ISOLATION AND CULTIVATION OF CYANOBACTERIA FROM KARST CAVES IN THAILAND FOR PHYCOBILIPROTEIN PRODUCTION AND BIOACTIVITY EVALUATION

KITTAKORN LIMRUJIWAT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN APPLIED BIOLOGY

FACULTY OF SCIENCE AND TECHNOLOGY

RAJAMANGALA UNIVERSITY OF TECHNOLOGY THANYABURI

ACADEMIC YEAR 2021

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การคัดแยกและเพาะเลี้ยงไซยาโนแบคทีเรียจากถ้ำเขาหินปูนในประเทศไทยเพื่อผลิตไฟโคบิลิโปรตีน และทดสอบฤทธิ์ทางชีวภาพ

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Program	Applied Biology
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หัวข้อวิทยานิพนธ์ การคัดแยกและการเพาะเลี้ยงไซยาโนแบคทีเรียจากถ้ำเขาหินปูนในประเทศไทยเพื่อผลิตไฟโคบิลิโปรตีนและการประเมินฤทธิ์ทางชีวภาพ

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บทคัดย่อ

ไฟโคบิลิโปรตีนเป็นโปรตีนเชิงซ้อนสำหรับเก็บเกี่ยวแสงพบได้บนเยื่อหุ้มไทลาคอยด์ของไซยาโนแบคทีเรียซึ่งสามารถดูดซับแสงและถ่ายโอนพลังงานไปยังระบบการสังเคราะห์ด้วยแสงของเซลล์ได้ ปัจจุบันมีการรายงานว่าไฟโคบิลิโปรตีนที่ได้จากไซยาโนแบคทีเรียจัดเป็นผลิตภัณฑ์ธรรมชาติที่มีมูลค่าสูง ซึ่งได้รับความสนใจในการนำไปใช้ประโยชน์ใช้ในอุตสาหกรรมต่าง ๆ เช่น อุตสาหกรรมยา อาหารสัตว์ และเครื่องสำอาง เป็นต้น ประเทศไทยมีพื้นที่ถ้ำเขาหินปูนกระจายอยู่เกือบทั่วทุกภูมิภาคของประเทศ สภาพแวดล้อมของถ้ำเขาหินปูนถูกพบว่ามีลักษณะที่จำเพาะและเป็นสภาวะวิกฤต คือ มีปริมาณแสงธรรมชาติน้อย และมีความชื้นสูง มีสารประกอบแคลเซียมคาร์บอเนตเป็นองค์ประกอบหลักและบางพื้นที่มีปริมาณสารประกอบฟอสฟอรัสและไนโตรเจนสูงเนื่องจากมูลของสัตว์ที่อาศัยอยู่ในถ้ำ ด้วยเหตุนี้จึงทำให้ถ้ำเขาหินปูนเป็นเป็นพื้นที่ที่น่าสนใจในการศึกษาความหลากหลายของไซยาโนแบคทีเรีย

โดยงานวิจัยนี้มุ่งเน้นการศึกษาความหลากหลายของไซยาโนแบคทีเรียจากถ้ำเขาหินปูนในประเทศไทยเพื่อผลิตไฟโคบิลิโปรตีน โดยการเก็บตัวอย่างมาจากถ้ำเขาหินปูนทั้งหมด 23 ถ้ำ จากการศึกษาลักษณะสัณฐานวิทยา พบว่าสามารถคัดแยกสายพันธุ์ไซยาโนแบคทีเรียได้ทั้งหมด 86 สายพันธุ์ จาก 12 สกุล และพบว่าสกุล *Leptolyngbya* sp. มีสายพันธุ์ที่พบได้มากสุดในตัวอย่างถ้ำเขาหินปูน ตัวอย่างที่คัดแยกทั้งหมดสามารถผลิตไฟโคบิลิโปรตีนได้ และมีไซยาแบคทีเรีย 6 สายพันธุ์ ได้แก่ *Leptolyngbya* sp. SCOM01, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14 และ *Nostoc* sp. SW02 สามารถผลิตไฟโคบิลิโปรตีนได้มากกว่า 400 มิลลิกรัมต่อกรัมโปรตีน ดังนั้น ทั้ง 6 สายพันธุ์ถูกนำไปยืนยันสายพันธุ์ด้วยเทคนิคทางชีวโมเลกุล และใช้เครื่องมือทาง

ชีวสารสนเทศศาสตร์ในการศึกษาสายสัมพันธ์เชิงวิวัฒนาการของไซยาโนแบคทีเรีย ทำการขยายขนาดการผลิตและศึกษาคุณสมบัติและความบริสุทธิ์ของไฟโคบิลิโปตีนต่อไป

ผลการทดลองพบว่า ไฟโคบิลิโปรตีนทั้งหมดที่ผ่านการทำให้บริสุทธิ์แสดงคุณสมบัติความสามารถในการดูดกลืนคลื่นแสงและความคงตัวที่แตกต่างกัน โดยไซยาโนแบคทีเรีย *Nostoc* sp. SW02 ให้ผลผลิตไฟโคบิลิโปรตีนสูงสุดคิดเป็นร้อยละ 31.92 และมีอัตราส่วนชนิดไฟโคอิริทรินต่อไฟโคไซยานินต่อออโลไฟโคไซยานิน เท่ากับ 3.4: 1.9: 1.0 นอกจากนั้น สารสกัดไฟโคบิลิโปรตีนจาก *Nostoc* sp. SW02 ยังแสดงคุณสมบัติการมีฤทธิ์ทางชีวภาพที่น่าสนใจ โดยมีฤทธิ์ต้านอนุมูลอิสระชนิด DPPH ที่ IC_{50} เท่ากับ 30.56 ไมโครกรัมต่อมิลลิลิตร สารสกัดไฟโคบิลิโปรตีนนี้ยังสามารถยับยั้งเชื้อแบคทีเรียก่อโรคได้โดยมีค่า MIC ของสารสกัดที่ความเข้มข้น 1000 ไมโครกรัมต่อมิลลิลิตร รวมทั้งสามารถยับยั้งเซลล์มะเร็งปากมดลูก (Hela) โดยมีค่า IC_{50} เท่ากับ 140 ไมโครกรัมต่อมมิลลิลิตร เมื่อบ่มเซลล์มะเร็งกับสารสกัดไฟโคบิลิโปรตีนเป็นเวลา 72 ชั่วโมง โดยที่ความเข้มข้นนี้ไม่ส่งผลความเป็นพิษต่อเซลล์ผิวหนังปกติ ดังนั้น จากผลงานวิจัยนี้สามารถช่วยทำให้ทราบข้อมูลความหลากหลายทางชีวภาพของไซยาโนแบคทีเรียในพื้นที่ถ้ำเขาหินปูนของประเทศไทย ตลอดจนไซยาโนแบคทีเรียที่คัดแยกถือได้ว่าเป็นแหล่งใหม่สำหรับผลิตได้ไฟโคบิลิโปตีนที่มีศักยภาพในการนำไปประยุกต์ใช้ประโยชน์ต่อไป

คำสำคัญ: ไซยาโนแบคทีเรีย, ถ้ำเขาหินปูน, ความหลากหลายทางชีวภาพ, ไฟโคบิลิโปรตีน, สารออกฤทธิ์ทางชีวภาพ

Thesis Title Isolation and Cultivation of Cyanobacteria from Karst Caves in Thailand for Phycobiliprotein Production and Bioactivity Evaluation

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Program Applied Biology

Thesis Advisor Assistant Professor Wanthanee Khetkorn, Ph.D.

Academic Year 2021

ABSTRACT

Phycobiliproteins are light-harvesting protein complexes found on cyanobacteria's thylakoid membranes which absorb light and transfer it to the photo system. Nowadays, phycobiliproteins are highly valuable natural products produced by cyanobacteria. Their use in a variety of industries such as pharmaceuticals, feed, and cosmetics has garnered attention. Thailand has many karst cave areas spread throughout the country that are considered distinct extreme environments due to their low natural lighting and high humidity. In these karst cave areas, calcium carbonate and compounds of phosphorus and nitrogen result from the excrement of animals. Therefore, this is an ideal environment to study cyanobacteria biodiversity.

This study examined the production of phycobiliproteins by cyanobacteria from Thailand karst caves. Samples were taken from 23 karst caves and all morphotype isolates were identified as 86 species from 12 genera. Those species were the *Leptolyngbya* sp. which are commonly found in karst cave samplings. All isolates could produce phycobiliproteins and six cyanobacterial strains, including *Leptolyngbya* sp. SCOM01, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, and *Nostoc* sp. SW02 could produce phycobiliprotein content above 400 mg g protein^{-1}. These six strains have been identified using molecular techniques and

bioinformatics tools for phylogenic tree analysis. Additionally, production has been scaled up and analyzed for phycobiliprotein properties and purity.

The results of this research showed that all purified phycobiliproteins exhibited distinct spectral characteristics and stabilities as the greatest yield of phycobiliprotein (31.92%) was obtained from cyanobacterium *Nostoc* sp. SW02, in which the ratio of phycoerythrin: phycocyanin: allophycocyanin was 3.4: 1.9: 1.0. The extracted phycobiliprotein from *Nostoc* sp. SW02 showed some interesting properties of biological activities such as DPPH antioxidant activity at IC_{50} value was 30.56 μg mL^{-1}. The inhibition of bacterial pathogens with the MIC values of phycobiliproteins extracted at a concentration of 1,000 μg mL^{-1} as well as the inhibition of Hela cancer cells by the IC_{50} value was 140 μg mL^{-1} when incubated with PBPs extract for 72 h without cytotoxicity on normal cell line. The results show that the biodiversity of cyanobacteria in karst caves of Thailand including isolated cyanobacteria could be considered as a new source for phycobiliprotein with potential for further applications.

Keywords: Cyanobacteria, Karst cave, Biodiversity, Phycobiliprotein, Bioactivity

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Kittakorn Limrujiwat

Date 28 Month May Years 2022

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List of Abbreviations

PBPs	Phycobiliproteins
PC	Phycocyanin
PE	Phycoerythrin
APC	Allophycocyanin
TOC	Total organic carbon
DOC	Dissolved organic carbon
LPS	Lipopolysaccharide
EPS	Exocellular polysaccharide
ML	Maximum likelihood
NJ	Neighbor joining
TEM	Transmission electron microscope
SEM	Scanning electron microscope

CHAPTER 1
INTRODUCTION

1.1 Background and Statement of the Problems

Cyanobacteria are among the oldest organisms living on earth (more than 2.5 billion years old). These prokaryotes can be found in oceans, lakes, estuaries, rivers, swamps, desserts, tropical rainforests, hot springs and karst caves [1]. Cyanobacteria are very resistant to severe conditions in cave, including even darkness. This research has been interested to study cyanobacterial biodiversity in the karst caves of Thailand. Since, the karst caves are highly specific environments scattered all over the country, where are considered a specific case of extreme environment characterizing low natural light, high humidity and the temperature is consistent throughout the year [2–4]. Importantly, the main components of karst cave contain calcium carbonate and compound of phosphorus achieved from animal excrement inside the cave. It is interesting that cyanobacterial species has a high adaptability to cave environment by interacting with minerals there and they might play an important role in reshaping the mineral structure of the cave walls, floors, and ceilings to form features such as stalactites and stalagmites. Therefore, the karst cave is an interesting area to study the biodiversity of rare cyanobacteria and finding the newly cyanobacteria strains capable to produce capable substances in the secondary metabolites group such as phycobiliprotein compound.

Cyanobacteria are oxygenic photosynthesis microorganism. The cyanobacterial photosynthetic apparatus consists of three principal light-harvesting systems: two main photosystems (also found in other photosynthetic organisms) and a phycobilisome [5]. In cyanobacteria, light harvesting is mediated by phycobilisomes (PBSs) by absorbing light and transfer energy to the photosystems [6]. Phycobilisomes are largely composed of phycobiliproteins (PBPs) located on the thylakoid membranes, and in cyanobacteria these proteins can represent up to 50% of the total cellular proteins [7]. The phycobiliproteins can be divided into three major groups such as phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) [8]. Phycobiliproteins have been reported that showing the

bioactivities as antioxidant, anticancer, antimicrobial and anti-inflammatory. Nowadays, phycobiliproteins are the high-value natural products from cyanobacteria which have attracted attention for their potential use in different industries, such as nutraceutical, pharmaceutical, food, feed and cosmetics [9].

Therefore, this research aimed to focus a cyanobacterial biodiversity existing in Thai karstic caves. The taxonomy of isolated cyanobacteria was identified using morphological and molecular techniques. In addition, the cyanobacterial sampling was screened for the production of high value-added compounds focusing on phycobiliproteins. Moreover, phycobiliproteins characterization and their biological activities were also investigated in this study.

1.2 Purpose of the Study

This thesis consists of three objectives as following:

1.2.1 To isolate and identify cyanobacteria from karst cave in Thailand using morphological and molecular techniques.

1.2.2 To screen phycobiliproteins content in isolated karst cave cyanobacteria for upscale production.

1.2.3 To purify and characterize properties of phycobiliproteins in biological activities.

1.3 Scope of Study

The scope of research corresponding to 2 year-proposal can be divided into four parts. The first part, the sampling site in the 23 karst caves widespread along of Thailand was selected to isolate and identify cyanobacterial karstic cave habitat. The second part, the isolated cyanobacteria were grown in BG11 medium to enhance biomass for screening phycobiliprotein production. The third part, the selected cyanobacteria were upscale cultivation for phycobiliprotein extraction and purification. And the last part, the biological activities consisting of antioxidant, antimicrobial and anticancer were investigated from purified phycobiliprotein.

1.4 Contribution to Knowledge

The expected benefits of this research are to gain more understanding the biodiversity of cyanobacteria in karst cave of Thailand, and finding newly cyanobacterial stain showing a high potential phycobiliprotein production which can be applied in further commercial performance.

CHAPTER 2
REVIEW OF THE LITERATURE

2.1 An Overview of Cave Environment

Caves have been studied for hundreds of years. The particularly interesting research is to gain more understanding of relation between biotechnology and microbiology together. Cave environments can be highly variable, limited availability of organic matter, variable levels of light and humidity, lack of limited connectivity to the surface, low or high temperatures, the nature of the hydrological connection between the cave, the surface and the groundwater, exposure to human or other animal visitations, air flow and pressure conditions, and the types and concentration of minerals in the matrix of rock surrounding the cavern [10,11]. Each cave is unique in its biological, chemical, and physical characteristics. There are many types of caves, depending on the method of speleogenesis. These types can be categorized on the basis of various factors, including 1) the types of minerals and bedrock surrounding them 2) their geometric structure and morphology 3) the time they were created in relation to the rock in which they are found and 4) the mechanism by which they were formed [10]. Depending on the amount of light that enters, the cave's interior environment can be divided into four main zones: 1) the entrance zone (where the surface and underground environments meet); 2) the twilight zone (where light gradually fades to none and no plants can grow beyond this point); 3) the transition zone (where there is no light, but surface environmental fluxes such as temperature and moisture are still sensed) and 4) the deep zone (where it is completely dark, with high humidity and constant temperature) (Figure 2.1).

2.2 Cave Habitats

Each cave is unique in terms of its biological, chemical and physical characteristics, factors that influence the amount of heterogeneity within and among cave habitats [10]. Depending on the transitional conditions (ecotones) in caves, one can know roughly where the energy and nutrients originate and how they flow in these nutrient-limited ecosystems. Typically, microbial trophic structures in caves are less complex than their counterparts in

surface environments due to the low amount of light and nutrient sources and the reduced fluctuations in temperature and humidity. In these natural habitats, autotrophic activity can be easily observed in the entrance zone due to light is still available as the source of energy. Therefore, deeper into caves, the oligotroph and heterotrophs can be found because of the source and amount of energy available. Any environments that contain less than 2 mg/L of total organic carbon (TOC) are regarded as oligotrophic [12]. Caves commonly receive organic carbon in the form of dissolved organic carbon (DOC). DOC can be presented in the caves as a result of allochthonous input from soil-derived ecosystems on the surface. In this case, the types and amount of DOC entering a cave will depend on its depth and proximity to, and relationship with, the surface [10].

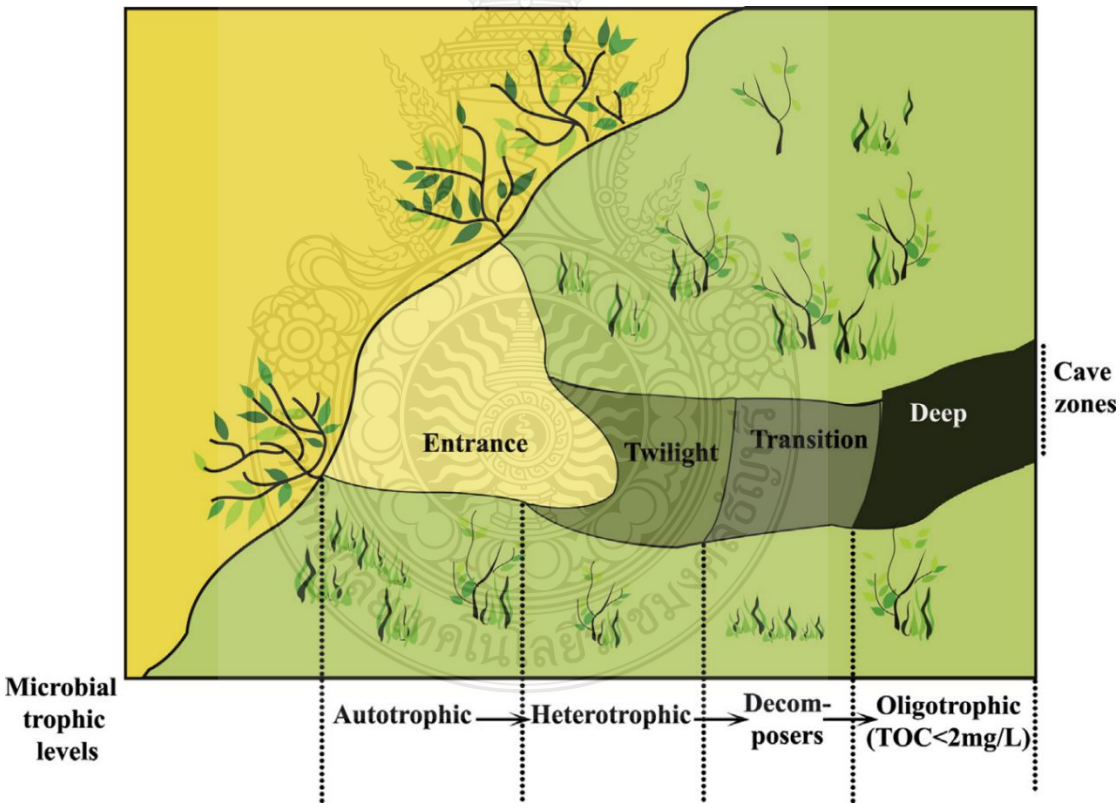


Figure 2.1 Schematic representation of the energy flow from the trophic levels in clave zone [10].

Figure 2.1 shows a schematic of potential energy flow and microbial trophic levels in a typical cave system. There have intentionally not included the microbial trophic levels that might be found in ecotones, which are the areas of transition between adjacent zones of cave interior environments (entrance, twilight, transition and deep zones). The structure and species composition of the microorganisms that live across these ecotones vary with conditions in which they live. However, it also means that a great number of unique species and communities are waiting to be discovered. Although cave habitats have been shown to be exceptionally nutrient-limited, diverse microbes are still able to thrive. Caves have been interested to study of microbial ecology for the last few years. Caves have also been shown to harbor microorganisms that display variable enzymatic and antimicrobial activities, which are different from those observed in other extreme environments, and thus are of great interest to researchers [13,14]. The cultivation of these microorganisms has proven to be challenging. Initially, researchers formulated rich nutrient media and incubated them at 37 ºC, which often resulted in poor recovery. This is because these microorganisms are adapted to living in nutrient poor environments, where they are almost starving, and must scavenge to obtain food. The abundance of nutrients media leads to osmotic stress in cells from caves, causing microbial death. Adopted strategies include the use of low-nutrient media and low temperature (15 ºC) for isolating and growing cave microbes had been prior experiment. The study of cave microbiomes has occurred mostly in limestone (calcium carbonate) and lava tube (basalt) formations, since these types of caves have diverse mineral deposits, harboring considerable microbial diversity. Cave environments are mostly colonized by unicellular filamentous microorganisms (Figure 2.2) such as bacteria, archaea, fungi, algae and cyanobacteria [14].

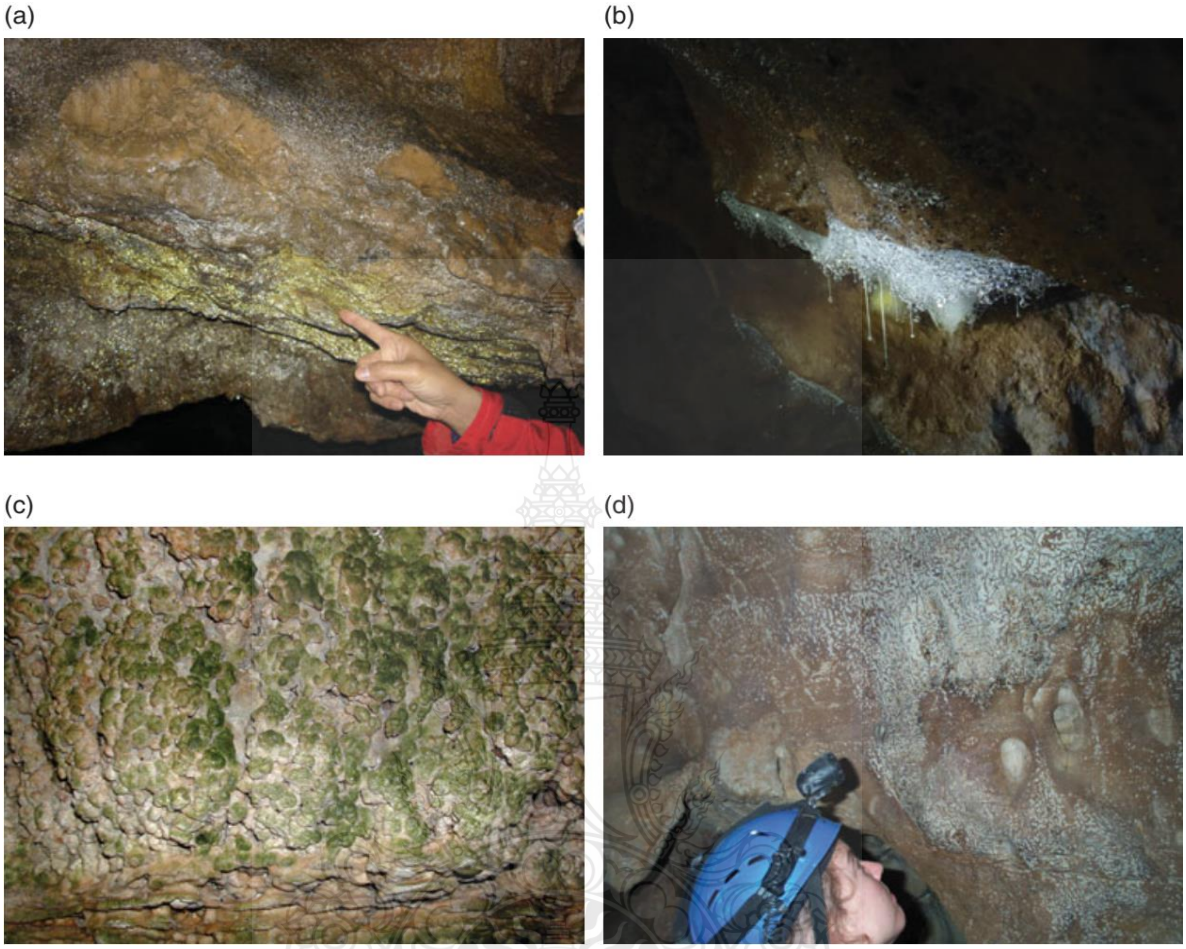


Figure 2.2 Microbial growth on walls in caves: (a) colonies of white and gold actinomycetes and other bacteria on a cave wall, (b) fungal snottites on cave, (c) cyanobacterial growth, (d) biovermiculations on the cave wall composed of clays and organic matter, are thought to be formed by microbial and nematode [15]

2.3 Karst Cave in Thailand

The karst area is widespread in Thailand, which covers 18% of land area (93,000 km^2), except in the northeastern region (Figure 2.3). The karst formed on limestones deposited from the Ordovician to Jurassic periods with Permian limestones occurring most extensively [16].

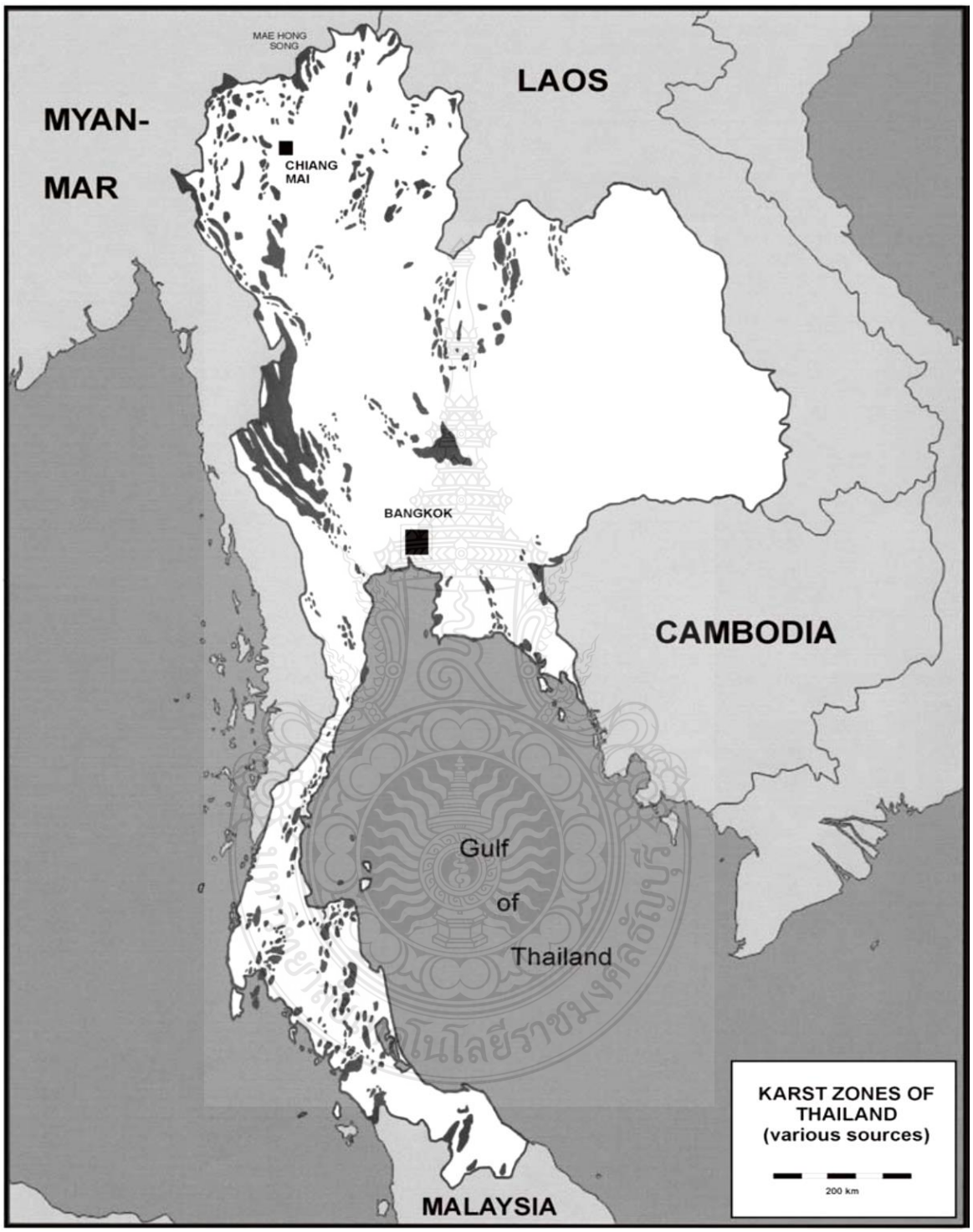


Figure 2.3 Karst map of Thailand [16].

The exploration and database of Thai caves has been particularly active since the early 1980s, with the majority of the exploration being done by European, Australian and American cavers on small scale expeditions and by Western expatriates living in Thailand. While the karst cave has been not widely interested by Thai academics. These explorations leading to increases in speleological knowledge during this period. Currently, 5000 sites have been recorded in a database of Thai caves. There are 16 caves longer than 3 km and 24 caves with a vertical range greater than 120 m [16]. The Tham Phra Wang Daeng located in Phitsanulok province, is one of the longest karst caves in Thailand at 13.75 km. While Tham Pha Phueng located in Chaiyaphum province, which is the karst cave that the largest vertical range at 476 m. Cave exploration is still active in a number of karst areas around Thailand, while other karst areas remain virtually unexplored with regard to speleology. Surveys of the cave biodiversity have resulted in over 400 species being recorded, of which 169 were discoveries new to science [16]. Due to the karst cave environment is a critical condition or "extreme environment", which is low light, consistent low temperature throughout the year and high humidity. The environment of the karst cave in Thailand contains calcium carbonate as the main component causing the alkaline area. Some karst cave has a high amount of phosphorus and nitrogen compounds resulted in the excrement of bats. Therefore, the karst cave is an interesting area to study the biodiversity of rare cyanobacteria. Surprizingly, the cyanobacteria in karst cave are very few studies and there have not reported cyanobacteria surverying from karst cave in Thailand. This may be caused by separation and cultivation that is quite difficult.

2.4 Cyanobacteria

Cyanobacteria commonly known as blue green algae. They are oxygenic photoautotrophic prokaryotes which among the oldest organisms (more than 2.5 billion years old) living on earth. These prokaryotes can be found in oceans, lakes, estuaries, rivers, swamps, desserts, tropical rainforests, hot springs, Antarctic dry valleys and cave [17,18]. Remarkably, cyanobacteria could adapt themselves under atmospheric low concentration of CO_2 by developing function of carbon fixation [19,20]. The main catalyzer for carbon

fixation in cyanobacteria and other aerobic photosynthetic organisms is the ribulose-1,5-biphosphate carboxylase (RuBisCO) enzyme [17]. Cyanobacteria are primarily aerobic photoautotrophs but can also perform anaerobic photosynthesis. Some of them can also grow as photoheterotrophs or chemoheterotrophs [21]. Many species of cyanobacteria are also diazotrophs playing a remarkable role in the nitrogen fixation, where nitrogenase catalyzes the reduction of N_2 to ammonium.

2.4.1 Cyanobacterial Diversity and Classification

Cyanobacteria are the most diverse groups of prokaryotes, showing unique cell morphologies, variable size, and different colors. They can be unicellular cells or complex filamentous cells with a high level of differentiation. Unicellular cyanobacteria can be spherical, rod-like shaped, or aggregated. Filamentous cyanobacteria are organized in the form of branched or linear chains of cells called trichomes that resemble smooth threads surrounded by a slimy sheath [22]. While cyanobacteria reproduce via binary fission as seen in other prokaryotes. However, cyanobacteria have evolved some interesting reproductive strategies. For instance, some unicellular cyanobacteria may produce baeocytes and exocytes, which are differentiated from the mother cell by size, shape, and successive multiple fission with subsequent release to the environment. Filamentous cyanobacteria may produce short, often motile filaments called hormogonia. Furthermore, Nostocalean cyanobacteria may produce long-term or overwintering reproductive cells called akinetes [23]. Akinetes are usually larger than vegetative cells, and with large amounts of stored nutrients visible as granules. Akinetes are reproductive cells, which may find in cyanobacteria adapted in unfavorable conditions such as drought, low temperatures and fall turnover in dimictic lakes [23,24].

Cyanobacteria occur in a wide range of morphologies and ecological forms. Hence, historically their classification has been based on simple morphological characteristics. They have been classified in term of morphological study in five subsections closely representing five orders of cyanobacteria. Subsection 1 includes unicellular species reproducing by binary fusion (order *Chroococcales*) as shown in Figure 2.4. Subsection 2 includes the order *Pleurocapsales*, unicellular organisms reproducing by multiple division

with daughter cells called baeocytes. Filamentous and mostly undifferentiated cyanobacteria belonging to the order *Oscillatoriales* compose Subsection 3, 4 and 5 include members of the orders *Nostocales* and *Stigonematales* (Figure 2.5 and 2.6). Both subgroups include filamentous cyanobacteria capable of cellular differentiation but only members of subsection 5 present true branching and division in more than one plane. Branched cyanobacteria have the most advanced structure among prokaryotes [25]. However, during the last several decades, ecological characteristics, ultrastructural features, and molecular evidence have substantially influenced our knowledge and understanding of cyanobacterial groups. The cellular-morphological approach including ecospecies are not enough for identification. The molecular and ultrastructural studies explain relationships well, but until now include only a small portion of the cyanobacterial diversity and variability that occur in natural habitats [26,27]. Therefore, the polyphasic approach, using a combination of molecular, cytomorphological, and ecological criteria for taxonomic evaluation, has been used recently to correct cyanobacterial classification and has produced convincing arguments and criteria for the objective definition of numerous new taxonomic genera based on phylogenetic relationships [27].

2.4.2 Cyanobacteria Found in Karst Cave Habitat

The diversity and peculiarities of cave habitats might be a host of hazards (e.g., water and air pollution, microclimatic changes, dripstone damage), particularly since caves generally are characterized by extreme conditions and low nutrient availability [28,29]. Nevertheless, some organisms prefer those conditions for colonization and growth. Algae are the most common flora of caves which frequently play a key role in the trophic networks and colonization processes of rocky habitats, producing colorful patches on cave walls (Figure 2.2) [29,30]. Cyanobacteria principally often occur on cave walls and rocks in caves, particularly in the entrance zone where growth conditions are best for them. The average annual temperature in caves is usually correlated with bedrock temperature, and its annual fluctuations are small (ambient temperature in caves ranges from 5 to 8 °C). Humidity in caves is also in narrow range (85 - 95%); light, both natural and electric, is a decisive factor for development of algae and cyanobacteria [30–32].

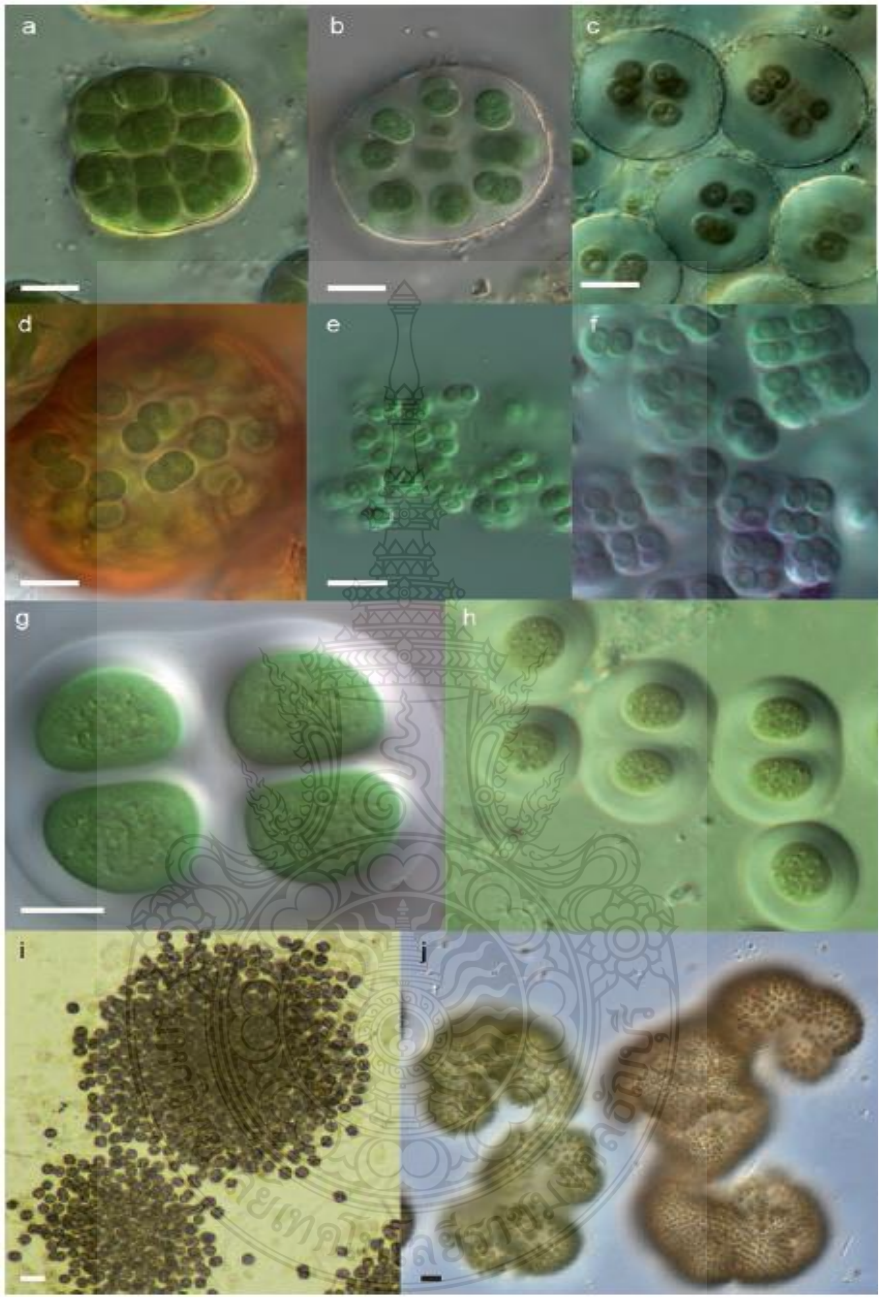


Figure 2.4 Microphotographs of Chroococcales;. (a) *Asterocapsa divina*, (b) *Asterocapsa* sp., (c) *Asterocapsa* sp., (d) *Gloeocapsa novacekii*, (e, f) *Gloeocapsa* sp., (g) *Chroococcus subnudus*, (h) *Chroococcus* sp., (i) *Microcystis novacekii*, (j) *Woronichinia naegeliana*. Scale bar 10 μm, [23].

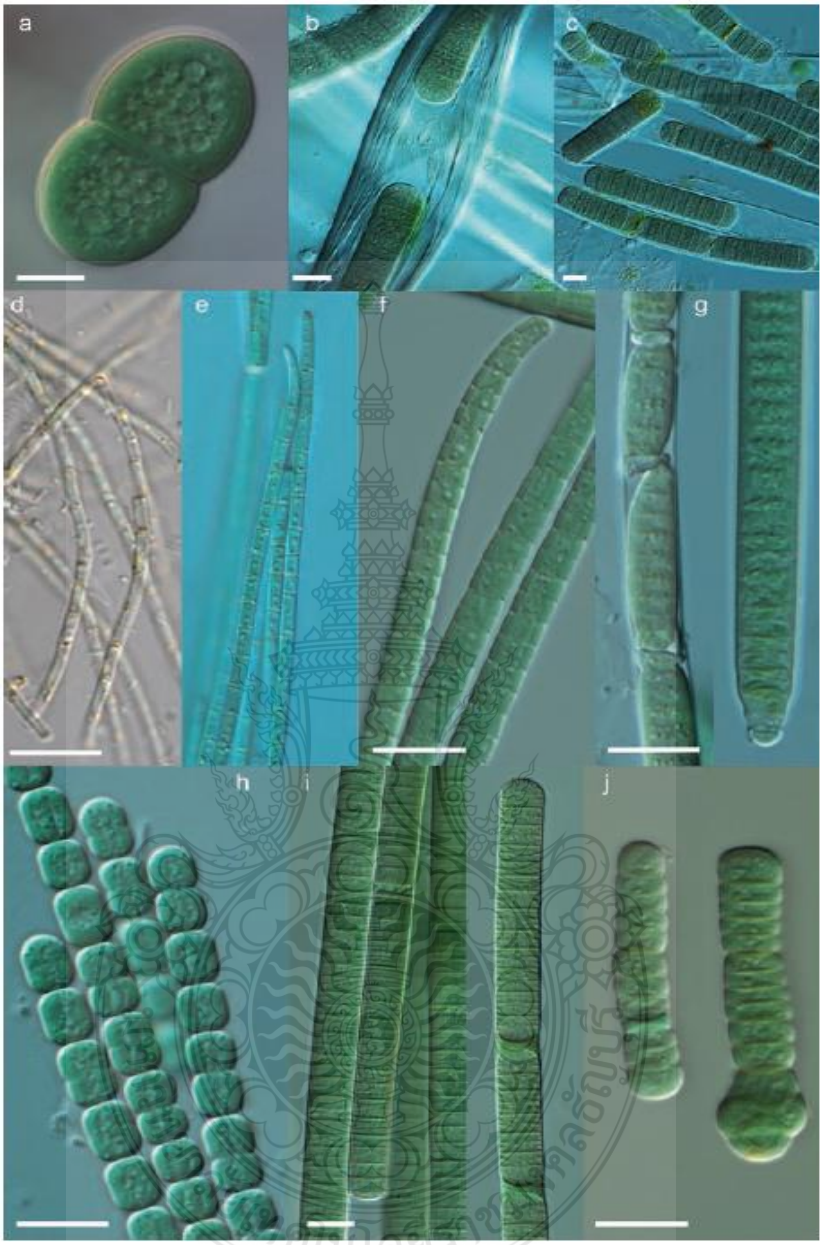


Figure 2.5 Microphotographs showing Oscillatoriales; (a) *Cyanothece aeruginosa*, (b, c) *Blennothrix* sp., (d) *Geitlerinema carotinosum*, (e) *Geitlerinema pseudacutissimum*, (f) *Kamptonema animale*, (g) *Phormidium* sp., (h) *Johanseninema constrictum*, (i) *Oscillatoria* sp., (j) *Crinalium* sp. Scale bar 10 μm [23].

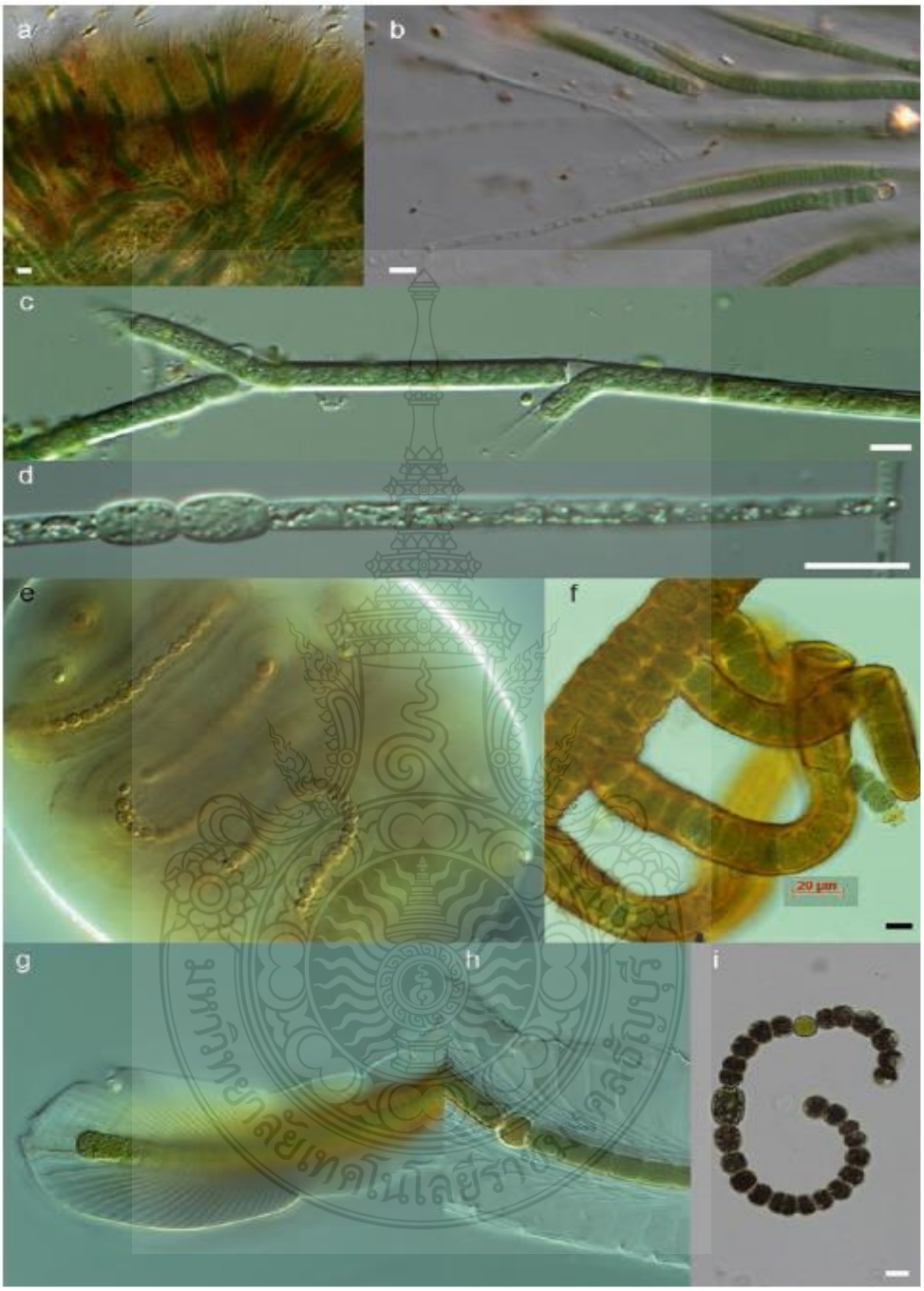


Figure 2.6 Microphotographs of Nostocales; (a, b) *Rivularia* sp., (c) *Tolypothrix* sp., (d) *Cuspidothrix issatschenkoi*, (e) *Nostoc microscopicum*, (f) *Stigonema* sp., (g, h) *Petalonema alatum*, (i) *Dolichospermum* sp. Scale bar 10 µm [23].

The most common species among cyanobacteria were found in caves namely, *Chroococcus* sp., *Nostoc* sp., *Gloeocapsa* sp., *Microcoleus* sp., and *Pseudanabaena* sp. [33]. (Figure. 2.7)**.** Cyanobacteria were most often observed in places more intensity of natural daylight at entrances to caves and slightly deeper parts which illuminated by artificial light (open cave for tourists), where they were prominent due to massive growth.

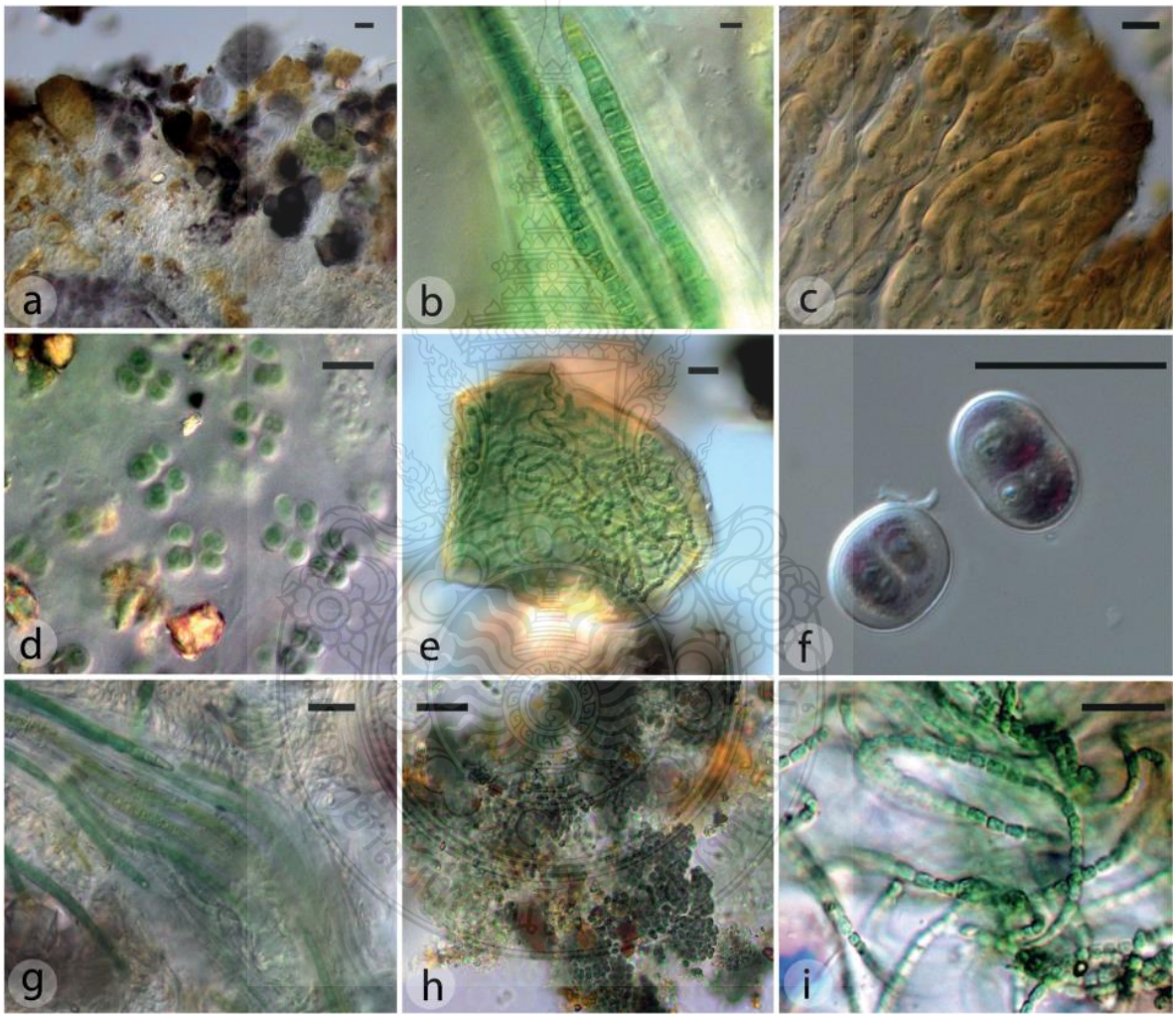


Figure 2.7 Microphotographs of cyanobacteria found in caves; (a) Cyanobacteria in karst cave profile, (b, g) *Microcoleus* sp., (c, e) *Nostoc* sp., (d) *Chroococcus* sp., (f, h) *Gloeocapsa* sp., (i) *Pseudanabaena* sp. Scale bar = 20 μm [33].

2.5 Cellular Organization in Cyanobacteria

Cyanobacteria are prokaryotes, which their cell configuration is also different from bacteria and archaea [25]. The general cyanobacterial cell organization is presented in (Figure. 2.8). The cyanobacterial cell wall is formed by an outer membrane containing lipopolysaccharide (LPS) and one inner layer of peptidoglycan separated by periplasm. Other exclusive components of the outer membrane are carotenoids, hydroxylated fatty acids, and Toc75 homologue porins responsible for protein translocation in photosynthetic organisms [19,34–36]. Cyanobacteria are also surrounded by sheaths, capsules, and slimes. These biofilms are formed by the release of exocellular polysaccharides (EPS) through the junctional pore complex (JPC) connecting the cytoplasm with the exterior. Capsules and sheaths play a protective role under extreme environmental conditions and extruded slime is necessary for gliding movement [37]. The cell wall is separated from the cell membrane by the inner periplasmic space. The thylakoids are intracellular membranes that occupy most of the cytoplasm, which organized as lipid bilayers [38]. The space inside the thylakoids is the lumen and the space outside them is a prolongation of the cytoplasm. The thylakoids are the centers for light reactions in photosynthesis, but are not present in some cyanobacteria (e.g. *Gloeobacter violaceus*). In those cases, light reactions are directly associated to the cytoplasmic membrane without formation of grana like in higher plants resulting as one of the main morphological differences between cyanobacterial and higher plants [37]. The cyanobacterial cytoplasm contains carboxisomes and genetic material (Figure. 2.8) [39]. Carboxisomes are organelles consist of polyhedral proteins housing Ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO) enzymes involving carbon fixation [40]. The cytoplasm of cyanobacteria also contains polymer granules and gas vesicles. Granules are used as reserves of storage polymers (i.e., cyanophycin and polyhydroxyalkanoates) and gas vesicles are used for buoyant movements [15,19,48]. The chromosomal DNA of most cyanobacteria is organized in circular chromosomes with wide range of ploidy levels. Cyanobacterial chromosomes are commonly single or double copies in monoploid and diploid marine species, but highly polyploid organisms also exist. As an example, the model cyanobacterium *Synechocystis* sp. PCC 6803 may have up to 218 chromosome copies per

cell. On the other hand, cyanobacterial plasmids may be completely absent (in *Prochlorococcus* and *Synechococcus*) or present as multiple copies with sizes reaching hundreds of kilobases [41].

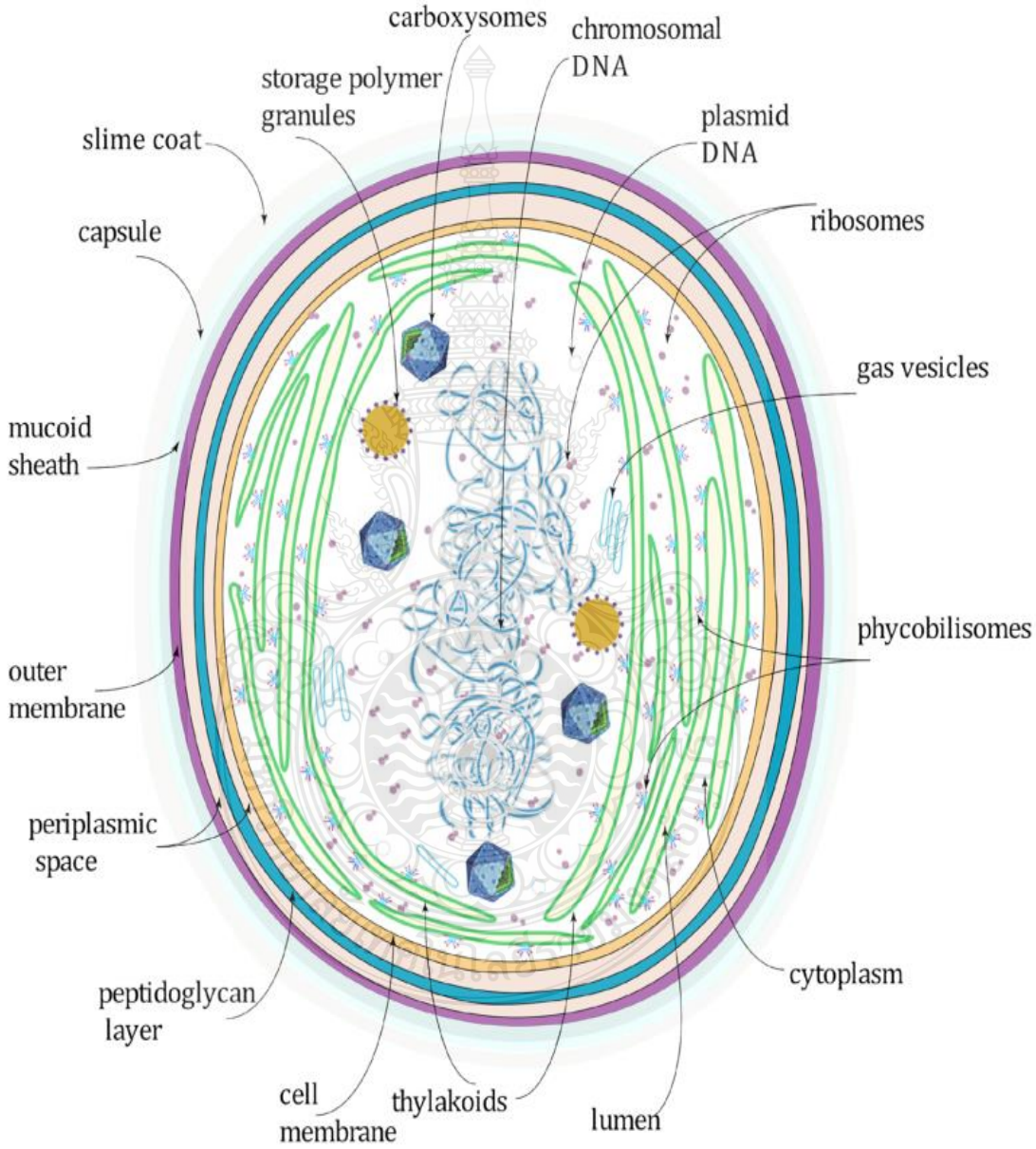


Figure 2.8 Cellular organization in cyanobacteria [1].

2.6 Cyanobacterial Pigments

Pigments are light absorbing compounds which absorb light in different range of the visible spectrum. These pigments have a complex conjugative structure which enables them to absorb and excite electrons at different energy level (Figure. 2.9). The backbone of pigments consists of isoprene and tetrapyrrole rings. Chlorophyll and phycobiliproteins have tetrapyrrole ring structure, whereas carotenoids are made of isoprene [42,43].

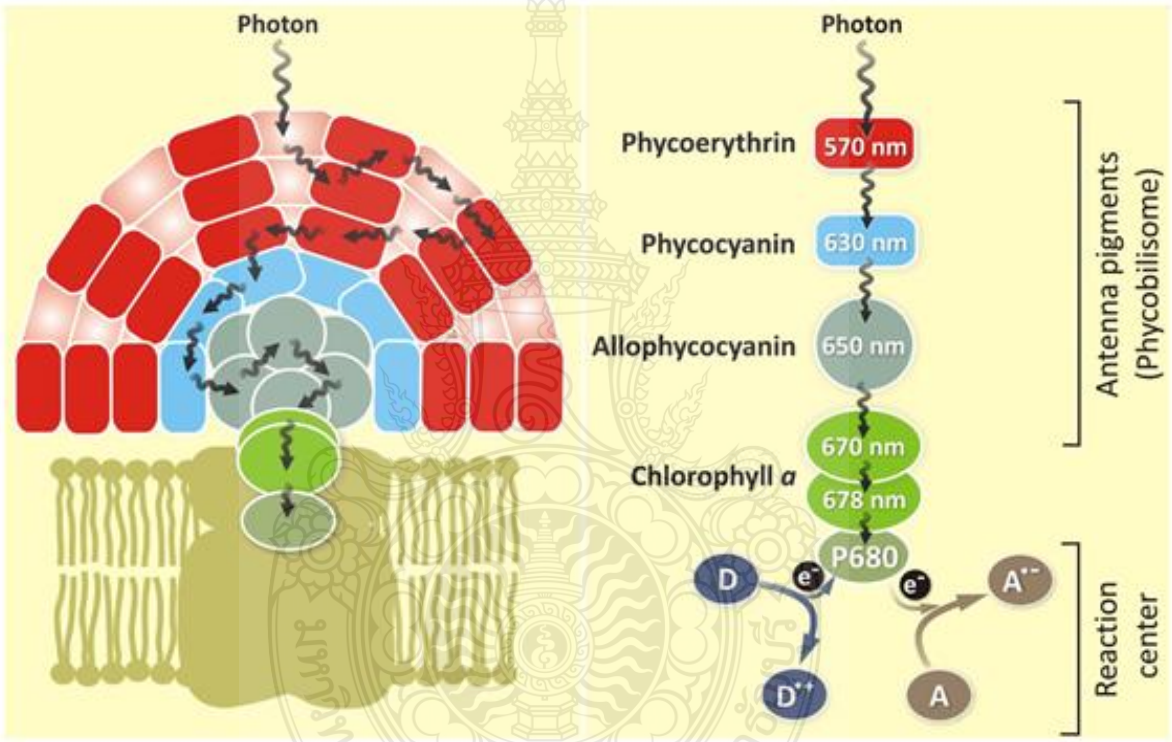


Figure 2.9 Schematic drawing of photosynthetic apparatus and energy transfer steps including photochemical reaction organized in the antenna system of PSII for cyanobacteria [44].

2.6.1 Chlorophylls

Chlorophylls are the leading light-harvesting pigment in cyanobacteria and other photoautotrophic organisms. Six different types of chlorophylls (Chls *a, b, d. f* and divinyl-chls *a* and *b*) naturally occur in cyanobacteria, but chl *a* was most plenty chlorophyll pigment in most cyanobacterial species [45]. Chlorophyll is an excellent photoreceptor because of the presence of interconnecting single, double bonds, which allow delocalization of electron in their structure [46]. This delocalization of electron permits polyene structures to absorb light from different band of the visible spectrum of sunlight and start the electron transfer chain reaction which ultimately transfer to carbon dioxide. It might be cause of the chlorophyll pigment is present in the center of the redox reaction of water photolysis and CO_2 formation [47].

2.6.2 Carotenoids

Carotenoids are light harvesting pigments which are found with chlorophylls. The carotenoid structures are formed by 40 carbon isoprene unit. These are divided into different groups by the presence or absence of oxygen at the terminal end. The non-oxygenated derivatives are called carotene, and their oxygenated derivatives are xanthophylls (primarily hydrocarbon). Xanthophylls are hydrophilic due to the presence of hydroxyl and keto groups at the end of the rings [48,49]. Carotenoids are sensitive to light, heat, and oxygen, which leads to problems in their storage and utilization. The absorption spectrum of different type of carotenoids is described in Table 2.1. Carotenoids have commercial value because of antioxidant property and health promoting factors. The different kinds of carotenoids produced by cyanobacteria microalgae include β-carotene, astaxanthin, lutein (with zeaxanthin), lycopene, canthaxanthin and fucoxanthin etc.

Table 2.1 Cyanobacterial pigments and their applications

Pigments	Absorption spectrum	Molecular weight (kDa)	Color	Uses and activity	Ref.
Chlorophyll *a*	662, 430 nm	892	Green	Pharmaceutical and cosmetics (Deodorant)	[50]
Phycobiliproteins					
Phycoerythrin	490–570 nm	240	Red	Immunofluorescence techniques, antibody labeling	[51]
Phycoerythro-cyanin	560–600 nm	232	Orange		[52]
Phycocyanin	610–625 nm	232	Blue	Food colorant (ice cream, sweets); cosmetics; Immunofluorescence techniques; Antibody labeling,	[53]
Allophycocyanin	650–660 nm	105	Bluish green		[54]
Carotenoids					
β-Carotene	425, 450, 480 nm	536.87	Orange	Antioxidants, used as pro-vitamin A	[55]
Astaxanthin	477 nm	105	Red	Antioxidant, Anti-cancer	[56, 57]

Table 2.1 Cyanobacterial pigments and their applications (Cont.)

Pigment	Absorption spectrum	Molecular weight (kDa)	Color	Uses and activity	Ref.
Fucoxanthin	420, 444, 467 nm	658.91	Olive-green	Anti-obesity, anti-proliferative activity	[58]
Lutein	425, 448, 476 nm	568.87	Yellowish red		[46]
Zeaxanthin	428, 454, 481 nm	568.87	Yellow	Used as in age related eye diseases, anti-cancerous activity	[59]
Scytonemin	384 nm	544.56	Yellow, brown	Anti-proliferative, anti-inflammatory.	[54, 60]

2.6.3 Phycobiliproteins

Phycobiliproteins are colored tetrapyrrole biliprotein which absorbs light in the region of the visible spectrum in which chlorophyll show low absorption. These pigments are arranged in specific arrangement called phycobilisomes [61]. as shown in Figure. 2.9. These proteins are generally divided into different categories by their absorption spectrum, namely phycoerythrin (PE), phycocyanin (PC), and Allophycocyanin (APC) having absorption maxima of 565 nm, 620 nm, and 650 nm, respectively. These are further classified into two categories by color, Phycoerythrin (red) and phycocyanin (blue), the absorption spectrum and intensity of different phycobilliproteins are given in Table 2.1.

Phycocyanin contains natural blue pigment and is present in almost every phycobiliproteins containing organisms predominately in cyanobacteria. Phycocyanin divided into three types namely, C-phycocyanin (615–620 nm), Phycoerythrocyanin (575 nm) and R-phycocyanin (615 nm). The different species of cyanobacteria, which are reported to produce phycocyanin are *Arthrospira* (*Spirulina*) *plantesis*, *Arthrospira* (*Spirulina*) *maxima*, *Pyrophyridium* sp., *Synechocystis* sp. etc [9]. Phycocyanin has application in a different domain, such as food additives, health food, cosmetic, pharmaceutical and medicine. It has pharmaceutical application, as it is known to have antiinflamtory, anticancerous and antiantioxidant activities [9,62]. Phycoerythrin is a red colored phycobiliproteins which are also divided into different classes like R-PE, B-PE, and C-PE [63]. These names were given on the basis of organism from which these were first isolated (R-PE from rhodophytes, B-PE from *Baigiales* (Red sea alga) and C-PE from cyanophytes). Their absorption maxima are 565, 545 and 563 nm respectively.

2.7 Phycobiliproteins Characteristics

Cyanobacteria are microalgae lack chlorophyll *b*, absorbing light mainly in the blue and red region of the visible spectrum due to chlorophyll *a*. Then, phycobilisomes (PBSs) act as molecular complexes to compensate for the large absorption gap and optimize light energy gathering. The phycobilisomes are complex macromolecular structures attached to the cytoplasmic surfaces of thylakoid membranes (Figure 2.10). Interestingly, each phycobilisome is made up of colored proteins called phycobiliproteins (PBPs), such as the blue colored phycocyanin and the red-colored phycoerythrin. These molecules are arranged in an antenna-like shape; perform in light harvesting and energy transfer to the center of the reaction of photosystem II with an efficiency higher than 95% (Figure 2.9) [44]. Phycobiliproteins are considered accessory pigments of chlorophyll, and they are composed primarily by two subunits: α subunit (MW 12 - 19 kDa) and β subunit (MW 14 - 21 kDa), each type consisting of 160 - 180 amino acids depended on cyanobacterial strain [64]. The color of these molecules is given by the bilin chromophore (phycobilin), which is a linear tetrapyrrole prosthetic group that binds the apoprotein through a thioether bond to cysteine

residues and its absorption spectrum depends on the protein attachment as in figure 2.11 [65]. The prosthetic group linked to the apoprotein can be a phycocyanobilin (phycocyanin) or phycoerythrobilin (phycoerythrin) as in Figure 2.12

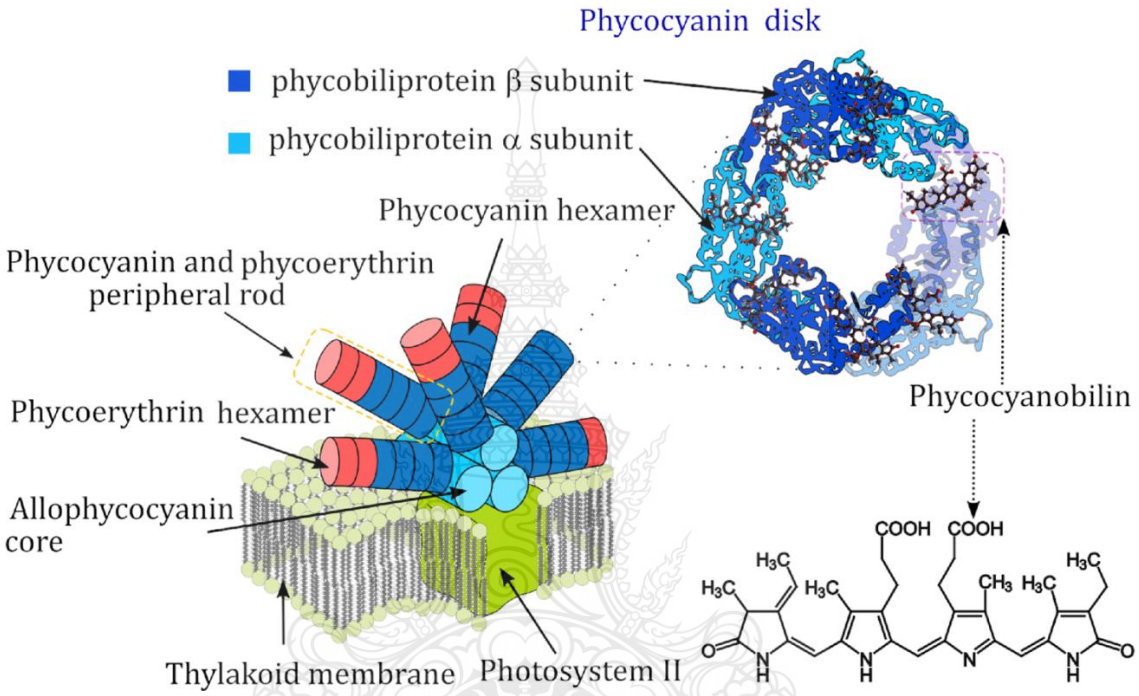


Figure 2.10 The general structure of a phycocyanin rich phycobilisome of cyanobacteria which attached to tetrapyrrole prosthetic groups, phycocyanobilin [1].

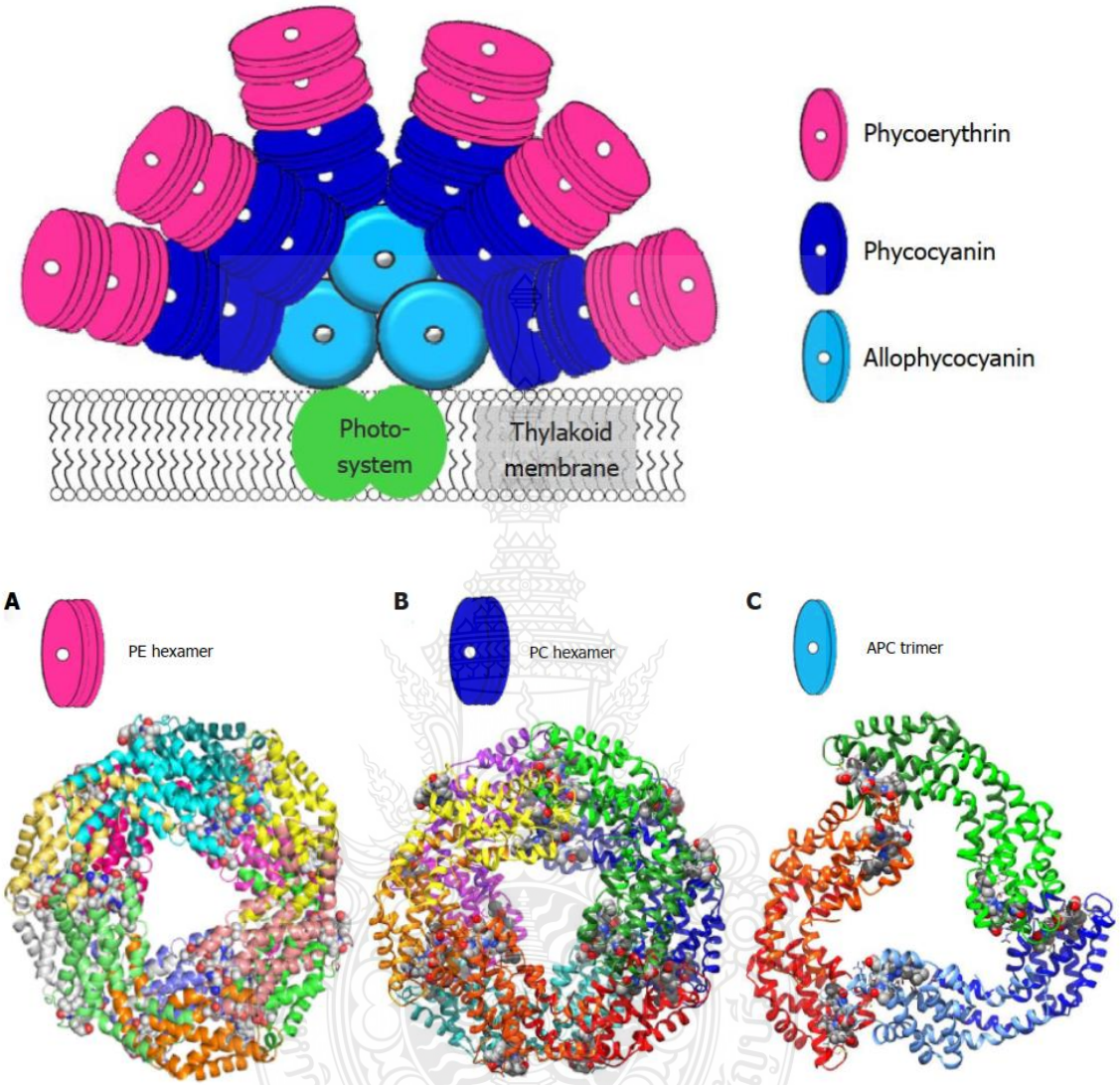
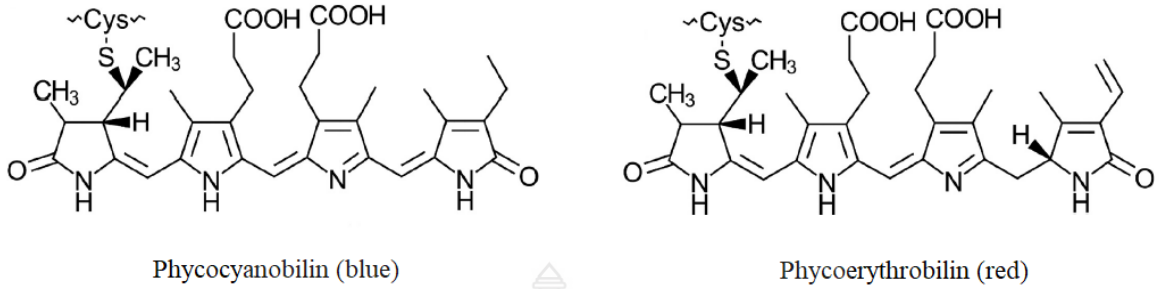


Figure 2.11 The phycobiliptotein is mainly made up of hexameric and trimeric disks of phycobiliptoteins. Schematic diagram and ribbon model of the structure of A: Phycoerythrin-hexamer; B: Phycocyanin-hexamer; C: Allophycocyanin-trimer. The apoprotein and chromophores are shown by ribbon and ball-stick model, respectively [66].



Phycocyanobilin (blue) Phycoerythrobilin (red)

Figure 2.12 Chemical structure of bilin chromophores in phycobiliproteins [65].

Phycobilins are noncyclic tetrapyrroles, linked by α, β, and γ single carbon bridges. (Figure 2.13) [67]. These compounds have molecular masses of about 600 $g.mol^{-1}$. Unlike hemes and chlorophylls, phycobilin molecules do not have metal atoms. In terms of structure, four types of phycobilins are found in cyanobacteria: phycocyanobilin, phycoerythrobilin, phycourobilin and phycoviolobilin [68,69]. Phycocyanobilin ($C_{33}H_{38}N_4O_6$) is an isomer of mesobiliverdin. This compound is present in the chromophore of the biliprotein phycocyanin and also in allophycocyanin, and it is covalently linked to the phycobiliproteins by a thioether bond [70]. Phycoerythrobilin ($C_{33}H_{38}N_4O_6$) is a red phycobilin which is the terminal acceptor of energy of phycoerythrin. Phycoerythrobilin may be also present in phycocyanin in some rhodophytes or oceanic cyanobacteria. Phycoerythrobilin is covalently linked to the phycobiliproteins by a thioether bond. The ratio of phycoerythrobilin in phycoerythrin varies according to the species [71]. Phycourobilin ($C_{33}H_{42}N_4O_6$) is an orange colored molecule involved in the phycoerythrin protein, thus, this chromophore is always an electron donor to phycoerythrin. It was first observed in red algae and represents an important adaptation of marine organisms due to its maximum absorption at 495 nm, the part of light that goes deeper in the water [72,73]. Phycoviolobilin ($C_{33}H_{42}N_4O_6$) is an isomer of phycocyanobilin which absorbs light of shorter wavelengths, which permits the organism to survive in deeper layers of the water column [74,75].

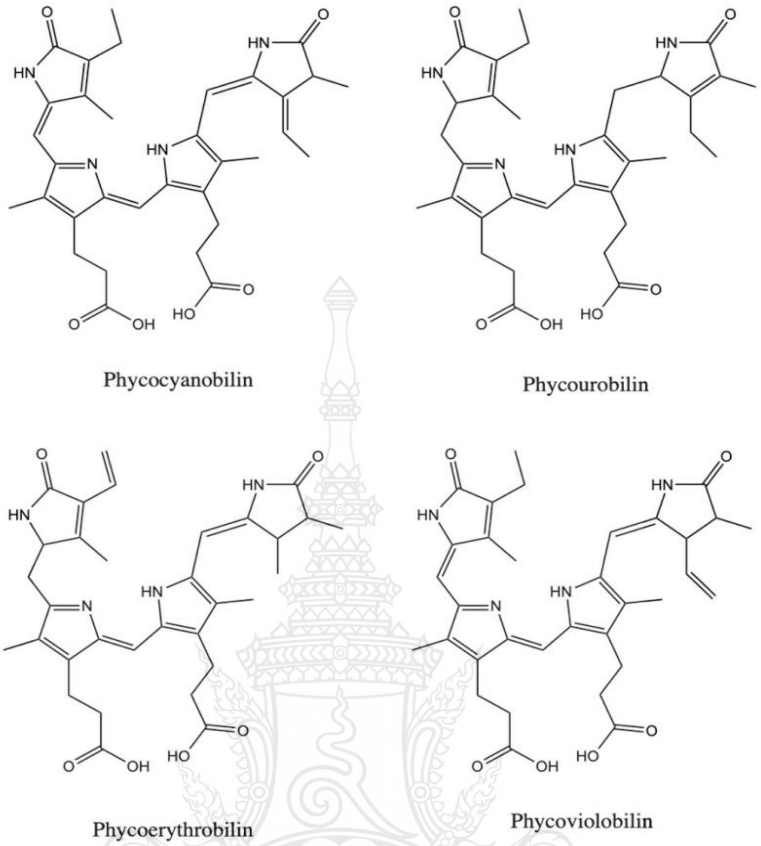


Figure 2.13 Structure of the four phycobilins found in cyanobacteria and red algae: phycocyanobilin (M = 588.6 g·mol^{-1}); phycoerythrobilin (M = 586.6 g·mol^{-1})

Besides light harvesting action, PBSs also play an important role in photoprotection mechanisms under high irradiances [76]. Moreover, PBSs may also be utilized as nutrient source under nitrogen and phosphorus starvation conditions [77], which is the family of brilliantly colored water soluble pigment proteins. PBPs are classified based on their spectral characteristics. Phycoerythrin (PE, λA max = 540 - 570 nm; λF max = 575 - 590 nm), phycocyanin (PC, λA max= 610 - 620 nm; λF max: 645 - 653 nm) and allophycocyanin (APC, λA max = 650 - 655 nm; λF max = 657 – 660 nm) are the majorly found PBPs (Figure 2.14) [51,78]. The bright color, nontoxic protein nature and antioxidant virtues of PBPs open up the doorway for their potential application in various industries.

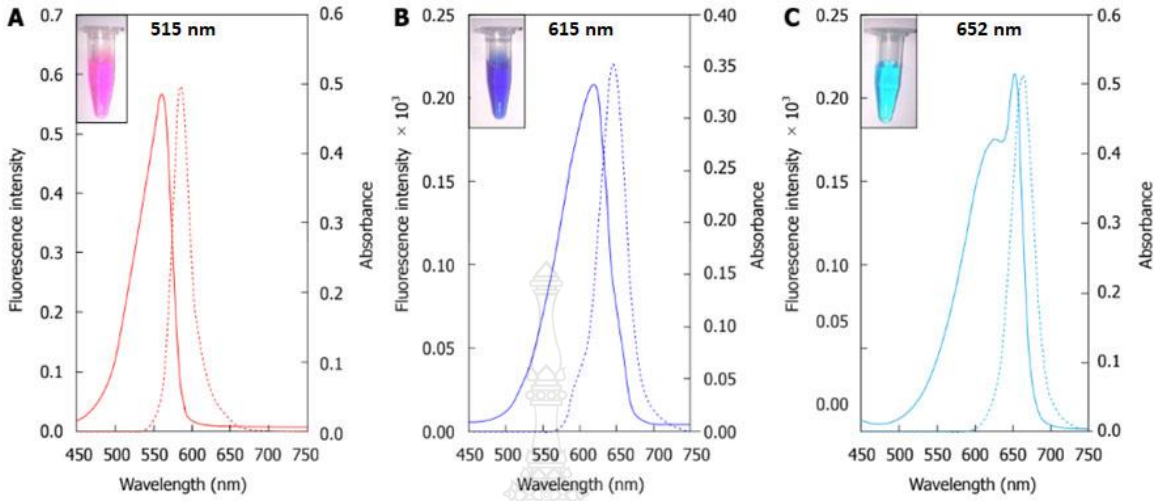


Figure 2.14 UV-visible absorbance (solid line) and fluorescence emission (dotted line) spectra, and appearance (inset) of A: Phycoerythrin, B: Phycocyanin and C: Allophycocyanin [66].

2.8 Phycobiliproteins Biosynthetic Pathway

Biosynthesis of phycobiliproteins in cyanobacterial cells is carried out through the transcription, translational and posttranslational pathways through which there are synthesis of amino acids, proteins and phycobilins, and finally, the ligation of phycobilins synthesized to apoproteins to form the phycobiliproteins during the posttranslational phase [79,80]. The mechanisms of phycobilins (i.e., chromophores of phycobiliproteins) biosynthesis that are ligated to the apoproteins to form the phycobiliproteins as depicted in Figure 2.15. Phycobilins are linear tetrapyrrole pigments that function as light harvesting entities in photosynthetic mechanisms of microalgae [81,82]. Phycobilins are biosynthesized from heme by the action of heme oxygenase, which converts heme to biliverdin, followed by the action of other enzymes that convert biliverdin to the phycobilins [83] and finally, phycobilins are covalently linked to different specific cysteine residues via thioether bonds by the bilin lyases; the latter process is considered as last step of phycobiliprotein biosynthesis. Heme is a cofactor with diverse biological functions including modulation of

enzymes activities etc. [84]. Heme molecule is believed as the suitable metabolic pathway of all biosynthetic pathways of all billins [85]. The heme synthesis has the core center on aminolevulinic acid (ALA) synthesis from either on glutamate or pyruvate or succinyl CoA as immediate precursor of heme but the succinyl coenzyme A (CoA) is more immediate precursor as shown (Figure. 2.15). Heme synthesis is carried out in the cytoplasm and mitochondria through eight main steps in case the succinyl coenzyme A is immediate precursor [86]. The heme mechanism starts from the condensation of succinyl CoA and glycine to form ALA [86,87]. The latter condensation is followed by other enzymatic reactions that lead to different intermediate molecules such as porphobilinogen (PGB) containing pyrrole ring and two side genes, acetate group A and propionate group P [88], uroporphyrinogen III, corproporphyrinogen III [89], protoporphyrinogen, Protoporphyrin IX, the heme synthesis is accomplished when Ferrochelatase intervene in addition of the ferrous to Protoporphyrin IX to form heme. The heme undergoes an oxidative cleavage by heme oxygenase to form biliverdin IX (BV) [85]. The biliverdin is an intermediate, i.e., common biosynthetic precursor molecule, in the biosynthesis of phycobilins; but there some cases where the biliverdin can be or not the intermediate molecule for the phycocyanobilin biosynthesis. If heme cleavage is directly to bile pigment before reduction, then biliverdin would be an intermediate in phycocyanin synthesis. In case one or both of the reduction step(s) occur(s) before heme cleavages then biliverdin would not be an intermediate [90]. Three main phycobilins are directly formed from the biliverdin: Phycocyanobilin synthase (PcyA) catalyzes the biliverdin to form phycocyanobilin, biliverdin reduction catalyzed by ferrodoxin oxidoreductase (PebA) lead to 15, 16-dihydrobiliverdin, which undergoes further reduction by ferrodoxin oxidoreductase (PebB) catalyst to form phycoerythrobilin; the third phycobilin formed from the biliverdin is phytochromobilin through the Ferrodoxin oxidoreductase (H2Y) catalysis. Apart of this first group of phycobilins produced from heme via biliverdin (i.e., commonly known ones: phycocyanobilin, phycoerythrobilin and photochromobilin), there is another second group of phycobilin which includes phycoviolobilin (PVB) and phycourobilin (PUB). The second group of phycobilins containing a vinyl group at C3 and they cannot be cleaved directly from the phycobiliprotein

but they should be produced by other mechanisms such as isomerization [90]. During the posttranslational phase, the phycobilins tetrapyrrole formed are usually attached to specific cysteine residues within the alpha and beta subunits via thioether linkages by bilin lyases [69,91].

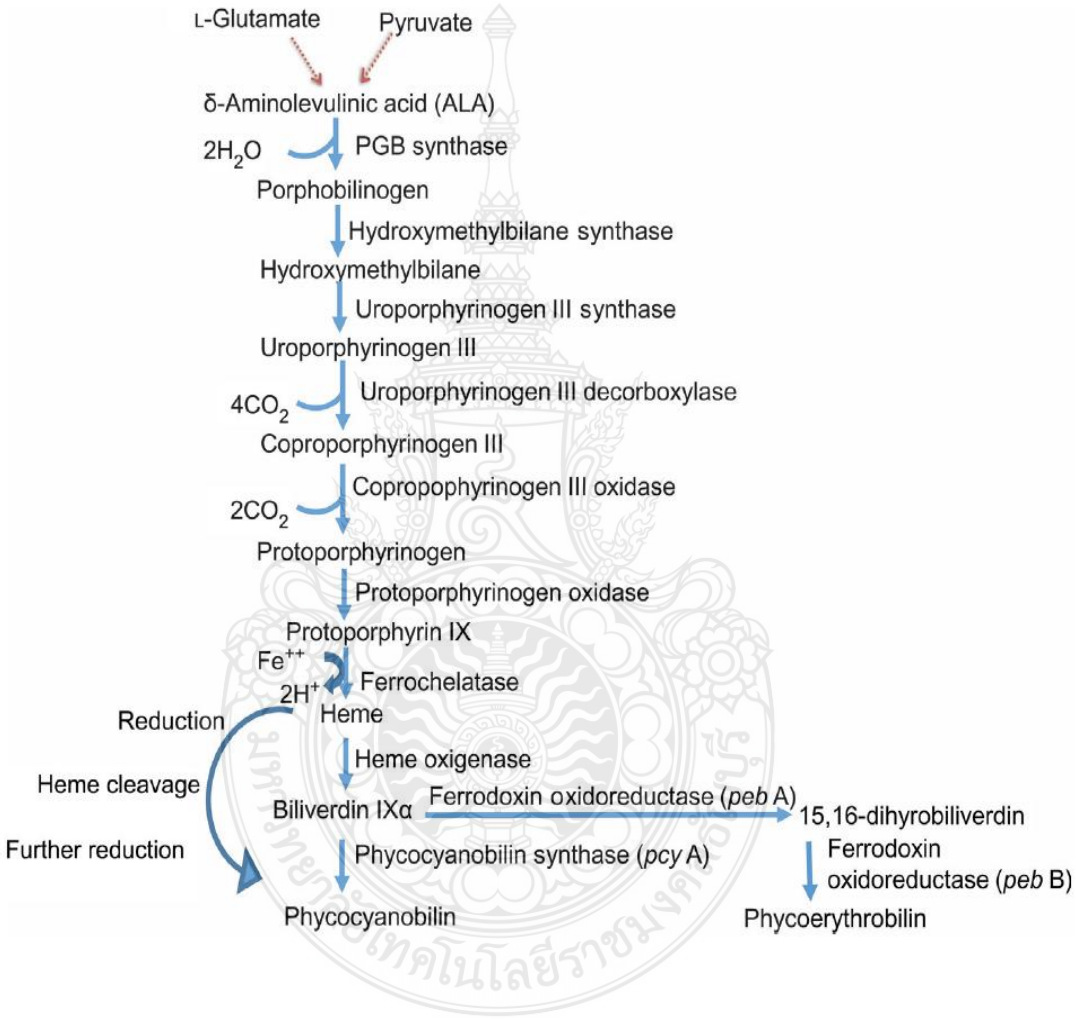


Figure 2.15 The biosynthetic pathway of phycobilins (chromophores of phycobiliproteins)

2.9 Production of Phycobiliproteins

Microalgae can be cultivated in open (raceway type or open circular ponds tanks) or closed systems (Photobioreactors (PBR)). Open systems are inexpensive and easy to operate, however, they have some disadvantages such as the high risk of contamination, poor illumination, and lower biomass productivity compared to closed systems [92]. Furthermore, the cultivation of microalgae can be carried out under autotrophic, heterotrophic or mixotrophic conditions (a combination of the first two), depending on the metabolism of the species used. In autotrophic cultivation, sunlight is commonly used as energy source and atmospheric air as a CO_2 source, therefore, the microalgal biomass production can be economically viable [93]. However, the autotrophic cultivation depends mainly on light intensity, hence the production of biomass can decrease if the light level is too low (photo-limitation) or too high (photo-inhibition). In the case of heterotrophic cultivation, microalgae assimilate available organic compounds, such as glucose and acetate, as a source of carbon in the dark [94]. The growth depends on the organic substrate and the low or high organic carbon substrate concentration may limit cell growth. Organic carbon sources lead to increased microalgae production but also contamination risks hence the compounds obtained may not be suitable for application in the food industry [95]. It is noteworthy that not all microalgal strains have the adaptability to grow under heterotrophic or mixotrophic conditions. Nowadays, phycocyanin is produced commercially in autotrophic cultures of cyanobacteria *Arthrospira platensis*, mainly in open ponds and raceways. *Arthrospira platensis* is often chosen as a phycocyanin producing strain due to its ubiquity rather than its pigment content [96]. Phycoerythrin, on the other hand, is mainly produced from the microalgae *Porphyridium* spp. [97,98], however, there is no commercial process established for large scale production so far [98]. PBR may be the method of choice for the production of phycoerythrin due to the high purity required as a reagent for immunoassays [93]. Even though microalgae could be a potential source of phycobiliproteins, large scale production of phycobiliproteins is limited due to low biomass production and low pigment accumulation in its cells [95]. This problem can be overcome by using optimal growth conditions and develop economically viable processes. In order to improve phycocyanin and phycoerythrin

production as well as microalgae growth, various physicochemical parameters such as nutrients, organic and inorganic carbon source, light source, pH and temperature have been studied [95,99]. It is possible to maximize the produced phycobiliprotein amount by modifying the aforementioned physicochemical parameters. For example, the phycocyanin content (mg·g biomass^{-1}) of *Arthrospira platensis* obtained using red light can be 5–7 times higher compared to blue light mixed with red light [100].

2.10 Utilization of Phycobiliproteins

Over past few decades, microalgae have been used in conventional agriculture for production of value-added biomass for human welfare. The demand of incredible color of PBPs has been increased in value-added science. In current scenario, PBPs have commercialized for the development of value-added products in various sectors such as food, pigments, nutraceuticals, pharmaceutics, biomedical, and biotechnology industries [101]. It has been reported that food grade, reagent grade, and analytical grade PC considered on the basis of their purity ratio (A620/280) ≤0.7, 0.7–3.9, and ≥4.0, respectively [102,103].

2.10.1 Natural Dye and Nutraceuticals

The applications of synthetic dyes in food products are potential threats for human life. Continuous increasing of threatened disease by food toxicants, a causal demand of natural dye has been increased for coloring food products which help in the reduction of life-threatened disease. Recently, the role of natural dye in food products for health beneficial effects, minimum side effects, and potential candidate for treatment of certain disease has been demonstrated [104–106]. In certain country, PBPs are frequently used as natural coloring agents for food, dairy products, beverages, candy, sweet gum, and ice cream. In red algae, most of the pink dye (PE) is obtained from *Porphyridium* sp. and used as coloring agent for gelatin deserts and confectionary products [107]. Commercially, three strains are available such as *Porphyridium* sp., *Rhodella* sp., and *Spirulina* sp. for extraction of dye for food additives [108]. In European country, PBPs have also been involved in traditionally supplied food products like pasta, cereals, biscuits, and ice cream as nutraceuticals. In PBPs subunits, PC is used most frequently as food dye and food storage due to great ability of

preservation [109,110]. In ultraviolet radiation, PE exhibits intense yellow fluorescence that is used for special effects on foods in dark condition. PC or PE has been used in certain food products like candies, soft drinks, lollipops, and various beverages for special effects under UV radiation [107]. Most of the PC products are thermal sensitive, thus blue color may fade by increasing temperature in candy preparation. However, color of PC is retained inside the jelly gum [111]. Sometimes, long exposure of solar radiation reduces the blue color of jelly gum. PE is more tolerable than PC but it is sensitive toward light and oxygen concentration [112]. PC shows strong antioxidative activity, therefore, it is widely adopted as antioxidants in food supplement [113]. The food-grade preservatives are important ingredients that play significant role in stability of food products for long duration. The self-life of PC could be improved by addition of certain preservatives such as citric acid, sodium chloride, and sucrose [114]. In addition, benzoic acid is effective preservative agent for expanding self-life of PC, PE by their prominent behavior of antimicrobial and antioxidant activity [110]. In the current scenario, most of the companies are involved in the production of additive food and neutraceutical products from cyanobacteria. DIC (Earthrise Farms) is the major industry in California, United States, that covers an area of about 444×103 (m^2) for the production of biomass of *Spirulina* sp. and organic tablets for utilization as nutraceuticals. The products of *Spirulina* biomass are marketed in more than 20 countries. Similarly, Cyanotech is another company located in Hawaii Island (Kona district). They have used cyanobacterial biomass for the production of value-added products [101,115].

2.10.2 Pharmaceutical Agents

Recently, eco-friendly approach for the generation of antibiotics, pharmaceutics products, and fluorescent tags has been widely accepted due to continuous incident of microbial drug resistance. The multifunctional colored pigments such as PC, PE, and APC have more advantage and potential over synthetic pharmacological products including various nutraceuticals supplements, antioxidants, antiinflammatory, antitumor and antiaging property (Table 2.2). It has been clearly demonstrated that PC from *Aphanizomenon* sp. shows strong antioxidative property [116]. In addition, PC has an ability to inhibit peroxyl radicals induced by hemolysis of blood vessels. Thus, PC has an ability to

inhibit the progressive oxidation process inside blood for prevention of aging and several pathological diseases. Apart from antioxidative property, they also inhibit the development process of edema, histamine, and leukotriene in inflammation [101].

Table 2.2 Pharmaceutical potentials of PBPs

Pharmaceutical potentials and its consequences	References
Antioxidants (inhibition of free radicals and lipid peroxidation-induced damage)	[101, 117]
Antiinflammatory (inhibition of glucose oxidase)	[117]
Antiatherosclerosis (antioxidant activity enhancer and free radicals inhibition)	[118]
Neuroprotective (inhibition of neural damage)	[119]
Nephroprotective (inhibition of lipid peroxidation and oxalic acid formation)	[120]
Anticancer potential (apoptosis activator, leukemia inhibitors)	[121]
Eye protection (inhibition of sodium selenite-induced cataract)	[122]
Lung protection (inhibition of paraquat-induced free radical generation with lipid peroxidation)	[123, 124]
Cardio protection (inhibition of ischemia-reperfusion-induced damage by free radicals)	[125]

Adapted and modified from [96].

2.10.3 Fluorescent Agents

Autofluorescent nature of PBPs has been widely used in scientific research and commercial biotechnology industries (Table 2.3). Indispensable fluorescence property of PBPs is associated with chromophores, high stokes shift, rate of quenching, high quantum

yield, fluorescence emission rate, and medium solubility [117]. Thus, it might act as a strong fluorophore for the commercial utilization. The fluorescence properties of PBPs have been utilized as marker in antibody receptor and donor in immunological research laboratories. Moreover, PBPs are used for the conjugation with proteins A, avidin, shorting out of single cells (fluorescence-activated cell sorting, FACS), histochemistry, biotinlabeled DNA fluorescence, and immunoassays [96]. In contrast to PC, PE is widely used as second fluorescent probes by the presence of intense yellow fluorescence and high quantum yield [126]. The fluorescence property of PBPs has been utilized for monitoring of HIV and developments of cancer. Recently, multicolor fluorescence analyses are formulated by utilization of energy-transfer mechanism in PE chromophores among Cy5 and Cy7 dye [127]. In cryptomonads, PBPs have lower molecular weight, thus it is great advantage for application in flow cytometry [128]. Although, fluorescent properties of PC have also been used for monitoring of ecological diversity [129]. And identification of toxic cyanobacteria [130].

2.10.4 Cosmetics

In modern research and development, cosmetics are crucially important and most economical ever demanding field for beauty care products [131]. A cosmetic product from synthetic source is highly toxic and causes a number of pathological diseases in human. Pigments of PBPs exhibit sustainable and toxin free approaches for the development of cosmetics products with negligible side effects [132]. In Japan, food and drug administration (FDA) has mandate for the utilization of PBPs as a coloring material for food products. The DIC Company (Dainippon Ink & Chemicals) has launched a Lina-Blue commercial dye for the development of cosmetic products [101]. Recently, a number of companies have developed several products like colored soaps, fairness cream, body lotion, lipsticks, eyeliner, etc.

Table 2.3 Fluorescence potentials of PBPs and its commercial utilization

Fluorescence applications	References
Fluorochromes, fluorescent probes, multimodel color fluorescence	[123, 137, 139, 140]
DNA fluorescent probes	[135]
Fluorescence resonance energy transfer, fluorescence activated cell sorter	[117]
Fluorescent probes for immunoassays	[128]
Photocurrent and fuel cell	[136]

Adapted and modified from [101]

2.10.5 Clinical Significance

Clinical significance of PC has been recorded in heart, kidney, lungs, cancer, neurological disease, antiinflammation, cataract, and liver diseases (Figure 2.16). Interestingly, fluorescence nature of PC has been widely accepted for the inhibitory role on malignant cell of leukemia (K562) [137]. PC exhibits remarkable inhibition for heart malfunctions by alteration of blood enzymes aspartate amino transferase, alanine amino transferase, and malondialdehyde [138]. PC had the ability to control thyroxin hormone by regulating nitrite molecules [139]. The activity of PC is widely accepted as potent anticancer in various cell types like breast cancer [140], lung cancer [141], and bone marrow cancer [142]. In colon cancer, PC had reduced cell viability (HT-29, A549 cell lines) by induction of apoptosis phenomena [143]. PC had also been used as antiplatelet therapy in arterial thrombosis [144]. It was found that PC inhibits the CCl4-induced damage in liver cells [145]. The protective effects of PC have also been found from atherogenesis disorder inhibit by scavenging of free radicals generation and reduction of high-density cholesterol [118].

PC has also reduced ischemia reperfusion-induced malfunction in heart by inhibition of free radicals [125]. The lung injury has also been prevented by PC molecules [124]. PC has the ability to reduce the formation of calcium oxalate and protect kidney from the damage by stone [120]. However, in vivo administration is hindered due to heavy molecular weight of PC. Thus, PC is needed to disintegrate into monomeric form prior to administration in living body [146]. The heteromeric components of PC (α- and β-subunits) show distinct effects related to growth response, apoptosis, and cell cycle arrest on cell lines [147].The utilization of PC as food-grade proteins helps in the inhibition of inflammation of osteoarthritis by regulation of interleukin-6 and cytokines factors [148]. The synthesis of nitric oxide (NO) is a key component for catalytic regulation of inflammation and pathogenesis by generation of free radicals and lipid peroxidation [149]. It was found that PC also inhibits release of histamine of mast cells which play an important role in allergic inflammation [139]. PC has a potent neuroprotective agent which shows significant protective role induced by tributyltin chloride and *N*-acetylcysteine [150]. PC has been found to be effective in the treatment of neurodegenerative diseases such as Alzheimer's, ischemia, and Parkinson's disease [151]. The regular consumption of PC in food material significantly inhibits the liver toxicity by antioxidative hepatoprotective function of P450 enzymes, glucose-6-phosphatase, and aminopyrine-*N*-demethylase [147]. PC has inhibited cisplatininduced renal toxicity and oxidative damage triggered by oxalic acid-mediated free radical stress in kidney [120,143]. The strong antioxidative feature of PC had inhibited the development of cataract [152] by regulation of mRNA expression, redox potential, and apoptotic pathway for maintenance of eye lens [153]. PC also regulates COX-2 enzyme which leads to development of tumor [154], angiogenesis [155], and poststage cancer. Recently, PC has been proposed for the utilization as photosensitizer in photodynamic therapy for the treatment of cancer cells [146].

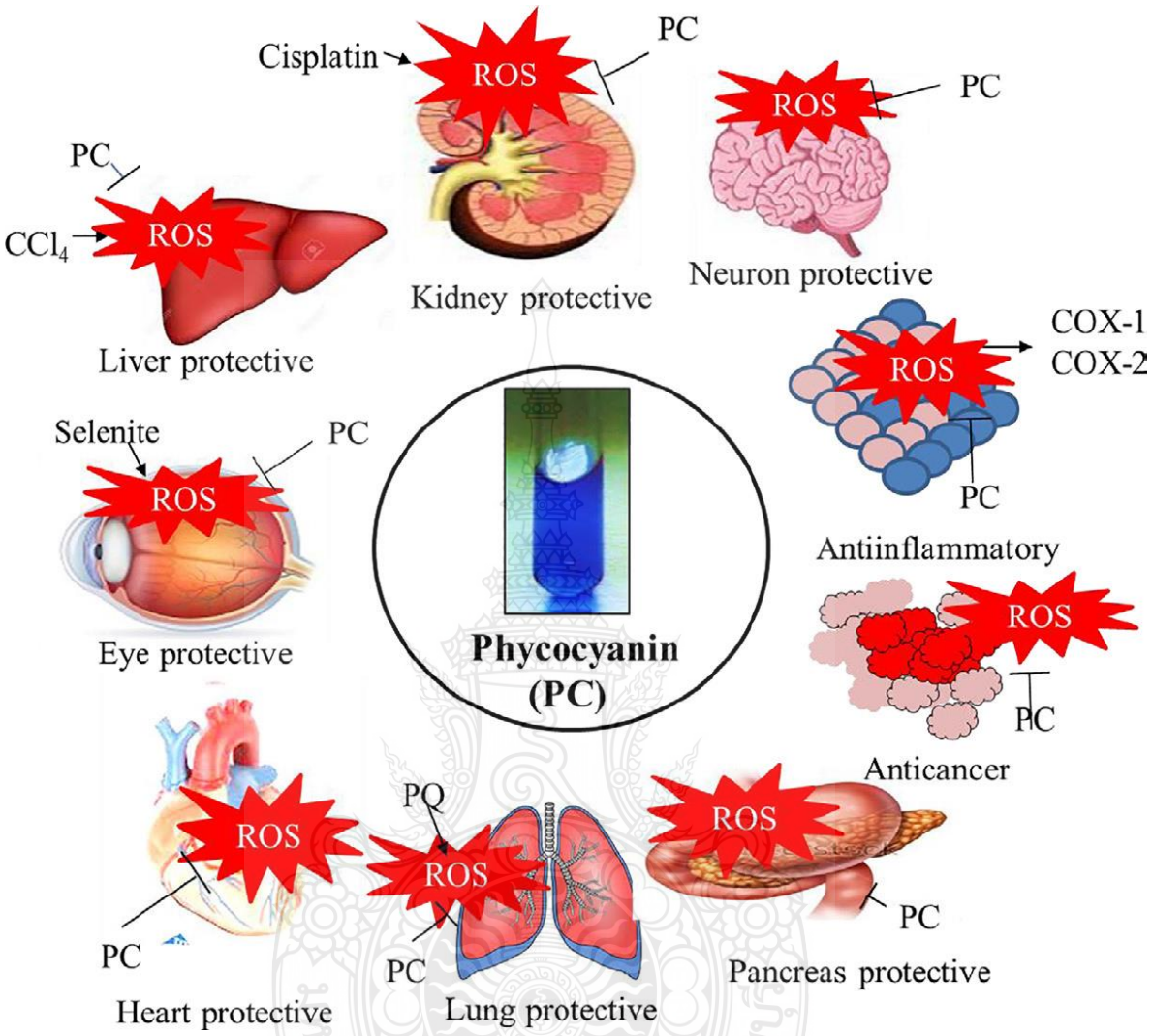


Figure 2.16 Antioxidative and biomedical potentials of phycocyanin (PC) [143].

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Materials

3.1.1 Equipment

3.1.1.1	Autoclave	Model NB-1100, N-BIOTEK, Korea
3.1.1.2	Biosafety cabinet	Mars Safety Class 2, SCANLAF, Denmark
3.1.1.3	Centrifuge	A Vanti centrifuge, J-26, USA
3.1.1.4	Chlorophyll fluorescence microscopy	Olympus DP72, Japan
3.1.1.5	DNA Thermal cycle	Model C1000, BIO-RAD, USA
3.1.1.6	Gel document	BIO-RAD, USA
3.1.1.7	Hot air oven	Model THEROTEC 2000, CONTHERM, New Zealand
3.1.1.8	Incubator shaker	Model Innova 42, NEW BRUNSWICK, USA
3.1.1.9	Light microscope	Olympus, Japan
3.1.1.10	Lyophilizer	OPERON, Korea
3.1.1.11	Fluorescence spectrophotometer	Hitachi, Japan
3.1.1.12	Mini-PROTEIN Tetra cell	BIO-RAD, USA
3.1.1.13	Scanning electron microscope (SEM)	JEOL, Japan
3.1.1.14	Spectrophotometer	GENESYS 10S UV-Vis Thermo SCIENTIFIC, USA
3.1.1.15	Transmission electron microscope	JEOL, Japan

3.1.1.16	Water bath	Model XUBA3, Grant, UK
3.1.1.17	GFC membrane	Whatman, UK
3.1.1.18	PCR tube	BIOLOGIX, USA
3.1.1.19	96-well microplate reader	EZ Read 2000, Biochrom, Germany

3.1.2 Chemicals

3.1.2.1	Acetic acid	KEMAUS, Osteria
3.1.2.2	Agar powder	BIO AGAR
3.1.2.3	Agarose powder	BIO AGARS
3.1.2.4	Ammonium sulfate	KEMAUS, Osteria
3.1.2.5	Ascorbic acid	Sigma-aldrich, Germany
3.1.2.6	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)	SRL, India
3.1.2.7	Butylated hydroxytoluene	KEMAUS, Osteria
3.1.2.8	Coomassie blue G-250	BIO-RAD, USA
3.1.2.9	Citric acid	KEMAUS, Osteria
3.1.2.10	N, N-dimethyl formamide (DMF)	Sigma-aldrich, Germany
3.1.2.11	Dimethyl sulfoxide (DMSO)	Sigma-aldrich, Germany
3.1.2.12	dNTPs mix	Invitrogen, Thermo scientific USA
3.1.2.13	2,2-diphenyl-1-picrylhydrazyl (DPPH)	SRL, India
3.1.2.14	Ethanol	RCi Labscan, Thailand
3.1.2.15	Ferric chloride	KEMAUS, Osteria
3.1.2.16	Glycerol	KEMAUS, Osteria
3.1.2.17	Methanol	RCi Labscan, Thailand

3.1.2.18	10X PCR buffer	Invitrogen, Thermo scientific USA
3.1.2.19	Phosphate buffered saline (PBS)	Sigma-aldrich, Germany
3.1.2.20	Potassium persulfate	KEMAUS, Osteria
3.1.2.21	Sodium chloride	KEMAUS, Osteria
3.1.2.22	Sodium nitrate	KEMAUS, Osteria
3.1.2.23	Tag DNA polymerase	Invitrogen, Thermo scientific USA
3.1.2.24	Trichloroacetic acid (TCA)	SRL, India
3.1.2.25	2,4,6-Tri(2-pyridyl)-s triazine (TPTZ)	SRL, India

3.1.3 Microorganisms

3.1.3.1 *Bacillus subtillis* TISTR 152

3.1.3.2 *Candida albicans* SU. 28

3.1.3.3 *Escherichia coli* ATCC 25922

3.1.3.4 *Propionibacterium acnes* DMFT 14916

3.1.3.5 *Staphylococcus aureus* ATCC 25923

3.2 Study Site and Sampling

The 23 karst caves in four regions widespread along of Thailand were selected for sampling locations as shown in Table 1. Map location and general view of sampling sites were represented in Figure 3.1 - 3.7. Samples were collected from June to September 2019 which was favorable season for cyanobacterial growth. Four different sampling sites were selected inside the cave including rocks and cave walls. Samples were scraped off with a scalpel and spatula and transferred to a sterile plastic container in order to preserve the natural form of the community. Two zones were distinguished in each cave: (i) the light zone, comprising the entrance (usually lit by sunlight and well-oxygenated); and (ii) the twilight zone of further chambers and rooms illuminated only by weak natural daylight or artificial light. Samples were inoculated in sterilized vial containing 10 ml of enriched algal medium (BG11) maintained in appropriate cultured condition with light intensity lower than 1,000 lux without shaking. The physical parameters, temperature (°C), relative humidity (%RH) and light intensity (lux) were measured in each sampling sites. The samples were examined with a light microscope (Olympus, Japan), and chlorophyll fluorescence microscopy (Olympus DP72, Japan). The accuracy of genus identification of karst cave cyanobacteria was based on morphological features. Nomenclature follows mainly by [156 - 167]. All studied samples were obtained in the Department of Biology, Faculty of science and technology, Rajamangala University of Technology Thanyaburi, Thailand.

Table 3.1 List of sampling locations along the karst cave in this study.

No.	Name of cave	Code	Coordinate location	Locality description
1	Rakang cave	RK	14°42'57.2"N 100°47'50.3"E	Khun Khlon, Phraphutthabat, Saraburi
2	Khao Wong cave	KW	14°40'14.2"N 100°49'44.1"E	KhaoWong, Phaphutthabat, Saraburi
3	Sap ChaOm cave	SCOM	14°41'55.9"N 100°50'36.9"E	Khun Khlon, Phraphutthabat, Saraburi
4	Si Wilai cave	SWL	14°40'14.2"N 100°49'44.1"E	KhaoWong, Phaphutthabat, Saraburi
5	LiangPha cave	LP	14°34'28.9"N 101°08'50.6"E	Mittraphap, Muaklek, Saraburi
6	Suwannakhuha cave	SWKH	8°25'42.1"N 98°28'24.0"E	Kra Som, TakuaThung, Phangnga
7	Phung Chang cave	PC	8°26'33.8"N 98°30'56.5"E	Thai Chang, Mueang, Phangnga
8	Phaya Nak cave	PYN	8°10'06.6"N 98°52'54.3"E	Thap Prik, Mueang, Krabi
9	Thep Prathan cave	TPT	8°10'06.6"N 98°52'54.3"E	Thap Prik, Mueang, Krabi
10	Lang Rongrian cave	LRR	8°10'50.3"N 98°52'48.9"E	Thap Prik, Mueang, Krabi
11	Khao Pi Na cave	KPN	7°44'00.2"N 99°30'58.0"E	NaWong, HuaiYot, Trang
12	Le Khao Kop cave	LKK	7°47'37.4"N 99°34'19.9"E	Khao Kop, HuaiYot, Trang

Table 3.1 List of sampling locations along the karst cave in this study (Cont.)

No.	Name of cave	Code	Coordinate location	Locality description
13	Khao Chakan cave	CHK	13°39'39.4"N 102°05'06.5"E	KhaoChakan, KhaoChakan, Sakaeo
14	Phet Pho Thong cave	PPT	13°24'49.1"N 102°19'31.2"E	KhlongHat, KhlongHat, Sakaeo
15	Nam Khao Siwa cave	SW	13°19'15.1"N 102°19'40.1"E	KhlongKaiThuean, KhlongHat, SaKaeo
16	Sa Rika cave	SRK	13°02'19.0"N 102°16'52.6"E	Saikhao, Soidao, Chanthaburi
17	Khao Wong Kot cave	KWK	12°53'11.4"N 101°49'06.2"E	Khao Wongkot, Kaeng-HangMaeo, Chanthaburi
18	Cha Ang cave	CA	13°09'41.6"N 101°35'51.3"E	PhluangThong, BoThong, Chon Buri
19	Kra Sae cave	KS	14°06'21.8"N 99°09'56.7"E	Lum Sum, Sai Yok, Kanchanaburi
20	Chaloei cave	CHL	14°05'40.5"N 99°10'43.0"E	LumSum, SaiYok, Kanchanaburi
21	Khao Pun cave	KP	13°59'59.4"N 99°30'12.6"E	NongYa, Mueang, Kanchanaburi
22	Khao Bin cave	KB	13°35'34.8"N 99°40'01.1"E	HinKong, Mueang, Ratchaburi
23	Kai Lon cave	KL	12°36'47.3"N 99°42'36.6"E	KhaoKrapuk, ThaYang, Phetchaburi

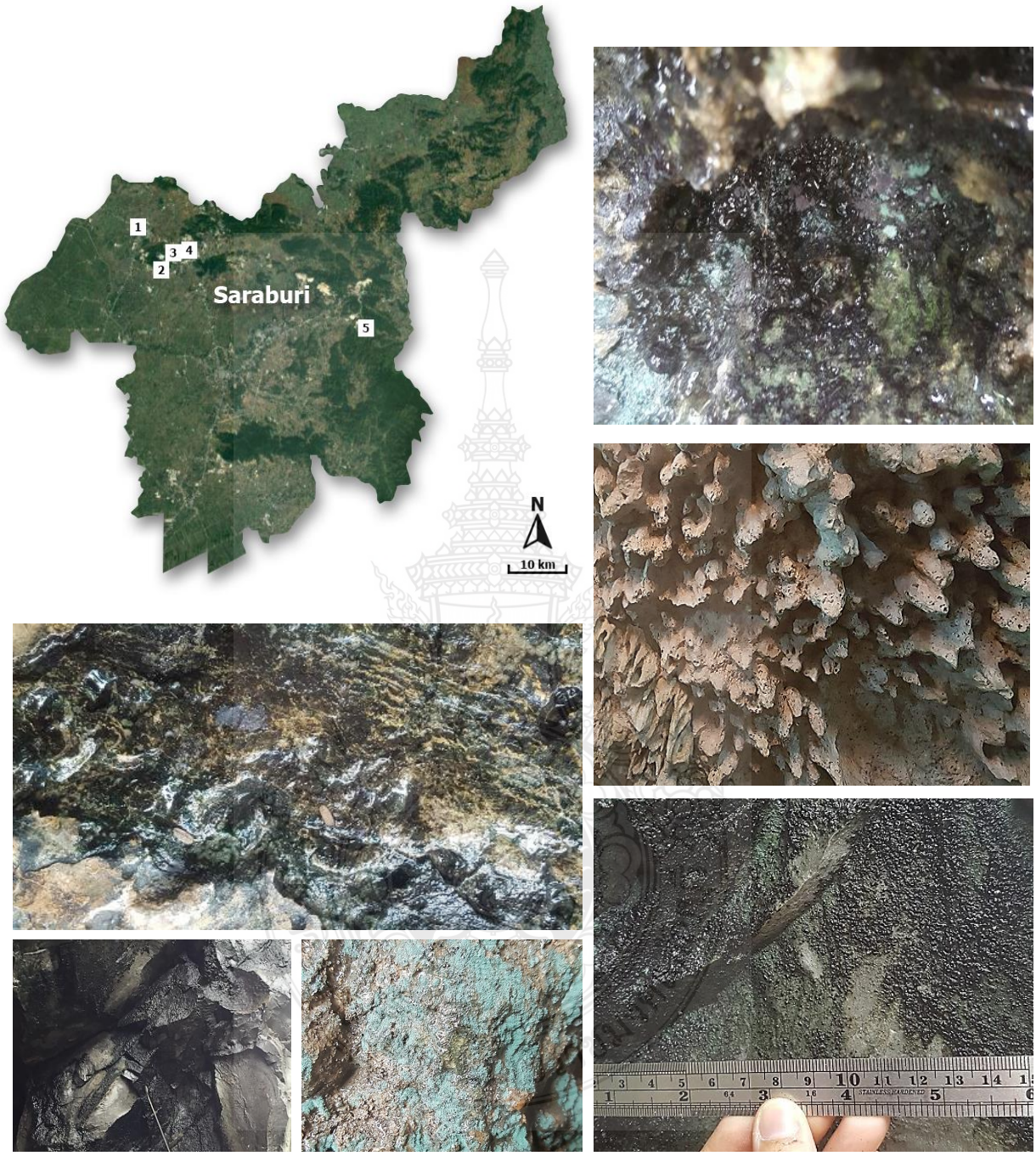


Figure 3.1 Map showing locations and general view of sampling sites in Central region of Thailand;1 Rakang cave, 2 Khao Wong cave, 3 Sap ChaOm cave, 4 Si Wilai cave, 5 LiangPha cave.

Figure 3.2 Map showing locations and general view of sampling sites in Southern region of Thailand; 6 Suwannakhuha cave, 7 Phung Chang cave.

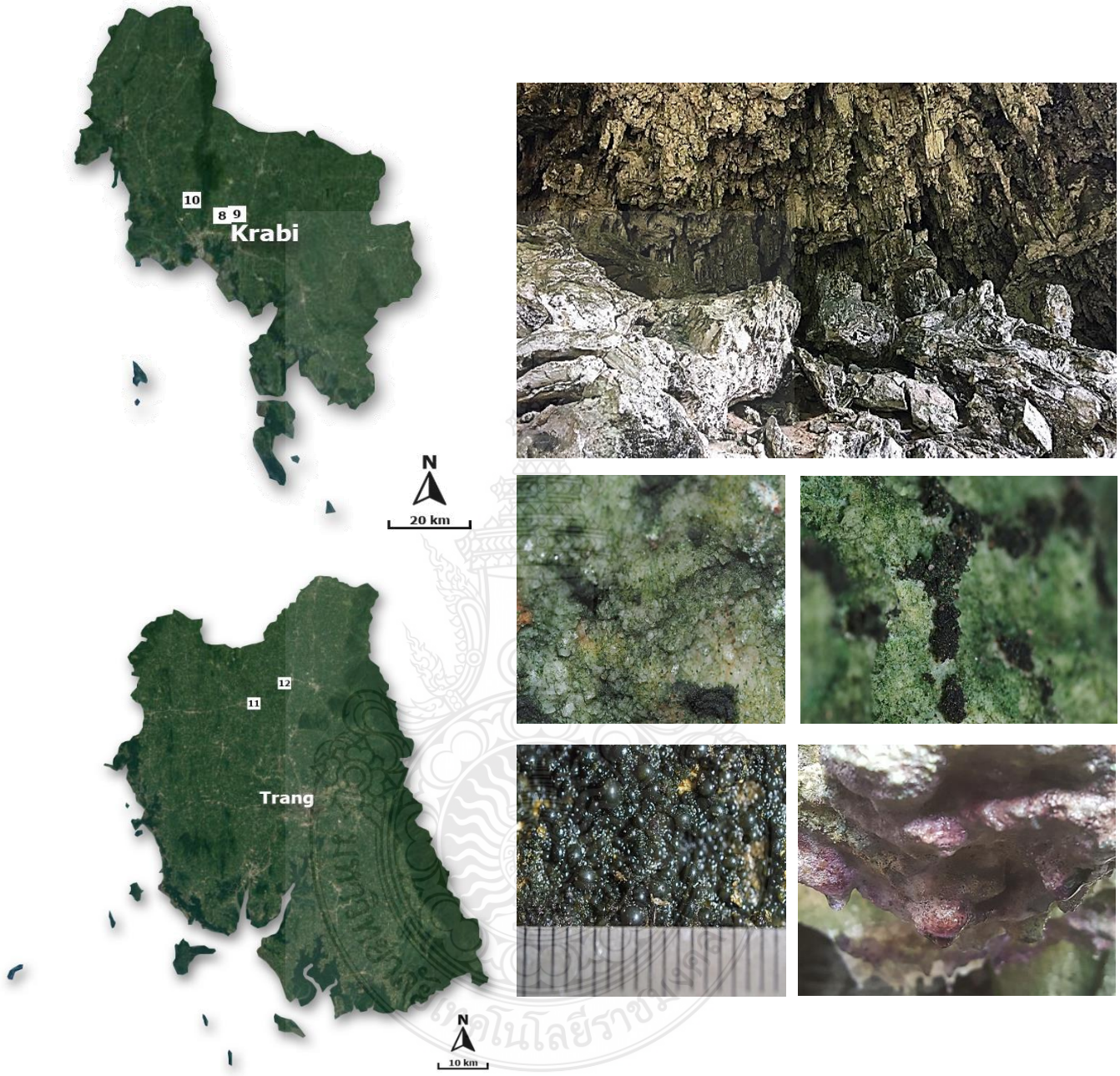


Figure 3.3 Map showing locations and general view of sampling sites in Southern region of Thailand; 8 Phaya Nak cave, 9 Thep Prathan cave, 10 Lang Rongrian cave, 11 Khao Pi Na cave, 12 Le Khao Kop cave.

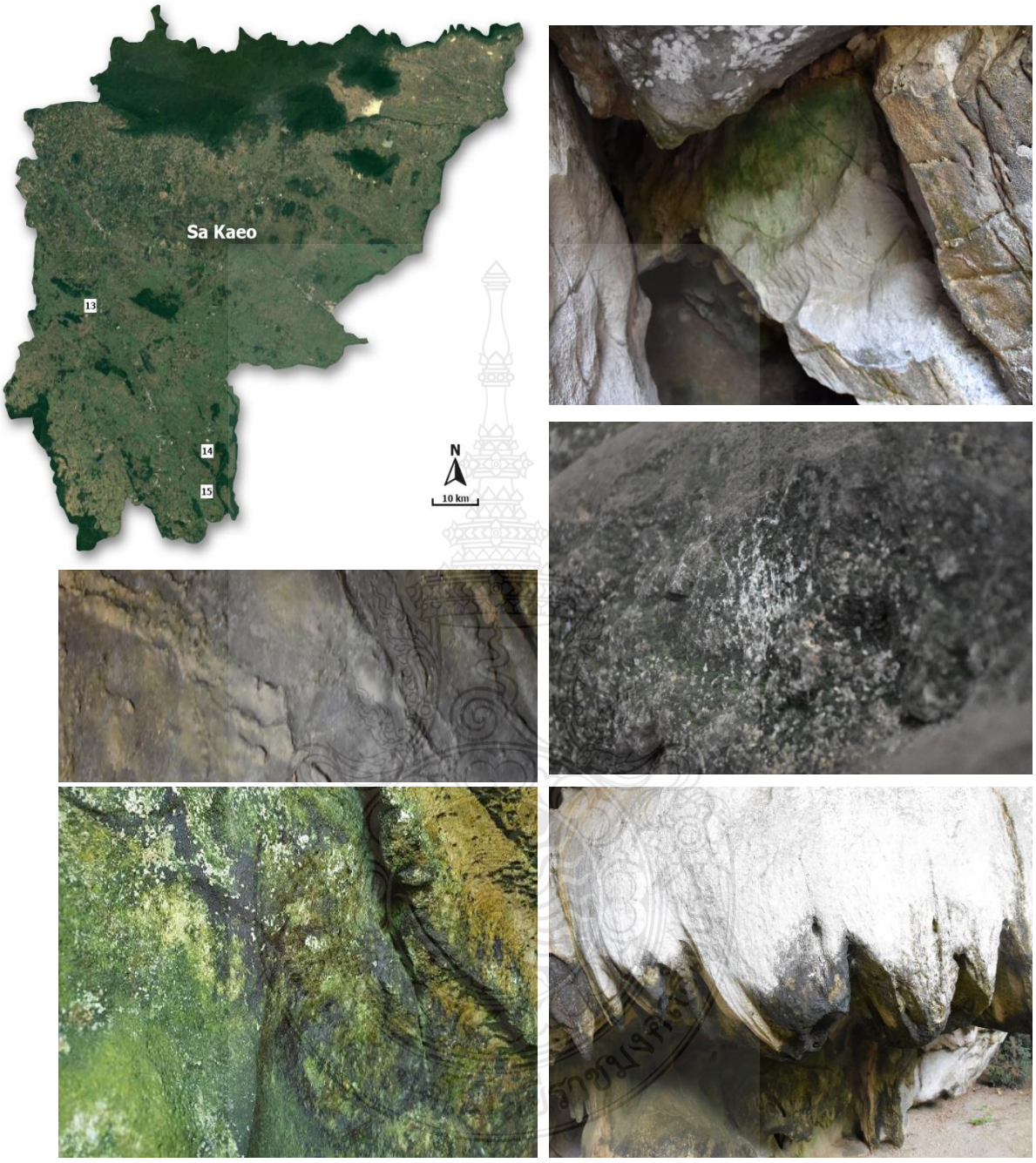


Figure 3.4 Map showing locations and general view of sampling sites in Eastern region of Thailand; 13 Khao Chakan cave, 14 Phet Pho Thong cave, 15 Nam Khao Siwa cave.

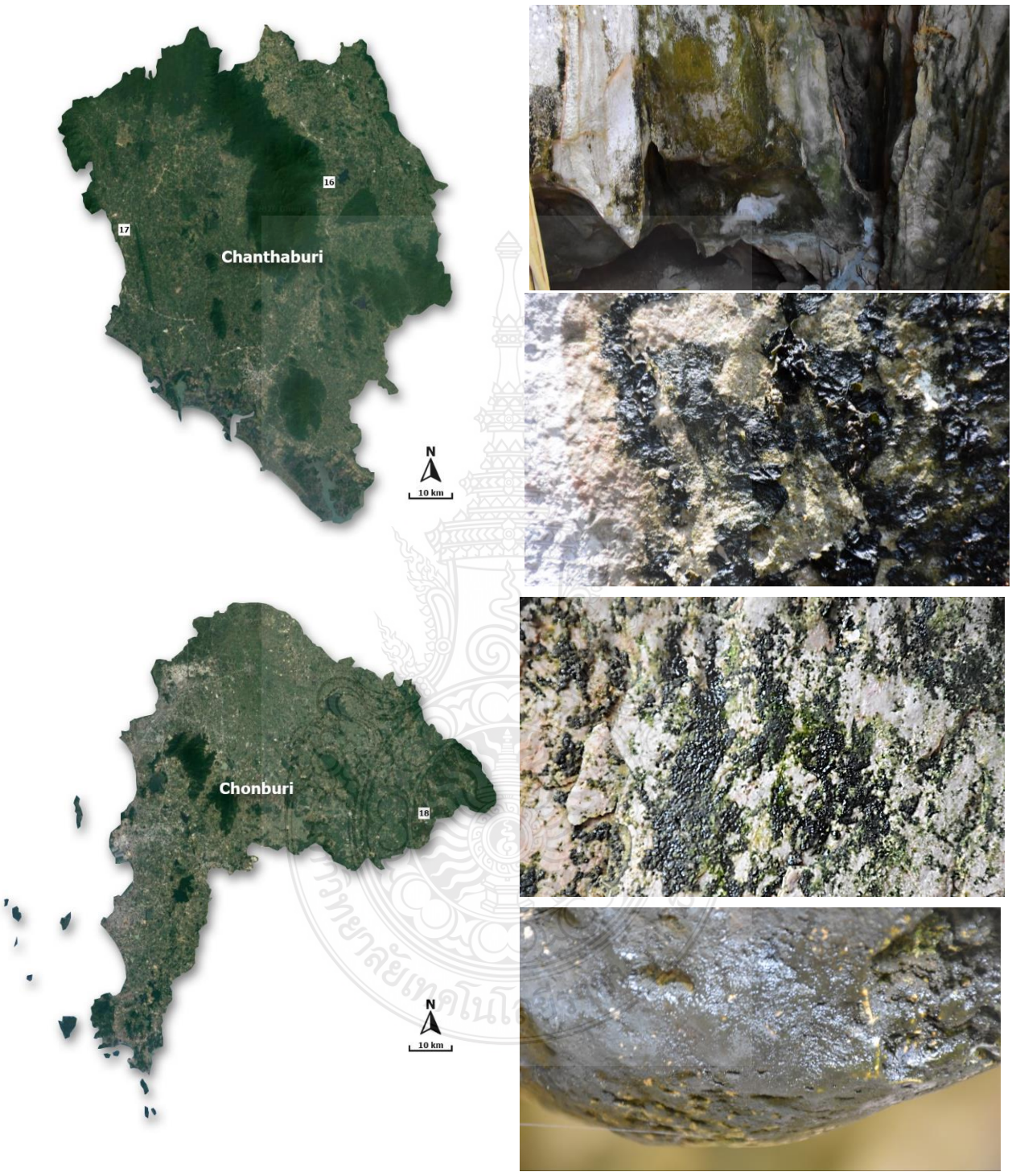


Figure 3.5 Map showing locations and general view of sampling sites in Eastern region of Thailand; 16 Sa Rika cave, 17 Khao Wong Kot cave, 18 Cha Ang cave.

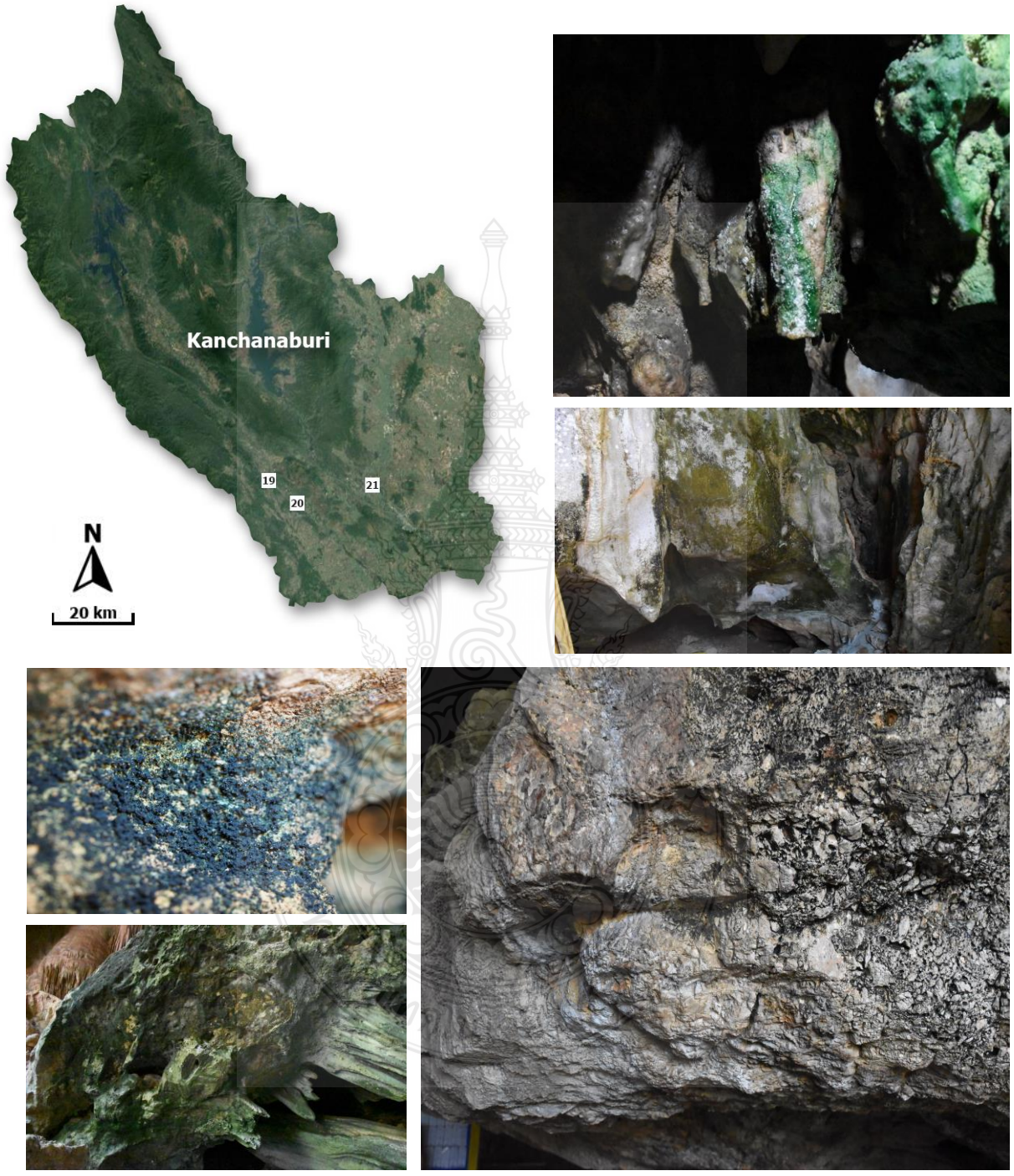


Figure 3.6 Map showing locations and general view of sampling sites in Western region of Thailand; 19 Kra Sae cave, 20 Chaloei cave, 21 Khao Pun cave.

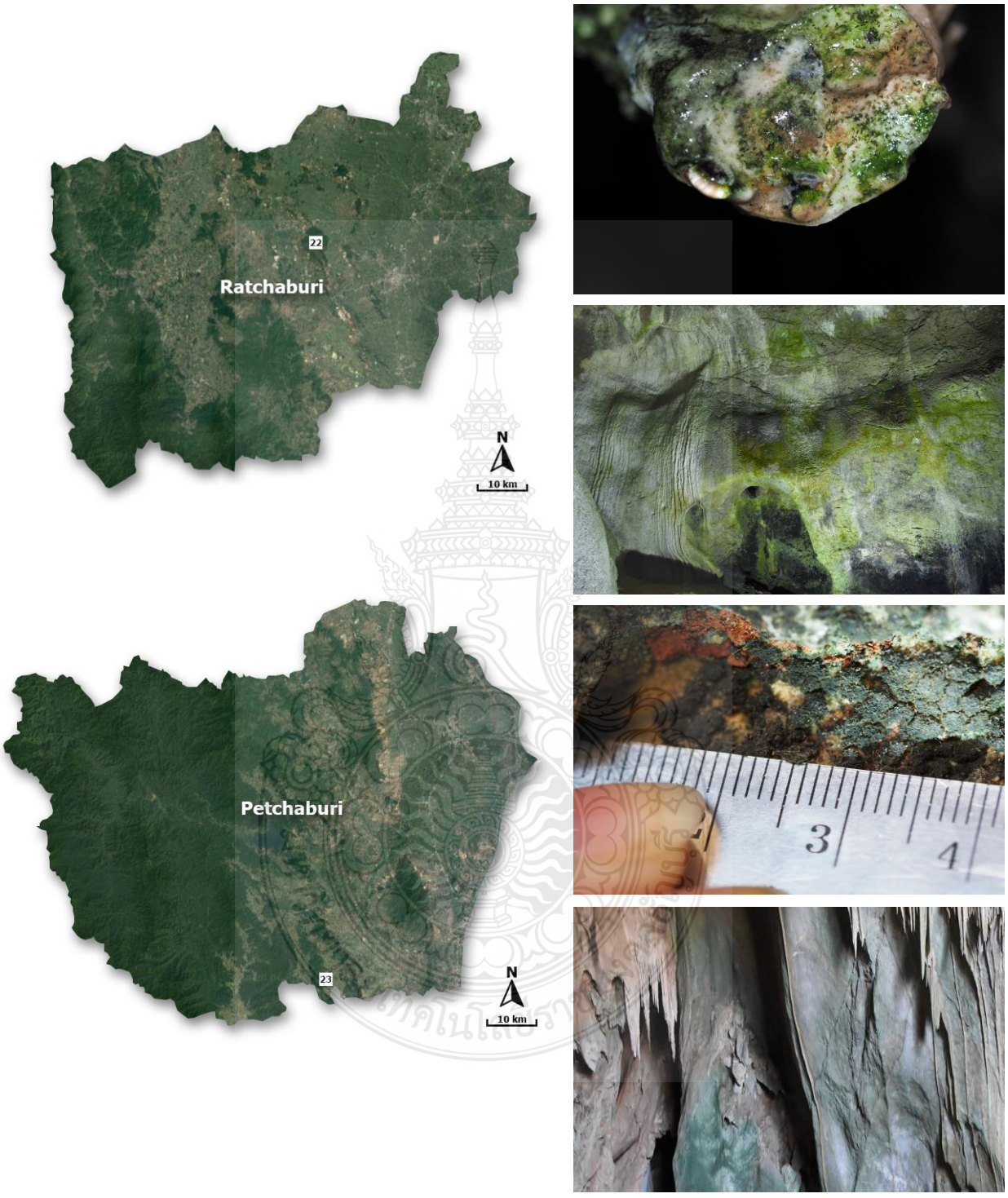


Figure 3.7 Map showing locations and general view of sampling sites in Western region of Thailand; 22 Khao Bin cave, 23 Kai Lon cave.

3.3 Isolation and Culture Conditions

Using aseptic technique under a flow cabinet, the karst cave cyanobacteria incubated in vial were re-cultured in 50 mL of BG-11 medium kept in 125 mL Erlenmeyer flask. The cyanobacterial cells were incubated for 30 days at 27 ± 2 °C under the constant light of a fluorescence lamp (10 µmol photon m^{-2} s^{-1}) on a rotatory shaker at 150 rpm. The appearance colonies were spread on agarized (1.5% w/v) BG11 medium and repeated streaking onto the fresh solid medium. Microscopic observations were performed until monocultures of cyanobacteria were obtained. After culture purification, all cyanobacterial strains were cultured and maintained in 125 mL Erlenmeyer flasks filled with 50 mL of BG11 medium before further analysis. They were also cryopreserved in a -80 °C freezer with glycerol (40% v/v), and stored at cryoprotective agents at the Department of Biology, Faculty of science and technology, Rajamangala University of Technology Thanyaburi, Thailand.

3.4 Morphological Identification

Morphological classification of cultivated cyanobacteria was based on characters observable under microscopic techniques. Morphotypes were identified to the genus level on the basis of identification systems proposed by Anagnostidis and Komarek [156,158,159].

3.4.1 Light Microscopy

Cells were observed directly using light microscope equipped with a DP72 high sensitivity color camera (Olympus, Japan). The morphological features observed were trichomes structure, size and shape of vegetative and heterocyst cells, diameter and length of each cell.

3.4.2 Chlorophyll Fluorescence Microscopy

Chlorophyll autofluorescence was observed under fluorescence microscope equipped with a digital camera (Olympus DP72, Japan), using a filter cube with 535 excitation wavelength and emission wavelength in the range of 500 - 550 nm.

3.4.3 Scanning Electron Microscopy (SEM)

Cells were collected by centrifugation and immediately fixed in 2% (w/v) of glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) and washed in double strength buffer before dehydration with a series of ethanol (25 - 100% v/v) and dried in the Leica EM CPD300 critical point dryer. Samples were coated with a gold thin film before observed under scanning electron microscope JSM-IT-500HR (JEOL, Japan)

3.4.4 Transmission Electron Microscopy (TEM)

Cells were collected by centrifugation and immediately fixed in 2% (w/v) of glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h and postfixed overnight with 2% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2) before embedding in a mixture of propyleneoxide and Epon resin. Thin sections of cells were contrasted with a Leica EM UC7 ultramicrotome and then observed under JEM-1400Flash (120kV) electron microscope equipped with a high-sensitivity CMOS camera (JEOL, Japan).

3.5 Molecular Identification

3.5.1 DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted using Soil DNA Isolation Plus Kit #64000 (Norgen, Canada), according to the manufacturer's established protocol. The taxonomic identities of the morphotypes were determined by sequencing the conserved *16S* rDNA gene. The *16S* rDNA gene was amplified by using 2 pair primers (i) 16SAPF (5′-GCA ATC TGA ACT GAG GAG AG-3′), 16SAPR (5′-CTT AAC ACA TGC AAG TCG A-3′), and (ii) 16SUinF (5′-GCC GGA CGG GTG AGT AAC GCG-3′), 16SUinR (5′-ACG GGC GGT GTG TAC AAG GC-3′). The 50 µl reaction mixture contained 50 ng of template DNA, 1× PCR buffer, 1 mM dNTPs, 1 U Taq DNA polymerase and 10 µM of each primer. A negative control (PCR mixture without DNA template) was included for each PCR reaction. The amplification was carried out in a thermal cycler (C1000^{TM} Thermal Cycler, BIO-RAD, USA) under the following amplification conditions: initial denaturation of 3 min at 94 °C, followed by 29 cycles of 30 s at 94 °C, 30 s at 60-62 °C, and 1 min at 72 °C and a final extension period of 10 min at 72 °C. About 1,200 bp of PCR products was checked by gel

electrophoresis (0.8% w/v) then analyzed sequencing by BTSeq^{TM} (Barcode-Tagged Sequencing) method [168]. BTSeq^{TM} is an NGS-based platform with high-fidelity and can acts as a suitable replacement for Sanger sequencing DNA sequences were refined and compared with basic sequence alignment with the database in the National Center for Biotechnology Information Blast (www.ncbi.nlm .nih.gov/BLAST).

3.5.2 Phylogenetic Tree Analysis

For the phylogenetic analyses, the *16S* DNA gene sequences were compared with sequence information available in the National Center for Biotechnology Information (NCBI) database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Assignations with an identity value above 97.5% and other representative cyanobacteria sequences were subsequently downloaded, and multiple alignments of all these sequences were generated using the Clustal W multiple alignment function in BIOEDIT Program. The phylogenetic trees were generated using MEGA (version 7.0.21) [169]. Therefore, distances for the maximum likelihood (ML) and Neighbor-Joining (NJ) using 1,000 replications. The 16S gene sequences obtained from this study were deposited in the NCBI GenBank database.

3.6 Determination of Biomass, Chlorophyll *a* and Carotenoid Contents

The isolated cyanobacterial strains were cultured in 50 ml of BG11 medium containing and incubated aerobically under continuous illumination of 10 µmol photon m^{-2} s^{-1} with cool white fluorescent lamps from two sides on a rotatory shaker at 160 rpm and 27 ± 2 ºC for at least three weeks until cells into steady state. The biomass content (cdw) was determined by separating cells from culture using 0.45 µm glass microfiber filter and dried in a 60 °C oven until a constant weight was obtained.

The total amount of chlorophyll *a* (Chl *a*) was extracted from biomass cells using 90% (v/v) of methanol, centrifuged at 8,000 rpm for 5 min and removed the green supernatant to measure the absorbance at 665 nm. The chlorophyll *a* content was calculated by following an equation, (1) where 12.7 is the extinction coefficient of 78.74 L g^{-1} cm^{-1}. The total amount of carotenoids was determuned by *N*, *N*-dimethylformamide (DMF) with vortexing vigorously (Jantaro *et al*., 2006). After centrifugation at 8,000 rpm for 5 min, removed cell

debris, the absorbance of supernatant was measured at 461 and 664 nm, respectively. The carotenoid content was calculated according to following an equation (2)

$$\text{Chlorophyll } a \text{ content } (\mu g/cdw/ml) = \frac{12.7 \times A_{665} \times \text{dilution}}{\text{g cdw}} \quad (1)$$

$$\text{Carotenoid content } (\mu g/cdw/ml) = \frac{[(A_{461} - (0.046 \times A_{664})) \times \text{dilution}]}{\text{g cdw}} \quad (2)$$

3.7 Extraction of Phycobiliproteins from Fresh Biomass

An aliquot of cyanobacterial culture was centrifuged at 8,000 rpm for 10 min to remove medium. The cyanobacterial pellet was resuspended in 1 mL of phosphate buffer (pH 7.0) and used freeze-thawing method for phycobiliprotein extraction [170]. The freeze-thawing treatment was performed at least four cycles between -20 ºC and room temperature (27 ± 2 ºC). After the freeze-thawing treatment, the suspension was maintained at 4 °C for 24 h and then centrifuged for 10 min (10,000 rpm, 4 °C). The supernatant (crude extract) was collected to determine phycobiliproteins (PBPs) and protein contents for phycobiliproteins.

3.8 Spectrophotometric Determination of the Phycobiliproteins

After centrifugation, samples were measured for absorbance at 652, 615, 652 and 280 nm by a UV–vis spectrophotometer (GENESYS 10S UV-VIS spectrophotometer, Thermo scientific, USA) against buffer as a blank. Phycobiliprotein concentration as a combination of PC, PE and APC was calculated using the following Eqs (3-5) by using different extinction coefficients [171]. Purity indices of the phycobiliproteins were determined spectrophotometrically and calculated by the following Eqs (6-8). This ratio indicates the extract purity with respect to forms of contaminating proteins. The PC, APC, and PE concentrations were indicated at absorbance wavelengths of 615, 652 and 562 nm, respectively, whereas the total concentration of proteins in solution was considered at an absorbance wavelength of 280 nm. Finally, phycobiliprotein extraction yield (%Yield) was determined by applying Eq (9).

$$\text{Phycocyanin [PC] concentration (mg/ml)} = \frac{(A_{615} - (0.474 \times A_{652}))}{5.34} \quad (3)$$

$$\text{Allophycocyanin [APC] concentration (mg/ml)} = \frac{(A_{652} - (0.208 \times A_{615}))}{5.09} \quad (4)$$

$$\text{Phycoerythrin [PE] concentration (mg/ml)} = \frac{(A_{562} - (2.41 \times [PC]) - (0.849 \times [APC]))}{9.26} \quad (5)$$

$$\text{Phycocyanin purity index} = \frac{(A_{615})}{(A_{280})} \quad (6)$$

$$\text{Allophycocyanin purity index} = \frac{(A_{652})}{(A_{280})} \quad (7)$$

$$\text{Phycoerythrin purity index} = \frac{(A_{562})}{(A_{280})} \quad (8)$$

$$\%\text{Yield} = \frac{[PBPs] \times V}{\text{mg cdw}} \times 100 \quad (9)$$

Where [PBPs] is phycobiliproteins concentration in mg/mL, V is the volume of buffer solvent in mL and cdw is cell dry weight in mg.

3.9 Phycobiliprotein purification

3.9.1 Phycobiliprotein Purification Steps

The extracted PBPs extract in 0.01 M phosphate buffer (pH = 7.0) was purified by following steps as show in Figure 3.8. In the crude extract, ammonium sulphate was added slowly for achieving 60% saturation while continuous stirring and resulting solution was allowed to stand at 4 ºC to precipitate protein for overnight before centrifugation at 10,000 rpm for 30 min. The dissolved precipitate was dialyzed overnight using dialysis tubing cellulose membrane (molecular weight cut of, 12 kDa) against ten times volume of the phosphate-buffered saline (PBS). For characterizing PBPs, each purified step was analyzed by absorption spectroscopy and Native/SDS-PAGE analysis. Recovery yield of purified PBPs was then calculated using Eq (10).

$$\text{Recovery yield (\%)} = \frac{[\text{PBP}]c \times Vc}{[\text{PBP}]i \times Vi} \times 100 \quad (10)$$

Where [PBP]c is PBP concentration of collect samples (mg/mL), [PBP]i is initial PBP concentration (mg/mL), Vc and Vi are collected volume and initial volume (mL), respectively.

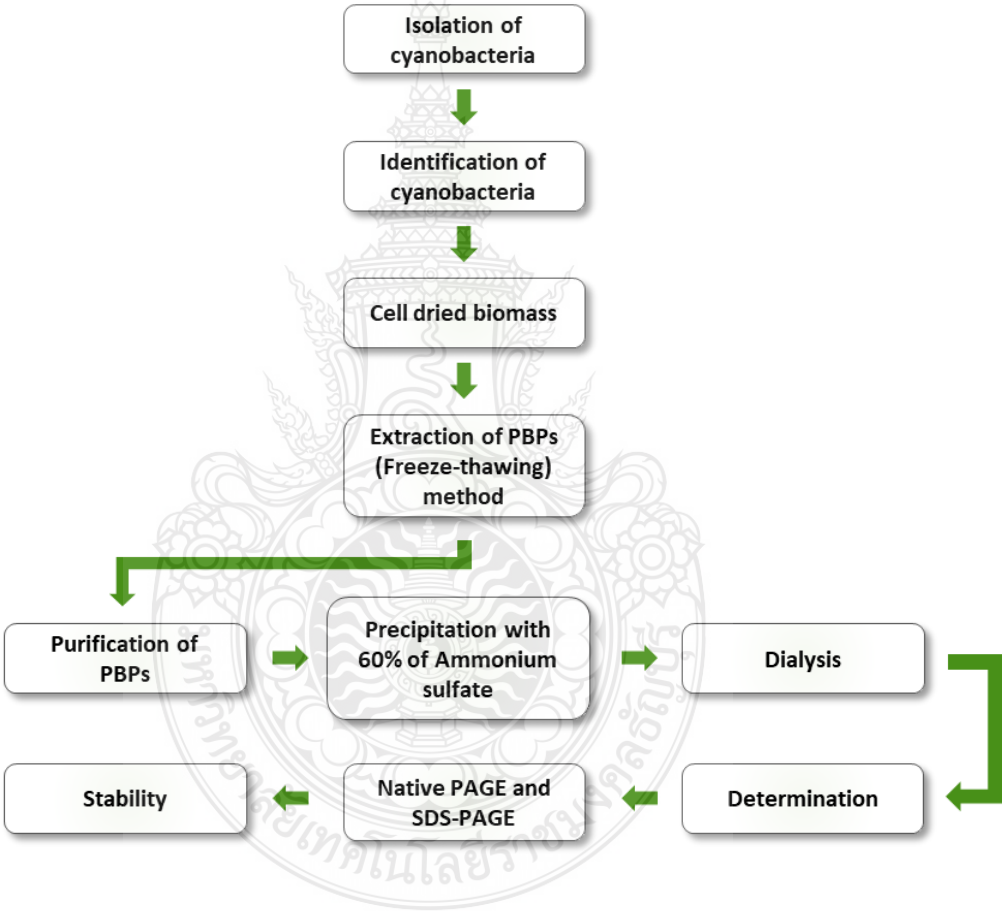


Figure 3.8 Steps of phycobiliproteins purification from karst cave cyanobacterial strains.

3.9.2 Phycobiliprotein scanning spectrum

The purified PE, PC and APC were analyzed by recording the absorbance spectrum over 300–800 nm on UV–Visible Spectrophotometer (GENESYS 10S UV-VIS spectrophotometer, Thermo scientific, USA). For fluorescence spectroscopy analysis, the purified PBPs were measured fluorescence spectrum using fluorescence spectrophotometer (Perkin-Elmer luminescence spectrophotometer LS55, England). The measurements were preformed using excitation wavelength of 560 nm (PE), 660 nm (APC), and 615 nm (PC) respectively and emission spectra from 510-800 nm at speed of 1200 nm/min. The slits width of excitation and emission were set at 5 mm, respectively [171].

3.9.3 Native PAGE and SDS-PAGE Analysis

The Native PAGE and SDS–PAGE used a TGX Stain-Free FastCast Acrylamide Kit (BIO-RAD, USA) with 12% polyacrylamide gels. Electrophoresis was performed at room temperature for 40 min at 200 V. After electrophoresis, both gels were washed in distilled water in a plastic container. The SDS-PAGE gel was stained with 0.3% Coomassie blue G-250 for 4 hours and de-stained for 24 hours in a solution containing 20% methanol and 10% acetic acid until the background was clear. Using protein BLUeye Prestained Protein Ladder was estimated standard protein in the wide range of molecular weight 10-245 kDa (GeneDireX, USA).

3.10 Phycobiliprotein Stability

To evaluate the effect of temperature on PBPs stability, 100 µg mL^{-1} of purified phycobiliprotein dissolved in 0.01M phosphate buffer at pH 7.0. The solutions were incubated in a temperature gradient with ten temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 ◦C) for 30 min before analysis of remaining concentration. To evaluate the effect of pH on PBPs stability, 100 µg mL^{-1} of purified phycobiliprotein was dissolved in different pH gradient buffers (0.01M) namely, citrate buffer (pH 3.0, 4.0, 5.0), phosphate buffer (pH 6.0, 7.0, 8.0), and glycine-NaOH buffer (pH 9.0, 10.0), respectively. Then, each sample was incubated under room temperature (27 ± 2 ºC) for 30 min before analysis. The PBPs stability

was analyzed in term of the relative concentration of PBPs (%CR), which is the remaining concentration of PBPs as percentage of the initial concentration as show in Eq (11).

$$\text{Relative concentration CR (\%)} = \frac{C_t}{C_0} \times 100 \quad (11)$$

Where C_t is the concentration at time t, C_0 is the initial concentration

3.11 Antioxidant Activity Measurement

3.11.1 DPPH-Scavenging activity

For DPPH assay was determined using 2, 2 diphenyl-1-Dipicrylhydrazyl (DPPH) solution was conducted following method of [172]. An equal volume of PBP sample (0 - 100 µg mL^{-1}) and 0.1 mM DPPH in 99% (v/v) ethanol were mixed and incubated at room temperature for 40 min in dark condition. The absorbance was measured at 517 nm and DPPH scavenging activity was calculated by using Eq (14). Ascorbic acid (100 µg mL^{-1}) was used as positive control [173].

$$\text{\% Scavenging activity} = \frac{(A_0 - A_b) - (A_1 - A_{1b})}{A_0 - A_b} \times 100 \quad (14)$$

where A_1, A_{1b}, A_0, and A_b are the absorbance of the sample, the blank sample, the control, and the blank control, respectively.

3.11.2 Ferric Ion Reducing Ability of Plasma (FRAP)

The FRAP assay was performed according to [174]. Reagent solution for FRAP assay was prepared freshly by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyls-triazine (TPTZ) in 40 mM hydrochloric acid and 20 mM ferric chloride in 10:1:1 ratio. The reagent solution was pre-warmed at 37 ºC before mixing with sample in 9:1 ratio. Reaction mixture was incubated at 37 ◦C for 4 min. Absorbance at 593 nm was recorded relative to a reagent blank as in Eq (15). Ascorbic acid was used as positive control.

$$\% \text{ FRAP} = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (15)$$

where A_0 and A_1 are the absorbance of control and sample, respectively.

In addition, the results were also expressed in term of IC_{50} meaning value of sample concentration can inhibited 50% of free radical.

3.12 Antimicrobial Activity Measurement

3.12.1 Disc diffusion assay

The antimicrobial activity of tested PBPs extracts was performed with CLSI, (formerly NCCLS) standard using disc diffusion and minimum inhibitory concentration MIC method [175]. Briefly, about 40 µl of different PBPs concentrates were dropped on 6 mm Whatman filter paper No. 1 disc and placed aseptically on the agar plate seeded with testing microorganism (0.5 McFarland turbidometry) namely, gram-positive bacteria (*Bacillus subtillis* TISTR 1528, *Propionibacterium acnes* DMFT 14916, and *Staphylococcus aureus* ATCC 25923, gram-negative bacteria (*Escherichia coli* ATCC 25922), and yeast (*Candida albicans* SU 28). All the test and standard control plates were incubated at 37 °C (bacterial growth), and 30 °C (yeast growth) for 24 h. and the zone of inhibition was measured. The phosphate buffer was used as solvent control and Chloramphenicol (10 µg) were used as antibiotic standards. All the experiments were performed in triplicates and the average with standard deviation was calculated [176,177].

3.12.2 MIC assay

All the active PBPs extracts were screened for their MIC values. The serial dilution of PBPs fraction were prepared from stock to get final concentrations of 1000 µg mL^{-1}, 500 µg mL^{-1}, 250 µg mL^{-1}, 125 µg mL^{-1}, and 0 µg mL^{-1} (control) by two-fold dilution along with 0.01M phosphate buffer (pH 7.0). The 1 mL of each concentration was added in medium both containing tested microorganism. Then, test tubes were incubated for 24 h. and

examined for lowest concentration of active fraction that prevented the microbial growth, which that concentration was considered to be the MIC value [178,179]**.**

3.13 *In vitro* Anticancer Activity Measurement

3.13.1 HeLa sulphorhodamine B assay

Briefly, HeLa cervical cancer cells were cultured in a 96-well plate ($1x10^4$ cells/well) for 24 h and new medium containing various concentrations of extracts (0-1000 µg/mL) was added. After 24, 48, 72 h, cells were fixed with ice-cold 10% trichloroacetic acid (TCA) at 4°C, stained with 0.4% SRB for 30 minutes at room temperature, and cells were dissolved with 10 mM Tris-base solution. The absorbance intensity was read with a filter wavelength of 540 nm.

3.13.2 HFF-1 MTT assay

Briefly, HFF-1 skin fibroblast cells were cultured in a 96-well plate at the density of $4x10^3$ cells/well for 24 h and new medium containing various concentrations of extracts was added. After 24, 48, 72 h, cells were added MTT (final concentration 0.5 mg/mL) for 3 h and then washed with PBS buffer. Cells were solubilized with 100 µL DMSO by pipetting up and down until cell break. The absorbance intensity was read with a filter wavelength of 540 nm.

3.14 Statistical Analysis

All experiments were performed in three biological replicates. The data were compared using the one-way analysis of variance (ANOVA) test to evaluate the differences between multiple groups. The differences in means values were identified by Duncan's multiple range test to determine whether significant difference ($p < 0.05$) existed among different treatments. All the statistical analyses were carried out using SPSS software.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Survey and Study of Cyanobacterial Growing in Karst Caves of Thailand

In this research, 23 karstic caves along of four regions of Thailand, namely western region, central region, southern region, and eastern region, were surveyed and collected natural samples as described in table 3.1. The karst cave was found that the most of algal species living in the cave are cyanobacteria. Cyanobacterial communities were mainly abundantly on cave walls, stalagmites, and stalactites, especially in the entrance zone, with large amounts of light. The appearance of cyanobacterial colonies grew in clusters embedded in mucilage with various cell colors including green, black, brown and greenish pink, as shown in Figure 4.1 (A - D).

4.2 Isolation and Diversity of Cyanobacteria in Karst Caves of Thailand

All samples collected from 23 karst caves were cultured in glass vial and incubated under low light intensity (10 µmol photon $m^{-2}s^{-1}$). The cyanobacterial colonies were appeared after 3 weeks of cultivation. Then, the single cell was picked up for isolation using streak plate technique. A total of 86 cyanobacteria strains were isolated from the sampling studies and identified base on morphological features as reported by [180]. They could be classified into 10 families, 12 genera such as *Nostoc*, *Oscillatoria*, *Mastigocladus*, *Scytonema*, *Anabaena*, *Hapalosiphon*, *Leptolyngbya*, *Phomidesmis*, *Scytolyngbya*, *Chroococcus*, *Synechococcus*, and *Chroococcidiopsis* (Figure 4.2 A and B). The most common cyanobacteria found in the four regions of Thailand were two genera, *Leptolyngbya* and *Oscillatoria* as shown in Figure 4.3.

All isolated cyanobacteria from karst caves showed that abundant cyanobacteria belonging of *Leptolyngbya* genus. A total of 37 species of *Leptolyngbya* were isolated in this study. The morphological characteristics of the *Leptlyongbya* genus were filamentous non-heterocyst cyanobacteria. Trichomes are cylindrical internodes, and each tri-column measures approximately 2.0 - 3.5 µm in length when cells were observed under a scanning

electron microscope (SEM) found that their filament contained thin outer sheath throughout the filament (Figure 4.4 A, B). Moreover, under a transmission electron microscope (TEM), the apical cell was a conical shape with a nucleus in the middle of the cell. Irregular thylakoids arrangement and various gas vacuoles were found and scattered throughout the cytoplasm (Figure 4.4 C, D). Cyanobacteria species are closely related to environmental factors and physical habitat characteristics. The *Leptolyngbya* genus has been reported that well adapted under changing of both physical and chemical environmental factors of sedimentary soils [181]. It may be the cause of this genus was frequently found in all sampling area.

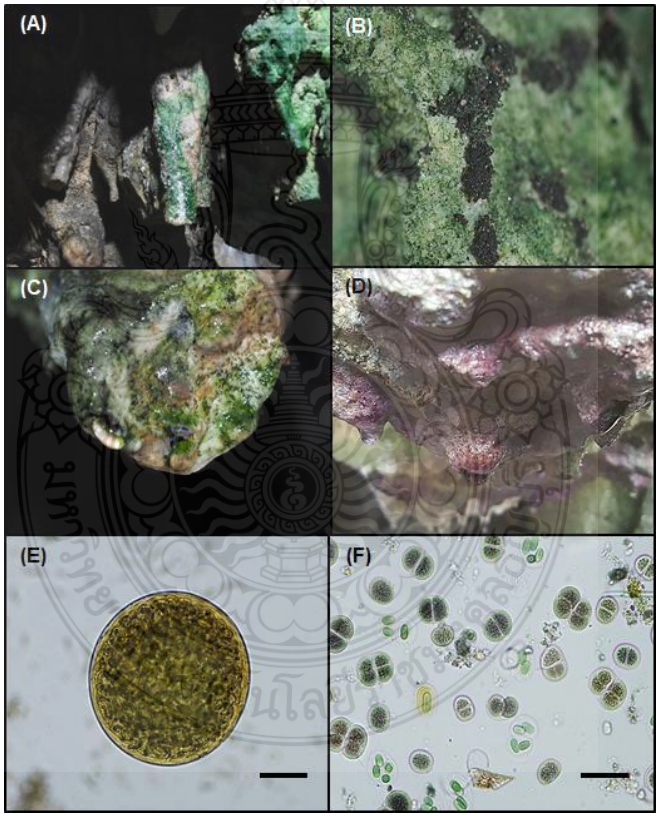


Figure 4.1 Photograph of cyanobacterial colonies attached to the karst cave wall (A-D) and diversity of cyanobacterial morphotypes found in the study site (E, F). Scale bars = 50 µm

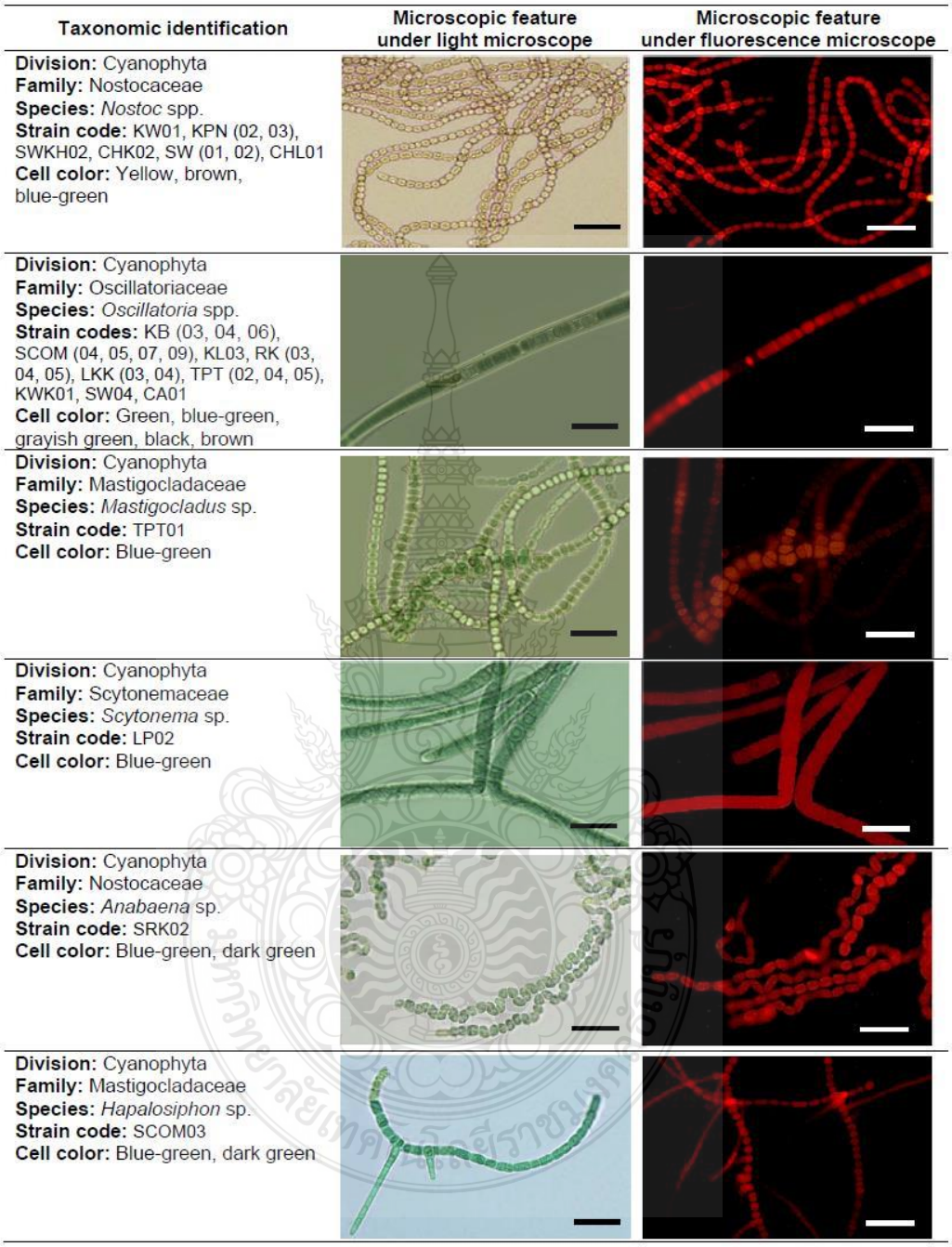


Figure 4.2A Morphological identification and features of isolated cyanobacterial genera *Nostoc*, *Oscillatoria*, *Mastigocladus*, *Scytonema*, *Anabaena,* and *Hapalosiphon*. Scale bare = 20 µm.

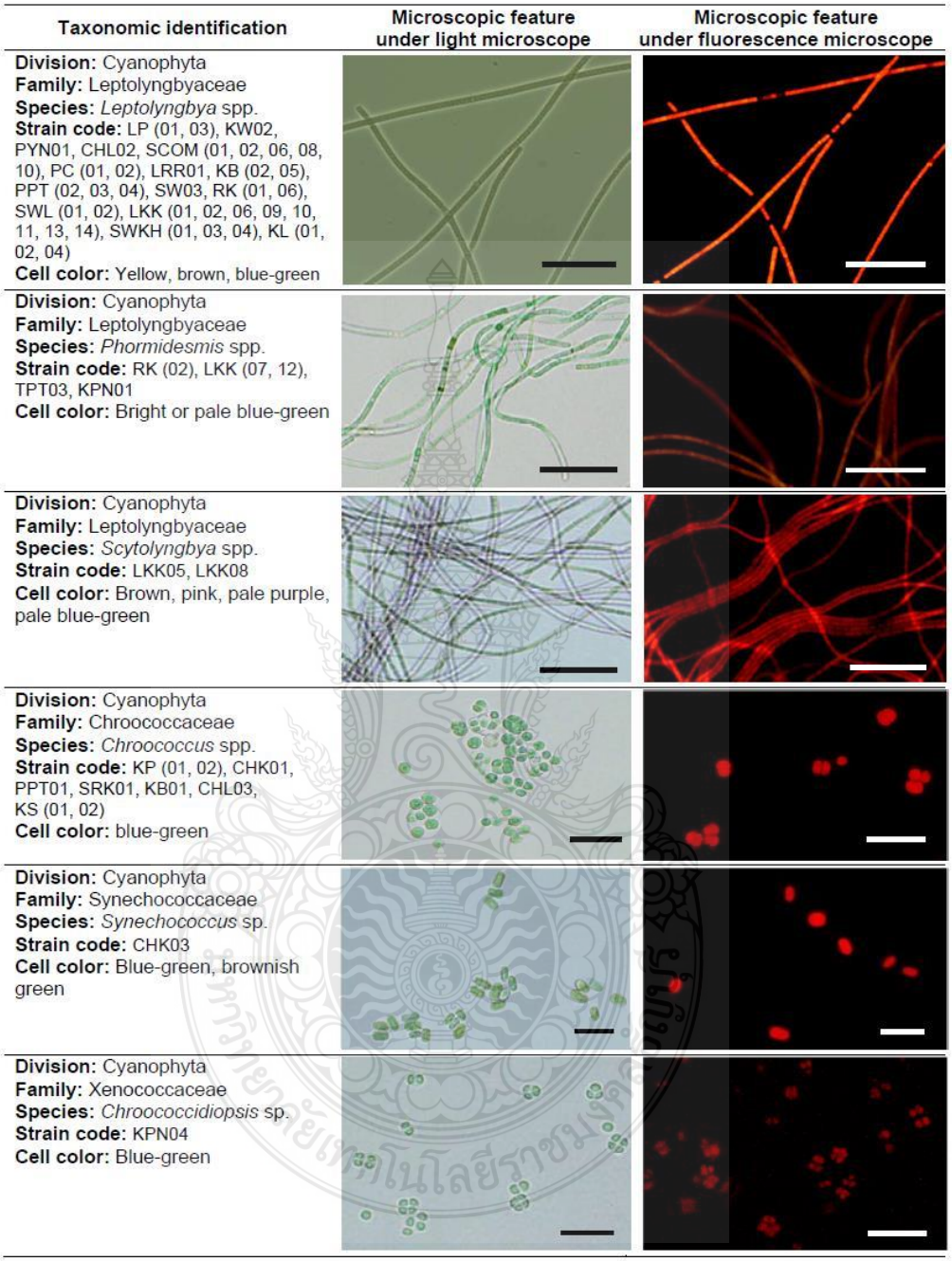


Figure 4.2B Morphological identification and features of isolated cyanobacterial genera *Leptolyngbya*, *Phomidesmis*, *Scytolyngbya*, *Chroococcus*, *Synechococcus,* and *Chroococcidiopsis*. Scale bare = 20 µm.

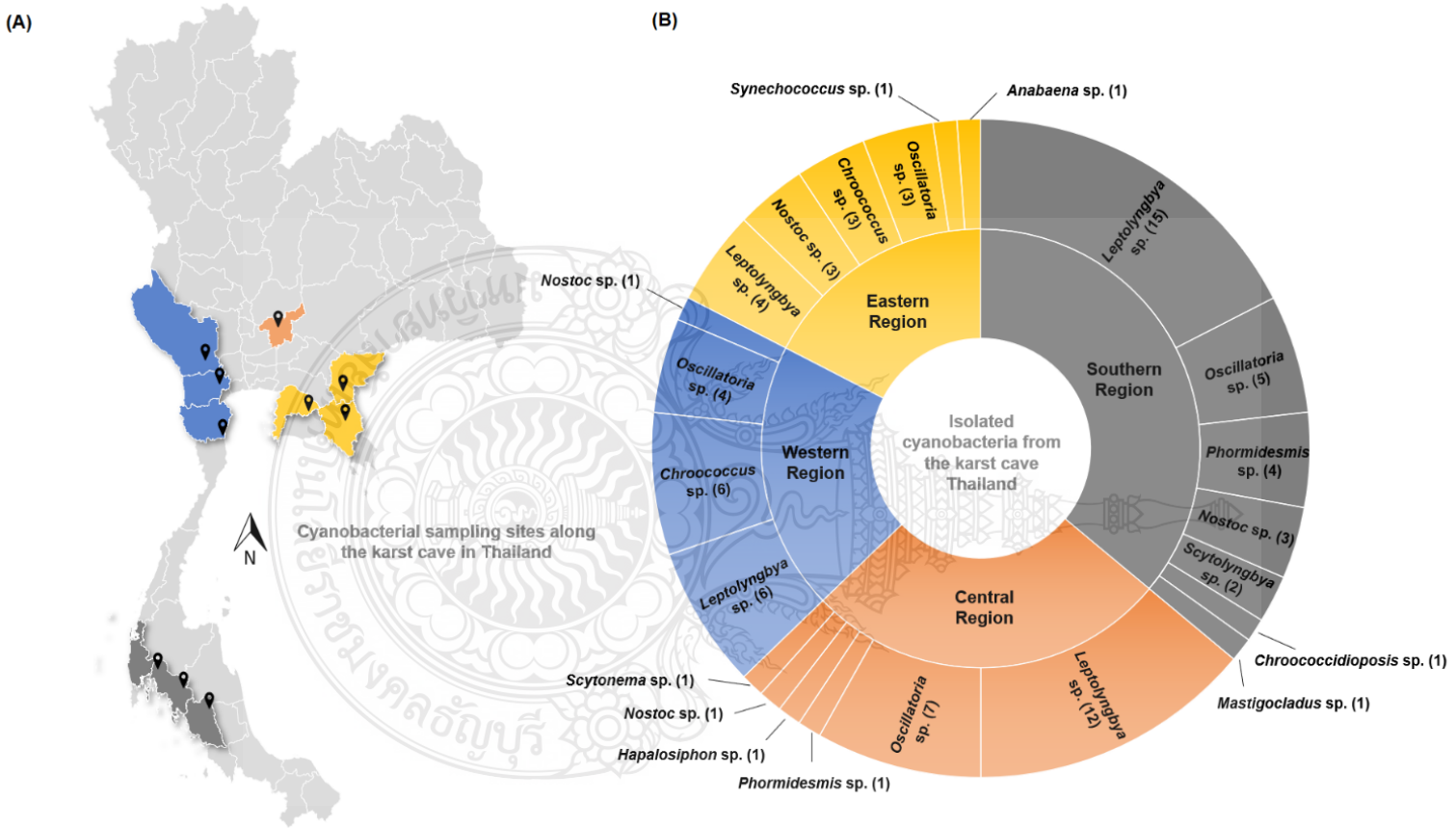


Figure. 4.3. (A) Location map of the sampling sites of the karst caves in Thailand. (B) The circle chart is showing the diversity of cyanobacterial strains isolated from four regions of Thailand. The number of each group species was represented in the bracket.

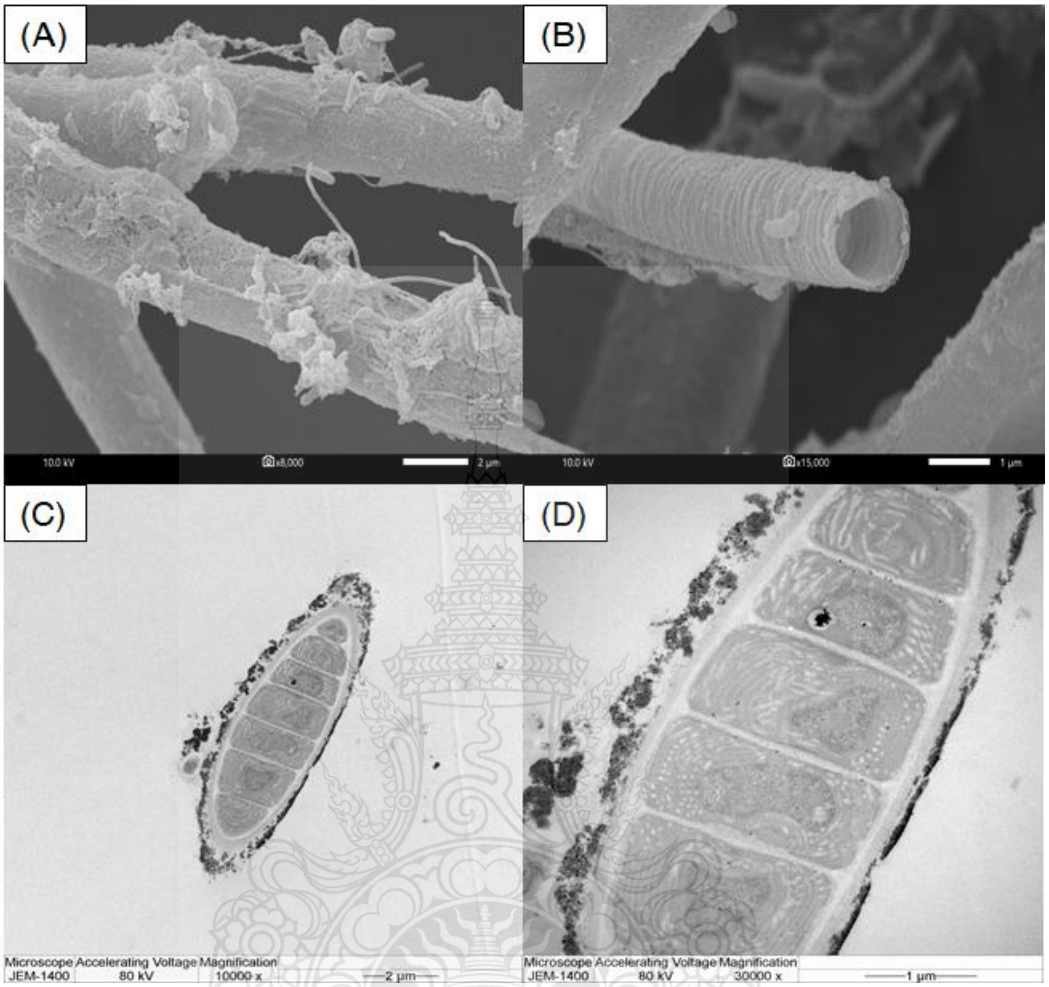


Figure 4.4 Morphology of *Leptolyngbya* genus under SEM (A, B) and TEM (C, D).

4.3 Study on the Relationship of Environmental Conditions to Cyanobacteria Growth in Karst Caves

In general, the characterization of karst cave is a critical environment, which is not suitable condition for living organisms. Because there are low available nutrient and less light exposure within the caves [180], therefore, cyanobacteria have to adapt themselves for survival in karst cave, such as mucilaginous biofilms creation and colony formation on cave walls. Their adaptation can reduce water loss and prevent cell drying due to cell desiccation [181]. However, in this study, the distribution of environmental factors, namely light

intensity, (%) relative humidity and temperature were analyzed for study of factors affecting on the diversity of cyanobacteria found in karst caves, as shown in Figure 4.5.

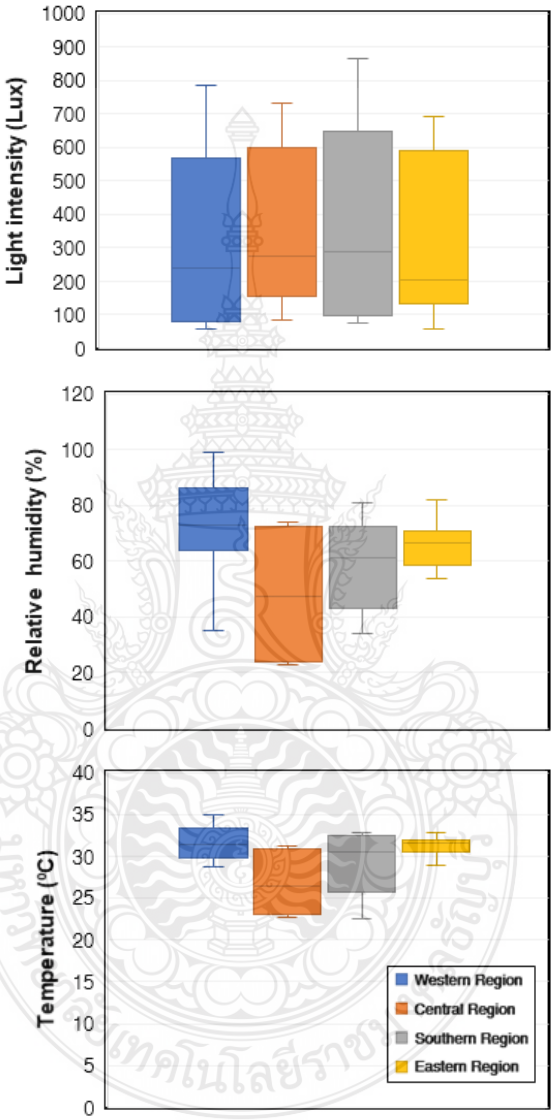


Figure 4.5 Boxplot showing the distribution of the various environmental parameters, light intensity, % relative humidity, and temperature of sampling sites in each region.

The results revealed that all sampling sites in different regions have similar light intensities ranging from 50 - 850 lux. However, the central and southern regions had a fragmented datasets of the (%) relative humidity and temperature than the western and eastern regions. It might be assumed that the percentage of relative humidity and temperature influenced on the diversity of cyanobacteria existing in the karst caves of Thailand. This data was consistent with the experimental reporting by [181] that water content was a significant factor in spreading cyanobacteria in different karst caves in the Peloponnese peninsula in Greece.

4.4 Study of Biomass, Chlorophyll *a*, and Carotenoids Contents in Isolated Cyanobacteria

The 86 strains of cyanobacteria isolated from karst caves were cultured in BG11 medium for three weeks under low light intensity (10 µmol photon $m^{-2}s^{-}$). Then cells were harvested to measure the dry cell weight, chlorophyll *a*, and carotenoid contents as shown in Figure 4.6. The results showed that almost isolated cyanobacteria could produce amounts of chlorophyll *a*, and carotenoid pigments. Suggesting, low light did not effective on photosynthesis of karst cave cyanobacteria. Moreover, the highest contents of chlorophyll *a* and carotenoid were found in cyanobacteria strain code KB01 (15.4 mg g cdw^{-1}) and KB05 (1456 µg g cdw^{-1}), respectively. When screening for the potential on biomass production, there were eight isolated strains showed biomass contents above 0.5 g L^{-1}, which were SCOM01, SWL01, KB05, LKK05, LKK11, SW02, and LKK12 strains. The *Leptolyngbya* sp. strain LKK05 showed highest biomass production with the rate of 0.71 g L^{-1}.

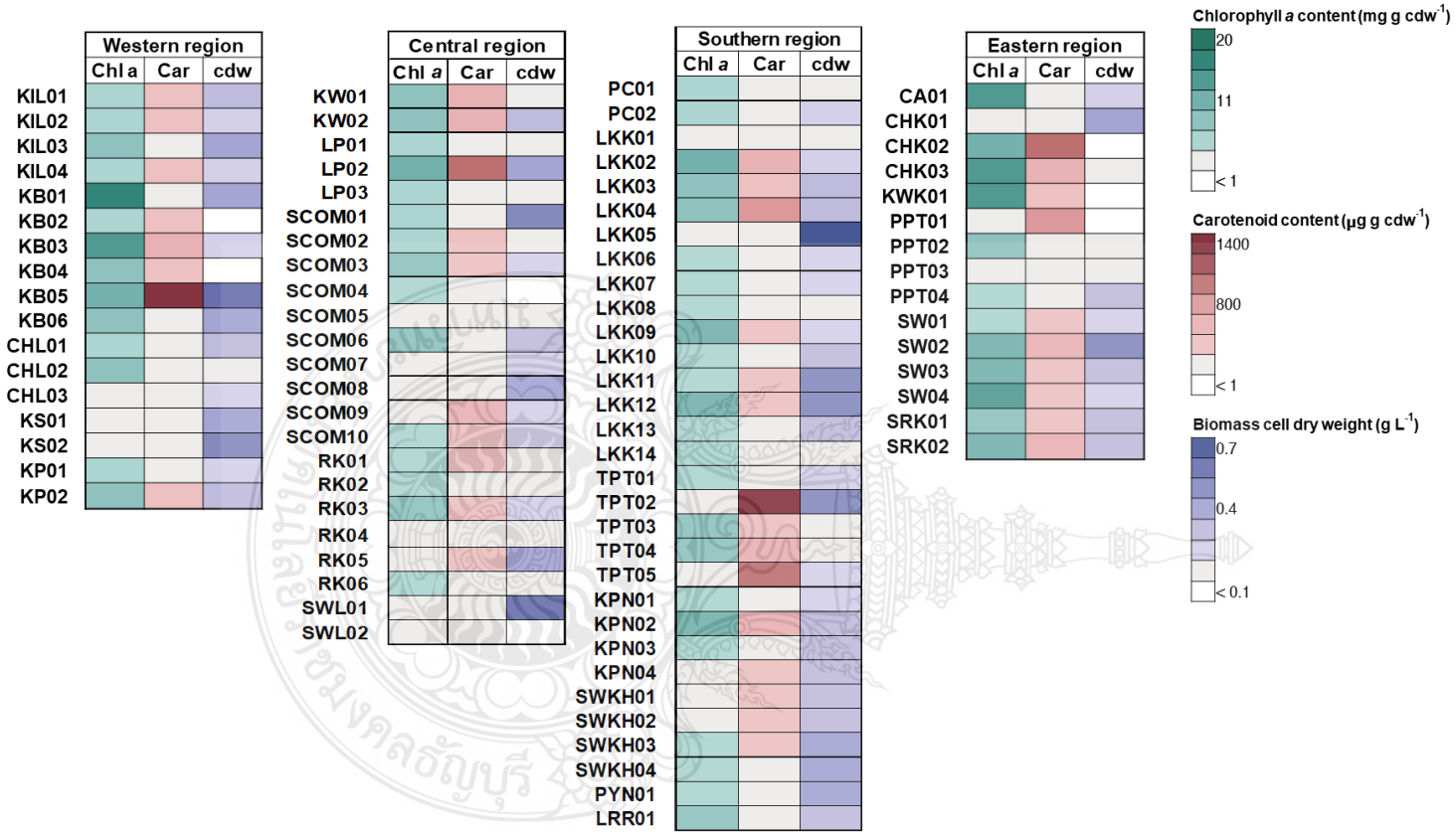


Figure. 4.6 Screening of chlorophyll *a*, carotenoid and biomass (cell dry weight) contents in cyanobacterial strains isolated from karst caves in four different regions of Thailand. Strain codes were related to morphotype as in Fig. 4.2. Abbreviations; Chl *a*: chlorophyll *a*, Car: carothenoid and cdw: biomass cell dry weight

4.5 Cyanobacterial Screening for Phycobiliprotein Production

Cyanobacteria living in caves present a unique group of microorganisms. They are particularly relevant for producing pigments and bioactive metabolites, such as antioxidants, due to their adaptation mechanisms to resist extreme conditions such as desiccation and darkness [184]. Morphological observation using a fluorescent microscope revealed cell samples glow in red color (Figure. 4.2A, B), indicating that the presence of phycobiliprotein (PBP) and chlorophyll *a* [185]. Phycobiliproteins are useful substances. They can be used in the food coloring industry as a substitute for synthetic dyes including in pharmaceuticals and cosmetics products [186]. In addition, phycobiliproteins had been known have biological activities as antioxidant, anti-inflammatory and help prevent brain-cell damage [187]. Therefore, all isolated cyanobacteria were screened for their ability to produce phycobiliprotein as shown in Figure 4.7. Interestingly, the 55 isolates could produce PBP content over 50 mg g protein^{-1}. Among cyanobacteria tested, there were six species of filamentous cyanobacteria, *Leptolyngbya* sp. SCOM01, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14 and *Nostoc* sp. SW02 showed PBP production as high yielders (over 400 mg g protein^{-1}). According to this results, six cyanobacteria representing high PBP yielders were selected for species identification using molecular technique and characterization of phycobiliprotein properties for biological activity.

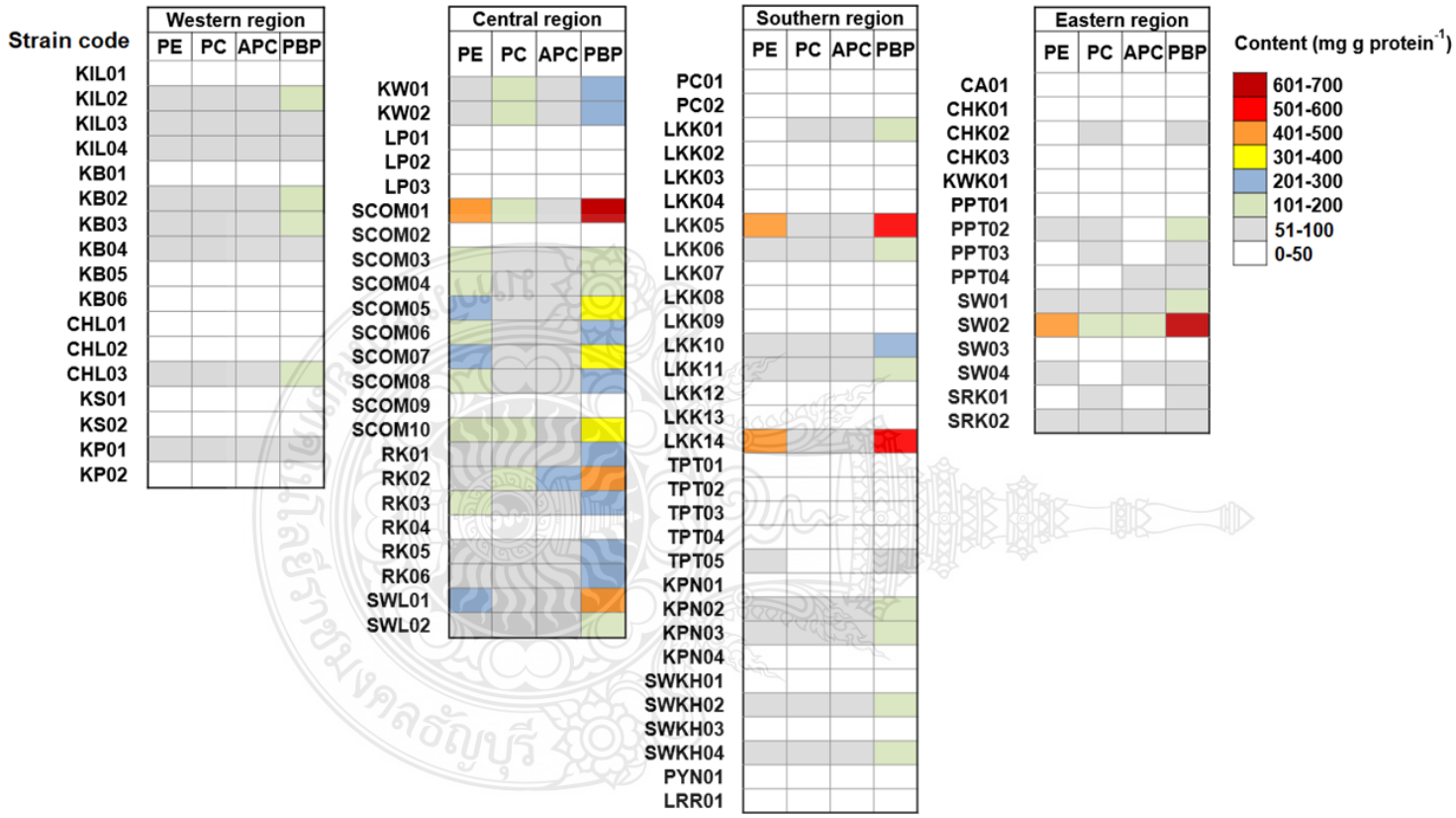


Figure 4.7 Screening of phycobiliprotein production in cyanobacterial strains isolated from karst caves in four different regions of Thailand. Strain codes were related to morphotype as in Fig. 4.2. Abbreviations; PE: phycoerythrin, PC: phycocyanin, APC: allophycocyanin, PBP: total phycobiliproteins.

4.6 Phylogenetic Tree Analysis Base on *16S* rDNA Gene Sequence of Selected Cyanobacteria

Molecular identification base on *16S* rDNA gene was used to confirm the taxonomic position of six selected cyanobacteria isolated from karst cave in Thailand. According to method 3.5.1, six selected strains were extracted their genomic DNA as show in Figure 4.8. Then, genomic DNA was used as a template for *16S* rDNA gene amplification. The *16S* rDNA partial sequences (1153 – 1470 bp) were amplified using specific primers as represented in Figure 4.9. All PCR products were done DNA sequencing by BTSeqTM (Barcode-Tagged Sequencing) method and queried against the GenBank database of cyanobacteria using a BLASTN search. The results revealed that all *16S* rDNA sequences showed approximately 96.47 - 99.66% identities against the top best hit available in NCBI public database (Table 4.3). The phylogenetic tree was created for study of evolutionary relationship based on *16S* rDNA gene as shown in Figure 4.10. Interestingly, the *16S* rDNA sequences were distributed in different five clusters in the phylogenetic reconstruction, namely Cluster I and III (*Leptolyngbya* groups), Cluster II (*Scytolyngbya* group), Cluster IV (*Phormidesmis* group), and Cluster V (*Nostoc* group). Where six isolates are separated into different groups. This phylogenetic tree analysis could suggest that six isolated SCOM01, SWL01, RK02, LKK05, SW02 and LKK14 should belong to *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Phromidesmis* sp. RK02, *Scytolyngbya* sp. LKK05, *Nostoc* sp. SW02 and *Leptolyngbya* sp. LKK14, respectively. In addition, the isolated strains, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SCOM01, and *Leptolyngbya* sp. SWL01 showed less than 97% similarity (*16S* rRNA sequence) with their closest sequence-related in the GenBank database (Table 4.3), suggested that they might be classified as new species isolated from the karst cave of Thailand.

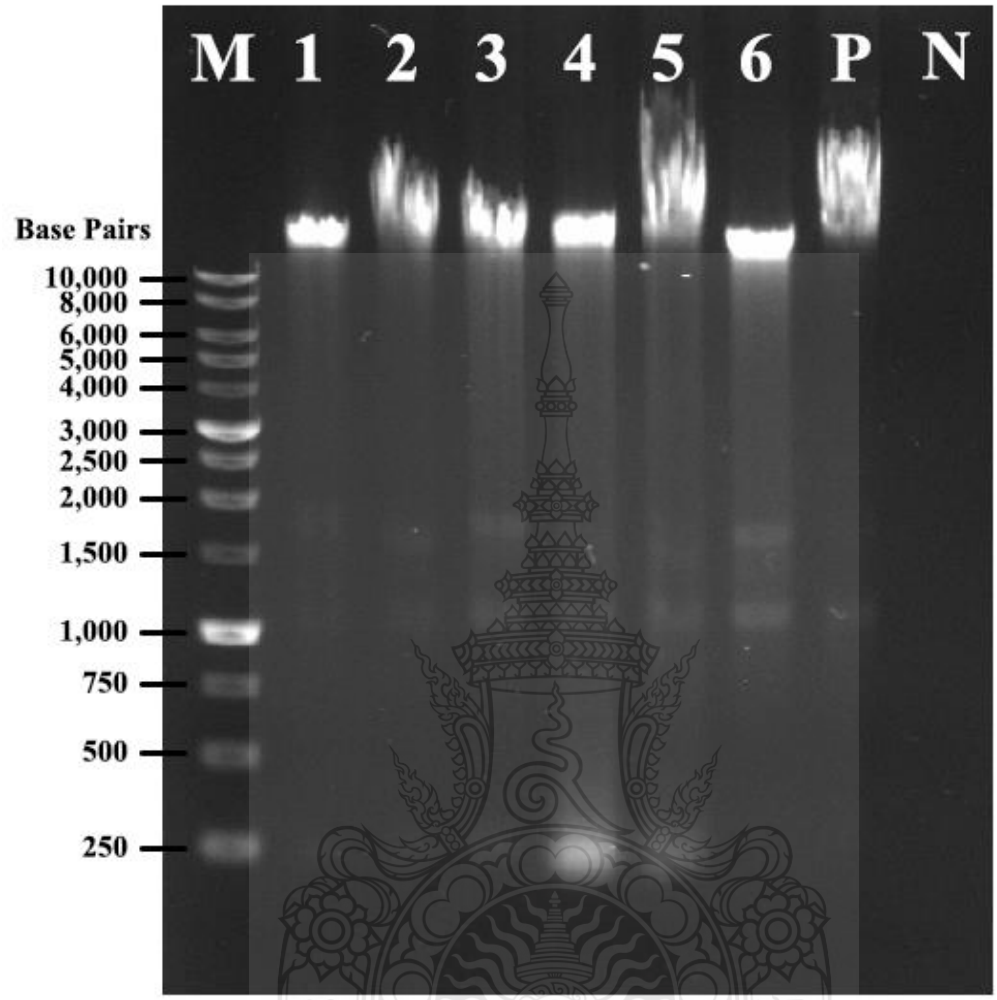


Figure 4.8 Agarose gel electrophoresis (0.8% w/v) analysis of genomic DNA isolated from six cyanobacterial strains Lane 1: SCOM01; Lane 2: RK02; Lane 3: SWL01; Lane 4: SW02; Lane 5: LKK05; Lane 6: LKK14; Lane P: positive control; lane N: negative control; and lane M: 1Kb DNA ladder RTU (GeneDireX, USA) used as marker.

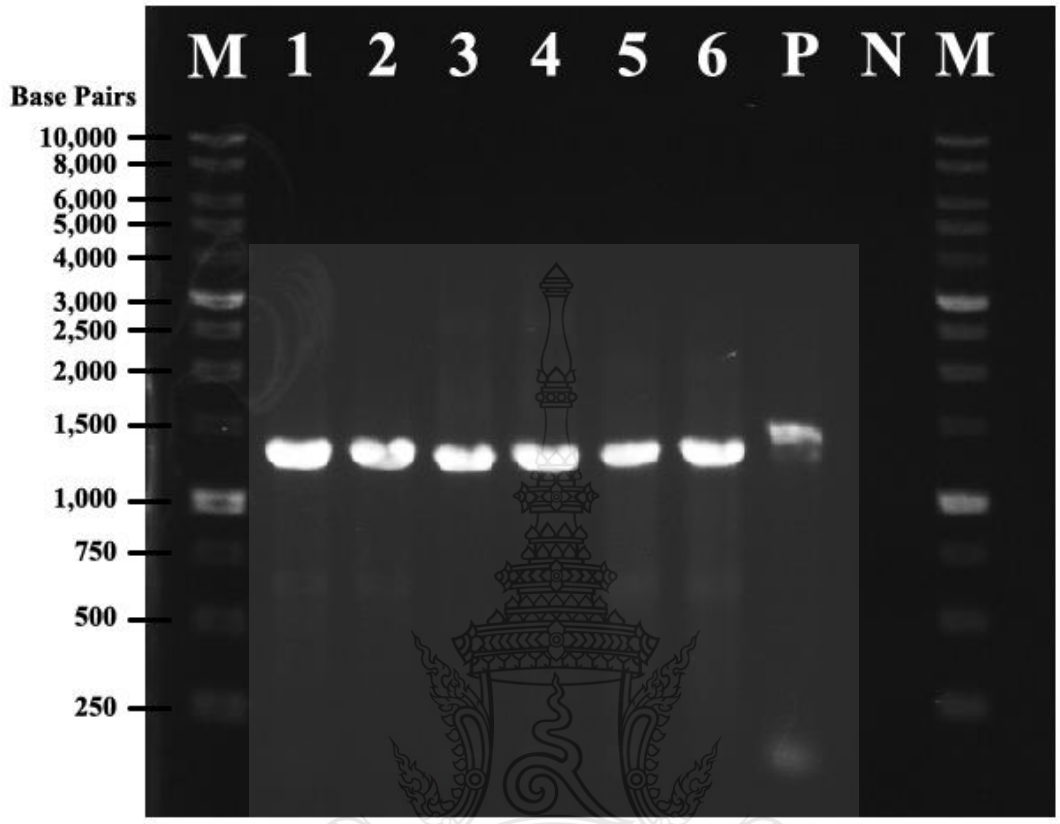


Figure 4.9 Agarose gel electrophoresis (0.8% w/v) analysis of *16S* rDNA gene amplified using genomic DNA of isolated cyanobacterial strain as a template. Lane 1: SCOM01; Lane 2: RK02; Lane 3: SWL01; Lane 4: SW02; Lane 5: LKK05; Lane 6: LKK14; Lane P: positive control; Lane N: negative control; and Lane M: 1Kb DNA ladder RTU (GeneDireX, USA) used as marker.

Table 4.3 Sequence identity (%) of *16S* rRNA gene sequences obtained from six cyanobacteria isolated from karst cave of Thailand compared to other cyanobacterial sequences available in GenBank.

Morphotypic isolated strains	Nucleotide fragment length (bp)	Query coverage (%)	Sequence identity (%)	The closest match in GenBank (accession number)
Phormidesmis sp. RK02	1412	100	96.47	*Phormidesmis* sp. ACSSI 324 (MT425940)
Leptolyngbya sp. SCOM01	1153	100	96.89	*Leptolyngby*a sp. CENA131 (KP835534)
Leptolyngbya sp. SWL01	1161	99	96.90	*Leptolyngby*a sp. CENA131 (KP835534)
Leptolyngbya sp. LKK14	1242	100	98.63	*Leptolyngbya* sp. Greenland_9 (DQ431004)
Nostoc sp. SW02	1247	99	99.03	*Nostoc* sp. PCC 8976 (AM711525)
Scytolyngbya sp. LKK05	1470	100	99.66	*Scytolyngbya timoleontis* XSP1 (KP688588)

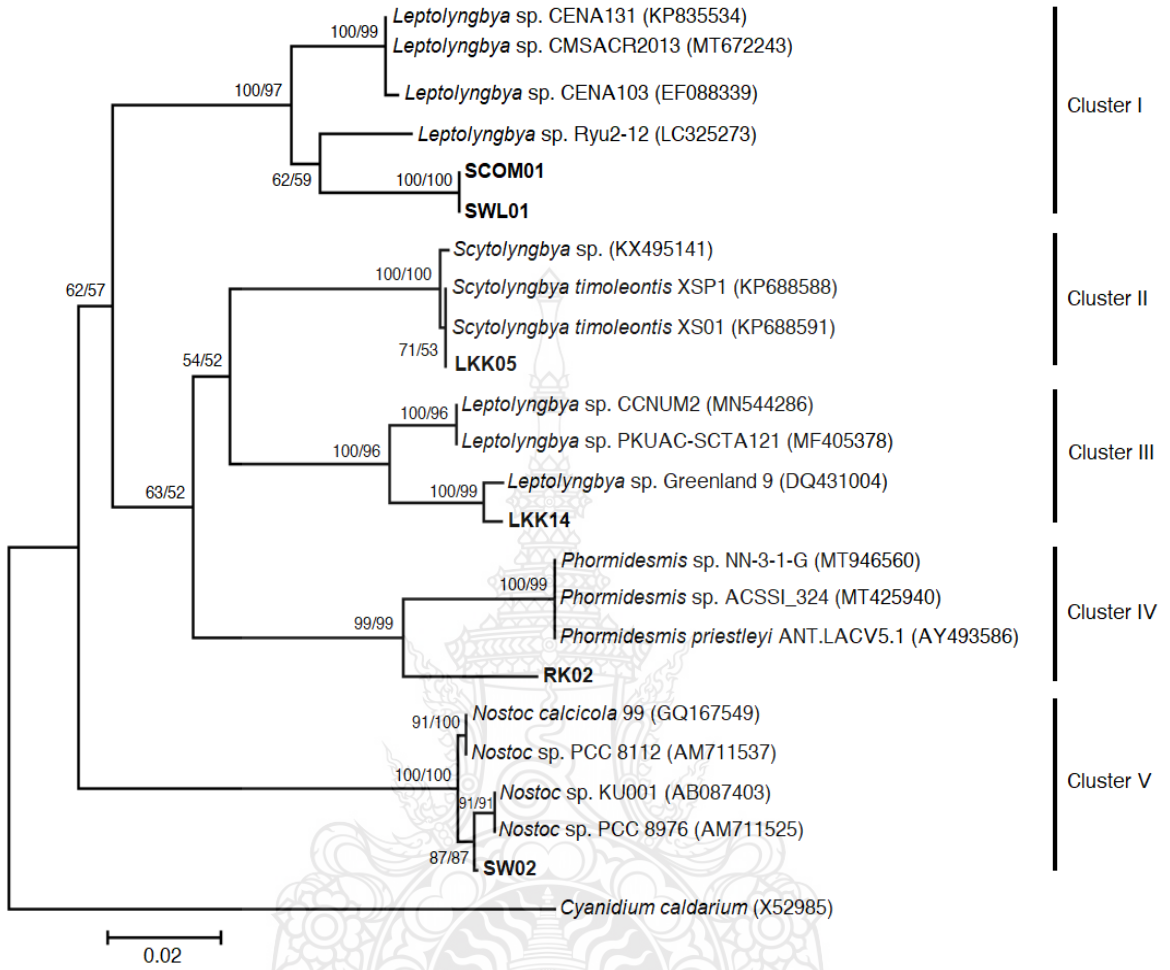


Figure 4.10 Phylogenetic tree based on *16S* rRNA gene sequences of cyanobacteria and reconstructed using the Maximum-Likelihood (ML) analysis. Numbers above branches indicate the bootstrap value (as percentages of 1,000 replications) for NJ/ML methods. The topologies of ML and NJ analyses were the same. Six isolated strains of this study are represented in bold, GenBank accession numbers are indicated in brackets. The 0.02 bar indicates substitutions per nucleotide position.

4.7 Upscale Phycobiliprotein Production in Six Selected Cyanobacteria

Phycobiliproteins (PBPs) extracted from cyanobacteria are classified as soluble pigment molecules composed of proteins and chromophores, called phycobilins [189]. Phycobilin can be divided into four major groups based on their absorbance spectra: Red; λmax: 490 - 570 nm), Phycoerythrocyanin (PEC; Orange; λmax: 560 - 600 nm), phycocyanin (PC; dark blue λmax: 610 - 625 nm), and allophycocyanin (APC; Blue green λmax: 650 - 660 nm) [188-189]. Then cells were harvested for PBPs extraction and characterization. Six strains of cyanobacteria were enlarged for cultivation in a 2-liter glass bottle and continuously aerated under low light intensity (10 µmol photon $m^{-2}s^{-1}$) at 27±2 °C for approximately three weeks. Six extracts were analyzed the type of produced phycobiliproteins. The results showed that five strains of cyanobacteria *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Nostoc* sp. SW02 and *Leptolyngbya* sp. LKK14 had a higher phycoerythrin contents than other phycobiliprotein type, whereas *Phormidesmis* sp. RK02 was found allophycocyanin over than other types (Figure 4.11A). In addition, 16.9% of the highest phycoerythrin yield was obtained from filamentous cyanobacteria *Nostoc* sp. SW02 with a PE: PC: APC ratio of 3.4:1.9:1.0 (Figure 4.11B).

Table 4.4 shows that *Nostoc* sp. SW02 had the highest phycobiliprotein yield of 31.92% (w/w), followed by *Scytolyngbya* sp. LKK05, which has an average phycobiliprotein yield of 13.42% (w/w). When comparing the phycobiliproteins produced in other cyanobacteria, isolated cyanobacterium *Nostoc* sp. SW02 and *Scytolyngbya* sp. LKK05 could produce higher phycobiliprotein yields compared to the yield of reference cyanobacteria strains (Table 4.5). In addition, both strains showed a high potential of biomass yields above 0.5 g L^{-1} when cultured in BG11 medium for three weeks (Table 4.4). These results show that the karst caves are good sampling sites for survey of cyanobacteria species capable of producing high-value compounds, such as phycobiliprotein pigments. The low light intensity within the cave may be the ideal condition for inducing phycobiliprotein synthesis in cyanobacteria.

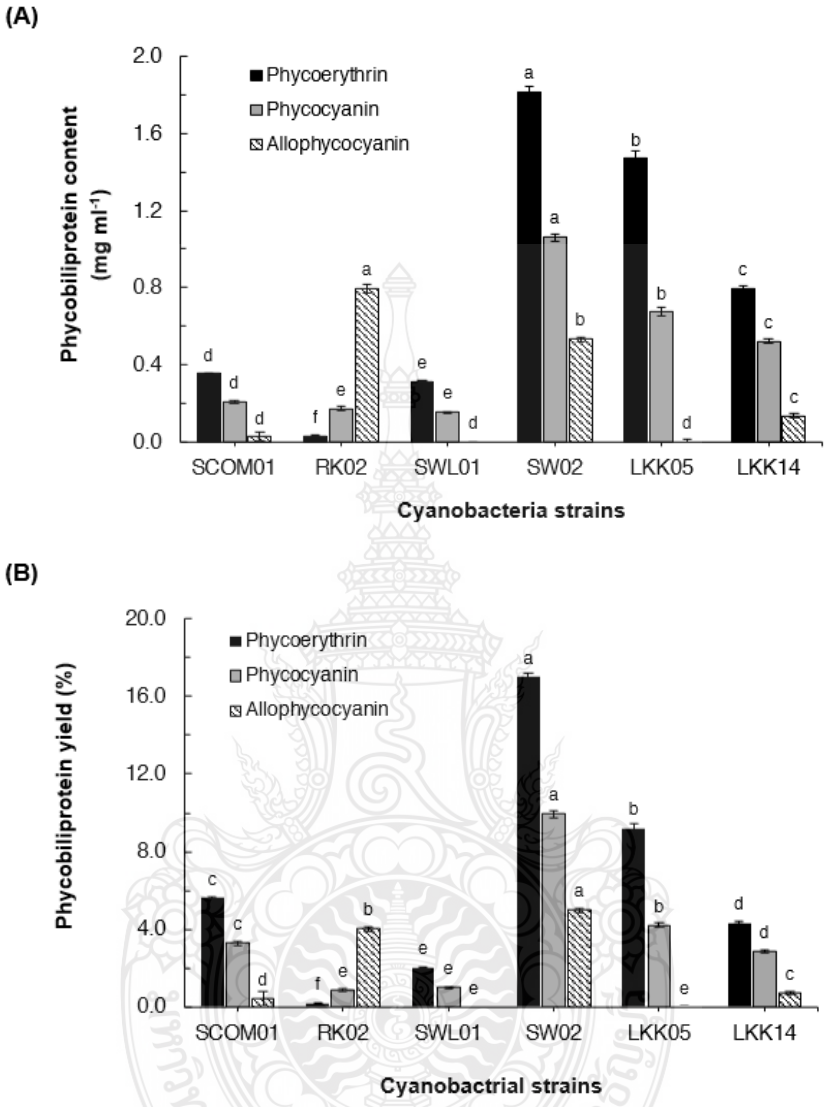


Figure 4.11 Study of phycobiliprotein concentration and yield (%) in six cyanobacteria isolated from karst cave of Thailand when cells were grown in BG11 medium for 21 days before analysis, mean ± S.D. (n = 3). According to Duncan's test, different letters within the same type of phycobiliprotein indicate significant differences ($p < 0.05$).

Table 4.4 Biomass and total phycobiliprotein yield of six cyanobacteria isolated from karst cave of Thailand

Isolated strains	Cell size (μm)		Biomass ($g\ L^{-1}$) *	Total phycobiliprotein (%yield) *
	Length	Width		
Leptolyngbya sp. SCOM01	3.71 ± 0.59	1.31 ± 0.15	0.62 ± 0.024^{a}	9.31 ± 0.540^{c}
Leptolyngbya sp. SWL01	3.48 ± 0.26	1.19 ± 0.08	0.67 ± 0.028^{a}	2.98 ± 0.042^{f}
Leptolyngbya sp. LKK14	3.35 ± 0.76	1.32 ± 0.17	0.17 ± 0.053^{c}	7.93 ± 0.213^{d}
Phormidesmis sp. RK02	3.39 ± 0.56	0.97 ± 0.23	0.17 ± 0.029^{c}	5.06 ± 0.185^{e}
Scytolyngbya sp. LKK05	3.22 ± 0.26	1.12 ± 0.15	0.71 ± 0.057^{a}	13.42 ± 0.388^{b}
Nostoc sp. SW02	5.44 ± 0.72	4.19 ± 0.94	0.55 ± 0.102^{b}	31.92 ± 0.515^{a}

* Mean ± S.D. (n = 3), Different letters within the same column indicate significant differences according to Duncan's test ($p < 0.05$).

Table 4.5 Comparison of phycobiliprotein production by different cyanobacteria under low light conditions. The percentage is relative to the cell dry weight of biomass.

Cyanobacterial strain	Growth condition	Phycobiliprotein production (%DW)	Reference
Nostoc sp. SW02	BG11 medium for 21 days, 27 ± 2 °C, 10 μmol photons m^{-2} s^{-1}	31.92	Present study
Scytolyngbya sp. LKK05	BG11 medium for 21 days, 27 ± 2 °C, 10 μmol photons m^{-2} s^{-1}	13.42	Present study
Arthrospira plantensis	Zarrouk medium for 21 days, 30 °C, 28 μmol photons m^{-2} s^{-1}	12.5	[182]
Anabaena variabilis NCCU-441	BG11 medium for 27 days, 30 ± 1 °C, 25 μmol photons m^{-2} s^{-1}	7.9	[183]
Anabaena NCCU-9	BG11 medium for 27 days, 30 ± 1 °C, 25 μmol photons m^{-2} s^{-1}	9.2	[183]
Michrochaete NCCU-342	BG11 medium for 27 days, 30 ± 1 °C, 25 μmol photons m^{-2} s^{-1}	9.1	[183]
Chroococcidiopsis sp.	BBM medium for 21 days, 20 °C, 10 μmol photons m^{-2} s^{-1}	20.4	[184]

4.8 Phycobiliprotein Purification in Six Selected Cyanobacteria

Phycobiliprotein extracted from six cyanobacterial strains were purified before properties analysis. These extracts were precipitated using 60% ammonium sulfate for enhanced the separating efficiency and yield of proteins due to the salting out effect. Then, proteins were separated through permeable membrane according to the method of dialysis before protein analysis using Native and SDS-PAGE techniques. Figure 4.12 represented protein separation in Native-PAGE electrophoresis, all crude protein extracts of six selected cyanobacteria were successfully purified and confirmed as phycobiliproteins. Based on Native-PAGE analysis, the results showed that *Leptolyngbya* sp. SCOM01 and *Leptolyngbya* sp. SWL01 expressed a single band of phycoerythrin. The other three strains, *Nostoc* sp. SW02, *Scytolyngbya* sp. LKK05, and *Leptolyngbya* sp. LKK14 showed two bands of phycoerythrin and phycocyanin, respectively. The last strain, *Phormidesmis* sp. RK02 expressed only single band of allophycocyanin (Figure 4.12).

SDS-PAGE analysis was used for determined the polypeptide compositions of purified PE, PC, and APC after running in tris-glycine-SDS buffer (pH 8.3) and Coomassie Brilliant Blue G-250 staining. The results indicated that phycobiliprotein from cyanobacterial *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc* sp. SW02, and *Scytolyngbya* sp. LKK05 showed three different protein bands (14.3, 18.2, and 19.8 kDa), which divided into α-subunit (MW 12-19 kDa) and β-subunit (MW 14-21 kDa), respectively (Figure 4.13). While *Leptolyngbya* sp. LKK14 showed protein bands approximately 18.2 and 19.8 kDa *Phormidesmis* sp. RK02 was also contained protein bands approximately 16.2 and 19.5 kDa. The Figure 4.13 revealed that each phycobiliprotein extracted from karst cave cyanobacteria is made up of heteromonomer of α and β subunit. After purification, the phycobiliprotein concentrations, purity index and % recovery were analyzed as shown in Table 4.6. The results showed that the cyanobacterium *Nostoc* sp. SW02 and *Scytolyngbya* sp. LKK05 had the phycobiliprotein recovery above 80% after purification, while the other strains had lower 50% recovery of phycobiliprotein (Table 4.6). In addition, the highest concentration and purity index were found in PE extract of *Nostoc* sp. SW02, which were 6.33 mg mL^{-1} and

2.62, respectively. However, the purity index of PE and APC in all six selected cyanobacteria were above 0.7, which was adequate purity for use as food grade product.

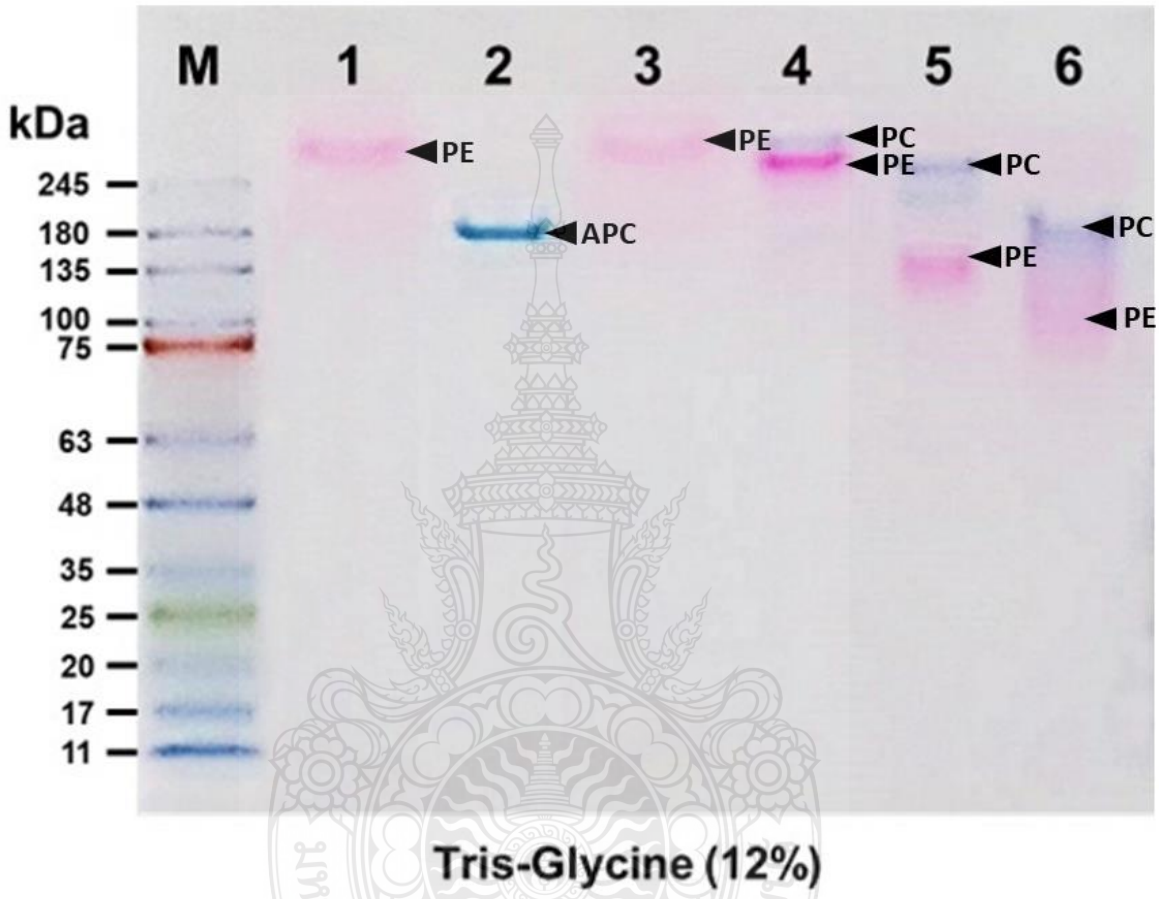


Figure 4.12 Native PAGE of the purified PBPs from six isolated cyanobacteria performed in electrophoresis of purified phycobiliproteins extracts in 12% tris-glycine without SDS buffer. Lane 1: SCOM01, Lane 2: RK02, Lane 3: SWL01, Lane 4: SW02, Lane 5: LKK05, Lane 6: LKK14, and Lane M: Blueye pre-stained protein ladder maker (GeneDireX, USA).

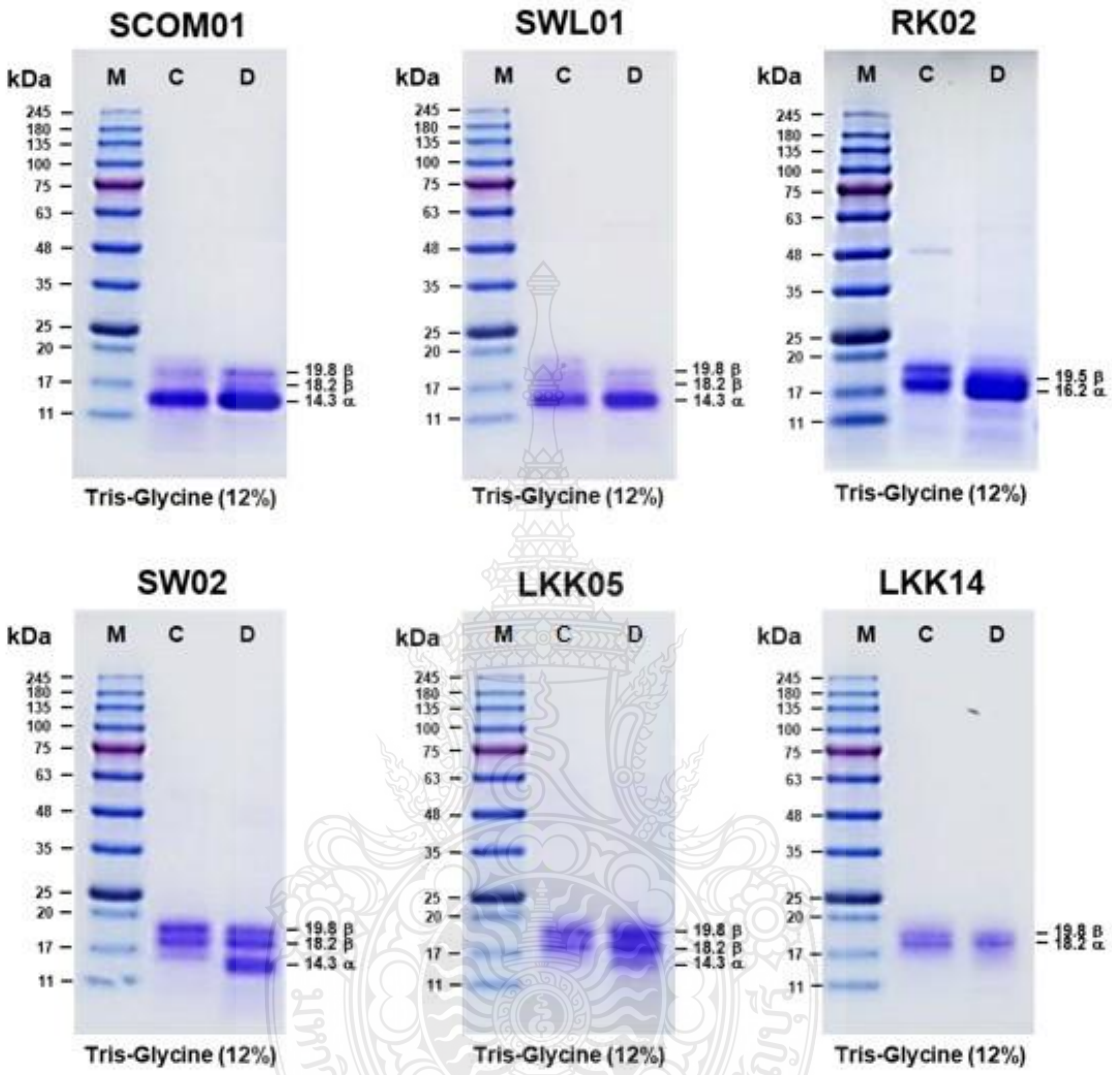


Figure 4.13 SDS-PAGE of the crude extract and purified PBPs from six selected cyanobacteria performed in this-glycine with SDS buffer.

Lane M: Blueeye pre-stained protein ladder molecular (GenDireX, USA)

Lane C: PBPs in crude extract step

Lane D: PBPs in purification step after dialysis

Table 4.6 Purification of phycobiliproteins in six selected cyanobacterial from karst cave of Thailand.

Cyanobacterial strain	Type of PBP	Purification step	Volume (mL)	PBP concentration (mg/mL)	Purity index	Recovery (%)
Leptolyngbya sp. SCOM01	PE	Crude extract	40	0.353±0.004	1.071±0.013	100
		60% APS + Dialysis	5.5	1.071±0.013	1.273±0.015	41.67±0.62
Phormidesmis sp. RK02	APC	Crude extract	40	0.939±0.028	0.747±0.003	100
		60% APS + Dialysis	5.4	1.008±0.012	1.371±0.010	28.97±0.73
Leptolyngbya sp. SWL01	PE	Crude extract	40	0.312±0.004	1.221±0.008	100
		60% APS + Dialysis	6.7	0.766±0.002	1.627±0.008	41.11±0.43
Nostoc sp. SW02	PE	Crude extract	40	1.816±0.025	1.374±0.006	100
		60% APS + Dialysis	10.5	6.331±0.009	2.621±0.010	91.54±1.15
	PC	Crude extract	40	1.062±0.018	0.535±0.001	100
		60% APS + Dialysis	10.5	3.691+0.019	0.307±0.001	91.27±1.67
Scytolyngbya sp. LKK05	PE	Crude extract	40	1.468±0.042	1.915±0.009	100
		60% APS + Dialysis	9.5	5.181±0.059	2.201±0.055	83.90±3.11
	PC	Crude extract	40	0.674±0.021	0.497±0.003	100
		60% APS + Dialysis	9.5	2.642±0.048	0.694±0.019	93.23±4.61
Leptolyngbya sp. LKK14	PE	Crude extract	40	0.794±0.017	1.959±0.013	100
		60% APS + Dialysis	5.5	2.332±0.055	2.403±0.017	40.39±0.32
	PC	Crude extract	40	0.522±0.011	0.775±0.004	100
		60% APS + Dialysis	5.5	1.826±0.047	0.150±0.005	48.12±1.74

4.9 Characterization of Phycobiliprotein Properties in Six Selected Cyanobacteria

Phycobiliproteins (PBPs) extracted from cyanobacteria are water-soluble pigment molecules composed of proteins and chromophores, called phycobilins [65]. These phycobilins can absorb light at different wavelengths and they have been reported to play action as bioactive roles of phycobiliprotein. Therefore, each purified PBPs extracts was studied for absorption and emission spectra.

Figure. 4.14 shows the results of the absorbance and fluorescence analysis of purified phycobiliprotein extracts obtained from cyanobacteria *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Nostoc*. sp. SW02 and *Leptolyngbya* sp. LKK14. The absorbance spectra of purified phycoerythrin from *Leptolyngbya* sp. SCOM01 and *Leptolyngbya* sp. SWL01 were reddish pink in solution. The highest absorption was at a wavelength of 562 nm and also found the shoulder peaks at 540 nm (Fig. 4.14 A-B). While the purified phycobiliprotein extracts from strains *Scytolyngbya* sp. LKK05, Nostoc sp. SW02 and *Leptolyngbya* sp. LKK14 were a pinkish-purple solution. They had a maximum absorbance of phycoerythrin and phycocyanin at 562 and 615 nm (Fig. 4.14 C-E). In contrast, emission wavelength spectra were 580 nm in all five strain (Fig. 4.14 A-E).

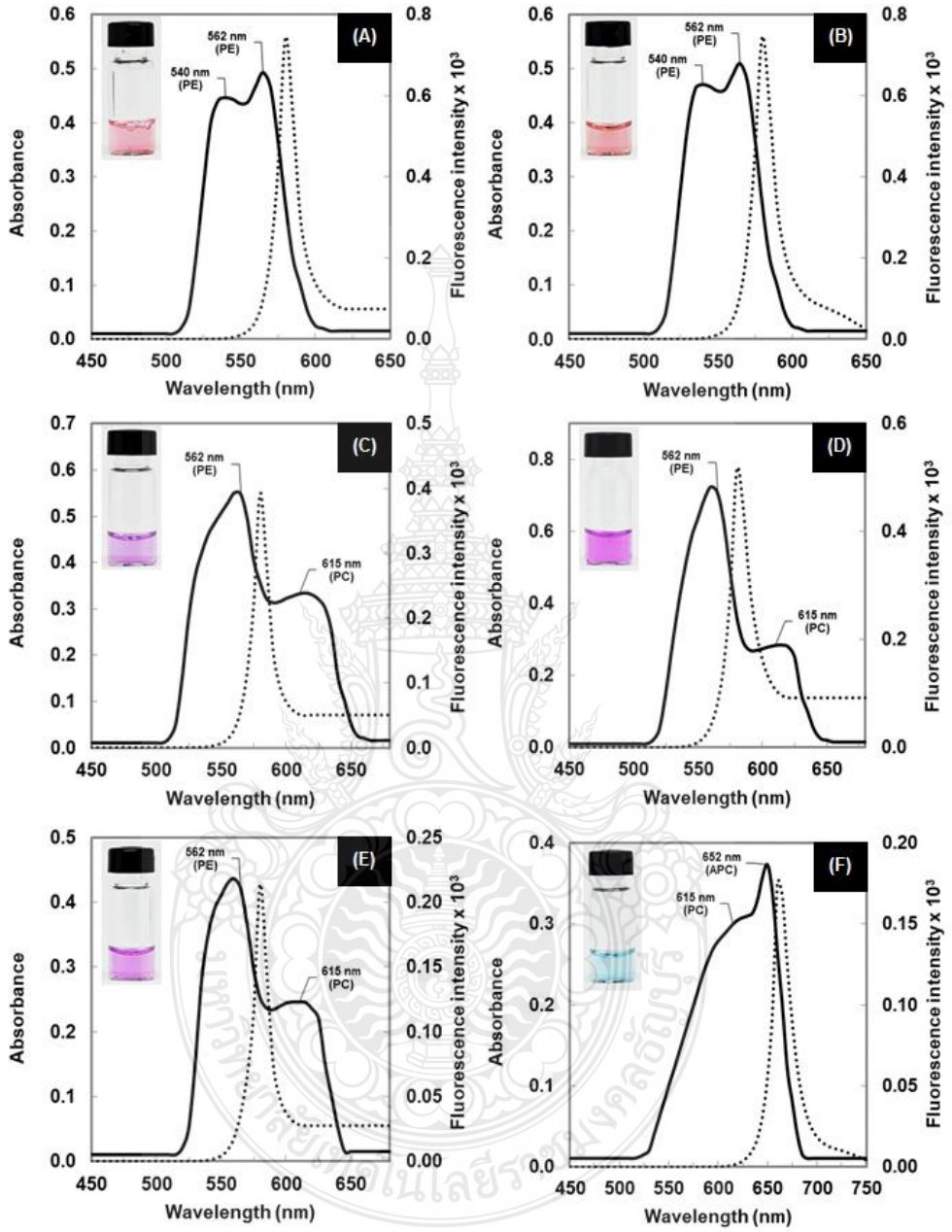


Figure 4.14 The absorbance (solid line) and fluorescence emission (dotted line) spectra with pigment appearance (inset) of purified phycobiliprotein (100 µg mL^{-1}) of six cyanobacterial strain codes SCOM01(A), SWL01 (B), LKK05 (C), SW02 (D), LKK14 (E) and RK02 (F).

The results showed that extracts containing phycoerythrin could emit fluorescence at a wavelength of 580 nm. The purified allophycocyanin extract from *Phormidesmis* sp. RK02 was a blue solution. It achieved maximum absorption at wavelengths of 615 nm and 652 nm (Fig. 4.14F). Moreover, the fluorescence emission of allophycocyanin showed at a wavelength of 660 nm. These results suggested that each type of phycobiliprotein had a different pattern of absorbance at different wavelengths. Indicating cyanobacteria isolated from the karst caves have different phycobiliprotein properties, resulting in different biological activities.

Phycoerythrin is phycobiliproteins classified as accessory pigments and available when environmental changes. The phycoerythrin can absorb light at a more comprehensive wavelength range than other phycobiliproteins. It can absorb red wavelengths to the green wavelengths of the visible light spectrum [196]. Indicating, when cyanobacteria are in low-light conditions in karst caves. Cyanobacteria might produce phycoerythrin to act as a critical protein to absorb light used for the photosynthesis process of cyanobacteria. As a result, almost cyanobacteria found in karst caves produce phycoerythrin as the main type of phycobiliprotein.

4.10 Study of Phycobiliprotein Stability in Six Selected Cyanobacteria

The effect of pH and temperature on the stability of purified phycobiliprotein from six cyanobacterial stains, *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc.* sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, and *Phormidesmis* sp. RK02 were performed as shown in Figure 4.15 and 4.16. The pH ranges from 3.0 - 10.0 using different buffer; citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 9.0-10.0) were studied. The PBPs stability was analyzed in term of the relative concentration of PBPs (%CR), the remaining concentration of PBPs as a percentage of the initial concentration. The results showed that PBPs from five stains, *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc* sp. SW02, *Scytolyngbya* sp. LKK05 and *Leptolyngbya* sp. LKK14 had been loosed stability at pH 3.0, 4.0 and 10.0. The relative

concentrations (%CR) were significantly decreased lower than 50% at pH 3.0 and 10.0 (Figure 4.15). It indicated that low and high pH affects stability of PBPs. The effect of temperature on the stability of purified phycobiliprotein from six strains of cyanobacteria was studied as shown in Figure 4.16. The results revealed that increasing of temperature above 50 °C affected PBPs stability. All purified PBPs were gradually lost their color. The phycoerythrins of *Leptolyngbya* sp. SCOM01, and *Leptolyngbya* sp. SWL01 were significantly decreased concentration when incubated under temperature above 50 °C. The %CR of both strains were below 60 at 60 °C. While the PBPs containing PE and PC in three cyanobacteria, *Nostoc* sp. SW02, *Scytolyngbya* sp. LKK05 and *Leptolyngbya* sp. LKK14 had various stabilities at different temperature. In addition, *Nostoc* sp. SW02 had the best capacity resistant to higher temperature. The purified PBPs could be stable until 55 °C (Figure 4.16). Moreover, the PBPs of *Scytolyngbya* sp. LKK05 showed lowest temperature tolerance. It started to decline %CR when reached 40 °C. Interestingly the purified allophycocyanin in *Phormidesmis* sp. RK02 retained the color up to 50 °C before completely lost when increasing higher temperature. Furthermore, this instability of phycobiliprotein samples might probably be due to the absence of intact protein, which makes the phycobilisome stable. In addition, Jespersen *et al*. [111] has also reported that the incubation at 45, 50 and 55 ◦C led to protein degradation. Furthermore, there have been reported that the CR value was above 80% when incubated phycobiliproteins at room temperature for ten days, but turbidity and odor of the solution were observed. This finding was also discussed by Doke [185], due to phycocyanin being a natural protein, microorganisms such as bacteria can easily utilize it at room temperature. However, the results of this study indicated that the phycobiliprotein extract was stable in pH 5.0 - 7.0, but with low stability at pH 3.0 and 10.0. The pH value and the phycobiliprotein concentration are the main factors controlling phycocyanin aggregation and dissociation to form monomers, trimers, hexamers and other oligomers in solution, while the hexameric structure is protected against denaturation, they dissociate to trimers at a higher pH value [114].

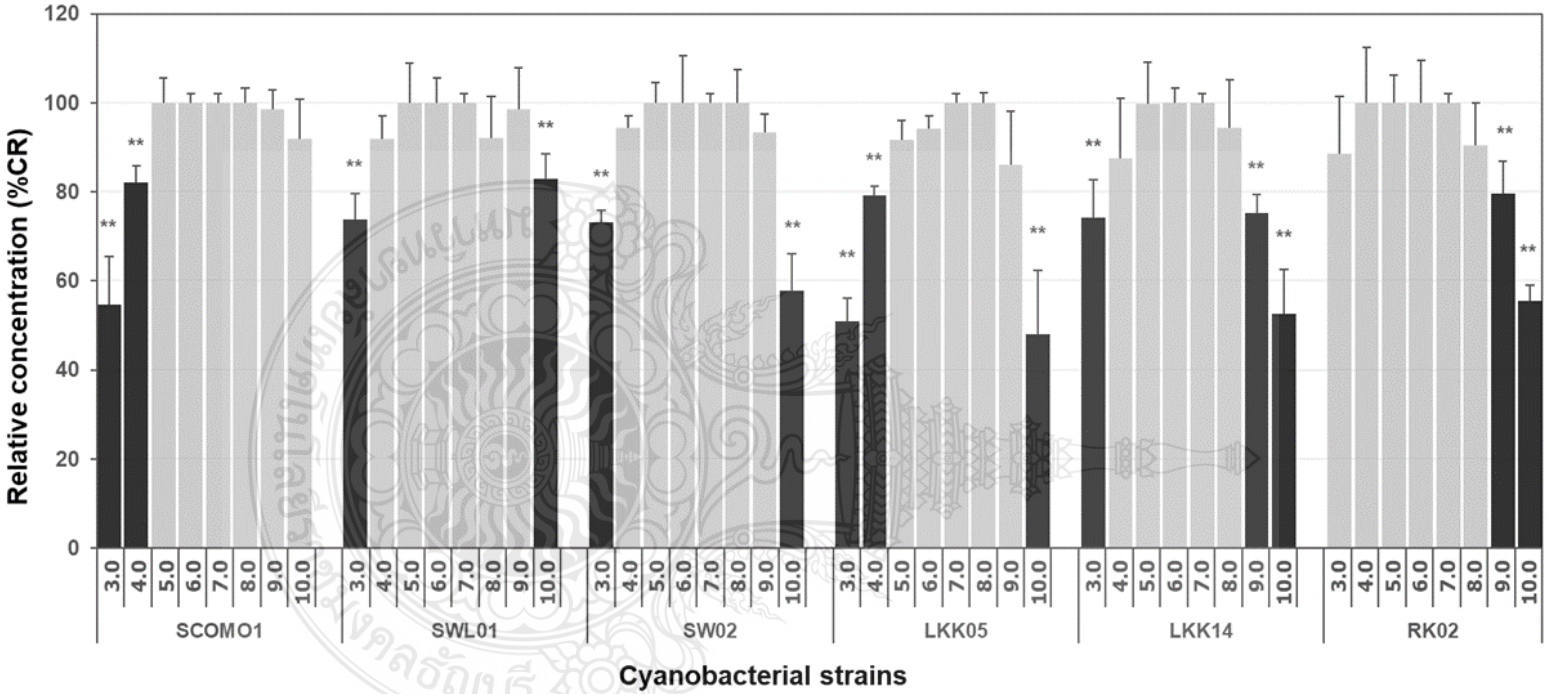


Figure 4.15 The relative concentration (%CR) of phycobiliprotein with various pH of six cyanobacterial strain *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc*. sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, and *Phormidesmis* sp. RK02. Mean ± S.D. (n = 3), asterisks indicate significant difference (** $p < 0.01$).

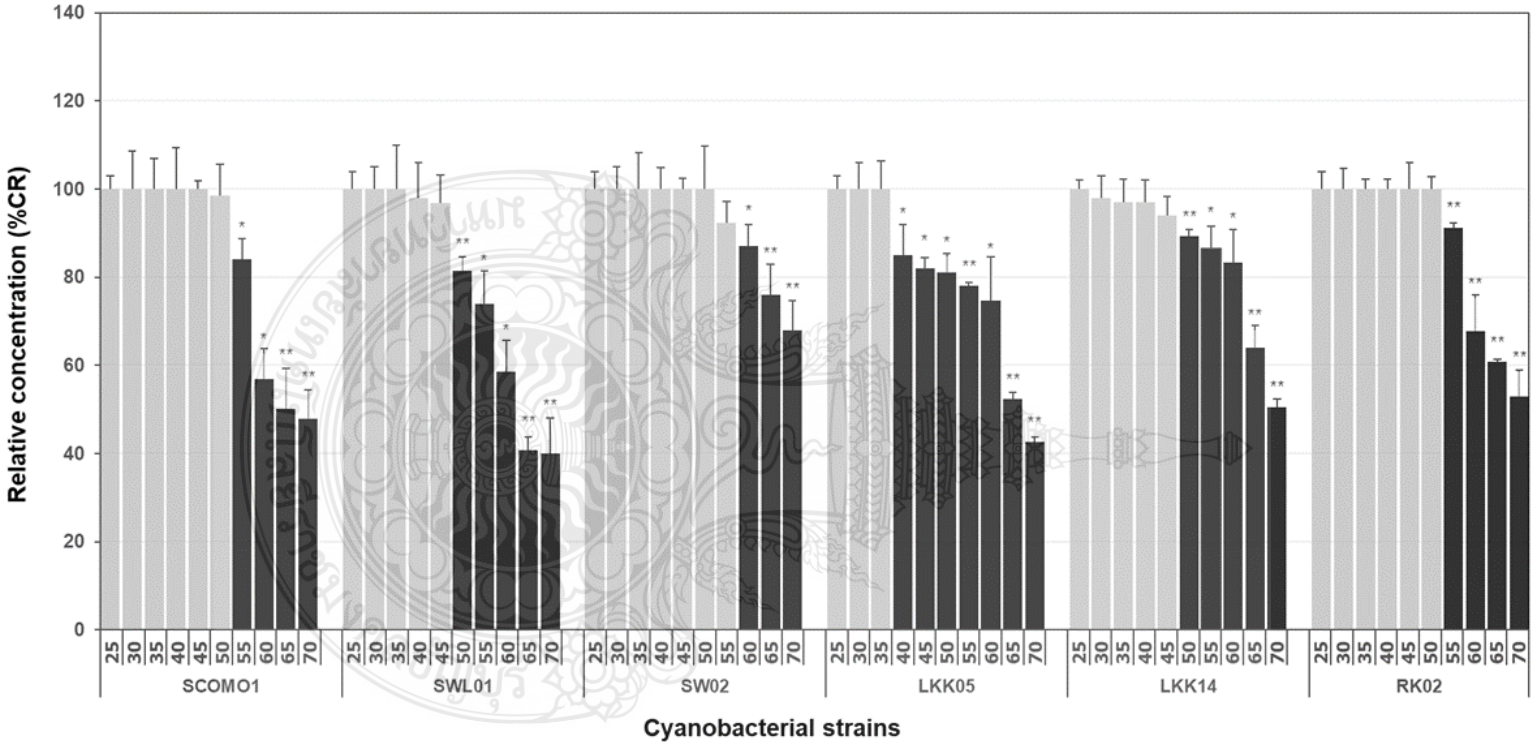


Figure 4.16 The relative concentration (%CR) of phycobiliprotein with various temperatures of six cyanobacterial strains *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc*. sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, and *Phormidesmis* sp. RK02. Mean ± (n = 3), asterisks indicate significant difference (* $p < 0.05$, ** $p < 0.01$).

4.11 Study of Antioxidant Activity of Phycobiliproteins in Six Selected Cyanobacteria

4.11.1 DPPH Activity

The DPPH assay is usually used as a reagent to evaluate the free radical scavenging activity of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [197]. The antioxidants donate electrons or hydrogen to reduce the stable radical DPPH to DPPH-H, non-radical form. A color change can visualize the reduction from purple to yellow and the reduction capability of the DPPH radical, which determined by the decrease in absorbance at 517 nm. The phycobiliprotein pigment was able to reduce the stable radical DPPH to the yellow-colored diphenyl picryl hydrazine. The vitamin C (ascorbic acid) was used as a standard to compare the antioxidant activity with purified phycobiliprotein extracts from six selected cyanobacteria. The results showed that phycobiliprotein extracts derived from six strains had antioxidant activity similar to the vitamin C standard. The various concentrations of PBPs (0-100 µg mL^{-1}) were tested. Purified PBPs from all six selected cyanobacteria exhibited high free radical scavenging activity in term of DPPH activity at 100% when using PBP extract at concentration of 100 µg mL^{-1}. Furthermore, the maximum DPPH activity at IC_{50} value was found in PBPs purified from *Nostoc*. sp. SW02, which was 30.56 µg mL^{-1}. It was almost similar activity when compared to vitamin C (Figure 4.17). However, the minimum DPPH activity (IC_{50} 58.19 µg mL^{-1}) was found in PBPs purified from *Leptolyngbya* sp. LKK14.

4.11.2 FRAP Activity

The principle of this method measures the electronegativity of antioxidants (reductant properties) in FRAP solutions; it contains Fe^{3+} and 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ). Under acidic conditions Fe^{3+} in FRAP reagent will receive electrons from antioxidant and changed into Fe^{2+} which can react with a compound TPTZ. Then, this complex is able to absorption light at the wavelength at 595 nm. The increase in absorbance at this wavelength is used as a measure of the antioxidant activity in term of FRAP activity. The results showed that when using the purified PBPs at concentration of 20 µg mL^{-1}, the cyanobacterium *Nostoc* sp. SW02 had the highest optical absorption at 595 nm, compared to

other strains (Figure 4.18). However, it was found that the electron transferred to Fe^{3+} was only half that of the standard vitamin C. The efficacy of the cyanobacterial bilin is comparable to that of other phytochemicals, indicating a great potential for antioxidant activity.

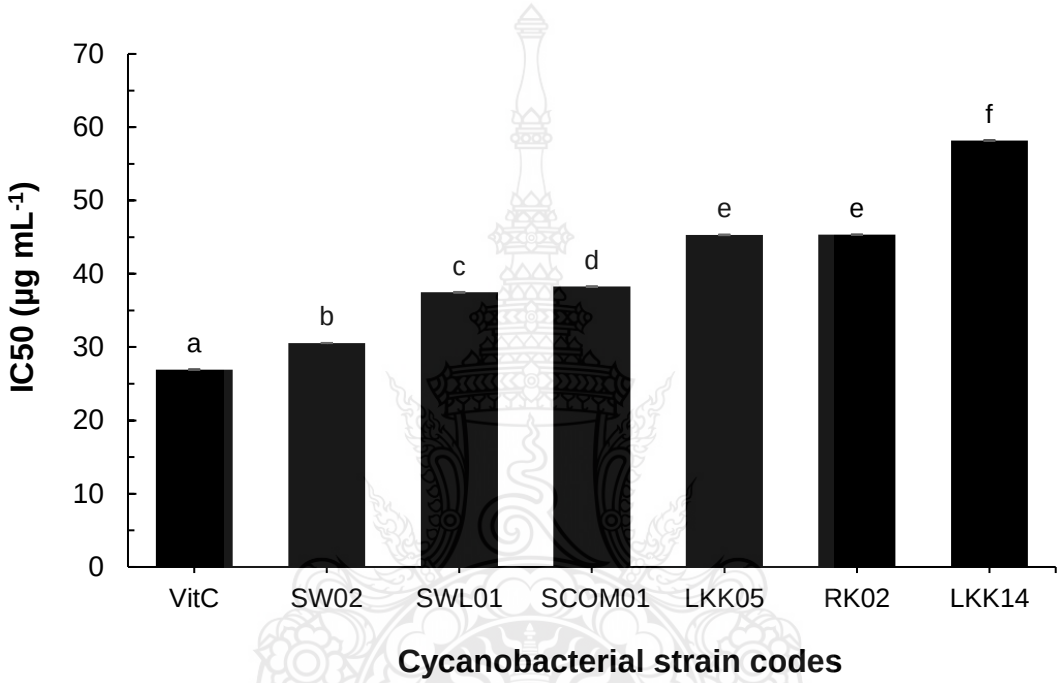


Figure 4.17 DPPH activity of purified PBPs from six selected cyanobacteria *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc*. sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, *Phormidesmis* sp. RK02, and vitamin C as a positive control. Mean ± S.D (n = 3).

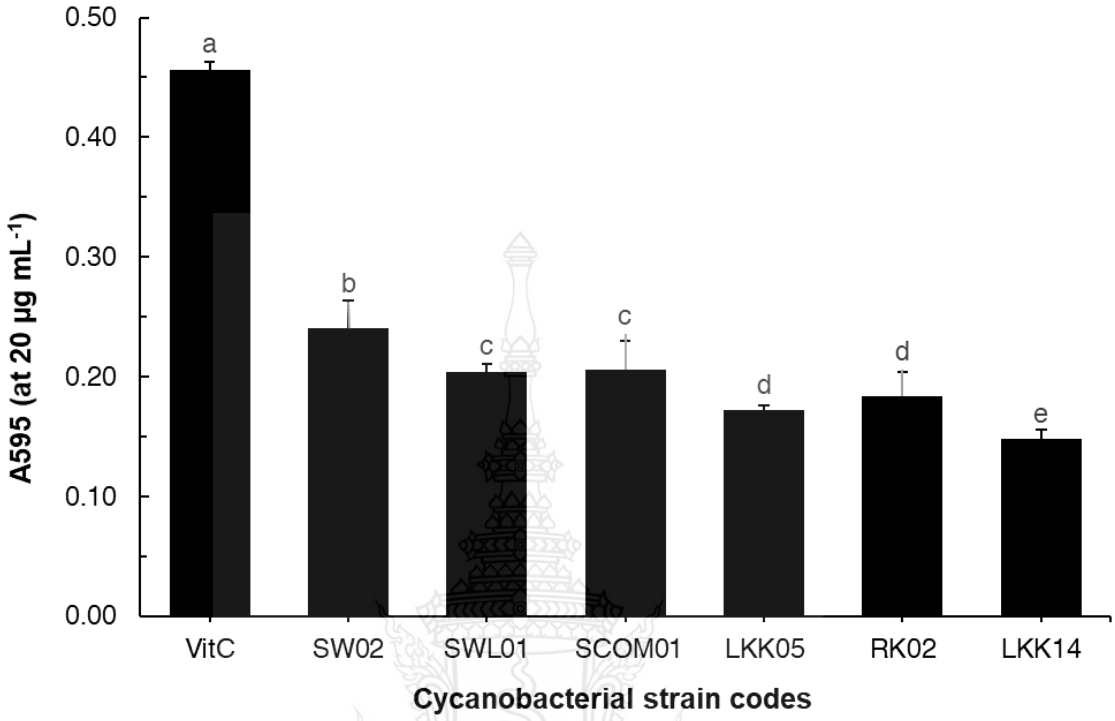


Figure 4.18 FRAP activity of purified PBPs from six selected cyanobacteria *Leptolyngbiya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc*. sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, *Phormidesmis* sp. RK02, and vitamin C as a positive control. Mean ± S.D (n = 3).

4.12 Study of Antimicrobial Activity of Phycobiliprotein in Six Selected Cyanobacteria

4.12.1 Paper Disc Diffusion Test

The method of disc diffusion used for preliminary testing antimicrobial activity of purified PBPs. The maximum content of purified PBPs of six strains *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Nostoc* sp. SW02, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14, and *Phormidesmis* sp. RK02 were 42.52, 30.56, 253.28, 175.32, 24.24, and 39.80 µg, respectively, which dropped to disc before testing. The microbial organisms used in this study were three gram-positive bacteria, *Bacillus subtillis*

TISTR 1528, *Propionibacterium acnes* DMFT 14916 and *Staphylococcus aureus* ATCC 25923; one gram-negative bacteria, *Escherichia coli* ATCC 25922; and one yeast *Candida albicans* SU 28 at 0.5 McFarland turbidometry. And chloramphenicol (10 µg) was used as antibiotic standards. The results showed that the extracts at the highest concentration of all species could not inhibit the microorganisms tested (data not shown). Therefore, cyanobacteria strain *Nostoc* sp. SW02 was selected for further MIC testing due to its good antioxidant activity.

4.12.2 Minimum Inhibitory Concentration (MIC) Assay

Cyanobacteria strain *Nostoc* sp. SW02 showed potential for phycobiliprotein production with a yield of phycobiliprotein equal to 31.92%. It also had similar antioxidant activity as compared to vitamin C. Therefore, this strain was tested for minimum inhibitory concentration (MIC) values as shown in Table 4.7. *Nostoc* sp. SW02 was able to inhibit human bacterial pathogens with the MIC values of PBPs extracts at a concentration of 1000 µg mL^{-1}. The percentage of inhibition were 17.75, 24.42, 14.81, and 6.60 on *Bacillus subtillis* TISTR 1528, *Propionibacterium acnes* DMFT 14916, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, respectively. However, this study showed that the phycobiliprotein extract could not inhibit *Candida albicans* SU 28 at the same concentrations.

Table 4.7 Antimicrobial activity of phycobiliprotein extracted from cyanobacteria strain *Nostoc* sp. SW02 showing %inhibition against microorganisms tested

Microorganisms	%Inhibition				
	1000 µg	500 µg	250 µg	125 µg	0 µg (control)
Gram positive bacteria					
Bacillus subtillis TISTR 1528	17.753±2.259	-	-	-	-
Propionibacterium acnes DMFT 14916	24.429±1.556	-	-	-	-
Staphylococcus aureus ATCC 25923	14.813±1.573	-	-	-	-
Gram negative bacteria					
Escherichia coli ATCC 25922	6.609±0.972	-	-	-	-
Yeast					
Candida albicans SU 28	-	-	-	-	-

- = no inhibition

4.13 Study of Anticancer Activity of Phycobiliproteins in *Nostoc* sp. SW02

4.13.1 Effect of Purified PBPs on Normal Cell

The human skin fibroblast cell (HFF-1) was used for study of cytotoxicity of purified PBPs from *Nostoc* sp. SW02 on normal cell line. The concentration of PBPs extract were varied at 0-1000 μg mL^{-1}. The results showed that when incubated HFF-1 cells with phycobiliprotein extract for 24 h, no cytotoxicity on normal cell line was found in every concentration of PBPs (Figure 4.19A). However, when incubated cell longer time, there was also found effect of toxic at high concentration of 500 and 1000 μg mL^{-1}, respectively. Therefore, it is noted that the dose of PBPs extract should not over than 500 μg mL^{-1} for preventing cytotoxicity.

4.13.2 Effect of Purified PBPs on Cancer Cell

The cytotoxicity of PBPs extracted from *Nostoc* sp. SW02 on cancer cell was studied using sulfhorodamine B assay. The cancer cell was tested in this study as human cervical cancer Hela cell. The Hela cell was mixed with PBPs extract at different concentration ranging of 0-1000 μg mL^{-1} and incubated for 24, 48, 72 h before determination. The result revealed that PBPs extract from *Nostoc* sp. SW02 had a potential on anticancer activity, at 1000 μg mL^{-1} could inhibit Hela cell growth since 24 h of incubation (Figure 4.20A). Moreover, PBPs extracts showed strongly effect on cell viability at 500 μg mL^{-1} when incubated longer time above 48 h (Figure 4.20B). The IC_{50} value was 140 μg mL^{-1} when incubated of Hela cell with PBPs extract for 72 h as shown in Figure 4.20C. The mechanisms of action of an anticancer compound can be characterized by three basic characteristics of cancer cells, proliferation, deregulated apoptosis and hyperplasia. Therefore, the anticancer drug may be able to inhibit the cell proliferation and/or induce the tumor cell apoptosis. To understand the action of phycocyanin in the apoptotic pathway, it is important to consider that during the apoptosis the main processes that occur are the activation of endonuclease and DNA fragmentation and the formation of apoptotic bodies as well as the activation of the caspase enzymes which are responsible for the apoptotic pathway [186–188].

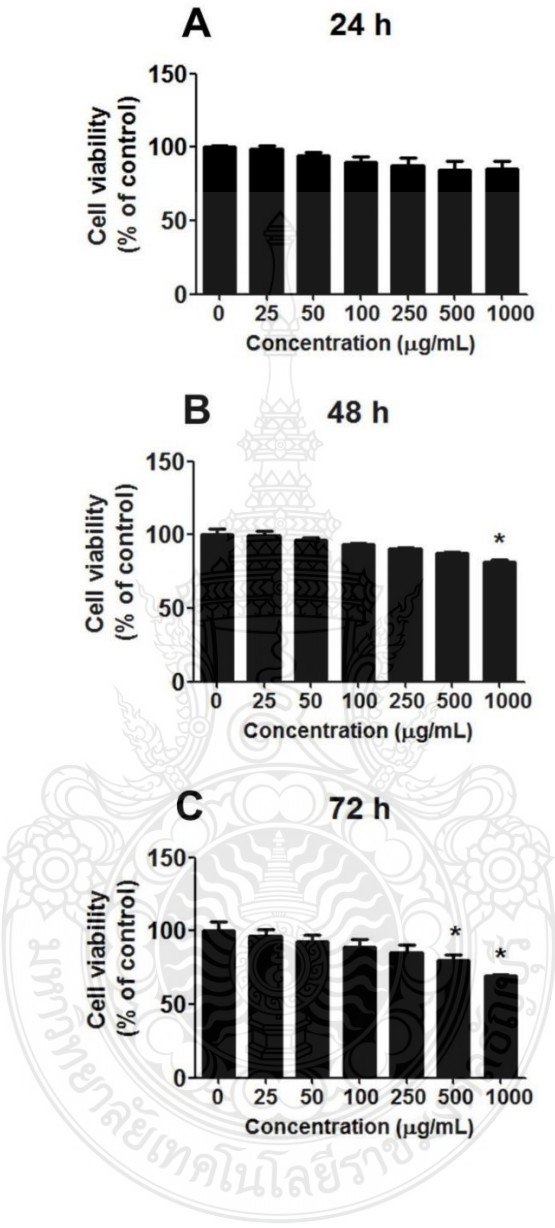


Figure 4.19 Effect of PBPs extract from *Nostoc* sp. SW02 on normal cell line (HFF-1) viability after incubation for 24 h (A), 48 h (B), 72 h (C). * Mean ± S.D. (n = 3), significant differences according to Duncan's test ($p < 0.05$).

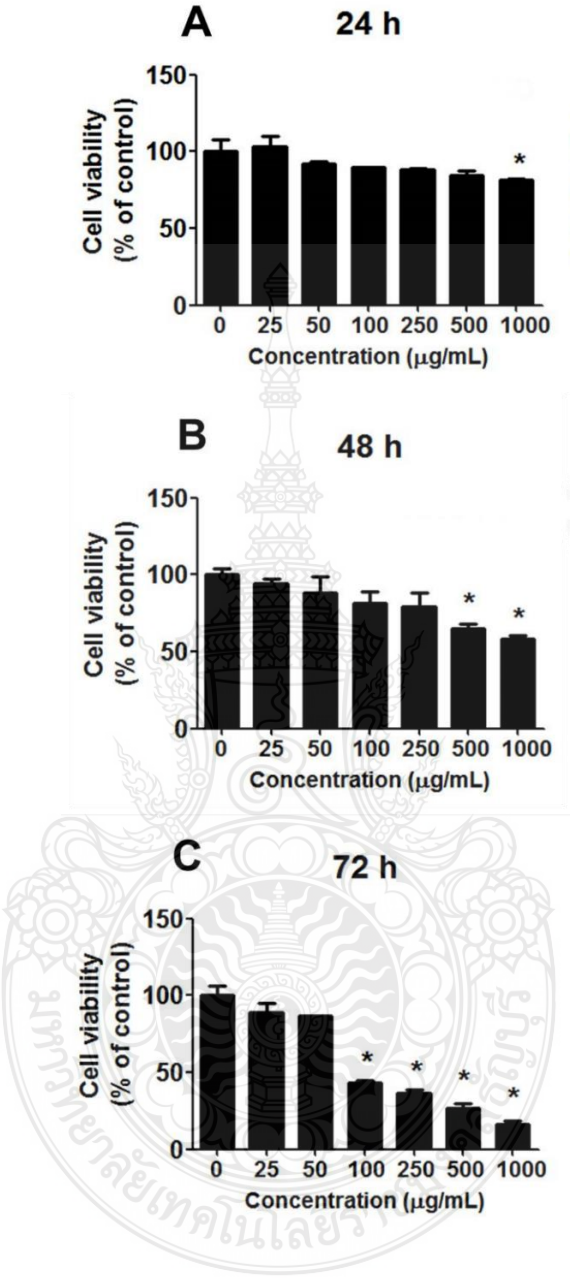


Figure 4.20 Effect of PBPs extract from *Nostoc* sp. SW02 on human cervical cancer Hela-cell after incubation for 24 h (A), 48 h (B), 72 h (C). * Mean ± S.D. (n = 3), significant differences according to Duncan's test ($p < 0.05$).

CHAPTER 5
CONCLUSION

1. A total of 86 cyanobacteria strains were isolated and cultivated from 23 karstic caves in Thailand. They could be classified into 10 families, 12 genera including *Nostoc*, *Oscillatoria*, *Mastigocladus*, *Scytonema*, *Anabaena*, *Hapalosiphon*, *Leptolyngbya*, *Phomidesmis*, *Scytolyngbya*, *Chroococcus*, *Synechococcus*, and *Chroococcidiopsis*.
2. All isolated cyanobacteria from karst caves showed that abundant cyanobacteria belonged to *Leptolyngbya* genus, which were 37 species distributing along to all sampling sites.
3. The % relative humidity and temperature might be influence on the diversity of cyanobacteria existing in the karst caves of Thailand.
4. Among cyanobacteria tested, there were six species of cyanobacteria, namely *Leptolyngbya* sp. SCOM01, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14 and *Nostoc* sp. SW02 showed a high potential for PBP production as high yielders (over 400 mg g protein^{-1}).
5. Cyanobacterium *Nostoc* sp. SW02 had the highest phycobiliprotein yield of 31.92% (w/w) with a PE: PC: APC ratio of 3.4:1.9:1.0.
6. The phylogenetic tree analysis could suggest that six isolated SCOM01, SWL01, RK02, LKK05, SW02 and LKK14 should belong to *Leptolyngbya* sp. SCOM01, *Leptolyngbya* sp. SWL01, *Phromidesmis* sp. RK02, *Scytolyngbya* sp. LKK05, *Nostoc* sp. SW02 and *Leptolyngbya* sp. LKK14, respectively.
7. The isolated strains, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SCOM01, and *Leptolyngbya* sp. SWL01 showed less than 97% similarity (*16S* rRNA sequence), they might be classified as new species.
8. The selected cyanobacterial *Leptolyngbya* sp. SCOM01, *Phormidesmis* sp. RK02, *Leptolyngbya* sp. SWL01, *Scytolyngbya* sp. LKK05, *Leptolyngbya* sp. LKK14 and *Nostoc* sp. SW02 were upscaled for phycobiliproteins production and purification, and the cyanobacterial *Nostoc* sp. SW02 and *Scytolyngbya* sp. LKK05 had the

phycobiliprotein recovery above 80% after purification, while the other strains had below 50% recovery of phycobiliprotein.

9. The highest concentration and purity index were found in PE extract of *Nostoc* sp. SW02, which were 6.33 mg mL^{-1} and 2.62, respectively.
10. The purity index of PE and APC in all six selected cyanobacteria had higher than 0.7, which purity for use as food grade product.
11. The Native-PAGE could confirm that all extracts from six selected cyanobacteria were phycobiliproteins.
12. The SDS-PAGE analysis indicated that phycobiliproteins from six selected cyanobacteria containing three different protein sizes, which divided into α-subunit (MW 12-19 kDa) and β-subunit (MW 14-21 kDa), respectively.
13. The study of phycobiliprotein characterization showed that all selected cyanobacteria had different patterns of absorbance at different wavelengths, indicating that the property of phycobilins containing phycobiliproteins might effect on different biological activities.
14. The pH 3.0, 4.0, and 10.0 resulted in phycobiliprotein stability from six selected cyanobacterial strains.
15. The temperature above 50 °C could influent on phycobiliprotein stability by reduced the properties of extracted PBPs.
16. All purified phycobiliprotein from selected karst cave cyanobacteria showed biological activity with the purified PBPs from *Nostoc*. sp. SW02 had a potential as antioxidant, antimicrobial and anticancer activities.
17. The purified phycobiliprotein from *Nostoc*. sp. SW02 showed the maximum DPPH activity at IC_{50} of 30.56 µg mL^{-1}.
18. The purified phycobiliprotein from *Nostoc*. sp. SW02 was able to inhibit human bacterial pathogens with the MIC values at a concentration of 1,000 µg mL^{-1}.
19. The purified phycobiliprotein from *Nostoc*. sp. SW02 could toxic on Hela cancer cells by the IC_{50} value of 140 µg mL^{-1} when incubated of Hela cancer cell with PBPs extract for 72 h with no cytotoxicity on normal cell line.

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APPENDICES

APPENDIX A

BG11 medium

BG11 medium

Nutrient composition of BG11 medium for growing cyanobacteria

Nutrients	Amount	Stock solution concentration
KH_2PO_4	1 mL L^{-1}	40 g L^{-1}
$MgSO_4.7H_2O$	1 mL L^{-1}	75 g L^{-1}
$CaCl_2.2H_2O$	1 mL L^{-1}	38 g L^{-1}
$Na_2EDTA.2H_2O$	1 mL L^{-1}	1 g L^{-1}
$FeSO_4.7H_2O$	1 mL L^{-1}	6 g L^{-1}
$NaCO_3$	1 mL L^{-1}	20 g L^{-1}
Citric acid	1 mL L^{-1}	6 g L^{-1}
$NaNO_3$	10 mL L^{-1}	150 g L^{-1}
Trace element*	1 mL L^{-1}	-

*Preparation stock solution by H_3BO_3 2.68 g, $CuSO_4.5H_2O$ 0.079 g, $Na_2MoO_4.2H_2O$ 0.39 g, $ZnSO_4.7H_2O$ 0.22 g, $MnCl_2.4H_2O$ 1.81 g, $Co(NO_3)_2.6H_2O$ 0.049 g and adjust volume to 1,000 ml with deionized water.

APPENDIX B

Bacterial Medium

Nutrient agar (NA)

Liquid media, composition per 1 liter

Peptone	2	g
Beef extract	3	g

Solid media, composition per 1 liter

Peptone	2	g
Beef extract	3	g
Agar	15	g

Yeast malt (YM)

Liquid media, composition per 1 liter

Malt extract	3	g
Yeast extract	3	g
Peptone	5	g
Glucose	10	g

Solid media, composition per 1 liter

Malt extract	3	g
Yeast extract	3	g
Peptone	5	g
Glucose	10	g
Agar	15	g

All compositions were dissolved together with 1 liter of distilled water. The medium was sterilized by autoclave.

APPENDIX C

Buffers

0.01 M Citrate buffer

pH 3.0

$C_6H_9Na_3O_9$ (mw: 294.10 g/mol)	276 mg
$C_6H_8O_7$ (mw: 192.12 g/mol)	1.741 g

pH 4.0

$C_6H_9Na_3O_9$ (mw: 294.10 g/mol)	993 mg
$C_6H_8O_7$ (mw: 192.12 g/mol)	1.273 g

pH 5.0

$C_6H_9Na_3O_9$ (mw: 294.10 g/mol)	1.71 g
$C_6H_8O_7$ (mw: 192.12 g/mol)	804 mg

0.01 M Phosphate buffer

pH 6.0

$Na_2HPO_4.7H_2O$ (mw: 268.07 g/mol)	367 mg
$NaH_2PO_4.H_2O$ (mw: 137.99 g/mol)	1.191 g

pH 7.0

$Na_2HPO_4.7H_2O$ (mw: 268.07 g/mol)	1.549 g
$NaH_2PO_4.H_2O$ (mw: 137.99 g/mol)	583 mg

pH 8.0

Na_2HPO_4 (mw: 141.96 g/mol)	27.61 g
$C_6H_8O_7.H_2O$ (mw: 210.14 g/mol)	580 mg

0.01 M Glycine-NaOH buffer

pH 9.0

$C_2H_5NO_2$ (mw: 75.07 g/mol)	750 mg
NaOH (mw: 40.0 g/mol)	70 mg

pH 10.0

$C_2H_5NO_2$ (mw: 75.07 g/mol)	750 mg
NaOH (mw: 40.0 g/mol)	260 mg

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