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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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SCREENING OF HYDROGEN-PRODUCING ALGAE AND OPTIMIZATION FOR ENHANCED HYDROGEN PRODUCTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Field of Study	Biochemistry
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น้ำจากแหล่งน้ำจืดในธรรมชาติและจากนาข้าวที่อยู่ในพื้นที่ภาคกลางของประเทศไทยถูกเก็บและนำมาใช้ในการคัดกรองเพื่อหาจุลสาหร่ายที่ผลิตก๊าซไฮโดรเจน สาหร่ายสายพันธุ์หนึ่งจากทั้งหมด 210 สายพันธุ์ถูกนำมาแสดงลักษณะสัณฐานวิทยาภายใต้กล้องจุลทรรศน์แบบใช้แสงพบว่าเป็นสายพันธุ์ *Tetraspora* พร้อมตั้งชื่อสายพันธุ์ว่า *Tetraspora* sp. CU2551 ผลการยืนยันจากการหาลำดับเบสดีเอ็นเอของยีน *18S* rDNA พบว่าสาหร่ายนี้มีความใกล้เคียงกับสาหร่ายสีเขียวเซลล์เดี่ยว สาหร่ายสายพันธุ์นี้ใช้เวลาของการเพิ่มจำนวนเป็นสองเท่าสั้นที่สุดเมื่อเลี้ยงในอาหาร Tris-Acetate-Phosphate (TAP) ภายใต้ความเข้มแสงช่วง 37 - 92 ไมโครโฟตอนต่อตารางเมตรต่อวินาที ที่อุณหภูมิ 36 องศาเซลเซียส สาหร่ายที่อายุ 24 ชม.เลี้ยงภายใต้ความเข้มแสงที่ 37 ไมโครโฟตอนต่อตารางเมตรต่อวินาที แสดงความสามารถในการผลิตก๊าซไฮโดรเจนที่เหมาะสมเมื่อบ่มเซลล์ที่ 35 องศาเซลเซียส การผลิตก๊าซภายใต้แสงจะเพิ่มขึ้นเมื่อ pH เพิ่มจาก 5.75 ถึง 9.30 อย่างไรก็ตาม การผลิตลดลงอย่างมากเมื่อลด pH ถึง 5.25 การเติม 0.5 มิลลิโมล่าร์ของเบต้าเมอแคปโตเอทธานอล ลงในอาหารเลี้ยงเชื้อจะช่วยกระตุ้นอัตราการผลิตก๊าซไฮโดรเจนได้ประมาณสองเท่า การขาดแหล่งไนโตรเจนและซัลเฟอร์ส่งผลให้อัตราการผลิตก๊าซเพิ่มขึ้นประมาณ 50% ซึ่งผลนี้ต่างจากการขาดแหล่งไนโตรเจนหรือซัลเฟอร์เพียงอย่างใดอย่างหนึ่งซึ่งจะส่งผลให้เซลล์ผลิตก๊าซในอัตราเพิ่มขึ้นเพียงเล็กน้อย นอกจากนี้ผลการเพิ่มอัตราการผลิตก๊าซไฮโดรเจนจากการเติม 0.5 มิลลิโมล่าร์ของเบต้าเมอแคปโตเอทธานอล ในภาวะขาดทั้งแหล่งไนโตรเจนและซัลเฟอร์จะเกิดขึ้นเมื่อความเข้มแสงไม่เกิน 5 ไมโครโฟตอนต่อตารางเมตรต่อวินาที หากแสงเข้มขึ้นจะไม่ส่งผลให้ผลิตได้สูงขึ้นตาม การบ่มเชื้อให้ผลิตก๊าซไฮโดรเจนจะเกิดสูงสุดเมื่ออยู่ในภาวะขาดทั้งแหล่งไนโตรเจนและซัลเฟอร์ภายใต้ความเข้มแสงประมาณ 29 ไมโครโฟตอนต่อตารางเมตรต่อวินาที คำนวณได้ค่าอัตราการผลิตได้ประมาณ 17.3 – 61.7 ไมโครโมลต่อมิลลิตกรัมคลอโรฟิลเอต่อชั่วโมง ซึ่งถือได้ว่าเป็นค่าการผลิตที่สูงเมื่อเทียบกับสาหร่ายสีเขียวชนิดอื่น ส่งผลให้ *Tetraspora* sp. CU2551 เป็นสายพันธุ์ที่น่าสนใจต่อการผลิตก๊าซไฮโดรเจนโดยวิธีทางชีวภาพที่ใช้แสง

ยีน sulfate permease (*sulP*) ที่แปลรหัสให้โปรตีนทำหน้าที่ขนส่งซัลเฟตเข้าสู่คลอโรพลาสต์และยีน hydrogenase (*hydA*) ที่แปลรหัสให้เอนไซม์เร่งปฏิกิริยาการสร้างก๊าซไฮโดรเจน ได้ถูกค้นพบในสาหร่ายชนิดนี้ ยีน *sulP* มี ORF ขนาด 1,014 เบส โดยที่มีขนาดของ 5'UTR และ 3'UTR เป็น 285 และ 225 เบส ตามลำดับ ยีน *sulP* นี้ประกอบด้วยอินตรอน 4 ชิ้นในระดับดีเอ็นเอ ในขณะที่สาหร่ายสีเขียวชนิดอื่นนั้นไม่พบอินตรอนในยีนยกเว้นในสาหร่าย *Chlamydomonas reinhardtii* นอกจากนี้โปรตีนที่แปลรหัสจาก *sulP* ของ *Tetraspora* ได้ถูกทำนายว่ามี chloroplast transit peptide ทางด้านปลาย N ส่งผลให้โปรตีนนี้มีความใกล้เคียงกับโปรตีนที่แปลรหัสจาก *sulP* ของสาหร่าย *Chlamydomonas reinhardtii* โดยยืนยันจากการทำ phylogenetic tree นอกจากนี้แล้ว พบว่ายีน *hydA* ของสาหร่าย *Tetraspora* มีขนาด 878 เบสซึ่งจะแปลรหัสเป็นโปรตีนประกอบด้วย 292 กรดอะมิโน อินตรอนสองชิ้นขนาด 261 และ 282 เบสตามลำดับถูกพบในลำดับเบสระดับดีเอ็นเอ โปรตีนที่แปลรหัสจาก *hydA* มีความใกล้เคียงกับโปรตีนที่แปลรหัสจาก *hydA* ของสาหร่าย *Chlorella fusca* ระดับการแสดงออกของยีนทั้ง *sulP* และ *hydA* ของ *Tetraspora* เพิ่มขึ้น 2.3 เท่าหลังจากอยู่ในภาวะขาดแหล่งซัลเฟอร์ ในขณะที่การเติมแหล่งซัลเฟอร์กลับคืนจะทำให้การแสดงออกของสองยีนนี้ลดลง ผลการผลิตก๊าซไฮโดรเจนและกิจกรรมของระบบแสงที่สอง (PSII) ลดลงเมื่อเซลล์อยู่ในภาวะขาดแหล่งซัลเฟอร์ ทั้งสองกิจกรรมนี้จะถูกฟื้นฟูคืนเมื่อเพิ่มแหล่งซัลเฟอร์ในอาหารเลี้ยงเชื้อ ผลการทดลองเน้นย้ำให้เห็นถึงความสำคัญของซัลเฟอร์ต่อการควบคุมวิถีการผลิตก๊าซไฮโดรเจนในสาหร่าย *Tetraspora* sp. CU2551

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2554.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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CHERDSAK MANEERUTTANARUNGROJ : SCREENING OF HYDROGEN-PRODUCING ALGAE AND OPTIMIZATION FOR ENHANCED HYDROGEN PRODUCTION. ADVISOR : PROF. ARAN INCHAROENSAKDI, Ph.D., CO-ADVISOR : PROF. PETER LINDBLAD, Ph.D., 171 pp.

Water samples collected from fresh water sources and rice paddy fields located in central part of Thailand were used in screening for hydrogen-producing green microalgae. One out of 210 isolates was identified as belonging to the genus *Tetraspora* under light microscope for morphology identification. Confirmation by phylogenetic analysis of *18S* rDNA sequence revealed that the green alga, identified as *Tetraspora* sp. CU2551, is closely related to other unicellular green algal species. *Tetraspora* sp. CU2551 had the shortest doubling time when grown in Tris-Acetate-Phosphate (TAP) medium under a light intensity of 37 - 92 $\mu E/m^2/s$ and a temperature of 36 ^oC. A 24 h culture under light intensity of 37 $\mu E/m^2/s$ showed the optimal hydrogen production rate when cells were incubated at 35 ^oC. The production under light incubation increased with increasing pH from 5.75 to 9.30; however, almost no production was observed at a pH of 5.25. Addition of 0.5 mM β-mercaptoethanol to the TAP medium stimulated hydrogen production rate about 2-fold. During the hydrogen production phase, the use of TAP medium lacking both nitrogen and sulfur resulted in about 50% increase in the hydrogen production. This was in contrast to only a small increase in the production when either nitrogen or sulfur was omitted in TAP medium. The stimulation of hydrogen production by 0.5 mM β-mercaptoethanol under nitrogen- and sulfur-deprived conditions occurred only when the cells were grown at a light intensity lower than 5 $\mu E/m^2/s$ with no effects at higher intensities. Incubating the culture in TAP-N-S medium under a light intensity of 29 $\mu E/m^2/s$ gave a maximal calculated hydrogen production of 17.3 - 61.7 μmol/mg Chl a/h. This is a very high production rate compared to other green algae and makes *Tetraspora* sp. CU2551 an interesting model strain for photobiological hydrogen production.

The newly identified chloroplast envelope-localized sulfate permease gene (*sulP*) and the hydrogenase gene (*hydA*) from the green alga *Tetraspora* sp. CU2551 are reported in this study. The *sulP* showed an open reading frame of 1,014 bp with the 5'- and 3' UTR being 285 and 225 bp, respectively. *Tetraspora sulP* also contained four introns, whereas other known photosynthetic organisms, except *Chlamydomonas reinhardtii*, show no intron at the DNA level. The deduced amino acid sequence of SulP revealed an extended N-terminus where the putative chloroplast transit peptide was identified. This suggests a close relationship between *Tetraspora* and *Chlamydomonas reinhardtii* SulPs, as confirmed by phylogenetic tree analysis. In addition, the *Tetraspora hydA* was identified. The cDNA sequence showed an 878 bp encoding 292 amino acid residues. Two introns of 261 and 282 bp, respectively were also found in *hydA* structural gene. The deduced amino acid sequence of *Tetraspora* HydA is closely related to HydA of *Chlorella fusca*. The transcript levels of both *sulP* and *hydA* of *Tetraspora* showed an up-regulation of about 2.3 times after sulfur deprivation, whereas upon sulfur repletion the expression of both genes decreased. The production of H_2 and PSII activity decreased in cells grown under sulfur-deprived condition. These two activities could be restored upon transferring the cells to sulfur-replete medium. Our results highlight the importance of sulfur for the regulation of hydrogen metabolism in *Tetraspora* sp. CU 2551.

Department :…….Biochemistry…..…Student's Signature…….…….
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LIST OF ABBREVIATIONS

H_2	Hydrogen
H_2ase	hydrogenase
Chl *a*	Chlorophyll *a*
bp	Base pair
^oC	Degree Celsius
EDTA	Ethylenediamine tetraacetic acid
g	Gram
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
A	Absorbance

CHAPTER I

INTRODUCTION

1.1 General introduction

Nowadays, global energy consumption tends to increase from 1988 (Rühl, 2008) and mostly are from oil, coal, and natural gas, as a result this will eventually lead to the depletion of limited natural energy resources. Presently, the utilization of fossil fuels in turn causing global climate change is mainly due to the emission of pollutant, sulfur dioxide (SO_2), nitrogen oxide (NO_x), especially carbon dioxide (CO_2) released into the atmosphere upon their combustion (Kapdan and Kargi, 2006). 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. These problems caused the scientist all around the world to search for alternative choices which is clean and renewable source.

In January of 2010, many countries were faced with extremes of temperature, heavy snowfall in European countries and heavy rainfall cause severe flood in Asian countries. These 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.

Hydrogen gas is one of most interesting renewable energy source since it is a clean energy, produce no carbon dioxide during combustion, and the heat of 141.65 MJ kg^{-1}, or 12.65 MJ m^{-3} (Perry, 1963) which is about 2.75 folds higher than that from other

hydrocarbon source. Moreover, hydrogen gas showed the highest energy produced per unit mass compared to other fuel types as shown in Table 1.1. Nowadays, hydrogen gas can be produced by 4 processes: stream reforming using fossil fuel, water electrolysis, thermochemical process, and biological process (Lay *et al*., 1999). Only the production from water electrolysis can sustainably supply to the need of industries. However, high cost of production is required with the huge amount of electricity, which is produced from fossil fuels. In addition, more than 96% of the world production of hydrogen gas is from fossil fuels (Wünschiers and Lindblad, 2002). Thus, the hydrogen production process is not the truly clean energy source.

Scientists aimed to produce the gas through the biological process instead of electrolysis process. The so-called biohydrogen is the gas produced by the living microorganisms where the organic wastes were utilized during gas production process. Until now, the bioproduction can be divided into 5 different approaches: direct biophotolysis, indirect biophotolysis, biological water-gas shift reaction, photo-fermentation, and dark fermentation.

Table 1.1 The energy comparison from different types of fuel source (modified from http://www.techno.msu.ac.th/bt/pdfdocxls/b.doc)

Fuel type	Energy per unit mass (MJ/kg)
Hydrogen gas	120
Oil	42-45
Diessel	42.8
Petrol	40-43
Bio-diessel	37
Natural gas	33-50
Charcoal	30
Ethanol	21
Coal sub-bituminous	27-30
Coal anthracite	15-19
Agricultural residue	10-17
Wood	15

1.2 H_2 production

1.2.1 Production from stream reforming using fossil fuels

Traditionally, H_2 is mainly produced from natural gas or fossil fuels by stream reforming in industrial scale. This process consists of two steps: 1) reformation of the substrate under high temperature steam supplied by burning the natural gas, and 2) using a water-gas shift reaction to form hydrogen and carbon dioxide from the CO produced in the first reaction.

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

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

However, these processes have some disadvantages such as using non-renewable energy sources, generates substantial CO_2 emission, and based on expensive techniques. In fact, most of the H_2 production processes relied on fossil fuels releases CO_2 in double amount of H_2 molar ratio (Navarro *et al.,* 2009).

1.2.2 Production from water electrolysis

In the process of electrolysis, water was used to be broken down and generated hydrogen and oxygen. To begin of the process, electricity passes through the positive electrode and negative electrode to electrolyte solution generated e.g. H_2SO_4 and KOH. By this method, electrical current up to 90 KW is needed to produce 1,000 ft^3 hydrogen gas. However, the energy obtained from the hydrogen combustion is lower than the electrical power that used in water electrolysis.

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

If the electricity generated from fossil fuels was used for water electrolysis, then CO_2 would be released to atmosphere. However, renewable technologies, such as solar cell and wind turbines can generate electricity with zero greenhouse gas emissions. In some countries, an abundance of nuclear power produces large amount of electricity making a logical process for water electrolysis (http://www.hydrogenassociation.org).

1.2.3 Production from thermochemical process

Thermochemical process has been developed by using the HgBr and calcium under the temperature of 200-700 $^{\circ}$C. Heat generated in nuclear reactor can be mostly recycled for other process. This process, however, has disadvantages from heavy metal waste which can cause severe pollution to the environment.

1.2.4 Production from biological process

The biohydrogen production is taking place in microorganisms, which can metabolize the organic compounds to generate the hydrogen gas. The bioproduction can be classified into 5 different methods (Levin *et al*., 2004): direct biophotolysis, indirect biophotolysis, biological water-gas shift reaction, photo-fermentation and dark fermentation.

1.2.4.1 Direct biophotolysis

This method based on the utilization of the microalgae to function as an antenna absorbing the solar radiation as a sole energy source for production process.

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

Microalgae, green algae and cyanobacteria, have an ability to fix atmospheric carbon dioxide, and absorb the solar radiation which is unlimited on the earth surface. Thus, the bioproduction by this process could help reduce the atmospheric carbon dioxide, which is the majority of green house gas. The production process involved in photosystem II (PSII) and photsystem I (PSI) where the electron will flow to ferredoxin (Fd). Hydrogenase enzyme is the last protein, which accepts electron and proton to form

hydrogen molecule (Figure 1.1). Although PSII system generates proton and electron which are the substrate of hydrogenase enzyme in microalgae, above 0.1% of molecular oxygen level can inhibit the activity of enzyme (Hallenback and Benemann, 2002).

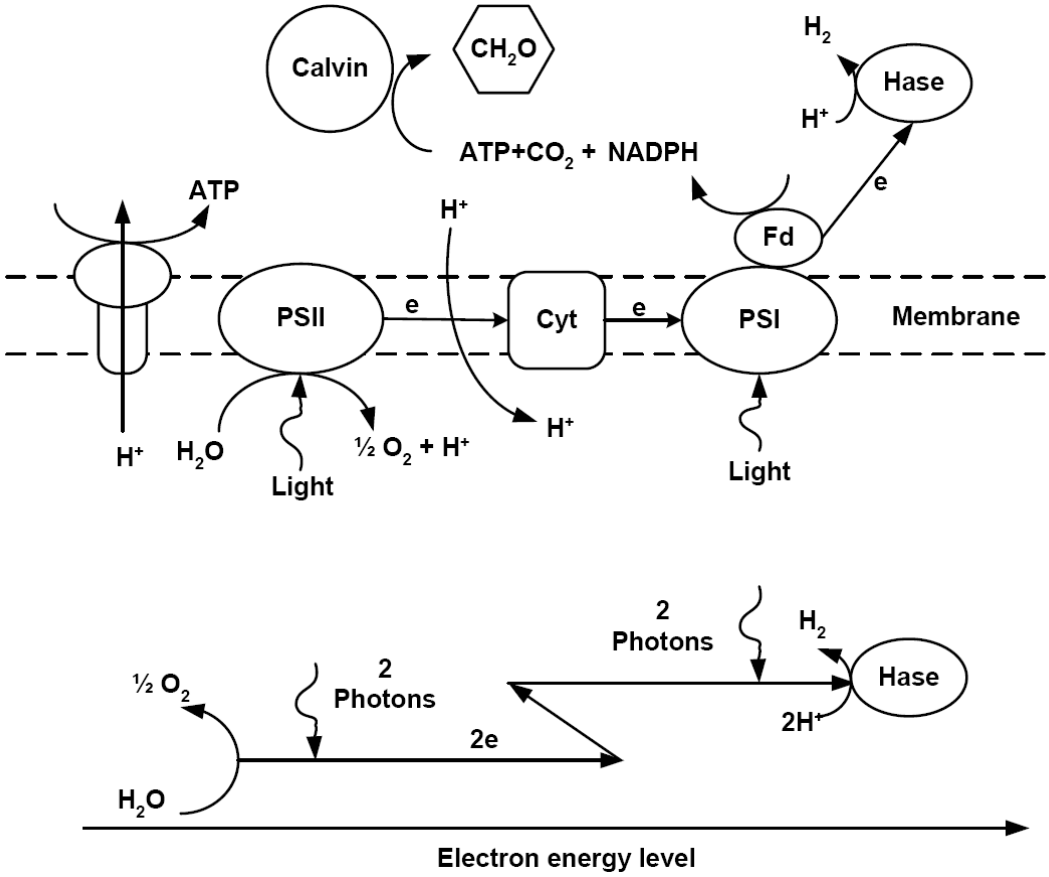


Figure 1.1 The schematic mechanisms of photosynthesis and biophotolysis of photoautotrophic microorganisms. The electrons or reducing equivalents from water oxidation is raised by the adsorbed photons at PSII and PSI. The reducing equivalent (NADPH) is used for CO_2 reduction in photosynthesis and carbohydrate (CH_2O) are accumulated inside the cells. The reducing power (Fd) could also be directed to hydrogenase (H_2ase) for H_2 evolution. (Yu and Takahashi, 2007)

1.2.4.2 Indirect biophotolysis

The indirect biophotolysis is a term mostly called for the hydrogen production in cyanobacteria. The chemical reaction is expressed as in equation 1.1 – 1.3. The process comprises two-phase system: growth phase and production phase. In the growth phase, cells are grown aerobically under the normal metabolic state with photosynthesis. Glucose is synthesized during dark cycle (as shown in equation 1.1). In the second phase, production phase, cells are transferred to anaerobic condition to allow hydrogenase active in the absent of oxygen. Glucose is metabolized to acetate in double molar ratio and hydrogen in 4 times molar ratio (as shown in equation 1.2). Acetate molecule is further metabolized to produce hydrogen in 4 times molar ratio (as shown in equation 1.3). Stoichiometry of all equations is shown in equation 1.4. One mole of glucose can be converted to form molecular hydrogen for 12 moles, however, the process is now being developed (Yokoi *et al*., 2002 and Tanisho and Ishiwata, 1995).

$$6\ H_2O + 6\ CO_2 \longrightarrow C_6H_{12}O_6 + 6O_2 \quad (1.1)$$

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2 \quad (1.2)$$

$$2\ CH_3COOH + 4\ H_2O \longrightarrow 8\ H_2 + 4\ CO_2 \quad (1.3)$$

$$12\ H_2O \longrightarrow 12\ H_2 + 6O_2 \quad (1.4)$$

1.2.4.3 Biological water-gas shift reaction

The biological water-gas shift reaction is the process to produce hydrogen gas via photoheterotrophic bacteria such as *Rhodospirillum rubrum*. Carbon monoxide (CO) can be used as a carbon source and metabolized to produce ATP coupled with water reduction reaction (Yokoi *et al*., 2001). Hydrogen molecule can be formed as shown in the following equation;

$$CO + H_2O \longrightarrow CO_2 + H_2 \qquad \Delta G^0 = -20.1 \text{ kJ/mol}$$

Gram negative bacteria such as *Rubrivax rubrum*, *Rubrivax gelatinosus*, and gram positive bacteria such as *Carboxydothermus hydrogenoformans* can produce biohydrogen through biological water-gas shift reaction. The bioproduction by this process, however, is studied in lab scale.

1.2.4.4 Photo-fermentation

The photo-fermentation is the bioproduction process found in photosynthetic bacteria containing nitrogenase enzyme. The pathway begins with the organic acids e.g. acetic acid, lactic acid, and lactic acid is metabolized under anaerobic condition. Electron can flow to nitrogenase enzyme via ferredoxin protein (Fd) as shown in Figure 1.2. Nitrogenase, however, consumes large amount of ATP to function, thus the efficiency of energy production is still low.

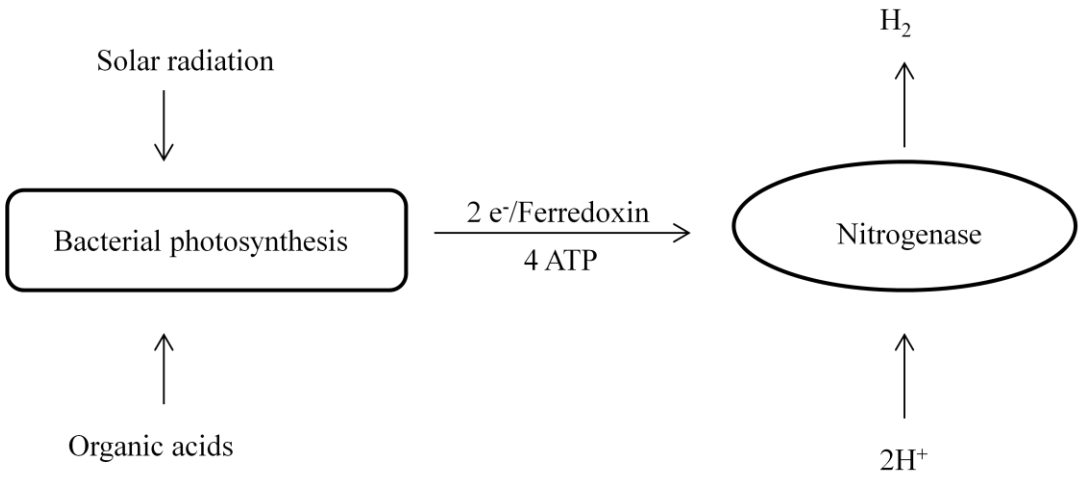


Figure 1.2 The schematic of hydrogen production by photo-fermentation process

1.2.4.5 Dark fermentation

The dark fermentation is the fermentation process under the darkness found in anaerobic bacteria and microalgae. The reaction can be metabolized through two reactions as shown in equation 1.5 and 1.6. The bioproduction by dark fermentation produce carbon dioxide (CO_2) as a by-product, while the photo fermentation only produces hydrogen gas.

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 2\ CH_3COOH + 4\ H_2 + 2\ CO_2 \quad (1.5)$$

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow CH_2CH_2CH_2OOH + 2\ H_2 + 2\ CO_2 \quad (1.6)$$

The production by this method is widely used because of the high efficiency, need less arable land, and no effect by the climate change.

Overall, there are advantages and disadvantages for each process as listed in Table 1.2. The direct biophotolysis by biological process shows the highest possibility for sustainable production since it has high efficiency in energy conversion, the process use low cost of production from culture and temperature control for the process, and green organisms can trap atmospheric carbon dioxide which is the majority of green house gas. The study will mainly focus on the biohydrogen production in green algae.

Table 1.2 Advantages and disadvantages of different biological processes for H_2 production (Das and Veziroglu, 2001)

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

1.3 Green algae

The green algae are a typical group of algae, which appeared from the higher plants (embryophytes) (Jeffrey *et al*., 2004). As such, they form a group consisting of all hypothetical closest common ancestors, although the group including both green algae and embryophytes is monophyletic (and often just known as kingdom Plantae). The green algae include unicellular and colonial flagellates. Two flagella per cell are usually found but not all species such as various colonial cells, coccoid-shaped, filamentous forms, and macroscopic seaweeds. There are about 6,000 species of green algae (Thomas, 2002). Many species live mostly as single cells during their life cycle, while other species form long filaments, colonies, coenobia, or highly differentiated to macroscopic scale.

1.3.1 Cellular structure

Chloroplasts can be found in almost all forms of green algae. Chlorophylls a and b give them a bright green color (as well as the other accessory pigments: beta-carotene and xanthophylls) (Burrows. 1991) and have thylakoid stacking (Hoek, 1995). All green algae also have flat cristae-form mitochondria. Their movement is based on flagella which are basically anchored by the cross-shaped system of fibrous strands and microtubules, but these structure are absent in higher plants and charophytes, the common name for green plants in the order Charophyceae where a raft of microtubules is found instead. Green algae themselves use flagella to move. They normally have cellulose-containing cell walls, and use no centrioles in mitosis during cell division.

1.3.2 Origins

Chloroplasts of green algae are surrounded by a double layer of lipid membrane, so probably they are fused by endosymbiosis of cyanobacteria during the evolution. Many cyanobacteria show similar pigment composition to the green algae such as *Prochloron* sp. Cyanobacterial endosymbiosis occurs to have more than one pigmentation, as found in the Rhodophyta, red algae. Certainly, the green algae probably adopted their chloroplasts from a Prochloron-type cyanobacterial ancestor, and evolved separately from the red algae.

1.3.3 Classification

Green algae are usually classified with a number of embryophyte descendants in the green plant phylogenetic tree of Viridiplantae, the green algae and land plants. Viridiplantae form a supergroup the so-called Primoplantae, which also known as Archaeplastida comprising green algae, red algae, and land plants (Figure 1.3). Classification systems of Protista may include green algae into the Protista or in the Plantae Kingdoms (Cavalier-Smith, 1993).

Mesostigma is one of the most basal flagellate green algae, although it is still unclear whether it is sister to all other green algae, or one of the more basal members of the Streptophyta (Jeffrey *et al*., 2004 and Simon *et al*., 2006).

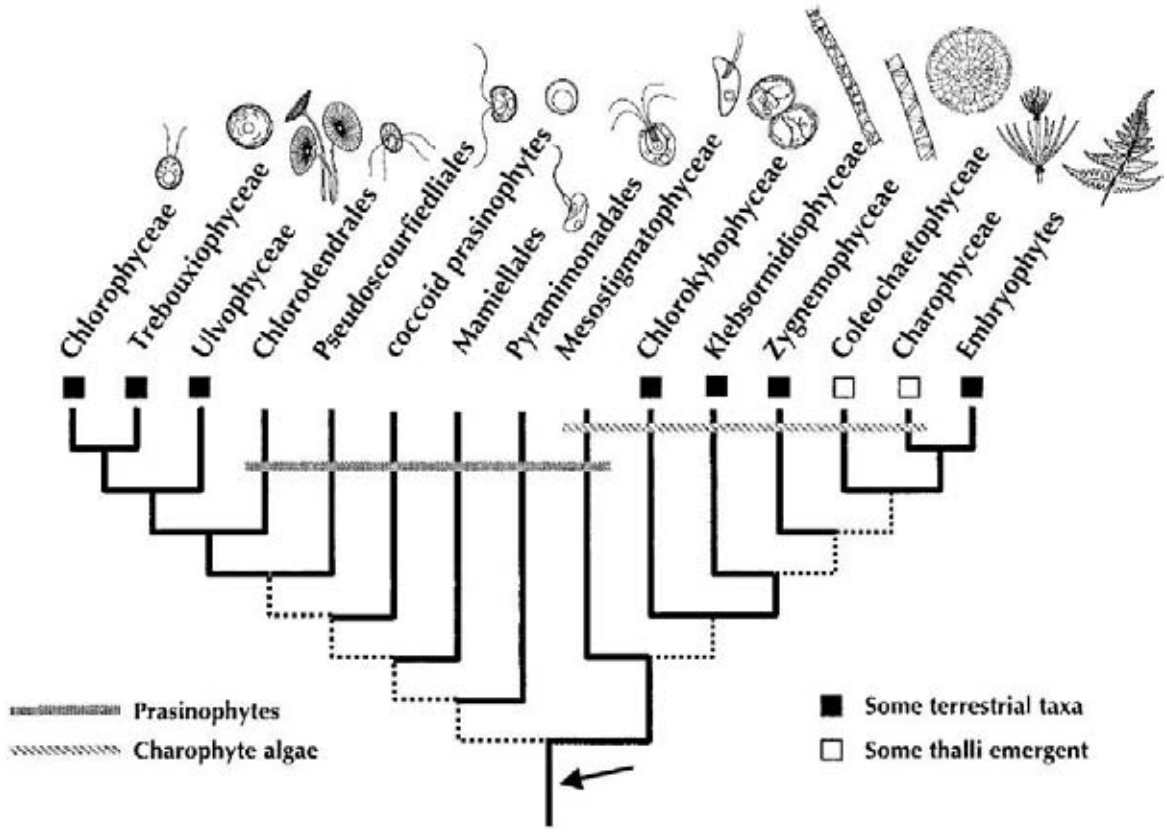


Figure 1.3 Summary of the phylogenetic determination among the major relatives of green algae analyzed by DNA sequence analysis. Branches of the tree indicated by dotted lines show relationships that are weakly supported by molecular data. Light gray bar represented the charophyte algae indicate barely resolved regions based on Karol *et al*. (2001). The arrow at the origin of the tree indicates the possibility of Mesostigma placement supported by Lemieux *et al*. (2000) and Turmel *et al*. (2002). Boxes at the edges of branches indicate the lineages containing at least some physical taxa (solid boxes) or emergent taxa (open boxes). No box shows taxa in aquatic group. The drawings are thumbnail views meant to show taxa representative (Lewis, L.A. and McCourt, R.M. 2004)

1.4 H_2 production in green algae

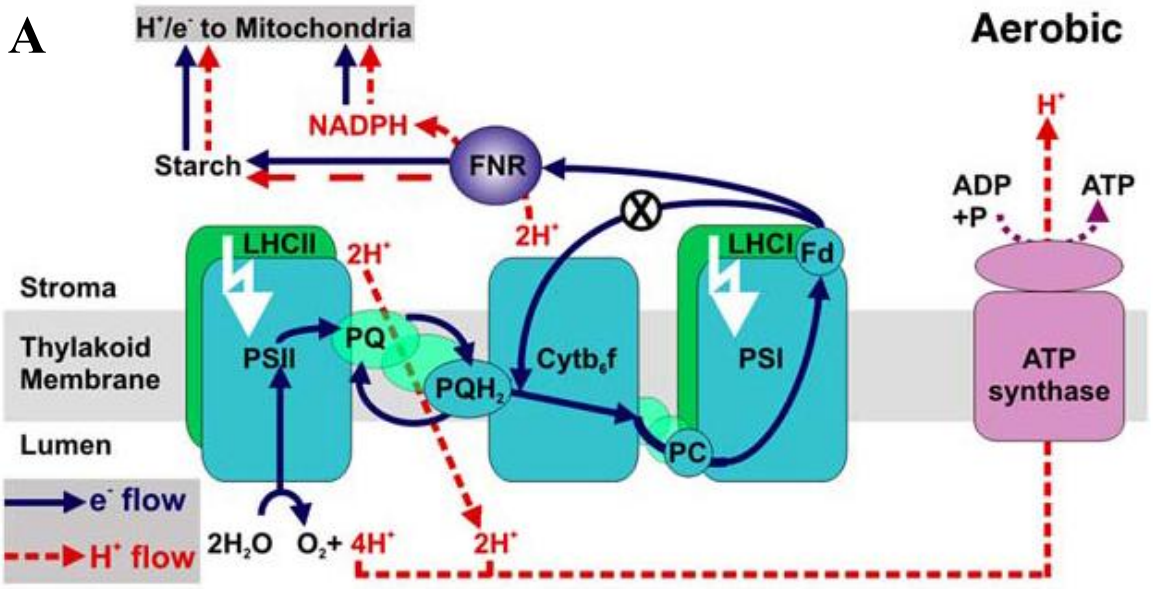
1.4.1 Background

Phototrophic algae have the notable ability to generate low-potential electrons from water oxidation by the photosynthetic system II (PSII) in photosynthesis system. Those electrons are partly used to reduce protons into hydrogen molecule. Consequently, the renewable biohydrogen production can be obtained in these photosynthetic microorganisms using solar radiation as energy source. When hydrogen gas is used as an energy carrier in fuel cell system, only environmentally friendly water vapor is emitted from the combustion reaction. By the microalgae system, the sustainable biohydrogen production can be realized to reach sufficient quantities of H_2 in a cost effective means. However, many researches *in vivo* and *in vitro* aim to understand the biological production process. Many proteins/enzymes are involved in algal hydrogen metabolism.

Historically, hydrogen production in a green alga was firstly discovered over 65 years ago in experiments performed by Hans Gaffron and teams (Gaffron and Rubin, 1942, Homann, 2003, and Melis and Happe, 2004). Subsequently, several diverse phototrophic microorganisms including a number of *Chlamydomonas* species have been shown to produce hydrogen gas through the catalysis of hydrogenase enzyme. To date, many green algae show the hydrogenase activity; however, not all of genera of green algae contain the activity (Melis A and Happe T, 2004 and Boichenko *et al*., 2004).

The production process by this manner sometimes called two-phase system comprises growth phase under aerobic condition and production phase under anaerobic condition. During the growth phase, the generated electrons from PSII system mainly flow to ferredoxin (Fd) followed by ferredoxin–NAD^+ reductase (FNR) prior to being accepted by starch metabolism, while the protons flow to ATP synthase forming ATP

molecules (Figure 1.1A). In production phase, the atmospheric oxygen is replaced by argon gas resulting in no oxygen molecule, which acts as hydrogenase inhibitor. Moreover, the PSII-generated oxygen can be overcome by the respiratory chain in mitochondria. The biohydrogen production starts when electrons flow to hydrogenase (Hyd) instead of FNR during aerobic condition (Figure 1.1B).



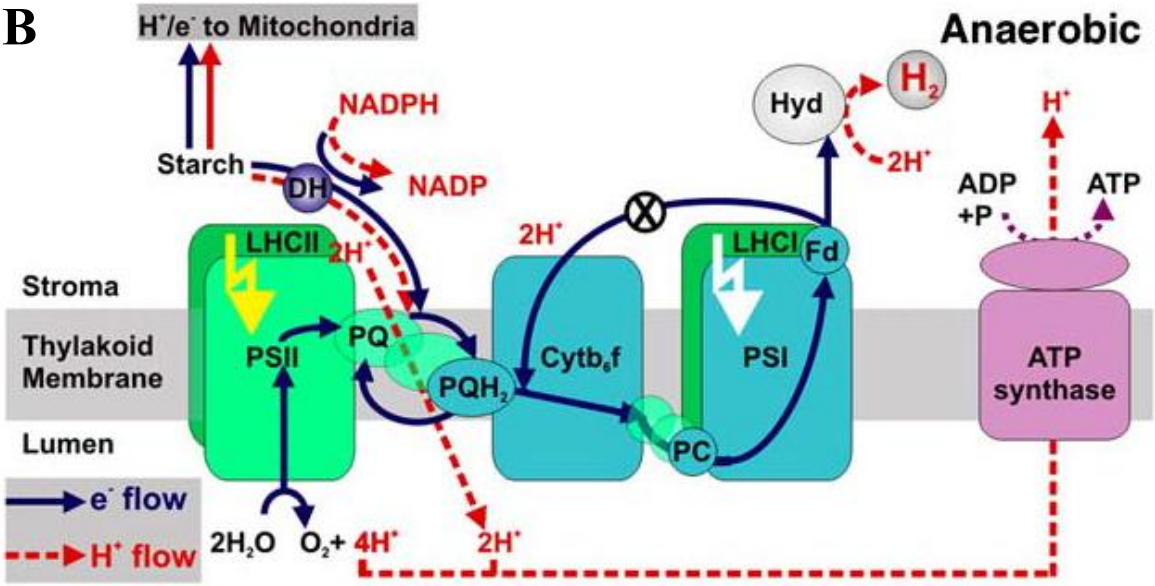


Figure 1.4 Schematic hydrogen metabolism during (a) the growth phase (aerobic condition) and (b) the production phase (anaerobic condition) in hydrogen production process occurred the chloroplast of the microalga *C. reinhardtii*. Both direct substrate supply from photosynthesis and indirect substrate supply via starch degradation are shown. PSII = Photosystem II; Cytb6f = cytochrome b6f; PSI = Photosystem I;PQ = plastoquinone; PC = plastocyanine; FD = ferredoxin;ATPase = ATP synthase; HydA = Hydrogenase A. X indicates the cyclic electron transport pathway that competed with HydA for electrons and that is blocked in Stm6 *C. reinhardtii* mutant (Kruse and Hankamer, 2010).

1.4.2 Hydrogenases

Biohydrogen production process in photosynthetic microorganism is metabolized by hydrogenase or nitrogenase enzymes (Weaver *et al*., 1980, Boichenko and Hoffmann, 1994, Tamagnini *et al*., 2002, Boichenko *et al*., 2004, and Kruse *et al*., 2005b). However, the nitrogenase is only found in filamentous cyanobacteria such as *Anabaena* sp., only hydrogenase will further be discussed. Two classes, [FeFe]- and [NiFe]- hydrogenases, enzymes are mostly found in green algae catalyzing the reversible reaction of hydrogen oxidation. The class of enzyme is classified from their respective active sites. To date, only [FeFe]-hydrogenases are found in green algae, and [NiFe]-hydrogenases are found in cyanobacteria (Ludwig *et al*., 2006). Although both classes of hydrogenase have no relationship genetically, they share some common features (1) the novel CO and CN^- ligands are found in active site, and (2) the active site is composed of binuclear metal center. Basically, [NiFe]-hydrogenase catalyzes the reversible hydrogen oxidation,

whereas [FeFe]-hydrogenase catalyzes only the formation of hydrogen gas (Frey, 2002). In addition, the turnover rate of [FeFe]-hydrogenase is generally 10 - 100 times higher than that of [NiFe]-hydrogenase (Frey, 2002). This probably makes green algae more efficient H_2 production than cyanobacteria.

The *Scenedesmus obliquus* algal [FeFe]-hydrogenase gene sequences were firstly published (Florin *et al*., 2001 and Wünschiers *et al*., 2001). Additional sequences have subsequently been reported for *Chlamydomonas hydA1* (Happe and Kaminski, 2002) and *hydA2* (Forestier *et al*., 2003), *Chlorella fusca* (formerly *Scenedesmus vacuolatus*) *hydA* (Winkler *et al*., 2002a), *Scenedesmus obliquus hydA* (Florin *et al*., 2001), *Chlamydomonas noctigama hydA1-3* (Skjånes K *et al*., 2010), and *Chlamydomonas moewusii hydA2* (Winkler *et al*., 2004). All reported algal [FeFe]-hydrogenase genes encode a monomeric protein with the size of about 48 kD, which are among the smallest characterized [FeFe]-hydrogenases (Happe *et al*., 2002). Green algae having hydrogenase activity often contain two isoforms of the [FeFe]-hydrogenase enzyme, *hydA1* and *hydA2* (Forestier *et al*., 2003 and Winkler *et al*., 2004), except *C. noctigama* containing 3 genes. Although the precise function of each enzyme to H_2 metabolism is currently unknown, three proposed involvements are: redox balance, photoprotection, and fermentative metabolism.

1.4.3 Oxygen sensitivity

Based on the *Chlamydomonas* system, the hydrogenases are sensitive to O_2 and are irreversibly inactivated within minutes after exposure to a level of O_2 in atmosphere (Abeles, 1964, Erbes *et al*., 1979, Happe and Naber, 1993, Ghirardi *et al*., 1997, Cohen *et al*., 2005, and King *et al*., 2006). Moreover, those enzymes show the most O_2-sensitive hydrogenases among all tested hydrogenases from other species under the same assay

condition including clostridial [FeFe]-hydrogenases (Cohen *et al*., 2005 and King *et al*., 2006). It is understandable that hydrogenases in green algae evolved O_2 hypersensitivity to control hydrogenase activity under the presence of O_2, when the cells prefer to uses higher energy-yielding metabolic pathway that require O_2 as the terminal electron acceptor. Lee and Greenbaum (2003) proposed the levels of about 0.1% O_2 can inhibit hydrogenase activity *in vivo* and an undefined metabolic pathway is activated, perhaps related to CO_2 fixation, to compete with hydrogenase for photosynthetic reductant contributing to the observed attenuation of H_2 photoproduction activity in the presence of O_2. The assay of hydrogenase activity in whole cells and cell-free extracts of *Scenedesmus* and *Chlamydomonas* showed the losts of activity within 5 minutes after exposure to O_2 (Urbig *et al*., 1993). In whole cell assay, reversible inactivation occurred upon repeated degassing and readapting cells with similar production rates to those measured after the first anaerobic adaptation. In contrast, the activity of enzymes in cell-free lysate could not be recovered even after prolonged degassing, consistent with the previously reported irreversible inactivation of [FeFe]-hydrogenases by O_2 (Adams, 1990).

Among the different enzymes tested to date, the [FeFe]-hydrogenases inactivation by O_2 are shown in variable rates. For purified enzymes expressed in *E. coli* system, the time required for 50% inhibition of hydrogenase activity after exposure to atmospheric levels of O_2 ranged from 2 seconds to 3 seconds for the *Chlamydomonas hydA1*, and for about 100 seconds for *Clostridium acetobutylicum* [FeFe]-hydrogenase (Cohen *et al*., 2005 and King *et al*., 2006). A truncated form of the *C. acetobutylicum* hydrogenase lacking FeS cluster binding domain at the N terminus was generated. This enzyme showed the same structure to algal hydrogenase, which contained H-cluster catalytic domain to catalyze the formation of hydrogen gas. The N-terminus truncated enzyme

remains less O_2 sensitivity indicating that the presence of the N-terminal domain of the native *C. acetobutylicum* enzyme is not required for the comparatively prolonged hydrogenase activity observed after exposure to O_2 (Cohen *et al*., 2005 and King *et al*., 2006).

1.4.4 Sulfur deprivation

Short-term method to avoid the O_2 sensitivity of algal H_2 production is developed by Melis *et al*. in 2000. The sustainable H_2 production can be achieved in approximately 4 days, demonstrating the potential of large scale for H_2 production (Happe *et al*., 2002, Melis *et al*., 2004, Melis *et al*., 2007, Ghirardi, 2006, and Seibert *et al*., 2008). Sulfur deprivation results in the partial and reversible inhibition of O_2 evolution. In the absence of sulfate, protein synthesis is geared towards those products that are necessary for viability under conditions of decreased metabolic activity (Wykoff *et al*., 1998). As a consequence, the D1 reaction center of PS II cannot be replaced quickly due to the rapidly photodamage, leading to the gradual loss of O_2 evolution. Simultaneously, as a response to nutrient depletion stress, sulfur-deprived algae start to accumulate starch in 8 – 20 times higher than that under normal conditions (Tsygankov *et al*., 2002 and Zhang *et al*., 2002). When the rate of O_2 evolution decreases below that of O_2 consumption in respiratory, the cultures rapidly shift to anaerobic condition (Antal *et al*., 2003). Under this condition, the cultures trigger several effects (Melis and Happe, 2001): (1) HydA1 and HydA2 are activated, and the activity can be observed after 3 - 4 hours (Forestier *et al*., 2003), (2) measurable H_2 production is monitored after another 5 hours (Antal *et al*., 2003), (3) starch accumulation is terminated and is replaced by degradation (Tsygankov *et al*., 2002 and Zhang *et al*., 2002), and (4) starch degradation generates reductant for H_2

production. It is known that no CO_2 fixation occurs during sulfur deprivation (Melis *et al*., 2000), as demonstrated by the early loss of Rubisco activity (Zhang *et al*., 2002).

1.5 Objectives of this research are:

1. To screen novel hydrogen-producing green microalgae for alternative choice of production
2. To optimize the condition for growth and hydrogen production process
3. To determine gene(s) involved in hydrogen production metabolism and understand those functions

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

All-glass vacuum filter holder, 47/50mm, Sartorius, Belgium

Autoclave, Model HA-30, Hirayama Manufactering, Cooperation, Japan

Autoclave, Model MLS-3020, Sunyo, Japan

Autopipette, Pipetman, Gilson, France

Balance, Mettler Toledo AB 204-S, USA

Balance, Mettler Toledo PB 303-S, USA

Balance, Sartorius LC 620 S, USA

Bead Beater, Precellys Bead Beater, Bertin technologies, France

Biolistic® PDS-1000/He Particle delivery system, Bio-Rad, USA

Centrifuge, Model 5804R, Eppendorf

Centrifuge, Refridgerated centrifuge, model 5417R, Eppendorf

Desiccator, Pyrex, USA

Digital lux meter, model FT-710 Faithful, Taiwan

Electrophoresis Unit, Horizontal chamber, Bio-Rad, USA

Filter membrane 0.45 μm, Millipore, USA

Gas Chromatography, Model GC-15A, Shimadzu, Japan

Gel documentation (gel doc), Syngene, UK

Incubator, Model OB-28L Fisher Scientific Inc., USA

Laminar flow, BVT-124 International Scientific Supply, Thailand

Light microscope, SEEK model SK-100, USA

Light source unit, Prekeo S250 Zeiss IKON, Japan

Magnetic stirrer with heater, Model IKAMA®GRH, Japan

Microcentrifugefuge tubes, Bioactive, Thailand

Orbital shaker, Gallenkamp, Germany

PCR apparatus, Mastercycler Gradient, Eppendorf

pH meter, Model PHM95, Radiometer Copenhegen, Denmark

pH meter, ORION model 420A, USA

Photometer, LI-COR Model LI-185B, USA

Power supply, BIO-RAD POWER PAC 1000, USA

Power supply, Model EC 139-90, E-C Apparatus Corporation

Scanning Electron Microscope, JEOL model JSM-5410LV, Japan

Shaker InnovaTM, 2100 PLATFORM SHAKER, USA

Spectrophotometer, Jenway 6400, England

Spectrophotometer, SPECTRONIC® GENESYSTM2, USA

Transformation apparatus, Bio-RAD, USA

Vortex mixer,Fisher Scientific model 202, USA

Vortex, Model K-550-GE, Scientific Industries, USA

Water bath, Charles Hearson Co. Ltd., England

Water bath, THERMOMIX® B B.BRAUN, USA

2.2 Chemicals

Acetic acid, BDH, England

Agarose, Promega Corporation, USA

Ammonium chloride, Sigma, USA

Ammonium sulfate, Sigma, USA

Bromophenol blue, Sigma, USA

Calcium chloride, Sigma, USA

Chloroform, Merck, Germany

Cobolt nitate, Sigma, USA

Copper chloride, Sigma, USA

Copper sulfate, Sigma, USA

Dithiothreitol (DTT), Sigma, USA

EDTA, Sigma, USA

Ethanol, Scharlau Chemie S.A., Spain

Ethidium bromide, Sigma, USA

Fructose, Sigma, USA

Galactose, Sigma, USA

Glucose, Sigma, USA

Glycerol, Scharlau Chemie S.A., Spain

Guanidine hydrochloride, Sigma, USA

Imidazol, Sigma, USA

Iron (II) sulfate, Sigma, USA

Isoamylalcohol, Sigma, USA

Isopropanol, Sigma, USA

Lactose, Sigma, USA

Lithium chloride, Sigma, USA

Magnesium acetate, Sigma, USA

Magnesium chloride, Sigma, USA

Magnesium sulfate, Sigma, USA

Maltose, Sigma, USA

Manganese chloride, Sigma, USA

β-mercaptoethanol, Sigma, USA

Phenol, Merck, Germany

Potassium chloride, Sigma, USA

Potassium di-hydrogen phosphate, Sigma, USA

Potassium hydrogen carbonate, Sigma, USA

di-Potassium hydrogen phosphate, Sigma, USA

Potassium iodide, Sigma, USA

Potassium nitrite, Sigma, USA

Potassium sodium tartrate, Sigma, USA

Sodium acetate, Sigma, USA

Sodium bicarbonate, BDH, England

Sodium bromide, Sigma, USA

Sodium carbonate, Sigma, USA

Sodium chloride, APS, Australia

Sodium citrate, Sigma, USA

Sodium dodecyl sulfate, Sigma, USA

Sodium hydrogen carbonate, Sigma, USA

Sodium molybdate, Sigma, USA

Sodium nitrate, Sigma, USA

Sodium nitrite, Sigma, USA

Sodium sulfite, Sigma, USA

Sodium thiosulfate, Sigma, USA

Sorbitol, Sigma, USA

Sucrose, Sigma, USA

Tris base, USB Corporation, USA

Zinc acetate, Sigma, USA

Zinc sulfate, Sigma, USA

2.3 Enzymes

Cellulase ONOZUKA R-10, Phyto Technology Laboratories, USA

Dream Taq polymerase, Fermentas, Canada

Lysosyme, Sigma, USA

Quick ligase™ Kit, BioLabs® Inc, USA

Restriction enzymes, Fermentas, Canada

Shrimp Alkaline Phosphatase (SAP), Fermentas, Canada

T4 DNA ligase, Fermentas, Canada

T4 DNA Polymerase, Fermentas, Canada

Taq DNA polymerase, Invitrogen, USA

2.4 Antibiotics

Ampicillin, Sigma, USA

Chloramphenicol, Sigma, USA

Neomycin, Sigma, USA

Paromomycin, Sigma, USA

2.5 Kits and suppliers

5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Invitrogen

PCR purification kit NucleoSpin® Extract II, Machery-Nagel, USA

GeneRuler™ 1 kb DNA Ladder, Fermentas, Canada

GeneJET™ Plasmid Miniprep Kit, Fermentas, Canada

GeneJET™ Gel Extraction Kit, Fermentas, Canada

λ DNA/*Hind*III Marker, Fermentas, Canada

iScript™ cDNA Synthesis Kit, Bio-Rad, USA

2.6 Bacterial strains

Escherichia coli DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–) was used for DNA manipulation.

Escherichia coli Top10 (F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-) was used for DNA manipulation.

Escherichia coli XL1-Blue (endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)) was used for DNA manipulation.

2.7 Plasmid vectors

The pGEM T-easy and pCR®2.1-TOPO® vector were used as an alternative vector for TA cloning. See the maps in appendix A.

The pSB1AC3 was used as a vector providing Chloramphenicol resistance cassette.

The pUC4K was used as a vector providing Neomycin/Kanamycin resistance cassette.

The pSI103-1 was used as a vector providing Paromomycin resistance cassette.

2.8 Oligonucleotides

Table 2.1 Primer listed used in this study

No.	Primer name	Sequence in 5' to 3' direction
1	18SF	CAG CAG CCG CGG TAA TTC
2	18SR	CAT CTA AGG GCA TCA CAG ACC
3	18SWalkto5	ATA GGC GGA CCG CTA GAAC
4	18SWalkto3	GTT GAT TCC GGT AAC GAA CG
5	YL	CGG TAG GAT CCC GCA GAA CGA CGG CCA G
6	YA	CGG TAG GAT CCC GCA GAA C
7	YS	GTT CTG CGG GAT CCT ACC G
8	SdegF	GAK GCD GCB RTD GAY YTD CC
9	SnestedF	CTG CCG TTC GCK CTG C
10	SdegR	RTA RTC RTA YTG YTC YAR
11	C3RF	CCA CAT CAG TAG CTG GAT TGA C
12	C3RR	TCA ACA CTG GCG CTA TCA AG
13	SMutNdeIF	TGC AGC CTG TTC ATA TGG TAA GAC CAG C

Table 2.1 (continued)

14	SMutNdeIR	GCT GGT CTT ACC ATA TGA ACA GGC TGC A
15	5UTR	TGC TCC TGA CAC TCG ATT TG
16	SMutSmaIF	TTT CTG TTG GCC CGG GTG CTG GTG AA
17	SMutSmaIR	TTC ACC AGC ACC CGG GCC AAC AGA AA
18	polyT-TAG	CAA CAG ACG CAC GAC GCA GCA GAC TTT TTT TTT TTT TTT TTT TT
19	TAG	CAA CAG ACG CAC GAC GCA GCA GAC
20	sulp3RACE	CCA CAT CAG TAG CTG GAT TGA C
21	sulP5RACE	TCA GCA AAC GAC CAA GAA TG
22	PmFClaI	ATC CAT CGA TGC CGC TCT AGA ACT AGT GGA
23	PmRNruI	ATC GTC GCG AAC GCC AAG CTC GAA ATT AAC
24	SulPForClaI	ATC CAT CGA TAA TGC AGC CTG TTC TTC AGG
25	SulPRevNruI	ATC GTC GCG ACT GCC ATG GTA CGT CAC TGC
26	In2F	CTT AGG CTG GCT CAC TCC TG
27	Ex3F	AGC AGT CAT GTT GGT GTT GC
28	Ex4R	CTG AAG AAC AGG CTG CAT TG
29	PmF	ACG GTG ACC TCC ACT TTC AG
30	PmF1	ACG GTG ACC TCC ACT TTC AG
31	PmR	AAC ACC ATC AGG TCC CTC AG
32	HydF	TGC CCA TGT TCA CCA G

Table 2.1 (continued)

33	HdegR	ACR TAG TGD GTG TGC AG
34	SulRTF	AGC AGT CAT GTT GGT GTT GC
35	SulRTR	TCA GCA AAC GAC CAA GAA TG
36	HydRTF	AAC TTC GAC AAC CCT GTT GG
37	HydRTR	TTC TTA GCA TTG CCC AAA CC
38	18SRTF	TGC GTA AAT CCC GAC TTC TG
39	18SRTR	AAC CCT AAT CCT CCG TCA CC

2.9 Green algae screening for H_2 production

2.9.1 Sampling and purification

The sterile 200 mL sampling bottles were prepared containing 20 mL of 10-fold dilution BG11 medium in order to maintain the nutrient sources for microorganisms. Water samples from fresh water sources including natural ponds and paddy fields were collected. An equal volume of collected water was poured into sampling bottles, loosely capped and placed in shaker under continuous light illumination at 30 $^{\circ}$C for several weeks until the culture become light greenish. A 300 uL of greenish culture was plated on BG11 agar medium plate and placed under light for several weeks until green colonies were observed. Every single colony was picked and streaked onto a new solid agar medium plate. The re-streaking process was repeated to purify the culture until the contaminant was eliminated.

2.9.2 Screening for H_2 production

A purified culture was inoculated into a flask containing 100 mL liquid BG11 medium. Culture was placed with shaking at 160 rpm under continuous light illumination at 30 °C for a week. Culture was then concentrated for 20 fold by centrifugation at 6000xg for 5 min, and resuspended by the old medium. A 5 mL of concentrated culture was transferred to 13 mL gas-tight vial, capped and bubbled with argon gas at 0.2 bar for 5 min to eliminate atmospheric oxygen in solution and head space of vial. The vial was then blocked from light by using aluminium foil wrapping, and incubated at 30 °C for 4 h prior to biohydrogen production measurement.

2.9.3 Measurement of H_2 production

H_2 concentration in the head space of gas-tight vial was determined using a Gas Chromatography. A 0.4 mL of headspace gas was withdrawn by 0.4 mL argon gas replacement, and injected into gas chromatograph using a gas tight syringe. The samples were separated and analyzed by a Gas Chromatograph (GC-15A, Shimadzu, Kyoto, Japan, 2 m stainless column packed with molecular Sieve 5A (pore size 5 Å), Mesh 60/80) equipped with a thermal conductivity detector. The injector and detector were maintained at 100 °C. The column oven temperature was 50 °C with argon carrier gas at the flow rate of 30 ml min^{-1}. The calculation method was noted in appendix B.

2.10 Strain identification

2.10.1 Morphology observation

2.10.1.1 Light microscope observation

Sample culture of the interesting isolate was sent to the Thailand Institute of Scientific and Technological Research (TISTR) to determine its identity by light microscopic observation for 21 days. Culture was grown for a week in liquid BG11 medium and aliquot for 10 mL into screw-cap glass tube prior sending to TISTR by post.

2.10.1.2 Scanning Electron Microscope observation

To determine the morphology surface observation by Scanning Electron Microscope (SEM), 5 mL of the same culture was sent to Testing Laboratory Scientific and Technological Research Equipment Center, Chulalongkorn University. Samples were filtered through 0.45 μM membrane and fixed by submerging the membrane in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for overnight. The membrane was then washed twice with the same phosphate buffer in the absent of glutaraldehyde followed by deionized water once for 10 min. The sample was dehydrated by submerging the membrane in each ethanol solution for 5 min with the concentration as the following step: 30%, 50%, 70%, 90%, and 100%, respectively. The sample was then dry to eliminate trace ethanol using Critical point dryer. The membrane was then attached to the stub and coated with gold using ion sputter and visualized under Scanning Electron Microscope (JEOL model JSM-5410LV, Japan).

2.10.2 18S rDNA identification by molecular technique

2.10.2.1 Primer design

To confirm the selected strain of culture, the *18S* ribosomal DNA gene determination was performed. The algal cells were used as template for PCR amplification of the *18S* rDNA with a pair of oligonucleotide primers (18SF and 18SR). The two primers were designed based on the conserved region when aligned with the *18S* rDNA gene sequences available in the NCBI nucleotide database from seven different green algae: *Ankistrodesmus gracilis* (Acc No. Y16937), *Monoraphidium dybowskii* (Acc No. Y16939), *Scenedesmus regularis* (Acc No. AB037095), *Chlorella ellipsoidea* (Acc No. X63520), *Chroomonas caudate* (Acc No. AB240963), *Rhodomonas duplex* (Acc No. AB240960), *Chlamydomonas reginae* (Acc No. DQ009749). The positions of 18SF and 18SR primers were indicated in Figure 3.3 and their sequences were listed in section 2.8.

2.10.2.2 Polymerase Chain Reaction (PCR) and cloning

PCR amplification was performed as followed: pre-denaturation at 94 ºC for 2 minutes, 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 30 seconds, extension at 72 ºC for 1 minute. The final extension step was performed at 72 ºC for 3 minutes. An expected PCR product size of *18S* rDNA was approximately 882 base pairs. A band was cut and purified by GeneJET™ Gel Extraction Kit. After column elution, the DNA fragment solution was ligated to the pGEM-TEasy vector. The recombinant plasmid ligation reaction was transformed into an *E. coli* strain DH5α using calcium chloride method (Sambrook and Russel, 2001). The transformants were selected using the blue/white screening on the ampicillin agar plates containing 40 μg/mL X-gal. A single white colony of *E. coli* harboring a recombinant plasmid was grown in 1.5 ml

LB broth containing 100 μg/ml of ampicillin and incubated with shaking at 37 ºC for 16 h. The recombinant plasmid was prepared by a GeneJET™ Plasmid Miniprep Kit. The plasmid was cut to check the size of an insert by using EcoRI restriction enzyme digestion. The fragments were analyzed by 0.8% agarose gel electrophoresis. The size corresponded fragment containing plasmid was sent to sequence**.**

2.10.2.3 Genomic DNA extraction

A loop of scraped culture was inoculated into fresh 100 mL TAP medium in 250 mL e-flask. Culture was then grown at 36 ^{o}C for 2 days and harvested by centrifugation in 50 mL plastic tube at 4,500x*g* for 2 min. Cells were washed once with equal volume of 40 mM sodium acetate buffer pH 5.0 and resuspended in 5 mL of the same buffer. A 10 μL of 50 mg/mL cellulase was added to the mixture. The tube was incubated at 37 ^{o}C for 30 min with gently mixing on every 10 min to resuspend the precipitated cells. After incubation, a 1 mL of 20% SDS and 0.5 μL of 50 mg/mL RNaseA were added to the mixture and gently mixed then incubated at 37 ^{o}C for 30 min. Consequentially, 6 mL of TE-saturated phenol was added and gently mixed by rotatory shaking at room temperature for 30 min. The tube was then centrifuged at 4,500x*g* for 10 min at 25 ^{o}C to remove cell debris. The colorless upper aqueous phase was transferred to a new microcentrifuge tube. An equal volume of chloroform was added to the tube and mixed gently prior to centrifugation. The DNA in an aqueous phase was precipitated by the addition of 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volume of absolute ethanol. The mixture was stored at -20^{o}C for overnight and centrifuged to collect the DNA at 12,000x*g* for 10 min at 4^{o}C. The DNA pellet was washed once with 70 % ice-cold ethanol and then air-dried for 30 min. The genomic DNA was dissolved with 100 μl

of TE buffer, determined and estimated the concentration by measuring the absorbance at 260 nm, and calculating in μg/ml unit, using the following equation:

$$[DNA] = A_{260} \times \text{dilution factor} \times 50^*$$

* The absorbance at 260 nm of 1.0 corresponds to the DNA of approximately 50 μg/ml (Sambrook and Russel, 2001).

2.10.2.4 18S rDNA walking by YADE method

A partial 18S rDNA sequence was used as a template to design the walking specific primer to 5'- and 3'- direction. The walking primer, 18SWalkto5 and 18SWalkto3, sequences were listed in section 2.8

The extended sequence of *18S* rDNA was obtained following the YADE method (Xiao Y.H. *et al*. 2002). A 1 μg of genomic DNA was digested by 4 blunt-end restriction enzymes in a total mixture volume of 40 μL. The reaction was incubated at 37 ^{o}C for 1 h prior to directly do the blunt-end ligation reaction to the YADE adaptor. The adaptor of YADE method was prepared by using the YL and YS primers.

A 10 μM of YL and YS primers were annealed at the following condition: 1X annealing buffer with 90 ^{o}C for 10 min and then cooled down to room temperature. The 1 X buffer composed of 100 mM NaCl, 10 mM Tris pH 7.0, 1 mM EDTA.

The PCR was performed by using adaptor-specific primer (YA) with either 5'- or 3'- walking primer. PCR amplification was performed as follows: pre-denaturation at 94 ºC for 3 min, 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 55 ºC for 30 sec, extension at 72 ºC for 1 min. The final extension step was performed at 72 ºC for 3 min. The PCR reaction was then electrophoresed, cut, and cloned prior to sequencing.

2.11 Phylogenetic tree determination

The required sequence of algal DNA/protein sequence was used to generate the phylogenetic tree against other known sequences available in NCBI database. All sequences for desired gene were retrieved and prepared in Fasta format in the same file and used in doing the complete alignment by Clustal-X program version 1.83 (Jeanmougin *et al*. 1998). The alignment output file was further used in Bootstrap NJ phylogenetic tree determination by the same program. The tree was viewed by TreeView (win32) program version 1.6.6 (Roderic, 2001).

2.12 Growth optimization

2.12.1 Media optimization

Culture will be optimized for suitable growing culture media including normal culture media and nitrogen-deprived media to check whether the cell can fix atmospheric nitrogen. Cells were grown in BG11 liquid medium at 30 ^{o}C for a week, and then harvested by centrifugation at 6,000x*g* for 1 min. Cells were washed twice with equal volume of sterilized water prior to measuring optical density at 730 nm by spectrophotometry. Cells were then inoculated to the final optical density of 0.1 into various media including Tris-Acetate-Phosphate (TAP) (Harris, 1989), BG11 (Rippka, 1979), N8 (Vonshak, 1986), N-free (Stewart, 1962), $BG11_0$ (BG11 minus NaNO3), Allen & Arnon's (AA) (Arnon, *et al*. 1974), and Zarrouk media (Zarrouk, 1966). Media compositions were listed in appendix C. The cultures were placed in shaker with speed of 160 rpm under continuous light illumination at 30 ^{o}C. The turbidity of culture was monitored on every 3 days by the optical density observation at 730 nm for 18 days long.

2.12.2 Physical parameters optimization

After media optimization, cells were then grown in TAP medium for 3 days, and inoculated to the fresh TAP medium to the final concentration of 0.01 and placed in shaker with the fixed speed of 160 rpm under various light intensity ranging from 0 to 92 μE/m^2s at desired temperature. Each culture was monitored the optical density of 730 nm on every 12 h for a total of 3 days. The set of experiment was also performed at several temperatures of growing ranging from 32 ^oC to 40 ^oC. To compare the growth in each condition, doubling time (dt) was determined by using an equation as expressed in (1): where *t* is cultivation time; *OD*730 is final optical density of cell culture, and $OD730_0$ is starting optical density of cell culture when incubated at the time of *t*.

$$dt = \frac{t \log(2)}{\log\left(\frac{OD730}{OD730_0}\right)} \qquad (1)$$

2.13 Optimization for H_2 production conditions

2.13.1 Optimization during growth phase

With the condition resulted in the shortest doubling time, cells were inoculated in TAP medium with the initial OD730 of 0.01. The culture was placed in the shaker under the continuous light illumination of 10 μE/m^2s at 36 ^oC for 24 h, with the shaking speed of 160 rpm. Two following parameters, culture age and light intensity, were tested to optimize the biohydrogen production affected during growth phase. A 5 mL of culture was directly placed in a gas-tight vial, and further caped The aluminium foil was used to wrap around the vial. After that, such vial was bubbled with argon gas for 5 min. The vial was incubated at 30^oC for 4 h prior to GC injection.

2.13.2 Optimization during production phase

To enhance the production, the following parameters were tested: temperature, buffer solution, additive carbon sources, additive salts, light intensity, additive reducing agents, and adapting deprivation media. Cells were grown in the optimized condition during growth phase. The additives were then separately added to the culture before placing the culture in gas-tight vial to further check the production rate. The vials were prepared in the same manner as describe in section 2.11.1, but the aluminium foil wrapping was omitted for light-dependent experiment. Vials were incubated for 4 h before the gas injection to GC.

For the deprivation condition, cells were grown in TAP medium for 24 h and then washed twice with sterile water prior to transferring to S- and N- deprived media. Sulfur and nitrogen deprivation conditions were obtained by omitting NH_4Cl (N deprivation) and by using $MgCl_2$, $FeCl_2$, $(CH3COO)_2Zn$, and $CuCl_2$ instead of corresponding sulfate salts (S deprivation).

2.14 cDNA library construction

2.14.1 Total RNA preparation

Cells were grown in TAP medium for 24 h and harvested at 4,500x*g* for 2 min. The cells pellet was washed twice with equal volume of sterilized water and then transferred to S-deprivation medium (TAP-S) for 6 h. The culture was centrifuged to collect the cells and kept in 2 mL screw-cap tube. One ml of TRI reagent was added to the tube and briefly homogenized by Precellys Bead Beater (Bertin technologies, France) for 20 seconds with the speed of 5,500 rpm. The homogenate was stored at room

temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Consequently, 200 µl of chloroform was added and vigorously shaken for 15 sec. The mixture was incubated at room temperature for 2-5 min and centrifuged at 12,000×*g* for 15 min at 4 ºC. The colorless upper aqueous phase was transferred to a new microcentrifuge tube. RNA was precipitated by the addition of 0.25 mL high salt solution (1.2 M sodium chloride and 0.8 M sodium citrate) and 0.25 mL of isopropanol. The mixture was left at room temperature for 10 min and centrifuged at 12,000×*g* for 10 min at 4 ºC. After centrifugation, the pellet was washed with 2 mL of 75% (v/v) cold ethanol. Then, the pellet was collected by centrifugation at 12,000×*g* for 10 min at 4 ºC and briefly air-dried for 5-10 min. The total RNA was dissolved with 20 µl of diethylpyrocarbonate (DEPC)-treated water, determined and estimated the concentration by measuring the absorbance at 260 nm, and calculating in µg/ml unit, using the following equation:

$$[\text{RNA}] = A_{260} \times \text{dilution factor} \times 40^{*}$$

*The absorbance at 260 nm of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook and Russel, 2001).

2.14.2 First-stranded cDNA synthesis

The first-strand cDNA was generated using the method according to the manufacturer protocol of iScript^{TM} cDNA Synthesis Kit (Bio-Rad). A microgram of the total RNA sample and 1 unit of DNaseI were mixed and incubate at 37 °C for 30 min. Five microliter of DNA-free RNA and 4 µL of 5X iscript buffer were mixed. Sterile water was added and adjusted the final volume to 19 µl, then a microliter of RNase H+ MMLV reverse transcriptase was added. The reaction was incubated at 25°C for 5

minutes followed by 42°C for 30 minutes. Consequently, the reaction was incubated at 85 ºC for 5 minutes to terminate the reverse transcription activity. The cDNA can be used as a template for PCR reaction, and stored at -20 ºC until used.

2.15 Gene investigation

2.15.1 Sulfate permease (sulP) investigation

The cDNA from the S-deprivation cells was used as template for PCR amplification of the *SulP* gene with a pair of degenerate oligonucleotide primers. The degenerate primers were designed to flank around the DNA region corresponding to the conserved regions in SulP protein sequence alignment. Five sequences of SulP protein were obtained from NCBI nucleotide database: *Nephroselmis olivacea* (Acc No. NP_050928), *Chlorella vulgaris* (Acc No. NP_045890), *Chlamydomonas reinhardtii* (Acc No. XP_001692459), *Mesostigma viride* (Acc No. NP_038441), *Chlorokybus atmophyticus* (Acc No. YP_001019170). Two forward (SdegF and nestedF) and a reverse (SdegR) degenerate primers were designed for first PCR and the following semi-nested PCR.

PCR cycles for the first reaction were conducted in 20 µL reaction mixtures containing 1X DreamTaq^{TM} buffer (Fermentas), 8 pmol of each primer (SdegF and SdegR primers), 4 nmol of dNTP, 2 µL of cDNA and 0.8 U of DreamTaq^{TM} polymerase (Fermentas). The PCR program was 3 min at 94 ^{o}C followed by 30 cycles of 94 ^{o}C for 30 sec, 50 ^{o}C for 30 sec, 72 ^{o}C for 30 sec, and then a final extension at 72 ^{o}C for 3 min. A 1 µL of 200 fold dilution from the first reaction was used as a template for semi-nested PCR (nestedF and SdegR primers) and used the same PCR profile as in the first reaction. PCR reaction mixture was electrophoresed in 1.0 % agarose gel and visualized by

ethidium bromide staining. PCR fragments were purified prior to ligation into pCR®2.1-TOPO® Vector Systems (Invitrogen) and then transformed into *E. coli* strain TOP10 according to the manufacturer's instruction. Recombinant plasmid was extracted using GeneJET™ Plasmid Miniprep Kit (Fermentas) and further used in DNA sequencing.

2.15.2 Full-length sulP determination by 5'RACE and 3'RACE

The 3'RACE reactions utilized the poly-A tail of mRNA transcript as a initiation point in cDNA library construction. An adaptor-linked oligo(dT) primer (polyT-TAG) and gene specific primer (sulp3RACE) were used in amplification for the first reaction at the annealing temperature of 50 ^{o}C with the same PCR profile as described previously using cDNA as a template. A microlitre of 200 fold dilution solution from the first reaction was used as a template for semi-nested PCR using sulp3RACE and adaptor-specific primers (TAG). The 3' RACE fragment was purified, cloned, and further sequenced. In addition, the sequence of polyT-TAG primer was slightly modified from previous study (Skjånes et al., 2010). The sequence from 3'RACE reactions was used to design gene specific primer for further sequence determination at genomic DNA level.

The 5'RACE reactions were conducted by the 5′ RACE System, Version 2.0 (Invitrogen). Gene specific primer (sulP5RACE) was used to synthesize the first stranded cDNA and used as a reverse primer in PCR amplification. Seminested PCR was conducted by using sulP5RACE primer and adaptor specific primer provided in the system kit. The 5' RACE fragment was purified, cloned, and later sequenced. This sequence was used as a template to design gene specific primer for further sequence determination at genomic DNA level.

2.15.3 Hydrogenase (hydA) investigation

Based on the same approach to investigate the *sulP* gene, without nested PCR, the forward (HydF) and degenerate reverse (HdegR) primers were designed from the conserved sequence, when aligned with other five algal *hydA* cDNA sequences: *Chlorella fusca hydA* (Acc No. AJ298228), *Chlamydomonas reinhardtii hydA1* (Acc No. AY055755), *Chlamydomonas reinhardtii hydA2* (Acc No. AY055756), *Chlamydomonas moewusii hydA2* (Acc No. AY578072), and *Scenedesmus obliquss hydA* (Acc No. AF276706), and used in PCR amplification by the same PCR profile as in *sulP*. The PCR product was purified, cloned, and sequenced.

2.16 Algal transformation

2.16.1 Cell wall digestion

Cells were inoculated to TAP medium with an initial OD of 0.01 and grown for 24 h at 36 °C under continuous light illumination before cell harvesting by centrifugation. Cell pellet was resuspended in one-tenth volume 50 mM sodium acetate, pH 5.0. Ten microlitre of 50 mg/mL of Cellulase (ONOZUKA R-10, PhytoTechnology Laboratories® company, USA) and 2 µL of 20 mg/mL RNase A were added to the culture suspension. The mixture was incubated at 37 °C for 30 minutes, the tube was gently swirled to resuspend the precipitated cells at the bottom of tube. After incubation, the culture was centrifuged to collect the cellulase-free algae by centrifugation at 2,000x*g* for 1 minute. Cells were washed by TAP medium for five times with the same speed of centrifugation.

2.16.2 Electroporation

The electroporation method was modified from the optimization electroporation for *C. reinhardtii* (Shimogawara *et al*., 1998). The transformation reaction was conducted in 250 µL TAP medium comprising about 10^8 cellulase-treated algal cells, 5 µg of required linearlized exogenous DNA, 50 µg of carrier DNA (Calf thymus DNA: DNA was sheared by sonication and denatured by heating at 100°C for 10 min.), and 40 mM sucrose. The reaction was incubated at 15 °C for 3 min before placing the reaction to 2-mm electroporation cuvette. The electroporation cell was pulsed by a voltage of 2,000 V/cm for 2.5 msec at 15 °C using MicroPulser Electroporator (Bio Rad). Transformation reaction was placed in a microcentrifuge tube and incubated under darkness at temperature of 36 °C without shaking for 3 h before plating on a selective medium.

2.16.3 Glass bead vortexing

The modified method was based on the optimal condition for *C. reinhardtii* transformation studied by Kindle (1990). A 250 µL TAP-containing transformation reaction composed of 10^8 cellulase-treated cells, 5% PEG 4,000, 5 µg required linearlized plasmid, and about 150 mg glass bead (212-300 µm diameter). The reaction was incubated at 15 °C for 3 minutes before vortex mixing at the highest speed for 15 seconds. Cell suspension was directly spreaded on the selective medium, and incubated at optimal condition until green colonies were observed.

2.16.4 Biolistic microparticle bombardment

The 0.6 µm gold particle was coated with the required linearlized plasmid. About 3 mg of gold particle was added in a 1.5 mL microcentrifuge tube followed by 5 µg of linearlized plasmid, 34 µL of 2.5 M calcium chloride, and 14 µL of spermine (free base).

The final volume was adjusted to 96 µL by water. The mixture was briefly vortexed in few seconds, and placed on ice for 30 minutes with occasional vortexing. Two hundred microlitre of 70% ethanol was added and vortexed the mixture briefly prior to collect the gold pellet by centrifugation at 13,000x*g* for 10 sec. The pellet was further washed with 250 µL absolute ethanol for 4 times before resuspending. The gold particle was finally dissolved in 60 µL absolute ethanol and kept on ice for use within 4 h.

Algae of approximately 10^5 cells were spreaded on 0.45 µm cellulose acetate membrane filter with a diameter of 47 mm (Whatman, London, UK). A 12 µL of gold particle suspension was poured equally onto the macrocarrier to a scarcely visible film. Then, ethanol was allowed to evaporate prior to placing in macrocarrier holder. The optimal parameters using in bombardment module were obtained from Lerche K and Hallman A (2009). The pressure on helium tank gauge was set to 1300 psi above the strength of the ruptor disk used with 1,100 psi. The gap between macrocarrier and stopping screen was set to 8 mm. The space between rupture disk and macrocarrier was adjusted to 7 mm. The membrane filter with the layer of algal cell was adjusted in the bombardment chamber, the distance between the target cells and stopping screen was 6 cm, and the chamber was evacuated to 27 inch Hg. After bombardment, the algal cells were washed off from the membrane filter with TAP medium. Bombarded culture was incubated under darkness for 6 h at 30 $^{\circ}$C before directly spreading on the selective medium containing 5 µg/mL paromomycin. After about 2 weeks of incubation in medium contains antibiotic, green colonies were obtained and further re-streaked for the next generation with 5 µg/mL stepwise increasing of antibiotic concentration until reaching 30 µg/mL paromomycin.

2.17 Fluorescence analysis

An equal optical density of culture was diluted with the corresponding growth medium for fluorescence measurement using fluorescence spectrometer (Luminescent spectrometer 55, Perkin Elmer, USA). The diluted culture was placed under darkness for 5 minutes before placing in cuvette holder. The culture was activated by the incoming light with the wavelength of 430 nm and scanned for the emission light with the wavelength ranging from 610 to 750 nm, using cell-free culture as a blank. The fluorescence value at the wavelength of 683.5 nm peak expressing in relative unit was plotted against the time.

2.18 Gene expression analysis

The sequences obtained at a cDNA level were used as templates for *sulP* and *hydA* primer designs. All primer sequences were indicated in Table 2.1. Primers SulRTF and SulRTR were used in *sulP* amplification, HydRTF and HydRTR were used in *hydA* amplification, and 18SRTF and 18SRTR for *18S* which was used as a reference gene. PCR cycles were conducted using 20 µL reaction mixtures containing 1X DreamTaq^{TM} buffer (Fermentas), 8 pmol of each forward and reverse primer, 4 nmol of dNTP, 2 µL of cDNA and 0.4 U of DreamTaq^{TM} polymerase (Fermentas). The PCR program consisted of 30 sec at 94 ^{o}C followed by 27 cycles (for *sulP* and *hydA*) and 15 cycles (for *18S*) of 94 ^{o}C for 15 sec, 60 ^{o}C for 15 sec, 72 ^{o}C for 15 sec, and a final extension at 72 ^{o}C for 30 sec. The PCR reaction was run in 1 % agarose gel electrophoresis and visualized by ethidium bromide staining under Gel documentation (gel doc) machine, Syngene, UK. The band intensity was calculated by using Gene tools program obtained from Syngene

company. The expression level was reported in relative unit and calculated with the respect to 18S rDNA, and this ratio at 0 h was set to 1.

2.19 General techniques

2.19.1 Preparation of E. coli competent cells

A single, well isolated colony of *E. coli* strain DH5α was inoculated into 2 mL of LB media and incubated at 37 ^{o}C for overnight with shaking at 250 rpm on a rotary shaker. A 0.5% inoculum volume of the overnight culture was transferred to 100 mL of LB media and grown until the OD_{600} nm reached between 0.3 - 0.4. The cells were chilled on ice for 15 minutes followed by 4 ^{o}C centrifugation at 3,500x*g* for 10 minutes. The supernatants were discarded and cells were resuspended in 10 mL of 0.1 M ice-cold $CaCl_2$. Cell suspensions were chilled on ice for 5 minutes followed by 4^{o}C centrifugation at 3,500x*g* for 10 min. The supernatant was discarded and the pellets were resuspended in 1/20 volume of 0.1 M ice-cold $CaCl_2$ supplemented with 15% (v/v) glycerol. Aliquots of 100 µl cell suspension in sterile 1.5 mL microcentrifuge tubes were stored immediately at -80 ^{o}C.

2.19.2 Restriction enzyme digestion

Restriction enzyme(s) and the required plasmid with the proper amount were incubated at the optimum temperature of restriction enzymes for 30 minutes. The total volume of the reaction was 10 µl containing 1 x proper restriction buffer, 5-10 U of restriction enzyme, and the DNA sample. The digested plasmid was analyzed by 1 % agarose gel electrophoresis and visualized by ethidium bromide staining.

2.19.3 Ligation

A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3, and blunt-end ligation is 1:1. 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 10 µl ligation reaction was composed as described in Table 2.2

Table 2.2 The composition of the ligation reaction

Component	Reaction (µl)
2x Quick ligation reaction buffer	5
Vector (50 ng)	0.5
Insert	X*
Quick Ligase (3U/µl)	0.5
Nuclease free water to a final volume of	10

The reaction was incubated at 25 ºC for 5 minutes. Two microlitre of the ligation mixture was transformed into competent cells of *E. coli* DH5α.

2.19.4 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. On the next day, obtained colonies were randomly selected for plasmid isolation.

CHAPTER III

RESULTS

3.1 Green algae screening for H_2 production

Twenty milliliter of water from rice paddy fields and fresh water ponds was collected in six provinces locating around central part of Thailand: Nakornsawan, Chainat, Singhburi, Angthong, Ayuthaya, and Pathumthani. By re-streaking technique, a total of 210 isolates were obtained from 12 natural water sources. The biohydrogen evolution of each isolate was measured and plotted in the same graph with a unit of $\mu molH_2$/mg chl a/h as shown in Figure 3.1. The production rate of each isolate was listed in appendix D.

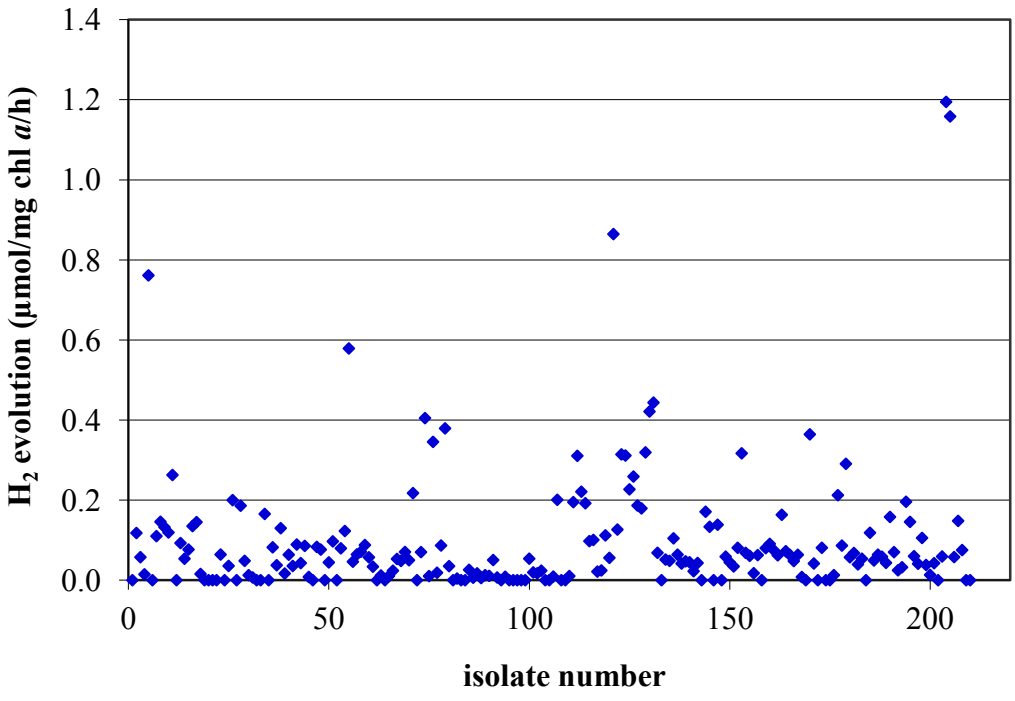


Figure 3.1 The biohydrogen production distribution of screened isolates

From the distribution, the results showed that 45 isolates (21.4 %) have no production ability; the rest demonstrates the production rate up to the maximum of 1.19 μmolH_2/mg chl *a*/h. One strain with the highest production rate was isolated from water source in Pathumthani province, about 20 km north of Bangkok, was selected for study in all experiments.

3.2 Strain identification

3.2.1 Morphology observation

The selected isolate was then sent to identify the strain by cell morphology observation at Thailand Institute of Scientific and Technological Research (TISTR). Under light microscope observation for 21 consecutive days, single cells, tetra cells surrounded by transparent sheath and truncated transparent sheath can be observed. Moreover, the cells showed the average diameter of 6.74±0.92 μm (Means ± S.D., n =3). Figure 3.2A shows the morphology of selected isolate observed under a light microscope. This selected isolate was named *Tetraspora* sp. CU2551. By taxonomic classification, *Tetraspora* belongs to the phylum chlorophyta. The taxonomic definition of *Tetraspora* sp. CU2551 is detailed as follows:

Domain Eukaryota

Kingdom Viridiplantae

Phylum Chlorophyta

Class Chlorophyceae

Order Tetrasporales

Family Tetrasporaceae

Genus Tetraspora

Furthermore, the higher resolution of cell morphology was observed by a scanning electron microscope (SEM). The SEM image of the cells where a disruption of the sheath is evident shown in Figure 3.2B.

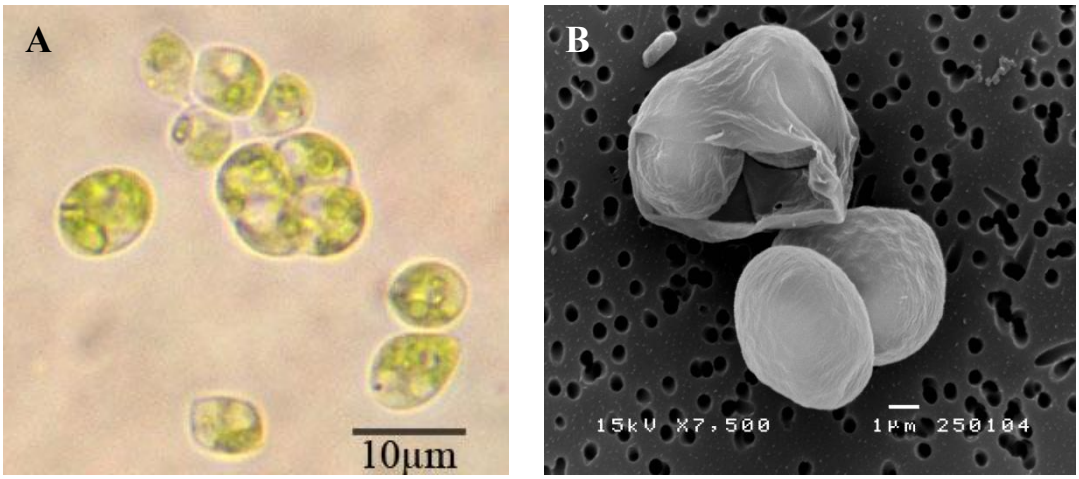


Figure 3.2 Cell morphology of *Tetraspora* sp. CU2551 cells observed under a light Microscope (A) and a Scanning Electron Microscope (SEM) (B).

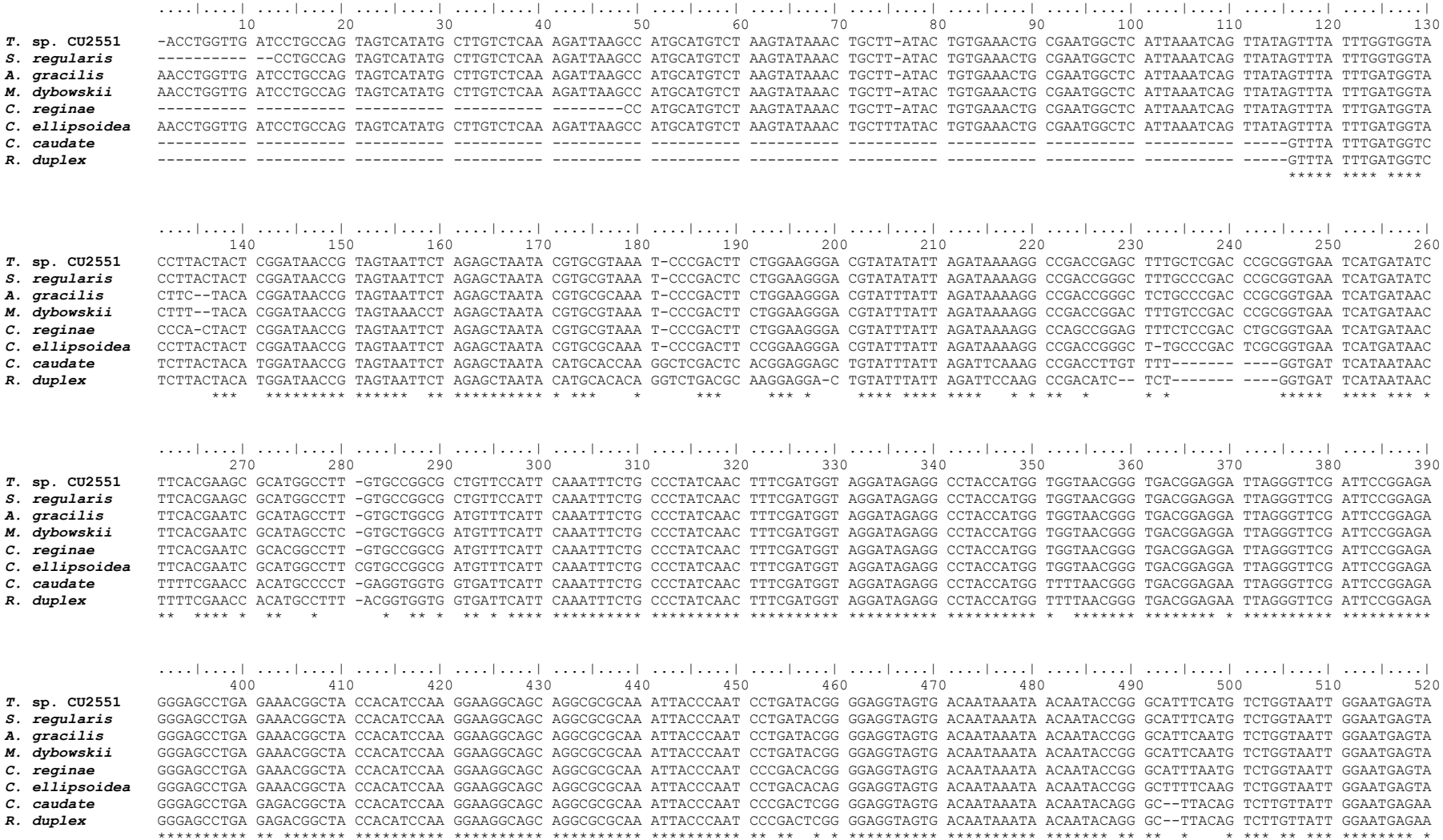
3.2.2 Molecular technique by 18S rDNA identification

18S rDNA gene specific primers given as 18SF and 18SR were designed from the conserved regions of the *18S* rDNA complete alignment as mentioned in section 2.9.2.1. Amplification using 18SF and 18SR primers gave a PCR product size of 1,348 bp. The fragment was cloned into pGEM®-T easy vector prior to sequencing. This sequence was used as a template for primer design in gene walking to 5' and 3' direction. 18SWalkto5 and 18SWalkto3 primers were designed and used as a primer in gene walking to 5' and 3' direction, recpectively.

Genomic DNA of *Tetraspora* sp. CU2551 was extracted and cut with 4 blunt-end restriction enzymes: *BsaBI, HincII, NruI*, and *SmaI*. The digested genomic DNA fragment was then ligated to YADE adapters. To extend the sequence in 5' and 3'

directions, this ligated reaction was used in PCR reaction using YADE-specific and 18SWalk primers. The fragments from these reactions were obtained, cloned, and sequenced. Using 18SWalkto5 primer resulted in 563 bp extra sequence in 5' direction where the 18SWalkto3 primer resuted in 747 bp extra sequence in 3' direction. A total of 2,658 bp was obtained for 18S rDNA sequence of *Tetraspora* sp. CU2551.

Figure 3.3 showed the alignment of 18S rDNA sequences from *Tetraspora* sp. CU2551 and other 7 algal 18S rDNA sequences that were used in primer design. Arrows indicated the regions of all primers used in 18S rDNA sequence determination. This *Tetrasposa* sp. 18S rDNA sequence was subjected to alignments (CLUSTAL-X) prior to Bootstrap NJ phylogenetic tree determination against 13 other species as shown in Figure 3.4. The 18S rDNA sequences of three identified *Tetraspora* isolates, accession numbers TSU83121, AF006314, and AF306793 (underlined) are included for comparative purposes. The tree shows several groups of algae where the *Tetraspora* isolates were separated into different groups. Even though the phylogenetic tree does not classify *Tetraspora* into a single group, we presume that this alga should belong to *Tetraspora*. The distance between each group in the tree is very close. Thus, few differences in the DNA sequence may lead to a separation into different groups. However, the present strain, CU2551, clusters with *Scenedesmus, Scotiellopsis terrestris,* and *Schroederiella*.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S. regularis ---------- --CCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC TGCTT-ATAC TGTGAAACTG CGAATGGCTC ATTAAATCAG TTATAGTTTA TTTGGTGGTA
A. gracilis AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC TGCTT-ATAC TGTGAAACTG CGAATGGCTC ATTAAATCAG TTATAGTTTA TTTGATGGTA
M. dybowskii AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC TGCTT-ATAC TGTGAAACTG CGAATGGCTC ATTAAATCAG TTATAGTTTA TTTGATGGTA
C. reginae ---------- ---------- ---------- ---------- --------CC ATGCATGTCT AAGTATAAAC TGCTT-ATAC TGTGAAACTG CGAATGGCTC ATTAAATCAG TTATAGTTTA TTTGATGGTA
C. ellipsoidea AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT AAGTATAAAC TGCTTTATAC TGTGAAACTG CGAATGGCTC ATTAAATCAG TTATAGTTTA TTTGATGGTA
C. caudate ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----GTTTA TTTGATGGTC
R. duplex ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----GTTTA TTTGATGGTC
***** **** ****
140 150 160 170 180 190 200 210 220 230 240 250 260
T. sp. CU2551 CCTTACTACT CGGATAACCG TAGTAATTCT AGAGCTAATA CGTGCGTAAA T-CCCGACTT CTGGAAGGGA CGTATATATT AGATAAAAGG CCGACCGAGC TTTGCTCGAC CCGCGGTGAA TCATGATATC
S. regularis CCTTACTACT CGGATAACCG TAGTAATTCT AGAGCTAATA CGTGCGTAAA T-CCCGACTC CTGGAAGGGA CGTATATATT AGATAAAAGG CCGACCGGGC TTTGCCCGAC CCGCGGTGAA TCATGATATC
A. gracilis CTTC--TACA CGGATAACCG TAGTAATTCT AGAGCTAATA CGTGCGCAAA T-CCCGACTT CTGGAAGGGA CGTATTTATT AGATAAAAGG CCGACCGGGC TCTGCCCGAC CCGCGGTGAA TCATGATAAC
M. dybowskii CTTT--TACA CGGATAACCG TAGTAAACCT AGAGCTAATA CGTGCGTAAA T-CCCGACTT CTGGAAGGGA CGTATTTATT AGATAAAAGG CCGACCGGAC TTTGTCCGAC CCGCGGTGAA TCATGATAAC
C. reginae CCCA-CTACT CGGATAACCG TAGTAATTCT AGAGCTAATA CGTGCGTAAA T-CCCGACTT CTGGAAGGGA CGTATTTATT AGATAAAAGG CCAGCCGGAG TTTCTCCGAC CTGCGGTGAA TCATGATAAC
C. ellipsoidea CCTTACTACT CGGATAACCG TAGTAATTCT AGAGCTAATA CGTGCGCAAA T-CCCGACTT CCGGAAGGGA CGTATTTATT AGATAAAAGG CCGACCGGGC T-TGCCCGAC TCGCGGTGAA TCATGATAAC
C. caudate TCTTACTACA TGGATAACCG TAGTAATTCT AGAGCTAATA CATGCACCAA GGCTCGACTC ACGGAGGAGC TGTATTTATT AGATTCAAAG CCGACCTTGT TTT------- ----GGTGAT TCATAATAAC
R. duplex TCTTACTACA TGGATAACCG TAGTAATTCT AGAGCTAATA CATGCACACA GGTCTGACGC AAGGAGGA-C TGTATTTATT AGATTCCAAG CCGACATC-- TCT------- ----GGTGAT TCATAATAAC
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270 280 290 300 310 320 330 340 350 360 370 380 390
T. sp. CU2551 TTCACGAAGC GCATGGCCTT -GTGCCGGCG CTGTTCCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
S. regularis TTCACGAAGC GCATGGCCTT -GTGCCGGCG CTGTTCCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
A. gracilis TTCACGAATC GCATAGCCTT -GTGCTGGCG ATGTTTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
M. dybowskii TTCACGAATC GCATAGCCTC -GTGCTGGCG ATGTTTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
C. reginae TTCACGAATC GCACGGCCTT -GTGCCGGCG ATGTTTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
C. ellipsoidea TTCACGAATC GCATGGCCTT CGTGCCGGCG ATGTTTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TGGTAACGGG TGACGGAGGA TTAGGGTTCG ATTCCGGAGA
C. caudate TTTTCGAACC ACATGCCCCT -GAGGTGGTG GTGATTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TTTTAACGGG TGACGGAGAA TTAGGGTTCG ATTCCGGAGA
R. duplex TTTTCGAACC ACATGCCTTT -ACGGTGGTG GTGATTCATT CAAATTTCTG CCCTATCAAC TTTCGATGGT AGGATAGAGG CCTACCATGG TTTTAACGGG TGACGGAGAA TTAGGGTTCG ATTCCGGAGA
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400 410 420 430 440 450 460 470 480 490 500 510 520
T. sp. CU2551 GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCTGATACGG GGAGGTAGTG ACAATAAATA ACAATACCGG GCATTTCATG TCTGGTAATT GGAATGAGTA
S. regularis GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCTGATACGG GGAGGTAGTG ACAATAAATA ACAATACCGG GCATTTCATG TCTGGTAATT GGAATGAGTA
A. gracilis GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCTGATACGG GGAGGTAGTG ACAATAAATA ACAATACCGG GCATTCAATG TCTGGTAATT GGAATGAGTA
M. dybowskii GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCTGATACGG GGAGGTAGTG ACAATAAATA ACAATACCGG GCATTCAATG TCTGGTAATT GGAATGAGTA
C. reginae GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCCGACACGG GGAGGTAGTG ACAATAAATA ACAATACCGG GCATTTAATG TCTGGTAATT GGAATGAGTA
C. ellipsoidea GGGAGCCTGA GAAACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCTGACACAG GGAGGTAGTG ACAATAAATA ACAATACCGG GCTTTTCAAG TCTGGTAATT GGAATGAGTA
C. caudate GGGAGCCTGA GAGACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCCGACTCGG GGAGGTAGTG ACAATAAATA ACAATACAGG GC--TTACAG TCTTGTTATT GGAATGAGAA
R. duplex GGGAGCCTGA GAGACGGCTA CCACATCCAA GGAAGGCAGC AGGCGCGCAA ATTACCCAAT CCCGACTCGG GGAGGTAGTG ACAATAAATA ACAATACAGG GC--TTACAG TCTTGTTATT GGAATGAGAA
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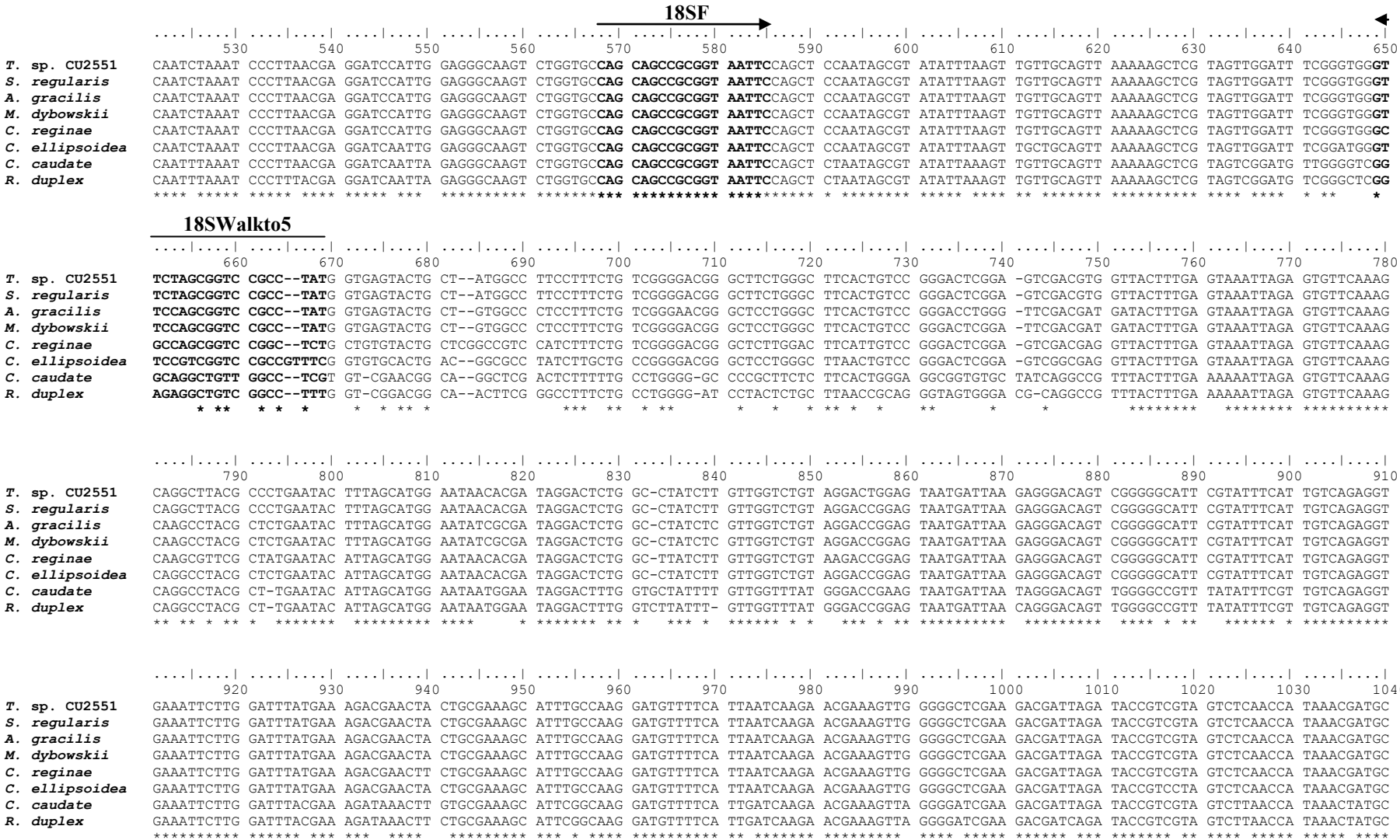
18SF
18SWalkto5
T. sp. CU2551
S. regularis
A. gracilis
M. dybowskii
C. reginae
C. ellipsoidea
C. caudate
R. duplex

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            1050       1060       1070       1080       1090       1100       1110       1120       1130       1140       1150       1160       1170
T. sp. CU2551       CGACTAGGGA TTGGCGAATG TTTTTTTAAT GACTTCGCCA GCACCTTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
S. regularis        CGACTAGGGA TTGGCGAATG TTTTTTTAAT GACTTCGCCA GCACCTTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
A. gracilis         CGACTAGGGA TTGGAGGATG TTCTTTTGAT GACTTCTCCA GCACCTTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
M. dybowskii        CGACTAGGGA TTGGAGGATG TTCTTTTGAT GACTTCTCCA GCACCTTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
C. reginae          CGTCTCGAAC TTGGTGGGCG TTTCGTTGAT GACCCTGCCA GTGTCGTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
C. ellipsoidea      CGACTAGGGA TTGGTGGATG TT-TATTGAT GACTTCACCA GCACCTTATG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
C. caudate          CGACTAGGGA TCAGTGGATG TCAATTTG-C GACTCCATTG GCACCTTGTG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
R. duplex           CGACTAGGGA TCAGTGGATG TCAATTTG-C GACTCCATTG GCACCTTGTG AGAAATCAAA GTTTTTGGGT TCCGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG GGCACCACCA
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                            1180       1190       1200       1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
T. sp. CU2551       GGCGTTAAAT ACATAGCTGA ACATAGCTCT AGCGCCAGTG GAGAGGAAAC TCTCTGCTAG TCAGTGTGGC ATAATAGTCA CATTGGCAAC ACCTTCAAAT TGCTGGGAAA CCCTAAAGCC ATTGCATACC
S. regularis        GGCGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis         GGCGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii        GGCGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae          GGCGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea      GGCGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. caudate          GGAGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex           GGAGT----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                    ** **

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                            1310       1320       1330       1340       1350       1360       1370       1380       1390       1400       1410       1420       1430
T. sp. CU2551       AAGGCAGACA TGGAAACATT CTGCTGGCCA GGTTAACGAC CTCGGGTACG GTGACAACTG CAATGGATTT AGTAGTTACA ACTGCTAGAA TGGGCAACCA GCAGCCAAGT CCTAAACATG GGCAGACAGC
S. regularis        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. caudate          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            1440       1450       1460       1470       1480       1490       1500       1510       1520       1530       1540       1550       1560
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S. regularis        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. caudate          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex           ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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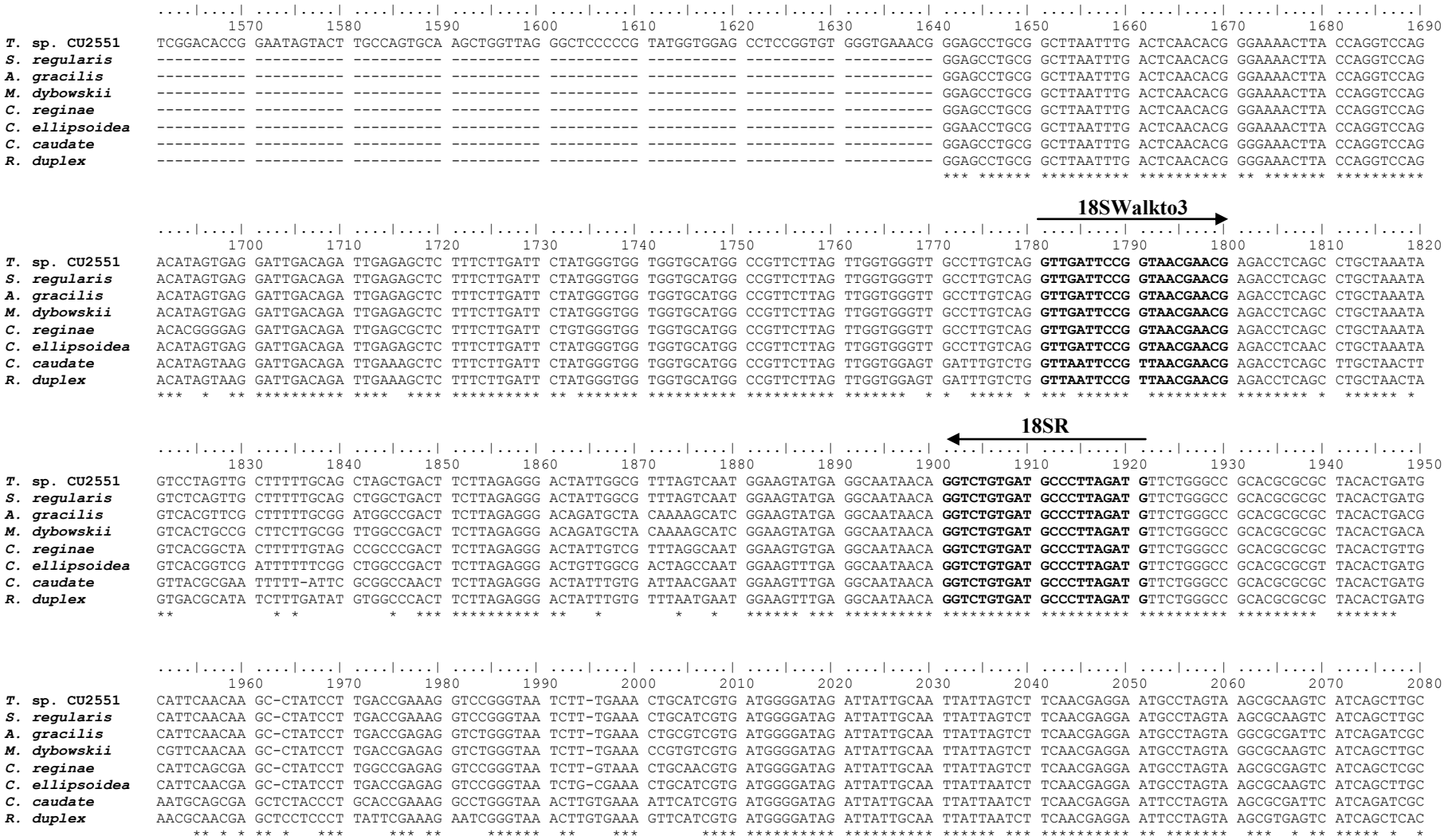
18SWalkto3
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C. reginae
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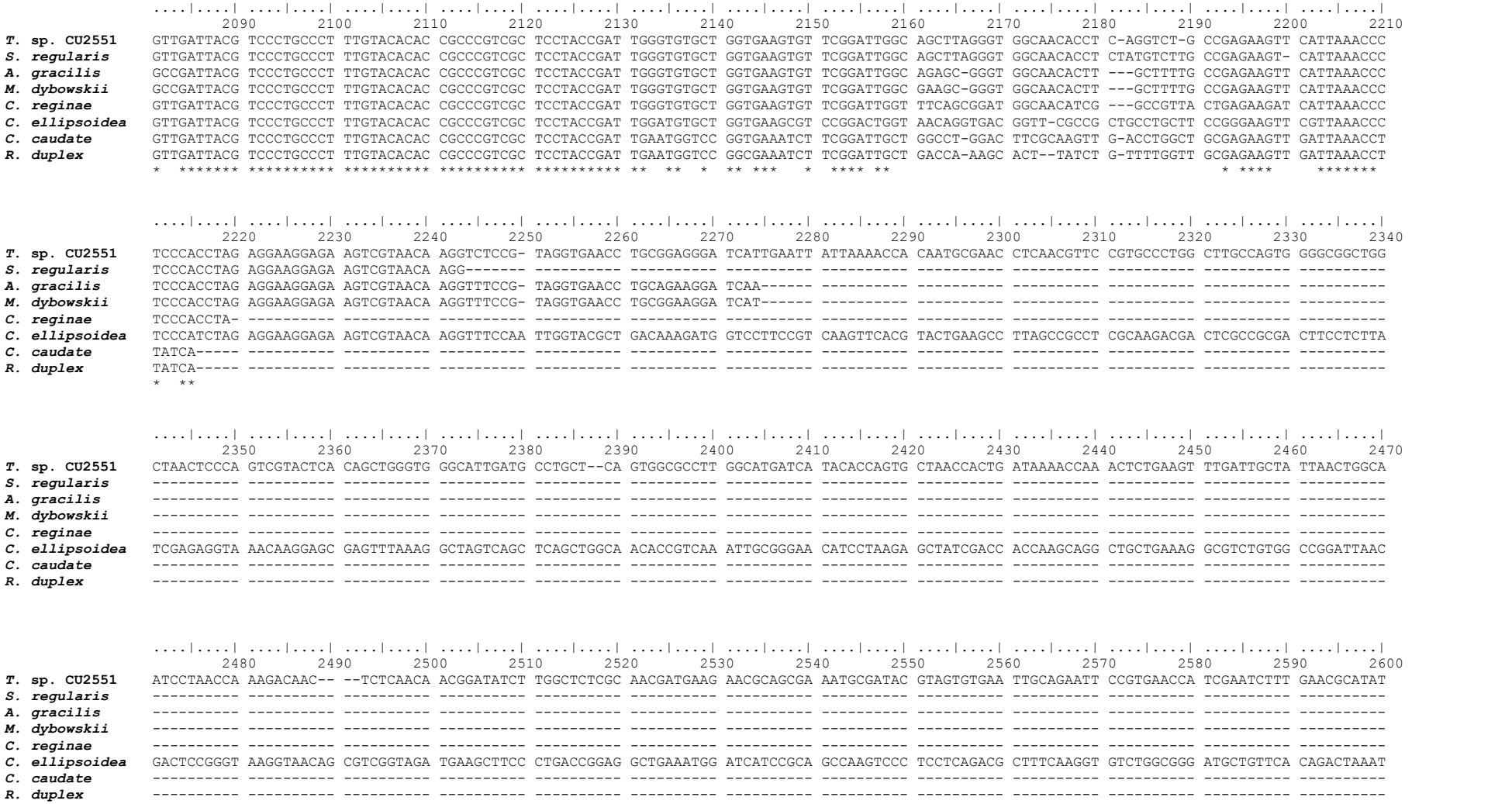
R. duplex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S. regularis GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGGGTGTGCT GGTGAAGTGT TCGGATTGGC AGCTTAGGGT GGCAACACCT CTATGTCTTG CCGAGAAGT- CATTAAACCC
A. gracilis GCCGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGGGTGTGCT GGTGAAGTGT TCGGATTGGC AGAGC-GGGT GGCAACACTT ---GCTTTTG CCGAGAAGTT CATTAAACCC
M. dybowskii GCCGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGGGTGTGCT GGTGAAGTGT TCGGATTGGC GAAGC-GGGT GGCAACACTT ---GCTTTTG CCGAGAAGTT CATTAAACCC
C. reginae GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGGGTGTGCT GGTGAAGTGT TCGGATTGGT TTCAGCGGAT GGCAACATCG ---GCCGTTA CTGAGAAGAT CATTAAACCC
C. ellipsoidea GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGGATGTGCT GGTGAAGCGT CCGGACTGGT AACAGGTGAC GGTT-CGCCG CTGCCTGCTT CCGGGAAGTT CGTTAAACCC
C. caudate GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGAATGGTCC GGTGAAATCT TCGGATTGCT GGCCT-GGAC TTCGCAAGTT G-ACCTGGCT GCGAGAAGTT GATTAAACCT
R. duplex GTTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT TGAATGGTCC GGCGAAATCT TCGGATTGCT GACCA-AAGC ACT--TATCT G-TTTTGGTT GCGAGAAGTT GATTAAACCT
2220 2230 2240 2250 2260 2270 2280 2290 2300 2310 2320 2330 2340
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S. regularis TCCCACCTAG AGGAAGGAGA AGTCGTAACA AGG------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis TCCCACCTAG AGGAAGGAGA AGTCGTAACA AGGTTTCCG- TAGGTGAACC TGCAGAAGGA TCAA------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii TCCCACCTAG AGGAAGGAGA AGTCGTAACA AGGTTTCCG- TAGGTGAACC TGCGGAAGGA TCAT------ ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae TCCCACCTA- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea TCCCATCTAG AGGAAGGAGA AGTCGTAACA AGGTTTCCAA TTGGTACGCT GACAAAGATG GTCCTTCCGT CAAGTTCACG TACTGAAGCC TTAGCCGCCT CGCAAGACGA CTCGCCGCGA CTTCCTCTTA
C. caudate TATCA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex TATCA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
2350 2360 2370 2380 2390 2400 2410 2420 2430 2440 2450 2460 2470
T. sp. CU2551 CTAACTCCCA GTCGTACTCA CAGCTGGGTG GGCATTGATG CCTGCT--CA GTGGCGCCTT GGCATGATCA TACACCAGTG CTAACCACTG ATAAAACCAA ACTCTGAAGT TTGATTGCTA TTAACTGGCA
S. regularis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea TCGAGAGGTA AACAAGGAGC GAGTTTAAAG GCTAGTCAGC TCAGCTGGCA ACACCGTCAA ATTGCGGGAA CATCCTAAGA GCTATCGACC ACCAAGCAGG CTGCTGAAAG GCGTCTGTGG CCGGATTAAC
C. caudate ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
2480 2490 2500 2510 2520 2530 2540 2550 2560 2570 2580 2590 2600
T. sp. CU2551 ATCCTAACCA AAGACAAC-- --TCTCAACA ACGGATATCT TGGCTCTCGC AACGATGAAG AACGCAGCGA AATGCGATAC GTAGTGTGAA TTGCAGAATT CCGTGAACCA TCGAATCTTT GAACGCATAT
S. regularis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. gracilis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
M. dybowskii ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reginae ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. ellipsoidea GACTCCGGGT AAGGTAACAG CGTCGGTAGA TGAAGCTTCC CTGACCGGAG GCTGAAATGG ATCATCCGCA GCCAAGTCCC TCCTCAGACG CTTTCAAGGT GTCTGGCGGG ATGCTGTTCA CAGACTAAAT
C. caudate ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
R. duplex ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

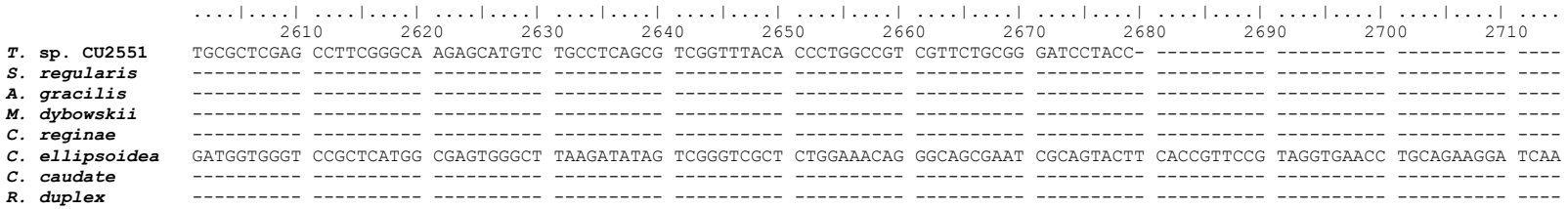


Figure 3.3 Alignment of 18S rDNA sequences from *Tetraspora* sp. CU2551 and other 7 algal 18s rDNA sequences that used in primer design. Arrows indicated the regions of primer used in 18S rDNA sequence determination. Seven 18S rDNA of green algae obtained from NCBI database: *Ankistrodesmus gracilis* (Acc No. Y16937), *Monoraphidium dybowskii* (Acc No. Y16939), *Scenedesmus regularis* (Acc No. AB037095), *Chlorella ellipsoidea* (Acc No. X63520), *Chroomonas caudate* (Acc No. AB240963), *Rhodomonas duplex* (Acc No. AB240960), *Chlamydomonas reginae* (Acc No. DQ009749).

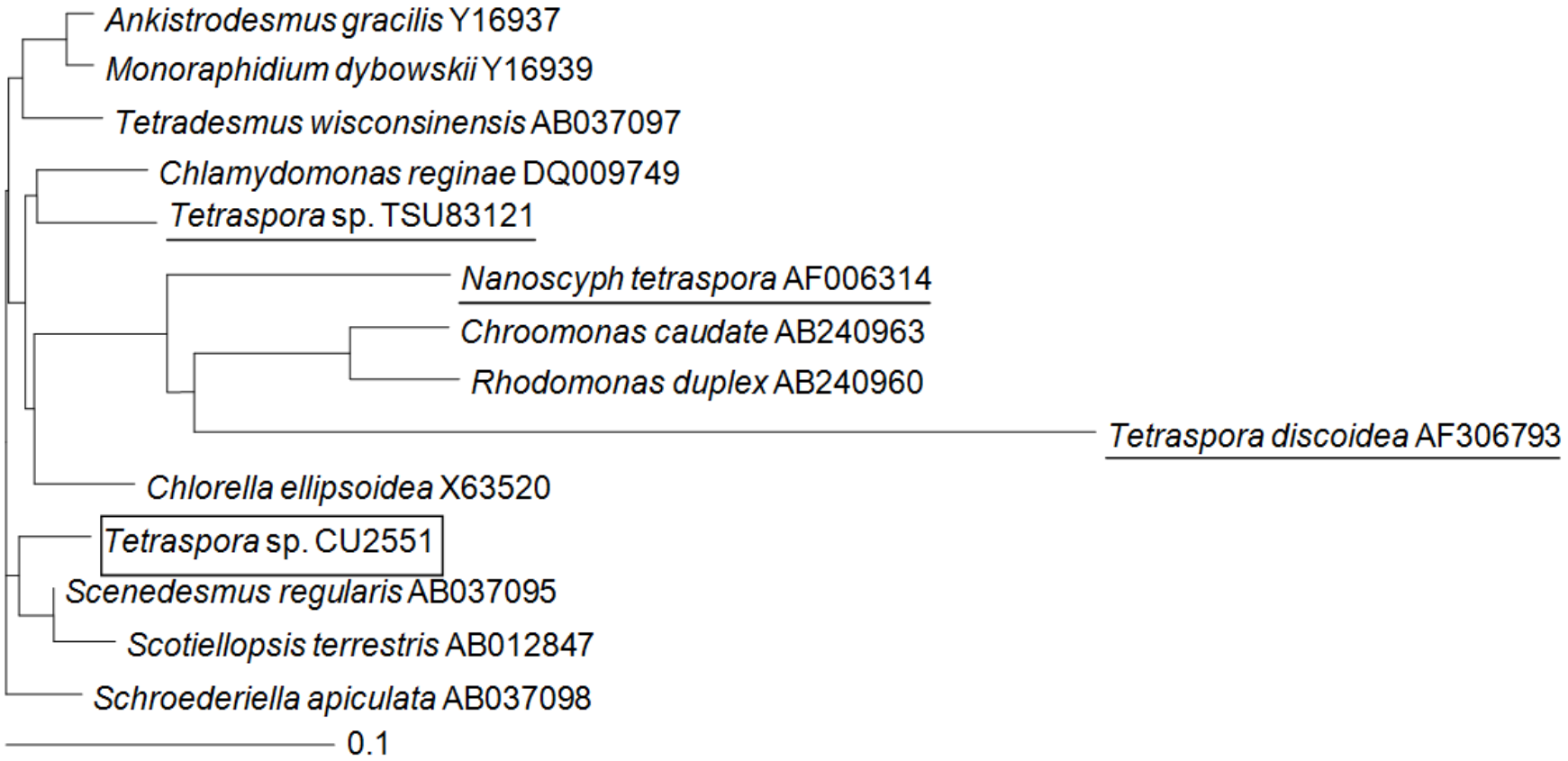


Figure 3.4 Phylogenetic tree using the obtained 18S ribosomal DNA sequences of *Tetraspora* sp. CU2551 (boxed) and 13 other green alga species including 3 named *Tetraspora* isolates (underlined). The number at the end of each name is the accession number available in the NCBI database.

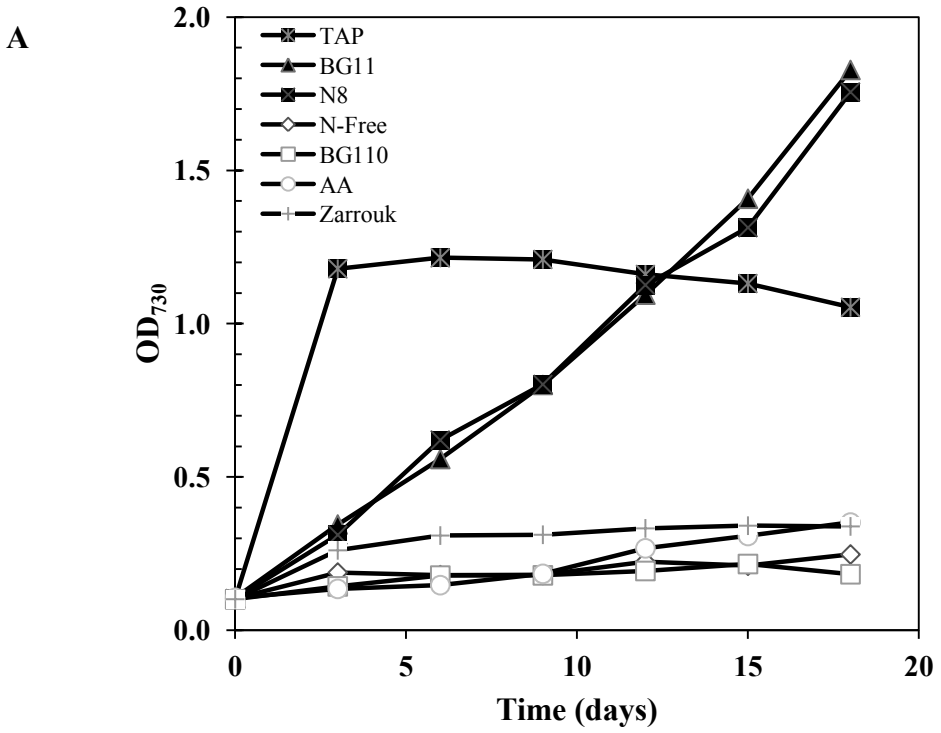
3.3 Growth optimization

There are several culture media available to grow the green organisms. Media optimization for cell culturing media was performed separately for *Tetraspora* sp. CU2551 in seven different media: N-free, BG11, $BG11_0$, N8, TAP (tris-acetate-phosphate), AA, and Zarrouk (see appendix C for media compositions). The starting culture was firstly grown in BG11 medium for a week before washing and inoculating into different media. The turbidity of culture was monitored by spectrophotometer with the wavelength of 730 nm. The growth curve was plotted and shown in Figure 3.5A. When grown in TAP medium, *Tetraspora* sp. CU2551 showed a fast initial growth rate in the first 3 d with no further growth observed afterwards, while the growth rates in BG11 and N8 media were similar and accounted for about half of that observed in TAP medium. It is noted that cells in BG11 and N8 media continued to grow at a similar rate up to 18 d. Slow growth rates were observed when cells were grown in N-free, $BG11_0$, AA, and Zarrouk media. TAP medium was identified as the most suitable for *Tetraspora* sp. CU2551 growth since high cells density can be obtained within 3 d compared to 12 d when grown in either BG11 or N8 medium to achieve equal cell density.

To identify the optimal condition for growth of *Tetraspora* in TAP medium, an initial OD was used to compare for S-curve of growth. The initial ODs were ranged from 0.005 to 0.2 and the culture density was monitored by measuring OD every 12 hours (Figure 3.5B). An initial OD of 0.01 showed the smooth pattern of S-curve. Then all further experiments used an initial OD_{730} of 0.01 when growing cells.

Doubling time was used to compare the effects of light intensity and temperature. Doubling time was calculated for cells grown under various light intensities ranging from 0 to 90 $\mu E/m^2s$ and the temperature ranging from 32 to 40 $^{\circ}C$. Cells could even grow

under darkness with longer doubling time. The minimal (shortest) doubling time was achieved when the cells were grown at 36 °C with light intensity ranging from 37 to 92 μE/m^2/s (Figure 3.5C). It should be noted that *Tetraspora* sp. CU2551 grows faster (shorter doubling time) under higher light intensity.



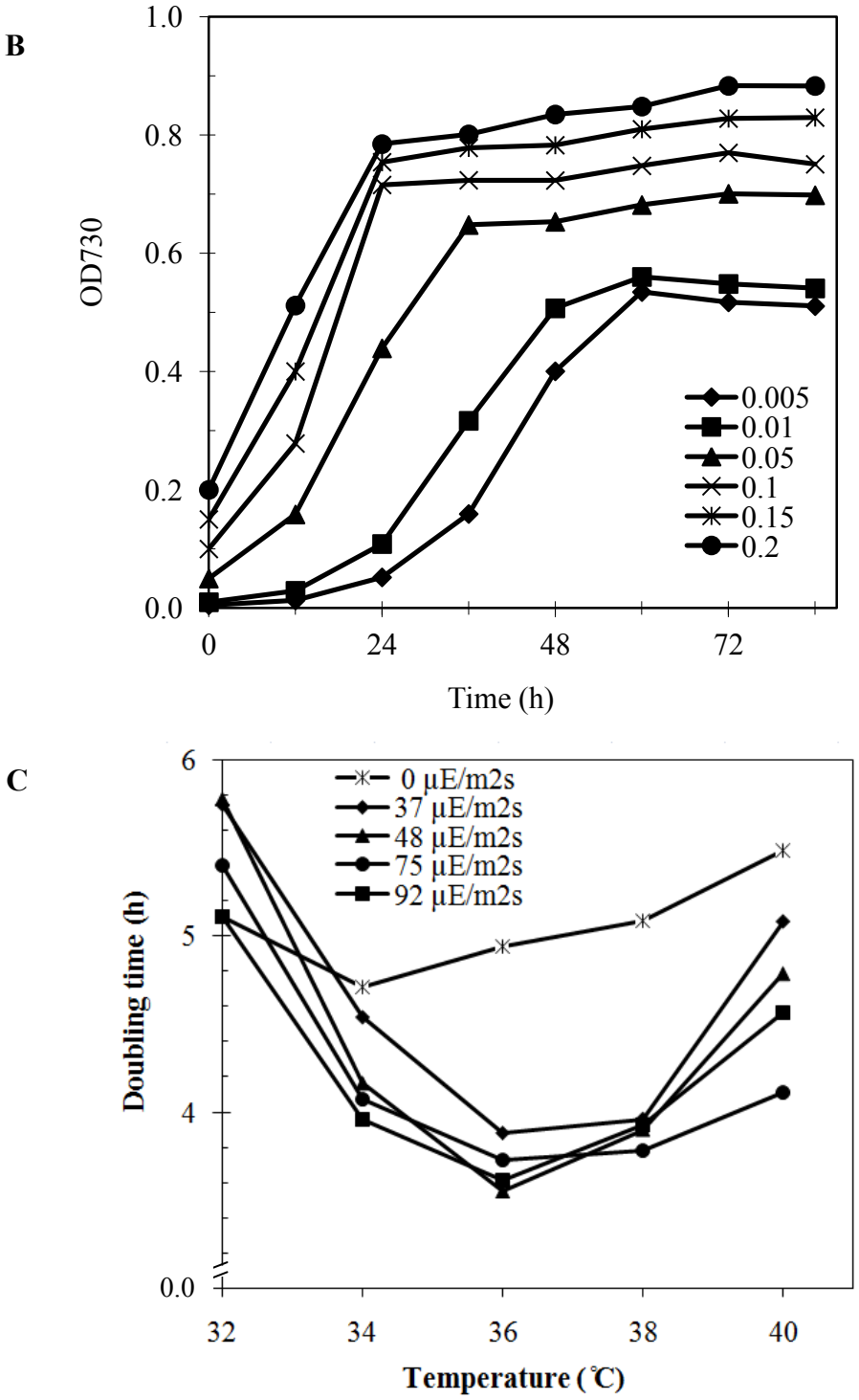


Figure 3.5 Growth curve of *Tetraspora* sp. CU2551. (A) Cells grown in seven different media, TAP (■), BG11 (▲), N8 (■), N-Free (◊), BG110 (□), AA (○), Zarrouk (+). (B) Growth curve when various initial OD_{730} was used. (C) The doubling time of alga when grown under different temperatures and at various light intensities

3.4 Optimization for hydrogen production

In screening step, the production unit was expressed as μmolH_2/mg chl *a*/h as described in section 3.1. To develop more reliable method, the production rate was changed from nmolH_2/mg Chl *a*/h to nmolH_2/mgDW/h or μmolH_2/mgDW/h. Using dry weight to express the production unit in *Tetraspora* sp. CU2551 resulted in higher accuracy and reproducibility than using the chlorophyll content. The standard curve between OD_{730} and dry weight was determined as shown in Figure 3.6. The dry weight was converted using and equation shown in the figure where y-axis is an optical density of the culture and x-axis is dry weight of *Tetraspora*.

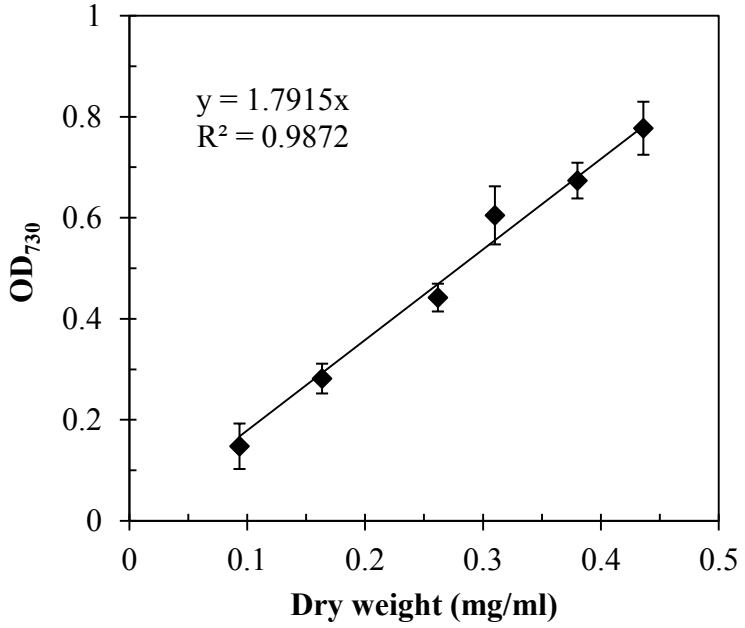


Figure 3.6 The standard curve of cell dry weight to the optical density. Means ± S.D. (n =3)**.**

The bioproduction process in green algae was designed as two-phase system: growth phase and production phase. Culture was optimized during growth phase to obtain the highest cell mass, while many physical parameters were optimized in the production phase such as temperature, pH, and chemical effects.

3.4.1 Optimization during growth phase

3.4.1.1 Culture age

Cells were grown for 72 h and made an aliquot to check the hydrogen production rate every 12 hours starting from 24 h. Figure 3.7 showed the production rate of cells in different culture age ranging from 24 h to 72 h. The 24-h cell culture showed the highest rate of hydrogen production. The older culture exhibited the slower rate. Growing culture longer than 48 h resulted in no significant difference in hydrogen production.

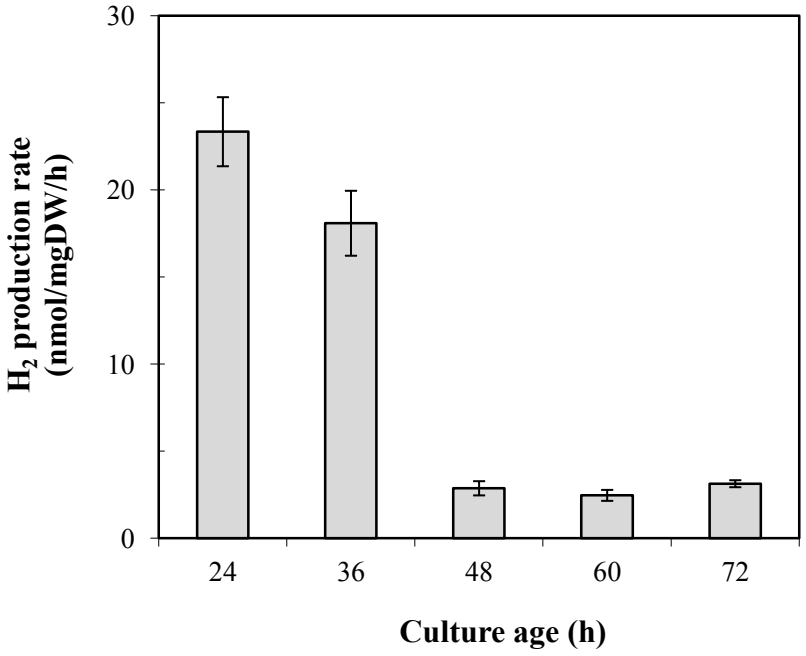


Figure 3.7 The production rate of cells at different culture age. Means ± S.D. (n =3).

3.4.1.2 Light intensity

Even cells can grow in the absence of light as described in section 3.3, the light intensity during culturing was also tested in order to compare the H_2 production. Cells were grown in different light intensities ranging from 0 to 90 μE/m^2s for 24 hours and further checked for the production rate as shown in Figure 3.8. The highest rate was significantly obtained from cells grown under darkness. Increasing light intensity resulted in lowering down the H_2 production rate.

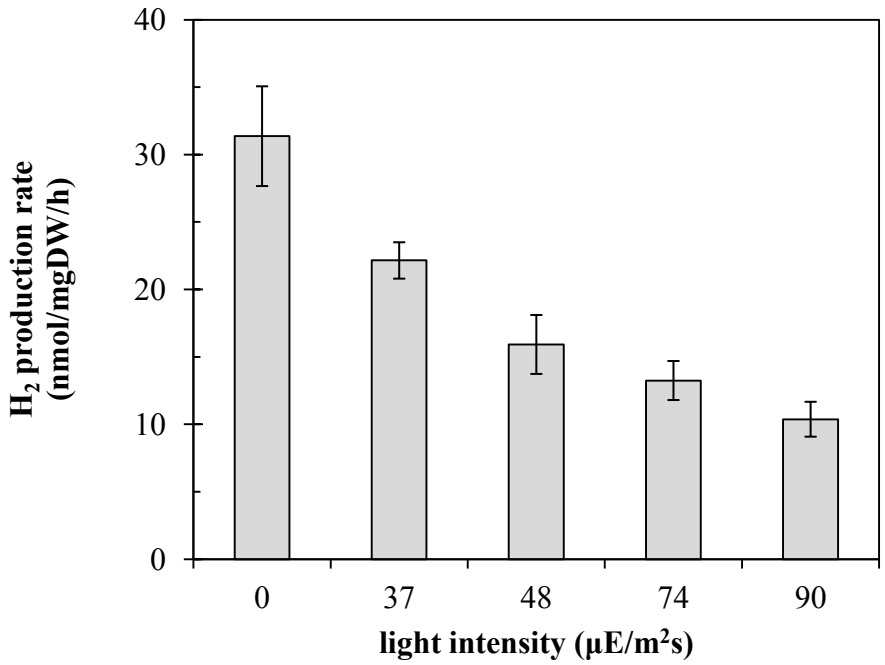


Figure 3.8 The production rate of cells at different light intensities during cultivation. Means ± S.D. (n =3).

3.4.2 Optimization during production phase

As two parameters, culture growth period and light intensity, were optimized during the growth phase, cells were grown at 36 °C for 24 h under the light intensity around 37 μE/m^2s to let cells grow. Culture under this condition was used for the production-phase optimization.

3.4.2.1 Temperature

On the first parameter optimization, culture was tested for the production of hydrogen gas at various temperatures. Gas-tight vials were incubated in water bath with various temperatures. Figure 3.9 showed the production rate when incubating cells at each temperature. *Tetraspora* sp. CU2551 demonstrated the optimal temperature of 35 °C for hydrogen production.

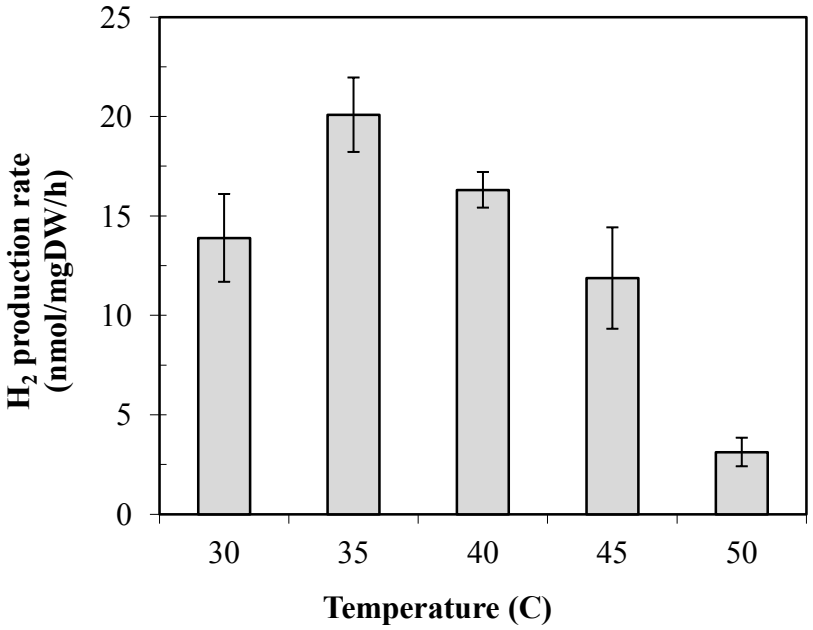


Figure 3.9 The production rate of cells in different incubation temperature. Means ± S.D. (n =3).

3.4.2.2 Light intensity

Tetraspora sp. was finally grown under the optimal condition. After transferring cells to the gas-tight vials, the vials were unwrapped from aluminium foil and let light pass through the culture vial during incubation. The light intensity was varied during incubation; and the production rate of culture under each condition was measured as shown in Figure 3.10. Increasing light intensity from darkness to 9 $\mu E/m^2s$ resulted in the corresponding increase of hydrogen production rate. Interestingly, light strikingly increase the production yield more than 10 times when compared to that under the dark incubation. From this result, the presence of light under incubation step was further performed as light-incubation during the hydrogen production in all experiments.

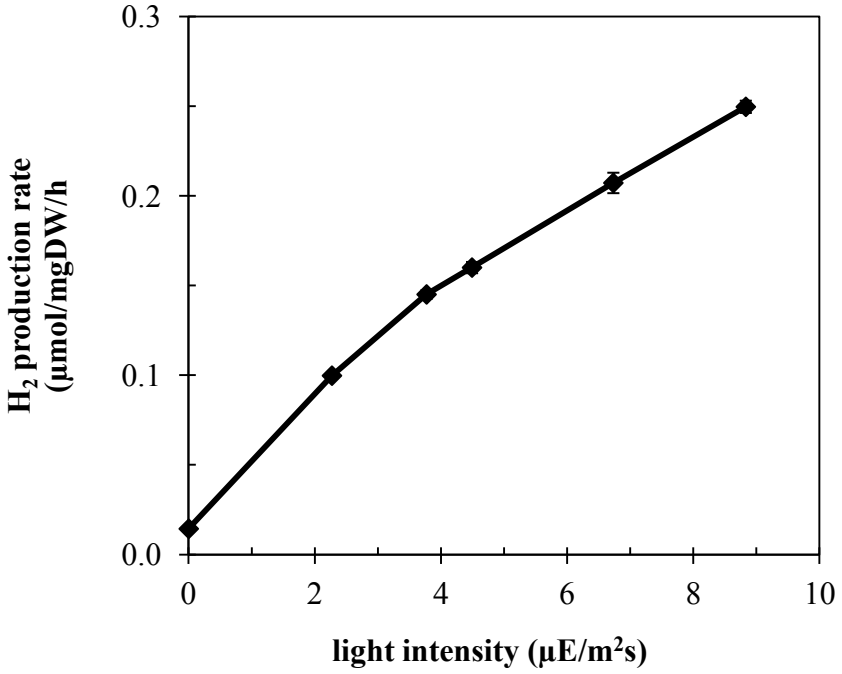


Figure 3.10 The production rate of cells at different light intensities during production. Means ± S.D. (n =3).

3.4.2.3 Additive carbon sources

Many carbon sources with the stock concentration of 20% were added to the culture to obtain the final concentration of 0.1% before starting the hydrogen production (Figure 3.11A). Glycerol was used as C-3 sources. Glucose, fructose, galactose, and sorbitol were used as C-6 source. Maltose, sucrose, and lactose were used as C-12 sources. The hydrogen production was compared with the rate of culture without any carbon source addition (control). Under darkness incubation, the results in Figure 3.11A showed that addition of extra carbon sources could not stimulate cells to produce biohydrogen at a higher rate. Then, the experiment was re-designed to add the carbon sources at the beginning of the growth phase. The C-sources were added to the culture with the same final concentration and the culture was grown at the same condition. The hydrogen production of each condition was measured as shown in Figure 3.11B. The hydrogen production was not significantly stimulated when carbon sources were added during growth phase, whereas glucose caused a significant decrease on the hydrogen production.

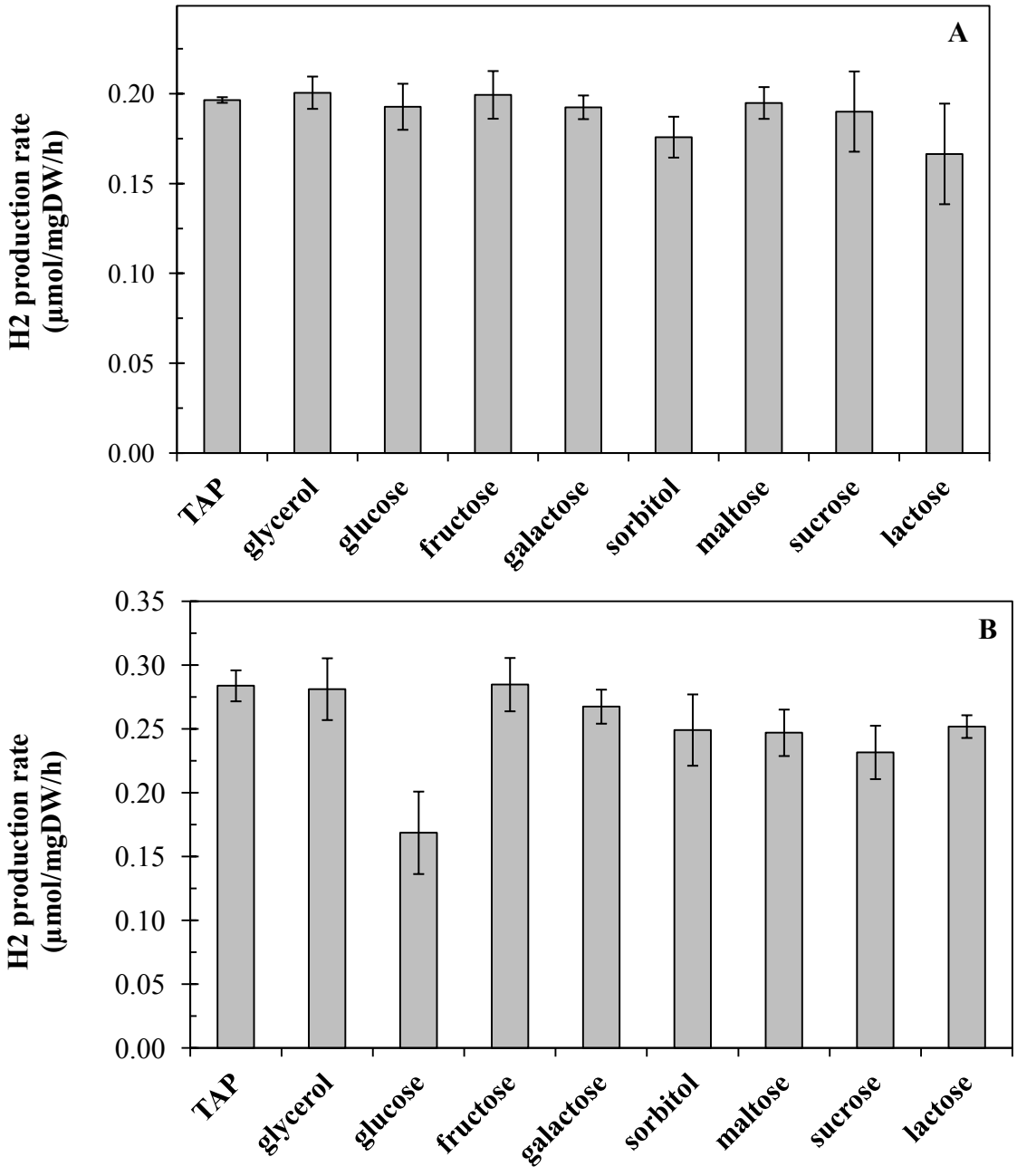


Figure 3.11 The production rate of cells under the presence of 0.1% various carbon sources during production phase (A) and during growth phase (B). Means ± S.D. (n =3).

3.4.2.4 Additive salts

A 500 mM salt stock solution was prepared and added to culture after cells growing in TAP for 24 h and made a final concentration of 50 mM of each salt type. Under 4 h light incubation, hydrogen production of each condition was measured as shown in Figure 3.12. The results revealed that all kinds of salt could not promote the hydrogen production compared to that without any salt (TAP). Especially, the carbonate salts including Na_2CO_3, $NaHCO_3$ and $KHCO_3$ inhibited the production by about 50%. The inhibition was also observed when nitrite salts, $NaNO_2$ and KNO_2, were present in the culture. The same pattern is also noted when the culture contained the denaturing agent, guanidine hydrochloride (GnHCl).

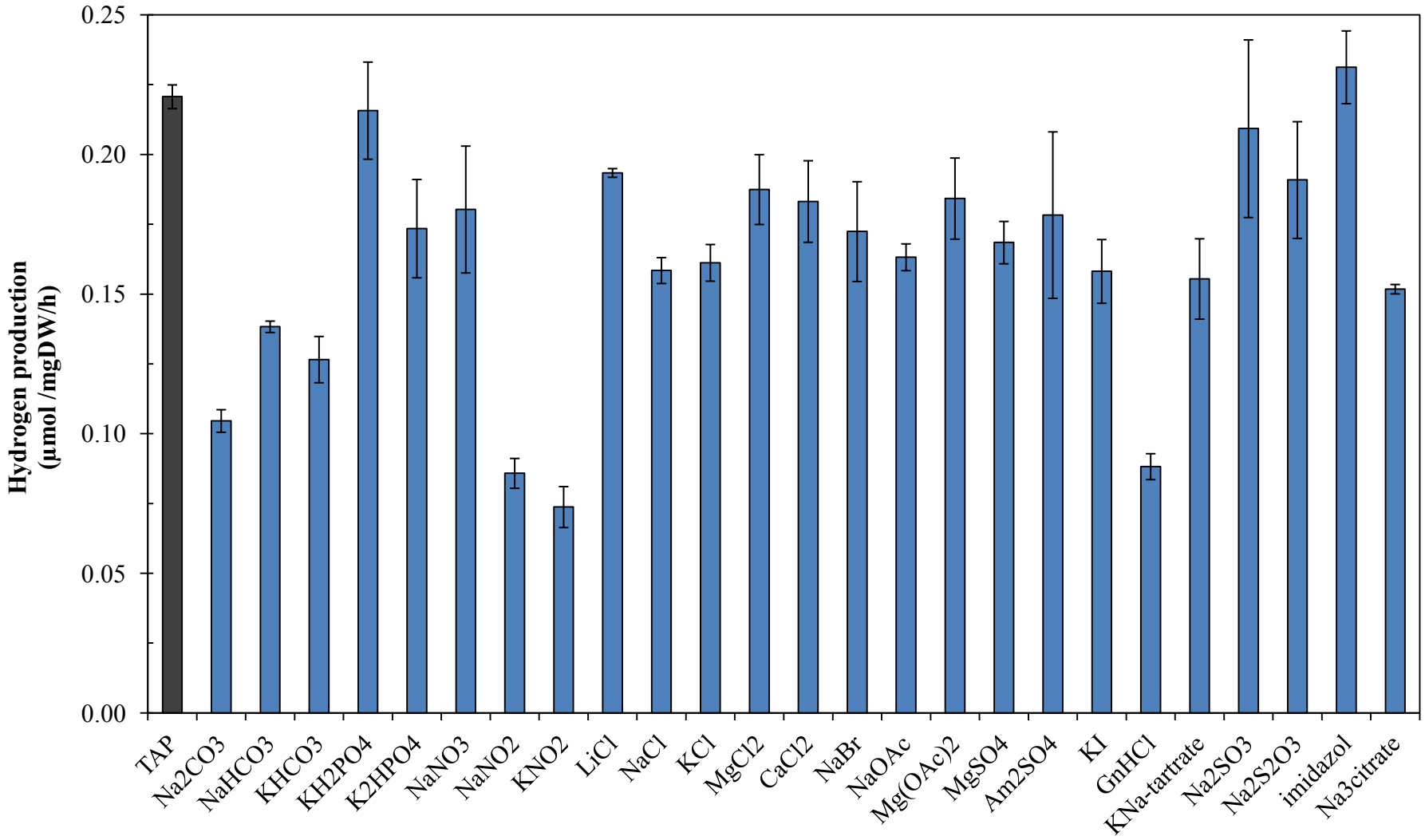


Figure 3.12 The production rate of *Tetraspora* sp. CU 2551 when different salts (50 mM) were present in culture medium

3.4.2.5 pH

The 24-h culture was aliquoted to the same volume and then buffered with 500 mM buffer solution to the final concentration of 50 mM. Acetate salt solution was used as buffer solution at pH 5.25 and 5.75. Citrate was used to buffer the culture at pH 5.9 and 6.4. Tris was used to buffer the solution at pH 7.3, 7.8, 8.3, 8.8 and 9.3. In Figure 3.13, H_2 production by *Tetraspora* sp. CU2551 under light condition showed in broad range and increased when increasing the pH from 5.75 to 9.30. In contrast, the production was dropped when lowering pH to 5.25 where almost no detectable H_2 evolution was observed.

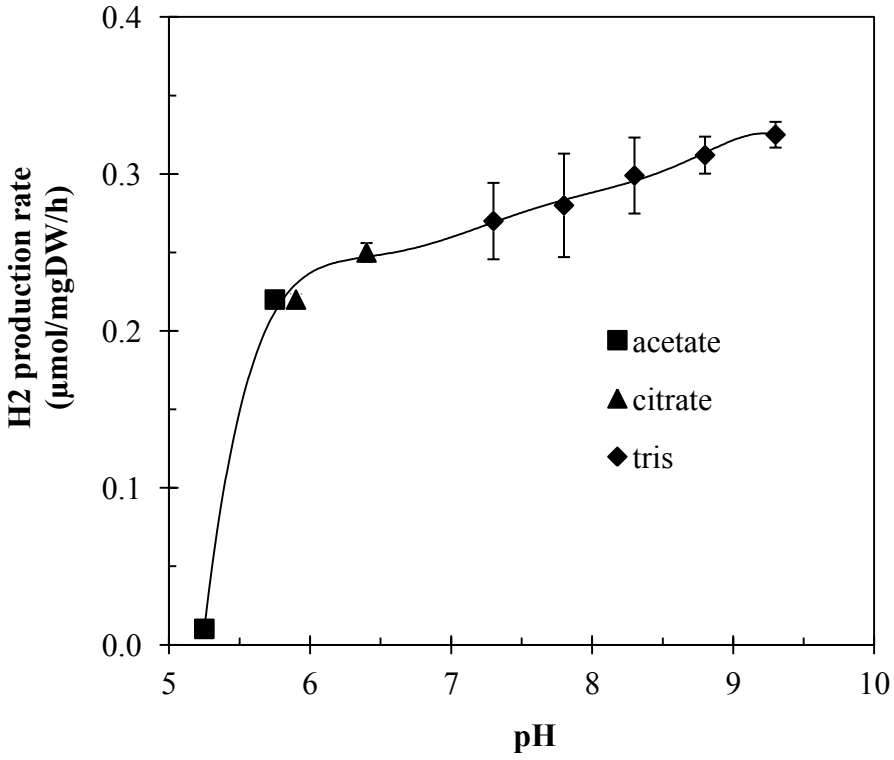


Figure 3.13 Effect of pH on hydrogen production by *Tetraspora* sp. CU2551. The cultures were buffered with 50 mM acetate buffer (■), citrate buffer (▲), and tris-HCl buffer (●). Means ± S.D. (n=3).

3.4.2.6 Additive reducing agents

Naturally, a reducing agent is a good source of electrons to common acceptors. Some studies have shown that hydrogenases may function as electron sink controlled by the redox status (Kruse, O. and Hankamer, 2010). When *Tetraspora* CU2551 was grown in TAP medium supplemented with 0.5 mM β-mercaptoethanol, a two-fold increase in H_2 production was observed with a maximal value of 0.42 μmol/mgDW/h (Figure 3.14). On the contrary, addition of dithiothreiol up to 1.25 mM had no effect on the H_2 production.

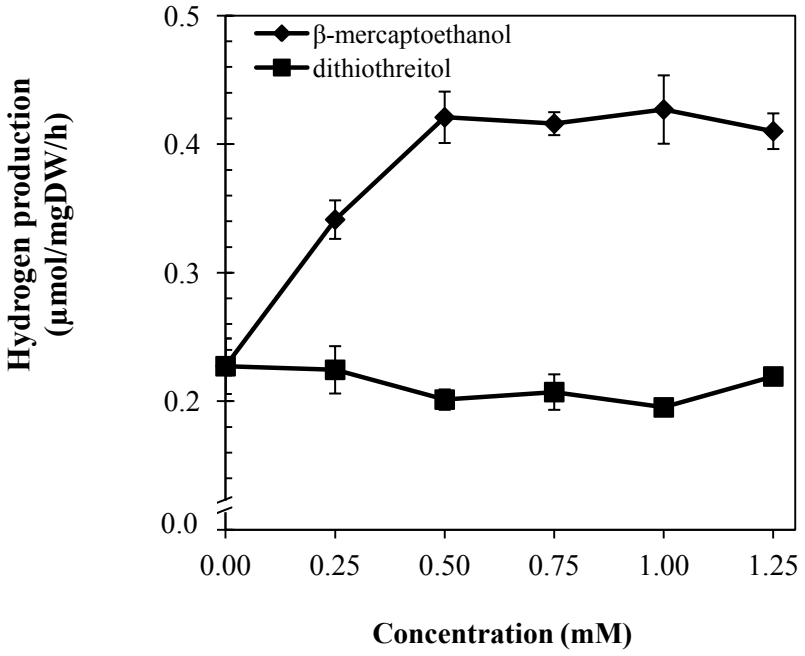


Figure 3.14 Effect of the addition of reducing agents on hydrogen production by *Tetraspora* CU2551. β-Mercaptoethanol (♦) or dithiothreiol (■) was added to the cells of *Tetraspora* CU2551. Means ± S.D. (n=3).

3.4.2.7 Adapting media

The deprivation of some nutrient sources causes an enhanced effect on the hydrogen production production in other green algae. Cells were grown in TAP medium for 24 h, then replaced by sulfur-deprived medium (TAP-S), nitrogen-deprived medium (TAP-S) and nitrogen-sulfur-deprived medium (TAP-N-S). The production was measured as shown in Figure 3.15A. Sulfur or nitrogen deprivation condition promoted the production by about 1.2 fold, but the combination of nitrogen and sulfur sources increase the production about 1.5 fold.

The result from section 3.4.2.6 indicated that 0.5 mM β-mercaptoethanol efficiently help cells produce the biohydrogen at higher rate. The experiment also monitored the effect of nutrient deprivation on cells grown in the presence of β-mercaptoethanol. After cells were grown in TAP medium supplemented with 0.5 mM β-mercaptoethanol (TAP+β) for 24 h, the medium was replaced by TAP-N-S, and would be so called TAP+β-N-S culture. Interestingly, when cells were incubated under both nitrogen- and sulfur- deprived conditions, there was no effect of the reducing agent on hydrogen production (Figure 3.15B)

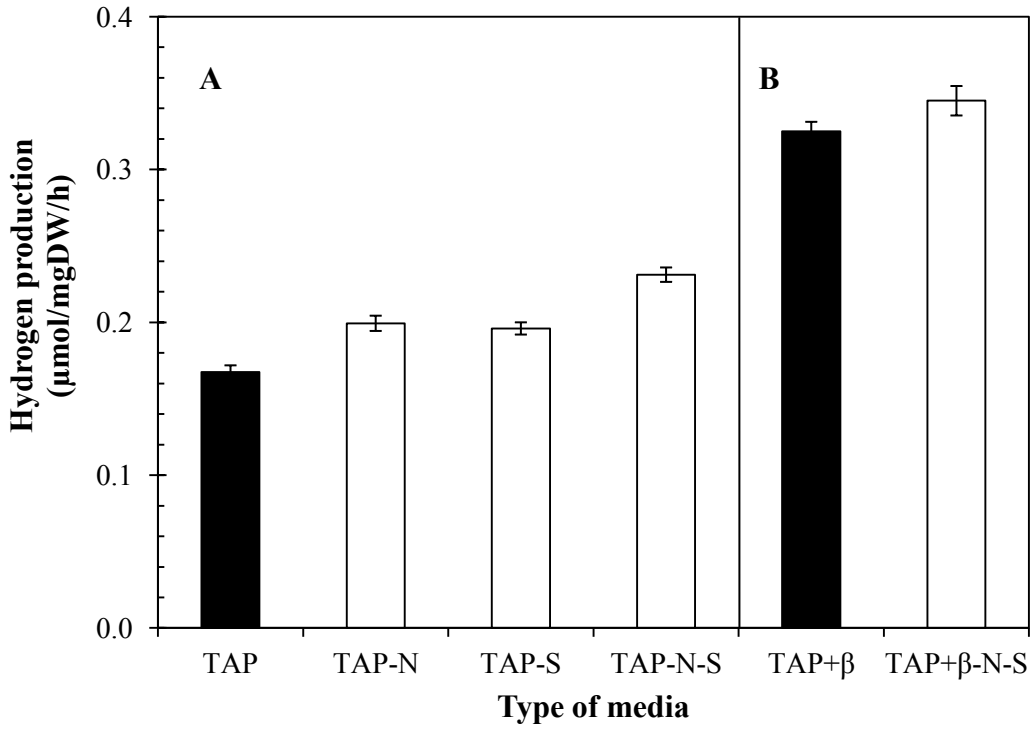


Figure 3.15 Effect of nutrient deprivation on hydrogen production by *Tetraspora* sp. CU2551. (A) The production rate of cells grown in TAP medium (solid bar) and nutrient-deprived media (open bar): nitrogen-deprivation (TAP-N), sulfur-deprivation (TAP-S), and nitrogen- and sulfur-deprivation (TAP-N-S). (B) The production rate of cells grown in TAP medium supplemented with either 0.5 mM β-mercaptoethanol (TAP+β) (solid bar) or a nitrogen- and sulfur-deprived medium (TAP-N-S) (open bar). Means ± S.D. (n=3).

3.4.2.8 Combination effect on H_2 production

Cells of *Tetraspora* sp. CU2551 were further examined for H_2 production under the combination of positive parameters. The production was observed when cells were incubated under the increase of light intensity (Figure 3.16). As the result indicated in section 3.4.2.6 that *Tetraspora* sp. grown in the presence of 0.5 mM β-mercaptoethanol produced biohydrogen in double rate of production, the increase pattern was observed when low light was applied to the culture ranging from 0 to about 5 $\mu E/m^2s$. After light intensity was higher than 7 $\mu E/m^2s$, the production from TAP+β cells (▲) reached the maximum capacity yielding about 0.28 $\mu molH_2$ (mg DW)$^{-1}$ h^{-1}. This is in contrast to cells grown in normal TAP medium (●) in which the production increased when high ligh was applied up to 39 $\mu E/m^2s$

Cultures of TAP and TAP+β were further examined under the nitrogen and sulfur deprivation effect indicated by TAP-N-S (○) and TAP+β-N-S (Δ). Culture TAP+β-N-S (Δ) showed higher capacity than that in TAP-N-S (○) under the low ligh as found in the pattern of nutrient-rich medium from the previous experiment. When light intensity was higher than 6 $\mu E/m^2s$, the increase capacity was only found in TAP-N-S (○).

The result suggested that β-mercaptoethanol efficiently help *Tetraspora* cell produce biohydrogen gas at a high rate during the low light ranging from 0 to about 6 $\mu E/m^2s$. But, nitrogen and sulfur deprivation was more effective than the reducing power when cells were incubated under light intensity above 6 $\mu E/m^2s$.

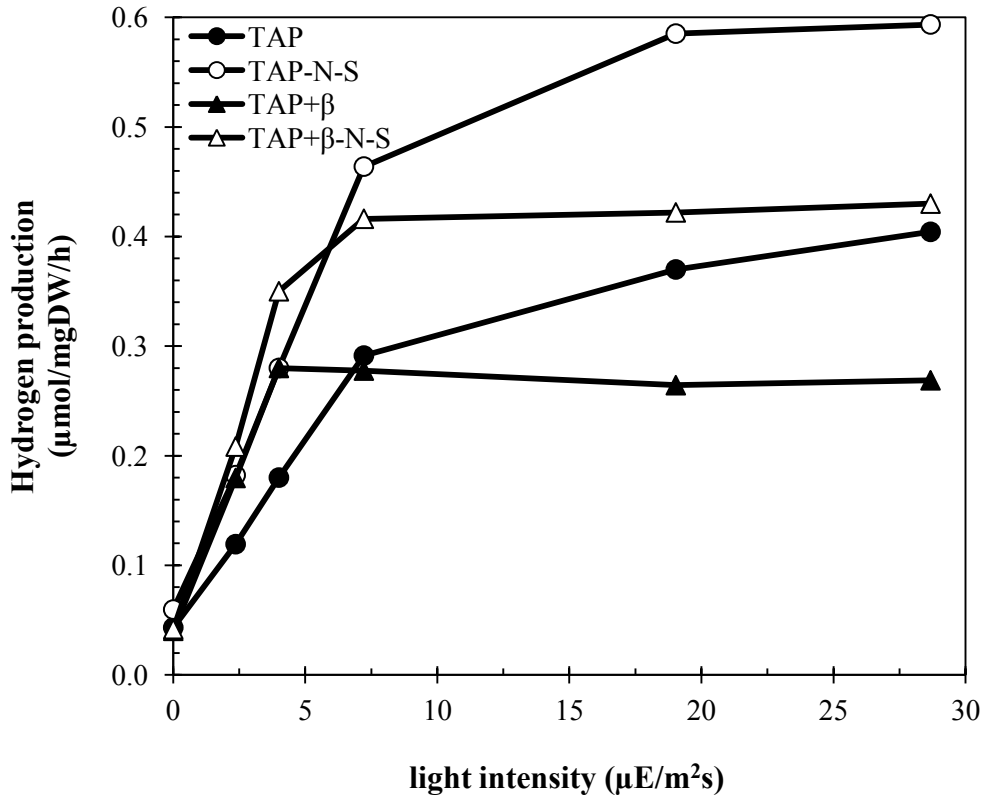


Figure 3.16 Effect of light intensity on hydrogen production by *Tetraspora* sp. CU2551. The hydrogen production rates were measured under various light intensities using cells grown in normal TAP (●) before transferring to nitrogen- and sulfur-deprived medium (TAP-N-S: ○) during the hydrogen production phase or when grown in TAP supplemented with 0.5 mM β-mercaptoethanol (▲) before transferring to nitrogen- and sulfur-deprived medium (TAP+β-N-S: Δ).

In summary, we identified the novel hydrogen-producing green alga as *Tetraspora* sp. CU 2551. *Tetraspora* sp. has high potential to produce an alternative energy source, molecular biohydrogen. They grow fast at 36 $^{\circ}$C with the low-cost medium (TAP) under light condition. With the optimal condition, *Tetraspora* can evolve the significant amount of hydrogen yielding about 0.6 μmol/mgDW/h.

3.5 Molecular study for biohydrogen metabolism

The aim of this molecular study part is to enhance the capacity of the production in wild-type *Tetraspora* sp. under the optimal condition. Based on the pathway studied in *Chlamydomonas reinhardtii*, several proteins are involved in biosynthesis pathway of molecular biohydrogen. One of these proteins, sulfate permease (SulP), was selected. Several reports indicated that this protein was embedded on the inner membrane of chloroplast and functioned as the sulfate transporter. Sulfate molecules can be transported from cytoplasm to stroma through this channel. Sulfate will be further used in biosynthesis pathway of cysteine which is a building block for other sulfur-containing amino acids biosynthesis pathway.

D1 protein is located in the reaction center of photosynthesis system II (PSII) where the water is splitted to oxygen, proton and electron. Oxygen can function as an inhibitor of hydrogenase enzyme which catalyzing formation of molecular hydrogen from proton and electron. Inhibition of D1 protein resulted in low level of molecular oxygen. This level of oxygen can be consumed by respiratory chain in mitochondria resuling in no inhibitor of hydrogenase in the stroma of chloroplast. The hydrogen production should be obtained in high yield.

One way to inactivate D1 protein turnover was to slow down the protein biosynthesis pathway. Low level of stroma sulfate can be controlled by the sulfate uptake channel (SulP). The SulP determination was started, and further studied with respect to the inactivation by the genetic engineering technique.

3.5.1 Sulfate permease gene (sulP) determination

As the cDNA sequences of green algal *sulP* were used in the multiple alignments, there was no usable conserved region to design either specific primer or degenerate primer. Thus, the strategy was changed to do the protein alignment. Five protein primary sequences of algal *sulP* were retrieved from NCBI database: *Nephroselmis olivacea* (Acc No. NP_050928), *Chlorella vulgaris* (Acc No. NP_045890), *Chlamydomonas reinhardtii* (Acc No. XP_001692459), *Mesostigma viride* (Acc No. NP_038441), *Chlorokybus atmophyticus* (Acc No. YP_001019170). The alignment showed the conserved regions on the middle and the C-terminus. The cDNA sequences correspondinding to the conserved parts were used as templates in degenerate primer design. More than a thousand combinations of degenerate forward primers (SdegF), two combinations of nested forward primers (SnestedF) and over a hundred combinations of degenerate reverse primers (SdegR) were designed.

The PCR was performed using *Tetraspora* sp. gDNA as a template with optimization on many parameters, but all reactions failed in amplification. Complementary DNA was used as a template instead of gDNA. The reaction was successfully amplified with 20-fold amount of both forward (SdegF) and reverse (SdegR) primers indicated by the presence of the expected band size of 450 bp as shown in lane 1 (Figure 3.17). However, there was another non-specific product in the reaction showing the size of about 800 bp. The more specific reaction was performed by seminested PCR using nested forward (SnestedF) and the same reverse (SdegR) primers. Along with the gradient annealing temperature, only one specific band was obtained when the annealing temperature ranged from 45 to 60 $^{\circ}$C as shown in lane 2-5 (Figure 3.17).

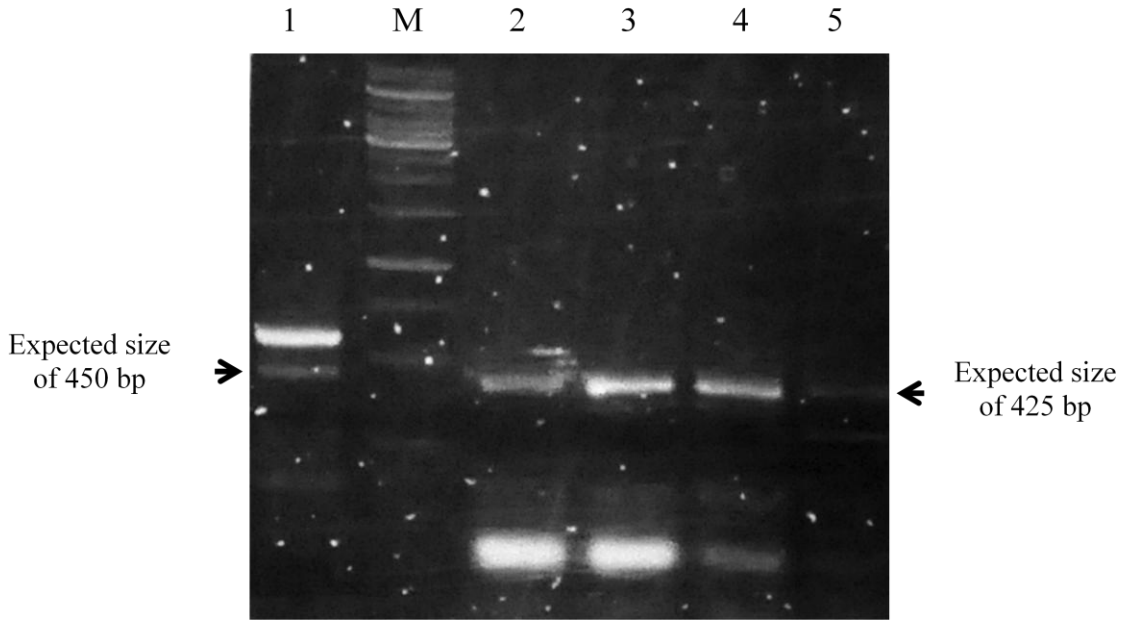


Figure 3.17 PCR product of putative *sulP* gene.

Lane 1: the first reaction using SdegF and SdegR primers

Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas)

Lane 2-5: gradient annealing temperature ranging from 45 – 60 °C (5°C increment) using SnestedF and SdegR primers.

The PCR fragments in lane 2 – 4 were pooled, purified and cloned to pCR®2.1-TOPO® cloning vector (Invitrogen) prior to sequencing. The sequencing result showed a fragment size of 432 bp. This sequence was subjected to the basic local alignment search tool (BLAST) in NBCI database. Blastn result showed no significant target to other sequence, but tBlastx program revealed the homology to *Chlamydomonas reinhardtii* chloroplast transport system permease (SulP) (Accession No. AF481828) with 3e-65 of E-value by 100% query coverage (as of 02 May 2012).

Moreover, based on the fragment sequenced, the gene specific primers were designed (C3RF and C3RR) for amplification using gDNA as a template in order to get a

fragment of this gene at DNA level. This PCR fragment was sequenced and showed larger size than that when using cDNA as a template. The result indicated the existence of intron inside this fragment. Comparison of gDNA and cDNA sequence revealed an intron fragment with a size of 231 bp (Figure 3.18). This fragment was ligated to pCR®2.1-TOPO® cloning vector and it was later called pTgSulP.

> Putative *Tetraspora* sp. CU 2551 *sulP* (mRNA)

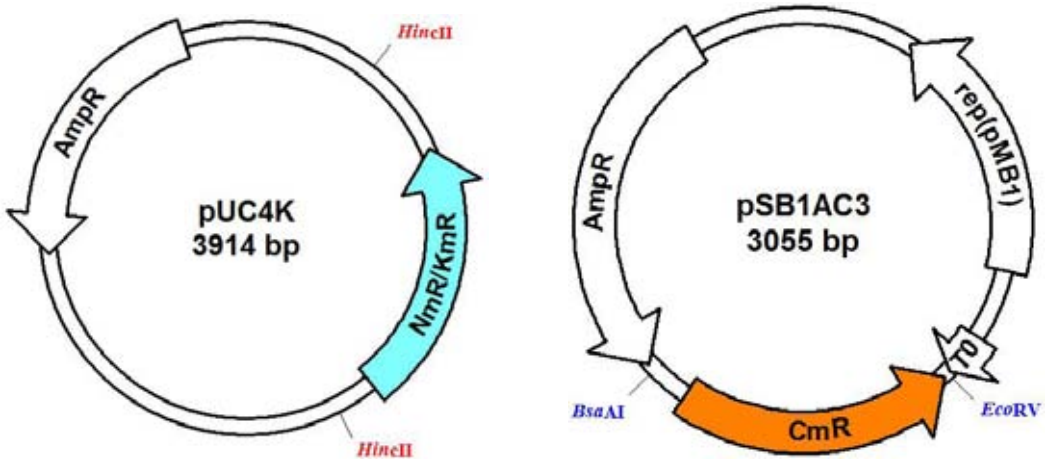
CCACATCAGTAGCTGGATTGACACTCGCCACAGTATACGGTGAAGAAGGCATTCTTGGTC
GTTTGCTGATGAGCTTGGGTGTAAATGTTGTGTACACCTGGCTGGGAGTAGCAGTGGCCA
TGGTGTTTGTGTCGTTCCCATTTGTAGTACGAACAATGCAGCCTGTT**CTTCAG**GTAAGAC
CAGCAGAGATGAGTGCGTCGACCTGTCGAATGGTTTGTGATTTTTTTGGCAGTGGGATAT
TGTTTACAACAAGGCTGAGTCAAGTGGCAAATATGAATCAGTCACAAGGTTCTTTCAGCG
CTGCACAGCAACAGCAATGGTTATGCATGTAGCAAGCACTGCAAGGCAGTATACCAGCAT
CTCATGTCACCAATGCTCATTGACTGTCATCTACATGCCTGCAGGAGATGGAGAAAGAGG
TTGAGGAAGCAGCTTGGTCTTTAGGAGCATCTCCCTGGTACACCTTCACCAAAGTCCTAC
TGCCACCTCTCCTACCACCCCTACTCACAGGCACAGCACTGGCATTCAGCAGGGCACTAG
GAGAGTTTGGCAGCATTGTGATAGTGTCTAGCAACTTCCCCTTTAAGGACTTGATAGCGC
CAGTGTTGA

Figure 3.18 A putative sequence of *Tetraspora* sulP. Specific forward (C3RF) and specific reverse (C3RR) primers are underlined. Shaded area showed the intron fragment with a size of 231 bp. Bold letters show the proper site for *NdeI*-site directed mutagenesis.

3.5.2 Construction of a sulP inactivation strain

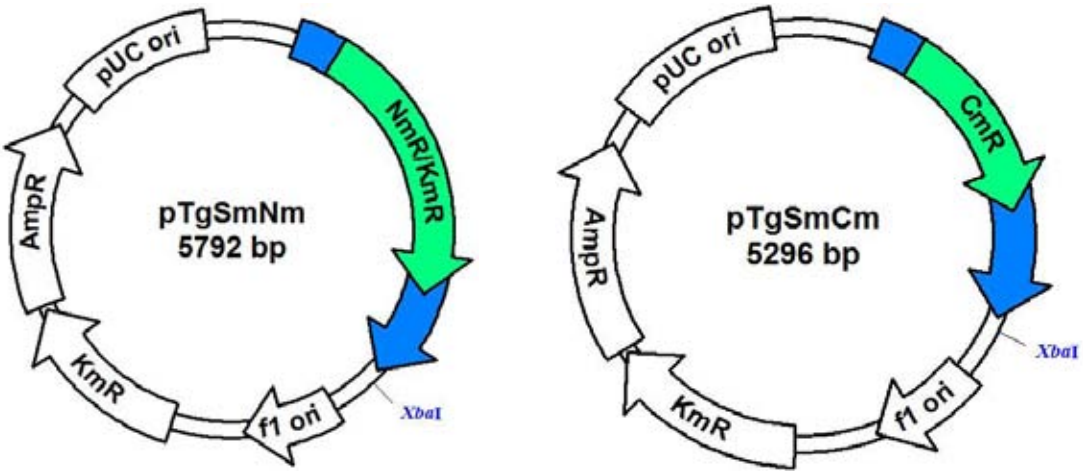
Based on the recombination by homologous DNA moving close together during DNA replication in cell cycle, the exogenous DNA can be introduced to the *Tetraspora* sp. genome by transforming an antibiotic resistance cassette flanked by the particular gene which is homologue to DNA sequence in algal genome. Searching for the restriction enzyme digestion on pTgSulP showed no suitable position for antibiotic resistance cassette insertion. Thus the NdeI restriction site was introduced to position of -7 before the intron site (indicated by bold letters in Figure 3.18). The sequence of CTTCAG was replaced with CATATG by site directed mutagenesis. The new plasmid was called pTgSm and further digested with *NdeI*. The pTgSm/*NdeI* linearlized plasmid was introduced to do the blunt-end tailing by T4 DNA polymerase followed by dephosphorylation by Shrimp alkaline phosphatase (SAP) to protect self ligation of vector.

Neomycin (Nm) and Chloramphenicol (Cm) antibiotic resistance cassettes were selected and used in the gene-inactivated plasmid construction. The Nm^R/Km^R fragment with a size of 1,252 bp was obtained from pUC4K digested with *HinCII* (Figure 3.19A). And, the Cm^R fragment with a size of 759 bp was obtained from pSB1AC3 digested with *EcoRV* and *BsaAI* (Figure 3.19B). Each gene-inactivated plasmid was separately constructed by Nm^R- pTgSm/*NdeI* and CmR- pTgSm/*NdeI* ligation. The new plasmids with Nm^R/Km^R and Cm^R were named pTgSmNm and pTgSmCm, respectively. The plasmid maps of both constructs were confirmed by sequencing and shown in Figure 3.20.



A **B**

Figure 3.19 Plasmid maps containing antibiotic resistance cassette. (A) Nm^R/Km^R was obtained from pUC4K digested with *HincII*. (B) Cm^R was obtained from pSB1AC3 digested with *EcoRV* and *BsaAI*.



A **B**

Figure 3.20 Plasmid maps for pTgSmNm (A) and pTgSmCm (B). The map showed the single cut by *XbaI* to linearlized the plasmids.

Due to the fact that the transformation method for *Tetraspora* sp. was not developed yet, many possibilities of transformation were used for trial. The traditional transformation methods, electroporation and glass bead vortexing, were selected. The *XbaI*-linearlized pTgSmNm and pTgSmCm recombinant plasmids were transformed to cellulase-treated *Tetraspora* sp. by those methods.

The TE buffer which is the plasmid solvent was used in transformation as negative control. After transformation of *XbaI*-linearlized pTgSmNm plasmid to algal cells by those methods, cells were spreaded on selective medium containing 30 µg/ml neomycin starting to bleach in 2 days and recovering in green colonies within 2 weeks. Both sets of transformation gave about 30 colonies on plate. Increasing the Neomycin concentration up to 60 µg/ml resulted in no colonies grown on the selective medium. However, the colonies on the selection of 30 µg/ml neomycin were picked and used in colony PCR to check the integration of antibiotic cassette to the algal genome. *SulP* gene specific primers (C3RF and C3RR) were used in colony PCR to track the up-shift band from *Tetraspora* sp. Forty-eight colonies from the 3rd generation were randomly picked and used as a template in colony PCR. The result showed no upshift band for the integration of antibiotic cassette to the algal genome, only wild-type band of *sulP* with a product size of 609 bp was observed.

Meanwhile, *XbaI*-linearlized pTgSmCm was transformed to *Tetraspora* sp. cells. Only a couple of colony grew on the selective medium containing 30 µg/ml chloramphenicol. These colonies were picked and streaked for the next generation on the selective medium with the same concentration of antibiotic. The third generation of transformants could not survive any longer. The confirmation of mutant strain was not analyzed.

Due to both pTgSmNm and pTgSmCm plasmids could not create the mutant strain, the length of homologous sequence was considered to have longer sequence than that in the present plasmids. The full-length of *sulP* was further determined.

3.5.3 Full-length sulP determination

As a *Tetraspora sulP* partial sequence was obtained, the cDNA sequence was used as a template for primer design using in 5'RACE and 3'RACE. 5'RACE and 3'RACE reactions provided the extended sequences in 5'- and 3'-direction of *Tetraspora sulP*. The cDNA sequence was shown in Figure 3.21. The transcript of *Tetraspora sulP* contained the coding sequence of 1,014 bp, and showed 5'- and 3'- UTR with 285 bp and 225 bp, respectively. This full-length fragment was introduced to blast against NCBI database. Blastn result showed no significant similarity to any other sequence in the database. However, tblastx results showed a high similarity to *Chlamydomonas reinhardtii* chloroplast sulfate transport system permease (*SulP*) with e-value of 1e-120 (as of 13 February 2012). The deduced amino acid sequence was used as an input sequence in protein alignment along with 14 other SulP sequences as shown in Figure 3.22. The shaded box ranging from a scale of 236 to 258 showed the conserved regions that used as a template in the corresponding to cDNA sequences for forward and nested forward primers design, and the region from a scale of 376 to 384 showed the region that used as a template for reverse primers. Moreover, the deduced amino acid sequences was predicted for chloroplast transit peptide (cTP) sequence by ChloroP 1.1 program. *C. reinhardtii* SulP and SulP2 sequences encoded from genomic DNA contained chloroplast transit peptides (cTP) with the length of 54 and 82 residues, respectively, while SulPs from other organisms showed no signal peptide. Based on the same strategy, the

Tetraspora SulP also showed the cTP with the length of 28 residues (underlined in Figure 3.22).

After alignment by ClustalX program, the Bootstrap NJ phylogenetic tree was determined as shown in Figure 3.23. Setting the *M. polymorpha* as an outgroup sequence, the map can be classified into several groups. *Tetraspora* SulP was grouped into the green algal SulP and showed close relationship to *C. reinhardtii* SulP. The map also classified the same group of SulP from cyanobacteria. *C. vulgaris* SulP was excluded from both green algal and cyanobacterial group, while the *C. reinhardtii* SulP2 was grouped to bacterial SulP which has close relationship to the liverwort *M. polymorpha*.

>*Tetraspora* sp. CU 2551 *sulP* (cDNA)

TTTTGTTATTCTGCCGCAGCACACCCCTACAACACCTTATGCACTTCTTTGGCTGAATAG
CTGTACCTGTTGCCCTGGATTTTTCCGTTCCGTGCGTCTTTTTCGAAACGAACTTTCACG
CTTGGTAGTGATATATAACTTGCTTTTGTGCTCCTGACACTCGATTTGGTCTGTCCTCCG
TTTCACCGCCCGACTTCTCAGATGGACAGGTTGAGCATTCCACAGATCTACAGTGATAGC
AGATGAGCGATAACAGTGAGAAACCTTGATAGATAAGCTTGGTATATGGAGCGATCGATT
CTGCATGGAGCGCAGTACCAAGCAGGACTGCCAGTGCTGACAGTTGCTAGACAGCAGCAG
AGAGTGGCGGCAAGCACGCCAGCAAAAGGCCCTCTATTGGCAGCATGCAGGCATCCGCTA
GCTGCAGCGGTCCTGCAAAGTAGCAGAATTCGGCCATGCAGCTTGCACTATGGGAATCAG
CAACAAAGTCAAGTTCTGCAACCAGTAGCAGCAGTTGGTTCTATAGGCAGCAGCAGTGAC
GTACCATGGCAGCCCCCACAACAAGCAAGCGGCCTAAGGCTACCCAAGATATCTCTCTGG
GACTTGGGTGGCCCCTTGGCTTGGTTGTATATGCTGGGCTACCTAGCAGTCATGTTGGTG
TTGCCCATCAGTGCGCTGCTGGCAAAGTCAAGCTTGGTACCCTTAGAGCAGTTTATAGCT
AGAGCCACAGAGCCAGTAGCTTTATCAGCTTATTATGTGTCCTTCAGTATGGCCCTCATG
GCTGGCGCCATCAACGCAGTGTTTGGGTTTCTGTTGGCCTGGGTGCTGGTGAAGTTTGAG
TTTCCAGGCAAGAAGTGGATAGATGCTGCAGTAGATCTTCCCTTTGCTCTACCCACATCA
GTAGCTGGATTGACACTCGCCACAGTATACGGTGAAGAAGGCATTCTTGGTCGTTTGCTG
ATGAGCTTGGGTGTAAATGTTGTGTACACCTGGCTGGGAGTAGCAGTGGCCATGGTGTTT
GTGTCGTTCCCATTTGTAGTACGAACAATGCAGCCTGTTCTTCAGGAGATGGAGAAAGAG
GTTGAGGAAGCAGCTTGGTCTTTAGGAGCATCTCCCTGGTACACCTTCACCAAAGTCCTA
CTGCCACCTCTCCTACCACCCCTACTCACAGGCACAGCACTGGCATTCAGCAGGGCACTA
GGAGAGTTTGGCAGCATTGTGATAGTGTCTAGCAACTTCCCCTTTAAGGACTTGATAGCG
CCAGTGTTGATATTCCAGTGTCTGGAACAATACGACTATGTTGGTGCTACTGTGATTGGG
ACTGTGTTGCTGCTCATCAGTTTGGTCATGATGGTGGGAGTCAACTGGCTGCAGTCCTAT
GCACAGCGCTTCAGGAGGTAGCTTGGCACTTCGTGTTAGCAAGTGCTTACCTATCGCTGA
AGTGTGACTGGAGAAGTTGGTGTTTTTACTCTGCAGATGTGGTATTCCTACTCTGCAGAT
GCAACAATGTAACATGTGCCATATAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.21 Full-length of putative *Tetraspora* sp. CU2551 *sulP* (cDNA). Shaded boxes showed a predicted open reading frame of 1,014 bp. Underlines show primer positions used in reverse transcription PCR analysis.

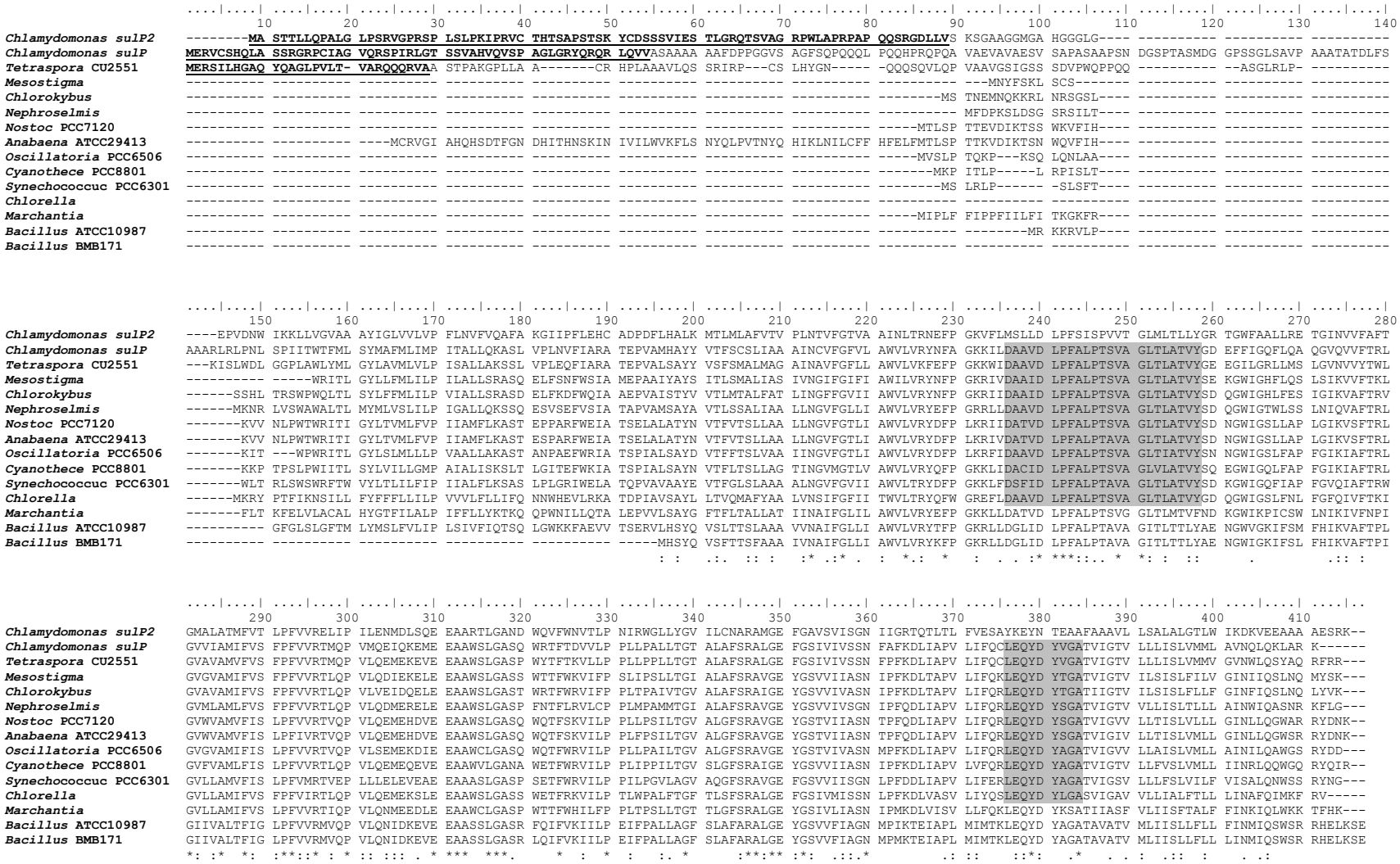


Figure 3.22 Sulfate permease protein alignments with other 14 different sequences.

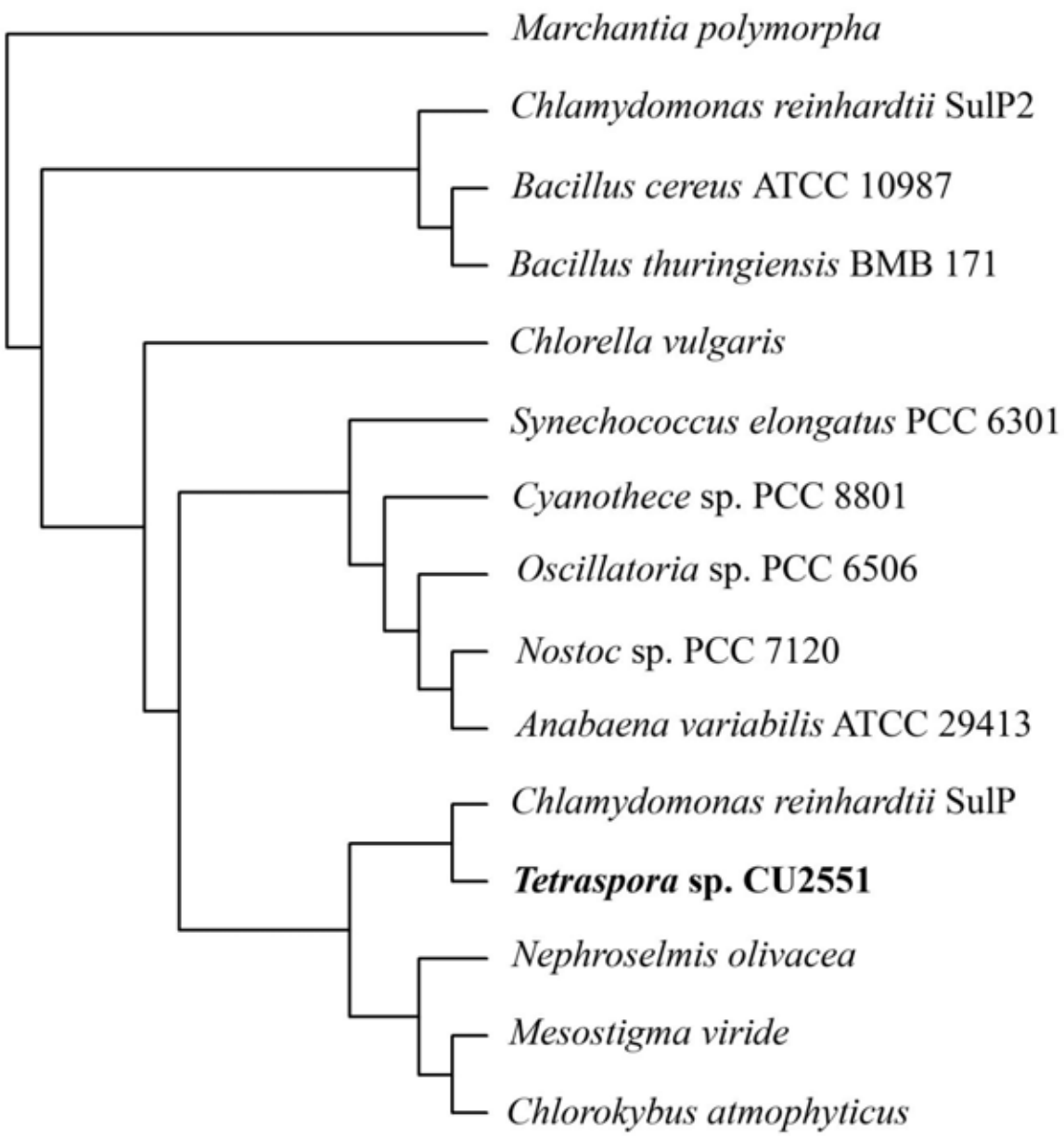


Figure 3.23 Bootstrap NJ phylogenetic tree of sulfate permease protein sequences using the deduced amino acid sequence of *Tetraspora* sp. CU2551 (bolded) and other 14 species

The *Tetraspora* sp. full-length sulP sequence was also determined at DNA level. Gene specific primers were designed to prime at the 5' and 3' end of cDNA sequence. Using gDNA as a template in PCR reaction, a specific band size of 2,271 bp was obtained and sequenced (Figure 3.24). The scaled position of introns as shown in Figure 3.25 was confirmed by comparison to the cDNA sequence. The gene contains four introns.

>*Tetraspora* sp. CU 2551 sulP (genomic DNA)

TTTTGTTATTCTGCCGCAGCACACCCCTACAACACCTTATGCACTTCTTTGGCTGAATAG
CTGTACCTGTTGCCCTGGATTTTTCCGTTCCGTGCGTCTTTTTCGAAACGAACTTTCACG
CTTGGTAGTGATATATAACTTGCTTTTGTGCTCCTGACACTCGATTTGGTCTGTCCTCCG
TTTCACCGCCCGACTTCTCAGATGGACAGGTTGAGCATTCCACAGATCTACAGTGATAGC
AGATGAGCGATAACAGTGAGAAACCTTGATAGATAAGCTTGGTATATGGAGCGATCAATT
CTGCATGGAGCGCAGTACCAAGCAGGACTGCCAGTGCTGACAGTTGCTAGACAGCAGCAG
AGAGTGGCGGCAAGCACGCCAGCAAAAGGTATGCGCTCACAACCTCGATCGCGGAGGTAG
TAGGAATACTGGTTCTCAACCCATGACTGTCTTGCAGGCCCTTTATTGGCAGCATGCAGG
CATCCGCTAGCTGCAGCAGTCCTGCAAAGTAGCAGAATTCGGCCATGCAGCTTGCACTAT
GGGAATCAGCAACAAAGTCAAGTTCTGCAACCAGTAGCAGCAGTTGGTTCTATAGGTGGG
TGCAACGTTGCATTCCTGTTACTTCATCTTCGCCGTATTTCGAAGCACACTGTCCTGGGA
CTTAGGCTGGCTCACTCCTGCACTTGCTGTTTTCAGTCTTTCCGATATGCTGCAAGTCTA
GCTTTGTCTTCGCCCTGCAACCGCTTTGTTTAGTCAAACATGGCATCGGCTGCATAGCTG
GCCAACAGCTGTACATACCTGTATCATCTGCACTCCACTACAGGCAGCAGCAGTGACGTA
CCATGGCAGCCCCCACAACAAGCAAGCGGCCTAAGGCTACCCGAGATATCTCTCTGGGAC
TTGGATGGCCCCTTGGCTTGGTTGTATATGCTGGGCTACCTAGCAGTCATGTTGGTGTTG
CCCATCAGTGCGCTGCTGGCAAAGTCAAGCTTGGTACCCTTAGAGCAGTTTATAGCTAGA
GCCACAGAGCCAGTAGCTTTATCAGCTTATTATGTGTCCTTCAGTATGGCCCTCATAGCT
GGCGCCATCAGCGCAGTGTTTGGGTTTCTGTTGG**CCTGGG**TGCTGGTGAAGTTTGAGTTT
CCAGGTAAGTAAAAAACCGCAGCAGCAGTGGCTGTATTTGTGAGCTGAGTTGTGGTTGTT
GTAGTACATCCCACAAGACCTCCATATGGTTGCACCTAGCATGCAATGCCCATGTGTCAC
AGAGCCCTGCATTCTTGCTGTTGCTTGTCAGTCGGTCTGCTCTTTGTACTACAGCAGATA
GTCGTTGCCTGAGCTGCTGACACCATGCAATGGTTCCCTGCAGGCAAGAAGTGGATAGAT
GCTGCAGCAGATCTTCCCTTTGCTCTACCCACATCAGTAGCTGGATTGACACTCGCCACA

GTATACGGTGAAGAAGGCATTCTTGGTCGTTTGCTGATGAGCTTGGGTGTAAATGTTGTG
TACACCAGGCTGGGAGTAGCAGTGGCCATGGTGTTTGTGTCGTTCCCATTTGTAGTACGA
ACAATGCAGCCTGTTCTTCAGGTAAGACCAGCAGAGATGAGTGCGTCGACCTGTCGAATG
GTTTGTGATTTTTTTGGCAGTGGGATATTGTTTACAGCAAGGCTGAGTCAAGTGGCAAAT
ATGAATCAGTCACAAGGTTCTTTCAGCGCTGCACAGCAACAGCAATGGTTATGCATGTAG
CAAGCACTGCAAGGCAGTATACCAGCACCTCATGTCACCAATGCTCATTGACTGTCATCT
ACATGCCTGCAGGAGATGGAGAAAGAGGTTGAGGAAGCAGCTTGGTCTTTAGGAGCATCT
CCCTGGTACACCTTCACCAAGGTCCTACTGCCACCTCTCCTACCACCCCTACTCACAGGC
ACAGCACTGGCATTCAGCAGGGCACTAGGAGAGTTTGGCAGCATTGTGATAGTGTCTAGC
AACTTCCCCTTTAAGGACTTGATAGCGCCAGTGTTGATATTCCAGTGTCTGGAACAATAC
GACTATGTTGGTGCTACTGTGATTGGGACTGTGTTGCTGCTCATCAGTTTGGTCATGATG
GTGGGAGTCAACTGGCTGCAGTCCTATGCACAGCGCTTCAGGAGGTAGCTTGGCACTTCG
TGTTAGCAAGTGCTTACCTATCGCTGAAGTGTGACTGGAGAAGTTGGTGTTTTTACTCTG
CAGATGTGGTATTCCTACTCTGCAGATGCAACAATGTAACATGTGCCATAT

Figure 3.24 Full-length of putative *Tetraspora* sp. CU2551 *sulP* (DNA). Shaded boxes showed the introns fragments. Bold letters showed the site-directed mutagenesis site for *SmaI*-restriction enzyme site introducing. Underlines are the primer-specific regions used in amplification for partial *sulP* fragment amplification.

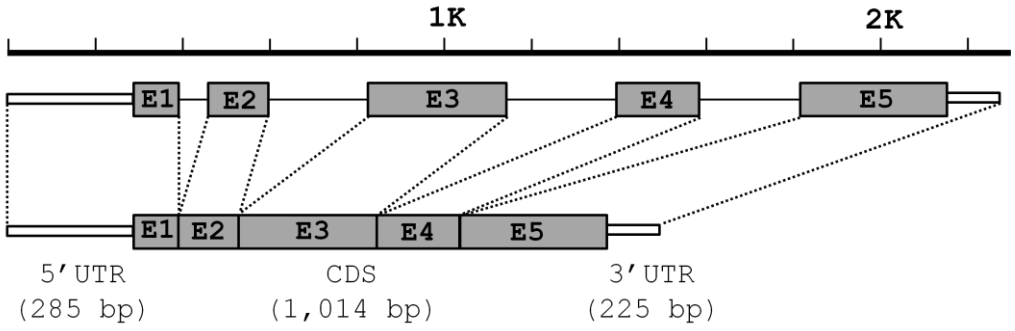
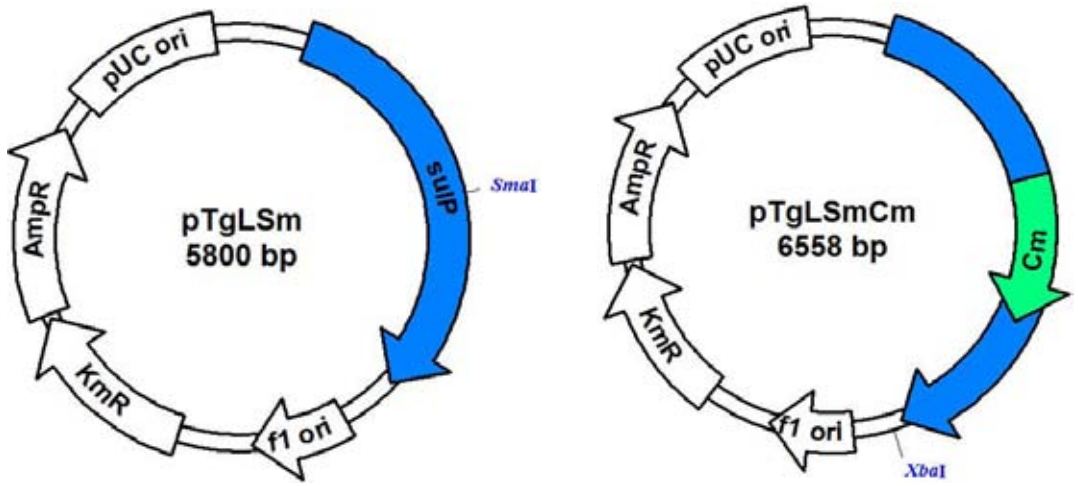


Figure 3.25 The structural gene of *sulP* in both cDNA and DNA levels along with the ruler indicating the length on the top. 'E' represents exon fragments.

3.5.4 Construction of a sulP inactivation strain (2)

As the full-length *sulP* was determined, a new long *sulP* fragment was amplified by using gene-specific forward (5UTR) and reverse (C3RR) primers as underlined in figure 3.23. A fragment size of 1,868 bp was produced and ligated to pCR®2.1-TOPO® vector. The sequence and orientation were confirmed by sequencing. The *SmaI*-restriction site was introduced to position of -27 before the 3rd intron (indicated by bold letters in Figure 3.24). The sequence of CCTGGG was replaced to CCCGGG by site directed mutagenesis. The new plasmid was called pTgLSm (Figure 3.26A) and further digested with *SmaI*. The linearlized plasmid was purified and treated with Shrimp Alkaline Phosphatase (SAP) to protect the self-ligation during the ligation step. Cm^R fragment obtained previously in section 3.5.2 was used to ligate to produce pTgLSmCm plasmid as shown in Figure 3.26B.



A **B**

Figure 3.26 Plasmid maps of *sulP* gene on cloning vector (left) and CmR-inactivated construct (right). *SmaI* and *XbaI* shows the position of single cut on pTgLSm and pTgLSmCm, respectively.

XbaI-linearlized pTgLSmCm was transformed to cellulase-treated *Tetraspora* sp. cells by glass bead vortexing method. About 10 colonies grew on the selective medium containing 30 µg/ml chloramphenicol. These colonies were picked and streaked for the next generation on the selective medium with the same concentration of antibiotic. Six transformants of the fourth generation were used as a template in colony PCR to check the segregation of the antibiotic resistance cassette to algal genome (Figure 3.27). Another pair of primers were designed (3F and CmR) to track for the segregation to algal genome. 3F and CmR primers primed to the upstream and inside of Cm^R-cassette, respectively (Figure 3.27 upper panel). The expected product was 850 bp as shown in lane 'P' using pTgLSmCm plasmid as a template for positive control (Figure 3.27). A product size of 850 bp was observed in transformant number 3 after PCR for 30 cycles. This transformant, however, could not grow any longer to the 6^{th} generation. Searching for new antibiotic cassette was done. Paromomycin antibiotic cassette, which is well

known cassette used in *Chlamydononas reinhardtii* transformation, was chosen as an alternative choice.

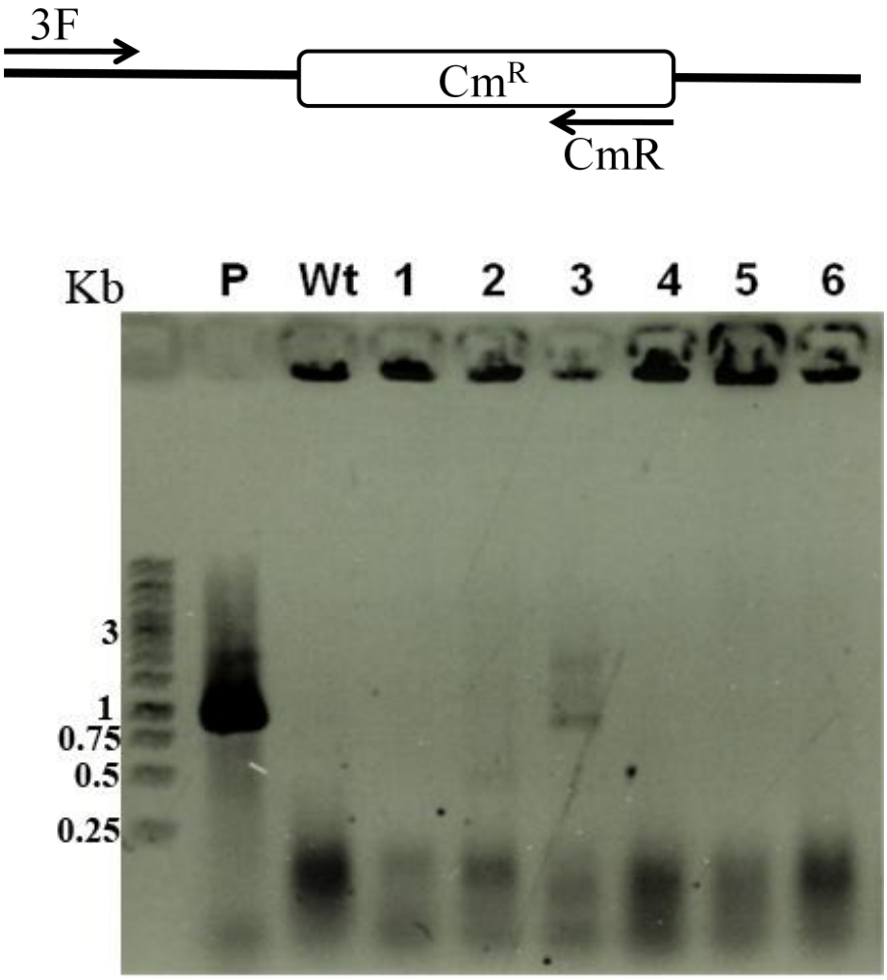


Figure 3.27 The negative exposure of PCR pattern after ethidium bromide staining. (upper) the schematic represented the primer positions on the pTgLSmCm. (lower) Colony PCR of 6 pTgLSmCm-transformants in the 4th generation.

P: positive control using pTgLSmCm plasmid as a template

Wt: negative control using wild-type cells as a template

1-6: 6 4th-generation transformants of *XbaI*-linearlized pTgLSmCm

Paromomycin antibiotic cassette (Pm^R) was carried on pSI103-1 plasmid purchased from Chlamy center (USA). The Pm^R cassette with the size of 1,809 bp was inserted between the *PstI* -- *KpnI* sites on pBluescript II KS (+). Figure 3.28 showed the paromomycin antibiotic cassette components developed previously (Sizova *et al*., 2001).

Figure 3.28 The Paromomycin resistance cassette components:

hsp70A: heat shock inducing promoter from *C. reinhardtii*

5'rbcS2: 5' untranslated region of the endogenous Rubisco small subunit gene from *C. reinhardtii*

In1: rbcS2 first intron of rbsc2 gene from *C. reinhardtii*

aph VIII: aminoglycoside 3'-phosphotransferase gene (*aph*) from *Streptomyces rimosus*

3'rbcS2: 3' untranslated region of the endogenous Rubisco small subunit gene from *C. reinhardtii*

To obtain the Pm^R fragment, the band of 1,809 bp was purified from pSI103-1 double digestion with *PstI* and *KpnI* (Figure 3.29A). Pm^R fragment was further treated with T4 DNA polymerase to make blunt-end termini prior to use in ligation with *SmaI*-linearlized pTgLSm plasmid (Figure 3.29B). At the same time, in order to increase the possibility to obtain a *sulP*-inactivated plasmid, a part of *sulP* with a size of 490 bp was removed by pTgLSm/*SmaIHincII* double digestion (Figure 3.29C). These vectors were further treated with Shrimp Alkaline Phosphatase (SAP) to get rid of self ligation during

ligation reaction in the presence of Pm^R fragment. After optimization of both reactions, the reactions unsuccessfully produced the recombinant plasmid. The strategies were changed to use PCR-base ligation. Gene specific primers were designed to amplified in the inverse PCR using pTgLSm as a template to produce a fragment with restriction enzyme sites at both ends (Figure 3.30A). The Pm^R fragment was also amplified with gene specific primer containing the same restriction sites (Figure 3.30B). Restriction enzymes, *ClaI* and *NruI*, treated fragments were purified and used in ligation reaction. Ligation reaction was optimized to get the proper recombinant plasmid, but no *E. coli* transformant was obtained after transformation.

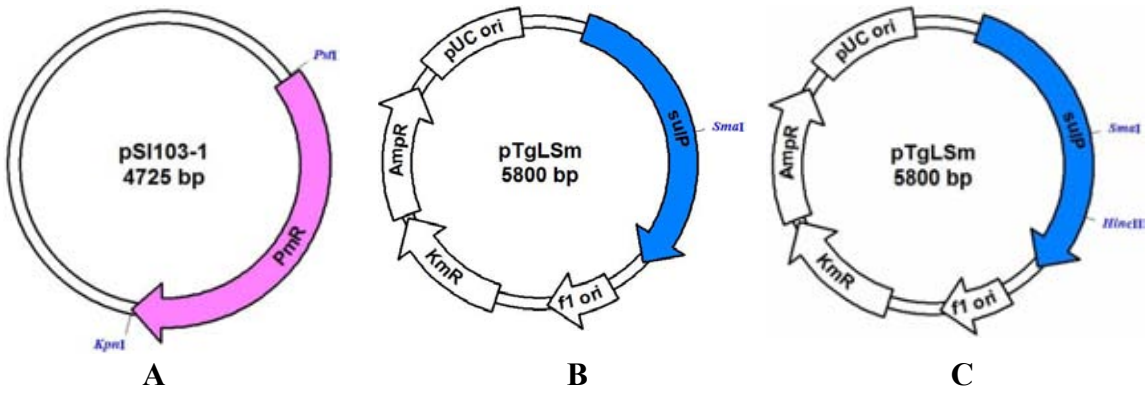


Figure 3.29 Schematic shows plasmids used in Pm^R:*sulP*-inactivated plasmid construction. (A) Pm^R fragment was purified from Pm^R-carrying plasmid (pSI103-1) by double digestion of *KpnI* and *PstI* prior to blunt-end treatment by T4 DNA polymerase. (B) A single digestion of *sulP*-carrying plasmid (pTgLSm) by *SmaI*. (C) A double digestion of *sulP*-carrying plasmid (pTgLSm) by *SmaI* and *HincII* to remove a fragment of 490 bp of *sulP*.

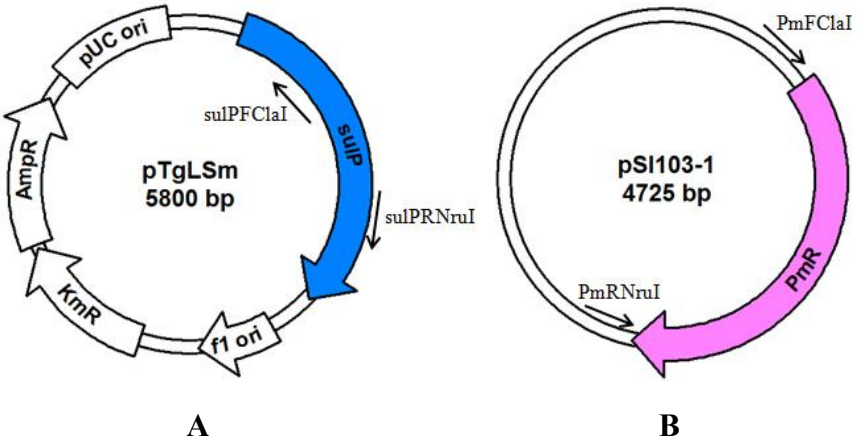


Figure 3.30 Schematic shows the primer positions on plasmids used in inverse PCR.

An indirect ligation was considered to solve the problem. The *sulP* fragment was divided into two fragments and further ligated to the flanking region of pSI103-1, Pm^R containing plasmid. The schematic of ligation strategies is shown in Figure 3.31. Fragment so called A was purified from pTgLSm/*KpnI* and fragment so called C was purified from pTgLSm/*PstI*. Each fragment was separately used in ligation to either pSI103-1/*KpnI* or pSI103-1/*PstI* to get the first flanking part on Pm^R. Fragment C was successfully cloned to pSI103-1 at the *PstI* cloning site. This new recombinant plasmid was called PmC plasmid. The PmC was then digested with *KpnI* and used in ligation with fragment A. The final construct was called PmCA plasmid. Figure 3.32 showed the flow chart of PmCA plasmid construction.

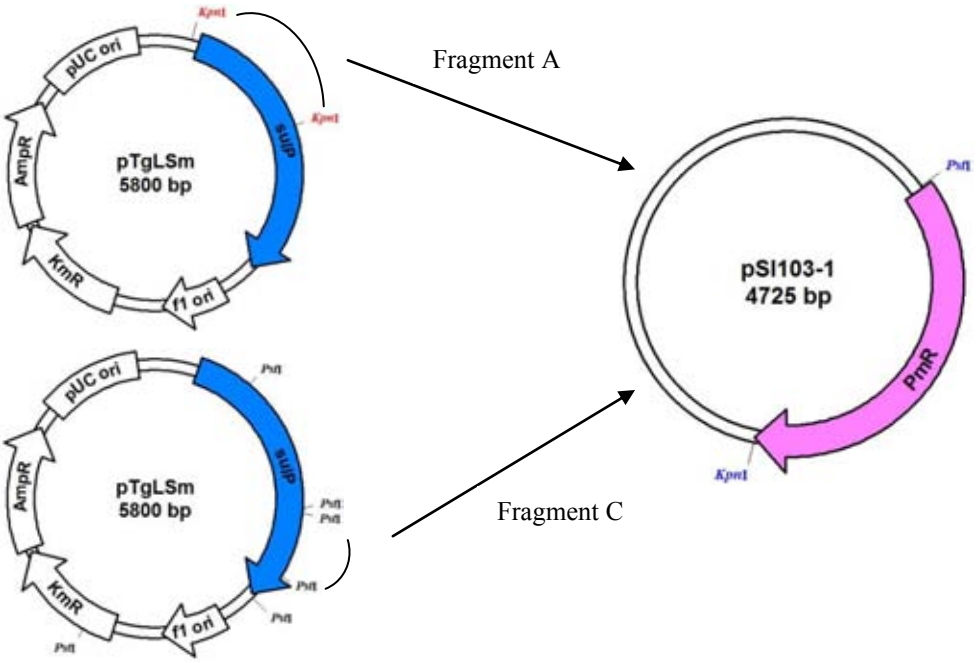
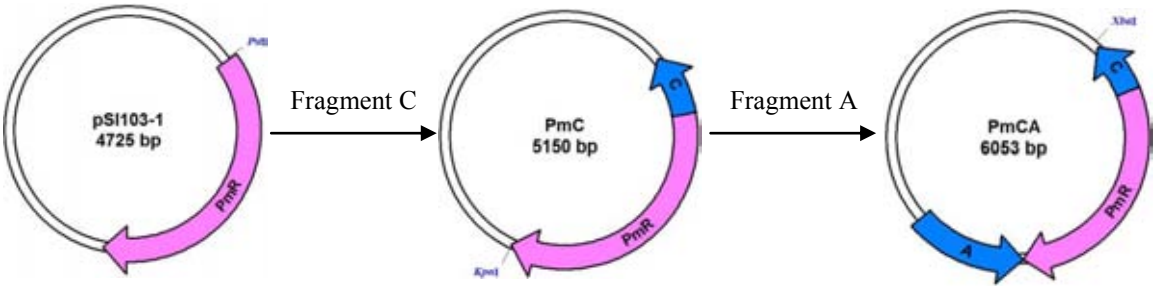
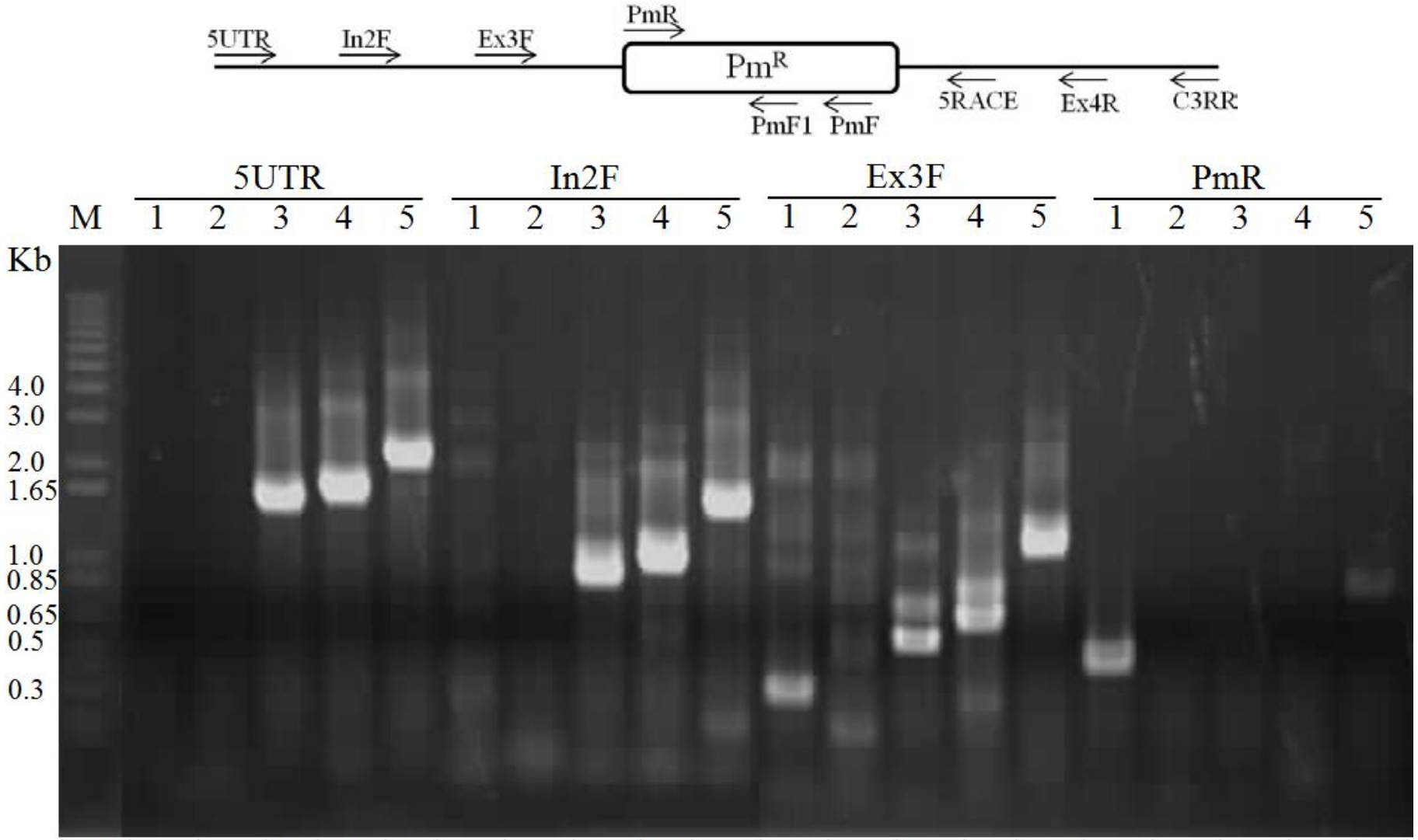


Figure 3.31 Schematic showing indirect ligation strategies. Fragment size of 899 bp from pTgLSm/*KpnI* was called fragment A and Fragment size of 425 bp from pTgLSm/*PstI* was called fragment C.



Fugure 3.32 Schematic showing the indirect ligation to obtain Pm^R:*sulP*-inactivated plasmid (PmCA). pSI103-1 was flanked by fragment C insertion followed by fragment A. *XbaI* position shows the single cut to linearlize plasmid for transformation.

XbaI-linearlized PmCA plasmid was transformed to cellulase-treated *Tetraspora* sp. CU2551. Glass bead vortexing transformation yielded no mutant by PmCA plasmid. The transformation was then changed to use biolistic microparticle bombardment method. After transformation, the transformants culture were spreaded on TAP agar medium containing 10 µg/ml paromomycin. Many colonies were picked and further streaked on 5 µg/ml paromomycin-containing agar stepwise with increasing concentration until the 5^{th} generation. The final concentration of paromomycin was maintained at 30 µg/ml paromomycin afterwards. One transformant had survived to 12^{th} generation. Colony PCR did not show any promising band in agarose gel. Then, genomic DNA of this transformant was isolated and used as a template in PCR to confirm the availability of mutant. Twenty combinations from 4 forward and 5 reverse primers around the recombination sites were used for the test (Figure 3.33).



WT	-	-	1329	1433	1869	-	-	817	921	1357	-	-	536	640	1076	-	-	-	-	-
Mut	1558	2478	2761	2865	3354	1076	1966	2249	2353	2788	795	1685	1968	2072	2507	447	1337	1620	1724	2159

Figure 3.33 PCR pattern from genomic DNA of a 12th generation transformant. Upper panel shows the schematic of primers around recombination sites. Four forward and five reverse primers were used. Middle panel showed the PCR pattern when 20 combinations of primers were used. Lower panel shows the expected product sizes from each reaction in bp unit from both wild (WT) type and mutant (Mut).

The lane numbers were listed as following:

M: 1 Kb Plus DNA Ladder (Invitrogen)

1: PmF1 reverse primer

2: PmF reverse primer

3: 5RACE reverse primer

4: Ex4R reverse primer

5: C3RR reverse primer

The gel pattern tended to show the promising strain of wild type alga except the reaction amplified with PmR and PmF1 primers, while the wild type genomic DNA did not produce this fragment. A part of Paromomycin resistance cassette with expected band was observed with a size of 447 bp. This band was purified, cloned and sequenced, and showed the size of 451 bp. Figure 3.34 showed the local alignment of a 451-bp band against the 447-bp known Pm cassette sequence between PmR and PmF1 sites. Alignment showed 52% similarity with identical primers sequence of 451-bp fragment to known sequence of Pm^{R} cassette. Sequencing result confirmed no mutant creation from Pm resistance cassette transformation by biolistic microparticle bombardment method.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60         70         80
451bp           GTCCGTTCGA TCGCAGTCTA GTAACTCTCG ATGTTATGCA GGTGCTGTCA GTGGTGGTGT GCAAGCCTAC TCCTCTTCAG
cassette        GTCCGTTCGA TCGCAGTCTC GCGG-TGACG GTGCC--GCA GGCG--GCCC GTGCTGTCGC TGAAGGGAGC GTCGACTTGG
                ********** *********  *    *  **  **    *** ** *  * *  *** **  *    ***    *   *   *  *

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      90        100        110        120        130        140        150        160
451bp           CAGAAGCTGT ATGAACACTT TTTGTTGTCA A-ATGCTACC CGAAGGTTAC TGTCTGGTAG CAAAG-CAGC TGGAGTCCTA
cassette        -AGGATCTGG ACGAGGAGCG GAAGGGGTGG TCGGGGGAGC GGCTTCTCGC CGAGCTGGAG CGGACTCGGC CTGCGGACGA
                 ** * ***  * **  *       *  **       *  * *  *    *  *  *    * ** *  *  * **   * *  * *

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      170        180        190        200        210        220        230        240
451bp           AGTGCTATCA -CCTGCTTGA AGAAGCTGTG CAACCACCCC AAGCTGATCT ATGACACCCT GCATAGCAAG ACACAGGTAG
cassette        GGATCTGGCG GTTTGCCACG GTCACCTGTG CCCGGACAAC GTGCTGCTCG AC--CCTCGT ACCTGCGAGG TGACCGGGCT
                 *  **  *     ***        * ***** *    **  *   **** **  *   *  * *  * *   * *   ** **

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      250        260        270        280        290        300        310        320
451bp           GGCTGGGGAC TGCAGGG--G GTTGGGTGGG GTTGCAGCTC ACTCCATGGC TAGATGTTGT ATTTGCTTGT TTGTT--GCA
cassette        GATCGACGTG GGGCGGGTCG GCCGTGCGGA CCGGCACTCC GATCTCGCGC TGG-TGCTGC GCGAGCTGGC CCACGAGGAG
                *   *  *    *  ***  * *  * * **     ***   *   **    ** * * ** **      *** *         *

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      330        340        350        360        370        380        390        400
451bp           TCTCCCTGCA TGATATCAGA GATGATGTCA GCTTCTCCAG CACTGCTACT TATTGCCAGC AGCTTGAAGT G-TCTAGTGA
cassette        GACCCGTGGT TCGGGCCGGA G-TGTTCCGC GGCGTTCCTG CGGGAGTACG GGC-GCGGGT GGGATGGGGC GGTATCGGAG
                   ** **   *     * ** * ** *     *    *** * *     ***      **  *   *  **  *  * * * *

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
                      410        420        430        440        450
451bp           GTGTCTCATG TGTATGACTG CAGGCTGATG GCACTGTGTC TGAGGGACCT GATGGTGTT
cassette        GAAAAGCTGG CGTTTTACCG GCTGTTGGAC GAGTTCT-TC TGAGGGACCT GATGGTGTT
                *     *  *  ** * ** *    * **    *   * * ** ********** *********
```

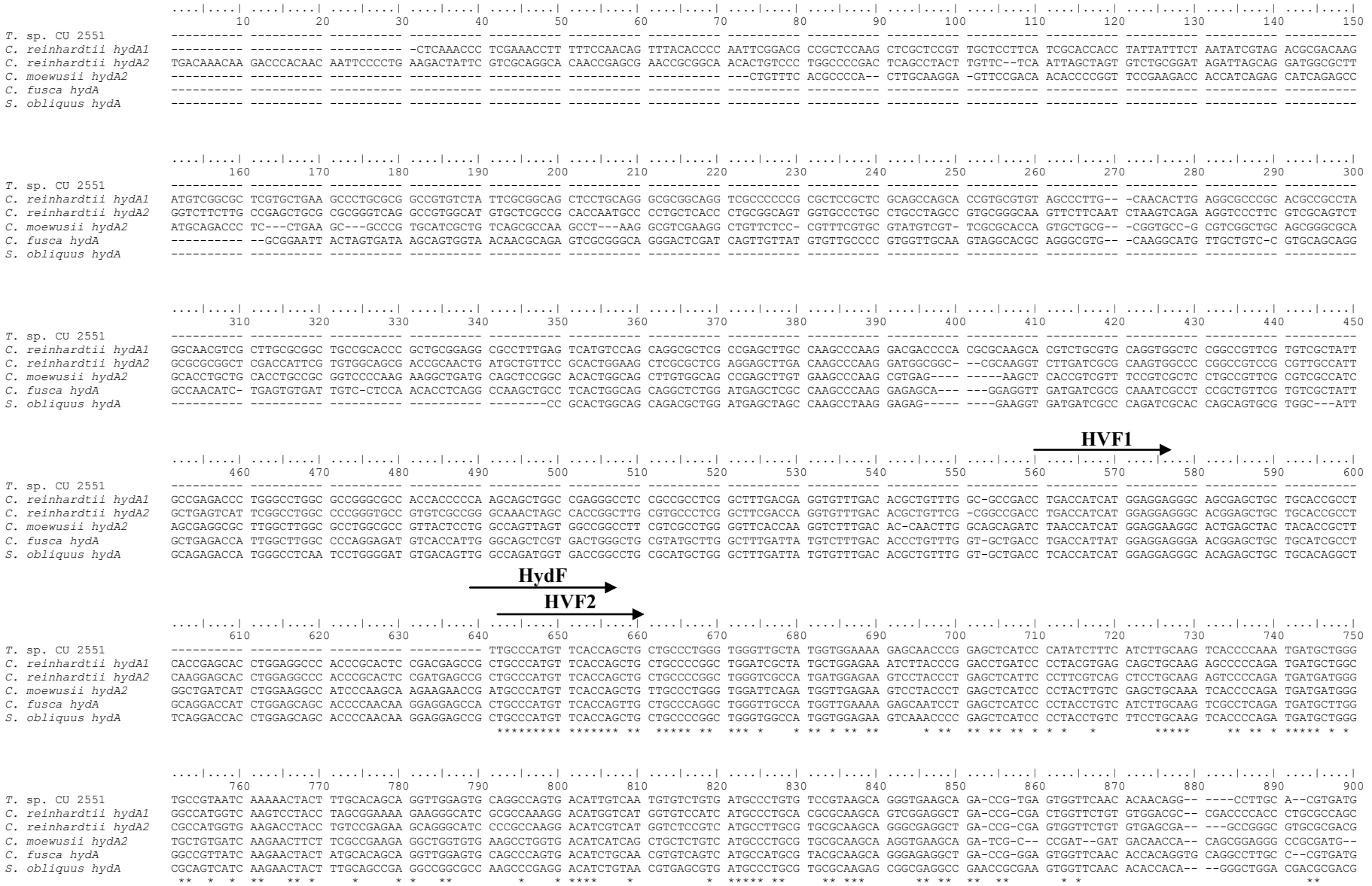
Figure 3.34 Alignment of a 451-bp fragment amplified by PmR and PmF1 primers using transformant genomic DNA as a template against the known Pm^R cassette sequence between PmR and PmF1 sites.

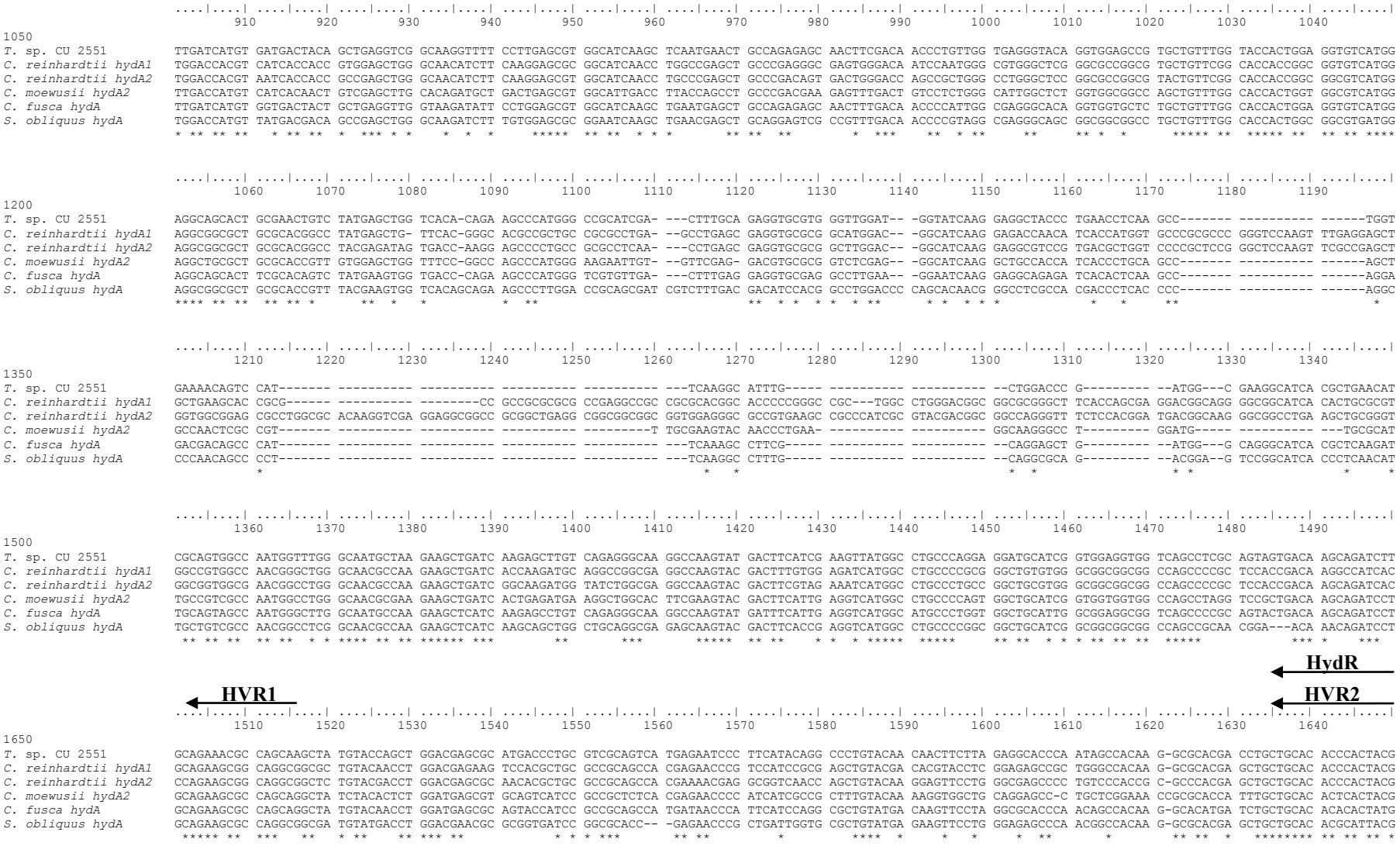
The overall results in mutant creation suggested that the reliable method to transform the exogenous DNA to *Tetraspora* sp. genome was unsuccessfully developed. The aim of this part is to investigate the gene(s) involved in hydrogen metabolism and understand their function; however, the mutant could not be created. Alternatively, the gene expression profile was investigted to understand the hydrogen production pathway in the green alga *Tetraspora* sp. CU2551. Sulfate permease (*sulP*) and hydrogenase (*hydA*) expression profiles were further studied.

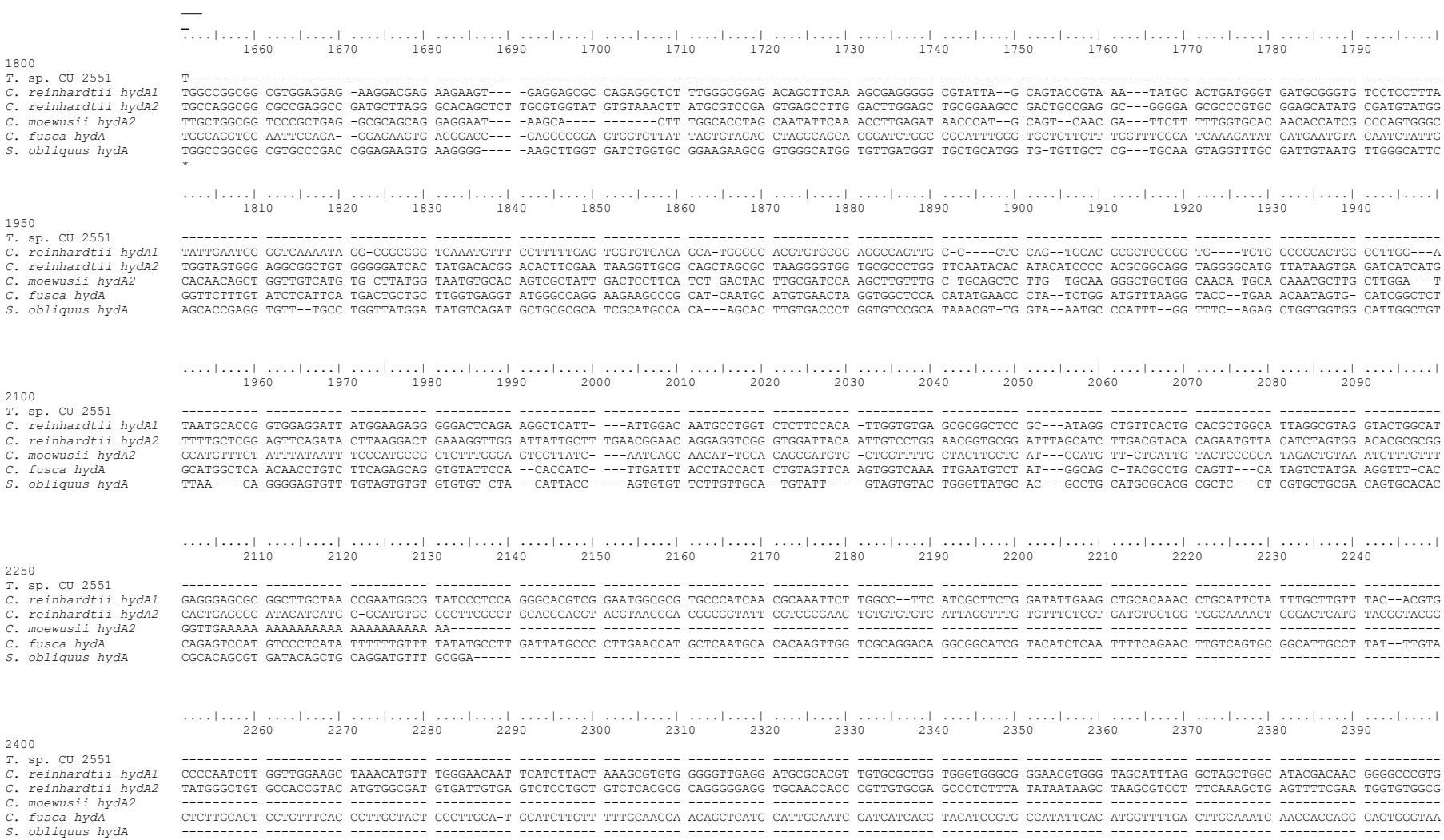
3.6 Sulfur deprivation

3.6.1 hydrogenase gene (hydA) investigation

Unlike *sulP* gene, the sequence of *hydA* from green algae showed high similarity at DNA level. The cDNA sequences of *hydA* from several green algal strains were obtained from NCBI. Five *hydA* sequences from *Chlorella fusca* (Acc No. AJ298228), *Chlamydomonas reinhardtii hydA1* (Acc No. AY055755), *Chlamydomonas reinhardtii hydA2* (Acc No. AY055756), *Chlamydomonas moewusii hydA2* (Acc No. AY578072), and *Scenedesmus obliquss* (Acc No. AF276706) were aligned, and the conserved regions were located. Although *hydA* showed higher level of conserved region than that in *sulP*, few numbers of degenerate primers were still designed from these conserved parts. Three forward and three reverse primers were synthesized according to sequence under arrows indicated in Figure 3.35. Sequence of 6 primers were listed in Table 1. Nine combinations of primer pairs were tested in PCR amplification using cDNA as a template and one of them showed a promising band in agarose gel electrophoresis. The primers giving PCR product of the *hydA* fragment were HvF2 and HvR2. PCR band was purified, cloned, and sequenced. A partial sequence of *Tetraspora hydA* was obtained. A full-length of this gene was not available, however, the sequence we obtained was usable for further experiment in gene expression analysis. By the same primer pair, the PCR reaction produced a fragment at genomic DNA level. The band was purified, cloned, and sequenced. Comparison between cDNA and gDNA sequences showed the existence of two introns inside the gene with the size of 261 and 282 bp, respectively, as shown in shaded regions (Figure 3.36). The sequence was also used as a template to design primer as underlined for gene expression analysis. Forward and reverse primers were underlined.


HVF1
HydF
HVF2
T. sp. CU 2551
C. reinhardtii hydA1
C. reinhardtii hydA2
C. moewusii hydA2
C. fusca hydA
S. obliquus hydA


HydR
HVR1
HVR2


1660 1670 1680 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790
1800
T. sp. CU 2551 T--------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reinhardtii hydA1 TGGCCGGCGG CGTGGAGGAG -AAGGACGAG AAGAAGT--- -GAGGAGCGC CAGAGGCTCT TTGGGCGGAG ACAGCTTCAA AGCGAGGGGG CGTATTA--G CAGTACCGTA AA---TATGC ACTGATGGGT GATGCGGGTG TCCTCCTTTA
C. reinhardtii hydA2 TGCCAGGCGG CGCCGAGGCC GATGCTTAGG GCACAGCTCT TGCGTGGTAT GTGTAAACTT ATGCGTCCGA GTGAGCCTTG GACTTGGAGC TGCGGAAGCC GACTGCCGAG GC---GGGGA GCGCCCGTGC GGAGCATATG CGATGTATGG
C. moewusii hydA2 TTGCTGGCGG TCCCGCTGAG -GCGCAGCAG GAGGAAT--- -AAGCA---- -------CTT TGGCACCTAG CAATATTCAA ACCTTGAGAT AACCCAT--G CAGT--CAAC GA---TTCTT TTTGGTGCAC AACACCATCG CCCAGTGGGC
C. fusca hydA TGGCAGGTGG AATTCCAGA- -GGAGAAGTG AGGGACC--- -GAGGCCGGA GTGGTGTTAT TAGTGTAGAG CTAGGCAGCA GGGATCTGGC CGCATTTGGG TGCTGTTGTT TGGTTTGGCA TCAAAGATAT GATGAATGTA CAATCTATTG
S. obliquus hydA TGGCCGGCGG CGTGCCCGAC CGGAGAAGTG AAGGGG---- -AAGCTTGGT GATCTGGTGC GGAAGAAGCG GTGGGCATGG TGTTGATGGT TGCTGCATGG TG-TGTTGCT CG---TGCAA GTAGGTTTGC GATTGTAATG TTGGGCATTC
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920 1930 1940
1950
T. sp. CU 2551 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reinhardtii hydA1 TATTGAATGG GGTCAAAATA GG-CGGCGGG TCAAATGTTT CCTTTTTGAG TGGTGTCACA GCA-TGGGGC ACGTGTGCGG AGGCCAGTTG C-C----CTC CAG--TGCAC GCGCTCCCGG TG----TGTG GCCGCACTGG CCTTGG---A
C. reinhardtii hydA2 TGGTAGTGGG AGGCGGCTGT GGGGGATCAC TATGACACGG ACACTTCGAA TAAGGTTGCG CAGCTAGCGC TAAGGGGTGG TGCGCCCTGG TTCAATACAC ATACATCCCC ACGCGGCAGG TAGGGGCATG TTATAAGTGA GATCATCATG
C. moewusii hydA2 CACAACAGCT GGTTGTCATG TG-CTTATGG TAATGTGCAC AGTCGCTATT GACTCCTTCA TCT-GACTAC TTGCGATCCA AGCTTGTTTG C-TGCAGCTC TTG--TGCAA GGGCTGCTGG CAACA-TGCA CAAATGCTTG CTTGGA---T
C. fusca hydA GGTTCTTTGT ATCTCATTCA TGACTGCTGC TTGGTGAGGT ATGGGCCAGG AAGAAGCCCG CAT-CAATGC ATGTGAACTA GGTGGCTCCA CATATGAACC CTA--TCTGG ATGTTTAAGG TACC--TGAA ACAATAGTG- CATCGGCTCT
S. obliquus hydA AGCACCGAGG TGTT--TGCC TGGTTATGGA TATGTCAGAT GCTGCGCGCA TCGCATGCCA CA---AGCAC TTGTGACCCT GGTGTCCGCA TAAACGT-TG GTA--AATGC CCATTT--GG TTTC--AGAG CTGGTGGTGG CATTGGCTGT
1960 1970 1980 1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090
2100
T. sp. CU 2551 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reinhardtii hydA1 TAATGCACCG GTGGAGGATT ATGGAAGAGG GGGACTCAGA AGGCTCATT- ---ATTGGAC AATGCCTGGT CTCTTCCACA -TTGGTGTGA GCGCGGCTCC GC---ATAGG CTGTTCACTG CACGCTGGCA TTAGGCGTAG GTACTGGCAT
C. reinhardtii hydA2 TTTTGCTCGG AGTTCAGATA CTTAAGGACT GAAAGGTTGG ATTATTGCTT TGAACGGAAC AGGAGGTCGG GTGGATTACA ATTGTCCTGG AACGGTGCGG ATTTAGCATC TTGACGTACA CAGAATGTTA CATCTAGTGG ACACGCGCGG
C. moewusii hydA2 GCATGTTTGT ATTTATAATT TCCCATGCCG CTCTTTGGGA GTCGTTATC- ---AATGAGC AACAT-TGCA CAGCGATGTG -CTGGTTTTG CTACTTGCTC AT---CCATG TT-CTGATTG TACTCCCGCA TAGACTGTAA ATGTTTGTTT
C. fusca hydA GCATGGCTCA ACAACCTGTC TTCAGAGCAG GTGTATTCCA --CACCATC- ---TTGATTT ACCTACCACT CTGTAGTTCA AGTGGTCAAA TTGAATGTCT AT---GGCAG C-TACGCCTG CAGTT---CA TAGTCTATGA AGGTTT-CAC
S. obliquus hydA TTAA----CA GGGGAGTGTT TGTAGTGTGT GTGTGT-CTA --CATTACC- ---AGTGTGT TCTTGTTGCA -TGTATT--- -GTAGTGTAC TGGGTTATGC AC---GCCTG CATGCGCACG CGCTC---CT CGTGCTGCGA CAGTGCACAC
2110 2120 2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2230 2240
2250
T. sp. CU 2551 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reinhardtii hydA1 GAGGGAGCGC GGCTTGCTAA CCGAATGGCG TATCCCTCCA GGGCACGTCG GAATGGCGCG TGCCCATCAA CGCAAATTCT TGGCC--TTC ATCGCTTCTG GATATTGAAG CTGCACAAAC CTGCATTCTA TTTGCTTGTT TAC--ACGTG
C. reinhardtii hydA2 CACTGAGCGC ATACATCATG C-GCATGTGC GCCTTCGCCT GCACGCACGT ACGTAACCGA CGGCGGTATT CGTCGCGAAG TGTGTGTGTC ATTAGGTTTG TGTTTGTCGT GATGTGGTGG TGGCAAAACT GGGACTCATG TACGGTACGG
C. moewusii hydA2 GGTTGAAAAA AAAAAAAAAA AAAAAAAAAA AA-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. fusca hydA CAGAGTCCAT GTCCCTCATA TTTTTTGTTT TATATGCCTT GATTATGCCC CTTGAACCAT GCTCAATGCA CACAAGTTGG TCGCAGGACA GGCGGCATCG TACATCTCAA TTTTCAGAAC TTGTCAGTGC GGCATTGCCT TAT--TTGTA
S. obliquus hydA CGCACAGCGT GATACAGCTG CAGGATGTTT GCGGA----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
2260 2270 2280 2290 2300 2310 2320 2330 2340 2350 2360 2370 2380 2390
2400
T. sp. CU 2551 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. reinhardtii hydA1 CCCCAATCTT GGTTGGAAGC TAAACATGTT TGGGAACAAT TCATCTTACT AAAGCGTGTG GGGGTTGAGG ATGCGCACGT TGTGCGCTGG TGGGTGGGCG GGAACGTGGG TAGCATTTAG GCTAGCTGGC ATACGACAAC GGGGCCCGTG
C. reinhardtii hydA2 TATGGGCTGT GCCACCGTAC ATGTGGCGAT GTGATTGTGA GTCTCCTGCT GTCTCACGCG CAGGGGGAGG TGCAACCACC CGTTGTGCGA GCCCTCTTTA TATAATAAGC TAAGCGTCCT TTCAAAGCTG AGTTTTCGAA TGGTGTGGCG
C. moewusii hydA2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. fusca hydA CTCTTGCAGT CCTGTTTCAC CCTTGCTACT GCCTTGCA-T GCATCTTGTT TTTGCAAGCA ACAGCTCATG CATTGCAATC GATCATCACG TACATCCGTG CCATATTCAC ATGGTTTTGA CTTGCAAATC AACCACCAGG CAGTGGGTAA
S. obliquus hydA ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

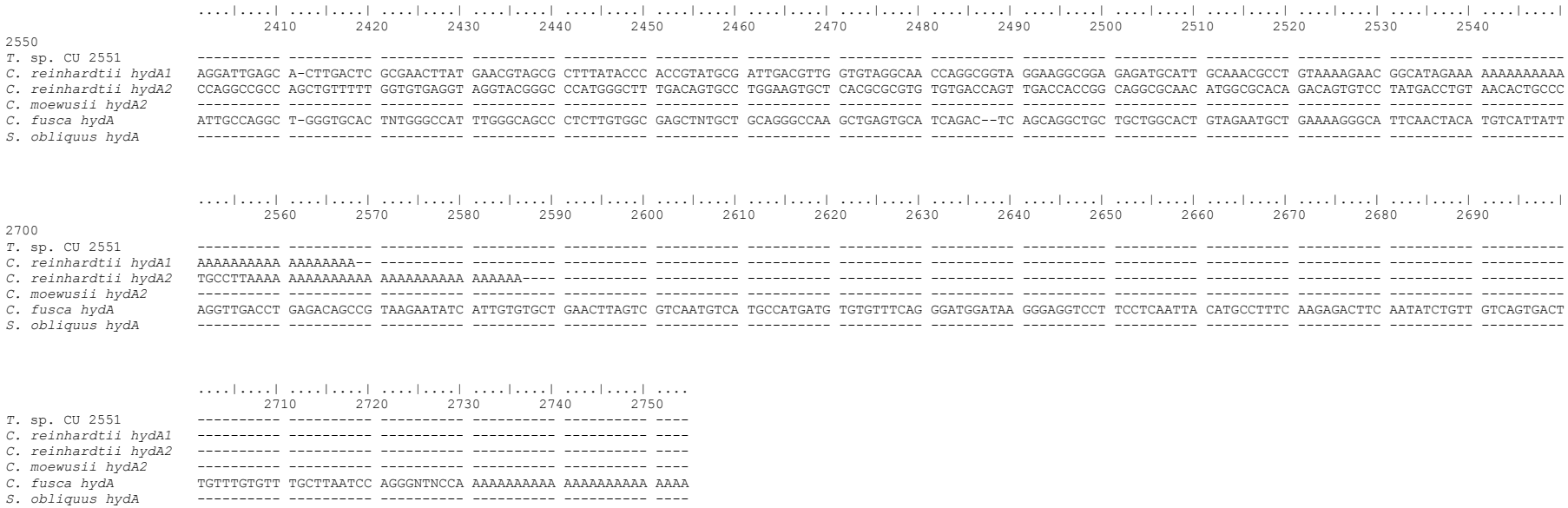


Figure 3.35 Alignment of hydrogenase (*hydA*) sequences from *Tetraspora* sp. CU2551 against other 5 algal hydrogenase sequences that used in primer design. Arrows indicated the regions that primers used in *hydA* investigation.

>*Tetraspora* sp. CU 2551 *hydA* (genomic DNA)

GCCCATGTTCACCAGCTGCTGCCCTGGGTGGGTTGCTATGGTGGAAAAGAGCAACCCGGA
GCTCATCCCATATCTTTCATCTTGCAAGTCACCCCAAATGATGCTGGGTGCCGTAATCAA
AAACTACTTTGCACAGCAGGTTGGAGTGCAGGCCAGTGACATTGTCAATGTGTCTGTGAT
GCCCTGTGTCCGTAAGCAGGGTGAAGCAGACCGTGAGTGGTTCAACACAACAGGTGGGTC
ACGCTTGTCTAGTGGGTTGCTGATACTGTATTGGTGATCCCACAGATAGCCTGAGCTACA
ACCGCTTGCTATTGTTGATGCACCCGACAGATTGATGGGGTGTGGCCCGATGTTTCCTTT
CTTTCCTTGCTCAAACTGTGTTGTCAACCAGCTAACAGGTTATAAGCTAGCACATTCATG
TCATTTGTGGCTACATCATACAGCAGGAGCGACATCAATTGACTGGCTGACCCTGCATGT
GTTGTGTTTCACAGGCCTTGCACGTGATGTTGATCATGTGATGACTACAGCTGAGGTCGG
CAAGGTTTTCCTTGAGCGTGGCATCAAGCTCAATGAACTGCCAGAGAGCAACTTCGACAA
CCCTGTTGGTGAGGGTACAGGTGGAGCCGTGCTGTTTGGTACCACTGGAGGTGTCATGGA
GGCAGCACTGCGAACTGTCTATGAGCTGGTGAGTGGATGCAATTGCAGGAAAGCGTACTT
TGTAGGCTGTAGCAGGTCATGCTCCAACCTGCTGACATTATCATGACGTTGCAACAGATA
TGTTGGCAGTAATCGTTTCCAATATCATCCAGTCTGGTCCCTGGCAATGAGTGTACATCT
AGCTGGTTGGGTTTTTGCTTGGCAGCCCTAGGGTTGGGTGTTGCATGCTGTCTACCTGGC
CAGATCATCAAGCAAGTAGCGGCATAACAGCTCAATAAGCCGCCTGCACTTGTGCAAATT
TCATGTACAGGTCACACAGAAGCCCATGGGCCGCATCGACTTTGCAGAGGTGCGTGGGTT
GGATGGTATCAAGGAGGCTACCCTGAACCTCAAGCCTGGTGAAAACAGTCCATTCAAGGC
ATTTGCTGGACCCGATGGCGAAGGCATCACGCTGAACATCGCAGTGGCCAATGGTTTGGG
CAATGCTAAGAAGCTGATCAAGAGCTTGTCAGAGGGCAAGGCCAAGTATGACTTCATCGA
AGTTATGGCCTGCCCAGGAGGATGCATCGGTGGAGGTGGTCAGCCTCGCAGTAGTGACAA
GCAGATCTTGCAGAAACGCCAGCAAGCTATGTACCAGCTGGACGAGCGCATGACCCTGCG
CCGCAGTCATGAGAATCCCTTCATACAGGCCCTGTACAACAACTTCTTAGAGGCACCCAA
TAGCCACAAGGCGCACGACCTGCTGCACACCCACTATGT

Figure 3.36 Partial sequence of putative *Tetraspora* sp. CU2551 *hydA* (gDNA). Shaded boxes shows introns with a size of 261 and 282 bp, respectively. Underlined shows primer positions used in reverse transcription PCR analysis.

The deduced amino acid sequence was used in protein alignment along with five other sequences that available in NCBI database. However, some proteins contain the Narf-like domain, which was proposed to play a role in Fe-S assembly in some eukaryotes, and they were believed to have no hydrogenase activity (Balk *et al*., 2004). Three sequences were included to the alignment as an outgroup: *C. reinhardtii* Hyd3/prelamin A binding protein, *H. sapiens* nuclear prelamin A recognition factor (Narf), and *S. cerevisiae* Nuclear architecture related protein (Nar1p). As a part of *Tetraspora hydA* sequence obtained, a local alignment of deduced amino acid sequences was performed. The alignment showed the conserved regions found among all green algal sequences as indicated by shaded areas among all hydrogenase genes from green algae (Figure 3.37). The Bootstrap NJ phylogenetic tree determination was performed as shown in Figure 3.38. The tree classified HydA proteins into several groups, and showed the closet relation of *Tetraspora* HydA to *C. fusca* HydA.

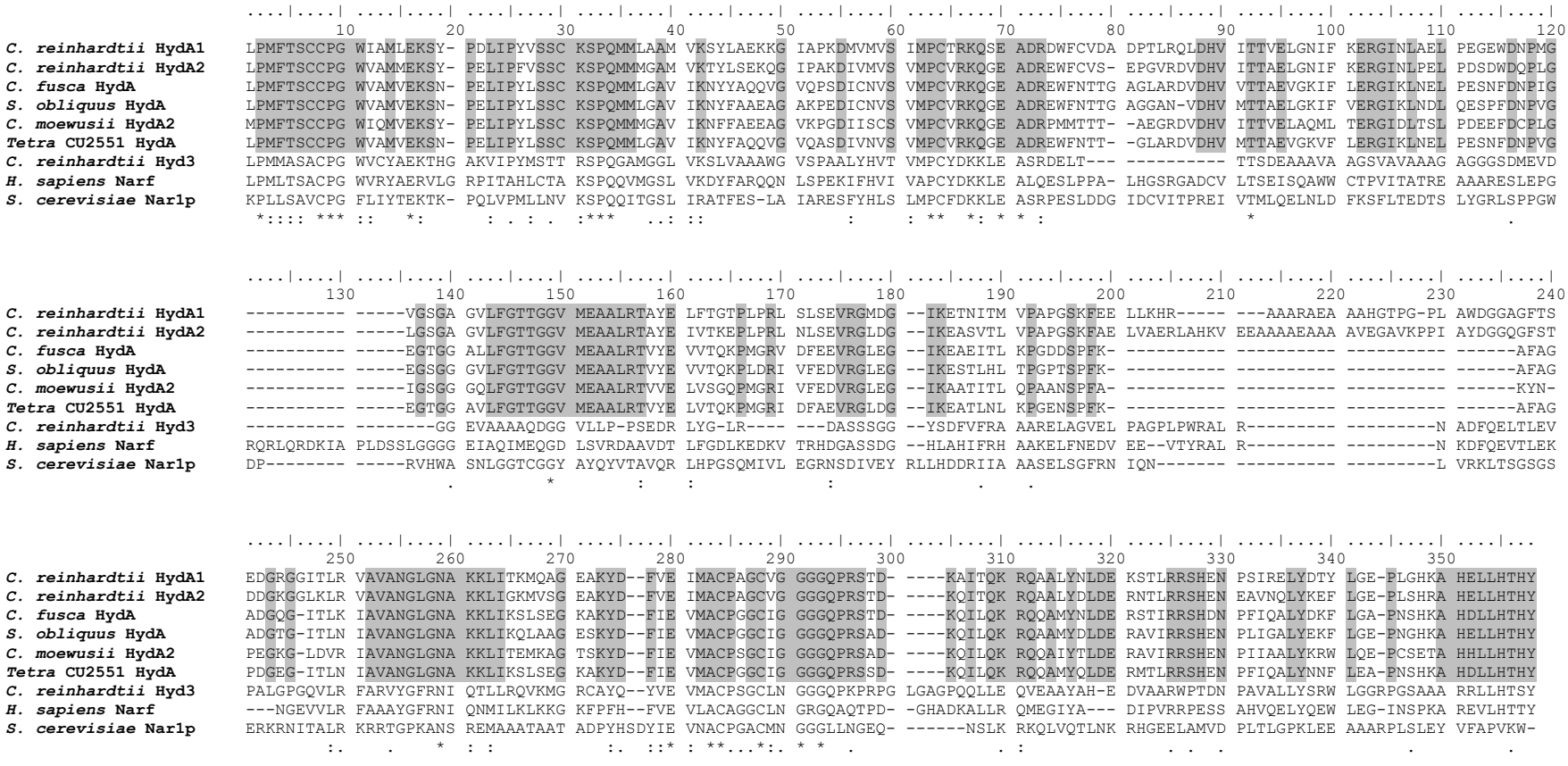


Figure 3.37 Proteins alignment *Tetraspora* HydA with other 5 different sequences, along with other 3 outgroups sequences. The shaded boxes shows the conserved regions among all green algal sequences.

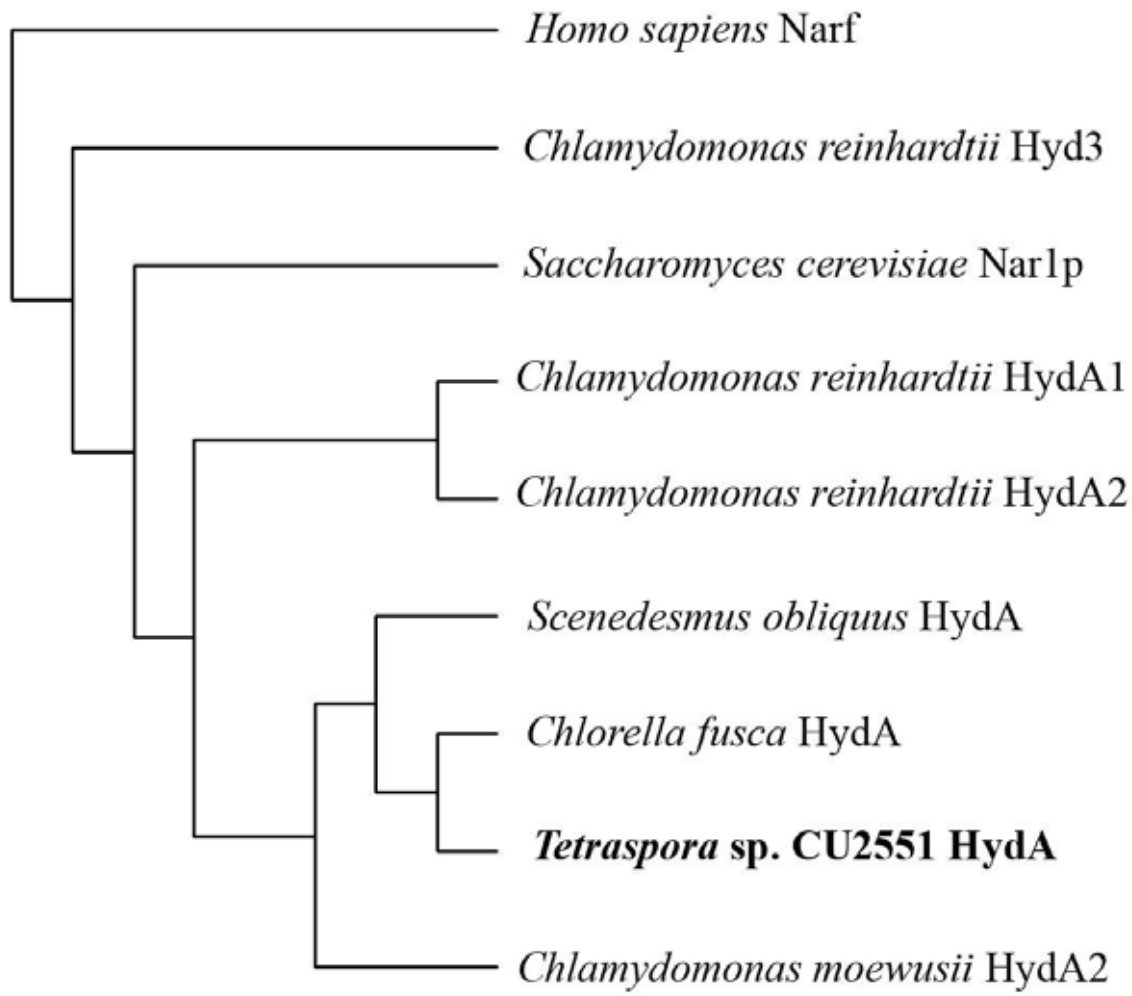


Figure 3.38 Bootstrap NJ phylogenetic tree of hydrogenase protein sequences using the deduced amino acid sequence of *Tetraspora* sp. CU2551 (bolded) and other 5 HydA sequences. The tree also showed three outgroup name CrHyd3, HsNarf, and ScNar1p.

3.6.2 Sulfur-deprivation condition

Cells were grown in normal TAP medium under continuous light illumination for 24 h. The culture medium was then replaced with TAP-S medium. Hydrogen production and fluorescence were monitored. The *Tetraspora* sp. CU2551 rapidly lost the hydrogen production ability under sulfate-deprived condition, and the production was hardly detectable after 24 h (Figure 3.39A).

The result was also confirmed by fluorescence measurement using fluorescence spectroscopy. A fluorescence value at 683.5 nm emission peak was used to compare the fluorescence of chlorophyll in *Tetraspora*. As shown in Figure 3.39B, the trend of fluorescence was increasing when cells were adapted under S-deprived condition indicating that the damage of PSII occurred. It should be also noted that the relative fluorescence value dropped by 20% for the control where sulfate was present in TAP medium.

The gene expression profiles were also examined to investigate the response of these genes to the sulfur-free condition. The relative expression level was calculated with respect to the *18S* rRNA expression level, and the relative expression at time 0 h was set as 1. The results showed the increase of *sulP* and *hydA* expression when cells were adapted in TAP-S medium and the expression almost reached the maximum after 6 h (Figure 3.40). The up-regulation of both genes was about 2.3-fold.

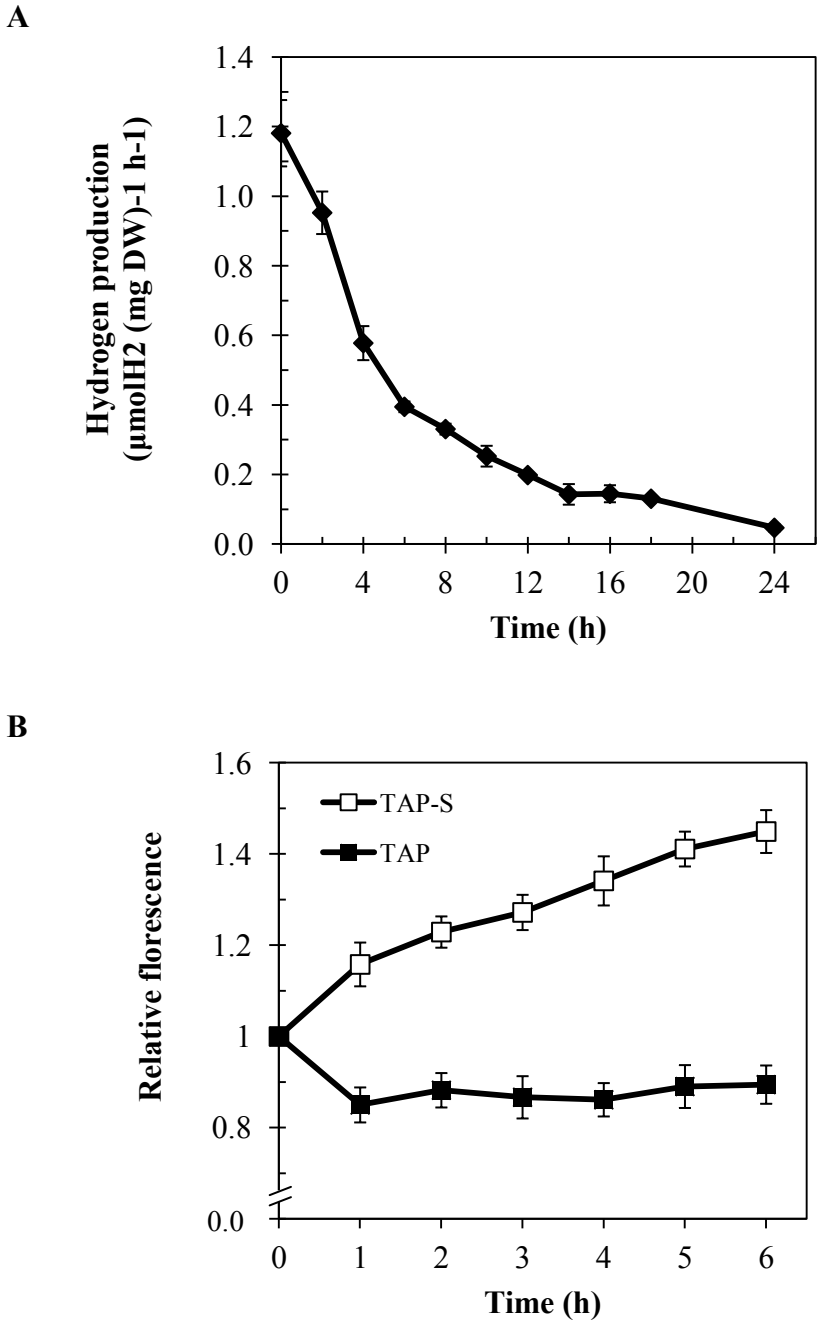


Figure 3.39 Effect of sulfate-deprived condition on hydrogen production (A) and fluorescent (B) using fresh TAP (■) as a control set. Means ± S.D. (n =3).

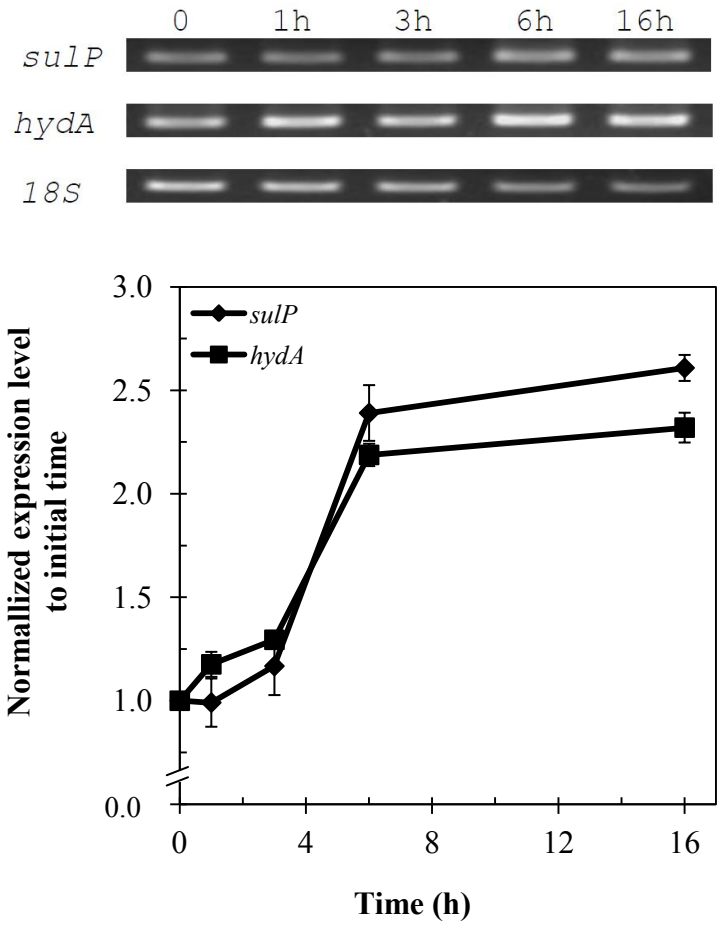


Figure 3.40 Relative transcription levels of *sulP* (♦) and *hydA* (■) with respect to *18S* sulfur-deprived condition were shown, setting the level at 0 h as 1. Upper panel shows the gel pattern of transcripts. Means ± S.D. (n =3).

3.6.3 Sulfur-repletion condition

After cells were adapted in sulfur-deprived condition for 16 h, the culture medium was replaced by pre-warm TAP medium. Interestingly, the biohydrogen production was rapidly restored within 4 h yielding about 0.93 μmol/mg DW/h (Figure 3.41A). This capacity, however, did not reach the normal rate before sulfur deprivation. The PSII activity was also indirectly monitored by fluorescence spectroscopy. The rapid decrease of fluorescence by about 30% occurred when cells were put to normal TAP medium. This data also supported that the PSII in *Tetraspora* sp. CU 2551 could restore the activity within 2 h (Figure 3.41B).

The *sulP* and *hydA* expression profile showed similar pattern with an initial sharp increase 1 h after sulfur repletion followed by a trend of decreased expression at longer duration of sulfur repletion (Figure 3.42). The initial burst was observed in first 1 h after media changing.

Overall results indicated that the existence of sulfur can help mediate the up and down regulation of the transport of sulfur as well as the production of biohydrogen in *Tetraspora* sp. CU 2551.

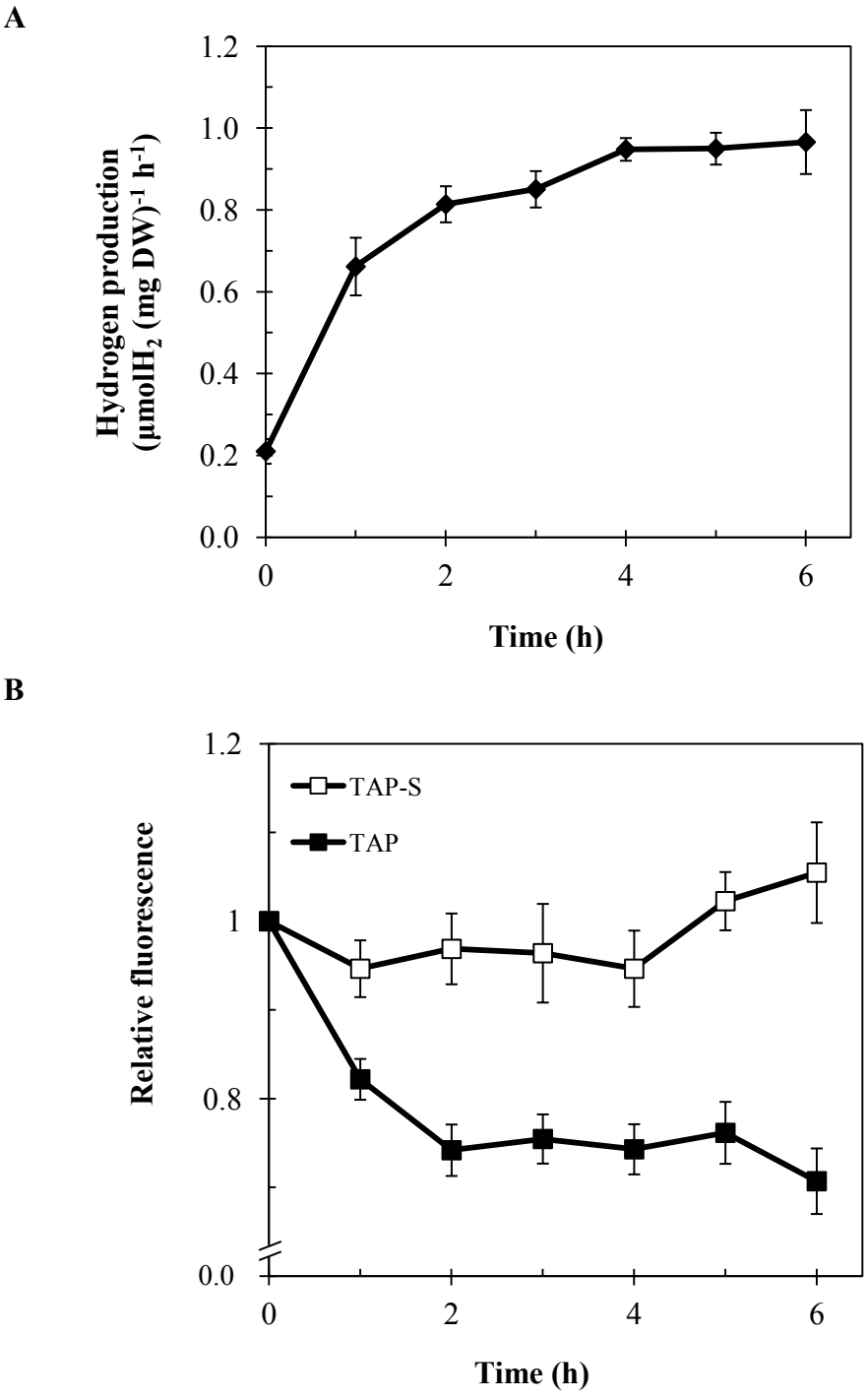


Figure 3.41 Effect of sulfate-replete condition on hydrogen production (A) and fluorescent (B) using fresh TAP-S (□) as a control set. Means ± S.D. (n =3).

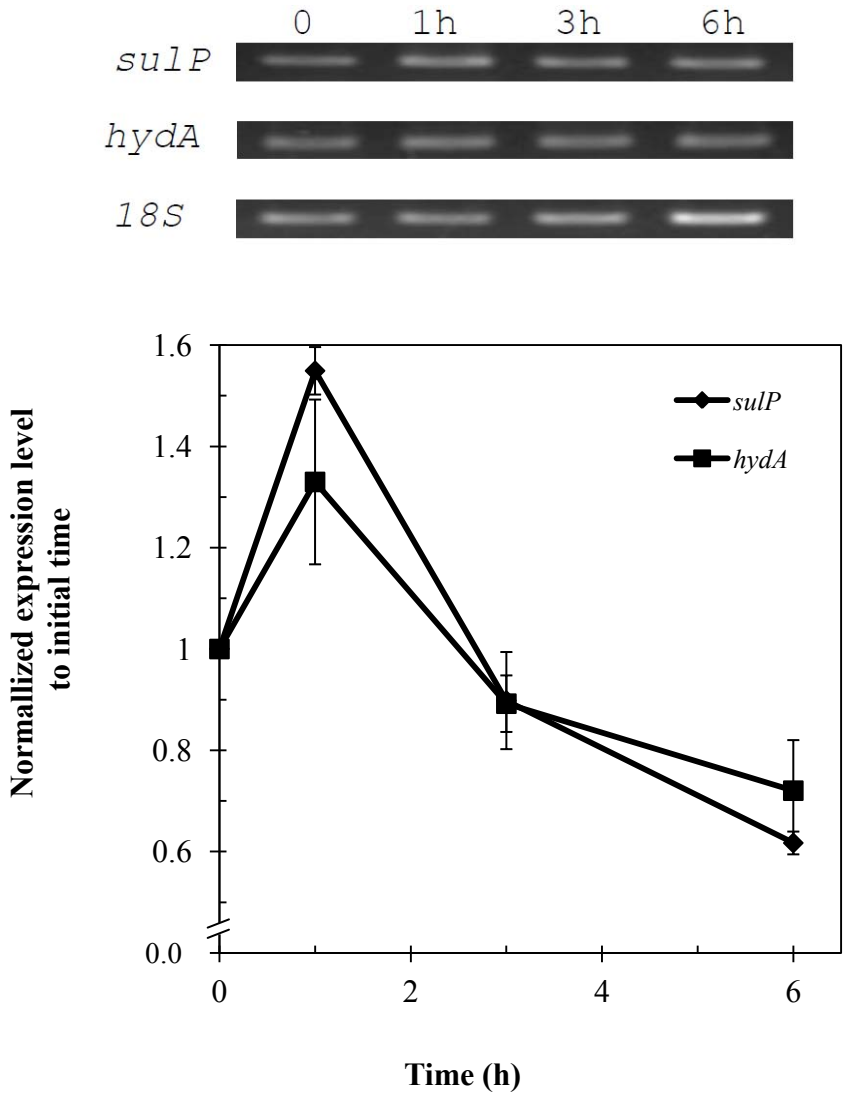


Figure 3.42 Relative transcription levels of *sulP* (♦) and *hydA* (■) with respect to *18S* under sulfur-replete condition were shown, setting the level at 0 h as 1. Upper panel shows the gel pattern of transcripts. Means ± S.D. (n =3).

CHAPTER IV

DISCUSSION

4.1 Background

Sunlight is a clean energy source directly reached to the earth surface from the sun. The total solar energy absorbed by Earth's atmosphere, oceans and landmasses is approximately 3.85 x 10^{24} joules per year. Such a high amount of energy is enough for all year round energy consumption of a whole world. However, the sunlight energy could not be stored; several methods were developed to convert the light energy to other forms of energy e.g. electricity and chemical. A solar cell is another way to convert the light energy based on the physical chemistry aspect. The biological process is considered as another way to convert the sunlight energy to be stored in chemical bonding of molecule. Hydrogen molecule is one of interesting molecules since (i) the energy bonding of H-H is high accounting for 436 kJ/mol which is higher than other energy bonding between hydrogen atom to other atoms except for fluorine atom (Table 4.1).

Table 4.1 The energy bonding of hydrogen atom to others

Bond	Energy (kJ/mol)
H-F	598
H-H	436
H-Cl	432
H-C	413

Bond	Energy (kJ/mol)
H-N	391
H-O	366
H-Br	366
H-I	298

(ii) Its combustion process produces heat and water as a by-product, which is environmentally friendly to our world. (iii) The combustion reaction produces no global warming gas e.g. carbon dioxide. (iv) It can be produced through the electrolysis of water and through the biological process from hydrogen-producing organisms. For the biological approach, chlorophyll in green organisms will function as an antenna absorbing the light to use in water breaking down in PSII system. Besides, growing green organisms can help reduce the atmospheric carbon dioxide, which is the major problem of the global warming. This work supports and enables the opportunity to produce an alternative clean energy source.

4.2 Screening and identification

Thailand has warm temperature in all year round with the average range of 25°C to 35 °C, and high intensity of sun light. Culture isolated from Thailand should have the optimal condition for growth under these parameters continuously. The continuous hydrogen bioproduction process could take place under Thailand weather.

Hydrogenase is an enzyme catalyzing the formation of hydrogen molecule from proton and electron. Previous reports showed that the *Chlamydomonas* hydrogenases are sensitive to oxygen molecules and are irreversibly inactivated within minutes after exposure to atmospheric O_2 (Abeles 1964, Erbes *et al*., 1979, Happe and Naber 1993, Ghirardi *et al*., 1997, Cohen *et al*., 2005 ; King *et al*., 2006). Under anaerobic condition by argon gas bubbling, the atmospheric oxygen is eliminated. As a result, no hydrogenase inhibitor is present. To screen culture, the anaerobic condition was used to determine the production yield of each isolate.

Culture screening gives the opportunities to find the new strains of hydrogen-producing green organisms in Thailand nature. In this work, 210 isolates were obtained and 80% of them showed the hydrogen production ability. This result suggested the availability of culture in Thailand nature to produce the hydrogen gas from green organism. Less than 20% of isolates showed no production. This was similar to the previous works reported by Melis and Happe (2004) and Boichenko *et al*. (2004). The selected strain showed the production capacity of about 1.2 μmol/mg chl a/h under anaerobic condition. Several reports showed the screening of cyanobacteria cultures from Baltic Sea and Finnish lakes (Allahverdiyeva *et al*., 2010). The highest rate was found in filamentous cyanobacterium *Calothrix* XSPORK 27A under microaerobic with continuous light illumination of 70 μmol photons/m^2/s yielding 0.737 μmol/mg chl a/h. In 2012, the hydrogen-producing micro algae from natural source in China (He *et al*., 2012) were also screened. A hundred isolates were obtained and 52% showed the biohydrogen production ability. Among these H_2 producing isolates, freshwater *Chlorella protothecoides* showed the highest H_2 producing capacity yielding 2.93 ml H_2/l/ h.

The selected strain was identified as *Tetraspora* sp. CU2551 by the morphology observation under light microscope and Scanning Electron Microscope (SEM). The strain was confirmed by 18S rDNA sequence and phylogenetic tree determination. Although the tree did not classify *Tetraspora* sp. into the same group, the distance bar showed the close relation among all green algae, thus, changing in some bases could cause the different classification in the tree.

4.3 Growth condition

Culture was previously maintained in BG11 medium during screening step, but each photosynthetic green organism might be able to grow in other medium. After medium optimization, *Tetraspora* sp. CU2551 was able to grow well in Tris-Acetate-Phosphate (TAP), and showed slower growth in BG11 and N8 media. Nitrogen-free media, N-free, BG110 and AA media, were also tested for the nitrogen fixing ability by *Tetraspora* sp. since filamentous cyanobacteria showed nitrogenase activity. The results showed that the growth was hardly observed when cells were kept in these media suggesting that there was no nitrogen fixing capacity of *Tetraspora* sp. Zarrouk medium was optimized for growing *Spirulina* sp. where the pH of culture was about 10; however, that *Tetraspora* sp. could not grow normally in zarrouk medium under strong alkaline condition.

It was known that the initial optical density could affect the S-curve of growth. Initial OD_{730} of 0.01 showed a typical curve of sigmoid pattern. Under the optimal parameters of light intensity and temperature, cells mass could be increased 30 – 40 times within 24 h. These conditions of growing could be easily found in Thailand in all year round making this *Tetraspora* sp. growth with low cost to produce cell mass during growth phase.

4.4 Optimization for hydrogen production

The production unit was changed since many reliable methods were developed for this strain. After 90% methanol extraction was done, chlorophyll could not be extracted out completely, This can be noticed by the presence of green pellet after centrifugation. Freeze-thaw repeating, glass bead vortexing, and cell breaking by mortar could not make

cells completely broken. Some researchers also used the production unit by weight of dried cell mass (Kumazawa and Mitsui, 1985), or the amount of protein (Antal and Lindblad, 2005, and Troshina *et al*., 2002). Unfortunately, *Tetraspora* sp. CU2551 could not be broken by 5% phosphoric acid of Bradford's reagent. Using cell dry weight resulted in more reliable and reproducible result for *Tetraspora* sp. CU2551. To reduce the error of standard curve determination of cell dry weight and optical density of 730 nm, 20 mL of culture was filtered and used in dry weight calculation per 1 mL.

4.4.1 Optimization during growth phase

The culture age of 24 h showed the highest production rate when compared to longer growing culture. This is probably due to the fact that during this mid-log phase of culturing *Tetraspora* sp. has the highest metabolism rate, together with light intensity effect on the production rate. Even though growing cell under the darkness can make cells produce biohydrogen in the highest rate, the cell amount is very low. The growth result can be confirmed by the doubling time of above 5 h. Thus we suggested to grow *Tetraspora* sp. under the presence of light with the intensity about 37 $\mu E/m^2s$. The amount of hydrogen gas can be compromised by the double or triple amount of cells when grown in the presence of light.

4.4.2 ***Optimization during production phase***

As a biocatalyst, the hydrogenase enzyme uniquely showed the optimal temperature for catalysis of the biohydrogen formation from proton and electron. *Tetraspora* sp. CU2551 showed the optimal temperature for hydrogen evolution at 35 $^{\circ}C$, while the purified hydrogenase from several green organisms was determined. Even the optimal production temperature was not reported, purified hydrogenase from green alga

Scenedesmus obliquus showed the optimal temperature of 50 °C (Schnackenberg *et al*., 1993), while some marine green algae *Tetraselmis subcordiformis* (Bhosale *et al*., 2009) and *Chlorococcum littorale* (Ueno *et al*., 1999) showed the optimal temperature of 55 °C and 50 °C, respectively. Unfortunately, the isolated hydrogenase from *Chlorella fusca* was only used to study in terms of molecular characterization (Winkler *et al*., 2002b).

We previously determined the production yield from *Tetraspora* sp. CU2551 under the darkness to avoid the molecular oxygen production from PSII system under light condition. Interestingly, the light incubation showed the unexpected result that increasing light intensity enhanced the hydrogen evolution. Light acts as a powerful energy source for water splitting in PSII system. As a result, molecular oxygen, proton, and electron are produced. Generated oxygen molecules also function as an inhibitor to hydrogenase activity. This obstacle can be overcome by the turnover rate of [FeFe]-hydrogenase from green algae which showed 10-100 times higher than that in [NiFe]-hydrogenase normally found in cyanobacteria (Frey, 2002). In addition, Lee and Greenbaum (2003) proposed that 0.1% of O_2 could inhibit hydrogenase activity *in vivo*. Moreover, some reports showed that different hydrogenases from different sources respond to atmospheric level of oxygen with different time ranges (Cohen *et al*., 2005 and King *et al*., 2006). Thus, the generated oxygen from PSII system might not reach the threshold concentration to inhibit the hydrogenase activity in *Tetraspora* sp. CU2551. Nevertheless, the produced oxygen molecules can be also consumed by mitochondria during respiration and this would reduce the level of oxygen inside the cells.

It was known that the carbohydrate in green algae can be metabolized and the reducing equivalents can be delivered to plastoquinone prior to PSI system (Antal *et al*., 2009, Hemschemeier and Happe, 2011). This would cause higher amount of electron to flow to hydrogenase enzyme. The higher hydrogen production should be obtained

theoretically. Under light incubation, addition of carbon sources to *Tetraspora* sp. culture within 4 h during adaptation time did not increase the rate of hydrogen production. The result did not include the time course analysis to monitor up/down regulation in long term. To improve the result, culture was grown for 24 h in the presence of many carbon sources with the final concentration of 0.1% to saturate the cells with carbohydrate. The hydrogen production showed the same rate as that in the control where extra carbon source was absent. It should be noted that the production rate was lower when glucose was used. This is due to the fact that glucose promoted the cell growth (data not shown), then the cells amount in this condition was high. As a result, the production rate per cell dry weight of glucose-grown cells was lower than others with lower cells amount. However, the total hydrogen production by glucose-grown cells showed the highest amount when compared to those grown in other carbon sources.

Salt was used to generate both osmotic and ionic stresses. With the final concentration of 50 mM, no salt can enhance the production capacity. In fact, some salts can inhibit the production by 50% e.g. carbonate salts: Na_2CO_3, $NaHCO_3$, and $KHCO_3$. It is possible that these salts may alter the pH of the culture due to the carbonate system and this reduces cells ability to themselves in a short time. Nitrite salt ($NaNO_2$ and KNO_2) and guanidine hydrochloride also inhibit hydrogen production more than 50%.

Tetraspora sp. CU2551 showed the wide pH range of biohydrogen production ranging from pH 6.5 to 9.5. This indicates the opportunity to produce hydrogen gas from *Tetraspora* sp. using water in many places so that the cost of pH control of water can be reduced. Like in the temperature optimization section, the purified hydrogenase from *Scenedesmus obliquus* showed the optimal pH of 6.3 (Schnackenberg *et al*., 1993), whereas some marine green algae *Tetraselmis subcordiformis* (Bhosale *et al*., 2009) and *Chlorococcum littorale* (Ueno *et al*., 1999) showed the optimal pH of 7.5.

As proposed, the hydrogenase activity is involved in redox balancing, photoprotection, and fermentative metabolism *in vivo*. Therefore, the reducing agent was used as a good source of electrons to any acceptors. Kruse and Hankamer (2010) showed that the hydrogenase enzyme functions as electron sink which is controlled by redox status. β-mercaptoethanol could deliver electrons to some acceptors in *Tetraspora* sp. making about double rate of hydrogen production, while dithiothreiol could not promote the H_2 evolution.

Melis *et al*. (2000) have developed a short-term method to avoid the O_2 sensitivity of hydrogen production from green algae. They could achieve a sustainable hydrogen production for a period of about 4 days. It is well known that *Chlamydomonas reinhardtii* showed high production under sulfur deprivation condition (Laurinavichene *et al*., 2006 and Jorquera *et al*., 2008). Nutrient deprivation can cause metabolic changes. In our study, either nitrogen or sulfur deprivation slightly promoted hydrogen production. On the other hand, nitrogen-deprivation could stimulate cells under S-deprived condition to increase biohydrogen production about 30%; however, this was obtained within 4 h after anaerobic adaptation. Though there is no report on the effect of nitrogen deprivation on hydrogen production in green algae, some studies showed that the lack of phosphate did not help *C. reinhardtii* during sulfur-deprivation cultivation (Batyrova *et al*., 2012). The experiments were also extended to include the effect of β-mercaptoethanol along with the nutrient deprivation. Since reducing agent was considered as an electron donor for any acceptor, the culture was saturated with the reducing agent to generate high redox potential inside the cells. Unexpectedly, nitrogen and sulfur deprivation condition, where the PSII system could not generate the molecular oxygen molecules, showed the enhanced production by 6% approximately.

All positive parameters were combined to test the production yield. Under the reducing condition, β-mercaptoethanol could promote the production when light intensity was below 7 μE/m^2s, but not when light intensity was above this level. Under the nutrient deprivation, β-mercaptoethanol also enhanced the production when light intensity was below 6 μE/m^2s, but no enhancement was observed when light intensity was higher than this level. These results provide a chance to produce biohydrogen from *Tetraspora* sp. CU2551 in any place of the world. For the place with low light intensity, β-mercaptoethanol is needed to enhance the production rate, while this reducing agent was not needed for high light intensity area. All in all, nitrogen and sulfur deprivation was necessary for improving the biohydrogen production from *Tetraspora* sp. CU2551.

In summary, *Tetraspora* sp. CU2551 produced high amount of biohydrogen within 24 h after cultivation yielding about 0.6 μmol/mgDW/h at the optimal condition. This unit was unusable to compare with other studies due to unreliable method to completely extract chlorophyll from *Tetraspora* sp. The comparison of this yield was estimated on the chlorophyll content distributed in green algae (Piorreck *et al*., 1984). By 0.03% (w/v) NH_4Cl in TAP medium, the chlorophyll content was measured to be 0.96 and 3.43% dry weight in *Chlorella vulgaris* and *Scenedesmus obliquus*, respectively. If we assume that *Tetraspora* CU2551 has a chlorophyll content ranging from 0.96 to 3.43 % dry weight, cells can produce H_2 at a rate of 17.3 - 61.7 μmol/mg Chl a/h or 423 - 1,511 μl/mg Chl a/h. This is a very high production rate compared to those from other green algae.

4.5 Molecular study for hydrogen metabolism

Among many parameters listed to optimize for the increased capacity of biohydrogen production from *Tetraspora* sp., the genetic engineering was used to manipulate the strain to extend the capacity of the production yield. Knocking down some genes involved in oxygen production pathway was considered. Membrane-bound sulfate permease is involved indirectly to reduce the molecular oxygen generating pathway. In stroma, blocking this channel resulted in no sulfate supply for cysteine biosynthesis pathway which is used as a precursor for sulfur-containing amino acid biosynthesis. As a result, protein turnover could not continue due to the lack of some important proteins particularly D1- the reaction center of PSII system (Makarova *et al*., 2007). Water could not be broken down to generate oxygen which is an inhibitor to hydrogenase activity.

4.5.1 Sulfate permease (sulP) investigation

The *sulP* fragment was unsuccessfully amplified by the degenerate primers though many parameters were optimized. Nevertheless, the fragment was successfully amplified at the cDNA level. This may be due to the fact that the algal genomic DNA has high complexity for primer binding specifically, then the low complexity of cDNA resulted in *sulP* amplification after semi nested PCR, where the first reaction showed 2 major bands. However, the cDNA sequence has the degenerate primer sequence at the priming site, and the fragment was probably not suitable for use in exogenous DNA transformation by the DNA recombination. Another pair of specific primer was designed to amplify *sulP* at DNA level. The result showed the existence of intron inside this fragment, and supported the hypothesis that the cDNA sequence might not be sufficient for DNA recombination.

4.5.2 Construction of a sulP inactivation strain

León and Fernández (2007) reviewed many reporter genes and transformation methods normally used in eukaryotic micro algae. For the green alga model organism *C. reinhardtii*, many selectable markers were used such as adenylyl transferase (resistance to spectinomycin) (Cerutti *et al*., 1997), aminoglycoside 3'phosphotransferase (resistance to paramomycin) (Sizova *et al*., 2001), bleomycin binding protein (resistance to zeocin) (Lumbreras *et al.*, 1998), chloramphenicol acetytransferase (resistance to chloramphenicol) (Tang *et al*., 1995), neomycin phosphotransferase 11 (resistance to G418) (Poulsen *et al*., 2005), and acetolactate synthase (resistance to sulfonylurea herbicides) (Kovar *et al*., 2002). The transformation methods that have been used in *C. reinhardtii* were: glass beads vortexing (Kindle, 1990), electroporation (Shimogawara *et al*., 1998), silicon carbide whiskers (Dunahay, 1993), biolistic microparticle bombardment (Debuchy *et al*., 1989 and Kindle *et al*, 1989), and agrobacterium tumefaciens-mediated gene transfer (Kumar *et al*., 2994).

In this work, since the exogenous DNA transformation methods for *Tetraspora* sp. were undeveloped, several common transformation methods were used as trial. They were electroporation and glass bead vortexing. Cell wall might act as significant barrier for DNA transformation, thus cells were treated by cellulase before the transformation. Unlike *C. reinhardtii* that the cell wall composition was explored, the composition of *Tetraspora* sp. cell walls was unknown. The mixed-enzyme cellulase was considered for use in cell wall digestion of *Tetraspora* sp. Cellulase, ONOZUKA R-10, was purchased from PhytoTechnology Laboratories® company. The powder is composed of cellulase, hemicellulase, pectinase, α-mylase, and protease. This enzyme mixture showed an optimal pH over the range from 3 to 7, with a peak at 4 to 5, under the incubation temperature of 40 °C (data from the company).

XbaI-linearlized Nm:*sulP*-inactivated plasmid (pTgSmNm) was successfully constructed and transformed to cellulase-treated cells by both electroporation and glass bead vortexing methods. The results showed an approximately equal amount of transformant on the test set and on the control set. Colony PCR confirmed that no mutant was obtained after keeping cells to the 3rd generation. Neomycin resistance cassette might not be suitable for *Tetraspora* sp. transformant selection, then this cassette will not be further discussed.

The construction of *XbaI*-linearlized Cm:*sulP*-inactivated plasmid (pTgSmCm) also followed the same strategies as that in Nm:*sulP*-inactivated plasmid. Unfortunately, transformants was not observed by electroporation transformation, but few colonies could survive up to the 3rd generation by glass bead vortexing method. Although, the reliable explanation was unclear for the instability of these transformants, there are some possibilities to explain these negative results. One could be the efficiency of transformation method. The plasmid might not enter the host cells resulting in no transformant on selective medium, or they entered but could not integrate completely to genome due to the length of *sulP* upstream Cm^R fragment. This part of *sulP* has a length of 169 bp, which is not too long for recombination. Some studies showed that the length of up-stream and down-stream sequence of resistance cassette could effect the integration efficiency in cyanobacterial *Synecococcus* R2 (Kolowsky *et al*., 1984). These results were similar to the recombination in bacteria as reviewed by Oliveira *et al*. (2008). Secondly, if Cm^R integrated to algal genome, the resistance cassette might be incompatible to the genome. Host cells need to eliminate an alien DNA.

4.5.3 Full-length sulP determination

A full-length *sulP* was successfully determined at both cDNA and genomic DNA levels. The cDNA sequence showed an open reading frame of 1,041 bp encoding 346 amino acid residues with the 5' and 3'untranslated regions size of 285 and 225 bp, respectively. This genomic *sulP* showed the existence of 4 introns with a size of 69, 228, 219, and 227 bp, respectively. We identified *Tetraspora* s*ulP* as the second strain having introns fragment in DNA sequence after the first strain intron-containing *C. reinhardtii sulP*. Deduced amino acid residues showed the predicted chloroplast transit peptide (cTP) of 28 residues. The results showed the coherence of *C. reinhardtii* SulPs: SulP and SulP2. These two protein sequences reported by Lindberg and Melis (2008) showed the cTP length of 54 and 82 residues, respectively, while others showed no putative signal peptide from ChloroP 1.1 program (Emanuelsson *et al*., 1999) prediction. Besides, many reports revealed the existence of *sulP* in chloroplast. These results indicated the possibility of the existence of sulP in *Tetraspora* nuclear DNA. A long discussion was provided by Chen *et al*. (2003) followed by Melis and Chen (2005) that during the evolution, it is possible that the sulfate permease genes of the liverwort *Marchantia polymorpha* SulP and the *Chlamydomonas reinhardtii* SulP2 migrated from the symbiosis of the prokaryote, e.g. *Bacillus*.

4.5.4 Construction of a sulP inactivation strain (2)

The long flanking regions of antibiotic cassette was used to construct the new gene-inactivated plasmid. XbaI-linearlized plasmid (pTgLSmCm) was transformed to *Tetraspora* sp. CU2551. Interestingly, the transformant number 3 showed the promising band with a size of 850 bp corresponding to the size in positive control. Unfortunately, this fragment was not sequenced to check whether it is the target band or a fault positive band. Moreover, this transformant could not grow to the 6^{th} generation. Again, the

explanation is still unclear and there is no report revealing the incompatiblility of chloramphenicol resistance cassette to algal genome; we proposed the possibility of incompatibility of this cassette to *Tetraspora* sp. genome.

Paromomycin resistance cassette was developed by Sizova *et al*. (2001). However, this cassette was developed for *C. reinhardtii* system since hsp70A, 5'rbsc2, In1, and 3'rbsc2 were obtained from *C. reinhardtii* genome sequence. They also reported that the existence of hsp70A promoter along with intron1 fragment could promote the expression level of *aph VIII* by 140 folds. Even though this system was developed for *C. reinhardtii*, this cassette was selected for use in *Tetraspora* sp. transformation.

Since the Pm^R in pSI103-1 could not be expressed in *E. coli* system due to the promoter upstream the *aph VIII* derived from *C. reinhardtii*, the recombinant plasmids were screened from the plasmid extracted from *E. coli* followed by restriction enzyme digestion. Several methods were used to initiate ligation using optimized parameters, but no positive construct was obtained. In addition, the PCR-product obtained also contained no expected construct. In this case, some investigations was performed to address the cause. We found the high GC content of 62% along the Pm^R cassette, especially high in *aph VIII* of 69%. We also found that the normal PCR using gene specific primer could not produce the expected band, but the band was found when 3% DMSO was present in PCR reaction. This result confirmed that the high GC content affected the topology of Pm^R fragment and the DMSO could help stabilize the topology of the fragment for suitable amplification.

An indirect ligation was used to get the Pm^R:*sulP*-inactivated construct. Each portion of sulP was ligated to flank to Pm^R in pSI103-1 in order to destroy some topology in the cassette. Fragment C of *sulP* was firstly cloned to flank at the upstream of Pm^R where the GC island of *aph VIII* region stayed far from this cloning site. After PmC

plasmid was constructed, fragment A could ligate to another end with the coherence direction to fragment A. Finally, PmCA plasmid was obtained and ready to transform to algal genome.

To make sure that an exogenous DNA can be delivered to cells, biolistic microparticle bombardment method was selected. The green alga *Gonium pectoral* has the morphology suitable for cell attachment to other cells. In addition, many optimized parameters for this technique were obtained from the study by Lerche and Hallmann (2009) to be applied in *Tetraspora* sp. transformation. After transformation, many transformants were obtained on the selective medium plates and only one transformant was grown to the 12^{th} generation. The mutant confirmation was initially proved by colony PCR, but the reaction failed to produce any promising band. Using genomic DNA as a template, all 9 primers were combined to get a total of 20 combinations of primer pairs to check the segregation at recombination sites. The PCR pattern showed no product when one of primers primed to the PmR resistance cassette except Ex3F/PmF1 and PmR/PmF1. Ex3F/PmF1 primer pairs did not produce the promising band, but the size of about 300 bp was obtained. This fragment was not sequenced. While PmR/PmF1 gave the expected PCR product size, the sequence result did not show the correct sequence. It should be noted that the wild type genomic DNA failed to produce this expected band size of 447 bp using PmR/PmF1 primers.

Overall results indicated that even many developed mutant creation methods were applied to *Tetraspora* sp., the stable mutant was not obtained in this study. However, the new approach was used to understand the genes involved in the metabolism of biohydrogen production. The gene expression analysis study of some genes was used to unveil the mechanism of cells response under sulfur deprivation condition.

4.6 Sulfur deprivation

4.6.1 hydrogenase gene (hydA) investigation

Since the *sulP*-deficient mutant strain was unsuccessfully construct by optimization in this study, the gene expression profile analysis was selected to understand the biohydrogen production pathway in green alga *Tetraspora* sp. CU2551. The study would like to connect between the hydrogen production catalyzed by hydrogenase enzyme (HydA) under sulfur deprivation condition. A *sulP* full-length sequence was determined previously and used as a template for primer design for use in gene expression analysis. Unlike *sulP* investigation, hydrogenase gene (*hydA*) investigation was started in both cDNA and gDNA level at the same time. Primers were designed around the conserved regions and one combination of them gave a PCR product at both cDNA and gDNA level. Structural gene showed two introns with the size of 261 and 282 bp, respectively. The deduced amino acid sequence of this partial sequence was subjected to the phylogenetic tree determination. *Tetraspora* HydA showed close relationship to other green algal HydAs. However, the cDNA sequence is sufficient for primer design for use in gene expression profile analysis.

4.6.2 Sulfur deprivation condition

Under S-deprivation the protein biosynthesis is geared towards those products that are necessary for maintaining under the conditions of reduced metabolic activities (Wykoff, *et al*. 1998). As a consequence, the D1 protein which is essential to the reaction center of the PSII system cannot restore the activity of PSII during rapidly continuous photodamage resulting in low electrons flow to hydrogenase. *Tetraspora* sp. showed the decrease of hydrogen production after sulfur deprivation; however, the long term

cultivation in 100 h of *C. reinhardtii* under sulfur deprivation condition could promote cells to produce biohydrogen in large amount, while *Scenedesmus obliquus* showed almost no detectable production (Winkler *et al*., 2002a). PSII activity during sulfur deprivation was indirectly monitored by fluorescence measurement. The fluorescence increased when *Tetraspora* sp. was adapted in S-deprived TAP medium. The control set showed the decrease fluorescence by 20% when fresh TAP was replaced within 1 h. This may be due to the resumption of PSII activity caused by the presence of sulfate in fresh TAP medium. This result was similar to *C. reinhardtii* when deprived the culture of sulfate (Volgusheva *et al*., 2007). Both *sulP* and *hydA* were sharply up-regulated and saturated within 6 h. This is in agreement with the previous study by Lindberg and Melis (2008) revealed that the *C. reinhardtii sulP* transcript level rapidly increased and saturated within 3 h under sulfur deprivation. Although the report from Winkler *et al*.(2002) showed the increase of *C. reinhardtii hydA* for more than 1,000 folds under anaerobic condition, *Tetraspora hydA* showed the up-regulated for about 2.3 folds under aerobic condition. This was from the fact that oxygen acts as the most powerful inhibitor for *hydA* expression (Melis 2007).

4.6.3 Sulfur replete condition

From the sulfur deprivation study, the hydrogen was hardly detectable at 16 h, it was assumed that the electron flow existed at very low level. Then, the culture was replaced by pre-warm fresh TAP medium to allow cells to restore all activities. Interestingly, hydrogen production was restored in 4 h, but the rate of production did not reach the production rate that cells attained before adapting in S-deprived medium. The cell culture showed the different maximum capacity of production when cells were repeated using +/- sulfate cycle (Kim *et al*., 2010). PSII activity was also restored within

2 h after repletion suggesting that this is a result of the *sulP* expression in high amount on the chloroplast surface during S-deprived adaptation. Then, after sulfate was present in culture medium, cell could uptake sulfate to reach an equilibrium point in short time. The expression level of *sulP* also indicated the possibility of SulP protein translation in short time to expedite sulfate uptake restoring cells in equilibrium status before the expression of sulP declined afterwards. This process also affected the *hydA* expression profile. The expression profile declined and the production was restored in short time.

In conclusion, *Tetraspora* sp. CU2551 has high potential for biohydrogen production in Thailand where light is plentiful with optimal temperature for cells to grow. Cells showed the highest production rate when nitrogen and sulfur deprivation condition was adapted to the culture under the light intensity of about 30 $\mu E/m^2s$. Compared to other studies, *Chlamydomonas reinhardtii* showed the highest capacity of production to produce hydrogen gas under sulfur deprivation condition; however, *Tetraspora* sp. CU2551 could still produce the gas in high level under normal condition. Genetic manipulation could be applied to enhance the production from this strain even the method could not be developed in this study. Gene expression analysis allowed us to understand the hydrogen production metabolism in *Tetraspora* sp. Sulfate permease and hydrogenase genes were up regulated when sulfur was deprived from the culture resulting in low electron flow from PSII followed by low biohydrogen production. The gene expression level was restored upon replacing the medium with sulfur-containing TAP which resulted in the increase of electron flow from PSII followed by the increase of hydrogen production. Gene expression analysis results provided the understanding of the genes response involved in H_2 metabolism, and fluorescence results highlight the importance of electron flow from PSII to hydrogenase enzyme that resulted in enhanced hydrogen production.

CHAPTER V

CONCLUSIONS

The present study of the hydrogen production in green microalgae has revealed the following findings:

1. Screening showed the distribution and availability of hydrogen-producing microalgae in nature of Thailand.
2. The high-producing selected strain was named as *Tetraspora* sp. CU2551, which belonged to green algae group confirmed by 18S rDNA determination.
3. *Tetraspora* sp. rapidly grows in TAP medium at 36 $^{\circ}$C under continuous light illumination ranging from 37 – 92 $\mu E/m^2/s$, and competent to produce hydrogen gas at high rate when cells were grown for 24 h and produced the gas at 35 $^{\circ}$C.
4. Increasing the pH ranging from 5.75 to 9.3 resulted in an increased hydrogen production rate, whereas almost no production was observed when pH was dropped to 5.25.
5. Under light incubation, supplementation of carbon sources and salts could not promote the production, while high rate hydrogen production for *Tetraspora* sp. was obtained when cells were incubated under sulfur and nitrogen deprivation.
6. β-mercaptoethanol increased the hydrogen production capacity when light intensity was below 7 $\mu E/m^2/s$.
7. The full-length of new chloroplast envelope-localized sulfate permease (*sulP*) was investigated. This study showed that *Tetraspora* sp. CU2551 is the second strain after *Chlamydomonas reinhardtii* where *sulP* contains intron fragments after *sulP*

in. The putative gene product also shows the close relationship to each other with the existence of chloroplast transit peptide.

8. Several methods were used to create the mutant, but no reliable method was developed for the transformation of exogenous DNA to *Tetraspora* sp. genome.
9. Hydrogenase gene (*hydA*) was investigated showing the existence of two intron regions in *hydA* at DNA level. The putative protein encoded from this 878-bp gene contains 292 amino acid residues which shows the closet relationship to hydrogenase from *Chlorella fusca*.
10. Under sulfur deprivation condition, the hydrogen production was decreased as a result of less electrons flow to hydrogenase enzyme indirectly measured by fluorescence of PSII system. However, sulfate permease gene and hydrogenase gene were increased about 2.3 folds to the saturation point within about 6 h.
11. Hydrogen production was rapidly restored in 4 h and the fluorescence of PSII system was recovered in 2 h when the culture medium was replaced by new sulfur-containing medium. The gene expression profiles of *sulP* and *hydA* showed the decrease of both genes after sulfur source was restored.
12. In summary, this study highlights the importance of the hydrogen production in *Tetraspora* sp. CU2551 which depends on the electron flow from PSII system to hydrogenase enzyme.

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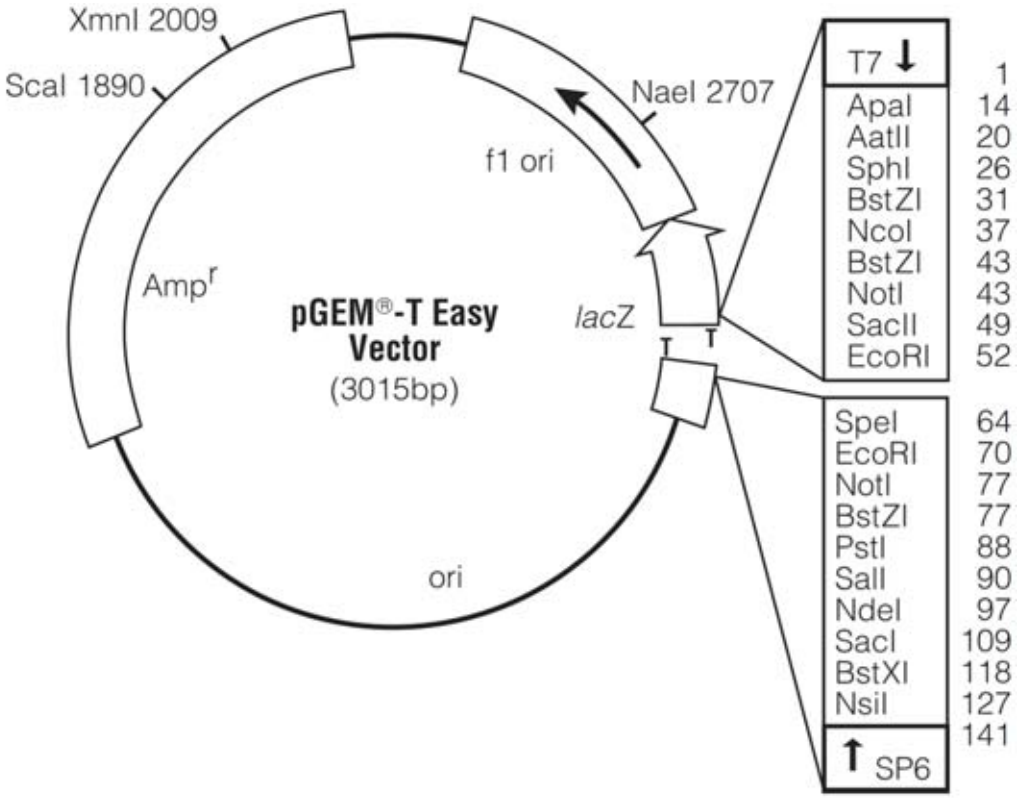
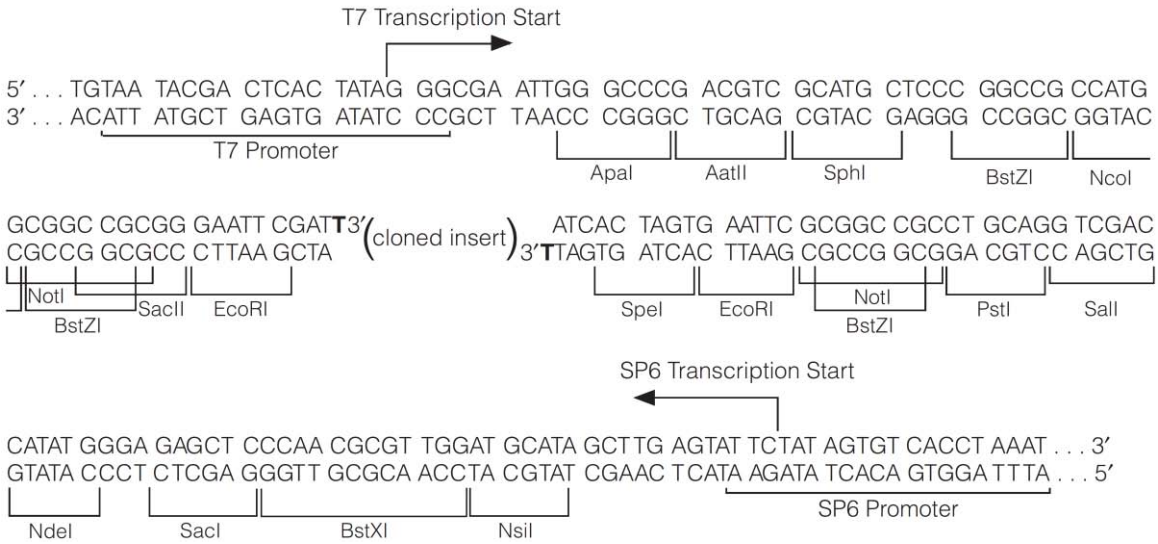
APPENDICES

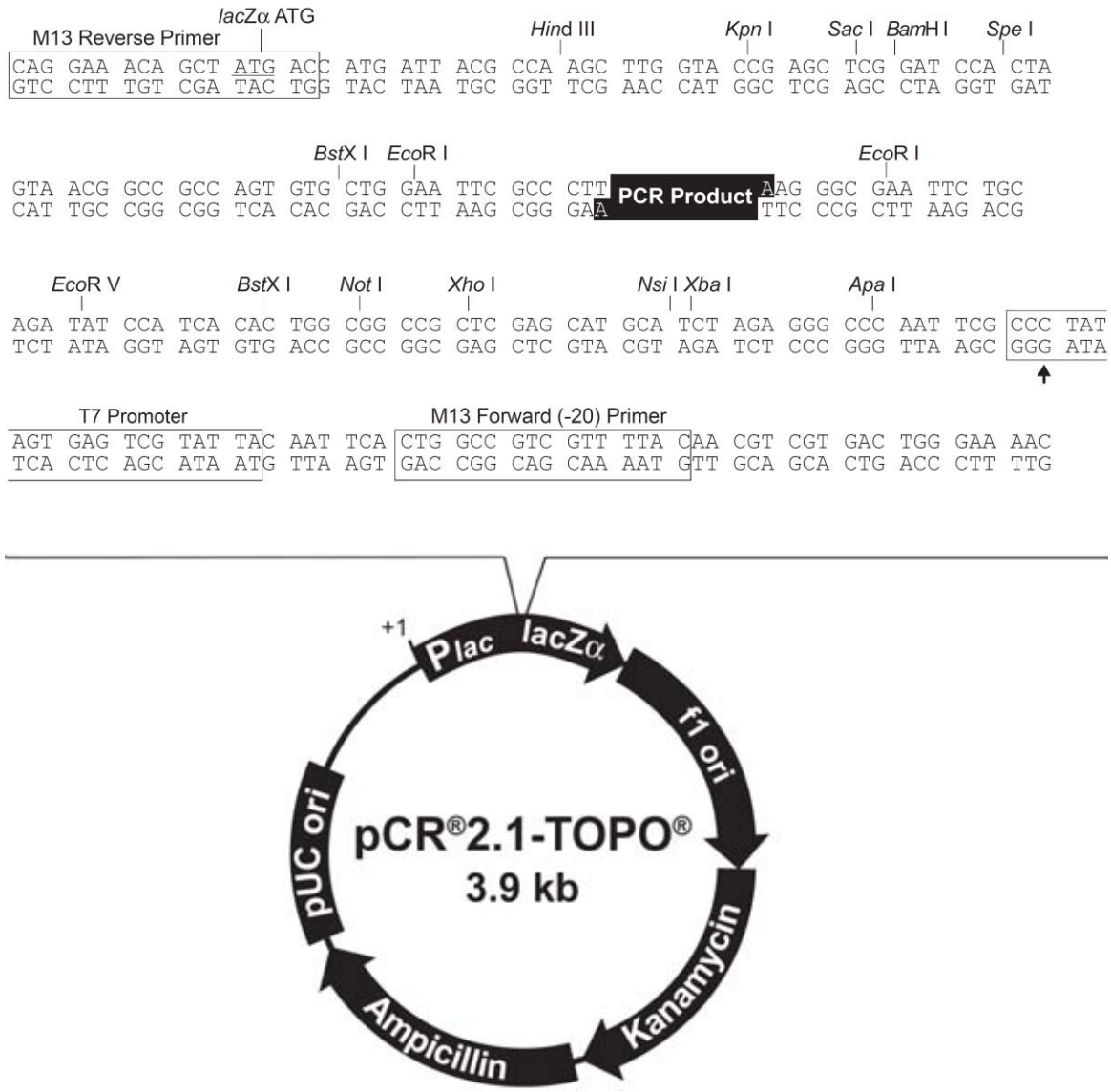
APPENDIX A

Cloning vector maps: pGEM®-T Easy Vector





Cloning vector maps: pCR®2.1-TOPO®Vector



APPENDIX B

H_2 content calculation

Determine the amount of H_2 corresponding to the peak area for each time point against 4% Ar-balanced hydrogen gas.

Calculate the concentration of hydrogen produced in vial head space (X %) by using the following equation:

$$X\ \% = \frac{\text{Peak area of sample}}{\text{Peak area of 4\% standard hydrogen gas}}\ (4\%)$$

22.4 dm^3 (L) = 1 mol H_2, thus the volume in head space was used to calculated.

Normalize the hydrogen content by using the dry weight amount.

Express the H_2 evolved in μmol/mg DW/h unit.

APPENDIX C

Media compositions

N-Free

NaCl	70.00	mg
$MgSO_4.7H_2O$	380.00	mg
$CaCl_2$	80.00	mg
K_2HPO_4	600.00	mg
$Fe_2(SO_4)_3.6H_2O$	10.00	mg
Na_2-EDTA	27.00	mg
H_3BO_3	3.00	mg
$MnSO_4.4H_2O$	2.00	mg
$NaMoO_4.2H_2O$	8.00	mg
$ZnSO_4.7H_2O$	0.30	mg
$CuSO_4.5H_2O$	0.08	mg
$CoCl_2$	0.02	mg
Diltilled water to	1.00	L

N8

$Na_2HPO_4.2H_2O$	260.00	mg
K_2HPO_4	740.00	mg
$CaCl_2$	10.00	mg
Fe.EDTA	10.00	mg
$MgSO_4.7H_2O$	50.00	mg
KNO_3	1000.00	mg
Trace element mixture*	1.00	mL
Diltilled water to	1.00	L
Trace element mixture for N8		
$Al_2(SO_4)_3.18H_2O$	3.58	g
$MnCl_2.4H_2O$	12.98	g
$CuSO_4.5H_2O$	1.83	g
$ZnSO_4.7H_2O$	3.20	g
Diltilled water to	1.00	L

BG11

$NaNO_3$	1.50	g
$K_2HPO_4.3H_2O$	40.00	mg
$MgSO_4.7H_2O$	75.00	mg
$CaCl_2$	36.00	mg
Citric acid	6.00	mg
Ferric Ammonium citrate	6.00	mg
Mg_2-EDTA	1.00	mg
Na2CO3	20.00	mg
Trace metal mix A5 +Co*	1.00	mg
Diltilled water to	1.00	mL
Trace metal mix A5 +Co		
H_3BO_3	2.86	g
$MnCl_2.4H_2O$	1.81	g
$ZnSO_4.7H_2O$	220.00	mg
$NaMoO_4.2H_2O$	390.00	mg
$CuSO_4.5H_2O$	79.00	mg
$Co(NO_3)_2.6H_2O$	49.00	mg
Diltilled water to	1.00	L

Zarrouk

NaHCO3	16.80	g
K_2HPO_4	0.50	g
$NaNO_3$	2.50	g
NaCl	1.00	g
$MgSO_4.7H_2O$	0.20	g
$FeSO_4.7H_2O$	0.01	g
$CaCl_2.2H_2O$	0.04	g
EDTA	0.08	g
K_2SO_4	1.00	g
A_5 solution*	1.00	mL
B_6 solution*	1.00	mL
Diltilled water to	1.00	L
A_5 solution (g/L)		
H_3BO_3	2.86	g
$MnCl_2.2H_2O$	1.80	g
$ZnSO_4.4H_2O$	0.22	g
MoO_3	0.01	g

$CuSO_4.5H_2O$	0.08	g
Diltilled water to	1.00	L
B_6 solution (mg/L)		
NH4NO3	22.90	g
K2Cr2(SO4)4.24H2O	96.00	g
NiSO4.7H2O	47.80	g
Na2SO4.2H2O	17.90	g
Ti2(SO4)3	40.00	g
$Co(NO_3)_2.6H_2O$	4.40	g
Diltilled water to	1.00	L

Tris-Acetate-Phosphate (TAP)

NH_4Cl	0.40	g
$CaCl_2.2H_2O$	0.05	g
$MgSO_4.7H_2O$	0.10	g
KH_2PO_4	0.05	g
K_2HPO_4	0.10	g
EDTA	0.05	g
Tris (base)	2.42	g
Glacial acetic acid	1.00	mL
$FeSO_4.7H_2O$	5.60	mg
$ZnSO_4.4H_2O$	22.08	mg
H_3BO_3	11.40	mg
$MnCl_2.2H_2O$	5.52	mg
$CuSO_4.5H_2O$	0.16	mg
$NaMoO_4.2H_2O$	0.75	mg
$CoCl_2.6H_2O$	0.17	mg
Diltilled water to	1.00	L

AA

solution A*	25.00	mL
solution B*	6.25	mL
KNO_3	0.25	g
$NaNO_3$	0.21	g
Diltilled water to	1.00	L
Solution A		
4% $MgSO_4.7H_2O$	500.00	mL

1.2% $CaCl_2.2H_2O$	500.00	mL
3.8% NaCl	500.00	mL
Microelements stock solution *	500.00	mL
Solution B		
K_2HPO_4	28.00	
Diltilled water to	500.00	mL
Microelements stock solution		
A&A Fe-EDTA solution*	160.00	mL
$MnCl_2.2H_2O$	360.00	mg
MoO_3	36.00	mg
$ZnSO_4.4H_2O$	44.00	mg
$CuSO_4.5H_2O$	15.80	mg
H_3BO_3	572.00	mg
NH_4VO_3	4.60	mg
$CoCl_2.6H_2O$	8.00	mg
Diltilled water	1090.00	mL

A&A Fe-EDTA solution

Dissolve 5.2g KOH in 186 ml distilled water, then add 20.4 g $Na_2EDTA \cdot 2H_2O$

Dissolve 13.7g $FeSO_4 \cdot 7H_2O$ in 364 ml

Mix these two solutions

Bubble filtered air through solution until color changes (minutes to hours)

Final pH of FeEDTA solution approximately 3.5

APPENDIX D

Hydrogen production of each isolate in section 3.1

Isolate No.	Isolate name	Production rate (µmol/mg chl a/h)
1	A11	0.00
2	A12	0.12
3	A21	0.06
4	A22	0.01
5	A23	0.76
6	A24	0.00
7	A25	0.11
8	A26	0.15
9	A27	0.13
10	A28	0.12
11	A29	0.26
12	A31	0.00
13	A32	0.09
14	A33	0.05
15	A34	0.08
16	A35	0.14
17	A36	0.15
18	A41	0.02
19	A42	0.00
20	A43	0.00
21	A44	0.00
22	A45	0.00
23	A46	0.06
24	A47	0.00
25	A48	0.04
26	A51	0.22
27	A61	0.00
28	A62	0.19
29	A63	0.05
30	A64	0.01
31	ACV	0.01
32	ACV-W-01F	0.00
33	ACV-W-02F	0.00
34	*Ankistrodesmus falcatus*	0.17
35	*Aphanocapsa biformis*	0.00
36	ATG-S-01	0.08
37	ATG-S-02	0.04
38	ATG-S-03	0.13
39	ATG-S-04	0.02
40	ATG-S-05	0.06
41	ATG-S-06	0.04
42	ATG-S-10	0.09
43	ATG-S-11	0.04
44	ATG-S-12	0.09
45	ATG-S-13	0.01
46	ATG-S-15	0.00
47	ATG-S-16	0.08
48	ATG-S-17	0.08

Isolate No.	Isolate name	Production rate (µmol/mg chl a/h)
49	ATG-S-18	0.00
50	ATG-S-19	0.04
51	ATG-S-20	0.10
52	ATG-S-21	0.00
53	AYT-S1-01F	0.08
54	AYT-S1-03	0.12
55	AYT-S2-01	0.58
56	AYT-W0-01	0.05
57	AYT-W0-03	0.06
58	AYT-W1-02	0.07
59	AYT-W1-04	0.09
60	AYT-W1-09F	0.06
61	AYT-W2-03	0.03
62	AYT-W2-04F	0.00
63	AYT-W2-05F	0.01
64	AYT-W2-06F	0.00
65	AYT-W2-07F	0.01
66	BOT-S-01	0.02
67	BOT-S-02	0.05
68	BOT-S-03	0.05
69	BOT-S-04	0.07
70	BOT-W-01	0.05
71	BOT-W-02	0.22
72	BOT-W-05	0.00
73	BOT-W-06	0.07
74	BOT-W-07	0.40
75	BOT-W-09	0.01
76	BOT-W-10	0.35
77	BOT-W-11	0.02
78	BOT-W-12F	0.09
79	BOT-W-14F	0.38
80	BPT-S-01	0.04
81	BPT-S-05	0.00
82	BPT-W-02	0.00
83	BPT-W-03	0.00
84	BPT-W-04	0.00
85	BPT-W-06	0.03
86	BPT-W-07	0.01
87	BPT-W-08	0.02
88	BPT-W-09	0.01
89	BPT-W-10	0.01
90	BPT-W-11	0.01
91	BPT-W-12	0.05
92	BPT-W-13	0.01
93	BPT-W-14	0.00
94	BPT-W-15	0.01
95	BPT-W-16	0.00
96	BPT-W-17	0.00
97	*Calothrix elenkinii*	0.00
98	*Chlorogloea fritschii*	0.00
99	*Chrorococcus turgidus*	0.00
100	CMU-W-01	0.05
101	CMU-W-02	0.02
102	*Coccomonas orbicularis*	0.02

Isolate No.	Isolate name	Production rate (µmol/mg chl a/h)
103	Finnland	0.02
104	*Fischerella muscicola*	0.00
105	*Gleocapsa atrata*	0.00
106	JET-S-01	0.01
107	JET-S-02	0.20
108	JET-S-05F	0.00
109	JET-S-06F	0.00
110	KK (BG11)	0.01
111	LG1	0.19
112	LG3	0.31
113	LG4	0.22
114	LG6	0.19
115	LG7	0.10
116	LPD-W-01	0.10
117	LPD-W-03	0.02
118	LPD-W-08	0.02
119	MAY-W-01	0.11
120	MAY-W-02	0.06
121	MAY-W-04	0.86
122	MAY-W-04.1	0.13
123	MAY-W-04.2	0.31
124	MAY-W-04.3	0.31
125	MAY-W-04.4	0.23
126	MAY-W-04.5	0.26
127	MAY-W-04.6	0.19
128	MAY-W-04.7	0.18
129	MAY-W-04.8	0.32
130	MAY-W-04.9	0.42
131	MAY-W-07	0.44
132	*Microcystis pulvaria*	0.07
133	*Myxosarcina burmensis*	0.00
134	NFP-W-02	0.05
135	NFP-W-03	0.05
136	NFP-W-07	0.10
137	NFP-W-10	0.06
138	NKN-S-01	0.04
139	NKN-S-02	0.05
140	NKN-S-03	0.04
141	NKN-S-04	0.02
142	NKN-S-05	0.04
143	NKN-S-06	0.00
144	NKN-S-07	0.17
145	NKN-S-08	0.13
146	*Nostoc calcicola*	0.00
147	*Oscillatoria salina*	0.14
148	*Phormidium calcicola*	0.00
149	PK8-W-01	0.06
150	PK8-W-04	0.04
151	PK8-W-05	0.03
152	PK8-W-06	0.08
153	PK8-W-07	0.32
154	PK8-W-09	0.07
155	PK8-W-10	0.06
156	PK8-W-11	0.02

Isolate No.	Isolate name	Production rate (µmol/mg chl a/h)
157	PK8-W-12	0.06
158	*Plectonema gracillimum*	0.00
159	PTH-F-01	0.08
160	PTH-F-03	0.09
161	PTH-F-04	0.07
162	PTH-F-05	0.06
163	PTH-F-06	0.16
164	PTH-F-07	0.07
165	PTH-F-08	0.06
166	PTH-F-09	0.05
167	PTH-S-01	0.06
168	PTH-S-02	0.01
169	PTH-S-03F	0.00
170	PTH-W-01	0.36
171	PTK-W-02	0.04
172	PTK-W-03	0.00
173	PTK-W-04	0.08
174	PTK-W-05F	0.00
175	PTK-W-06F	0.00
176	PTK-W-08	0.01
177	SBR-S-01	0.21
178	SBR-S-02	0.09
179	SBR-S-03	0.29
180	SBR-S-04	0.06
181	SBR-S-05	0.07
182	SBR-S-06	0.04
183	SBR-S-07	0.05
184	SBR-S-08	0.00
185	SBR-S-09	0.12
186	SBR-S-10	0.05
187	SBR-S-11	0.06
188	SBR-S-12	0.06
189	SBR-S-13	0.04
190	SBR-S-14	0.16
191	*Scenedesmus acuminatus*	0.07
192	*Scytonema bohneri*	0.02
193	SS-2	0.03
194	SS-3	0.20
195	SS-4	0.15
196	STH-W-01	0.06
197	STH-W-03	0.04
198	STH-W-04	0.11
199	TPS-W-01	0.04
200	TPS-W-02	0.01
201	TUR-dark green	0.04
202	TUR-filament	0.00
203	TUR-green	0.06
204	TUR-light green	1.00
205	TUR-W-20	0.96
206	TUR-W-21	0.06
207	TUR-W-22	0.15
208	TUR-W-24F	0.08
209	WBU-W-02	0.00
210	WBU-W-03	0.00

APPENDIX E

PERSONAL INFORMATION

FIELD OF RESEARCH INTEREST

I have started my PhD since 2008 in Cyanobacterial Biotechnology Laboratory, Biochemistry Department, Faculty of Science, Chulalongkorn University, Thailand. I have focused on the finding of new energy source for our world, and the green energy source like the hydrogen production by biophotolysis process interests me. For my work, I have screened several hydrogen-producing green microalgae and optimized the production condition for use as an alternative energy source. Genes involved in hydrogen metabolism in green algae were investigated and were monitored under nutrient deprivation to understand their function *in vivo.*

In the past, I worked as research assistant at the Shrimp Molecular Biology and Genetics Laboratory (SMBGL) Bangkok Thailand, the same place where I performed my Master thesis work. My work is focused on an antimicrobial peptide gene isolated from the hemocyte cDNA library of the black tiger shrimp (*P. monodon*). Alternatively, I have additionally developed 30 type I microsatellite markers which were extended from my thesis area. I have done these works since June 2005. After relocated to a new job in January 2007, I have joined the Protein-Ligand Engineering and Molecular Biology Laboratory, National Center for Genetics Engineering and Biotechnology (BIOTEC), Thailand. At BIOTEC, my researche was mainly focused on drug screening for inhibiting malarial key enzyme (DHFR-TS) in *Plasmodium falciparum* via X-ray crystallography technique. In addition,I also earned experiences in data collection from National Synchrotron Radiation Research Center, Taiwan in 2008.

During my Master thesis research, I worked on type I microsatellite markers to extend the genome mapping of the black tiger shrimp (*Penaeus monodon*). The work is intended to provide the additional markers to the previous *P. monodon* genetic linkage map of AIMS (The Australian Institute of Marine Science). I had developed 50 type I microsatellite markers in my study and found that 36 markers could be placed on the previous genetic linkage map.

SCHOLARSHIPS

- June 2009- May 2010

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

- June 2008 - May 2011

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

- June 2004 - May 2005

  Local Graduate Scholarship (LGS). This scholarship was funded by National Center for Genetics Engineering and Biotechnology (BIOTEC) for local master degree student.

AWARDS

- 20 April 2012

  Best presentation award from The Science Forum 2012 (Applies Science and Technology session). Mahamakut Building, Faculty of Science, Chulalongkorn University, Thailand: April 19 – 20, 2012.

- 03 April 2011

  Outstanding presentation award from RGJ-Ph.D. Congress XII, Jomtein Palm Beach Resort Pattaya, Chonburi, Thailand: April 1 -3, 2011.

- 17 June 2010

  Recognized as best graduate student, Chulalongkorn University, Thailand

- 21 August 2005

  The excellent graduate student award, the Prof. Dr. Tab Nilanidhi Foundation (the award was presented to the student who received the highest GPA above 3.70 in each department).

ACADEMIC EXPERIENCES

1. Science Forum 2012, Faculty of Science, Chulalongkorn University, Thailand: April 19 – 20, 2012. High Rates of Hydrogen Production by a Novel Green Alga *Tetraspora* sp. CU2551 (Oral presentation).
2. RGJ–Ph.D. Congress XII, Jomtein Palm Beach Resort Pattaya, Chonburi, Thailand: April 1 – 3, 2011. High Rates of Hydrogen Production by a Novel Green Alga *Tetraspora* sp. CU2551 (Oral presentation).
3. CAP meeting, Department of Photochemistry and Molecular Science, Uppsala University, Sweden: October 05, 2010. Identification and characterization of a novel hydrogen-producing green alga *Tetraspora* sp. CU2551 isolated from natural pond in Thailand (Oral presentation).
4. The 9th International Hydrogenase Conference, Uppsala, Sweden: June 27 – July 2, 2010. Identification and characterization of a hydrogen-producing green alga *Tetraspora* sp. TURW 20.6 isolated from natural pond in Thailand (Poster presentation).

5. The 14th Biological Sciences Graduate Congress. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. December 10 – 12, 2009. Screening for photosynthetic hydrogen-producing bacteria and optimization for the hydrogen production (Poster presentation).
6. The 13th Biological Sciences Graduate Congress. Faculty of Science, National University of Singapore, Singapore: December 15 – 16, 2008. Screening of hydrogen producing cyanobacteria from natural ponds in Thailand (Poster presentation).
7. The 16th Annual Meeting of the Thai Society for Biotechnology on "Innovative Biotechnology: The Opportunity for Kitchen of the World". 12 – 15 December 2004, Phitsanulok, Thailand (Poster presentation).

PUBLICATIONS

1. **Maneeruttanarungroj, C.**, Lindblad, P. and Incharoensakdi, A. (2012) Sulfate permease (SulP) and hydrogenase (HydA) in the green alga *Tetraspora* sp. CU2551: Dependence of gene expression on sulfur status in the medium. International Journal of Hydrogen Energy (submitted)
2. **Maneeruttanarungroj, C.**, Lindblad, P. and Incharoensakdi, A. (2010) A newly isolated green alga, *Tetraspora* sp. CU2551, from Thailand with efficient hydrogen production. International Journal of Hydrogen Energy 35:13193-13199.
3. Nzila, A., Rottmann, M., Chitnumsub, P., Kiara, S. M., Kamchonwongpaisan, S., **Maneeruttanarungroj, C**., Taweechai, S., Yeung, B. K., Goh, A., Lakshminarayana, S. B., Zou, B., Wong, J., Ma, N. L., Weaver, M., Keller, T. H., Dartois, V., Wittlin, S., Brun, R., Yuthavong, Y. and Diagana, T. T. (2010) Preclinical evaluation of the antifolate QN254, 5-chloro- N060-(2,5-dimethoxy-

benzyl)-quinazoline-2,4,6-triamine, as an antimalarial drug candidate. Antimicrob. Agents Chemother54, 2603–2610.

4. Dasgupta, T., Chitnumsub, P., Kamchonwongpaisan, S., **Maneeruttanarungroj, C**., Nichols, SE., Lyons, TM., *et al*. (2009) Exploiting structural analysis, *in silico* screening, and serendipity to identify novel inhibitors of drug-resistant Falciparum malaria. ACS Chem Biol 4:29–40.
5. Supungul, P., Tang, S., **Maneeruttanarungroj, C**., Rimphanitchayakit, V., Hirono, I., Aoki, T., *et al*., 2008. Cloning, expression and antimicrobial activity of crustinPm1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. Dev. Comp. Immunol. 32, 61–70.
6. **Maneeruttanarungroj, C.**, Pongsomboon, S., Wuthisuthimethavee, S., Klinbunga, S., Wilson, K.J., Swan, J., Li, Y., Whan, V., Chu, K.H., Li, C.P., Tong, J., Glenn, K., Rothschild, M., Jerry, D., Tassanakajon, A., 2006. Development of polymorphic expressed sequence tag-derived microsatellites for the extension of the genetic linkage map of the black tiger shrimp (*Penaeus monodon*). Anim. Genet. 37, 363–368.

TRAINING & ACTIVITIES

2010 Organizing committee of Cyanobacteria and algae biotechnology symposium. Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand

2009 Organizing committee of the 14th Biological Sciences Graduate Congress. Faculty of Science, Chulalongkorn University, Bangkok, Thailand

2009	The president of Biochem Lover Club, Department of Biochemistry, Chulalongkorn University
2008	X-ray diffraction data collection at National Synchrotron Radiation Research Center (NSRRC), Hsinchu Taiwan (April 04-06, 2008)
2007	Training course on the "School on Use of Synchrotron Radiation" (April 23 – May 4, 2007) at the National Synchrotron Research Center (NSRC), Nakhon Ratchasima, Thailand
2004	Registrar of the 17th FAOBMB Symposium/ 2nd IUBMB Special Meeting/ 7th A-IMBN Conference, Bangkok, Thailand
2003	Equipment supplier staff of Graduated Committee of Chulalongkorn University
2002	Student trainee at Institute of Nutrition, Mahidol University (March - May), Thailand
2001	Deputy President (academic affairs) of Science Study Club of Chulalongkorn University
2000	Editor of science magazine (SCIMAX) of Science Study Club of Chulalongkorn University, Faculty of Science, Chulalongkorn University
1999 - 2002	Staff of Young Leader Scientist Camp 6th - 9th of Science Study Club of Chulalongkorn University (in the position of Academic affairs, Assessment affairs, and Ceremony affairs)

REFERENCES

Professor Aran Incharoensakdi, Ph.D. Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

E-Mail: aran.i@chula.ac.th

Professor Peter Lindblad, Ph.D. Photochemistry and Molecular Science, Department of Chemistry – Ångström Laboratory, Uppsala University, Box 523, SE-75120, Uppsala, Sweden

E-Mail: peter.lindblad@fotomol.uu.se

BIOGRAPHY

Mr. Cherdsak Maneeruttanarungroj was born on December 11, 1981 in Nakornratchasrima, Thailand. He graduated with Bachelor degree and Master degree in Biochemistry from the department of Biochemistry, Faculty of Science, Chulalongkorn University in 2003 and 2005, respectively. He has further studied for the Doctor of Philosophy (Ph.D.) degree in Program of Biochemistry, Chulalongkorn University since 2008.