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ENHANCEMENT OF HYDROGEN PRODUCTION BY UNICELLULAR HALOTOLERANT CYANOBACTERIUM *Aphanothece halophytica* BY CELL IMMOBILIZATION AND USE OF INHIBITORS

สุนิษา ปั้นสุข
SUNISA PANSOOK

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
ภาควิชาชีววิทยา คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
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SUNISA PANSOOK

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DEPARTMENT OF BIOLOGY FACULTY OF SCIENCE
KING MONGKUT'S INSTITUTE OF TECHNOLOGY LADKRABANG

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ชื่อนักศึกษา	นางสาวสุนิษา ปั้นสุข
รหัสประจำตัว	56605017
ปริญญา	ปรัชญาดุษฎีบัณฑิต (เทคโนโลยีชีวภาพ)
ภาควิชา	ชีววิทยา
พ.ศ.	2562
อาจารย์ที่ปรึกษาวิทยานิพนธ์	ผศ.ดร. สรัญญา พันธุ์พฤกษ์
อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม	ศ.ดร. อรัญ อินเจริญศักดิ์

บทคัดย่อ

ไฮโดรเจนเป็นพลังงานทางเลือกหนึ่งที่ได้รับความสนใจเป็นอย่างมากในปัจจุบัน เนื่องจากการเผาไหม้ไฮโดรเจนจะให้ค่าความร้อนสูงและไม่ก่อให้เกิดผลิตภัณฑ์ที่เป็นมลพิษต่อสิ่งแวดล้อม ไซยาโนแบคทีเรียเป็นจุลินทรีย์ชนิดหนึ่งที่มีความสามารถในการผลิตไฮโดรเจน โดยผลิตไฮโดรเจนจากการทำงานของเอนไซม์ไนโตรจีเนสผ่านการตรึงไนโตรเจน และ/หรือ จากการทำงานของเอนไซม์ไฮโดรจีเนสผ่านกระบวนการแตกตัวของน้ำทางตรงและทางอ้อม *Aphanothece halophytica* เป็นไซยาโนแบคทีเรียทนเค็มเซลล์เดียวที่มีศักยภาพในการผลิตไฮโดรเจน โดยส่วนใหญ่ *A. halophytica* ผลิตไฮโดรเจนจากการทำงานของเอนไซม์ไบไดเรคชันนาลไฮโดรจีเนส ผ่านการย่อยสลายไกลโคเจนที่สะสมภายในเซลล์ภายใต้สภาวะมืดและปราศจากอากาศ งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาการเพิ่มการผลิตไฮโดรเจนของไซยาโนแบคทีเรีย *A. Halophytica* โดยการตรึงเซลล์และการใช้สารยับยั้ง ผลการศึกษาพบว่า เซลล์ตรึงของ *A. halophytica* ผลิตไฮโดรเจนในปริมาณที่สูงกว่าเซลล์อิสระ ในบรรดาวัสดุที่นำมาใช้ตรึง อะการ์หรือวุ้นเป็นวัสดุตรึงที่มีประสิทธิภาพสูงสุดสำหรับการผลิตไฮโดรเจนจาก *A. halophytica* สภาวะที่เหมาะสมในการตรึงเซลล์ *A. halophytica* เพื่อผลิตไฮโดรเจน คือ ความเข้มข้นของอะการ์ 3 เปอร์เซ็นต์ (น้ำหนักต่อปริมาตร) ความหนาแน่นของเซลล์ 0.2 มิลลิกรัมน้ำหนักเซลล์แห้งต่อมิลลิลิตรของสารละลายเจล และขนาดของชิ้นวุ้นปริมาตร 0.125 ลูกบาศก์เซนติเมตร ความเข้มข้นของสารอาหารและแร่ธาตุที่เหมาะสมในอาหาร BG11 ที่เสริมด้วยสารละลาย Turk Island salt solution ต่อการผลิตไฮโดรเจนของเซลล์ตรึง คือ อาหารที่ปราศจากแหล่งไนโตรเจน ที่มีความเข้มข้นของโซเดียมคลอไรด์ 0.5 โมลาร์ แมกนีเซียมซัลเฟตเฮปตะไฮเดรต 30 มิลลิโมลาร์ เหล็กไอออน 4 ไมโครโมลาร์ พีเอชเริ่มต้นและอุณหภูมิของการบ่มที่เหมาะสมต่อการผลิตไฮโดรเจนของเซลล์ตรึง คือ พีเอช 7.4 และอุณหภูมิ 40 องศาเซลเซียส ตามลำดับ การผลิตไฮโดรเจนของเซลล์ตรึงจะเพิ่มขึ้นเมื่อบ่มเซลล์ตรึงในขวดแก้วที่มีปริมาตรมากขึ้น และมีปริมาตรของ headspace เพิ่มขึ้น สุดท้าย ทำการศึกษาการผลิตไฮโดรเจนของเซลล์ตรึงจำนวน 3 รอบ พบว่าเซลล์ตรึงสามารถผลิตไฮโดรเจนได้ในระดับสูงสุดในรอบที่ 2 เมื่อนำเซลล์ตรึงใหม่ปริมาณ 50 เปอร์เซ็นต์ มาแทนที่เซลล์ตรึงเดิม ในการเพิ่มการผลิตไฮโดรเจนของ *A. halophytica* โดยการใช้สารยับยั้ง จะทำการคัดเลือกชนิดและความเข้มข้นของสารยับยั้งที่ทำให้ *A. halophytica* ผลิต

ไฮโดรเจนได้สูงภายใต้สภาวะที่มีแสงและสภาวะมืด จากการทดลองพบว่า สารคาร์บอนิล-ไซยาไนด์ เอ็ม-คลอโรฟีนิลไฮดราโซนที่มีความเข้มข้น 0.5 ไมโครโมลาร์ เป็นสารยับยั้งที่มีประสิทธิภาพสูงสำหรับการเพิ่มการผลิตไฮโดรเจน การบ่มเซลล์กับ CCCP และ 3-(3,4-ไดคลอโรฟีนิล)-1,1-ไดเมธิลยูเรีย (DCMU) ที่มีความเข้มข้นสูงและเป็นระยะเวลานาน มีผลทำให้จำนวนเซลล์และความเข้มข้นของคลอโรฟิลล์ลดลง สาร CCCP และ DCMU สามารถเพิ่มการผลิตไฮโดรเจนของ *A. halophytica* ภายใต้สภาวะที่มีแสง ในขณะที่มีเพียง CCCP เท่านั้นที่สามารถเพิ่มการผลิตไฮโดรเจนในที่มืด สาร CCCP สามารถลดการผลิตออกซิเจนทั้งในที่มีแสงและที่มืด ในขณะที่สาร DCMU ลดการผลิตออกซิเจนในที่มีแสง ภายใต้สภาวะที่มีแสง กิจกรรมของเอนไซม์ไบไดเรคชันนาลไฮโดรจีเนสของ *A. halophytica* มีค่าสูงสุด เมื่อบ่มใน CCCP ที่มีความเข้มข้น 0.5 ไมโครโมลาร์และใน DCMU ที่มีความเข้มข้น 50 ไมโครโมลาร์ ภายใต้สภาวะที่มีแสง CCCP และ DCMU ยับยั้งคลอโรฟิลล์ฟลูออเรสเซนต์ ส่งผลให้มีปริมาณออกซิเจนลดลง และกระตุ้นกิจกรรมของเอนไซม์ไบไดเรคชันนาลไฮโดรจีเนสของ *A. halophytica* นอกจากนี้ CCCP ยังสามารถเพิ่มอัตราการหายใจ ซึ่งส่งผลให้ปริมาณของออกซิเจนลดลงอีกด้วย ในทางตรงกันข้าม DCMU ลดอัตราการหายใจใน *A. halophytica* เซลล์ *A. halophytica* ที่บ่มด้วยสาร CCCP ความเข้มข้น 0.5 ไมโครโมลาร์ สะสมไฮโดรเจนสูงสุด เท่ากับ 254.29 ± 16.33 ไมโครโมลไฮโดรเจนต่อกรัมน้ำหนักเซลล์แห้ง ซึ่งสูงกว่าเซลล์ที่ปราศจากการบ่มด้วย CCCP ประมาณ 2.6 เท่า งานวิจัยนี้แสดงความเป็นไปได้ในการเพิ่มการผลิตไฮโดรเจนของไซยาโนแบคทีเรีย *A. halophytica* โดยวิธีตรึงเซลล์และการใช้สารยับยั้ง

คำสำคัญ : การผลิตไฮโดรเจน, ไซยาโนแบคทีเรีย, การตรึงเซลล์, สารยับยั้ง

Thesis Title	Enhancement of hydrogen production by unicellular halotolerant cyanobacterium *Aphanothece halophytica* by cell immobilization and use of inhibitors
Student Name	Sunisa Pansook
Student ID	56605017
Degree	Doctor of Philosophy (Biotechnology)
Department	Biology
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Thesis Advisor	Asst. Prof. Dr. Saranya Phunpruch
Thesis Co-advisor	Prof. Dr. Aran Incharoensakdi

Abstract

H_2 is one of the alternative energy carriers that has been increasingly interesting nowadays because the combustion of H_2 provides a high heating value and its product does not cause pollution to the environment. Cyanobacteria are one of microorganisms capable of H_2 production. They can produce H_2 by nitrogenase activity via N_2 fixation and/or by hydrogenase activity via a direct and an indirect photolysis. The unicellular halotolerant cyanobacterium *Aphanothece halophytica* has been shown as a potential H_2 producer. It mainly produces H_2 by bidirectional hydrogenase activity via the catabolism of storage glycogen under dark anaerobic condition. This work aimed to study the enhancement of H_2 production by *A. halophytica* by cell immobilization and by treatment with Inhibitor. The result showed that immobilized cells of *A. halophytica* produced higher H_2 than free cells. Among all support materials for immobilization, agar was the most effective support material for H_2 production by *A. halophytica*. The optimal conditions of cell immobilization of *A. halophytica* for H_2 production were 3% (w/v) agar concentration, 0.2 mg dry cell weight mL^{-1} of gel solution and 0.125 cm^3 of agar cube volume. The optimum nutrient and mineral concentrations in BG11 supplemented with Turk Island salt solution for H_2 production of agar-immobilized cells were nitrogen-free medium containing 0.5 M NaCl, 30 mM $MgSO_4.7H_2O$, 4 μM Fe^{3+}. The optimum initial pH of medium and incubation temperature for H_2 production by agar-immobilized cells was pH 7.4 and 40 °C, respectively. An increase in H_2 production by agar-immobilized cells was found in larger volumes of glass vial and headspace. Finally, H_2 production by agar-immobilized cells was determined for three consecutive cycles. H_2 production could be maintained at the highest level at the second cycle when half of immobilized cells were replaced with fresh immobilized cells. To enhance H_2 production of *A. halophytica* by use of inhibitors, various kinds and

concentrations of inhibitors were screened for H_2 production under dark and light conditions. It was found that carbonyl cyanide m-chlorophenyl hydrazone (CCCP) at concentration of 0.5 μM was the most effective inhibitor to enhance H_2 production. The treatment with higher concentrations of CCCP and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and long-term incubation resulted in the lower cell concentration and chlorophyll a content. CCCP and DCMU could enhance H_2 production by *A. halophytica* under the light whereas only CCCP could enhance H_2 production under darkness. CCCP reduced O_2 production under light and dark conditions while O_2 production of cells treated with DCMU was decreased under light condition. Under illumination, bidirectional hydrogenase activity of *A. halophytica* was highest in the presence of 0.5 μM CCCP and 50 μM DCMU. Under illumination, CCCP and DCMU inhibited chlorophyll fluorescence, resulting in a low level of O_2, which promoted bidirectional hydrogenase activity in *A. halophytica* cells. In addition, only CCCP enhanced the respiration rate, further reducing the O_2 level. In contrast, DCMU reduced the respiration rate in *A. halophytica*. The maximum H_2 accumulation of *A. halophytica* treated with 0.5 μM CCCP was 254.29 ± 16.33 μmol H_2 g^{-1} dry weight which was approximately 2.6 folds higher than that without CCCP treatment. This study shows the possibility of enhancement of H_2 production by *A. halophytica* by cell immobilization and by use of inhibitor.

Keywords : H_2 production, Cyanobacteria, Cell immobilization, Inhibitor

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Chapter 1

Introduction

1.1 Research Motivation

Energy is important for all living organisms in the world. Energy has been used in many ways such as in a daily life, for transportation and in various types of industries. An increase in urban growth, the world population growth and economic growth has resulted in a higher energy demand. Nowadays, the major energy sources are come from non-renewable resources such as petroleum, coal, natural gas, nuclear fuel, oil shale and oil sands; however, the amount of these energy resources are limited and tend to decrease over a period. In addition, the consumption of these energy sources leads to environmental problems especially air pollution and greenhouse effect, resulting in a global warming and damage to human public health. To solve these problems, alternative energy sources, such as wind energy, biomass energy, water energy and hydrogen, have been increasingly paid attention.

Hydrogen (H_2) is one of interesting alternative energy sources. H_2 is colorless, odorless and tasteless. When H_2 is burned, it provides a high heating value of 141.6 MJ kg^{-1} (Perry, 1963). H_2 gas is composed of two hydrogen atoms. Hydrogen atom is the simplest element and is not found as a free element in nature. Instead, in nature at least, it exists in compounds with other elements. H_2 gas can be used either as the fuel for direct combustion in an internal combustion engine or as the fuel for a fuel cell. H_2 combustion generates water as a main product. Therefore, H_2 combustion not only has the potential to meet a wide variety of end use applications but also does not contribute the environmental pollution. There are several processes for H_2 production generated from fossil fuels such as a catalytic steam reforming of naphtha or natural gas, a gasification of coal, and an electrolysis of water. In addition, H_2 can be produced by various biological processes from different kinds of microorganisms.

Cyanobacteria are one of many microorganisms that are capable of highly efficient H_2 production. They can produce H_2 via a direct and an indirect photolysis (Pinto *et al*., 2002). Cyanobacteria require the simplest nutritional components, just only nitrogen and carbon dioxide available in open air, for their growth. They can use water as a reductant or a source of electrons (Tiwari and Pandey, 2012). By H_2 production via a direct photolysis, cyanobacteria produce H_2 via hydrogenase activity

by accepting electrons from water-splitting in a photosynthesis whereas by H_2 production via an indirect photolysis, cyanobacteria produce H_2 via hydrogenase activity by accepting electrons from a hydrolysis of accumulated glycogen. Their H_2 production mostly occurs under dark and anaerobic conditions. Moreover, some heterocystous N_2-fixing cyanobacteria can produce H_2 as a by-product via the N_2 fixation process using nitrogenase activity. Hydrogenases involved in H_2 metabolism of cyanobacteria are always NiFe-hydrogenases (Przybyla *et al*., 1992; Cammack, 1999; Vignais *et al*., 2001; Hallenbeck, 2012).

The unicellular halotolerant cyanobacterium *Aphanothece halophytica* is one of cyanobacteria that has been previously shown to produce potential H_2. *A. halophytica* can grow in a medium containing high sodium chloride concentration. It mainly produces H_2 via the catabolism of storage glycogen under dark anaerobic condition by bidirectional hydrogenase activity (Taikhao *et al*., 2013; Taikhao *et al*., 2015; Phunpruch *et al*., 2016). But it hardly produces H_2 under illumination because bidirectional hydrogenase is very sensitive to molecular oxygen which is produced from the water-splitting in photosystem II during photosynthesis (Mckinlay and Harwood, 2010).

This work focuses on the enhancement of H_2 production by unicellular halotolerant cyanobacterium *A. halophytica* by methods of cell immobilization and using inhibitors. An advantage of cell immobilization is a protection of enzyme activity and/or cells from an external environment by separating cells from a liquid phase (Bickerstaff, 1995). Moreover, immobilization avoids cells from forming clumps and prevents a breakage of the filaments. In addition, inhibitor can also enhance H_2 production by cyanobacteria due to an inhibition of O_2 evolution from photosynthesis. Some inhibitors can block electron transfer in many metabolic pathways; therefore, electrons are shifted to H_2 production pathway instead.

1.2 Objectives of the study

The objectives of this study are

1) To study the effect of support material type on H_2 production by immobilized unicellular cyanobacterium *A. halophytica*.

2) To investigate the optimization of H_2 production by immobilized cells of unicellular cyanobacterium *A. halophytica* by studying agar concentration, cell concentration, size of immobilized cells, nutrient and mineral concentration, initial pH, temperature and volumetric size of glass vial, headspace and immobilized cells.

3) To study cycle of H_2 production of immobilized unicellular cyanobacterium *A. halophytica.*

4) To screen inhibitors affecting H_2 production by *A. halophytica*.

5) To study the effect of the selected inhibitors on cell concentration, chlorophyll a content, bidirectional hydrogenase activity, photosystem II activity and dark respiration rate of *A. halophytica.*

1.3 Scope of the study

Scope of this study could be divided into two parts. Firstly, cell immobilization of *A. halophytica* for H_2 production was optimized. The effects of several parameters such as support material types and concentrations, cell concentrations, nutritional and mineral concentrations in medium, pH, temperature, sizes of vial, volumes of head space and cell cycling, on H_2 production by immobilized cells of unicellular cyanobacterium will be investigated. Secondly, many kinds of inhibitors (inhibitors for PSII, oxidative phosphorylation, respiration, CO_2 fixation and Krebs' cycle) were screened for H_2 production. In addition, the effect of the effective inhibitor on H_2 metabolism and other involving metabolisms of *A. halophytica* was investigated.

1.4 Benefits of the study

This research has benefits for understanding H_2 metabolism by unicellular cyanobacterium *A. halophytica* from studying the effect of inhibitor and for enhancement of H_2 production by cell immobilization method. A high potential H_2 production by this cyanobacterium is one of the most promising ways in the successful of fuel evolution in future.

Chapter 2

Theory and Literature Reviews

2.1 Hydrogen (H_2)

Nowadays, the demand for energy is rapidly increasing due to the enlargement of the electricity and fuel consumption for transportation, for agriculture, in a household and in industries. It is a result from an increase in human population. However, an existence of energy sources used nowadays, such as natural gas, coal, and crude oil, is limited and predicted to be insufficient for use in the near future. Therefore, much effort has been attempted to search and study alternative and renewable energy sources which are highly efficient fuels and do not release toxic and hazardous substances or gases to the environment when they are burned. One of interesting alternative energy sources is hydrogen gas (H_2).

Hydrogen atom (H) is a chemical element commonly found in the universe. Hydrogen atom is the lightest element with an atomic number as 1. It has a standard atomic weight of 1.00794 and the density of 0.08988 g mol^{-1}. A hydrogen atom consists of a nucleus which is made up of a proton and neutrons with electrons bound to its orbitals, similarly to other elements. There are three isotopes of hydrogen atom; (1) protium consisting of 1 proton and no neutrons with a molecular weight of 1.0078 g mol^{-1}, (2) deuterium consisting of 1 proton and 1 neutron with a molecular weight 2.0141 g mol^{-1} and (3) tritium consisting of 1 proton and 2 neutrons with a molecular weight of 3.0161 g mol^{-1} (Fig. 2.1) (Gurov *et al.,* 2005).

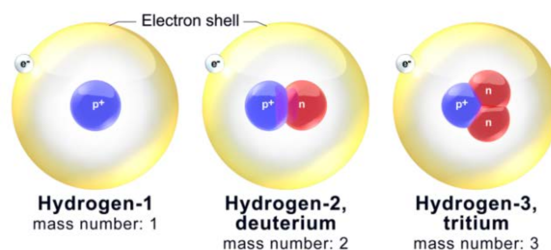


Figure 2.1 Isotopes of hydrogen atom (https://simple.wikipedia.org/wiki/Isotope)

Hydrogen gas (H_2) is one of interesting alternative energy carriers which is composed of two hydrogen atoms. H_2 is colorless, odorless and tasteless. It has a hydrogen bond strength of 1.04 kcal mol^{-1}. When hydrogen is used as a catalyst for the reaction, it needs a high energy to breakdown a hydrogen bond between two hydrogen atoms. H_2 combustion provides a high heating value of 141.6 MJ kg^{-1} (Perry, 1963) that is higher than combustion energy from other hydrocarbon sources (Fig. 2.2). Moreover, H_2 combustion releases only environmental-friendly products and does not generate toxic gases such as carbon dioxide (CO_2) or sulfur dioxide (SO_2), and other non-metallic compounds. The boiling point of H_2 is -252.77 °C and the melting point of H_2 is -259.2 °C (Pant *et al.,* 2009; Subramani *et al.,* 2015).

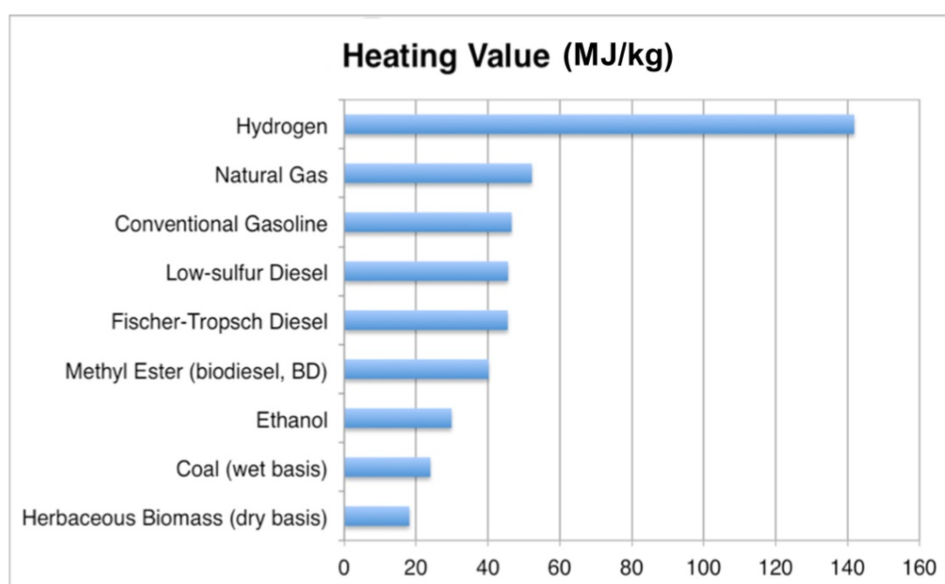


Figure 2.2 Heating value from H_2 combustion compared to other hydrocarbon fuels (https:// energyclub.stanford.edu/solar-fuels-as-versatile-energy-solutions/)

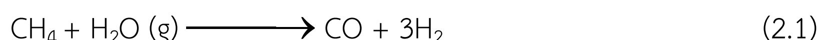
2.2 H_2 production processes

Three main processes have been involving in H_2 production nowadays; thermochemical process, electrochemical process and biological process.

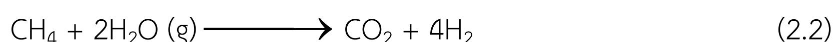
2.2.1 H_2 production by thermochemical process

H_2 production by thermochemical process is the process which uses high heat to turn biomass, natural gas and coal, into mixed gases comprising hydrogen gas (H_2), carbon monoxide (CO), carbon dioxide (CO_2), water (H_2O) and methane (CH_4). H_2 is then separated from the components of these mixed gases via combustion process. The thermochemical process can be divided into two processes; steam

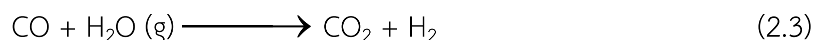
reforming and gasification. Currently, the most common process of H_2 production is steam reforming which produces H_2 as a main product (more than 90 % of all products) (Palmová and Schöngut, 2004). The steam reforming process has two stages. In the first stage, hydrocarbon raw material is fed into steam at high temperature from 500 to 900 °C and high pressure from 0.3 to 2.5 MPa. During its reaction, H_2 and CO are produced along with a lower proportion of CO_2 (Equation 2.1 and 2.2). In the second stage, the cooled gas is led into the converters, where CO is converted by use of steam into CO_2 (Equation 2.3). The nascent carbonic gas is removed by a reversible exothermic reaction (Equation 2.4) (Bicakova and Straka, 2010). The disadvantage of this process is the production of pollutant by-products from toxic residues and non-combustible hydrocarbons.



$$CH_4 + H_2O\,(g) \longrightarrow CO + 3H_2 \qquad (2.1)$$



$$CH_4 + 2H_2O\,(g) \longrightarrow CO_2 + 4H_2 \qquad (2.2)$$



$$CO + H_2O\,(g) \longrightarrow CO_2 + H_2 \qquad (2.3)$$

$$CO_2 + H_2 \longleftrightarrow CO + H_2O \qquad (2.4)$$

2.2.2 H_2 production by electrochemical process

H_2 production by electrochemical process is a process of separating water molecules into O_2 and H_2 using electricity. The reaction takes place in the so-called “electrolyzer” which has been created in a small size and is responsible for the electrolysis reaction. The anode and the cathode separate H_2O into H_2 and O_2. The produced H_2 is attached to the cathode and the produced O_2 is bound to the anode (Fig. 2.3). This process uses electricity up to 90 kW and can produce H_2 up to 1,000 cubic feet with a high purity. There are many types of H_2 production by electrochemical process depending on the type of electrolyzers, for instance, polymer electrolyzer membrane, alkaline electrolyzer, and solid oxide electrolyzer. The advantage of H_2 production by this process is production of H_2 and O_2 without greenhouse gas emission (Turner *et al.,* 2008). The disadvantages of this process are the requirement of a large amount of electricity and the loss of electricity at each stage of water decomposition. This process requires very high temperatures (above 2,500 °C) to separate the water molecules into H_2 and O_2.

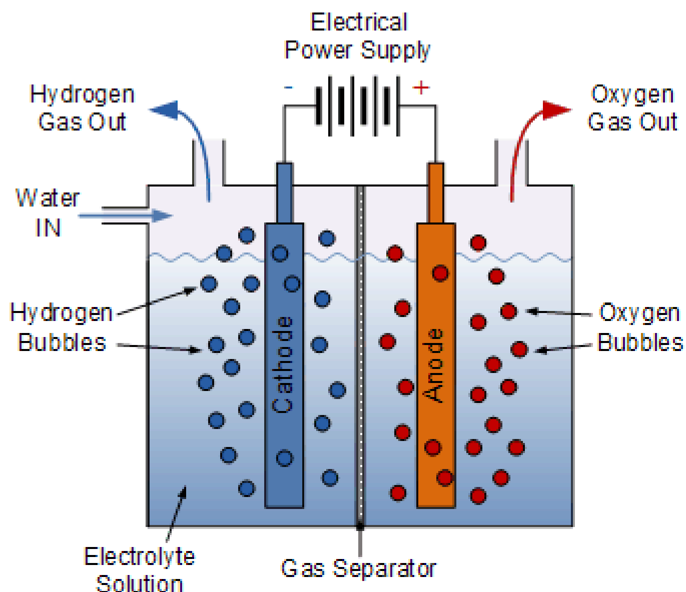


Figure 2.3 H_2 production by electrochemical process
http://www.alternative-energy-tutorials.com/energy-articles/hydrogen-energy.html)

2.2.3 H_2 production by biological process

Biological H_2 production is a process of H_2 production using various typical metabolic pathways by several kinds of organisms. Microorganisms such as fermentative bacteria, photosynthetic bacteria, green algae and cyanobacteria are capable of producing H_2. Photosynthetic bacteria use light energy and organic compounds to produce H_2 via nitrogen fixation by nitrogenase activity under nitrogen-deficient condition (Liu *et al.*, 2006). Fermentative bacteria can convert the fermentative organic substrates into H_2 (Hallenbeck and Benemann, 2002). The reaction of H_2 production does not require O_2 and light energy. Green algae and cyanobacteria can produce H_2 by hydrogenase activity via photosynthesis by using light and water as raw materials. They can fix atmospheric CO_2 and N_2 in the air and use unlimited sunlight energy to produce H_2 via photosynthesis. Most of them can also produce H_2 using electrons obtained from a dark fermentation process. Some N_2-fixing cyanobacteria can produce H_2 as by-product via nitrogen fixation by nitrogenase activity.

2.3 H_2 production of cyanobacteria

Cyanobacteria are unique prokaryotes with diverse range of characteristics. Cyanobacteria are one of organisms that show a potential for H_2 production. H_2

production has been studied in a wide variety of cyanobacterial species and strains (Pinto *et al.,* 2002). Cyanobacteria capable of H_2 production are categorized into three groups; heterocystous, non-heterocystous and marine cyanobacteria (Table 2.1). H_2 production of cyanobacteria can be devided into two main processes as following.

Table 2.1 Type of H_2 producing cyanobacteria (Tiwari and Pandey, 2012)

Heterocystous cyanobacteria	Non-heterocystous cyanobacteria	Marine cyanobacteria
Anabaena flos-aquae	*Synechococcus* sp.	*Oscillatoria brevis*
Anabaena cylindrica	*Microcystis* sp.	*Oscillatoria limosa*
Anabaena variabilis	*Gloebacter* sp.	*Oscillatoria* sp. Miami BG7
Anabaena azollae	*Synechocystis* sp.	*Calothrix scopulorum*
Anabaena sp. PCC 7120	*Aphanocapsa montana*	*Calothrix membranacea*
Nostoc muscorum	*Gloeocapsa alpicola* CALU 743	*Cyanothece* 7822
Nostoc linckia	*Chroococcidiopsis thermalis*	*Anabaena cylindrica* B-629
Nostoc commune	CALU 758	
Anabaenopsis circularis	*Microcystis* PCC 7806	
	Microcoleus chthonoplasts	

2.3.1 H_2 production of cyanobacteria by photosynthesis

Cyanobacteria produce H_2 by catalyzing reaction with hydrogenase enzyme using reduced electron sources form photosynthesis via a direct photolysis pathway and an indirect photolysis pathway. In a direct photolysis, H_2 production of cyanobacteria by photosynthesis occurs when photosystem II (PSII) captures sunlight energy. The energy is transferred to the reaction center of PSII (P680). Electrons from an excited state of the reaction center (P680*) are transferred to reduce the plastoquinone (PQ) pool. Then, water-splitting occurs and generates O_2, protons and electrons. PQ is used to maintain the chemiosmotic potential to generate ATP via the ATP synthase complex. The obtained electrons are transferred via plastoquinone (PQ), cytochrome *b6f* (cyt *b6f*), plastocyanin (PC) and photosystem I (PS I) to reduce ferredoxin (Fd), the central hub of electron flow in photosynthetic organisms. Finally, the reduced Fd donates electrons to Fd-$NAD(P)^+$ oxidoreductase (FNR) to reduce oxidized nicotinamide adenine dinucleotide (phosphate) ($NAD(P)^+$) to reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H). Then NAD(P)H is oxidized by [NiFe]-hydrogenase enzyme to produce H_2 (Fig. 2.4). In addition, the energy obtained by photosynthesis in form of ATP and NAD(P)H are also used in CO_2 fixation to

synthesize and store carbohydrates within the cell. Cyanobacteria accumulate carbohydrates in the form of glycogen. In an indirect photolysis, under darkness and nitrogen deprivation, accumulated glycogen within cells is degraded to glucose and finally generating a large amount of electrons. The obtained electrons are transferred to reduced $NAD(P)^+$ to NAD(P)H and then NAD(P)H is oxidized by [NiFe]-hydrogenase enzyme to produce H_2.

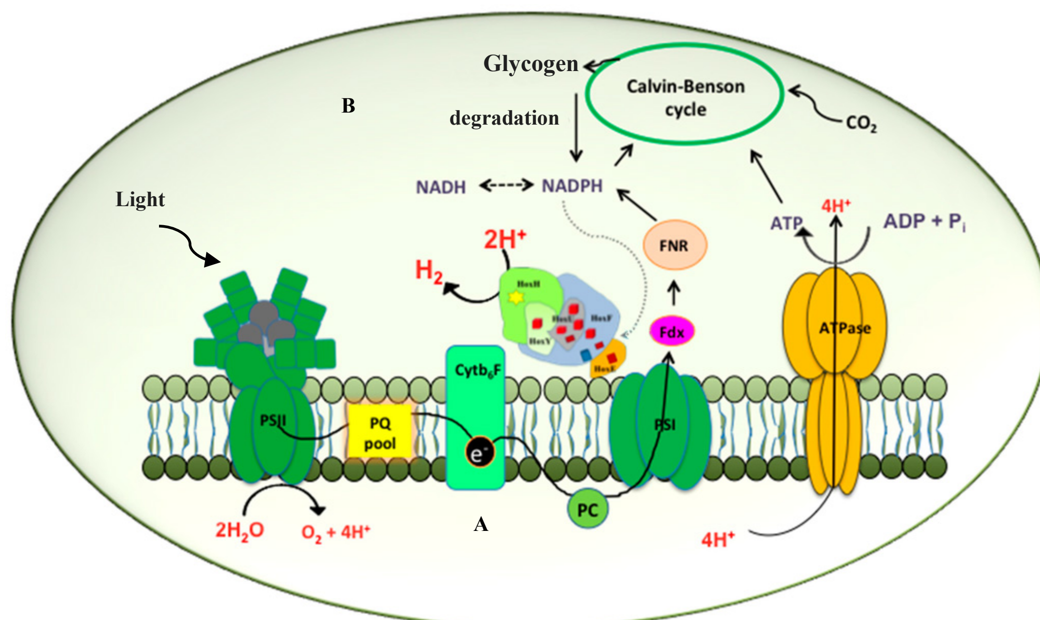


Figure 2.4 H_2 production by cyanobacteria via direct photolysis (A) and indirect photolysis (B) (modified from Khanna and Lindblad, 2015).

2.3.2 H_2 production of cyanobacteria by nitrogen fixation

H_2 production by nitrogen fixation is found in filamentous N_2-fixing cyanobacterial species such as *Nostoc* sp. PCC 73102, *Anabaena variabilis* PK84, and *Nostoc* sp. ARM 411 and *Anabaena* sp. PCC 7120 (Tamagnini *et al*., 1997; Tsygankov *et al*., 1998; Dawar *et al*., 1999; Masukawa *et al*., 2001). H_2 production occurs in special cells called “heterocyst”. These cyanobacteria fix atmospheric N_2 to produce ammonia as a main product and H_2 as a by-product.

This pathway is energetically expensive since two molecules of ATP are required for each electron being transferred. Then, electrons and ATP are fed to nitrogenase to produce H_2 (Fig. 2.5). In addition, H_2 produced by nitrogen fixation process is utilized by uptake hydrogenase. The electrons will return to the electron transport chain via the plastoquinone pool (Appel and Schulz, 1998).

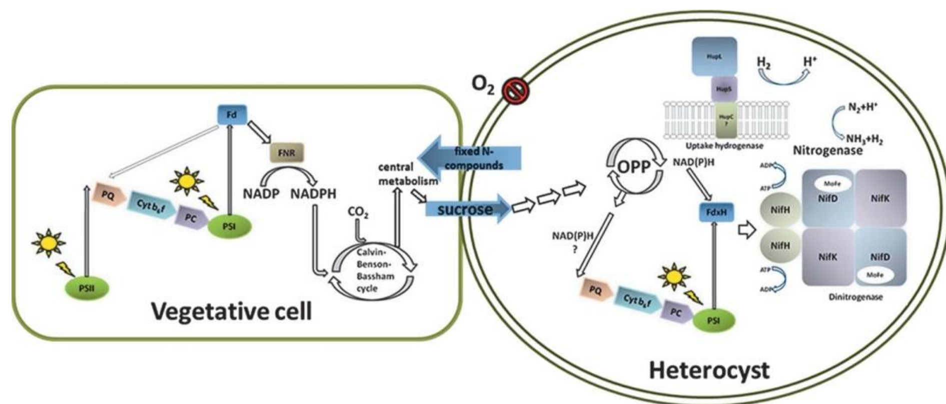


Figure 2.5 H_2 production by N_2-fixing cyanobacteria via nitrogen fixation process (modified from Hallenbeck, 2012)

2.4 Enzymes involving in H_2 metabolism of cyanobacteria

Cyanobacteria contain various enzymes involving in H_2 metabolism. Each enzyme has different and typical characteristics.

2.4.1 Nitrogenase

Nitrogenase is responsible for fixing atmospheric N_2 to generate ammonia and H_2. Nitrogenase is composed of two subunits. The first subunit, dinitrogenese, is an iron containing protein (Fe-protein) with molecular mass of 60-70 kDa. It contains two subunits, α and β, which are encoded by *nifD* and *nifK*, respectively (*nif* from *nitrogen fixation*). These subunits act to fix N_2 and generate H_2 molecules. The second subunit, dinitrogenase reductase, is an iron and molybdenum containing protein (MoFe-protein) with molecular weight of 220-240 kDa. It is encoded by *nifH*. This enzyme acts as the electron acceptor and uses ATP to transfer electrons into the former subunit. Two hydrogen ions can be reduced from two electrons, which is responsible for the reduction of N_2 into diamine (HN=NH). The reduction of diamine (HN=NH) into hydrazine (H_2N-NH_2) will repeat the cycle until the reduced hydrazine turns into 2 molecules of ammonia. The total N_2 fixation of ammonia is based on the use of all eight electrons, six electrons in the reduction process from N_2 to ammonia and two electrons to reduce H_2. The synthesized ammonia is mostly used in the metabolism of nucleic acids (Fig. 2.6).

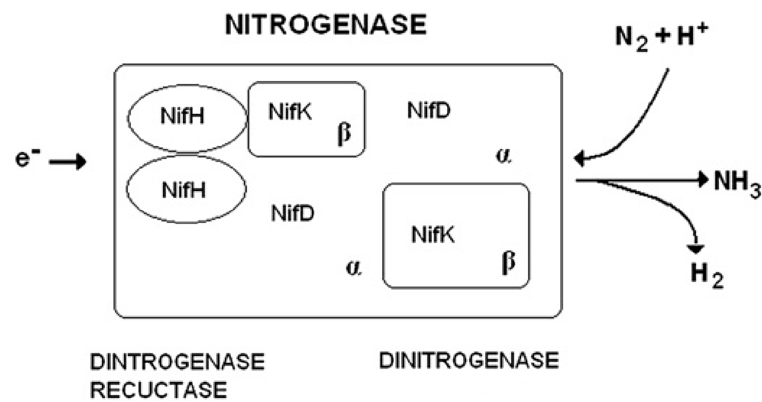
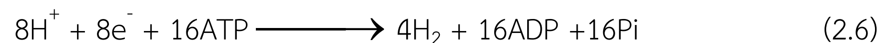


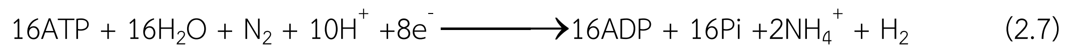
Figure 2.6 Mechanism and composition of nitrogenase enzyme (Tiwari and Pandey, 2012)

This enzyme is found in N_2-fixing organisms, especially in heterocyst cells of cyanobacteria under nitrogen deprivation. In the N_2 fixation process, ATP energy at least 16 molecules is used to reduce N_2 to form ammonia and H_2 (Rao and Hall, 1996) (Equation 2.5, 2.6 and 2.7).

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (2.5)$$



$$8H^+ + 8e^- + 16ATP \longrightarrow 4H_2 + 16ADP + 16Pi \quad (2.6)$$



$$16ATP + 16H_2O + N_2 + 10H^+ + 8e^- \longrightarrow 16ADP + 16Pi + 2NH_4^+ + H_2 \quad (2.7)$$

2.4.2 Hydrogenase

2.4.2.1 Unidirectional or uptake hydrogenese

The unidirectional or uptake hydrogenese is found attached to the thylakoid membrane of heterocyst cells in filamentous cyanobacteria. It consists of two subunits, a large subunit, HupL, encoded by *hupL* (*hup* is from *hydrogen uptake*) which breaks down the H_2 molecules from the N_2-fixing process into protons and electrons, and the small protein subunits, HupS, encoded by *hupS*, which serves to promote the function of HupL protein. H_2 production from nitrogenase enzyme is oxidized immediately by uptake hydrogenase (Fig. 2.7). This reaction is called “the Knallgas reaction”. Therefore, there is no H_2 production from cyanobacteria in the species that have full activity of uptake hydrogenese (Tamagnini *et al*., 2002).

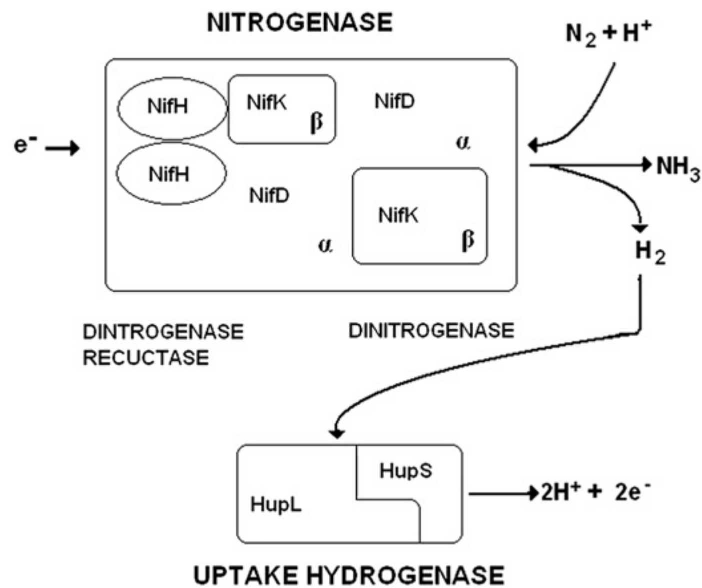


Figure 2.7 Mechanism and composition of nitrogenase and uptake hydrogenase (Tiwari and Pandey 2012)

2.4.2.2 Bidirectional or reversible hydrogenase

Bidirectional or reversible hydrogenase is found in all cyanobacteria including unicellular, non-heterocystous and heterocystous cyanobacteria. Bidirectional hydrogenase produces or consumes H_2 via the reversible reaction (Equation 2.8) (Eisbrenner *et al*., 1978; Tamagnini *et al*., 2002)

$$2H^+ + 2e^- \longleftrightarrow H_2 \quad (2.8)$$

It is a heterotetrameric enzyme consisting of 4 different subunits. The σ and β subunits are combined to form hydrogenase and encoded by *hoxY* and *hoxH*, respectively. The remaining two subunits, α and γ subunits, are combined to form diaphorase and encoded by *hoxF* and *hoxU*, respectively. Both subunits transfer electrons from NAD(P)H to protons (Fig. 2.8). Besides HoxF and HoxU, HoxE encoded by *hoxE* is found in some cyanobacterial strains; for example, *Synechocyctis* sp. PCC 6803 (Appel and Schulz, 1996), *Synechococcus* sp. PCC 6301 (Boison *et al*., 1998), and *Anabaena* sp. PCC 7120 (Tamagnini *et al*., 2002). The bidirectional hydrogenase enzyme is very sensitive to O_2 (Mckinlay and Harwood, 2010).

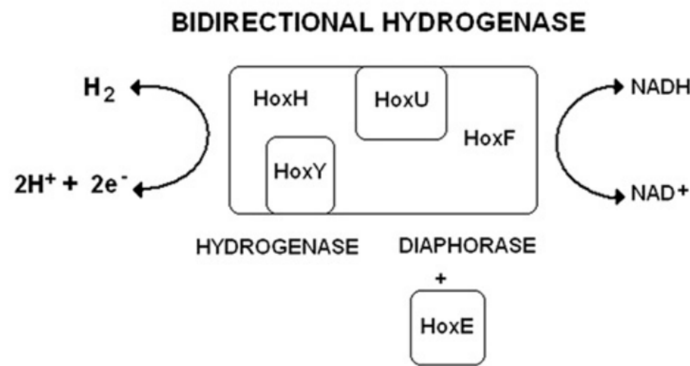


Figure 2.8 Mechanism and composition of bidirectional hydrogenase (Tiwari and Pandey 2012)

2.5 Cyanobacteria

Cyanobacteria are considered as the first living organism on earth. There is an evidence of the discovery of bacterial fossils in rock sediments in the ocean. Geologists expected its age as about 3,500 million years old.

2.5.1 Characteristics of cyanobacteria

Cyanobacteria are categorized as microorganisms in the division of Cyanochloronta. Cyanobacteria are classified in a prokaryotic organism as same as bacteria, but they belong different properties. Cyanobacteria contain a main pigment, chlorophyll a, which allows them to conduct a photosynthesis. They have a simple cell structure without nuclear membrane which allows genetic materials to be distributed within the cell. Normally, they show asexual reproduction. The world in about 3,500 million years ago is extremely hot and has not much O_2. There is only CO_2 and substances or elements residual from the volcanic eruptions such as nitrogen, methane, ammonia, etc. Cyanobacteria are highly adaptive organisms, creating a cellular mucus with air bag for assistance in floating in water to find the optimal conditions for photosynthesis. They also develop pigments that help to resist damages caused by ultraviolet light from the sun. Chlorophyll inside their cells allows them for O_2 production to the Earth. It is assumed that this is the beginning process of creation an environment conducive to the birth of other organisms.

2.5.2 Morphology

Cyanobacteria are separated into two groups; (1) non-filamentous form or unicellular cyanobacteria and (2) filamentous form. The non-filamentous cyanobacteria are spherical in shape (coccoid form) which is found both in single cell

and palmelloid colonies with firm mucilaginous envelopes (Fig. 2.9). In the filamentous cyanobacteria, cells are rearranged into a strand called "trichome". This trichome may be straight and smooth (homocystous form) such as *Oscillatoria* sp. and *Lyngbya* sp. or a chain containing regular cells with heterocysts at the end of trichrome (heterocystous form) such as *Nostoc* sp. and *Anabaena* sp. (Fig. 2.10). Heterocysts belong thick cell walls. The inside of the cell is light yellow. The heterocyst cells which occur between vegetative cells in the filamentous line are called "intercalary heterocysts". The heterocyst cells found at the end of each filament are called "terminal heterocysts", which are divided into 3 types; (1) basal heterocyst found in filamentous cyanobacterium *Calothrix* sp., (2) pedicellate heterocyst formed at the end of short filaments containing 1 to 3 cells and (3) lateral heterocyst formed beside the filaments attaching to the filamentous strand itself. Pedicellate and lateral heterocyst can be found in cyanobacterium *Nostochopsis*.

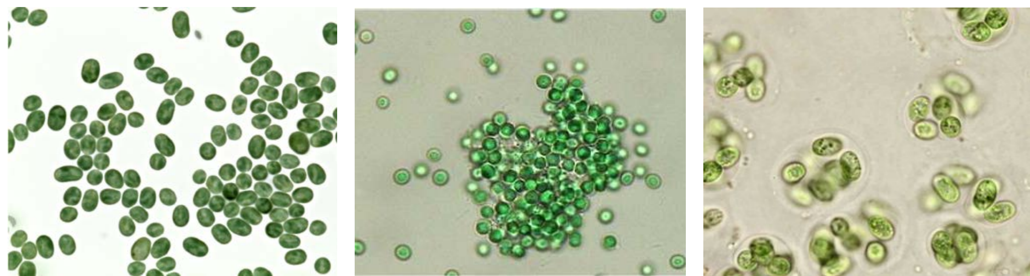


Figure 2.9 Unicellular or non-filamentous form of cyanobacteria
(http://irrigation.rid.go.th/rid14/water/library/shelf/data/page/science/science_6.html)

Figure 2.10 Filamentous form of cyanobacteria
(http://irrigation.rid.go.th/rid14/water/library/shelf/data/page/science/science_6.html)

2.5.3 Reproduction of cyanobacteria

Cyanobacteria have normally asexual reproduction by dividing cells and sporulation.

1) Cell division

Unicellular cyanobacteria divide cells within the same cell wall. Cells obtained from the division are then removed from the cell wall to grow into new cells. Cyanobacteria that form colonies can grow larger and later separate into smaller groups which then grow to new cells. Cyanobacteria with trichomes will separate these trichomes into smaller units of 2-3 cells called "hormogonium" and grows into larger strands. The hormogonium shows more movement compared with the original trichrome. The separation from the point of dead cell is called "the separation disk or necridia". The filamentous cyanobacteria also produce connected heterocyst and thick-walled cells, similar to spores, called "akinete" containing cyanophycin granules. The cells will proliferate when there is a new akinete production. Akinete can withstand harsh environments such as drought or high temperature for a certain period of time until the environment is suitable for the new akinete to proliferate.

2) Sporulation

There are two types of spore produced by cyanobacteria, endospore and exospore. Spore has no flagellum whcih helps cell movement. The endospore is formed inside the cell from protoplast division. Each part can develop into spores after falling off from the cellular wall and grow into a new thallus. The exospore is formed from the separation of the ends.

2.5.4 Cyanobacterium *Aphanothece halophytica*

The unicellular cyanobacterium *Aphanothece halophytica* can be found in high salinity environments. *A. halophytica* cells are round or cylindrical in shape with a size from 2-10 μm (Fig. 2.11). The varying in size and shape of the cells allows it to be more tolerant to salt concentration (Berland *et al.,* 1989). *A. halophytica* accumulates an osmoprotectant glycine betaine at high salinity (Ishitani *et al*., 1993). Therefore, it can grow in a wide range of salinity from 0.25 to 3.0 M NaCl and in extreme alkaline conditions up to an external pH of 11.0 (Hibino *et al*., 1999; Waditee *et al*., 2003). *A. halophytica* has been shown as cyanobacterial strain that has potential to produce H_2 (Taikhao *et al.,* 2013; Taikhao *et al.,* 2015). It mainly produces H_2 via a catabolism of storage glycogen in dark anaerobic condition by

bidirectional hydrogenase activity and produces less H_2 via photosynthesis (Taikhao *et al.,* 2015; Phunpruch *et al.,* 2016) In addition, *A. halophytica* can cultivate in natural seawater supplemented with 1.76 mM $NaNO_3$, and can provide long term H_2 production in natural seawater (Taikhao *et al.,* 2015).

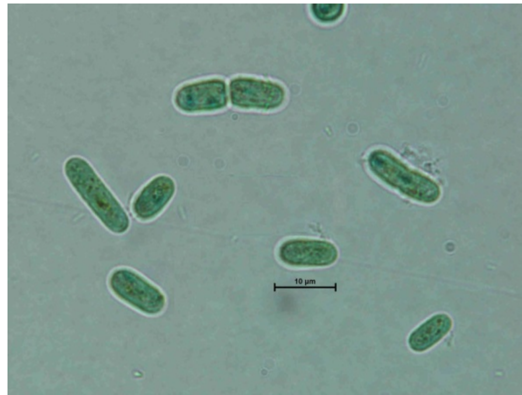


Figure 2.11 Cell morphology of *A. halophytica* under microscope

2.6 Cell immobilization

Cell immobilization systems have been applied for many biochemical processes for a long time (Durand and Navarro, 1978; Klein and Wagner, 1978). Immobilized cells are defined as cells that by natural or artificial means are prevented from moving independently of their neighbors to all parts of the aqueous phase of the system (Tampion and Tampion, 1987).

Immobilization methods can be divided into five methods based on the immobilization principles as followed;

2.6.1 Immobilization by absorption method

Immobilization by physical adsorption is the most commonly used technique in immobilization. This method is mild and easy to perform. Adsorption usually requires no special adjustment of the support. It is performed directly in the container by introducing the inoculum and the support to the medium. Cells become immobilized simply. Because of the low cost of the support and ease of preparation, adsorption onto porous and/or fibrous materials is very useful in several applications. The supports used include celite, sand, porous brick, glass beads, clay, ceramics, wood chips, ion exchange resins, plastic materials, organic materials such as cellulosic materials and activated carbon (Zhu, 2007). Adsorption is a process in which the cells are attached to the surface of organic or inorganic support matrix. (Messing, 1976; Woodward, 1985). The forces involving in this method are electrostatic forces, ionic

bond and hydrogen bonding interactions. These forces are weak but strong enough for bonding cell and surface of support material (Fig. 2.12). In the procedure of mixing cells and supports, pH, ionic strength, a period of incubation, the collection of the immobilized material and the removal of non-bound biological components by washing should be considered. The advantages of this method are (1) little or no damage to cells, (2) cheap, (3) quick to obtain immobilization, (4) no chemical changes to support or cells and (5) reversible to allow regeneration with fresh cells. The disadvantages of this method include (1) leakage of cells from the support, (2) contamination to product and (3) nonspecific binding. Desorption can occur under many conditions. Environmental changes in pH, temperature, and ionic strength will also promote desorption (Bickerstaff, 1997). Different chemical and physical characteristics of the support surface, including chemical composition, hydrophobicity and its morphology, and cell characteristics such as size, hydrophobicity and surface charge can significantly affect cell adsorption (Merritt and An, 2000).

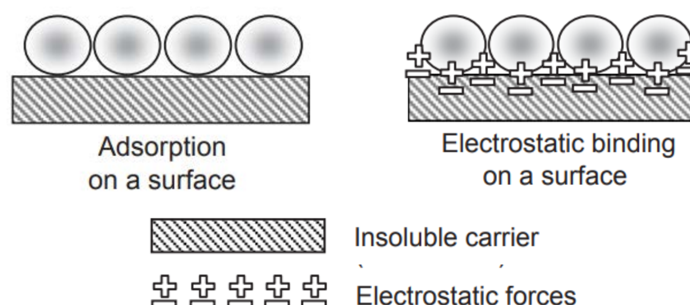


Figure 2.12 Immobilization by adsorption method (Kourkoutas *et al*., 2004)

2.6.2 Immobilization by covalent binding

Covalent binding immobilization depends on covalent bond formation between a support material and functional groups of amino acid residues on the surface of enzyme or cells in the presence of a binding agent (Fig. 2.13). Several amino acid functional groups are suitable for participation in covalent bond formation. Those functional groups most frequently found are the amino group (NH_2) of lysine or arginine, the carboxyl group (COOH) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine, and the sulfhydryl group (SH) of cysteine (Srere and Uyeda, 1976). Chemical modification of the support surface is necessary for the specific binding. Some coupling reagents, α-amino propyl triethoxy silane, isocyanate, hydroxyethyl acrylate, and sodium periodate, can be used to activate the

support for covalent bonding. Other agent, such as cyanuric chloride, can also couple yeasts and bacteria on a cellulose support by a double bond with the hydroxyl groups of the cells and the cellulose (Zhu, 2007). Compared to adsorption method, much stronger cell-support binding is formed, which reduces cell loss due to cell removal from the support. There are the formation of bonding of the support material to surface of the cell such as isourea linkage, diaso linkage, peptide linkage, and alkylation reaction (Bickerstaff, 1997). The disadvantages of this method for cell immobilization are the risk of cell damage and the loss of activity because the cell membrane is involved in covalent bonding to the support (Groboillot *et al*., 1994).

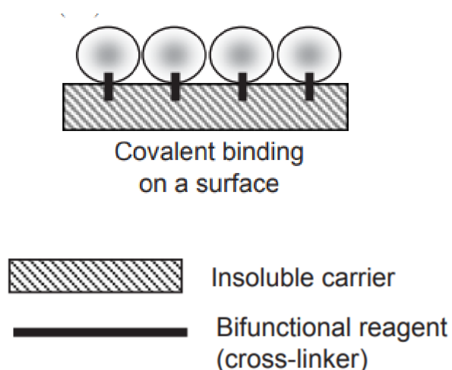


Figure 2.13 Immobilization by covalent binding method (Kourkoutas *et al*., 2004)

2.6.3 Immobilization by entrapment

Entrapment is the most extensively studied method in cell immobilization. It is based on the enclosure of cells within a rigid network. Immobilization by entrapment differs from adsorption and covalent binding in that cells are free in solution, but restricted in movement by the lattice structure of a gel (Fig. 2.14) (O'driscoll, 1976; Bickerstaff, 1995). The porosity of the gel lattice is tight enough to prevent the release of the cells while it still allows the diffusion of substrates and products. Entrapment method has been widely applied in biotransformation and fermentation for production of antibiotics, organic acids, enzymes, and alcohols (Ramakrishna and Prakasham, 1999). There are some drawbacks of cellular entrapment due to that entrapment systems reduce the mass transfer kinetics of the uptake of metal ions (Aksu *et al.,* 2002). In addition, entrapment limits O_2 and/or CO_2 transfer from the liquid environment through the immobilization matrix, which would cause difficulties for immobilization of aerobic microorganisms (Toda and Sato, 1985).

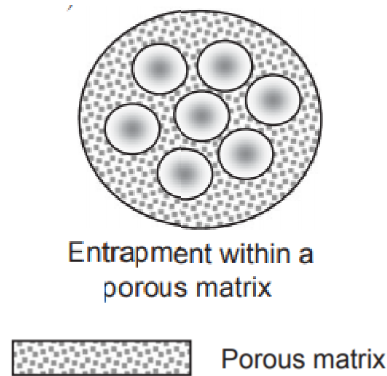


Figure 2.14 Immobilization by entrapment method (Kourkoutas *et al*., 2004)

2.6.4 Immobilization by encapsulation

Encapsulation of cells can be achieved by enveloping cells within various forms of semipermeable membranes (Fig. 2.15) (Groboillot *et al.,* 1994). In this method, there is no covalent bonds associatiated between the network and the cells. This method is similar to the immobilization by entrapment that cells are free in solution but restricted in space. Large cells or enzymes cannot penetrate into and out of the capsule but small substrates and products can pass freely across the semipermeable membrane. Many support materials from 10-100 μM in diameter, such as nylon and cellulose nitrate, have been used to construct microcapsules. The problems of encapsulation method are acute diffusion of substrates and products that can break the membrane if products from a reaction accumulate rapidly. Besides, combination between cells and support materials may interrupt substrates and products passing across the membrane (Bickerstaff, 1997).

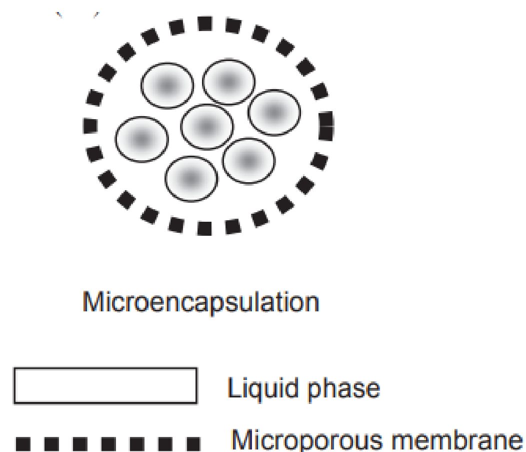


Figure 2.15 Immobilization by encapsulation method (Kourkoutas *et al*., 2004).

2.6.5 Immobilization by crosslinking

Immobilization by crosslinking is a support-free method and involves joining the cells or the enzymes to each other to form three-dimensional complex structure by chemical or physical methods (Fig. 2.16) (Broun, 1976). Chemical method of crosslinking involves interaction of covalent bond between the cells by using reagents, such as glutaraldehyde and toluene diisocyanate. However, the toxicity of such reagents is limitation for immobilization cells and enzymes. Physical crosslinking of cells by flocculation is used in the biotechnological industry and lead to high cell densities. Flocculating agents, such as polyamines, polyethyleneimine, polystyrene sulfonates, and various phosphates, have been extensively used and are well characterized. Crosslinking is rarely used for immobilization due to the absence of mechanical properties and poor stability.

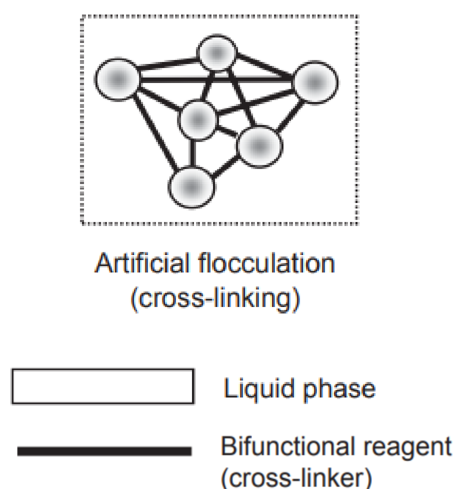


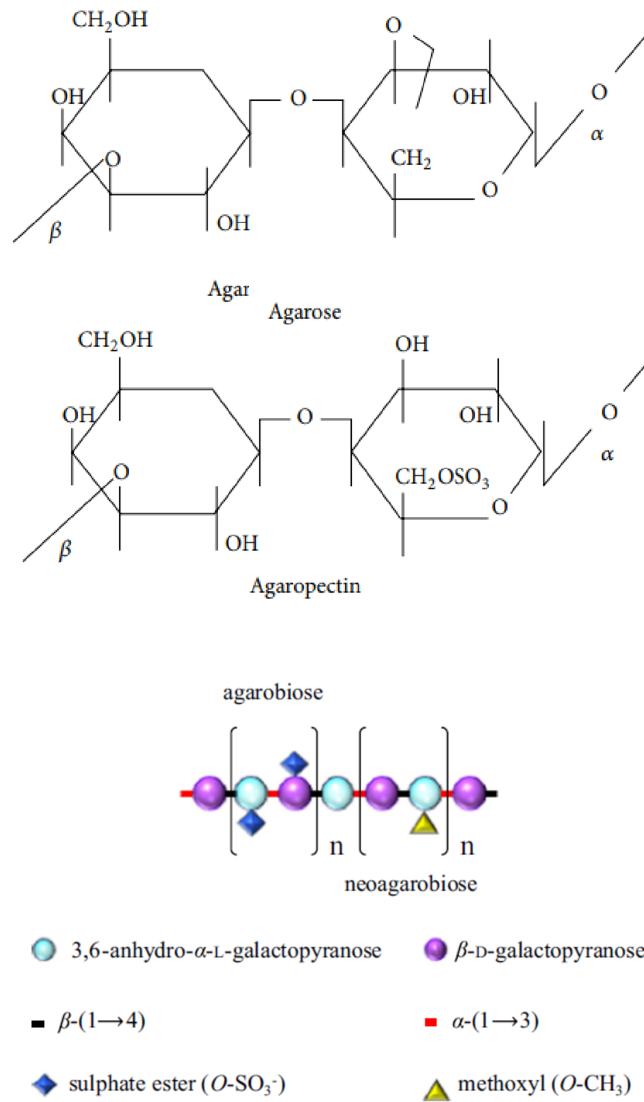
Figure 2.16 Immobilization by crosslinking method (Kourkoutas *et al*., 2004)

2.7 Support materials in immobilization by entrapment method

Entrapment method of immobilization is mostly used in cell immobilization procedures because of the simplicity and excellent cell containment. Cell entrapment can be achieved through *in situ* immobilization in the presence of the porous matrix. There are many natural gel polymers used in entrapment, such as agar, alginate, and carrageenan. In addition, there are synthetic polymeric networks formed from monomeric precursors, such as acrylamide, acrylonitrile, urethane, vinyl alcohol, and hydroxyethyl methacrylate (HEMA). Immobilization by using natural polysaccharide is a widely used technique for microorganisms, especially algae and cyanobacteria.

2.7.1 Agar and agarose

Agar and agarose are sulphated galactans obtained from some species of red algae (mainly from genus *Gelidium*, *Pterocladia* and *Gracilaria*) (Burdin and Bird, 1994). Agar is a thermo-reversible gel. It consists of two major polysaccharides, 70% agarose and 30% agaropectin (Fig. 2.17A) (Araki and Arai, 1967). Agarose is a linear polysaccharide composed of repeating agarobiose units consisting of 1,3-linked β-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactopyranose (Fig. 2.17A). Agarose exhibits high gelling capacity. Agaropectin is a more complex structure than agarose, containing in addition to D-galactopyranose and 3,6-anhydro-α-L-galactopyranose units by D-gluconic acid, pyruvic acid, and a much higher proportion of sulphate ester groups that exhibits low gelling capacity (Varshosaz *et al.,* 2015). Backbone structure of agarose is repeating disaccharide units are called agarobiose and neoagarobiose (Fig. 2.17B) (Duckworth and Yaphe, 1971). The ratio of agarose to agaropectin varies depending on the seaweed species and isolation conditions. Agar is insoluble in cold water and for dissolution it requires heating at temperatures of above 85 °C. The viscosity of agar solutions at 45 °C is not affected by ionic strength or pH range between 4.5-9.0 (Stanley *et al.,* 2006). The gelation of agar commences with a heating step (85-95 °C) where chains adopt random coil conformation. Cooling the system to gelling temperature (33-45 °C) results in the sol-gel transition that proceeds in two steps (Armisén and Gaiatas, 2009; Normand *et al.,* 2000). Initially, randomly distributed, single agarose coils join to form a double helical association via intra-molecular H-bonding. This is followed by aggregation of double helices via inter-molecular H-bonding into a three-dimensional gelled network (Fig. 2.18).



(B)

Figure 2.17 Agar structure including agarose and agaropectin and backbone structure of agar (Varshosaz *et al.,* 2015; Alba and Kontogiorgos, 2015)

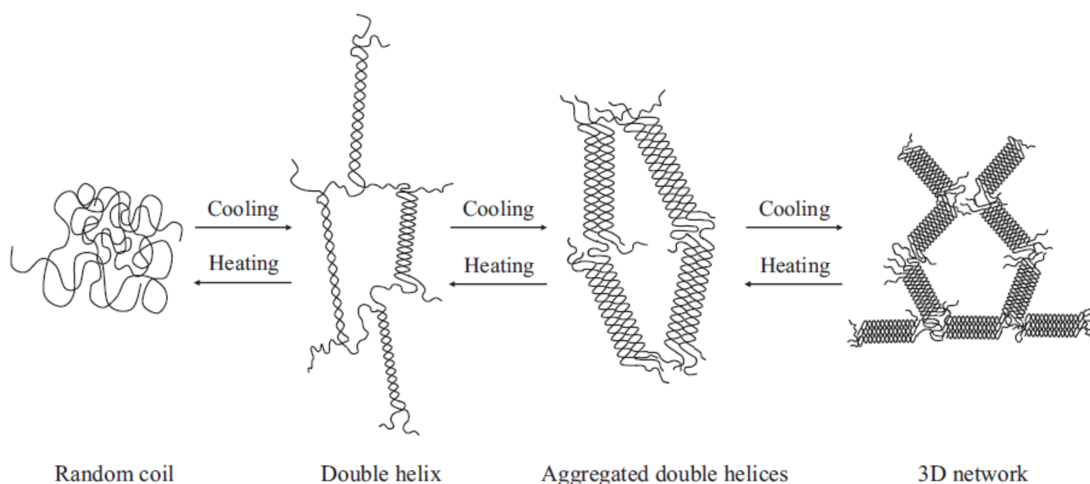


Figure 2.18 Gelation mechanism of agar (Alba and Kontogiorgos, 2015)

2.7.2 Carrageenan

Carrageenan is one of the most widely used materials for cell immobilization by gel entrapment because it is cheap, simple, and reproducible with mild conditions. Carrageenan is a polysaccharide extracted from some Rhodophyceae (red algae) in Families of Gigartinacieae and Solieriaceae. Carrageenan is a linear, sulphated galactan that is composed of alternating 3-linked β-D galactopyranose and 4-linked α-D-galactopyranose or 4-linked 3,6-anhydro-α-D-galactopyranose, thus forming their disaccharide repeating unit (Knutsen *et al.,* 1994). There are three types of carrageenan, kappa (κ), lambda (λ), and iota (ι) (Fig. 2.19). Type of carrageenan is related to number and position of sulfate ester substituents on the sugars and the extent to which the 1,4-linked residues exist as the 3,6-anhydro derivatives (Imeson, 2000). The carrageenan concentration depends on the type of carrageenan and at least must be sufficient to produce a firm gel. All carrageenan types are soluble in hot water at temperatures above its gel melting temperature. Carrageenan dispersions are heated to 80 °C and chains attain random coil conformation. On cooling to about 40 to 60 °C, they undergo coil-to-double helix conformational transition. The final sol–gel transition occurs in the presence of cations such as K^+, Na^+ and Ca^{2+} that leads to the helix–helix aggregation of the adjacent chains containing sulphate groups. Gels are thermo-reversible and melt on heating (Fig. 2.20). Carrageenan can be used to immobilize cells by forming gels in various shapes such as cubes, beads, or membranes.

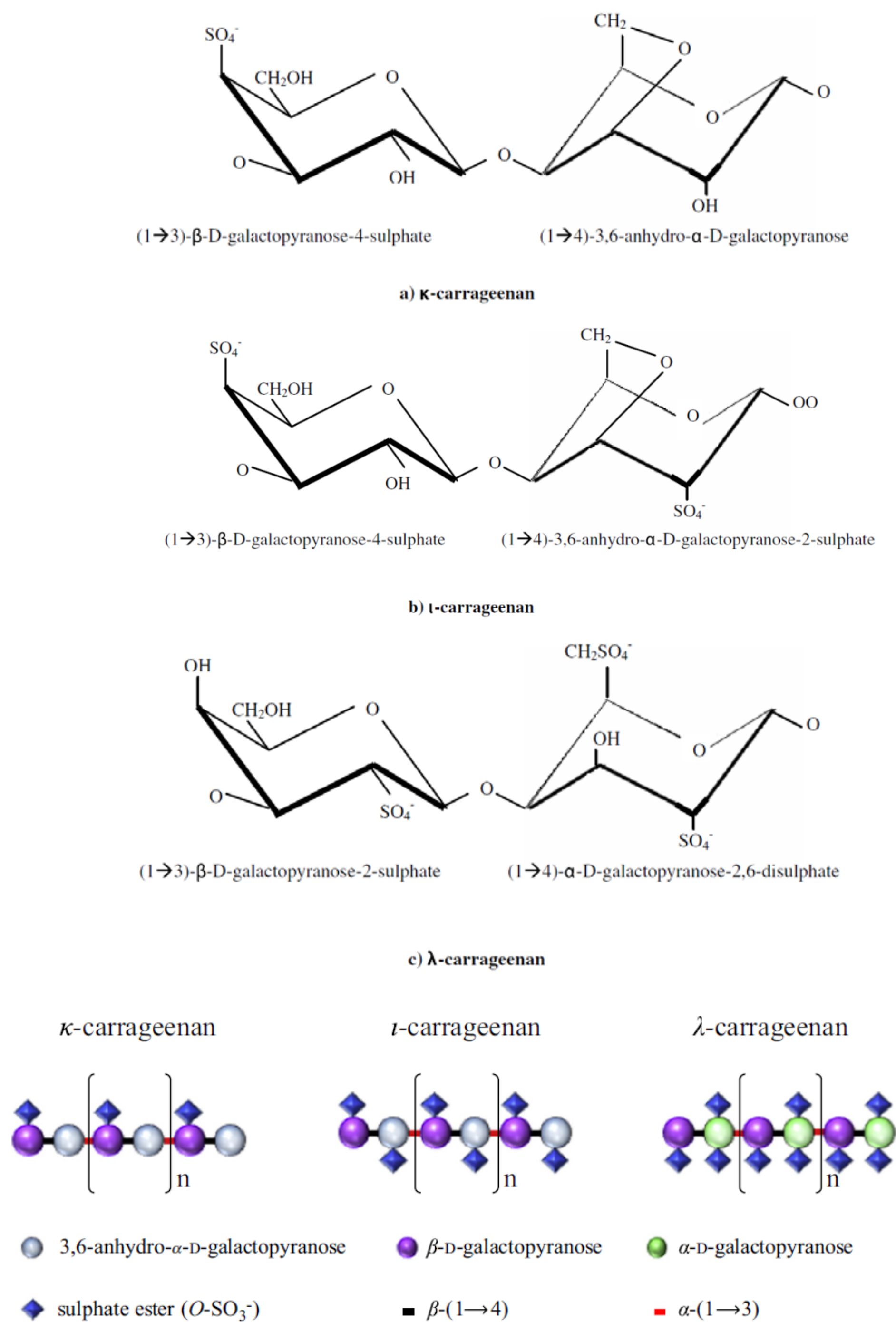


Figure 2.19 Carrageenan structural types (Burey *et al.,* 2008; Alba and Kontogiorgos, 2015)

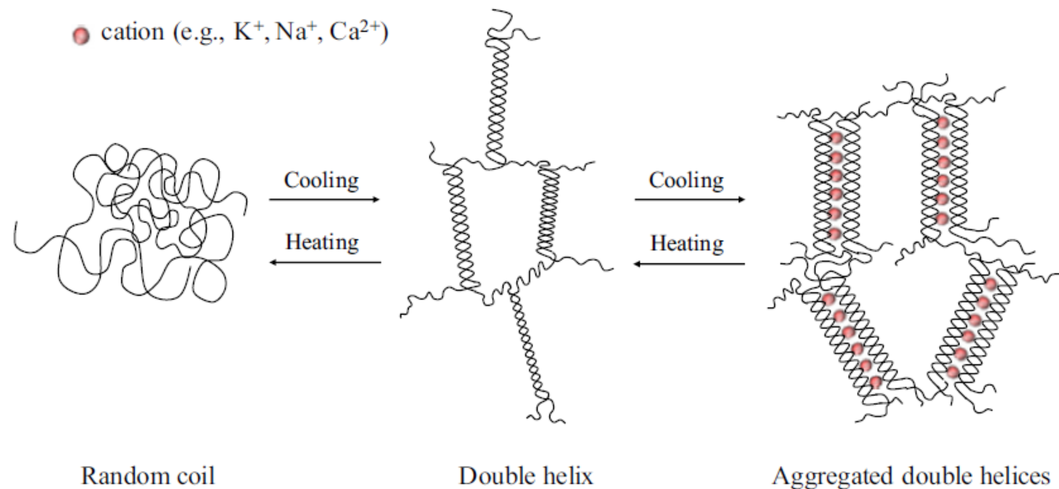


Figure 2.20 Gelation mechanism of carrageenan (Alba and Kontogiorgos, 2015)

2.7.3 Alginate

Entrapment of cells in alginate is one of the simplest methods for immobilization. Alginate is extracted from brown algae, mainly from different species of the genus *Laminaria*, and other species such as *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Lesonia negrescens* or species in the genus *Sargassum*. All brown algae contain alginate in different proportions reaching up to 40% of dry weight (Ertesvåg and Valla, 1998). Alginate is an anionic polysaccharide composed of α-(1,4)-linked-L-guluronic (G) and β-(1,4)-linked-D-mannuronic acid residues (M). Alginate backbone consists of sequences of mannuronic acid block (M-block) or guluronic acid block (G-block), and regions of alternating sequences (e.g., MG, MMG, GGM) (Fig. 2.21) (Grasdalen *et al.,* 1979). The gel formation occurring at room temperature causes an electrostatic interaction between the carboxylic groups on the guluronic acid residues and the divalent ions (Ca^{2+} and Ba^{2+}). G-blocks integrate divalent cations into pocket-like structures formed between adjacent chains of guluronate residues leading to formation of a network. This is known as the "egg box" model (Fig. 2.22). The polymerization process of alginate solution between 3-6% (w/v) is stable (Moreno, 2007). Alginate is also cheap and can be produced from a renewable resource in a large scale. However, the mechanical stability of alginate polymers still needs improvement, and different approaches have been utilized to overcome this problem.

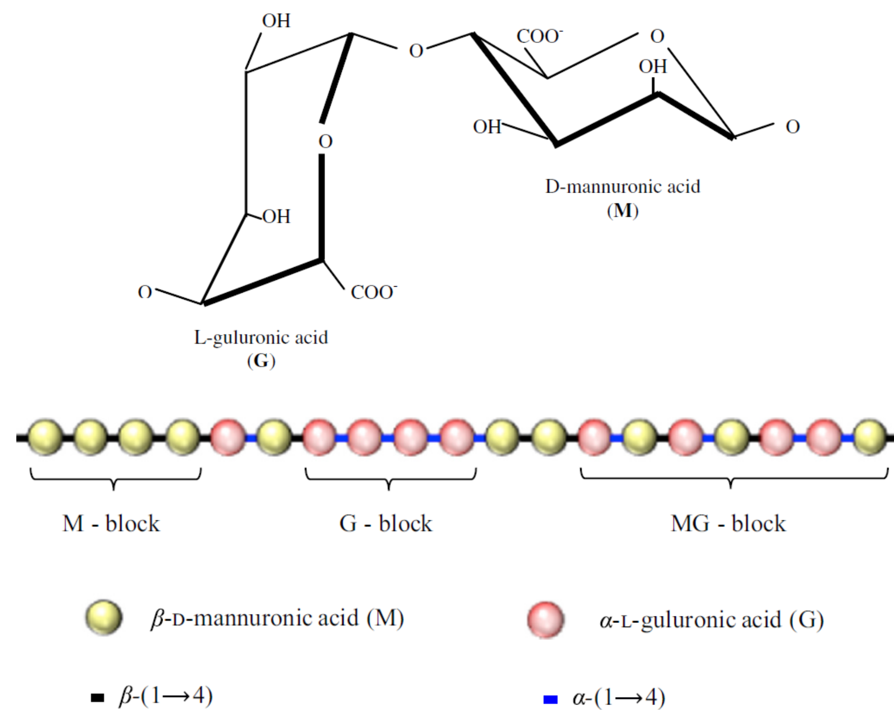


Figure 2.21 Structure of alginate (Burey *et al.,* 2008; Alba and Kontogiorgos, 2015)

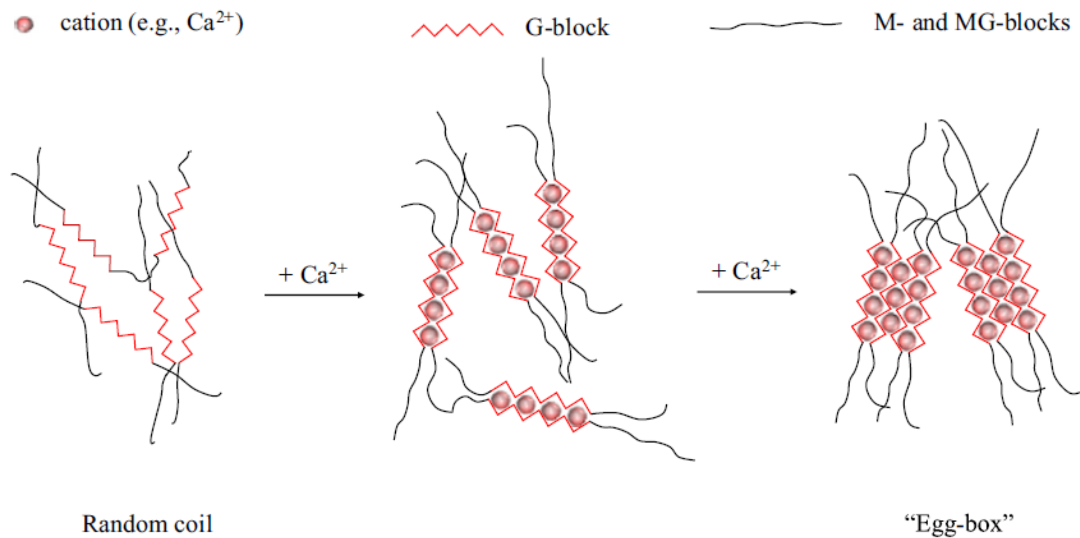


Figure 2.22 Gelation mechanism of alginate (Alba and Kontogiorgos, 2015)

2.8 Applications of immobilized cyanobacterial cells

The use of immobilized cells of oxygenic phototrophic microorganisms, including cyanobacteria and eukaryotic microalgae, in biotechnology shows an

upward trend during recent years. The main advantages of immobilized cells compared with free cells are facilitation of biomass harvesting and increase of cell tolerance to unfavorable factors (e.g. extreme temperatures, acidity, toxicants). Currently, immobilized cells of cyanobacteria are employed in various applications as following.

2.8.1 Removal of heavy metals

The problem of heavy metal pollution is becoming increasingly important nowadays. Cyanobacteria can accumulate many heavy metals at high concentrations; therefore, they are widely used for removal of metals from wastewater. Metal ion adsorption on the surface of cells accounts for heavy metals to cell wall and/or cytoplasmic membrane, surface structures and extracellular compounds. Both natural (carrageenan, alginate, chitosan, agarose) and synthetic (polyacrylamide, polypropylene, polysulfone, various copolymers) carriers are widely used for immobilization for bioremoval of heavy metals. The biomass of cyanobacterium *Phormidium laminosum* immobilized in polysulfone and epoxy resin beads was used for Cu, Fe, Ni and Zn sequestering (Blanco *et al.,* 1999). It was found that the number of biosorbed metal increased with the amount of metal and biomass. The effective adsorption of heavy metals was maintained during 10 cycles of biosorption-acid mediated desorption. The chromium removal of 82% was achieved using immobilized cyanobacterium *Lyngbya putealis* in alginate gel (Anjana *et al.,* 2007).

2.8.2 Nutrient biocapture from wastewater

Biological treatment with microalgae is the most promising biotechnological method for wastewater remediation. Some strains of cyanobacteria can cultivate in wastewater enriched with nutrients. They can remove nitrogen and phosphorus from wastewater and generate biomass. The cyanobacterium *Phormidium laminosum* immobilized with polyurethane or polyvinyl successfully could remove nitrate from the medium (Garbisu *et al.,* 1991). The entrapped cyanobacteria *Anabaena* CH3 by using calcium alginate could remove nitrogenous compounds from wastewater (Lee *et al.,* 1995). Removal of nitrate and phosphate ions was also found in the thermophilic cyanobacterium *Phormidium laminosum* immobilized on hollow cellulose fibers and incubated in a tubular photobioreactor at 43 °C (Sawayama *et al*., 1998).

2.8.3 Biohydrogen Production

Several cyanobacteria are capable of generating H_2 through hydrogenase enzyme whereas N_2-fixing cyanobacteria are able to produce H_2 mainly via nitrogenase enzyme. However, hydrogenase and nitrogenase are sensitive to O_2 which is generated by water splitting process of photosystem II (PSII). H_2 production by cyanobacteria.

immobilized with various kinds of support material have been reported in many strains (Table 2.2).

Table 2.2 H_2 production by immobilized cyanobacteria

Cyanobacteria	Support materials	References
Anabaena azollae	Alginate/polyurethane foam	Rao and Hall, 1984
Anabaena cylindrica	Polyurethane foam	Jeanfils and Loudeche, 1986
Anabaena cylindrica CCAP 1403/2a	Alginate	Muallem *et al.,* 1983
Anabaena cylindrica UTEX B629	Polyurethane foam	Lambert *et al.,* 1979
Anabaena sp. N-7363	Glass beads	Kayano *et al.,* 1981
Anabaena variabilis	Agar	Markov *et al.,* 1995
Chlorogloea fritschii	Fiber glass matrix	Muallem *et al.,* 1983
Gleocapsa olpicola	Polyurethane foam	Antal and Lindblad, 2005
Mastigocladus laminosus	Agar	Rao and Hall, 1984
Mastigocladus laminosus	Alginate/agar	Muallem *et al.,* 1983
Nostoc muscorum	Polyurethane foam	Rao and Hall, 1984
Nostoc muscorum	Agar	Rao and Hall, 1984
Oscillatoria lemnitica	Polyurethane foam	Muallem *et al.,* 1983
Phormidium laminosum	Polyurethane foam	Muallem *et al.,* 1983
Synechocystis sp. PCC 6803	Polyurethane/alginate	Antal and Lindblad, 2005

It has been reported that H_2 production of cyanobacterium *Anabaena* N-7363 immobilized in 2% carrageenan gel was 2.4 times higher than that by free cells (Kayano *et al.,* 1981). Immobilized cells of filamentous cyanobacterium *Calothrix* 336/3 with sodium alginate showed the improvement of H_2 production and H_2 was prolong produced over several cycles (Leino *et al.,* 2009). The cyanobacterium *Microcystis aeruginosa* entrapped with agar allowed the maintenance of stable H_2 production (Rashid *et al.,* 2009). H_2 production by filamentous cyanobacterium *Lyngbya perelegans* immobilized with agar and alginate was higher than that of free cells (Anjana and Kaushik, 2014).

2.9 Inhibitors

The electrons from photosynthesis in cyanobacteria are transferred to many pathways in metabolic system, such as CO_2 fixation, photosynthesis, respiratory electron transport chain (Eroglu and Melis, 2011; Srirangan *et al.,* 2011). Thus, electrons are not entirely shuttled to H_2 metabolism. A use of inhibitors is one of methods to enhance H_2 production. Inhibitors should hinder electron transfer to other pathways. As a result, electrons are directed towards to bidirectional hydrogenase and finally enhancing H_2 production. There are various types of inhibitor involving in H_2 metabolism of cyanobacteria, such as photosystem II inhibitor, respiratory inhibitor, uncoupling agent of oxidative phosphorylation inhibitor, CO_2 fixation inhibitor and Krebs cycle inhibitor.

2.9.1 Photosystem II Inhibitors

There are many kinds of chemical compounds functioning as photosystem II inhibitors. In this study, four compounds are used as following.

2.9.1.1 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (Atrazine)

Atrazine binds to the D1 protein by hydrogen bonds, van der Waals forces and hydrophobic interactions (Michel *et al.,* 1986; Trebst, 1987) and prevents the binding of plastoquinone, disallowing the electron transport chain. Therefore, photosystem activity and water splitting are failed (Fig. 2.23). However, in case of cyanobacteria, the effect of atrazine depends on concentration of atrazine, duration time of incubation and species of cyanobacteria.

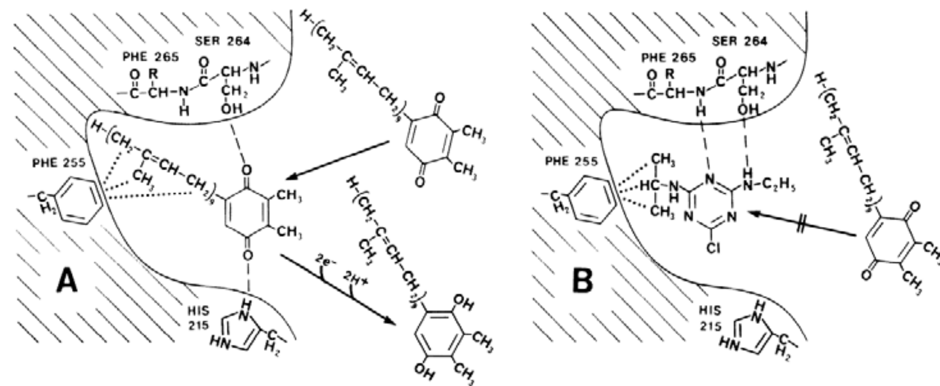


Figure 2.23 Schematic figure of binding of plastoquinone to the D1 protein. Plastoquinone binds to D1 protein, accepts two electrons and two protons, and releasing as plastohydroquinone (A). Atrazine binds to the D1 protein and prevents the binding of plastoquinone (B) (Fuerst and Norman, 1991)

2.9.1.2 Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)

In cyanobacteria and green algae, CCCP is known as a photosystem II inhibitor. The structure of CCCP is shown in Fig. 2.24A. It can accelerate the deactivation reaction of water-splitting enzyme system Y (ADRY agent) in photosystem II (Fig. 2.24B) (Samuilov and Barsky, 1993). The enzyme comprising manganese molecules can activate the reaction of important water splitting into O_2, protons and electrons. CCCP binds to the active site of enzyme. As a result, the enzyme becomes an inactive compound and the procedure of water splitting is failed (Renger, 1970

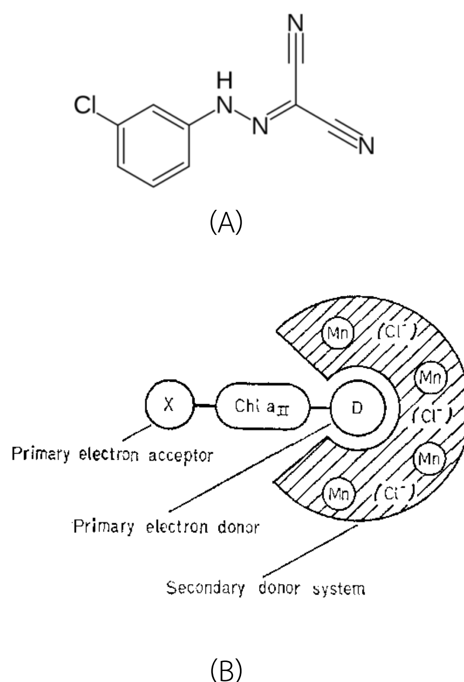
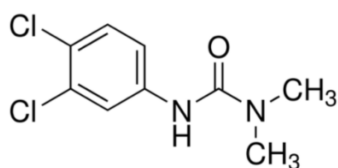


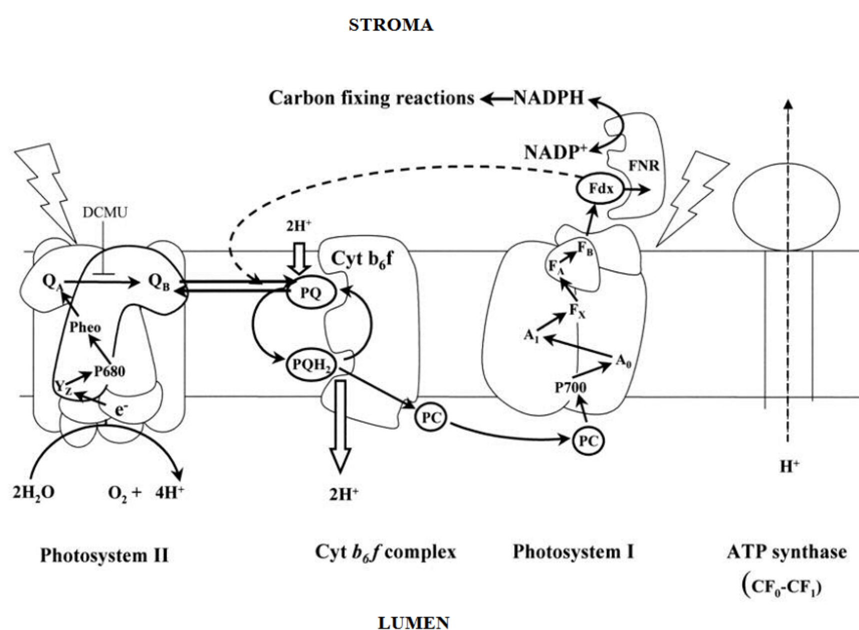
Figure 2.24 The structure of CCCP (A) and water splitting enzyme system Y consisting of manganese molecules (B) (Renger, 1970)

2.9.1.3 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU)

Photosystem II can be stimulated by photons causing the unstable of the reaction center molecule (P680*). Thus, electrons are transferred to the second electron receptor, called pheophytin quinone A (Q_A) and quinone B (Q_B) prior to plastoquinone. Then, photosystem II obtains electrons from water splitting. However, the inhibitor DCMU can block electron transport form Q_A to Q_B at protein D1 in photosystem II. As a result, electron transfer is interrupted to the electron transport chain and water splitting is also inhibited (Fig. 2.25) (Dean, 2014).



(A)



(B)

Figure 2.25 DCMU structure (A) and the schematic diagram of incapable electron transfer process to plastoqinone of DCMU in photosystem II (B) (Dean, 2014)

2.9.1.4 N-(phosphonomethyl)-glycine (glyphosate)

Glyphosate is a broad-spectrum systemic herbicide and crop desiccant used to kill weeds. Glyphosate causes damage of cellular structures and other biochemical processes, such as disruption of chloroplasts, membranes and cell walls, reduction in chlorophyll content and changes in nucleic acid synthesis, photosynthesis, and respiration (Ali and Fletcher, 1978; Hernando *et al*., 1989; Schaffer and Sebetich, 2004). Glyphosate interrupts the target enzyme, 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) in the shikimate pathway that inhibits biosynthesis of the essential aromatic amino acids, phenylalanine, tryptophan and tyrosine (Duke and Powles, 2008). Glyphosate affects the photosynthetic electron transport indirectly by inhibiting chlorophyll biosynthesis (Fedtke and Duke,

2005) or inducing chlorophyll degradation (Gomes *et al.,* 2016). In cyanobacteria, glyphosate inhibits the photosynthetic electron transport (Hill reaction) and O_2 evolution (Singh and Singh, 2004).

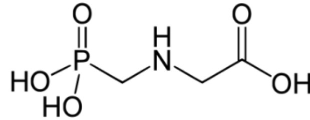


Figure 2.26 Glyphosate structure
(https://en.wikipedia.org/wiki/Glyphosate)

2.9.2 Respiratory Inhibitors

There are many kinds of chemical compounds functioning as respiratory inhibitors. The known respiratory inhibitors are malonate, potassium cyanide, sodium azide and retenone.

2.9.2.1 Malonate

The respiratory electron transport system contains complex II or succinate dehydrogenase which is attached on the membrane surface. This enzyme activates the reduction reaction of succinate into fumarate and concomitantly reduces FAD into $FADH_2$ (Fig. 2.27). Malonate is the strong competitive inhibitor of succinate dehydrogenase. It binds at the active site of succinate dehydrogenase. As a result, enzyme becomes inactive. Therefore, the inactive enzyme is unable to change succinate molecule into fumarate and hindering the respiratory electron transport (Fig. 2.28).

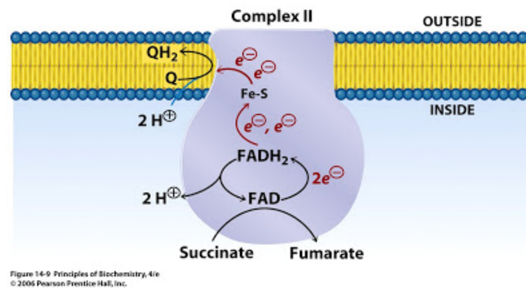
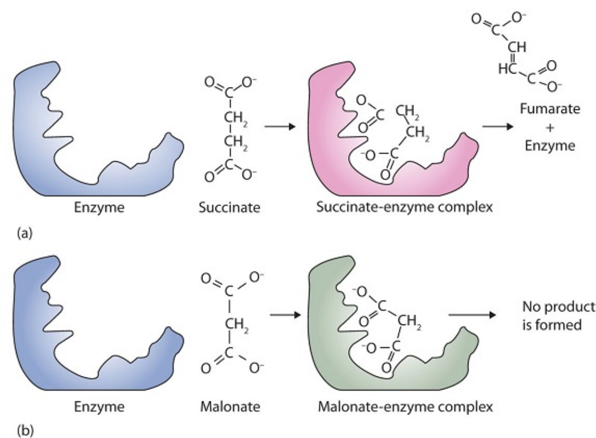


Figure 2.27 Succinate dehydrogenase reaction
(http://www.studydroid.com/index.php?page=viewPack&packId=128877)

(A)



(B)

Figure 2.28 Reaction of succinate dehydrogenase with succinate (A) and malonate (B) (http://2 0 1 2 books.lardbucket.org/books/introduction-to-chemistry-general-organic-and-biological/s21-08-enzyme-inhibition.html)

2.9.2.2 Potassium cyanide (KCN) and Sodium azide (NaN_3)

Potassium cyanide (KCN) inhibits the electron transport chain to O_2 molecule at the last receptor, quinol oxidase, and cytochrome c oxidase whereas sodium azide (NaN_3) disallows only the electron transport chain from cytochrome c oxidase to water. The photosystem and respiration mechanisms in cyanobacteria are nearby at the thylakoid or intracytoplasmic membranes (ICM). However, only the respiration pathway is occurring at the cytoplasmic membrane (CM). At the CM, NAD(P)H loses electrons to NAD(P)H dehydrogenase. On the other hand, succinate dehydrogenase gains electrons from succinate. Finally, electrons from both pathways

are transferred to plastoquinone and O_2 via cytochrome *b6f* complex and cytochrome c oxidase, respectively. Both KCN and NaN_3 can block the electron flow from cytochrome c to O_2 (Fig 2.29). At the ICM, plastoquinone obtains electrons from NAD(P)H dehydrogenase and photosystem II. Then, electrons are transferred to O_2 via quinol oxidase activity. Moreover, electrons are also transferred to O_2 by cytochrome c-553 and cytochrome c oxidase. After that, NADH(P)H is utilized via photosystem II. As above mention, KCN is capable to obstruct the electron transport chain from quinone oxidase and cytochrome c to O_2. Meanwhile, NaN_3 can directly block the electron flow from cytochrome c to oxygen (Fig 2.29) (Pils and Schmetterer, 2001).

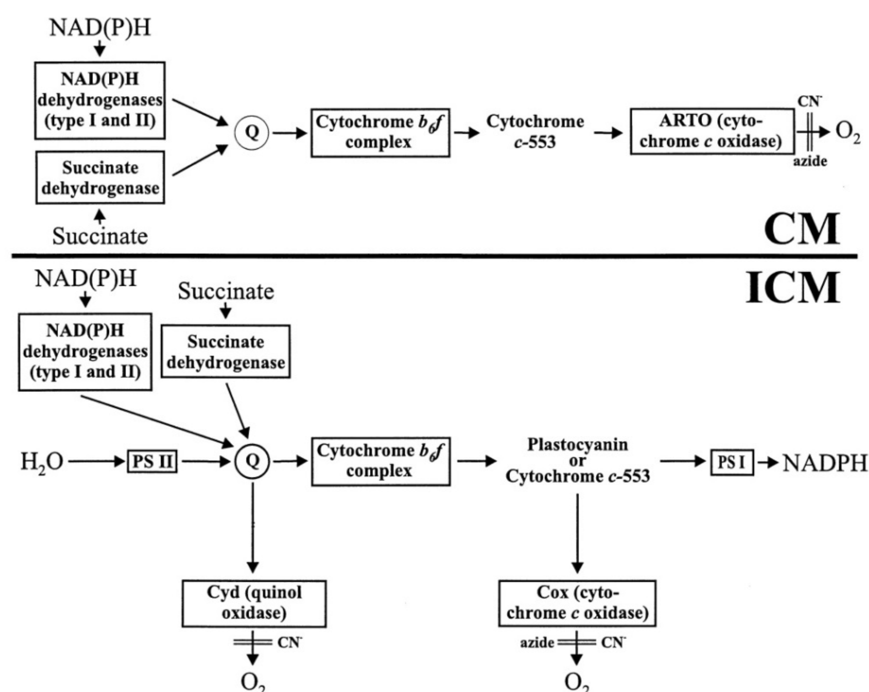


Figure 2.29 Effect of potassium cyanide and sodium azide on the electron transportation in the respiration of cyanobacteria (Pils and Schmetterer, 2001)

2.9.2.3 Rotenone

NAD(P)H in the respiration chain transfers electrons to NAD(P)H dehydrogenase. After that electrons are shuttled to plastoquinone, plastocyanin, and photosystem I. Rotenone disallows the electron transfer to plastoquinone by inactivating NAD(P)H dehydrogenase. As a result, the respiration mechanism is inhibited (Fig. 2.30)

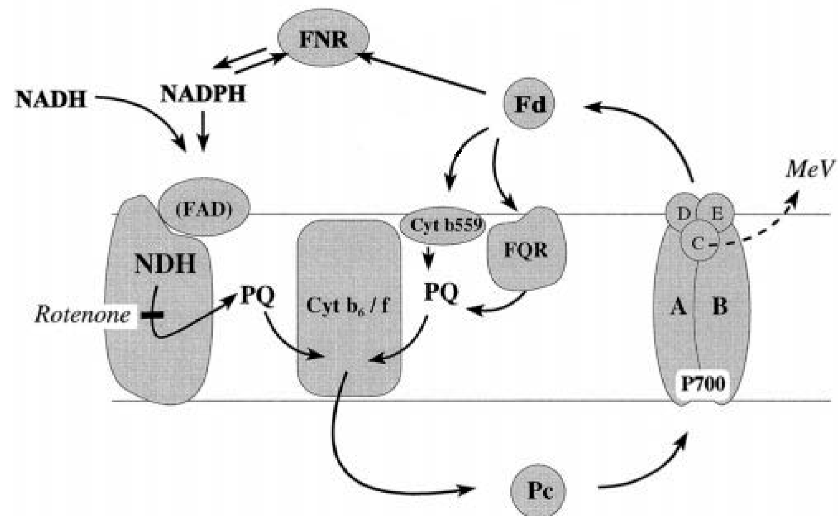


Figure 2.30 Block of rotenone in electron flowing from NAD(P)H dehydrogenase to plastoqinone (Teicher and Scheller, 1998)

2.9.3 Uncoupling oxidative phosphorylation

There are many kinds of chemical compounds functioning as inhibitors of uncoupling oxidative phosphorylation. The known uncoupling oxidative phosphorylation inhibitors are carbonyl cyanide m-chlorophenyl hydrazone and 2,4-dinitrophenol.

2.9.3.1 Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is categorized as both photosystem II inhibitor and the uncoupling oxidative phosphorylation inhibitor. CCCP can destroy the proton motive force by attracting to protons. Then, CCCP with attached protons can pass throughout the membrane without ATP synthase activity (Fig. 2.31). This process interrupts the ATP production (Hopfer *et al.,* 1968).

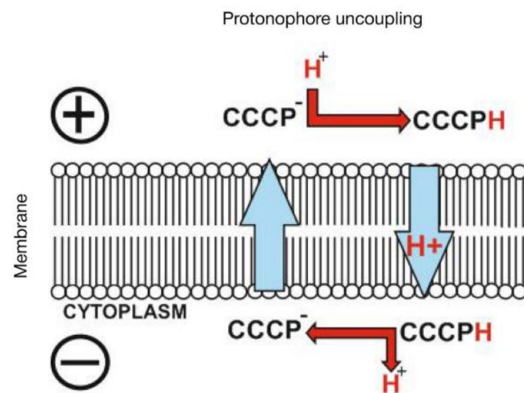


Figure 2.31 Uncoupling oxidative phosphorylation by CCCP (https://vestnikrgmu.ru/archive/2018/1/2/media?lang=en)

2.9.3.2 2,4-Dinitrophenol (DNP)

2,4-dinitrophenol (DNP) inhibits ATP synthesis via the proton pumping without ATP synthase activity. In the respiration pathway and photosystem, there is proton transferring through membrane, resulting from proton gradient at the part of matrix and intermembrane space. The protons are usually pumped throughout the membrane by ATP synthase. Meanwhile, ATP is created. However, when DNP is capable to bind with protons instead, the DNP with attached protons can pass through the membrane without ATP pumping via ATP synthase. So, ATP is not produced (Fig. 2.32) (Heytler, 1979).

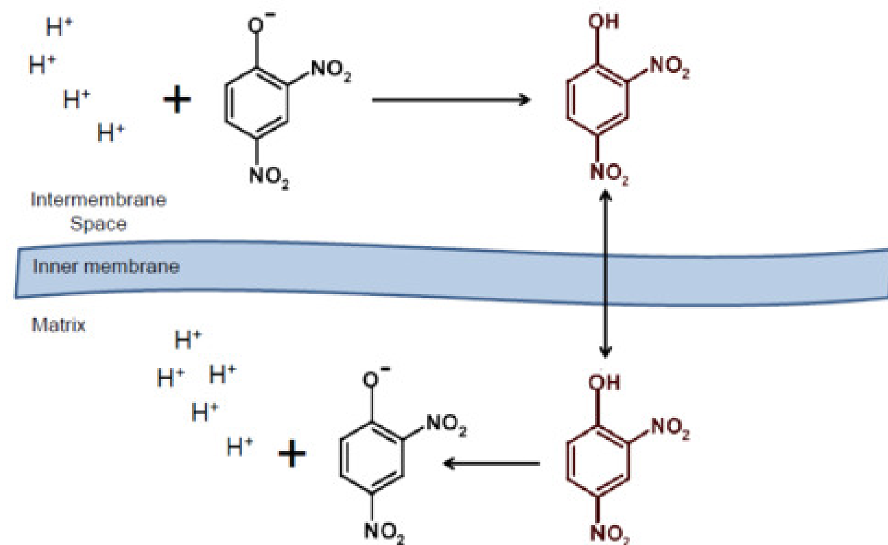


Figure 2.32 Proton transportation via DNP activity
(https://quizlet.com/2934325/etc-flash-cards/)

2.9.4 Inhibitor of CO_2 fixation

In this study, DL-glyceraldehyde is used as inhibitor of CO_2 fixation. DL-glyceraldehyde can interrupt the electron transport chain in CO_2 fixation. The ribulose-1,5-bisphosphate, 5 carbon atoms, condenses with CO_2, then creates an unstable 6 carbon atoms. The unstable molecule will be separated into 3-phosphoglycerate via RuBisco enzyme. After, 3-phosphoglycerate comprising 3 carbon atoms will be utilized for CO_2 fixation (Fig. 2.33). DL-glyceraldehyde is the competitive inhibitor of phosphoribulokinase enzyme which inhibits the phosphorylation reaction of ribulose-5-phosphate to ribulose-1,5-bisphosphate. As a result, CO_2 fixation is blocked.

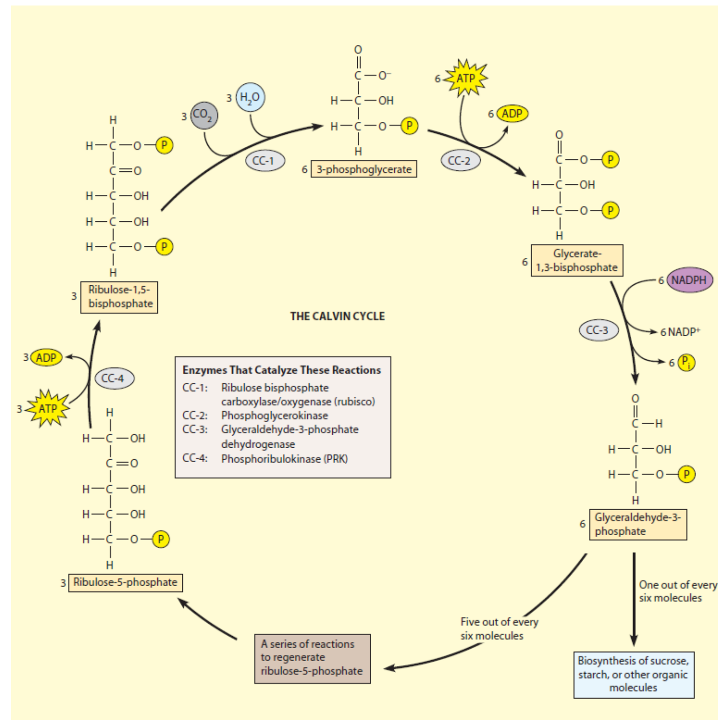


Figure 2.33 Reactions in carbon dioxide fixation
(http://reasonandscience.heavenforum.org/t2164-the-calvin-benson-cycle)

2.9.5 Inhibitor in Krebs cycle

Sodium arsenate ($NaAsO_2$) and potassium arsenate ($KAsO_2$) are herbicides that inhibit pyruvate dehydrogenase activity (Tretter and Vizi, 2000). Sodium arsenate can bind with -SH groups of pyruvate dehydrogenase (Fig. 2.34). Thus, pyruvate cannot be changed into acetyl-CoA. Therefore, the condensation of acetyl-CoA and oxaloacetate is blocked in Krebs cycle.

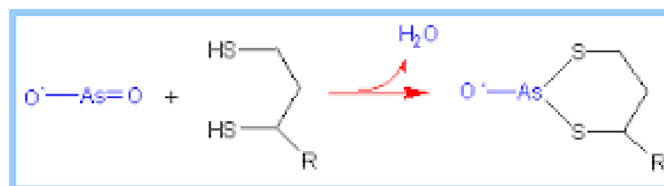


Figure 2.34 Reaction of sodium arsenate and pyruvate dehydrogenase
(https://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/krebs.htm)

Chapter 3

Research Methodology

3.1 Cyanobacterial strain

The unicellular halotolerant cyanobacterium *Aphanothece halophytica* was obtained from the Laboratory of Cyanobacterial Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Thailand

3.2 Culture medium

Blue green 11 (BG11) medium (Rippka *et al*., 1979) supplemented with Turk Island salt solution (Garlick *et al*., 1977) was used to cultivate *A. halophytica* in this study.

3.3 Chemical reagents

3.3.1 Chemicals for cultivation

3.3.1.1 Boric acid (H_3BO_3) (Merck, Germany)
3.3.1.2 Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Carlo Erba, Italy)
3.3.1.3 Citric acid ($C_6H_8O_7$) (Analar, England)
3.3.1.4 Cobalt choride hexahydrate ($CoCl_2.6H_2O$) (Fluka, Switzerland)
3.3.1.5 Cobalt nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) (Ajex, Australia)
3.3.1.6 Copper sulfate pentahydrate ($CuSO_4.5H_2O$) (Carlo Erba, Italy)
3.3.1.7 Cupric chloride dihydrate ($CuCl_2.2H_2O$) (Mallinckrodt Baker, USA)
3.3.1.8 Diaminoethylene tetraacetic acid disodium salt (Na_2EDTA) (Promega, USA)
3.3.1.9 Dipotassium hydrogen phosphate (K_2HPO_4) (Carlo Erba, Italy)
3.3.1.10 Ferric ammonium citrate ($FeNH_4$ citrate) (British Drug House, England)
3.3.1.11 Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) (Carlo Erba, Italy)
3.3.1.12 Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Carlo Erba, Italy)
3.3.1.13 Manganese chloride tetrahydrate ($MnCl_2.4H_2O$) (Ajex, Australia)
3.3.1.14 Nickel chloride ($NiCl_2$) (Merck, Germany)
3.3.1.15 Sodium carbonate (Na_2CO_3) (Carlo Erba, Italy)

3.3.1.16 Sodium chloride (NaCl) (Ajax, Australia)
3.3.1.17 Sodium hydroxide (NaOH) (Carlo Erba, Italy)
3.3.1.18 Sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (British Drug House, England)
3.3.1.19 Sodium nitrate ($NaNO_3$) (Carlo Erba, Italy)
3.3.1.20 Potassium chloride (KCl) (Merck, Germany)
3.3.1.21 Potassium dihydrogen phosphate (KH_2PO_4) (Carlo Erba, Italy)
3.3.1.22 Zinc chloride ($ZnCl_2$) (Fluka, Switzerland)
3.3.1.23 Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (Fluka, Switzerland)

3.3.2 Support materials for immobilization

3.3.2.1 Agar (Difco, USA)
3.3.2.2 Agarose (BioWhittaker Molecular Application, USA)
3.3.2.3 Calcium alginate (Sigma, USA)
3.3.2.4 κ-Carrageenan (Marcel Carrageenan, Republic of the Philippines)

3.3.3 Inhibitors

3.3.3.1 Atrazine ($C_8H_4ClN_5$) (Sigma, Germany)
3.3.3.2 Carbonyl cyanide m-chlorophenylhydrazone (CCCP) ($C_9H_5ClN_4$) (Sigma, Spain)
3.3.3.3 2,4-Dinitrophenol ($C_6H_4N_2O_3$) (Sigma, Spain)
3.3.3.4 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) ($C_9H_{10}Cl_2N_2O$) (Sigma, Israel)
3.3.3.5 DL- Glyceraldehyde ($C_3H_4O_4$) (Sigma, Switzerland)
3.3.3.6 Glyphosate ($C_3H_8NO_5P$) (Sigma, USA)
3.3.3.7 Malonic acid ($C_3H_4O_4$) (Sigma, China)
3.3.3.8 Rotenone ($C_{23}H_{22}O_6$) (Sigma, China)
3.3.3.9 Sodium arsenate ($Na_2HAsO_4.7H_2O$) (Sigma, India)
3.3.3.10 Sodium azide ($Na\ N_3$) (Sigma, Germany)

3.3.4 Chemicals for quantitative analyses

3.3.4.1 Methanol (CH_3OH) (Scharlau, Spain)
3.3.4.2 Methylviologen dichloride hydrate ($C_{12}H_{14}Cl_{12}N_2.H_2O$) (Sigma, USA)
3.3.4.3 Sodium dithionite ($Na_2O_4S_2$) (Sigma, USA)

3.3.5 Gases for quantitative analyses

3.3.5.1 Argon (Ar) (99.999%) (Thonburiwatthana Ltd., Thailand)

3.3.5.2 Standard hydrogen (H_2) (4% (v/v) Hydrogen in argon) (Praxair Ltd., Thailand)

3.4 Instruments

3.4.1 Autoclave (Hirayama Manufacturing Corporation, HV-50, Japan)
3.4.2 Clark-type oxygen electrode (Hansatech, UK)
3.4.3 Desiccator (Duran, Germany)
3.4.4 Erlenmeyer flask (Pyrex, USA)
3.4.5 Gas chromatograph (Hewlett-Packard HP890A GC, Japan)
3.4.6 Glass microfiber filter GF/C (47 mm diameter) (Whatman, UK)
3.4.7 Glass plate (Pyrex, USA)
3.4.8 Glass ware (Pyrex, USA)
3.4.9 Hemocytometer (Boeco, Germany)
3.4.10 Hot air oven (Delta Laboratory, 1375FX, Thailand)
3.4.11 Incubator shaker (Gallenkamp, T490188, UK)
3.4.12 Laminar air flow cabinet (International Scientific Supply, HS123, Thailand)
3.4.13 Light incubator shaker (Vision Scientific, Green SSeriker II, Korea)
3.4.14 Light microscope (Olympus, CH30, Japan)
3.4.15 Microcentrifuge (Labnet, Spectrafuge 16M, USA)
3.4.16 pH meter (Denver Instrument 215, USA)
3.4.17 Refrigerated centrifuge (Hermle Labortech, Z383K, Germany)
3.4.18 Semimicro cuvette rectangular 10 mm (Hella, USA)
3.4.19 Spectrofluorometer (Jasco, Model FP-6300, Japan)
3.4.20 Suction pump (LabTech, GM-0.33 II, USA)
3.4.21 Test tube (Pyrex, USA)
3.4.22 UV-VIS spectrophotometer (Shimadzu, UV-1601, Japan)
3.4.23 Vortex (Scientific Industries, Genie2, USA)
3.4.24 Waterbath (Heto-Holten, CBN 28-30, Denmark)

3.5 Cultivation

The unicellular halotolerant cyanobacterium *A. halophytica* was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) (Rippka *et al.,* 1979) supplemented with Turk Island salt solution (Garlick *et al.,* 1997). An initial cell concentration was adjusted to the optical density at 730 nm of approximately 0.1. Cells were shaken in an incubator shaker with a speed of 120 rpm at 30 °C under a white-light illumination of 30 μmol photons m^{-2} s^{-1} (for 18 h light and 6 h dark $days^{-1}$)

for 7 days. The cyanobacterial cells were maintained by sub-culturing cells in a fresh medium every 2-3 weeks.

3.6 Analytical methods

3.6.1 Optical density measurement

The optical density of *A. halophytica* was examined by measurement at wavelength 730 nm using a spectrophotometer (Simon, 1977). Triplicate measurements were done for each experiment.

3.6.2 Dry cell weight measurement

Dry cell weight of *A. halophytica* was measured by filtration 10 mL of cell culture through a glass microfiber filter GF/C (47 mm diameter) (Whatman, UK). The filter was washed twice with distilled water and dried at 70 °C in an oven for overnight. The filter containing cells was cooled in a desiccator for 1 h and its weight was then measured by the 4 digit analytical balance. Then, the filter paper was dried with the same process and weighted again. This process was repeatedly performed until a constant weight was obtained**.** The dry cell weight was calculated by a subtraction between the weight of filter containing cells and the weight of filter only.

3.6.3 Chlorophyll a measurement

One mL of cell culture was transferred to a microcentrifuge tube. The cells were harvested by centrifugation at 12,000 xg at room temperature for 5 min. The supernatant was subsequently removed. Chlorophyll a was extracted from the cell pellet by adding 1 mL of 90 % (v/v) methanol. The mixture was mixed by vortexing, incubated under darkness at room temperature for 1 h and then mixed by vortexing again. Cell debris was removed from chlorophyll extract by centrifugation at 12,000 xg for 5 min. The chlorophyll a content of extract was determined by measuring an absorbance at wavelength 665 nm and cultivated according to the formula by MacKinney (1941).

3.6.4 H_2 production measurement

The unicellular halotolerant cyanobacterium *A. halophytica* was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution under previously described conditions (Topic 3.5). Cells were then harvested by centrifugation at 8,000 ×g at 4 °C for 10 min, washed twice and resuspended in $BG11_0$ medium (pH 7.4) supplemented with Turk Island salt solution. For H_2 production by free cells, five mL of cell suspension

was transferred to a 12-mL glass vial. Glass vial was sealed with a rubber stopper with an aluminum rim. For H_2 production by immobilized cells, cells were immobilized following protocols in Topic 3.7.1. Six mL of immobilized cells containing six milligrams dry cell weight were transferred to a 20-mL glass vial. Nine mL of $BG11_0$ medium (pH 7.4) supplemented with Turk Island salt solution were added into glass vial. Glass vial was also sealed with a rubber stopper with an aluminum rim. Then, air in glass vial was removed by purging argon gas for 10 min. The glass vial was incubated under darkness at 30 °C to enter anaerobiosis for induction of H_2 production. H_2 evolution of free and immobilized cells was determined by analyzing 500 µL of gas phase in headspace using Gas chromatograph (Hewlett-Packard HP5890A, Japan) with a molecular sieve 5 Å 60/80 mesh packed column and thermal conductivity detector. The GC condition for H_2 determination is shown in Table 3.1. H_2 peak area obtained by Gas chromatograph was calculated compared with that of 4% (v/v) standard H_2 in argon. The values of H_2 production rate and H_2 production yield are shown as a terms of H_2 evolved per dry cell weight per time (µmolH_2 g^{-1} dry weight h^{-1}) and H_2 evolved per dry cell weight (µmolH_2 g^{-1} dry weight), respectively. Each measurement in all experiments was performed in triplicate.

Table 3.1 Gas chromatograph conditions used for determination of H_2 production

Type	Condition
Detector	Thermal conductivity detector (TCD)
Column	Packed SS column 2m x 4mm OD x 3mm ID p/w
	Molecular sieve 5 Å 60/80 mesh
Temperature program	Injector temperature at 100 °C
	Column temperature at 50 °C
	Detector temperature at 100 °C
Carrier gas	Argon gas (99.999% purity) with flow rate 20 mL min^{-1}

3.6.5 O_2 production measurement

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution under previously described conditions (Topic 3.5.) Cells were harvested by centrifugation at 8,000 ×g at 4 °C for 10 min, washed twice and resuspended in 5 mL of $BG11_0$ medium (pH 7.4) supplemented with Turk Island salt solution. The cell suspension was then transferred to a 12-mL glass vial. Glass vial was sealed with a rubber stopper with an aluminum rim. After that, the vial was purged with argon gas for 10

min. O_2 evolution in system was determined by analyzing 500 µL of gas phase in headspace using gas chromatograph with the same conditions as shown in Table 3.1. O_2 production rate was shown as a term of O_2 evolved per dry cell weight per time (µmolO_2 g^{-1} dry weight h^{-1}) form comparing O_2 peak area in experiment with the O_2 peak area from air injection (20.95% O_2 (v/v)). Each measurement was performed in triplicate.

3.6.6 Bidirectional hydrogenase activity measurement

Bidirectional hydrogenase activity of *A. halophytica* was determined in the presence of sodium dithionite-reduced methyl viologen following Baebprasert and coworker (2010). *A. halophytica* grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. The cell suspension was incubated in $BG11_0$ medium at 30 °C under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h. Cells were harvested by centrifugation and resuspended in $BG11_0$ medium. Five mL of cell suspension was transferred to glass vial and selected inhibitors were added to the cell suspension. The vial was incubated at 30°C under the light 2 h. One mL of cells treated with selected inhibitor and without inhibitor treatment were mixed with 1 mL of 25 mM phosphate buffer (pH 7.0) containing of 2.5 mM methyl viologen and of 10 mM sodium dithionite that purged with argon gas for 10 min before mixing with the cells. The reaction was incubated at 25 °C under dark anaerobic condition for 15 min before H_2 measurement by gas chromatograph. Bidirectional hydrogenase activity was calculated as a term of H_2 produced per dry cell weight per time (µmolH_2 g^{-1} dry weight min^{-1}).

3.6.7 Fluorescence emission spectra measurement

Chlorophyll fluorescence emission spectra measurement was determined at room temperature by spectrofluorometer (Jasco, Model FP-6300, Japan). *A. halophytica* grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. The cell suspension was incubated in $BG11_0$ medium at 30 °C under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h. Cells were harvested by centrifugation and resuspended in 5 mL of $BG11_0$ medium. Cell suspension was transferred to glass vial and the selected inhibitor was added to the cell suspension. The vial was incubated at 30°C under the light for 2 h. One mL of *A. halophytica* culture with and without inhibitor treatment was added into chamber and incubated under white-light intensity of 2,000 µmol photons m^{-2} s^{-1} for 10 min. Chlorophyll fluorescence measurement was carried out by using excitation wavelength at 437

nm. Chlorophyll fluorescence emission spectra was recorded in the range 600–800 nm (Joshua *et al*., 2005).

3.6.8 O_2 consumption by dark respiration rate measurement

Dark respiration rate was analyzed using a Clark-type oxygen electrode (Hansatech, UK) at 25 °C. *A. halophytica* grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. The cell suspension was incubated in $BG11_0$ medium at 30 °C under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h. Cells were harvested by centrifugation and resuspended in 5 mL of $BG11_0$ medium. Cell suspension was transferred to glass vial and the selected inhibitor was added to the cell suspension. The vial was incubated at 30°C under the light for 2 h. Before O_2 measurement, distilled water was added into chamber following the addition of about 1 g of sodium dithionite to achieve the zero oxygen signal. After that, two mL of *A. halophytica* cell suspension with and without selected inhibitor treatment were added into the chamber and illuminated under the white-light illumination of 300 µmol photons m^{-2} s^{-1} for 15 min. Then, the respiratory rate was measured as O_2 consumption in the dark for 15 min. O_2 consumption inside the O_2 electrode chamber was calculated as a term of µmol O_2 g^{-1} dry weight min^{-1} by the O_2 view Software.

3.7 Improvement of H_2 production of *A. halophytica* by cell immobilization method

3.7.1 Cell immobilization with various types of support material

3.7.1.1 Cell preparation

A. halophytica grown in 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation at 8,000 ×g at 4 °C for 10 min, washed twice and resuspended in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution before immobilization. The cyanobacterial cells were immobilized with four support materials; agar, agarose, sodium alginate and κ-carrageenan. The cell concentration was adjusted to approximately 1 mg dry cell weight mL^{-1} of gel solution.

3.7.1.2 Cell immobilization by agar

Agar was weighted and added with $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. Agar was dissolved by heating on a hot plate until the

agar was dissolved completely. Then agar solution was sterilized by autoclaving. One mL of cell suspension from topic 3.7.1.1 was mixed with 9 mL of the autoclaved agar solution in waterbath at 50 °C that a final agar concentration was 1.5 % (w/v). The mixture was cooled down to become a solid gel. The gel was subsequently cut into square pieces with same each size of 0.5 cm.

3.7.1.3 Cell immobilization by agarose

Agarose was weighted and added with $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. Agarose was dissolved by heating on a hot plate until the agar was dissolved completely. Then agarose solution was sterilized by autoclaving. One mL of cell suspension from topic 3.7.1.1 was mixed with 9 mL of the autoclaved agarose solution in waterbath at 50 °C that a final agarose concentration was 1.5 % (w/v). The mixture was cooled down to become a solid gel. The gel was subsequently cut into square pieces with same each size of 0.5 cm.

3.7.1.4 Cell immobilization by κ-carrageenan

κ-carrageenan was weighted and added with $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. κ-carrageenan was dissolved by heating on a hot plate until the agar was dissolved completely. Then κ-carrageenan solution was sterilized by autoclaving. One mL of cell suspension from topic 3.7.1.1 was mixed with 9 mL of the autoclaved κ-carrageenan solution in waterbath at 50 °C that a final κ-carrageenan concentration was 1.5 % (w/v). The mixture was cooled down to become a solid gel. The gel was subsequently cut into square pieces with same each size of 0.5 cm. κ-carrageenan gels were immersed in 0.3 M potassium chloride solution for 30 min before immersion in $BG11_0$ until use.

3.7.1.5 Cell immobilization by alginate

Sodium alginate was weighted and dissolved in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. Sodium alginate was dissolved by heating on a hot plate until the agar was dissolved completely. The alginate solition was then sterilized by autoclaving. One mL of *A. halophytica* cell suspension (prepared form 3.7.11) was mixed with 9 mL of the autoclaved sodium alginate solution that a final alginate concentration was adjusted at 4 % (w/v). The mixture was dropped into autoclaved 100 mM calcium chloride solution by using a 10-mL syringe. The gel beads containing immobilized cells were immersed in calcium chloride solution for at least 2 h and washed twice with $BG11_0$ before use.

3.7.2 Effect of support material type and concentration on H_2 production by immobilized cells

A. halophytica was prepared and immobilized with 1.5 % (w/v) agar, 1.5 % (w/v) agarose, 1.5 % (w/v) κ-carrageenan, and 4 % (w/v) alginate as described in Topic 3.7.1. Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. The immobilized cells were adapted under nitrogen deprivation by incubating at 30 °C under the white-light illumination of 30 μmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4. The optimal support material type was selected. The effect of concentration of the optimal support material on H_2 production was investigated.

3.7.3 Effect of cell concentration on H_2 production by immobilized cells

A. halophytica was immobilized using the selected support material type and concentration for H_2 production (Topic 3.7.2). Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. To investigate effect on cell concentration on H_2 production by immobilized cells, cell concentrations were varied from 0.2 to 5 mg dry cell weight mL^{-1} of gel solution. The immobilized cells were adapted under nitrogen deprivation by incubating at 30 °C under the white-light illumination of 30 μmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4.

3.7.4 Effect of size of immobilized cells on H_2 production by immobilized cells

A. halophytica was immobilized with the optimal type and concentration of support material (Topic 3.7.2) and cell concentration (Topic 3.7.3). Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. To investigate size of immobilized cells on H_2 production by immobilized cells, gel was cut into square pieces of 0.25 cm x 0.25 cm x 0.25 cm, 0.5 cm x 0.5 cm x 0.5 cm, 0.75 cm x 0.75 cm x 0.75 cm and 1 cm x 1 cm x 1 cm. The immobilized cells were adapted under nitrogen deprivation by incubating at 30 °C under the white-light illumination of 30 μmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4.

3.7.5 Effect of nutrient and mineral concentration on H_2 production by immobilized cells

A. halophytica was immobilized with the optimal type and concentration of support material (Topic 3.7.2), cell concentration (Topic 3.7.3) and the optimal size of immobilized gel beads (Topic 3.7.4). Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing various nutrient and mineral concentrations of $NaNO_3$ (0, 0.0176, 0.176, 1.76, 17.6 and 176 mM), $MgSO_4.7H_2O$ (0, 1.5, 15, 30, and 150 mM), NaCl (0.25, 0.5, 0.75 and 1 M), Fe^{3+} (0, 0.04, 0.4, 4, 40 and 400 µM) and Ni^{2+} (0, 0.01, 0.1, 1, 10 and 100 µM). The immobilized cells were adapted at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4.

3.7.6 Effect of initial pH of medium on H_2 production by immobilized cells

A. halophytica was immobilized with the optimal type and concentration of support material (Topic 3.7.2), cell concentration (Topic 3.7.3) and size of immobilized gel beads (Topic 3.7.4). Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ supplemented with Turk Island salt solution. To investigate the effect of initial pH of medium on H_2 production by immobilized cells, immobilized cells were incubated in medium with initial pH 6, 7, 7.4, 8, 9 and 10. The immobilized cells were adapted at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4.

3.7.7 Effect of temperature on H_2 production by immobilized cells

A. halophytica was immobilized with the optimal type and concentration of support material (Topic 3.7.2), cell concentration (Topic 3.7.3) and size of immobilized gel beads (Topic 3.7.4). Six mL of immobilized cells were transferred to a 20-mL glass vial containing 9 mL of $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. To investigate effect of temperature on H_2 production by immobilized cells, immobilized cells were incubated at temperature 20, 25, 30, 35, 40 and 45 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. H_2 production of immobilized cells was analyzed as previously described in Topic 3.6.4.

3.7.8 Effect of volume of glass vial, immobilized cells and headspace on H_2 production by immobilized cells

A. halophytica was immobilized with the optimal conditions. The immobilized gel beads were transferred to a 20-mL of glass vial containing $BG11_0$ supplemented with Turk Island salt solution. The immobilized beads were adapted at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. Air in glass vial was removed by purging argon gas for 20 min. The immobilized beads were incubated under dark for H_2 production. H_2 production was analyzed as previously described in Topic 3.6.4. To investigate effect of immobilized cells and headspace volumes on H_2 production by immobilized cells, various volumes of immobilized cells (15,24, 36 and 54 cm^3) and medium and headspace (15, 18, 20, 24, 30 and 40 mL) were used.

3.7.9 Cycle of H_2 production by immobilized cells

A. halophytica was immobilized with the optimum conditions. Thirty-six mL of immobilized gel beads and free cells were transferred into a 120-mL glass vial containing 54 mL of $BG11_0$ supplemented with Turk Island salt solution. They were adapted with N-deprivation at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. Then, the air in glass vial was removed by purging argon gas for 20 min. H_2 production was induced under darkness at 40 °C for 24 h and analyzed for three cycles. For each cycle, H_2 production was analyzed for 36 h. After finishing H_2 production measurement in each cycle, the medium in glass vial was removed and replaced with the fresh medium. In some cases, different volumes (30, 50 and 100 %) of immobilized cells after the first cycle were removed and replaced with the same respective volume of fresh immobilized cells for the analysis of H_2 production in the second and third cycle. For free cells experiments, the culture after each cycle was centrifuged to harvest the cells before suspension with the new fresh medium for the next cycle. Similarly, the different volumes (30, 50 and 100 %) of free cells after cycle were removed and replaced with the same respective volume of fresh free cells obtained from 7-day culture before analyzing H_2 production. Then, cells were purged with argon gas to remove O_2. H_2 production was analyzed for three cycles.

3.8 Improvement of H_2 production of *A. halophytica* by use of Inhibitors

3.8.1 Screening of inhibitors affecting H_2 production by *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 (pH 7.4) supplemented with Turk Island salt solution as described in Topic 3.5. Cells were harvested by centrifugation at 8,000 xg at 4 °C for 10 min, washed twice and resuspended in 100 ml of $BG11_0$ supplemented with Turk Island salt solution. Cells were then incubated at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 24 h. After that cells were harvested by centrifugation and resuspended in 5 mL of $BG11_0$ supplemented with Turk Island salt solution**.** Five mL of cell suspension was transferred to a 12-mL glass vial and inhibitors were applied to the cells suspension. The vial was sealed with a rubber stopper with an aluminum rim. The vial was incubated at 30 °C under the white-light illumination of 30 µmol photons m^{-2} s^{-1} for 2 h. The vial was purged with argon gas for 10 min and incubated at 30 °C under light or darkness for 2 h. After that, H_2 was analyzed as methods previously described in Topic 3.6.4. For screening the inhibitors affecting H_2 production by *A. halophytica*, cells were treated with various types and concentrations of inhibitors as summarized in Table 3.2.

Table 3.2 Type and concentration of inhibitors

Type	Name	Concentration
Photosystem II inhibitors	atrazine	0, 0.05, 0.5, 5, 25 and 50 µM
	CCCP	0, 0.01, 0.1, 0.5, 1 and 5 µM
	DCMU	0, 0.5, 5, 50, 125 and 250 µM
	glyphosate	0, 0.03, 0.3, 3, 150 and 300 µM
Respiration inhibitors	malonic acid	0, 0.01, 0.1, 1, 10, 50 and 100 mM
	rotenone	0, 0.01, 0.1, 1, 10, 50 and 100 µM
	sodium azide	0, 0.01, 0.1, 1, 5 and 10µM
Uncoupling agent of oxidative phosphorylation inhibitor	2,4-dinitrophenol	0, 0.1, 1, 10, 50 and 100 µM
Carbon dioxide fixation inhibitor	DL- glyceraldehyde	0, 0.5, 1, 5, 10, 50 and 250 µM
Krebs' cycle inhibitor	sodium arsenate	0, 0.1, 1, 10, 50 and 100 µM

3.8.2 Effect of inhibitors on cell concentration, chlorophyll a content of *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation at 8,000 ×g at 4 °C for 10 min, washed twice and resuspended in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. The cell suspension was incubated at 30 °C under light intensity of 30 μmol photons m^{-2} s^{-1} by shaking at 120 rpm for 24 h. After incubation, cells were harvested by centrifugation and resuspended in $BG11_0$ medium. Five mL of cell suspension was transferred to a 12-mL glass vial. The optimal selected inhibitors were added to the cell suspension. The vials was sealed with a rubber stopper with an aluminum rim and incubated at 30°C under the white-light illumination of 30 μmol photons m^{-2} s^{-1} 2 h. The vial was purged with argon gas for 10 min to establish an anaerobiosis. The vials were further incubated under the light at 30 °C. An aliquot of cell suspension after incubation for 2, 24, 48, 72 and 96 h was collected for cell and chlorophyll concentration measurement. Cell concentration was analyzed by 10 μL of cell suspension was pipetted into a hemocytometer and counted cells under a microscope. The cell concentration was calculated as a term of cells mL^{-1}. Chlorophyll a content was analyzed as described in Topic 3.6.3.

3.8.3 Effect of inhibitors on H_2 production and O_2 production of *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation, washed twice and resuspended in $BG11_0$ medium. The cell suspension was incubated at 30 °C under light for 24 h. After that, cells were harvested by centrifugation and resuspended in $BG11_0$ medium. Five mL of cell suspension was transferred to a 12-mL glass vial. The optimal selected inhibitors were added to the cell suspension. The vials was sealed with a rubber stopper with an aluminum rim and incubated at 30°C under light for 2 h. The vial was purged with argon gas for 10 min to establish an anaerobiosis. H_2 and O_2 production were analyzed after incubation cells under both light and dark conditions for 2 h as described in Topic 3.6.4 and 3.6.5, respectively.

3.8.4 Effect of inhibitors on bidirectional hydrogenase activity of *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation, washed twice and resuspended in $BG11_0$ medium. Cells were incubated at 30 °C under light for 24 h. Then, cells were harvested by centrifugation and resuspended in $BG11_0$ medium agan. Five mL of cell suspension was transferred to a 12-mL glass vial. The optimal selected inhibitors were added to the cell suspension. The vials was sealed with a rubber stopper with an aluminum rim and incubated at 30 °C under light for 2 h. Bidirectional hydrogenase activity was measured as methods previously described in Topic 3.6.6.

3.8.5 Effect of inhibitors on fluorescence emission spectra of *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation, washed twice and resuspended in $BG11_0$ medium. The cell suspension was incubated at 30 °C under light for 24 h. Then, cells were harvested by centrifugation and resuspended in $BG11_0$ medium. Five mL of cell suspension was transferred to a 12-mL glass vial. The optimal selected inhibitors were added to the cell suspension. The vials was sealed with a rubber stopper with an aluminum rim and incubated at 30 °C under light for 2 h. fluorescence emission spectra was performed as methods previously described in Topic 3.6.7.

3.8.6 Effect of inhibitors on O_2 consumption by dark respiration of *A. halophytica*

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation, washed twice and resuspended in $BG11_0$ medium. The cell suspension was incubated at 30 °C under light for 24 h. After that, cells were harvested by centrifugation and resuspended in $BG11_0$ medium again. Five mL of cell suspension was transferred to a 12-mL glass vial. The optimal selected inhibitors were added to the cell suspension. The vials was sealed with a rubber stopper with an aluminum

rim and incubated at 30 °C under light for 2 h. O_2 consumption by dark respiration was measured as methods previously described in Topic 3.6.8.

3.8.7 Effect of inhibitor on long term of H_2 production

A. halophytica was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of BG11 medium (pH 7.4) supplemented with Turk Island salt solution as previously described condition as described in Topic 3.5. Cells were harvested by centrifugation, washed twice and resuspended in $BG11_0$ medium. The cell suspension was incubated at 30 °C under light intensity of 30 μmol photons m^{-2} s^{-1} for 24 h. After that, cells were harvested by centrifugation and resuspended in $BG11_0$ medium again. Five mL of *A. halophytica* in $BG11_0$ medium was incubated with inhibitor, without inhibitor and negative control without cells but containing inhibitor. The vial was purged with argon gas for 10 min and incubated at 30 °C under darkness. H_2 production was analyzed for 240 h as described in Topic 3.6.4

3.9 Statistical analysis

Data values in these experiments were statistically compared using a one-way ANOVA with Duncan's post hoc test. Differences between treatment means were considered significantly if $p < 0.05$. Data were analyzed by IBM SPSS statistic 23 (IBM Corp, USA).

Chapter 4

Main Results and Discussion

In this study, the enhancement of H_2 production by the unicellular cyanobacterium *A. halophytica* was investigated by using two methods; cell immobilization and inhibitor.

4.1 Improvement of H_2 production by *A. halophytica* by using cell immobilization

In this experiment, the improvement of H_2 production by *A. halophytica* was performed by using the method of cell immobilization. The support material type and concentration, cell concentration, volumetric size of immobilized cells, nutrient and mineral concentrations, initial pH, incubation temperature, size of vial, volume of headspace and immobilized cells were optimized. Finally, cycle of H_2 production by immobilized cells were investigated in three cycles.

4.1.1 Effect of support material types on H_2 production by immobilized cells

To study the effect of support material types on H_2 production by immobilized cells, *A. halophytica* was immobilized with four support materials; agar, agarose, alginate and κ-carrageenan. *A. halophytica* grown in BG11 (pH 7.4) supplemented with Turk Island salt solution was harvested by centrifugation and resuspended in nitrogen-deprived BG11 or $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution. Cells were immobilized with final concentrations of 1.5% (w/v) agar, 1.5% (w/v) agarose, 4% (w/v) alginate and 1.5% (w/v) κ-carrageenan. The cell concentration was adjusted to approximately 1 mg dry cell weight mL^{-1} of gel solution. Agar-, agarose- and κ-carrageenan-immobilized cubes including alginate-immobilized beads are shown in Fig. 4.1.

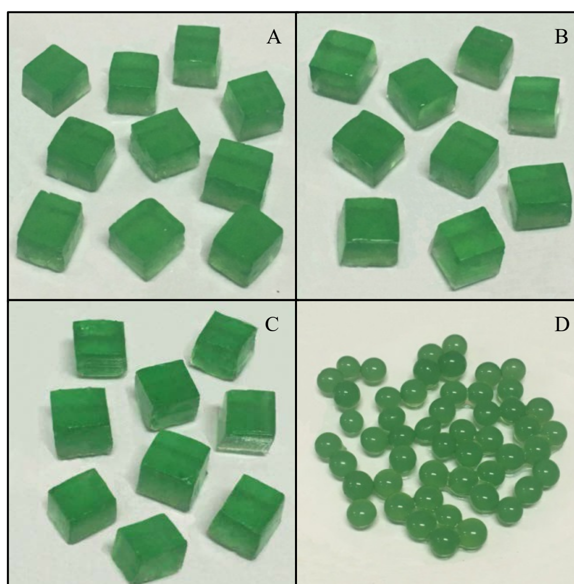


Figure 4.1 Immobilized cells of *A. halophytica* with four support materials; 1.5% (w/v) agar (A), 1.5% (w/v) agarose (B), 1.5% (w/v) carrageenan (C) and 4% (w/v) alginate (D).

For H_2 production measurement, cubes or beads containing immobilized cells were incubated in N-deprived medium under the light for 24 h before purging with argon and further incubation in darkness. H_2 production by immobilized cells was measured for 7 days compared to that of free cells. It was found that *A. halophytica* immobilized cells with 1.5% (w/v) agar gave the highest H_2 production rate with 6.16 ± 0.08 µmolH_2 g^{-1} dry weight h^{-1} and accumulated the maximum H_2 production with 164.08 ± 4.76 µmolH_2 g^{-1} dry weight h^{-1} after dark anaerobic incubation for 2 days, followed by cells immobilized with alginate, κ-carrageenan and agarose, respectively (Fig. 4.2). Free cells of *A. halophytica* were obviously shown to produce the lowest H_2 yield (Fig. 4.2).

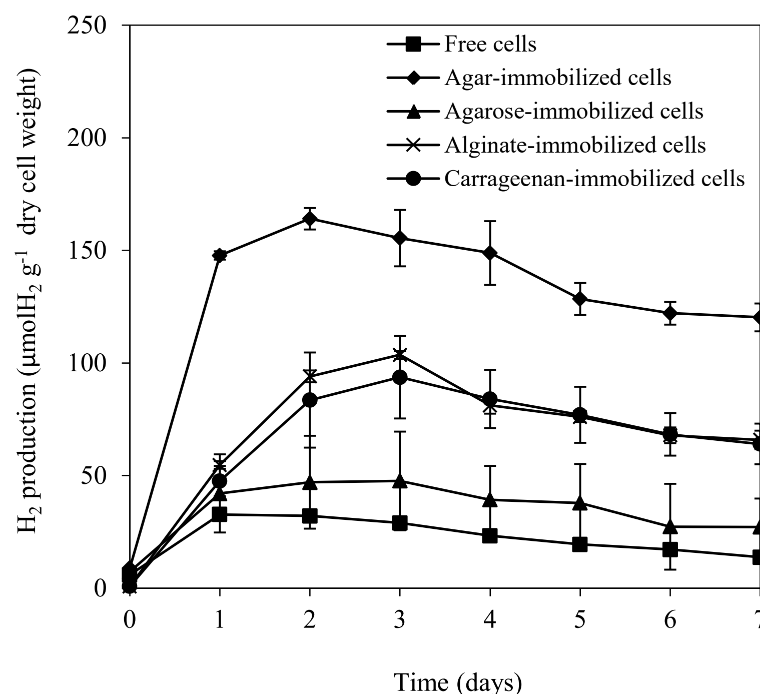


Figure 4.2 H_2 production by *A. halophytica* cells immobilized with 1.5% (w/v) agar, 1.5% (w/v) agarose, 4% (w/v) alginate and 1.5% (w/v) κ-carrageenan under nitrogen deprivation. H_2 production was measured under dark anaerobic condition for 7 days.

H_2 production of immobilized *A. halophytica* cells per total volume of immobilized cubes and beads was investigating. It was found that *A. halophytica* immobilized cells with 1.5% (w/v) agar gave the highest H_2 production rate with 64.64 ± 0.80 nmolH_2 cm^{-3} and accumulated H_2 production with 143.57 ± 4.17 nmolH_2 cm^{-3} after dark anaerobic incubation for 2 days, followed by cells immobilized with alginate, κ-carrageenan and agarose, respectively (Fig. 4.3).

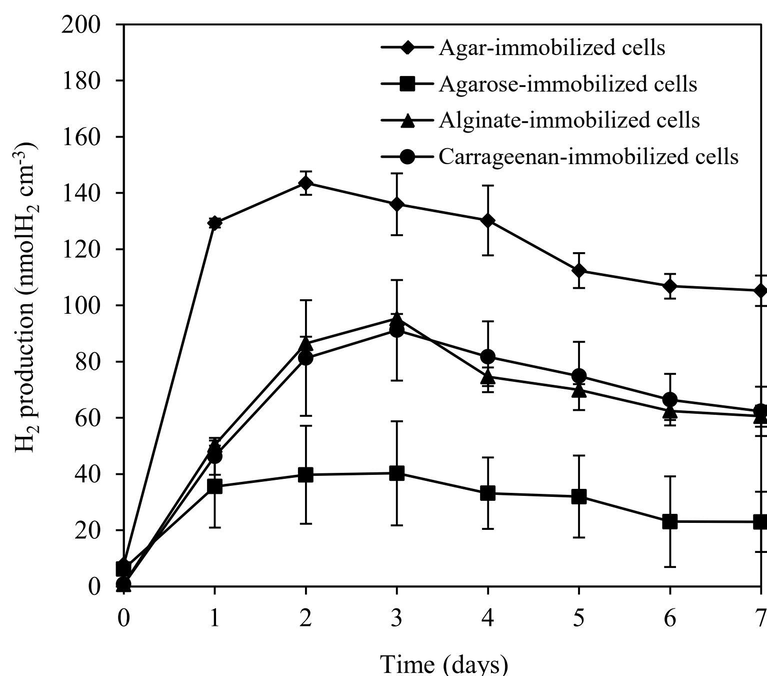


Figure 4.3 H_2 production per volume of *A. halophytica* cells immobilized with 1.5% (w/v) agar, 1.5% (w/v) agarose, 4% (w/v) alginate and 1.5% (w/v) κ-carrageenan under nitrogen deprivation. H_2 production was measured under dark anaerobic condition for 7 days.

In this study, cells immobilized with all support material types showed higher H_2 production than free cells (Fig. 4.2). It could be explained that cells immobilized in support materials were enclosed in a rigid network to prevent cells from the entry of nutrients and the release of products into the surrounding medium. Therefore, cell immobilization in all support materials could separate cells from a liquid phase and could protect bidirectional hydrogenase from O_2, a strong cyanobacterial hydrogenase inhibitor, in an external environment. As a result, this promoted hydrogenase activity and finally increasing H_2 production. In addition, cells immobilized with support materials were located in the restricted area, thus making it difficult for nutrients to access into cells. Therefore, their growth was lower than that of free cells. Consequently, excess accumulated reducing powers and electrons were then transferred to bidirectional hydrogenase to generate H_2 and thus promoting H_2 production. On the other hand, free cells of *A. halophytica* gave the lowest H_2 production because they were easily contacted with the surrounding medium including O_2 in the system, resulting in the inhibition of bidirectional hydrogenase activity. The results of the present study were in agreement with those from previous studies where immobilized cells of many species of cyanobacteria

such as N_2-fixing cyanobacteria *Anabaena* N-7363 and *Anabaena siamensis* TISTR 8012 (Karube *et al*., 1986; Taikhao and Phunpruch, 2017), filamentous cyanobacterium *Lyngbya perelegans* (Anjana and Kaushik, 2014), filamentous marine cyanobacteria *Oscillatoria* sp. Miami BG7 and *Oscillatoria subbrevis* strain 111 (Phlips and Mitsui, 1986; Kumar *et al*., 1991) gave higher H_2 production than free cells.

A. halophytica cells immobilized with agar gave the highest H_2 production compared to cells immobilized with other support materials (Fig. 4.2). The maximum H_2 production by agar-immobilized cells might be attributed to the high ability of agar gel to protect cells against O_2 available in the $BG11_0$ medium. In addition, agar gel showed the highest consistency of gel strength compared to other types of support materials (Armisén, 1991). Agar is shown to form solidity and stability gel under both alkaline and acidic conditions, and even in the addition of salts, minerals and chelating agents (Scott, 2012). In this study, cell immobilization was performed in $BG11_0$ supplemented with Turk Island salt solution which is a solution for suspension of cyanobacterial cells and solubility of support materials. This medium contains various kinds and high concentrations of minerals and salts. The composition and concentration of minerals and salts affects the stability of some types of gel bead or cube. In case of alginate, alginate gel bead was found to be unstable in a presence of cations chelating agents such as citrate, lactate, and phosphate or competing cations such as sodium or potassium (Castro *et al*., 2009). Na-EDTA, citrate and phosphate are chelating agents available in $BG11_0$ medium supplemented with Turk Island salt solution, making alginate bead too soft. In case of carrageenan, NaCl in medium decreased strength of the carrageenan gel, resulting in a low stability of carrageenan gel. Comparing the stability of agar and agarose gels, it was found that agar gel showed higher stability than agarose gel (Semenchuk *et al*., 2000). Our results were similar to the previous report showing that 1.5% (w/v) agar-immobilized cells of non-diazotrophic cyanobacterium *Oscillatoria subbrevis* strain 111 produced higher H_2 than 1.5% (w/v) alginate-immobilized cells (Kumar *et al*., 1991). However, type of support materials is dependent on the microorganism types and species. In cyanobacterium *L. perelegans*, cells immobilized with 2% (w/v) alginate slightly gave higher H_2 production than 2% (w/v) agar-immobilized cells (Anjana and Kaushik, 2014). In the heterocystous cyanobacterium *Anabaena* N-7363, cells immobilized with 2% (w/v) carrageenan produced higher H_2 than cells immobilized with 2.5% (w/v) agar and 3% (w/v) alginate (Karube *et al*., 1986). From these results, agar is a suitable support material for cell immobilization of *A. halophytica* in order to produce H_2. Agar-immobilized cells were used in optimization of cell immobilization for H_2 production in further experiments.

4.1.2 Effect of agar concentration on H_2 production by agar-immobilized cells

A. halophytica cells grown in BG11 medium (pH 7.4) supplemented with Turk Island salt solution for 7 days were harvested by centrifugation and resuspended in N-deprived medium. *A. halophytica* cells were immobilized by agar with final concentrations of 1, 1.5, 2, 2.5 and 3% (w/v). The cell concentration used in this study was adjusted equivalent to approximately 1 mg dry cell weight mL^{-1} of gel solution. Six mL (cm^3) of immobilized gel cube were transferred into a 20-mL glass vial containing 9 mL of $BG11_0$ medium (pH 7.4) supplemented with Turk Island salt solution. The agar-immobilized cells were incubated at 30 °C under the light for 24 h before purging with argon gas. H_2 production was determined under darkness. The result showed that H_2 production rates of agar-immobilized cells were increased with higher agar concentrations (Fig. 4.4). *A. halophytica* cells immobilized with 3% (w/v) agar gave the highest H_2 production rate with 19.72 ± 0.76 μmolH_2 g^{-1} dry weight h^{-1} after dark anaerobic incubation for 1 day (Fig. 4.4). When agar concentrations for cell immobilization were lower than 3% (w/v), H_2 production rates were decreased (Fig. 4.4).

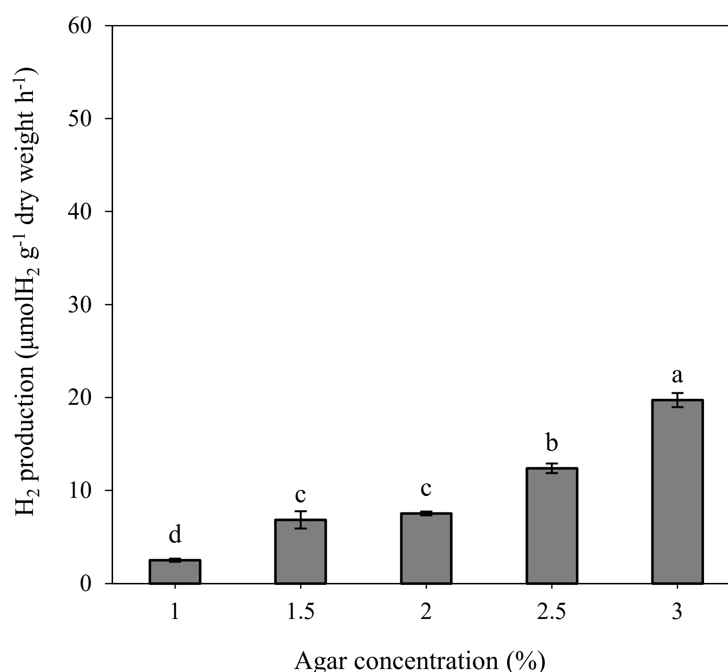


Figure 4.4 H_2 production by *A. halophytica* cells immobilized with various agar concentrations. The cell concentration was approximately 1 mg dry cell weight mL^{-1} of gel solution. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

The results showed that agar concentration was relevant for H_2 production by immobilized cells. It could be explained that higher agar concentrations resulted in an increased degree of polymerization. A high degree of polymerization led to a decrease in pore size of gel. The pore size of 3% (w/v) agar gel was small enough to prevent O_2 permeating into the gel, thus increasing bidirectional hydrogenase activity and finally resulting in the enhancement of H_2 production. In addition, it was reported that an increase of agar concentration might enhance an efficacy of cell survival and also enhance mechanical stability of gel (Seol *et al*., 2011). In this study, it was found that cell immobilization with 1% (w/v) agar concentration resulted in a difficulty to handle due to the gel softness and break. Higher agar concentrations than 3% (w/v) made cell immobilization also trouble since gels showed a lot of bubbles, a high viscosity and too rapid solidification during immobilization. From previous studies, agar concentrations used in immobilized cyanobacterial cells for H_2 production were prepared from 1.5 to 2.5% (w/v) (Karube *et al*., 1986; Phlips and Mitsui, 1986; Ramachandran and Mitsui, 1987; Kumar *et al*., 1991; Anjana and Kaushik, 2014). This result suggested that the mechanical stability of immobilization system was increased when agar concentration was increased, lead to the decreasd porosity that allows more difficult diffusion of O_2 into the matrix. Therefore, agar concentration at 3% (w/v) was used in optimization of cell immobilization for H_2 production in further experiments.

4.1.3 Effect of cell concentration on H_2 production by agar-immobilized cells

To investigate the effect of cell concentration on H_2 production by immobilized cells, *A. halophytica* cells were immobilized with 3% (w/v) agar by using various final cell concentrations at 0.2, 0.5, 1, 2, 3 and 5 mg dry cell weight mL^{-1}. Then, agar-immobilized cells were incubated in $BG11_0$ at 30 °C under the light for 24 h before purging with argon gas. H_2 production was measured under darkness. The result showed that immobilization with higher cell concentrations decreased H_2 production rate (Fig. 4.5). Agar-immobilized cells containing 0.2 mg dry cell weight mL^{-1} gave the highest H_2 production with 38.85 ± 1.91 μmolH_2 g^{-1} dry weight h^{-1} (Fig. 4.5). The higher cell concentrations than 0.2 mg dry cell weight mL^{-1} in agar-immobilized cells led to a decrease in H_2 production (Fig 4.5).

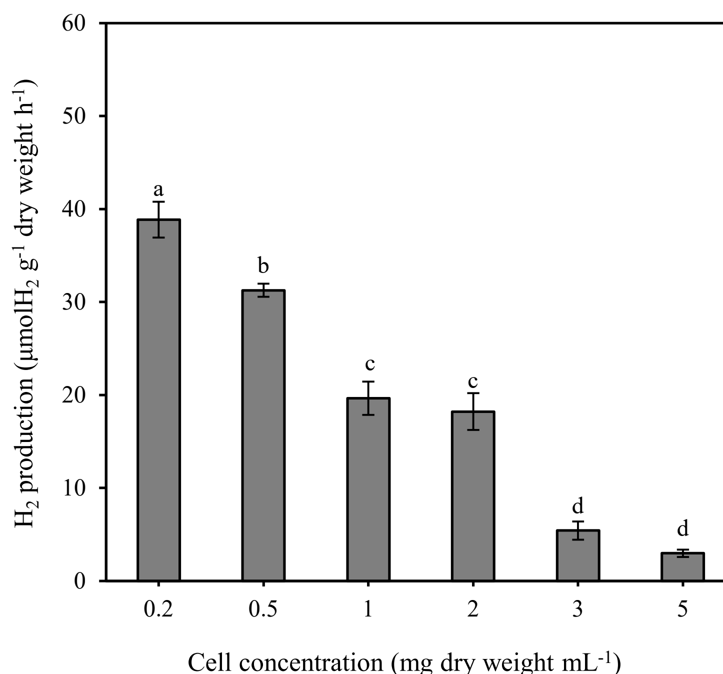


Figure 4.5 H_2 production by 3% (w/v) agar-immobilized cells of *A. halophytica* with various final cell concentrations. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

This result showed that cell concentration influenced H_2 production of agar-immobilized cells. The highest H_2 production rate was obtained in agar-immobilized cells with the lowest cell concentration (Fig 4.5). The decreased of H_2 production rate by immobilized cells with higher cell concentrations might be due to the availability of too many cell numbers. This led to the reduction of metabolic activity within cells including the reduction of electron flow towards bidirectional hydrogenase, finally resulting in a decrease in H_2 production. In addition, higher cell concentrations in immobilized cells resulted in the competition of substrate binding and also an insufficient amount of substrate or cofactor. This result was in line with previous studies showing that an increase in cell density of cyanobacteria in gel led to a decrease of H_2 production (Phlips and Mitsui, 1986; Ramachandran and Mitsui, 1987; Kumar *et al*., 1991; Anjana and Kaushik, 2014). However, these results of previous studies were all related to photohydrogen production. The reduction of H_2 production was due to either the photoinhibition or the effect of self-shading of cells in the rear portion of the gel layer during light exposure (Phlips and Mitsui, 1986; Ramachandran and Mitsui, 1987; Kumar *et al*., 1991). In this study, the measurement of H_2 production by agar-immobilized cells of *A. halophytica* was carried out under

darkness, thus eliminating the influence of light on H_2 production, suggesting the similar effect of cell concentration on dark fermentative and photo H_2 production by agar-immobilized cells of *A. halophytica.* Therefore, final cell concentration at 0.2, mg dry cell weight mL^{-1} was used in optimization of cell immobilization for H_2 production in further experiments.

4.1.4 Effect of volumetric size of cubes with agar-immobilized cells on H_2 production

To study the effect of volumetric size of agar-immobilized gels on H_2 production, *A. halophytica* cells were immobilized with 3% (w/v) agar and final cell concentration at 0.2 mg dry cell weight mL^{-1}. Then, agar gel cubes were cut into square pieces with different diameter sizes of 0.25, 0.50, 0.75 and 1 cm corresponding volumetric sizes of 0.0156, 0.125, 0.422 and 1 cm^3. The immobilized cells were incubated under N-deprivation at 30 °C under the light for 24 h before purging with argon gas. H_2 production was measured under darkness. The result showed that agar-immobilized gels with the volumetric size of 0.125 cm^3 showed the highest H_2 production rate with 39.14 ± 1.51 μmolH_2 g^{-1} dry weight h^{-1} (Fig. 4.6). Higher and lower volumetric size than 0.125 cm^3 decreased H_2 production by agar-immobilized cells of *A. halophytica*.

The agar-immobilized cells containing cubes with the volumetric size of 0.125 cm^3 showed the highest H_2 production (Fig. 4.6), suggesting that this size was suitable for contact a gas or medium in vial and optimal for H_2 to diffuse conveniently through the agar square cube. The volumetric sizes higher than 0.125 cm^3 showed a decrease of H_2 production due to the difficulty of medium or H_2 diffusion in or out of the gel. On the other hand, the smaller size of agar cube at 0.0156 cm^3 showed the lowest H_2 production, resulting from the decreased stability and strength of agar gel and the easy diffusion of O_2 through the cells, thus inhibiting bidirectional hydrogenase activity for H_2 evolution. In most previous reports, H_2 production by cyanobacteria was investigated in the cubes of agar-immobilized cells normally cut into square pieces with 0.5 cm of each side (or volumetric size of 0.125 cm^3) (Karube *et al*., 1986; Kumar *et al*., 1991; Rashid *et al*., 2009). However, it was found that 1 mm^3 agar cube of immobilized cells of *L. perelegans* gave the highest H_2 production compared to 0.125 or 8 mm^3 agar cube of those cells (Anjana and Kaushik, 2014). The volumetric size of 0.125 cm^3 was optimum for maximum H_2 production by *A. halophytica* and was used in next experiments.

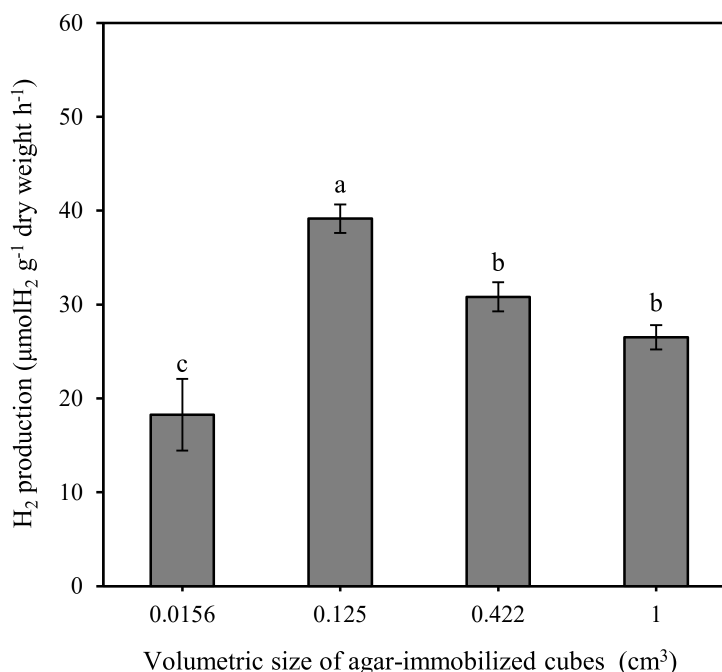


Figure 4.6 H_2 production by 3% (w/v) agar-immobilized cubes with different volumetric sizes. The final cell concentration was controlled at 0.2 mg dry cell weight mL^{-1}. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

4.1.5 Effect of nutrient and mineral concentrations on H_2 production by agar-immobilized cells

4.1.5.1 Effect of $NaNO_3$ concentration on H_2 production by agar-immobilized cells

To examine the effect of $NaNO_3$ concentration on H_2 production, *A. halophytica* cells immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 0.0176, 0.176, 1.76, 17.6 and 176 $NaNO_3$ at 30 °C under light for 24 h. Immobilized cells were purged with argon gas before measuring H_2 production in the dark. The result showed that the agar-immobilized cells incubated nitrogen-free medium gave the highest H_2 production with 39.26 ± 0.38 µmolH_2 g dry weight^{-1} h^{-1} (Fig. 4.7). When $NaNO_3$ concentrations were increased, H_2 production by agar-immobilized cells was significantly decreased (Fig. 4.7).

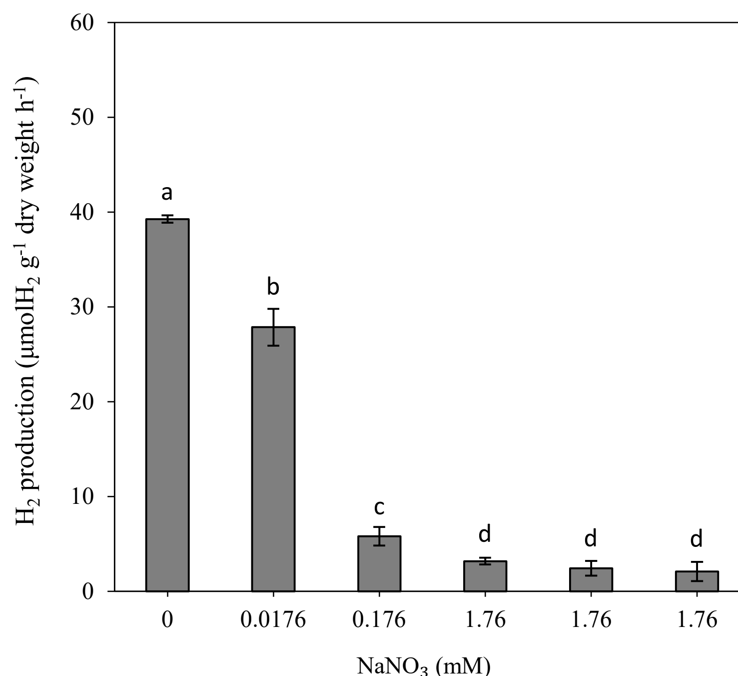


Figure 4.7 H_2 production by agar-immobilized cells of *A. halophytica* under various $NaNO_3$ concentrations. Immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 0.0176, 0.176, 1.76, 17.6 and 176 mM $NaNO_3$. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

The result showed that nitrogen deprivation led to the highest H_2 production by immobilized cells of *A. halophytica* and higher $NaNO_3$ concentrations caused a decrease in H_2 production (Fig. 4.7). It could be explained that under nitrogen deprivation *A. halophytica* changed its metabolism by inhibiting protein synthesis and accumulating glycogen instead. When cells entered H_2 production period under anaerobic condition, endogenous storage glycogen was consumed as an electron donor. The released electrons were used to generate H_2 molecules by a function of the bidirectional hydrogenase enzyme (Troshina *et al*., 2002). This result was similar to the previous studies investigated in the immobilized cells of filamentous, non-heterocystous *Plectonema boryanum* which produced H_2 when cells were incubated in NO_3^--free Chu-10 medium (Sarkar *et al*., 1992), in the immobilized cells of unicellular *Gloeocapsa alpicola* CALU 743 and *Synechocystis* sp. PCC 6803 which produced H_2 when cells were incubated in $BG11_0$ medium (Serebryakova and Tsygankov, 2007) and $BG11_0$-Tris medium (Touloupakis *et al*., 2016), respectively. In case of free cells, nitrogen deprivation resulted in the highest

H_2 production rate in several cyanobacteria such as *Oscillatoria* sp. Miami BG7, *Gloeocapsa alpicola*, *Arthrospira maxima* and *A. halophytica* (Kumazawa avd Mitsui, 1981; Serebryakova *et al*., 1998; Troshina *et al*., 2002; Ananyev *et al*., 2008; Taikhao *et al*., 2013).

4.1.5.2 Effect of NaCl concentration on H_2 production by agar-immobilized cells

A. halophytica cells immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0.25, 0.5, 0.75 and 1 M NaCl at 30 °C under light for 24 h. After adaptation, immobilized cells were purged with argon gas. H_2 production was measured under darkness. The result showed that the highest H_2 production with 39.09 ± 4.29 μmol H_2 g^{-1} dry weight h^{-1} was obtained in agar-immobilized cells incubated in $BG11_0$ containing 0.5 M NaCl (Fig. 4.8). H_2 production was decreased when immobilized cells were incubated in medium containing 0.25, 0.75 and 1 M NaCl (Fig. 4.8).

Under various NaCl concentrations, the maximum H_2 production rate was observed in immobilized cells treated with 0.5 M NaCl containing medium (Fig. 4.8). This concentration is normally found in the composition of BG11 medium. Normally, H_2 production on salinity is dependent on cyanobacterial species. A freshwater cyanobacterium *Anabaena variabilis* was highly sensitive to Na^+ concentration in the medium in an absence of nitrogen source. Its H_2 production was not shown in presence of NaCl (Shah *et al*., 2001). Similarly, freshwater cyanobacteria *Anabaena variabilis* SPU 003 and *Nostoc muscorum* gave the highest H_2 production in cells incubated in NaCl-free medium and their H_2 production was decreased when NaCl concentrations were increased (Shah *et al*., 2003). On contrary, marine cyanobacterium *Lyngbya* sp. strain 108 gave the highest H_2 production in medium containing 3% (w/v) or 0.5 M NaCl (Kuwada and Ohta, 1989) and marine non-heterocystous cyanobacterium *Phormidium valderianum* BDU 20041 showed the optimal H_2 production in salinity level up to 2.5% or 0.4 M NaCl (Prabaharan and Subramanian, 1996).

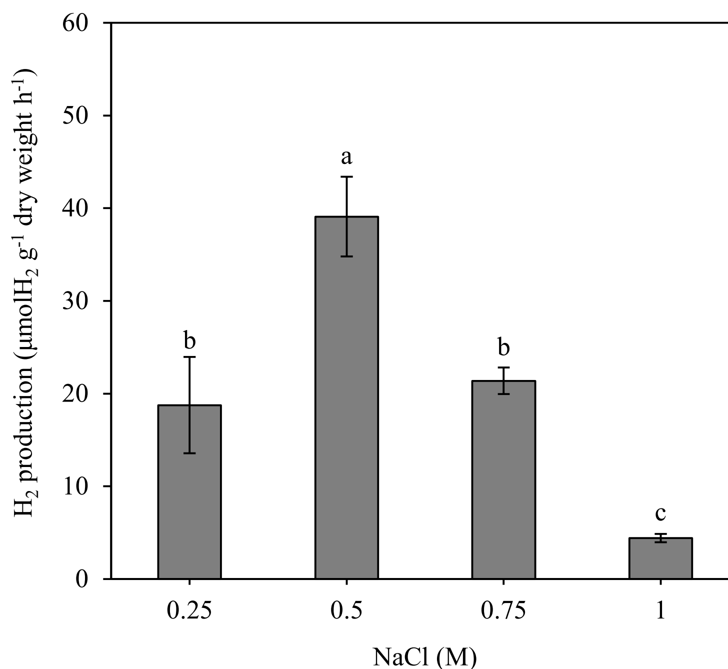


Figure 4.8 H_2 production by agar-immobilized cells of *A. halophytica* under various NaCl concentrations. Immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0.25, 0.5, 0.75 and 1 M NaCl. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

4.1.5.3 Effect of $MgSO_4.7H_2O$ concentration on H_2 production by agar-immobilized cells

To study effects of $MgSO_4.7H_2O$ concentration on H_2 production by immobilized cells, *A. halophytica* cells immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 1.5, 15, 30, 150 mM $MgSO_4.7H_2O$ at 30 °C under light for 24 h. After adaptation, immobilized cells were purged with argon gas. H_2 production was measured under darkness. The result revealed that no significant differences of H_2 production were shown in agar-immobilized cells incubated in $BG11_0$ containing $MgSO_4.7H_2O$ concentrations from 0 to 30 mM (Fig. 4.9). The significant decrease of H_2 production was found in agar-immobilized cells incubated in $BG11_0$ containing 150 mM $MgSO_4.7H_2O$ (Fig. 4.9).

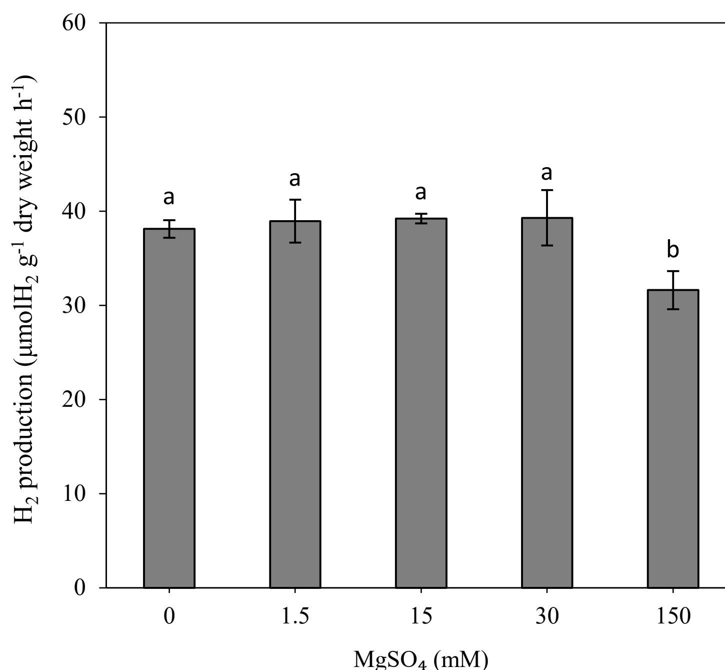


Figure 4.9 H_2 production by agar-immobilized cells of *A. halophytica* under various $MgSO_4.7H_2O$ concentrations. Immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 1.5, 15, 30, 150 mM $MgSO_4.7H_2O$. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

Sulfur is essential for all living organisms. It is a minor constituent of fats, body fluids, and skeletal minerals. Sulfur is a key component in most proteins since it is contained in the amino acids, methionine and cysteine. Sulfur-sulfur interactions are important in determining protein tertiary structure. In cyanobacteria, sulfur is an important constituent of D1 protein in Photosystem II. In this study, sulfur deprivation does not affect dark fermentative H_2 production by immobilized cells of *A. halophytica*. It might due to that H_2 production in this study was measured under dark anaerobic condition where the main electron sources used as H_2 generating substrate were obtained from the degradation of storage glycogen but not from the water splitting of PS II activity during photosynthesis. Although there were some cellular metabolic changes due to sulfur deprivation, it might not involve in H_2 metabolism. However, the effect of sulfur deprivation on H_2 production by cyanobacteria is species dependent. On contrary, it was reported that H_2 production by immobilized cyanobacterial cells of *M. aeruginosa* and *A. siamensis* TISTR 8012 was enhanced in cells incubated in sulfur deprived medium (Rashid *et al*., 2009; Rashid *et al*., 2012; Taikhao and Phunpruch, 2017). They suggested that an increase

of H_2 production by cyanobacteria under sulfur deprivation was resulted from the inactivation of photosystem II activity, resulting in inhibits O_2 evolution.

4.1.5.4 Effect of Fe^{3+} concentration on H_2 production by agar-immobilized cells

To study effects of Fe^{3+} concentration on H_2 production by immobilized cells, *A. halophytica* cells immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0. 0.04, 0.4, 4, 40 and 400 μM Fe^{3+} at 30 °C under light for 24 h. After adaptation, immobilized cells were purged with argon gas. H_2 production was measured under darkness. The result revealed that no significant differences of H_2 production by *A. halophytica* were found in immobilized cells incubated in $BG11_0$ containing 0-40 μM Fe^{3+} (Fig 4.10). An increase in Fe^{3+} concentration to 400 μM resulted in a significant decrease in H_2 production by immobilized cells (Fig 4.10).

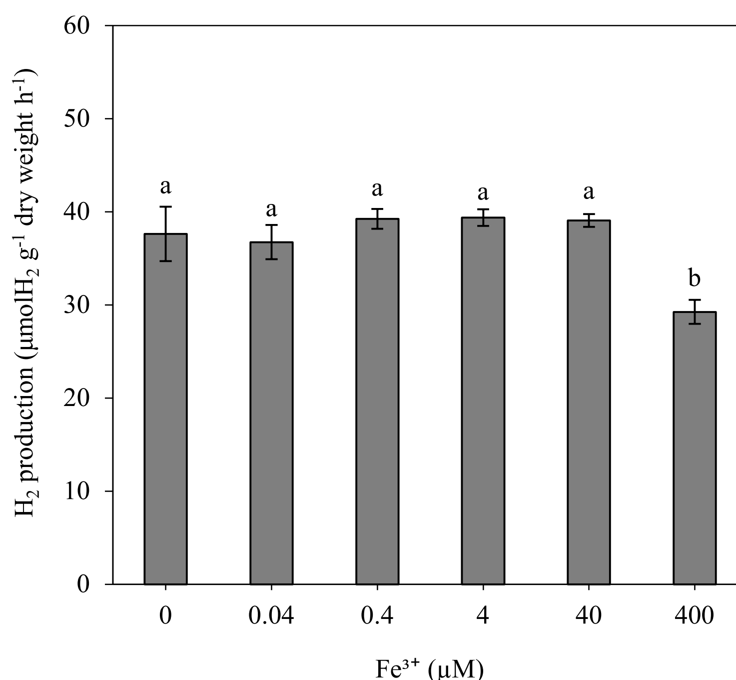


Figure 4.10 H_2 production by agar-immobilized cells of *A. halophytica* under various Fe^{3+} concentrations. Immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0. 0.04, 0.4, 4, 40 and 400 μM Fe^{3+}. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

In this experiment, iron concentrations had not significant effect on H_2 production by agar-immobilized cells of *A. halophytica*, especially Fe^{3+} concentration between 0-40 µM (Fig 4.10). Iron is one of important cofactors of many enzymes. It is also a cofactor of NiFe-hydrogenase (Peters *et al*., 2015). It functions to transport electrons from photosynthetic and/or dark fermentative pathways to hydrogenase for H_2 production (Lin and Stewart, 1997). In addition, iron has a role in electron transport system in photosynthesis and respiration in cyanobacteria (Raven *et al*., 1999). In free cells of *A. halophytica*, it has been reported that Fe^{3+} concentrations affected H_2 production. The optimal Fe^{3+} concentration for H_2 production was 0.4 µM. Higher and lower Fe^{3+} concentrations than 0.4 µM reduced H_2 production (Taikhao *et al*., 2013). Like other previous studies in free cells of *Anabaena cylindrica*, *A. siamensis* TISTR 8012 and *Synechocystis* sp. PCC 6803, an increase in iron concentrations also enhanced H_2 production (Jeffries *et al*., 1978; Baebprasert *et al*., 2011; Khetkorn *et al*., 2012). However, an increase in concentrations of Fe^{3+} did not increase H_2 production by immobilized *A. halophytica* cells. It might be due to the difficulty of Fe^{3+} permeability in agar cubes.

4.1.5.5 Effect of Ni^{2+} concentration on H_2 production by agar-immobilized cells

To study effects of Ni^{2+} concentration on H_2 production by immobilized cells, *A. halophytica* cells immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1} were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 0.1, 1, 10 and 100 µM Ni^{2+} at 30 °C under light for 24 h. After adaptation, immobilized cells were purged with argon gas. H_2 production was measured under darkness. The result showed that the agar-immobilized cells incubated in Ni^{2+}-free medium gave the highest H_2 production with 39.25 ± 0.14 µmolH_2 g dry weight^{-1} h^{-1} (Fig. 4.11). When Ni^{2+} concentration was increased, H_2 production by agar-immobilized cells was markedly decreased (Fig. 4.11).

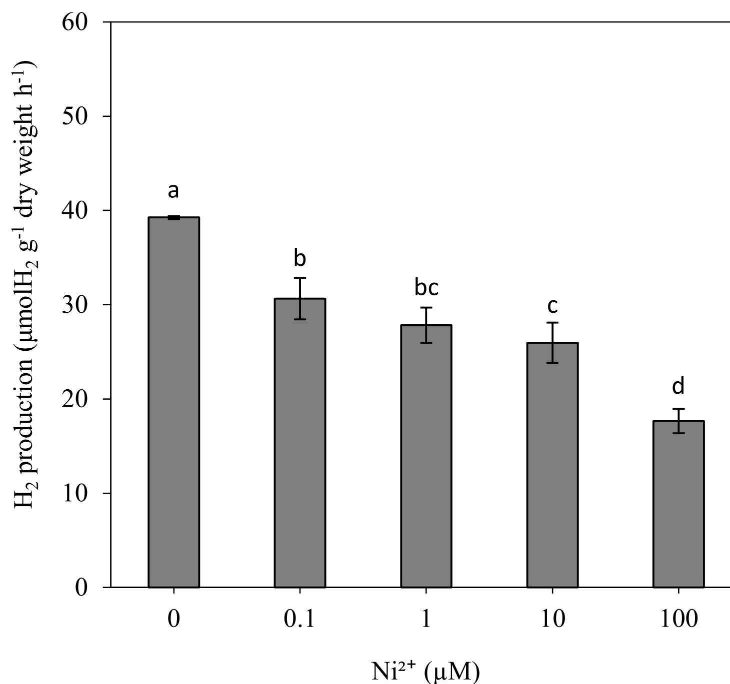


Figure 4.11 H_2 production by agar-immobilized cells of *A. halophytica* under various Ni^{2+} concentrations. Immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution containing 0, 0.1, 1, 10 and 100 µM Ni^{2+}. H_2 production was measured under darkness. Data are means ± SD (n = 3). Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

Nickel is a cofactor of NiFe-hydrogenase in cyanobacteria (Peters *et al*., 2015). Theoretically, the availability of nickel is important for function of hydrogenase activity. In previous reports, the optimal Ni^{2+} concentration for H_2 production by free cells of many kinds of cyanobacteria was ranged from 0.5 to 10 µM Ni^{2+} (Axelsson and Lindblad, 2002; Gutekunst *et al*., 2006; Rai and Raizada, 1986; Serebryakova *et al*., 1998). In *A. halophytica*, free cells produced the highest H_2 when 10 µM of external Ni^{2+} was added (Taikhao *et al*., 2013). In this study, it was suggested that the external nickel ion might not be required for H_2 production in immobilized cells of *A. halophytica*. It was possible that immobilized cells needed only little concentration of nickel ion and this low Ni^{2+} concentration has been already available in immobilized cells. In addition, too high concentrations of Ni^{2+} decreased H_2 production in agar-immobilized cells of *A. halophytica* due to the toxicity of nickel to cyanobacterial cells (Babich and Stotzky, 1983).

The effect of nutrient and mineral concentrations on H_2 production by agar-immobilized cells can be summarized in Table 4.1. The optimum nutrient and mineral concentrations of agar-immobilized *A. halophytica* for H_2 production were 0 mM $NaNO_3$, 0.5 M NaCl, 30 mM $MgSO_4.7H_2O$, 4 µM Fe^{3+} and 0 µM Ni^{2+}.

Table 4.1 H_2 production by immobilized cells of *A. halophytica* incubated in $BG11_0$ supplemented with Turk Island salt solution under various $NaNO_3$, NaCl, $MgSO_4{\cdot}7H_2O$, NaCl, Fe^{3+} and Ni^{2+} concentrations.

Composition	Concentration	H_2 production (µmol H_2 g^{-1} dry weight h^{-1})
$NaNO_3$ (mM)	0	39.26 ± 0.38
	0.0176	27.85 ± 1.95
	0.176	5.78 ± 0.96
	1.76	3.19 ± 0.37
	17.6	2.10 ± 1.01
	176	2.43 ± 0.78
NaCl (M)	0.25	18.75 ± 5.19
	0.5	39.09 ± 4.29
	0.75	21.37 ± 1.43
	1	4.41 ± 0.45
$MgSO_4{\cdot}7H_2O$ (mM)	0	38.11 ± 0.94
	1.5	38.94 ± 2.28
	15	39.20 ± 0.51
	30	39.29 ± 2.94
	150	31.62 ± 2.02
Fe^{3+} (µM)	0	37.63 ± 2.91
	0.04	36.74 ± 1.84
	0.4	39.23 ± 1.06
	4	39.37 ± 0.91
	40	39.06 ± 4.31
	400	29.25 ± 1.30
Ni^{2+} (µM)	0	39.25 ± 0.14
	0.1	30.66 ± 2.20
	1	27.84 ± 1.86
	10	25.97 ± 2.14
	100	17.65 ± 1.30

4.1.6 Effect of physical factors on H_2 production by agar-immobilized cells

4.1.6.1 Effect of initial pH of medium on H_2 production by agar-immobilized cells

A. halophytica cells were immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1}. The agar-immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution, whose initial pH of medium was adjusted to pH 6-10 by universal buffer (Britton and Robinson, 1931), at 30 °C under the light for 24 h. After adaptation, immobilized cells were purged with argon gas. H_2 production was measured under darkness. The result showed that the highest H_2 production with 39.13 ± 1.15 μmolH_2 g^{-1} dry weight h^{-1} was obtained in immobilized cells incubated in medium at initial pH 7.4 but this H_2 production did not show significant differences with that of immobilized cells incubated in medium at initial pH 8.0 (Fig. 4.12). Higher and lower initial pH than 7.4 and 8.0 resulted in a significant decrease in H_2 production (Fig. 4.12). Moreover, the lowest H_2 production of agar-immobilized cells was found when incubated in medium with initial pH 6 (Fig. 4.12).

In this study, agar-immobilized cells of *A. halophytica* incubated in $BG11_0$ supplemented with Turk Island salt solution at initial pH 7.4 gave the highest H_2 production (Fig. 4.12). The initial pH at 7.4 of medium is normally found in BG11 and suitable for cyanobacterial growth and intracellular metabolism (Rippka *et al*., 1979). It is suggested that bidirectional hydrogenase activity of agar-immobilized cells of *A. halophytica* might be favorable at neutral pH. However, it has been previously reported that H_2 production by free cells of *A. halophytica* was highest when cells were incubated in optimal seawater at pH 6.0 (Taikhao *et al*., 2015). Therefore, cell immobilization and type of media might alter the response of cells to changes of pH with respect to H_2 production.

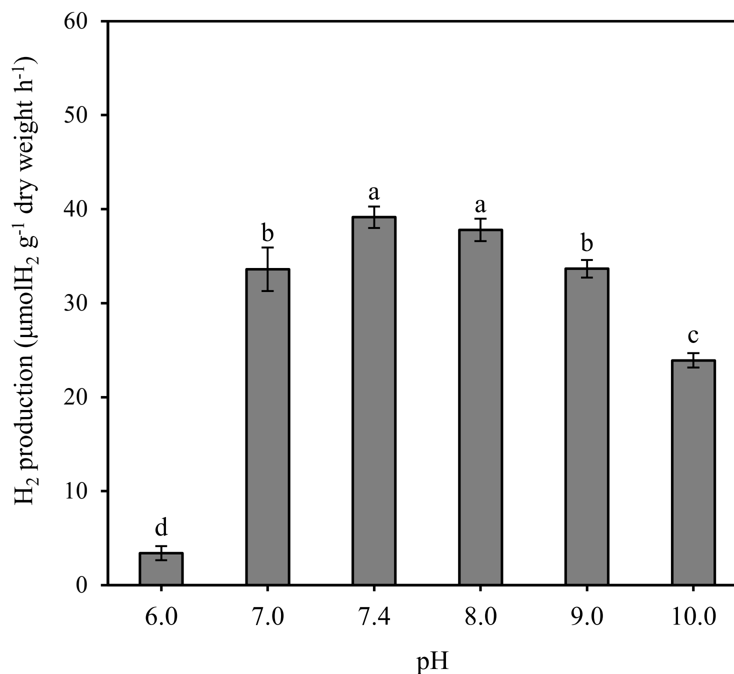


Figure 4.12 H_2 production by agar-immobilized cells of *A. halophytica* under various initial pH values of medium. Agar-immobilized cells were incubated in $BG11_0$ supplemented with Turk Island salt solution with initial pH values at 6.0, 7.0, 7.4, 8.0, 9.0 and 10.0. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

4.1.6.2 Effect of incubation temperature on H_2 production by agar-immobilized cells

A. halophytica cells were immobilized with 3% (w/v) agar concentration and final cell concentration of 0.2 mg dry cell weight mL^{-1}. The agar-immobilized cells were incubated in $BG11_0$ (pH 7.4) supplemented with Turk Island salt solution at 30 °C under the light for 24 h. After adaptation, immobilized cells were purged with argon gas before dark anaerobic incubation at temperature 20 to 45 °C. H_2 production was measured under darkness. The result showed that the highest H_2 production rate with 54.30 ± 1.38 $\mu molH_2\ g^{-1}$ dry weight h^{-1} was observed in immobilized cells incubated at 40 °C (Fig. 4.13). H_2 production rate was decreased when immobilized cells were incubated at higher or lower temperature than 40 °C (Fig. 4.13).

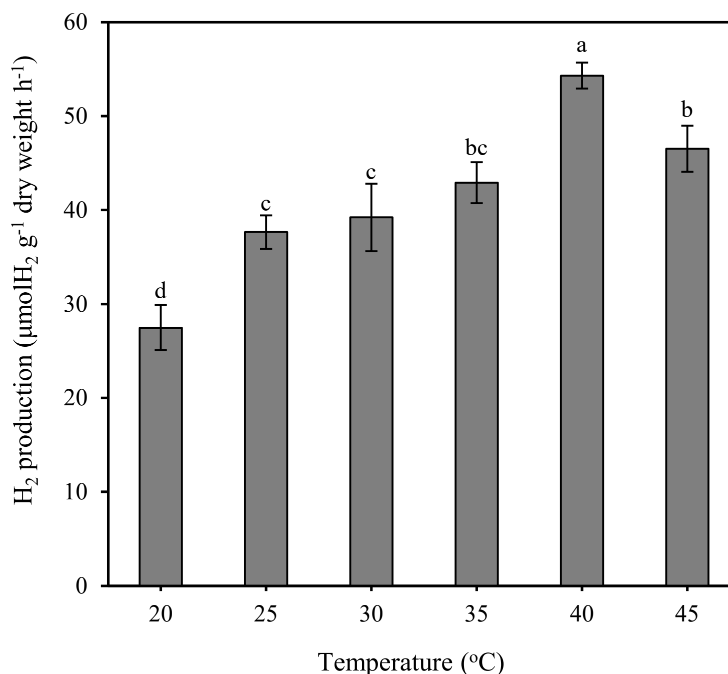


Figure 4.13 H_2 production by agar-immobilized cells of *A. halophytica* under various incubation temperatures. Agar-immobilized cells were incubated in $BG11_0$ supplemented with Turk Island salt solution (pH 7.4) under darkness anaerobic condition at temperature from 20 to 45 °C. H_2 production was measured under darkness. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

In this study, it was shown that the temperature at 40 °C was favorable for H_2 production by agar-immobilized cells of *A. halophytica* (Fig. 4.13). However, too high temperature (45 °C) decreased H_2 production by agar-immobilized cells. It was suggested that cells could not survive after incubation under high temperature for a long time. In addition, agar gel was not stable and did not show a strength and flexibility at higher temperature. This result was consistent with previous studies showing that H_2 production was increased with an increase in temperature and reached the maximum when agar-immobilized cells of *Oscillatoria* sp. Miami BG7 and *M. aeruginosa* were incubated at 37 °C and 40 °C, respectively (Phlips and Mitsui, 1986; Rashid *et al*., 2009). For free cells of *A. halophytica*, the optimal temperature for H_2 production was at 35 °C (Taikhao *et al*., 2013), indicating that the agar-immobilized cells were slightly more tolerant to high temperature than free cells.

4.1.6.3 Effect of volumetric size of glass vial, headspace and immobilized cells on H_2 production by agar-immobilized cells

A. holophytica was immobilized in 3 % (w/v) agar gels with final cell concentration of 0.2 mg dry cell weight mL^{-1}. To investigate the effect of volumetric size of glass vial, agar-immobilized cubes with total volumes of 6 and 36 cm^3 were transferred to 20-mL and 120-mL of glass vial containing 9 and 54 mL of $BG11_0$ medium, respectively, in order that the constant volumetric ratio of immobilized cells, medium and headspace was obtained at 6:9:5. The immobilized cubes were incubated at 30 °C under for 24 h before purging with argon gas. The agar-immobilized cubes were incubated at 40 °C under dark anaerobic condition. The result showed that H_2 production rates of agar-immobilized cells in both volumetric sizes of glass vial were quite similar. The maximum H_2 production rate of agar-immobilized cells in 20- and 120-mL glass vial were 63.03 ± 3.10 and 67.44 ± 9.27 μmol H_2 g^{-1} dry weight h^{-1}, respectively (Table 4.2). By consideration of H_2 production yield, it was found that the maximum H_2 production yield of agar-immobilized cells in a 120-mL glass vial was a 2-fold higher than that that in a 20-mL glass vial (Table 4.2). The maximum H_2 production of agar-immobilized cells in 120-mL glass vial was 2,169.87 ± 191.66 μmol H_2 g^{-1} dry weight (Table 4.2).

To investigate the effect of headspace volume on H_2 production, 36 cm^3 of immobilized cells were added into a 120-mL glass vial and medium was added into vials with the different volumes in order to adjust headspace volume tos 15-40 mL. The result revealed that H_2 production of immobilized was related to the headspace volume. Higher headspace volumes resulted in higher H_2 production. The maximum H_2 production rate and yield with 77.03 ± 1.46 μmol H_2 g^{-1} dry weight h^{-1} and 2,209.15 ± 19.38 μmol H_2 g^{-1} dry weight, respectively, were found in agar-immobilized cells in a 120-mL glass vial and 40 mL of headspace volume (Table 4.2).

Table 4.2 Effect of sizes of glass vial, headspace volume and immobilized cells volume on maximum H_2 production by immobilized cells of *A. halophytica.*

Volumetric size (mL)				Maximum H_2 production rate (μmol H_2 g^{-1} dry weight h^{-1})	Maximum H_2 production yield (μmol H_2 g^{-1} dry weight)
Glass vial	Immobilized cells	Medium	Headspace		
20	6	9	5	63.03 ± 3.10	1,034.72 ± 32.16
120	36	54	30	67.44 ± 9.27	2,169.87 ± 191.66
120	36	69	15	38.96 ± 3.78	1,428.82 ± 8.96
120	36	66	18	51.97 ± 6.15	1,644.28 ± 212.29
120	36	64	20	57.45 ± 4.47	1,847.13 ± 100.76
120	36	60	24	63.97 ± 1.06	1,965.45 ± 108.63
120	36	54	30	67.44 ± 9.27	2,169.87 ± 191.66
120	36	44	40	77.03 ± 1.46	2,209.15 ± 19.38
120	54	36	30	55.94 ± 0.99	1,868.30 ± 120.23
120	36	54	30	67.44 ± 9.27	2,169.87 ± 191.66
120	24	66	30	117.44 ± 0.72	3,497.03 ± 131.76
120	15	75	30	135.54 ± 1.92	4,410.88 ± 56.84

To study the effect of immobilized cell volume on H_2 production, agar-immobilized cells with total volumes of 15, 24, 36 and 60 cm^3 were added into 120-mL glass vials and different volumes of medium in order that headspace volume was fixed at 30 mL. It was found that the maximum H_2 production rate and yield with 135.54 ± 1.92 μmol H_2 g^{-1} dry weight h^{-1} and 4,410.88 ± 56.84 μmol H_2 g^{-1} dry weight, respectively, were obtained when 15 cm^3 of immobilized cells and 70 mL of medium were used (Table 4.2). The maximum H_2 production rate and yield were decreased when immobilized cell volume was increased (Table 4.2).

In this study, the volumetric sizes of glass vial, headspace volume and immobilized cells affect H_2 production by agar-immobilized cells (Table 4.2). Higher glass vial volume did not change H_2 production rate but increased H_2 production yield. It could be explained that cells produced H_2 at the normal rate but there was more headspace volume in larger glass vial volume. Therefore, cells provided higher saturation of H_2 concentration in larger vial. In addition, when the same sizes of glass vial and immobilized cells were used, an increase in the headspace volume caused an increase in H_2 production (Table 4.2), indicating that H_2 production was dependent

on the headspace volume. The reason for this increase might due to the equilibrium of hydrogenase toward H_2 production rather than H_2 uptake when there is a larger headspace volume. Bidirectional hydrogenase is a reversible enzyme that is capable of both synthesizing and dissociating H_2. As any chemical reaction, the equilibrium between the H_2 production and H_2 uptake depends on the concentrations of substrates and products. In a larger headspace volume, H_2 accumulation was higher than H_2 accumulation in a small headspace volume, resulting from the low H_2 partial pressure in the headspace. Thus, bidirectional hydrogenase catalyzed H_2 evolution rather than H_2 consumption. This result was consistent with that found in three microalgal strains of *Chlamydomonas reinhardtii* (CC-125, CC-4169 and CC-4170) showing that H_2 production per volume of culture increased when the headspace volume was increased (Altimari *et al*., 2014). In addition, in *Clostridium acetobutylicum* ATCC 824, the enlarged headspace volume could also enhance H_2 production (Oh *et al*., 2009). Finally, the lowest volumetric size of immobilized cells gave the maximum H_2 production rate and H_2 accumulation (Table 4.1). This might due to that H_2 has facile diffusion when using a low number of immobilized cells.

Our results showed that the maximum H_2 production rate with 135.54 μmol H_2 g dry wt^{-1} h^{-1} or 12.71 μmol H_2 mg $chla^{-1}$ h^{-1} was obtained in agar-immobilized cells of *A. halophytica* incubated in $BG11_0$ medium in 120 mL glass at 40 °C under dark anaerobic condition. When compared with H_2 production by immobilized cells of other cyanobateria, it was found that agar-immobilized *A. halophytica* could produce higher H_2 than some immobilized cyanobacterial strains such as *Lyngbya perelegans* and *Anabaena siamensis* TISTR 8012 and could produce H_2 at the same rate with *Oscillatoria subbrevis* strain 111 and wild type of *Anabaena* sp. PCC 7120 (Table 4.3). However, it produced less H_2 than immobilized *Anabaena* N-7363 and *Synechocystis* sp. PCC 6803 (Table 4.3). Therefore, H_2 production of immobilized cells was suggested to be dependent on the type of cyanobacteria and physiological parameters including cultivation and immobilization conditions.

Table 4.3 Comparison of maximum H_2 production by various cyanobacterial species and strains

Species/Strains	Type of support material	Maximum H_2 production	Conditions	References
Aphanothece halophytica	3% (w/v) Agar	135.54 µmol H_2 g^{-1} dry wt h^{-1} 12.71 µmol H_2 mg^{-1} chla h^{-1}	$BG11_0$ (pH 7.4), 40 °C, dark anaerobic condition	This study
Oscillatoria sp. Miami BG 7	1.5% (w/v) Agar	1.4 mL H_2 mg^{-1} dry wt h^{-1}	AN (N-deprived), 35 °C, 120 µE m^{-2} s^{-1}, anaerobic condition	Phlips and Mitsui, 1986
Oscillatoria subbrevis strain 111	1.5% (w/v) Agar 1.5% (w/v) Alginate	16.97 µmol H_2 mg^{-1} chla h^{-1} 18.83 µmol H_2 mg^{-1} chla h^{-1}	BG11 (pH 7.5), 28 °C, 2,500 lux, anaerobic condition	Kumar *et al*., 1991
Anabaena N-7363	2% (w/v) κ-carrageenan	162 µmol H_2 g^{-1} dry wt h^{-1}	BG11 (pH 8), 35 °C, 6,000 lux, anaerobic condition	Karube *et al*., 1986
Microcystis aeruginosa	1.5% (w/v) Agar	48 mL L^{-1} h^{-1}	MA (S-deprived) (pH 8.6), 42 °C, dark anaerobic condition	Rashid *et al*., 2009
Anabaena sp. PCC 7120 wild type and Δ*hupL*	4% (w/v) alginate	13 µmol H_2 mg^{-1} chla h^{-1} 30 µmol H_2 mg^{-1} chla h^{-1}	Z8 (N-deprived), 26 °C, 130 µmol photons m^{-2} s^{-1}, anaerobic condition	Leino *et al*., 2012
Lyngbya perelegans	2.5% (w/v) Agar 2% (w/v) Alginate	22.06 µmol H_2 g^{-1} dry wt h^{-1} 24.06 µmol H_2 g^{-1} dry wt h^{-1}	BG11, 25 °C, 21 h light/3 h, dark anaerobic condition	Anjana and Kaushik, 2014
Synechocystis sp. PCC 6803	3% (w/v) Alginate	40.6 µmol H_2 mg^{-1} chla h^{-1}	$BG11_0$-Tris (pH 7.4), 28°C, 60 µmol photons m^{-2} s^{-1}, air/CO_2	Touloupakis *et al*., 2016
Anabaena siamensis TISTR 8012	1.5% (w/v) Agar 3% (w/v) Alginate	0.25 µmol H_2 mg^{-1} chla h^{-1} 0.34 µmol H_2 mg^{-1} chla h^{-1}	$BG11_0$-S medium, anaerobic dark condition, room temperature	Taikhao and Phunpruch, 2017

4.1.7 Cycle of H_2 production by immobilized cells

To investigate the reuse of agar-immobilized cells for H_2 production, three cycles of H_2 production were investigated by immobilized cells compared to those by free cells. *A. holophytica* cells were immobilized in 3 % (w/v) agar gels with final cell concentration of 0.2 mg dry cell weight mL^{-1}. Thirty-six mL of agar-immobilized agar cubes and free cells were transferred into a 120-mL glass vial containing 54 mL of $BG11_0$ supplemented with Turk Island salt solution and incubated at 30 °C under the light for 24 h. Then, the air in glass vial was removed by purging argon gas for 20 min. For each cycle, H_2 production was analyzed under darkness for 36 h. It was found that in the first cycle of H_2 production, agar-immobilized cells produced the highest H_2 yield with 1,294.27 ± 108.24, 1,271.84 ± 58.07, 1,272.99 ± 22.31 and 1256.44 ± 34.18 μmol H_2 g^{-1} dry weight, respectively. This H_2 production was approximately 3 times higher than that by free cells (Fig. 4.14), suggesting that immobilization could prevent cells from O_2 in the system. The O_2 reduction gave rise to the induction of bidirectional hydrogenase activity and thus resulting in the higher H_2 production. The H_2 production was highest at 24 h of dark anaerobic incubation and remained constant until 36 h of incubation (Fig. 4.14). In the second cycle, H_2 production of both agar-immobilized cells and free cells was decreased and increasingly decreased in the third cycle. It could be explained that *A. halophytica* mainly produces H_2 via a catabolism of storage glycogen in dark anaerobic condition by bidirectional hydrogenase activity (Taikhao *et al*., 2015; Phunpruch *et al*., 2016). The carbohydrates accumulated and reducing powers were consumed quickly under anaerobic and nitrogen-starvation conditions for generating H_2 in the first cycle. Therefore, these substances might not be sufficient to produce high H_2 for the second and third cycles. However, an attempt to increase H_2 production in the second and third cycles was performed. After first cycle, agar-immobilized cells and free cells were transferred into the fresh BG11 in order to refresh cells in the normal medium. Expectedly, cells might produce substrates of H_2 production and can use in the next cycle. Unfortunately, H_2 production in the second and third cycles was remained decreased.

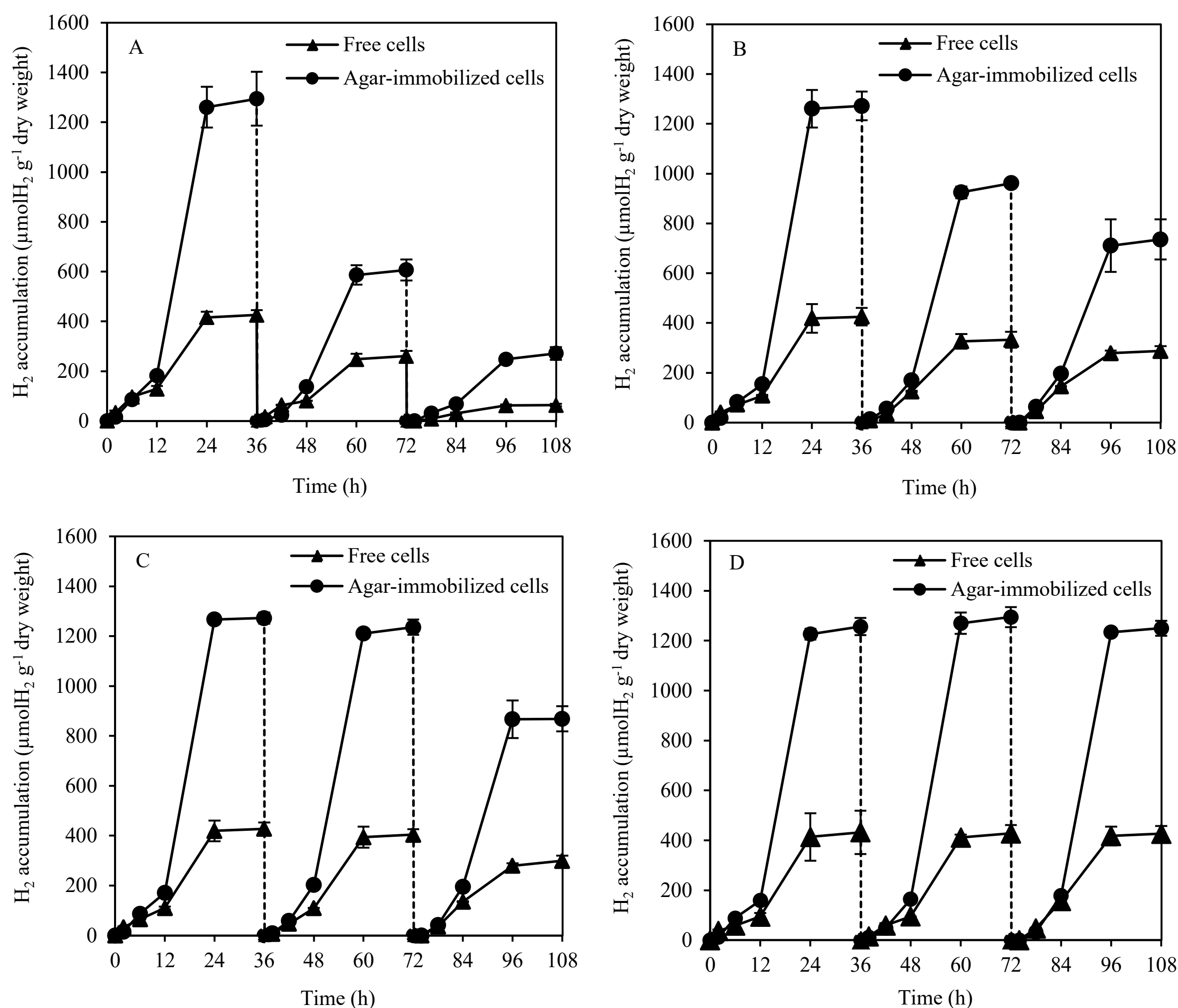


Figure 4.14 H_2 production cycles by immobilized cells of *A. halophytica* under N-deprivation. The cubes of immobilized cells were used in another two cycles of H_2 production without a replacement with fresh cubes of immobilized cells (A), with 30 % (B), 50 % (C) and 100 % (D) replacement with fresh agar cubes in cycles 2 and 3. Free cells experiments were done in the same manner but without a procedure of cell immobilization.

Another effort to recycle immobilized cells for H_2 production was achieved by the replacement by fresh immobilized cells. It can be a choice for sustainable H_2 production by immobilized cells of *A. halophytica*. After first cycle of H_2 production, 30, 50 and 100 % of immobilized cells and free cells were replaced with the newly fresh immobilized cells and free cells in the second and third cycle for H_2 production. The result showed that an increase in volume of fresh cells gave rise to the higher H_2 production by *A. halophytica* (Fig. 4.14 B-D). Interestingly, H_2 production of 50 and 100% of newly fresh immobilized cells replacement in second cycle was as high as H_2 production in the first cycle (Fig. 4.14 C,D). Whereas H_2

production by immobilized cells replaced with 30% of newly fresh immobilized cells was lower than that with 50 and 100% of newly fresh immobilized cells in the second cycle (Fig. 4.14 B). However, in the previous study, it was found that *Calothrix* 336/3 strain and Δ*hupL* mutant cells immobilized with alginate showed prolonged H_2 production over several cycles whereas H_2 production reduced significantly after the first cycle in suspension cultures (Leino *et al*., 2012). The agar-immobilized cells of cyanobacterium *Microcystis aeruginosa* produced H_2 up to 3 cycles and in each cycle, H_2 production was stably generated for more than 40 h (Rashid *et al*., 2009). The cyanobacterium *Gloeocapsa alpicola* CALU 743 immobilized in glass fiber could produce H_2 in a long-term cyclic regime and provided relatively stable H_2 production over a period of not less than 20 days (Serebryakova and Tsygankov, 2007).

4.2 Improvement of H_2 production of *A. halophytica* by use of Inhibitor

The study of improvement of H_2 production of *A. halophytica* by use of inhibitor was divided into two parts; (1) screening of inhibitors affecting H_2 production by *A. halophytica* and (2) effect of the effective inhibitor on H_2 metabolism and involving metabolisms of *A. halophytica.*

4.2.1 Screening of inhibitors affecting H_2 production by *A. halophytica*

H_2 production of *A. halophytica* cells treated with various kinds and concentrations of inhibitors was performed under dark and light anaerobic conditions for 2 h. *A. halophytica* cultivated in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested, washed twice and resuspended in 100 ml of $BG11_0$. Cells were then incubated at 30 °C under the light for 24 h before harvesting and resuspended in 5 mL of $BG11_0$. The cell suspension was transferred to glass vial and added with several inhibitors containing various concentrations. The vial was incubated at 30°C under the light 2 h before purging with argon gas followed by incubation at 30°C under the light or darkness for 2 h before H_2 measurement.

4.2.1.1 Effect of photosystem II inhibitors on H_2 production by *A. halophytica*

To investigate effect of photosystem II inhibitor on H_2 production by *A. halophytica*, four kinds of PSII inhibitor with different concentrations were added into the cell suspension, atrazine (0-50 μM), CCCP (0-5 μM), DCMU (0-250 μM), and glyphosate (0-300 μM). The result showed that *A. halophytica* cells incubated with and without inhibitor under darkness produced H_2 higher than those under the

light (Fig. 4.15). Under light illumination H_2 production by *A. halophytica* cells treated with all photosystem II inhibitors was higher than that without inhibitors (Fig. 4.15). However, under darkness H_2 production by *A. halophytica* cells treated with atrazine, CCCP and glyphosate seemed to be higher than that without those inhibitors (Fig. 4.15A,B,D). An increase in H_2 production related to higher concentrations of inhibitors (Fig. 4.15A,B,D). Only cells treated by DCMU did not show higher H_2 production when DCMU concentrations were increased (from 0-125 µM) (Fig. 4.15C). The highest H_2 production rate with 40.56 ± 1.35 µmol H_2 g^{-1} dry weight h^{-1} was found in cells treated with 0.5 µM CCCP in the dark (Fig. 4.15B). Whereas the highest H_2 production rates with 36.85 ± 0.90 and 23.44 ± 0.58 µmol H_2 g^{-1} dry weight h^{-1} were found in cells treated with 3 µM glyphosate and 0.5 µM atrazine, respectively (Fig. 4.15D,A).

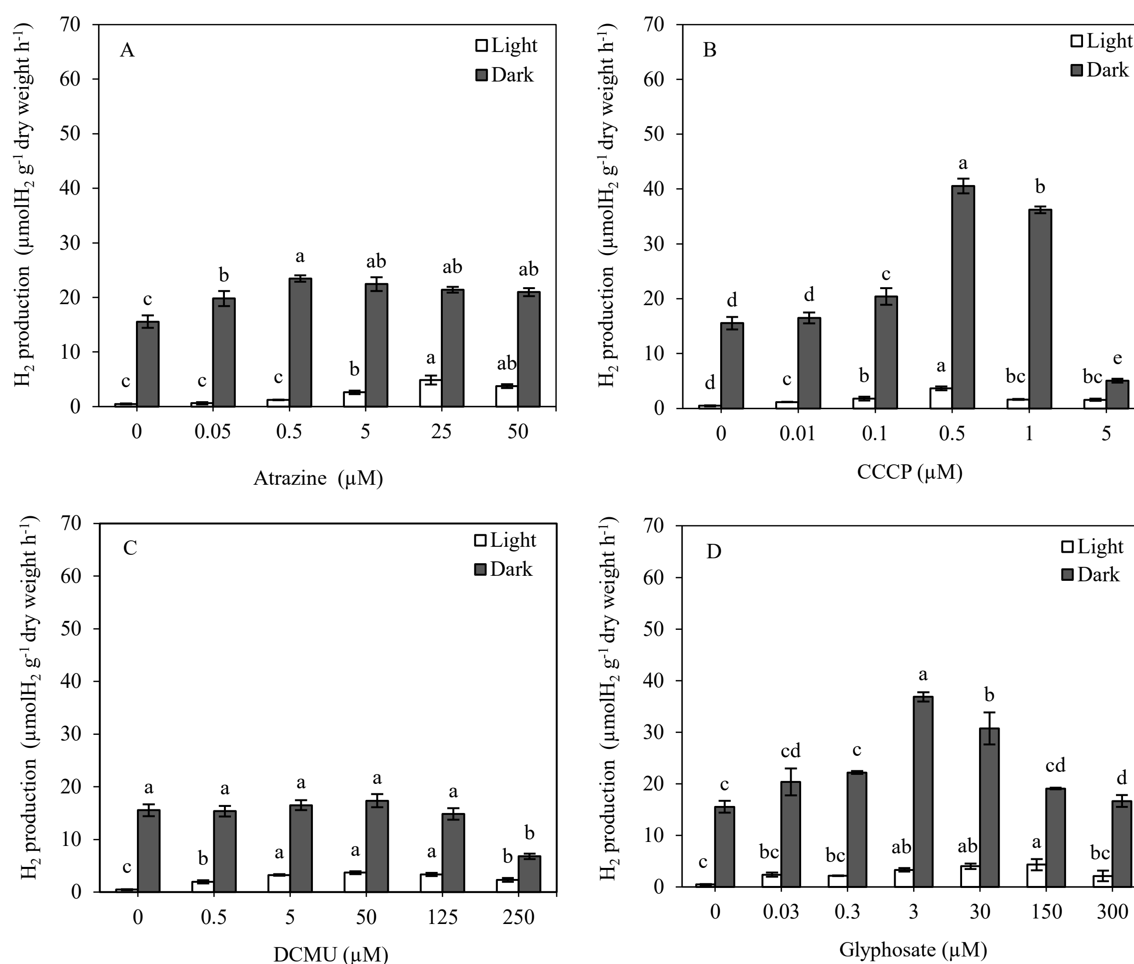


Figure 4.15 Effect of photosystem II inhibitors, atrazine (A) CCCP (B) DCMU (C) and glyphosate (D) on H_2 production by *A. halophytica* after incubation under light and dark anaerobic condition for 2 h. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

The study showed that the type and concentration of photosystem II inhibitor influenced H_2 production by *A. halophytica.* Moreover, the presence and absence of light also showed significant effect on H_2 production. Although all inhibitors investigated were classified as photosystem II inhibitors, but they might play different roles in H_2 metabolism and even photosynthesis. In *A. halophytica*, CCCP treatment to *A. halophytica* cells promoted the highest H_2 production under dark condition (Fig. 4.15B). CCCP is a known photosystem II inhibitor that accelerates the deactivation reactions of water-splitting enzyme system in photosystem II and finally reducing O_2 production (Samuilov and Barsky, 1993). The reduction of O_2 production led to improve bidirectional hydrogenase for producing H_2. Moreover, CCCP can also function as an uncoupling agent of oxidative phosphorylation inhibitor. It can disrupt the proton motive force by transporting protons across lipid bilayer component of membrane resulting in ATP synthesis inhibition. Therefore, a large number of electrons and protons can be transferred to bidirectional hydrogenase to enhance H_2 production (Ran *et al*., 2006). This study was consistency with the previous study in cyanobacteria *Oscillatoria chalybea*, *Synechocystis* PCC 6803, *Platymonas helgolandica* var. *tsingtaoensiswere* and green alga *Chlamydomonas reinhardtii* showing that cells treated with CCCP could enhance H_2 production (Abdel-Basset and Bader, 1998; Zang *et al*. 2012; Yang *et al*., 2014).

Other photosystem II inhibitor, atrazine has been found to affect algal responses with a wide variety depending on concentrations, duration of exposure and type of algae (Tang *et al*., 1998). Atrazine binds to the D1 protein in photosystem II and prevents the binding of plastoquinone, resulting in an inhibition of electron transport chain. Consequently, photosystem activity and water splitting are failed (Fuerst and Norman, 1991). It has been reported that cyanobacterium *Nostoc muscorum* treated with atrazine gave enhanced H_2 production (Spiller *et al*., 1978). Other photosystem II inhibitor, glyphosate has been reported to inhibit the photosynthetic electron transport and O_2 evolution in both wild type and mutant cells of *Anabaena doliolum* (Singh and Singh, 2004). Inhibition of photosystem II activity led to the decreased O_2 evolution that enhanced bidirectional hydrogenase activity. The last photosystem II inhibitor, DCMU blocks electron transfer between the Q_A and Q_B on the reducing side of photosystem II (Metz *et al*., 1986). This interrupts the photosynthetic electron transport chain in photosynthesis and thus reduces molecular oxygen from water-splitting in photosystem II. H_2 photoevolution by a new marine green alga, *P. helgolandica* var. *tsingtaoensis* was increased when treated with DCMU due to the complete PSII inhibition by DCMU (Zhang *et al*., 2012).

Likewise, the cyanobacterium *Anabaena cylindrica* treated with DCMU improved H_2 production resulting from the low level of O_2 content (Chen *et al*., 2013).

4.2.1.2 Effect of respiration inhibitors on H_2 production by *A. halophytica*

To study effect of respiration inhibitors on H_2 production by *A. halophytica*, malonic acid (0-100 mM), rotenone (0-100 μM), and sodium azide (0-10 μM) were added into the cell suspension and incubated under light and dark anaerobic conditions for 2 h before analyzing H_2. The result showed that *A. halophytica* treated with rotenone could improve H_2 production under both dark and light conditions (Fig. 4.16). The highest H_2 production rate with 30.96 ± 1.34 μmol H_2 g^{-1} dry weight h^{-1} was found in cells treated with 1 μM rotenone under dark condition (Fig. 4.16B). On the other hand, *A. halophytica* could not enhance H_2 production when cells were treated with malonic acid and sodium azide (Fig. 4.16A,C). H_2 production of *A. holophytica* treated with all respiration inhibitors under light was slightly increased (Fig. 4.16A-C).

The result in this study showed that among respiratory inhibitors, rotenone was the effective inhibitor which could obviously enhance H_2 production by *A. holophytica* under both light and dark conditions. It might be due to that rotenone disallows the electron transfer to plastoquinone by inactivating NAD(P)H dehydrogenase activity. As a result, the respiration mechanism is inhibited (Teicher and Scheller, 1998). In the previous report, rotenone treatment significantly increased H_2 production in N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012 under N-deprivation (Khetkorn *et al*., 2012). Respiratory electron transport competes for utilization of electrons that potentially may flow to the H_2 metabolic pathway. Thus, inhibition of respiratory would result in redirecting electron flow to bidirectional hydrogenase and hence improving H_2 production. On contrary, treatment of malonic acid and sodium azide did not show significantly effect on H_2 production by *A. holophytica.* It might be suggested that type of inhibitors was important and responsible for different respiratory mechanisms. The result in this study was contrast to what found in *Synechocystis* sp. PCC 6803 showing that cells treated with malonate increased H_2 production about 30 folds (Burrows *et al*., 2001).

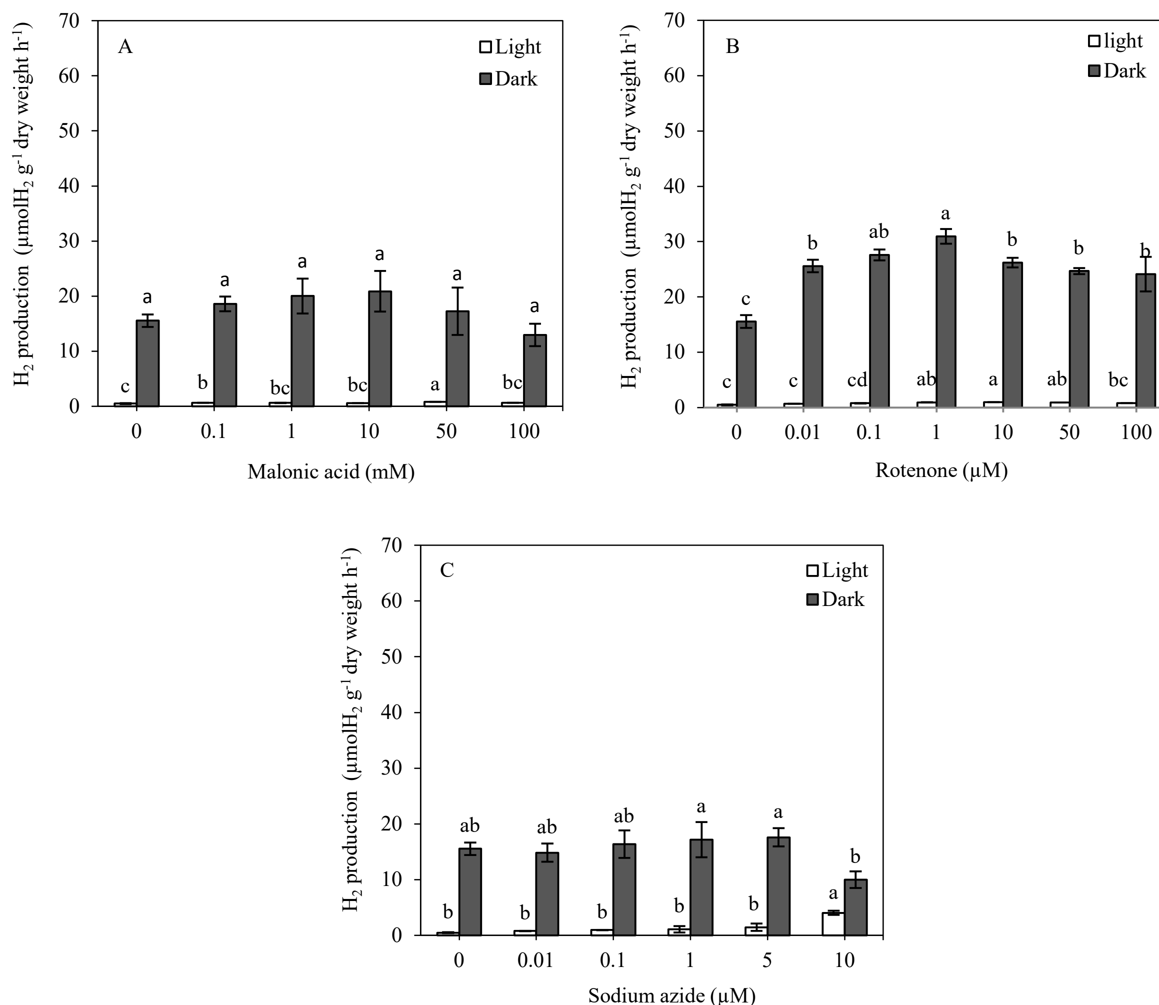


Figure 4.16 Effect of respiration inhibitors, malonic acid (A), rotenone (B) and sodium azide (C) on H_2 production by *A. halophytica* after incubation under light and dark anaerobic conditions for 2 h. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

4.2.1.3 Effect of inhibitor of uncoupling agent of oxidative phosphorylation on H_2 production by *A. halophytica*

To study effect of inhibitor of uncoupling agent of oxidative phosphorylation on H_2 production by *A. halophytica*, cells were treated with various concentrations of 2,4-dinitrophenol (0-100 µM) under light and dark anaerobic conditions for 2 h before analyzing H_2. The result showed that *A. halophytica* treated with 10 µM 2,4-dinitrophenol gave the highest H_2 production with 19.75 ± 1.89 µmol H_2 g^{-1} dry weight h^{-1} under darkness (Fig. 4.17). H_2 production was decreased when cells were treated with higher concentrations of 2,4-dinitrophenol (50 and 100 µM) (Fig. 4.17). Under light condition, *A. halophytica* produced the highest H_2 with 5.53 ±

1.26 μmol H_2 g^{-1} dry weight h^{-1} when cells were treated with 100 μM 2,4-dinitrophenol (Fig. 4.17).

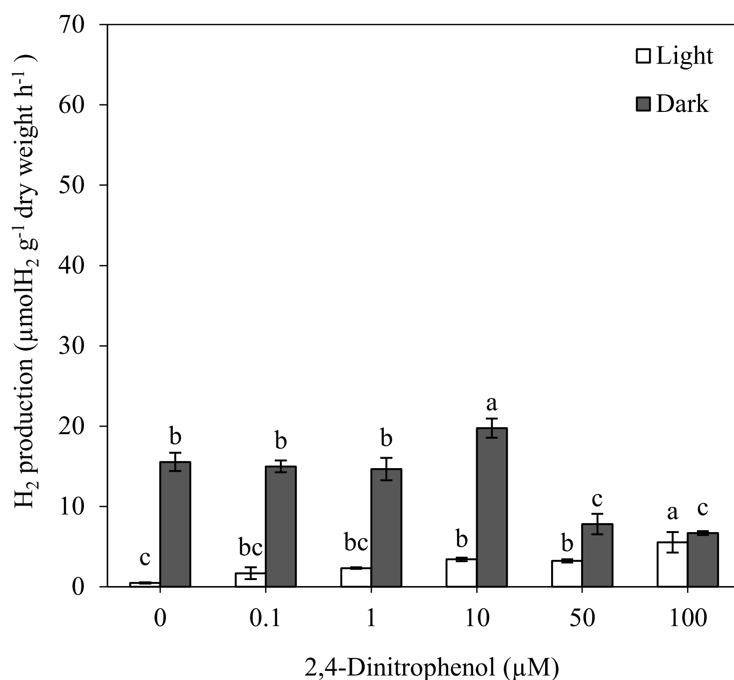


Figure 4.17 Effect of inhibitor of uncoupling agent of oxidative phosphorylation, 2,4-dinitrophenol, on H_2 production by *A. halophytica* after incubation light and dark anaerobic conditions for 2 h. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

In the respiratory and photosynthetic pathways, 2,4-dinitrophenol is capable to bind with protons and then 2,4-dinitrophenol attached with protons can pass through the membrane without proton pumping via ATP synthase (Heytler, 1979). Therefore, protein synthesis, CO_2 fixation and oxidative phosphorylation were also inhibited. The excess reducing power and NAD(P)H were used as substrate source for H_2 evolution by bidirectional hydrogenase instead. This result was correlated to the previous study in *Cyanothece* sp. found that cells treated with 50 μM 2,4-dinitrophenol increased dark H_2 production rate (Skizim *et. al.*, 2011).

4.2.1.4 Effect of carbon dioxide fixation inhibitor on H_2 production by *A. halophytica*

To investigate effect of carbon dioxide fixation inhibitor on H_2 production by *A. halophytica*, cells were treated with various concentrations of DL-

glyceraldehyde (0-250 μM) under light and dark anaerobic conditions for 2 h before H_2 measurement. The result revealed that the highest H_2 production was 27.56 ± 0.57 μmol H_2 g^{-1} dry weight h^{-1} was obtained in cells treated with 0.5 μM DL-glyceraldehyde under dark condition (Fig. 4.18). Cells treated with higher concentration than 0.5 μM DL-glyceraldehyde under darkness gave a decrease in H_2 production (Fig. 4.18). Under the light, *A. halophytica* was treated with 250 μM DL-glyceraldehyde gave the highest H_2 production with 3.35 ± 1.32 μmol H_2 g^{-1} dry weight h^{-1} (Fig. 4.18).

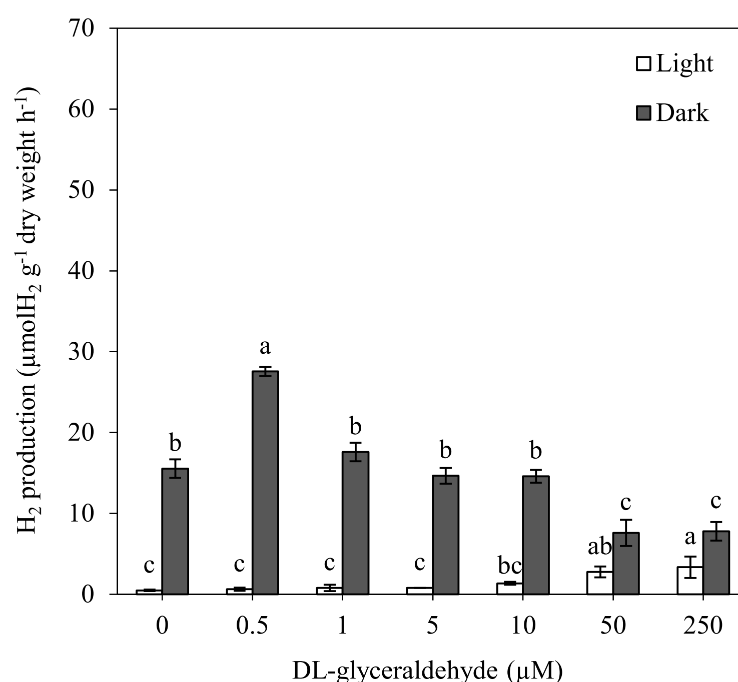


Figure 4.18 Effect of carbon dioxide fixation inhibitor, DL-glyceraldehyde, on H_2 production by *A. halophytica* after incubation under light and dark anaerobic conditions for 2 h. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

The carbon dioxide fixation inhibitor, DL-glyceraldehyde, is the competitive inhibitor of phosphoribulokinase enzyme which inhibits the phosphorylation reaction of ribulose-5-phosphate to ribulose-1,5-bisphosphate, leading to the inhibition of CO_2 fixation (Stokes and walker, 1972). Therefore, electrons and reducing powers are directly shuttled to bidirectional hydrogenase to produce H_2. This result was agreed with the previous report found in *A. siamensis*

TISTR 8012 showing that H_2 production was enhanced when cells were treated with DL-glyceraldehyde (Khetkorn *et al*., 2012).

4.2.1.5 Effect of Krebs cycle inhibitor on H_2 production by *A. halophytica*

To investigate effect of Krebs cycle inhibitor on H_2 production by *A. halophytica*, cells were treated with various concentrations of sodium arsenate (0-100 μM) under light and dark anaerobic conditions for 2 h before analyzing H_2. The result indicated that cells treated with 10 μM sodium arsenate gave the highest H_2 production with 27.56 ± 0.57 μmol H_2 g^{-1} dry weight h^{-1} under darkness (Fig. 4.19). H_2 production was decreased in cells treated with higher concentration of sodium arsenate than 10 μM (Fig. 4.19). Under light condition, *A. halophytica* treated with 50 and 100 μM sodium arsenate produced high H_2 production with 3.31 ± 0.69 and 3.72 ± 0.94 μmol H_2 g^{-1} dry weight h^{-1}, respectively (Fig. 4.19).

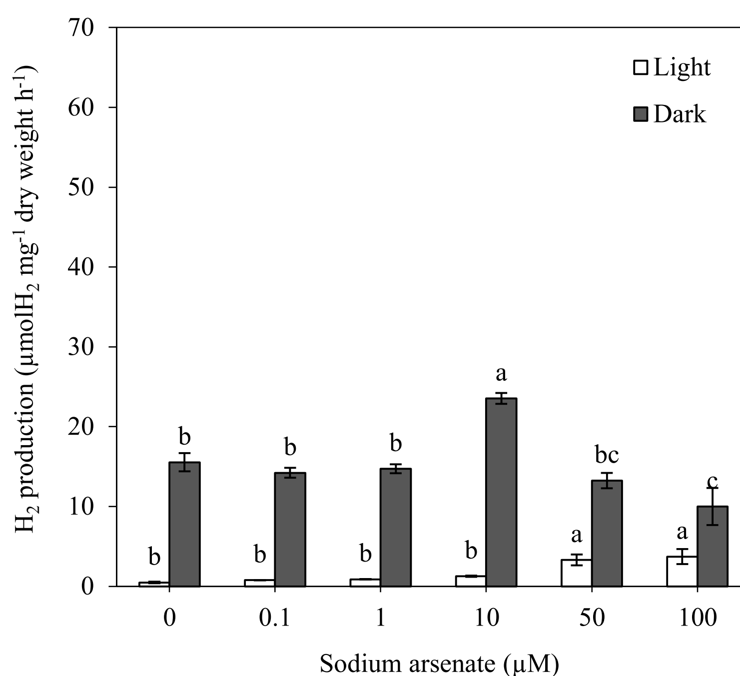


Figure 4.19 Effect of Krebs cycle inhibitor, sodium arsenate, on H_2 production by *A. halophytica* after incubation under light and dark anaerobic conditions for 2 h. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

Sodium arsenate inhibits pyruvate dehydrogenase activity in Krebs cycle by binding with -SH group of pyruvate dehydrogenase, resulting that pyruvate cannot be changed into acetyl-CoA. Therefore, the condensation of acetyl-CoA and oxaloacetate is blocked in Krebs cycle (Tretter and Vizi, 2000). The result in this study was similar to that previously reported in *A. siamensis* TISTR 8012, cells treated with sodium arsenate gave higher H_2 production than those without sodium arsenate treatment (Khetkorn *et al*., 2012).

In this study, *A. halophytica* treated with CCCP, the photosystem II inhibitor and uncoupling agent of oxidative phosphorylation inhibitor, gave the highest H_2 production under dark and light conditions. Therefore, CCCP was the most effective inhibitor for H_2 production of *A. halophytica* and was selected for further study compares with DCMU, a known photosystem II inhibitor that is widely used to study the improvement of H_2 production by cyanobacteria and green algae.

4.2.2 Effect of CCCP and DCMU on H_2 metabolism of *A. halophytica*

4.2.2.1 Effect of CCCP and DCMU on cell concentration and chlorophyll a content of *A. halophytica*

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$. CCCP and DCMU with final concentrations of 0-5 μM and 0-250 μM, respectively, were added to the cell suspension. After incubation for 2, 24, 48, 72 and 96 h, cells were collected to determine cell and chlorophyll concentration. The result showed that cell concentration and chlorophyll a content of cells treated with CCCP and DCMU were slightly decreased after the first 2 h of incubation and continuously decreased until 96 h of incubation (Fig. 4.20). The higher concentrations of CCCP and DCMU led to an obvious decrease of cell and chlorophyll a concentrations of *A. halophytica* (Fig. 4.20)

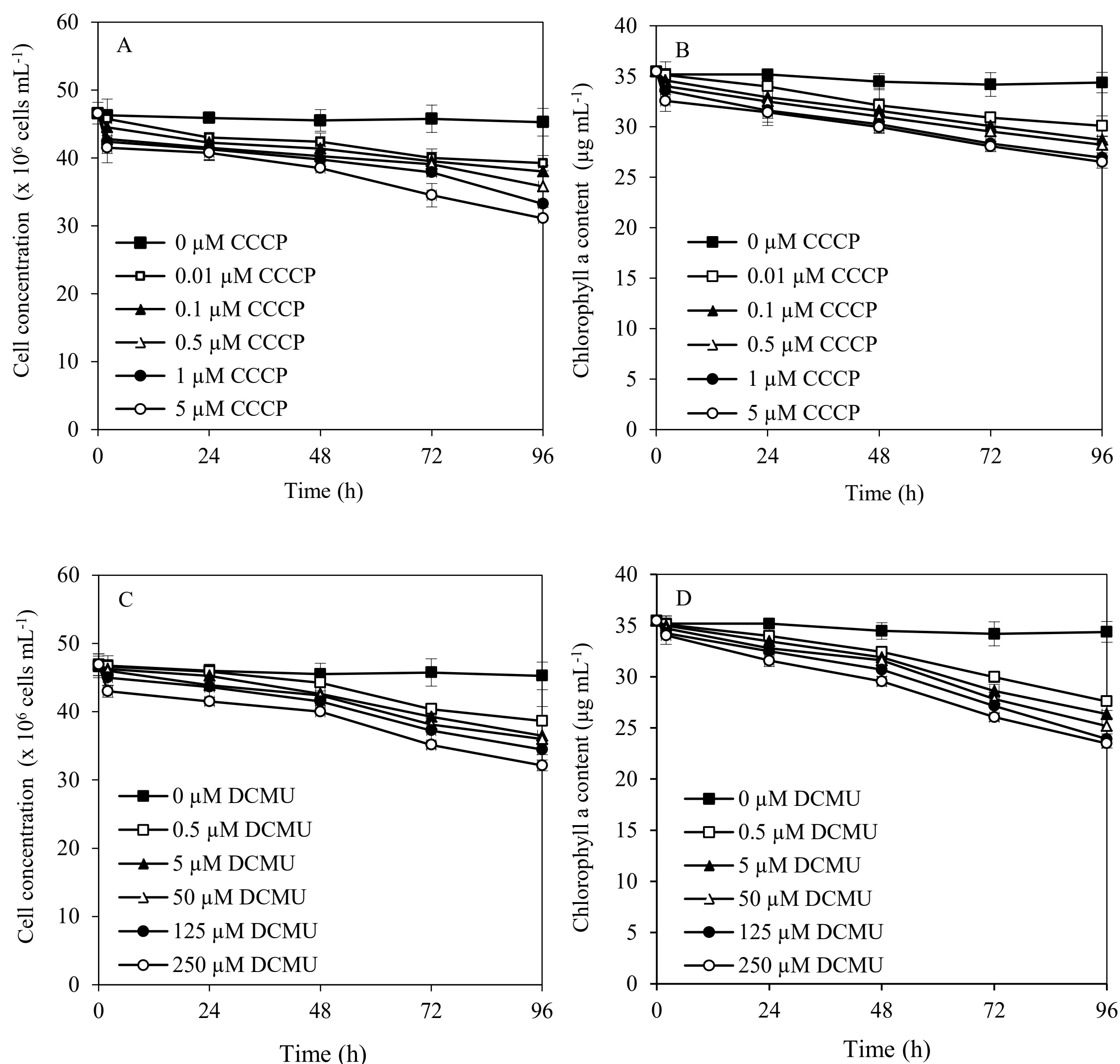


Figure 4.20 Cell concentration and chlorophyll a content of *A. halophytica* treated with various concentrations of CCCP (A, B) and DCMU (C, D) at a period of incubation time.

The result indicated that high concentration of CCCP and DCMU and long-term of incubation decreased cell concentration and chlorophyll a content (Fig. 4.20). *A. halophytica* without CCCP and DCMU treatment did not decrease cell concentration and chlorophyll a content (Fig. 4.20). Without inhibitor treatment, cell concentration and chlorophyll a content kept constant because cells were incubated in $BG11_0$ which lacks a nitrogen source $NaNO_3$ for cyanobacterial growth. CCCP is photosynthesis inhibitor that interrupts electron flow through the photosynthetic electron transport chain in thylakoid membrane of cyanobacteria by inhibiting water

splitting in photosystem II (Pisciotta *et al*., 2010). In addition, CCCP is an uncoupling agent of oxidative phosphorylation that inhibits ATP synthesis by disrupting the proton motive force by transporting protons across lipid bilayer (Ran *et al.,* 2006). With these reasons, cells concentration and chlorophyll a content of *A. holophytica* were decreased when treated with various concentrations of CCCP. This result agreed with previous study in unicellular green alga *C. reinhardtii* showing that cells showed an inhibition of growth when treated with 14.6 μM for CCCP under heterotrophic and photoautotrophic growth conditions (Mottley and Griffiths, 1977). In addition, the unicellular cyanobacterium *Synechococcus* sp. treated with 10 μM CCCP led to a decrease in optical density at 750 nm and cell concentration (Kallas and Castenholz, 1982). Chlorophyll content of *P. helgolandica* var. *tsingtaoensis* and *C. reinhardtii* were decreased when cells were treated with 15 μM CCCP (Zhang *et al*., 2012; Yang *et al*., 2014) Moreover, it has been reported that the glycolytic pathway and starch biosynthesis were rapidly blocked with CCCP treatment in *C. reinhardtii*, leading to a decrease in cell concentration and chlorophyll a content (Yang *et al.*, 2014).

DCMU is an inhibitor of photosynthesis. DCMU blocks electron transfer between Q_A and Q_B on the reducing side of photosystem II, leading to the inhibition of electron flow through the photosynthetic electron transport chain in thylakoid membrane of cyanobacteria (Allen *et al*., 1983; Pisciotta *et al*., 2010). In addition, DCMU affects other cellular processes, such as cyclic phosphorylation, chlorophyll synthesis, and galactolipid and fatty acid biosynthesis (Laval-Martin *et al*., 1977). The treatment of DCMU might lead to the inhibition of photosynthesis and other involving metabolisms, resulting in a decrease of cell concentration and chlorophyll a content of *A. holophytica*. This result was consistent with the previous studies that algae *Eudorina elegans* and *Nannochloropsis* treated with 0.1 μM DCMU reduced the growth of cells (Orr *et al*., 1976; Gonen-Zurgil *et al*., 1996). Chlorophyll concentration of N_2-fixing cyanobacteria *Nostoc* sp. G3 and *Anabaena variabilis* was decreased when cells were treated with DCMU (Gadkari, 1988; Singh *et al*., 2011). The treatment of cyanobacterium *Synechocystis* sp. PCC 6803 with 0.1 μM DCMU resulted in a decrease in optical density at 730 nm (Burrows *et al*., 2011). The result of the present study suggested that high concentrations of CCCP and DCMU and long-term incubation caused cell toxicity and death.

4.2.2.2 Effect of CCCP and DCMU on H_2 and O_2 production by *A. halophytica*

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$**.** CCCP and DCMU with final concentrations of 0-5 μM and 0-250 μM, respectively, were added to the cell suspension. The vial was incubated at 30°C under the light 2 h and purged with argon gas for 10 min before H_2 and O_2 measurement. The result revealed that cells with CCCP treatment could enhance H_2 production under light and dark conditions (Fig. 4.21A). H_2 production of cells with and without CCCP treatment under dark condition was higher than that under light condition (Fig. 4.21A). Under darkness cells treated with 0.1, 0.5 and 1 μM CCCP increased H_2 production. The highest H_2 production at 39.50 ± 2.13 μmol H_2 g^{-1} dry weight h^{-1} was found in the presence of 0.5 μM CCCP and H_2 production was decreased at 5 μM CCCP (Fig. 4.21A). Under light condition, H_2 production of cells treated with 0.5 μM CCCP gave the highest H_2 production with 2.26 ± 0.26 μmol H_2 g^{-1} dry weight h^{-1} (Fig. 4.21A). O_2 production of *A. halophytica* cells under dark condition was lower than that under light condition (Fig. 4.21B). In addition, an increase of CCCP concentration decreased O_2 production under dark and light conditions (Fig. 4.21B).

In the presence of DCMU, H_2 production by *A. halophytica* treated with DCMU under light condition was higher than that under the light (Fig. 4.21C). *A. halophytica* treated with DCMU did not significantly enhance H_2 production under dark condition but cells treated with DCMU could produce higher H_2 than those without DCMU treatment under light condition (Fig. 4.21C). For O_2 production of *A. halophytica*, cells treated with DCMU under light condition gave lower O_2 production than those without DCMU treatment (Fig. 4.21D). Higher concentrations of DCMU resulted in lower O_2 production in the light whereas no significant difference of O_2 production was found under darkness (Fig. 4.21D).

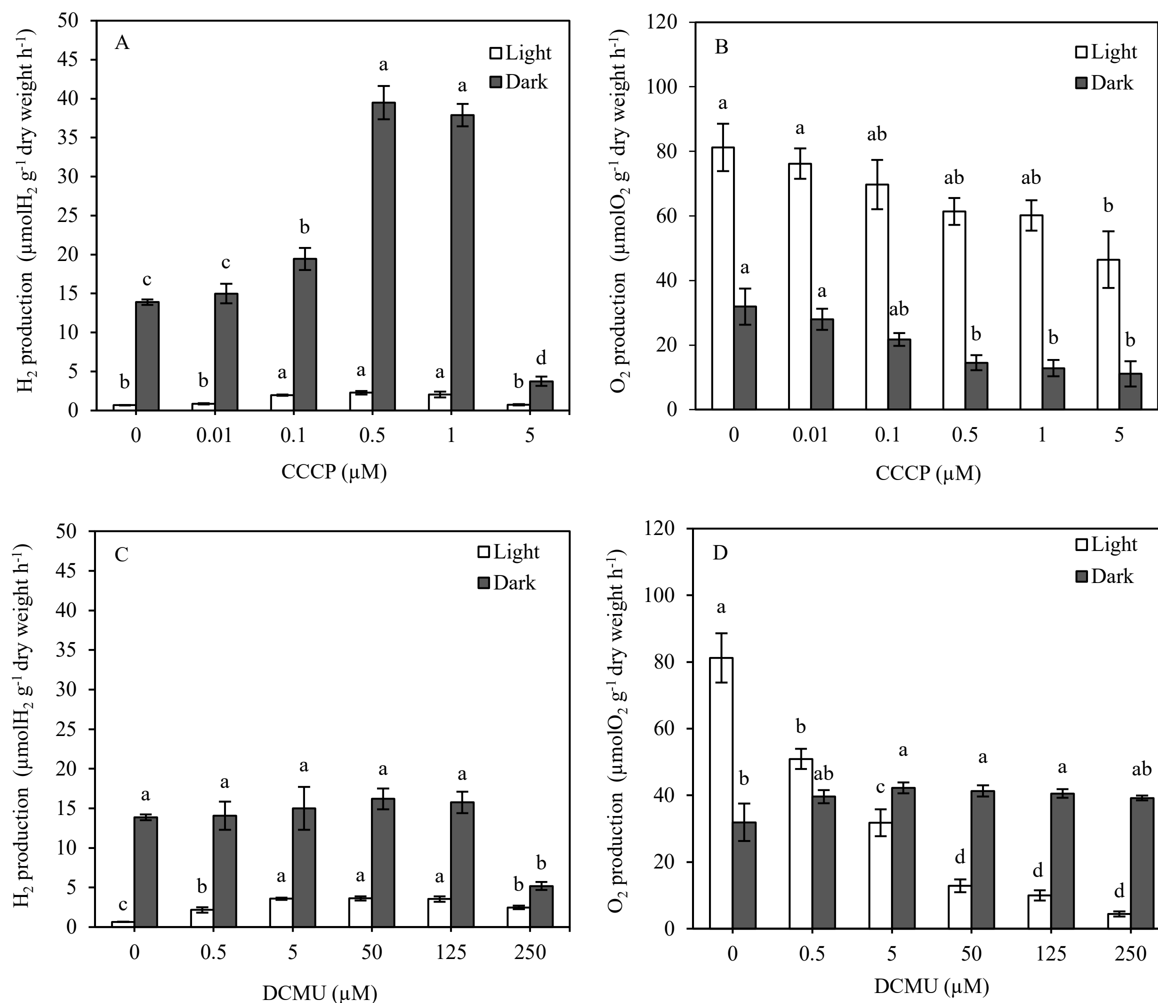


Figure 4.21 H_2 production rate and O_2 production rate by *A. halophytica* treated with various concentrations of CCCP (A,B) and DCMU (C,D). Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

This study was investigated to confirm the function of CCCP and DCMU as photosystem II inhibitor. When CCCP and DCMU interrupt the electron transport in PSII, O_2 is lower produced especially under illumination. The result in this study correlated to the expected result that a decrease in O_2 concentration was found in cells treated with CCCP and DCMU under the light. Moreover, H_2 production was related with O_2 production. *A. halophytica* treated with higher concentration of CCCP under light and darkness increased H_2 production while its O_2 production decreased. However, cells treated with 5 μM CCCP gave decreased H_2 and O_2 production due to the toxicity of CCCP at high concentration.

In case of DCMU, H_2 production by *A. halophytica* under light depended on DCMU concentration since DCMU blocks electron transfer in photosystem II resulting in a decrease in O_2 production. The lower O_2 concentration promoted bidirectional hydrogenase activity, leading to an increase in H_2 production. This result agreed with previous studies in new marine green alga *P. helgolandica* var. *tsingtaoensis* which H_2 production was increased when treated with DCMU due to the inhibition of PSII photochemical activity during illumination (Zhang *et al*., 2012). The cyanobacterium *A. cylindrica* incubated with DCMU showed improvement of H_2 production due to the low level of O_2 content (Chen *et al*., 2013). Under dark condition, no significant differences of H_2 and O_2 production of *A. halophytica* treated with various concentrations of DCMU and without DCMU treatment (Fig. 4.21C,D). It was suggested that DCMU does not affect H_2 production of *A. halophytica* under dark condition resulting in a constant H_2 production rate compared with the untreated cells. These results contrasted with those of studies in *Synechocystis* sp. PCC 6803, cells gave higher H_2 production when treated with 75 μM DCMU under dark and anaerobic conditions (Cournac *et al*., 2002; Cournac *et al*., 2004).

4.2.2.3 Effect of CCCP and DCMU on bidirectional hydrogenase activity of *A. halophytica*

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$**.** CCCP and DCMU with final concentrations of 0-5 μM and 0-250 μM, respectively, were added to the cell suspension. Cells were incubated at 30°C under the light 2 h before bidirectional hydrogenase activity measurement. The result showed that bidirectional hydrogenase activity of *A. halophytica* treated with CCCP was higher than that of cells without CCCP treatment (Fig. 4.22A). The highest bidirectional hydrogenase activity was 27.32 ± 2.73 μmolH_2 g^{-1} dry weight min^{-1} when cells were treated with 0.5 μM CCCP (Fig. 4.22A). Higher CCCP concentration than 0.5 μM led to a decrease in bidirectional hydrogenase activities (Fig. 4.22A). The result of bidirectional hydrogenase of *A. halophytica* with and without CCCP treatment correlated with the result of H_2 production (Fig. 4.21A). This result indicated that concentration of CCCP at 0.5 μM is optimal for promoting bidirectional hydrogenase activity in *A. halophytica* and leading to the highest H_2 production. It was suggested that photosystem II of cells treated with CCCP was inhibited. The inhibition of photosynthetic activity led to a decrease in O_2 production and finally enhanced bidirectional hydrogenase activity. This result was consistent with previous study that *A. siamensis* TISTR 8012 treated

with 10 μM CCCP increased bidirectional hydrogenase activity (Khetkorn *et al*., 2012). However, CCCP concentration influencing bidirectional hydrogenase activity might be species dependent.

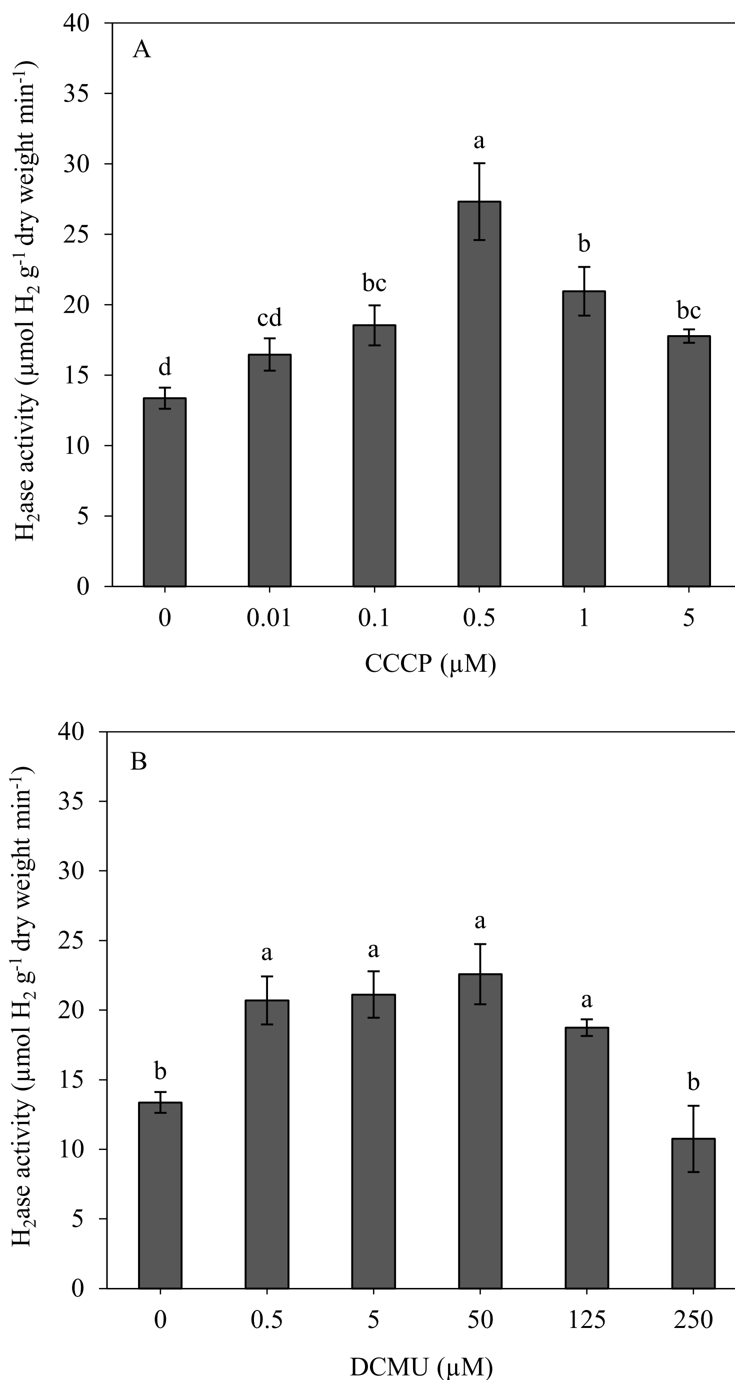


Figure 4.22 Bidirectional hydrogenase activities of *A. halophytica* cells treated with various concentrations of CCCP (A) and DCMU (B) after 2 h of treatment under illumination. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$.

In case of DCMU, the highest bidirectional hydrogenase activity with 22.58 ± 2.15 μmolH_2 g^{-1} dry weight min^{-1} was found in *A. halophytica* treated with 50 μM DCMU (Fig. 4.22B). Bidirectional hydrogenase activity was significantly decreased in cells treated with 250 μM DCMU (Fig. 4.22B). As expected, bidirectional hydrogenase activities were related to H_2 production rates (Fig. 4.21A). This result was consistent with the previous study found that the highest bidirectional hydrogenase activity of *A. siamensis* TISTR 8012 was obtained when cells treated with 50 μM DCMU under nitrogen deprivation (Khetkorn *et al*., 2012). It could be explained that DCMU blocks electron transfer in photosystem II (Metz *et al*., 1986) resulting in a decrease in O_2 evolution and thus promoting bidirectional hydrogenase activity.

4.2.2.4 Effect of CCCP and DCMU on chlorophyll fluorescence emission spectra of *A. halophytica*

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$**.** CCCP and DCMU with final concentrations of 0-5 μM and 0-250 μM, respectively, were added to the cell suspension. Cells were incubated at 30°C under the light 2 h. Chlorophyll fluorescence emission spectra of cells was measured by spectrofluorometer at room temperature. The result revealed that *A. halophytica* treated with CCCP and DCMU gave lower fluorescence intensity of chlorophyll fluorescence emission spectra than cells without CCCP and DCMU treatment (Fig. 4.23A,B). The fluorescence intensities of chlorophyll fluorescence emission spectra were obviously lower in cells treated with higher concentration of CCCP and DCMU (Fig. 4.23A,B).

In this study, it was shown that treatment of CCCP and DCMU resulted in the lower photosynthetic activity by observation of chlorophyll fluorescence spectra. This led to the lower ability of PSII activity and a decrease in the O_2 production under light condition. Finally, H_2 production was induced. This result agreed with previous results reported in many cyanobacterial and green algal strains, indicating that CCCP reduced PSII photochemical activity (Williams and Dominy, 1990; Ran *et al*., 2006; Guo *et al*., 2008; Ji *et al*., 2011; Yang *et al*., 2014).

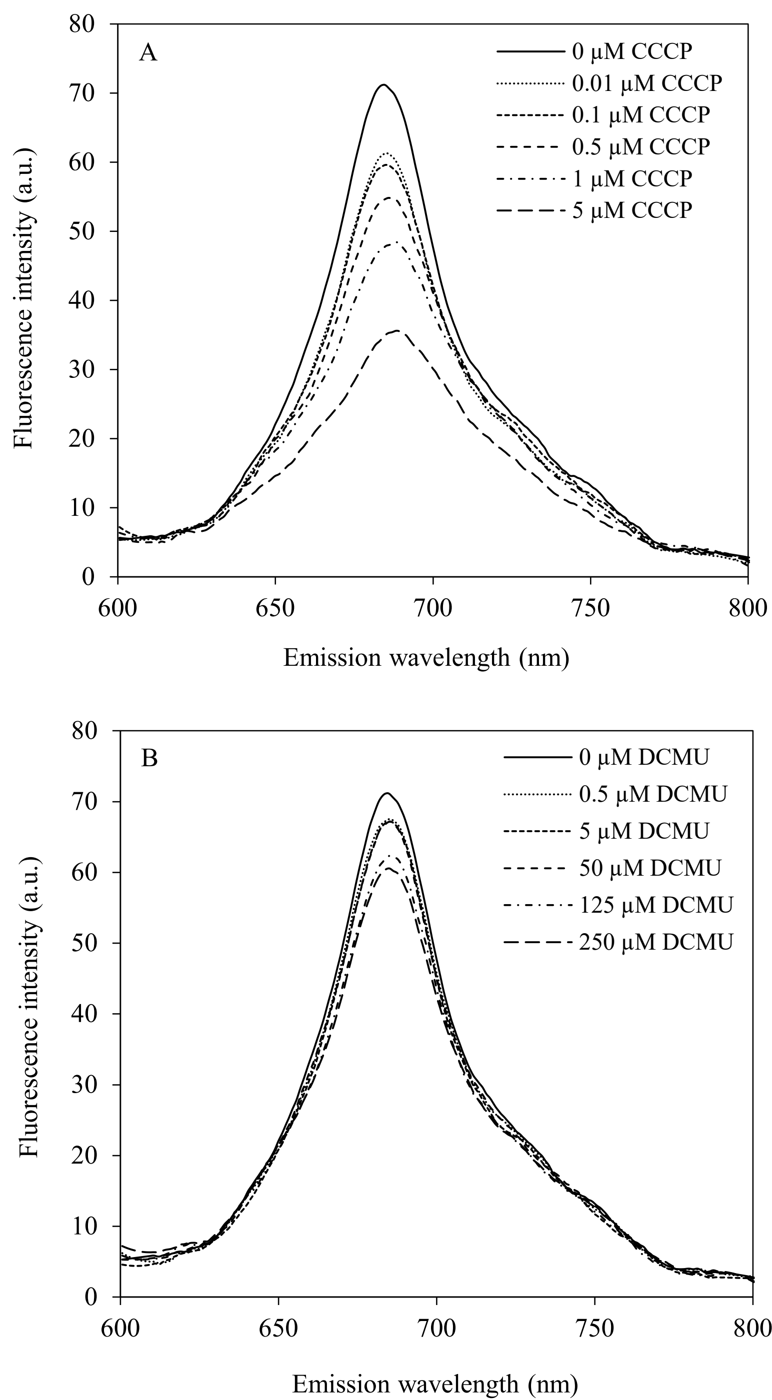


Figure 4.23 Fluorescence emission spectra of *A. halophytica* treated with various concentrations of CCCP (A) and DCMU (B) after 2 hours of treatment under illumination.

4.2.2.5 Effect of CCCP and DCMU on O_2 consumption by dark respiration rate measurement of *A. halophytica*

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$**.** CCCP and DCMU with final concentrations of 0-5 µM and 0-250 µM, respectively, were added to the cell suspension. Cells were incubated at 30°C under the light 2 h. O_2 consumption by dark respiration rate was monitored at 25 °C using a Clark-type oxygen electrode. It was shown that dark respiration rate of cells increased in cells treated with 0.1-1 µM CCCP (Fig. 4.24A). Cells treated with 0.5 µM CCCP gave the highest O_2 consumption by dark respiration rate at 335.30 ± 3.32 µmol O_2 g^{-1} dry weight min^{-1} (Fig. 4.24A). O_2 consumption by dark respiration rate of cells significantly decreased in cells treated with 5 µM CCCP (Fig. 4.24A). In case of DCMU, *A. halophytica* treated with higher DCMU concentrations than 0.5 µM gave lower O_2 consumption by dark respiration rate than cells without DCMU treatment (Fig. 4.24B).

It has shown in topic 4.2.2.2 that under darkness O_2 production of cells treated with CCCP was lower than cells treated with DCMU (Fig. 4.21B). A decrease in O_2 level might come from other mechanisms apart from photosynthesis, participating in an enhancement of H_2 production. One possible way is the O_2 consumption by dark respiration. Therefore, O_2 consumption by dark respiration was investigated in this study. Interestingly, CCCP enhanced dark respiration rate by *A. halophytica* (Fig. 4.24A) whereas DCMU decreased dark respiration rate (Fig. 4.24B). It could be explained that CCCP inhibited ATP synthesis from working as an uncoupler of oxidative phosphorylation and subsequently increased the respiration rate. An increase O_2 consumption by dark respiration led to decrease O_2 concentration that promoted bidirectional hydrogenase to produce H_2. This result correlated the previous study showing that *A. variabilis* and *A. nidulans* treated with 5 and 10 µM CCCP enhanced the rate of dark respiration (Imafuku and Katoh, 1976; Shyam *et al*., 1993). In green algae *C. reinhardtii* treated with 2.5 µM CCCP increased the dark respiration rate by 40% without influence in photosynthesis (Singh *et al*., 1996). On the other hand, *A. halophytica* with DCMU treatment showed a significant decrease in respiration rate, especially in an increased DCMU concentrations (Fig. 4.24B). This led to an increase in O_2 production and subsequently no enhancement of H_2 production under dark condition (Fig. 4.21C,D). This result was contrasted with CCCP treatment, indicating that DCMU and CCCP possess different mechanisms involving in the respiration under the dark. This result was similar to previous results reported in

A. flos-aquae and *Chlorella* sp. that cells treated with DCMU inhibited dark respiration rates (Yallop, 1982; Sargent and Taylor, 1972).

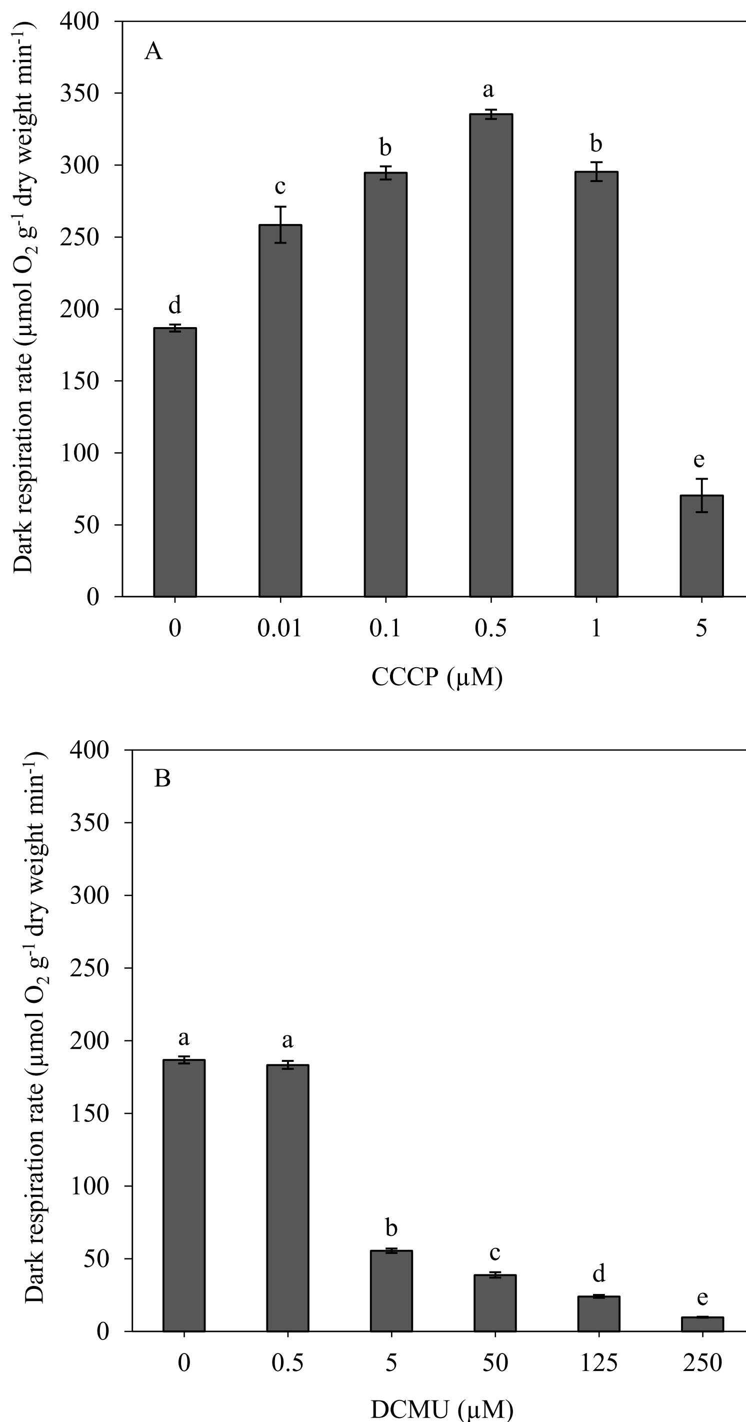


Figure 4.24 Dark respiration rate of *A. halophytica* treated with various concentrations of CCCP (A) and DCMU (B) after 2 h of treatment under illumination. Data are means ± SD (n = 3). Different letters indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range tests at $p < 0.05$

4.2.2.6 Effect of CCCP on long-term H_2 production

A. holophytica grown in BG11 (pH 7.4) supplemented with Turk Island salt solution for 7 days was harvested by centrifugation, washed twice and resuspended in 100 ml of $BG11_0$ medium. Then, cells were incubated at 30 °C under light for 24 h before harvesting and resuspending in 5 mL of $BG11_0$. Cells were treated with and without 0.5 μM CCCP under dark for 240 h. For negative control, the medium containing 0.5 μM CCCP without cells was used. It was shown that *A. halophytica* treated with 0.5 μM CCCP showed higher H_2 production than that without CCCP treatment under dark condition (Fig. 4.25). *A. halophytica* gave the maximum H_2 production rate and H_2 accumulation with 40.46 ± 1.16 μmol H_2 g^{-1} dryweight h^{-1} at the first 2 h of incubation and 254.23 ± 16.33 μmolH_2 g^{-1} dry weight h^{-1} after 24 h of incubation, respectively (Fig. 4.25). The maximum H_2 accumulation under dark condition was approximately 2.6 folds higher than cells without CCCP treatment. H_2 production was decreased when cells were incubated longer than 24 h (Fig. 4.25) In negative control, CCCP solution without cells did not show H_2 yield (Fig. 4.25).

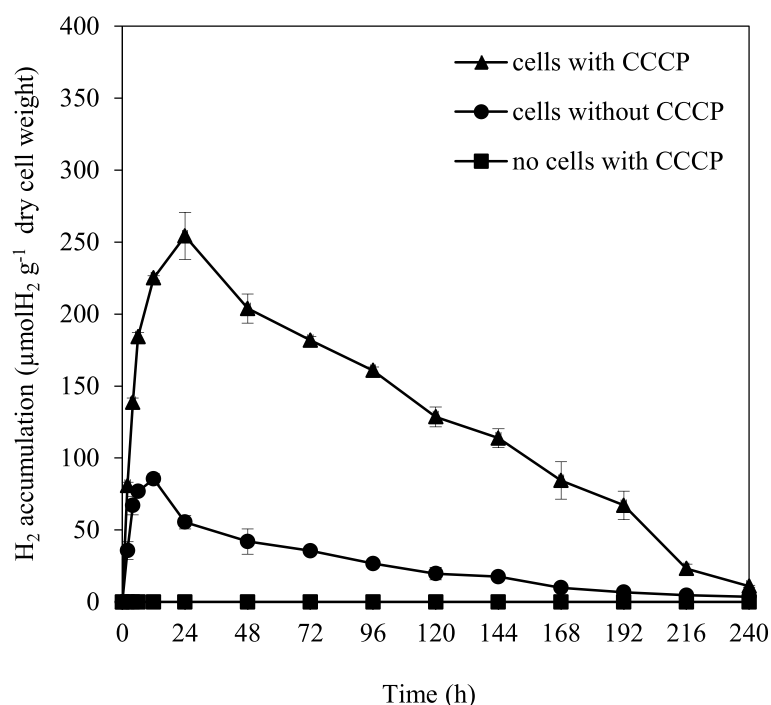


Figure 4.25 Long-term H_2 production of *A. halophytica* treated with and without 0.5 μM CCCP for 240 h under darkness. Medium containing 0.5 μM CCCP without cells was used as a negative control.

This study confirmed the capability of the effective inhibitor CCCP for enhancement of dark fermentative H_2 production by *A. halophytica*. However,

after 24 h of CCCP treatment cells reduced dark fermentative H_2 production due to the decreased action of CCCP and the toxicity of CCCP to growth and cellular metabolism during long-term incubation as shown in Fig. 4.20

Chapter 5

Conclusions

In this study, the enhancement of H_2 production by unicellular cyanobacterium *A. halophytica* was investigated by two methods; (1) using cell immobilization and (2) using inhibitor. The results in this study can be summarized as following:

5.1 Improvement of H_2 production by *A. halophytica* by cell immobilization method

5.1.1 The immobilized cells of *A. halophytica* with four support materials; agar, agarose, alginate and κ-carrageenan showed higher H_2 production than free cells.

5.1.2 Among *A. halophytica* immobilized with all support materials, cells immobilized with 1.5% (w/v) agar gave the highest H_2 production after dark anaerobic incubation in $BG11_0$ supplemented with Turk Island salt solution for 2 days, followed by those immobilized with 4% (w/v) alginate, 1.5% (w/v) κ-carrageenan and 1.5% (w/v) agarose, respectively.

5.1.3 Higher agar concentrations for immobilization of *A. halophytica* resulted in an increase in H_2 production. Cells immobilized with 3% (w/v) agar gave the highest H_2 production rate after anaerobic dark incubation in $BG11_0$ supplemented with Turk Island salt solution for 1 day.

5.1.4 Higher cell concentrations for immobilization of *A. halophytica* resulted in a decrease in H_2 production. H_2 production of 3% (w/v) agar-immobilized cells containing 0.2 mg dry cell weight mL^{-1} gave the highest H_2 production rate in $BG11_0$ supplemented with Turk Island salt solution under dark anaerobic condition for 1 day.

5.1.5 The volumetric size at 0.0125 cm^3 or the diameter at 0.5 cm of 3% (w/v) agar-immobilized cubes containing 0.2 mg dry cell weight mL^{-1} showed the highest H_2 production rate in $BG11_0$ medium under dark anaerobic condition for 1 day.

5.1.6 The optimum nutrient and mineral concentrations of agar-immobilized *A. halophytica* for H_2 production were 0 mM $NaNO_3$, 0.5 M NaCl, 30 mM $MgSO_4.7H_2O$, 4 μM Fe^{3+} and 0 μM Ni^{2+}.

5.1.7 H_2 production of 3% (w/v) agar-immobilized cells was highest when immobilized cells were incubated in $BG11_0$ medium pH 7.4 and at temperature 40 °C.

5.1.8 The volumes of glass vial, immobilized cells and headspace influenced H_2 production of 3% (w/v) agar-immobilized cells of *A. halophytica*. Agar-immobilized cells accumulated maximum H_2 production yield in larger volumes of glass vial and headspace but lower volumes of immobilized cubes.

5.1.9 Agar-immobilized cells of *A. halophytica* could recover H_2 production for three cycles. H_2 production was decreased in each cycle. The replacement of 50% of newly fresh immobilized cells and medium in cycle 2 could be maintained H_2 production by 3% (w/v) agar-immobilized cells of *A. halophytica*.

5.2 Improvement of H_2 production by *A. halophytica* by use of Inhibitor

5.2.1 Type and concentration of inhibitors affected H_2 production by *A. halophytica*. Among inhibitors investigated, treatment of *A. halophytica* with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) at 0.5 µM gave the highest dark fermentative H_2 production under anaerobic condition for 2 h.

5.2.2 Higher CCCP and DCMU concentrations and long term of incubation of inhibitors led to a decrease in cell concentration and chlorophyll a content of *A. halophytica*.

5.2.3 CCCP and DCMU enhanced H_2 production by *A. halophytica* under light condition whereas only CCCP enhanced H_2 production under darkness. An increase in H_2 production was related to a decrease in O_2 production.

5.2.4 Under light illumination, bidirectional hydrogenase activity of *A. halophytica* increased when cells were treated with 0.1-5 µM CCCP and 0.5-125 µM DCMU. The highest bidirectional hydrogenase activity was found in cells incubated with 0.5 µM CCCP. Too high concentrations of DCMU (250 µM DCMU) did not significantly increase bidirectional hydrogenase activity in *A. halophytica*.

5.2.5 Under light illumination, the fluorescence intensity of chlorophyll in *A. halophytica* treated with CCCP and DCMU under light 2 h was lower than that without CCCP and DCMU treatment. Higher concentrations of CCCP and DCMU decreased the fluorescence intensity of chlorophyll fluorescence emission spectra in *A. halophytica*.

5.2.6 CCCP increased O_2 consumption rate by dark respiration but DCMU decreased O_2 consumption rate by dark respiration in *A. halophytica*. The treatment

of CCCP concentrations at 0.01-1 μM led to an increase in O_2 consumption rate by dark respiration but at 5 μM CCCP dark respiration rate was inhibited.

5.2.7 For long term incubation, the maximum H_2 accumulation of *A. halophytica* cells treated with 0.5 μM was approximately 2.6 folds higher than that without CCCP treatment at 24 h of dark anaerobic incubation. H_2 production was decreased when cells were incubated after 24 h.

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Appendices

Appendix A

Blue-green 11 (BG11) medium (Ripka *et al*., 1979)

Trace metal mix (1000 x)

H_3BO_3	46.30	mM
$MnCl_2.4H_2O$	4.15	mM
$ZnSO_4.7H_2O$	0.77	mM
$NaMoO_4.2H_2O$	1.61	mM
$CuSO_4.5H_2O$	0.32	mM
$Co(NO_3)_2.6H_2O$	0.17	mM

Composition of BG11 (100 x)

$NaNO_3$	1.76	M
$MgSO_4.7H_2O$	30.40	mM
$CaCl_2.2H_2O$	24.50	mM
Citric Acid	3.12	mM
Na_2EDTA	279	mM

Composition of BG11

BG11 (100 x)	10	mL
Na_2CO_3 (2 g/100 mL)	1	mL
K_2HPO_4 (3.05 g/100 mL)	1	mL
$FeNH_4$.Citrate (0.60 g/100 mL)	1	mL
Trace metal mix (1000 x)	1	mL

The volume was adjusted to 1 L with deionized before autoclaving

Appendix B

Blue-green 11 (BG11) medium supplemented with Turk Island Salt Solution (Garlick *et al.*, 1977)

Composition of BG11) medium supplemented with Turk Island Salt Solution

100xBG11 (Appendix A)	10 mL
*Stock A solution	100 mL
**Stock B solution	100 mL
Na_2CO_3 (2g/100 mL)	1 mL
K_2HPO_4 (3.05 g/100mL)	1 mL
$FeNH_4$.Citrate (0.60 g/100 mL)	1 mL
Trace metal mix (1000 x)	1 mL
NaCl	28.16 g

Adjust volume to 1 L with deionized water. Adjust pH of medium to 7.4 by adjusting with 2 N NaOH.

***Stock A solution**

Composition per liter

KCl	6.6 g
$MgCl_2.6H_2O$	55 g
$CaCl_2.2H_2O$	14.66 g

****Stock B solution**

Composition per liter

$MgSO_4.7H_2O$	74.48 g

Appendix C

Chlorophyll a determination (MacKinney, 1941)

Chlorophyll a content (µg chla mL^{-1}) = $12.7 \times A_{665}$

A_{665} = Chlorophyll a absorbance at wavelength 665 nm

Author biography

Name	Miss Sunisa Pansook
Date of birth	25 July, 1983
Address	210/88 Moo 1 bangpleng, Bangboo, Samutprakan Thailand 10560
Education	(2006) Bachelor of Science in Biotechnology GPA 3.20 Prince of Songkla University, Hat Yai (2009) Master of Science in Food Science GPA 3.41 King Mongkut's Institute of Technology Ladkrabang (2019) Doctor of Philosophy in Biotechnology King Mongkut's Institute of Technology Ladkrabang

Academic Publications

1. Pansook, S., Incharoensakdi, A. and Phunpruch, S. 2019. “Enhanced dark fermentative H_2 production by agar-immobilized cyanobacterium *Aphanothece halophytica*.” *Journal of Applied Phycology*. (DOI.org/10.1007/s10811-019-01822-9)
2. Pansook, S., Incharoensakdi, A. and Phunpruch, S. 2019. “Effects of the photosystem II inhibitors CCCP and DCMU on hydrogen production by the unicellular halotolerant cyanobacterium *Aphanothece halophytica*.” *The Scientific World Journal*. (DOI.org/10.1155/2019/1030236)