การผลิตไฮโดรเจนและการนำเข้าไนเตรตในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาว วิภาวี แบบประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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HYDROGEN PRODUCTION AND NITRATE UPTAKE IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

Miss Wipawee Baebprasert

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	HYDROGEN PRODUCTION AND NITRATE UPTAKE IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803
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วิภาวี แบบประเสริฐ: การผลิตไฮโดรเจนและการขนส่งไนเตรตในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (HYDROGEN PRODUCTION AND NITRATE UPTAKE IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.ดร.อรัญ อินเจริญศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: Prof. Peter Lindblad, Ph.D., อ.ดร.อภิชาติ กาญจนทัต, 122 หน้า

ศึกษาการผลิตไฮโดรเจนและกิจกรรมของเอนไซม์ไบไดเรกชันนัล ออกซ์ ไฮโดรจีเนส ในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 ที่ไม่สามารถตรึงไนโตรเจนได้ พบว่าทั้งกลูโคสและออสโมลาริตีภายนอกเซลล์กระตุ้นให้เซลล์มีการผลิตไฮโดรเจนเพิ่มสูงขึ้น โดยค่าที่เหมาะสมต่อการผลิตก๊าซไฮโดรเจนคือที่กลูโคสความเข้มข้น 0.4 เปอร์เซ็นต์ และออสโมลาริตีที่ 20 มิลลิออสโมลต่อกิโลกรัม เซลล์ที่อยู่ในอาหารเลี้ยงเชื้อที่ขาดไนเตรต ($BG11_0$) และไร้อากาศเป็นเวลา 24 ชั่วโมง มีกิจกรรมของเอนไซม์ไบไดเรกชันนัลไฮโดรจีเนสสูงขึ้นอย่างมีนัยสำคัญ แสดงให้เห็นว่าเอนไซม์ดังกล่าวมีความไวต่อก๊าซออกซิเจนและไนเตรตมีผลยับยั้งการทำงานของเอนไซม์ ค่าพีเอชและอุณหภูมิที่เหมาะสมต่อการทำงานของเอนไซม์ไบไดเรกชันนัลไฮโดรจีเนสคือ 7.5 และ 70 องศาเซลเซียสตามลำดับ เมื่อศึกษาผลของการเติมรีดักเทนท์ได้แก่ ไดไทโอทรีออล และบีตา เมอแคปโตเอทานอล ในเซลล์ที่อยู่ในอาหารเลี้ยงเชื้อสูตรปกติ (BG11), อาหารเลี้ยงเชื้อที่ขาดไนเตรต ($BG11_0$) และอาหารเลี้ยงเชื้อที่ขาดทั้งไนเตรตและซัลเฟอร์ ($BG11_0$-S-deprived) พบว่า บีตาเมอแคปโตเอทานอลกระตุ้นให้เซลล์ที่อยู่ในอาหารสูตรที่ขาดทั้งไนเตรตและซัลเฟอร์เป็นเวลา 24 ชั่วโมง มีการผลิตไฮโดรเจนสูงขึ้นอย่างมีนัยสำคัญ โดยมีการผลิตไฮโดรเจนสูงสุดที่ความเข้มข้นของบีตา เมอแคปโตเอทานอล 750 ไมโครโมลาร์ และเมื่อศึกษากิจกรรมของเอนไซม์ไบไดเรกชันนอลไฮโดรจีเนสที่อุณหภูมิ 70 องศาเซลเซียส พบว่าเซลล์ที่อยู่ในอาหารที่ขาดทั้งไนเตรตและซัลเฟอร์เป็นเวลา 24 ชั่วโมง โดยเติมบีตา เมอแคปโตเอทานอลความเข้มข้น 750 ไมโครโมลลาร์ มีกิจกรรมของเอนไซม์ดังกล่าวสูงสุดที่ 14.32 ไมโครโมลไฮโดรเจนต่อมิลลิกรัมคลอโรฟิลด์เอต่อนาที นอกจากนี้พบว่าในสายพันธุ์กลายที่ขาดยีนไนเตรตรีดัคเทส ($\Delta narB$), ไนไตรทรีดักเทส ($\Delta nirA$) และสายพันธุ์กลายสองจุดทั้งไนเตรตรีดักเทสและไนไตรทรีดักเทส (Δnar:$\Delta BnirA$) มีการผลิตไฮโดรเจนสูงกว่าในสายพันธุ์ดั้งเดิม แสดงให้เห็นว่าขบวนการดูดซึมไนเตรตเป็นขบวนการหนึ่งที่มีผลยับยั้งการผลิตไฮโดรเจนในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

เมื่อทำการศึกษาจลนพลศาสตร์การนำเข้าไนเตรตเข้าสู่เซลล์ *Synechocystis* sp. PCC 6803 ที่ไม่สามารถตรึงไนโตรเจนได้ ที่ภาวะปกติและภาวะความเครียดจากเกลือ 20 มิลลิโมลลาร์ มีค่า K_s เท่ากับ 46 และ 79 ไมโครโมลาร์ตามลำดับ และอัตราเร็วสูงสุด V_{max} เท่ากับ 1.37 และ 2.45 µmol $(min)^{-1}$ mg Chl^{-1} ตามลำดับ โดยที่ความเข้มข้นออสโมติกภายนอกเซลล์ที่เกิดจากซอร์บิทอลที่ 30 มิลลิออสโมลต่อกิโลกรัม และโซเดียมคลอไรด์ที่ 40 มิลลิออสโมลต่อกิโลกรัม ส่งผลให้มีการนำเข้าไนเตรตสู่เซลล์เพิ่มขึ้น 3.5 และ 4.5 เท่า ตามลำดับ แสดงให้เห็นว่า โซเดียมไอออนกระตุ้นการนำเข้าไนเตรตสู่เซลล์ จากการศึกษาผลของตัวยับยั้งการนำเข้าไนเตรตโดยแอมโมเนียม และตัวยับยั้งการตรึงก๊าซคาร์บอนไดออกไซด์โดยดีแอล-กลีเซอรอลดีไฮด์ พบว่าการนำเข้าไนเตรตสู่เซลล์ลดลง เมื่อนำเซลล์ที่บ่มภายใต้ภาวะมืดก่อนนำไปวิเคราะห์การนำเข้าไนเตรต พบว่ามีการนำเข้าไนเตรตสู่เซลล์ลดลง 70 เปอร์เซ็นต์ แสดงให้เห็นว่า *Synechocystis* sp. PCC 6803 ต้องการพลังงานในการขับเคลื่อนไนเตรตเข้าสู่เซลล์ การขนส่งไนเตรตมีความไวต่อตัวยับยั้งกระบวนการเมแทบอลิซึมที่เกี่ยวข้องกับการกระจายของโปรตอนเกรเดียนท์และความต่างศักย์ของเมมแบรน จากผลงานวิจัยข้างต้นแสดงให้เห็นว่า การนำเข้าไนเตรตใน *Synechocystis* sp. PCC 6803 ถูกกระตุ้นโดยโซเดียมไอออนและต้องการพลังงานซึ่งเกิดจากอิเล็กโทรเคมิคอลโพเทนเชียลซึ่งเกิดจากการขนส่งอิเล็กตรอน

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2552.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....อภิชาติ กาญจนทัต

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WIPAWEE BAEBPRASERT: HYDROGEN PRODUCTION AND NITRATE UPTAKE IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. THESIS ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., THESIS CO-ADVISOR : PROF. PETER LINDBLAD, Ph.D., APHICHART KARNCHANATAT, Ph.D., 122 pp.

The effects of external factors on both H_2 production and bidirectional Hox-hydrogenase activity were examined in the non-N_2 fixing cyanobacterium *Synechocystis* PCC 6803. Exogenous glucose and increased osmolality both enhanced H_2 production with optimal production observed at 0.4% and 20 mosmol kg^{-1}, respectively. Anaerobic condition for 24 h induced significant higher H_2ase activity with cells in $BG11_0$ showing highest activities. Increasing the pH resulted in an increased Hox-hydrogenase activity with an optimum at pH 7.5. The Hox-hydrogenase activity gradually increased with increasing temperature from 30°C to 60°C with the highest activity observed at 70°C. A low concentration at 100 µM of either DTT or β-mercaptoethanol resulted in a minor stimulation of H_2 production. β-mercaptoethanol added to nitrogen and sulfur deprived cells stimulated H_2 production significantly. The highest Hox-hydrogenase activity was observed in cells in $BG11_0$-S-deprived condition and 750 µM β-mercaptoethanol measured at a temperature of 70 °C; 14.32 µmol H_2 mg chl a^{-1} min^{-1}. The nitrate assimilation mutant strains Δ*narB*, Δ*nirA* and double mutant, Δ*nar:*Δ*BnirA* gave higher H_2 production than the wild type when cells were adapted in normal BG11 for 24 h.

Nitrate uptake in response to osmotic upshifts in the non-diazotrophic cyanobacterium *Synechocystis* sp. strain PCC 6803 was studied. A small increase of osmolality by 30 and 40 mosmol kg^{-1} sorbitol and NaCl resulted in about 3.5- and 4.5- fold increase of nitrate uptake, respectively. At 25 mosmol kg^{-1} or higher, NaCl exhibited higher nitrate uptake than sorbitol suggesting a stimulatory effect of Na^+ on the uptake activity. External 20 mM NaCl stimulated nitrate uptake with K_s and V_{max} values of 79 µM and 2.45 µmol min^{-1} mg Chl^{-1}, respectively which were about 2-fold higher than those without NaCl. Ammonium and DL-glyceraldehyde, an inhibitor of CO_2 fixation, caused a reduction of nitrate uptake. Cells pre-incubated in darkness showed drastic reduction of uptake activity by 70% suggesting energy-dependent nitrate uptake systems in *Synechocystis* sp. strain PCC 6803. Nitrate transport was sensitive to various metabolic inhibitors including those dissipating proton gradients and membrane potential. Altogether, the results suggest that nitrate uptake in *Synechocystis* sp. strain PCC 6803 is dependent on and stimulated by Na^+ ions and that the uptake requires energy provided by electrochemical potentials generated by electron transport.

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Field of Study :....Biotechnology..... Advisor's Signature....
Academic Year :....2009..... Co-Advisor's Signature .....
Co-Advisor's Signature Aphichart Karnchanatat

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LIST OF ABBREVIATIONS

H_2	Hydrogen
H_2ase	Bidirectional hydrogenase
Chl *a*	Chlorophyll *a*
bp	Base pair
^oC	Degree Celsius
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
g	Gram
HEPES	Hydroxyethyl piperazineethanesulfonic acid
kb	Kilo base
h	hour
L	Liter
mM	Milimolar
min	Minute
µg	Microgram
µl	Microliter
mL	Milliliter
mM	Millimolar
M	Molar
nm	nanometer
OD	Optical density
PCR	Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

Presently, the utilization of fossil fuels, as a consequence on the global climate is mainly due to the emission of pollutant released into the atmosphere upon their combustion. In January of 2010, many countries were faced with extremes of temperature, heavy snowfall in European countries and heavy rainfall become to water flood in Asian countries were because of Global Warming. The global energy requirements are heavily dependent on fossil fuels (around 80% of the present world energy demand), as a result this will eventually lead to the foreseeable depletion of limited fossil energy resources which are likely to be depleted in the next 50-100 years (Veziroglu, 1987). Therefore, researchers are trying to find clean, renewable and sustainable energy resources.

H_2 is considered an ideal energy carrier for the future. Compare to fossil fuels as traditional energy sources, H_2 is a promising candidate as a clean energy carrier in the future because of its higher heating values 142 MJ kg^{-1} for H_2 vs. 42 MJ kg^{-1} for oil (Perry, 1963). Water is a main product of combustion thus, this is the greatest advantage of H_2 that environmental friendly and reduces global greenhouse gas emissions. H_2 can also be converted directly to electricity in fuel cells, with a variety of applications in transportation and stationary power generation.

1.1 H_2 production

Presently, H_2 can be produce in three primary methods:

1.1.1 Production from fossil fuels and biomass

Conventionally, H_2 is produced mainly form fossil fuels or natural gas by stream reforming at industrial scale. This process consists of two steps: 1) reformation of the feedstock with high temperature steam supplied by burning natural gas to obtain a synthesis gas, and 2) using a water-gas shift reaction to form H_2 and CO_2 from the CO produced in the first step.

Step 1 $CH_4 + H_2O \rightarrow CO + 3H_2$

Step 2 $CO + H_2O \rightarrow CO_2 + H_2$

However, these processes are based on non-renewable energy sources, generates substantial sulfur and CO_2 emission and relia on expensive techniques. In fact, most of the H_2 producing processes based on fossil fuels release approximately twice as much CO_2 than of H_2 molar ratio (Navarro *et al.*, 2009).

1.1.2. Production from water through non-biological methods

In the process for generating H_2 (and O_2) from water is called “Electrolysis”, electricity passes through water in an ionic transfer device to separate water into H_2 and O_2.

$$2H_2O \xrightarrow{\text{electricity}} O_2 + 2H_2$$

If the electricity used for electrolysis is generated from fossil fuels, then CO_2 would be emitted in support of our electrolysis process. Renewable technologies, such as wind turbines and solar cell can generate electricity to produce hydrogen from electrolysis with zero greenhouse gas emissions. In France, an abundance of nuclear power makes electrolysis a logical, and their most common, method for producing H_2 (http://www.hydrogenassociation.org).

These industrial methods mainly consume fossil fuel as energy source, and sometimes hydroelectricity (Lodhi, 1987; Rosen and Scott, 1998). Both of thermochemical and electrochemical hydrogen generation processes are energy intensive and generate CO_2 and pollutants as by-products, not always environment friendly. On the other hand, the hydrogen production by photosynthesis organisms is one method to capture and accumulate the energy from sunlight to produce clean and renewable sources of hydrogen.

1.1.3. Biological H_2 production

H_2 can be produced biologically various processes; such as dark fermentation and photo fermentation, direct and indirect biophotolysis. Another optional method for producing H_2 is by photosynthetic microorganisms that harvesting solar energy and converts it into H_2. This biological approach is environmentally friendly.

Microbes produce H_2 for two principle reasons. Firstly, to dispose of excess reducing equivalents during fermentative metabolism either carried out in a dark anaerobic process or associated with the anoxic photosynthetic activity. For example is the production of hydrogen during the phosphoroclasic reaction of pyruvate and other 2-

oxoacids. Secondly, H_2 is a by-product of atmospheric N_2-fixation (Rao and Cammack, 2001).

1.1.3.1 Photo-fermentative H_2 production

This method found in photosynthetic bacteria evolves molecular hydrogen catalyzed by nitrogenase enzyme under nitrogen-deficient conditions using light energy and reducing compounds (organic acids). Photosynthetic bacteria are able to use simple organic acid as e^- donors under anaerobic conditions. The overall reaction of H_2 production can be given as

$$C_6H_{12}O_6 + 6H_2O \xrightarrow{\text{Light energy}} 12H_2 + 6CO_2$$

1.1.3.2 Dark-fermentative H_2 production

Dark-fermentative H_2 production found in anaerobic bacteria can produce H_2 when they were grown in darkness and anaerobic conditions by using carbohydrate as substrate. The majority of microbial H_2 poduction is driven by the anaerobic metabolism of pyruvate. Pyruvate is the key intermediate in the principle fermentation pathways leading to hydrogen from the glycolytic breakdown of carbohydrate derived sugars, which two enzymes are implicated in breakdown of pyruvate to intermediates involved in hydrogen production depending on the metabolic type (Manish and Banerjee, 2008; Hallenbeck, 2009):

1. *Pyruvate: formate lyase (Pfl), in enteric-type mixed-acid fermentation.*

   Pyruvate + CoA → acetyl-CoA + formate

2. *Pyruvate: ferredoxin oxido reductase (Pfor), in clostridial-type fermentations.*

   Pyruvate + CoA + 2Fd (ox) → acetyl-CoA + CO_2 + 2Fd (red)

In both cases, acetyl-CoA produced can be converted to acetate with concomitant ATP synthesis from the acetyl-phosphate intermediate.

1.1.3.3 Biophotolysis

Photoautotrophic microorganisms, green algae and cyanobacteria, process chlorophyll a to capture solar energy and use photosynthetic systems, PSI and PSII to carry out oxygenic photosynthesis. Biohydrogen production is often conducted in two stages under different atmospheric conditions, the first stage for cell growth followed by the second stage for H_2 evolution. In the photosynthesis process the pigments in PSII absorb the photons, oxygen is related when H_2O is spit and generated electrons are channeled through the so called “electron transport chain”. These electrons are led through several enzymes to the PSI (Figure 1). The electrons or reducing equivalents are transferred through a series of electron carrier and cytochrome complex to PSI. The pigments in PSI absorb the light which further raises the energy level of the e^- to reduce the oxidized ferredoxin (Fd) and/or nicotinamide adenine dinucleotide phosphate ($NADP^+$) into their reducing forms. The proton gradient formed across the thylakoid membrane drives adenosine triphosphate (ATP) production via ATP synthase. CO_2 is reduced with ATP and NADPH via a reductive pentose phosphate pathway or Calvin cycle for cell growth. The excess reduced carbon is stored inside the cells as carbohydrate (CH_2O) and/or lipid. Under special conditions, the reducing equivalents (ferredoxin) can also be used by hydrogenase or nitrogenase to reduce protons for production and evolution of H_2 ($2H^+ + 2Fd \leftrightarrow H_2 + 2\ Fd$) (Madamwar et al., 2000; Manish and Banerjee; 2008; Yu and Takahashi, 2007).

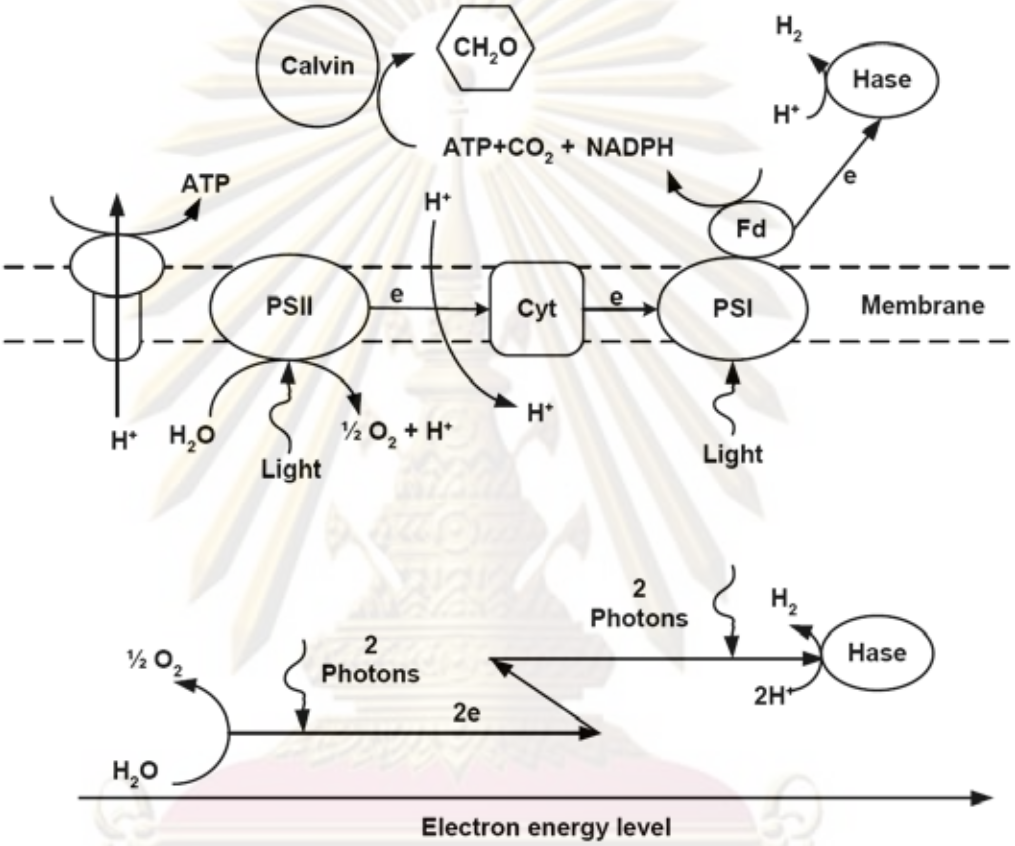

Figure 1 The schematic mechanisms of photosynthesis and biophotolysis of photoautotrophic microorganisms. The electrons or reducing equivalents from water oxidation is raised by the adsorbed photons at PSII and PSI. The reducing equivalent (NADPH) is used for CO_2 reduction in photosynthesis and carbohydrate (CH_2O) are accumulated inside the cells. The reducing power (Fd) could also be directed to hydrogenase (Hase) for H_2 evolution. (Yu and Takahashi, 2007)

1.1.3.3.1 Direct biophotolysis

This is an attractive process since solar energy is used to convert a readily available substrate, water, to O_2 and H_2 via photosynthetic reactions as following equations.

$$2H_2O \xrightarrow{\text{Light energy}} 2H_2 + O_2$$

The light energy is absorbed by the pigments of PSII or PSI, which raises the energy level of e^- from water oxidation when they are transferred from PSII via PSI to ferredoxin. A portion of the light energy is directly stored in H_2 gas. Cyanobacteria are potential microbial species for producing H_2 via direct biophotolysis, using nitrogenase in filamentous N_2-fixing cyanobacteria *Anabaena variabilis* PK84 (Sveshnikov *et al.*, 1997; Borodin *et al.*, 2000) or bidirectional hydrogenase in unicellular non-N_2-fixing cyanobacteria in *Synechococcus* PCC 6307 and *Aphanocapsa montana* (Howarth and Codd, 1985). Moreover, several reports found in green algae such as *Chlamydomonas* (Frenkel, 1952; Roessler and Lien, 1984; Wykoff *et al*., 1998; Melis and Happe, 2004).

1.1.3.3.2 Indirect biophotolysis

Indirect biophotolysis or dark fermentation on endogenous carbohydrate is consists of two stages in series: photosynthesis for carbohydrate accumulation (starch in microalgae and glycogen in cyanobacteria), and dark fermentation of the carbon reserve for H_2 production. Gaffron and Rubin (1942) found that e^- or reducing equivalents of hydrogenase and nitrogenase may sometimes originate from the intracellular energy reserve including carbohydrate. The overall mechanism of H_2 production in cyanobacteria

can be represented by the following reactions (Yu and Takahashi, 2007; Manish and Banerjee, 2008):

$$\textit{Photosynthesis}: \quad 12H_2O + 6CO_2 \xrightarrow{\text{Light energy}} C_6H_{12}O_6 + 6O_2$$

$$\textit{Dark fermentation}: \quad C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH$$

Cyanobacteria are one of the groups of microorganisms suitable for the photosynthetic production of hydrogen (Hansel and Lindblad, 1998). In cyanobacteria, hydrogen is evolves as a by-product of photosynthesis process by hydrogenase enzyme (Rao and Hall, 1996; Hansel and Lindblad, 1998). Moreover, cyanobacteria can induce a NiFe-hydrogenase that use reduced NAD(P)H as the substrates in H_2 evolution different from green algae that only use Fe-hydrogenase and reduced ferredoxin as the substrate for producing H_2 (Troshina *et al*, 2002). The photobiological H_2 production is the great public interest since it promises a renewable energy carrier from nature's most plentiful resources, solar energy and water.

As seen in table 1, it has been found that most of the biological processes are operated at an ambient temperature (30-40 °C) and normal pressure. So, these processes are not energy intensive. In addition, fermentative H_2 production production processes produce some useful fatty acids, such as lactic acid, acetic acid, butyric acid, etc. These acids have to be separated, otherwise, they will pose water pollution problems. A photosynthetic bacterium on the other hand produces low concentration of fatty acids (Das and Veziroglu, 2001). Therefore, H_2 production by cyanobacteria is an alternative way for clean energy.

Table 1 Advantages and disadvantages of different biological processes for H_2 production *

Type of microorganism	Advantages	Disadvantages
Green algae	-Can produce H_2 from water -Solar conversion energy increased by 10 folds as compared to trees, crops	-Require light for H_2 production -O_2 can be dangerous for the system
Cyanobacteria	-Can produce H_2 from water -Nitrogenase enzyme mainly produces H_2 -Has the ability to fix N_2 from the atmosphere	-Uptake hydrogenase enzymes are to be removed to stop the degradation of H_2 -Require sun light -About 30% O_2 present in the gas mixture with H_2 -O_2 has inhibitory effect on nitrogenase -CO_2 present in the gas
Photosynthetic bacteria	-Can use different waste materials like, whey, distillery, etc. -Can use wide spectrum of light	-Require light for the H_2 production -Fermental broth will cause water pollution problem
Fermentative bacteria	-It can produce H_2 all day long without light -It can utilize different carbon source like, starch, cellobiose, sucrose, xylose, etc. and so different types of raw materials can be used -It produces valuable metabolites such as butyric acid, lactic acid, acetic acid, etc. as by products -It is anaerobic process, so there is no O_2 limitation problems	-The fermented broth is required to undergo further treatment before disposal otherwise it will create water pollution problem -CO_2 present in the gas

* Das and Veziroglu, 2001

1.2 Cyanobacteria

Cyanobacteria are prokaryotes that belong to the bacteria domain and are characterized by the ability to performed oxygenic photosynthesis. They were known as blue-green algae, blue-green bacteria and classified in the phylum Cyanophyta, phylum of bacteria that obtain their energy through photosynthesis. The name "cyanobacteria" comes from the color of the bacteria (Greek word: kyanós which means blue). They are a significant component of the marine nitrogen cycle and an important primary producer in many areas of the ocean, but are also found in almost every conceivable habitat, from oceans to fresh water to bare rock to soil and extremes habitats (Witton and Potts, 2000). They can live in extremes of temperatures -60°C to 85°C, and a few species are halophilic or salt tolerant (as high as 27%, for comparison, percentage of salt in seawater is 3%). The ability of cyanobacteria to perform oxygenic photosynthesis is thought to have converted the early reducing atmosphere into an oxidizing one, which dramatically changed the composition of life forms on Earth by provoking an explosion of biodiversity and leading to the near-extinction of oxygen-intolerant organisms. According to endosymbiotic theory, Chloroplasts in plants and eukaryotic algae have evolved from cyanobacteria via endosymbiosis.

Cyanobacteria include unicellular and colonial species. Colonies may form filaments, sheets or even hollow balls. Some filamentous colonies show the ability to differentiate into four different cell types; 1) Vegetative cells, the normal, photosynthetic cells that are formed under favorable growing conditions. 2) Homogonia, involved in mobility and symbiosis are short and mobile filaments formed in response to different environmental stress. 3) Akinetes, the climate-resistant spore-like cell that may form when environmental conditions become harsh. Akinetes contain large reserves of

carbohydrates, and owing to their density and lack of gas vesicles, eventually settle to the lake bottom. They can tolerate adverse conditions such as the complete drying of a pond or the cold winter temperatures, and, as a consequence, akinetes serve as "seeds" for the growth of juvenile filaments when favorable conditions return. (Meeks *et al.*, 2002). 4) Heterocysts, cells will a thick cell wall which contain the enzyme nitrogenase, vital for nitrogen fixation. In heterocysts, which are basically anaerobic cells due to (1) There lack PSII resulting no O_2-evolution activity, (2) heterocyst has a tick envelope consisting of an inner layer composed of glycolipids and an outer layer composed of polysaccharides protecting the rate of oxygen diffusion from within is decreased, and (3) then have an increased level of respiration (Meeks *et al*., 2002; Meeks and Elhai, 2002; Tamagnini *et al*., 2007). Heterocyst-forming strain are specialized for nitrogen fixation and are able to fix nitrogen gas into ammonia (NH_3), nitrites (NO_2^-) or nitrates (NO_3^-) at ambient atmosphere which can be absorbed by plants and converted to protein and nucleic acids atmospheric nitrogen cannot be used by plants directly.

1.2.1 Cyanobacterium *Synechocystis* sp. PCC 6803

The cyanobacterium, *Synechocystis* sp. PCC 6803 is the best characterized strain, unicellular non-nitrogen (N_2)-fixing cyanobacterium growing in fresh water. *Synechocystis* PCC 6803 is capable of both autotrophic and heterotrophic growth. Under photoheterotrophic conditions, and diminished sunlight, *Synechocystis* PCC 6803 can obtain energy from glucose which is used as a carbon source, and light as an energy source (Bullerjashn *et al*., 1985; Der-Vartanian *et al*., 1981). It has been one of the most used model organisms for genetic and physiological studies of photosynthesis for reasons; it is naturally transformable organism and grows heterotrophically at the expense of glucose (Rippka e*t al*., 1979; Williams, 1988), and genetically well characterized with a

sequence genome determined in 1996 (Kaneko *et al*., 1996). This was the first genome of a photoautotrophic organism to be fully sequenced. Sequencing was carried out using a clone-by-clone strategy based on the physical map of the genome, resulting in a highly accurate sequence, originally deduced to be 3,573,470 bp (Nakamura *et al*., 2000) (Figure 2). The availability of the entire genome sequence has allowed closer studies on the structure of each gene, ORF and the organization of the entire genome, resulting in a better understanding of the unique genetic characteristic of phototrophic organisms. Since then, a tremendous amount of information has been generated through functional genomics, and proteome and transcriptome studies.

The cyanobacterium *Synechocystis* sp. PCC 6803 has been defined as a unicellular coccoid, or spherical cyanobacterium lacking gas vesicles or a sheath. It divides by binary fission at two or three successive planes. *Synechocystis* sp. PCC 6803 is considerably more complex than other Gram-negative bacteria in that it contains an internal membrane system, the thylakoid membranes, where active photosynthesis occurs. In standard thin section electron micrographs, thylakoid membranes appear as internal membraneous structures organized in concentric layers within the cell (Figure 3). Cyanobacteria owe the ubiquitous distribution to remarkable capacities to adapt to varying environmental conditions. A few data are available regarding adaptation of natural populations, most studies having been performed on a few model strains selected for specific adaptive capacities to a chosen environmental or stress factors as well as for solving agricultural problems (Ikeuchi, and Tabata, 2001). Therefore, *Synechocystis* is a useful tool when education the biochemistry and genetics of cyanobacteria (Joset *et al*., 1996).

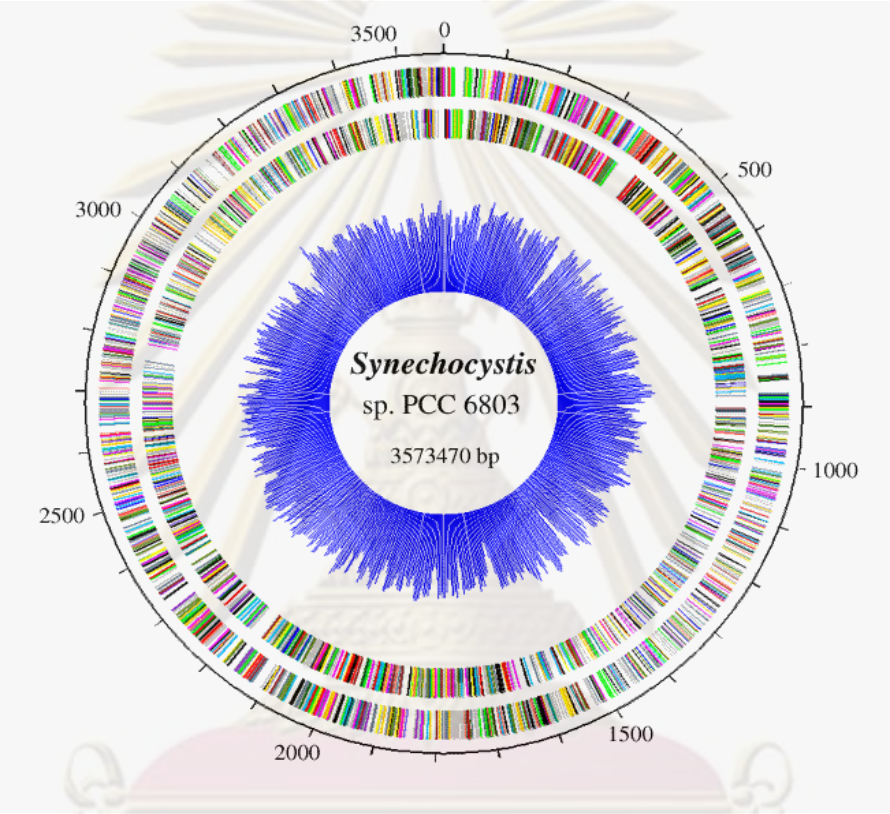

Figure 2 The circular genome of *Synechocystis* sp. PCC 6803 according to cyanobase (www.kazusa.or.jp/cyano/Synechocystis).

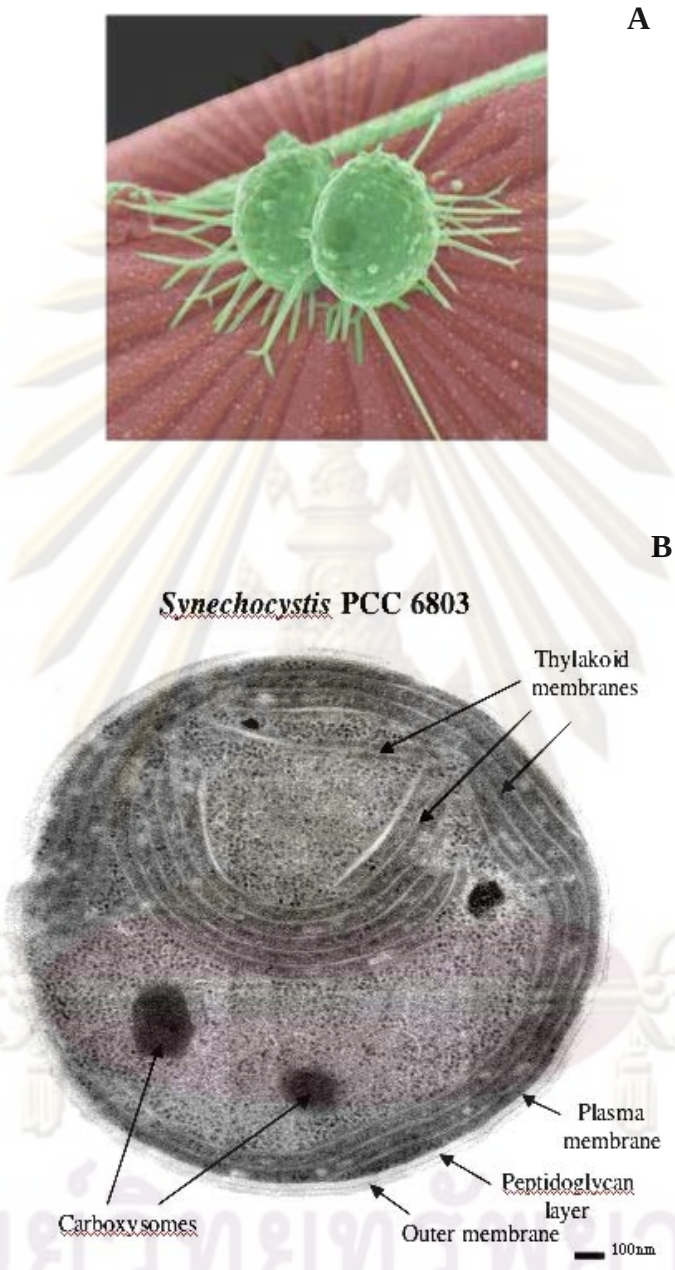

Figure 3 (A) Cell of the unicellular cyanobacterium *Synechocystis* and (B) schematic representation of a thin section (Transmition Electron Microscope) of a cell. (http://cosmology.com/Cosmology3.html)

(http://www.danforthcenter.org/imf/pakrasi/animation.htm)

1.3 Enzyme involved in H_2 metabolism by cyanobacteria

Cyanobacteria carry out both hydrogen consumption and evolution functions. Two classes of enzymes, hydrogenase and nitrogenase, are involved in catalyses H_2 metabolism.

1.3.1 Nitrogenase

Nitrogenase (EC class 1.18.6.1) is found in the heterocysts filamentous cyanobacteria such as *Anabaena variabilis* IAMM-58 (Masukawa *et al.*, 2001) but also present in some unicellular strains, *Synechococcus* sp. (Mitsui *et al*, 1986), *Cyanothece* sp. (Reddy *et al*., 1993) and *Gloeothece* sp. (Maryan *et al*., 1986) The activity of this multiprotein enzyme complex for nitrogen fixation is essential for maintenance of the nitrogen cycle. This enzyme reduces atmospheric of nitrogen to ammonia, and produces hydrogen gas as a by-product. The reaction consumes ATP and has the general form (Hansel and Linblad, 1998).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

H_2 evolution by nitrogenase requires considerable of electrons, reductants and ATP provided from photosynthesis or by degradation of energy with carbon countering molecule in the cell. The quanta required for H_2 evolution by nitrogenase would be double those required for CO_2 fixation with an overall minimum quantum requirement for approximately 9-10 quanta/H_2. It is a metalloenzyme complex consisting of a dinitrogenase (MoFe protein: $\alpha_2\beta_2$, encoded by *nifD* and *nifK*, α and β respectively) and a dinitroganase reductase (Fe protein: γ_2, encoded by *nifH*) (Figure 4A). There are three

types of dinitrogenases depending on the metal content. Type one contains molybdenum (Mo) (Thiel, 1993), type two contains vanadium (V) instead of Mo (Kentemich *et al.*, 1988) and type three has neither Mo nor V but it contains iron (Fe) (Bishop and Premakumar, 1992). The dinitrogenase catalyse reduction of substrate molecule whereas the dinitrogenase reductase accepts electrons from donors such as ferredoxin or flavodoxin and transfers electron to dinitrogenase with the concomitant hydrolysis of two molecules of ATP per electron transferred.

1.3.2 Hydrogenases

Hydrogenases are the key enzyme in biological H_2 metabolism which catalyse the oxidation of hydrogen to protons and the reduction of protons to hydrogen (Smith, 1990).

$$H_2 \leftrightarrow 2H^+ + 2e^-$$

Hydrogenase in microorganism is classified according to the metal composition of the active site into three major groups, Fe-H_2ase, NiFe-H_2ase or metal-free-H_2ase (Vignasis *et al.*, 2001). Cyanonacteria may have two functionally different NiFe-H_2ase (Apple and Schulz, 1998; Schlegel and Schneider, 1985), an uptake hydrogenase and bidirectional enzyme.

1.3.2.1 Uptake hydrogenase

The uptake hydrogenase, found in all N_2-fixing cyanobacteria strains, its located at the cytoplasmic face of the cell membrane or thylakoid membrane, where it utilizes H_2 evolved by nitrogenase enzyme. Previous reports shown that uptake hydrogenase is a membrane-bound enzyme (Houchin and Burris, 1981; Lindblad and

Sellstedt, 1990; Rai *et al.*, 1992). The uptake hydrogenase is encoded by the *hup*, hydrogen uptake, genes is a heterodimeric enzyme with a large subunit of about 60 kDa containing the active site (HupL) encoded by *hupL* and a small subunit (HupS) encoded by *hupS* playing a role in e^- transfer (Tamagnini *et al.*, 2007; Madamwar *et al.*, 2000). The main physiological function of the uptake hydrogenase is take up H_2 and split it into H^+ and e^- before feeding the e^- back into the e^- transport chain for preventing the highly reducing environmental within the cell or to save reducing equivalents (Figure 4B). This recycling has been suggested to have at least three beneficial functions, (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 removes O_2 from nitrogenase (Robson and Postage, 1980; Howarth and Codd, 1985; Weisshaar and Böger, 1985; Smith, 1990).

1.3.2.2 Bidirectional/Reversible hydrogenase

Apart from the nitrogenase enzyme, cyanobacteria have another hydrogen producing enzyme, the reversible or bidirectional hydrogenase. This enzyme is a heteropentameric, NAD^+-reducing enzyme, consisting of hydrogenase structural complex (HoxY and HoxH encoded by *hoxY* and *hoxH*, respectively) and a diaphorase component (HoxE, HoxF and HoxU encoded by *hoxE*, *hoxF* and *hoxU*, respectively) (Figure 4C). The bidirectional hydrogenase is located at the cytoplasmic membrane and common found in both N_2-fixing and non-N_2-fixing cyanobacteria (Kentamich *et al.*, 1991, Ghirardi *et al.*, 2007). The bidirectional hydrogenase can both uptake and produce hydrogen (Eisbrenner *et al.*, 1978; Tamagnini *et al.*, 2002). The physiological function of this enzyme has been a matter of speculation and is still unclear. One possible is that

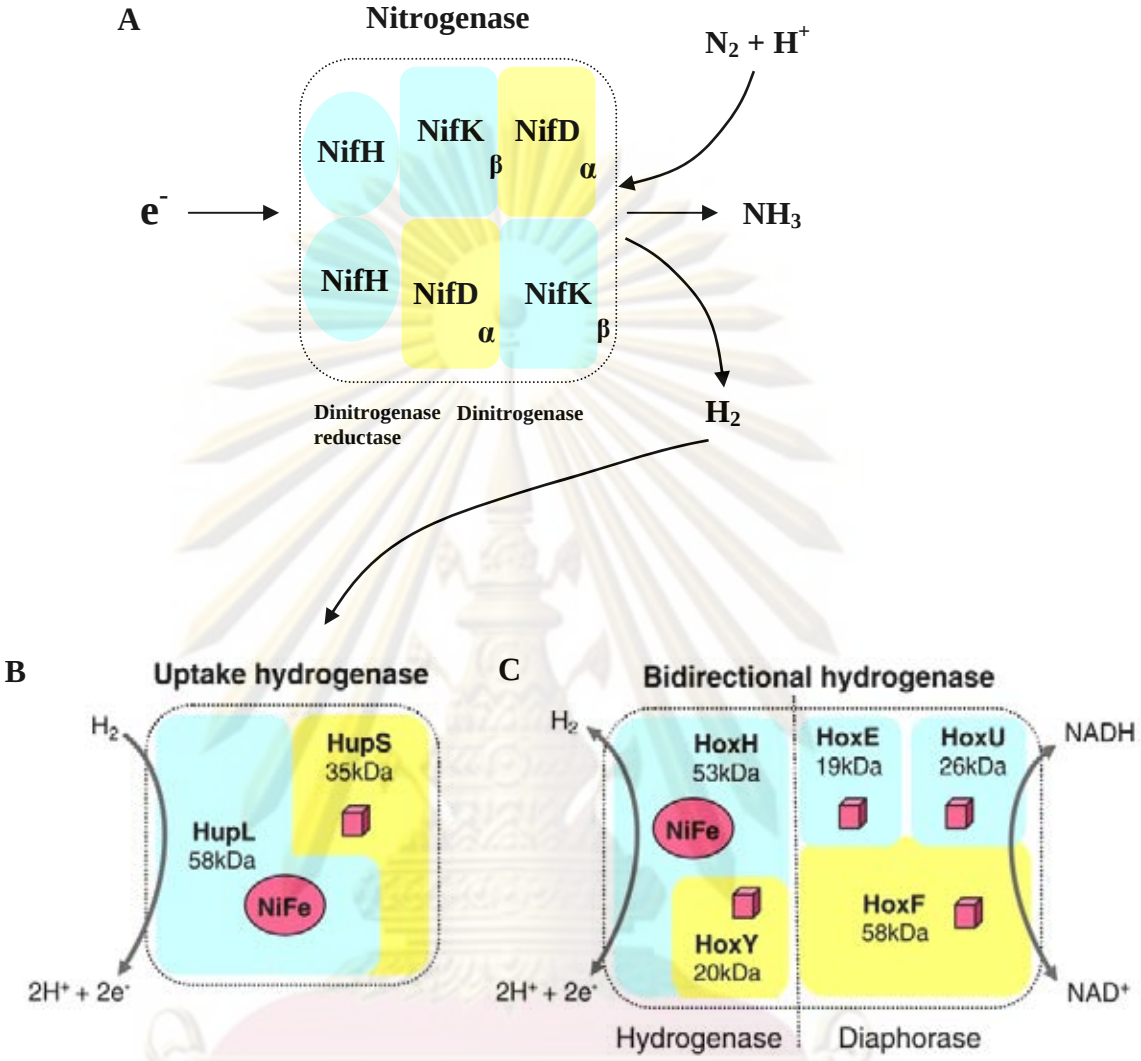

Figure 4 Enzymes directly involved in hydrogen metabolism in cyanobacteria. The nitrogenase (A) and uptake hydrogenase (B) is present in all nitrogen fixing strains tested so far, the bidirectional hydrogenase (C) is distributed among both nitrogen-fixing and non-nitrogen-fixing cyanobacteria. (Modified from Tamagnini *et al*., 2002 and Ghirardi *et al*., 2007)

bidirectional hydrogenase function as a valve for low potential e^- generated during light reaction of photosynthesis (Apple *et al*., 2000).

1.4 H_2 production in cyanobacteria

Various parameters influence hydrogen production in different ways, (1) the environmental conditions such as light, temperature, salinity, nutrient availability, gaseous atmosphere play a role in hydrogen production. Requirement of different cyanobacterial species are different for optimum hydrogen production. (2) Intrinsic factors affecting hydrogen production such as genetic components or sensitive proteins, i.e. uptake hydrogenase in cyanobacteria, the present of oxygen was inhibited the activity of nitrogenase and hydrogenase enzymes (Dutta *et al*., 2005, Kemner and Zeikus, 1994;). Different cyanobacterial species have different requirement for optimum hydrogen production.

Filamentous cyanobacteria may produce H_2 by the activity of the enzyme nitrogenase and bidirectional hydrogenase. Initial work by several scientists focused on the heterocystous flamentous cyanobacterium *Anabaena cylindrical* B-629. The highest hydrogen evolution was observed under growth conditions Air + CO_2 (0.3%), total incident light was 1.8×10^4 erg cm^{-2} s^{-1} and 32 µl mg dry wt^{-1} h^{-1} (1.33 µmol H_2 (mg dry w)$^{-1}$ h^{-1}) (Weissman and Benemann, 1977). Howard and Codd (1985) reported that the non nitrogen fixing cyanobacterium *Gloebacter* PCC 7421 demonstrated high hydrogen evolution rate (1.38 µmol H_2 (mg chl *a*)$^{-1}$ h^{-1}) when incubated under low light condition (20–30 µE m^{-2} s^{-1}) with CO and C_2H_2. This hydrogen evolution value was comparable with hydrogen evolution values of nitrogen fixing heterocystous cyanobacteria such as *Anabaena fos-aquae* UTEX 1444 (1.7 µmol H_2 (mg chl *a*)$^{-1}$ h^{-1}), *Anabaena cylindrica*

UTEX B 629 (0.91 µmol H_2 (mg chl *a*)$^{-1}$ h^{-1}) (Masukawa *et al*., 2001), *Anabaena cylindrica* (1.3 mmol H_2 (g dry cell)$^{-1}$ h^{-1})(Weissmen and Bonemann, 1977), *Anabaena variabilis* (0.6 mmol H_2 (g dry cell)$^{-1}$ h^{-1}) (Markov, et al., 1997)**.** However, the H_2 production rate was higher (2.14 mmol H_2 (g dry cell)$^{-1}$ h^{-1}) when the heterocysts of *Anabaena* sp. strain CA were isolated and measured (Kumar and Kumar, 1992). Moreover, the uptake hydrogenase is a negative waste enzyme for H_2 production. Therefore, a *Anabaena* sp. PCC 7210 deficient uptake hydrogenase mutant (Δ*hupL*) was constructed and it was shown that the cells produced H_2 at a rate 4-7 times higher than that of the wild type under optimum conditions (Masukawa *et al*., 2002). Moreover, the capacity to metabolize hydrogen via hydrogenase may be widespread among non-nitrogen fixing cyanobacteria.

Several marine cyanobacteria have a potential in hydrogen production such as *Oscillatoria brevis* B-1567 (0.168 µmol H_2 (mg dry wt)$^{-1}$ h^{-1}), *Calothrix scopulorum* 1410/5 (0.128 µmol H_2 (mg dry wt)$^{-1}$ h^{-1}) and *Calothrix membranacea* B-379 (0.108 µmol H_2 (mg dry wt)$^{-1}$ h^{-1}) grown under Air + CO_2 (5%), 7,000 Lux at the surface of the culture vessels (Lambert and Smith, 1977).

The fresh water cyanobacterium *Synechocystis* sp. PCC 6803 is a unicellular, non-nitrogen fixing cyanobacterium. Complete genome sequence and a survey based on a molecular approach show the absence of both genes encoding a nitrogenase and an uptake hydrogenase and the presence of genes encoding a bidirectional hydrogenase (Tamagnini *et al*., 2000), observed to be associated to the thylakoid membranes (Apple *et al*., 2000). The bidirectional hydrogenase enzyme in *Synechocystis* is a pentameric NiFe-H_2ase utilizing NAD(P) as substrate (Apple and Schulz, 1998; Schmitz *et al.*, 2002). The corresponding structural genes, *hoxEFUYH* are cluster together with three open reading frames (ORFs) of

unknown function(s) (Kaneko *et al*., 1996). The HoxY and HoxH subunit form the NiFe-H_2ase moiety, while three other subunits (HoxU, HoxF and HoxE) homologous to subunits of complex I of respiratory chains, contain NAD(P), flavin mononucleotide, and FeS binding sites (Appple and Schulz, 1998; Boison *et al*., 1998; Kaneko *et al*., 1996; Schmitz *et al*., 1995; Schmitz *et al*., 2002). A previous showed that there is no H_2ase activity in a hydrogenase deletion of *Synechocystis* sp. PCC 6803 mutant (Δ *hoxH*), concluding that in *Synechocystis* sp. PCC 6803 there is only a single the bidirectional HoxEFUYH hydrogenase functioning in both H_2 uptake and H_2 production (Apple *et al*., 2000).

H_2 production in cell of *Synechocystis* sp. PCC 6803 have been examined and found that they can produced hydrogen at a rate of 0.13 μmol H_2 (mg chl a)$^{-1}$ h^{-1} (Howard and Codd, 1985). In the fermentation processes under sulfur deprived condition, 100% CH_4 caused substantially increased hydrogen production by at least 4 times (Antal and Linblad, 2005). The bidirectional hydrogenase in this organism may function as a valve for low potential electrons generated during the light reaction of photosynthesis, thus preventing a slowing down of electron transport (Apple *et al.,* 2000). Cournac *et al.* (2002) reported that the PS II-independent hydrogen production is limited by recycling of NAD(P)H through the NDH dehydrogenase complex. In 2002 this enzyme was purified 467 fold and found to have a specific activity of hydrogen evolution of 46 μmol H_2 (mg protein)$^{-1}$ min^{-1}. The hydrogen evolution of the purified enzyme was the highest at 60° C and pH 6.3 (Schmitz *et al*., 2002). Using response surface methodology to determine the optimum conditions, the highest H_2 production, a close to 150-fold increase was observed (Burrows *et al.*, 2008).

For hydrogen production by the activity of bidirectional hydrogenase, electrons are required to combine with protons to form hydrogen. The protons are plenty within the

cells, thus the number of electrons available is the main limitation of hydrogen production. Nevertheless, electrons are used by competing pathways such as Calvin cycle. In consequence, one strategy for an enhanced production of hydrogen is to direct the electron flow toward bidirectional hydrogenase enzyme and away from competing pathway (Tamagnini *et al*., 2007). However, nitrate assimilation is one of competing pathway. Figure 5 shows the relation between nitrate assimilation and hydrogen production in cyanobacteria via the electron transport pathway. The pathway shows that nitrate reduction to nitrite and nitrite reduction to ammonium are competing and withdrawing electrons from hydrogen production. The cyanobacterial nitrate and nitrite reductases rely on reduced ferredoxin as an electron donor (Flores and Herrero, 1994; Flores and Herrero, 2005). Northern blot analysis of *hox* gene expression in *Synechocystis* sp. PCC 6803 under nitrogen limiting growth condition indicated an increased in transcription level (Antal *et al*., 2006). Similar results were observed by Osanai *et al*. (2006) who reported that *Synechocystis* sp. PCC 6803 under 4 hour of nitrogen starvation conditions showed an increase of the *hox* gene transcription. On the contrary, *Gloeocapsa alpicola* CALU 743 growing under nitrate limiting conditions showed an increased bidirectional hydrogenase activity, but no regulation at the transcription level of *hoxY* and *hoxH* (Sheremetieva *et al*., 2002). In addition, Gutthann *et al.* (2007) studied the relationship between nitrate assimilation and hydrogen metabolism in *Synechocystis* sp. PCC 6803. They showed an increased fermentative hydrogen production and a decreased hydrogen uptake when nitrate reductase was inhibited in cells grown in medium without nitrate. From many previous reports, besides oxygen, nitrate assimilation was found to be a most important electron sink.

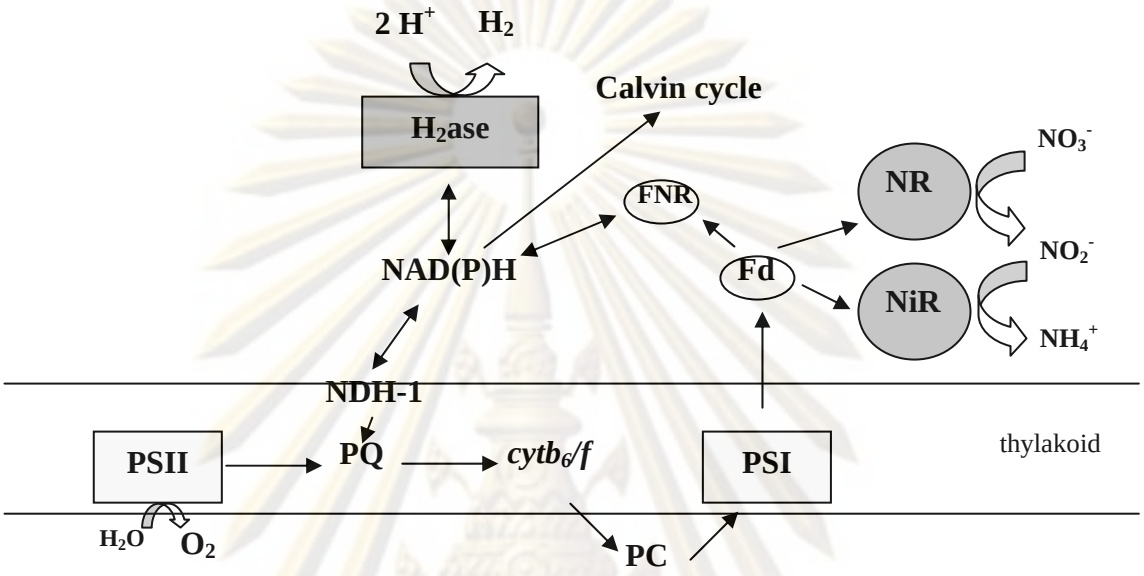

Figure 5 Schematic model of electron transport pathway in hydrogen production in cyanobacteria. Abbreviations: H_2ase: hydrogenase; PSI: photosystem I; PSII: photosystem II; *cytb$_6$/f*: cytochrome b_6/f complex; NDH-1: Complex I-like NAD(P)H dehydrogenase; PQ: plastoquinone; PC: plastocyanin; Fd: ferredoxin; FNR: Ferredoxin-$NADP^+$ reductase; NR: nitrate reductase; NiR: nitrite reductase. (Modified from Gutthann *et al*., 2007)

1.5 Nitrate metabolism in cyanobacteria

Nitrate, ammonium, urea and dinitrogen are the most abundant of nitrogen source in nature for plants and microorganisms, and nitrate assimilation is the most common mode of nitrogen nutrition in cyanobacteria. Cyanobacteria are able to grow with nitrate as the nitrogen source and commonly culture media use nitrate as a source of combined nitrogen (Rippka *et al*., 1979).

1.5.1 Nitrate uptake

Nitrate is the most commonly used for nitrogen source by cyanobacteria (Guerrero *et al*., 1981). The nitrate assimilation involves entrance of nitrate into the cells by an active transport system (nitrate/nitrite transporter, NRT), the same transporter mediates the uptake of nitrate and nitrite (Madueño *et al*., 1987). The nitrate is reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) (Flores *et al*., 1983). The resulting ammonium is reduced into carbon skeletons through the pathway commonly known as GS-GOGAT, by the sequential action of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) (Flores *et al*., 2005; Muro-Paster *et al*., 2005) as shown in Figure 6.

Three distinct types of NRTs have been identified in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (Omata *et al*., 1993), while in the denitrifying bacterium *Paracoccus pantotrophus*, there are two distinct nitrate transport systems, one operating as a nitrate-proton symporter while the second as an energy-independent nitrate/nitrite antiporter (Wood *et al*., 2002). Both these functions were proposed to be associated with one membrane protein. In fact, several genes additionally involved in

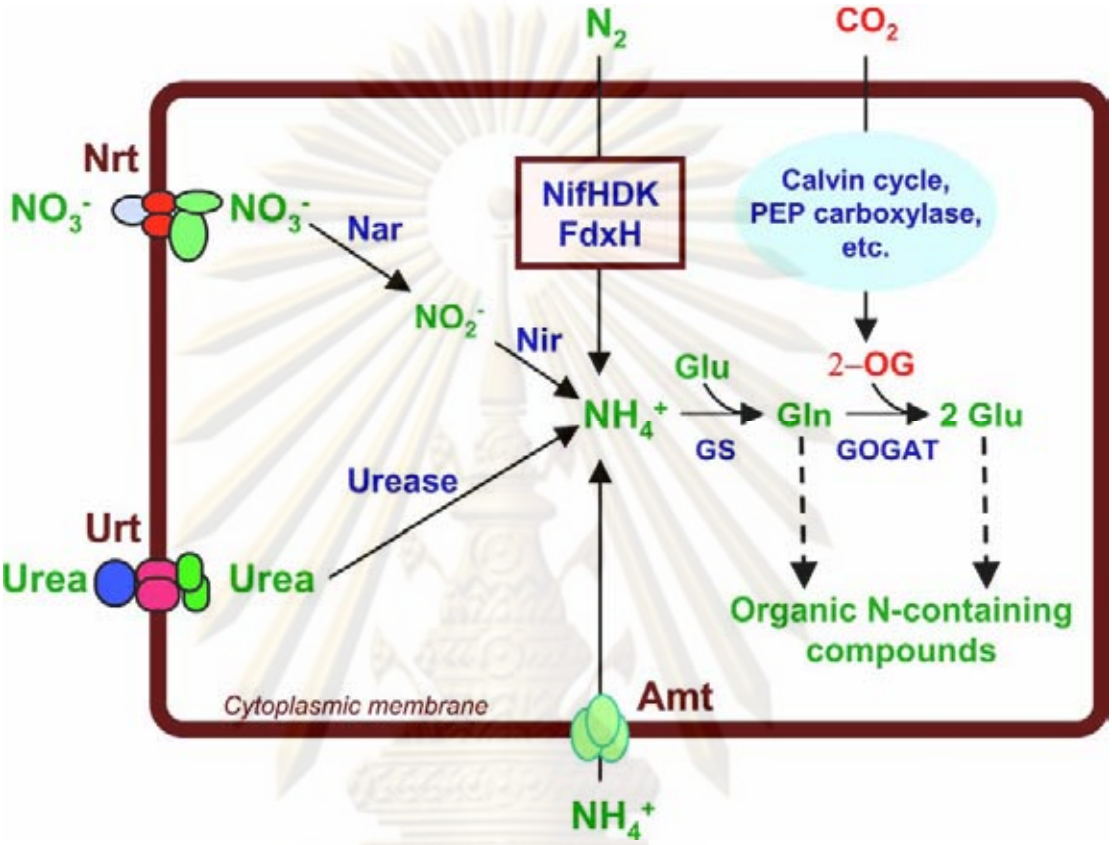

Figure 6 Main nitrogen assimilation pathways in cyanobacteria. Nrt, ABC-type nitrate/nitrite transporter; Urt, ABC-type urea transporter; Amt, ammonium permease; Nar, nitrate reductase; Nir, nitrite reductase; NifHDK, nitrogenase complex; FdxH, heterocyst-specific ferredoxin; PEP carboxylase, phosphoenolpyruvate carboxylase; 2-OG, 2-oxoglutarate; GS, glutamine synthetase; GOGAT, glutamate synthase. (Flores and Herrero, 2005)

nitrate transport have been described in the green alga *Chlamydomonas reinhardtii* and in plants (Galván *et al*., 1996; Quesada *et al*., 1994; Daniel-Vedele *et al*., 1998).

Although there is a general reliance on the involvement of nitrate transport systems in nitrate utilization, little is known of biochemistry of nitrate transport in photosynthetic bacteria. The uptake of nitrate in cyanobacterial cells has been widely studied by monitoring nitrate disappearance from the external medium (Flores *et al*., 1983; Meek *et al*., 1983; Tischner and Schmidt, 1984). At high external nitrate concentration, a low affinity transport system (LATS, Km > mM), when the low external concentration of nitrate (0-0.5 mM) is present, two high affinity transport system (HATS, 5 < Km < 200 µM) operate (Aslam *et al*., 1992). Differences from nitrate, external nitrite at the low concentrations is transport into the cyanobacterial cell by two distinct mechanism; (1) by active transport of nitrite (NO_2^-), which is sensitive to N,N'-dicyclohexylcarbodiimide (DCCD), and (2) by passive diffusion of nitrous acid (NHO_2)(Flores *et al.*, 1987). The assimilation of nitrate is not regulated by ammonium itself, but is related to its assimilation products in several cyanobacteria, i.e., *Aphanothece halophytica* (Incharoensakdi and Wangsupa, 2003), *Anacystis nidulans* (Lara *et al*., 1987), and *Anabaena cycadeae* (Bagchi *et al*., 1985). The energetic component for nitrate uptake was ascribed to contribution by the pH gradient in the halotolerant cyanobacterium *A. halophytica* (Incharoensakdi and Laloknam, 2005). In cyanobacterium *Synechocystis* sp. PCC 6803, the genes encoding nitrate transport (*nrtA*, *nrtB*, *nrtC* and *nrtD*) are clustered together with NR (encoding by *narB*) called *nrtABCD-narB*. The proteins involved in nitrate assimilation are organized into two transcription units, *nrtABCD-narB* and *nirA* (encoding NiR) (Aichi *et al.*, 2001).

1.5.2 Nitrate assimilation enzymes/genes

1.5.2.1 Nitrate reductase

Cyanobacterial nitrate reductase (EC. 1.7.7.2) has been purified to homogeneity and characterized as a molybdo-iron protein consisting of a single peptide chain with a molecular mass about 75 kDa in unicellular cyanobacterium *Synechococcus* sp. (Candau, 1979; Guerrero *et al*., 1981), 58 kDa in salt-tolerant unicellular cyanobacterium *Aphanothece halophytica* (Thaivanich and Incharoensakdi, 2007) and 83 to 85 kDa in filamentous non-heterocystuos cyanobacterium *Plectonema boryanum* (Mikami and Ida, 1984). This enzyme is associated with the thylakoid membranes in both uni-cellular and filamentous cyanobacteria (Manzano *et al*., 1976; Ida and Mikami, 1983). Ferredoxin is believed to be the physiological electron donor for the cyanobacterial nitrate reductase. When the enzyme catalyzes the reduction of nitrate to nitrite, two mol of ferredoxin are oxidized per mol of nitrate reduced (Mikami and Ida, 1984). Interestingly, *Synechocystis* sp. strain PCC 6803 contains only one copy of the NR gene, *narB*, in its genome (http://www.kazusa.or.jp/cyano/Synechocystis/index.html). In all cyanobacteria tested to date, the *narB* gene is located just downstream of nitrate transporter genes, and the encoded NR is subjected to repression by ammonium present in the growth medium.

1.5.2.2 Nitrite reductase

Nitrite reductase (EC. 1.7.7.1), consists of a single polypeptide of 52-68 kDa. Cyanobacterial nitrite reductase catalyzes the assimilatory 6-electron reduction of nitrite to ammonium. The molecular weight of this enzyme is 52-56 kDa, which contains a [4Fe-4S] cluster and a siroheme as prosthetic groups. Reduced ferredoxin or flavodoxin

from photosynthesis pathway are physiological e^- donor for nitrite reductase (Manzano *et al*., 1976). This enzyme was found firmly bound to thylakoid membrane of cyanobacteria *Synechococcus* sp. (Guerrero *et al*., 1974).

The strategies used by cyanobacteria to survive under environmental changes with high salinities have been much studied by scientist. However, there have been very few studies on relationship of nitrate metabolism to salt or osmotic stress in cyanobacteria. The effects of NaCl caused an increased rate of nitrate uptake in *A. torulosa* and *Anabaena* sp. PCC 7120 (Reddy *et al*., 1989; Rai and Tiwari, 1999) but not in *A. halophytica* (Incharoensakdi and Wangsupa, 2003).

Objectives of this research are:

1. To examine the conditions giving rise to high production of hydrogen using wild-type *Synechocystis* sp. PCC 6803
2. To investigate nitrate assimilation with emphasis on nitrate uptake
3. To determine hydrogen production in nitrate reductase mutant strain (Δ*narB*), nitrite reductase mutant strain (Δ*nirA*) and double mutant strain of nitrate reductase and nitrite reductase (Δ*nar*:Δ*BnirA*) compared with wild type 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
Centrifuge	Jouan MR 1812, USA
	SORVALL® MC 12V DUPONT, USA
	HERMLE Z233 MK, USA
Electrophoresis Unit	BIO-RAD PROTEIN® II xi Cell, USA
Gas Chromatography	Model GC-15A, Shimadzu, Japan
High Performance Liquid Chromatography	Series 1050, Hewlett Packard, USA
Laminar flow	BVT-124 International Scientific Supply, Thailand
Light source unit	Prekeo S250 Zeiss IKON, Japan
PCR apparatus	PERKIN ELMER DNA Thermal Cycler, Japan
pH meter	ORION model 420A, USA
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
Wescor Vapor Pressure Omometer	Model 5520
Ultracentrifuge	BECKMAN COULTER OPTIMA™ L-100XP, USA
Vortex	Model K-550-GE, Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.1.2 Chemicals

2,4-Dinitrophenol(DNP)	Merck, Germany
3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)	Sigma, USA
Acetic acid	BDH, England
Acetone	Merck, Germany
Agarose	Promega Corporation, USA
β-mercaptoethanol	Sigma, USA
Brilliant blue	Sigma, USA
Bromophenol blue	Sigma, USA
Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)	Sigma, USA
Chloroform	Merck, Germany
Dithiothreitol (DTT)	Sigma, USA
Dimethylformamide (DMF)	Sigma, USA
DL-Glyceroldehyde	Sigma, USA
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
Gramicidine D	Sigma, USA
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Mercaptoethanol	Sigma, USA
Methanol	Scharlau Chemie S.A., Spain
Metylene blue	Sigma, USA
Monensin	Sigma, USA
Methyl viologen	Sigma, USA
N, *N*′-dicyclohexyl carbodiimide (DCCD)	Sigma, USA
N-2-hydroxyethylpiperazine- *N*'-2-ethanesulfonic acid (HEPES)	Sigma, USA
Nicotinamide Adenosine Dinucleotide (NADH)	Sigma, USA
Nigericin	Sigma, USA
Phenol	Merck, Germany
Sodium arsenate	Sigma, USA
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
Toluene	BDH, England
Tris base	USB Corporation, USA
Tris HCl	Sigma, USA
Triton X-100	Packard, USA
Tween-20	BIO-RAD, USA
Urea	Sigma, USA
Valinomycin	Sigma, USA

2.1.3 Enzymes

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

2.1.4 Antibiotics

Ampicillin	Sigma, USA
Chloramphenicol	Sigma, USA
Kanamycin	Sigma, USA

2.1.5 Kits and suppliers

Nylon membrane filter 0.45 and 0.22 μm	Sartorius, Germany

PCR purification kit NucleoSpin® Extract II	Machery-Nagel, USA
GeneRuler™ 1 kb DNA Ladder	Fermentas, Canada
GeneRuler™ 100 bp DNA Ladder	Fermentas, Canada
λ DNA/*Hind*III Marker	Fermentas, Canada
Quick ligation™ Kit	BioLabs
pGEM®-T Easy vector System	Promega, Canada

2.1.6 Organisms

2.1.6.1 The unicellular cyanobacterium *Synechocystis* sp. PCC 6803

Cells of the cyanobacterium *Synechocystis* sp. PCC 6803 wild type (Williams, 1988), were obtained from the Laboratory of Plant Physiology and Molecular Biology, Department of Biology, University of Turku, Finland.

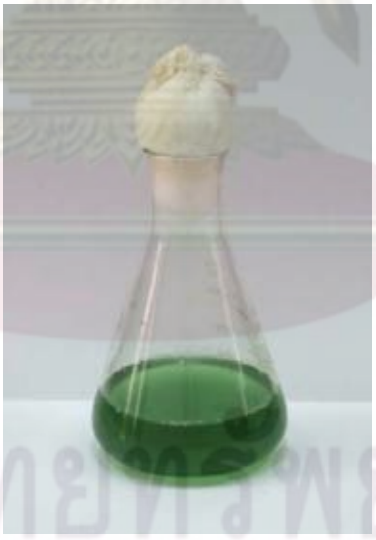

Figure 7 *Synechocystis* sp. PCC 6803 in BG11 medium

2.1.6.2 *Escherichia coli* strain DH5α

The *E. coli* strain DH5α was obtained from Department of Photochemistry and Molecular Science, The Ångström Laboratorie, 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 *thi*-1 *gyr*A96 *rel*A1 λ-) was used for DNA manipulation.

2.1.7 Plasmids

The three plasmids, namely pGEM®T Easy, pSB2K3 and pBR325 vector were used in this study. Circle maps are shown in APPENDIX I, J and K, respectively.

2.1.8 Oligonucleotides

Table 2 PCR primers for *Synechocystis* sp. PCC 6803 chromosomal DNA

Target gene	Name	Oligo sequence	Amplified fragment length (bp)
narB	narBF	5'- TGGGCTAGCCCTCAGGACAAA-3'	2,440
	narBR2	5'-CAGAGGCTATCTCAGATGGCTA-3'	
nirA	nirAF	5'-ACTGCATATGCCTTGGCTGA -3'	1,646
	nirAR	5'-ACATTAACGATGGGGAAGCG-3'	

Table 3 PCR primers for antibiotic cassette

Target gene	Name	Oligo sequence	Amplified fragment length (bp)
Kanamycin -cassette	KnSBKF1	5'-ATAGGTACCTTGGCGGGATATCAAACTTC-3'	1,376
	KnSBKR1	5'-ATAGGTACCTATCTGAAATTCTGCCTCGT-3'	
Chloramphenicol-cassette	CmF3-pBR325	5'-ATACCTAAGGATAATACCGCGCCACATAGC-3'	1,558
	CmR2	5'-ATACCTAAGGCGGCTATTTAACGACCCTGCCCTGA-3'	

2.2 Methods for *Synechocystis* sp. PCC 6803

2.2.1 General methods

2.2.1.1 Culture conditions

The cyanobacterium *Synechocystis* sp. strain PCC 6803 was grown photoautotrophically in BG11 medium containing 17.6 mM $NaNO_3$ as nitrogen source buffered with 20 mM HEPES-NaOH (pH 7.5) (Rippka, 1979). In addition, the mutant strains, Δ*narB*, Δ*nirA* and double mutant Δ*narB*:Δ*nirA* were grown in $BG11_0$ (BG11 medium without nitrogen source) containing 3.75 mM of $(NH_4)_2SO_4$ as nitrogen source, supplemented with 30 μg mL^{-1} kanamycin or 30 μg mL^{-1} chloramphenicol, and both of kanamycin and chloramphenicol for Δ*narB*, Δ*nirA* and double mutant Δ*narB:*Δ*nirA* respectively. The initial and cell concentration was adjusted to an OD_{730} of 0.1 and cultures of 100 mL volume were grown in 250 mL Erlenmeyer flasks on a rotatory shaker at 160 rpm, 30 °C under continuous illumination of 30 μE m^{-2} s^{-1} with cool white fluorescent lamps. Two-mL samples were taken from a culture flask every other day to determine growth rate by measuring the optical density of the culture at 730 nm with a Spectronic® Genesys™2 spectrophotometer. Other growth media used: BG11-C-deprived (BG11 medium without Na_2CO_3), BG11-C-deprived plus different sugars (sucrose, fructose or glucose), and $BG11_0$-S-deprived ($BG11_0$ medium with the following modifications: $MgSO_4 \cdot 7H_2O$ replaced with $MgCl_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$ replaced with $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ replaced with $CuCl_2$ and $ZnSO_4 \cdot 7H_2O$ replaced with $ZnCl_2 \cdot 2H_2O$, all in equal molarity). For long-term storage, the strains were stored at -80°C in BG-11liquid medium containing 15% glycerol.

2.2.1.2 Chlorophyll *a* measurement

The total amount of chlorophyll a (Chl *a*) were determined spectrophotometrically at 665 nm in 90% (v/v) methanol extracts (Mackinney, 1941).

2.2.1.3 Cells preparation for measuring H_2 production

Cells were prepared by culturing in BG11 medium until OD_{730} ~ 0.75 before harvested by centrifugation at 8,000 rpm for 15 min at room temperature. The pellets were washed twice in desired medium and resuspended in 100 mL of each desired medium. After 24 h of preculture stage, the cells were harvested, the pellet resuspended in 5 mL of medium and transferred to a 10 mL glass vial, before sealed with a rubber septum and a proper screw lid. The vial was bubbled with argon gas for 5 min to get rid of oxygen before the vials were put upside down in darkness under anaerobic condition at room temperature.

2.2.1.4 H_2 production assay

At time intervals, H_2 concentration in the head space of the vial was measured via 400 µl of head space gas samples withdrawn from the vials with a gas tight syringe. The sample was analyzed by a Gas Chromatograph (GC-15A, Shimadzu, Kyoto, Japan, 2 m stainless column packed with molecular Sieve 5 °A, Mesh 60/80) equipped with a thermal conductivity detector. The injector and detector were maintained at 100 ^{o}C. The column oven temperature was 50 ^{o}C. The argon carrier gas flow rate was maintained at 30 mL min^{-1}.

2.2.1.5 Bidirectional hydrogenase activity assay

The activity of the bidirectional hydrogenase is measured as evolution of hydrogen in the presence of methyl viologen reduced by sodium dithionite, using gas chromatography. The reaction mixture (2.0 mL) contains 1 mL cells suspension, 2 mM methyl viologen, 20 mM sodium dithionite in 25 mM phosphate buffer, pH 7.0 under anaerobic condition. The reactions were carried out with shaking at 30 °C. Activity is expressed as μmol H_2 (mg chl *a*)$^{-1}$ min^{-1}.

2.2.1.6 Nitrate uptake assay

Uptake of nitrate was measured by monitoring the decrease in the extracellular concentration of nitrate. *Synechocystis* sp. PCC 6803 cells were harvested by centrifugation, washed twice with 25 mM Tris-HCl pH 8.0, supplemented with 12 mM $NaHCO_3$ and resuspended in same buffer at chlorophyll concentration of 10 μg mL^{-1}. The reaction is started by the addition of 100 μM $NaNO_3$ to the suspension kept at 30 ^{o}C in the light with an irradiance of 60 μE m^{-2} s^{-1}. At regular intervals the cells suspension is rapidly filtered through a 0.45 μm membrane filter. The nitrate content remaining in the filtrate was determined by anion exchange HPLC (Hypersil-10 Sax column, 250 mm × 4.6 mm).

2.2.2 Molecular biology methods

2.2.2.1 Chromosomal DNA extraction

Total DNA was extracted from exponentially growing cultures of *Synechocystis* sp. PCC 6803 in BG11 medium (OD_{730} > 0.8). The pellets were collected by centrifugation at 4,000 x g for 10 min and resuspended in 535 μl of TE-Buffer, pH 8.0 (10

mM Tris-HCl and 1 mM EDTA). Then 60 µl of 20% SDS and 60 µl of proteinase K (1 mg mL^{-1}) were added into the mixture and incubated at 37 ^{o}C for 2 h. After incubation, 100 µl of 5M NaCl and 80 µl of CTAB:NaCl (10% CTAB in 0.7 M NaCl) were added and incubated at 65^{o}C for 10 min. The lysate was then centrifuged (10 min, 10,000 x g) to remove major amount of polysaccharides, and the clear supernatants (containing total DNA) were transferred to a new microcentrifuge tube. After that, 800 µl (1:1 volume) of Chloroform:Isoamyl alcohol (24:1) was mixed and centrifuged at 12,000 rpm, room temperature for 5 min. Kept viscous supernatant in new microcentrifuge tube, added 1:1 volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and then centrifuged at 12,000 rpm, room temperature for 5 min. Kept supernatant in new microcentrifuge tube, added 0.6 volume of isopropanol then incubated on ice for 15 min and centrifuged at 12,000 rpm, 4^{o}C for 10 min. Pellets were kept and then added 70% ethanol. After gentle mixing by inversing, the mixture was centrifuged at 12,000 rpm, 4^{o}C for 2 min. Washed pellets were air dried at room temperature. 100 µl of TE-Buffer pH 8.0 was suspended and then 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 (Sambrook and Russell, 2001). DNA sample was run in 0.8% agarose gel electrophoresis with 0.5xTBE buffer pH 8.0.

2.2.2.2 Primers design

Primers for amplification of *narB* and *nirA* gene of *Synechocystis* sp. PCC 6803 were designed from the genome database for cyanobacteria (http://genome.kazusa.or.jp/cyanobase/) and primers for amplification kanamycin resistance cassette of pSB2K3 were designed from the BioBrick plasmid database

(http://partsregistry.org/Part:pSB2K3) using the program primer3 (v.0.4.0) (http://frodo.wi.mit.edu/primer3/).

2.2.2.3 Polymerase Chain Reaction

The PCR was performed in a 1**x** PCR mix (1**x** Taq buffer $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.5 pmol of each forward and reverse primer) using 0.1-1 µg of template DNA (or picked colony for colony PCR) and 0.05 U of *Taq* polymerase, making to 20 µl of reaction mixture with sterile DI water. The PCR reaction involved an initial denaturing step of 5 min at 94°C followed by 30 cycles of 30 s denaturing at 94°C, 1 min annealing at 45-65°C, depending on the oligonucleotides used, 1-3 min extension at 72°C, depending on length of expected PCR product, and 30 s denaturing at 94°C. The reaction ended with a final extension at 72°C for 5 min. the PCR product was analyzed by electrophoresis.

2.2.2.4 Natural transformation into *Synechocystis* sp. PCC 6803

The natural transformation was performed as follows: 50 mL of an exponentially growing culture (OD_{730} ~ 0.5) were centrifuged at room temperature at 5,000 rpm for 5 min. The supernatants were discarded and the pellets were resuspended in 2 mL fresh $BG11_0$ containing 3.75 mM $(NH_4)_2SO_4$ buffered by 20 mM HEPES-NaOH, pH 7.5. Aliquots of 0.5 mL cell suspensions with an OD_{730} of ~ 2.5 were distributed into 2 mL steriled eppendrof tubes. The exogenous plasmid DNA of pMnarB19 and pMnirA3 were purified by NucleoSpin® Extract II (Machery-Nagel, USA) to a final concentration of 20 µg mL^{-1} before being mixed with cells suspension. The mixture of cells and DNA was incubated for 6 h under light at 30 ºC, and shaken once tube once after 3 h. Cells were spread onto membrane filters placed on $BG11_0$ (containing 3.75 mM $(NH_4)_2SO_4$ as

nitrogen source, 0.3% Na-thiosulfate, buffered with 20 mM HEPES) do not contain any antibiotics. The plates were transferred to a growth chamber at 30 ºC, light illumination at 30 μE m^{-2} s^{-1} for 18 h. After 18 h, the transfromants were plated onto medium agar plates containing 20 μg mL^{-1} of antibiotic. After 3 days, the filters were transferred onto medium new agar plates containing the antibiotic at the concentration 30 μg mL^{-1}. To ensure the complete segregation of obtained gene knockouts were performed colony PCRs.

2.3 Methods for *Escherichia coli*

2.3.1 General methods

2.3.1.1 Culture condition

Cells of *Escherichia coli* were grown in LB medium at 37 ºC with shaking at 250 rpm using a rotary shaker.

2.3.1.2 Gel electrophoresis

DNA sample were analysed by electrophoresis in 0.8% agarose gel. The agarose gels were run in 0.5x TBE buffer. 1 μl of DNA loading buffer was added to the DNA sample, which were run at 100 volts for 45 minutes. The DNA samples were stained for 10 min in distilled water containing ethidium bromide at a final concentration of 1 ng/mL. Bands were visualized using a Geldoc® system with DNA visualization UV light.

2.3.2 Molecular biology methods

2.3.2.1 Preparation of competent cells

A single, well isolated colony of *E. coli* strain DH5α was inoculated into 10 mL of LB media and incubated at 37^{o}C overnight with shaking at 250 rpm on a rotary shaker. A 2 mL aliquot of the overnight culture was transferred to 100 mL of LB media and grown until the OD_{600} nm was between 0.3-0.4. The cells were chilled on ice for 15 min followed by 4^{o}C centrifugation at 3,500 x g for 15 minute. The supernatants were discarded and resuspended cells in 10 mL of $CaCl_2$ buffer. Cells suspensions were chilled on ice for 15 minute followed by 4^{o}C centrifugation at 3,500 x g for 15 minute. The supernatant was discarded and the pellets were resuspended in 1.5 mL of $CaCl_2$ buffer, 0.3 mL of 86% Glycerol. Aliquots of 100 µl in sterile 1.5 mL micro centrifuge tubes were stored immediately at -80^{o}C.

2.3.2.2 Restriction digestion

Single or double restriction digestions were incubated at the optimum temperature of restriction enzymes for 60 min. The total volume of the reaction was 10 µl containing 1 x proper restriction buffer, 1-2 U of restriction enzyme, and the DNA sample. The digested plasmid was analyzed by 0.8% agarose gel electrophoresis. The clone containing the correct DNA fragment was sequencing to confirm correct vector and insert.

2.3.2.3 Ligation

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was used

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

A 20 µl ligation reaction was composed as described in Table 4

Table 4 The composition of the ligation reaction

Component	Reaction (µl)
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

The reaction was incubated overnight at 22 ºC, 10 minutes. Two microlitre of the ligation mixture was transformed into competent cells of *E. coli* DH5α.

2.3.2.4 Heat-shock transformation

An aliquot of competent *E. coli* DH5α was gently thawed on ice for 5-10 minutes. The cells suspension was gently mixed with 2 µl of ligation reaction mixture and incubated on ice for 30 minutes. The cells were transformed by heat-shock at 42 ºC for 1 minute, then placed on ice for 2 minutes following by adding 900 µl of LB medium and

incubated at 37 ºC with shaking at 250 rpm for 1 hour. The mixture was spread on the LB agar plates containing appropriate antibiotic and incubated at 37 ºC overnight. The next day, obtained colonies were randomly selected for plasmid isolation.

2.3.2.5 Alkaline lysis extraction of plasmid

A single, well-isolated colony of the transformed *E. coli* DH5α strain was inoculated into 2 mL of LB media containing appropriate antibiotic and grown overnight shaking at 37 ºC, 250 rpm. The entire culture was centrifuged for 30 s at 12,000 rpm. Obtained pellet was resuspended in 100 µl of Solution I to which 20 µg mL^{-1} RNase was added and incubated at room temperature for 5 min. The sample was placed on ice for 5 min, 200 µl of Solution II was added, shaken vigorously and further incubated on ice for 5 min. This was followed by the addition of 150 µl of Solution III and 5 min incubation on ice. After centrifuging for 5 min at 12,000 rpm, approximately 350 µl of the supernatant was transferred to a fresh microcentrifuge tube and extracted with 100 µl of 1:1 Phenol:Chloroform:Isoamyl alcohol(25:24:1), mix. The sample was centrifuged for 5 min at 12,000 rpm; the 300 µl upper phase was transferred to a new microcentrifuge tube. After that 180 µl of 100% isopropanol was added to the sample and incubated at room temperature for 10 min. The sample was centrifuged for 10 min at 12,000 rpm. The supernatant was discarded and the pellet washed with 800 µl of cold 70% ethanol before drying at 37^{o}C. The final pellet was resuspended in 50 µl of TE buffer. One µl of this sample was analyzed and confirmed by running on a 0.8% agarose gel in 0.5x TBE buffer.

CHAPTER III

RESULTS

3.1 Charactrization of H_2 production by *Synechocystis* sp. PCC 6803 under various conditions

3.1.1 Dependence of H_2 production on stage of cell growth and time course of H_2 production

The unicellular non-nitrogen fixing cyanobacterium *Synechocystis* sp. strain PCC 6803 was grown in BG11 medium and the growth monitored over three weeks. Both optical density (OD_{730}) and chlorophyll *a* content increased with an increase in cultivation time (Figure 8A). After nine days the increase of chlorophyll *a* occurred at a faster rate than the increase of OD_{730}. Stationary phase of growth was not reduced even at day 21.

Cells after one-week cultivation gave the highest H_2 production up to at least 24 h followed by those of two-week and three-week cultivation, respectively (Figure 8B). A fast and linear increase of H_2 production was observed during the first 6 h for all three cultures with a saturation of H_2 production after 12 h.

3.1.2 Effect of different nitrogen source

Synechocystis sp. PCC 6803 were grown in BG11 medium without nitrogen source (hereafter called $BG11_0$) and $BG11_0$ medium containing various nitrogen sources, NH_4Cl, $NaNO_2$ and $NaNO_3$ (control). The results showed similar growth pattern when NH_4Cl or $NaNO_3$ was used as nitrogen source. When $NaNO_2$ was used as nitrogen source, cells have longer lag-phase. No growth was observed when cells were grown in the medium without nitrogen source ($BG11_0$ medium) (Figure 9A).

Cells cultivated in BG11 medium for one week were further adapted 24 in BG11 medium without nitrogen source and with different nitrogen sources including NH_4Cl, $NaNO_2$ and $NaNO_3$ (control) at the same concentration. Figure 9B shows that cells adapted in $BG11_0$ medium gave the highest H_2 production rate (0.194 ± 0.072 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}). For cells grown in different types of nitrogen sources, $NaNO_3$, NH_4Cl and $NaNO_2$, the H_2 production rate was 0.080 ± 0.011, 0.056 ± 0.013 and 0.016 ± 0.022 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}, respectively.

3.1.3 Effect of different carbon sources and glucose concentration

The growth rate of *Synechocystis* sp. PCC 6803 grown in BG11 with or without Na_2CO_3 (BG11-C-deprived) as carbon sources, and BG11-C-deprived supplemented with three different types of sugars; sucrose, fructose and glucose at 0.1% concentration was studied. Cells grown in the medium containing different types of carbon source gave the same pattern of growth rate (Figure 10A).

Cells grown after one week were further adapted for another 24 h in BG11 medium with or without Na_2CO_3 (C-deprived) as carbon source and in BG11 medium where Na_2CO_3 was replaced by different carbon sources. Glucose at 0.1% yielded the highest H_2 production (0.114 ± 0.011 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}) followed by sucrose (0.050 ± 0.004 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}) and fructose (0.034 ± 0.002 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}), respectively (Figure 10B). Cells grown in BG11 with or without Na_2CO_3 as carbon source gave similar H_2 production (0.025 ± 0.001 and 0.027 ± 0.002 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}, respectively). Replacing Na_2CO_3 in BG11 medium with 0.1 % glucose led to a 5-fold increase in H_2 production. Moreover, an increase of glucose concentration to 0.4 % could further enhance H_2 production to the highest level (0.161 ± 0.010 μmol H_2 (mg chl *a*)$^{-1}$ h^{-1}, Figure 11). H_2 production decreased with glucose concentrations higher than 0.4 %.

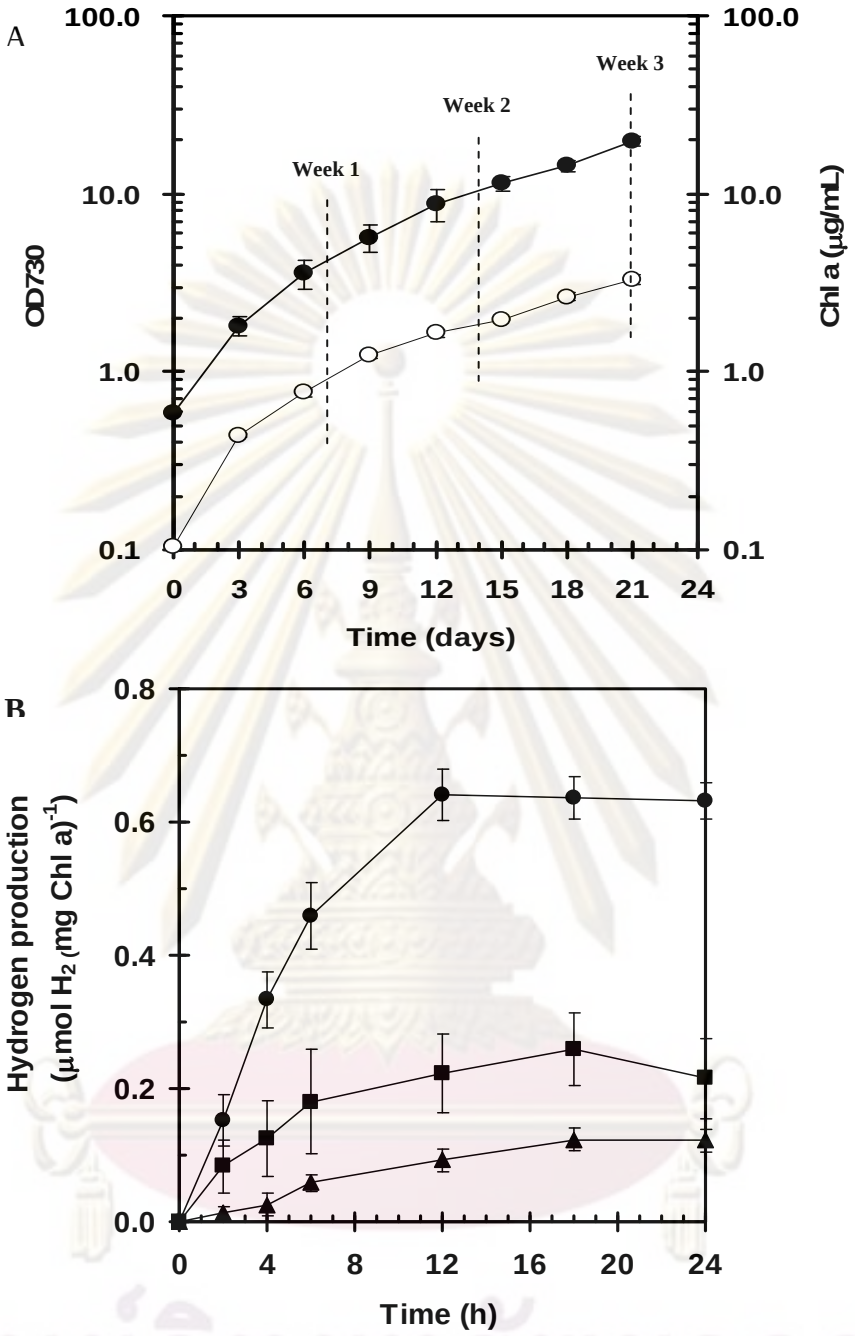

Figure 8 Effect of growth of cells of *Synechocystis* sp. strain PCC 6803 on H_2 production. (A) Growth in BG11 medium monitored by measuring the optical density of the culture at 730 nm (○) and by the chlorophyll a content (●). (B) H_2 production by cells grown for 1(●), 2 (■) and 3 (▲) weeks under dark, aerobic conditions. The hydrogen content was determined at indicated times for 24 h. Means ±S.D. (n=3).

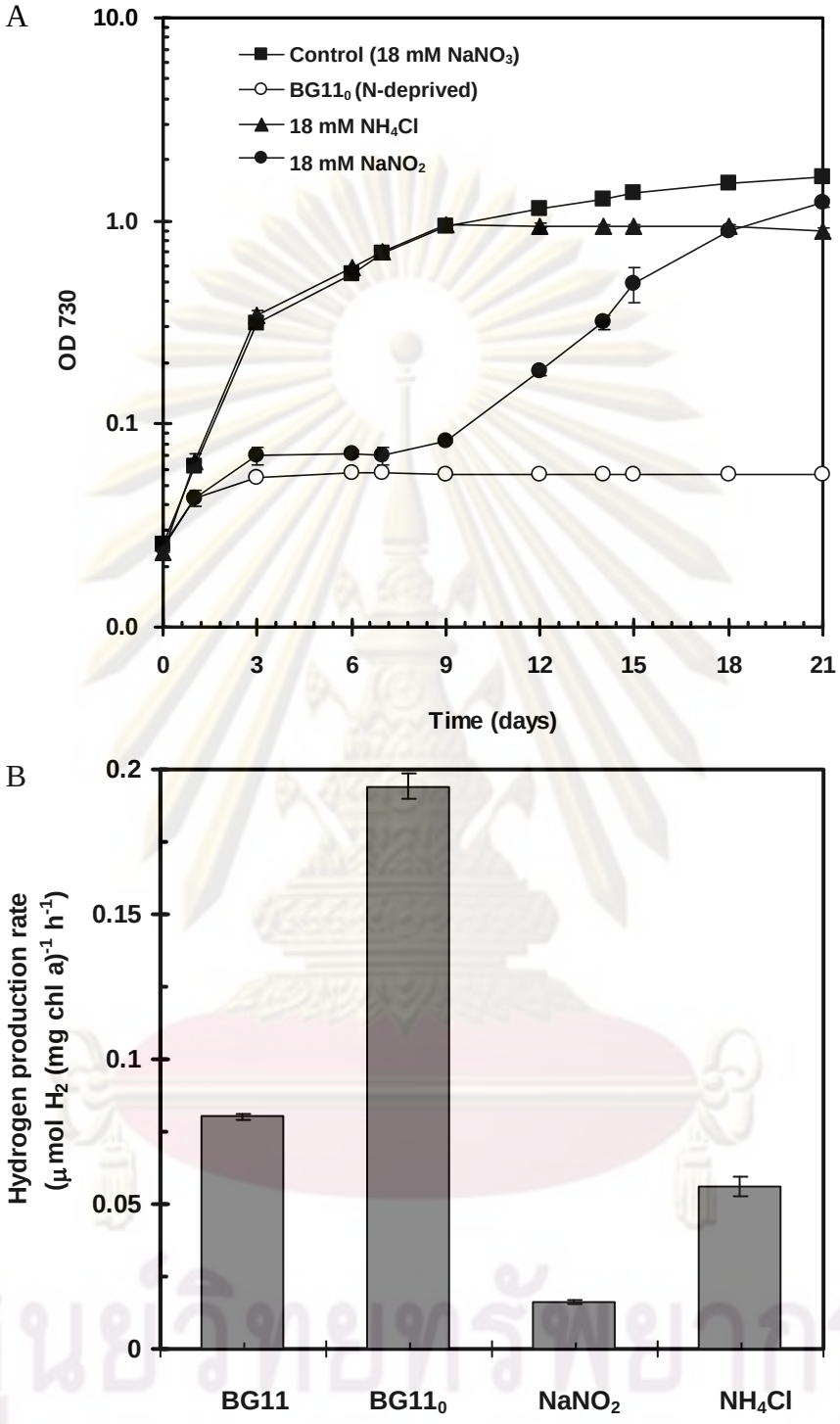

Figure 9 Growth of *Synechocystis* sp. PCC 6803 (A) and H_2 production (B) in $BG11_0$ and $BG11_0$ containing three different nitrogen sources, $NaNO_3$(control or BG11), NH_4Cl and $NaNO_2$.

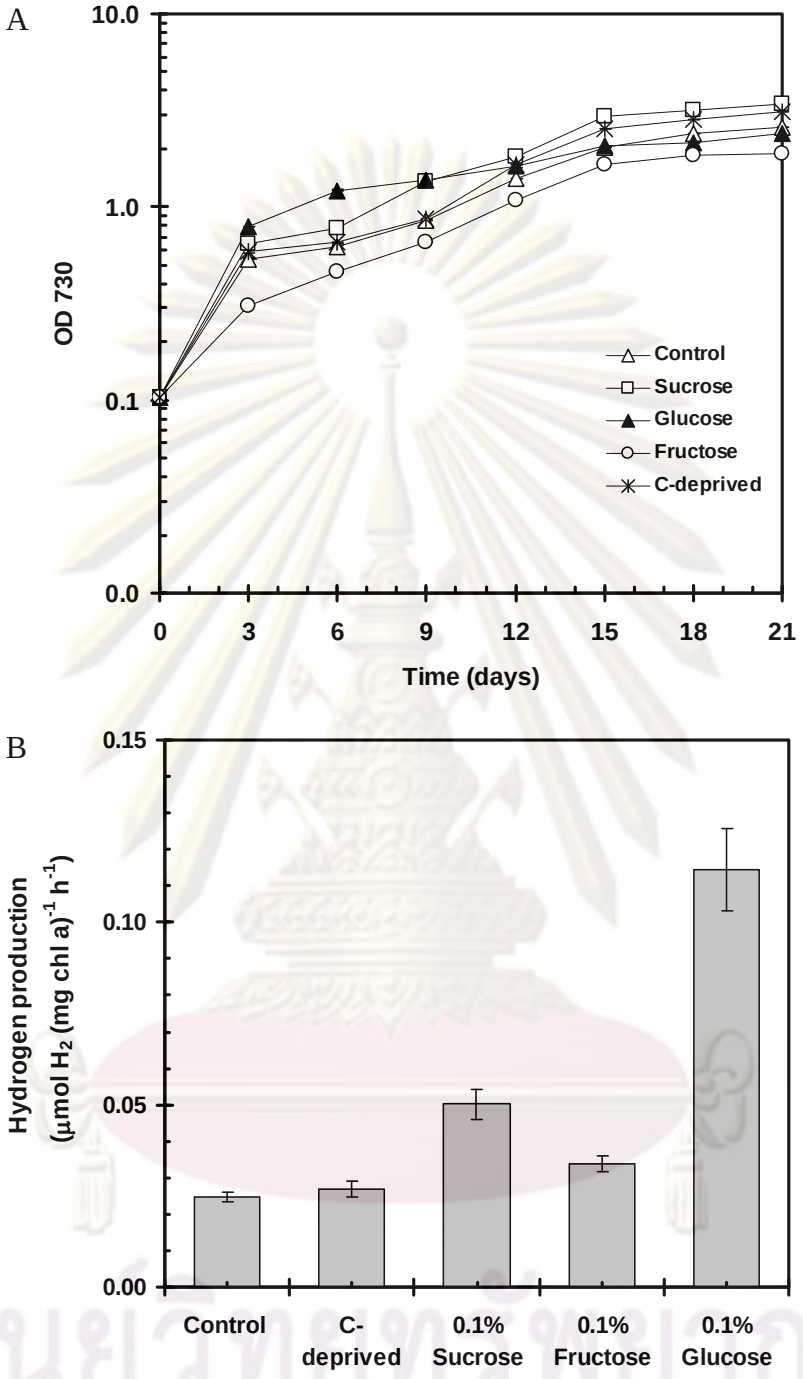

Figure 10 Growth of *Synechocystis* sp. PCC 6803 (A) and H_2 production (B) in BG11-C-deprived and BG11 containing three different sugars; sucrose, fructose and glucose at the same concentration 0.1%. Using BG11 medium containing Na_2CO_3 (carbon source) as a control.

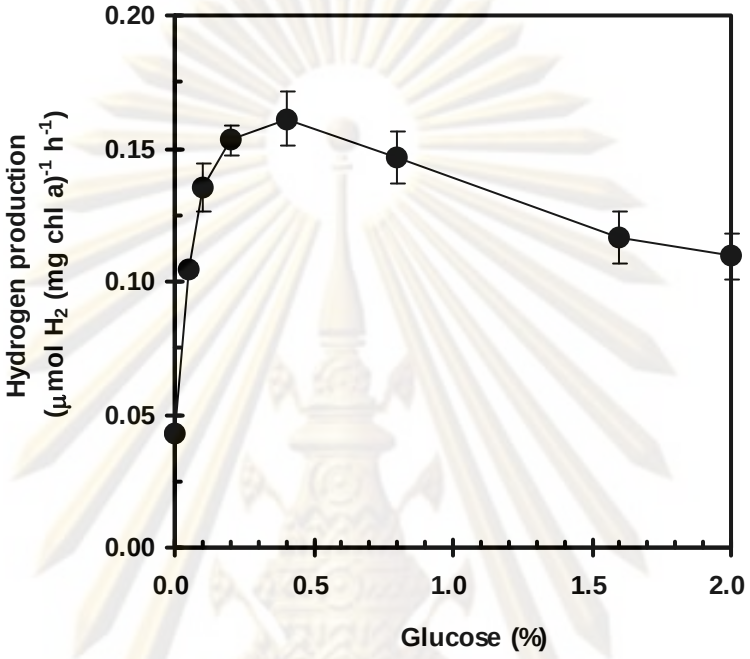

Figure 11 Effect of glucose concentration on H_2 production under dark and anaerobic conditions by *Synechocystis* sp. PCC 6803.

3.1.4 Effect of osmolality on growth and H_2 production

Synechocystis sp. PCC 6803 grown in BG11 medium at different external osmolalities generated by NaCl and sorbitol showed nodifference pattern of cell growth during a 21-days of culture (Figure 12A, B).

At one-week cells were adapted for another 24 h in BG11 medium at different osmolalities up to 100 mosmol kg^{-1} generated by NaCl or sorbitol. H_2 production was stimulated by low osmolality generated by either NaCl or sorbitol up to about 20 mosmol kg^{-1} (Figure 12C). A further increase in osmolality by NaCl resulted in a sharp decrease of H_2 production. This was not the case for sorbitol where only a slight decrease was observed at higher than 20 mosmol kg^{-1}.

3.1.5 Effect of light illumination

Effect of different light illumination on cell growth was determined. Cells were grown in BG11 medium under various light illuminations at 0, 1,000, 1,500, 1,700 and 2,600 Lux. The result showed that cells could not grow under dark condition. The culture under light illumination at 1,700 and 2,600 Lux reached stationary phase at day 12, whereas, the culture under light illumination at 1,000 and 1,500 Lux was still in late log phase (Figure 13A).

H_2 production was determined under dark and anaerobic conditions. Using cells culture in BG11 medium under various light illumination. Cells culture under light illumination at 1,500 and 1,700 Lux gave the highest H_2 production (Figure 13B).

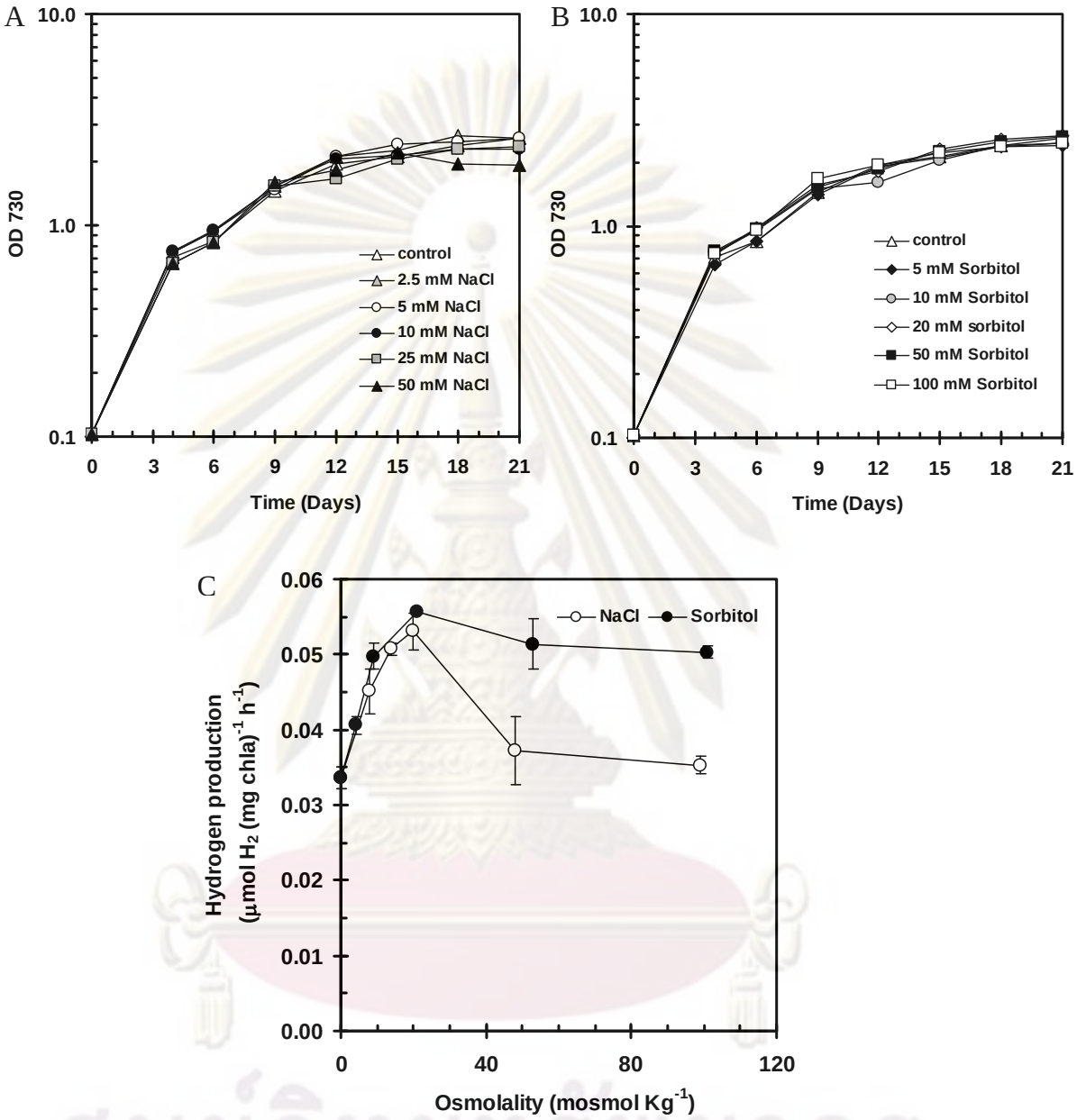

Figure 12 Growth of *Synechocystis* sp. PCC 6803 in BG11 medium under various external osmolality generated by NaCl (A) or sorbitol (B) and H_2 production (C).

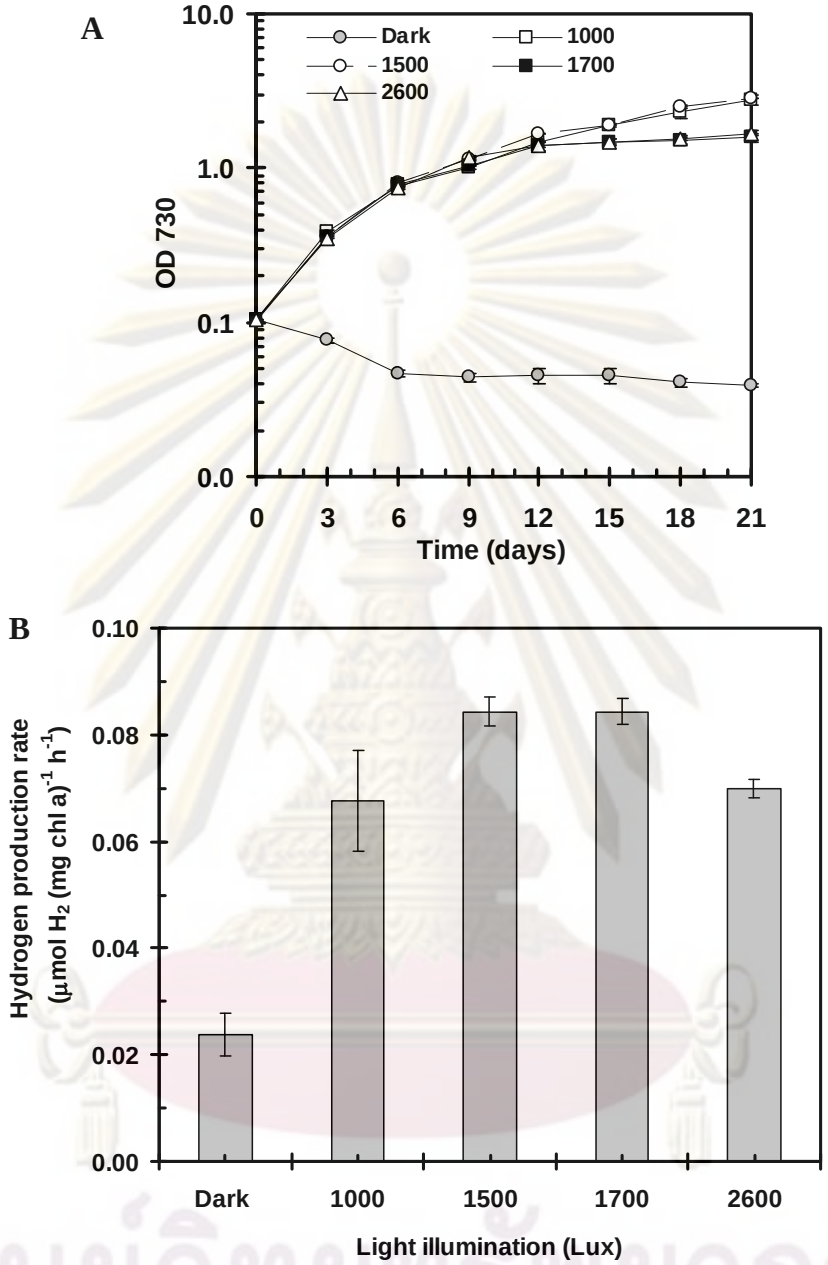

Figure 13 Growth of *Synechocystis* sp. PCC6803 (A) and H_2 production (B). Cells were grown in BG11 medium under various light illumination conditions.

3.1.6 Effect of nitrogen source and sulfur on H_2 production

The experiment was performed by cells were adapted in four different media, BG11, BG11-S-deprived, $BG11_0$ and $BG11_0$-S-deprived for 24 hour. The highest H_2 production found in cells were adapted in $BG11_0$-S-deprived following with cells were adapted in $BG11_0$, BG11-S-deprived and BG11 at H_2 production rate 0.505 ± 0.014, 0.305 ± 0.026, 0.235 ± 0.015, and 0.071 ±0.014, respectively (Figure 14)

3.1.7 Effect of reductants on H_2 production

Cells adapted in $BG11_0$ and $BG11_0$-S-deprived medium for 24 h showed higher H_2 production than cells adapted under BG11 and BG11-S-deprived medium (Figure 14). Therefore, $BG11_0$ and BG110-S-deprived were then chosen as the starting medium to test the effect of various reductants on H_2 production. A low concentration at 100 µM of both DTT and β-mercaptoethanol could slightly stimulate H_2 production with the latter having a slightly stronger effect (Figure 15). At higher than 100 µM, both reductants gave no further increase in the H_2 production. Cells adapted under S-limitation (S-deprived) in combination with β-mercaptoethanol showed an increased H_2 production with increased concentration of β-mercaptoethanol, with an approximately 4-fold increase of H_2 production at 750 µM β-mercaptoethanol. It is worth mentioning that the stimulation of H_2 production was not observed under conditions of S-deprivation in combination with DTT.

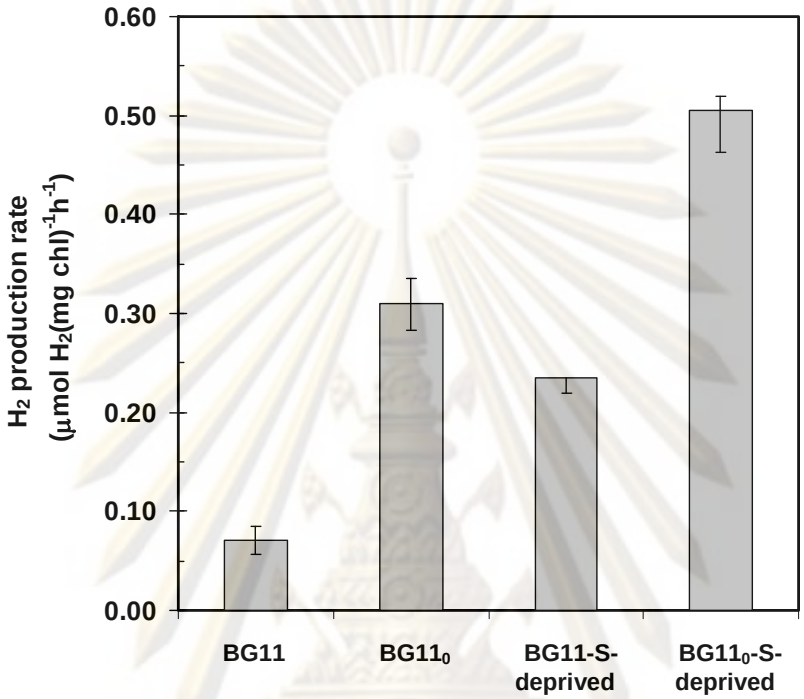

Figure 14 H_2 productions by *Synechocystis* sp. PCC 6803. Cells were adapted in BG11, BG11-S-deprived, $BG11_0$, and $BG11_0$-S-deprived before being measured H_2 production under dark and anaerobic conditions.

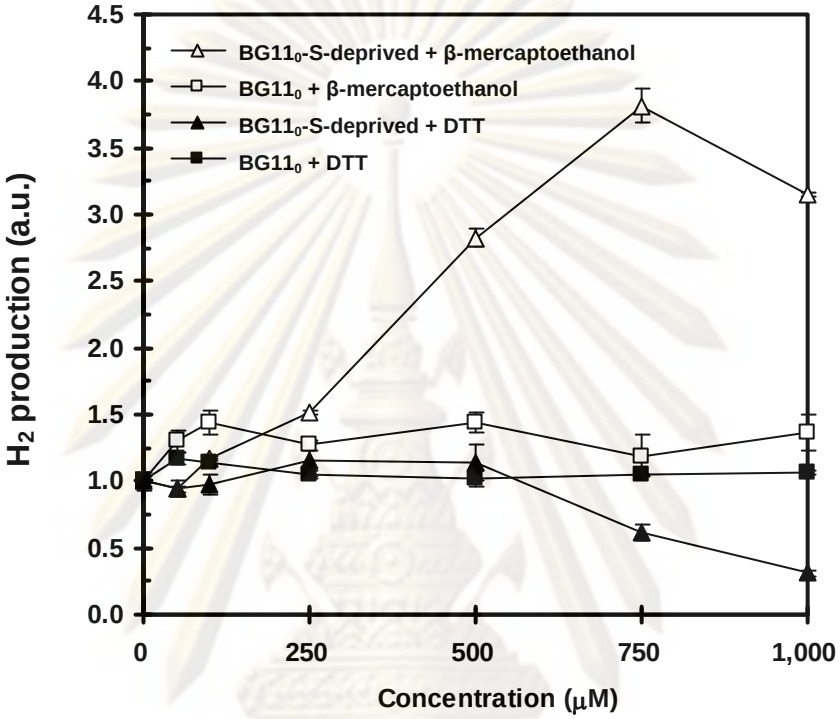

Figure 15 Effect of physiological reductants on H_2 production by cells of *Synechocystis* sp. strain PCC 6803. Cell suspensions in $BG11_0$ or $BG11_0$ S-deprived were treated with various reductants at indicated concentrations for 24 h before being analyzed for hydrogen production as described in Materials and methods. Means ± S.D. (n=3).

3.1.8 Effect of temperature on H_2 production

Cells were adapted for 24 hour in $BG11_0$ and $BG11_0$-S-deprived medium before being measured H_2 production under various temperatures from 30 up to 80 °C, dark and anaerobic conditions. The H_2 production rate increased when temperature increased from 30 °C to 40 °C and slightly decreased when temperature over than 40 °C (Figure 16).

3.2 Determined the effects on bidirectional hydrogenase (H_2ase) activity

3.2.1 Time course of H_2ase activity

The culture were adapted in BG11 and $BG11_0$ medium for 24 hour, cells were collected and measured H_2ase activity by the reaction of methyl viologen reduced by Na-dithionite as an e^- donor as described in materials and methods. Linearly increased of H_2ase activity was observed in first 15 minute of incubation time in cells culture were adapted in both BG11 and $BG11_0$ medium as shown in Figure 17. However, the culture were adapted in $BG11_0$ medium gave H_2ase activity twice times higher than the culture adapted in BG11 medium at 10 min. From these results the incubation 10 minute of incubation time was chosen to do next further an experiments.

3.2.2 Effect of aerobic and anaerobic conditions on H_2ase activity

O_2 is a by product of the photosynthetic PSII activity and strongly inhibits the bidirectional hydrogenase activity. Cells grown under anaerobic condition had higher hydrogenase activity than those grown under aerobic condition (Table 5). In addition, an increase of 1.23- and 1.45-fold was observed for cells grown in BG11 and $BG11_0$, respectively. Interestingly, presence of nitrogen in the growth medium influenced the hydrogenase activity, $BG11_0$ yielded higher activity than BG11.

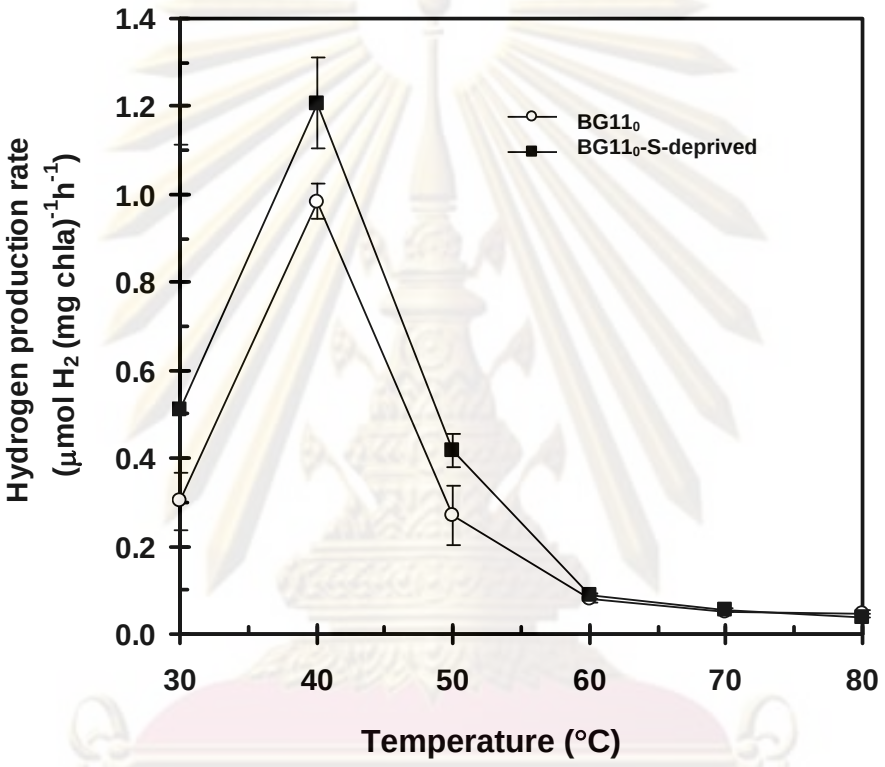

Figure 16 Effect of temperature on H_2 production by *Synechocystis* sp. PCC 6803. Cells were adapted for 24 hour in $BG11_0$ and $BG11_0$-S-deprived before being measured H_2 production.

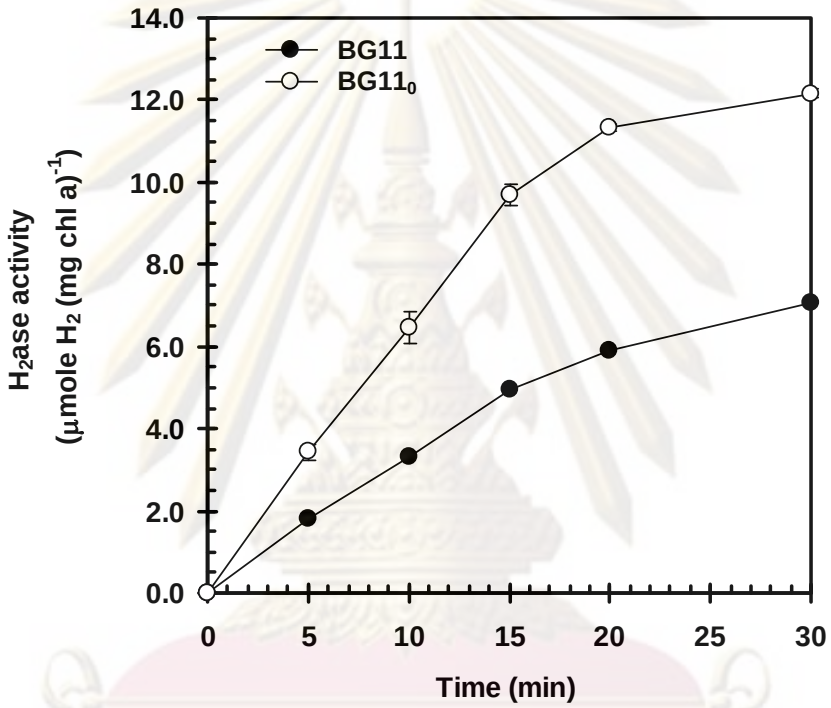

Figure 17 Time course of H_2ase activity by *Synechocystis* sp. PCC 6803. Cells were adapted in BG11 and $BG11_0$ medium for 24 hour before being measured H_2ase activity under anaerobic condition.

Table 5 Hydrogenase activity in intact cells of *Synechocystis* sp. strain PCC 6803 grown in different medium under aerobic and anaerobic conditions.

Growth	**Hydrogenase activity (μmol H_2 (mg chl *a*)$^{-1}$ min^{-1})**	
	Air (Aerobic)	**Argon (Anaerobic)**
BG11	$0.162^{a} \pm 0.004$	$0.199^{b} \pm 0.020$ (1.23×)
$BG11_0$	$0.233^{a} \pm 0.011$	$0.339^{b} \pm 0.043$ (1.45×)

Each sample was incubated at room temperature for 10 min before being analyzed for hydrogen production. Means ± S.D. (n=3); Number in bracket indicates increase under anaerobic compared to aerobic condition. a,b The data represents significance of 95% (*P-value* $\leq$ 0.05).

3.2.3 Effect of pH and temperature on H_2ase activity

Cells were cultivated in BG11 or $BG11_0$ medium 24 h before examining the hydrogenase activity under different pH ranging from 6.5-9.5 for 10 min. Increasing the pH from 6.5 to 7.5 resulted in an increased hydrogenase activity (Figure 18A). A pH higher than 7.5 resulted in a decrease of the activity. The optimal pH for bidirectional hydrogenase activity at room temperature was 7.5 for cells cultivated in both BG11 and $BG11_0$.

The bidirectional hydrogenase activity gradually increased with increasing temperature from 30 °C to 60 °C (Figure 18B). The highest activity occurred at 70 °C. Interestingly, high activity of H_2ase was observed even up to 90 °C. Similar results were observed for both cells cultivated in BG11 and $BG11_0$ medium, with the latter having higher activity.

3.2.4 Effect of β-mercaptoethanol on H_2ase activity

Our previous result (Figure 15) showed an increased H_2 production in the presence of β-mercaptoethanol and the highest H_2ase activity at 70 °C (Figure 18B). We therefore determined the effect of 750 µM β-mercaptoethanol in different growth media and measured the activity at 70 °C. The addition of 750 µM β-mercaptoethanol to BG11 or $BG11_0$ medium had no stimulatory effect on H_2ase activity (Table 6). Interestingly, the presence of β-mercaptoethanol in $BG11_0$-S-deprived medium resulted in about 3-fold higher H_2ase activity compared to that observed in BG11 medium. Figure 19 shows image of Scanning Electron Microscope of *Synechocystis* sp. PCC 6803. Cells adapted for 24 hour under presence and absence of 750 µM β-mercaptoethanol in BG11, $BG11_0$ and $BG11_0$-S-deprived medium. The result showed the same image of cell surface in all conditions.

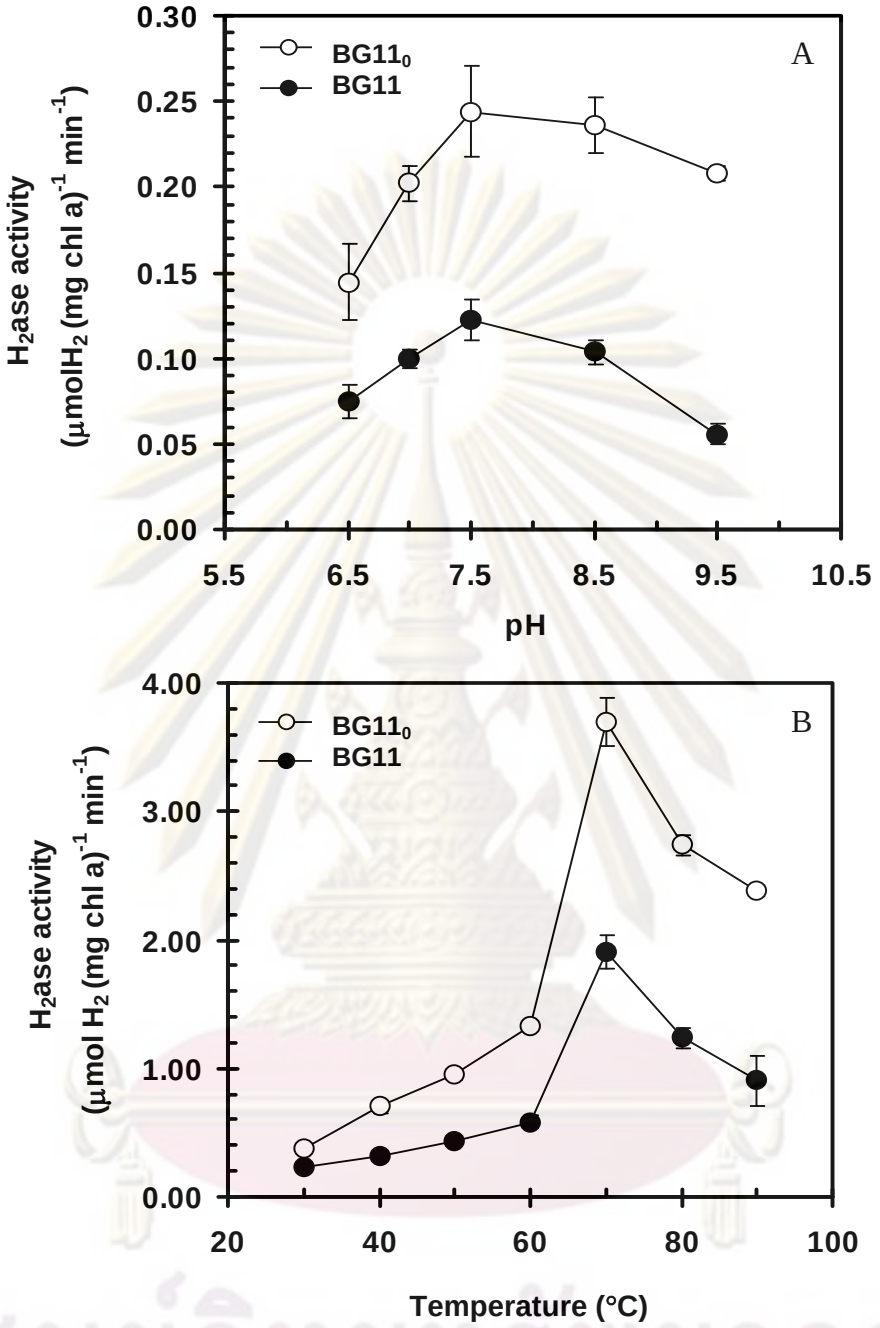

Figure 18 Effect of pH and temperature on hydrogenase activity in intact cells of *Synechocystis* sp. strain PCC 6803 grown in BG11 and $BG11_0$ (N-deprived). (A) Samples were incubated at various pHs and room temperature for 10 min before being analyzed for hydrogenase activity. (B) Samples were incubated at various temperatures and pH 7 for 10 min before being analyzed for hydrogenase activity. Means ± S.D. (n=3).

Table 6 Effect of β-mercaptoethanol on hydrogenase activity by intact cells of *Synechocystis* sp. strain PCC 6803 grown in BG11, $BG11_0$ and $BG11_0$-S deprived medium for 24 hour

Growth medium	**Hydrogenase activity ($\mu mol\ H_2 (mg\ chl\ a)^{-1}\ min^{-1}$)**
BG11	$5.56^a \pm 1.42$
BG11+750 mM β-mercaptoethanol	$3.71^a \pm 0.80$
$BG11_0$	$8.60^a \pm 0.61$
$BG11_0$+750 mM β-mercaptoethanol	$9.05^a \pm 1.12$
$BG11_0$-S deprived	$8.10^a \pm 1.84$
$BG11_0$-S deprived+750 mM β-mercaptoethanol	$14.32^a \pm 0.61$

Each sample was incubated at 70 °C for 10 min before being analyzed for hydrogen production. Means ± S.D. (n=3). a The data represents significance of 95% (*P-value* $\leq$ 0.05).

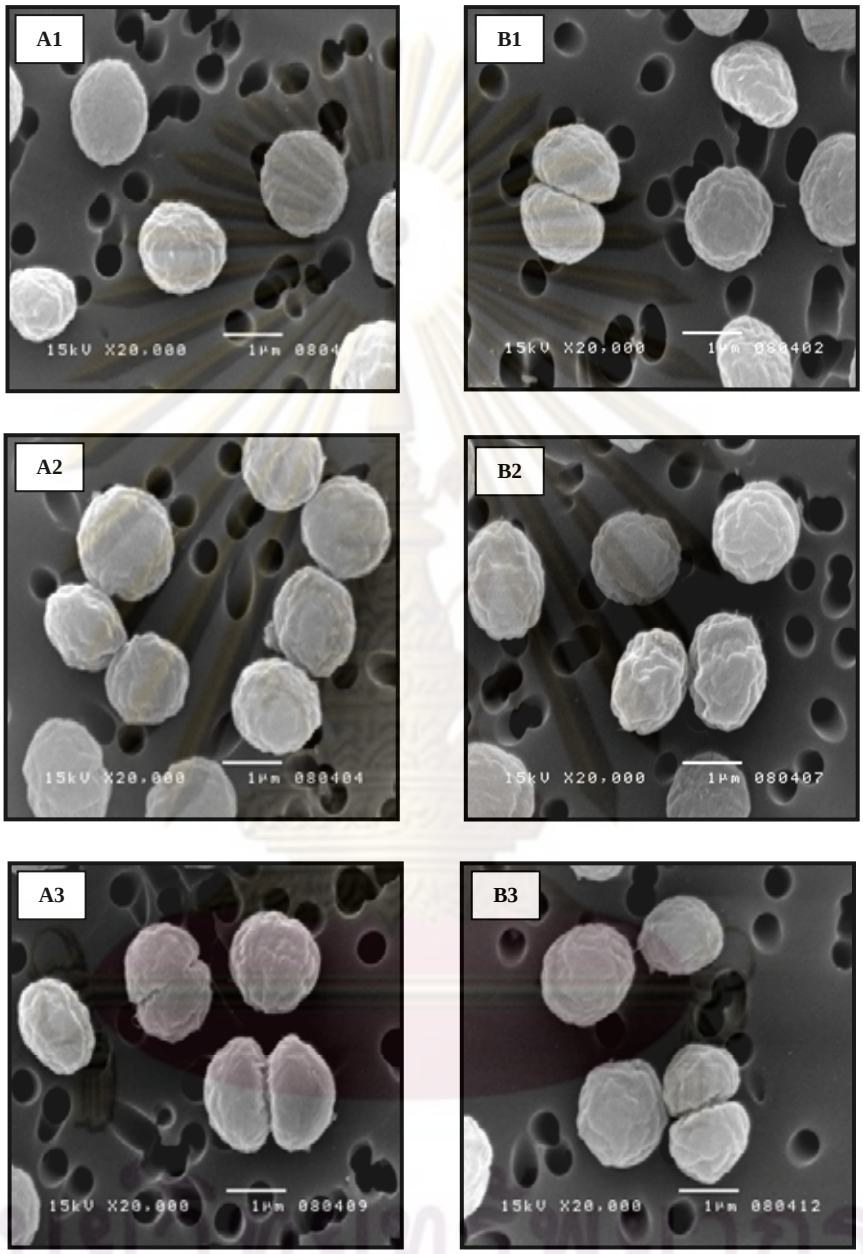

Figure 19 Image of Scanning Electron Microscope by *Synechocystis* sp. PCC 6803 cells for 24 hour under presence (A) and absence (B) of 750 µM β-mercaptoethanol in BG11 (A1, B1), $BG11_0$ (A2, B2) and $BG11_0$-S-deprived (A3, B3) medium.

3.2.5 Effect of reductants on H_2ase activity

The bidirectional hydrogenase activity was determined by measuring the evolution of H_2 using methyl viologen as the redox mediator reduced by sodium dithionite. Under this condition, cells grown in BG11 medium yielded a high activity of 0.45 μmol H_2 (mg chl *a*)$^{-1}$ min^{-1} (Table 7). When replacing dithionite with either DTT or β-mercaptoethanol, no hydrogenase activity could be detected. Low enzyme activity was observed with NADH and ferredoxin either alone or in combination. Under control condition with addition of ferredoxin, a slight stimulation of hydrogenase activity was observed, 0.57 versus 0.45 μmol H_2 (mg chl *a*)$^{-1}$ min^{-1}. Similar results were obtained for cells growing in $BG11_0$ medium. However, in all conditions cells grown in $BG11_0$ medium gave higher activity than those grown in BG11 medium.

3.2.6 Effect of light illumination on H_2ase activity

Different light illumination from 300 to 4,500 Lux were tested for the effect on H_2ase activity by cells adapted in both BG11 and $BG11_0$ medium. The results in Figure 20 showed H_2ase activities increased following the increase of light illumination. The highest H_2ase activity was observed at 2,100 Lux of light illumination. Over light illuminated 2,100 Lux reduced H_2ase activity (only $BG11_0$).

Table 7 Effect of physiological reductants on hydrogenase activity by intact cells of *Synechocystis* sp. strain PCC 6803

Physiological reductant	**Hydrogenase activity (µmol H_2(mg chl a)$^{-1}$ min^{-1})**	
	BG11 medium	**$BG11_0$ medium**
Controls (No addition, cells suspension only)	ND	ND
+ 5mM MV	ND	ND
+ 20 mM Na-dithionite	ND	ND
+ 5mM MV & 20 mM Na-dithionite	$0.45^a \pm 0.04$	$0.78^b \pm 0.03$
+ 5 mM MV& 20 mM DTT	ND	ND
+ 5 mM MV& 20 mM β-mercaptoethanol	ND	ND
+ 5 mM NADH	$0.04^a \pm 0.01$	$0.12^b \pm 0.01$
+ 0.2 µM ferredoxin	0.03 ± 0.01	0.05 ± 0.01
+ 0.2 µM ferredoxin & 5 mM NADH	$0.09^a \pm 0.01$	$0.15^b \pm 0.01$
+ 0.2 µM ferredoxin & 5 mM NADH & 20 mM Na-dithionite	$0.57^a \pm 0.02$	$1.12^b \pm 0.09$

Each sample was incubated at room temperature for 10 min before being analyzed for hydrogen production. Means ± S.D. (n=3). ND= Not detectable. a, b The data represents significance of 95% (*P-value* ≤ 0.05)

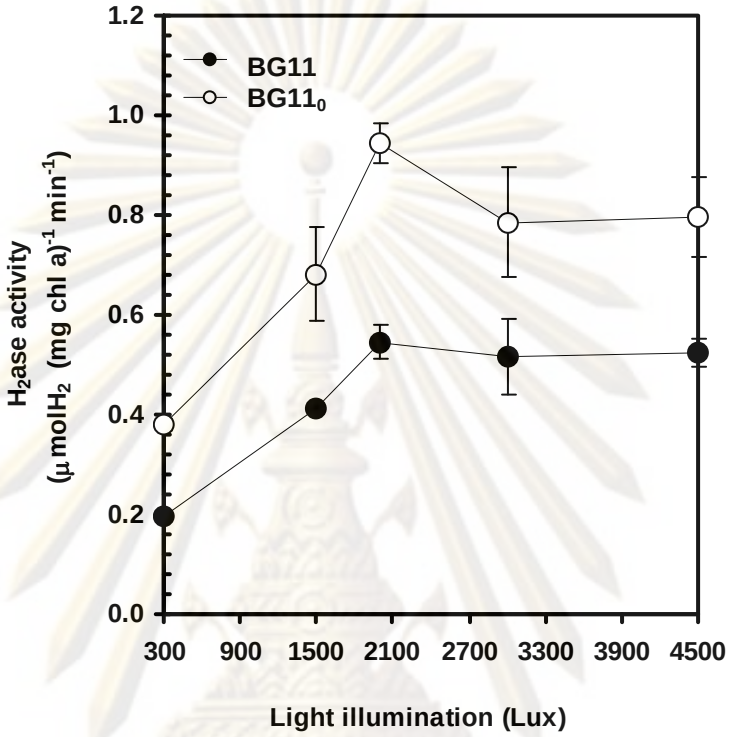

Figure 20 The effect of light illuminationon H_2ase activity. Cells culture were adapted in BG11 and $BG11_0$ medium for 24 hour before being analyzed for H_2ase activity under anaerobic condition.

3.3 Study of nitrate uptake in *Synechocystis* sp. PCC 6803

3.3.1 Effect of osmotic upshifts on nitrate uptake

The effect of osmolality generated by NaCl and sorbitol on nitrate uptake was extensively examined. The uptake was sensitive to osmotic upshifts (Figure 21). The highest nitrate uptake occurred at 30 mosmol kg^{-1} sorbitol with an approximately 3.5-fold increase of nitrate uptake and at 40 mosmol kg^{-1} NaCl with an approximately 4.5-fold increase of nitrate uptake. However, when increased external osmolalities to above 30 and 40 mosmol kg^{-1} by sorbitol and NaCl respectively, a progressive reduction of nitrate uptake was observed.

The stimulation of nitrate uptake by Na^+ as shown in Figure 21 is further supported by results shown in Figure 22. At a fixed osmolality of 120 mosmol kg^{-1} generated by sorbitol, an increase in NaCl concentration resulted in a progressive increase of nitrate uptake with an approximately 3-fold increase observed at 10 mM NaCl. The stimulation of nitrate uptake was slightly decreased at higher than 10 mM NaCl.

3.3.2 Kinetics of nitrate uptake

Nitrate uptake in *Synechocystis* sp. strain PCC 6803 as a function of external nitrate concentrations ranging from 0-400 μM is shown in Figure 23. The nitrate uptake rates increased with increasing concentrations of nitrate for both unstress and moderate salt-stress conditions. The uptake rates increased with applying 20 mM external NaCl. Lineweaver-Burk transformations of the data for both unstress and moderate salt-stress conditions showed K_s values of 46 and 79 μM with maximal velocities (V_{max}) of 1.37 and 2.45 μmol (mg Chl a^{-1}) min^{-1}, respectively (Figure 23).

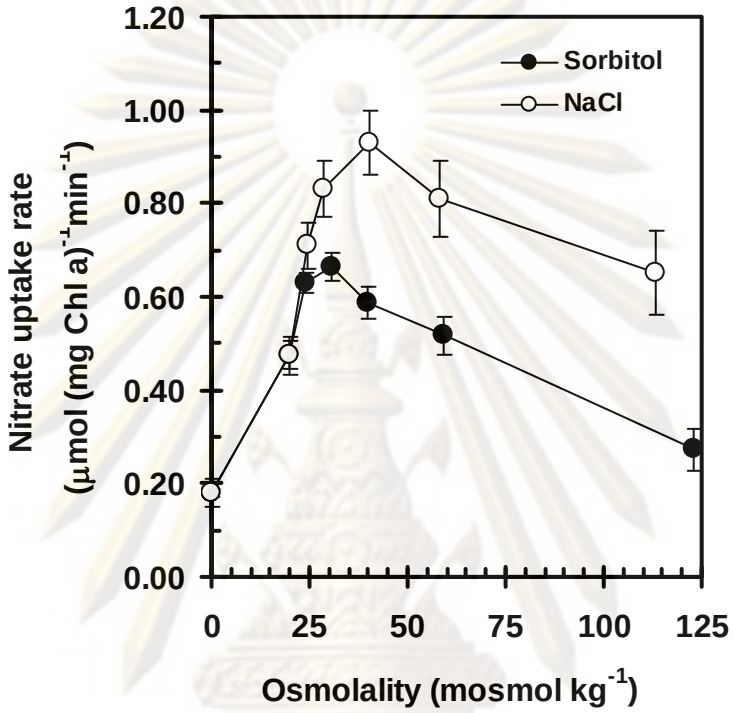

Figure 21 Effect of external osmolality on nitrate uptake (A). Initial uptake rate was determined in the presence of increasing osmolality generated by NaCl (○) and sorbitol (●).

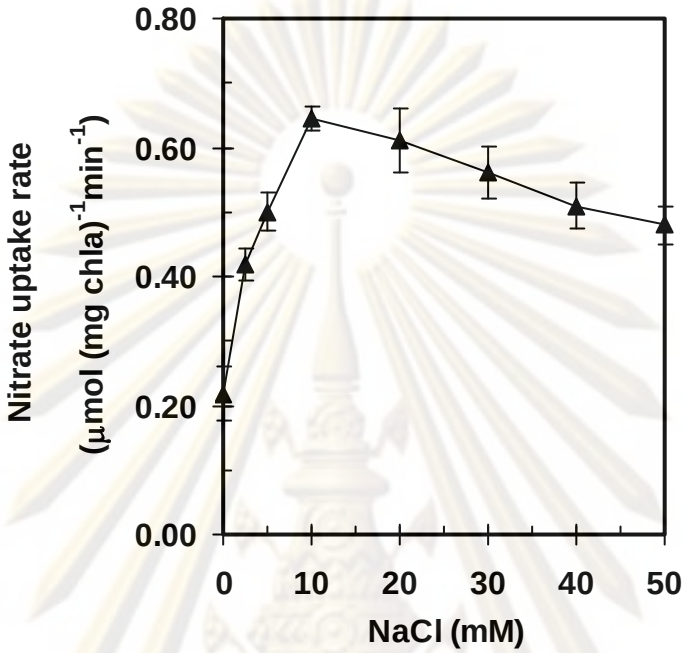

Figure 22 Stimulatory effect of external NaCl on nitrate uptake. An initial uptake rate was determined in the presence of initially fixed osmolaltiy at 120 mosmol kg^{-1} generated by sorbitol. The assay was carried out using 50 μM KNO_3 and without 10 mM $NaHCO_3$.

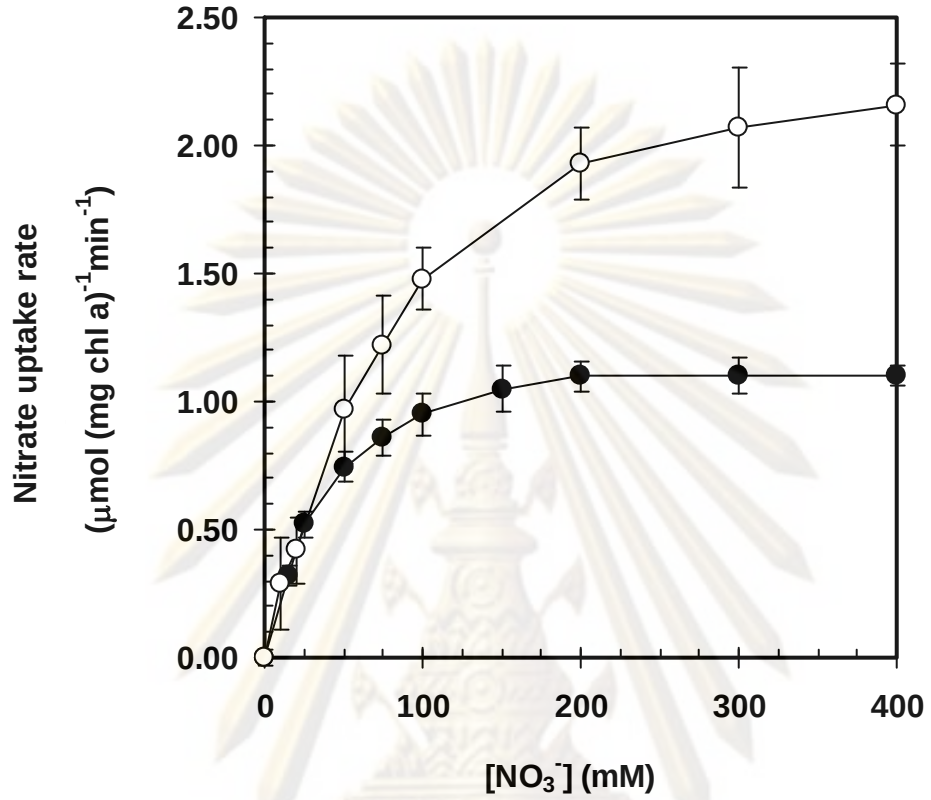

Figure 23 Initial rates of nitrate uptake as a function of nitrate concentrations. The assay of nitrate uptake was done with (○) or without (●) externally added 20 mM NaCl. The data shown are means from three independent experiments with vertical bars representing standard errors of the means.

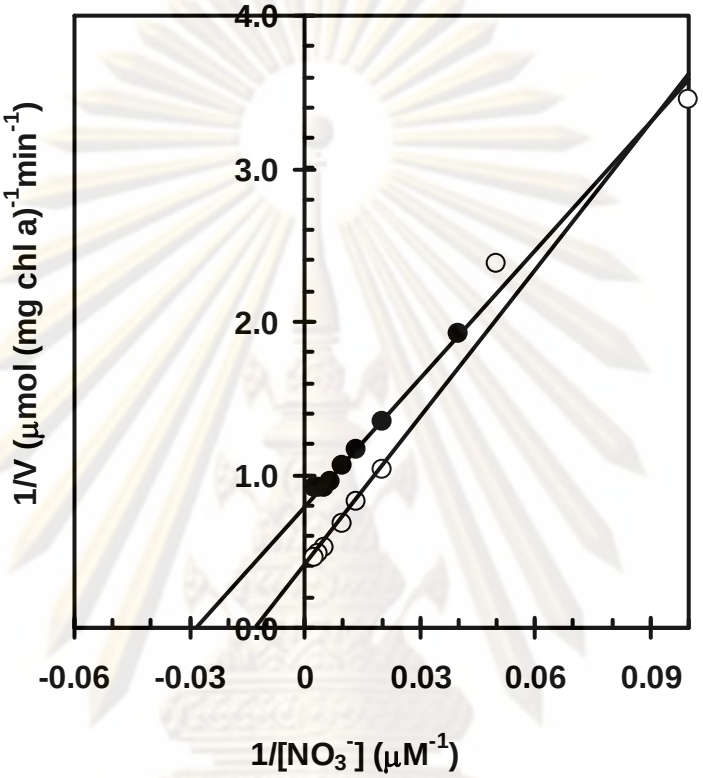

Figure 24 Lineweaver-Burk plots of the kinetics of nitrate uptake. The assay of nitrate uptake was done with (○) or without (●) externally added 20 mM NaCl. The data shown are means from three independent experiments.

3.3.4 Inhibition of nitrate uptake by NH_4Cl and CO_2 fixation inhibitor

The presence of NH_4Cl in the assay medium resulted in a decline of the nitrate uptake (Figure 25). Ammonium was slightly more inhibitory to nitrate uptake under moderate salt- stress condition than under unstress condition, i.e., at 100 mM NH_4Cl about 50% of the nitrate uptake was inhibited under the moderate salt-stress condition, whereas 30% was inhibited under unstress condition. At higher concentration of external ammonium ions, the nitrate uptake constantly declined under both conditions.

Figure 26 shows that DL-glyceraldehyde, an inhibitor of CO_2 fixation, inhibited nitrate uptake in *Synechocystis* sp. strain PCC 6803 with cells under salt-stress condition being more sensitive than cells under unstress condition, i.e., about 50% inhibition of nitrate uptake occurred at 50 and 100 mM DL-glyceraldehyde for salt-stress and unstress conditions, respectively. A concentration of DL-glyceraldehyde higher than 100 mM slightly increased the inhibition of nitrate uptake for both conditions.

3.3.5 Effect of metabolic inhibitors on nitrate uptake

The results in Table 8 show that the nitrate uptake was strongly inhibited in dark-incubated cells. The uptake was sensitive to *N, N'*-dicyclohexylcarbodiimide (DCCD), a H^+ gradient ATPase inhibitor and orthovanadate, an inhibitor of P-type ATPase. The protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), which dissipates both the chemical hydrogen ion concentration gradient (ΔpH) and the proton electrochemical gradient, markedly reduced the nitrate uptake. Similarly, a transport uncoupler, dinitrophenol (DNP) and nigericin, an ionophore that collapses ΔpH could significantly inhibit nitrate uptake. Valinomycin, an ionophore which permeabilises cells membrane to

potassium ions and has been proposed to affect the membrane electrical potential ($\Delta\psi$), strongly inhibited the nitrate uptake. Gramicidin D and monensin which are K^+ and Na^+ ionophores respectively, also inhibited the nitrate uptake.

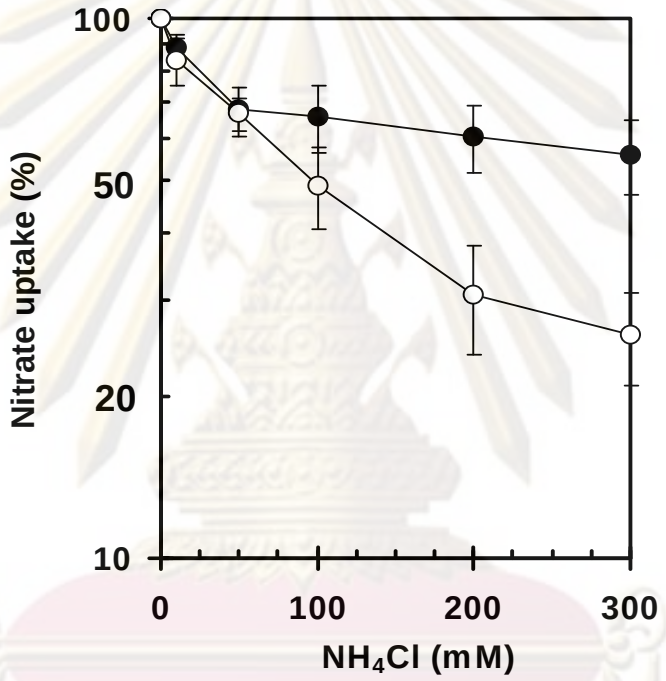

Figure 25 Effect of NH_4Cl on nitrate uptake assayed with (○) or without (●) externally added 20 mM NaCl. NH_4Cl was simultaneously added 30 min before the addition of 50 μM $NaNO_3$ to initiate the uptake. The data shown are means from three independent experiments with vertical bars representing standard errors of the means.

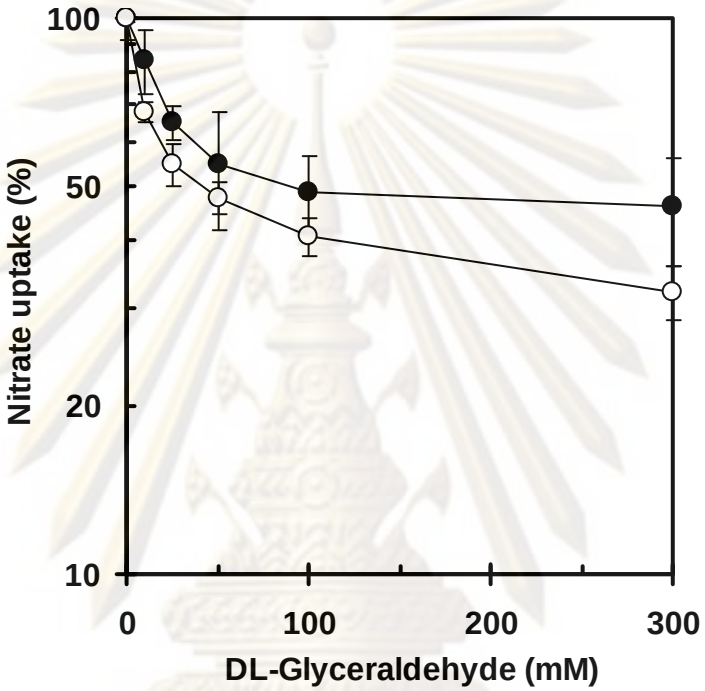

Figure 26 Effect of DL-glyceraldehyde on nitrate uptake assayed with (○) or without (●) externally added 20 mM NaCl. The reaction mixture without nitrate was preincubated with DL-glyceraldehyde 30 min before the addition of 50 μM $NaNO_3$ to initiate the uptake. The data shown are means from three independent experiments with vertical bars representing standard errors of the means.

Table 8 Effect of metabolic inhibitors on nitrate uptake in *Synechocystis* sp. strain PCC 6803. Cells were pre-incubated with inhibitors in the dark for 30 min before the addition of 50 µM $NaNO_3$ to initiate the uptake. Potassium phosphate buffer was used when testing the effect of valinomycin. Uptake rate of control = 0.44 µmol (mg Chl *a*)$^{-1}$ min^{-1}.

Inhibitor	**Concentration**	**Nitrate uptake (%)**
Control		100 ± 2
Control (in dark)		30 ± 3
DCCD	20 µM	68 ± 4
	40 µM	41 ± 2
Orthovanadate	1 mM	47 ± 1
	2 mM	39 ± 1
CCCP	20 µM	72 ± 1
	40 µM	43 ± 3
DNP	1 mM	82 ± 2
	2 mM	46 ± 2
Nigericin	40 uM	54 ± 1
	100 uM	50 ± 3
Valinomycin	20 µM	26 ± 2
	40 µM	5 ± 3
Gramicidin D	50 µM	17 ± 5
	100 µM	2 ± 3
Monensin	20 µM	61 ± 2
	40 µM	40 ± 1

3.4 Cloning and Characterization of recombinant plasmid

3.4.1 Amplification of the *narB* and *nirA* genes from *Synechocystis* chromosomal DNA

The chromosomal DNA was extracted and purified from the *Synechocystis* sp. PCC 6803 cells as described in materials and methods 2.2.6.1. The PCRs were performed using chromosomal DNA as the template and DNA polymerase (taq polymerase, Promega). The initial denaturation at 95^{o}C for 5 min was done, followed by 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 60 ^{o}C for 1 min and extension at 72 ^{o}C for 2 min, followed by final extension at 72 ^{o}C for 5 min. The intensities of chromosomal DNA and PCR products were analyzed by 0.8% agarose gel in 0.5x TBE buffer for electrophoresis and autoradiography as shown in Figure 27

3.4.2 Construction of a pGEM-T easy recombinant plasmid containing *narB* and *nirA* genes

The recombinant plasmid containing the *narB* and *nirA* genes was organized. Firstly, PCR product of *narB* and *nirA* genes from the previous step was purified using NucleoSpin® Extract II. The fragments were ligated together with commercial cloning plasmid pGEM-T easy vector. The ligation reactions were transformed into the *E. coli* DH5α cells. The recombinant clone was selected on LB agar plate containing ampicilin supplemented with X-Gal and IPTG.

The white colonies were randomly selected and cultured in LB broth media containing ampicillin overnight. The cultures were subjected to plasmid extraction and digestion with *Eco*RI restriction enzyme to prove the correction of recombinant plasmid. Subsequently, these reactions were analyzed by 0.8% agarose gel electrophoresis. The

result showed that the recombinant plasmid namely pWBB1.3 and pWBA1.4 were pGEM-T Easy vector contained a *narB* gene of an approximate size of 2.4 kb (Figure 28) and *nirA* gene of an approximate size of 1.6 kb (0.94 kb + 0.66 kb), (Figure 29) respectively.

3.4.3 Construction of knockout *narB* and *nirA* genes recombinant plasmid

The recombinant plasmid pWBB1.3 (pGEM-T Easy vector containing *narB* gene) and plasmid pWBA1.4 (pGEM-T Easy vector containing *nirA* gene) were interrupted with kanamycin cassette and chloramphenicol cassette, resulting in plasmids pMnarB19 and pMnirA3, respectively.

Figure 30A shows the result of plasmid pMnarB19 cut with restriction enzyme *Kpn*I and determined with 0.8% agarose gel electrophoresis showing in two bands of pGEM-T Easy vector + *narB* gene (5.4 kb) and kanamycin cassette (1.4 kb). The constructed plasmid pMnarB19 (6.8 kb) was confirmed by sequencing and the plasmid map is shown in Figure 30B.

The pMnirA3 was cut with restriction enzyme *Hinc*II, the result of 0.8% agarose gel electrophoresis showed two bands of 3.9 kb and 2.8 kb (Figure 31A). The sequence of constructed plasmid was confirmed by sequencing and the map of pMnirA3 is shown in Figure 31B.

The inactivation mutant strains nitrate reductase (Δ*narB*), nitrite reductase (Δ*nirA*) and double mutant strain nitrate and nitrite reductase (Δ*narB*:Δ*nirA*) in *Synechocystis* sp. PCC 6803 were constructed by natural transformation.

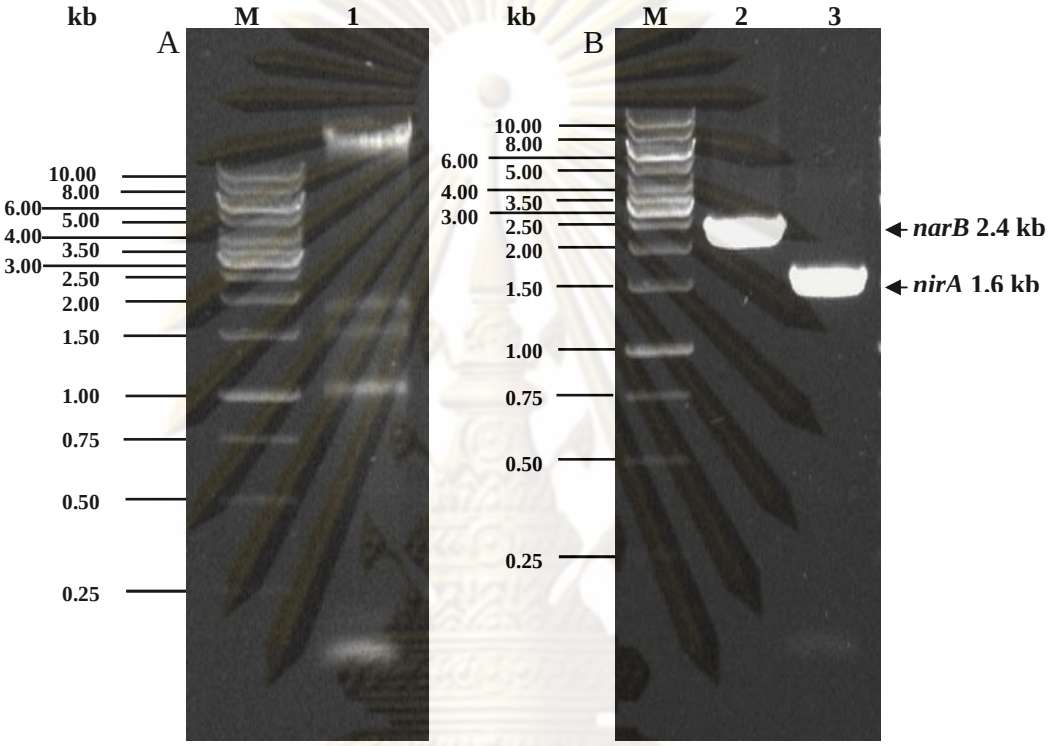

Figure 27 Chromosomal DNA of *Synechocystis* sp. PCC6803 (A) and PCR product of *narB* and *nirA* genes (B) were electrophoresed on 0.8% agarose.

Lane M: 1Kb Ladder

Lane 1: Genomic DNA of *Synechocystis* sp. PCC 6803

Lane 2: PCR product of *narB* gene

Lane 3: PCR product of *nirA* gene

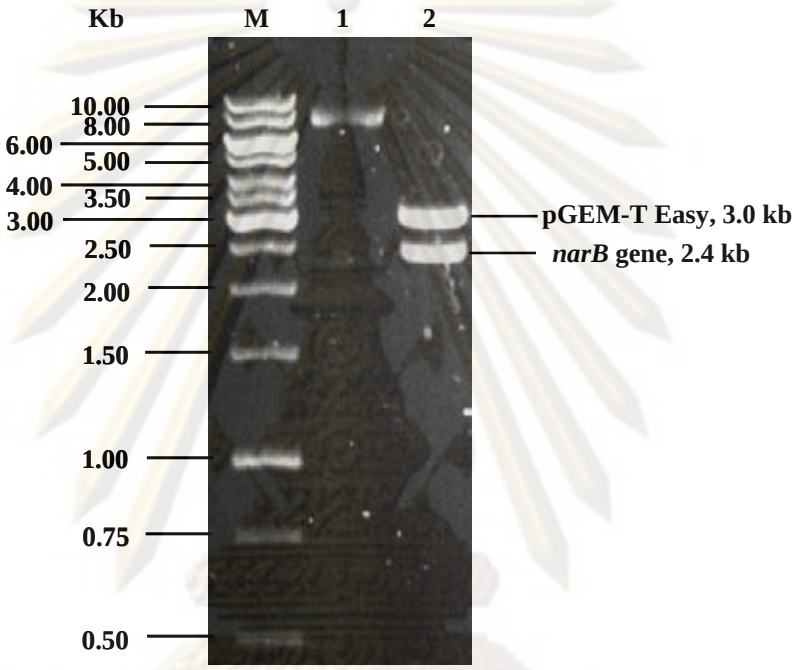

Figure 28 0.8% agarose gel electrophoresis of plasmid pWBB1.3 cut with restriction enzyme *Eco*RI

Lane M1: 1 Kb ladder

Lane 1: pWBB1.3

Lane 2: pWBB1.3 cut with *Eco*RI

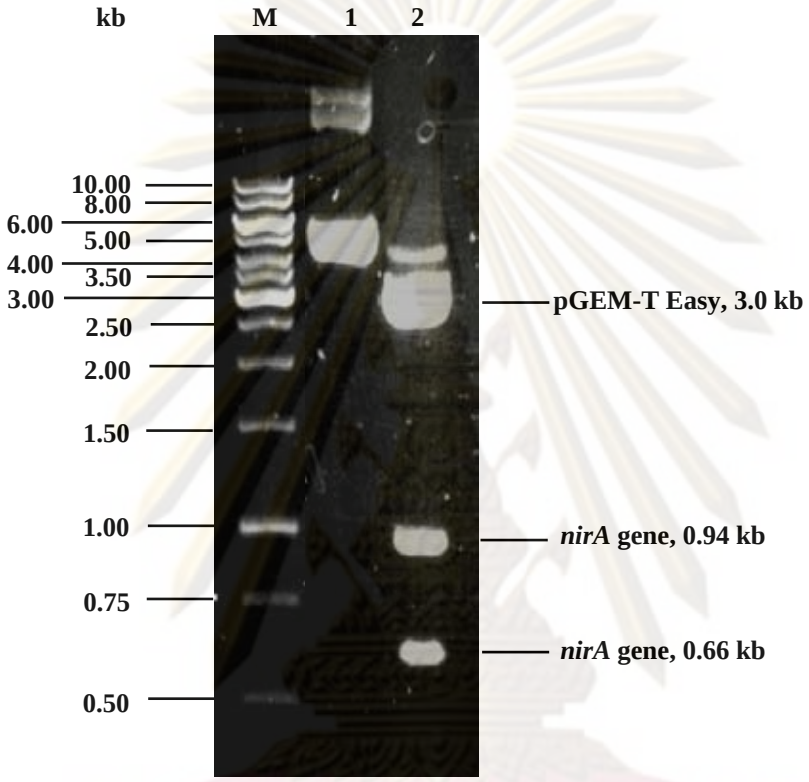

Figure 29 0.8% agarose gel electrophoresis of plasmid pWBA1.4 cut with restriction enzyme *Eco*RI

Lane M1: 1 Kb ladder

Lane 1: pWBB1.4

Lane 2: pWBB1.4 cut with *Eco*RI

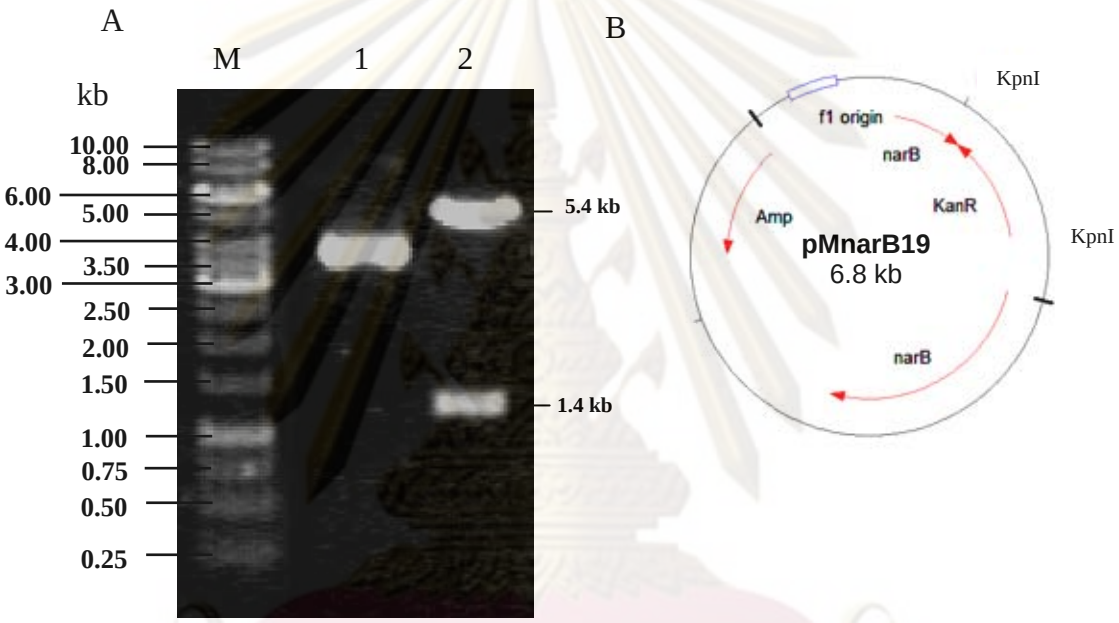

Figure 30 0.8% agarose gel electrophoresis of plasmid pMnarB19 cut with restriction enzyme *Kpn*I (A) and map of pMnarB19 (B).

Lane M1: 1 Kb ladder

Lane 1: pMnarB19

Lane 2: pMnarB19 cut with *Kpn*I

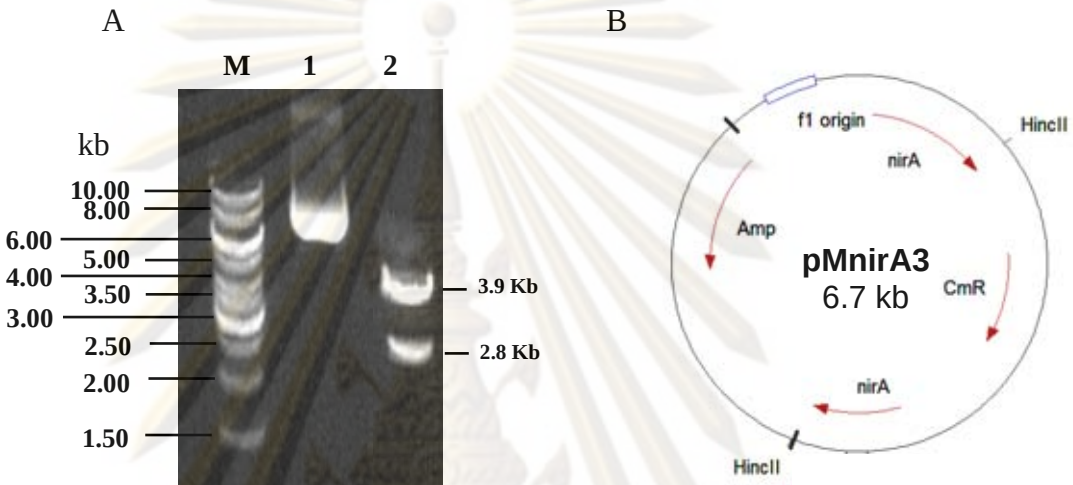

Figure 31 0.8% agarose gel electrophoresis of plasmid pMnirA3 cut with restriction enzyme *Hinc*II (A) and map of plasmid pMnirA3 (B)

Lane M1: 1 Kb ladder

Lane 1: pMnirA3

Lane 2: pMnirA3 cut with *Hinc*II

3.4.4 Construction of Δ*narB*, Δ*nirA* and Δ*narB*:Δ*nirA* in *Synechocystis* sp. PCC 6803 mutant strains

The plasmid pMnarB19 and pMnirA3 were transformed into *Synechocystis* cells. Mutant strains Δ*narB*, Δ*nirA* and Δ*narB*:Δ*nirA* were grown in $BG11_0$ medium using 3.75 mM $(NH_4)_2SO_4$ as nitrogen source and supplemented with 30 μg ml^{-1} of kanamycin and 30 μg ml^{-1} of chloramphenicol for Δ*narB* and Δ*nirA*, respectively. For double mutant strain Δ*narB*:Δ*nirA* both kanamycin and chloramphenicol were supplemented in the medium. To ensure the complete segregation of obtained gene knockouts, colony PCR was performed by using primers pair of *narB*, *nirA*, Kn^R and Cm^R gene (Table2, 3). PCR products were analysed by 0.8% agarose gel electrophoresis as shown in Figure 32. The PCR result shows that the DNA bands of *narB* and *nirA* genes in Δ*narB*, Δ*nirA* and Δ*narB*:Δ*nirA* mutant strains were upshifted (gene + antibiotic cassette) comparing with wild type strain.

3.4.5 Comparison of H_2 production in wide type, Δ*narB*, Δ*nirA* and Δ*narB:*Δ*nirA* mutants strain

Synechocystis sp. PCC 6803 wild type and mutants strain, Δ*narB*, Δ*nirA* and Δ*narB:*Δ*nirA* were grown in $BG11_0$ medium containing 3.75 mM $(NH_4)_2SO_4$ as a nitrogen source for 7 days. The culture at logarithmic phase (OD_{730}~0.7) were collected and adapted in new BG11 medium containing 18 mM $NaNO_3$ as nitrogen source for 24 hour before being measured for H_2 production. Table 9 shows the result of H_2 production in wild type compared with mutant strains. The H_2 production in Δ*narB*, Δ*nirA* and Δ*narB:*Δ*nirA* mutant strains were higher than that in wild type with an approximately increase of 6, 12 and 20 fold, respectively.

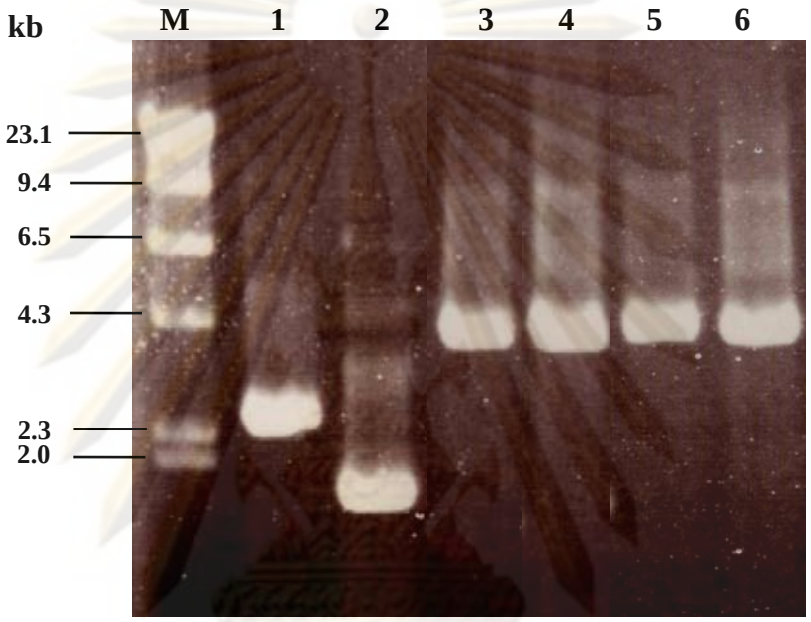

Figure 32 0.8% agarose gel electrophoresis of PCR product, for checking complete segregation in Δ*narB*, Δ*nirA* and Δ*narB:*Δ*nirA* mutant strain

Lane M: Lambda DNA/*Hind*III marker

Lane 1: PCR product of *narB* gene in wild type strain (2.4 kb)

Lane 2: PCR product of *nirA* gene in wild type strain (1.6 kb)

Lane 3: PCR product of *narB* gene in Δ*narB* strain (3.8 kb)

Lane 4: PCR product of *nirA* gene in Δ*nirA* strain (3.7 kb)

Lane 5: PCR product of *narB* gene in double mutant Δ*narB:*Δ*nirA* strain (3.8 kb)

Lane 6: PCR product of *nirA* gene in double mutant Δ*narB:*Δ*nirA* strain (3.7 kb)

Table 9 The H_2 production in *Synechocystis* sp. PCC 6803 wild type and Δ*narB*, Δ*nirA* and Δ*narB:*Δ*nirA* mutant strain

***Synechocystis* sp. PCC 6803 strain**	**H_2 production ($\mu mol\ H_2\ (mg\ chl\ a)^{-1}\ h^{-1}$)**
Wild type	$0.015^a \pm 0.01$
ΔnarB	$0.086^a \pm 0.01$
ΔnirA	$0.174^a \pm 0.03$
ΔnarB:ΔnirA	$0.300^a \pm 0.02$

Each sample was incubated at room temperature for 4 hour under dark and anaerobic conditions before being analyzed for hydrogen production. Means ± S.D. (n=5). a The data represents significance of 95% (*P-value* ≤ 0.05).

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CHAPTER IV

DISCUSSION

In photosynthetic bacteria H_2 is mainly produced through the nitrogenase enzyme in the cells and induction of nitrogenase activity is affected by nitrogen source (Takahashi, 1984). Photosynthetic bacteria *Rhodobacter sphaeroides* produced large amount of H_2 when cells used glutamate-Na as a nitrogen source (Sasaki, 1997).

The unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803, cultivated in BG11 medium for three weeks, showed a pattern of no lag-phase, a short log-phase in first three days of cultivation followed by a stationary-phase. The growth pattern in first three days of cultivation is similar to what has been reported in the marine unicellular cyanobacterium *Synechococcus* sp. strain Miami BG 043511. The pattern of H_2 production in *Synechocystis* PCC 6803 showed the highest H_2 production during the first growth stage compared with the second and third growth stage (Figure 7). 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. Another explanation is *Synechocystis* sp. PCC 6803 in the early stage of cultivation have highest photosynthesis activity and the lowest respiration (Apple *et al*., 2000).

Many cyanobacterial strains are able to metabolize sugars as a source of carbon. Previous studies have shown that the N_2-fixing cyanobacteria *Anabaena variabilis* and *Anabaena* sp. CH3 are able to use fructose as a substrate for producing H_2. In the present study, we examined sucrose, fructose and glucose as a source of external carbon. Our result showed that C-deprived cells did not produce as much H_2 as cells in BG11 medium (Figure 10). In agreement, Burrows *et al* (1998) reported that HCO_3^- alone does not

appear to be significant for H_2 production. Interestingly, the highest H_2 production was observed at 0.4% glucose, demonstrating distinct difference among cyanobacterial strains. This indicated that glucose causes an increased level of reduced NAD(P) for bidirectional hydrogenase and thereby plays a significant role in H_2 production. At higher glucose concentrations, the cells may use energy to drive the sugar out of the cells and thereby lower the capacity for H_2 production.

A high concentration of external NaCl have a negative effect on growth in *Anabaena doliolum*. In *Synechocystis* PCC 6803, high concentrations of external sorbitol significantly reduced growth. We analysed the short-term osmotic stress in which external osmolalities were generated by either NaCl or sorbitol at iso-osmolar concentrations and showed low osmolality stimulated H_2 production in *Synechocystis* PCC 6803 (Figure 12). However, a further increase in osmolality by NaCl resulted in a sharp decrease of H_2 production while this was not the case for sorbitol where only a slight decrease was observed. In *Synechocystis* PCC 6803 the cells synthesize the natural solute glucosylglycerol (GG) to balance the osmotic potential. The cells may either switch to synthesize GG to protect proteins and membranes or to drive both energy and reductant to prevent an influx of Na^+ under salt-stress conditions (Mikkat *et al*., 1996). Previously, the synthesis of GG was shown to be strictly salt dependent in contrast to non-ionic osmotic-stress by e.g. sorbitol that accumulates in the form of sorbitol instead of GG in *Synechocystis* PCC 6803 (Marin *et al*., 2006). As a consequence external NaCl will be negative for H_2 production compared to external sorbitol.

Nitrogen and sulfur are required nutrients for cell growth and there are several reports on how to optimize H_2 production in *Synechocystis* PCC 6803 by balancing the different nutrient levels (Antal *et al*., 2006; Burrows *et al*., 2008; Chen *et al*., 2007). Higher H_2 production was observed when switching the nitrogen source from NO_3^- to N-

deprived as well as under sulfur deprived conditions. Our results clearly demonstrate that nitrogen deprived conditions result in significantly enhanced H_2 production compared with changing nitrogen sources (NO_3^-, NH_4^+, NO_2^-) (Figure 9B) or depriving the cells of sulfur (Figure 14). Moreover, the H_2 production resulted in single mutant, Δ*narB* or Δ*nirA*, and double mutant, Δ*narB:*Δ*nirA*, (Table 9) could be confirmed that nitrate assimilation pathway is the competing pathway of H_2 production in *Synechocystis* sp. PCC 6803.

The result of H_2 production under nitrogen deprived is in agreement with a previous report that showed a higher transcript level of the structural *hox* genes in cells facing N starvation. There has been a recent review on the transcriptional regulation of the *hox* genes with a special focus on the transcriptional factors CyLexA and CyAbrB. For short time (24 h), a combination of nitrogen and sulfur deprived conditions gave the highest H_2 production (Figure 14). Unexpectedly, the presence of the reductant β-mercaptoethanol leads to an additional significant stimulation of the H_2 production activity. Thus our results suggest that nitrogen deprived condition is an electron sink, providing more electrons to the bidirectional hydrogenase enzyme. Zhang *et al* (2008) found that under sulfur starvation, both light-harvesting and photosynthetic activity were reduced in *Synechocystis* PCC 6803. At the same time the gene encoding a high-affinity sulfate transporter was significantly induced and genes encoding components of phycobilisomes, photosystems I and II, cytochrome $b_{6/f}$, and ATP synthase were all down regulated. The glycogen accumulation increases and reversibly inactivates PSII to stop O_2 evolution and promotes an anaerobic condition under sulfur deprivation. In addition, DTT, a reducing agent did not cause an increased in H_2 production. This might be due to the fact that the molecule of DTT is larger than β-mercaptoethanol.

The bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 is oxygen sensitive. As a consequence, cells grown under anaerobic condition for 24 h showed a much higher H_2ase activity. Presence of light, even under anaerobic conditions inhibits the hydrogenase activity. However, addition of DCMU, resulted in the maintenance of constant hydrogenase activity even under a light regime. One competing pathway of H_2 production is nitrate assimilation, the H_2ase activity increased significantly when nitrate was withdrawn from the growth media. Under the optimal conditions for H_2 production all electrons should be directed to H_2 production rather than to other competing pathways and the redox status of the cells is essential in order to actively maintain the highest H_2 production. The hydrogenase activity was stimulated by the presence of both reductants, ferredoxin and NADH as a source of external electrons (Table 7). The ability of these reductants to provide e^- to H_2ase enzyme for the production of H_2 suggests that the activity for H_2 production is not the hydrogenase enzyme but rather the supply of NADH and/or ferredoxin.

Also pH and temperature influenced the hydrogenase activity with an optimum at rather neutral pH (7.5) and an increased hydrogenase activity with increasing temperature at 70 ºC (Figure 18). Interestingly, the hydrogen evolution of the purified enzyme was highest at 60° C and pH 6.3 and Dutta *et al* (2005) reported an optimum temperature for cyanobacterial based hydrogen production between 30–40 °C. A probable explanation for our observation is an increased influx of the sodium dithionite-reduced methyl viologen. Moreover, these temperature and combined with the effect of pH are optimum for protein formation of H_2ase enzyme.

Synechocystis belongs to a group of moderately halotolerant cyanobacteria. *Synechocystis* sp. PCC 6803 cannot grow in media without a fixed nitrogen source.

Nitrate is a usual nitrogen source used in culturing *Synechocystis*. To assess whether an external osmotic upshift could affect nitrate transport, the effect of osmolality generated by NaCl and sorbitol on nitrate uptake was extensively examined. The uptake was stimulated by osmotic upshifts up to about 120 mosmol kg^{-1} (Figure 21). It is remarkable that the uptake of nitrate responded to changes in osmolality with similar manner regardless of the source generating the osmotic upshift. A large increase of uptake was observed at low osmolality followed by a smaller increase of uptake at higher osmolality. However, it is noted that higher nitrate uptake occurred with osmotic upshift generated by NaCl than by sorbitol suggesting additional ionic effect by NaCl. The stimulation of nitrate uptake by Na^+ as shown in Figure 21 is further supported by results shown in Figure 20 at a fixed osmolality of 120 mosmol kg^{-1}. Similar studies in *Synechocystis* sp. PCC 6803 on the uptake of putrescine, a polycationic compound, have shown that the stimulation of the uptake of putrescine by NaCl or sorbitol was a result of an osmotic effect rather than an ionic effect (Raksajit *et al*., 2006). The stimulation of nitrate uptake by NaCl or sorbitol as shown in Figure 21 might reflect a better physiological state of *Synechocystis* cells rather than a direct effect on the uptake system. An inhibition of nitrate uptake was evident when the medium osmolalities were 200 mosmol kg^{-1} or higher (data not shown). Previous studies showed that nitrate uptake in at least two cyanobacteria, *Anacystis nidulans* R2 and *Aphanothece halophytica*, is Na^+ -dependent (Rodriguez *et al*., 1992; Incharoensakdi and Wangsupa, 2003). In the present study, Na^+ -dependency of nitrate uptake was also confirmed for *Synechocystis* sp. PCC 6803 (Figure 21, 22).

The results of K_s values and maximal velocities (V_{max}) (Figure 23) presented here clearly demonstrate the existence of an active transport system for exogenous nitrate in cyanobacterium *Synechocystis* sp. PCC 6803. The transport system was saturable with the

substrate, and a Michaelis-Menten type relationship was observed (Figure 24). Most cyanobacteria contain only one nitrate uptake system with either a low or a high affinity which is in contrast to higher plant systems where both low and high affinity nitrate uptake systems exist (Incharoensakdi, 2006). Previous reports showed that *Anacystis nidulans* R2 (Rodriguez *et al*., 1998) and *Anabaena* sp. PCC 7120 (Rai and Tiwari, 1999) contain high affinity nitrate transport systems with K_s values of 1.3 and 31 μM, respectively. The low affinity nitrate uptake has been observed in the halotolerant cyanobacterium *Aphanothece halophytica* with a K_s value of 416 μM (Incharoensakdi and Wangsupa, 2003). The half saturation value (K_s) of nitrate uptake in non-nitrogen fixing cells of *Synechocystis* sp. PCC 6803, clearly indicated the presence of a high affinity uptake system.

The inhibitory effect by ammonium assimilation products which act principally on the entrance of nitrate into the microorganisms has been previously studied (Revilla *et al*., 1986; Omata, 1995). Our results demonstrating a depression of nitrate uptake in *Synechocystis* sp. strain PCC 6803 by ammonium (Figure 25) are in agreement with previous observations in other cyanobacteria (Ohmori *et al*., 1977; Omata, 1998; Sakamoto and Bryant, 1999). There exists the possibility that the inhibitory effect by ammonium on nitrate uptake was through stimulation of nitrate efflux rather than inhibition of nitrate influx (Kronzucker *et al*., 1999). However, like other cyanobacteria *Synechocystis* sp. PCC 6803 can assimilate several forms of nitrogen such as ammonium (NH_4^+), nitrite (NO_2^-) and urea. The assimilation of most of these components provides intracellular ammonium, which itself is a preferred nitrogen source. Thus, in the presence of ammonium, the genes encoding both the permease and the enzymes for assimilation of nitrogen sources alternative to ammonium are repressed (Flores and Herrero, 2005).

As previous studies on cyanobacterial nitrate transport showed a positive correlation between photosynthetic assimilation of nitrate and carbon skeleton from CO_2 fixation (Flores *et al*., 1983; Incharoensakdi and Wangsupa, 2003; Lara and Romero 1986; Rodriguez *et al*., 1998), we tested whether the inhibition of CO_2 fixation affected the nitrate uptake and found that DL-glyceraldehyde inhibited nitrate uptake for both unstress and moderate salt-stress conditions (Figure 26). Altogether, the results indicate a strict dependency of nitrate uptake in *Synechocystis* sp. PCC 6803 on active CO_2 fixation and the overall results of the effect of metabolic inhibitors (Table 8) suggest that the nitrate uptake in *Synechocystis* sp. PCC 6803 is an energy requiring process with the proton motive force providing the contribution by both ΔpH and $\Delta\psi$.

CHAPTER V

CONCLUSIONS

The present study of the hydrogen production and nitrate assimilation in cyanobacterium *Synechocystis* sp. PCC 6803 has revealed the following findings:

1. Various external factors could affect both H_2 production and bidirectional Hox-hydrogenase activity examined in the non-N_2 fixing cyanobacterium *Synechocystis* PCC 6803.
2. Exogenous glucose and increased osmolality both enhanced H_2 production with optimal production observed at 0.4% and 20 mosmol kg^{-1}, respectively.
3. Anaerobic condition for 24 h induced significantly higher H_2ase activity with cells in $BG11_0$ showing highest activities.
4. Increasing the pH resulted in an increased Hox-hydrogenase activity with an optimum at pH 7.5. The Hox-hydrogenase activity gradually increased with increasing temperature from 30°C to 60°C with the highest activity observed at 70°C.
5. A low concentration at 100 µM of either DTT or β-mercaptoethanol resulted in a minor stimulation of H_2 production. β-mercaptoethanol added to nitrogen and sulfur deprived cells stimulated H_2 production significantly.
6. The highest Hox-hydrogenase activity was observed in cells in $BG11_0$-S-deprived condition and 750 µM β-mercaptoethanol measured at a temperature of 70 ºC; 14.32 µmol H_2 mg chl a^{-1} min^{-1}. The nitrate assimilation mutant strains Δ*narB*, Δ*nirA* and double mutant, Δ*narB*:Δ*nirA* gave the H_2 production higher than in wild type when cells were adapted in normal BG11 for 24 hour.

7. A small increase of osmolality by 30 and 40 mosmol kg^{-1} sorbitol and NaCl resulted in about 3.5- and 4.5- fold increase of nitrate uptake, respectively.
8. At 25 mosmol kg^{-1} or higher, NaCl exhibited higher nitrate uptake than sorbitol suggesting a stimulatory effect of Na^+ on the uptake activity.
9. External 20 mM NaCl stimulated nitrate uptake with K_s and V_{max} values of 79 µM and 2.45 µmol (mg Chl *a*)$^{-1}$ min^{-1}, respectively which were about 2-fold higher than those without NaCl.
10. Ammonium and DL-glyceraldehyde, an inhibitor of CO_2 fixation, caused a reduction of nitrate uptake. Cells pre-incubated in darkness showed drastic reduction of uptake activity by 70% suggesting energy-dependent nitrate uptake systems in *Synechocystis* sp. strain PCC 6803.
11. Nitrate transport was sensitive to various metabolic inhibitors including those dissipating proton gradients and membrane potential.
12. Nitrate uptake in *Synechocystis* sp. strain PCC 6803 is dependent on and stimulated by Na^+ ions and that the uptake requires energy provided by electrochemical potentials generated by electron transport.

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APPENDICES

APPENDIX A

BG11 medium

Liquid media, composition per litter

1.	$NaNO_3$ (1.77M)	10	mL
2.	*KH_2PO_4 (0.29M)	1	mL
3.	$MgSO_4.7H_2O$ (0.3M)	1	mL
4.	$CaCl_2.2H_2O$ (0.244M)	1	mL
5.	Na_2CO_3 (0.19M)	1	mL
6.	Citric acid (mM)	1	mL
7.	Na_2EDTA (2.7mM)	1	mL
8.	$FeSO_4.7H_2O$ (21mM)	1	mL
9.	**Trace element A5 solution	1	mL

* KH_2PO_4 should be autoclaved separately to avoid precipitation after autoclaving

** Trace element A5 solution preparation:

1.	H_3BO_3 (27mM)	2.68	g
2.	$ZnSO_4.7H_2O$ (0.76mM)	0.22	g
3.	$CuSO_4$ (0.3mM)	0.079	g
4.	$MnCl_2.4H_2O$ (9.1mM)	1.81	g
5.	$Na_2MoO_4.2H_2O$ (1.6mM)	0.39	g
6.	$Co(NO_3)_2.6H_2O$ (0.17mM)	0.049	g

Adjust volume to 1 liter with deionized water. Adjust the pH of the medium to 7.6 by slowly adding 2N NaOH.

Solid media, composition per litter

1.	TES (1M)	10	mL
2.	$Na_2S_2O_3$ (30%)	10	mL
3.	Becto-agar	15	g

Are add in BG11 liquid medium

APPENDIX B

LB medium

Liquid media, composition per 1 litre

1. Bacto tryptone	10	g
2. NaCl	10	g
3. Yeast extract	5	g

Solid media, composition per 1 litre

Bacto tryptone	10	g
NaCl	10	g
Yeast extract	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 NaOH. The total volume of solution was then adjusted to 1 litre with deionized water. The medium was sterilized by autoclaving at 15 Ib/in^2 for 15 minute.

APPENDIX C

Chlorophyll *a* content determination

1. Transfer 100 µl of cells suspension to 2 mL micro-centrifuge tube (make two replicates).
2. Add 900 µl of Methanol to each of the tube.
3. Vortex the tubes for 45 seconds and leave them for at least 1 hour under dark. Chlorophyll *a* is a photosynthetic pigment and becomes degraded when extracted; thus, the need of protecting the sample from light.
4. Centrifuge the samples at 14,000 rpm, for 5 minutes, at 4 ^{o}C.
5. Remove the green phase without disturbing the pellet and transfer it to a quartz cuvette.
6. Measure the absorbance at 665 nm, using a blank made of 90% methanol and 10% BG11.
7. To calculate the chlorophyll *a* concentration, use the following formula:

$$[\text{Chlo } a - \mu g\ mL^{-1}] = 12.7 * Abs_{665nm} * 10$$

12.7: based on the extinction coefficient 78.74 L g^{-1} cm^{-1}
(ref. Meeks and Castenholz, 1974. *Archives of Microbiology*, 78: 25-41).
10: dilution factor.

APPENDIX D

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/minute

APPENDIX E

H_2 standard graph

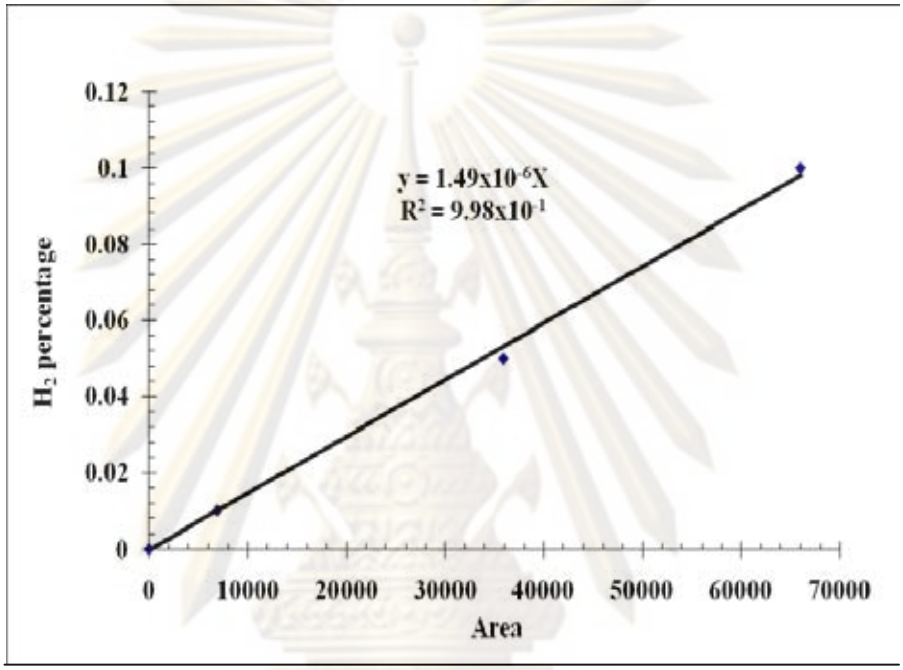

Figure A.1 Standard curve of H_2 standard gas

APPENDIX F

H_2 content calculation

Determine the amount (in percentage) of H_2 corresponding to the peak area for each time point. Use the calibration curve and formula supplied in appendix E.

Calculate the amount of produced hydrogen considering the following:

X% H_2 corresponds to X mL of H_2 in 100 mL gas phase.

1 mol = 22.4 dm^3 (L)

Normalize the hydrogen content by using the Chl *a* content.

Express the H_2 evolved in: nmol H_2 h^{-1} μg^{-1} Chl *a*

APPENDIX G

TBE buffer

Working solution

0.5X: 0.045 M Tris-borate

0.001 M EDTA

Concentrated stock solution (per liter)

5X: 54 g Tris base

27.5 g Boric acid

20 ml 0.5 M EDTA (pH 8.0)

APPENDIX H

Reagents for alkaline lysis

Solution I (100 ml)

5.0 ml 1.0 M Glucose

2.5 ml 1.0 M Tris-HCl, pH 8.0

2.0 ml 0.5 M EDTA, pH 8.0

After autoclave 20 μg/ml of RNase was added and stored at $4^{o}C$

Solution II (25 ml)

0.5 ml 10 M NaOH

1.25 ml 20% SDS

Solution III (500 ml)

147 g potassium acetate

57.5 ml glacial acetate

Autoclave and store at $4^{o}C$

TE buffer (500 ml)

5 ml 1 M Tris-HCl, pH 8.0

1 ml 0.5 M EDTA

Autoclave and store at room temperature

APPENDIX I

pGEM-T Easy Vector

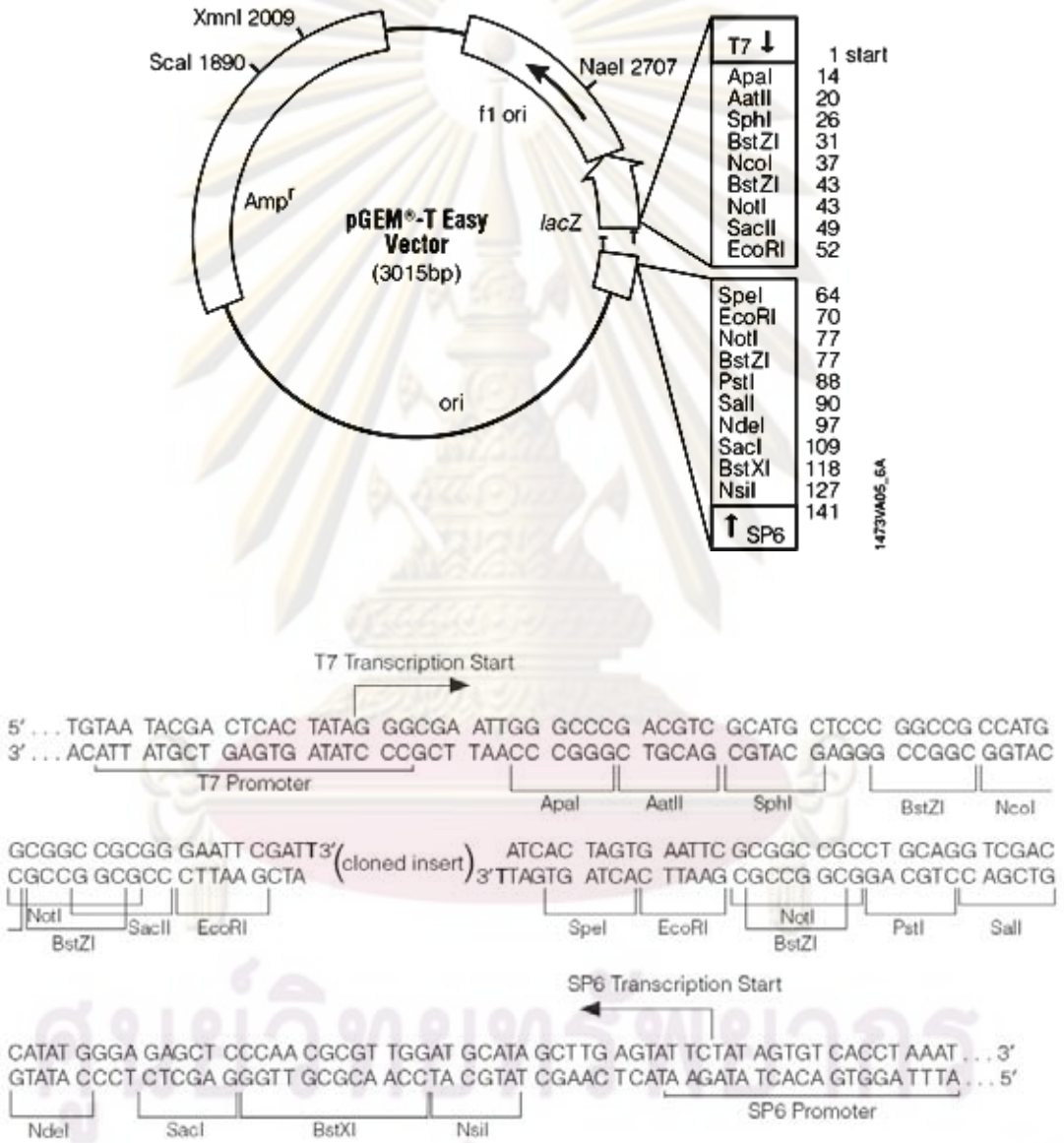

Figure A.3 Map of pGEM®-T Easy vector and cloning/expression region

APPENDIX J

pSB2K3

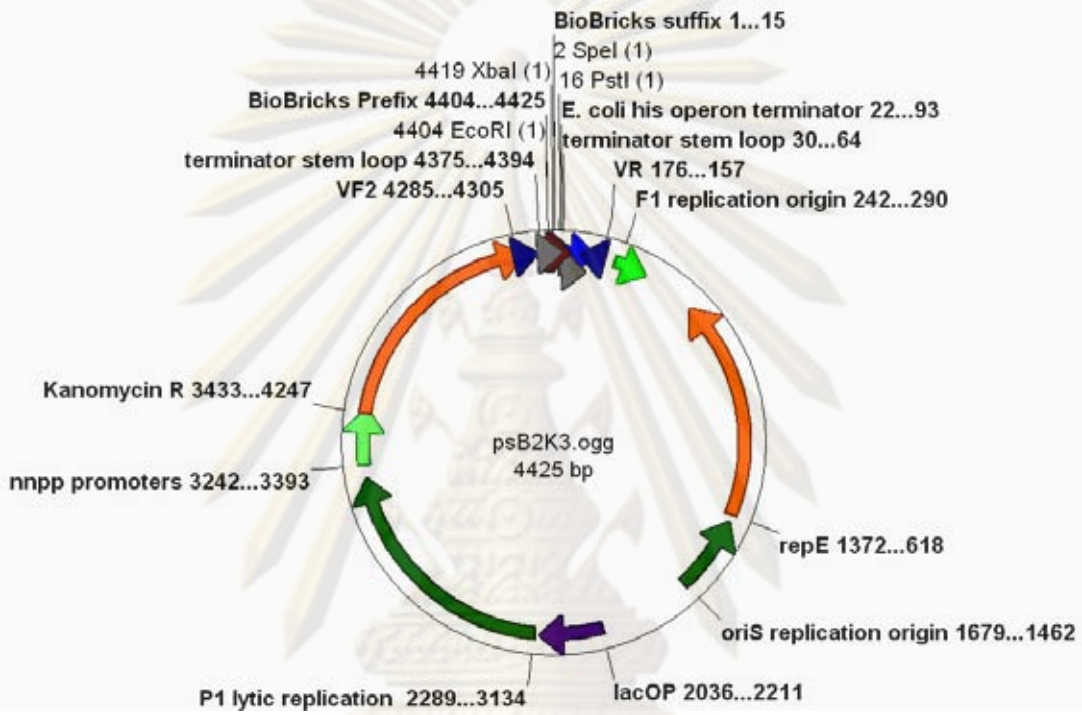

Figure A.4 Map of psB2K3 vector

APPENDIX K

pBR325

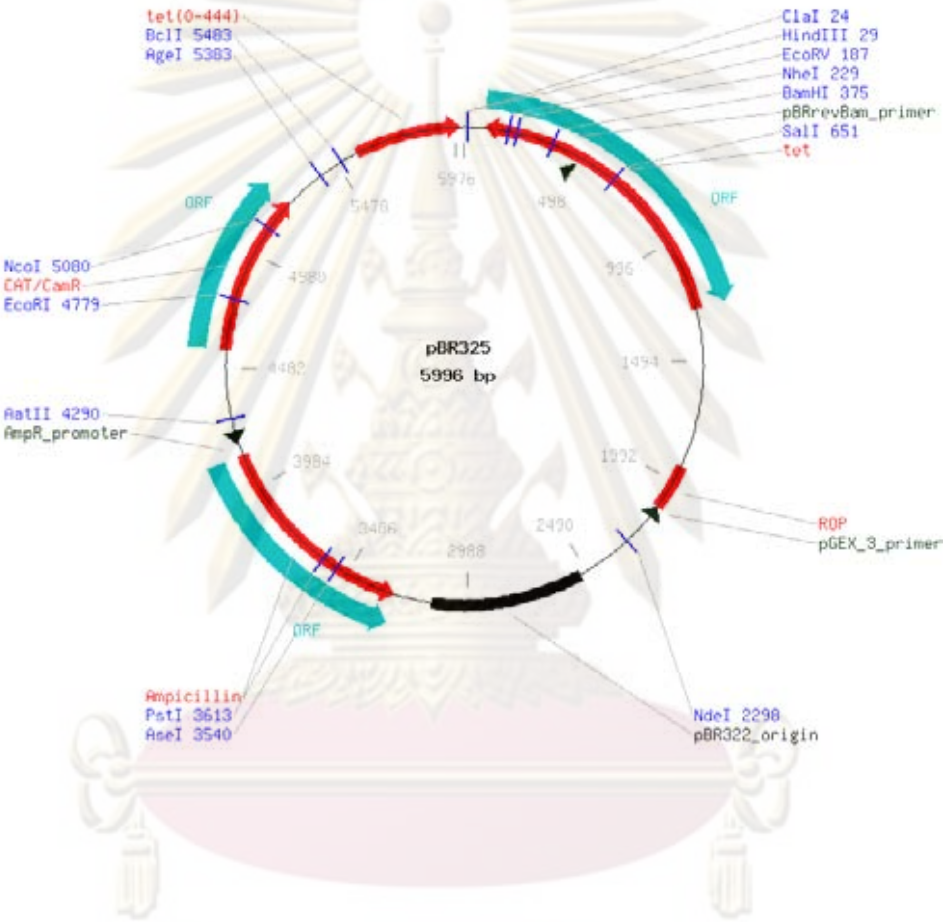

Figure A.5 Map of pBR325 vector

APPENDIX L

PERSONAL INFORMATION

FIELD OF RESEARCH INTEREST

My dissertation focused on H_2 production and nitrate uptake in cyanobacterium *Synechocystis* sp. PCC 6803 under environmental stresses. Methodologically, I am familiar with biochemical laboratory techniques such as DNA and RNA extraction, PCR, RT-PCR, DNA cloning, mutant strain construction, GC, HPLC, and Microsoft Office as well as Photoshop Adobe and CorelDraw programs.

SCHOLARSHIPS

June 2007 - May 2010

Academic scholarship from Thailand Research Fund through the Royal Golden Jubilee Ph.D. program, Bangkok, Thailand

June 2009- May 2010

Academic scholarships from the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphote Endowment Fund), Bangkok, Thailand

ACADEMIC EXPERIENCES

1. The Examination for Radiation Protection Course Level II. Office of Atoms for Peace Ministry of Science and Technology, Bangkok Thailand 17-28 July 2006.
2. The 32nd Congress on Science and Technology of Thailand Science and Technology for Sufficiency Economy to celebrate the 60th Anniversary of His Majesty the King's Accession to the Throne, Queen Sirikit National Convention Center, Bangkok Thailand 10-12 October 2006. “Nitrate and phosphate uptake by cyanobacterium *Synechocystis* sp. 6803 under non-stress and salt-stress conditions”
3. The 33rd Congress on Science and Technology of Thailand Science and Technology for Global Sustainability, Walailak University, Nakhon Sri Thammarat, Thailand 18-20 October 2007. “Nitrate transport system in response to osmotic stress by the cyanobacterium *Synechocystis* sp. PCC 6803”.
4. The 13th Biological Sciences Graduate Congress. Faculty of Science, National University of Singapore, Singapore. December 15-16, 2008.

5. RGJ-Ph.D. Congress X, Jomtein Palm Beach Resort Pattaya, Chonburi. April 3-5, 2009. “Optimal conditions for H_2 production in *Synechocystis* sp. PCC 6803’’.
6. The 14th Biological Sciences Graduate Congress. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. December 10-12, 2009 (organizing committee)
7. RGJ-Ph.D. Congress XI, Jomtein Palm Beach Resort Pattaya, Chonburi. April 1-3, 2010. “Enhancement of H_2 production in *Synechocystis* sp. PCC 6803 by the physiological reductant’’.

PUBLICATIONS

1. **Baebprasert, W**., Lindblad, P. and Incharoensakdi, A. (2010) Response of H_2 production and Hox-hydrogenase activity to external factors in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. International Journal of Hydrogen Energy. (Accepted)
2. Agervald, Å., **Baebprasert, W**., Incharoensakdi, A., Lindblad, P. and Stensjö, K. (2010) The CyAbrB transcription factor CalA regulates the iron superoxide dismutase in *Nostoc* sp. strain PCC 7120. Environmental Microbiology and Environmental Microbiology Reports. (Accepted)
3. **Baebprasert, W**., Karnchanatat., A., Lindblad, P. and Incharoensakdi, A. (2010) Stimulation of nitrate uptake by osmotic upshifts and sodium in the cyanobacterium *Synechocystis* sp. strain PCC 6803. (Preparing)
4. Phunpruch, S., **Baebprasert, W**., Thongpeng, C. and Incharoensakdi, A. (2006) Nucleotide sequencing and transcriptional analysis of uptake hydrogenase genes in the filamentous N_2-fixing cyanobacterium *Anabaena siamensis*. Journal of Applied Phycology 18:713-722.

REFERENCES

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BIOGRAPHY

Miss Wipawee Baebprasert was born on March 9, 1978 in Bangkok, Thailand. She graduated with a Bachelor of Science degree in Biology, Faculty of Science, Burapha University in 2000 and Master of Science in Biotechnology from Faculty of Science, King Mongkut's Institute of Technology Ladkrabang in 2004. She has further studied for the Doctor of Philosophy (Ph.D.) degree in Program of Biotechnology, Chulalongkorn University since 2005.