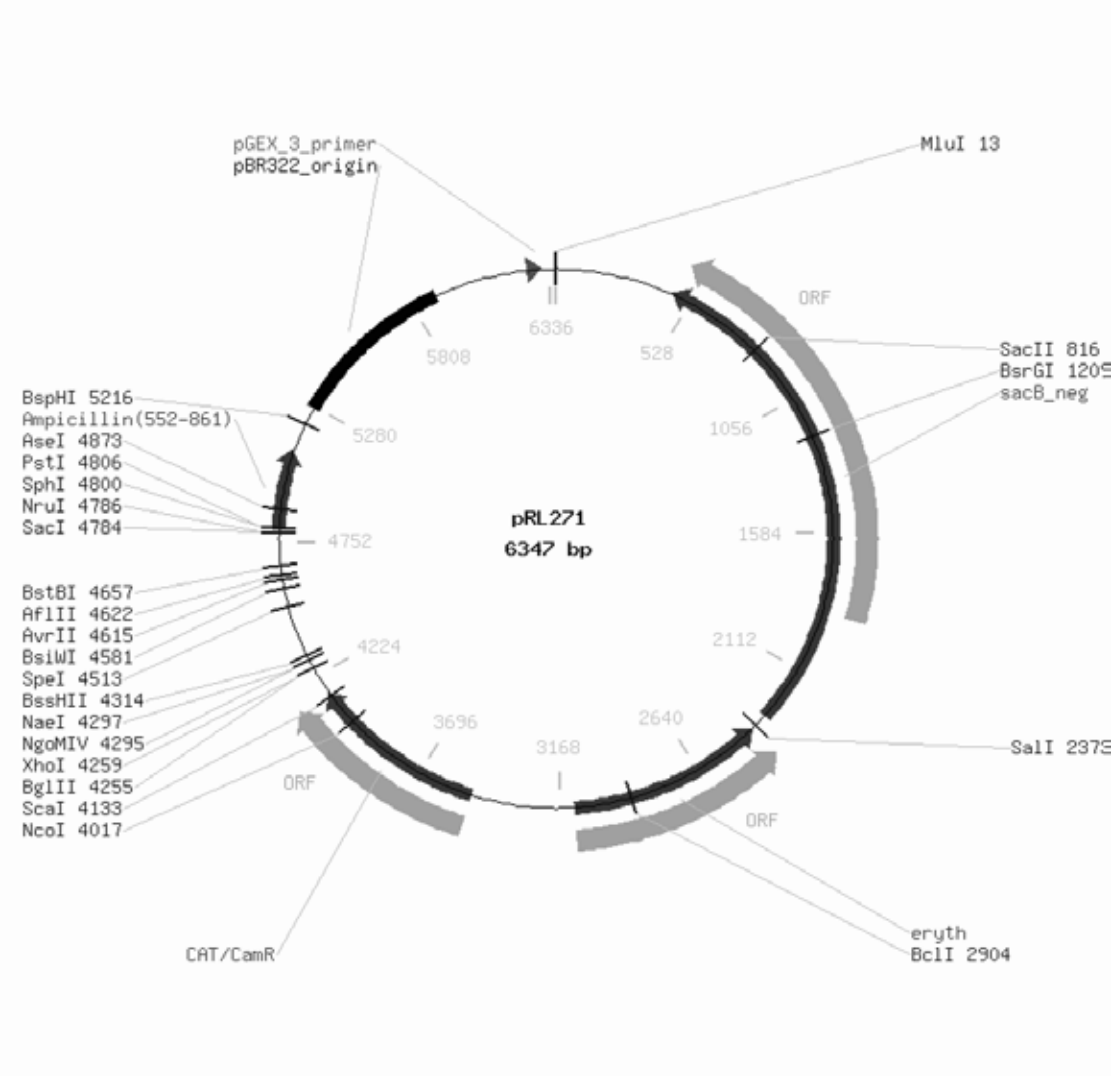

Figure A.3 Map of pRL 271 vector

APPENDIX D

LB medium

LB medium, composition per 1 L

Nutrients composition	Liquid medium	Solid medium
Bacto tryptone	10 g	10 g
NaCl	10 g	10 g
Yeast extract	5 g	5 g
Agar	-	15 g

All compositions were dissolved together with 800 mL of distilled water, and then the mixture was adjusted to pH of 7.0 with 6 M of NaOH. The total volume of solution was then adjusted to 1 L with deionized water. The medium was sterilized by autoclaving at 15 Ib/in^2 for 15 min.

APPENDIX E

Buffers for cell adaptation

Universal buffer solution (1 M); pH 3.5-12.0

1 L of mixture for titration contains

Citric acid	6.008	g
KH_2PO_4	3.893	g
H_3BO_3	1.769	g
Diethyllarlituric acid	5.266	g

The 100 mL of mixture is titrated with 0.2 N of NaOH to give the required pH. The final concentration of Universal buffer in the medium is 80 mM.

Other buffers were prepared by using Henderson Hasselbalch equation below:

$$pH = pK_a + \log \frac{[conjugate\ Acid]}{[conjugate\ Base]}$$

The pK_a values of different types of buffer

Type of buffer	pK_a values
Phosphate	2.15, 7.20, 12.33
HEPES	7.48
TES	7.40
Tris-HCl	8.55

APPENDIX F

Buffers for agarose gel electrophoresis

5X TBE buffer, composition per 1 L

Distilled water	800	mL
0.5 M EDTA, pH 8.0	20	mL
Boric acid	27.5	g
Tris base	54.0	g

Mix the compositions together and adjust volume up to 1 L, mix again in glass bottle and then store at room temperature. 0.5X TBE buffer is used for running gel electrophoresis.

20X SB buffer, composition per 1 L

SB buffer is a buffer solution used in agarose gel electrophoresis, it has a lower conductivity, produces sharper bands, and can be run at higher speeds than can gels made from TBE buffer. 20X SB buffer containing $Na_2B_4O_4$ at concentration of 100 mM and adjusted pH at 7.8 with boric acid. 1X SB buffer is used for running gel electrophoresis.

APPENDIX G

Buffer for preparation of competent cells

CCMB80 buffer, 1L

10 mM KOAc, pH 7.0	10	mL of a 1 M stock solution
80 mM $CaCl_2.2H_2O$	11.8	g
20 mM $MnCl_2.4H_2O$	4.0	g
10 mM $MgCl_2.6H_2O$	2.0	g
10% glycerol	100	mL
Distilled water	up to a final volume of 1 L	

Mix the compositions together and adjust pH down to 6.4 with 0.1 N HCl, if necessary, adjusting pH up will precipitate manganese dioxide from Mn containing solution. Slight dark precipitate appearance has no effect on its function.

APPENDIX H

DNA digestion

DNA digestion

1. Combine the reaction components by following below:

Reaction components	Plasmid DNA	PCR product
Water, nuclease-free	15 μL	17 μL
10X restriction enzyme buffer	2 μL	2 μL
DNA	2 μL (up to 1μg)	10 μL (~0.2 μg)
Restriction enzyme	1μL (1 U)	1μL (1 U)
Total volume	20 μL	30 μL

2. Mix gently and spin down.
3. Incubate at appropriate temperature of each restriction enzyme for optimum time.
4. Inactivate the enzyme by heating of 10 min at 80 ºC or depends on the enzyme recommendation.
5. Double and multiple digestion of DNA are performed by using 1 μL of each enzyme and scale up the reaction condition appropriately. Using the proper buffer. If the enzyme require different reaction temperatures, start with the enzyme that requires a lower temperature, then add the second enzyme and incubate at the higher temperature.

APPENDIX I

Modified Blunt-ending and DNA ligation

Modified blunt-ending

For filling recessed 3' termini	For removing protruding 3' termini
1 μL solution containing 1 mM dNTPs and 1U Klenow per 1 μg of DNA, incubate for 15 min at room temperature. After incubation, heat inactivate at 75 ºC for 10 min	2 μL solution containing 1 mM dNTPs and1-2U T4 DNA polymerase per 1 μg of DNA, incubate for 15 min at 12 ºC. After incubation, heat inactivate at 75 ºC for 10 min

DNA Ligation

To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation.

$$\frac{\text{ng of vector x size of insert}}{\text{kb size of vector}} \text{ x insert:vector molar ratio = ng of insert}$$

Where, a 3:1 insert:vector molar ratio is desired.

Components for ligation	Reaction (μL)	The reaction was incubated overnight at 22 ºC, 10 minutes. 2 μL of the ligation mixture was transformed into competent cells
2x Quick ligation reaction buffer	10	
Vector (50 ng)	1	
Insert	X	
Quick T4 DNA Ligase (3U/μL)	1	
Nuclease free water to a final volume of	20	

APPENDIX J

Acid washed glass beads

Methods: acid washed glass beads

1. Weigh 50 g of 0.5 mm glass beads (Sigma G-9268) into a 100 mL of Erlenmeyer flask, the volume of glass beads should not be more than 1/5 the volume of the flask that is used.
2. Add enough 5.8 M HCl to cover the glass beads.
3. Swirl the flask to wash the glass beads with acid.
4. Carefully pour 5.8 M HCl into another bottle.
5. Add distilled water to wash the glass beads by volume of distilled water should be at least 5X the volume of the beads in flask. Swirl the bottle for 10 sec to stir up the glass beads.
6. Pour off the distilled water wash and repeat wash steps for 10 times to reduce the acid to below 10 mM.
7. Move the glass beads to a beaker, cover with foil and autoclave th beads for 20 min.
8. Dry glass beads at 50 ºC overnight.

APPENDIX K

H_2 production determination

Gas chromatography condition:

Column type	: Packed column, 2 m. Molecular Sieve 5Aº
Detector type	: Thermal Conductivity Detector (TCD)
Detector temperature	: 100 °C
Column oven temperature	: 50 °C
Injector port temperature	: 100 °C
Carrier gas	: Argon
Flow rate of carrier gas	: 20 mL/min

Calculation of the H_2 content:

Calculate the amount of produced H_2 (in percentage) of H_2 corresponding to the peak area for each time point. Use the calibration curve and formula below:

- X% H_2 corresponds to X mL of H_2 in 100 mL gas phase
- 1 mol = 22.4 dm^3 (L) ; assume H_2 is an ideal gas at STP
- Normalize the H_2 content by using the Chl *a* content

The H_2 production rate was expressed as μmol H_2 per chlorophyll *a* per h.

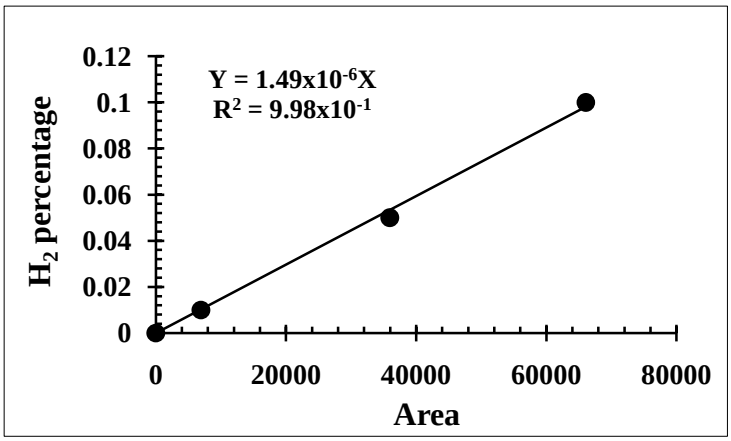

Figure A.4 Standard curve of H_2 standard gas

APPENDIX L

O_2 accumulation determination

Calculation of the O_2 content:

Calculate the amount of produced O_2 (in percentage) of O_2corresponding to the peak area for each time point. Use the calibration curve and formula below:

- X% O_2 corresponds to X mL of O_2 in 100 mL gas phase
- 1 mol = 22.4 dm^3 (L) ; assume O_2 is an ideal gas at STP

The O_2 accumulation rate was expressed as nmol O_2 per mL.

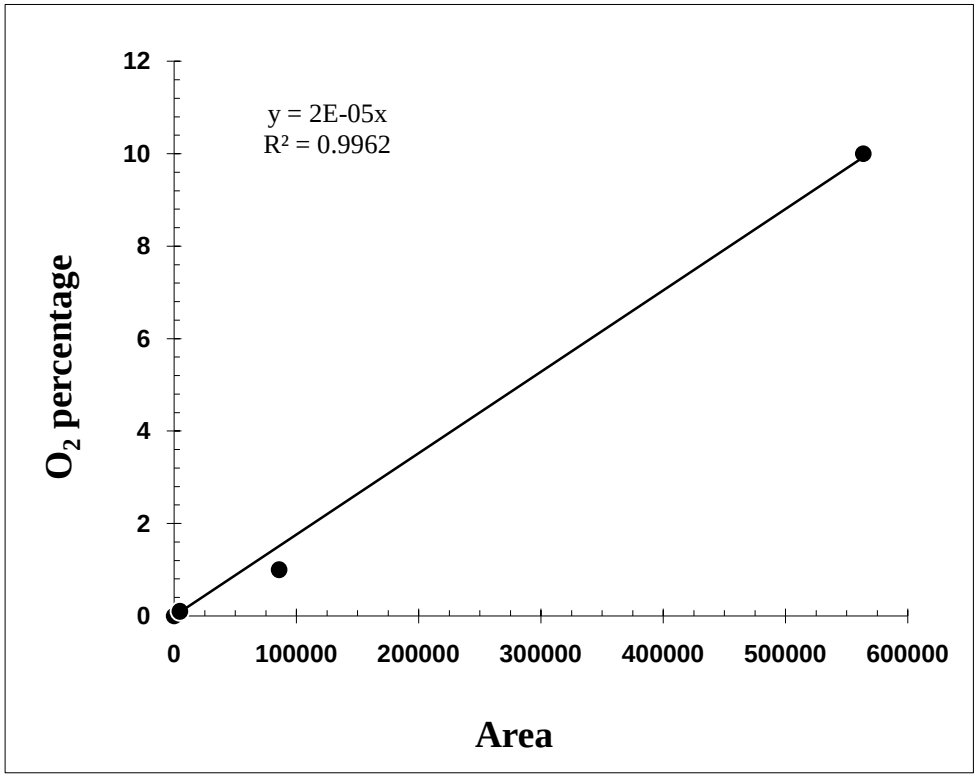

Figure A.5 Standard curve of O_2 standard gas

APPENDIX M

Uptake hydrogenase activity assay

Methods: H_2 uptake assay

1. Bubble distilled water with H_2 gas for 15 min to make a H_2-saturated water.
2. Run H_2-standard in the electrode at 30 ºC by following below:

   - Set the electrode as described in manual protocol and adjust temperature of the water-jacketed electrode chamber at 30 ºC.

   - Add 0.9 mL distilled H_2O in electrode chamber follow by 0.1 mL of H_2-saturated water and adjust the plunger.

   - Close the electrode with the gas-tight Teflon lid.
3. H_2 uptake assay:

   - Add 0.9 mL of *A. siamensis* cells suspension (10-20 μg chl a^{-1} mL^{-1}) in the reaction chamber and adjust the plunger.

   - Close the electrode with the gas-tight Teflon lid and wait 1-2 min to establish a stable base line.

   - Add 0.1 mL of H_2-saturated water, close the electrode with the gas-tight Teflon lid and start the stirring.

   - Take away the cover, turn on the light source, the changes in the amount of H_2 gas present in the monitor of recorder.

4. Calculate the amount of consumed H_2 by using known amounts of H_2 as standards (100 μL of H_2-saturated water, at 30 ºC contains 77.4 nmol of H_2) and expressed as μmol H_2 per chlorophyll *a* per h.

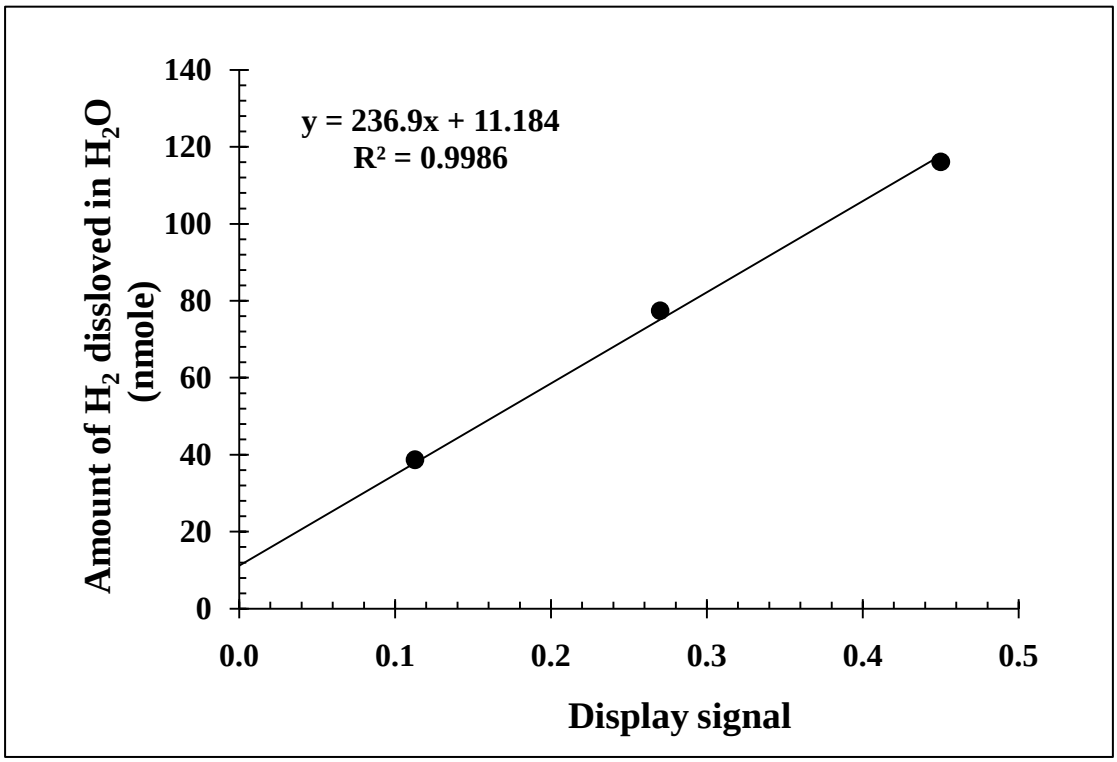

Figure A.6 Standard curve of H_2-saturated water

APPENDIX N

Nitrogenase activity assay

Acetylene reduction assay

Gas chromatography condition:

Column type	: Packed column, 50/80 mesh, Porapak Q
Detector type	: Flame ionization detector (FID)
Detector temperature	: 150 °C
Column oven temperature	: 100 °C
Injector port temperature	: 150 °C
Carrier gas	: Argon
Flow rate of carrier gas	: 20 mL/min

Calculation of the ethylene (C_2H_4) content:

Calculate the amount of produced C_2H_4 (in moles of gas) of C_2H_4 corresponding to the peak area for each time point.

- Calculate C_2H_4 standards (assume C_2H_4 is an ideal gas) by using equation below:

$$n = PV/RT$$

When: P = Atmospheric pressure at 25º C = 1 atm or 101.3 Kpa
V = Volume of gas (L)
R = Universal gas constant = 8.314
T = Temperature (K)
n = Mole of gas

- 1 L of 100% C_2H_4 will be X moles
- Then 1L of 10% standard C_2H_4 used will be 10*X/100 moles
- Convert standard peak area to mole of C_2H_4
- Normalize the C_2H_4 content by using the Chl *a* content

The C_2H_4 production rate was expressed as μmol C_2H_4 per chlorophyll *a* per h.

APPENDIX O

Phenol-sulfuric acid assay

Materials:

1. 5%(w/v) phenol: 50 g phenol (reagent grade) in 1 liter distilled water, store up to 6 months at room temperature
2. 96% sulfuric acid (reagent grade)

Methods: the general phenol-sulfuric acid assay for carbohydrate

1. Prepare the reagent by dissolving phenol in water (5% w/v).
2. Mix samples (200 μL containing up to 100 μg carbohydrate) with 200 mL of phenol reagent.
3. Add 1.0 mL of concentrated sulfuric acid rapidly and directly to the solution surface without allowing it to touch the sides of the tube.
4. Leave the solutions undisturbed for 10 min before shaking vigorously.
5. Determine the absorbance at 490 nm after a further 30 min.
6. Calculate the sugar uptake (in percentage) by comparing with control, the percentage of sugar utilized by the cells, representing sugar uptake.

APPENDIX P

PERSONAL INFORMATION

Personal Information:

Date of Birth: July 11st, 1984

Nationality: Thai Race: Thai Religion: Buddhism

Education:

June 2007- Ph.D. Candidate in Biotechnology, Program of Biotechnology, Faculty
May 2012 of Science, Chulalongkorn University, Bangkok, Thailand
Ph.D. Dissertation title: Biohydrogen Production by Nitrogen Fixing Cyanobacterium *Anabaena siamensis* TISTR 8012

June 2004- B.Sc. (Biotechnology), King Mongkut's Institute of Technology
May 2007 Ladkrabang, Bangkok, Thailand, with First Class Honours (GPA 3.68)
B.Sc. Thesis title: Study of Hydrogenase Gene of Cyanobacteria and Green Algae by Polymerase Chain Reaction.

Scholarships:

June 2007- Academic scholarship from Thailand Research Fund through the Royal
May 2012 Golden Jubilee Ph.D. program, Bangkok, Thailand

June 2010- Academic scholarships from the 90th Anniversary of Chulalongkorn
May 2012 University Fund (Ratchadaphiseksomphote Endowment Fund), Bangkok, Thailand

Honors and Awards:

- Honorable Mention Award for Poster Presentation "Combination of optimum condition to biohydrogen production by nitrogen fixing cyanobacterium *Anabaena siamensis* TISTR 8012" In: "The 14th Biological Sciences Graduate Congress", 10-12 December 2009, Chulalongkorn University, Bankok, Thailand.

Publications:

1 **Khetkorn, W**., Lindblad, P. and Incharoensakdi, A. (2010) Enhanced biohydrogen production by the N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012. International Journal of Hydrogen Energy. 35: 12767-12776.

2 Baebprasert, W., Jantaro, S., **Khetkorn, W**., Lindblad, P. and Incharoensakdi, A. (2011) Increased H_2 production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. Metabolic Engineering. 13: 610-616.

3 **Khetkorn, W**., Baebprasert, W., Lindblad, P. and Incharoensakdi, A. (2012) Increasing electron flow towards hydrogenase and nitrogenase using specific inhibitors results in enhanced H_2 production by *Anabaena siamensis* TISTR 8012. Bioresource Technology. (Revised)

4 **Khetkorn, W**., Lindblad, P. and Incharoensakdi, A. (2012), Sustained H_2 production through enhanced activity of PSII in hydrogen uptake deficient mutant of *Anabaena siamensis* TISTR 8012. (In preparation)

A**cademic experiences:**

1 Staff of the 14th Biological Sciences Graduate Congress", 10-12 December 2009, Chulalongkorn University, Bangkok, Thailand.

2 CAP Meeting, 2 November 2010, Kemicentrum, Lund University, Uppsala, Sweden. (Oral presentation).

3 The 9th International Hydrogenase Conference", 27 June-2 July 2010, Uppsala Concert and Congress Hall, Uppsala, Sweden. (Poster presentation). "Optimum condition for enhanced biohydrogen production by nitrogen fixing cyanobacterium *Anabaena siamensis* TISTR 8012"

4 The 14th Biological Sciences Graduate Congress", 10-12 December 2009, Chulalongkorn University, Bangkok, Thailand. (Poster presentation). "Combination of optimum condition to biohydrogen production by nitrogen fixing cyanobacterium *Anabaena siamensis* TISTR 8012"

5 The 13rd Biological Sciences Graduate Congress", 15-16 December 2008, National University of Singapore, Singapore. (Poster presentation) "Effect of several factor on hydrogen production by nitrogen fixing cyanobacterium *Anabaena siamensis* TISTR 8012"

6 The 34th Congress on Science and Technology of Thailand (STT.34)", 31 October- 2 November 2008, Queen Sirikit National Convention Center, Bangkok, Thailand. (Poster presentation). "Hydrogen Production in N_2-Fixing cyanobacterium *Anabaena siamensis* TISTR 8012 and Non-N_2 Fixing cyanobacterium *Synechocystis* sp. PCC 6803 under Different Carbon and Nitrogen Source"

7 RGJ-Ph.D. Congress X, Jomtein Palm Beach Resort Pattaya, Chonburi. April 3-5, 2009. (Oral presentation). "Effect of Culture Medium on Growth and Hydrogen Production by N_2-Fixing Cyanobacterium *Anabaena siamensis* TISTR 8012''

Field of research interest:

My dissertation focused on photobiological H_2 production, is the one method to produce clean renewable H_2 by using nature's most plentiful resources, solar energy and water. I am familiar with biochemical laboratory techniques such as DNA and RNA extraction, PCR, RT-PCR, DNA cloning, mutant strain construction, Protein purification, GC, Bioinformatic and Metabolic pathway and Microsoft Office as well as Photoshop Adobe.

References:

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BIOGRAPHY

Miss Wanthanee Khetkorn was born on July 11, 1984 in Uthaitani, Thailand. She graduated with a Bachelor of Science degree in Biotechnology, Faculty of Science, King Mongkut's Institute of Technology in 2007. After graduating with Bachelor's Degree of Science, she has further studied for the Doctor of Philosophy (Ph.D.) degree in Program of Biotechnology, Chulalongkorn University since 2007.