การคัดแยกไซยาโนแบคทีเรียจากแหล่งน้ำทะเลเพื่อการผลิตไฮโดรเจน

ISOLATION OF CYANOBACTERIA FROM MARINE SOURCES FOR HYDROGEN PRODUCTION

ณิชนันทน์ ทินปราณี
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คณะวิทยาศาสตร์
สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
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NICHANAN TINPRANEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

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ใบรับรองวิทยานิพนธ์

หัวข้อวิทยานิพนธ์ การคัดแยกไซยาโนแบคทีเรียจากแหล่งน้ำทะเลเพื่อการผลิตไฮโดรเจน
Isolation of cyanobacteria from marine sources for hydrogen production

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หัวข้อวิทยานิพนธ์ การคัดแยกไซยาโนแบคทีเรียจากแหล่งน้ำทะเลเพื่อการผลิตไฮโดรเจน
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บทคัดย่อ

ในปัจจุบัน ไฮโดรเจนจัดเปนแหลงพลังงานทางเลือกชนิดหนึ่งที่น่าสนใจ ซึ่งให้พลังงานจากการเผาไหม้สูงและกอใหเกิดมลพิษทางอากาศนอยกวาแหล่งพลังงานอื่นๆ งานวิจัยนี้มีวัตถุประสงค์ในการศึกษาการผลิตไฮโดรเจนของไซยาโนแบคทีเรียที่คัดแยกไดจากน้ำทะเลฝั่งอันดามันและอ่าวไทยของประเทศไทย จากการทดลองพบว่า สามารถแยกไซยาโนแบคทีเรียจากน้ำทะเลของประเทศไทยและทำให้บริสุทธิ์ได้จำนวน 54 ไอโซเลท จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของยีน 16S rDNA พบว่าไซยาโนแบคทีเรียที่แยกได้ส่วนใหญ่อยู่ในจีนัส *Geitlerinema* และมีลักษณะทางสัณฐานวิทยาที่ไม่แตกต่างกัน จากการวิเคราะห์ลำดับนิวคลีโอไทด์และสร้างแผนภูมิต้นไม้ของยีน 16S rDNA, 16S-23S ITS และ *cpcB-cpcA* IGS พบว่า *Geitlerinema* ที่แยกได้จากน้ำทะเลในประเทศไทยจัดอยู่ในกลุ่มเดียวกัน เช่นเดียวกับ *Geitlerinema* sp. Flo1 และ *Geitlerinema* sp. PCC7105 แต่แตกต่างอย่างเห็นได้ชัดกับ *Geitlerinema* ที่แยกได้จากน้ำจืด นอกจากนี้ ไซยาโนแบคทีเรีย *Geitlerinema* ที่แยกได้สามารถแบ่งกลุ่มในระดับประชากรได้เป็น 8 กลุ่ม แต่การจัดแบ่งกลุ่มนี้ไม่มีความสัมพันธ์กับตำแหน่งทางภูมิศาสตร์ของไซยาโนแบคทีเรีย จากการคัดเลือกไซยาโนแบคทีเรียที่มีประสิทธิภาพในการผลิตไฮโดรเจนสูงพบว่า ไซยาโนแบคทีเรียเส้นสาย *Geitlerinema* sp. RMK-SH10 มีการผลิตไฮโดรเจนสูงที่สุดภายใต้สภาวะปราศจากอากาศในที่มีแสงและไม่มีแสง จากนั้น นำ *Geitlerinema* sp. RMK-SH1 ไปศึกษาสภาวะที่เหมาะสมต่อการผลิตไฮโดรเจนพบว่า ไซยาโนแบคทีเรีย *Geitlerinema* sp. RMK-SH10 มีการผลิตไฮโดรเจนสูงที่สุด เมื่อทำการบ่มอาหาร ASN III ที่ปราศจากโซเดียมไนเตรทที่มีความเข้มข้นของแมกนีเซียมซัลเฟตเฮปตะไฮเดรต 1.4 มิลลิโมลาร์ ความเข้มข้นของโซเดียมคลอไรด์ 0.2 โมลาร์ ความเข้มข้นของเหล็กไอออน 2 ไมโครโมลาร์ ความเข้มข้นของนิกเกิลไอออน 0.1 ไมโครโมลาร์ และใช้กลูโคสเป็นแหล่งคาร์บอนที่มีความเข้มข้นของกลูโคส 18.9 มิลลิโมลคาร์บอนอะตอมต่อลิตร ความเข้มแสง อุณหภูมิ และพีเอชของอาหารที่

เหมาะสมต่อการผลิตไฮโดรเจนของไซยาโนแบคทีเรีย *Geitlerinema* sp. RMK-SH10 คือ ในที่มืด อุณหภูมิ 40 องศาเซลเซียส และพีเอช 8 ตามลำดับ ไซยาโนแบคทีเรีย *Geitlerinema* sp. RMK-SH10 สามารถเจริญเติบโตได้ในน้ำทะเลมาที่มีการการเติมโซเดียมไนเตรทความเข้มข้น 9 มิลลิโมลาร์ *Geitlerinema* sp. RMK-SH10 มีการผลิตไฮโดรเจนสูงสุดเท่ากับ 1,724.098±47.976 ไมโครโมล ไฮโดรเจนต่อกรัมน้ำหนักเซลล์แห้ง ในเซลล์ที่บ่มในน้ำทะเลที่มีกลูโคสความเข้มข้น 18.9 มิลลิโมล คาร์บอนอะตอมต่อลิตร เซลล์สามารถสะสมไฮโดรเจนสูงสุดถึง 2,237.631±151.453 ไมโครโมล ไฮโดรเจนต่อกรัมน้ำหนักเซลล์แห้ง หลังจากวันที่ 4 ของการบ่มในที่มืดและปราศจากอากาศ แสดงให้เห็นว่าเซลล์สามารถใช้น้ำทะเลเพื่อการเพาะเลี้ยงและผลิตไฮโดรเจนได้

คำสำคัญ : การผลิตไฮโดรเจน ไซยาโนแบคทีเรียทะเล *Geitlerinema*

Thesis Title	Isolation of cyanobacteria from marine sources for hydrogen production
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Degree	Doctor of Philosophy
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Abstract

Nowadays, Hydrogen (H_2) is one of alternative interesting energy sources that provides high energy during combustion and causes less air-pollution than other energy sources. This research aims to study H_2 production by cyanobacteria isolated from the Gulf of Thailand and the Andaman Sea in Thailand. The results showed that fifty-four cyanobacterial isolates could be isolated from seawater in Thailand and purified. By identification of 16S rDNA sequencing, most of isolated cyanobacteria were found to belonging in genus *Geitlerinema*. No morphological differences were observed in the *Geitlerinema* isolates. Nucleotide sequencing and phylogenetic tree analyses of the 16S rDNA, the 16S-23S rRNA internal transcribed spacer (16S-23S ITS), and the *cpcB-cpcA* intergenic spacer (*cpcB-cpcA* IGS) showed that the marine *Geitlerinema* isolates isolated from Thailand belonged to a single cluster that includes marine *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 but they were obviously distinct from true freshwater *Geitlerinema*. In addition, using random amplification of polymorphic DNA (RAPD), the marine *Geitlerinema* isolates in this study could be classified into eight clades; however, this classification revealed no correlation with the geographic locations. By screening for high H_2-producing marine cyanobacterial strains isolated in Thailand, the filamentous cyanobacterium *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production under anaerobic/dark and microaerobic/light conditions. *Geitlerinema* sp. RMK-SH10 showed the maximum H_2 production rate when cells were incubated in $NaNO_3$–free ASN III medium containing

1.4 mM $MgSO_4$. $7H_2O$, 0.2 M NaCl, 2 µM Fe^{3+}, 0.1 µM Ni^{2+} and 18.9 mmol C-atom glucose L^{-1}. The optimal light intensity, temperature and pH for H_2 production in *Geitlerinema* RMK-SH10 were under darkness, 40 °C and pH 8, respectively. *Geitlerinema* sp. RMK-SH10 could grow in seawater containing 9 mM $NaNO_3$. The maximum H_2 production of *Geitlerinema* sp. RMK-SH10 with 1,724.098±47.976 µmolH_2 g^{-1} dry weight was shown in cells incubated in seawater containing 18.9 mmol C-atom glucose L^{-1}. The highest H_2 accumulation of *Geitlerinema* sp. RMK-SH10 with 2,237.631±151.453 µmolH_2 g^{-1} dry weight was observed after 4 days of dark incubation under anoxic condition, suggesting that possibility of utilizing natural seawater for growth and H_2 production.

Keywords : H_2 production, marine cyanobacteria, *Geitlerinema*

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Miss Nichanan Tinpranee

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Chapter 1

Introduction

1.1 Research motivation

Nowadays, energy is essential for all living organisms. Energy is usually used for electricity production, household consumption, industrial consumption and transportation. The main energy sources used at the present time come from natural gas, coal and crude oil. These energy sources are finite fossil fuels that have been increasingly diminished in the near future. In addition, incomplete burning of fossil fuels causes environmental pollution by releasing the air pollutants such as CO_2, CO, SO_2 and other toxic substances, etc. From fuel reserve survey in Thailand, there are 0.362 billion barrels reserve of crude oil, 14,241 billion cubic feet reserve of natural gas and 0.422 billion barrels of condensate oil that can be used for about 6, 10 and 13 years, respectively. In addition, energy consumption of Thailand accounts 2,127 billion barrels per day whereas energy production and energy importation are 972 and 1,427 billion barrels per day, respectively (Ministry of Energy, 2017). Therefore, for prevention of an energy shortage in the future, many researchers have to search for alternative renewable energy which should not originate from carbon-based energy sources and should not affect environmental problems.

Hydrogen (H_2) is one of alternative interesting energy sources because it provides the highest heating value per unit weight with 141.6 MJ kg^{-1} (Perry, 1963), an approximately 3-7 times of heating value, compared with that of fossil fuels (Gupta, 2009). H_2 is a colorless and odorless gas. When it is burned with O_2, the obtained products are water and heat whereas when it is burned in the air, only oxide of nitrogen is produced as a by-product. It is demonstrated that the consumption of H_2 causes less air pollution than that of fossil fuels. H_2 is therefore assigned as a renewable clean fuel. Until now, there are several processes for H_2 production; steam reforming of natural gases, water electrolysis and biological process. H_2 production by the two former processes gives a lot of disadvantages that they use limited petroleum fuels as a feedstock and need a supply of high pressure during H_2 producing process. In addition, they release greenhouse gases affecting the

environmental problems. Biological H_2 production is very much interesting as an efficient and alternative method for sustainable H_2 production.

Biological H_2 production is a biological process that relies on the H_2 production ability of microorganisms such as photosynthetic bacteria, dark fermentative bacteria, green algae and cyanobacteria. Among H_2 producing microorganisms, prokaryotic cyanobacteria provide a number of advantages since they can fix atmospheric CO_2 and N_2 in the air and use unlimited sunlight energy to evolve H_2 via photosynthesis, N_2 fixation and dark fermentation using hydrogenase and nitrogenase enzymes. In addition, cyanobcteria can grow well in culture medium containing cheap substrates, have various methods and techniques for an easy manipulation and mass cultivation, and have intensive investigation for industrial applications.

In cyanobacteria, H_2 is produced by a direct photolysis and an indirect photolysis. During photosynthesis they use a solar light as an energy source to produce H_2 via hydrogenase activity. Under dark fermentation they produce H_2 by hydrogenase activity from a hydrolysis of accumulated glycogen. In addition, some heterocystous N_2-fixing cyanobacteria can produce H_2 as a by-product via the N_2 fixation process using nitrogenase activity. Hydrogenase involved in H_2 metabolism of cyanobacteria is NiFe-hydrogenase (Przybyla *et al.*, 1992; Cammack, 1999; Vignais *et al.*, 2001; Hallenbeck, 2012). Many cyanobacterial strains have been shown to produce H_2 under darkness. H_2 metabolism is studied in various types of cyanobacteria such as *Oscillatoria* sp. Miami BG7, *Calothrix scopulorum*, *Cyanothece* sp. PCC 7822, *Anabaena cylindrica* B-629, *Synechocystis* sp. PCC 6308, *Phormidium valderianum*, and *Aphanothece halophytica*. (Phlips and Mitsui 1983; Howarth and Codd, 1985; Lambert and Smith, 1977; Van der Oost *et al.*, 1989; Prabaharan *et al.*, 2010; Taikhao *et al.*, 2013; Taikhao *et al.*, 2015).

Cyanobacteria can be found in various habitats such as freshwater, brackish water, soil or seawater ecosystem. Cyanobacteria found in seawater or marine cyanobacteria are more diverse than those found in other habitats. Seawater is composed of 96.5% water and 3.5% mineral salts (Smith, 1974; Garrison, 2007). Many researchers are interested in an investigation of H_2 production by cyanobacteria,

especially freshwater cyanobacteria while the study of H_2 production by marine cyanobacteria is rather rare. It has been previously reported that marine cyanobacteria show higher H_2 production than freshwater cyanobacteria (Kothari, 2012).

This work focuses on the study of H_2 production by marine cyanobacteria isolated from seawater in Thailand. Marine cyanobacteria are organisms which are found or abundant in the ocean or marine environments. They can tolerate and survive under the extreme salt concentrations and are possible to grow and produce H_2 in natural seawater. In this study, cyanobacteria are isolated from the Gulf of Thailand and the Andaman Sea in Thailand, and then they are identified by morphological and genetic characteristics. Isolated cyanobacteria are screen for high H_2 production under different conditions. Afterthat, the optimization for H_2 production by the selected strain is performed under several physiological parameters. Finally, an attempt for utilization of natural seawater as a culture medium for growth and H_2 production in the selected marine cyanobacterial isolate is shown.

1.2 Objectives of the study

The objectives of this study are

1) To isolate and study the morphological and genetic characterization of marine cyanobacteria isolated from the Gulf of Thailand and the Andaman Sea in Thailand.

2) To screen for high H_2 producing marine cyanobacteria isolated from the Gulf of Thailand and the Andaman Sea in Thailand.

3) To optimize H_2 production by the selected high potential marine cyanobacterial isolate.

4) To utilize natural seawater as a culture medium for growth and H_2 production in marine cyanobacterial isolate.

1.3 Scope of the study

Marine cyanobacterial strains are isolated from samples of seawater, stones, sand and shells collected from 24 coastline locations along the Gulf of Thailand and 16 coastline locations along the Andaman Sea of Thailand. Then, isolated marine cyanobacteria are purified using a single cell isolation technique under a stereomicroscope. The specie identification of purified cyanobacterial isolates is performed by morphological study and molecular analysis by 16S rDNA sequencing. The genetic diversity of marine cyanobacterial isolates is investigated using sequencing analysis of 16S rDNA, 16S-23S rRNA internal transcribed spacer region (16S-23S ITS), intergenic spacer region of the phycocyanin locus *(cpcB-cpcA* IGS*)* and random amplification of polymorphic DNA (RAPD) method. Marine cyanobacterial isolates are grown in ASN III medium and their H_2 production rate is measured under four conditions; (1) anaerobic/dark condition, (2) microaerobic/light condition, (3) aerobic/dark condition and (4) aerobic/light condition. The highest H_2 producing strain is selected and its optimization for H_2 production is investigated. The effect of several physiological parameters such as cultivation time, nutrient and mineral compositions, salinity, light intensity, medium pH and incubation temperature on H_2 production is investigated. Finally, natural seawater is used as a culture medium for cultivation the selected marine cyanobacterial isolate and its H_2 production is examined.

1.4 Benefits of the study

The results of this study will be of great benefit to researchers and renewable energy organizations in searching for high potential H_2 producing marine cyanobacterial strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand. In addition, this study provides the informations about the optimal conditions for H_2 production and the possibility for cultivation in natural seawater by the selected high potential marine cyanobacterail isolate.

Chapter 2

Theory and Literature Reviews

2.1 Hydrogen (H_2)

Nowadays, energy is essential for all living organisms in a daily life. Energy is used for human in many purposes; for example, electricity in a household consumption, electricity in an industrial consumption, and fuel for transportation. The main energy sources come from fossil fuels such as natural gas, coal and crude oil. These fossil fuels are finite and are going to diminish in the near future. In addition, incomplete combustion of fossil fuels causes environmental pollution by a release of air pollutants such as CO_2, CO, SO_2 and other toxic substances, etc. In Thailand, there is a high demand of energy consumption but there is not enough energy production within a country; therefore it is unavoidable to import these fuels from foreign countries. From fuel reserve survey in Thailand, there are 0.362 billion barrels reserve of crude oil, 14,241 billion cubic feet reserve of natural gas and 0.422 billion barrels of condensate oil that can be used for about 6, 10 and 13 years, respectively. In addition, energy consumption of Thailand accounts 2,127 billion barrels per day whereas energy production and energy importation are 972 and 1,427 billion barrels per day, respectively (Fig. 2.1) (Ministry of Energy, 2017). For prevention of an energy shortage in the future, many researchers have to search for alternative renewable energy sources which should be non-carbon-based energy and should not affect environmental problems. The examples of these renewable energy sources are wind, water, geothermal heating, solar, biomass and hydrogen.

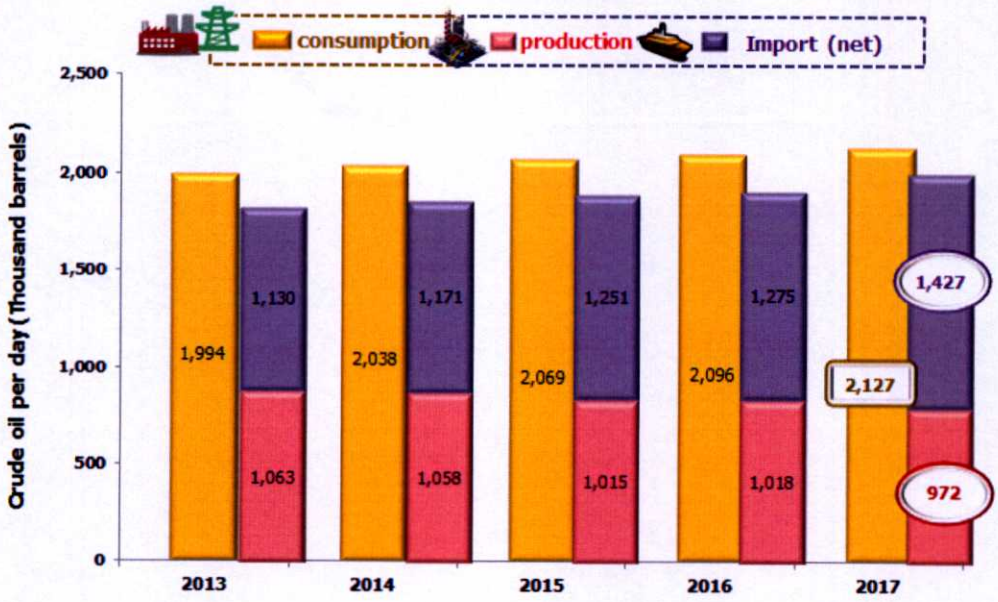


Figure 2.1 Consumption, production and import (net) of crude oil per day in Thailand between 2013 to 2017 (Ministry of Energy, 2017)

Hydrogen (H_2) is one of alternative interesting energy sources because it provides the highest heating value per unit weight with 141.6 MJ kg^{-1} (Perry, 1963), an approximately 3-7 times of heating value compared with that of fossil fuels (Gupta, 2009) (Fig. 2.2). H_2 is a colorless, odorless, tasteless gas known as the lightest gas in the universe. H_2 can change from a gas phase to a liquid phase at a temperature of -252.77 °C (-422.99 °F) and it can change from a liquid phase to a solid phase at a temperature of -259.2 °C (-434.6 °F) (Pant *et al.*, 2009; Subramani *et al.*, 2015). It can slightly soluble in water, alcohol and some other common liquids (Brunner, 1985). When H_2 is burned with O_2, the obtained products are water and heat whereas when it is burned in the air, oxide of nitrogen is produced as a by-product. When H_2 is combined with nitrogen gas (N_2) at high pressure and temperature, ammonia (NH_3) is produced whereas when it is combined with carbon monoxide (CO), methanol (CH_3OH) is generated (Hwang and Mebel, 2003; Martin *et al.*, 2016). In addition, H_2 is a highly flammable and combustible diatomic gas. When H_2 is mixed with air and chlorine, its mixture can spontaneously explode by sparking and heating (Subramani *et al.*, 2015). From above mention about H_2 characteristics, it is demonstrated that H_2 consumption causes less air pollution than that of fossil fuels.

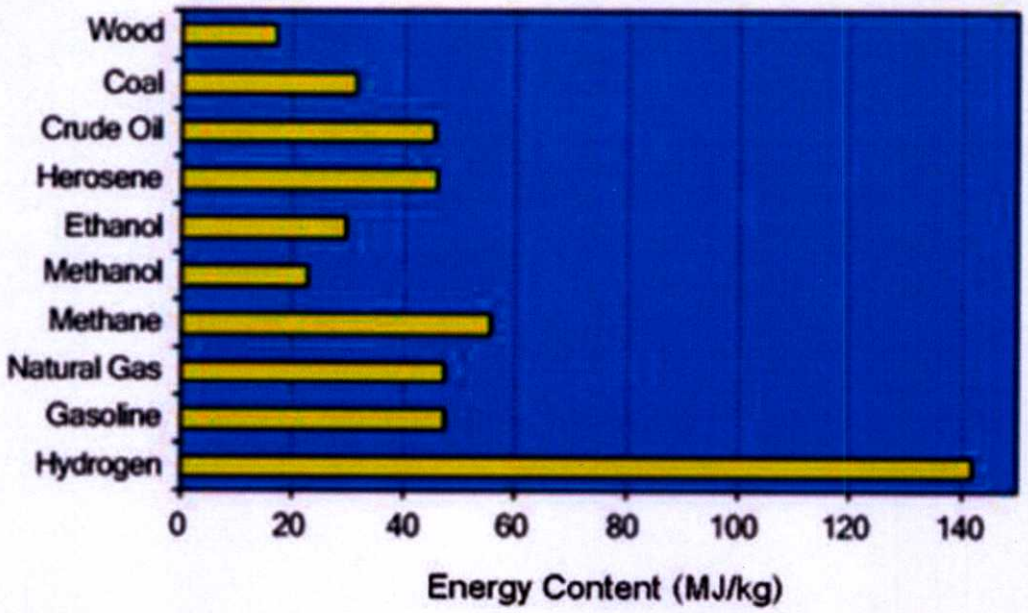


Figure 2.2 Energy content of H_2 compared to the other hydrocarbon fuels (http://www.people.hofstra.edu/geotrans/eng/ch8en/conc8en/energycontent.html)

2.2 H_2 production processes

H_2 production can be divided into 3 main processes; H_2 production by steam reforming of natural gas, H_2 production by water electrolysis and biological H_2 production.

2.2.1 H_2 production by steam reforming of natural gas

H_2 production by reforming of natural gas obtained from fossil fuels has been used as the main process for H_2 production, accounting for 50 % of the world's total H_2 production. The popularity of this process derives from its high conversion efficiency and cost-effectiveness in comparison with other processes (Chen *et al.*, 2008). There are two chemical reactions in this process. First is called "Steam reforming reaction". This reaction occurs at high temperatures (700-1,000 °C) in which the fuel (such as hydrocarbon or alcohol) reacts with steam and is converted into a gaseous mixture of H_2, CO, CO_2, unreacted hydrocarbon or alcohol, and unreacted steam (Equation 2.1). Another one is called "Water-gas shift reaction" that occurs at lower temperature (130 °C) in a shift reactor in which CO from the former reaction reacts with H_2O and produce the additional mixture of CO_2 and H_2 (Equation 2.2).

$$\text{Fuel} + H_2O \longrightarrow CO\,(g) + H_2\,(g) \qquad (2.1)$$

$$CO\,(g) + H_2O \longrightarrow CO_2\,(g) + H_2\,(g) \qquad (2.2)$$

H_2 production by steam reforming of natural gas provides high H_2 yield and high thermal efficiency; however, there are a lot of disadvantages of H_2 production by this process. It requires a large amount of energy for supplying a high temperature during steam reforming process. It still uses natural gases, petroleum refinery products from limited fossil fuel sources, as substrate. In addition, this process produces CO_2, the main greenhouse gas, other than H_2, causing the air pollutant and global warming.

2.2.2 H_2 production by water electrolysis

H_2 production by water electrolysis is a method of water splitting into H_2 and O_2 using electricity (direct current). This reaction takes place in a unit called "electrolyzer". The oxidation reaction takes place at anode. Water is hydrolyzed to oxygen (O_2), protons (H^+) and electrons (e^-) (Equation 2.3). At cathode the reduction reaction occurs, water is reacted with electrons and this generates H_2 gas and hydroxide ions as products (Equation 2.4).

$$2H_2O \longrightarrow O_2\,(g) + 4H^+ + 4e^- \qquad (2.3)$$

$$4H_2O + 4e^- \longrightarrow 2H_2\,(g) + 4OH^- \qquad (2.4)$$

By this process, the number of H_2 molecules is produced at cathode whereas the number of O_2 molecules is produced at anode (Fig. 2.3). The advantage of H_2 production by this process is that there is no need for a posterior compression of the produced H_2, although it reduces purity of the product gases caused by the fact that high pressures and high temperatures increase the permeability for gases through the membrane (Bhandari *et al.*, 2014). However, this process has an important disadvantage that it needs a large amount of electricity for decomposing H_2O, accounting for 80% of the operating cost of H_2 production (Kapdan and Kargi, 2006).

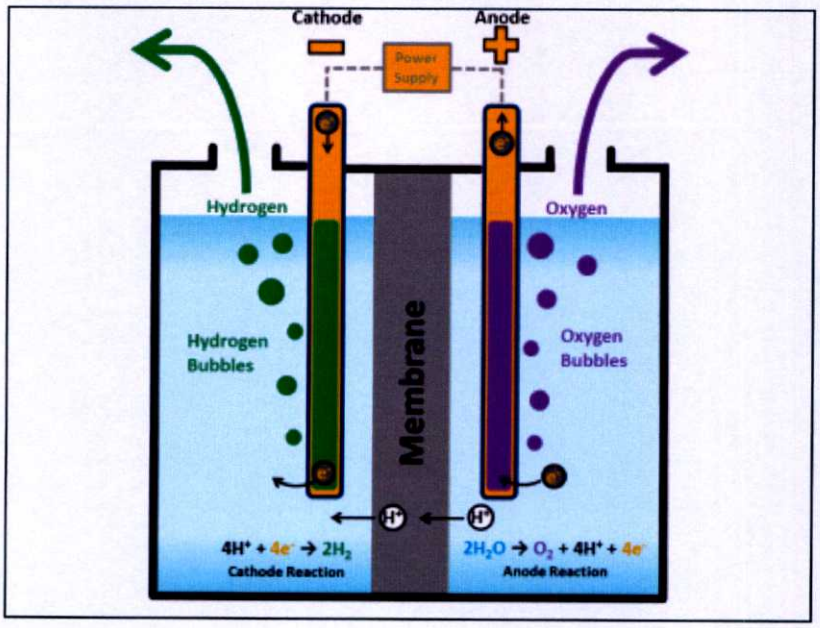


Figure 2.3 H_2 production by water electrolysis
(https://www.energy.gov/eere/fuelcells/hydrogen-production-electrolysis)

2.2.3 Biological H_2 production

Biological H_2 production is a biological process that relies on the H_2 producing ability of microorganisms such as photosynthetic bacteria, dark fermentative bacteria, green algae and cyanobacteria. These microorganisms are able to produce H_2 by hydrogenase activity via oxygenic photosynthesis and/or dark fermentation (Fig. 2.4). In addition, some heterocystous N_2-fixing cyanobacteria can produce H_2 as a by-product of N_2 fixation by nitrogenase activity (Kosaric and Lyng, 1988). The biological H_2 production shows some advantages compared with the chemical H_2 production. Some photosynthetic microorganisms can use atmospheric CO_2 and sunlight as carbon and energy sources for cell growth and H_2 production, resulting in a reduction of the main greenhouse gas CO_2 in the atmosphere. Some bacteria can use organic wastes as carbon source for growth, leading to the decrease of organic wastes in an environment. Some bacteria can use organic wastes as carbon source for growth, causing a decrease of organic wastes in nature. Therefore, H_2 produced by biological process is implied as an environmental-friendly fuel.

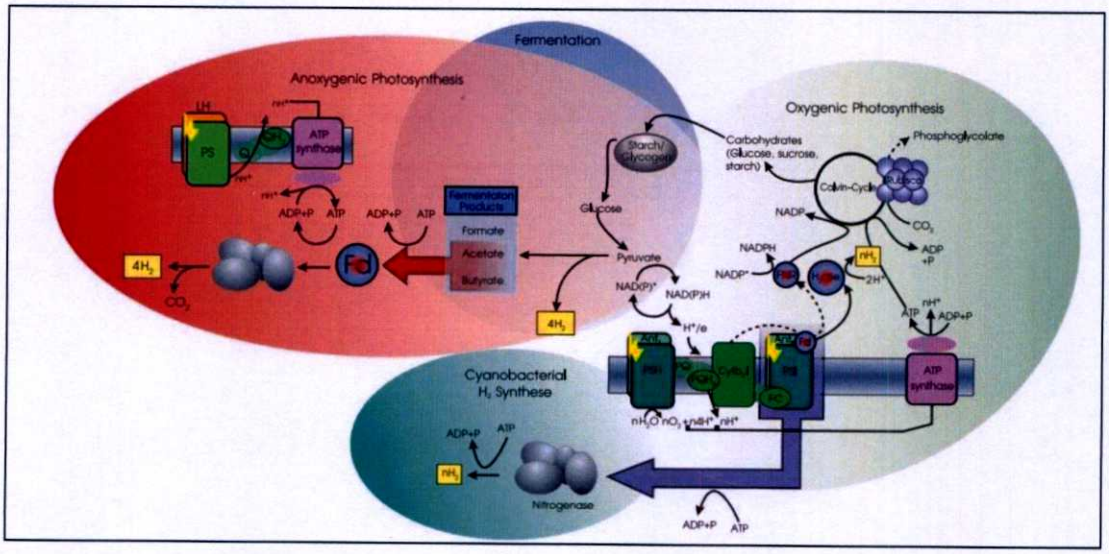


Figure 2.4 Biological H_2 production
(http://www.making-hydrogen.com/biohydrogen.html)

2.3 Biohydrogen production

H_2 can be produced by various types of microorganisms such as photosynthetic bacteria, dark fermentative bacteria, cyanobacteria and green algae. These microorganisms show different and unique pathways of H_2 metabolism as follows.

2.3.1 H_2 production by photosynthetic bacteria

Photosynthetic bacteria produce H_2 via nitrogen fixation and anoxygenic photofermentation. The purple non-sulfur (PNS) photosynthetic bacteria, including *Rhodobacter* species, are able to convert organic acids such as acetate, lactate, and butyrate, to H_2 and CO_2 under an anaerobic condition using nitrogenase activity in an absence of ammonium ions in medium (Basak and Das, 2007; Mohan and Pandey, 2013; Azwar *et al.*, 2014). The O_2-sensitive nitrogenase is not a problem for H_2 production process in purple bacteria because these organisms belong a nonoxygenic photosynthesis (Basak and Das, 2007). In H_2 production by purple non-sulfur bacteria, organic or inorganic compounds are used to produce biomass by oxidizing these compounds to NAD(P)H and CO_2. Electrons from NAD(P)H are transferred via ubiquinone (UQ) to the photosystem (PS) where they are energized by light. Electrons are repeatedly energized and cycled through the photosynthetic electron transport chain to produce a proton gradient. Energy from a proton gradient

is used to transfer electrons from the photosynthetic electron transport chain to ferredoxin via oxidoreductases (OR). The proton gradient is used to generate ATP. Ferredoxin and ATP are then used to generate H_2 via nitrogenase (N_2ase) (Fig. 2.5) (McKinlay and Harwood, 2010).

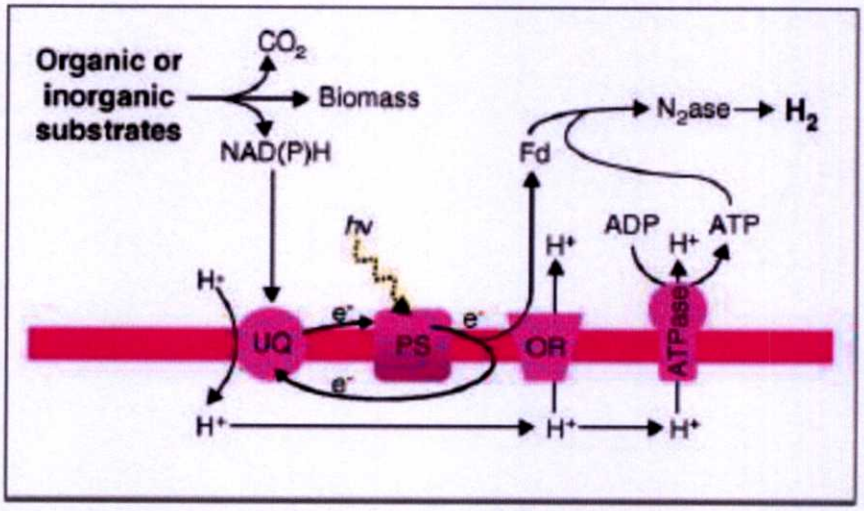


Figure 2.5 H_2 production by purple non-sulfur bacteria (McKinlay and Harwood, 2010)

2.3.2 H_2 production by dark fermentative bacteria

Dark fermentative H_2 production is the fermentative process of organic substrates conversion to H_2 (Hallenbeck and Benemann, 2002). This process was performed in facultative anaerobic bacteria such as *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli* and *Citrobacter intermedius*, and obligate anaerobic bacteria such as *Clostridium beijerinckii*, *Clostridium paraputrificum* and *Ruminococcus albus* (Hallenbeck and Ghos, 2009; Chandrasekhar and Mohan, 2014a, b). H_2 is produced in many types of fermentation such as acetic acid fermentation (Equation 2.5) and butyric acid fermentation (Equation 2.6) as a way to regenerate NAD^+ from NADH. Electrons are transferred to ferredoxin, which is in turn oxidized by hydrogenase to produce H_2.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad (2.5)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad (2.6)$$

2.3.3 H_2 production by cyanobacteria

Cyanobacteria are prokaryotic microorganisms capable of oxygenic photosynthesis. They can produce H_2 by hydrogenases using electrons derived either from water splitting process of photosynthesis in the light (Prince and Kheshgi, 2005; Allahverdiyeva *et al.*, 2010) or from the degradation of storage glycogen via a dark fermentation (Ananyev *et al*, 2008). Some N_2–fixing cyanobacteria can release H_2 as a byproduct by an action of nitrogenase via nitrogen fixation (Reddy *et al.*, 1996; Chen *et al.*, 2008). H_2 metabolism is studied in various species of cyanobacteria such as *Oscillatoria* sp. Miami BG7, *Calothrix scopulorum*, *Cyanothece* 7822, *Anabaena cylindrica* B-629, *Synechocystis* sp. PCC 6803, *Phormidium valderianum* and *Aphanothece halophytica* (Phlips and Mitsui, 1983; Howarth and Codd, 1985; Lambert and Smith, 1977; Van der Oost *et al.*, 1989; Prabaharan and Subramanian, 1996; Prabaharan *et al.*, 2010; Taikhao *et al.*, 2013; Taikhao *et al.*, 2015).

2.3.4 H_2 production by green algae

In green algae, H_2 is produced by a direct photolysis and an indirect photolysis. During photosynthesis they use a solar light as an energy source and use inorganic or organic compounds as an electron source to produce H_2 via hydrogenase activity (Iron hydrogenase type). Under dark fermentation they produce H_2 by hydrogenase activity from a hydrolysis of accumulated starch (Fig. 2.6) (Melis *et al.*, 2000; Melis and Happe, 2001). Green algal hydrogenases are also sensitive to O_2. However, Iron hydrogenases in green algae are more oxygen-tolerant than cyanobacterial hydrogenases (Ghirardi *et al.*, 2007). H_2 metabolism in green algae is intensively investigated in many species of green algae such as *Chlamydomonas reinhardtii* (Tsygankov *et al.*, 2006), *Chlorella protothecoides* (He *et al.*, 2012) and *Scenedesmus obliquus* (Abeliovich and Weisman, 1978). Amomg green algal strains, *C. reinhardtii* is a model organism for studying H_2 metabolism in green algae (Happe and Naber, 1993; Harris, 2001; Hemschemeier, 2009).

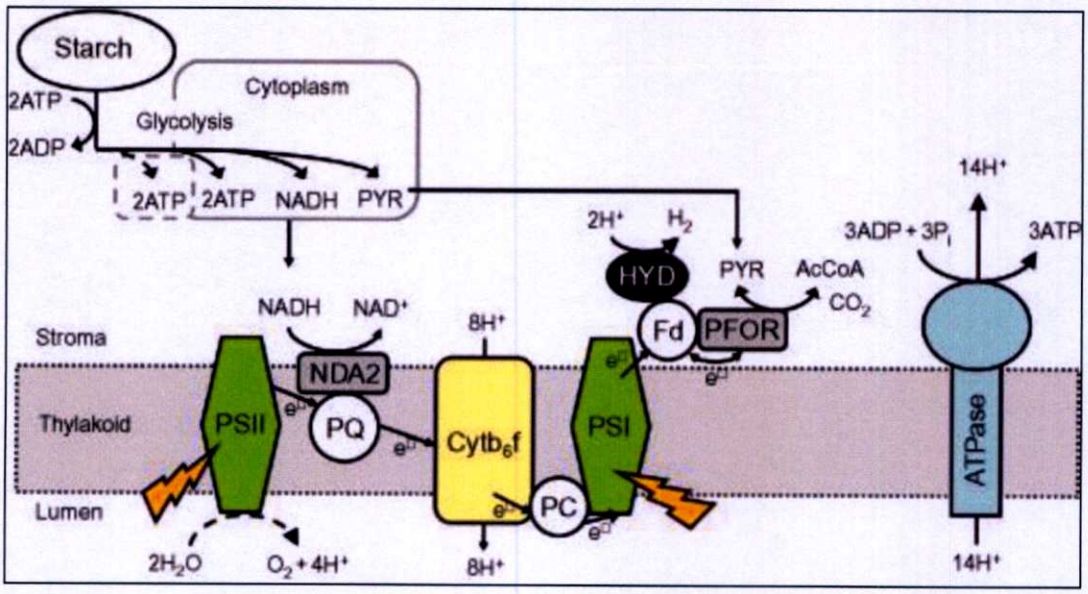


Figure 2.6 H_2 production by green algae via direct photolysis and indirect photolysis (Burgess *et al.*, 2011)

2.4 H_2 production by cyanobacteria

Cyanobacteria can produce H_2 via a direct photolysis and an indirect photolysis. In a direct photolysis cyanobacteria use a solar light as an energy source to produce H_2 by hydrogenase activity under the light. In an indirect photolysis they produce H_2 by hydrogenase activity from a hydrolysis of accumulated glycogen under darkness. In addition, some heterocystous N_2-fixing cyanobacteria can produce H_2 as a by-product via N_2 fixation by nitrogenase activity. Three types of cyanobacteria, heterocystous cyanobacteria, non-heterocystous cyanobacteria and marine cyanobacteria, have been reported to produce H_2 (Table 2.1).

Table 2.1 Type of H_2 producing cyanobacteria

Type of H_2 producing cyanobacteria	References
Heterocystous cyanobacteria	
Anabaena azollae	Peters *et al.*, 1976
Anabaenopsis circularis	Dutta *et al.*, 2005
Anabaena cylindrica	Hallenbeck *et al.*, (1978); Dutta *et al.*, 2005
Anabaena flos-aquae	Dutta *et al.*, 2005
Anabaena sp. PCC 7120	Lindblad *et al.*, 2002
Anabaena variabilis	Borodin *et al.*, 2000
Nostoc commune	Dutta *et al.*, 2005
Nostoc linckia	Mona *et al.*, 2011
Nostoc muscorum	Spiller *et al.*, 1978
Non-Heterocystous cyanobacteria	
Aphanocapsa montana	Dutta *et al.*, 2005
Chroococcidiopsis thermalis CALU 758	Serebryakova *et al.*, 2000
Gloebacter sp.	Dutta *et al.*, 2005
Gloeocapsa alpicola CALU 743	Troshina *et al.*, 2002
Microcystis sp. PCC 7806	Moezelaar and Stal, 1994
Microcoleus chthonoplasts	Dutta *et al.*, 2005
Synechococcus sp. PCC 602	Howarth and Codd, 1985
Synechocystis sp. PCC 6803	Appel *et al.*, 1996; Antal and Lindblad, 2005
Marine cyanobacteria	
Anabaena cylindrica B-629	Lambert and Smith, 1977
Calothrix membranacea B-379	Lambert and Smith, 1977
Calothrix scopulorum 1410/5	Lambert and Smith, 1977
Cyanothece sp. PCC 7822	Van der Oost *et al.*, 1989
Oscillatoria brevis	Lambert and Smith, 1977
Oscillatoria limosa	Heyer *et al.*, 1989
Oscillatoria sp. Miami BG7	Phlips and Mitsui, 1983
Phormidium valderianum BDU 20041	Prabaharan and Subramanian, 1996
Trichodesmium erythraeum IMS 101	Wilson *et al.*, 2010

2.4.1 H_2 production by photosynthesis

H_2 by cyanobacteria is produced via a direct photolysis and an indirect photolysis. In direct photolysis, photosystem II (PS II) captures sunlight energy. Then, water splitting occurs to generate O_2, protons and mobilized electrons. The electrons are transferred through plastoquinone (PQ), cytochrome *b6f* (cyt *b6f*), plastocyanin (PC) and photosystem I (PS I). Then electrons are transferred to oxidized nicotinamide adenine dinucleotide (phosphate) ($NAD(P)^+$) via ferredoxin (Fd). The reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) is formed by reduction reaction through ferredoxin oxidoreductase and subsequently transfer electrons to NiFe-hydrogenase to produce H_2 (Fig. 2.7). In addition, ATP and NAD(P)H are also used in CO_2 fixation to generate biosynthetic precursors and storage compounds in the form of a carbohydrate via the Calvin cycle. Indirect photolysis, the ATP and NAD(P)H generated during the photosynthesis electron flow are consumed in the Calvin–Benson cycle that fixes CO_2 to store endogenous carbohydrate (CH_2O) such as glycogen in cyanobacteria. H_2 is obtained from anaerobic glucose or glycogen) fermentation (Fig. 2.7) as shown in equation 2.8. The energy efficiency of the overall glucose fermentation can be roughly estimated from the heat of combustion of glucose, hydrogen and acetic acid. The energy obtained in 4 moles of H_2 is about 40% of the energy in one mole of glucose (Yu and Takahashi, 2007).

$$C_6H_{12}O_6+2H_2O \longrightarrow 2CH_3COOH+4H_2+2CO_2 \qquad (2.8)$$

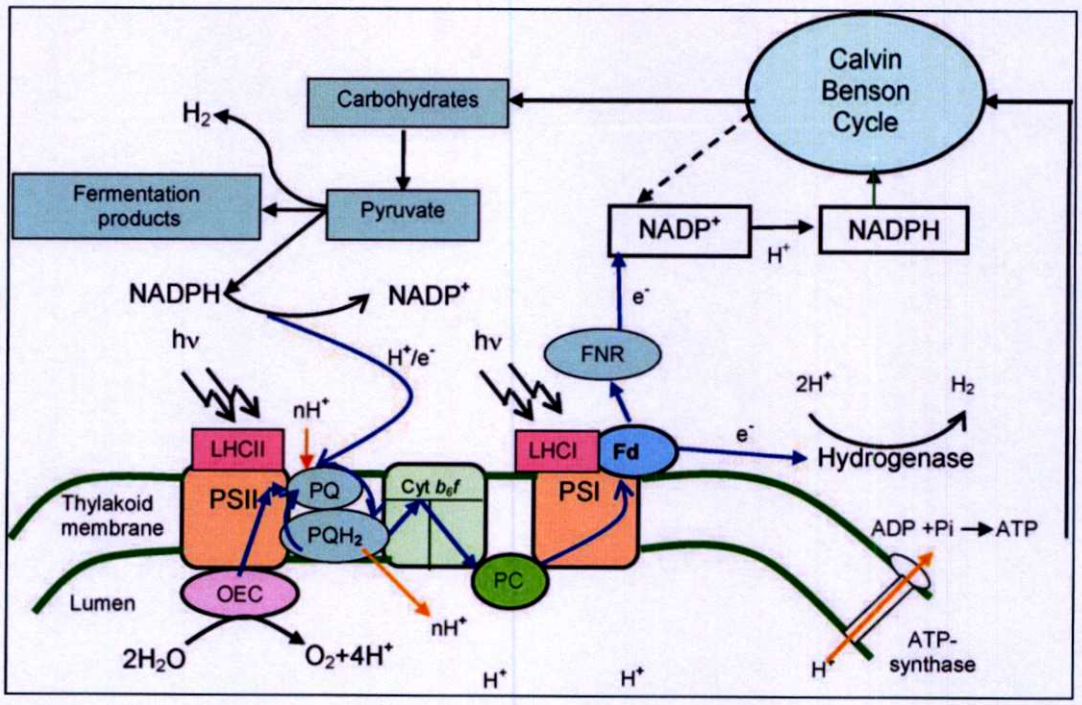


Figure 2.7 H_2 production by cyanobacteria via direct photolysis and indirect photolysis (Allakhverdiev *et al.*, 2010)

2.4.2 H_2 production by nitrogen fixation

H_2 production by N_2 fixation is mostly found in the filamentous cyanobacterial species that have specialized cells called "heterocyst". These cyanobacteria include *Anabaena* sp. PCC 7120, *Calothrix membranacea* B379, *Tolypothrix* sp. and *Nostoc muscorum* (Lambert and Smith, 1977; Kumar and Kumar, 1992; Masukawa *et al.*, 2001; Yeager, 2011). The heterocyst cells have a thick wall that shows O_2 impermeable ability, leading to an anaerobic (oxygen-free) environment. This condition is necessary for the operation of nitrogenase activity. Under an atmosphere of air and N_2, N_2 fixation is the predominant reaction at heterocyst cells. It can fix atmospheric N_2 by catalyzing of nitrogenase enzyme to produce NH_3 or NH_4^+ as a main product and H_2 as a by-product. When PS I in heterocyst cells absorbs sunlight energy, electrons are shuttled via ferredoxin to feed the Calvin cycle for CO_2 fixation in order to generate storage carbohydrate. Nitrogenase can accept electrons from ferredoxin and combine them with protons and N_2 to generate NH_3 and H_2 (Fig. 2.8).

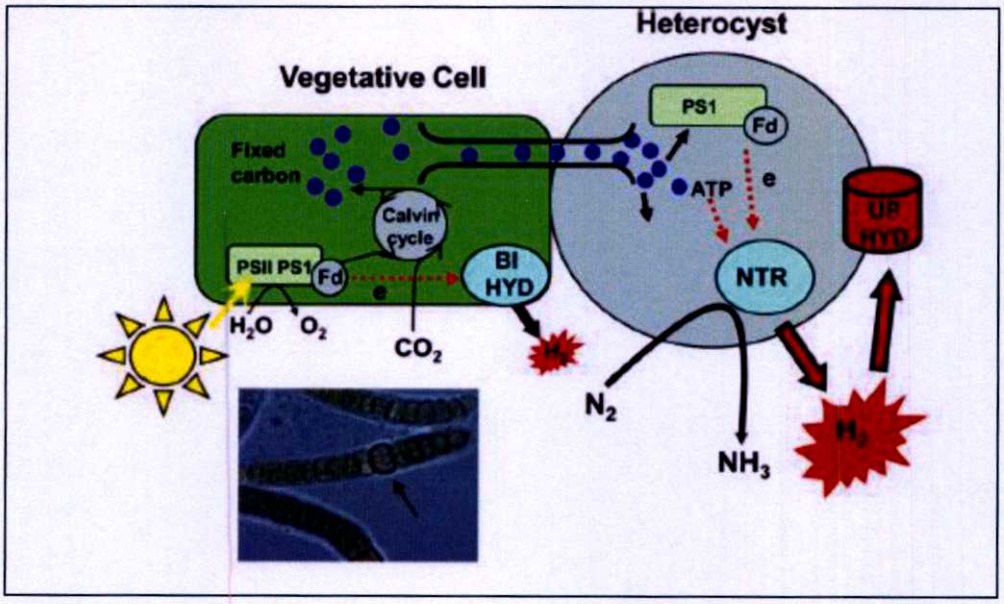


Figure 2.8 H_2 production by N_2-fixing cyanobacteria via nitrogen fixation process (Yeager *et al.*, 2011)

2.5 Enzymes involved in H_2 metabolism of cyanobacteria

There are three enzymes involved in H_2 metabolism of cyanobacteria including, nitrogenase, uptake hydrogenase and bidirectional hydrogenase.

2.5.1 Nitrogenase

Nitrogenase (EC 1.18.6.1, EC 1.19.6.1) is an enzyme complex catalyzing N_2 fixation found in the filamentous species that have heterocyst cells when grown in nitrogen limitation in the medium. This enzyme is responsible for the reduction of nitrogen (N_2) to ammonia (NH_3). H_2 is produced as a by-product of fixation of nitrogen into ammonia. The reaction consumes a numerous of ATP as shown in equation 2.9.

$$N_2+8H^++8e^-+16ATP \longrightarrow 2NH_3+H_2+16ADP+16Pi \qquad (2.9)$$

The nitrogenase complex comprises two main functional subunits, one is dinitrogenase (MoFe protein) composed of α and β subunits encoded by *nifD* and *nifK*, respectively, (*nif* is from *nitrogen fixation*) and another is dinitrogenase reductase (Fe Protein) encoded by *nifH*. Dinitrogenase is a $\alpha 2\beta 2$ heterotetramer, with a total molecular weight of about 220 to 240 kDa, respectively, and has two copies of P-cluster (an 8Fe-7S cluster) which is involved in the participation of MoFe cofactor (Fig.

2.9) (Rees and Haward, 2000). Dinitrogenase reductase is a homodimer with a molecular weight of about 60 to 70 kDa. The two Fe-protein subunits of dinitrogenase reductase bind with 4Fe-4S cluster and also bind Mg-ATP at one site in each subunit (Fig. 2.9) (Triplett, 2000). It plays the specific role in mediating the transfer of electrons from the external electron donor (a ferredoxin or a flavodoxin) to the dinitrogenase (Orme-Johnson, 1992; Flores and Herrero, 1994; Masepohl *et al.*, 1997).

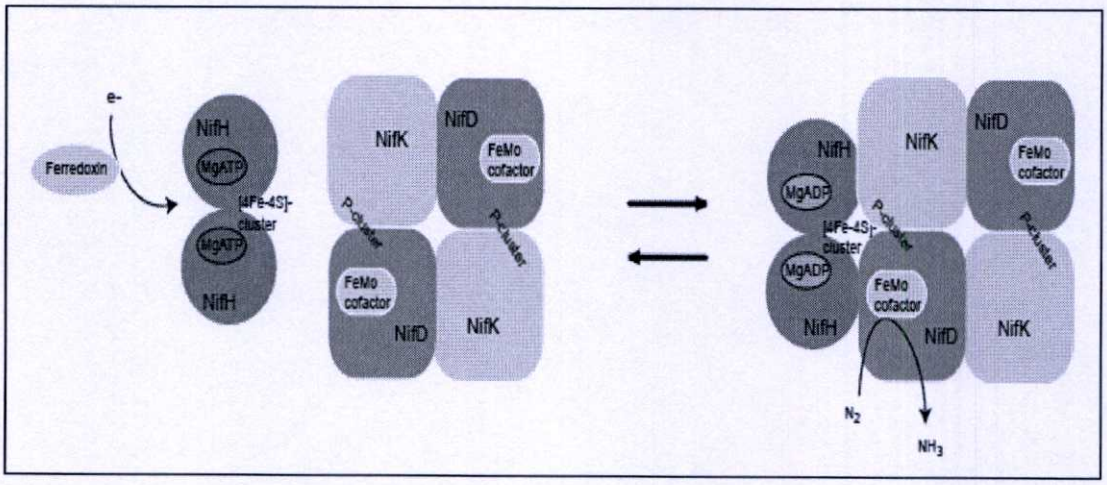


Figure 2.9 Nitrogenase enzyme complex and their mechanism (Lindberg, 2003)

There are three types of dinitrogenase depending on type of the metal content in the active site. Type one contains molybdenum (Mo) and is called "Molybdenum nitrogenase" (Thiel, 1993). Type two contains vanadium (V) instead of Mo and is called "Vanadium nitrogenase" (Kentemich *et al.*, 1988; Thiel, 1993). The last type has neither Mo nor V but it contains iron (Fe) in the active site and is called "Iron nitrogenase" (Kentemich *et al.*, 1991; Bishop and Premakumar, 1992). The vanadium and iron nitrogenases are known as an alternative nitrogenase that is not expressed when cells are incubated in molybdenum-sufficient condition but it promotes continuously by N_2 fixation in the limited molybdenum condition. The N_2-fixing cyanobacterium *Anabeana variabilis* has been reported to contain two nitrogenases, molybdenum nitrogenase and vanadium nitrogenase (Thiel, 1993; Thiel, 1996).

2.5.2 Hydrogenase

Hydrogenase (EC 1.12.1.2, 1.12.2.1 and 1.18.99) is an enzyme that reversibly catalyzes the reduction of protons to molecular H_2 and the oxidation of H_2 to protons as shown in equation 2.10.

$$H_2 \longleftrightarrow 2H^+ + 2e^- \qquad (2.10)$$

Hydrogenase can be divided into three major groups according to the metal composition in the active site, NiFe–hydrogenase, Fe-hydrogenase and metal-free hydrogenase (Vignais *et al.*, 2001). In cyanobacteria the mostly found hydrogenase is NiFe–hydrogenase (Przybyla *et al.*, 1992; Cammack, 1999; Vignais *et al.*, 2001; Hallenbeck, 2012) whereas hydrogenase found in green algae is Fe-hydrogenase (Gaffron and Rubin, 1942; Hartman and Krasma, 1963; Healey, 1970). Green algal and cyanobacterial hydrogenases are very sensitive to O_2. However, iron hydrogenase in green algae is more oxygen-tolerant than cyanobacterial hydrogenase (Ghirardi *et al.*, 2007). In addition, hydrogenase in cyanobacteria can be classified in two groups depending on the direction of the reaction; (1) Unidirectional or uptake hydrogenase, catalyzing the oxidation of H_2 into protons and (2) Bidirectional or reversible hydrogenase, catalyzing the oxidation of H_2 into protons and reduction of protons into H_2.

2.5.2.1 Unidirectional or uptake hydrogenase

The unidirectional or uptake hydrogenase is found attached to the thylakoid membrane of heterocyst cells in filamentous cyanobacteria. It functions only to catalyze the oxidation of H_2 into protons and electrons. The obtained electrons are transferred for the reduction of O_2 via the respiratory chain. This reaction is known as "Oxyhydrogenation or Knallgas reaction". Uptake hydrogenase is a heterodimeric protein consisting of two subunits, HupL and HupS (Fig. 2.10). HupL is a large subunit encoded by *hupL* (*hup* is from *hydrogen uptake*) and has a molecular weight of about 55-70 kDa. It contains a redox-active nickel plus iron-sulfur cluster in the active site. HupS is a small subunit encoded by *hupS* and has a molecular weight of about 25-35 kDa. It contains three iron-sulfer clusters bound by cysteine and/or histidine residues which play a role in an electron transfer.

The main physiological function of uptake hydrogenase is to reutilize and regain H_2 and electrons produced by H_2 evolution by nitrogenase (Tamagnini *et al.*, 2002). It is suggested that uptake hydrogenase gives three beneficial functions; (1) it provides additional electrons as reducing equivalents for various cell functions, (2) it provides ATP via the oxyhydrogen reaction resulting in minimizing the loss of energy and (3) it protects inactivation of nitrogenase by removing of O_2 (Bothe *et al.*, 1977; Howarth and Codd, 1985; Weisshaar and Böger, 1985; Smith, 1990; Kentemich *et al.*, 1991).

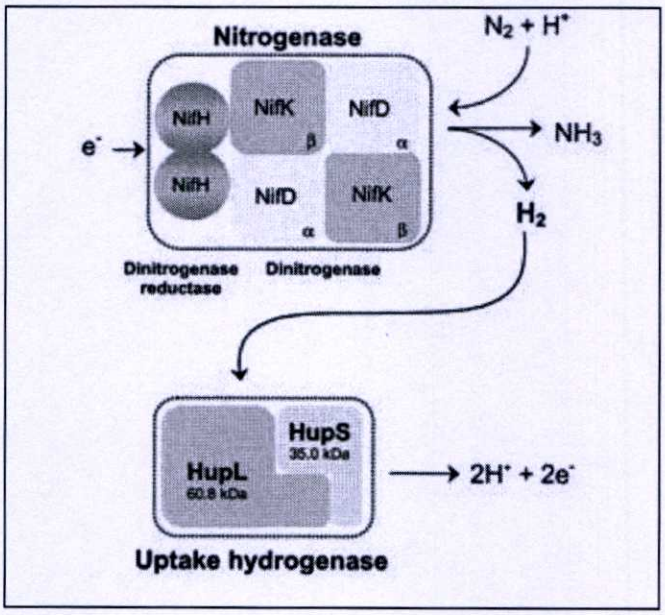


Figure 2.10 H_2 oxidation into protons and electrons by uptake hydrogenase (Lindberg, 2003)

2.5.2.2 Bidirectional or reversible hydrogenase

Bidirectional or reversible hydrogenase is a soluble enzyme or loosely attached membrane enzyme found in both N_2-fixing and non N_2-fixing cyanobacterial strains. Bidirectional or reversible hydrogenase is a heterotetrameric enzyme consisting of two main subunits, hydrogenase structural complex (HoxY and HoxH) and diaphorase component (HoxF and HoxU) (Fig. 2.11). HoxH and HoxY are encoded by *hoxH* and *hoxY*, respectively (*hox* is from *hydrogen oxidation*) whereas HoxF and HoxU are encoded by *hoxF* and *hoxU*, respectively. In addition, beside 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).

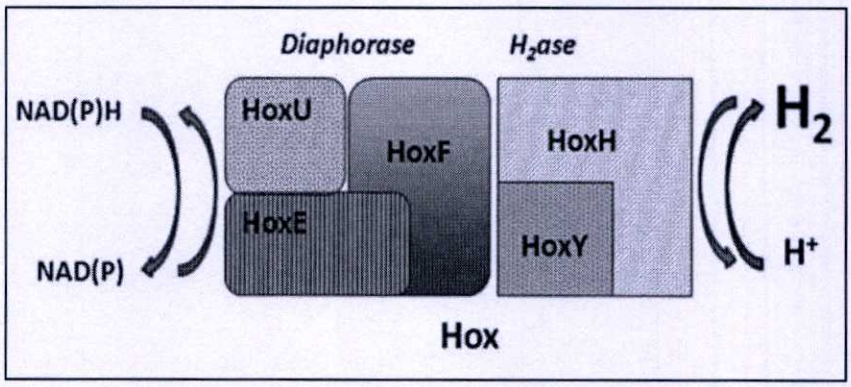


Figure 2.11 H_2 production and consumption by bidirectional hydrogenase (Hallenbeck, 2012)

The bidirectional or reversible hydrogenase can both take up H_2 and produce H_2 depending on the requirement of electrons or protons in cells (Eisbrenner *et al.*, 1978; Tamagnini *et al.*, 2002). This enzyme is involved in a variety of metabolic functions and exhibits a broad range of biochemical characteristics; (1) function as an electron valve during photosynthesis (Appel *et al.*, 2000) (2) play a role in fermentation as a mediator in the release of excess reducing power under anaerobic condition (Stal and Moezelaar, 1997; Troshina *et al.*, 2002) and (3) might be a part of the respiratory NADH dehydrogenase (Complex I) (Appel and Schulz, 1996; Schmitz and Bothe, 1996).

2.6 Cyanobacteria

Cyanobacteria or blue green algae are prokaryotic microorganisms that are classified as a phylum within Domain Bacteria. Cyanobacteria contain chlorophyll *a*, carotenoid and phycocyanin. They are capable of an oxygenic photosynthesis. Cyanobacteria can be found in water resources worldwide such as in fresh water, in marine ecosystem and in hot spring.

2.6.1 Characteristics of cyanobacteria

Prokaryotic cyanobacterial cells are different from eukaryotic plants. They contain pigments distributed in cytoplasm but do not contain in plastid as eukaryotic cells. Besides, they reproduce in an asexual reproduction. Cyanobacteria reproduce asexually by constructing the specialized cells such as an exospore, endospore, akenete or hormogonium. The unicellular cyanobacteria divide themselves by binary fission whereas filamentous cyanobacteria reproduce by creating the short motile filament from breaking the longer filaments. There are three main photosynthetic pigments in cyanobacteria; a green color chlorophyll *a*, a yellow-red color carotenoid including carotene such as β-carotene and xanthophylls including myxoxanthin, myxoxanthophyll, oscillaxanthin, zeaxanthin, lutein, fulvicin, and aphanizophyll, and the blue color phycobillin including C-phycocyanin, C-allophycocyanin and C-phycoerythrin. Cyanobacteria synthesize and accumulate cyanophycean starch during photosynthesis and CO_2 fixation. The cyanophycean starch is found in a small granule distributed in cytoplasm. This kind of starch is different from other starches by given a red brown color when interacted with Iodine (I_2).

Cell wall of cyanobacteria are divided into two layers; peptidoglycan layer and an outer membrane. Peptidoglycan comprises important compounds such as mucopeptide, sugar and amino acid similar to cell wall of Gram-negative bacteria. Outer of cyanobacterial cells is covered with mucilage called sheath which has different thicknesses and shows color or colorless with multi-layers. The function of sheath is to prevent cells from dryness and to relate with the process of gliding movement. All types of cyanobacterial cells (both vegetative cells and reproductive cells) lack flagella. They have a movement by gliding. Gliding motility requires a contact with a solid surface and occurs in a direction parallel to the long axis of the cell or filament. Gliding of cyanobacteria is controlled by a number of external stimuli, of which light seems to be the most important factor.

2.6.2 Cell Structure

Cyanobacteria are divided into two shapes; Non-filamentous form and filamentous form. For non-filamentous form, most of them are unicellular with coccoid shape (Fig. 2.12). The examples of these unicellular cyanobacteria are *Anacystis, Chroococcus, Eucapsis*, *Merismopedia*, *Synechococcus*, and *Synechocystis*. Beside coccoid shape, their shape can be spherical, oval, tube-rod or head-end sharp oval. Colonial palmella have spherical colonies with a flat, square or irregular shape. Unicellular cyanobacteria have a binary fission by dividing from 1 to 2 cells and from 2 to 4 cells. For filamentous form, cells produce an elongate hair-like structure called a trichome (Fig. 2.13). Single trichome filaments may further be classified into two types, homocystous cells (trichome with undifferentiated cells) such as *Oscillatoria* and *Lyngbya*, and heterocystous cells (trichome with differentiated cells or containing heterocyst cells such as *Nostoc* and *Anabaena*.

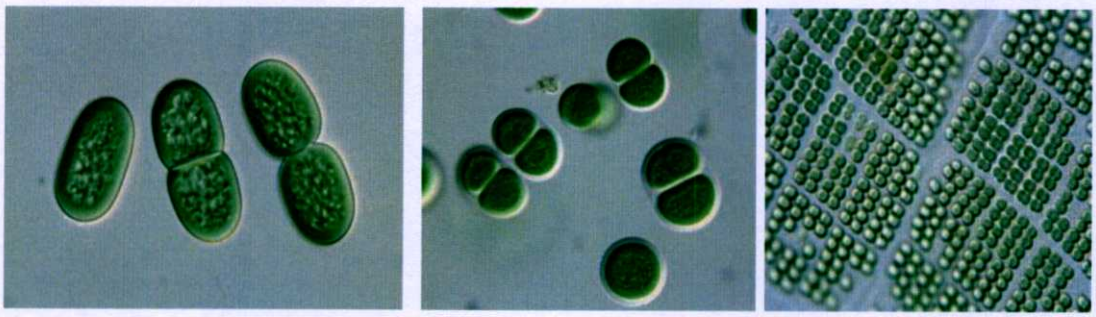


Figure 2.12 Unicellular or non-filamentous form of cyanobacteria
(http://protist.i.hosei.ac.jp/PDB/Images/Prokaryotes.html)

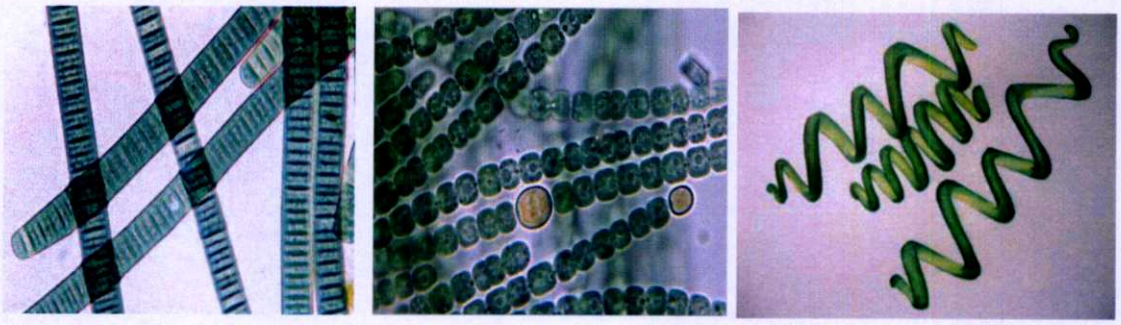


Figure 2.13 Filamentous form of cyanobacteria
(http://protist.i.hosei.ac.jp/PDB/Images/Prokaryotes.html)

2.6.3 Cell features

Cyanobacteria are gram-negative bacteria whose cell structure is composed of many organelles such as cell wall and sheath, cell membrane, cytoplasm including phycobilisome, ribosome, caboxysome, gas vacuole, thylakoid and genetic materials (Fig. 2.14).

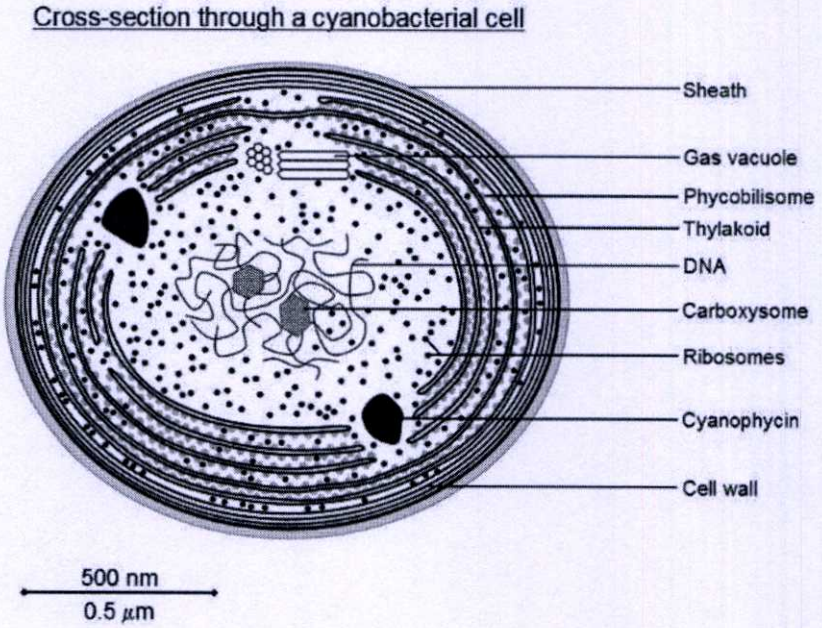


Figure 2.14 Structure of organelles in cyanobacteria (http://cronodon.com/BioTech/Cyanobacteria.html)

2.6.3.1 Cell wall and sheath

Cell wall of cyanobacteria comprises two layers including outer and inner cell wall. Outer cell wall layer structure depends on environmental conditions and amount of mucilage release. Cyanobacterial cell wall is similar to that of Gram-negative bacteria. The main chemical composition of cell wall is peptidoglycan composed of carbohydrates and short peptides. When cell wall is stained with crystal violet, cells will show a purple color. Outer cell wall always has mucilage layer that covers cell with sheath or capsule. The function of sheath is to prevent cells from dryness and to relate with the process of gliding movement. The inner layer lies in between outer wall layer and plasma membrane. It is made up of

mucopeptide and muramic acid, glucosamine, glanine, glutamic acid and diamino pamellic acid. This layer provides shape and mechanical strength to the cell wall.

2.6.3.2 Plasma membrane

The plasma membrane is selectively permeable living membrane enclosing the cytoplasm containing proteins and lipids, as this compartment differs significantly from the environment within the thylakoid lumen. The plasma membrane is an 80 nm thick electron-dense layer attributed to the phycobilisomes.

2.6.3.3 Cytoplasm

Cytoplasm is a region of many materials or organelles existing in living cells, excluding the cell nucleus. It is surrounded with membrane muti-layers. A thin layer membrane called "Lamella" has a wide variety of function depending on an individual layer. Photosynthesis lamellae contains phycobilisome which includes a red chomatophore, phycoerythrin and a blue chomatophore, phycocyanin. Both of them are combined as one unit called "Cyanosome or Phycobilisome" functioning for cyanophycean starch storage. Cyanophycean starch are distributed and collected in a granule called "Cyanophycin granule".

The 70S ribosome is distributed in cytoplasm and most of them is located at the center surrounding nucleoplasm. Gas vacuole is a small organelle distributing in cytoplasm. Generally, gas vacuole has an irregular shape and contains a gas making this organelle floating buoyancy and receiving light as well. Carboxysome is an organelle containing many enzymes of CO_2 fixation especially ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Thus, it has important roles in a storage of lipids, carbohydrates, proteins and a synthesis of source of energy or materials for nucleic acid and phospholipid formation. Thylakoid is a cell structure for absorbing the light energy for photosynthesis. It contains chlorophyll *a* and other pigments such as carotenoid and phycocyanin.

2.6.3.4 Genetic materials

DNA of cyanobacteria is a genetic material without histone binding and distributed in nucleoid of cells. Nucleoid in cyanobacteria contains a double strand DNA with a molecular size of about 1.6×10^{9}–8.6×10^{9} dalton and is similar to DNA found in bacteria containing a size of 1.0×10^{9}-1.6×10^{9} dalton and larger than that of mycoplasma containing a size of 0.4×10^{9}-0.5×10^{9} dalton.

2.6.4 Specialized cells

2.6.4.1 Akinete cells

Akinete cells or resting spores are developed from vegetative cells and play a major role in a reproduction. Akinete cells have a thick cell wall bounding with coat reaching to three layers resulting in tolerance to unsuitable environmental conditions. The mature akinete cells are larger than vegetative cells (Fig. 2.15). Akinete cells contain protoplasm which is plenty of food and chemical storage such as cyanophycin, glycogen, lipids and pigments. Most of them is arranged close to heterocyst cells that are found in Order Nostocales and Stigonematales such as *Nostoc* sp., *Gloeotrichia* sp. and *Anabaena* sp.

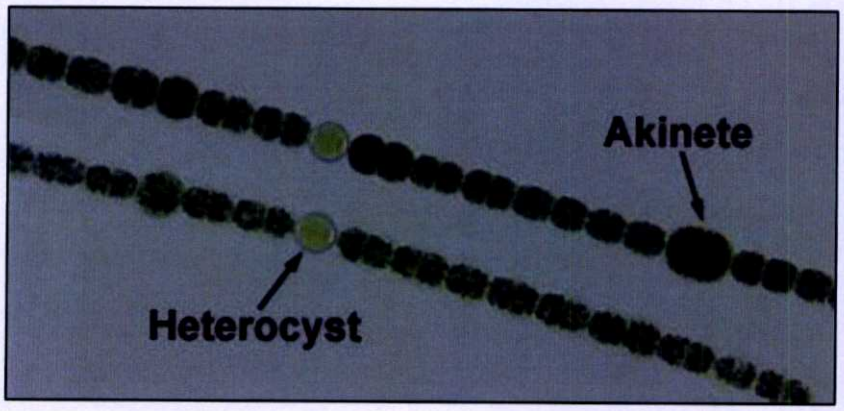


Figure 2.15 Akinete and heterocyst cells of cyanobacteria (http://fmp.conncoll.edu/Silicasecchidisk/LucidKeys/Carolina_Key/html/anabaena_Main.html)

2.6.4.2 Heterocyst cells

Heterocyst cells are specialized cells playing a role in N_2 fixation. They are larger than vegetative cells (Fig. 2.15). Intracellular heterocyst has a light-yellow mucilage due to a lack of chlorophyll *a*. Heterocyst cells are found located between vegetative cells in a filament or at one terminal or both ends of a filament. Heterocyst cells are found only in some species of cyanobacteria especially when there is a lack of nitrogen sources such as ammonia, nitrate and urea in medium. Heterocyst cell has a thick cell wall composed of polysaccharide and glycoprotein layers. The thickness of cell wall supports a decrease of O_2 diffusion that is important for protection nitrogenase enzyme from O_2. Heterocyst cells cannot photosynthesize and produce O_2. However, low level of O_2 is enough to permit N_2 fixation which is occurred within heterocyst cells. After N_2 fixation, various compounds such as glutamine or other amino acids are transported from heterocyst cells into vegetative cells. Whereas vegetative cells fix CO_2 from the atmosphere and transfer synthesized carbohydrate into heterocyst cells.

Hetereocysts can form in many positions of filament. Heterocysts which take place within the filament between vegetative cells are called "Intercalary heterocysts". Heterocysts located at the position in one or both of terminals are called "Terminal heterocysts". Terminal heterocysts can be divided into 3 types; the first type is "Basal heterocysts" found in unequal cells through the filament and mostly often occurred by attaching with the largest cells. Basal heterocysts can be found in the cyanobacterium *Calothrix* sp. The second type is "Pedicellate heterocysts" found in terminal of short branching filaments with only one-three cells. The third type is "Lateral heterocysts" occurring beside of filaments by attaching cells in each filament. The type of pedicellate and lateral heterocyst can be found in cyanobacterium *Nostochopsis* sp. and some filaments are possible to show terminal curve or spiral such as *Arthrospira* and *Spirulina*. In filamentous form, non-branching filaments can be observed in *Oscillatoria*, *Lyngbya*, *Anabaena* and *Anabaenopsis* whereas branching filaments with either true branching or false branching can be investigated in *Stigonema*, *Fischerella*, *Asterocystis*, *Tolypothrix* and *Coleodesmium*.

2.7 Taxonomy of cyanobacteria

Morphological analysis is normally used to classify a genus and species in cyanobacteria (Rippka, 1988). Morphological analyses such as growth and cellular characteristics are determined. For example, *Oscillatoria* and *Lyngbya* are simple filament cyanobacteria. Branching filament can be divided into two structures, (1) true branching such as *Fischerella* and *Stigonema* and (2) false branching such as *Plectonema* and *Scytonema*. In addition, the features of filament in some cyanobacteria occur differently. Both akinete and heterocyst cells can be found in some cyanobacteria such as *Gloeotrichia*. Sheath can be found in some cyanobacteria such as *Lyngbya* and *Phormidium*.

There are many systems used to classify taxonomy of cyanobacteria. Taxonomy of cyanobacteria is classified into 4, 3, 5, 2, 3 and 5 orders by Geitler (1932), Fremy (1934), Fritsch (1945), Smith (1950), Bold and Wynne's (1985) and Desikachary (1959), respectively.

Rippka and Colleages (1979) classified the taxonomy of cyanobacteria into five orders:

1) Order Chroococcales, cyanobacteria in this order can multiply in binary fission and budding.

2) Order Pleurocapsales, cyanobacteria in this group can be devided by multiple fission and baeocytes are occured.

3) Order Oscillatoriales, cyanobacteria in this order are filament and multiply by binary fission in single plane but neither heterocyst nor akinete is found in this group.

4) Order Nostocales, cyanobacteria are the same as the previous group but heterocyst and akinete cell can be found.

5) Order Stigonematales, another filament group of cyanobacteria, they are remarkable as they are able to devide by fission in many planes caused the true branching and specific cells.

Anagnostidis and Komarek (1988), Komarek and Anagnostidis (1989), Anagnostidis and Komarek (1990), Komarek and Anagnostidis (1999), and Komarek (2003) classified cyanobacteria into 4 orders.

1) Order Chroococcales, cyanobacteria in this order are unicellular cells, and their cell division is binary fission.

2) Order Oscillatoriales, cyanobacteria in this order are filaments found either true or false branching and sheath in some cyanobacteria. Cylinder form is found in most cells. Neither heterocyst nor akinete cells is occured. Their cell division is fragmentation.

3) Order Nostocales, cyanobacteria in this order are non-branching filaments with dense or thin sheath depending on the types. Their appearance is circular or cylinder and there are heterocyst and akinete cells that can form brand-new branches.

4) Order Stigonematales, cyanobacteria in this order have many branching types, true and false branching. Filament cells may comprise two cells or more. Sheath color is yellow or brown. Heterocyst but akinete is found. Cell division is fragmentation.

2.7.1 Molecular taxonomy

Cyanobacterial identification is dependent on environmental and cultivation conditions (Doers and Parker, 1988). Cyanobacterial cells can be identified in different species upto different growth and environmental conditions. These factors cause the number of genus and species of cyanobacteria excessively. Nowadays, many molecular techniques have been used to investigate the genetic diversity and phylogenetic relationships among cyanobacteria such as 16S rDNA sequencing (Komárek and Anagnostids, 1986; Komárek and Anagnostids, 1989), variable nucleotide sequence regions; the 16S-23S rRNA internal transcribed spacer region (16S-23S ITS) and the intergenic spacer region of the phycocyanin locus (*cpcB-cpcA* IGS) (Neilan *et al.*, 1995; Lu *et al.*, 1997; West and Adams, 1997; Scheldeman *et al.*, 1999; Iteman *et al.*, 2000; Premanandh *et al.*, 2006). In addition, random amplification of polymorphic DNA (RAPD), DNA fingerprinting, Denaturing gradient gel electrophoresis (DGGE), Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP) and short and long tandem repeat repetitive sequences (STRR and LTRR) have been used to examine the correlation of morphology, genetics and geography among strains of the cyanobacteria. In this

study, genetic diversity by 16S rDNA, 16S-23S and *cpcB-cpcA* IGS sequencing and RAPD analysis is analyzed.

1) 16S rDNA

The 16S rDNA is ribosomal RNA gene, the basic element of ribosome operating synthesis of the essential proteins in cell metabolism. It is a notable method to study the phylogenetic relatives because this gene occurs in all eubacteria. The 16S rRNA contains 1,500 bp with various base pair regions in the same types of eubacteria. The same and difference of nucleotide can used for studying the cyanobacterial diversity. The same region is used to design primer, which is useful for amplification of a number of DNA by PCR technique. These primers called universal primers because DNA sequences from such primers are similar in all organisms.

2) 16S-23S internal transcribed spacer (16S-23S ITS)

In prokaryote, rRNA gene is arranged as an operon. This operon is called "*rrn* operon". It is arranged specifically as 16S, 23S and 5S rRNA, respectively. Normally, it is found in many cassettes depending on type of organisms such as 7 and 10 cassettes in *E. coli* and *Bacillus subtilis*, respectively. DNA is located between genes called "internal transcribed spacer (ITS)". The 16S-23S ITS contain tRNA gene, for Isoleucine and alanine (Fig. 2.16) (Boyer *et al.*, 2002).

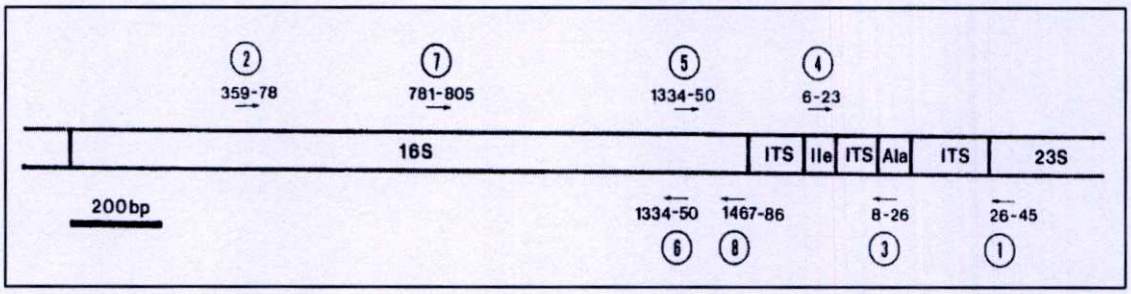


Figure 2.16 Gene diagram of 16S rRNA, 16S-23S rRNA ITS and 23S rRNA (Boyer *et al.*, 2002)

3) Phycocyanin intergenic sequence

Phycocyanin intergenic sequence (PC-IGS) is an accessory pigment promoting the chlorophyll function of photosynthesis in cyanobacteria. The structure of phycocyanin is composed of alpha and beta subunits encoded by *cpcA* and *cpcB*, respectively (Fig. 2.17). DNA located between these genes can be used to study genetic diversity of cyanobacteria.

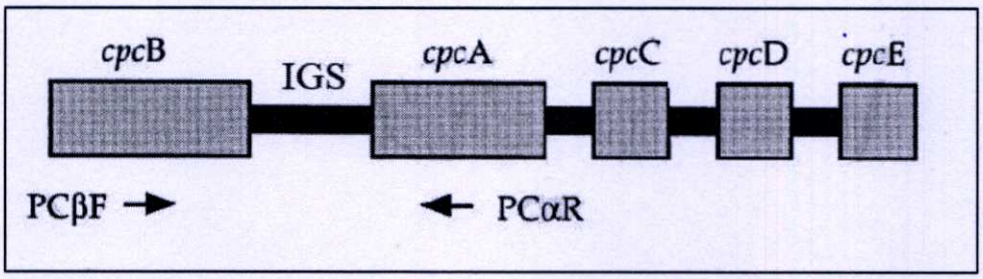


Figure 2.17 IGS region of phycocyanin operon located in between beta subunit encoded by *cpcB* and alpha subunit encoded by *cpcA* (Neilan *et al.*, 1995)

4) RAPD

RAPD is a technique using PCR for studying genetic diversity in organisms. The principle of this technique is to amplify randomly amount of DNA simultaneously. Primers with 10-12 nucleotides are plausible to approach to many regions of DNA template. Because primers of RAPD is able to amplify DNA randomly, the nucleotide data of organisms is not the matter. The amplification region contributes in whole genome randomly and DNA can be verified in the meantime. The verification can be performed by agarose gel electrophoresis. Ethidium bromide is a dye for detecting the different features of DNA.

2.7.2 Phylogenetic tree

Phylogenetic tree is used for studying the genetic evolution of the organisms. Its concept is involved in the traditional principle of taxonomy to classify the organisms. The similar organisms are in the same group indicated that these organisms are inherited from same ancestor (Page and Holmes, 1998). Phylogenetic tree refers to the evolution of gene and protein, making the information clear and

reliable. Besides, phylogenetic tree is one of polyphasic tool, which can be used to study particular information including species or upper order in the organisms.

Phylogenetic tree can be divided into two types: rooted and unrooted tree. Rooted tree is represented as common ancestor of all organisms, while unrooted tree refers to relatives of that without position of common ancestor (Fig. 2.18).

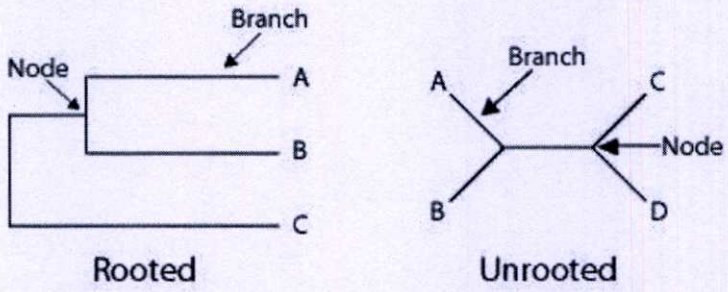


Figure 2.18 Rooted and unrooted phylogenetic tree
(http://scienceblogs.com/evolgen/2007/03/16/new-terms-in-phylogenetics/)

To appoint the outgroup and build appropriate root, DNA of irrelevant organisms is inserted into the tree. Generally, nucleotides of *Escherichia coli* are used as those of the outgroup. To study genetic diversity in prokaryote, data alignment is used many times until the information reaches to the most similarity and mismatch is rarely occurred. There are many types of tree diagram construction such as Distance matrix, Maximum likelihood, and Maximum parsimony.

2.8 Isolation of cyanobacteria

Cyanobacteria can be collected and isolated from water sources in nature. The cyanobacterial samples can be collected by a pull plankton net. The filamentous cyanobacteria are possible to collected by scraping them from rocks or water plant fragments such as stalks and leaves. In addition, they can be collected from soil, sand, shells and stones and other water ecosystems.

2.8.1 Techniques for isolation of cyanobacteria

There are many techniques for cyanobacterial isolation as described below:

1) Single cell isolation technique

Single cell isolation is the easiest technique for cyanobacterial isolation. A single cell is isolated under a microscope using Pasteur pipette. Each cell is sucked in Pasteur pipette and then is washed with a sterile medium for many times. For the beginning of isolation, one end side of Pasteur pipette is burnt with Bunsen burner until Pasteur pipette glass becomes weak. The glass tube is pulled to be lengthened with a smaller diameter using a forcep (Fig. 2.19A). When available suitable size is obtained, the unwanted part is discarded by breaking with a forcep. Another side of Pasteur pipette is added with the rubber stopper. Cell is sucked in the pipette under microscope (Fig 2.19B). Each cell is released into a well of tissue culture plate containing medium and sucked up and down at least 3-4 times for washing. Cyanobacterial cell is placed under light for growth.

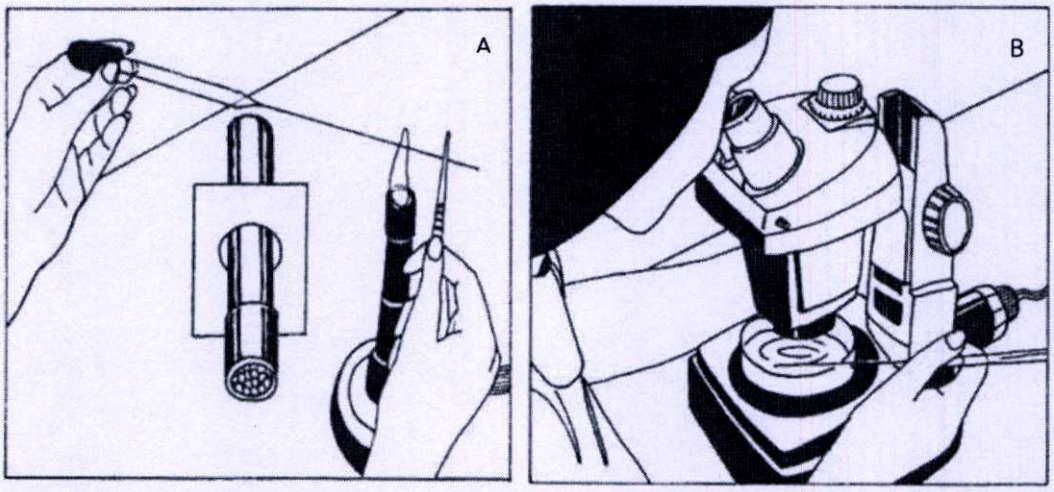


Figure 2.19 Single cell isolation technique. Pasteur pipette is prepared by heating with Bunsen burner (A) and cyanobacterial cell is sucked in Pasteur pipette under inverted microscope (B) (Stein, 1973)

2) Selective media technique

This technique is suitable for macroalgal isolation more than microalgae isolation. Fragment of algae is selected and other organisms are removed. Then, other remaining microorganisms are checked under low magnification microscope. If there are microorganisms remaining, a brush must be used to remove them. Afterthat, the single filament is cultivated in medium containing 10 mL of germanium oxide to prevent other diatom or algal growth.

3) Agar pour plate isolation technique

Algal sample is mixed with agar medium at various dilution levels. The mixture is mixed well and then is poured on sterile plate. The medium is left at room temperature until agar is solid. The agar plate is placed under light condition. The green color of algae on agar will appear within 7-14 days. Then, the small agar is cut by a sterile knife and put into glass tube containing medium (Fig. 2.20).

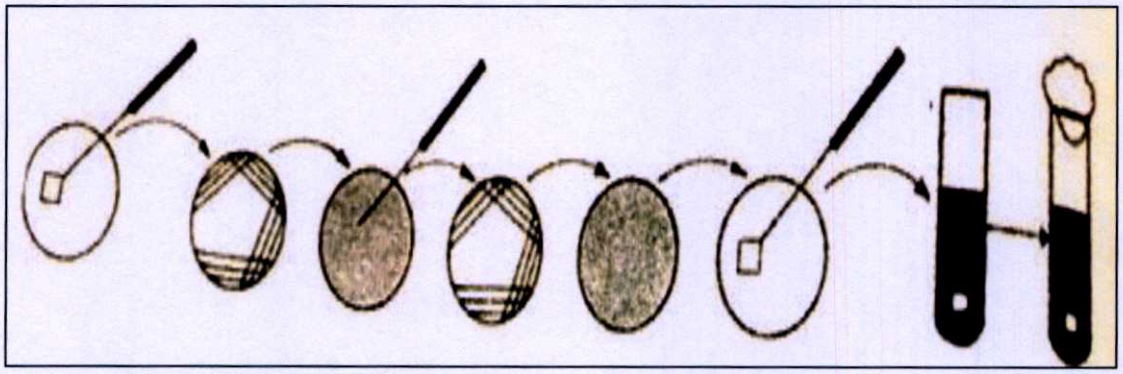


Figure 2.20 Agar pour plate isolation technique (Vonshak, 1986)

4) Osmotic balance technique

This technique is used for algal isolation depending on the differences of osmotic balance of algae. Algae are washed in saline solution at various osmotic concentrations and switched with washing in distilled water for many times. This process must be rapidly washed to deteriorate of the cells. So, it can use for isolation of filamentous algae from the protozoa.

2.8.2 Purification of cyanobacteria

When a single cell of cyanobacteria is obtained, it must be purified from bacterial contamination (become axenic culture). The purification methods include;

1) Washing by centrifugation

Bacterial cells can be separated from cyanobacteria by centrifugation at low speed (2,000 rpm) for 45-90 sec. Cyanobacterial cells are washed in sterilized medium and centrifuged again. Then, the supernatant is poured out. This procedure is repeatedly performed for at least 12 times.

2) Antibiotics

Antibiotic drugs such as penicillin, streptomycin, tetramycin and kanamycin, are used for inhibiting contaminant bacterial cells. Their dosage should be optimal and does not give a damage to cyanobacterial samples. In addition, mixed antibiotic drugs provide more success to free from pathogen.

3) UV light

UV light is suitable technique for purification of thick mucilage cyanobacteria. The ultraviolet light is set at 2,750 A°. Light bulb is placed over cyanobacterial culture container about 25 cm. The UV light is provided for 8-16 min before transferring cells into new agar plate.

4) Filtration

This technique uses a membrane filter to separate cyanobacteria from bacteria. The filamentous cyanobacteria are cut into short fragments. After that the short fragments are added into cultural container which has funnel and filter paper with a size of 8 μm. The fragments of cyanobacteria are filtrated using a suction pump. The paper containing cells is added into the bottle containing new medium and shaken. Cyanobacteria are poured on agar and placed in the light for 2-3 weeks. Then, agar is cut into small pieces and transferred to sterile medium for cultivation or other purposes.

2.9 Factors affecting H_2 production by cyanobacteria

There are a lot of environmental parameters influencing H_2 production by cyanobacteria such as nutrient and mineral composition in medium, light intensity and pattern, incubation temperature and medium pH. In addition, some intrinsic factors can affect H_2 production by cyanobacteria as well such as genetic components, sensitivity of enzyme and type of cyanobacteria.

2.9.1 Environmental parameters

There are several environmental parameters such as nutrient composition, mineral composition, salinity, light intensity, temperature and pH that play a role in H_2 production by cyanobacteria.

2.9.1.1 Nitrogen

Several inorganic nitrogenous compounds such as nitrite, nitrate and ammonium salt influences H_2 production by inhibiting nitrogenase activity in some N_2-fixing cyanobacteria such as *Anabaena variabilis* SPU 003 and *Anabaena cylindrica* (Lambert *et al.*, 1979; Datta *et al.*, 2000). It has been reported that an addition of all exogenous nitrogen sources in medium inhibits nitrogenase synthesis (Radway *et al.*, 1999). Some studies showed that there are significant differences of H_2 production depending on the nitrogen content in medium (Weissman and Benemann, 1977; Aoyama *et al.*, 1997; Yodsang, 2018). Under nitrogen deprivation an increase of H_2 production is ascribed to an increase of electron flow towards hydrogenase accompanying a degradation of the fermentative glycogen accumulated during photoautotrophic growth in *Gloeocapsa alpicola* (Serebryakova *et al.*, 1998; Troshina *et al.*, 2002) and *Arthrospira maxima* (Ananyev *et al.*, 2008) in marine cyanobacteria *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui, 1981), *P. valderianum* BDU 20041 (Prabaharan and Subramanian, 1996), *L. valderiana* BDU 20041 (Prabaharan *et al.*, 2010) and *A. halophytica* (Taikhao *et al.*, 2013).

2.9.1.2 Sulphur

Sulphur deprivation enhances H_2 production in several cyanobacterial species such as *G. alpicola*, *Synechocystis* sp. PCC 6803 and *A. halophytica* (Antal and Lindblad, 2005; Taikhao *et al.*, 2013). Sulfur is a very important component in the photosystem II repair cycle (Wykoff *et al.*, 1998). Lack of sulfur causes an inhibition of the oxygenic photosynthesis resulting in a decrease of O_2 and thus leads to an enhancement of H_2 production.

2.9.1.3 Salinity

Salinity affects H_2 production in freshwater cyanobacteria. H_2 production is highest in cells incubated in NaCl-free medium but it is decreased when NaCl concentration is increased (Shah *et al.*, 2003). In marine cyanobacterium *Lyngbya* sp. strain 108, the highest H_2 production is found at its optimal level of NaCl concentration (Kuwada and Ohta, 1989). Too high NaCl concentration reduces H_2 production in all types of cyanobacteria because they have to combat salinity stress by extrusion of Na^+ ions out of cells or by prevention of Na^+ influx into the cells (Tel-Or and Melhamed-Harel, 1981; Rai and Abraham, 1995).

2.9.1.4 Carbon source

The type of carbon source and its concentration in medium show the great influence on H_2 production by cyanobacteria. The presence of different carbon sources causes variation in electron donation capabilities by cofactor compounds to nitrogenase and hydrogenase thus influencing H_2 production (Datta *et al.*, 2000; Chen *et al.*, 2008).

2.9.1.5 Micronutrients

Micronutrients such as iron (Fe) and nickel (Ni) play a significant role in H_2 production by cyanobacteria (Ramchandran and Mitsui, 1984). Iron is normally a cofactor of NiFe-hydrogenase enzyme and it enhances the electron transport process towards hydrogenase (Lin and Stewart, 1997). In addition, iron is also involved in the electron transport system in cyanobacterial cells, such as photosynthesis and respiration (Raven *et al.*, 1999) and nitrogen fixation (Küpper *et al.*, 2008). Whereas

nickel is known as a metal cofactor of NiFe–hydrogenase in cyanobacteria; therefore, nickel is required for hydrogenase activity to produce H_2 (Axelsson and Lindblad, 2002; Gutekunst *et al.*, 2006). In addition, nickel may have other roles in cell function other than hydrogen metabolism (Daday *et al.*, 1985). However, too high concentrations of nickel results in a decrease of H_2 production that is toxic to the cells (Daday *et al.*, 1985).

2.9.1.6 Light

Light is important for growth and photosynthesis. Light requirement during H_2 production is variable depending on the cyanobacterial species (Dutta *et al.*, 2005). Most of cyanobacteria produce H_2 under anaerobic/dark conditions. Under light condition cells increase level of O_2 due to the water spitting via PS II. The generated O_2 inhibits the activity of bidirectional hydrogenase and nitrogenase resulting in a decrease in H_2 production (Fay, 1992; Tamagnini *et al.*, 2000).

2.9.1.7 Temperature

The optimum temperature for H_2 production is dependent on the species of cyanobacteria. The optimum temperature for for H_2 production varies from 30 °C to 40 °C (Ernst *et al.*, 1979; Dutta *et al.*, 2000; Dutta *et al.*, 2005). The maximum hydrogenase activity is also found at 30 °C to 40 °C (Baebprasert *et al.*, 2010).

2.9.1.8 pH

The pH affects H_2 production and hydrogenase activity. The optimal pH for H_2 production of cyanobacteria is between 5-9 (Antal and Linbld, 2005). The optimal pH leads to the maximum electron flow toward hydrogenase, resulting in the maximum H_2 production (Baebprasert *et al.*, 2010).

2.9.2 Intrinsic factors

There are several intrinsic factors such as genetic components, O_2 sensitivity of involving enzymes, affecting H_2 production by cyanobacteria.

2.9.2.1 Presence of uptake hydrogenase

H_2 production yield is decreased in cyanobacterial strains containing uptake hydrogenase due to loss of H_2 by the activity of uptake hydrogenase (Tamagnini *et al.*, 2002).

2.9.2.2 O_2 Sensitivity of hydrogenase and nitrogenase

O_2 is inhibitor of hydrogenase and nitrogense (Fay, 1992; Tamagnini *et al.*, 2000). The presence of O_2 in the environmental system causes a decrease of hydrogenase and nitrogense activities, thus resulting in a reduction of H_2 production.

2.9.2.3 Heterocystous cyanobacteria

The heterocystous cyanobacteria are normally more efficient to produce H_2 than cyanobacteria with vegetative cells (Radway *et al.*, 1999; Pinzon-Gamez *et al.*, 2005). They have ability to perform simultaneous O_2 and H_2 production coupled with CO_2 fixation (Radway, 1999).

2.10 H_2 production by marine cyanobacteria

Cyanobacteria can be found in various habitats such as freshwater, brackish water, soil or seawater ecosystem. Cyanobacteria found in seawater or marine cyanobacteria are more diverse than those found in other habitats. Seawater is about 97.2% of the total water surface of the world. It is composed of 96.5% water and 3.5% mineral salts (Fig. 2.21) that are less than 1 ppm by weight (Table 2.2) (Smith, 1974). Although mineral salts are less abundant in seawater but they are very important to the biochemical processes in marine organisms including growth and H_2 production of marine cyanobacteria.

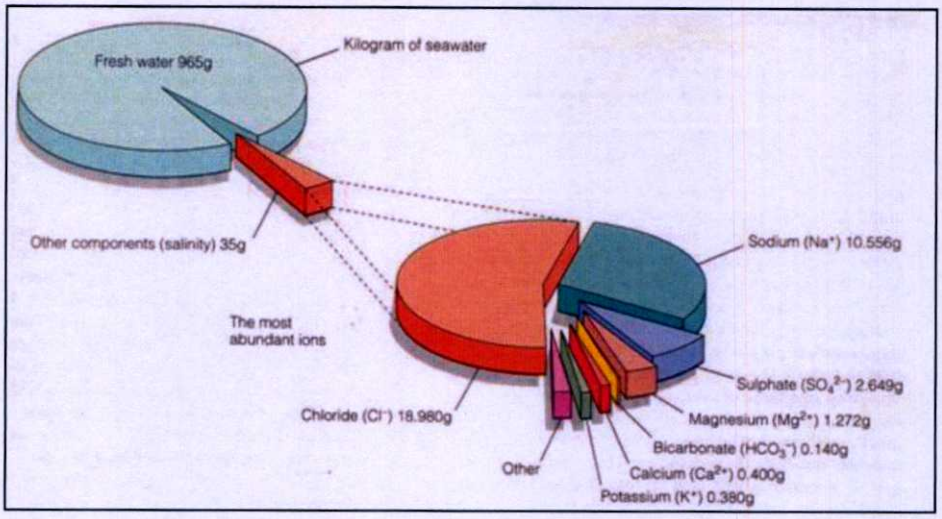


Figure 2.21 Proportion of salt and dissolved ions to water in the seawater (Garrison, 2007)

Table 2.2 Mineral concentrations in seawater (Smith, 1974)

Constituents	Concentration in parts per thousand	Concentration in parts per million	Concentration in parts per billion
Minor element			
Bromine (Br)	0.065	65	
Strontium (Sr)		8	
Boron (B)		4	
Silicon		3	
Fluorine		1	
Important trace elements			
Nitrogen (N)[a]		0.3	300
Lithium (Li)			170
Phosphorus (P)			70
Iodine (I)			50
Zinc (Zn)			10
Iron (Fe)			10
Aluminum (Al)			10
Manganese (Mn)			2
Lead (Pb)			0.04
Mercury (Hg)			0.03
Gold (Au)			0.000004

Until now, H_2 production has been investigated in a wide variety of cyanobacterial strains. Each cyanobacterial strain has differences in H_2 production yield and H_2 metabolism (Dutta *et al.*, 2005). Many researchers are interested in an investigation of H_2 production by cyanobacteria, especially freshwater cyanobacteria while the study of H_2 production by marine cyanobacteria is rather rare. It has been previously reported that marine cyanobacteria show higher H_2 production than freshwater cyanobacteria (Kothari, 2012). In addition, marine cyanobacteria show some advantages since they can grow in high salinity, making them possible to cultivate in natural seawater. H_2 production by marine cyanobacteria is shown in Table 2.3. They show the typical character of H_2 production depending on the specific H_2 metabolism of each strain.

Table 2.3 H_2 production by various kinds of marine cyanobacteria

Organisms	Maximum H_2 production rate	Growth conditions	H_2 evolution assay conditions	References
Unicellular				
Cyanothece sp. PCC 7822	0.920 mg chla^{-1} h^{-1}	BG11 medium+200 mg L^{-1} Na_2CO_3, 5% CO_2 in N_2, 30 °C	20 mM potassium phosphate buffer (pH 7.3), Ar, 30 °C	Van der Oost *et al.*, 1989
Synechococcus sp. Miami BG 043511	1.110 mg dry weight^{-1} h-1	A-N medium + 2.5 g L^{-1} Na_2CO_3, 4% CO_2 in Air, 150 µmol E m^{-2}s^{-1}, 30 °C	A-N medium + 10 mM HEPES + 25 pyruvate, 150 µmol E m^{-2}s^{-1}, 30 °C	Luo and Mitsui, 1994
Crocosphaera watsonii WH8501	0.090 µmolH_2 mg chla^{-1} h^{-1}	SO medium (pH8), 12 h light (45 µmol E m^{-2}s^{-1}) / 12 h dark, 26 °C	SO medium (pH8), 12 h light / 12 h dark, 26 °C	Wilson *et al.*, 2010a
Filamentous heterocystous				
Anabaena sp. CA	80 µL mg dry weight^{-1} h^{-1}	ASP-2-N medium +1% CO_2 in air, 450 µmol E m^{-2}s^{-1}, 39 °C	ASP-2-N medium +1% CO_2 in AR, 450 µmol E m^{-2}s^{-1}, 39 °C	Xiankong *et al.*, 1983
Anabaena cylindrica B-629	0.103 µmolH_2mg dry weight^{-1} h^{-1}	Air contained 5% CO_2, 7,000 lx at the surface of culture vessels	Ar with 3% CO_2 4,000 lx at surface of the culture vessel	Lambert and Smith, 1977
Calothrix scopulorum 1410/5	0.128 µmolH_2mg dry weight^{-1} h^{-1}	Air contained 5% CO_2, 7000 lx at the surface of culture vessels	Ar with 3% CO_2 4,000 lx at surface of the culture vessel	Lambert and Smith, 1977
Calothrix membrnacea B-379	0.108 µmolH_2mg dry weight^{-1} h^{-1}	Air contained 5% CO_2, 7000 lx at the surface of culture vessels	Ar with 3% CO_2 4,000 lx at surface of the culture vessel	Lambert and Smith, 1977
Oscillatoria brevis B1567	0.168 µmolH_2 mg dry weight^{-1} h^{-1}	AA medium+5% CO_2 in air, 7,000 lux, 25 °C	AA medium+3% CO_2, 4,000 lux, 25 °C	Lambert and Smith, 1977
Calothrix scopulorum 1410/5	0.128 µmolH_2 mg dry weight^{-1} h^{-1}	AA medium+5% CO_2 in air, 7,000 lux, 25 °C	AA medium+3% CO_2, 4,000 lux, 25 °C	Lambert and Smith, 1977
Calothrix membrnacea B379	0.108 µmolH_2 mg dry weight^{-1} h^{-1}	AA medium+5% CO_2 in air, 7,000 lux, 25 °C	AA medium+3% CO_2, 4,000 lux, 25 °C	Lambert and Smith, 1977
Anabaena sp. TU37-1	84 µmolH_2 mg chla^{-1} h^{-1}	A-N medium, Air, 150 µmol E m^{-2}s^{-1}, 30 °C	A-N medium, 150 µmol E m^{-2}s^{-1}, 30 °C	Kumazawa, 2003

Table 2.3 H_2 production in various marine cyanobacteria (Continued)

Organisms	Maximum H_2 production rate	Growth conditions	H_2 evolution assay conditions	References
Filamentous non-heterocystous				
Oscillatoria limosa Miami BG7	0.250 μmolH_2 mg dry weight^{-1} h^{-1}	A medium+25 mg L^{-1} NH_4Cl, 100 μmol E m^{-2}S^{-1}, 28 °C	A-N medium, Ar 90 μE/m^2 , 37°C	Philips and Mitsui, 1983
Phormidium valderianum BDU 20041	0.2 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, Light (5.5 μmol photon m^{-2} s^{-1}, 18 h dark~6 h light cycle, 27 °C, pH 7.5	Prabaharan and Subramanian, 1996
Arthrospira maxima CS-328	0.19 μL H_2 mg dry weight^{-1} h^{-1}	Zarrouk medium, Air, 1 μM Ni^+, 30 °C	Zarrouk medium, Ar, 1μM Ni^+, dark	Ananyev *et al*., 2008
Leptolyngbya valderiana BDU20041	0.02 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, Ar, 27±2 °C, dark	Prabaharan *et al*., 2010
Lyngbya confervoides BDU142001	0.02 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, N_2, 27±2 °C, dark	Prabaharan *et al*., 2010
Lyngbya confervoides BDU1420301	0.01 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, Ar, 27±2 °C, dark	Prabaharan *et al*., 2010
Microcoleus chthnoplasts BDU91212	0.017 μmolH2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, Ar, 27±2 °C, dark	Prabaharan *et al*., 2010
Plectonema terebrans BDU141311	0.013 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III –N medium, N_2, 27±2 °C, dark	Prabaharan *et al*. 2010)
Trichodesmium erythraeum IMS101	3 μmolH_2 mg chla^{-1} h^{-1}	YBC II-N medium, 12h light (44 μmolEm^{-2}S^{-1}) /12 dark, 26 °C	YBC II-N medium, 12h light (44 μmolEm^{-2}S^{-1}) /12 dark, 26 °C	Wilson *et al*., 2010b

Chapter 3

Research Methodology

3.1 Microorganisms

In this study, there are 54 cyanobacterial isolates isolated from samples of sand, shells, stones and seawater in the Gulf of Thailand and the Andaman Sea of Thailand (Table 3.1).

Table 3.1 Cyanobacterial isolates used in this study

Isolates	Origins	Habitat
KK	Koh Kood, Trad	seawater
LS-W2	Laemsing, Chanthaburi	seawater
JL-SH1	JaoLao, Chanthaburi	shell
JL-SA1	JaoLao, Chanthaburi	sand
JL-W2	JaoLao, Chanthaburi	seawater
LSD-SH2	Laemsadet, Chanthaburi	shell
LSD-W3	Laemsadet, Chanthaburi	seawater
KVM-W1	Kungviman, Chanthaburi	seawater
MP-SA1	Makhampom, Rayong	sand
MP-W	Makhampom, Rayong	seawater
PKR-ST1	Pakarang, Rayong	stone
PKR-W3	Pakarang, Rayong	seawater
PI-S1.1	Phai, Rayong	seawater
PR-SH1	Prao, Rayong	shell
PR-SH9	Prao, Rayong	shell
SKR-W2.2	Saikeaw, Rayong	seawater
VD-SH2.3	Vongdeuan, Rayong	shell
VD-W	Vongdeuan, Rayong	seawater
WI-SA1	Wai, Rayong	sand
WI-SH3	Wai, Rayong	shell
ST-ST1.5.1	Saitong, Rayong	stone
P-SA1	Phla, Rayong	sand
P-SH8.2.1	Phla, Rayong	shell

Table 3.1 Cyanobacterial isolates used in this study (Continued)

Isolates	Habitats	Origins
P-ST2.3	Phla, Rayong	stone
P-W2.1	Phla, Rayong	seawater
SS-ST6	Samaesan, Chonburi	stone
NR-SH2	Nangram, Chonburi	shell
TG-W2.3.3	Toeingam, Chonburi	seawater
SK-ST1.1	Saikeaw, Chonburi	stone
SK-ST1.2	Saikeaw, Chonburi	stone
SK-ST2.1	Saikeaw, Chonburi	stone
Y-SH8	Yao, Trang	shell
YL-SH4	Yonglin, Trang	shell
S-S	San, Trang	seawater
S-SH3	San, Trang	shell
SR-SH4	Samran, Trang	shell
MTN-SH5	Modtanoy, Trang	shell
MTN-SH9	Modtanoy, Trang	shell
CHL-SH1	Changlang, Trang	shell
CHL-SH10	Changlang, Trang	shell
PM-SH13	Pakmeng, Trang	shell
JM-SH2	Jaomai, Trang	shell
RMK-SH10	Rachmonkol, Trang	shell
LK-SH2	Laemkruat, Krabi	shell
NM-SA4	Nummao, Krabi	sand
NM-SH1	Nummao, Krabi	shell
NTR-S	Nopparattara, Krabi	seawater
N-ST1	Nang, Krabi	stone
N-ST2	Nang, Krabi	stone
SSH-SH5	Susanhoy, Krabi	shell
SSH-SH12	Susanhoy, Krabi	shell
TL-S	Thalane, Krabi	seawater
TL-SH2	Thalane, Krabi	shell
KM-ST9	Klongmuang, Krabi	stone

3.2 Chemical reagents

3.2.1 Culture media

3.2.1.1 Artificial Seawater Nutrient (ASN) III medium (Rippka *et al.*, 1979) (Appendex A)

3.2.1.2 Blue green (BG) 11 medium (Rippka *et al.*, 1979) (Appendex B)

3.2.1.3 Blue green (BG) 11 medium (Rippka *et al.*, 1979) supplemented with Turk Island salt solution (Garlick *et al.*, 1977) (Appendex C)

3.2.1.4 Luria-Bertani (LB) medium (Bertani, 1951) (Appendex D)

3.2.2 Enzymes

3.2.2.1 α-Amylase from *Bacillus* sp. Type II-A (Sigma, USA)

3.2.2.2 Amyloglucosidase (Sigma, USA)

3.2.2.3 *Taq* DNA polymerase (Promega, USA)

3.2.3 DNA markers

3.2.3.1 λDNA/*Hin*dIII fragment (Invitrogen, USA)

3.2.3.2 100 bp Ladder DNA (Fermentas, USA)

3.2.4 Chemicals for cultivation

3.2.4.1 Boric acid (H_3BO_3) (Merck, Germany)

3.2.4.2 Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Carlo Erba, Italy)

3.2.4.3 Citric acid ($C_6H_8O_7$) (Analar, England)

3.2.4.4 Cobalt nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) (Ajex, Australia)

3.2.4.5 Copper sulfate heptahydrate ($CuSO_4.5H_2O$) (Carlo Erba, Italy)

3.2.4.6 Diaminoethylene tetraacetic acid disodium salt (Na_2EDTA) (Promega, USA)

3.2.4.7 Dipotassium hydrogen phosphate (K_2HPO_4) (Carlo Erba, Italy)

3.2.4.8 Ferric ammonium citrate ($FeNH_4$ citrate) (British Drug House, England)

3.2.4.9 Fructose ($C_6H_{12}O_6$) (Carlo Erba, Italy)

3.2.4.10 Glucose ($C_6H_{12}O_6$) (Carlo Erba, Italy)

3.2.4.11 Manganese chloride tetrahydrate ($MnCl_2.4H_2O$) (Ajex, Australia)

3.2.4.12 Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) (Carlo Erba, Italy)

3.2.4.13 Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Carlo Erba, Italy)

3.2.4.14 Maltose monohydrate ($C_{12}H_{22}O_{11}.H_2O$) (Merck, Germany)

3.2.4.15 Nickel chloride ($NiCl_2$) (Merck, Germany)

3.2.4.16 Lactose monohydrate ($C_{12}H_{22}O_{11}.H_2O$) (Sigma, USA)

3.2.4.17 Sodium carbonate (Na_2CO_3) (Carlo Erba, Italy)

3.2.4.18 Sodium chloride (NaCl) (Ajex, Australia)

3.2.4.19 Sodium hydroxide (NaOH) (Carlo Erba, Italy)

3.2.4.20 Sodium hydrogen carbonate ($NaHCO_3$) (Carlo Erba, Italy)

3.2.4.21 Sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (British Drug House, England)

3.2.4.22 Sodium nitrate ($NaNO_3$) (Carlo Erba, Italy)

3.2.4.23 Sucrose ($C_{12}H_{22}O_{11}$) (Carlo Erba, Italy)

3.2.4.24 Potassium chloride (KCl) (Merck, Germany)

3.2.4.25 Potassium dihydrogen phosphate (KH_2PO_4) (Carlo Erba, Italy)

3.2.4.26 Vitamin B12 (Cyanocobalamin) (Sigma, USA)

3.2.4.27 Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) (Fluka, Switzerland)

3.2.5 Chemicals and gases for quantitative analyses

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

3.2.5.2 Glycogen (Sigma, USA)

3.2.5.3 Hydrogen (H_2) (4%) (Praxair Ltd., Thailand)

3.2.5.4 Methylviologen dichloride hydrate ($C_{12}H_{14}CL_{12}N_2.H_2O$) (Sigma, USA)

3.2.5.5 Methanol (CH_3OH) (Scharlau, Spain)

3.2.5.6 Phenol (Merck, Germany)

3.2.5.7 Potassium hydroxide (KOH) (Carlo Erba, Italy)

3.2.5.8 Sodium dithionite ($Na_2O_4S_2$) (Sigma, USA)

3.2.5.9 Sulfuric acid (Scharlau, Spain)

3.2.6 Chemicals for genetic studies

3.2.6.1 Agarose (Bio Whittaker Molecular Application, USA)

3.2.6.2 Chloroform ($CHCl_3$) (Analar®, USA)

3.2.6.3 Deoxyribonucleotide triphosphates) (dNTPs) (Promega, USA)

3.2.6.4 Ethanol (C_2H_5OH) (Fischer, USA)

3.2.6.5 Ethidium bromide ($C_{21}H_2OBrN_3$) (Bio Basic, USA)

3.2.6.6 Isoamylalcohol $(CH_3)_2$ $CHCH_2CH_2OH$) (Sigma, USA)

3.2.6.7 Magnesium chloride ($MgCl_2$) (Promega, USA)

3.2.6.8 Oligonucleotide primers (Bio Basic Inc., Thailand)

3.3.6.9 Sodium acetate (CH_3COONa) (Merck, Germany)

3.3.6.10 Sodium dodecyl sulfate (SDS) (Merck, Germany)

3.3.6.11 Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) (Scharlau, Spain)

3.2.7 Kit

Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan)

3.3 Instruments

3.3.1 Autoclave (Hirayama Manufacturing Corporation HV-50, Japan)

3.3.2 Desiccator (Duran, Germany)

3.3.3 DNA thermal cycler (Eppendoft, Mastercycler ep gradient S, USA)

3.3.4 Electrophoresis equipment (Advance, Mupid®-exu, Japan)

3.3.5 Filter paper No. 1 (55 mm diameter) (Whatman, UK)

3.3.6 Gas chromatograph (Hewlett-Packard HP890A GC, Japan)

3.3.7 Gel Documentation (Syngene, MD1 1019, Japan)

3.3.8 Glass wares (Pyrex, USA)

3.3.9 Hot air oven (Delta Laboratory, 1375FX, Thailand)

3.3.10 Incubator shaker (Gallenkamp, T490188, UK)

3.3.11 Laminar air flow cabinet (International Scientific Supply HS123, Thailand)

3.3.12 Light incubator shaker (Vision scientific, Green Seriker II, Korea)

3.3.13 Light microscope (Olympus, CH30, Japan)

3.3.14 Microcentrifuge (Labnet, Spectrafuge 16M, USA)

3.3.15 pH meter (Denver Instrument 215, USA)

3.3.16 Refrigerated centrifuge (Hermle Labortech, Z383K, Germany)

3.3.17 Semimicro cuvette rectabgular 10mm (Hella, USA)

3.3.18 Suction pump (LabTech, GM-0.33 II, USA)

3.3.19 Thermoblock (Labnet, Accu Block™ Digital dry bath, USA)

3.3.20 Ultrasonicate (Sonics and Materials, VC 505, USA)

3.3.21 UV-VIS spectrophotometer (Shimadzu, UV-1601, Japan)

3.3.22 Vortex (Scientific Industries, Genie2, USA)

3.3.23 Water bath (Heto-Holten, CBN 28-30, Denmark)

3.4 Collection, isolation and purification of cyanobacteria

Cyanobacterial strains in this study were isolated from samples of sand, shells, stones and seawater from two coastlines of Thailand, the Gulf of Thailand and the Andaman Sea of Thailand (Fig. 3.1). There were a total of 40 collection sample sites in six provinces of Thailand, including 24 coastlines of the Gulf of Thailand, Koh Kood island in Trad province, Laemsing beach, Krating bay, Jaolao beach, Laemsadet beach and Kungviman beach in Chanthaburi province, Makhampom bay, Maerampung beach, Samet island contained Cho bay, Pakarang bay, Phai bay, Prao bay, Saikaew beach, Vongdeuan bay and Wai bay, Saitong beach, Namrin beach, and Phla beach in Rayong province, Samaesan beach, Nangram beach, Toeingam beach, Saikaew beach, Jomtein beach, and Nuan bay (Larn island) in Chonburi province, and 16 coastlines of the Andaman Sea of Thailand, Yao beach, Yonglin beach, San beach, Samran beach, Modtanoy beach, Changlang beach, Pakmeng beach, Jaomai beach and Rachmongkol beach in Trang province, Laemkruat beach, Nummao bay, Nopparattara beach, Nang bay, Susanhoy beach, Thalane bay and Klongmuang beach in Krabi province. The approximately 20-40 samples from each collection site were randomly collected. Planktonic cyanobacterial strains were isolated from seawater whereas benthic cyanobacterial strains were isolated from stones, sand and shells. Samples from each location were inoculated in flasks containing liquid ASN III medium (Rippka *et al.*, 1979). The flasks were shaken with a speed of 120 rpm at 30 °C under light illumination of 30 μmol photons m^{-2} s^{-1} for 7-30 days or until green color of colonies or filaments from cyanobacteria in medium appeared. Each filament or colony of

microalgae from samples was subsequently isolated by a single cell isolation technique under a stereomicroscope (Hoshaw and Rosowski, 1973). The cyanobacterial isolates were washed several times with autoclaved ASN III medium and transferred onto new solid ASN III agar plates until a single colony or filament was obtained. Bacterial contamination was examined on LB agar by Gram staining technique (Gram, 1884). The axenic culture of cyanobacterial isolates were used in all experiments.

3.5 Cyanobacterial cultivation

Cyanobacterial isolates were cultivated in a 250-mL Erlenmeyer flask containing 100 mL of liquid ASN III medium. An initial cell concentration of cyanobacterial isolates was adjusted to the optical density at 730 nm of approximately 0.1. The cyanobacterial isolates were cultivated by shaking at 120 rpm under a white light illumination of 30 µmol photons m^{-2} s^{-1} at 30 °C for 14 days. All isolates were maintained by sub-culturing cells in a fresh ASN III medium every 2-3 weeks.

3.6 Morphological analysis of cyanobacterial isolates

Morphological analysis of cyanobacterial isolates was performed under a light microscope. In unicellular cyanobacterial isolates, dimension and the shape of colony was determined. In filamentous cyanobacterial isolates, the cellular width and length, shape of trichome and apical cells, cell wall constrictions, the availability of calyptras, and the number of cyanophycin granules were determined. In each isolate, dimension of ten vegetative cells per trichome was measured in twenty trichome cells by calculation magnification of images using the reference scale bar. Unicellular or trichome cells were photographed under magnification with video camera system (Nikon Bx51, Japan) using NIS-Elements Ver. 3.2 software (Nikon, Japan).

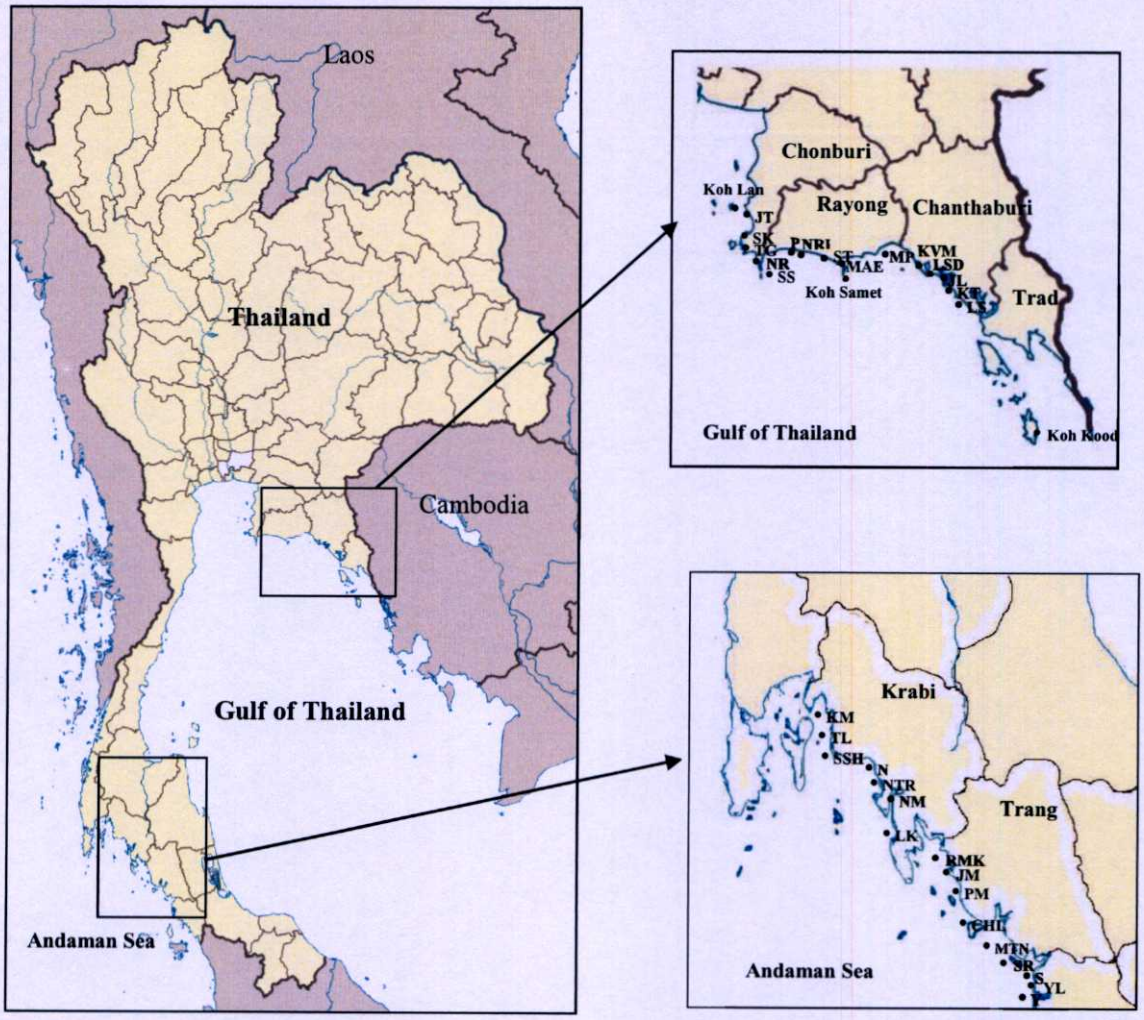


Figure 3.1 Map of sampling collection sites along 24 coastlines of the Gulf of Thailand, Koh Kood (KK), Laemsing (LS), Krating (KT), Jaolao (JL), Laemsadet (LSD), Kungviman (KVM), Makhampom (MP), Maerampung (MA), Samet Island (contained Cho (CH), Pakarang (PKR), Phai (PI), Prao (PR), Saikaew (SKR), Vongdeuan (VD), and Wai (W)), Saitong (ST), Phla (P), Namrin (NRI), Samaesan (SS), Nangram (NR), Toeingam (TG), Saikaew (SK), Jomtein (JT), and Larn island (contained Nuan (NE) and 16 coastlines of the Andaman Sea of Thailand, Yao (Y), Yonglin (YL), San (S), Samran (SR), Modtanoy (MTN), Changlang (CHL), Pakmeng (PM), Jaomai (JM), Rachmongkol (RMK), Laemkruat (LK), Nummao (NM), Nopparattara (NTR), Nang (N), Susanhoy (SSH), Talane (TL) and Klongmuang (KM).

3.7 Identification of cyanobacterial isolates by 16S rDNA sequencing analysis

3.7.1 Genomic DNA isolation

One loopful of cyanobacterial isolates grown on ASN III agar was suspended in 400 µL of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) in a microcentrifuge tube. About 100 µL of glass beads, 8 µL of 10% (w/v) SDS, 8 µL of 10% (w/v) SLS and 400 µL of TE saturated phenol were added to the cell suspension. The mixture was then vortexed for 10 s and immediately placed on ice for 3 min. This process was performed at least three times. Afterthat the mixture was centrifuged at 12,000×g at 4 °C for 10 min. The supernatant was transferred to a new microcentrifuge tube and was added with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1) (v/v). The aqueous DNA phase was separated by centrifugation and extracted again with an equal volume of chloroform-isoamylalcohol (24:1) (v/v). Total DNA was precipitated by adding with 0.1 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of ice-chilled absolute ethanol. The mixture was incubated at -70 °C for 30 min. Total genomic DNA was separated by centrifugation at 12,000×g at 4 °C for 10 min and washed once with 500 µL of ice-chilled 70% ethanol, dried, and resuspended in 20 µL of TE buffer.

3.7.2 DNA analysis by agarose gel electrophoresis

Genomic DNA isolated from cyanobacterial isolates was analyzed by agarose gel electrophoresis. Genomic DNA was separated on 0.8% (W/V) agarose gel in Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM borate and 2 mM EDTA, pH 7.4) by using the constant electric at 8 volts/cm for 30 min with an electrophoresis system (Mupid-EXU, Japan). The agarose gel was stained with 0.5 mg L^{-1} ethidium bromide (Bio Basic, USA). Finally, genomic DNA was visualized under UV light using gel documentation (Syngene, Japan). The genomic DNA size was estimated compare with the size of λDNA/*Hin*dIII marker (Invitrogen, USA).

3.7.3 Polymerase chain reaction (PCR) amplification

The DNA fragment of 16S rDNA of cyanobacterial isolates was amplified by polymerase chain reaction (PCR) using a forward primer F16S rDNA Cyano (5'-GCTCA GGATGAACGCTGGCG-3') and a reverse primer R16S rDNA Cyano (5'-CGGCTACCTTGTT

ACGACTCCA-3') (Phunpruch *et al.*, 2006) and genomic DNA as a template. The composition of PCR reaction used in this study was shown in Table 3.2. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Table 3.2 PCR reaction of 16S rDNA amplification for cyanobacterial isolates

Components	Volume (μL)
5X PCR buffer	10
$MgCl_2$ (25 mM)	3
dNTPs (10 mM)	1
Forward primer (5 μM)	2.5
Reverse primer (5 μM)	2.5
Taq DNA polymerase (5 U μL^{-1})	0.5
Genomic DNA (50 ng μL^{-1})	2
DI water	28.5
Total	50

3.7.4 Purification of PCR product

PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). Five volumes of DF buffer were added into PCR product. The mixture was mixed by vortexing and subsequently applied to a DF column placed into a 2-mL DF collection tube. The DF collection tube was centrifuged at 14,000xg for 30 s and a flow through was then discarded. The column was placed back into the collection tube. Six hundred μL of wash buffer (ethanol added) was added into center of DF column and the column was left standing for 1 min. Then, The column was repeatedly centrifuged and a flow through was discarded. The column was placed back into the collection tube again and was centrifuged for an additional 2 min. The DF column was placed into a new 1.5-mL microcentrifuge tube. PCR product was eluted from the column by adding 30 μL of EB buffer to the center of the DF column membrane, standing for 2 min followed by centrifugation for 2 min.

3.7.5 Nucleotide sequencing and analysis

The purified PCR product was sequenced in both directions with Big-DyeTM terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) using ABI PRISM® 3700 DNA analyzer at First BASE Laboratories (Malaysia). The taxonomic identification of cyanobacterial isolates was assessed by comparison of the obtained sequences to other cyanobacterial 16S rRNA genes available in National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST) (Altschul *et al.*, 1990).

3.8 Study of genetic diversity of selected cyanobacteria by 16S-23S ITS, *cpcB cpcA* IGS sequencing and RAPD method

3.8.1 16S-23S ITS and *cpcB-cpcA* IGS sequencing

The DNA fragments of 16S rDNA, 16S-23S rDNA internal transcribed spacer (16S-23S ITS) and the *cpcB-cpcA* intergenic spacer (*cpcB-cpcA* IGS) were amplified by PCR using primer pairs F16S-23S ITS Cyano (5'-TGTACACACCG CCCGTCAC-3') and R16S-23S ITS Cyano (5'-CTCTGTGTGCCTAGGTATCC-3') (Boyer *et al.*, 2002) and FcpcB-cpc IGS cyano (5'-TTGCCT(G/T)CGCGACATGGAAAT-3') and RcpcB-cpcA IGS cyano (5'-AGAGCTTCAAC (G/A)TACCAGCT-3') (Neilan *et al.*, 1995), respectively. Each 50 µL PCR reaction contained 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, 0.25 µM of each primer, 0.05 U of Taq DNA polymerase (Promega, USA) and 0.1 µg of genomic DNA. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55-60 °C for 1 min, and extension at 72 °C for 5 min with a final extension at 72 °C for 10 min. PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) as described in 3.7.4. The purified PCR products were sequenced in both directions as described in 3.7.5.

3.8.2 Random Amplification of Polymorphic DNA analysis

Ten universal random 10-mer primers were screened for random amplification of polymorphic DNA (RAPD) analysis. Their nucleotide sequences were shown in Table 3.3. The primers displayed polymorphism were chosen for RAPD analysis. RAPD

reaction was conducted in a total volume of 25 µL. It contained 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM of each primer, 0.05 U of Taq DNA polymerase (Promega, USA) and 0.05 µg of template DNA. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min. PCR products of RAPD were analyzed by 1.5% (w/v) agarose gel electrophoresis staining with 0.5 µg mL^{-1} ethidium bromide (Bio Basic, USA) as previously described 3.7.2.

Table 3.3 Primers for RAPD analysis used in this study

Primers	Nucleotide sequences (5' → 3')	References
OPA03	AGTCAGCCAC	El-Alfy *et al.*, 2009
OPA04	AATCGGGCTG	El-Alfy *et al.*, 2009
OPA05	AGGGGTCTTG	El-Alfy *et al.*, 2009
OPA07	GAAACGGGTG	El-Alfy *et al.*, 2009
OPA08	GTGACGTAGG	El-Alfy *et al.*, 2009
OPA09	GGGTAACGCC	El-Alfy *et al.*, 2009
OPA10	GTGATCGCAG	El-Alfy *et al.*, 2009
OPA13	CAGCACCCAC	Bardakci and Skibinski, 1994
OPA17	GACCGCTTGT	Bardakci and Skibinski, 1994
OPA19	CAAACGTCGG	Bardakci and Skibinski, 1994

3.8.3 Phylogenetic analysis

The obtained nucleotide sequences of 16S rDNA, 16S-23S ITS and *cpcB-cpcA* IGS fragments were compared with those other cyanobacteria available in the GenBank nucleotide database by multiple sequence alignment analysis using the ClustalW program (Thompson *et al.*, 1994). The phylogenetic tree using Neighbor Joining (NJ) and Maximum Likelihood (ML) estimation were constructed with molecular evolutionary genetics analysis (MEGA) software version 6.06 (Tamura *et al.*, 2013). The nucleotide substitution with complete-deletion gap treatment using

Tamura-Nei model was used for analysis. Bootstrap value was obtained from 1,000 replicates for each database. Each RAPD fingerprint established on an agarose gel was analyzed and RAPD pattern was generated. To construct binomial matrix, the presence of a DNA band at each position on a gel was scored as "1" while the absence of a DNA band was scored as "0". Genetic similarity between the selected isolates was calculated based on Jaccard's similarity coefficient and the dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) by the NTSYSpc 2.01e program (Rohlf, 1993).

3.8.4 Determination of tRNA secondary structure in 16S-23S ITS region

The secondary.structure of transfer RNA (tRNA) was predicted by using Mfold 3.2 software (http://www.bioinfo.rpi.edu/applications/mfold/) (Zuker, 2003). The folding temperature of tRNA was set at 37 °C.

3.9 Analytical methods

3.9.1 Dry cell weight measurement

Ten mL of culture were filtrated through a filter paper No. 1 (55 mm diameter) (Whatman, UK). The filter paper containing cells was subsequently washed twice with 10 mL of distilled water and dried at 60 °C in an oven for 1-3 days. Then, the filter paper was put in a desiccator for at least 1 h before weight measurement by the 4 digit balance. Dry cell weight was calculated from the subtraction of weight of filter paper containing cells and weight of filter paper without cells.

3.9.2 Determination of H_2 production

Cyanobacterial isolates were cultivated in ASN III medium for 14 days. The cultivation conditions were previously described in Topic 3.5. Cyanobacterial cells were harvested by centrifugation at 5,000xg at 4 °C for 10 min and washed twice with of $NaNO_3$-free ASN III medium. Then, cells were resuspended in 100 mL of $NaNO_3$-free ASN III medium and further incubated for 1 day before harvesting cells to assay H_2 production. The harvested cells were resuspended in 5 mL of $NaNO_3$-free ASN III medium. The cell suspension was transferred to a 10-mL gas-tight vial. The vial was sealed with a rubber stopper. The cell suspension in vial was purged with argon gas

for 5 min to enter an anaerobic adaptation. Cells were then shaken at 120 rpm at 30 °C under darkness for 24 h before analyzing H_2 production. After 24 h of incubation, 500 µL of gas phase in a vial was withdrawn by a gas-tight syringe and H_2 concentration in gas phase was analyzed by Gas Chromatograph (Hewlett-Packard HP890A GC, Japan) equipped with a thermal conductivity detector. The conditions for H_2 production determination by Gas chromatograph were shown in Table 3.4. The percentage concentration of H_2 from peak area obtained by Gas chromatograph was calculated compared with 4% (v/v) standard H_2. H_2 production rate and H_2 production yield was calculated as a term of H_2 evolved per dry cell weight per time ($\mu molH_2\ g^{-1}$ dry weight h^{-1}) and H_2 evolved per dry cell weight ($\mu molH_2\ g^{-1}$ dry weight), respectively.

Table 3.4 Gas chromatograph conditions used for determination of H_2 production with a thermal conductivity detector

Type	Condition
Detector	Thermal conductivity detector (TCD)
Column	Packed SS column 2m x 4mm OD x 3mm ID p/w
	Molecular sieve 5 ^{0}A 60/80 mesh
Temperature program	Injector temperature 100 °C
	Column temperature 50 °C
	Detector temperature 100 °C
Argon carrier gas	Flow rate 20 ml/min (99.999% purity)

3.9.3 Bidirectional hydrogenase activity assay

The *in vivo* bidirectional hydrogenase activity was determined by measuring H_2 produced in the presence of dithionite-reduced methylviologen. Two mL of reaction mixture contained 1 mL of cell suspension and 1 mL of the mixture containing 5 mM methyl viologen and 20 mM Na-dithionite (in 25 mM potassium phosphate buffer, pH 7.0). The reaction was incubated at 25 °C under dark anaerobic condition for 15 min before analysis of H_2 by Gas chromatograph. Hydrogenase activity was calculated in term of H_2 produced per dry cell weight per time ($\mu molH_2\ mg^{-1}$ dry weight min^{-1}).

3.9.4 Glycogen content measurement

Cyanobacterial cells were cultivated in ASN III medium for 14 days under conditions as described in Topic 3.5. Cells were subsequently harvested by centrifugation at 5,000xg at 4 °C for 10 min and washed twice with $NaNO_3$-free ASN III medium. Cells were resuspended in 100 mL of $NaNO_3$-free ASN III medium. The cell suspension was transferred in a 250 mL Erlenmeyer flask and further incubated at 30 °C for 0, 12, 24, 36, 24 and 48 h before harvesting cells to determine glycogen content. The extraction and hydrolysis of glycogen were performed as previously described (Ernst *et al.*, 1984). After incubation, cells were harvested by centrifugation at 5,000xg at 4 °C for 10 min and resuspended in 5 mL of $NaNO_3$-free ASN III medium. Then, 50 µL of 5 mL cell suspension was added to 200 µL of 30% KOH (w/v). The mixture was incubated in waterbath at 100 °C for 90 min and ultrasonicated for 5 min at 20% of pulse rate to hydrolyze cells before cooling down. To precipitate glycogen, 600 µL of absolute ethanol was added to the cooled extracts and the mixture was then kept on ice for 1 h. Glycogen was separated by centrifugation at 5,000xg at 4 °C for 5 min and washed twice by absolute ethanol before drying at 60 °C for 1 min. Glycogen was resuspended in 300 µL of 100 mM acetate buffer (pH 4.75) and digested with amyloglucosidase (4 U per assay) and amylase (8 U per assay) by incubation at room temperature for 1 h. After digestion, insoluble membrane fragments were removed by centrifugation at 5,000xg at 4 °C for 5 min. Fifty µL of supernatant was transferred to the new test tube and was then added with 500 µL of 4% (w/v) phenol reagent and 2.5 mL of 96% sulfuric acid. The reaction mixture was incubated in waterbath at 30 °C for 10 min and its absorbance was then measured at wavelength 490 nm using spectrophotometer (Dubois *et al.*, 1956). Glycogen content was calculated by using a standard calibration curve of glycogen content. The glycogen content was calculated in term of glycogen per dry cell weight.

3.10 Screening for high H_2-producing cyanobacterial isolates

Cyanobacterial cells were cultivated in ASN III medium under conditions previously described in Topic 3.5 for 14 days. Cells were harvested by centrifugation at 5,000xg at 4 °C for 10 min and washed twice with $NaNO_3$-free ASN III medium.

Then, cells were resuspended in 100 mL of $NaNO_3$-free ASN III medium and further incubated for 1 day before harvesting cells to determine H_2 production. The harvested cells were resuspended in 5 mL of $NaNO_3$-free medium and transferred to a 10-mL gas-tight vial. The vial was sealed with a rubber stopper. H_2 production was allowed to proceed for 24 h under four conditions; (1) anaerobic/dark condition, (2) microaerobic/light condition, (3) aerobic/dark condition and (4) aerobic/light condition. Under anaerobic/dark condition, cell suspension was purged with argon gas for 5 min to eliminate O_2 in a vial before incubation in a shaker with a speed of 120 rpm at 30 °C for 24 h under darkness. For microaerobic/light condition, cell suspension in a vial was also purged with argon gas for 5 min and incubated by shaking at 120 rpm at 30 °C under light intensity of 30 µmol photons m^{-2} s^{-1} for 24 h. Under aerobic condition, purging with argon gas was omitted. The vial was incubated by shaking at 30 °C either under the light intensity of 30 µmol photons m^{-2} s^{-1} (aerobic/light condition) or in the dark (aerobic/dark condition) for 24 h. After 24 h of incubation, 500 µL of gas phase in a vial was withdrawn by a gas-tight syringe and H_2 concentration in gas phase was analyzed by Gas Chromatograph as previously described 3.9.4.

3.11 Optimization of H_2 production by the selected cyanobacterial isolate

In the previous study, the most promising H_2-producing cyanobacterial strain was selected from the screening step of the work. Later using the selected cyanobacterial strain, several different conditions such as cultivation time, anaerobic adaptation time, medium composition and physical parameters, were investigated to better appreciate the H_2 production capacity of the organism. To determine the effect of cultivation time on H_2 production, the selected cyanobacterial cells were cultivated in ASN III medium for 7, 14 and 21 days. Cells were harvested by centrifugation at 5,000xg at 4 °C for 10 min and washed twice with $NaNO_3$-free ASN III medium. Then, cells were resuspended in 100 mL of $NaNO_3$-free ASN III medium and further incubated on a shaker for 1 day, subsequently harvested, resuspended in 5 mL of $NaNO_3$-free medium. Cells were transferred to the glass vial and incubated under darkness for 24 h before H_2 production measurement. To investigate the effect of medium compositions on H_2 production by the selected strain, 7-day grown cells

were harvested, washed twice and resuspended in $NaNO_3$-free ASN III medium containing various $NaNO_3$, $MgSO_4.7H_2O$, NaCl, Fe^{3+} and Ni^{2+} concentrations and incubated under darkness for 24 h before H_2 production measurement. In case of investigation on carbon sources and concentrations, Na_2CO_3 as a carbon source in ASN III medium was replaced by different types of sugar such as $NaHCO_3$, glucose, fructose, sucrose, lactose and maltose, with an equimolar concentration of C-atom (0.189 mmol C-atom L^{-1}). The concentrations of optimal sugar source were varied from 0 to 189 mmol C-atom L^{-1}. To investigate the effect of physical parameters on H_2 production by the selected strain, light intensity, medium pH and temperature were adjusted during H_2 production assay. All parameters used in this study were summarized in Table 3.5.

Table 3.5 Parameters used in this study for H_2 production optimization in ASN III medium

Parameter	condition
Cultivation time	7, 14, 21 days
Anaerobic adaptation time	0, 12, 24, 36, 48 hours
$NaNO_3$ concentration	0-88 mM
$MgSO_4.7H_2O$ concentration	0-140 mM
NaCl concentration	0-2 M
Carbon source	Sodium hydrogen carbonate, glucose, fructose, sucrose, lactose, maltose
Glucose concentration	0-189 mmol C-atom L^{-1}
Fe^{3+} concentration	0-200 μM
Ni^{2+} concentration	0-100 μM
Light intensity	0-150 μmol photons m^{-2} s^{-1}
pH	4-10
Temperature	20-70 °C

3.12 Method for cultivation the selected cyanobacterial strain in seawater for H_2 production

3.12.1 Cultivation of the selected strain in seawater

The seawater used in this study was collected from Namsai beach, Chonburi province, in the Gulf of Thailand. Before use, seawater was filtered through a filter paper No. 1 (55 mm diameter) (Whatman, UK) by using suction pump. The pH in seawater was adjusted to pH 7.5 with 2 M NaOH before sterilization by autoclaving. Cyanobacterial cells were cultivated in a 250-mL Erlenmeyer flask containing 100 mL of seawater, seawater containing 9 mM $NaNO_3$ and seawater containing 9 mM $NaNO_3$ and supplemented with Turk Island salt solution (Garlick *et al.*, 1977). Cells were cultivated by shaking at 120 rpm under a white light illumination of 30 µmol photons m^{-2} s^{-1} at 30 °C for 21 days. The growth of cyanobacteria by dry cell weight measurement was monitored every 2 days. In addition, growth of cells grown in seawater was compared with that of cells grown in normal ASN III medium and BG11 medium (Rippka *et al.*, 1979) supplemented with Turk Island salt solution.

3.12.2 H_2 production of the selected strain under various types of media

Cells grown in seawater containing 9 mM $NaNO_3$ by shaking at 120 rpm under a white light illumination of 30 µmol photons m^{-2} s^{-1} at 30 °C for 7 days were harvested by centrifugation, washed twice with seawater and resuspended in seawater containing concentrations of nutrients and minerals and further incubated in a 250-mL Erlenmeyer flask under light for 1 day before harvesting cells to determine H_2 production. The harvested cells were resuspended in the corresponding medium and transferred to a 10-mL gas-tight vial. The vial was sealed with a rubber stopper and subsequently purged with argon gas for 10 min to enter an anaerobic condition. The vial was shaken at 120 rpm in incubator shaker at 30 °C under darkness for 24 h before H_2 production, H_2 production rate and bidirectional hydrogenase activity measurements. The conditions of several type of media for optimization of H_2 production by the selected cyanobacterial strain. In addition, H_2 accumulation by *Geitlerinema* sp. RMK-SH10 under various conditions were determined for 14 days.

3.13 Statistical analysis

All H_2 production values were presented as means±SD from three independent biological replicates. Differences among treatments were analyzed by Duncan's multiple range test with the $P<0.05$ significance level using one way ANOVA of SPSS 24 software (IBM Corp., USA).

Chapter 4

Main Results and Discussion

4.1 Isolation of cyanobacteria

Cyanobacterial strains in this study were isolated from samples of sand, shells, stones and seawater from two coastlines of Thailand, the Gulf of Thailand and the Andaman Sea of Thailand. There were a total of forty collection sample sites in six provinces of Thailand during June to December in 2013. They include twenty-four coastlines of the Gulf of Thailand; Kohkood island in Trad province, Laemsing beach, Krating bay, Jaolao beach, Laemsadet beach and Khungwiman beach in Chanthaburi province, Makhampom bay, Maerampung beach, Cho bay, Pakarang bay, Phai bay, Prao bay, Saikaew beach, Vongdeuan bay, Wai bay, Saithong beach, Namrin beach and Phla beach in Rayong province, Samaesan beach, Nangram beach, Toeingam beach, Saikaew beach, Jomtien beach and Nuan bay in Chonburi province, and sixteen coastlines of the Andaman Sea of Thailand; Yao beach, Yonglin beach, San beach, Samran beach, Modtanoy beach, Changlang beach, Pakmeng beach, Jaomai beach and Rachamongkol beach in Trang province, Laemkruat beach, Nammao bay, Nopparatthara beach, Nang bay, Susanhoy beach, Talane bay and Klongmuang beach in Krabi province. The approximately 20-40 samples from each collection site were randomly collected and purified. The result showed that only 54 cyanobacterial isolates could be purified devoid of bacterial contamination isolated from the Gulf of Thailand and the Andaman Sea of Thailand (Table 4.1). There were four cyanobacterial isolates isolated from samples of sand, twenty-five cyanobacterial isolates isolated from samples of shells, eleven cyanobacterial isolates isolated from samples of stones and fourteen cyanobacterial isolates isolated from samples of seawater (Table 4.1).

Table 4.1 Samples of cyanobacterial isolates isolated from sand, shells, stones and seawater from the Gulf of Thailand and the Andaman Sea of Thailand.

Origins	Sand	Shell	Stone	Seawater	Total
Kohkood island, Trad	-	-	-	1	1
Laemsing beach, Chantaburi	-	-	-	1	1
Krating bay, Chantaburi	-	-	-	-	0
Jaolao beach, Chantaburi	1	1		1	3
Laemsadet beach, Chantaburi		1		1	2
Khungwiman beach, Chantaburi	-	-	-	1	1
Makhampom bay, Rayong	1	-	1	-	2
Maerampung beach, Rayong	-	-	-	-	0
Cho bay, Rayong	-	-	-	-	0
Pakarang bay, Rayong	-	-	1	1	2
Phai bay, Rayong	-	-	-	1	1
Prao bay, Rayong	-	2	-	-	2
Saikaew beach, Rayong	-	-	-	1	1
Vongdeuan bay, Rayong	-	1	-	1	2
Wai bay, Rayong	1	1	-	-	2
Saithong beach, Rayong	-	-	1	-	1
Namrin beach, Rayong	-	2	-	-	2
Phla beach, Rayong	-	1	1	1	3
Samaesan beach, Chonburi	-	-	1	-	1
Nangram beach, Chonburi	-	1	-	-	1
Toeingam beach, Chonburi	-	-	-	1	1
Saikaew beach, Chonburi	-	-	3	-	3
Jomtein beach, Chonburi	-	-	-	-	0
Nuan bay, Chonburi	-	-	-	-	0
Yao beach, Trang	-	1	-	-	1
Yonglin beach, Trang	-	1	-	-	1

Table 4.1 Samples of cyanobacteria isolates isolated from sand, shells, stones and seawater from the Gulf of Thailand and the Andaman Sea of Thailand (continued).

Origins	Sand	Shell	Stone	Seawater	Total
San beach, Trang	-	-	-	1	1
Samran beach, Trang	-	1	-	-	1
Modtanoy beach, Trang	-	2	-	-	2
Changlang beach, Trang	-	2	-	-	2
Pakmeng beach, Trang	-	1	-	-	1
Jaomai beach, Trang	-	1	-	-	1
Rachamongkol beach, Trang	-	1	-	-	1
Laemkruat beach, Krabi	-	1	-	-	1
Nammao bay, Krabi	1	1	-	-	2
Nopparatthara beach, Krabi		-	-	1	1
Nang bay, Krabi	-	-	2	-	2
Susanhoy beach, Krabi	-	2	-	-	2
Talane bay, Krabi	-	1	-	1	2
Klongmuang beach, Krabi	-	-	1	-	1
Total	**4**	**25**	**11**	**14**	**54**

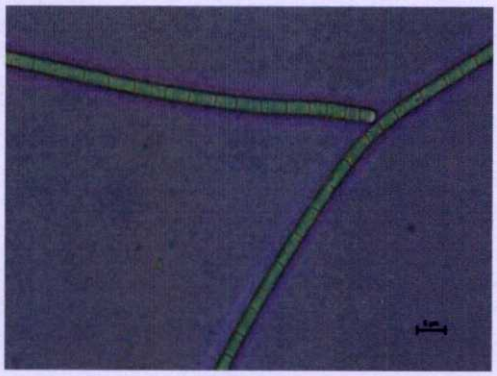
4.2 Morphology of cyanobacterial isolates

Cyanobacterial isolates were grown in ASN III medium for 14 days. The morphological analysis of these cyanobacterial isolates was performed under a light microscope. By morphological characteristics, these isolates can be divided into 5 groups (Appendix E, Fig. 4.1 and Table 4.2). Group 1 was filamentous cyanobacteria without sheath. They showed flexuous or straight filament containing a single trichome with one or more granules of cyanophycin which one of granules was close to the cross wall. The trichome was attenuated toward their ends with round cones at apical cell without calyptras at the outer cell wall. Trichome had a blue-green color. They did not show akinete or hetercystous cells. Each trichome had 2.6-6.4 μm cellular length and 1.7-2.6 μm cellular width (Fig. 4.1A). Group 2 was filamentous cyanobacteria with sheath. They showed rod or cylindrical filament with constricted

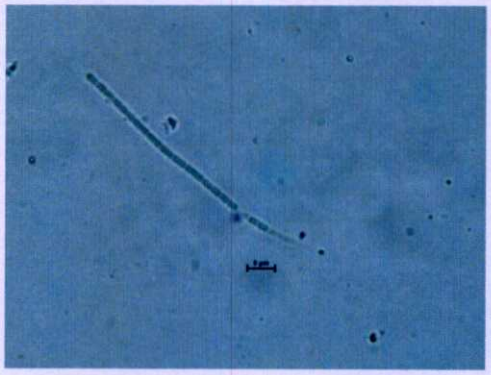
at the cross-walls, not attenuated at ends and a thin sheath. The filament was cylindrical or round apical cells without calyptras at the outer cell wall. The trichome had blue-green color, 1.4-2.7 μm cellular length and 0.5-0.9 μm cellular width (Fig. 4.1B). Group 3 was small round unicellular cyanobacteria. They showed a single cell or two-four cells with an oval or round shape and had a blue-green color with sizes varied from 0.77-2.2 μm of dimension (Fig. 4.1C). Group 4 was ovoid or cylindrical unicellular cyanobacteria. They were ovoid or cylindrical in shape. They had a blue-green color and had 4.1-4.5 μm in width and 7.7-8.6 μm in length (Fig. 4.1D). Group 5 was unicellular cyanobacteria found in spherical single cell or colonies of two to four cells. They showed a blue-green color, mucilage colorless and had 1.8-2.2 μm cell dimension (Fig 4.1E).

4.3 Identification of cyanobacterial isolates by 16S rDNA sequencing analysis

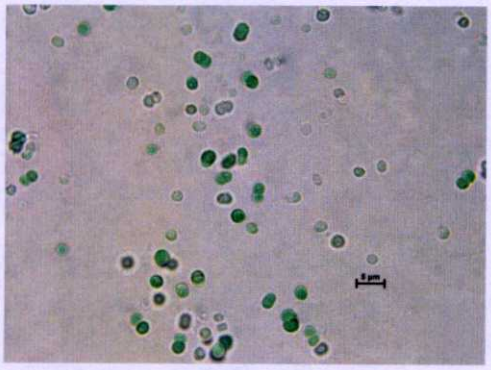
Cyanobacterial isolates were grown on ASN III agar under a white light illumination of 30 μmol photons m^{-2} s^{-1} at 30 °C for 14 days. Genomic DNA of cyanobacterial isolates was isolated by phenol-chloroform-isoamylalcohol method and analyzed by 0.8% (w/v) agarose gel electrophoresis. Genomic DNA was visualized under UV light using gel documentation (Syngene, Japan). The genomic DNA size was quantitatively analysed compared with the size of λDNA/*Hin*dIII marker. The result showed that only one dense DNA band was shown in each genomic DNA of cyanobacterial isolates (Fig. 4.2). The size of genomic DNA of cyanobacterial isolates was higher than 23,130 bp and genomic DNA concentration was about 200 ng μL^{-1} (Fig. 4.2). This concentration was sufficient to use as a template DNA in a PCR reaction.



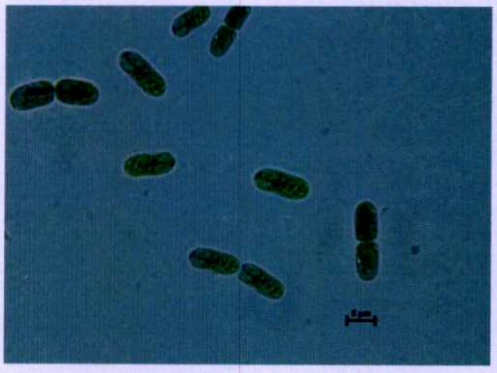
(A)



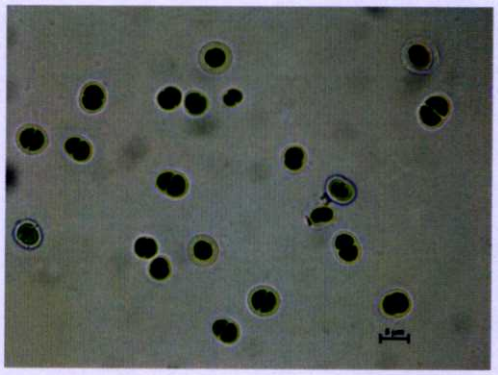
(B)



(C)



(D)



(E)

Figure 4.1 Morphology of cyanobacterial isolates isolated from seawater in the Gulf of Thailand and the Andaman Sea of Thailand.

Table 4.2 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand.

Group	Isolates	Morphology
1	CHL-SH1, JL-SH1, JL-SA1, JM-SH2, KVM-W1, LK-SH2, LS-W2, LSD-SH2, MTN-SH5, MTN-SH9, N-ST1, N-ST2, NM-SH1, NM-SA4, NR-SH2, P-ST2.3, P-W2.1, PI-S1.1, PKR-W3, PM-SH13, RMK-SH10, S-SH3, SK-ST1.1, SK-ST1.2, SK-ST2.1, SR-SH4, SS-ST6, SSH-SH12, ST-ST1.5.1, TG-W2.3.3, TL-SH2, VD-SH2.3, WI-SA1, WI-SH3 and YL-SH4	Filamentous, flexuous or straight filament, single trichome containing one or more granules of cyanophycin, one of which close to the cross wall, attenuated toward their ends, round-cones apical cell without calyptras at the outer cell wall, blue-green color, 2.6-6.4 μm cellular length, 1.7-2.6 μm cellular width
2	CHL-SH10, KK, KM-ST9, MP-SA1, NTR-S, PKR-SH1, PKR-SH9, PKR-ST1, P-SA1.1, S-S, SSH-SH5, TL-S, and Y-SH8	Filamentous, rod or cylindrical filament, constricted at the cross-walls, not attenuated at the ends, cylindrical or round apical cell without calyptra at the outer cell wall, thin sheath, blue-green, non-capitate, 1.4-2.7 μm cellular length, and 0.5-0.9 μm cellular width
3	LSD-W3, JL-W2, MP-W, and VD-W	Unicellular, oval or round shape, single cell or 2-4 cells, blue-green color, size varied from 0.77-2.2 μm of dimension
4	P-SH8.2.1	Unicellular, ovoid or cylindrical in shape, blue-green color, 4.1-4.5 μm in width and 7.7-8.6 μm in length
5	SKR-W2.2	Unicellular, spherical single cell or colonies of 2-4 cells, shape inside mucilaginous envelope, hemispherical shape when in group, blue-green color, mucilage colorless, 1.8-2.2 μm cell dimension

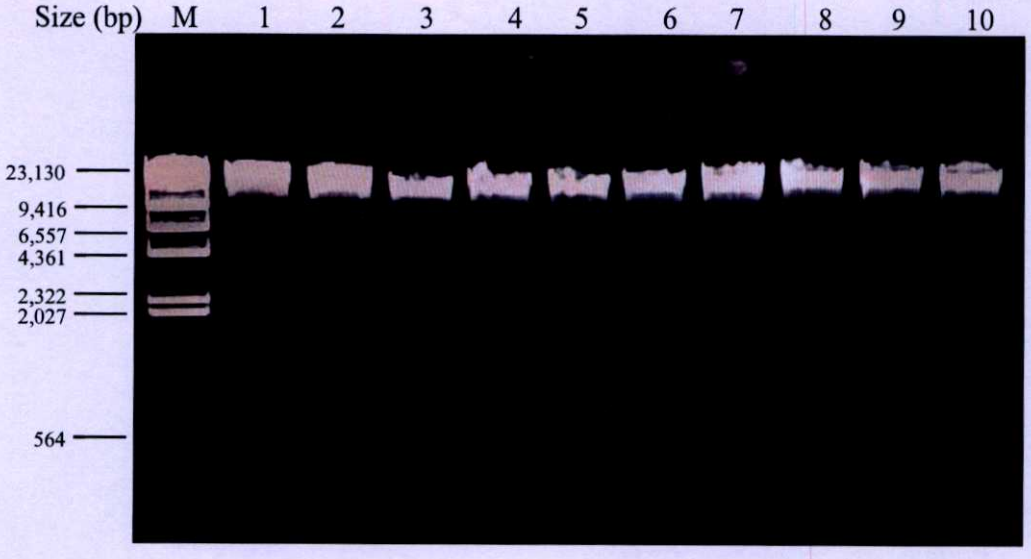


Figure 4.2 Genomic DNA of cyanobacterial isolates using 0.8% agarose gel electrophoresis.

Lane M : DNA marker/*Hin*dIII fragment

Lane 1 : Genomic DNA of cyanobacterial isolate ST-ST1.5.1

Lane 2 : Genomic DNA of cyanobacterial isolate NM-SH1

Lane 3 : Genomic DNA of cyanobacterial isolate S-SH3

Lane 4 : Genomic DNA of cyanobacterial isolate JM-SH2

Lane 5 : Genomic DNA of cyanobacterial isolate TL-SH2

Lane 6 : Genomic DNA of cyanobacterial isolate LK-SH2

Lane 7 : Genomic DNA of cyanobacterial isolate RMK-SH10

Lane 8 : Genomic DNA of cyanobacterial isolate MTN-SH5

Lane 9 : Genomic DNA of cyanobacterial isolate MTN-SH9

Lane 10: Genomic DNA of cyanobacterial isolate CHL-SH1

The DNA fragment of 16S rDNA of cyanobacterial isolates was amplified by polymerase chain reaction (PCR) using a forward primer F16S rDNA Cyano and a reverse primer R16S rDNA Cyano. The PCR reaction and condition used in this study is shown in Table 3. After amplification, the PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis. The result showed that only one specific band with a size of about 1,300 bp was observed in of all PCR products (Fig. 4.3). No PCR products were found in negative control using deionized instead of genomic DNA as template.

Figure 4.3 PCR product of 16S rDNA in cyanobacterial isolates using 0.8% agarose gel electrophoresis.

Lane M : DNA marker/*Hin*dIII fragment

Lane 1 : PCR product of 16S rDNA in cyanobacterial isolate PM-SH13

Lane 2 : PCR product of 16S rDNA in cyanobacterial isolate S-SH3

Lane 3 : PCR product of 16S rDNA in cyanobacterial isolate KM-SH3

Lane 4 : PCR product of 16S rDNA in cyanobacterial isolate TL-SH2

Lane 5 : PCR product of 16S rDNA in cyanobacterial isolate NM-SH1

Lane 6 : PCR product of 16S rDNA in cyanobacterial isolate NTR-S

Lane 7 : PCR product of 16S rDNA in cyanobacterial isolate N-ST1

Lane 8 : PCR product of 16S rDNA in cyanobacterial isolate PI-S1.1

Lane 9 : PCR product of 16S rDNA in cyanobacterial isolate SR-SH2

Lane 10: Negative control of 16S rDNA amplification

The PCR products were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). The purified PCR product was sequenced in both directions with Big-Dye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, USA) using ABI PRISM® 3700 DNA analyzer at First BASE Laboratories (Malaysia). The taxonomic identification of cyanobacterial isolates was assessed by comparison of the obtained sequences to other cyanobacterial 16S rRNA genes available in National Center for Biotechnology Information (NCBI) database using the basic local alignment search tool (BLAST). It was shown that thirty-five isolates were classified in genus *Geitlerinema*, nine isolates were classified in genus *Leptolyngbya*, and three isolates each were classified in genus *Phormidium* and genus *Synechococcus*, respectively. The remaining three isolates were each classified in genus *Chroococcus*, *Cyanothece*, *Pseudoanabaena* and *Synechocystis* (Table 4.3). The identity percentage of cyanobacterial isolates compared to other organisms reported in GenBank ranged from 92.77% to 99.71% (Table 4.3). Interestingly, the cyanobacterium isolate TL-S isolated from sample of seawater in Thalane bay at Krabi province in the Andaman Sea in Thailand showed 92.77% identity to *Pseudanabaena*. Because this identity was quite low (<95%), it might be classified in a distinct genus or classified in other genus of cyanobacteria (Stackebrandt and Goebel, 1994). The cut-off value at the species level was later reevaluated at 98.7% and then 98.65% (Stackebrandt and Ebers, 2006; Kim *et al.*, 2014; Rossi-Tamisier *et al.*, 2015). The identity of 16S rRNA in cyanobacteria could indicate just at genus level and was not sufficient for determination at species level. The DNA-DNA hybridization, G+C content and phenotypic characterization are also very important tests for the novel species determination (Staley, 2006; Goris *et al.*, 2007; Rosselló-Móra *et al.*, 2011).

In this study, most of cyanobacterial isolates were identified as *Geitlerinema*. *Geitlerinema* is a filamentous predominant benthic cyanobacterium found attached to shells, stones and widespread on sand. *Geitlerinema* could be found in both coastlines of Thailand; the Gulf of Thailand connected to the Pacific Ocean and the Andaman Sea connected to the Indian Ocean. Although samples were randomly collected from seawater, it was quite easy for isolation benthic cyanobacteria such as *Geitlerinema* or *Leptolyngbya* attached to shells and stones due to the trichome structure and the obvious blue-green color appearance.

Table 4.3 Cyanobacterial isolates, origins, habitats, genus and percentage of identity compared to other organisms reported in GenBank.

Isolates	Origins	Habitats	Genus	Identity (%)
KK	Koh Kood, Trad	seawater	*Leptolyngbya*	98.84
LS-W2	Laemsing, Chanthaburi	seawater	*Geitlerinema*	99.37
JL-SH1	JaoLao, Chanthaburi	shell	*Geitlerinema*	99.46
JL-SA1	JaoLao, Chanthaburi	sand	*Geitlerinema*	98.84
JL-W2	JaoLao, Chanthaburi	seawater	*Synechococcus*	98.03
LSD-SH2	Laemsadet, Chanthaburi	shell	*Geitlerinema*	98.04
LSD-W3	Laemsadet, Chanthaburi	seawater	*Synechocystis*	97.95
KVM-W1	Kungviman, Chanthaburi	seawater	*Geitlerinema*	98.40
MP-SA1	Makhampom, Rayong	sand	*Leptolyngbya*	98.76
MP-W	Makhampom, Rayong	seawater	*Synechococcus*	98.28
PKR-ST1	Pakarang, Rayong	stone	*Leptolyngbya*	98.94
PKR-W3	Pakarang, Rayong	seawater	*Geitlerinema*	99.46
PI-S1.1	Phai, Rayong	seawater	*Geitlerinema*	99.59
PR-SH1	Prao, Rayong	shell	*Leptolyngbya*	97.02
PR-SH9	Prao, Rayong	shell	*Leptolyngbya*	99.12
SKR-W2.2	Saikeaw, Rayong	seawater	*Chroococcus*	97.94
VD-SH2.3	Vongdeuan, Rayong	shell	*Geitlerinema*	97.69
VD-W	Vongdeuan, Rayong	seawater	*Synechococcus*	98.11
WI-SA1	Wai, Rayong	sand	*Geitlerinema*	99.64
WI-SH3	Wai, Rayong	shell	*Geitlerinema*	98.55
ST-ST1.5.1	Saitong, Rayong	stone	*Geitlerinema*	98.94
P-SH8.2.1	Phla, Rayong	shell	*Cyanothece*	98.11
P-SA1.1	Phla, Rayong	shell	*Phormidium*	96.54
P-ST2.3	Phla, Rayong	stone	*Geitlerinema*	98.93
P-W2.1	Phla, Rayong	seawater	*Geitlerinema*	99.02
SS-ST6	Samaesan, Chonburi	stone	*Geitlerinema*	98.02
NR-SH2	Nangram, Chonburi	shell	*Geitlerinema*	99.55

Table 4.3 Cyanobacterial isolates, origins, habitats, genus and percentage of identity compared to other organisms reported in GenBank (continued).

Isolates	Origins	Habitat	Genus	Identity (%)
TG-W2.3.3	Toeingam, Chonburi	seawater	*Geitlerinema*	99.12
SK-ST1.1	Saikeaw, Chonburi	stone	*Geitlerinema*	99.71
SK-ST1.2	Saikeaw, Chonburi	stone	*Geitlerinema*	98.73
SK-ST2.1	Saikeaw, Chonburi	stone	*Geitlerinema*	98.74
Y-SH8	Yao, Trang	shell	*Phormidium*	96.81
YL-SH4	Yonglin, Trang	shell	*Geitlerinema*	99.73
S-S	San, Trang	seawater	*Leptolyngbya*	99.05
S-SH3	San, Trang	shell	*Geitlerinema*	98.79
SR-SH4	Samran, Trang	shell	*Geitlerinema*	96.48
MTN-SH5	Modtanoy, Trang	shell	*Geitlerinema*	96.42
MTN-SH9	Modtanoy, Trang	shell	*Geitlerinema*	98.22
CHL-SH1	Changlang, Trang	shell	*Geitlerinema*	99.71
CHL-SH10	Changlang, Trang	shell	*Leptolyngbya*	99.20
PM-SH13	Pakmeng, Trang	shell	*Geitlerinema*	96.47
JM-SH2	Jaomai, Trang	shell	*Geitlerinema*	96.52
RMK-SH10	Rachmonkol, Trang	shell	*Geitlerinema*	98.47
LK-SH2	Laemkruat, Krabi	shell	*Geitlerinema*	99.70
NM-SA4	Nummao, Krabi	sand	*Geitlerinema*	98.02
NM-SH1	Nummao, Krabi	shell	*Geitlerinema*	99.47
NTR-S	Nopparattara, Krabi	seawater	*Leptolyngbya*	96.45
N-ST1	Nang, Krabi	stone	*Geitlerinema*	97.53
N-ST2	Nang, Krabi	stone	*Geitlerinema*	95.90
SSH-SH5	Susanhoy, Krabi	shell	*Leptolyngbya*	97.92
SSH-SH12	Susanhoy, Krabi	shell	*Geitlerinema*	98.58
TL-S	Thalane, Krabi	seawater	*Pseudanabaena*	92.77
TL-SH2	Thalane, Krabi	shell	*Geitlerinema*	95.96
KM-ST9	Klongmuang, Krabi	stone	*Phormidium*	96.54

4.4 Characterization of *Geitlerinema* using morphological analysis, 16S rDNA, 16S-23S ITS, *cpcB-cpcA* IGS sequencing and RAPD method

By isolation of cyanobacteria from samples of seawater, stones, sand and shells, randomly collected from coastal locations in Thailand, most of purified cyanobacterial isolates were classified in genus *Geitlerinema*. *Geitlerinema* is a filamentous cyanobacterium belonging to the order Oscillatoriales (Castenholz *et al.*, 2001). It was originally classified in the LPP-B "Oscillatorian" group (Rippka *et al.*, 1979) but was later simultaneously assigned to a subgenus of the genus *Phormidium* (Anagnostidis and Komárek, 1988) and designated as "*Oscillothrix*" (Rippka, 1988). *Geitlerinema* was later re-classified as a new genus of oscillatorialean cyanophytes in the family Pseudanabaenaceae. It can be found in different aquatic habitats such as freshwater or marine environments (Rippka *et al.*, 1979; Romo *et al.*, 1993; Silva *et al.*, 1996; Margheri *et al.*, 2003; Kirkwood *et al.*, 2006). *Geitlerinema* can also form blooms in reservoirs (Torgan and Paula, 1994). It is interesting whether the characterization of morphology and genetics among *Geitlerinema* populations in two different coastlines of Thailand are diverse. In addition, our *Geitlerinema* isolated from seawate might be distinct from the previously reported marine or freshwater *Geitlerinema* strains. Therefore, genetic diversity based on morphological and genetic characterization was performed in twenty representative *Geitlerinema* isolates comprising sixteen isolates from the Andaman Sea and four isolates from the Gulf of Thailand.

4.4.1 Morphological characteristics of *Geitlerinema*

Twenty *Geitlerinema* isolates were cultivated in ASN III medium with shaking at 120 rpm under white-light illumination of 30 μmol photons m^{-2} s^{-1} at 30 °C for 14 days. Their trichome shape, cellular width and length, cell wall constrictions, shape of apical cells, the presence or absence of calyptras, and the number of cyanophycin granules were determined under a light microscope (Olympus CX31, Japan). Under microscope, all isolates appeared in a filamentous, flexuous or straight filament composed of a single trichome, thin trichome and blue-green color (Fig. 4.4). The cylindrical trichome was not constricted at the cross walls and was attenuated toward their ends. Each individual trichome contained one or more granules of

cyanophycin (Table 4.4) which one was close to the cross wall. In addition, the apical cells were round cones without calyptras at the outer cell wall. Although all twenty *Geitlerinema* isolates showed the similar cellular shape, they appeared in a wide variety of trichome sizes. In this study, the average cellular length and width of the *Geitlerinema* strains were 2.59-6.36 μm and 1.67-2.64 μm, respectively, and the cellular length/width ratio ranged from 1.42 to 3.22 (Table 4.4). A single granule per cell was mostly observed in *Geitlerinema* (30-84%) whereas two or three granules per cell were less observed (16-50% and 0-21%, respectively). Four granules per cell were observed in only three strains (isolates N-ST2, SK-ST1.2 and ST-ST1.51) (Table 4.4).

The morphological and ultrastructural characteristics have been well-studied in some strains of *Geitlerinema* (Romo *et al.*, 1993; Komárek and Azevedo, 2000; Bittencourt-Oliveira *et al.*, 2009; Hašler *et al.*, 2012; Strunecký *et al.*, 2017). In this study, all isolates showed a single morphological type and all of them were similar to *Geitlerinema pseudacutissimum* and *Geitlerinema carotinosum* whose morphology has been previously reported (Hašler *et al.*, 2012). They were filamentous, flexuous or straight filament composed of a single trichome without constriction at the cross walls. Apical cells were round without calyptra outside the cell wall. However, all *Geitlerinema* isolates in this study were different from the type species of *Geitlerinema*, *G. splendidum* P014 and *G. splendidum* P017 which are bent or screw-like at the ends with capitate or round apical cells (Hašler *et al.*, 2012). Furthermore, *G. amphibium* and *G. unigranulatum* formed a flexuous to straight trichome with no constriction at the cross walls but with round conical apical cells. Their hooked apical cells were observed in cultures (Bittencourt-Oliveira *et al.*, 2009). In this study, the observed cellular length was similar to that of *G. amphibium* and *G. unigranulatum*, which are 2.2-7 μm long, but the observed cellular width was quite wider than those organisms, which are 1.02-1.91 μm wide, as well as the number of granules (Bittencourt-Oliveira *et al.*, 2009). Since all *Geitlerinema* strains investigated showed the similar morphological data, using genetic information might help to assess the variation among *Geitlerinema* strains in this study. In this reason, the genetic diversity and phylogenetic relationships among *Geitlerinema* strains were further investigated.

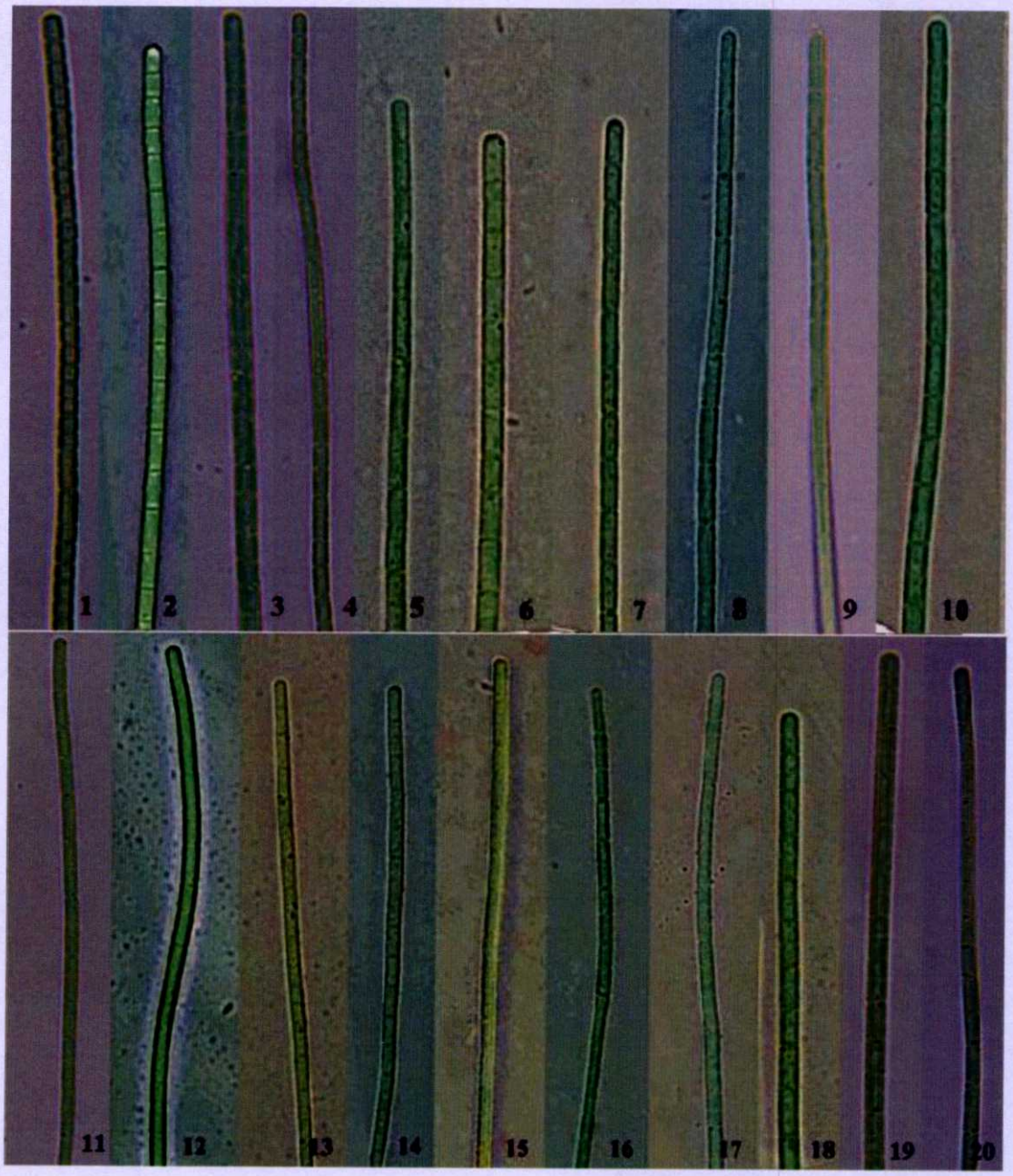


Figure 4.4 Morphology of *Geitlerinema* strains isolated from the Andaman Sea and the Gulf of Thailand in Thailand; *Geitlerinema* sp. CHL-SH1 (1), JM-SH2 (2), KM-SH3 (3), LK-SH2 (4), MTN-SH5 (5), MTN-SH9 (6), N-ST1 (7), N-ST2 (8), NM-SA4 (9), NM-SH1 (10), PI-S1.1 (11), PM-SH13 (12), RMK-SH10 (13), S-SH3 (14), SK-ST1.1 (15), SK-ST1.2 (16), SR-SH4 (17), SSH-SH12 (18), ST- ST1.5.1 (19) and TL-SH2 (20).

Table 4.4 Morphological characteristics comprising cellular width, cellular length, and number of cyanophycin granules per cell of *Geitlerinema* strains isolated from Thailand. Mean and standard deviation of cell width and length were calculated from twenty measurements. (L:W is a ratio of average length and width; *nG* is a total number of granules).

No.	Isolates	Width (μm)			Length (μm)			L:W	Granules				
		Min-Max	Mean	SD	Min-Max	Mean	SD		1	2	3	4	*n*G
1	CHL-SH1	2.22-2.50	2.28	0.11	4.09-6.80	5.09	1.01	2.23	15	7	2	0	24
2	JM-SH2	2.22-2.78	2.33	0.17	3.18-5.45	4.27	0.68	1.83	12	14	2	0	28
3	KM-SH3	1.67-2.22	1.82	0.19	1.82-3.18	2.59	0.46	1.42	18	9	1	0	28
4	LK-SH2	2.22-2.78	2.38	0.21	3.18-6.80	4.73	1.00	1.99	15	12	1	0	28
5	MTN-SH5	1.67-2.22	2.07	0.25	3.18-9.55	6.36	1.86	3.07	15	7	0	0	22
6	MTN-SH9	1.67-2.22	1.82	0.17	3.18-5.90	4.18	0.78	2.30	20	7	3	0	30
7	N-ST1	1.67-2.22	1.83	0.16	2.73-4.55	3.64	0.64	1.99	14	8	0	0	22
8	N-ST2	1.67-1.94	1.82	0.14	3.18-7.73	5.86	1.41	3.22	10	15	7	1	33
9	NM-SA4	1.67-2.22	1.89	0.28	3.64-5.45	4.45	0.60	2.35	16	8	1	0	25
10	NM-SH1	2.22-2.78	2.57	0.25	3.24-5.16	4.20	0.58	1.63	17	5	0	0	22
11	PI-S1.1	1.67-2.22	1.67	0.20	3.18-5.00	4.23	0.54	2.53	15	13	2	0	30
12	PM-SH13	1.94-2.22	2.10	0.14	3.64-5.45	4.86	0.58	2.31	18	6	1	0	25
13	RMK-SH10	1.67-1.94	1.82	0.14	2.73-4.55	3.64	0.54	2.00	12	14	2	0	30
14	S-SH3	1.67-2.22	2.13	0.16	2.73-5.45	4.18	0.86	1.96	15	11	2	0	28
15	SK-ST1.1	1.94-2.22	2.11	0.14	2.73-6.80	5.00	1.27	2.37	21	6	1	0	28
16	SK-ST1.2	1.67-2.22	1.94	0.13	3.64-6.80	5.45	0.86	2.81	11	7	4	1	23
17	SR-SH4	1.67-2.22	1.92	0.15	3.18-7.27	5.50	1.05	2.86	19	5	3	0	27
18	SSH-SH12	2.22-2.78	2.64	0.23	3.18-5.45	4.50	0.66	1.70	21	4	0	0	25
19	ST-ST1.5.1	1.67-2.22	1.88	0.24	3.18-5.00	4.05	0.66	2.15	12	8	0	2	22
20	TL-SH2	1.67-2.22	1.94	0.22	3.64-5.45	4.59	0.59	2.37	18	5	3	0	26

4.4.2 Phylogenetic tree analysis of 16S rDNA

DNA Fragments of 16S rDNA of all twenty *Geitlerinema* isolates were amplified by PCR using primers F16S rDNA Cyano and R16S rDNA Cyano and sequenced as previously described. The resulting 960 bp nucleotide sequences were deposited in GenBank under accession numbers KX955234-KX955253. The nucleotide sequences were compared with those of other *Geitlerinema* and cyanobacterial strains. The phylogenetic analysis of the 16S rDNA sequences by neighbor-joining (NJ) and maximum-likelihood (ML) methods was performed using 16S rDNA sequence of *Gloeobacter violaceus* as cyanobacterial outgroup and that of *E. coli* as distant outgroup. The resulting phylogenetic tree from both NJ and ML methods shows three clades of this genus (Fig. 4.5 and Fig. 4.6). All *Geitlerinema* isolates from Thailand (both from the Gulf of Thailand and the Andaman Sea) were clustered in the "Marine *Geitlerinema*". Interestingly, *Geitlerinema* sp. A28DM, *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 were also clustered into this clade. The second *Geitlerinema* clade comprised many strains of freshwater *Geitlerinema splendidum* strain P014, P017, SERB48 and PSE0519C. The third clade was composed of many strains of freshwater *Geitlerinema* species and *Leptolyngbya* species which were recently designated as *Anagnostidinema pseudacutissimum* and *Anagnostidinema amphibium* (Strunecký *et al.*, 2017) (Fig. 4.5 and Fig. 4.6).

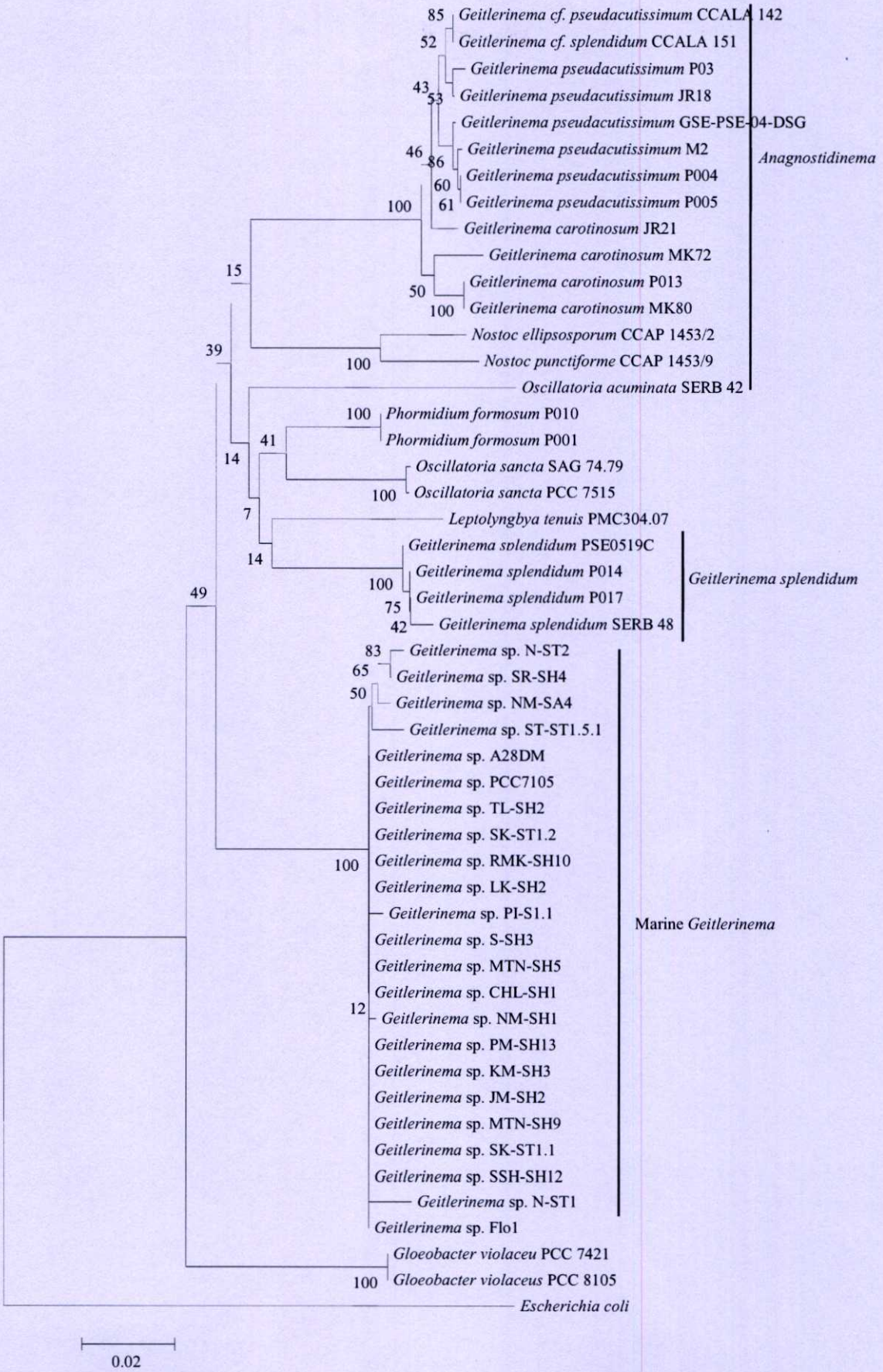


Figure 4.5 Phylogenetic tree based on 16S rDNA sequences of twenty marine *Geitlerinema* isolates and other cyanobacterial strains using NJ method with 1,000 bootstrap replicates.

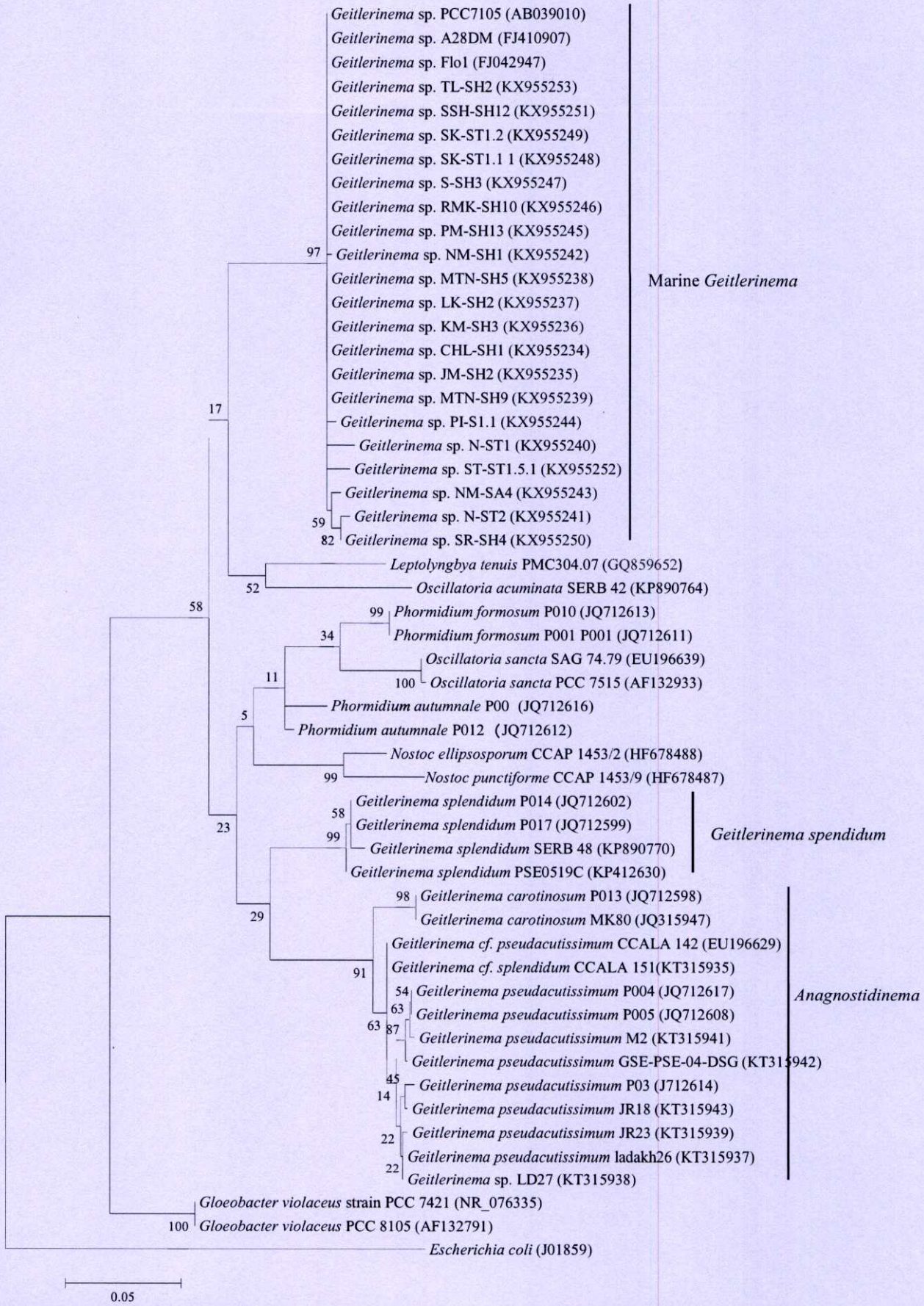


Figure 4.6 Phylogenetic tree based on 16S rDNA sequences of twenty marine *Geitlerinema* isolates and other cyanobacterial strains using ML method with 1,000 bootstrap replicates.

The 16S rDNA phylogenetic trees showed that all *Geitlerinema* isolates from Thailand were clustered in the same clade designated as "Marine *Geitlerinema*" (Fig. 4.5 and Fig. 4.6). These results indicate that marine *Geitlerinema* isolates from Thailand are genetically similar to each other and also closely related to many marine *Geitlerinema* strains, *Geitlerinema* sp. A28DM isolated from sandy shores parallel to the coast indented by the estuarine mouth of river Tapi, Gujarat, India (Parmar *et al.*, 2007), *Geitlerinema* sp. Flo1 (=*Oscillatoria limnetica* Flo1), present in the culture collection of the Department of Marine Microbiology at the University of Bremen (Schrübbers *et al.*, 2008) and *Geitlerinema* sp. PCC7105, recognized as the reference strain for the marine species of this genus according to Bergey's Manual (Castenholz *et al.*, 2001). This clade was phylogenetically distant from other freshwater *Geitlerinema* clades which are consistent with previous reports (Perkerson *et al.*, 2010; Strunecký *et al.*, 2017). This result clearly demonstrated that marine *Geitlerinema* is phylogenetically distant from other freshwater *Geitlerinema* and might be separated into other genera of cyanobacteria. The 16S rDNA sequences have been used as a target region for identification and phylogenetic analysis of several cyanobacteria; however, the data are insufficient to guarantee species identity and genetic diversity because of a lower evolutionary rate of variation (Fox *et al.*, 1992). With this reason other variable nucleotide sequence regions such as 16S-23S ITS and *cpcB-cpcA* were investigated.

4.4.3 Phylogenetic tree analysis of 16S-23S rRNA internal transcribed spacer (16S-23S ITS)

The DNA fragments of 16S-23S rRNA internal transcribed spacer (16S-23S ITS) were amplified by PCR using primers F16S-23S ITS Cyano and R16S-23S ITS. The PCR reaction was performed using PCR program as previously described in 3.8.1. After amplification, the PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis under UV light. The result showed that only one specific band of PCR products about 700-800 bp size was observed in all PCR reactions (Fig 4.7). No PCR products were found in the negative control using deionized water instead of genomic DNA as template (Fig. 4.7). The PCR products were purified using the Gel/PCR DNA fragments extraction kit and the purified PCR product was sequenced in both directions. From the obtained sequences of all twenty *Geitlerinema* isolates, there were approximately 711 bp nucleotide sequences containing partial sequences of 16S ribosomal RNA gene, complete sequences of 16S-23S ribosomal RNA and partial sequences of 23S ribosomal RNA gene. The 570-580 bp DNA fragments of 16S-23S ITS of twenty *Geitlerinema* isolates were deposited in the GenBank database under accession numbers KT186109-KT186128. Their 16S-23S ITS sequences showed 86.5-100.0% nucleotide identity to each other. When compared with other *Geitlerinema* strains deposited in GenBank, their sequences showed 41.9-98.7% nucleotide identity. In addition, the highest similarity of 86.6-98.7% was found when compared to *Geitlerinema* sp. Flo1 (accession no. FJ042948.1) and *Geitlerinema* sp. PCC7105 (accession no. FJ042946.1).

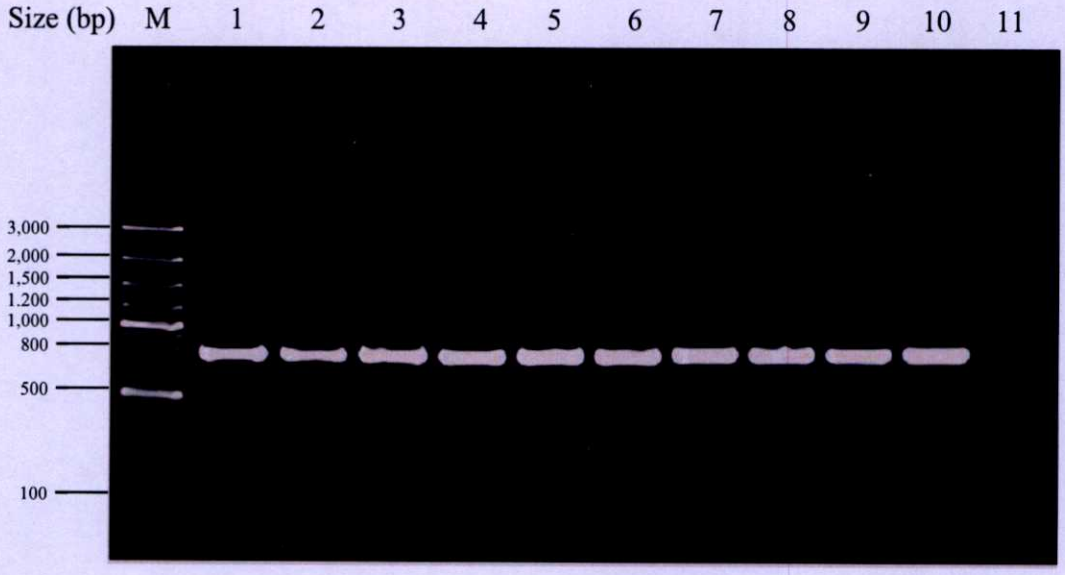


Figure 4.7 PCR products of 16S-23S ITS of *Geitlerinema* isolates using 1.5% agarose gel electrophoresis.

Lane M : 100 bp DNA ladder

Lane 1 : PCR product of *Geitlerinema* isolate ST-ST 1.5.1

Lane 2 : PCR product of *Geitlerinema* isolate NM-SH1

Lane 3 : PCR product of *Geitlerinema* isolate S-SH3

Lane 4 : PCR product of *Geitlerinema* isolate JM-SH2

Lane 5 : PCR product of *Geitlerinema* isolate TL-SH2

Lane 6 : PCR product of *Geitlerinema* isolate LK-SH2

Lane 7 : PCR product of *Geitlerinema* isolate RMK-SH10

Lane 8 : PCR product of *Geitlerinema* isolate MTN-SH5

Lane 9 : PCR product of *Geitlerinema* isolate MTN-SH9

Lane 10 : PCR product of *Geitlerinema* isolate CHL-SH1

Lane 11 : Negative control

The phylogenetic tree of the 16S-23S ITS sequences by neighbor-joining (NJ) and maximum-likelihood (ML) methods showed that *Geitlerinema* could be divided into four clades (Fig. 4.8 and Fig. 4.9). All marine *Geitlerinema* strains isolated from Thailand, *Geitlerinema* sp. Flo1, and *Geitlerinema* sp. PCC7105 were clustered into clade I; *G. pseudacutissimum* and *G. carotinosum* were clustered into clade II; *G. unigranulatum*, *Geitlerinema lemmermannii*, and *G. amphibium* were clustered into clade III; and *Geitlerinema acuminatum* and *G. splendidum* were clustered into clade IV. From these results, marine *Geitlerinema* isolates from Thailand are genetically similar to each other and are closely related to *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 but are different from other freshwater *Geitlerinema* strains found in central Europe such as *G. carotinosum*, *G. pseudacutissimum*, and *G. cf. acuminatum*. It was suggested that 16S-23S ITS sequence analysis could be used to primarily classify *Geitlerinema* strains at the species level based on the highly variable nucleotide sequences; however, this was not possible due to the limited genetic variation among the strains assessed in this study.

The 16S-23S ITS nucleotide sequences among *Geitlerinema* sp. collected from Thailand (clade I) were subdivided into 4 groups (Fig. 4.8 and Fig. 4.9). Sequences of each representative subgroup, *Geitlerinema* sp. RMK-SH10, LK-SH2, ST-ST1.5.1, and TL-SH2 were compared with that of *Geitlerinema* sp. PCC7105. The results showed that the 16S-23S ITS of *Geitlerinema* sp. RMK-SH10 is 9-10 nucleotides longer than that of other strains. All the sequences contain the conserved domains (D1, D1', D2, D3, D4, D5, and the antiterminator boxA), which are the regions involved in the formation of stem-loop structure (V2, V3 and the antiterminator boxB), and two tRNA sequences ($tRNA^{Ile}$ and $tRNA^{Ala}$) (Fig. 4.10). The $tRNA^{Ile}$ gene is located downstream of the 16S rDNA whereas the $tRNA^{Ala}$ is located upstream of the 23S rDNA (Fig. 4.10). The $tRNA^{Ala}$ secondary structure of *Geitlerinema* sp. TL-SH2, LK-SH2 and ST-ST1.5.1 have three loops (the small bubble above, the side loop, and the terminal loop) (Fig. 4.11A), whereas that of *Geitlerinema* sp. RMK-SH10 contains only two loops (Fig. 4.11B). *Geitlerinema* sp. RMK-SH10 may be genetically diverse from other strains in the subgenus. A comparative analysis of tRNA secondary structure might be a useful tool for studying genetic diversity among individual species of cyanobacteria.

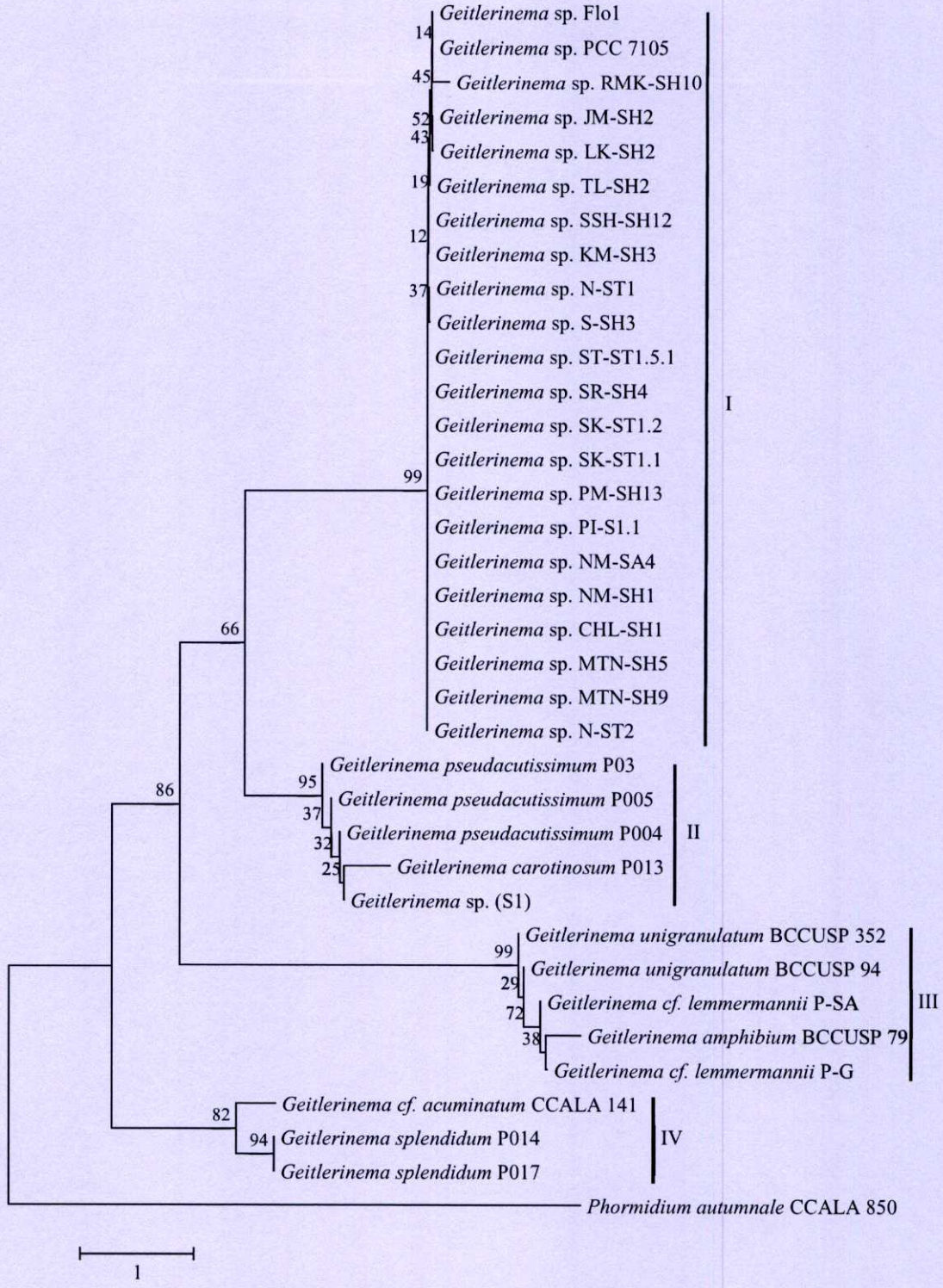


Figure 4.8 Phylogenetic tree based on the 16S-23S rRNA ITS sequences of twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains using NJ method with 1,000 bootstrap replicates.

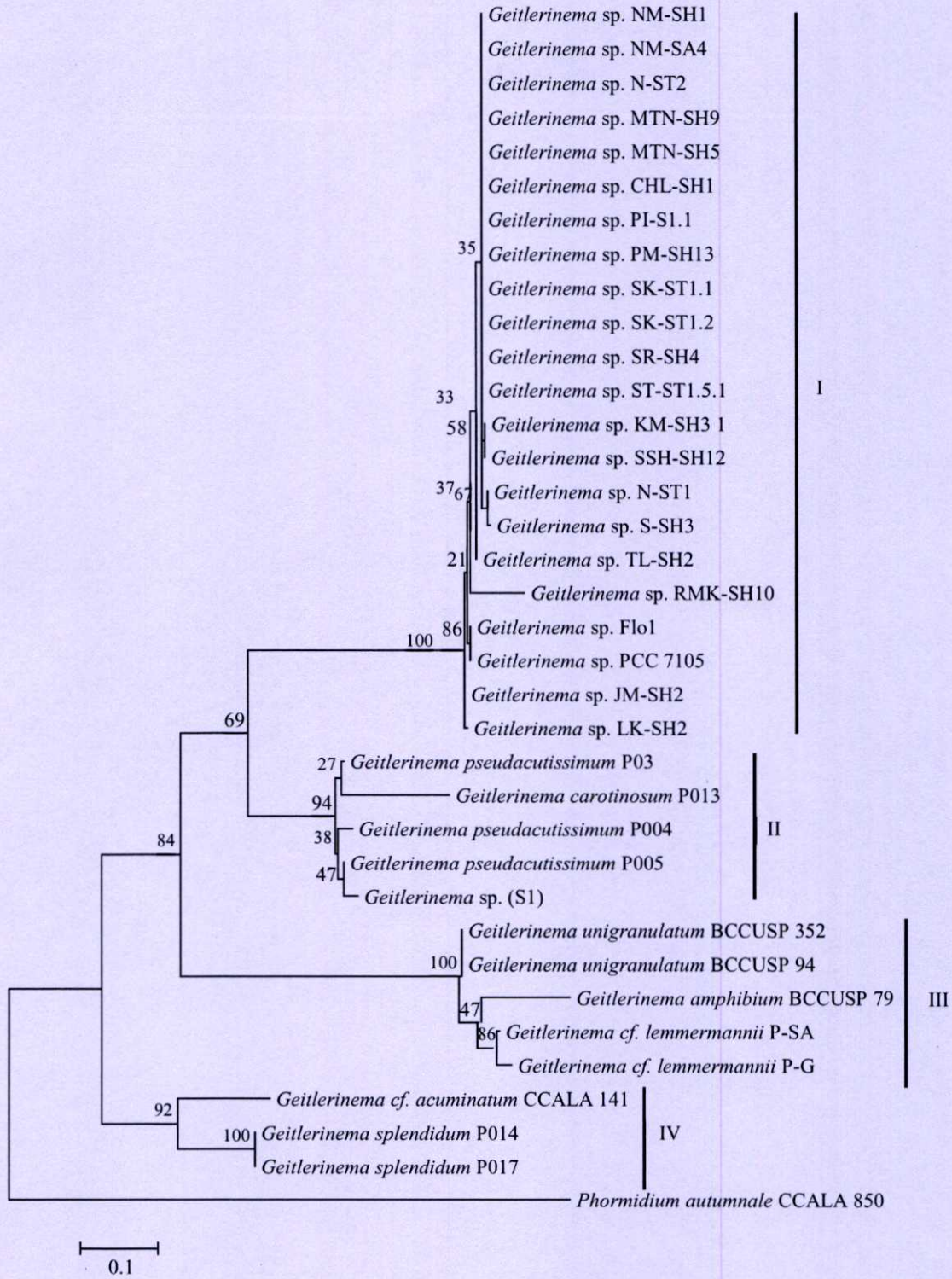
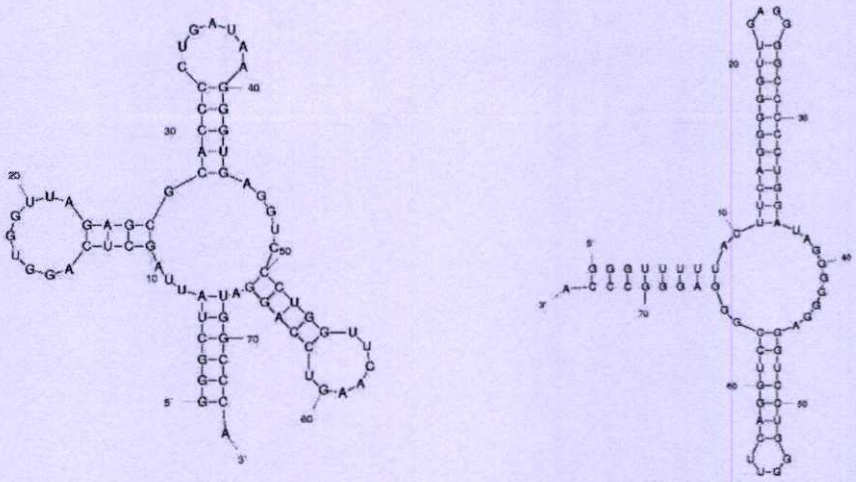


Figure 4.9 Phylogenetic tree based on the 16S-23S rRNA ITS sequences of twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains using ML method with 1,000 bootstrap replicates.

Figure 4.10 Alignment of 16S-23S rRNA ITS sequences of *Geitlerinema* sp. TL-SH2, LK-SH2, ST-ST1.5.1 and RMK-SH10 compared to that of *Geitlerinema* sp. PCC7105. All sequences contain $tRNA^{Ile}$, $tRNA^{Ala}$, the consensus domain sequences (D1-D5), the antiterminator (boxB and boxA) and the major variable stems V2 and V3.



(A) (B)

Figure 4.11 Predicted secondary structures of the $tRNA^{Ala}$ region in *Geitlerinema* sp. TL-SH2, LK-SH2 and ST-ST1.5.1 (A) and *Geitlerinema* sp. RMK-SH10 (B).

4.4.4 Phylogenetic analysis of the *cpcB-cpcA* IGS

The DNA fragments of *cpcB-cpcA* IGS were amplified by PCR using primer pair FcpcB-cpc IGS cyano and RcpcB-cpcA IGS cyano. The PCR reaction and the PCR program were used as previously described in 3.8.1. After amplification, PCR product was analyzed by 1.5% (w/v) agarose gel electrophoresis. The result showed that only one specific band of PCR product about 600-700 bp size was observed in the PCR reaction (Fig 4.12). No PCR products were observed in the negative control using deionized water instead of genomic DNA (Fig. 4.12). Then, all PCR products were purified using the Gel/PCR DNA fragments extraction kit and the purified PCR products were sequenced in both directions.

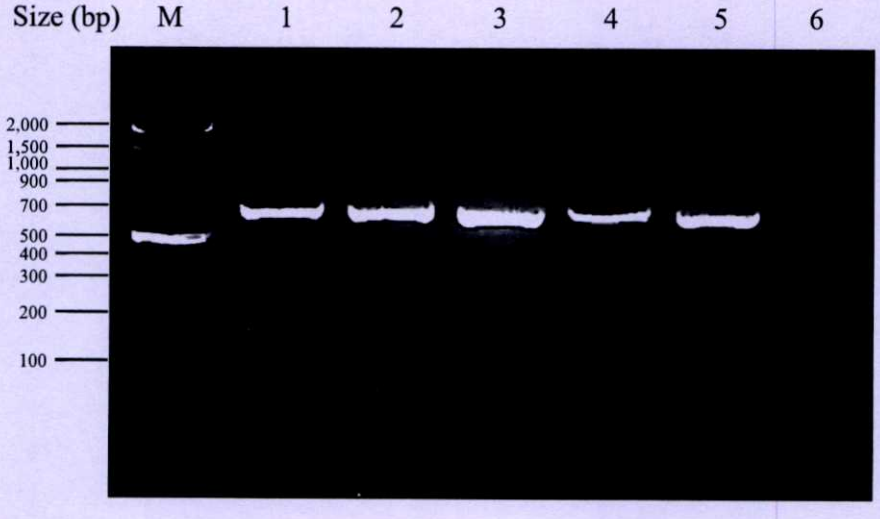


Figure 4.12 PCR product of *cpcB-cpcA* IGS of *Geitlerinema* isolates using 1.5% (w/v) agarose gel electrophoresis.

Lane M : 100 bp DNA ladder

Lane 1 : PCR product of *Geitlerinema* isolate PI-S1.1

Lane 2 : PCR product of *Geitlerinema* isolate MTN-SH5

Lane 3 : PCR product of *Geitlerinema* isolate N-ST1

Lane 4 : PCR product of *Geitlerinema* isolate RMK-SH10

Lane 5 : PCR product of *Geitlerinema* isolate LK-SH2

Lane 6 : Negative control

From the obtained *cpcB-cpcA* IGS gene sequences, it was found that the sequence of all *Geitlerinena* isolates was about 600 bp containing partial coding of phycocyanin beta subunit (*cpcB*) gene, complete sequence *of cpcB-cpcA* intergenic spacer and partial coding of phycocyanin beta subunit (*cpcA*) gene. Only 90-91 nucleotide sequences of *cpcB-cpcA* IGS were found. Their *cpcB-cpcA* IGS sequences were deposited in the GenBank database under accession numbers KT228281-KT228300. From a multiple sequence alignment, the obtained *cpcB-cpcA* IGS nucleotide sequences showed high identity with 95.6-100% to each other and showed 42.7-98.9% similarity to other *Geitlerinema* strains reported in GenBank. From the phylogenetic tree using neighbor-joining (NJ) and maximum-likelihood (ML) methods, *Geitlerinema* could be divided into three clades with a bootstrap value of >90% (Fig. 4.13 and Fig. 4.14). All *Geitlerinema* isolates from Thailand, *Geitlerinema*

sp. Flo1 (accession no. FJ042941.1), and *Geitlerinema* sp. PCC7105 (accession no. FJ042942.1) were clustered into Clade I. In addition, *Geitlerinema* sp. LK-SH2 and *Geitlerinema* sp. JM-SH2 were genetically similar. Clades II and III contained many strains of *G. unigranulatum* and *G. amphibium*. The *cpcB-cpcA* IGS region of *Geitlerinema* species in clade I comprised 86-91 nucleotides in length, and was distinct from those of clade II and clade III that contained 83 and 291-294 nucleotides, respectively.

In cyanobacteria, the photosynthetic pigments contain chlorophyll a and specific accessory pigments including allophycocyanin, phycocyanin, and phycoerythrin (Glazer, 1989). The phycocyanin and the other biliprotein pigments in the phycobilisome are the major light-harvesting antennae in photosystem II of cyanobacteria (Dubbs and Bryant, 1991; Glazer, 1989). The phycocyanin operon contains genes coding for two bilin subunits and three linker polypeptides (Belknap, and Hazelkorn, 1987). The IGS between the two subunit genes, *cpcB* and *cpcA* of the phycocyanin operon was chosen as a highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level (Neilan *et al.*, 1995).

In this study, all twenty marine *Geitlerinema* isolates from Thailand, *Geitlerinema* sp. Flo1, and *Geitlerinema* sp. PCC7105 were classified into Clade I (Fig. 4.12). This result is similar to the phylogenetic tree based on 16S rDNA and 16S-23S ITS sequences. It also confirmed the close genetic relationship among these *Geitlerinema* species. It was previously reported that the nucleotide sequences of cyanobacterial *cpcB-cpcA* IGS ranged from 69 to 298 nucleotides (Bittencourt-Oliveira *et al.*, 2012). Since the length of *cpcB-cpcA* IGS sequence is variable compared with that of the 16S-23S ITS sequence, the former might yield information enabling the differentiation at the strain level. However, this was not the case for the highly similar *Geitlerinema* isolates from Thailand assessed in this study.

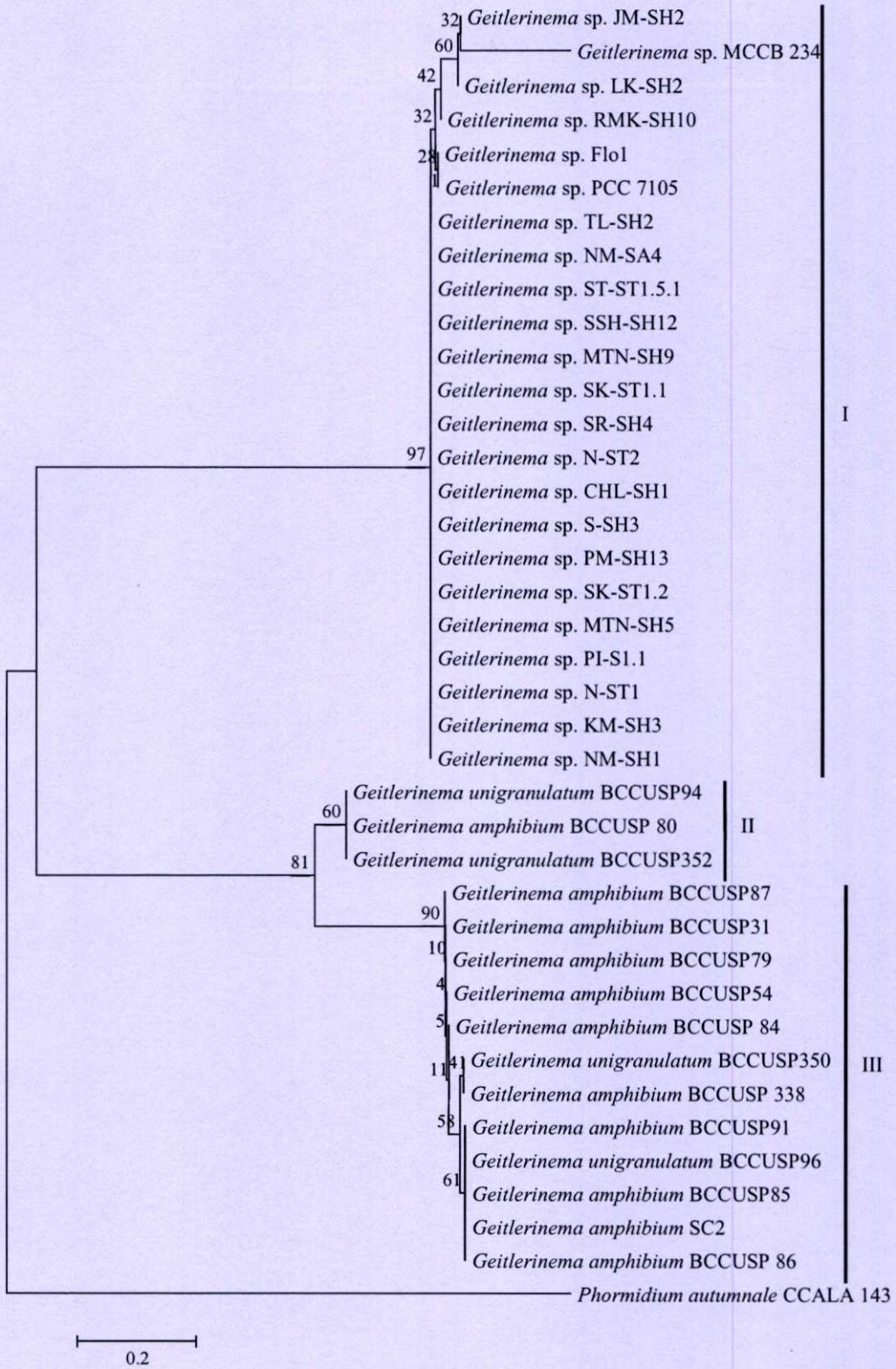


Figure 4.13 Phylogenetic tree based on the *cpcB-cpcA* IGS sequences of twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains using NJ method with 1,000 bootstrap replicates.

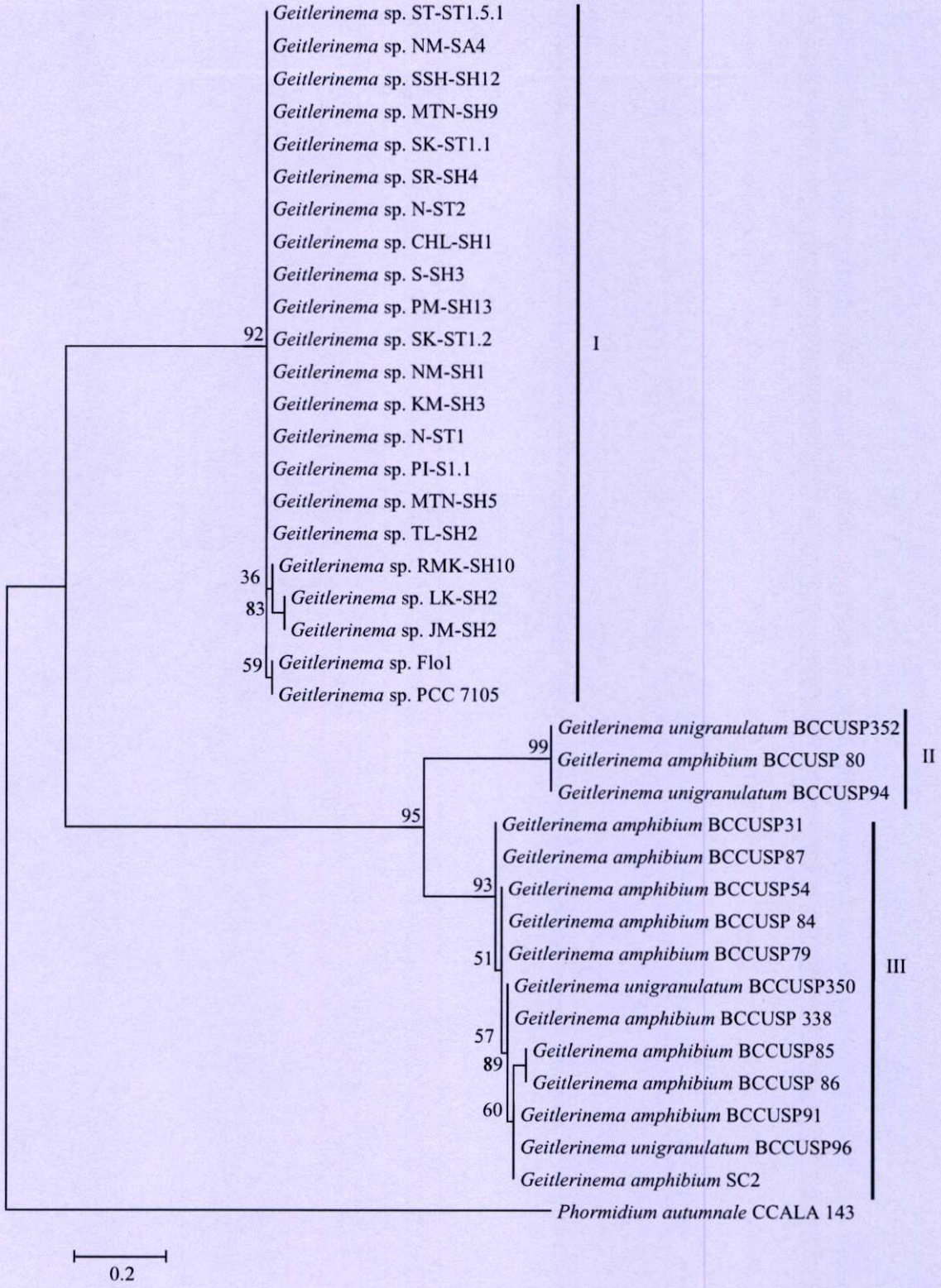
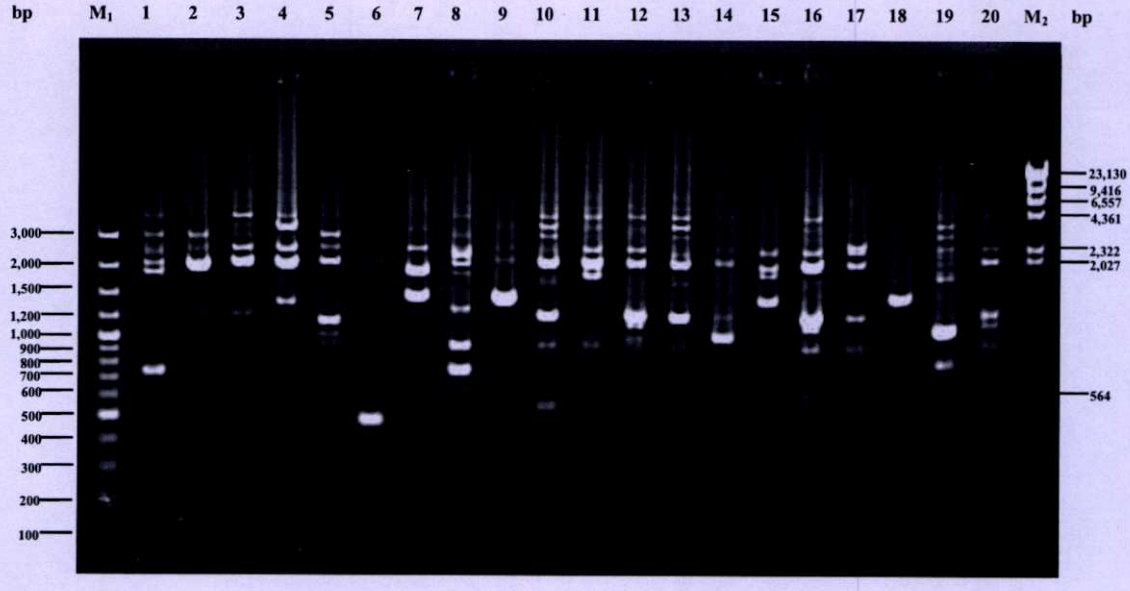


Figure 4.14 Phylogenetic tree based on the *cpcB-cpcA* IGS sequences of twenty marine *Geitlerinema* isolates and other *Geitlerinema* strains using ML method with 1,000 bootstrap replicates.

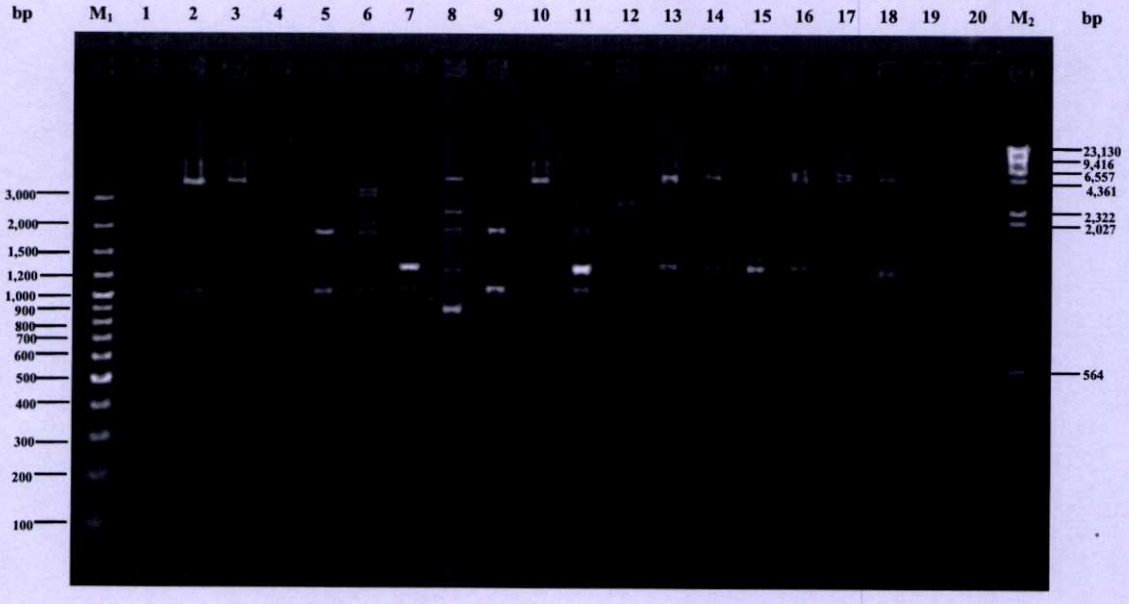
4.4.5 RAPD analysis

Ten universal random 10-mer primers were used for primary screening in random amplification of polymorphic DNA (RAPD) analysis. Five primers, OPA03, OPA05, OPA07, OPA10 and OPA19 displayed polymorphism were chosen for RAPD analysis. RAPD reaction was conducted with the PCR program previously described in the section 3.8.2. The PCR products of RAPD were analyzed by agarose gel electrophoresis. The result revealed that 85 different PCR product bands that yielded 100% polymorphism were constructed of RAPD pattern analysis. Each individual primer generated 14 to 17 identical PCR product bands (Fig. 4.15-4.17). The size of the PCR products ranged from 600 to 4,000 bp (Fig. 4.15-4.17). The RAPD similarity matrix of *Geitlerinema* isolates using five selected primers is shown in Table 4.5. The RAPD phylogenetic tree of *Geitlerinema* isolates based on UPGMA cluster analysis using Jaccard's similarity coefficient exhibited eight clades with an average similarity coefficient of 0.71 (Fig. 4.18). Clade I contained *Geitlerinema sp.* KM-SH3 and clade II contained *Geitlerinema* sp. PI-S1.1. Clade III contained twelve *Geitlerinema* strains, including three *Geitlerinema* isolates SK-ST1.2, ST-ST1.5.1 and SK-ST1.1 from the Gulf of Thailand and nine isolates from the Andaman Sea. The similarity within clade III ranged from 71.1-90.4%. The remaining six isolates from the Andaman Sea were classified into five clades: *Geitlerinema* sp. TL-SH2 in clade IV, *Geitlerinema* sp. N-ST1 in clade V, *Geitlerinema* sp. PM-SH13 in clade VI, *Geitlerinema* sp. CHL-SH1 in clade VII, and *Geitlerinema* sp. LK-SH2 and *Geitlerinema* sp. JM-SH2 in clade VIII (Fig. 4.18). The similarity within clade VIII was 73.0%.

In general, the DNA fingerprint obtained by RAPD is a molecular technique used for cluster analysis in order to study geographic patterns and to examine genetic diversity among cyanobacteria (Moschetti *et al.*, 1998; Casamatta *et al.*, 2003; Premanandh *et al.*, 2009). In this work, eight clades of marine *Geitlerinema* could be separated by this method, representing the genetic relationship among *Geitlerinema* populations. Within clade III, twelve *Geitlerinema* strains could be divided into many subgroups depending on the similarity coefficient. *Geitlerinema* sp. ST-ST1.5.1 was most closely related to *Geitlerinema* sp. SK-ST1.2 whereas *Geitlerinema* sp. MTN-SH5 was most closely related to *Geitlerinema* sp. MTN-SH9 (Fig. 4.18).

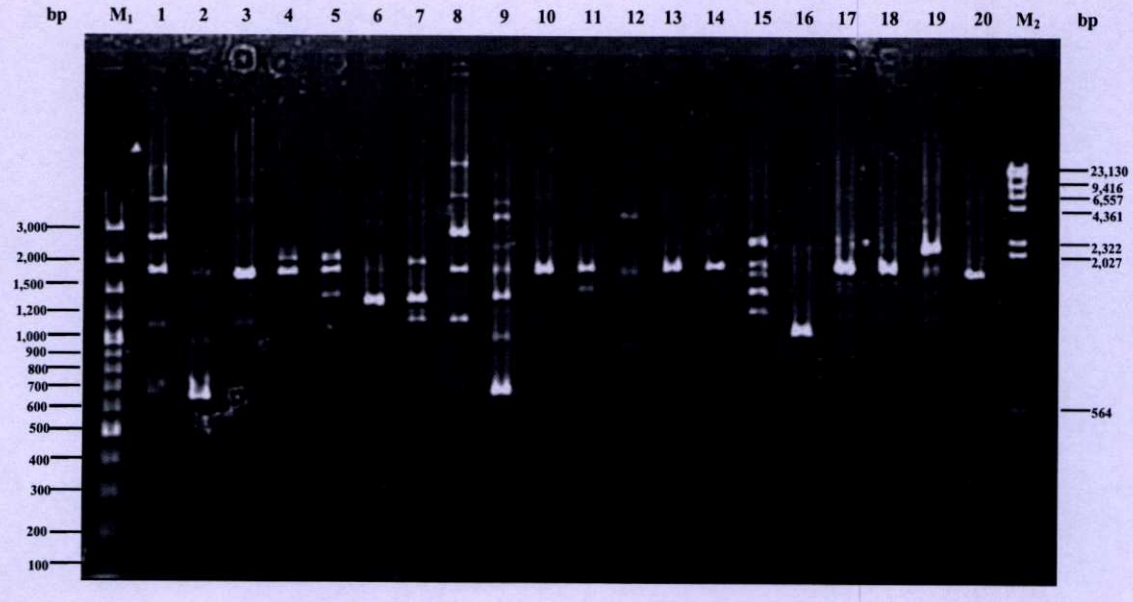


(A)

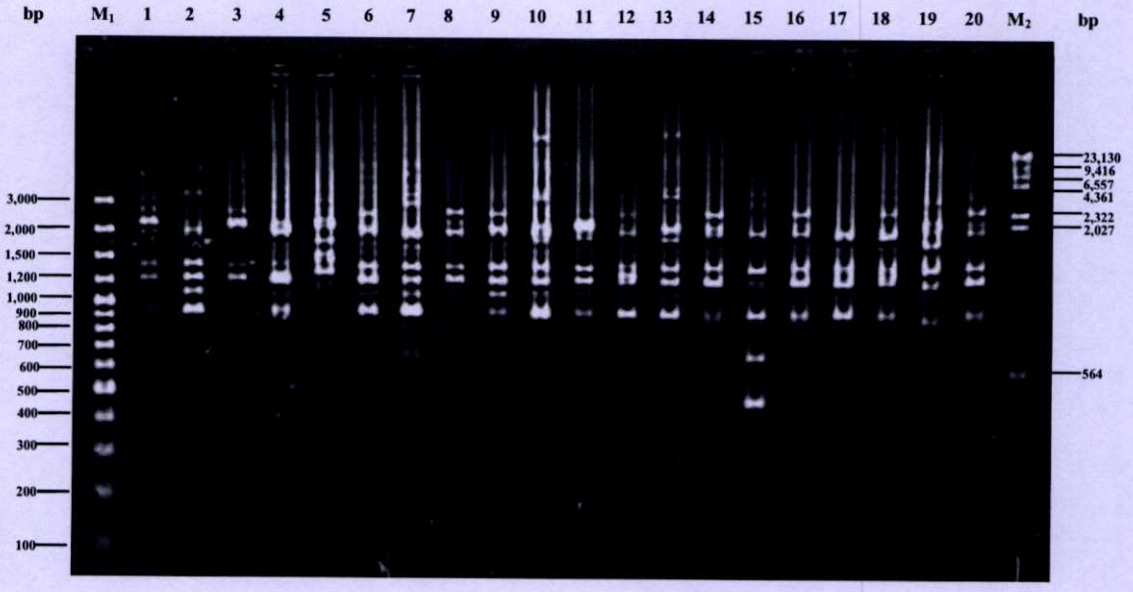


(B)

Figure 4.15 RAPD patterns of *Geitlerinema* isolates amplified by primer OPA03 (A) and OPA05 (B). Lane M1: 100 bp DNA ladder; M2: λDNA/*Hin*dIII fragments; 1: KM-SH3; 2: PI-S1.1; 3: NM-SH1; 4: N-ST2; 5: TL-SH2; 6: PM-SH13; 7: LK-SH2; 8: CHL-SH1; 9: N-ST1; 10: MTN-SH5; 11: S-SH3; 12: NM-SA4; 13: MTN-SH9; 14: SK-ST1.2; 15: JM-SH2; 16: ST-ST1.5.1; 17: SSH-SH12; 18: SR-SH4; 19: RMK-SH10; and 20: SK-ST1.1.



(A)



(B)

Figure 4.16 RAPD patterns of *Geitlerinema* isolates amplified by primer OPA07 (A) and OPA10 (B). Lane M1: 100 bp DNA ladder; M2: λDNA/ *Hin*dIII fragments; 1: KM-SH3; 2: PI-S1.1; 3: NM-SH1; 4: N-ST2; 5: TL-SH2; 6: PM-SH13; 7: LK-SH2; 8: CHL-SH1; 9: N-ST1; 10: MTN-SH5; 11: S-SH3; 12: NM-SA4; 13: MTN-SH9; 14: SK-ST1.2; 15: JM-SH2; 16: ST-ST1.5.1; 17: SSH-SH12; 18: SR-SH4; 19: RMK-SH10; and 20: SK-ST1.1.

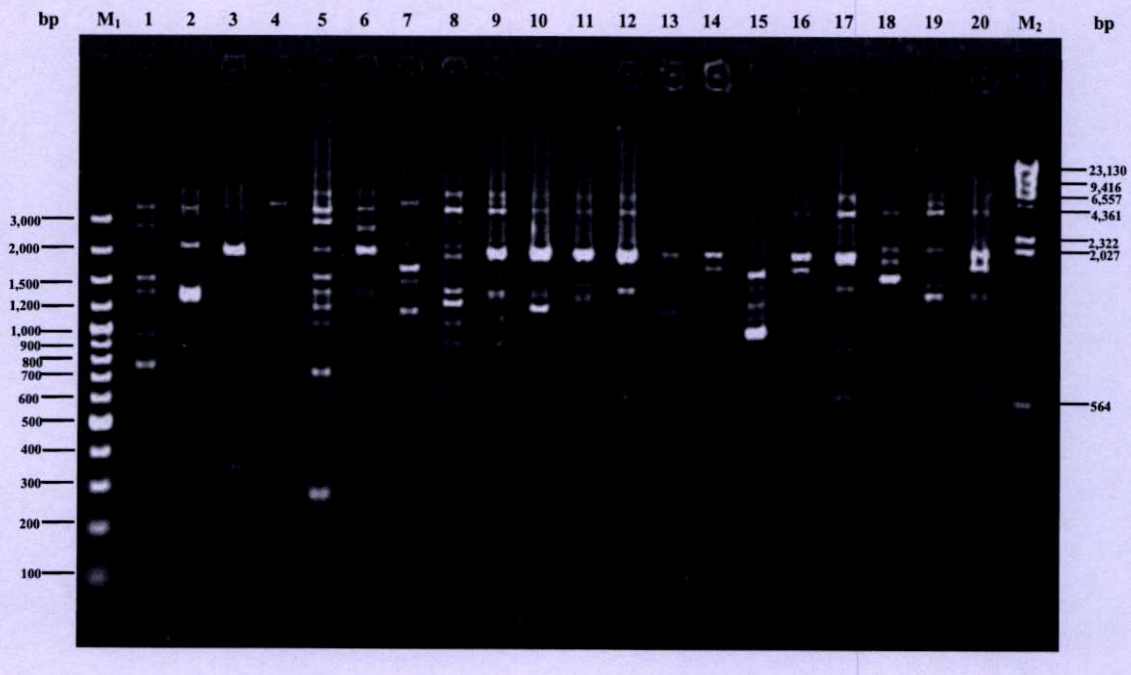


Figure 4.17 RAPD patterns of *Geitlerinema* isolates amplified by primer OPA19. Lane M1: 100 bp DNA ladder; M2: λ*Hin*dIII fragments; 1: KM-SH3; 2: PI-S1.1; 3: NM-SH1; 4: N-ST2; 5: TL-SH2; 6: PM-SH13; 7: LK-SH2; 8: CHL-SH1; 9: N-ST1; 10: MTN-SH5; 11: S-SH3; 12: NM-SA4; 13: MTN-SH9; 14: SK-ST1.2; 15: JM-SH2; 16: ST-ST1.5.1; 17: SSH-SH12; 18: SR-SH4; 19: RMK-SH10; and 20: SK-ST1.1.

Table 4.5 Similarity coefficient between *Geitlerinema* isolates on RAPD data using OPA03, OPA05, OPA07, OPA10 and OPA19 primers.

	KM-SH3	PI-S1.1	NM-SH1	N-ST2	TL-SH2	PM-SH13	LK-SH2	N-ST1	CHL-SH1	MTN-SH5	S-SH3	NM-SA4	MTN-SH9	SK-ST1.2	JM-SH2	ST-ST1.5.1	SSH-SH12	SR-SH4	RMK-SH10	SK-ST1.1
KM-SH3	1.000																			
PI-S1.1	0.663	1.000																		
NM-SH1	0.759	0.735	1.000																	
N-ST2	0.699	0.771	0.771	1.000																
TL-SH2	0.651	0.675	0.747	0.735	1.000															
PM-SH13	0.542	0.687	0.663	0.651	0.602	1.000														
LK-SH2	0.494	0.614	0.614	0.723	0.578	0.639	1.000													
N-ST1	0.627	0.699	0.675	0.614	0.663	0.578	0.482	1.000												
CHL-SH1	0.639	0.711	0.663	0.723	0.627	0.687	0.590	0.578	1.000											
MTN-SH5	0.687	0.735	0.759	0.795	0.699	0.687	0.687	0.675	0.663	1.000										
S-SH3	0.639	0.735	0.759	0.795	0.723	0.663	0.663	0.699	0.663	0.807	1.000									
NM-SA4	0.711	0.687	0.735	0.771	0.699	0.663	0.590	0.602	0.711	0.807	0.783	1.000								
MTN-SH9	0.663	0.735	0.783	0.819	0.675	0.663	0.687	0.651	0.614	0.904	0.759	0.735	1.000							
SK-ST1.2	0.735	0.759	0.855	0.843	0.699	0.663	0.663	0.675	0.639	0.783	0.759	0.807	0.855	1.000						
JM-SH2	0.663	0.663	0.687	0.747	0.675	0.566	0.735	0.530	0.639	0.663	0.663	0.639	0.711	0.735	1.000					
ST-ST1.5.1	0.687	0.711	0.807	0.795	0.675	0.639	0.639	0.651	0.614	0.807	0.759	0.783	0.855	0.904	0.663	1.000				
SSH-SH12	0.711	0.759	0.807	0.747	0.699	0.735	0.663	0.723	0.735	0.807	0.759	0.759	0.783	0.807	0.663	0.783	1.000			
SR-SH4	0.663	0.759	0.783	0.819	0.675	0.711	0.663	0.651	0.735	0.759	0.783	0.783	0.759	0.831	0.735	0.783	0.831	1.000		
RMK-SH10	0.711	0.687	0.711	0.771	0.699	0.639	0.614	0.651	0.639	0.759	0.759	0.831	0.735	0.783	0.639	0.783	0.759	0.759	1.000	
SK-ST1.1	0.723	0.699	0.795	0.735	0.759	0.675	0.602	0.735	0.651	0.795	0.795	0.795	0.771	0.795	0.651	0.795	0.819	0.843	0.795	1.000

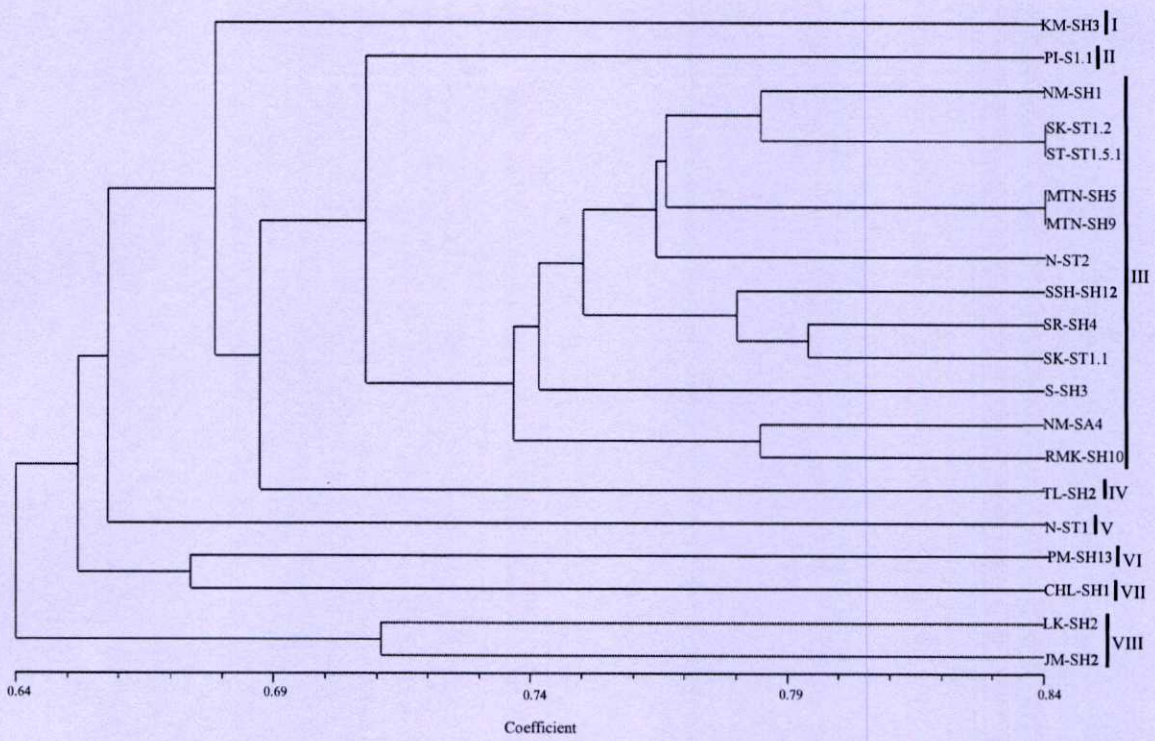


Figure 4.18 Dendrogram showing the relationship of *Geitlerinema* isolates based on UPGMA cluster analysis of the RAPD profiles derived using five primers and Jaccard's similarity coefficient.

Interestingly, *Geitlerinema* sp. LK-SH2 and JM-SH2 were not separated from other strains based on 16S-23S ITS sequence (Fig. 4.8-4.9) and *cpcB-cpcA* IGS sequences (Fig. 4.13-4.14) but could be separated by RAPD. It could be explained that RAPD technique uses one short primer to randomly amplify DNA fragments to generate many PCR bands in a pattern. The polymorphic profiles generated by RAPD analysis suggested a considerable degree of intra-species heterogeneity (Saker *et al.*, 1999). However, previous reports demonstrated that although RAPD patterns of the cyanobacteria *Leptolyngbya valderiana* and *Phormidium retzii* showed genetic diversity, they did not correspond to the distinct geographic area of isolation (Casamatta *et al.*, 2003; Premanandh *et al.*, 2009). In addition, previous study by Bittencourt-Oliveira *et al.* (2007) revealed that the HIP1 fingerprinting technique was a well-powerful tool to study genetic diversity for closely related taxa in *G. amphibium* and *G. unigranulatum*; however, this tool was not related to the geographic location. Thus, a combination of both morphological and molecular data may facilitate the establishment of taxonomic diversity.

4.5 Screening for high H_2-producing cyanobacterial isolates

Cyanobacterial cells were cultivated in ASN III medium for 14 days. Cells were harvested and washed twice with $NaNO_3$-free ASN III medium. Then, cells were resuspended in 100 mL of $NaNO_3$-free ASN III medium and further incubated for 1 day before harvesting cells to determine H_2 production. The harvested cells were resuspended in 5 mL of $NaNO_3$-free medium and transferred to a 10-mL gas-tight vial. The vial was sealed with a rubber stopper. H_2 production was allowed to proceed for 24 h under four conditions; (1) anaerobic/dark condition, (2) microaerobic/light condition, (3) aerobic/dark condition and (4) aerobic/light condition. After 24 h of incubation, 500 µL of gas phase in a vial was withdrawn by a gas-tight syringe and H_2 concentration in gas phase was analyzed by GC-TCD. The results revealed that all cyanobacterial isolates except *Leptolyngbya* sp. PKR-ST1 could produce H_2 under anaerobic/dark condition. Interestingly the cyanobacterium filamentous *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production with 273.701±7.451 and 141.252±11.845 µmolH_2 g^{-1} dry weight under anaerobic/dark and microaerobic/light conditions, respectively (Table 4.6). More than half of the tested isolates produced low H_2 production under aerobic/dark and aerobic/light conditions (Table 4.6). Under aerobic/dark condition, a unicellular cyanobacterium *Cyanothece* sp. P-SH8.2.1 gave the highest H_2 production of 142.422±0.806 µmolH_2 g^{-1} dry weight, whereas under aerobic/light condition very low H_2 production was detected whereby no cyanobacterial isolates showed H_2 production higher than 10 µmolH_2 g^{-1} dry weight (Table 4.6). From this result, *Geitlerinema* sp. RMK-SH10 was chosen for further optimization for H_2 production.

Table 4.6 H_2 production of cyanobacterial strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand

Strains	Origins	Habitats	H_2 production ($\mu molH_2\ g^{-1}$ dry wt.)			
			Anaerobic	Microaerobic	Aerobic	
			darkness	light	darkness	light
Chroococcus sp. SKR-W2.2	Saikeaw, Rayong	seawater	108.679±3.127	11.121± 0.806	59.751±4.290	nd
Cyanothece sp. P-SH8.2.1	Phla, Rayong	shell	171.728±2.291	114.206±7.376	142.422±0.806	5.978±0.326
Geitlerinema sp. CHL-SH1	Changlang, Trang	shell	81.885±4.108	22.873±0.982	nd	nd
Geitlerinema sp. JL-SH1	JaoLao, Chanthaburi	shell	5.908 ±0.112	83.764± 5.069	nd	0.626±0.075
Geitlerinema sp. JL-SA1	JaoLao, Chanthaburi	sand	28.312±1.669	1.679± 0.141	nd	nd
Geitlerinema sp. JM-SH2	Jaomai, Trang	shell	160.957±4.656	46.904±1.283	nd	nd
Geitlerinema sp. KVM-W1	Kungviman, Chanthaburi	seawater	212.098±5.221	2.871±0.178	23.055±1.187	nd
Geitlerinema sp. LK-SH2	Laemkruat, Krabi	shell	150.999±12.776	7.2733±0.726	nd	nd
Geitlerinema sp. LS-W2	Laemsing, Chanthaburi	seawater	30.240±1.541	4.520±0.220	1.658±0.155	0.674±0.050
Geitlerinema sp. LSD-SH2	Laemsadet, Chanthaburi	shell	193.313±1.006	22.087±0.120	94.484± 6.360	6.287±0.076
Geitlerinema sp. MTN-SH5	Modtanoy, Trang	shell	82.610±6.230	1.453±0.018	4.928±0.086	2.514± 0.033
Geitlerinema sp. MTN-SH9	Modtanoy, Trang	shell	114.670±9.476	64.232±2.309	21.366±1.577	1.146±0.064
Geitlerinema sp. N-ST1	Nang, Krabi	stone	83.135±1.037	6.886±0.346	0.888±0.013	1.702±0.133
Geitlerinema sp. N-ST2	Nang, Krabi	stone	109.765±5.980	13.034± 0.836	51.399±3.973	5.106±0.249
Geitlerinema sp. NM-SH1	Nummao, Krabi	shell	181.428±6.218	20.722±1.653	2.734±0.230	2.033±0.179
Geitlerinema sp. NM-SA4	Nummao, Krabi	sand	38.550±3.707	5.581±0.191	nd	nd
Geitlerinema sp. NR-SH2	Nangram, Chonburi	shell	56.624±2.439	24.769±1.596	nd	nd
Geitlerinema sp. P-ST2.3	Phla, Rayong	stone	34.371±2.159	1.552±0.076	nd	nd
Geitlerinema sp. P-W2.1	Phla, Rayong	seawater	74.9137±4.819	9.375±0.565	4.7512± 0.113	0.738±0.028
Geitlerinema sp. PI-S1.1	Phai, Rayong	seawater	34.668±2.840	nd	nd	nd

nd : non detected

Table 4.6 H_2 production of cyanobacterial strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand (continued)

Strains	Origins	Habitats	H_2 production ($\mu molH_2\ g^{-1}$ dry wt.)			
			Anaerobic	Microaerobic	Aerobic	
			darkness	light	darkness	light
Geitlerinema sp. PKR-W3	Pakarang, Rayong	seawater	75.150±3.045	nd	nd	nd
Geitlerinema sp. PM-SH13	Pakarang, Trang	shell	102.968±6.595	0.573±0.025	nd	nd
***Geitlerinema* sp. RMK-SH10**	**Rachmonkol, Trang**	**shell**	**273.701±7.451**	**141.252±11.845**	**1.251±0.050**	**1.408±0.013**
Geitlerinema sp. S-SH3	San, Trang	shell	114.066±1.244	2.015±0.147	nd	nd
Geitlerinema sp. SK-ST1.1	Saikeaw, Chonburi	stone	95.843±3.282	31.183±2.286	25.553±1.133	3.072±0.204
Geitlerinema sp. SK-ST1.2	Saikeaw, Chonburi	stone	119.4534±7.086	7.607±0.373	nd	0.292±0.003
Geitlerinema sp. SK-ST2.1	Saikeaw, Chonburi	stone	69.551±5.604	13.862±0.261	0.223±0.015	1.229±0.013
Geitlerinema sp. SR-SH4	Samran, Trang	shell	98.530±6.263	8.615±0.657	3.270±0.075	1.323±0.064
Geitlerinema sp. SS-ST6	Samaesan, Chonburi	stone	56.501±3.779	10.836±0.195	nd	nd
Geitlerinema sp. SSH-SH12	Susanhoy, Krabi	shell	180.619±6.703	33.308±0.595	7.598±0.388	3.435±0.144
Geitlerinema sp. ST-ST1.5.1	Saitong, Rayong	stone	108.694±1.847	120.970±2.814	1.251±0.050	1.418±0.001
Geitlerinema sp. TG-W2.3.3	Toeingam, Chonburi	seawater	113.827±0.556	0.919±0.076	1.495±0.124	nd
Geitlerinema sp. TL-SH2	Thalane, Krabi	shell	126.327±5.250	32.422±0.753	2.075±0.007	2.033±0.179
Geitlerinema sp. VD-SH2.3	Vongdeuan, Rayong	shell	46.967±1.261	18.585±1.051	0.176±0.005	0.337±0.017
Geitlerinema sp. WI-SA1	Wai, Rayong	sand	5.719±0.114	nd	nd	nd
Geitlerinema sp. WI-SH3	Wai, Rayong	shell	64.227± 4.639	1.620±0.107	nd	nd
Geitlerinema sp. YL-SH4	Yonglin, Trang	shell	68.3565±3.539	1.953± 0.056	nd	nd
Leptolyngbya sp. CHL-SH10	Changlang, Trang	shell	0.761±0.009	nd	nd	nd
Leptolyngbya sp. KK	Koh Kood, Trad	seawater	19.167±0.849	30.497±1.671	1.300± 0.093	nd
Leptolyngbya sp. MP-SA1	Makhampom, Rayong	sand	5.378±0.451	3.849±0.163	nd	nd

nd : non detected

Table 4.6 H_2 production of cyanobacterial strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand (continued)

Strains	Origins	Habitats	H_2 production ($\mu molH_2\ g^{-1}$ dry wt.)			
			Anaerobic	Microaerobic	Aerobic	
			darkness	light	darkness	light
Leptolyngbya sp. NTR-S	Nopparattara, Krabi	seawater	87.613±2.600	8.186±0.523	nd	nd
Leptolyngbya sp. PKR-ST1	Pakarang, Rayong	stone	nd	nd	nd	nd
Leptolyngbya sp. PR-SH1	Prao, Rayong	shell	112.989±8.679	nd	nd	nd
Leptolyngbya sp. PR-SH9	Prao, Rayong	shell	59.473±3.799	nd	nd	nd
Leptolyngbya sp. S-S	San, Trang	seawater	135.013± 5.670	121.332±7.783	0.312±0.029	0.327±0.018
Leptolyngbya sp. SSH-SH5	Susanhoy, Krabi	shell	70.399±2.927	nd	nd	nd
Phormidium sp. KM-ST9	Klongmuang, Krabi	stone	41.230±2.666	5.506±0.202	nd	nd
Phormidium sp. P-SA1.1	Phla, Rayong	sand	241.597± 2.715	103.658±0.188	5.440±0.120	1.087±0.006
Phormidium sp. Y-SH8	Yao, Trang	shell	75.963±2.641	12.803±1.045	13.112±0.751	3.371±0.181
Pseudoanabeana sp. TL-S	Thalane, Krabi	seawater	25.208±0.606	5.146±0.086	nd	nd
Synechococcus sp. JL-W2	JaoLao, Chanthaburi	seawater	5.440±1.045	1.789± 0.141	3.964±0.021	0.170±0.010
Synechococcus sp. MP-W	Makhampom, Rayong	seawater	8.582±0.694	nd	3.455±0.057	nd
Synechococcus sp. VD-W	Vongdeuan, Rayong	seawater	0.258±0.006	nd	nd	nd
Synechocystis sp. LSD-W3	Laemsadet, Chanthaburi	seawater	3.394±0.366	2.849±0.013	nd	nd

nd : non detected

In this study, all purified 54 cyanobacterial isolates were screened for high H_2 production under N-deprivation in four conditions. Most cyanobacterial isolates produced high amount of H_2 under anaerobic/dark condition (Table 4.6). Under N-deprivation, these cyanobacterial isolates could accumulate glycogen in the cells. During anaerobic fermentation glycogen is catabolized via glycolytic pathway to provide sufficient amounts of ATP and NAD(P)H for H_2 production by an activity of bidirectional hydrogenase (Troshina *et al.*, 2002). The primary screening process for H_2 production by cyanobacteria was mostly performed under N-deprivation (Ramana *et al.*, 1990; Allahverdiyeva *et al.*, 2010; He *et al.*, 2012). However, the deprivation of other compositions might also play an important role in cyanobacterial H_2 production (Antal and Lindblad, 2005; Raksajit *et al.*, 2012). Cyanobacteria in this study generally produced less H_2 in the light than in the dark because under light condition cells increase level of O_2 due to the water spitting via PS II. The generated O_2 inhibits the activity of bidirectional hydrogenase and nitrogenase resulting in a decrease in H_2 production (Fay, 1992; Tamagnini *et al.*, 2000).

In the preliminary screening, *Geitlerinema* sp. RMK-SH10 produced the highest H_2 production of 273.701±7.451 $\mu molH_2\ g^{-1}$ dry weight after incubation in $NaNO_3$-free medium under anaerobic/dark for 24 h (Table 4.6). Until now, no H_2 production by marine or freshwater *Geitlerinema* has been investigated. Although the *Geitlerinema* sp. PCC 9228, *Geitlerinema* sp. FC II and marine cyanobacterium *Geitlerinema* sp. BBD 1991 were shown to be capable of N_2 fixation in the culture (Stal and Krumbein, 1981; Den-Uyl *et al.*, 2016; Grim and Dick, 2016; Batchu *et al.*, 2018). They also contained a nitrogen fixation operon (*nifHDK*) and a bidirectional hydrogenase gene cluster (*hoxEFUYH*) but had no uptake hydrogenase genes (*hupSL*) (Den-Uyl *et al.*, 2016; Grim and Dick 2016; Batchu *et al.*, 2018). It need further investigation whether *Geitlerinema* sp. RMK-SH10 had nitrogenase activity.

In this study, *Geitlerinema* sp. RMK-SH10 was cultivated by two stage culture; growth and adaptation stages. In first stage, *Geitlerinema* sp. RMK-SH10 was grown in ASN III medium for accumulation biomass, then in adaptation stage, cells were adapted in N-deprived ASN III medium under the light for accumulation carbohydrate (glycogen). Under dark/anaerobic condition, the hydrogen productions of cells were assayed.

In a quantitative analysis of glycogen content under N-deprivation in *Geitlerinema* sp. RMK-SH10, cells were grown in ASN III medium for 14 days, harvested, washed twice and resuspended in 100 mL of $NaNO_3$-free ASN III medium. Then, cells were incubated for 0, 12, 24, 36 and 48 h under light by shaking before glycogen content determination. The result showed that *Geitlerinema* sp. RMK-SH10 gave the highest glycogen accumulation up to 40% of cell dry weight after 24 h of incubation in $NaNO_3$-free medium under light exposure (Fig. 4.19). Therefore, an adaptation time for $NaNO_3$ deprivation for 24 h under light condition was used for further experiments.

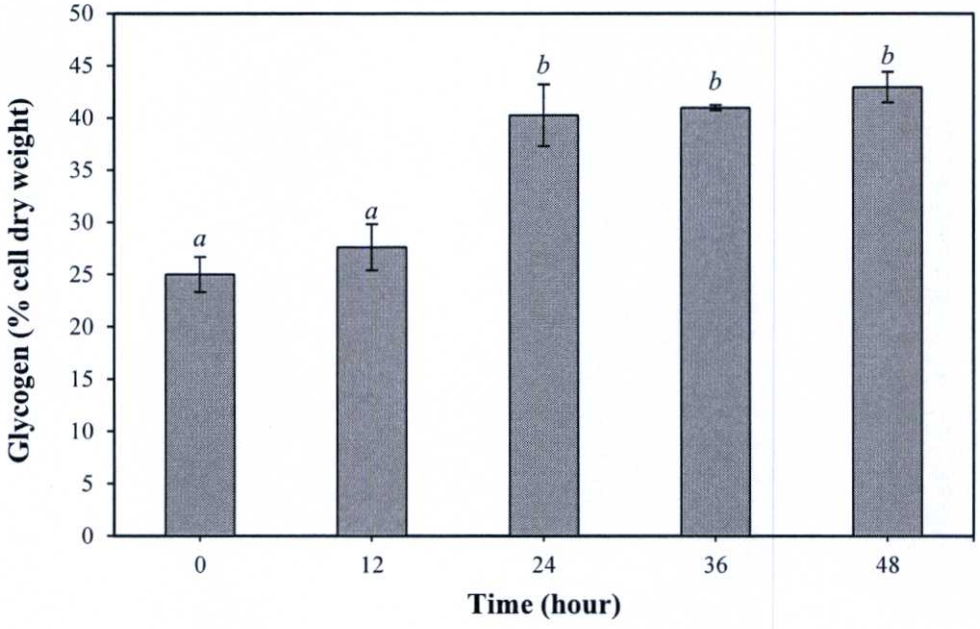


Figure 4.19 Glycogen accumulation of *Geitlerinema* sp. RMK-SH10 under nitrogen deprivation. Data are means±SD (n=3). Different letters on columns indicate the significant difference according to Duncan's multiple range test at $P < 0.05$

In cyanobacteria, glycogen is the main type of polysaccharide accumulated in the cells under unsuitable conditions (Ball and Morell, 2003; Suzuki *et al.*, 2010; Grundel *et al.*, 2012). In glycogen biosynthesis in cyanobacteria, glucose-6-P is converted into glucose-1-P by phosphoglucomutase, followed by the synthesis of ADP-glucose from glucose-1P and ATP by an activity of ADP-glucose pyrophospho rylase (AGP). This reaction generates PPi, which is converted into phosphate by a soluble pyrophosphatase. The glucose moiety of the ADP-glucose is then transferred to the non-reducing end of a linear α-1,4 glucan by glycogen synthase. Finally, glycogen branches are introduced by the branching enzyme, which adds α-1,6 glycosidic bonds (Yoo *et al.*, 2002; Ball and Morell, 2003; Miao *et al.*, 2003; Suzuki *et al.*, 2010; Xu *et al.*, 2013). The increase of glycogen content has been reported in *Synechocystis* sp. PCC 6803 incubating in nitrogen-deprived medium in the light (Díaz-Troya *et al.*, 2014; Monshupanee and Incharoensakdi, 2014; Welkie *et al.*, 2015). From the result, *Geitlerinema* sp. RMK-SH10 could accumulate glycogen up to 40% of cell dry weight after 24 h of incubation in $NaNO_3$-deprived medium under light condition correlated with *Spirulina platensis* NIES-6 that showed glycogen accumulation at 15-50% of cell dry weight after 24-72 h of incubation in nitrogen-deprived medium during photoautotrophic condition (Aoyama *et al.*, 1997) and the unicellular halotolerant cyanobacterium *Aphanothece halophytica* that showed 44% of cell dry weight after 48 h of incubation in seawater without an addition of $NaNO_3$ under light condition (Taikhao *et al.*, 2015).

4.6 Optimization of H_2 production by *Geitlerinema* sp. RMK-SH10

4.6.1 Effect of cultivation time on H_2 production by *Geitlerinema* sp. RMK-SH10

Geitlerinema sp. RMK-SH10 was grown in liquid ASN III for 21 days. The growth of *Geitlerinema* sp. RMK-SH10 by dry cell weight measurement was monitored every 2 days. *Geitlerinema* sp. RMK-SH10 showed a growth pattern of a logarithmic phase, a late logarithmic phase and a stationary phase within 7, 14 and 21 days of cultivation, respectively (Fig. 4.20). H_2 production rate of *Geitlerinema* sp. RMK-SH10 was determined in cells with an age of 7, 14 and 21 days. The result showed that 7-day old *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate of 13.382± 0.600 $\mu molH_2\ g^{-1}$ dry weight h^{-1} (Fig. 4.20). H_2 production rate of 14- and 21-day grown cells was decreased (Fig. 4.20). *Geitlerinema* sp. RMK-SH10 could grow in ASN III medium because ASN III or artificial seawater nutrient III medium contains many nutrients and mineral elements including NaCl required for marine cyanobacterial growth (Rippka *et al.*, 1979). On the other hand, *Geitlerinema* sp. RMK-SH10 was not able to grow well in BG11 medium (data not shown) because BG11 lacks NaCl and trace elements essential for growth of marine cyanobacteria (Rippka *et al.*, 1979).

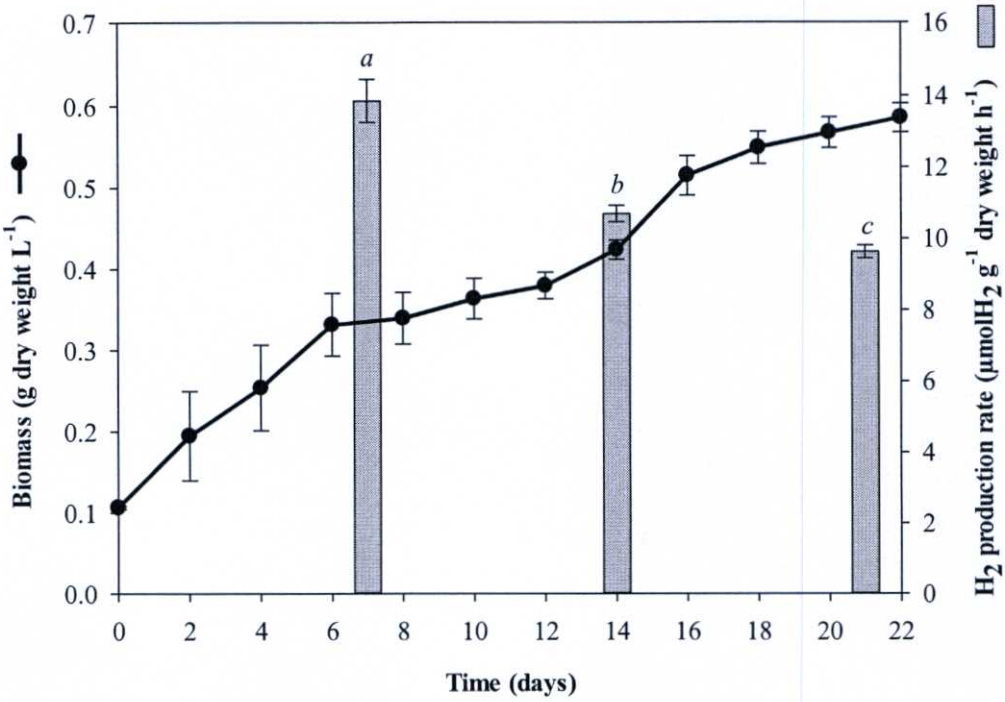


Figure 4.20 Biomass of *Geitlerinema* sp. RMK-SH10 cultivated in ASN III medium for 21 days and dark fermentative H_2 production rate of 7-, 14-, and 21-day grown cells under nitrogen deprived condition. Data are means±SD (n=3). Different letters on columns indicate the significant difference according to Duncan's multiple range test at $P<0.05$

From the result, *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate of 13.382±0.600 µmolH_2 g^{-1} dry weight h^{-1} in 7-day old cells (Fig. 4.20), which its growth was at logarithmic phase. This result is similar with the previous reports found in freshwater unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Baebprasert *et al.*, 2010) and halotolerant unicellular cyanobacterium *A. halophytica* (Taikhao *et al.*, 2013). The growth phase dependent H_2 production has also been shown in a marine filamentous cyanobacterium *Oscillatoria* sp. Miami BG7 (Phlips and Mitsui, 1983) and a marine unicellular cyanobacterium *Synechococcus* sp. Miami 04351 (Luo and Mitsui, 1994) with highest production observed at the beginning of stationary growth phase and at the early logarithmic phase, respectively. Therefore, the amounts of H_2 production depend on species of cyanobacteria and their growth phase. The 7-day old cells of *Geitlerinema* sp. RMK-SH10 were used for further H_2 production optimization.

4.6.2 Effect of anaerobic adaptation time on H_2 production by *Geitlerinema* sp. RMK-SH10

Geitlerinema sp. RMK-SH10 was grown in ASN III medium for 7 days. Cells were harvested, washed twice and resuspended in 100 mL of $NaNO_3$-free ASN III medium and further incubated for 24 h by shaking under the light before harvesting cells. The harvested cells were resuspended in 5 mL of $NaNO_3$-free medium and transferred to a gas-tight vial and purged with argon gas for 5 min. After purging with argon, cells were incubated under dark condition for 0, 12, 24, 36 and 48 h before determination of H_2 production. The result revealed that *Geitlerinema* sp. RMK-SH10 showed the highest H_2 production of 308.930±17.463 $\mu molH_2\ g^{-1}$ dry weight in cells incubated in $NaNO_3$-free ASN III medium after dark anaerobic incubation for 24 h (Fig. 4.21).

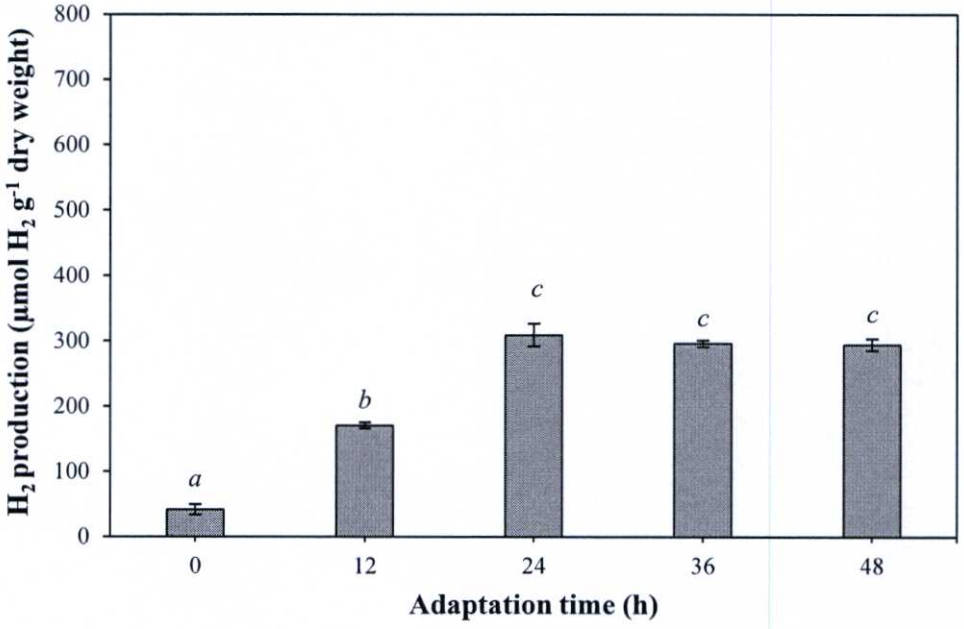


Figure 4.21 Effect of different adaptation time on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

From the result, H_2 production of *Geitlerinema* sp. RMK-SH10 was highest after 24 h of dark anaerobic incubation (Fig. 4.21), indicating that *Geitlerinema* sp. RMK-SH10, like most non-N_2-fixing cyanobacteria, produced H_2 after entering anaerobic phase at a short period of time and suitable for hydrogenase activity. In anaerobic dark condition, NAD(P)H-dependent NiFe hydrogenase evolved H_2 reversibly that led to a fermentation. In anaerobic dark condition, H_2 production by *Arthrospira* (*Spirulina*) *maxima* was shown in two temporary phases; phase 1 from 2-3 minutes to 2-4 h involving in the oxidation of NAD(P)H produced by the prior photosynthetic process, and phase 2 from 8-18 h related to the oxidation of NAD(P)H produced by the dark fermentation (Ananyev *et al.*, 2008). Our result is in agreement with *Lyngbya* sp. BLJ that showed the maximum H_2 concentration and hydrogenase activity in cells incubated for 24 h (Kothari *et al.*, 2012). Therefore, adaptation time under anaerobic/dark condition for 24 h was used for further optimization study on H_2 production by *Geitlerinema* sp. RMK-SH10.

4.6.3 Effect of nutrient and mineral concentrations on H_2 production by *Geitlerinema* sp. RMK-SH10

In this study, effects of concentration of nutrients and minerals such as $NaNO_3$, $MgSO_4.7H_2O$, NaCl, Fe^{3+}, Ni^{2+} and carbon sources on H_2 production by *Geitlerinema* sp. RMK-SH10 were investigated.

4.6.3.1 Effect of $NaNO_3$ on H_2 production

Cells were cultivated in ASN III medium for 7 days. Cells were harvested, washed twice and resuspended with ASN III medium containing 0, 0.088, 0.88, 8.8 and 88 mM $NaNO_3$. The culture was further shaken for 24 h in the light before harvesting cells. The harvested cells were resuspended in 5 mL of medium containing various concentrations of $NaNO_3$ (0-88 mM), transferred to a gas-tight vial and purged with argon gas for 5 min. Cells were then incubated under dark/anaerobic condition for 24 h before analyzing H_2 production. The result showed that H_2 production by *Geitlerinema* sp. RMK-SH10 was highest with 318.237±23.405 $\mu molH_2\ g^{-1}$ dry weight in cells incubated in $NaNO_3$-deprived medium, an approximately ten-fold increase compared to that of cells incubated in the normal ASN III medium containing 8.8 mM $NaNO_3$ (Fig. 4.22). The lower H_2 production was

observed in the higher concentration of $NaNO_3$ (0.088-88 mM) (Fig. 4.22). Therefore, all experiments for measurement of H_2 production in this study were performed under N-deprivation.

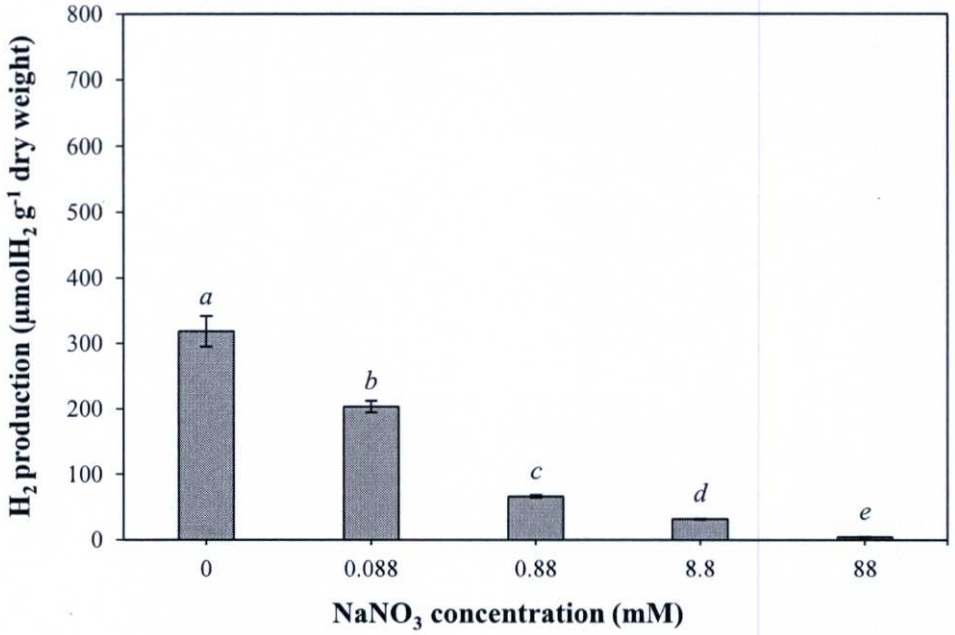


Figure 4.22 Effect of $NaNO_3$ concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

The nutrients and microelements play an important role in H_2 production by cyanobacteria (Datta *et al.*, 2000; Carrieri *et al.*, 2008). H_2 production by *Geitlerinema* sp. RMK-SH10 was highest under N-deprivation. It could be explained that under N-deprivation, H_2 production was increased due to the decrease of photosynthetic capacity, light harvesting chlorophyll complex (LHC) activity, RuBisCO activity and CO_2 fixation and oxygen evolving complex (OCE) activity. These resulted in a reduction of light absorption, quantum yield of PSII and photorespiration, and finally resulting in a decrease of O_2 production. When O_2, an inhibitor of hydrogenase, was decreased, hydrogenase activity was induced, leading to higher H_2 production.

In addition, in cyanobacteria, glycogen is accumulated within cells under N-deprivation (Ball and Morell, 2003; Suzuki *et al.*, 2010; Grundel *et al.*, 2012). During anaerobic fermentation glycogen is degraded via glycolytic pathway to provide amounts of ATP and NAD(P)H for H_2 production by an activity of bidirectional hydrogenase (Troshina *et al.*, 2002). This result is correlated with previous studies in freshwater cyanobacteria *Gloeocapsa alpicola* (Serebryakova *et al.*, 1998; Troshina *et al.*, 2002), *Arthrospira maxima* (Ananyev *et al.*, 2008), in marine cyanobacteria *Oscillatoria* sp. Miami BG7 (Kumazawa and Mitsui, 1984), *Phomidium valderianum* BDU 20041 (Prabaharan and Subramanian, 1996), *Leptolyngba valderiana* BDU 20041 (Prabaharan *et al.*, 2010) and *A. halophytica* (Taikhao *et al.*, 2013).

4.6.3.2 Effect of $MgSO_4.7H_2O$ on H_2 production

Cells grown in ASN III medium for 7 days were harvested, washed twice and resuspended with $NaNO_3$-free ASN III medium containing 0, 1.4, 14, 28 and 140 mM $MgSO_4.7H_2O$. The culture was further shaken under light for 24 h before harvesting cells. The harvested cells were resuspended in 5 mL of corresponding media. Then, cells were transferred to a gas-tight vial and purged with argon for 5 min, incubated under dark/anaerobic condition for 24 h before analyzing H_2 production. The result revealed that *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production with 387.383±17.378 $\mu molH_2\ g^{-1}$ dry weight when cells were incubated in $NaNO_3$-free ASN III medium containing 1.4 mM $MgSO_4.7H_2O$, whereas at 0 and 14 mM $MgSO_4.7H_2O$ no significant differences of H_2 production were found (Fig. 4.23). The lowest H_2 production was found in cells incubated in $NaNO_3$-free ASN III medium containing 140 mM $MgSO_4.7H_2O$ (Fig. 4.23).

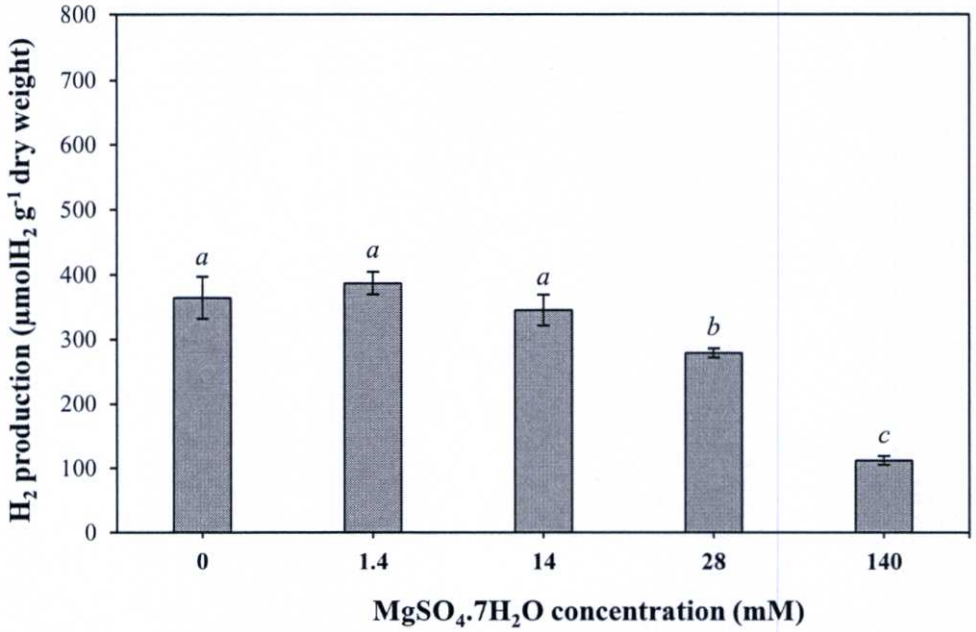


Figure 4.23 Effect of $MgSO_4.7H_2O$ concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

H_2 production by *Geitlerinema* sp. RMK-SH10 under sulfur starvation was not higher than that of cells incubated in 1.4 and 14 mM $MgSO_4.7H_2O$ (Fig. 4.23). On contrary, sulfur deprivation has been found to enhance the rate of H_2 production in other cyanobacterial species such as *Gloeocapsa alpicola*, *Synechocystis* sp. PCC 6803 and *Aphanothece halophytica* (Antal and Lindblad, 2005; Taikhao *et al.*, 2013). Sulfur is a very important component in the photosystem II repair cycle (Wykoff et al., 1998). Lack of sulfur causes an inhibition of oxygenic photosynthesis resulting in a decrease of O_2 and thus leads to an enhancement of H_2 production by *G. alpicola* and *Synechocystis* sp. PCC 6803 (Antal and Lindblad, 2005). Some green algae such as *Chlamydomonas reinhadtii* and *Tetraspora* sp. CU2551 showed an increase of H_2 production when cells were incubated in sulfur-deprived medium such as *Chlamydomonas reinhadtii* and *Tetraspora* sp. CU2551 (Melis *et al.*, 2000; Zhang *et al.*, 2002; Tsygankov *et al.*, 2006, Maneeruttanarungroj *et al.*, 2010).

4.6.3.3 Effect of NaCl on H_2 production

Cells grown in ASN III medium for 7 days were harvested, washed twice and resuspended with $NaNO_3$-free ASN III medium containing 0, 0.2, 0.4, 0.6, 0.8, 1 and 2 M NaCl. The culture was further shaken under light for 24 h before harvesting cells. The harvested cells were resuspended in 5 mL of indicated media. Then, cells were transferred to a gas-tight vial and purged with argon for 5 min, incubated under dark/anaerobic condition for 24 h before analyzing H_2 production. The maximum H_2 production of 434.705±25.337 $\mu molH_2\ g^{-1}$ dry weight was found in cells incubated in N-free ASN III medium containing 0.2 M NaCl, an approximately 1.2-fold increase compared to that of cells incubated in the normal medium containing 0.4 M NaCl (Fig. 4.24). Whereas lack of NaCl and a high NaCl concentration decreased H_2 production by this strain (Fig. 4.24). The lowest H_2 production was found in cells incubated in 2 M NaCl containing $NaNO_3$-free ASN III medium (Fig. 4.24).

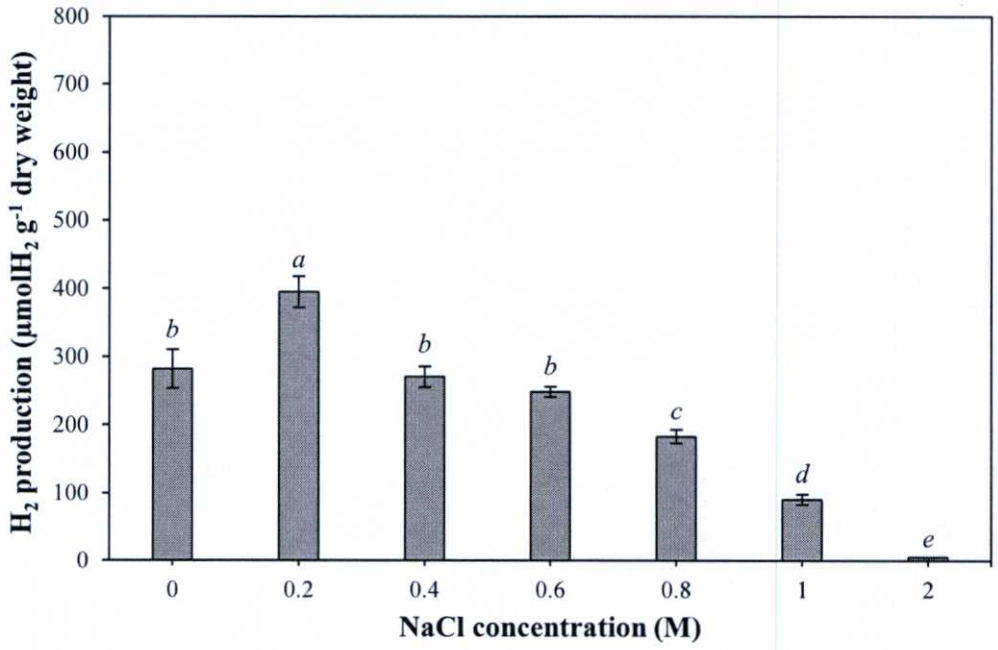


Figure 4.24 Effect of NaCl concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

In this study, it was found that NaCl concentration affected H_2 production of *Geitlerinema* sp. RMK-SH10. The highest H_2 production with 434.705 ± 25.337 $\mu molH_2\ g^{-1}$ dry weight was obtained in cells incubated in $NaNO_3$-free ASN III medium containing 0.2 M or 1.2% (w/v), an approximately 1.2 folds increase compared to that of cells incubated in the $NaNO_3$-free ASN III medium containing 0.4 M NaCl (Fig. 4.24). H_2 production was decreased when NaCl concentration was higher than 0.4 M (Fig. 4.24). Salinity decreased H_2 production in freshwater cyanobacteria *A variabilis* SPU 003 and *N. muscorum* (Shah *et al.*, 2001; Shah *et al.*, 2003). On the contrary, marine cyanobacteria produced highest H_2 at their optimal level of NaCl concentration. A marine cyanobacterium *Lyngbya* sp. strain 108 showed the highest H_2 production in medium containing 3% (w/v) NaCl (Kuwada and Ohta, 1989), a marine non-heterocystous cyanobacterium *Phormidium valderianum* BDU 20041 produced the highest H_2 production when salinity level was up to 2.5% or 0.4 M NaCl (Prabaharam and Subramanian, 1996), *A. halophytica* produced the highest H_2 production rate in 0.75 M NaCl containing medium (Taikhao *et al.*, 2013). Too high NaCl concentration reduces H_2 production in all types of cyanobacteria because they have to combat salinity stress by extrusion of Na^+ ions out of cells or by prevention of Na^+ influx into the cells (Tel-Or and Melhamed-Harel, 1981; Rai and Abraham, 1995).

4.6.3.4 Effect of Fe^{3+} on H_2 production

Geitlerinema sp. RMK-SH10 grown in ASN III medium for 7 days was harvested, washed twice and resuspended in N-free ASN III medium containing 0, 0.02, 0.2, 2, 20 and 200 μM of Fe^{3+}. The culture was further shaken under light for 24 h. The harvested cells were resuspended in indicated media. Then, cells were transferred to a gas-tight vial and purged with argon for 5 min, incubated under dark/anaerobic condition for 24 h before analyzing H_2 production. The result revealed that *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production with 391.501±8.278 $\mu molH_2\ g^{-1}$ dry weight when incubated cells in N-free ASN III medium containing 2 μM Fe^{3+} (Fig. 4.25). The highest Fe^{3+} concentration (200 μM) decreased drastically H_2 production (Fig. 4.25).

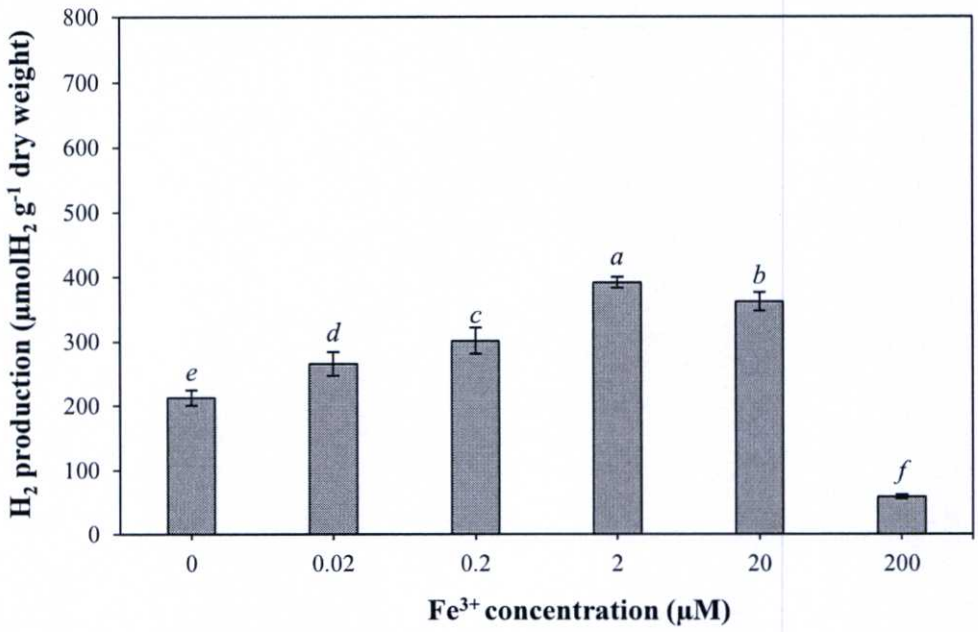


Figure 4.25 Effect of Fe^{3+} concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at *P<0.05*

Under various Fe^{3+} concentrations, the highest H_2 production was found in cells incubated in $NaNO_3$-free ASN III medium containing 2 μM Fe^{3+} (Fig. 4.25). This Fe^{3+} concentration is suitable for cyanobacterial growth. Iron (Fe) plays a significant role in H_2 production by cyanobacteria (Ramchandran and Mitsui, 1984). Iron is a cofactor of NiFe-hydrogenase and it enhances the electron transport process towards hydrogenase (Lin and Stewart, 1997). In addition, iron is also involved in the electron transport system such as photosynthesis and respiration (Raven *et al.*, 1999) and nitrogen fixation (Küpper *et al.*, 2008) in cyanobacterial cells. Previous studies have been reported that an addition of iron led to redirecting of electron flow towards nitrogenase and hydrogenase resulting in an increase in H_2 production in *Anabeana cylindrica*, *A. siamensis* TISTR 8012 and *Synechocystis* sp. PCC 6803 (Jeffries *et al.*, 1978; Baebprasert *et al.*, 2011; Khetkorn *et al.*, 2012) However, too high iron concentration at 200 μM drastically reduced H_2 production (Fig. 4.25), which might be due to the diversion of the energy and reducing power for the extrusion of excess iron out of the cells to alleviate iron toxicity.

4.6.3.5 Effect of Ni^{2+} on H_2 production

Cells grown in ASN III medium for 7 days were harvested, washed twice and resuspended in N-free ASN III medium containing 0, 0.1, 1, 10 and 100 μM of Ni^{2+}. The culture was further shaken under light for 24 h. The harvested cells were resuspended in indicated media. Then, cells were transferred to a gas-tight vial, purged with argon and incubated under dark/anaerobic condition for 24 h before analyzing H_2 production. The highest H_2 production with 359.826±6.836 μmolH_2 g^{-1} dry weight was observed in cell incubated in N-free ASN III medium containing 0.1 μM Ni^{2+} (Fig. 4.26). Higher Ni^{2+} concentration than 0.1 μM decreased H_2 production (Fig. 4.26).

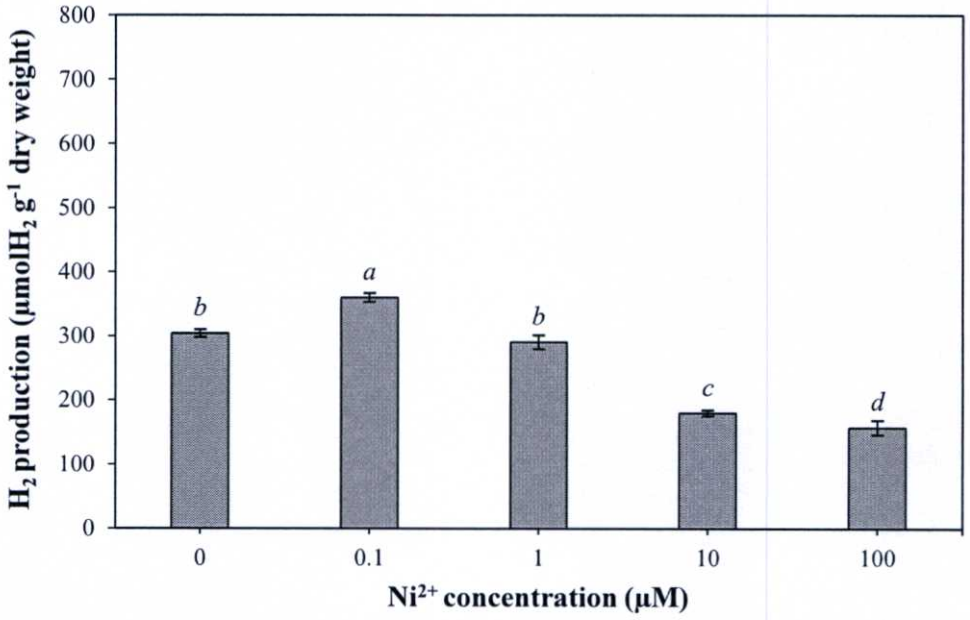


Figure 4.26 Effect of Ni^{2+} concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

The concentration of Ni^{2+} at 0.1 μM is shown to be suitable for H_2 production by *Geitlerinema* sp. RMK-SH10. Nickel also plays a significant role in hydrogenase activity in cyanobacteria because nickel is a metal cofactor of NiFe–hydrogenase. Therefore, nickel is required for hydrogenase activity to produce H_2 (Axelsson and Lindblad, 2002; Gutekunst *et al.*, 2006). In addition, nickel may have other roles in cell function other than hydrogen metabolism (Daday *et al.*, 1985). It has been reported that the addition of external Ni^{+} concentration ranged from 0.5-10 μM was optimal for growth and H_2 production in cyanobacteria (Rai and Raizada, 1986; Serebryakova *et al.*, 1999; Alexlsson and Lindblad, 2002; Gutekunst *et al.*, 2006). The high Ni^{2+} concentration at 100 μM reduced H_2 production in this strain (Fig. 4.26) suggesting that too high concentrations of nickel are toxicity to the cells.

4.6.3.6 Effect of carbon sources on H_2 production

Cells grown in ASN III medium for 7 days were harvested, washed twice and resuspended in N-free ASN III medium containing various carbon sources, Na_2CO_3, $NaHCO_3$, glucose, fructose, sucrose, lactose and maltose with a final concentration of 0.189 mmol C-atom L^{-1}. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested, resuspended in 5 mL of the indicated media and incubated under darkness for further 24 h before analyzing H_2 production. The highest H_2 production of 609.076±41.562 μmolH_2 g^{-1} dry weight was found in cells incubated in $NaNO_3$-free ASN III medium containing glucose as a carbon source, an approximately 1.9-fold increase compared to that of cells incubated in the normal medium containing 0.189 mmol C-atom L^{-1} of Na_2CO_3 (Fig. 4.27).

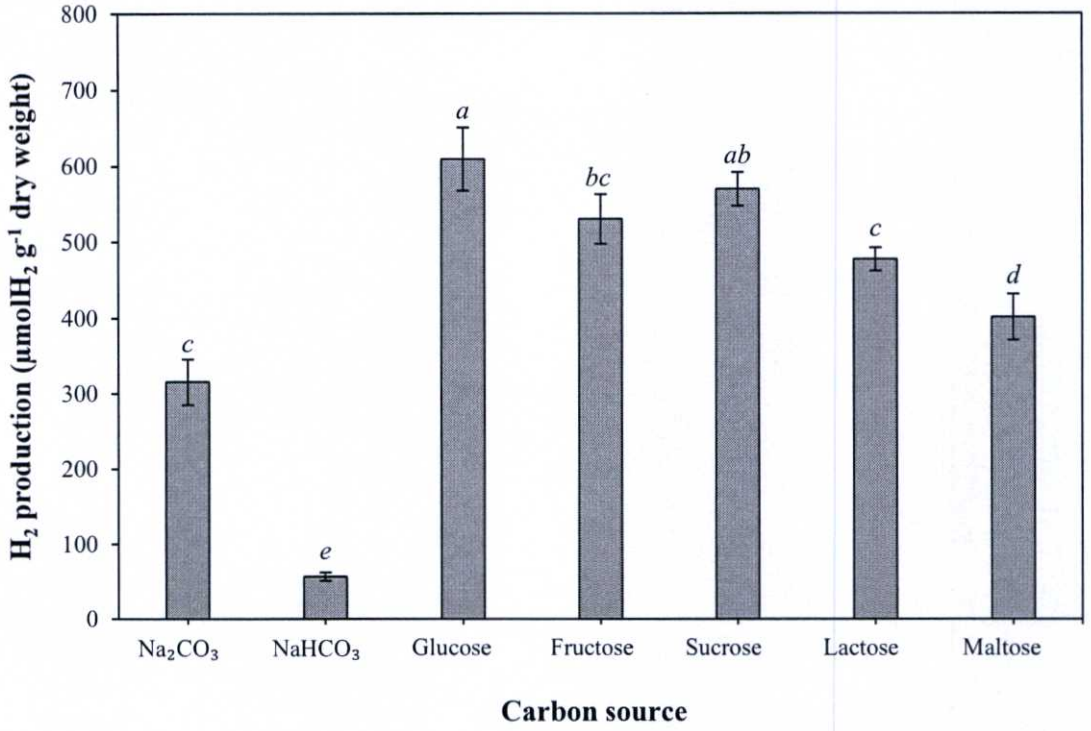


Figure 4.27 Effect of different carbon sources on H_2 production by *Geitlerinema*. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

Cyanobacteria are photoautotroph organisms that can produce their own food through the photosynthesis and CO_2 fixation. The type of carbon source and its concentration in medium shows the great influence on H_2 production by cyanobacteria. Previously studies have been reported that cyanobacteria were able to use different sugars; for example sucrose, fructose, and glucose, as a carbon source (Reddy *et al.*, 1996, Chen *et al.*, 2008, Raksajit *et al.*, 2012). The presence of different carbon sources causes variation in electron donation capabilities by cofactor compounds to nitrogenase and hydrogenase thus influencing H_2 production (Datta *et al.*, 2000; Chen *et al.*, 2008). Among different types of carbon source, glucose seemed to be an optimal carbon source for H_2 production by *Geitlerinema* sp. RMK-SH10.

In *Geitlerinema* sp. RMK-SH10, type of carbon source and its concentration had much greater influence on H_2 production than that by other nutrients and minerals. When glucose catabolism occurs, it leads to an increase of NAD(P)H and ATP which are used for H_2 production aided by nitrogenase or hydrogenase activity (Datta *et al.*, 2000; Chen *et al.*, 2008). The results of the present study are consistent with those of the previous reports that unicellular cyanobacteria *Microcystis aeruginosa* (Rashid *et al.*, 2009), *Synechocystis* sp. PCC 6803 (Baeprasert *et al.*, 2010), *A. halophytica* and a marine cyanobacterium *Synechococcus* sp. Miami BG 043511 used glucose as a carbon source for H_2 production (Luo and Mitsui, 1994; Taikhao *et al.*, 2013). In contrast, some cyanobacteria such as *A. siamensis* TISTR 8012, *Anabeana* sp. CH3, *A. variabilis* and *Nostoc* sp. ARM 411 were able to use fructose as a carbon source for H_2 production (Reddy *et al.*, 1996; Dawar *et al.*, 1999; Chen *et al.*, 2008; Khetkorn *et al.*, 2010).

4.6.3.7 Effect of glucose concentration on H_2 production

From the previously result, the maximum H_2 production was observed in cells incubated in nitrogen-free ASN III medium containing glucose. Therefore, effect of glucose concentration on H_2 production of *Geitlerinema* sp. RMK-SH10 was performed. Cells grown in ASN III medium for 7 days were harvested, washed twice and resuspended in N-free ASN III medium containing various concentrations of glucose from 0, 0.0189, 0.189, 1.89, 18.9 to 189 mmol C-atom L^{-1}. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested, resuspended in 5 mL of indicated media and incubated under darkness for further 24 h before analyzing H_2 production. The result showed that H_2 production was increased with the higher glucose concentration upto 18.9 mmol C-atom L^{-1} (Fig. 4.28). The maximum H_2 production of 1,458.094±34.751 $\mu molH_2\ g^{-1}$ dry weight was observed in cells incubated in $NaNO_3$-free ASN III medium containing 18.9 mmol C-atom L^{-1} glucose (Fig. 4.28).

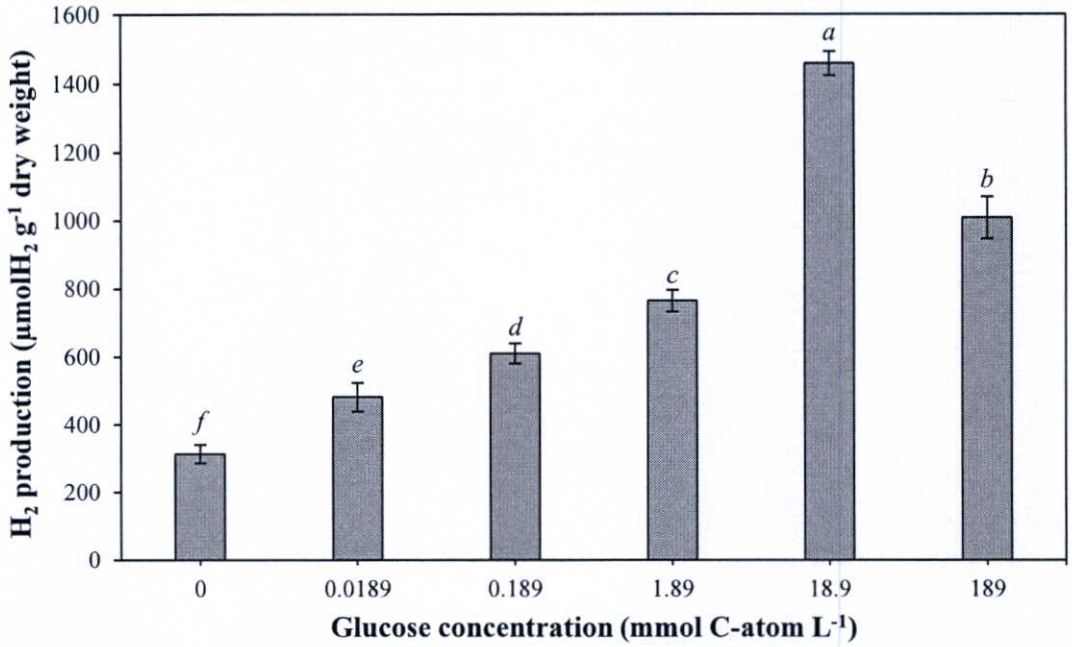


Figure 4.28 Effect of glucose concentration on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at $P<0.05$

4.6.4 Effect of physical parameters on H_2 production by *Geitlerinema* sp. RMK-SH10

In this study, effects of physical parameters such as light intensity, incubation temperature and initial medium pH, on H_2 production by *Geitlerinema* sp. RMK-SH10 were investigated.

4.6.4.1 Effect of light intensity on H_2 production

Geitlerinema sp. RMK-SH10 cultivated in ASN III medium for 7 days was harvested, washed twice and resuspended in $NaNO_3$-free ASN III medium. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested and resuspended in 5 mL of indicated medium. The cultures were purged with argon and incubated under various light intensities from 0, 15, 30, 100 and 150 µmol photons $m^{-2}s^{-1}$ for 24 h before analyzing H_2 production. The result showed that the highest H_2 production with 328.168±27.737 µmolH_2 g^{-1} dry weight was found in cells incubated in $NaNO_3$-free medium under darkness (Fig. 4.29). H_2 production was

decreased in cells incubated in $NaNO_3$-free medium under the higher light intensities (Fig. 4.29).

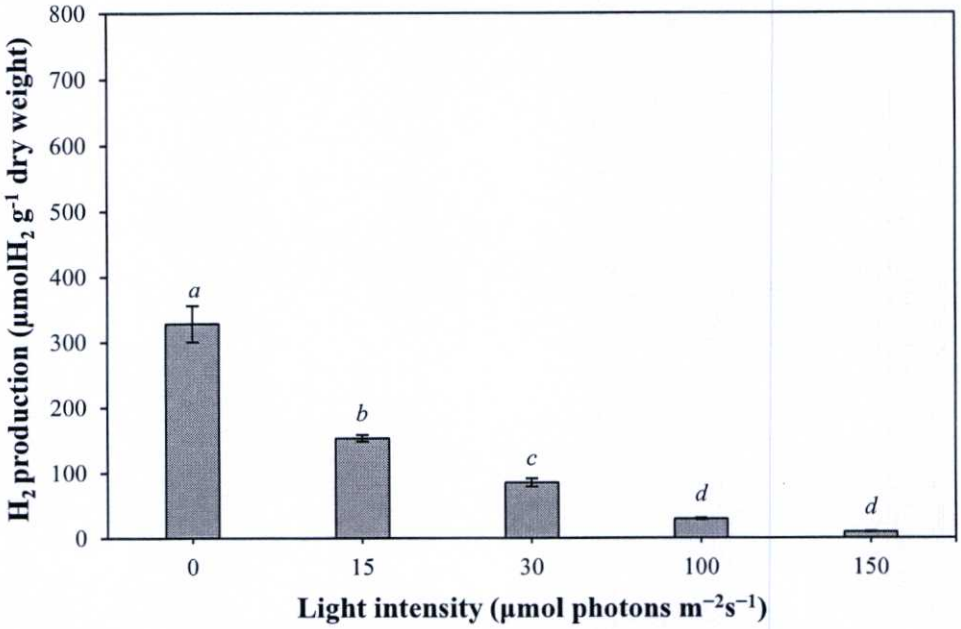


Figure 4.29 Effect of light intensity on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at *P<0.05*

There are several physical parameters such as light intensity, temperature and pH that play a role in H_2 production by cyanobacteria. Under various light intensities, the maximum H_2 production was found in cells grown under darkness (Fig. 4.29). This result indicates that under dark condition, O_2 was less produced than under light condition. A decrease of concentration of O_2, known as hydrogenase or nitrogenase inhibitor, leads to the reduction of H_2 production (Fay, 1992; Tamagnini *et al.*, 2000). This result is in agreement with the previous study in *Synechoccocus* PCC 942 and *Nostoc muscorum* that they produced H_2 only in the dark (Asada and Miyake 1999; Shah *et al.*, 2003). In marine cyanobacterium *Plectonema terebrans* BDU30343, it produced H_2 only in the dark, whereas *P. terebrans* BDU14131 produced H_2 only upon illumination (Prabaharan *et al.*, 2010) and *A. halophytica* provided the highest H_2 production rate in the darkness (Taikhao *et al.*, 2013; Taikhao *et al.*, 2015).

4.6.4.2 Effect of temperature on H_2 production

Geitlerinema sp. RMK-SH10 cultivated in ASN III medium for 7 days was harvested, washed twice and resuspended in $NaNO_3$-free ASN III medium. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested and resuspended in 5 mL of indicated medium. The cultures were purged with argon and incubated under darkness at the temperature from 20-70 °C for 24 h before analyzing H_2 production. The result showed that the highest H_2 production of 408.923±22.052 $\mu molH_2\ g^{-1}$ dry weight was found in cells incubated in $NaNO_3$-free ASN III medium under darkness at 40 °C (Fig. 4.30). It was significantly higher than that by cells incubated at other incubation temperatures. At lower or higher incubation temperature than 40 °C, cells produced less H_2 production (Fig. 4.30).

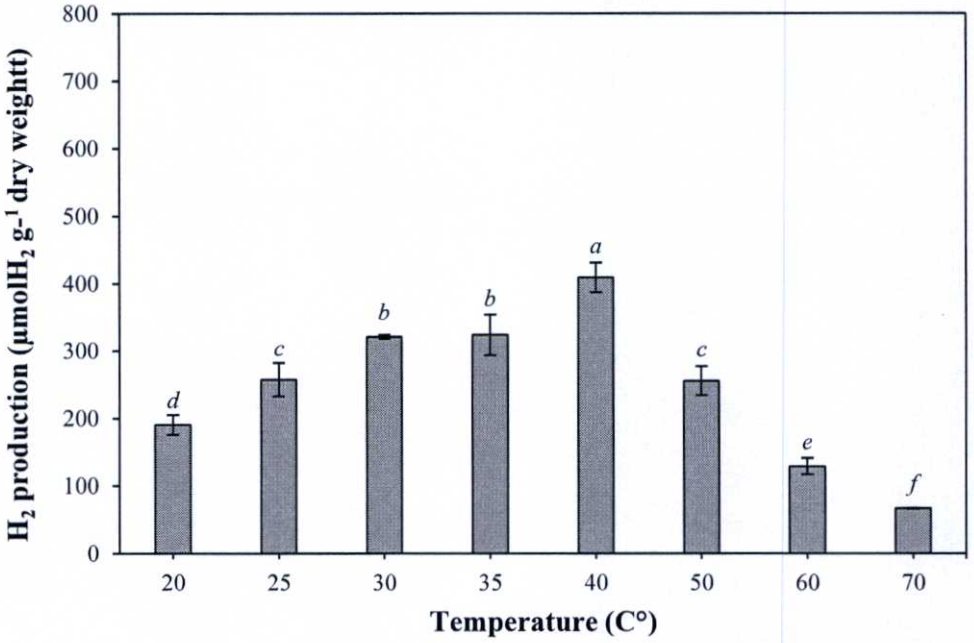


Figure 4.30 Effect of temperature on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan's multiple range test at *P<0.05*

Geitlerinema sp. RMK-SH10 gave the maximum H_2 production when incubated under N-deprivation at 40 °C (Fig. 4.30), indicating that this temperature was optimal for hydrogenase activity, thus resulting in the highest H_2 production yield. Normally the optimal temperature for H_2 production in cyanobacteria is between 30-40 °C varying in cyanobacterial species (Dutta *et al.*, 2005; Tiwari and Pandey, 2012); for example, *N. muscorum* SPU004 produced the highest H_2 at 40 °C (Datta *et al.*, 2000) and *A. variabilis* SPU003 produced the highest H_2 at 40 °C (Serebryakova *et al.*, 2000) whereas unicellular cyanobacterium *Synechocystis* produced the maximum H_2 at 50-70 °C (Baebprasert *et al.*, 2010). In marine cyanobacterium, *P. valderibilis* BDU 20041 the maximum H_2 production rate was observed at 27 °C (Prabaharan and Subramanian, 1996), in *Calothrix* 336/3, the highest H_2 production was observed at 30°C (Allahverdiyeva *et al.*, 2010) and *A. halophytica* was found at 35 °C (Taikhao *et al.*, 2013; Taikhao *et al.*, 2015).

4.6.4.3 Effect of pH on H_2 production

Geitlerinema sp. RMK-SH10 was cultivated in ASN III medium for 7 days, harvested, washed twice and resuspended in the $NaNO_3$-free medium. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested and resuspended in 5 mL of $NaNO_3$-free ASN III medium in which the pH was adjusted with universal buffer ranging from 4-10. The culture was purged with argon, and incubated under darkness/anaerobic condition in the previously medium for 24 h before analyzing H_2 production. The maximum H_2 production of 372.525±3.556 $\mu molH_2\ g^{-1}$ dry weight was found significantly higher than that by cell grown $NaNO_3$-free medium in darkness at the pH 8 (Fig. 4.31). H_2 production in cells grown at the pH at 7, 7.5 and 9 were subordinated significant (Fig. 4.31). At acidic pH 4, 5, 6 and the high pH 10, *Geitlerinema* sp. RMK-SH10 produced the lowest H_2 production (Fig. 4.31).

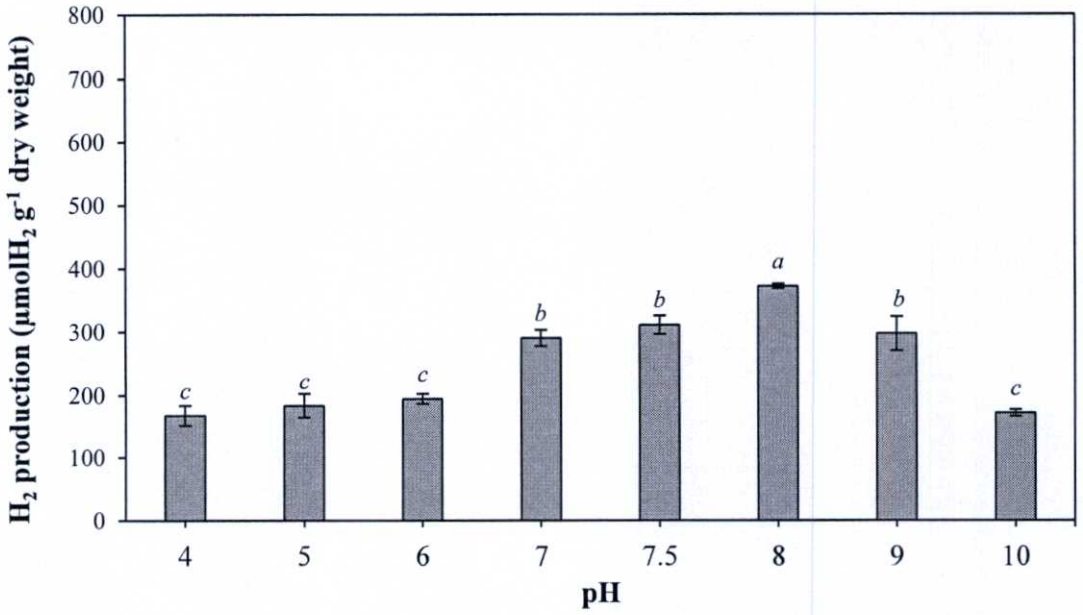


Figure 4.31 Effect of pH on H_2 production by *Geitlerinema* sp. RMK-SH10. Data are means±SD (n=3). Different letters on columns indicate the significant difference, and the same letter indicates no significant difference according to Duncan'smultiple range test at $P<0.05$

The H_2 production was highest in cells grown in $NaNO_3$-free medium at pH 8 and cells were produce less H_2 when cultivated in $NaNO_3$-free medium with pH 4, 5, 6 and the high pH 10 (Fig. 4.31). This result indicating that the optimal pH for H_2 production and hydroginase activity in *Geitlerinema* sp. RMK-SH10 was 8, the optimal pH leads to the maximum electron flow toward hydrogenase, resulting in the maximum H_2 production (Baebprasert *et al.*, 2010). Lower pH than 7.0 and higher than pH 9.0 led to the reduction of hydrogenase activity. This result correlated with previously reported in *Spirulina platensis*, it was produced H_2 decreasing at the lower pH due to inhibition of hydrogenase activity by acidic products within cells (Aoyama et al., 1997). Normally, the optimal pH for H_2 production of cyanobacteria is between 5-9 depends on species and growth condition (Antal and Linbld, 2005). In unicellular *Synechocystis* sp. PCC 6803 showed the increasing of hydrogenase activity when increasing pH ranging from 6.5 to 7.5 (Baebprasert *et al.*, 2010) whereas the optimal pH for H_2 production under sulfur deprivation was 5.0-5.5 (Antal and Lindblad, 2005). In *G. alpicola* gave the maximum H_2 production rate when cells incubated in sulfur deprived medium at pH 6.0 to 7.5 (Antal and Lindblad, 2005) whereas the optimal

pH for H_2 production under nitrate limitation and dark anoxic condition was 6.8-8.3 (Troshina *et al.*, 2002). In marine cyanobacteria, *Laminaria japonica*, the highest H_2 evolution was detected at the pH 7.5 (Park *et al.*, 2009), *Calothrix* XPORK5 provided the high H_2 production rate when cells incubated under nitrate-free medium at the pH 7.5 (Allahverdiyeva *et al.*, 2012) and *A. halophytica* was at pH 6 (Taikhao *et al.*, 2013; Taikhao *et al.*, 2013).

4.7 Effect of combined factors on H_2 production and hydrogenase activity of *Geitlerinema* sp. RMK-SH10

In order to maximize dark fermentative H_2 production, effects of combined factors on H_2 production and hydrogenase activity of *Geitlerinema* sp. RMK-SH10 incubated in various media containing different nutrients and minerals were investigated. Cells grown in ASN III medium for 7 days were then transferred into different types of N-free ASN III and ASN III media. Cells were further incubated for another 24 h before H_2 production analysis under dark anaerobic condition. The maximum H_2 production rate was determined at 4 h of incubation time whereas the maximum H_2 yield was measured after incubation for 24 h. The in *vivo* hydrogenase activity was determined from 4-h incubated cells by measuring H_2 produced in the first 15 min with the presence of dithionite-reduced methyl viologen. The results revealed that *Geitlerinema* sp. RMK-SH10 provided the highest H_2 production rate with 271.093±13.074 $\mu molH_2\ g^{-1}$ dry weight h^{-1} or 0.271±0.013 $\mu molH_2\ mg^{-1}$ dry weight h^{-1} and the highest hydrogenase activity of 0.389±0.012 $\mu molH_2\ mg^{-1}$ dry weight min^{-1} s when cells were incubated in $NaNO_3$-free ASN III medium containing 0.2 M NaCl, 18.9 mmol C-atom glucose L^{-1} and 0.1 μM Ni^{2+} for 4 h (Table 4.7). It should be noted that the maximum production of H_2 at 2,083.406±107.497 $\mu molH_2\ g^{-1}$ dry weight or 2.083±0.107 $\mu molH_2$ mg dry weight was obtained by cells incubated in the same medium for 24 h (Table 4.7). This H_2 production was approximately 70 and 6 folds higher than that of cells incubated in ASN III and $NaNO_3$-free ASN III media, respectively (Table 4.7). H_2 production by cells in each type of media corresponded well with hydrogenase activity. The result confirmed that the absence of nitrogen source and the presence of optimal concentrations of NaCl, glucose and Ni^{2+} in media promoted hydrogenase activity and H_2 production.

Table 4.7 Maximum H_2 production, H_2 production rate and bidirectional hydrogenase activity of *Geitlerinema* sp. RMK-SH10 in various types of media. Data are means±SD (n=3).

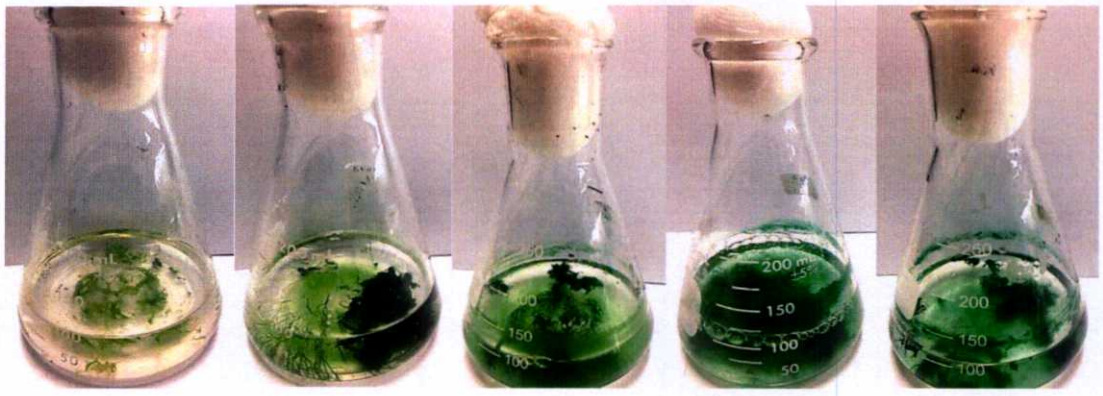
Type of media	Maximum H_2 production ($\mu molH_2\ g^{-1}$ dry weight)	Maximun H_2 production rate ($\mu molH_2\ g^{-1}$ dry weight h^{-1})	H_2ase activity ($\mu molH_2\ mg^{-1}$ dry weight min^{-1})
ASN III	30.647±0.453	13.819±0.574	0.057±0.002
N-free ASN III + 0.2 M NaCl	424.504±61.516	117.578±8.752	0.121±0.004
N-free ASN III + 0.2 M NaCl + 18.9 mmol C-atom glucose L^{-1}	1,802.118±122.779	237.717±8.317	0.345±0.004
N-free ASN III + 0.2 M NaCl + 18.9 mmol C-atom glucose L^{-1} + 0.1 μM Ni^{2+}	2,083.406±107.497	271.093±13.074	0.389±0.012
N-free ASN III + 0.4 M NaCl	353.705±7.693	69.266±2.222	0.097±0.002
N-free ASN III + 0.4 M NaCl + 18.9 mmol C-atom glucose L^{-1}	1,410.687±19.583	119.563±4.428	0.149±0.024
N-free ASN III + 0.4 M NaCl + 18.9 mmol C-atom glucose L^{-1} + 0.1 μM Ni^{2+}	1,536.721±5.138	160.409±7.836	0.252±0.010

4.8 Cultivation of *Geitlerinema* sp. RMK-SH10 in seawater for H_2 production

Geitlerinema sp. RMK-SH10 isolated from the Andaman Sea in Thailand showed the high potential for H_2 production under optimized conditions. In this section, the effort of cultivation of *Geitlerinema* sp. RMK-SH10 in natural seawater was attempted in order to reduce the cost of the medium for growth and H_2 production.

4.8.1 Growth of *Geitlerinema* sp. RMK-SH10 in natural seawater

The seawater used in this study was collected from Namsai beach, Chonburi province, in the Gulf of Thailand. NaCl content in seawater was determined by using salinity refractometer. It was found that the collected seawater contained approximately 3% (w/v) NaCl. Before use, seawater was filtered through a filter paper No. 1 (55 mm diameter) (Whatman, UK) using a suction pump. The pH in seawater was adjusted to pH 7.5 with 2 M NaOH before sterilization by autoclaving. *Geitlerinema* sp. RMK-SH10 was cultivated in seawater, seawater containing 9 mM $NaNO_3$ and seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution. Cells were cultivated by shaking in the light for 21 days. The growth of cyanobacteria by dry cell weight measurement was monitored every 2 days. In addition, growth of cells grown in seawater was compared with that of cells grown in normal ASN III medium and BG11 medium supplemented with Turk Island salt solution. The result showed that growth of *Geitlerinema* sp. RMK-SH10 grown in seawater was obviously lower than those of cells grown in seawater containing 9 mM $NaNO_3$, seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution, ASN III medium and BG11 medium supplemented with Turk Island salt solution (Fig. 4.32 and Fig. 4.33), Interestingly, *Geitlerinema* sp. RMK-SH10 could grow in seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution (Fig. 4.33). Its growth did not show any differences with that of cells cultivated in enriched ASN III and BG11 supplemented with Turk Island salt solution (Fig. 4.33).



(A) (B) (C) (D) (E)

Figure 4.32 Ten-day old cell cultures of *Geitlerinema* sp. RMK-SH10 in seawater (A), seawater containing 9 mM $NaNO_3$ (B), seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution (C), ASN III medium (D) and BG11 medium supplemented with Turk Island salt solution (E).

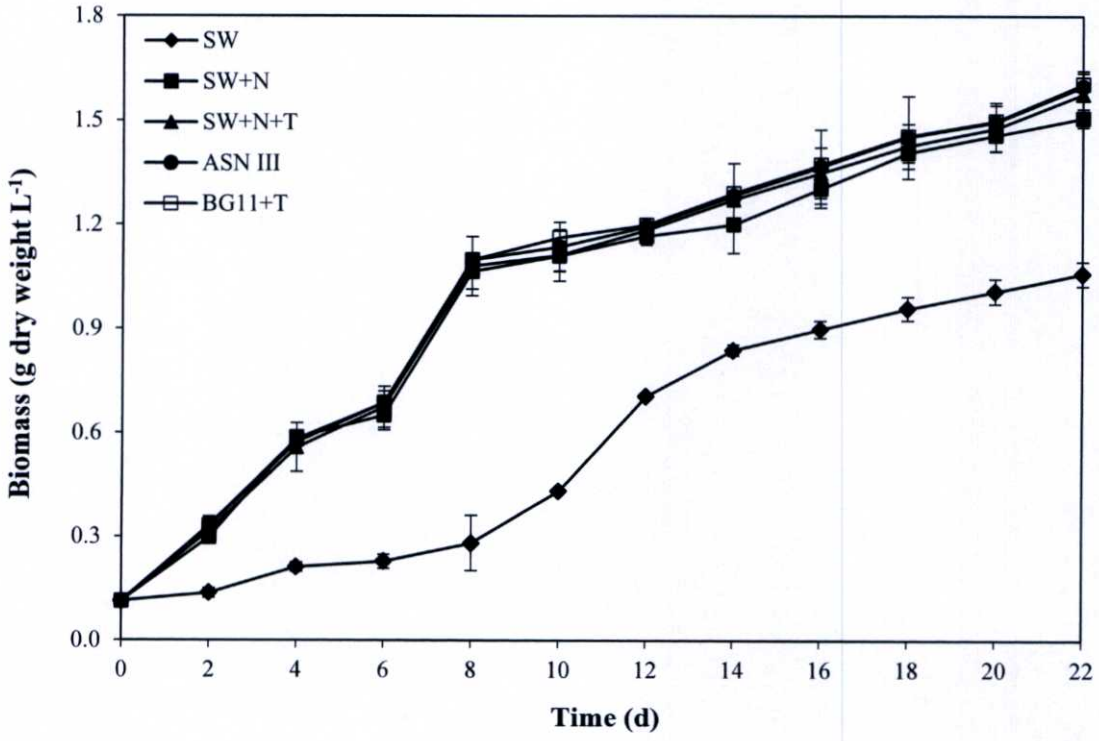


Figure 4.33 Biomass of *Geitlerinema* sp. RMK-SH10 in seawater, seawater containing 9 mM $NaNO_3$, seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution, ASN III medium and BG11 medium supplemented with Turk Island salt solution for 21 days.

In general, natural seawater is about 97.2% of the total water surface of the world. It is composed of 96.5% water and 3.5% mineral salts (Garrison, 2007), which is composed of more than 50 known elements and a large number of organic compounds (Harrison and Berges, 2005). The feasibility study of utilization natural seawater as a culture medium for growth in marine cyanobacteria has been attempted for a long time. However, their growth and biomass are quite low compared with cultivation in enrichment media. Therefore, cultivation of cyanobacteria in seawater is more difficult for growth and active compound production. *Geitlerinema* sp. RMK-SH10 showed a slow growth in natural seawater compared to that grown in the enriched ASN III medium and BG11 medium supplemented with Turk Island salt solution (Fig. 4.33), indicating that there are insufficient nutrients in seawater for growth of *Geitlerinema* sp. RMK-SH10. In order to increase a growth in seawater, nitrogen source should be added into the medium. From the result, *Geitlerinema* sp. RMK-SH10 could grow in seawater supplemented with $NaNO_3$, indicating that *Geitlerinema* sp. RMK-SH10 need nitrogen for its growth. ASN III medium and BG11 medium contain a sufficient amount of $NaNO_3$ (Rippka *et al.*, 1979) whereas seawater contains low inorganic and organic nitrogen sources (less than 0.007 mg L^{-1} NH_4-N and less than 0.002 mg L^{-1} NOx-N) (Ahernetal, 2007 Therefore, for H_2 production experiment, *Geitlerinema* sp. RMK-SH10 was cultivated in seawater containing 9 mM $NaNO_3$.

4.8.2 H_2 production of *Geitlerinema* sp. RMK-SH10 in natural seawater

Geitlerinema sp. RMK-SH10 grown in seawater containing 9 mM $NaNO_3$ for 7 days were harvested, washed twice with seawater and resuspended in seawater, seawater containing 9 mM $NaNO_3$, seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution, seawater containing 18.9 mmol C-atom glucose L^{-1}, seawater containing 9 mM $NaNO_3$ plus 18.9 mmol C-atom glucose L^{-1}, seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution plus 18.9 mmol C-atom glucose L^{-1}. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested and resuspended in 5 mL of corresponding media. Cells were further incubated for 24 h before H_2 production analysis under dark anaerobic condition. The maximum H_2 production rate was determined at 4 h of incubation time whereas the maximum H_2 yield was measured after incubation for 24 h; except

for cells grown in seawater containing 9 mM $NaNO_3$, seawater containing 9 mM $NaNO_3$ supplemented with Turk Island salt solution conditions whose the maximum H_2 yield was found at 4 h of incubation. The in *vivo* hydrogenase activity was determined from 4-h incubated cells by measuring H_2 produced in the first 15 min in the presence of dithionite-reduced methyl viologen. The result showed that *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate of 160.889±4.440 $\mu molH_2\ g^{-1}$ dry weight h^{-1} or 0.161±0.004 $\mu molH_2\ mg^{-1}$ dry weight h^{-1} and the highest hydrogenase activity of 0.178±0.004 $\mu molH_2\ mg^{-1}$ dry weight min^{-1} in cells incubated in seawater containing 18.9 mmol C-atom glucose L^{-1} (Table 4.8). The maximum production of H_2 at 1,724.098±47.976 $\mu molH_2\ g^{-1}$ dry weight or 1.724±0.048 $\mu molH_2$ mg dry weight was obtained in cells incubated in the same medium for 24 h (Table 4.8).

From this result, the cyanobacterium filamentous *Geitlerinema* sp. RMK-SH10 was able to grow in seawater supplemented with 9 mM $NaNO_3$ and it produced the highest H_2 production when incubated cells in seawater supplemented with 18.9 mmol C-atom glucose L^{-1} (Table 4.8). The maximum H_2ase activity as well as H_2 production were observed in cells incubated in seawater supplemented with 18.9 mmol C-atom glucose L^{-1} (Table 4.8). However, H_2ase activity in this study was not correlated to H_2 production of cells incubated in seawater and seawater containing 18.9 mmol C-atom glucose L^{-1} (Table 4.8). It might be suggested that H_2 production was aided by nitrogenase activity. *Geitlerinema* has been reported that it contained a nitrogen fixation operon (*nifHDK*) (Den-Uyl *et al.*, 2016; Grim and Dick 2016; Batchu *et al.*, 2018).

Table 4.8 H_2 production, H_2 production rate and bidirectional hydrogenase activity of *Geitlerinema* sp. RMK-SH10 grown in seawater. Data are means±SD (n=3).

Type of media	Maximum H_2 production ($\mu molH_2\ g^{-1}$ dry weight)	Maximun H_2 production rate ($\mu molH_2\ g^{-1}$ dry weight h^{-1})	H_2ase activity ($\mu molH_2\ mg^{-1}$ dry weight min^{-1})
Seawater	1,091.986±153.922	118.986±7.445	0.095±0.002
Seawater+ 9 mM $NaNO_3$	60.646±10.768	15.162±2.692	0.046±0.003
Seawater + 9 mM $NaNO_3$+Turk Island salt solution	47.905±2.325	11.976±0.581	0.044±0.004
Seawater + 18.9 mmol C-atom glucose L^{-1}	1,724.098±47.976	160.889±4.440	0.178±0.004
Seawater + 9 mM $NaNO_3$+ 18.9 mmol C-atom glucose L^{-1}	590.846±32.278	102.961±5.260	0.144±0.006
Seawater + 9 mM $NaNO_3$+Turk Island salt solution + 18.9 mmol C-atom glucose L^{-1}	450.395±31.715	98.407±9.312	0.140±0.003
Optimized ASN III medium (N-free ASN III medium + 0.2 M NaCl + 18.9 mmol C-atom glucose L^{-1} + 0.1 µM Ni^{2+})	2,018.227±57.586	269.582±17.882	0.346±0.006

4.9 H_2 accumulation by *Geitlerinema* sp. RMK-SH10 under optimal condition

Geitlerinema sp. RMK-SH10 was grown in ASN III medium and seawater supplemented with 9 mM $NaNO_3$ for 7 days. Cells grown in seawater supplemented with 9 mM $NaNO_3$ were harvested, washed twice and resuspended in various types of seawater media as described in 4.8.2 whereas cells grown in ASN III medium were washed twice and resuspended in N-free ASN III medium containing of 0.2 M NaCl, 18.9 mmol C-atom glucose L^{-1} and 0.1 μM Ni^{2+}. All cultures were cultivated by shaking in the light at 30 °C for 24 h. Then, the cultures were harvested, resuspended in 5 mL of corresponding medium, purged with argon and incubated for 24 h before H_2 production analysis under dark anaerobic condition everyday for 14 days. At the first day, the highest H_2 accumulation was observed in cells incubated in optimized ASN III medium (Fig. 4.34). Interestingly, cells cultivated in seawater supplemented with 18.9 mmol C-atom glucose L^{-1} gave the H_2 production yield of 2,237.631±151.453 μmolH_2 g^{-1} dry weight at day 4 of dark anaerobic incubation whereas cells grown in seawater produced the maximum production yield of 1,336.005±20.870 μmolH_2 g^{-1} dry weight at day 3 under anaerobic incubation (Fig. 4.34). Cells incubated in seawater supplemented with 9 mM $NaNO_3$ provided the highest H_2 production yield at day 4 of dark anaerobic incubation after 1 day, then H_2 production yield was dramatically decreased (Fig. 4.34). *Geitlerinema* sp. RMK-SH10 incubated in seawater supplemented 18.9 mmol C-atom glucose L^{-1} gave the similar H_2 production yield compared with cells grown in optimized ASN III medium (Fig. 4.34).

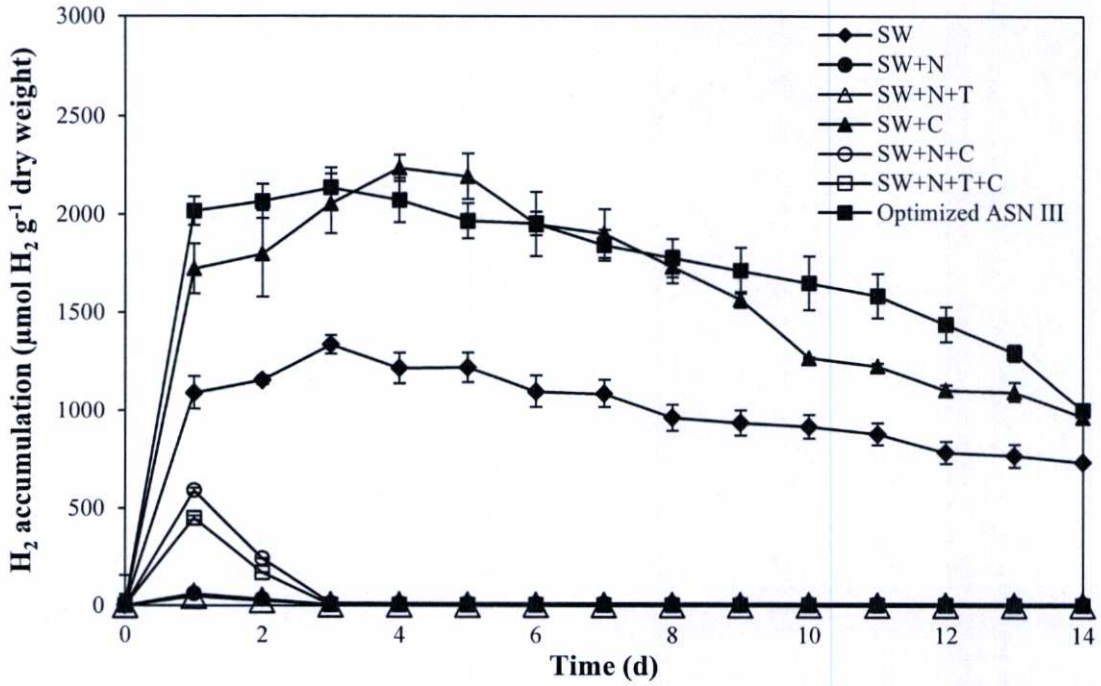


Figure 4.34 H_2 accumulation by *Geitlerinema* sp. RMK-SH10 incubated in various types of media.

For study H_2 accumulation by *Geitlerinema* sp. RMK-SH10, the maximum H_2 accumulation was found in cells incubated in optimized ASN III and in seawater supplemented with 18.9 mmol C-atom glucose L^{-1}. The maximum H_2 production yield of 2,237.631±151.453 µmolH_2 g^{-1} dry weight was shown in cells incubated in seawater supplemented with 18.9 mmol C-atom glucose L^{-1} at day 4 of dark anaerobic incubation, indicating that glucose as carbon source mainly affected H_2 production by this strain. Comparison H_2 production with other marine cyanobacterial strains, H_2 production by this strain is quite high. Table 4.9 shows dark fermentative H_2 production rate and conditions of various marine filamentous cyanobacterial strains in comparison with *Geitlerinema* sp. RMK-SH10 reported in this study. It is apparent that *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate, suggesting the possibility of using this strain for H_2 production.

Table 4.9 H_2 production by *Geitlerinema* sp. RMK-SH10 compared with other marine filamentous cyanobacterial strains

Filamentous cyanobacteria	Maximum H_2 production rate	Growth conditions	H_2 evolution assay conditions	References
Geitlerinema sp. RMK-SH10	0.271 μmolH_2 mg dry weight^{-1} h^{-1} or 6.072 mLH_2 g dry weight^{-1} h^{-1}	ASN III medium, 30 μmol photon m^{-2}s^{-1}, 30 °C	ASN III–N medium + 0.2 M NaCl + 18.9 mmol C-atom L^{-1} glucose + 0.1 μM Ni^{2+}, Ar, dark, 30 °C	This study
Oscillatoria brevis B1567	0.168 μmolH_2 mg dry weight^{-1} h^{-1}	AA medium, 5% CO_2 in air, 7,000 lux, 25 °C	AA medium, 3% CO_2, 4,000 lux, 25 °C	Lambert and Smith (1977)
Calothrix scopulorum 1410/5	0.128 μmolH_2 mg dry weight^{-1} h^{-1}	AA medium, 5% CO_2 in air, 7,000 lux, 25 °C	AA medium, 3% CO_2, 4,000 lux, 25 °C	Lambert and Smith (1977)
Calothrix membrnacea B379	0.108 μmolH_2 mg dry weight^{-1} h^{-1}	AA medium, 5% CO_2 in air, 7,000 lux, 25 °C	AA medium, 3% CO_2, 4,000 lux, 25 °C	Lambert and Smith (1977)
Oscillatoria limosa sp. Miami BG7	0.250 μmolH_2 mg dry weight^{-1} h^{-1}	A medium + 25 mg L^{-1} NH_4Cl, 100 μE m^{-2}s^{-1}, 28 °C	A-N medium, Ar, 90 μE m^{-2}s^{-1}, 37 °C	Phlips and Mitsui (1983)
Phormidium valderianum BDU 20041	0.20 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, pH 7.5, 5.5 μmol photon m^{-2} s^{-1}, 18 h dark~ 6 h light cycle, 27 °C	Prabaharan and Subramanian (1996)
Arthrospira maxima CS-328	13.3 mL H_2 g dry weight^{-1} d^{-1}	Zarrouk medium+1 μM Ni^+, Air, 30 °C	Zarrouk medium + 1 μM Ni^+, Ar, dark, 35 °C	Ananyev *et al.* (2008)
Leptolyngbya valderiana BDU20041	0.02 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, Ar, dark, 27±2 °C	Prabaharan *et al.* (2010)
Lyngbya confervoides BDU142001	0.02 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, N_2, dark, 27±2 °C	Prabaharan *et al.* (2010)
Lyngbya confervoides BDU1420301	0.01 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, Ar, dark, 27±2 °C	Prabaharan *et al.* (2010)
Microcoleus chthnoplasts BDU91212	0.017 μmolH2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, Ar, dark, 27±2 °C	Prabaharan *et al.* (2010)
Plectonema terebrans BDU141311	0.013 μmolH_2 mg dry weight^{-1} h^{-1}	ASN III medium, 13.7 W m^{-2}, 27±2 °C	ASN III–N medium, N_2, dark, 27±2 °C	Prabaharan *et al.* (2010)

Chapter 5

Conclusions

5.1 Isolation of cyanobacteria isolated from the Gulf of Thailand and the Andaman Sea in Thailand

A total of fifty-four cyanobacterial isolates were purified and identified by 16S rDNA sequencing, 35 isolates were classified in genus *Geitlerinema*, nine isolates were classified in genus *Leptolyngbya*, three isolates each were classified in genus *Phormidium* and genus *Synechococcus*, one isolate each classified in genus *Chroococcus*, *Cyanothece*, *Pseudoanabaena* and *Synechocystis*.

5.2 Morphological and genetic characterization by *Geitlerinema* sp. using morphological analysis, 16S rDNA, 16S-23S ITS, *cpcB-cpcA* IGS sequencing and RAPD method

5.2.1 All *Geitlerinema* isolates showed one single morphological type; filamentous, flexuous or straight filament composed of a single trichome, thin trichome, blue-green color, non-constricted cylindrical trichome at cross walls, attenuated trichome toward their ends, one or more granules of cyanophycin, round cones without calyptras at the outer cell wall.

5.2.2 By phylogenetic tree analysis of 16S rDNA, all *Geitlerinema* strains isolated from Thailand were clustered in the "Marine *Geitlerinema*" clade which were genetically similar to each other and closely related to other marine *Geitlerinema* strains, *Geitlerinema* sp. A28DM, *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105.

5.2.3 By phylogenetic tree analysis of 16S-23S ITS, *Geitlerinema* isolates could be divided into four clades; all marine *Geitlerinema* strains isolated from Thailand, *Geitlerinema* sp. Flo1, and *Geitlerinema* sp. PCC7105 were clustered into the same clade. In addition, *Geitlerinema* sp. RMK-SH10 might be genetically diverse from other strains in the subgenus due to the tRNA secondary structure analysis.

5.2.4 By phylogenetic tree analysis of the *cpcB-cpcA* IGS, *Geitlerinema* could be divided into three clades. All *Geitlerinema* isolates from Thailand, *Geitlerinema* sp. Flo1 and *Geitlerinema* sp. PCC7105 were clustered into Clade I whereas other freshwater *Geitlerinema* were clustered into Clade II and III.

5.2.5 The RAPD phylogenetic tree of all marine *Geitlerinema* strains isolated in Thailand based on UPGMA cluster analysis using Jaccard's similarity coefficient exhibited eight clades with an average similarity coefficient of 0.71; however, this classification revealed no correlation with the geographic locations.

5.3 Screening for high H_2-producing marine cyanobacteria isolated from the Gulf of Thailand and the Andaman Sea in Thailand

The filamentous cyanobacterium *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production with 273.701±7.451 and 141.252±11.845 μmolH_2 g^{-1} dry weight under anaerobic/dark and microaerobic/light conditions, respectively. It provided the maximum glycogen accumulation up to 40% of cell dry weight after 24 h of incubation in $NaNO_3$-free medium under light condition.

5.4 Optimization of H_2 production by *Geitlerinema* sp. RMK-SH10

5.4.1 The 7-day old cells or mid-logarithmic phase cells of *Geitlerinema* sp. RMK-SH10 gave the highest H_2 production rate.

5.4.2 *Geitlerinema* sp. RMK-SH10 showed the highest H_2 production in cells incubated in $NaNO_3$-free ASN III medium after incubation under anaerobic/dark condition for 24 h.

5.4.3 The optimal ASN III composition for H_2 production by *Geitlerinema* RMK-SH10 were 0 mM $NaNO_3$, 1.4 mM $MgSO_4.7H_2O$, 0.2 M NaCl, 2 μM Fe^{3+}, 0.1 μM Ni^{2+} and glucose as a carbon source with final concentration of 18.9 mmol C-atom L^{-1}.

5.4.4 The optimal light intensity, temperature and pH for H_2 production in *Geitlerinema* RMK-SH10 were 0 μmol photons m^{-2} s^{-1}, 40 °C and pH 8, respectively.

5.4.5 The highest H_2 production rate with 271.093±13.074 $\mu molH_2\ g^{-1}$ dry weight h^{-1} and the highest hydrogenase activity of 0.389±0.012 $\mu molH_2\ mg^{-1}$ dry weight min^{-1} s were found in cells incubated in $NaNO_3$-free ASN III medium containing 0.2 M NaCl, 18.9 mmol C-atom glucose L^{-1} and 0.1 μM Ni^{2+}.

5.5 Growth and H_2 production of *Geitlerinema* sp. RMK-SH10 in natural seawater

5.5.1 *Geitlerinema* sp. RMK-SH10 could grow in seawater containing 9 mM $NaNO_3$ with no differences from cells cultivated with ASN III medium or BG11 medium supplemented with Turk Island salt solution.

5.5.2 The maximum H_2 production of *Geitlerinema* sp. RMK-SH10 with 1,724.098±47.976 $\mu molH_2\ g^{-1}$ dry weight was shown in cells incubated in seawater containing 18.9 mmol C-atom glucose L^{-1} for 1 day.

5.5.3 H_2 accumulation of *Geitlerinema* sp. RMK-SH10 with 2,237.631±151.453 $\mu molH_2\ g^{-1}$ dry weight was observed after 4 days of dark incubation under anoxic condition. The high yield of H_2 was sustained at least up to 14 days, suggesting the possibility of utilizing natural seawater for growth and H_2 production of *Geitlerinema* sp. RMK-SH10.

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Appendices

Appendix A

Artificial Seawater Nutrient (ASN) III medium (Rippka *et al.*, 1979)

Composition of Trace metal mix 1000X

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 100X ASN III

$NaNO_3$	8.82	mM
$MgSO_4.7H_2O$	14.20	mM
$MgCl_2.6H_2O$	9.84	mM
$CaCl_2.2H_2O$	3.4	mM
KCl	6.7	mM
Citric Acid	0.14	mM
Na_2EDTA	1.2	μM

Composition of ASN III medium

ASN III 100X	10	mL
Trace metal mix 1000X	1	mL
Vitamin B_{12} (cyanocobalamin)	4×10^{-9}	M
Na_2CO_3 (2 g/100 mL)	1	mL
K_2HPO_4 (2 g/100 mL)	1	mL
$FeNH_4$.Citrate (0.30 g/100 mL)	1	mL
NaCl	25	g

Adjusted volume to 1 L with deionized water and adjust the pH of the medium to 7.5 with 2 N NaOH

Appendix B

Blue green (BG) 11 medium (Rippka *et al.*, 1979)

Composition of Trace metal mix 1000X

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 100X

$NaNO_3$	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 100X	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

The volume was adjusted to 1 L with deionized before autoclaving.

Appendix C

BG 11 medium supplemented with Turk Island salt solution (Garlick *et al.*, 1977)

Composition of BG11 medium supplemented with Turk Island salt solution

BG11 100 (Appendix B)	10	mL
*Stock A solution	100	mL
**Stock B solution	100	mL
Na_2CO_3 (2 g/100 mL)	1	mL
K2HPO4 (3.05 g/100 mL)	1	mL
FeNH4.Citrate (0.60 g/100 mL)	1	mL
NaCl	28.16	g

Adjusted volume to 1 L with deionized water and adjust the pH of the medium to 7.6 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 A solution**

Composition per liter		
$MgSO_4.7H_2O$	74.48	g

Appendix D

Luria-Bertani (LB) medium (Bertani, 1951)

LB broth

Composition per liter

Bacto-tryptone	10	g
NaCl	10	g
Yeast-extract	5	g

LB agar

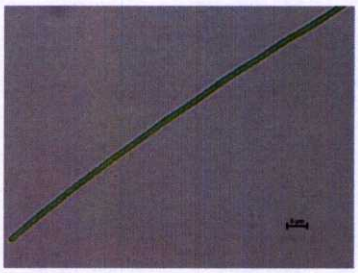
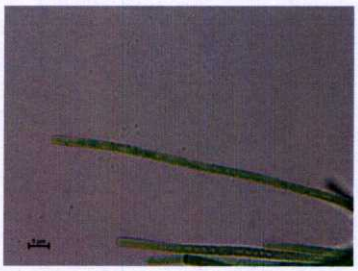
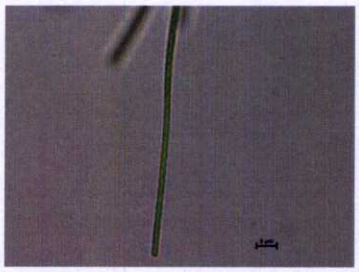
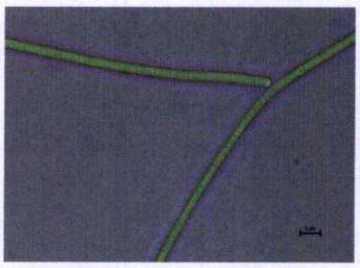
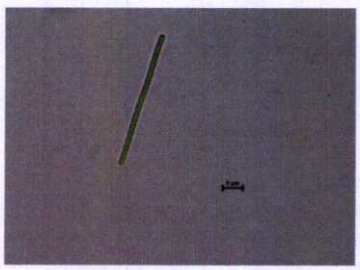
Composition per liter

Bacto-tryptone	10	g
NaCl	10	g
Yeast-extract	5	g
Agar	15	g

The pH was adjusted to 7.4 with 2 N NaOH and sterilized by autoclaving

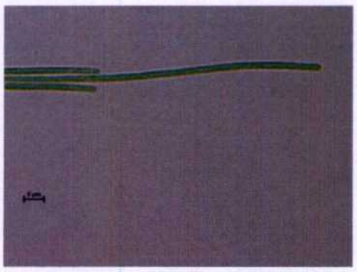
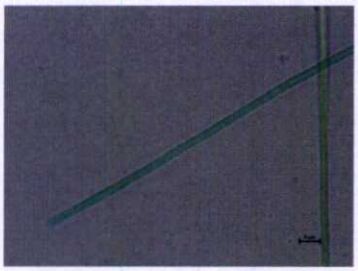
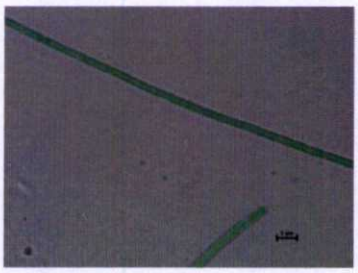
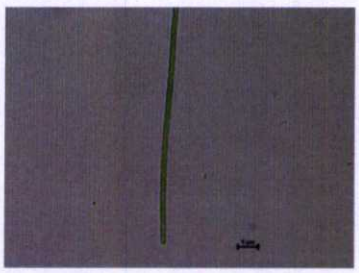
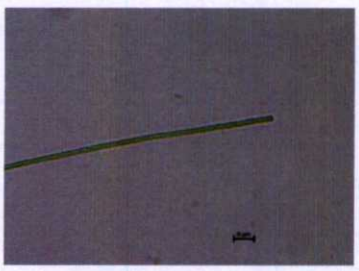
Appendix E

Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand.

Group	Isolates	Origin	Habitat	Morphology
1	CHL-SH1	Changlang beach, Trang	shell	
	JL-SH1	Jaolao beach, Chantaburi	shell	
	JL-SA1	Jaolao beach, Chantaburi	sand	
	JM-SH2	Jaomai beach, Trang	shell	
	KVM-W1	Khungwiman beach, Chantaburi	seawater	

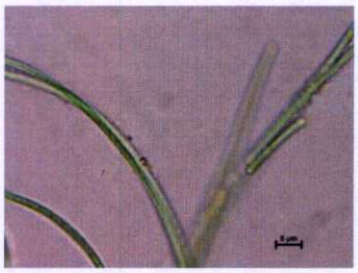
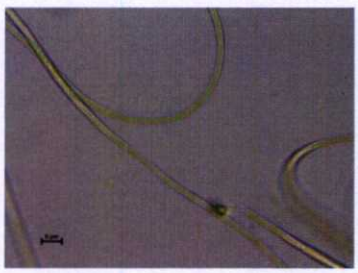
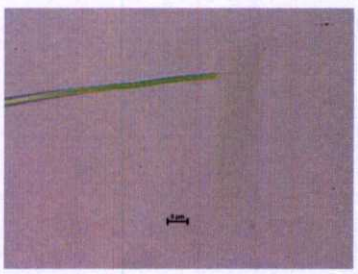
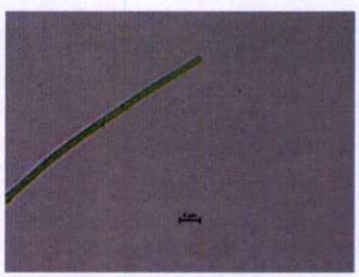
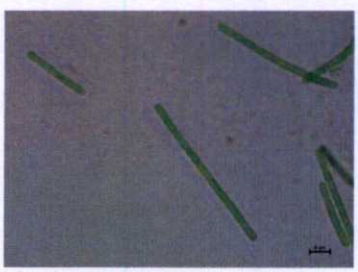
Appendix E

Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

Group	Isolates	Origin	Habitat	Morphology
1	LK-SH2	Laemkruat beach, Krabi	shell	
	LS-W2	Laemsing beach, Chantaburi	seawater	
	LSD-SH2	Laemsadet beach, Chantaburi	shell	
	MTN-SH5	Modtanoy beach, Trang	shell	
	MTN-SH9	Modtanoy beach, Trang	shell	

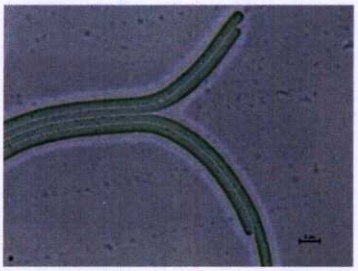
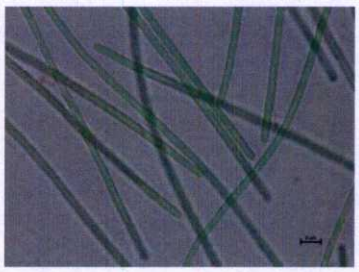
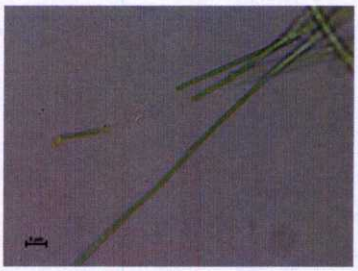
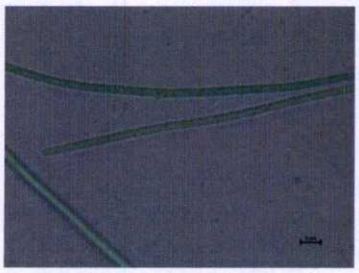
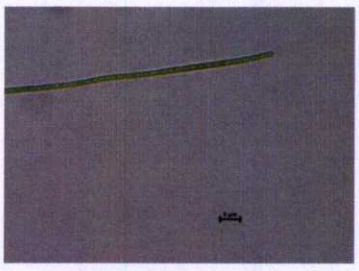
Appendix E

Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

Group	Isolates	Origin	Habitat	Morphology
1	N-ST1	Nang bay, Krabi	stone	
	N-ST2	Nang bay, Krabi	stone	
	NM-SA4	Nammao bay, Krabi	sand	
	NM-SH1	Nammao bay, Krabi	shell	
	NR-SH2	Nangram beach, Chonburi	shell	

Appendix E

Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

Group	Isolates	Origin	Habitat	Morphology
1	P-ST2.3	Phla beach, Rayong	stone	
	P-W2.1	Phla beach, Rayong	seawater	
	PI-S1.1	Phai bay, Rayong	seawater	
	PKR-W3	Pakarang bay, Rayong	seawater	
	PM-SH13	Pakmeng beach, Trang	seawater	

Appendix E

Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

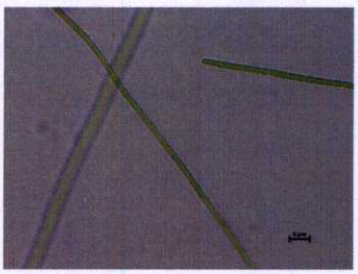
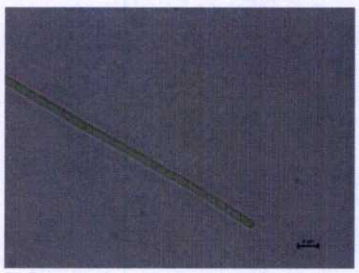
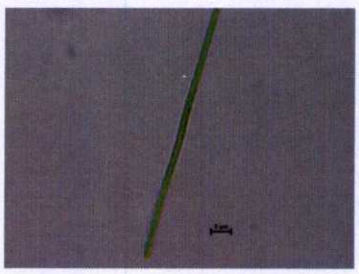
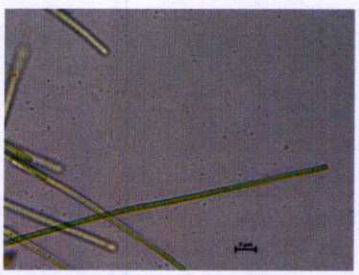
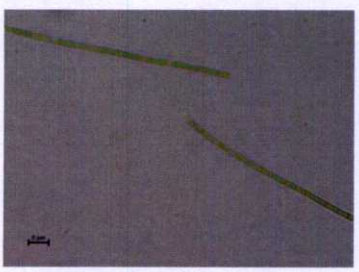
Group	Isolates	Origin	Habitat	Morphology
1	RMK-SH10	Rachamongkol beach, Trang	shell	
	S-SH3	San beach, Trang	shell	
	SK-ST1.1	Saikaew beach, Chonburi	stone	
	SK-ST1.2	Saikaew beach, Chonburi	stone	
	SK-ST2.1	Saikaew beach, Chonburi	stone	

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Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

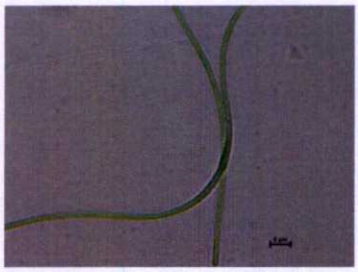
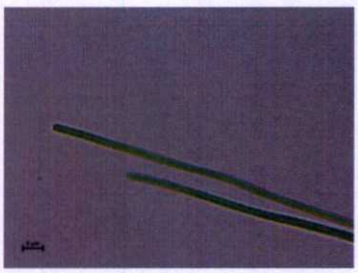
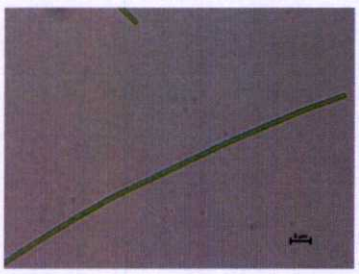
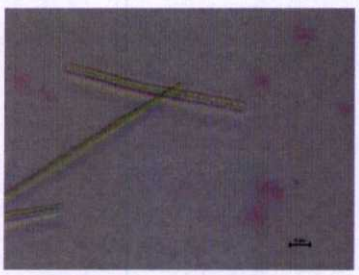
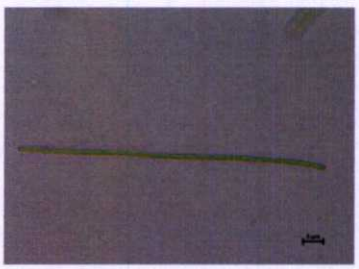
Group	Isolates	Origin	Habitat	Morphology
1	SR-SH4	Samran beach, Trang	shell	
	SS-ST6	Samaesan beach, Chonburi	stone	
	SSH-SH12	Susanhoy beach, Krabi	shell	
	ST-ST1.5.1	Saithong beach, Rayong	stone	
	TG-W2.3.3	Toeingam beach, Chonburi	seawater	

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Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

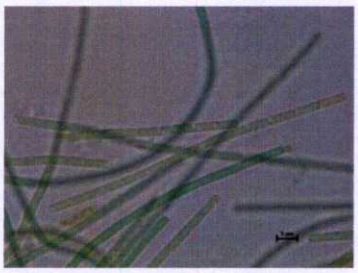
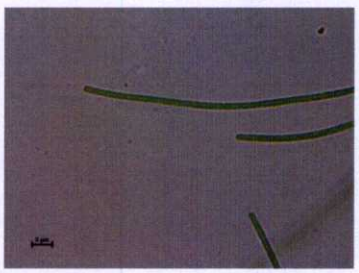
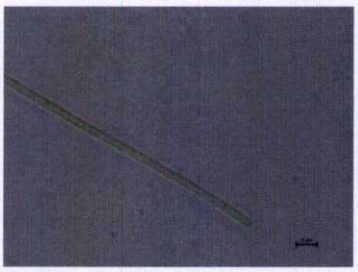
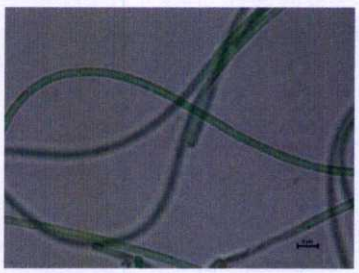
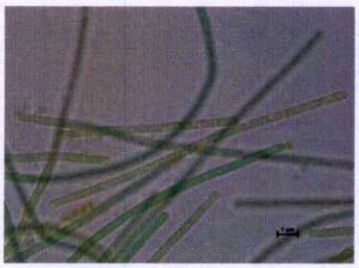
Group	Isolates	Origin	Habitat	Morphology
1	TL-SH2	Talane bay, Krabi	shell	
	VD-SH2.3	Vongdeuan bay, Rayong	shell	
	WI-SA1	Wai bay, Rayong	sand	
	WI-SH3	Wai bay, Rayong	shell	
	YL-SH4	Yonglin beach, Trang	shell	

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Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

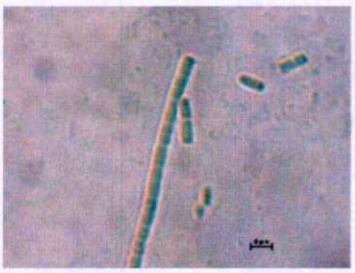
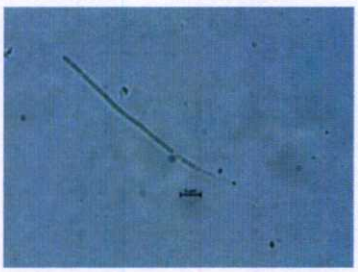
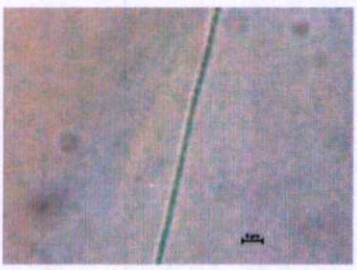
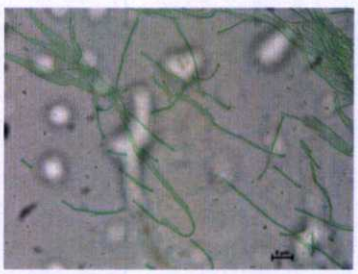
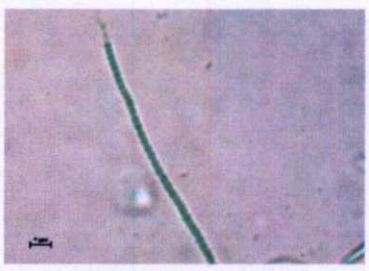
Group	Isolates	Origin	Habitat	Morphology
2	CHL-SH10	Changlang beach, Trang	shell	
	KK	Kohkood island, Trad	seawater	
	KM-ST9	Klongmuang beach, Krabi	stone	
	MP-SA1	Makhampom bay, Rayong	sand	
	NTR-S	Nopparatthara beach, Krabi	seawater	

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Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

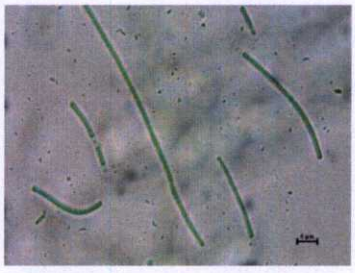
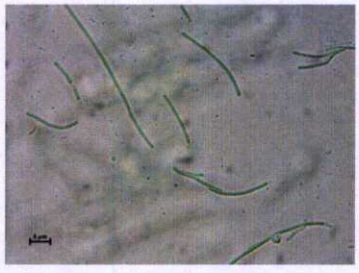
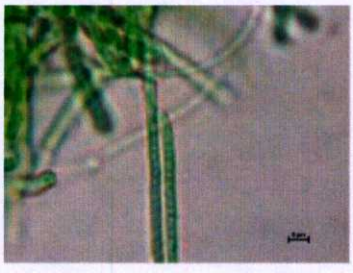
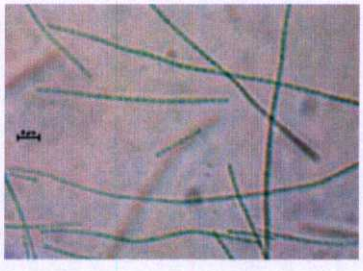
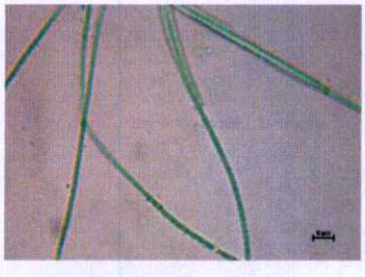
Group	Isolates	Origin	Habitat	Morphology
2	PKR-SH1	Pakarang bay, Rayong	shell	
	PKR-SH9	Pakarang bay, Rayong	shell	
	PKR-ST1	Pakarang bay, Rayong	stone	
	P-SA1.1	Phla beach, Rayong	sand	
	S-S	San beach, Trang	seawater	

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Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

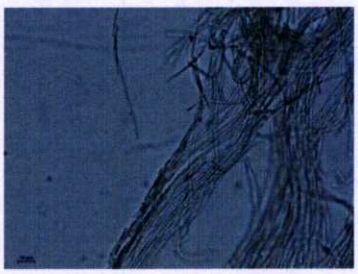
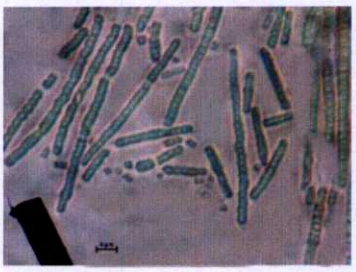
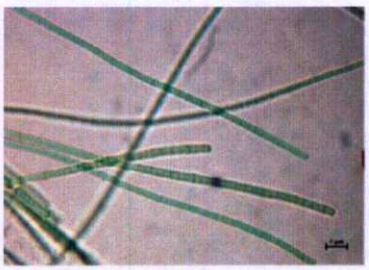
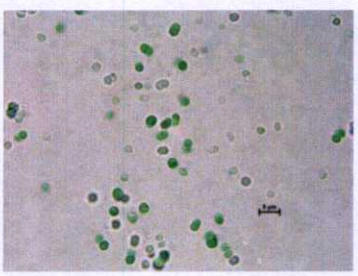
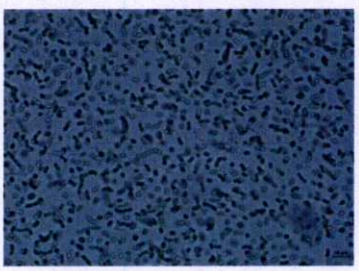
Group	Isolates	Origin	Habitat	Morphology
2	SSH-SH5	Susanhoy beach, Krabi	shell	
	TL-S	Talane bay, Krabi	seawater	
	Y-SH8	Yao beach, Trang	shell	
3	LSD-W3	Laemsadet beach, Chantaburi	seawater	
	JL-W2	Jaolao beach, Chantaburi	seawater	

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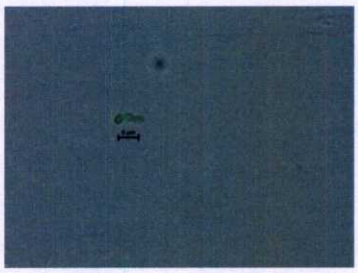
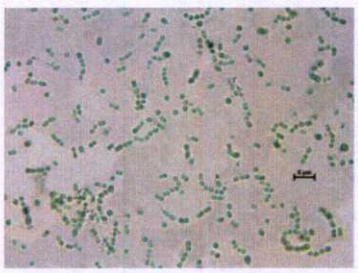
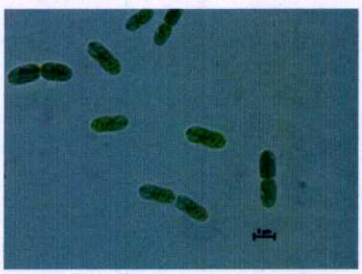
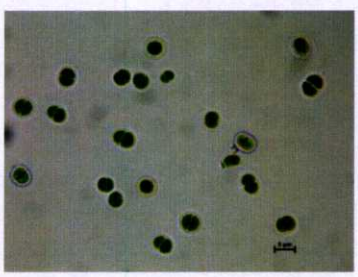
Table A1 Morphological characteristics of 54 purified cyanobacterial isolates isolated from seawater in Gulf of Thailand and the Andaman Sea of Thailand (Continued).

Group	Isolates	Origin	Habitat	Morphology
3	MP-W	Makhampom bay, Rayong	seawater	
	VD-W	Vongdeuan bay, Rayong	seawater	
4	P-SH8.2.1	Jaolao beach, Chantaburi	shell	
5	SKR-W2.2	Jaolao beach, Chantaburi	sand	

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Scholarship	Research grant by the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang

Academic Publications

1. **Tinpranee, N.**, Incharoensakdi, A. and Phunpruch, S. 2018. "Screening cyanobacteria from marine coastal waters of Thailand for biohydrogen production." *Journal of Applied Phycology*. Doi.org/10.1007/s10811-018-1490-6.
2. **Tinpranee, N.**, Worananthakij, W., Incharoensakdi, A. and Phunpruch, S. 2018. "Morphological and genetic characterization of marine filamentous cyanobacterium *Geitlerinema* (Oscillatoriales, Cyanophyta) isolated from Thailand." *ScienceAsia*.