Studies on Cultivation, Cell Wall Development, and Gene Analysis of Marine Microalgae *Pleurochrysis carterae* and *Isochrysis zhanjiangensis*

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ABSTRACT

In last decade, the mankind has been intensively facing the climate changes in the whole world, because the demand for fuel and greenhouse gas emissions is increasing dramatically, especially the amount of carbon dioxide. To reduce carbon dioxide in the environment, chemical immobilization, physical fixation, and biofixation are used. To solve this series of problems, except developing hydro energy, sources like wind energy, solar energy, humans began to exploit new alternative energy sources, such as bioethanol and biodiesel. Among biofixation, microalga is generally considered to be the most effective one, because of its faster growth rate (about 8 hours/generation), wider distribution range, and lower production cost. *Pleurochrysis carterae* is a marine microalga that not only absorbs carbon dioxide by photosynthesis but also absorbs HCO_3^- in the ocean and air to synthesize $CaCO_3$ through its unique calcification. Moreover, *Pleurochrysis carterae* has a lipid content as high as to 33 %, which also contains with high nutritional value. In other words, *Pleurochrysis carterae* not only has a high potential value in fixing carbon dioxide but also has a great potential in the production of biodiesel and some other high priced products, such as fucoxanthin. Another species of microalga, haptophyte *Isochrysis zhanjiangensis,* has tremendous potentials in producing high-value products, including fucoxanthin and docosahexaenoic acid (DHA).

The *P. carterae* cells are covered by coccoliths which are mainly composed of calcium carbonate. The coccoliths including scales are formed inside the cells first and then are

ejected to the outside of the cells. The *P. carterae* cells are surrounded by coccospheres consisting of a single layer of coccoliths and some loosely underlying layers of un-mineralized scales. This study showed *P. carterae* has four stages in its life cycle, and coccoliths occurred in single cell stages but not in colony stages. We revealed the development processes of coccolith and scale on the shells of *P. carterae* and the cells are only covered with tight multiple layers of scale coat without coccolith on its top surface. We call this as scale extracellular formation pathway, which is different from the coccolith and scale intracellular formation pathway.

In this study, 109,017 bp genome of *P. dentata* chloroplast (cp) were sequenced and analyzed. This is the first report of the cp genome analysis of the genus *Pleurochrysis,* just liking those found in other algae. The study of the phylogenetic relationship with other algal species at various genetic distance gives the insights into the molecular evolution of their cp genomes where *P. dentata* shows a close lineage with other two haptophyte *Phaeocystis antartica* and *Phaeocystis globose*. The results of this study can be used for the discovering the genome organization and evolution in other species of *Pleurochrysis.*

From this study, we cultivated the cell of *Isochrysis zhanjiangensis*, and discovered the effect of initial cell density and temperature on algal growth is obvious indoor and outdoor. The group of lower density has faster growth rate and more biomass accumulation. During the outdoor experiment, if the temperature is higher than 37℃, the algal growth and photosynthesis in chloroplast would be seriously inhibited.

Key words: *Pleurochrysis carterae*, life cycle, scale extracellular formation, chloroplast genome analysis, *Isochrysis zhanjiangensis*, photosynthesis efficiency, outdoor cultivation.

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ABBREBIATIONS

aa	amino acid
ACCase	acetyl CoA carboxylase
ALA	α-linoleic acid
bp	base plate
bs	body scale
C-cells	contain coccolith but no flagella coccolith-bearing cells
N-cells	without coccolith and flagella naked cells
S-cells	contain coccolith and flagella
cp	chloroplast genome
CT	cytoplasmic tongue
CV	coccolith vesicle
DCW	dry cell weight
DHA	docosahexaenoic acid
DMSO	dimethyl sulphoxide
DMSP	dimethyl sulfoniopropionate
DOM	dissolved organic matter
EPA	eicosapentaenoic acid
ESM	enriched sea medium
d4FAD	Δ4-desaturase
d5FAD	Δ5-desaturase
FAME	fatty acid methyl ester
FPPs	flat plate photobioreactors
Fv/Fm	photosynthesis efficiency
GLA	γ-linolenic acid
ICP	intracellular coccolith precursor
LC- PUFA	Long chain polyunsaturated fatty acids
MN	microtube network

MFPPs	modular flat-plate photobioreactors
OD	optical density
PDOM	particulate organic matter
PER	peripheral endoplasmic reticulum
IPER	inner peripheral endoplasmic reticulum
OPER	outer peripheral endoplasmic reticulum
PS1	acidic polysaccharides -1
PS2	acidic polysaccharides -2
PS3	acidic polysaccharides -3
PUFAs	polyunsaturated fatty acids
S	scale
ss	base plate scale
SDA	stearidonic acid
TAG	triacylglycerols
VCB	vertical column photobioreactor
VCPs	vertical column photobioreactors

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CHAPTER I

An Overview of Marine Microalgae *Pleurochrysis carterae* and *Isochrysis zhanjiangensis*

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Abstract

As the global industrialization accelerates process, the demand for fuel and greenhouse gas emissions are increasing dramatically, especially the amount of carbon dioxide. To solve this series of problems, humans began to develop new, alternative energy sources, such as bioethanol, biodiesel. To reduce carbon dioxide in the environment, chemical immobilization, physical fixation, or biofixation are used. Among them, microalga is generally considered to be most effective because of its faster growth rate, wider distribution range, and lower cost. *Pleurochrysis carterae* is a marine microalga that not only absorbs carbon dioxide by photosynthesis but also absorbs HCO_3^- in the ocean and air to synthesize $CaCO_3$ through its unique calcification. Moreover, *Pleurochrysis carterae* has a lipid content as high as to 33 %, which also contains docosahexaenoic acid (DHA) with high nutritional value. In other words, *Pleurochrysis carterae* not only has a high potential value in fixing carbon dioxide but also has extreme potential in the production of biodiesel and biomedicine. The algae database (https://www.algaebase.org/) shows that there is no any chloroplast genomes data of *Pleurochrysis*, there are only 8 species chloroplast genomes

message are not belong to genus *Pleurochrysis*.

Isochrysis zhanjiangensis is one of marine microalgae that widely distribute near costal area. There were several *Isochrysis spp*. that had been cultured as well bait to feed larval stage of molluscs and fishes, depending on the high fatty acid content with a variety of high-value products, including fucoxanthin and docosahexaenoic acid (DHA) within cell body.

Key words: docosahexaenoic acid, fucoxanthin, biofixation

1 Introduction

Billions of years ago, algae and cyanobacteria thriving in the ocean evolved the ability for capturing atmospheric carbon to synthesize carbohydrate. During photosynthesis, these microorganisms convert light energy to chemical energy via accumulation of various sugar. As the time went on, all organic materials in primary producers were mineralized into earth's crust and finally formed fossil energy. Since the development of mechanization, a large amount of fossil resource has been explored and utilized as industrial blood. With the development of global industrialization, the demand of fossil energy is consistently increasing. Prior the term of the industrial revolution, the volume of greenhouse gas in air was carbon dioxide and its content was 280 ppm for numerous thousand years (Galbe et al. 2002; Hoegh-Guldberg et al. 2007). However, at the end of the twentieth century, the concentration of this greenhouse gas (CO_2) in the atmosphere had increased to 367 ppm, and showed a continuous rising trend (Geider et al. 2001; Le Quéré et al. 2012). The bulk volume of CO_2 in atmosphere have been increasing continuously since then as the result of combustion of fossil fuels. Therefore, the excess greenhouse gas CO_2 plays a key role on global climate change and ocean acidification (Iglesias-Rodriguez et al. 2008). To comprehend the effect of climate change in the ocean, the flow of organic matters in ocean is a significant research aspect. The biological pump involves a set of metabolic pathways via carbon

fixation by photosynthesis, exported into ocean causing the increased stock of carbon. Generally, the biological pump deposits 300 million tons of carbon in the deep ocean annually. Moreover, vast amounts are suspended in the water as soluble organic carbon, much of which is converted into stable structure through the microbial carbon pump (Jiao et al. 2011).

Therefore, in the recent years, scientists preferred to fix atmospheric CO_2 via biological methods to achieve carbon mineralization. Compared with chemical or physical fixation, biological fixation is friendly to environment and is feasible for promotion. The organisms which have the ability of CO_2 fixation mainly include plants, cyanobacteria, photosynthetic bacteria and algae. The efficient way of carbon fixation is photosynthesis by higher plants. However, due to limitations in growth rate, water supply and area of land, the higher plants don't represent outstanding advantage for CO_2 bioremediation, therefore, the cultivation of photosynthetic microalgae has been proposed as an alternative strategy (Herzog & Drake, 1996). Compared with other carbon-fixing organisms including terrestrial plants, a series of features that algae have make them more attractive. Indeed, algae have a higher growth rate, photosynthesis efficiency and CO_2 fixation rate, thus, they can be regarded as an ideal carbon-fixing organism (Benemann 1997; Packer 2009).

Consequently, there are three kinds of microalgae dominating contemporary ocean: diatoms, dinoflagellates, and coccolithophores (Endo & Hirotoshi, et al. 2016). Coccolithophores, the members of phylum Haptophyta, are common groups of unicellular marine algae, widely distribute in tropical and temperate ocean areas as autotrophy phytoplankton. Coccolithophores have species-specific capacity that could yield calcium carbonates by intracellular calcification and cover themselves with $CaCO_3$ crystal (coccolith) as extracellular scales. The mechanisms involved coccolith formation have been mainly studied in the genera *Emiliania* and *Pleurochrysis*, which

are grouped in two separate phylogenetic lineages of the coccolithophorids (Fujiwara et al. 2001). According to this unique characteristic, coccolithophores play vital roles in carbon elemental biogeochemical cycles as main contributors of oceanic calcite (Holligan et al. 1993).

Based on the ability of producing coccolith scales, coccolithophorid algae could be a candidate for CO_2 bioremediation. In contrast to other species of coccolithophorid algae growing in the ocean, *Pleurochrysis carterae* and *Emiliania huxleyi* species have great advantage on the growth rate. In fact, *P. carterae* is a symbolic coccolithophorid species that widely distributes and blooms in coastal areas. As a single-cell marine microalga, *P. carterae* can accumulate CO_2 from the surrounding environment for photosynthesis and produce organic substances including carbohydrates, proteins, and lipids. Moreover, it can also convert CO_2 into calcium carbonate to form their outer shell by calcification. Therefore, *P. carterae* has a broad application prospect in carbon fixation. On the other hand, *Emiliania huxleyi* is the most significant global distributing and blooming species (Zhou & Chengxu, et al. 2016) and well-known as the largest long-term sink of inorganic carbon on the earth (Westbroek et al. 1994). The large-scale cultivation for coccolithophorid algae has recently been reported to be available. Nonetheless, due to the difficulties of commercial culture for the production of biofuels or other high value products, sustainable high productivities are an inevitable issue (Moheimani & Borowitzka, 2006).

There are many factors that influence the growth of microalgae. In general, the major factors can be divided in two groups, abiotic factors and biotic factors. However, the abiotic factors including appropriate light intensity, temperature, and nutrients bring positive effects on algal growth and multiplication. Furthermore, a few biotic factors like presence of predators and toxic material in aquatic environment are restricting the accumulation of algal biomass. In fact, it is hard to point out which single factor influences algal growth, since all of factors involve in operation

of life cycle (Abu-Rezq et al. 1999). Therefore, in order to developed renewable biofuels from microalgae, a study was run from 1978 to 1996 on the aquatic species program funded by U.S. Department of Energy. The initial purpose of this program was productivity of biofuels from high lipid content of aquatic flora. During this two decades, massive advances have been performed in the program on screening of algal strains and their characterization. In addition, the design of algal bioreactors and analysis of modifying metabolism in algae also have been developed (Sheehan et al. 1998).

1.1 Lipid in Coccolithophores

As a multiple group of microorganisms, eukaryotic algae have broad range of habitats from the Arctic to the Sahara (Irina A & Guschina, et al. 2006). On the bottom of food chain in ecosystem, more than half primary producers consist of microalgae. In the early period, algae were classified based on composition of photosynthetic pigments stored in different species.

Marine microorganisms, including microalgae, are well known to produce LC-PUFA (Long Chain- Polyunsaturated Fatty Acids). In the past ten years, scientists have successfully isolated the genes of enzymes which involve in LC- PUFA biosynthesis from marine phytoplankton (Robert SS, 2009). Compared with cyanobacteria, eukaryotic algae prefer to accumulate more unsaturated fatty acids. Among *Dunaliella salina* in Chlorophyceae have a tendency to store α-linolenic acid (ALA, 18:3 $\Delta^{9,12,15}$) as a component of lipid content (Shi H et al. 2018). Polyunsaturated fatty acids (PUFAs) contain more than three double bonds that are known as acyl-lipid with C18 to C22 carbons. According to the location of primary double bond proximal to the methyl end, PUFAS are classified into two groups of ω-6 and ω-3 forms (Chi & Zhanyou, et al. 2009). Generally, the nutritional benefits from seafood are due to two kinds of PUFA, eicosapentaenoic acid (EPA,

20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3) which are belong to ω-3 fatty acid. In order to acquire omega-3 fatty acid, the traditional way is deep-sea fishing. Indeed, the worldwide demand for these ω-3 fatty acids are related to health benefits from PUFA. Therefore, PUFAs are the major phospholipid component of cell membranes and work as a variety of important physiological functions in vertebrates (Marie & Vagner, et al. 2011). The position of the double bond in the PUFAs influences the properties of its derivatives. However, DHA is an essential component of algal cell membrane and scientists have paid much attention on DHA due to its various physiological functions that operate in the human body. For example, in the retina of rodent outer segment, 60% of the total fatty acids make up with DHA (Giusto et al. 2000). Additionally, DHA plays an important role on the development of structure of fatty acid and it is also involved on functions of nutrient for the primary stage of neurological growth of infants (Nettleton 1993; Mohney et al. 2003). In fact, human being doesn't have the ability to synthesize DHA themselves. So, people especially infants should intake this PUFA from diet. Moreover, some good evidence has proved the importance of EPA and DHA in the human diet, therefore, these fatty acids obtained from coccolithophores play a role in preventing cardiovascular disease and related precursor stage, including metabolic syndrome (Riediger et al. 2009).

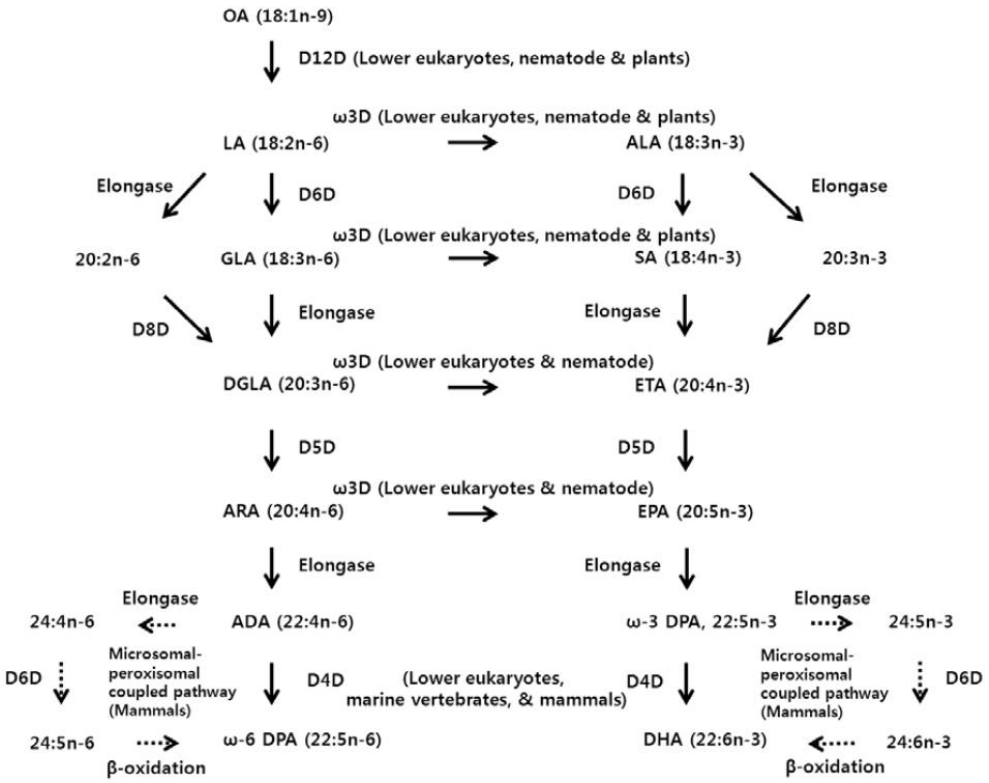

Fig 1. 1 PUFAs synthetic pathway from C18 to C22 carbon fatty acids in eukaryotic systems (Lee & Je Min, et al. 2016).

PUFAs are synthesized progressively through addition of carbon or double bonds in backbone of chains. As the primary member of Omega-3 fatty acid groups, ALA (Fig 1.1) can be converted to stearidonic acid (SDA) by delta-6 desaturase. Subsequently, SDA is catalyzed to form eicosatetraenoic acid (ETA, 20:4n-3) with addition of two-carbon via elongase. Then, with catalysis of two enzymes (delta-5 and delta-4 desaturases) and elongases, two metabolites, EPA and DHA, are progressively produced.

As the essential dietary molecules, the application of ω-3 fatty acid has been involved in massive articles, especially in fields of medical health. By large amounts of clinical tests, results have shown that plenty of diseases relate to the insufficient uptake of unsaturated fatty acids in

diet. By adding intake of ω-3 PUFAs, the risk of various diseases including cardiovascular disease (Kris-Etherton et al. 2002), inflammation (Calder PC 2006; 2008), and cancer (Roynette CE & Calder PC, et al. 2004) is apparently reduced,

The coccolithophorid algae are a group of unicellular marine algae, which accumulate a high lipid content (more than 30% of cell body) and generate calcite in the form of coccolith on its surface (Holligan et al., 1993). These algae are studied for biological CO_2 fixation and recycling. Their oil-rich biomass has great potential to be applied as a feedstock for energy production. Furthermore, the coccolith can be buried to 'fossilize' the carbon. This trait is not available with other non-calcareous algae. The lipids of these algae also have potential commercial advantages as 'marine oils'. They are rich in ω-3 polyunsaturated fatty acid (Volkman et al. 1981) and these marine oils have commercial value (Borowitzka 1988). One of the important species of coccolithophorid *Emilianea huxleyi* is a durable sink of inorganic carbon on earth (Westbroek et al. 1994). Consequently, *E. huxleyi* is notable for the accumulation of the DHA as well as EPA. Also, the species *E. huxleyi* could accumulate fish oils which is known to bring health-beneficial properties to humans, guarding against cardiovascular disease and related pathologies (Olga Sayanova et al. 2011). Several articles have been reported on ω-3 fatty acids health-beneficial compounds which is accumulated in food webs of marine ecosystem (Williams & Burdge, 2006). A recent study (Wu et al. 1999) also pointed out that the biomass of *E. huxleyi* has the ability to yield a high amount of pyrolytic gases with CH_4 being the major component. There is also the possibility to convert the lipids from coccolithophorid to methyl ester fuels – biodiesel (Nagle & Lemke, 1990).

1.1.1 Omega-3 desaturases

In order to clarify the metabolic pathway of omega-3 LC-PUFAs, it has been reported two

archetypal enzyme desaturases and lipid-specific elongases activities which enhance the aerobic synthesis of EPA and DHA, (Venegas-Calerón et al. 2010). In the last 20 years, few genes which code corresponding fatty acids desaturases and elongases had been successfully cloned, and their enzyme functions were characterized from several algal species. The predominant series of desaturation reactions start from the conversion of C18 fatty acids to C20 and PUFAs. The first step of conversion initializes with the introduction of a double bond at the 6th carbon position, followed by C2-chain elongation and a subsequent desaturation which occur at D5 position in the carbon chain of C20-PUFA, producing EPA from α-linolenic acid orα γ-linolenic acid (Olga Sayanova et al. 2011).

Because of differences in solubility, desaturases make up with two forms: soluble and membrane-bound. As on type of membrane-bound desaturases, ω-3 Desaturases are short of an N-terminal fused cytochrome b5 domain (Pereira et al. 2003). Moreover, as fusion proteins, frontend desaturases contain b5-like domain at same position. In the role of fundamental enzymes for desaturation, ω3-Desaturases promote the conversion of PUFA from LA to ALA and have been widely found in higher plants and microalgae including cyanobacteria. In microalgae, this pathway is reported among a few members of phytoplanktons including *Euglena gracilis* (Wallis & Browse, 1999) and *Isochrysis galbana* (Qi B et al. 2002). As shown in Fig. 1.2, the phylogenetic relationships between ω-3 and Δ12 membrane desaturase genes from various organisms were showed via using the neighbor-joining tree (Mingxuan Wang et al. 2013).

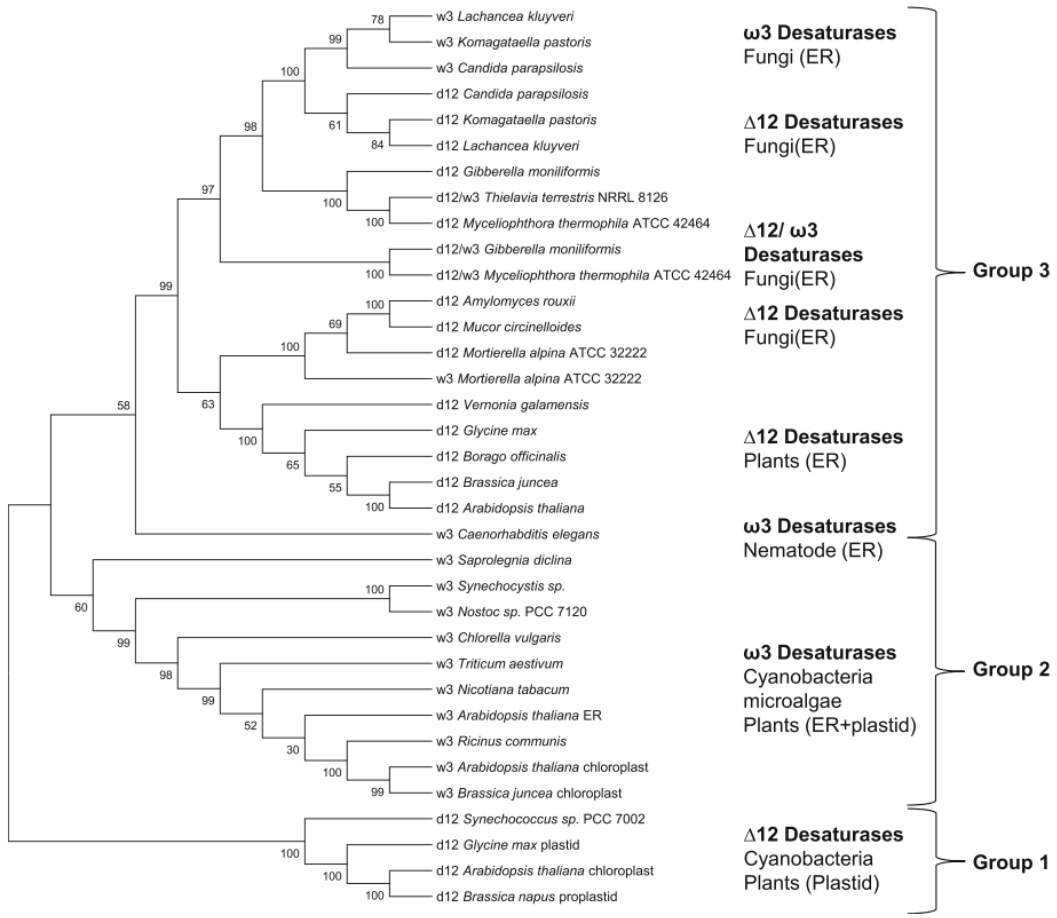

Fig 1. 2 The second cluster groups are built up with omega-3 desaturase from chloroplast of higher plants, and microalgae including cyanobacteria. The similarity among these genes indicates the ω-3 desaturase of cyanobacteria evolve two branches in higher plant and microalgae, respectively.

1.2 Coccolith and scale formation

As one genus of coccolithophorid, *Pleurochrysis carterae* which belongs to Haptophyta is an important marine phytoplankton (Paasche 1968). In the lineage of *Emiliania*, coccolith formation occurs in vacuolar system of the endoplasmic reticulum (ER) with direct attachment to

the nuclear membrane (Westbroek et al. 1989). On the other hand, coccolith formation in *Pleurochrysis* takes place in Golgi cisternae (van der Wal et al. 1983). Up to date, several articles indicated that acidic polysaccharides involve in coccolith processing and their functions in the synthesis of calcite have been analyzed. However, the mechanisms of coccolith formation have hardly been illuminated with enough information on genomes, which participate in coccolith formation (Paasche E 2001).

In mainstream opinion, the coccolith precursors, base plates and acidic polysaccharides are initially built in Golgi cisternae. Subsequently, both of them are transferred to the mineralizing vesicle for generation of calcite rings (Fig.1.3) (de Vrind-de Jong & de Vrind, 1997; Marsh 2003). In case of mineral deposition, the two acidic polysaccharides PS1 and PS2 produce 20 nm particles with calcium ions and then are adhered to the base plate rim (Marsh 1994; Outka & Williams, 1971; Van der Wal et al. 1983). Moreover, the two polysaccharides combine with calcium ions which form aggregates that transport within Golgi system. The formation of coccolith requires a galacturonomannan PS3, which is concentrated at the boundary area between the growing crystals and the membrane where mineralize vesicle. At the final stage, the crystals earn an amorphous polysaccharide coat, which comes from the dissociation of PS/PS2, packages with 20-nm crystal particles, and then coccoliths are exported into the coccosphere (Marsh et al. 2002).

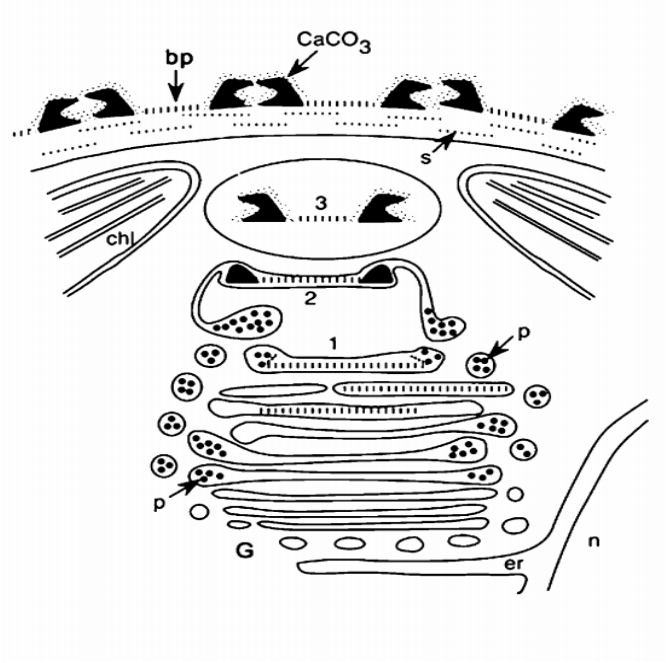

Fig 1. 3 Coccolith formation pathway in *Pleurochrysis carterae* (Marsh 1994). The observation of mineralizing vesicles at three progressive stages: (1) Prior to $CaCO_3$ deposition, (2) The period of crystal growth, (3) Since termination of crystal growth, (p) In *Pleurochrysis*, Acidic polysaccharides PS2-containing particles mediate mineralization, **(bp)** Base plates, **(chl)** Chloroplasts, **(G)** Golgi stacks, **(er)** Endoplasmic reticulum, **(n)** Nucleus, **(s)** unmineralized scales are revealed.

On the other hand, some authors have pointed out that the flagellar apparatus (containing several microtubules) and peripheral endoplasmic reticulum (PER) should involve in dynamics of coccolith deployment and secretion. The structure, named cytoplasmic tongue (Beech & Wetherbee, 1988) comes from the partial enveloped of PER.

1.3 Chloroplast genome analysis of *Pleurochrysis sp.*

Chloroplast is a double membrane bounded plastid and helps in the process of biosynthesis of amino acids and lipids in green plants and algae (Daniell et al. 2016). Haptophytes are photosynthetic microalgae found in marine environments and freshwater. The genus *Pleurochrysis*

as the photoautotrophic unicellular marine algae belong to the division Haptophyta which can produce and deposit $CaCO_3$ scales called coccolith (Chen et al. 2019). Coccolithophores are well known for their roles in the precipitation of biogenic carbonate and their contribution to marine primary production. As we know haptophyte have represented important ecological roles, only little is known regarding their genomic organization. The algae database (https://www.algaebase.org/) shows that there are only 8 species chloroplast genomes message are recorded, but not including any data in *Pleurochrysis spp*.

1.4 The application of microalgae

Some of the microalgae are used as a traditional food in the world and they have been used as a part of the human edible or health food for more than 2000 years. Therefore, three different species of *Nostoc* and *Spirulina* (*Arthrospira*) have been used in Asia (China) and Africa, respectively (Jensen 2001, Chacon-Lee 2010). In the last a few decades, microalgae have been in the preparation in cosmetics, nutraceuticals and functional foods. Moreover, a few high-value chemicals including β-carotene, astaxanthin, PUFAs (DHA, EPA), phycobiliprotein and algal extracts obtained from microalgae, are used in cosmetics (Borowitzka 2013). Nowadays, the commercial applications of microalgae include following aspects: (1) For functional foods such as algal powder, tablet and capsule in the nutraceuticals market; (2) For aquaculture and feed industries, especially as starter diet of the larvae; (3) Application in cosmetics industry. Some algae components or ingredients used in infant nutrition formula contain high level of polyunsaturated fatty acids (DHA and EPA). One of the Australian companies produces 1200 tons/year powders of *Dunaliella salina* with βeta-carotene. The biggest producer in Taiwan has the ability to produce 400 tons of dry powder of *Chlorella* per year. The company has different *Chlorella* products e.g.,

pasta, noodles, liquids and tablet with plenty of omega-3 PUFAs and antioxidants. As a result, the global *Chlorella* products rise to 5279.53 tons in 2016, with an average growth rate of>6.07% (Katiyar et al. 2020). The salmon feed industries would also need a supply of more than 100,000-ton dry algae year^{-1}. Algal pigments are also used as high-value natural dyes in cosmetics (Spolaore et al. 2006). The global demand amounts are estimated to be 5,000 tons of algae powder per year, the value is about $1.25 billion per year (Pulz & Gross, 2004). Recently, the microalgae that have been used for large-scale commercial applications are *Spirulina* (*Arthrospira*)*, Chlorella, Dunaliella, Heamatococcus, Euglena and Aphanizomenon flos-aquae*.

1.4.1 The applications of *Pleurochrysis carterae* and *Isochrysis zhanjiangensis*

P. carterae can utilize sunlight and carbon dioxide (CO_2) through photosynthesis to produce their organic substances. Also, *P. carterae* cell can accumulate $CaCO_3$ to constitute the coccolith on the cell or colony surface. On the surface of *P. carterae* cells, the content of $CaCO_3$ account for 10% of the algal dry weight, whereas the component of intracellular lipids account for 33% (Moheimani & Borowitzka, 2006). Moreover, the characteristic of lipids obtained from *P. carterae* are particularly rich in long-chain unsaturated fatty acids DHA (docosahexaenoic acid, 22:6ω-3), and the higher DHA content can reach 30% ~ 40% of total fatty acid (Bell & Pond, 1996). DHA has a wide range of applications as infant foods and in reducing hyperlipidemia, atherosclerosis and risk of heart disease; thus, it can fulfill the needs of the global market demand of billions of dollars (Katiyar 2020). Therefore, it is worthy of trust that *P. carterae* has broad commercial and social prospects.

Isochrysis sp. has a high growth rate and is adaptable under outdoor culture conditions (Devos et al. 2006; Lin et al. 2007; Liu et al. 2013), and it is able to produce certain high-value compounds such as pigments, protein and oils (Rawat 2011). Because of their nutritional values, *Isochrysis*

has fed to target larval organism in the aquaculture for decades. It was used as essential starter diet in early stage of marine bivalves, helping their transformation from juvenile to adult stages. Molina Grima (1994) cultivated one *Isochrysis* strain to produce eicosapentaenoic acid (EPA), while Hu and Richmond (1994) enhanced biomass productivity and DHA production in *Isochrysis* cultures. Moreover, *Isochrysis zhanjiangensis* exhibited higher growth rate and was considered as the most promising strain for co-production of fucoxanthin and DHA at commercial scale.

2. Research objectives

The research on coccolithophore is currently focused on *P. carterae* and *Isochrysis zhanjiangensis.* There are relatively few studies on the morphology and physiology *Pleurochrysis carterae* and even less on the mass culture of *Isochrysis zhanjiangensis* in outdoor condition. The main research objectives are as following:

1) Life cycle and pathway of extracellular scale formation of *Pleurochrysis carterae.*
2) Chloroplast genome analysis of *Pleurochrysis sp.*
3) The effect of environmental factors on growth of *Isochrysis zhanjiangensis.*in outdoor.

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CHAPTER II

Life Cycle and Cell wall development of *Pleurochrysis carterae*

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Abstract

Pleurochrysis carterae is a marine alga, universal distributes in oceans. The *P. carterae* cell is covered with a lot of individual coccoliths which are mainly composed of calcium carbonate ($CaCO_3$). As one of coccolithophorid algae, *P. carterae* has a strong ability to absorb CO_2 from the environment and mineralize it. Thus, it plays an important role in the carbon cycle for the global climate change. Generally, the coccoliths (including scales) are formed inside the cell and then are ejected to outside of cell. However, *Pleurochrysis* cell is surrounded by a coccosphere consisting of a single layer of coccoliths and several loosely underlying layers of unmineralized scales. In this study, we report four stages in the life cycle and a new pathway for the development of coccolith and scale formation on the shells of *P. carterae* through comparing morphological characters under light microscope, Transmission Electron Microscope and Scanning Electron Microscope. Therefore, we revealed a cell which was covered only with tightly multiple layers of scales coat without coccolith on its top surface and we named this scales coat formation pathway as scale extracellular formation pathway, which is different from the coccolith and scale intracellular formation pathway.

KEYWORDS: *Pleurochrysis carterae,* coccoliths, coccolithophorid species, apistonema, heterococcolith stage

2.1 Introduction

The ocean plays a key role in the global carbon cycle by absorbing carbon dioxide (CO_2) emission produced from anthropogenic activities. However, the dissolution of CO_2 is leading to a decrease of the pH in aquatic environments, especially in the oceans. One-third of the CO_2 is releasing in the environment from human activities, and it is absorbed by the oceans as a form of carbonic acid leading to ocean acidification, one of the major threats to marine ecosystems (Sabine et al. 2004; Fabry et al. 2008).

Pleurochrysis carterae (Braarud & Fagerland) Christensen, a typical coccolithophorid species is distributed in coastal areas by producing blooms. The species *P. carterae* belongs to the division Haptophyta, class Coccolithophyceae, order Syracosphaerales and family Syracosphaeraceae (Braarud & Fagerland, 1946; Christensen 1978; Beech & Wetherbee 1984; Guiry & Guiry, 2020). As a single-cell marine microalga, *P. carterae* can accumulate CO_2 from the surrounding environment for photosynthesis. It is not only absorbing CO_2 by photosynthesis, but also absorbs HCO_3^- from the ocean and air to synthesize $CaCO_3$ through its unique calcification. Therefore, it has a broad application prospect on carbon fixation in oceans (Paasche 1968).

P. carterae is considered a single cell species with two equal or slightly unequal length of flagella, and one organelle comprise of a comparatively short and bulbous structure called haptonema locates between the bottoms of flagella. Moreover, the cell contains two parietal chloroplasts with one pyrenoid and can form colonies at certain growth conditions (Hawkins et al. 2011). The most intensive research on life cycle has been found on species *Pleurochrysis scherffelii, Pleurochrysis carterae* and *Pleurochrysis dentata* under the genus *Pleurochrysis.*

(Brown 1969; van der Wal et al. 1983; Marsh 1994; Marsh 1999; de Vrind-de Jong & de Vrind, 1997; Paasche 2002; Marsh 2003).

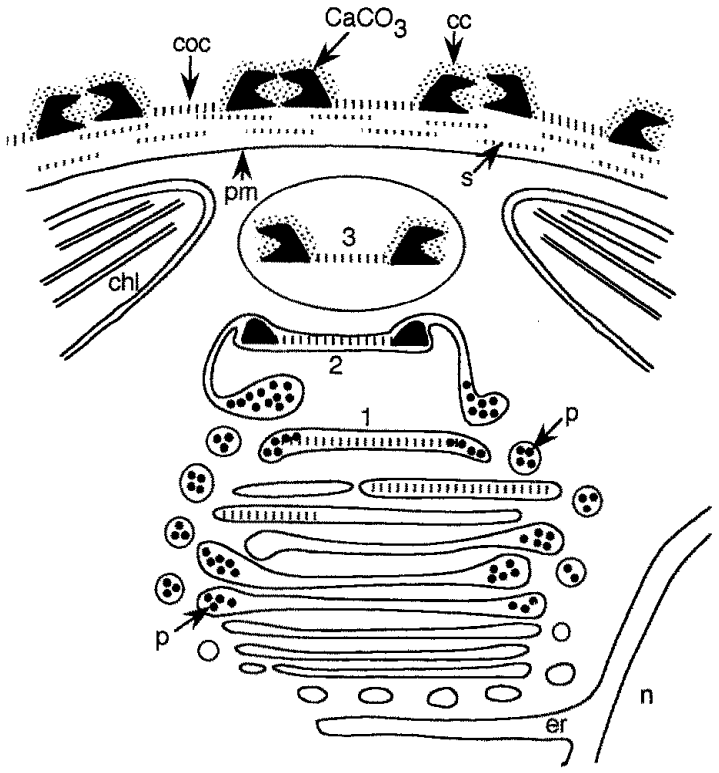


Fig 2. 1 Coccolith formation pathway in *Pleurochrysis carterae* (Marsh 1994).

Until now, the popular theory on the formation of coccolith and scale is believed to be intracellular in *P. carterae* and the coccoliths including scales are formed inside the cells first and then are ejected to the outside of the cells (Marsh 1994; Paasche 2002). The cell wall formation of vegetative cell of *Pleurochrysis scherffelii* is described in the Golgi apparatus (Brown 1969). The formation of coccolith is synthesized in a special Golgi vesicle (Marsh 1999). In *P. carterae*, the coccolith precursors, base plates and acidic polysaccharides are initially produced in Golgi cisternae (van der Wal et al. 1983) and subsequently, both of them are transferred to the mineralizing vesicle for generation of calcite rings (van der Wal et al. 1983; de Vrind-de Jong & de Vrind, 1997; Marsh 2003). Therefore, the coccolith is released to the cell surface by Golgi

vesicles. Acidic polysaccharides involve in coccolith processing and their functions in the synthesis of calcite have been analyzed. However, the mechanisms of coccolith formation have hardly been illuminated with enough information on genomes, which participate in coccolith formation (Paasche 2002). In the lineage of *Emiliania*, coccolith formation occurs in vacuolar system of the endoplasmic reticulum (ER) with direct attachment to the nuclear membrane (Westbroek et al. 1989). The other hypothesis is the coccolith and scale are also synthesized outside the cells. There is an alternative scale and coccolith formation pathway occurring on external surface of cells.

In case of mineral deposition, the two acidic polysaccharides PS1 and PS2 produce 20 nm particles with calcium ions and then are adhered to the base plate rim (Marsh 1994; Outka & Williams, 1971; Van der Wal et al. 1983). Moreover, the two polysaccharides combine with calcium ions which form aggregates that transport within Golgi system. The formation of coccolith requires a galacturonomannan PS3, which is concentrated at the boundary area between the growing crystals and the membrane where mineralize vesicle is present (Marsh 2003). At the final stage of the calcite growth phase, the crystals earn an amorphous polysaccharide coat, which comes from the dissociation of PS/PS2, packages with 20-nm crystal particles, and then coccoliths are exported into the coccosphere. On the other hand, some authors have pointed out that the flagellar apparatus (containing several microtubules) and peripheral endoplasmic reticulum (PER) should involve in dynamics of coccolith deployment and secretion. The structure, named cytoplasmic tongue (Beech & Wetherbee, 1988) comes from the partial enveloped of PER.

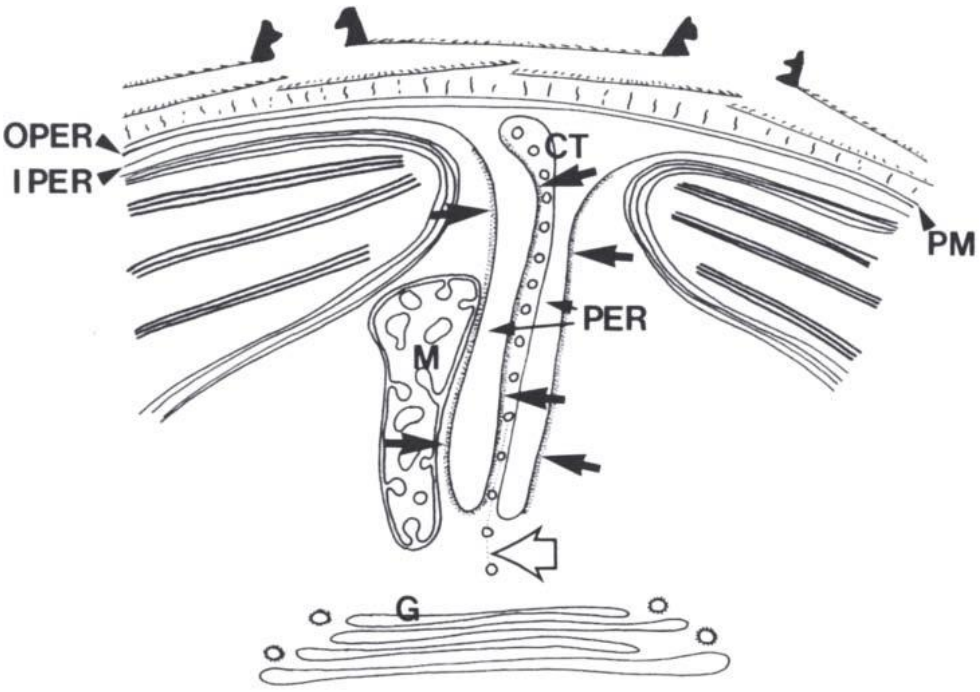


Fig 2. 2 Diagrammatic representation of the associations between plasma membrane (PM), the peripheral endoplasmic reticulum (PER), the cytoplasmic tongue (CT) and other cell organelles including Golgi body (G), mitochondrion (M); a fibrous component (open arrow) is visible interconnecting some of the microtubules of the CT (from Beech & Wetherbee, 1988).

Therefore, the principal aim of this research work is to discover new data supporting the scale extracellular formation pathway, which is not only relative to the coccolith and scale intracellular formation pathway, but also is different from the coccolith and scale intracellular formation pathway.

2.2 Materials and Methods

2.2.1 Algal strain and culturing

Pleurochrysis carterae was purchased from National Center for Marine Algae and Microbiota, East Boothbay ME, USA. (NCMA- https://ncma.bigelow.org/ccmp646). Two types of modified

f/2 culture media were used for the growth of algae. The first type of f/2 medium was prepared using the method described by Guillard (1975) with slight modification. Briefly, the concentration of $NaNO_3$ in the f/2 was 750 mg/L. The 100 ml trace metal solution was composed of 315 mg $FeCl_3 \cdot 6H_2O$, 436 mg $Na_2EDTA \cdot 2H_2O$, 9.8 mg $CuSO_4 \cdot 5H_2O$, 6.3 mg $Na_2MoO_4 \cdot 2H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, and 180 mg $MnCl_2 \cdot 4H_2O$. The vitamin solution per 100 ml was composed of 20 mg thiamine HCl (vitamin B_1), 10 mg biotin (vitamin H), and 10 mg cyanocobalamin (vitamin B_{12}). The second type f/2 medium contained the same ingredients of first type f/2 plus 36 mg/L $CaCl_2 \cdot 2H_2O$. For f/2 agar medium, 15 g/L of agar was added.

In order to obtain *P. carterae* without contamination, cells were inoculated on the first type f/2 agar plates. For inhibit the bacterial growth, 50 μg/ml of ampicillin was added in agar medium. After two weeks of incubation, single colonies of cells were picked up from agar culture, then inoculated in 50 ml flask, then transplanted to 250 mL, 600 mL, and 10L columns for cultivation, respectively. During cultivation, 24 hours illumination from cool white fluorescent lamp was provided. The light intensity was controlled between 20-25 $\mu mol.m^{-2}/s^{-1}$. Moreover, air pump also supplied aeration in 24 hours.

A. 50 mL Culture Tube B. 600 mL VCB C. 10L VCB

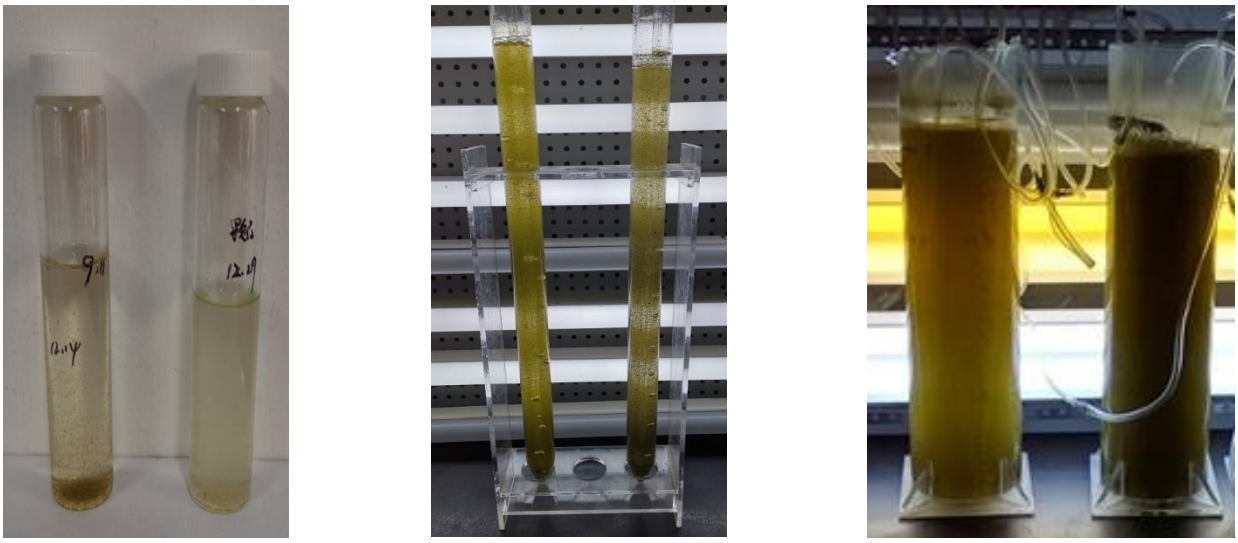


Fig 2. 3 Cultivation of single cells in 50mL culture tube (A), 600mL (B) and multi-cell colonies in 10L (C); Vertical Column Photobioreactors (VCB).

2.2.2 Light microscope and laser confocal microscope

Microphotographs were taken with an Olympus BX53 light microscope (Olympus Corp., Tokyo, Japan) using the differential interference contrast methods and were taken under an oil immersion objective lens. Microphotographs also were taken using laser confocal microscope NOL-LSM 710 (Carl Zeiss, Germany).

2.2.3 Scanning Electron Microscope (SEM)

Algal cells from 10 ml culture were harvested by centrifugation (5 minute). Samples used for SEM analysis were fixed in 2.5% glutaraldehyde in phosphate buffer (pH around 6.8 to 7.4) at 4 °C for 8–10 h, rinsed three times with distilled water. Microscopic slides (0.5 cm × 0.5 cm) were coated with three piles of poly-L-lysine solution to aid adhesion of the cells. Gradual dehydration of the cells was achieved by dipping into an ethanol series of 30, 50, 70, 90, 95, 99, and 100% for

15 minutes at each concentration. The dehydrated algal cell samples were kept it in a freezer at -80 ℃ until use. Subsequently, the algal cells were critical-point-dried with CO_2 and sputter-coated with gold. A Hitachi S-4800 (Tokyo, Japan) scanning electron microscope was used to visualize the fixed cells. Electron microscopic analysis was performed at Wuhan Institute of Virology and Institute of Hydrobiology, the Chinese Academy of Sciences, China.

2.2.4 Transmission Electron Microscope (TEM)

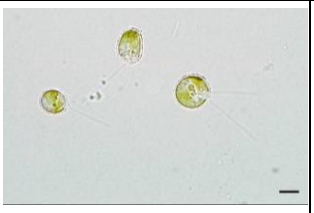
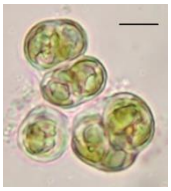
For Transmission electron microscopic observation, 10 ml of alga culture was taken, centrifuged for 5 minutes. Cells undergoing exponential growth were collected for transmission electron microscopy (TEM). The algal samples were fixed for 2 h at 5 °C in 2% glutaraldehyde and 0.05 M phosphate buffer. They were post-fixed for 2 h at 5 °C in 1% osmium tetroxide and 0.05 M phosphate buffer and then put the cells overnight at 5 °C in 1% uranyl acetate and methanol. After dehydration through an ethanol series, the samples were embedded in Spurr medium containing propylene oxide. Ultrathin sections were cut on a Leica UC7 (Leica, Wetzlar, Germany), post-stained with uranyl acetate and bismuth oxynitrate and examined using a Hitachi HT-7700 TEM (Hitachi, Tokyo, Japan) at 80 kV at the Wuhan Institute of Virology, the Chinese Academy of Sciences.

2.3 Results

Based on our observation during the cultivation of *P. carterae*, four stages in its life cycle were summarized in **Table 2.1**. Therefore, one clone of *P. carterae* was picked from solid f/2 agar plate, transferred to a 50 ml tube with fresh f/2 broth medium. After two weeks, large quantity of single cells with two flagella and a short haptonema were observed under the microscope. This type of single cell was defined as the first stage of the life cycle of *P. carterae* (Stage I) (Fig. 2.4).

Subsequently, these motile cells lost their flagella and developed to immotile single cells. Three days later, the size of motile cells became larger with coccoliths during this stage II (Fig. 2.5). Moreover, it was observed clearly that the single immotile cells were bigger than single motile cells (Fig. 2.6). When these immotile cells were cultured in 600 ml and 10 L vertical columns respectively under continuous aeration, a proportion of cells did not repeat the binary fission to form more immotile cells. Instead, several cells aggregated together to build multi-cell colonies (Stage III) (Fig. 2.6) and some of them were also called pseudo-filamentous stage or apistonema (Fig. 2.7). *P. dentata* has been exhibited an alternation between haploid and diploid generations with an alternation of a non-motile stage called apistonema stage, contained one or more motile forms. There were non-calcified cell showing the refrainment layer peeling away from the cell. With the increase of cell number and deprivation of nutrients in medium, more aggregated colonies were formed (Stage IV), and then some of cells were deposited on the bottom of bioreactors to became sediments (Figs 2.6 G, 2.7 G, H).

Table 2. 1 Life Cycle of *P. carterae* in the column photobioreactors.

Stage I motile single cell	Stage II immotile single cell	Stage III multi-cell colonies	Stage IV aggregated colonies
 Cells with two equal length flagella;	 Cells loss flagella, increasing size;	 **(bar = 10 µm)** Daughter cells do not separate, forming colonies with big cells.	 Sedimentation of colonies due to aggregation of big cells.
Grown in full medium	Grown in full medium	Grown in nutrient deprived medium	Grown in nutrient deprived medium

It was pointed out that a single cell of Stage I & II requires full nutrients media, while multi-

cell colonies of Stage III & IV grow on nutrient-deprived media. The single cells can be cultivated efficiently in 50 mL tubes and 600 mL column photobioreactors. The sedimentation of *P. carterae* were mostly aggregated colonies (Stage IV) and can be observed only in 10 L photobioreactors.

2.3.1 The cell wall development of *Pleurochrysis carterae*

It is interesting to point out that we observed a swim cell of *P. carterae* with four flagellates and coccoliths in the lab culture (Fig. 2.4 G). The cell size was around 10 μm which is bigger than normal swim cell (about 5-6μm) (Fig. 2.4 B). At the same time in the same culture, there were many swim cells with two flagellates in each anterior part of cell. It is not clear whether this big swimming cell came from two gametes united through syngamy process or not. It was possible that both gametes become a diploid zygote through the merging/fusing process, known as heterococcolith stage. This was the first record of such big swimming cell with four flagellates of *P. carterae*. Based on our observation, sometime coccoliths were observed only in motile and immotile single cell stage I and stage II (Figs 2.4 E, G, 2.5), however, no coccolith was observed in the colony stage of *P. carterae* (Figs 2.6, 2.7)

Therefore, the cells of *P. carterae* are vegetatively divided by the process of binary fission. After cell division, the daughter cells develop coccoliths (or scales) on outside surface through calcification. However, there has been an argument whether the cells produced coccoliths or scales in each stage of development during the life cycle of *P. carterae*. According to our observation, we found that: 1) The planktonic generation, such as a motile cell (Figs 2.4 A, E, F), non-motile cell (Figs 2.5 A, F, 2.6 B) and multi-cell colonies (Figs 2.6 C, F,) produced or not produced scales or coccolith outside the cell wall, depending on nutrition state in the medium; 2) aggregated

colonies or benthic pseudo-filamentous generations (apistonema) only produced motile cells with an haptonema without scale or coccolith (Fig. 2.4 A).

In the early stage, motile cells with coccoliths are spherical to ovate. When 4-6 weeks old cultures were transferred into fresh medium, the haploid pseudofilamentous cells or multi-cell colonies quickly released haploid non-coccolith-bearing motile cells in large quantity. Two of this type of motile cells generated syngamy to give rise to diploid coccolith-bearing motile cells (Fig. 2.4 G). The syngamy has been rare occurred and the environmental conditions controling this phenomenon is not clear at this time.

By analyzing SEM image (Figs 2.8 C, D, G), it was observed that the micro-tube networks (filamentous-nets) were mixed up with a few pieces of ovoid mucilage matter at the outside of a colony. In addition, a piece of *Pleurochrysis* cells coat was stripped of their coccospheres. In the central area of SEM picture, a matured cell commands to construct the shape of the irregular less-developed scale connecting mixture with micro-tube networks (Figs 2.8 C, D) and this structure was not clearly seen at the beginning. Consequently, the edge of scale appeared first, later the radial microfibers on proximal face and concentric spiral way of microfibers on the distal view were observed (Figs 2.8 C, D, H). At the outside of central area of SEM picture, it was appeared a water membrane or mucilage matter only with a round edge, which showed weak light reflection with no clear structure or ornament, not even micro-tubes connecting these types of less developed scales (Fig. 2.8 C). The external coat was probably stripped by some force in this moment. Therefore, the cells with more multi-layer scales (about 40-60 layers) (Figs 2.9 E, F, 2.10 F, G, 2.11 B) did not contain any coccolith on the top of outside coat. It was speculated that it is difficult for a single coccolith to penetrate and cross the thick and tight scale coating and move to the outside surface of a cell. In this situation, we could find the micro-tube network and scale

connecting each other in mixture way in three stratums (outer, middle and inner) of the external coat of a colony cells (Fig. 2.8). It could be postulated that the organic components have been transported from inside of the cells, could pass the plasma membrane and the cell wall through micro-tube network to outside surface of cells. The micro-tube networks and scales in the outside surface of the cells were constructed using organic components transported from inside of cells (Fig. 2.8). The clear pores in the developed circular scale could be an evidence to support such explanation. In a glance of cell coating, there were at least seven to nine scales connected in different layers (Figs 2.9 A, C).

The formation of water membrane or transparent mucilage scale was not related with micro-tube component, and there was no micro-tube network connecting with any of scales, possibly, they are the early-stage scales which were stripped from a scale coat of an old cell or colony. Sometimes, the compact scales coat was divided in several sub-coats at the outside of a cell, later they were stripped out gradually (Figs 2.11 A, B).

Based on our SEM and TEM images results of *P. carterae*, it was found that a plenty of the micro-tube networks were outside of cell, but not found inside of cell. At the same time, the young scales were composited directly from outside micro-tube networks (Figs 2.8 D, E, 2.9 B). We proposed that there was another scale formation pathway, we named it extracellular scales or coccolith formation pathway in *Pleurochrysis carterae* (Fig. 2.12). The main procedure of the extracellular scales (coccolith) formation pathway in *Pleurochrysis carterae* are summarized as: 1) upon the base of intracellular coccolith and scale formation pathway, which is the authorized process including golgi vesicle or cisternae, flagellar apparatus (with a lot of microtubules), reticulated mitochondrion and peripheral endoplasmic reticulum (PER) (containing the cytoplasmic tongue) all of them are related with and has implicated for the dynamics of coccolith

and scales deployment and ejection to the cell surface; 2) through the intracellular coccolith and scales formation system connecting with plasma membrane to transport the organic component from inside to outside; 3) after cell fast growth or being in old state the micro-tube networks start to generate in outside of cell to form the extracellular system to obtain organic component to construct the scale/coccolith in external space.

Pleurochrysis carterae is normally described as the motile single cells with biflagellates, however, immotile single cells and multi-cell colonies were also reported in our work. The life cycle of *Pleurochrysis carterae* consists of four stages and the cell wall consists of scales with or without coccoliths. Coccoliths occurred in single cell stages but not in colony stages. During a certain period of life cycle, the *P. carterae* cells form an elliptical ring structure composed of $CaCO_3$ crystals called coccolith on the surface of the cells through the process of calcification.

Therefore, it is the first-time report for images of the micro-tube networks and scale coat of *P. carterae* under SEM. The *P. carterae* cells are surrounded by coccospheres consisting of a single layer of coccoliths and several loosely underlying layers of un-mineralized scales. We also revealed the development process of coccolith and scale on the shells of *P. carterae*. Cells are only covered with tight multiple layers of scale coat without coccolith on its top surface, thus, we named this scale coat forming pathway as extracellular scale formation pathway, which is different from the coccolith and intracellular scale formation pathway.

Normally, the surface of calcified coccolithophore cells is often coated with a single layer or two layers (e.g., *Pleurochrysis carterae*) (Beech & Wetherbee, 1988), or multiple layers of coccolith (e.g., *E. huxleyi*) (Marsh 2007). Pienaar (1969) showed two to seven scales in different layer connected by micro-tube networks where the scales and coccoliths were transferred through a channel to the outside of cell in *Cricosphaera carterae* as a synonym of *Pleurochrysis carterae*.

However, he published these pictures about 50 years ago, and did not give the term of the micro-tube network, also did not explain micro-tube network structure characters. Moreover, cell of *Jomonlithus littoralis* surrounded by multiple layers loose arranged body scales without any coccolith in outside of cell (Probert 2014). Brown (1969) showed that vegetative cell illustrating the laminated cell wall in TEM of *P. scherffelii* (not called the scales as we do in this paper). *P. carterae* was covered by three types of scales; large elliptical calcified scales (called as the coccoliths), circular unmineralized scales and small elliptical to rectangular unmineralized scales (Pienaar 1969, Chen et al. 2019). In *P. carterae*, the coccolith precursors, base plates and acidic polysaccharides are initially produced in Golgi cisternae and subsequently, both of them are transferred to the mineralizing vesicle for generation of calcite rings (van der Wal et al. 1983; de Vrind-de Jong & de Vrind, 1997; Marsh 2003). Gayral & Fresnel (1983) have already revealed that the cytoplasmic tongue could be involved in the production of scales and they have also described a repeated association of branches of the mitochondrion with the peripheral endoplasmic reticulum, suggesting this area may be a region of intense metabolic activity. Beech & Wetherbee (1984) have proposed a single, reticulated mitochondrion branching throughout the cell and considered that respiratory products are sufficiently delivered to all regions of the cell.

Multilayer scaly cell walls and several possible pathways for the formation of coccoliths and scales have long been reported, but not much attention has been paid to them (Leadbeater 1971). All of these gave us the strong proof that the micro-tube network exists in previous study. We could know the relationship of micro-tube networks and scale during the scale development process in outside of cell.

Based on the multi-layer scales at the outer surface of the cells, it was considered that it is not possible to transport the bundle of scales (or stacked flat base plates) from intracellular to

extracellular. According to the fact of the simultaneous development of micro-tube network and scales the surface of cells, it is difficult to imagine how the full developed micro-tube networks and scales or coccoliths are transported from inside the cell passing through thick scales coating layer to the external surface. In another word, a single coccolith was very difficult to transit and cross the thick and tight scale coating layer to the external surface of cells. Therefore, according to the above exposed results, our report has for the first time proved that there is a new pathway for the formation of coccoliths and scales. We also revealed that the coccoliths and scales could be synthesized extracellularly.

2.4 Conclusion and Discussions

It is believed that the pathway for the formation of coccolith and scale of *P. carterae* is intracellular and the coccoliths including scales are formed inside the cells, however, our results prove that this pathway is extracellular. Moreover, in this report, we explained the complete lifecycle of *P. carterae*. In future experiments, we hope to obtain further research data to support our hypothesis on the scale extracellular formation pathway. We need to do further experiments for confirming the relationship between the thick mucilage cell wall (a single cell and colony cells) and compact scales coat of *P. carterae* in SEM and TEM. We should take the cell sample with the thick mucilage wall and follow the treatment process for SEM or TEM, obtain the SEM or TEM images in near future experiment. Further experimental and ultra-structural works are needed obviously to clarify the nature of endocytosis in the Prymnesiophyceae. Therefore, our work would lead to contribute on fully understand the lifecycle and cell wall development of *Pleurochrysis carterae*.

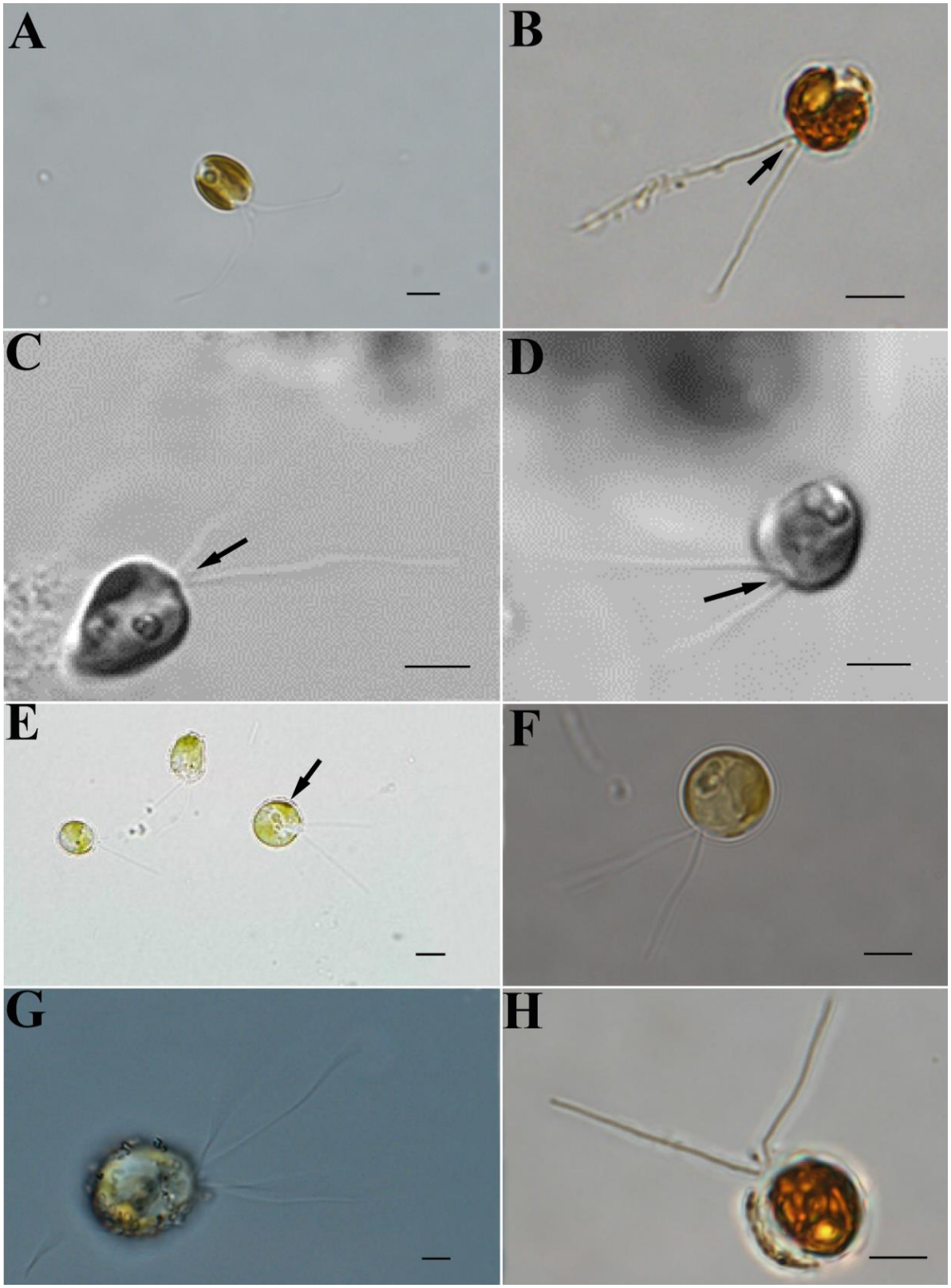


Fig 2. 4 Light micrographs of the zoospores of the *P. carterae*. **A-D, H.** showing the zoospores

with a short, bulbous haptonema (black arrow) and without coccolith; **C-D.** Confocal images; **F.** a zoospore without coccolith; **E, G.** zoospores with coccolith (arrow). **G.** a zygote with four flagellates (syngamy) in heterococcolith stage. **Scale bars:** 5 μm for A-H

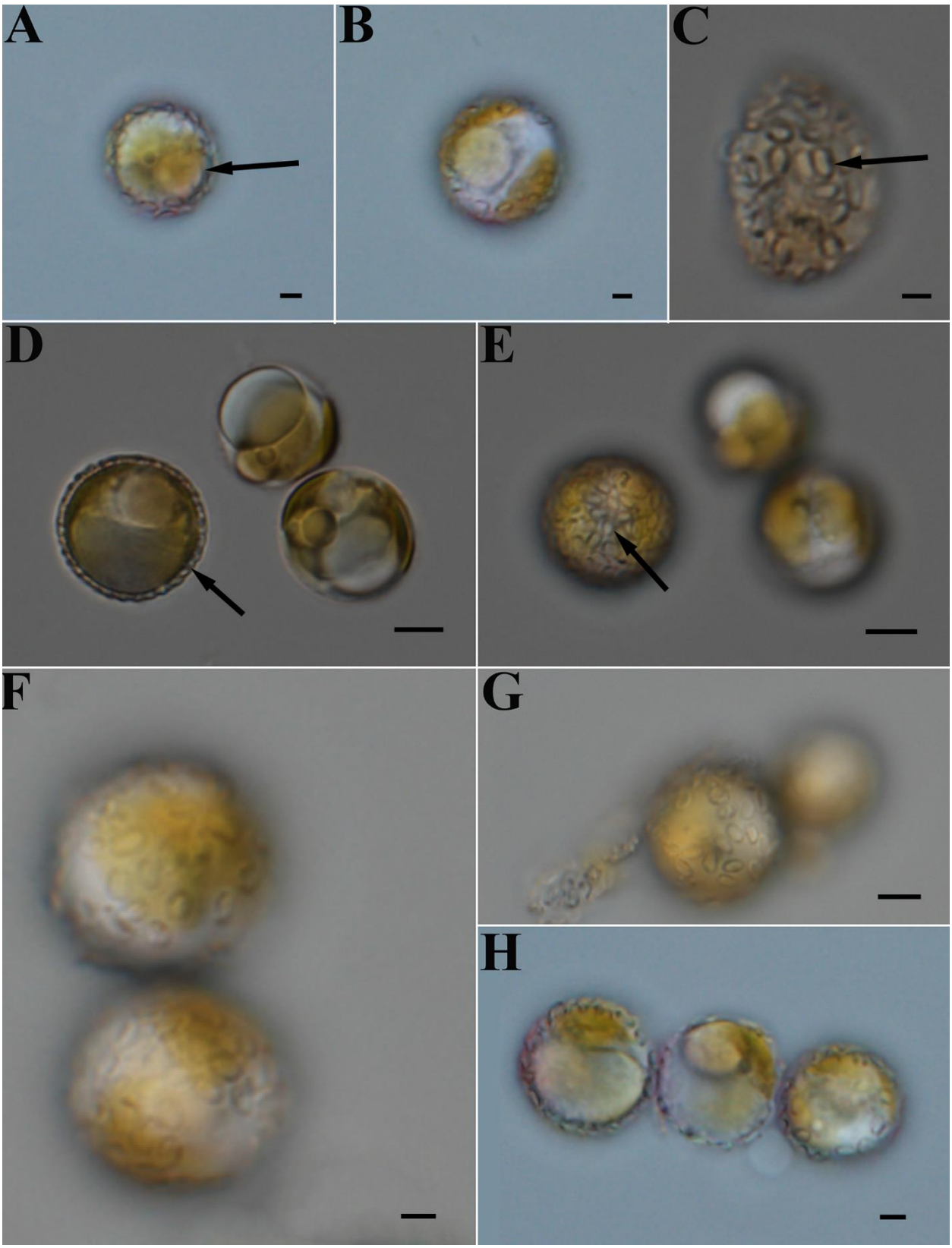


Fig 2. 5 A-H. Light micrographs of the non-flagellated vegetative cell with coccolith (arrow). **C**.

empty cell wall with coccolith; **D-E**. same cells with coccolith at different focus; **H.** three cells in a row. **Scale bars**: 2 μm for A-C, G, H and 5 μm for D-F.

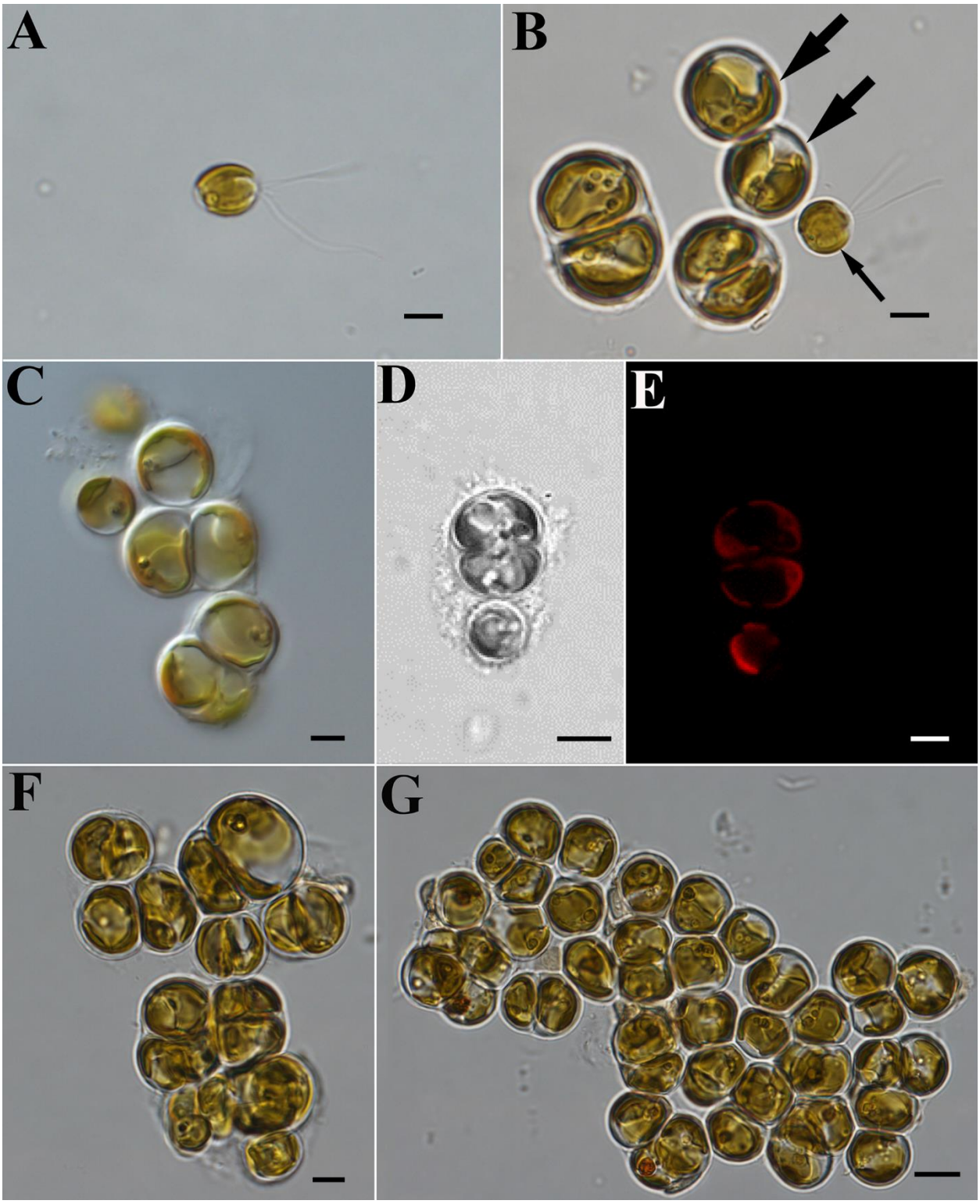


Fig 2. 6 Light micrographs showing four different stages of non-calcified cells; **A.** a motile with two flagellate and a short, bulbous haptonema (Stage I); **B.** immotile (big) (thick arrow) (Stage II)

and motile cells (small) (arrow); **C. F.** multi-cell colony (Stage III); **D-E.** immotile single cell by confocal; **G.** aggregated colonies in old culture (Stage IV). **Scale bars:** 5 μm for A-F and 10 μm for G.

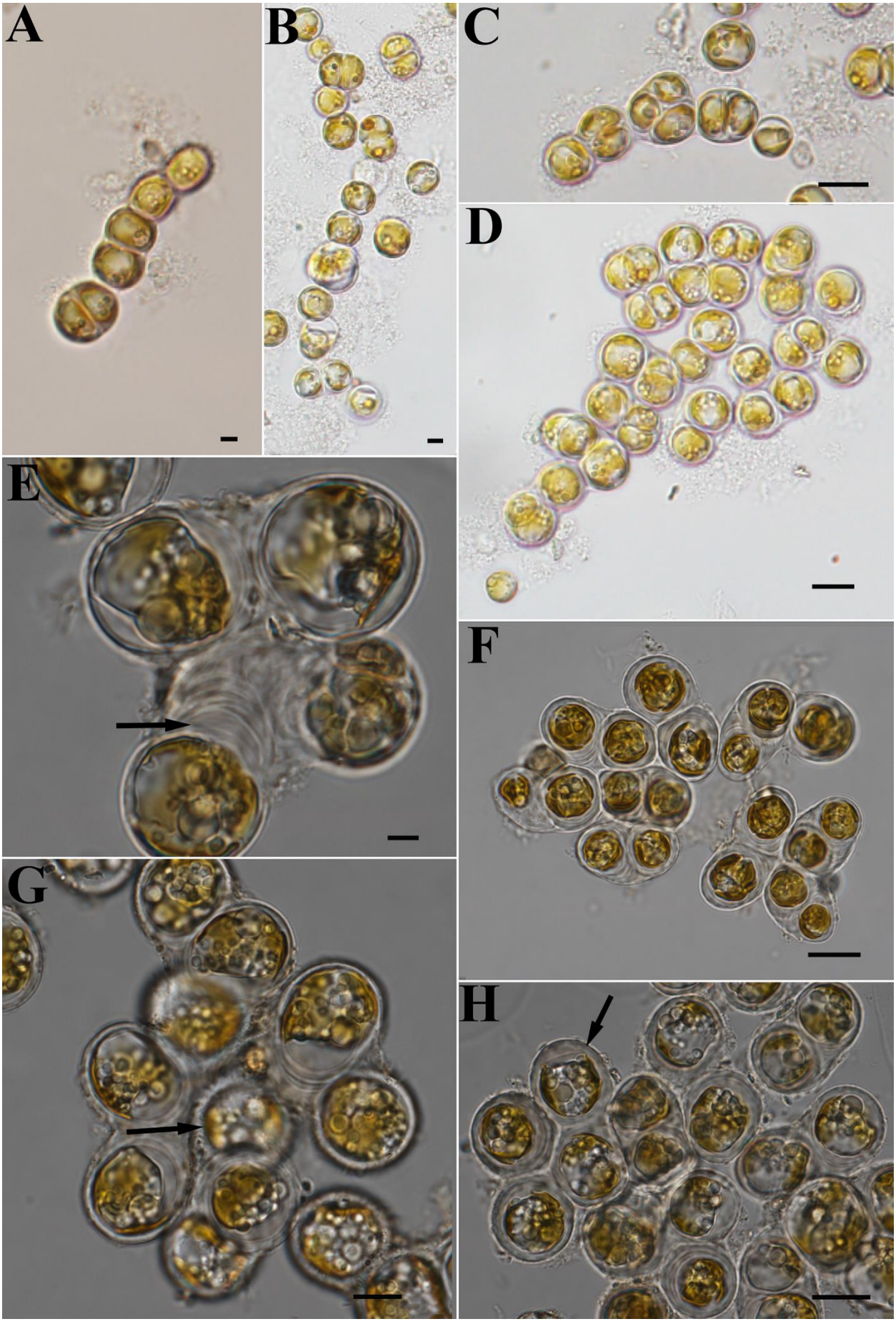
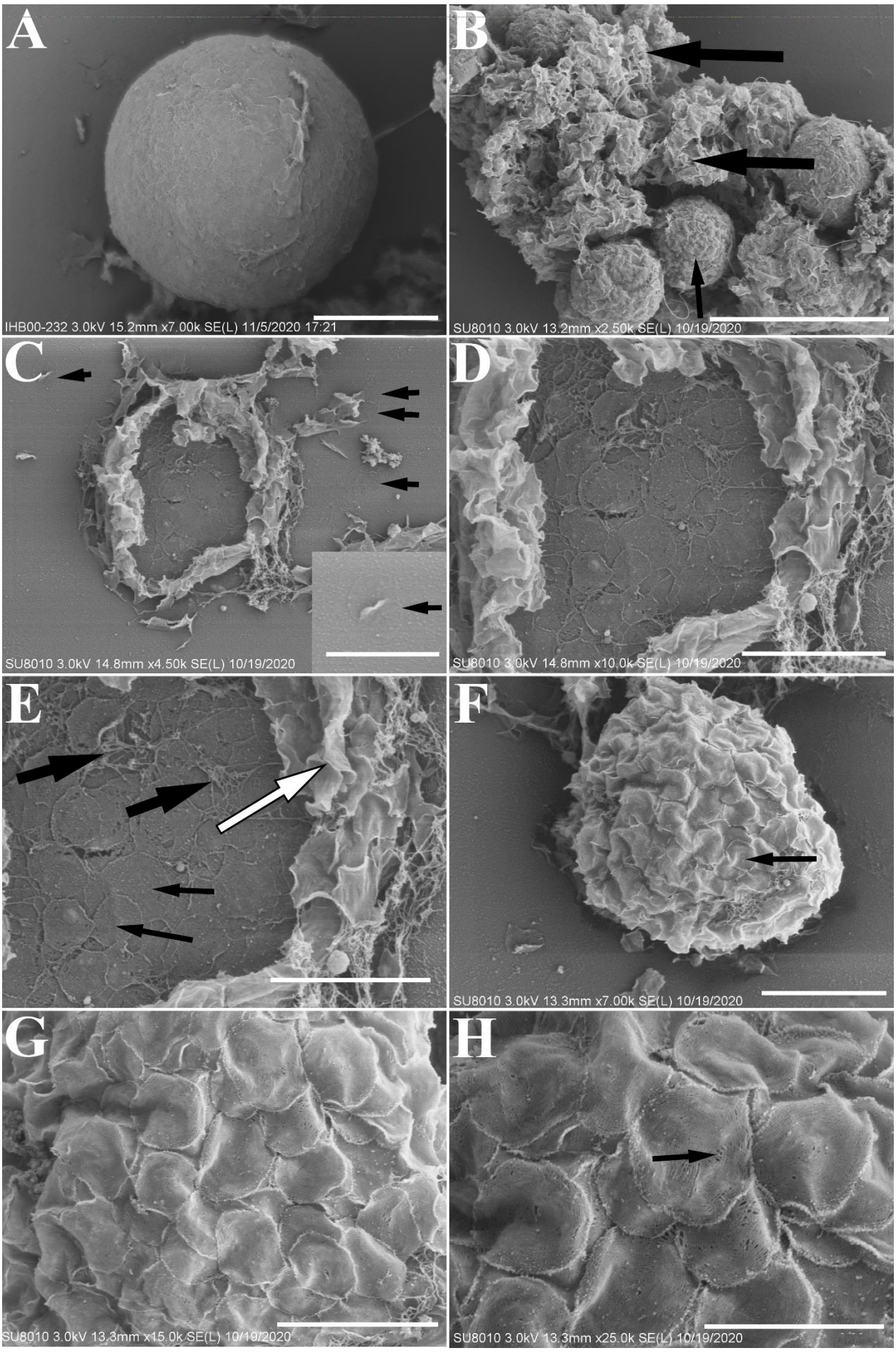

A
B
C
D
E
F
G
H

Fig 2. 7 A-D. Light micrographs show pseudo-filamentous stage or apistonema. **E-H.** gelatinous stalks/ thick cell wall in old culture (arrow) with packets of non-calcified cells (Probert 2014 fig.5). **Scale bars**: 5 μm for A, B, E and 10 μm for C, D, F-H.


A
IHB00-232 3.0kV 15.2mm x7.00k SE(L) 11/5/2020 17:21
B
SU8010 3.0kV 13.2mm x2.50k SE(L) 10/19/2020
C
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E
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G
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H
SU8010 3.0kV 13.3mm x25.0k SE(L) 10/19/2020

Fig 2. 8. Scanning electron micrographs show the multiple scales and micro-tube networks. **A.** a single cell covered by micro-tube networks and scales on the surface; **B.** colony covered by colony scale coat (thick arrow), cell also covered by cell scale coat (arrow), both of them with plenty of scales and micro-tube networks; **C-E.** a part of stripped micro-tube network with a few irregular less-developed scales in the center (the same image at different amplifications); **C.** a few of mucilage scales (arrow) without micro-tube networks, were stripped from the scale coat of an old colony. **E.** micro-tube networks (thick arrow) start to construct young scales (arrow) comparing with elder scales (white arrow); **F-H.** well-developed scales with concentric spiral way of microfibers (same image at different amplifications). H. the mature circular scale with lot of pores (arrow) and the deposited particles. Scale bars: A, D-F, 5 μm; B, 20 μm; C, 10 μm; G, 3 μm; H, 2 μm.

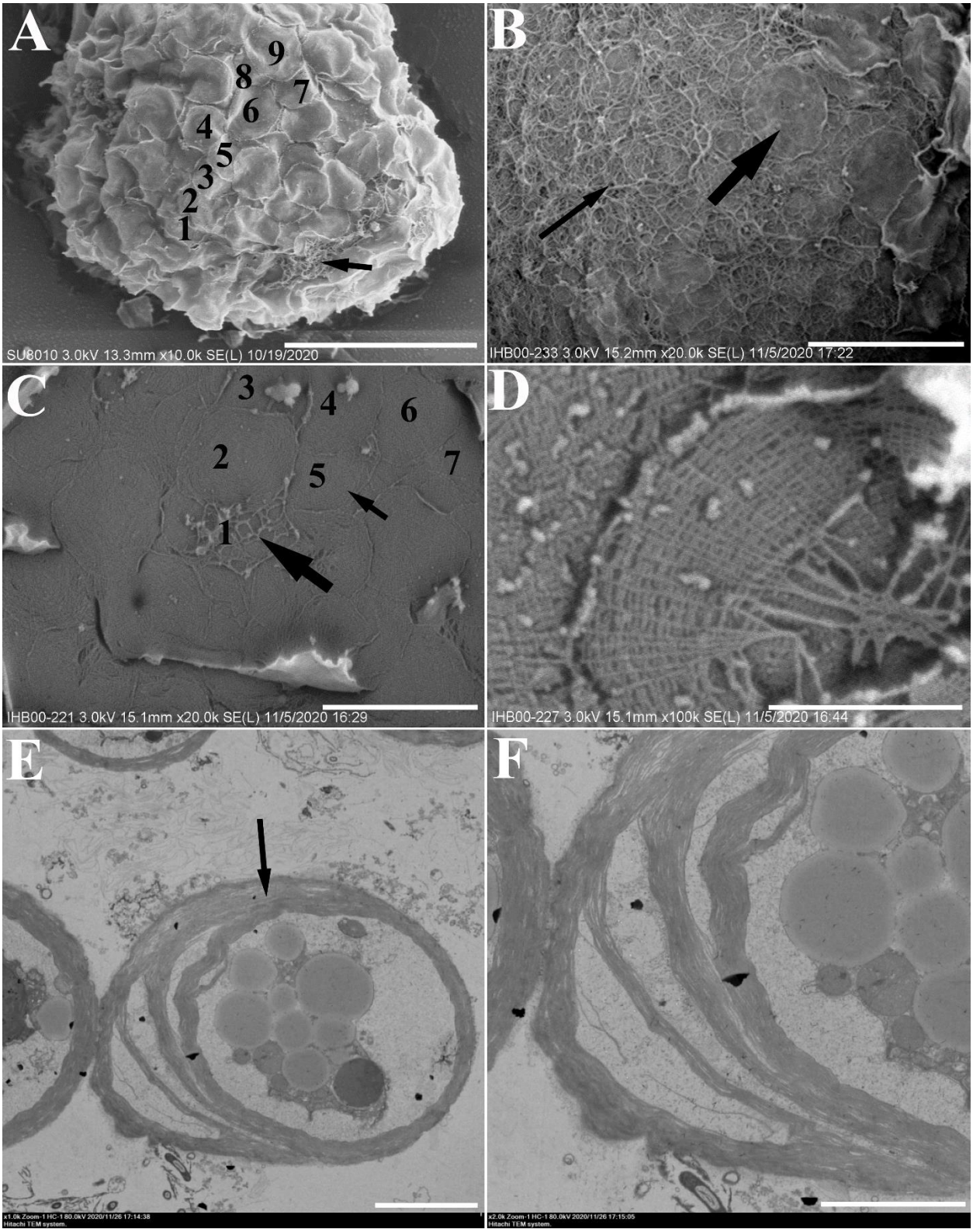

Fig 2. 9 SEM (**A-D**) and TEM (**E-F**) show the morphology of scales. **A.** a mixture of micro-tube networks (arrow) and adult scales coat in the nine different layers; **B.** position of micro-tube

networks (arrow) and young scales (thick arrow) in one cell scale coat; **C.** a mixture of micro-tube networks and scales coat in seven different layers, No.1 is the young micro-tube network (thick arrow) at top of center which will produce new micro-tube networks or scales in the up layer, No.2-7 are the six scales (arrow) in different layers; **D.** a scale center maintaining a net state; Showing net and scale keep with ability of exchange each other; **E-F.** TEM show one old cell with the thick scales coat which is divided in four layers of sub-coat in different amplifications. Scale bars: A, E, 5 μm; B, C, F, 2 μm; D, 500 nm.

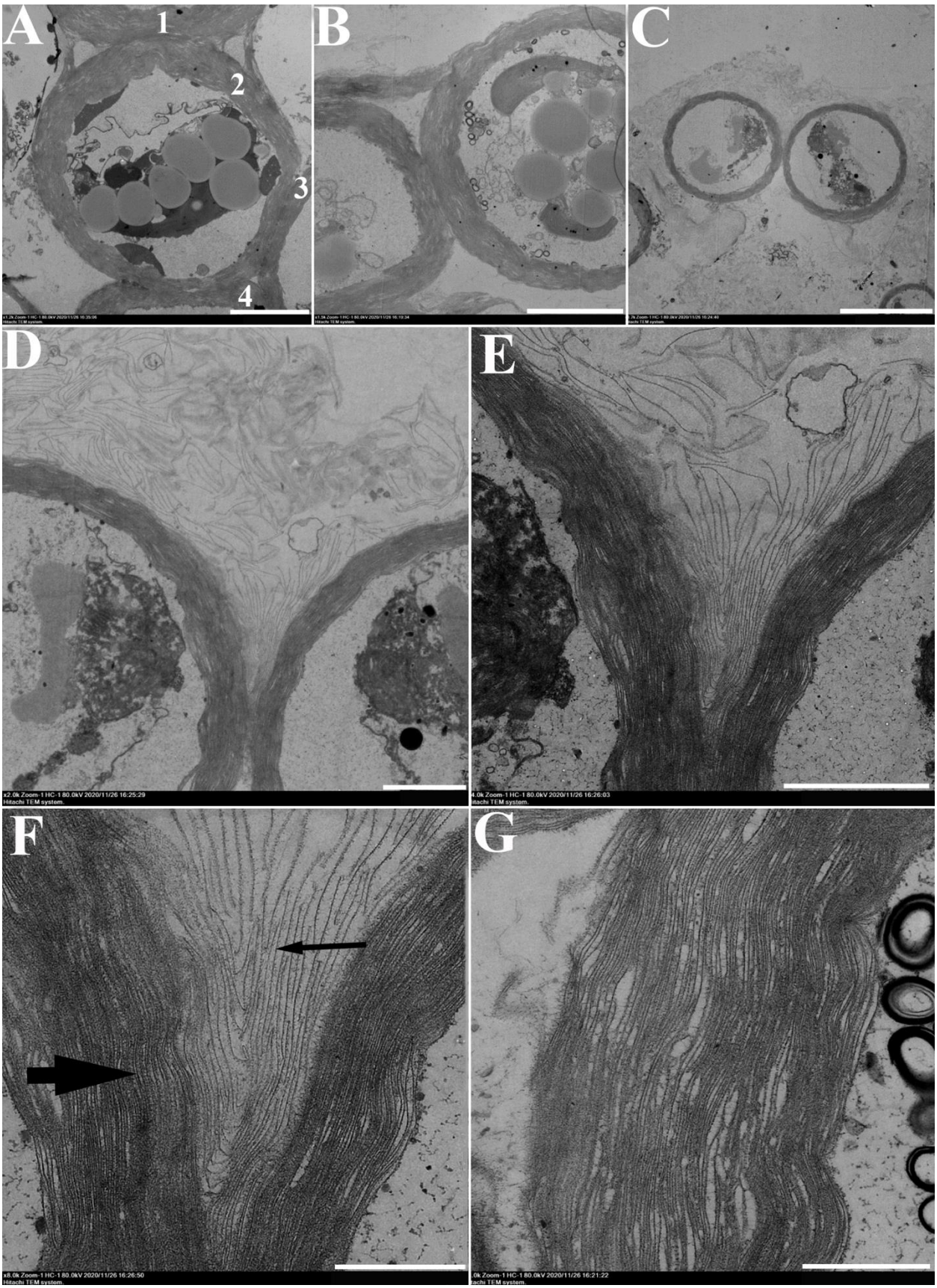
A
1
2
3
4
B
C
D
E
F
G

Fig 2. 10 Images (TEM) illustrate the positions of the unmineralized scales. **A.** four pseudo-filamentous cells with jointly compact scales coat (No.1-4); **B.** two cells with jointly compact scales coat; **C-F.** two same cells show compact and loose scales coat with different amplifications. The compacted scales coat (thick arrow) gradually changed to loose scales coat (arrow) from inside to outside, even to became a single scale; **G.** amplified partial scales coat of B. with about 60 layers of scales. Scale bars: A, B, 5 μm; C, 10 μm; D, E, 2 μm; F, G, 1 μm.

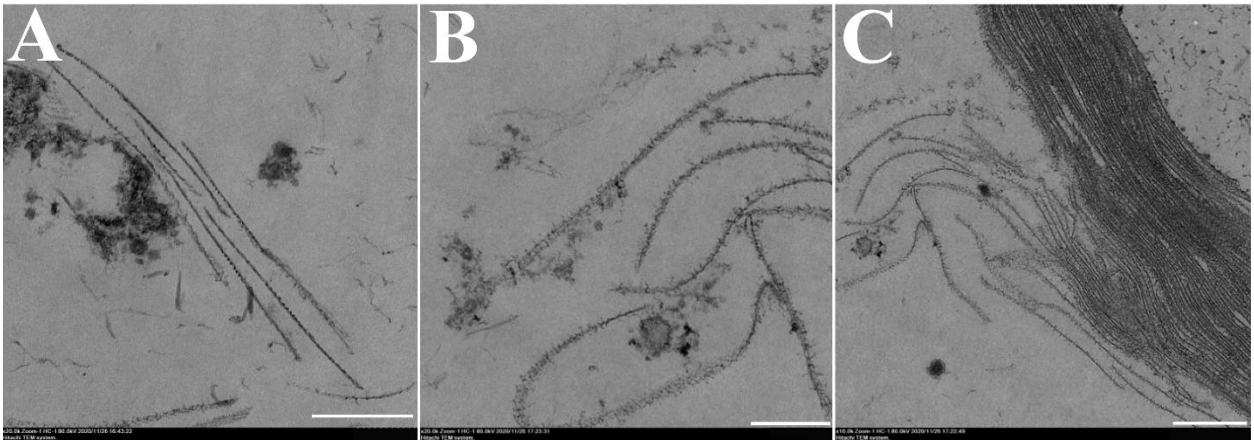

Fig 2. 11 Transmission electron micrographs (TEM). **A.** a few of the compacted scales coat are separated gradually becoming loose arrangement of scales to outside direction, even to become a single scale; **B-C.** the same image at different amplifications. Scale bars: A, B, C 500 nm.

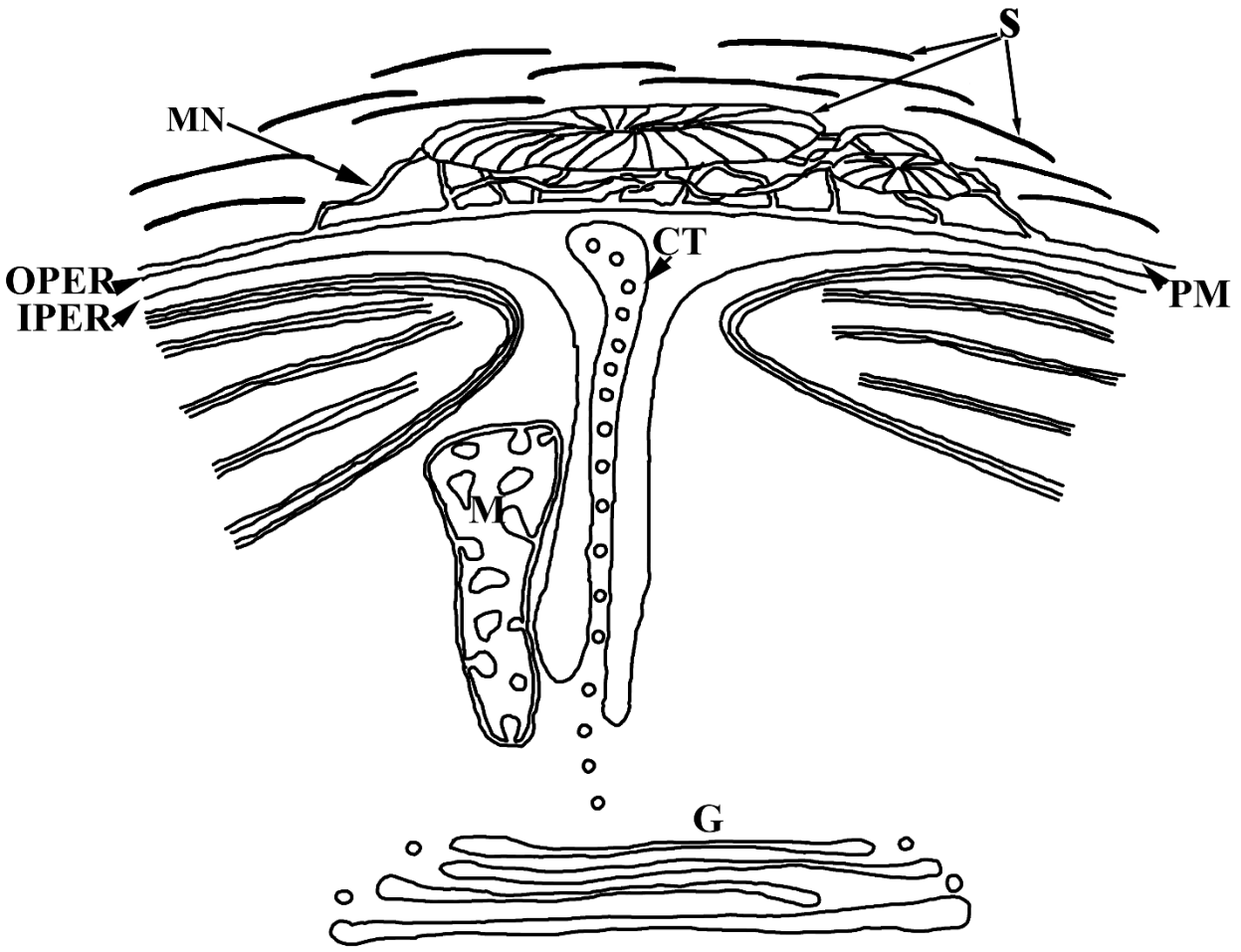

Fig 2. 12 Diagram shows the associations between the peripheral endoplasmic reticulum (PER), the cytoplasmic tongue (CT) and other cell organelles including micro-tube networks and unmineralized scales in outside of cell as seen in transverse cell section at a level just posterior to the basal bodies: there is a thin layer of cytoplasm between the plasma membrane (PM) and the outer envelope of the peripheral endoplasmic reticulum (PER); Around whole cell plasma membrane (PM) surface the IPER system forms the cytoplasmic tongue (CT) with ability to transmit the organic material passing the OPER and PM pass through outside of micro-tube network to construct the micro-tube networks (MN) and scales (S) (based on Beech & Wetherbee, 1988, revised by Mr. Hu and Dr. Qin, 2021).

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CHAPTER III

Chloroplast genome analysis of *Pleurochrysis dentata*

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Abstract

Pleurochrysis dentata is an ecologically important marine alga contributing to the coccolith formation. In the present study, a complete chloroplast (cp) genome of *P. dentata* was sequenced by using Illumina Hiseq and analyzed the sequenced data with the help of a bioinformatics tool, CPGAVAS2. The circular chloroplast genome of *P. dentata* has a size of 109,017 bp with two inverted repeat (IR) regions each (4513 bp each) which is a common feature in most land plants and algal species. The *P. dentata* CP genome consists of 61 identified protein-coding genes, 30 tRNA genes and 6 rRNAs with 21 microsatellites. The phylogenetic relationship with other select algal species revealed a close phylogeny of *P. dentata* with *Phaeocystis antarctica.* This is the first report of the cp genome analysis of genus *Pleurochrysis* and the results from this study will be helpful for understanding the genetic structure and function of chloroplast in other species of *Pleurochrysis.*

Key words: *Pleurochrysis dentata,* haptophyta, chloroplast, genome sequencing

3.1 Introduction

Chloroplast is a double membrane bounded plastid and helps in photosynthesis in green plants and algae. In addition to photosynthesis, the chloroplast also helps in the biosynthesis of amino acids and lipids (Daniell et al. 2016). The cp genome is circular in most of the plant species and the size of the gnome varies from 72 kb to 217 kb (Moore et al. 2007). In most cases, the chloroplast genome is a quadripartite structure consisting of a pair of inverted repeats (IRs) regions separating a large single copy (LSC) from small one-fold copy (SSC) regions. Haptophytes are photosynthetic microalgae found in marine. The genus *Pleurochrysis* represents haptophytes algae that consists of 9 species. *Pleurochrysis dentata* is a photoautotrophic unicellular marine alga belongs to the division Haptophyta and class Prymnesiophyceae. This group of the algae can produce and deposit $CaCO_3$ scales called coccoliths (Chen et al. 2019). This genus represents the marine coccolithophore producing algae and has a great economic significance because of their blooms which is released during the formation of coccoliths impacting on the carbon cycle (Reid et al. 2011). Coccolithophorids are well known for their roles in the precipitation of biogenic carbonate and their contribution to marine primary production. As we know haptophyte have represented important ecological roles, however, the study of chloroplast genome from other haptophytes only little is known regarding their genomic organization. The algae database (https://www.algaebase.org/) shows that there are 764 species of phylum haptophyta with the chloroplast genome analysis of only eight species including *Isochrysis galbana* (Fang et al. 2020), *Tisochrysis lutea* (Méndez-Leyva et al. 2019), *Phaeocystis antarctica* and *Phaeocystis globose*

(Smith et al. 2014), *Chrysochromulina tobin* (Hovde et al. 2014), *Chrysochromulina tobin* (Hovde et al. 2014) *Chrysochromulina parva* (GenBank Accession No.: MG520331.1), *Pavlova lutheri* (Baurain et al. 2010). The study of chloroplast genomes from other haptophytes will be very helpful to understand the functions of the genes and evolutionary lineage and their relationship with other species. This study reports the chloroplast genome sequencing and analysis of *P. dentata.*

3.2 Materials and Methods

3.2.1 DNA extraction and chloroplast genome sequencing, assembly, and annotation

Chloroplast DNA of *P. dentata* was isolated using a widely used extraction method (McPherson et al. 2013). The fragmented DNA was used to construct short-insert libraries and sequenced using Illumina Hiseq 4000 (Borgstrom et al. 2011). The data were trimmed using SOAPnuke 1.3.0 (Chen et al. 2018) and assembled with SPAdes 3.13.0 (Bankevich et al. 2012). The annotation of the cp genome was performed to predict the genes, rRNAs, and tRNAs in the genome using a bioinformatics tool, CPGAVAS2 (http://47.96.249.172:16019/analyzer/home) (Shi et al. 2019). The physical cp genome map was drawn using the OGDRAWv1.2 program with default parameters (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) (Greiner et al. 2019). Similarly, tRNAscan-SE (Schattner et al. 2005) was used to identify the tRNA genes.

3.2.2 Characterization of repeat structure and simple sequence repeats (SSRs)

The sizes and locations of forward, palindrome, reverse and complement sequences were analyzed using REPuter program (Kurtz et al. 2001) with the following parameters: minimum repeat size of 30 bp, maximum computed repeats of 5000 and a Hamming distance of 3 (a sequence

identity greater than 90%). Similarly, microsatellite identification tool MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html) was used for identifying the simple sequence repeats (SSRs). The setting for minimum number of SSRs was ten repeat units for mononucleotide, five repeat units for dinucleotide, four repeat units for trinucleotide. Similarly, three repeats units were used for tetra, penta and hexanucleotide.

3.2.3 Phylogenetic analysis

Available complete chloroplast sequence data for all related species of algae were downloaded from GenBank. Sequences were assembled and aligned using the Clustalw module in BioEdit v. 7.0.9.0 (Hall 1999) with default settings. Alignments were also checked and manually edited, if necessary. Maximum Likelihood (ML) analysis with 1000 bootstrap was conducted using MEGA 7 (Kumar et al. 2017).

3.3 Results and Discussion

3.3.1 Genome Analysis of *Pleurochrysis dentata*

The complete cp genome of *P. dentata* was assembled with a total of 109, 017 bp in size with the common quadripartite structure as seen found in most land plants that is divided in four regions, which included a large single-copy (LSC) region, a small single-copy (SSC) region, separated by two inverted repeat (IR) regions each (4513 bp each) (Fig. 3.1). The presence of identical IR regions is like most of the land and algal chloroplasts (Hovde et al. 2014; Liu et al. 2017). The GC content in *P. dentata* cp genome is 37.2 %. As shown in Table 3.1, the chloroplast of *P. dentata* consists of 61 protein-coding genes (6 ATP synthase, 9 ribosomal proteins, 4 cytochrome b/f complex, 3 RNA polymerase, 1 RubisCO large subunit, 3 photosystem I, 13 photosystem II, 12

ribosomal proteins (SSU), 3 hypothetical chloroplast reading frames and 7 other genes), 30 tRNA genes and 6 rRNA genes.

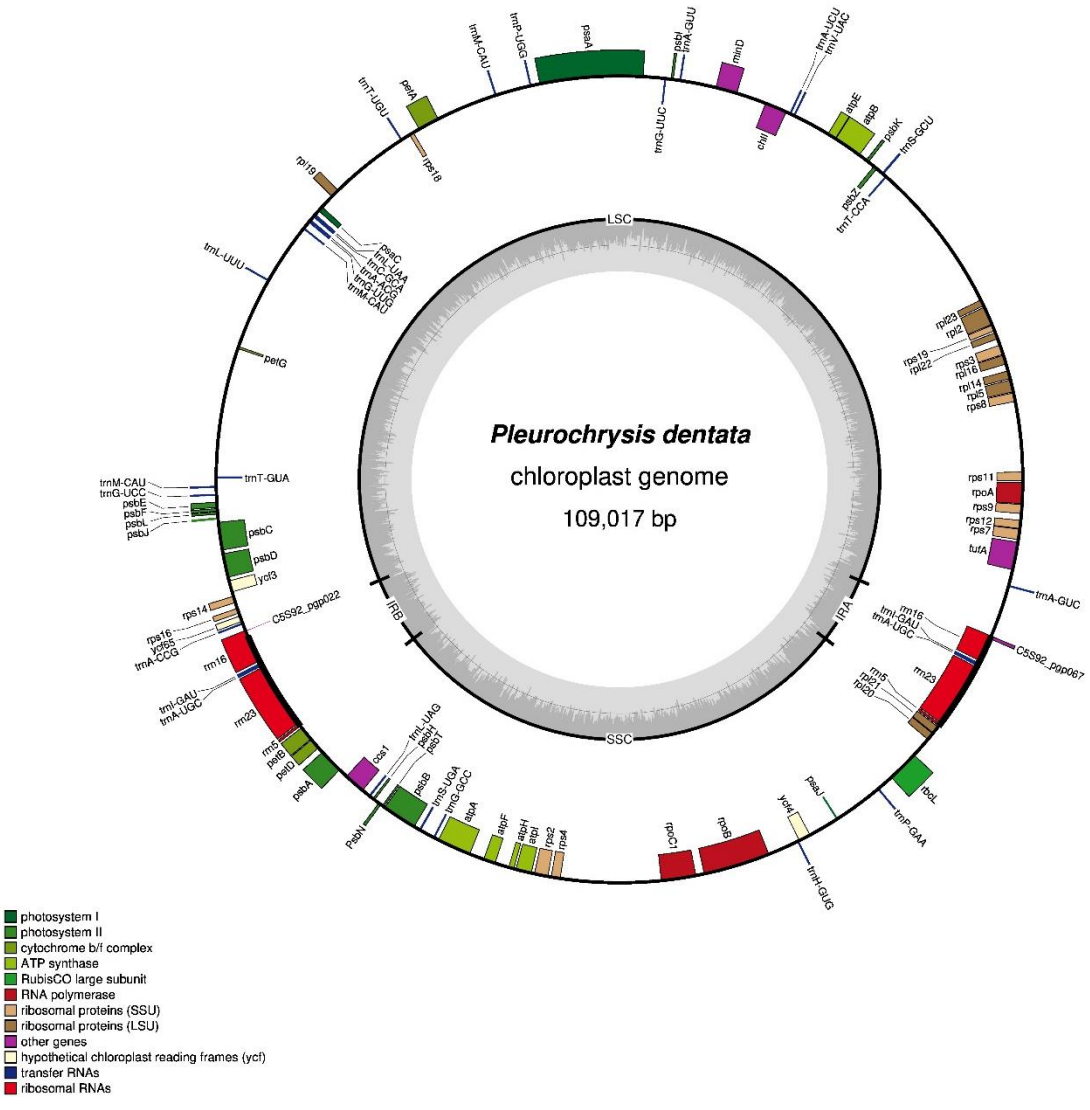

Fig 3. 1 Chloroplast genome map of *Pleurochrysis dentata*. Thick lines indicate the extent of the

inverted repeat regions (IRA and IRB), which separate the genome into small single copy (SSC) and large single copy (LSC) regions. Genes shown in inner circle transcribe clockwise whereas the genes in outer circle transcribe counterclockwise. The functional groups of the genes are represented by different colors.

Table 3. 1 Gene composition in *Pleurochrysis dentata* chloroplast genome.

Group of genes	Name of genes
Subunits of ATP synthase	*atpE, atpI, atpH, atpF, atpB, atpA*
ribosomal proteins(LSU)	*rpl14, rpl22, rpl2, rpl21, rpl20, rpl5, rpl16, rpl23, rpl19*
cytochrome b/f complex	*petB, petG, petA, petD*
RNA polymerase	*rpoC1, rpoB, rpoA*
Subunit of rubisco	*rbcL*
other genes	*ccs1, C5S92_pgp022, minD, chlI, C5S92_pgp067, tufA, psbN*
hypothetical chloroplast	*ycf65, ycf4, ycf3*
photosystem II	*psbZ, psbL, psbK, psbH, psbJ, psbT, psbA, psbC, psbB, psbE*
photosystem I	*psaJ, psaC, psaA*
ribosomal proteins(SSU)	*rps2, rps11, rps18, rps9, rps14, rps12, rps4, rps19, rps3, rps8*

Based on tRNA and protein-coding genes, the frequency of codon usage for *P. dentata* cp genome was estimated (Table 3.2 and Table 3.3) to have 8362 codons. Among these codons, 824 (9.85%) of the codons Glycine is the most frequent amino acid in the genome and the cysteines with 86 codons (1.02%) is the least frequent amino acid. The chloroplast genomes in algae have conserved features. However, the GC content varies in different algal species with the variation in

different regions of the chloroplast genomes. The study of codon usage is helpful for understanding the evolutionary relationship processes, and the selection pressure on genes and genome structure (Yang et al. 2014). The similarities in the codon usage seen in the *P. dentata* and other haptophytes (Fang et al. 2020) shows that these species might have gone through a similar environmental stress in their ecological niche.

Table 3. 2 Information of tRNA of the *Pleurochrysis dentata* chloroplast genome.

tRNA begin	Bounds end	tRNA type	Anticodon	tRNA begin	Bounds end	tRNA type	Anticodon
2243	2316	Asp	GTC	100723	100795	Phe	GAA
21783	21871	Ser	GCT	111260	111333	Asp	GTC
26355	26426	Val	TAC	107735	107662	Ile	GAT
26546	26618	Arg	TCT	107657	107585	Ala	TGC
31505	31576	Asn	GTT	80417	80346	Gly	GCC
37982	38055	Pro	TGG	79738	79653	Ser	TGA
39537	39610	Met	CAT	77123	77042	Leu	TAG
44033	44105	Thr	TGT	61371	61290	Tyr	GTA
52443	52514	Lys	TTT	49803	49730	Met	CAT
61705	61789	Met	CAT	49440	49369	Gln	TTG
62050	62120	Gly	TCC	49352	49279	Arg	ACG
67724	67796	Arg	CCG	49135	49065	Cys	GCA
69611	69684	Ile	GAT	49054	48971	Leu	TAA
69689	69761	Ala	TGC	32223	32151	Glu	TTC
96592	96664	His	GTG	21670	21598	Trp	CCA

Table 3. 3 Codon usage in *Pleurochrysis dentata* chloroplast genome.

Codon	Amino acid	Frequency	Number	Codon	Amino acid	Frequency	Number
GCA	Ala	38.636	324	AAC	Asn	23.849	200
GCC	Ala	6.201	52	AAT	Asn	12.402	104
GCG	Ala	12.163	102	CCA	Pro	24.326	204
GCT	Ala	35.058	294	CCC	Pro	0.954	8
TGC	Cys	1.431	12	CCG	Pro	4.293	36
TGT	Cys	8.824	74	CCT	Pro	16.456	138
GAC	Asp	10.017	84	CAA	Gln	25.28	212
GAT	Asp	28.858	242	CAG	Gln	8.109	68
GAA	Glu	26.711	224	AGA	Arg	9.54	80
GAG	Glu	19.079	160	AGG	Arg	1.669	14
TTC	Phe	32.435	272	CGA	Arg	5.247	44
TTT	Phe	25.042	210	CGC	Arg	4.77	40
GGA	Gly	17.648	148	CGT	Arg	24.565	206
GGC	Gly	8.824	74	AGC	Ser	4.293	36
GGG	Gly	5.962	50	AGT	Ser	13.117	110
GGT	Gly	65.824	552	TCA	Ser	24.088	202
CAC	His	12.163	102	TCC	Ser	0.954	8
CAT	His	12.163	102	TCG	Ser	6.678	56
ATA	Ile	3.339	28	TCT	Ser	15.502	130
ATC	Ile	18.364	154	ACA	Thr	26.473	222
ATT	Ile	46.029	386	ACC	Thr	1.669	14
AAA	Lys	24.565	206	ACG	Thr	6.439	54
AAG	Lys	11.448	96	ACT	Thr	19.318	162
CTA	Leu	29.573	248	GTA	Val	26.95	226
CTC	Leu	0.954	8	GTC	Val	2.146	18
CTG	Leu	2.385	20	GTG	Val	4.293	36
CTT	Leu	20.749	174	GTT	Val	37.443	314
TTA	Leu	37.92	318	TGG	Trp	23.134	194
TTG	Leu	4.054	34	TAC	Tyr	18.125	152
ATG	Met	23.611	198	TAT	Tyr	15.025	126

3.3.2 Repeat structure and simple sequence repeats (SSRs) analysis

REPuter analysis showed the presence of 18 pairs of repeats in the cp genome of *P. dentata* showing the copy size 30 or longer (Table 3.4). There are only 2 repeats containing forward repeats whereas 16 repeats are related to the palindromic repeats. There longer repeats are similar to those found in other algal species and such longer repeats might play an important role in sequence divergence of chloroplast genome (Cavalier-Smith, 2002).

Table 3. 4 Repeated structure in the chloroplast genome of *P. dentata*.

Repeat no.	Size(bp)	Type	Repeat 1 start	Repeat 2 Start
1	30	F	34854	37094
2	32	F	34050	36275
3	4514	P	68328	104503
4	146	P	1	68181
5	108	P	197	68022
6	106	P	95	68127
7	82	P	75389	75389
8	72	P	129	68127
9	53	P	148	68127
10	53	P	58933	58933
12	48	P	77708	77708
13	45	P	26709	26709
14	33	P	74084	74124
15	38	P	31995	31995
16	34	P	80527	80527
17	30	P	18056	180096
18	30	P	60750	60783

Simple sequence repeats (SSRs) are well known as microsatellites. They are short (1-6 bp), tandemly repeated DNA sequences in the genome. The analysis of cp of *P. dentata* revealed a total of 21 microsatellites consisting of 3 mononucleotide SSR (14.28%), 3 dinucleotide SSR (14.28%), 3 trinucleotide SSR (14.28%), 10 tetranucleotide SSR (47.61%), 1 pentanucleotide SSR (4.76%) and 1 hexanucleotide SSR (4.76%). Most of the SSRs are composed of A/T bases thus contributing to the AT richness of cp genome.

Table 3. 5 Simple sequence repeats in the cp genome of *P. dentata*.

SSR ID	Repeat Motif	Length (bp)	Start	End
1	(ATAA)3	3	5150	5161
2	(GATT)3	3	9046	9057
3	(TCCAAC)3	3	9521	9538
4	(AT)5	5	21024	21033
5	(TTTG)4	4	21297	21312
6	(A)11	11	21465	21475
7	(A)10	10	27890	27899
8	(TTAAA)3	3	35388	35402
9	(ATTT)3	3	39238	39249
10	(TTAT)3	3	39631	39642
11	(TAAT)3	3	41238	41249
12	(ATT)4	4	43652	43663
13	(A)10	10	49578	49587
14	(AAAG)3	3	53332	53343
15	(TAA)4	4	59150	59161
16	(ACCC)3	3	67881	67892
17	(AGC)5	5	74745	74759
18	(AT)5	5	76898	76907
19	(TATT)3	3	80028	80039
20	(TA)5	5	82138	82147
21	(TTAC)3	3	103481	103492

3.3.3 Phylogenetic analysis

Our phylogenetic data set included 27 species from three major taxonomic division: Haptophyta and Rhodophyta including Chlorophyta as an outgroup. The ML tree constructed with

1000 bootstraps support indicates that the *Pleurochrysis dentata* in this study was sister taxon of *Phaeocystis antarctica* (class: Coccolithophyceae) and *Phaeocystis globose* (Fig. 3.2). Species from Haptophyta and Rhodophyta lineages formed a monophyletic clade with high bootstrap support value (ML = 100). Our study is in line with the previous studies (Fang et al. 2020; Hovde, 2014) that reported a monophyletic clade of Haptophyta and Rhodophyta.

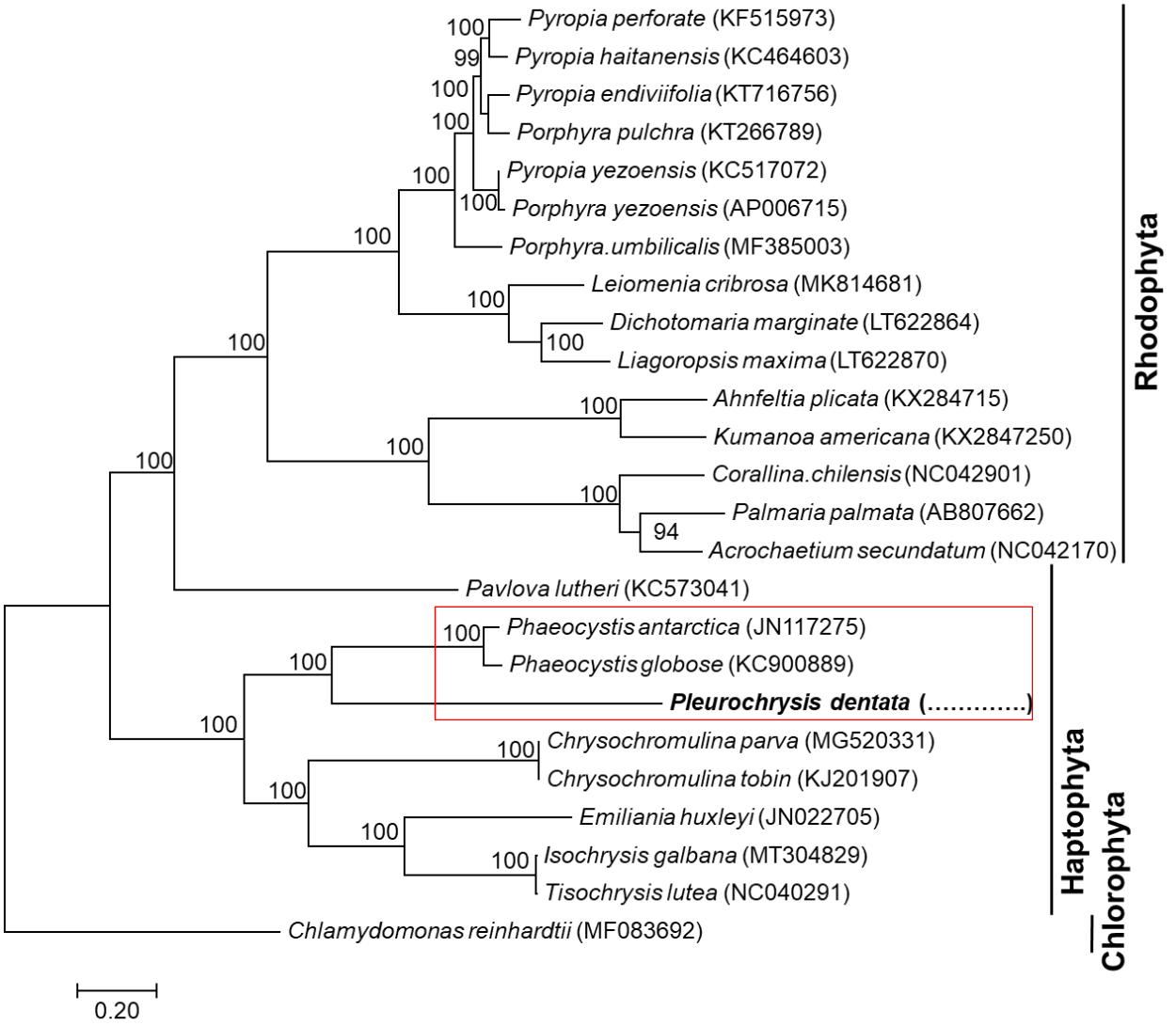

Fig 3. 2 ML tree based on complete chloroplast genome. Values on branches of the tree are

Maximum likelihood (ML).

3.3.4 Synteny analysis

Progressive Mauve 2.4.0 (Darling et al. 2010) was used to analyze synteny of *Pleurochrysis dentata, Phaeocystis antarctica* (JN117275) and *Phaeocystis globose* (KC900889). Fig.3.3 show the chloroplast genome synteny analysis. More than 21 locally collinear blocks (LCBs) were identified. The LCB connecting lines were confounding among the chloroplast genomes and considerable rearrangements and inversions were noted, especially in genus *Pleurochrysis* and genus *Phaeocystis*. *Phaeocystis antarctica* (JN117275) was highly similar to *Phaeocystis globose* (KC900889). The largest LCB was more than 24 kb, including 25 genes (*trnQ-atpA-atpD-atpF-atpG-atpH-atpI-rps2-rps4-rpoC2-rpoC1-rpoB-ycf27-trnH-ycf4-petL-ycf55-psaJ-ccs1-trnF-rbcL-rbcS-rpl27-rpl20-rpl21*). Only six LCBs were rearranged and inversed between *C. lobata* and *Chaetophora* sp., and involved *psbA, psbK, psbL, psbJ, rps11, cemA, rps14, psaA, atpI, petG, clpP, rps2, rpl23, rpl2, petD,* and *psbD* genes.

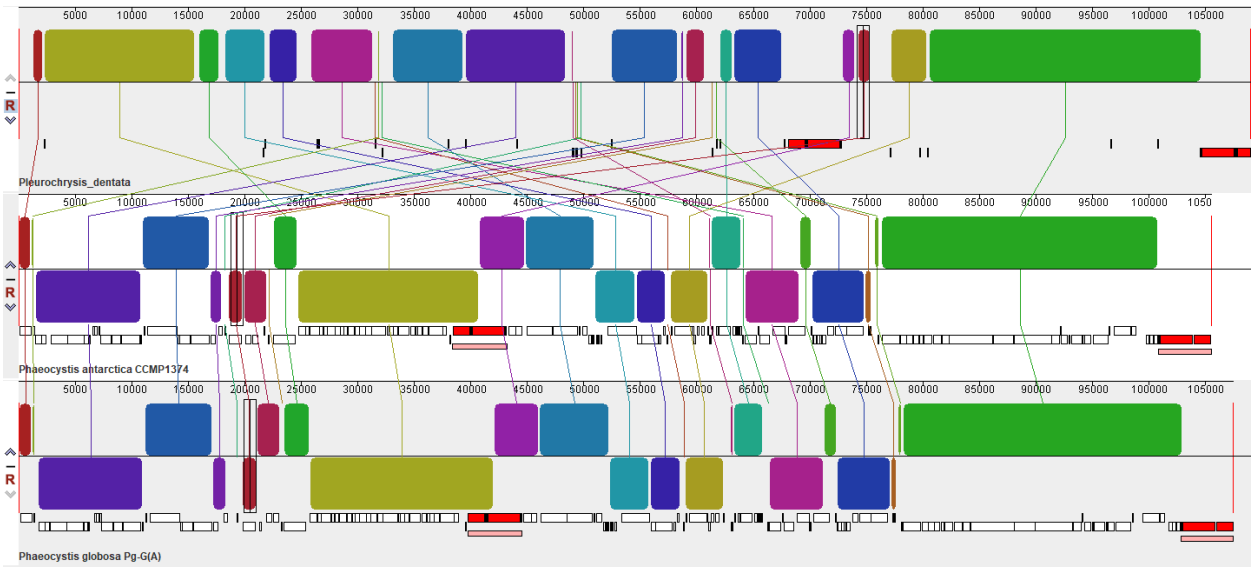

Fig 3. 3 Synteny comparison of the *Pleurochrysis dentata*, *Phaeocystis antarctica* (JN117275) and *Phaeocystis globose* (KC900889). The coloured syntenic blocks are local collinear blocks; blocks above the centre line indicate they are on the same strand, and blocks below the centre line indicate they are on the opposite strand.

3.4 Conclusion and discussion

In this study, we sequenced and analyzed the complete cp genome of *P. dentata* which is composed of 109,017 bp. This is the first report of the cp genome analysis of genus *Pleurochrysis* showing the gene contents and orientations like those found in other algae. The distribution and location of repeated structures and SSRs might be helpful for developing the microsatellite markers and to understand the cp organization of other species of *Pleurochrysis.* The study of phylogenetic relationship with other algal species at various genetic distance gives the insights into the molecular evolution of their cp genomes where *P. dentata* shows a close lineage with another haptophyte *Phaeocystis antartica*. The results of this study can be used for the discovering the genome organization and evolution in other species of *Pleurochrysis.*

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CHAPTER IV

Cultivation of marine microalga *Isochrysis zhanjiangensis* in indoor and outdoor conditions

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Abstract

Isochrysis zhanjiangensis is one of marine microalgae that widely distribute near costal area. There were several *Isochrysis spp*. that had been cultured as well bait to feed larval stage of molluscs and fishes, because of the high fatty acid content with a variety of high-value products, including fucoxanthin and docosahexaenoic acid (DHA) within cell body. In this study, the strain of *Isochrysis zhanjingensis* was cultured in outdoor and indoor bioreactors to analyze the effect of environment factors (temperature, light density) on growth and photosynthesis efficiency. When the temperature was higher than 37℃, the algal growth and photosynthesis would be seriously inhibited. Eventually, most of cells were crushed within 10 hours, when the culture temperature reached over 40℃. Meanwhile, the cells cultured under indoor conditions were growing well. The light intensity below 600 $\mu mol.m^{-2}.s^{-1}$ was found to be suitable for the growth.

Key words: *Isochrysis zhanjiangensis*, light density, photosynthesis efficiency, vertical column photobioreactor (VCB)

4.1 Introduction

In ancient period, the initial use of microalgae could be traced back to 2000 years ago by Chinese through collecting *Nostoc* as substitution of food during famine. Nevertheless, the development of biotechnology involved in mass culture of algae didn't start until the middle of last century (Spolaore et al. 2006). Since then, the biotechnology for mass cultivation and utilization of microalgae has been evolved. Until now, microalgae used for commercial applications are generally harvested from controlled cultivation processes in open or closed bioreactors (Tredici et al. 2009). As photosynthetic microorganisms, algae gain their energy via conversing light to organic matters. Most of algal species have the ability to capture inorganic carbon source from atmosphere or water body and fix it into cell biomass. So the microalgae play the key role in aquatic ecosystem as primary producers. Based on the ecological niche of algae, many predators are raised by these microorganisms, not only mollusks, but also fish and shrimps. In the stage of larval, both bivalves and shellfishes feed on algae as direct feeds. Some zooplanktons, like rotifers, artemia, and daphnia, capture microalgae as main nutrition source. These zooplanktons could be supplied to the young stage of some fish species, as well as to juvenile stage of crustacean species (Brown et al. 2013). Actually several marine algae are known as essential food source in certain stages of marine bivalves, enhancing transformation from juvenile to adult stages. During last two decades, the most popular genera of microalgae, such as *Chaetoceros*, *Thalassiosira*, *Tetraselmis*, *Isochrysis*, *Skeletonema* and *Nannochloropsis*, have been used directly or indirectly to feed aquaculture organisms (Kaparapu 2018). Compared to intake of a single diet that compose with sole algal species, there is definite improvement on the growth rates of target animals if these rearing species feed on combinations of several algae species. For

mass culture of these commercially valuable algae for aquaculture applications, producers need to assess some criteria for selecting these algae. For example, (1) the algal strains should have rapid growth rates and efficient accumulation of nutritious compounds, such as polyunsaturated fatty acids. (2) Residue of heavy metals or toxic compounds in algal cells must be at minimum level. (3) The contents of nutritional compounds must meet the requirements for raising the aquatic animals. (4) The production costs for these algae must be competitive to alternative feeds, and the targeted commercial market is strong demand for these algae. In the fact, hundred species have been trialed as feeds during last decades. However, less than twenty species have reached widespread application in aquaculture (Priyadarshani et al. 2012). After year 2000, there were plenty of articles that focused on the application of different algal strains in the field of aquaculture hatcheries, their improvements of biotechnology, design of bioreactors, and optimization of operation (Muller-Feuga et al. 2003; Muller-Feuga et al. 2004). Those literatures have comprehensively revealed not only the development of algal production and application in hatcheries, but also current manufacturing tendencies and advanced methods of detection.

Among previous published papers, scientists have found out that some microalgae can be used as feed for aquaculture species, as well as for terrestrial livestock. Robin J (2012) has summarized seventeen algal genera that had been successfully used in aquaculture and their breeding targets (Table 4.1). Only a few of strains have been successfully cultured in aquaculture hatcheries, due to difficulties in maintaining stability of strains , culture circumstances, viability of algae, concentration of available nutrients, and gazing threat from zooplankton (Guedes & Malcata, 2012; Tredici et al. 2009; Muller-Fuega et al. 2003a; Muller-Fuega et al. 2003b; Muller-Fuega et al. 2004;).

Table 4. 1 Major strains of microalgae utilized in aquaculture (Ref.)

Group	*Genus*	*Species*	*Area of application*
Cyanobacteria	Arthrospira	platensis	FFI
Chlorophyta	Tetraselmis	suecica, chui	B, CL
	Chlorella	sp., vulgaris, minutissima, virginica, grossii	R, FFI
	Dunaliella	sp., tertiolecta, salina	FFI
	Haematococcus	pluvialis	FFI
Eustigmatophyceae (Phylum Heterokontophyta)	Nannochloropsis	sp., oculata	R, GW
Labyrinthulea (Phylum Heterokonta)	Schizochytrium	sp.	RAD
	Ulkenia	sp.	RAD
Bacillariophyta (diatoms)	Chaetoceros	calcitrans, gracilis	B, CL
	Skeletonema	costatum	B, CL
	Thalassiosira	pseudonana	B, CL
	Nitzschia	sp.	GU
	Navicula	sp.	GU
	Amphora	sp.	GU
Haptophyta	Pavlova	lutheri	B
	Isochrysis	galbana, add. galbana "Tahiti" (T-iso)	B, GW
Dinophyta (dinoflagellates)	Crypthecodinium	cohnii	RAD

Key: FFI formulated feed ingredient; B bivalve molluscs (larvae/postlarvae/broodstock), C crustacean larvae (shrimps, lobsters); R rotifer live prey; RAD rotifer and Artemia live prey (dry product form); GU gastropod molluscs and sea urchins; GW "green water" for finfish larvae

Table 4.1. lists of the 17 major genera of algae currently feed to several groups of commercial aquatic organisms. The catalog includes groups of diatoms, chlorophyte and dinoflagellates, green algae as well as cyanobacteria. In the early juvenile stage of bivalve molluscs, the most widely used bait are *Isochrysis sp.*, *Pavlova lutheri*, and *Chaetoceros calcitrans*. They are generally mixed together to feed the raised aquatic animals (O'Connor & Heasman, 1997; Zhang 2014).

4.1.1 Nutritional compound of microalgae

As a function of the adapting environment, many microalgae have the ability to vary significantly in nutritional value. However, only small number of species has been tested for their nutritional compositions. Actually, the initial purposes of these tests may not focus on scientific

evidences of performance as supplement in the field of aquaculture. Thus, the components value in microalgal bait offer the opportunity to promote the development of nutritional compound supplied to larval animals. Indeed, several factors influence the nutritional value in microalga, including, but not limited to its size and shape, digestibility, nutritional composition, toxicity and the daily requirement of the aquatic animals. Thus, early studies mainly showed the differences of biochemical composition between microalgae (Parsons et al. 1961) and fatty acids (Webb & Chu, 1983). Several scientists have tried to correlate nutritional benefit of microalgae to their biochemical value. Yet results of feeding experiment are hard to be explained owing to confused influences from other formulation additives (Guedes & Malcata, 2012).

As PUFAs which derived from microalgae (DHA, EPA and ALA) are regarded as essential fatty acid for various larvae. The percentage of these PUFAs in 46 strains of microalgae is revealed (Volkman et al. 1989; Dunstan et al. 1993). The microalgae, like *Pavlova sp.* and *Isochrysis sp.*, have an obvious advantage on the accumulation of particular PUFAs. So these strains can be directly fed to zooplankton (Hemaiswarya et al. 2011). Compared with commercial oils, DHA content in several products which occupy 5-15% of dry weight have generate similar levels of DHA improvement within zooplankton (Gara et al. 1998).

4.1.2 Nutrients in *Isochrysis sp.*

Marine microalgae, like *Isochrysis* and *Nannochloropsis* genera, have been studied as the reliable diets and applied in mariculture due to their cell size, easy digestibility, high nutritional compounds and rich contents of polyunsaturated fatty acids in lipid body (PUFAs) (Kiffe & Matsunaga, 1995; Alonso et al. 1992). As essential nutrients, these PUFAs, including omega-3

fatty acids, promote growth and development of animals and human. The sufficient PUFAs, particularly omega-3 groups in diet, have been proved to enhance growth rates and feeding efficiency in aquaculture, while the effect of single PUFA is hard to prevent high mortality and poor growth. *Isochrysis galbana* accumulates large content of PUFAs and grows well in mass cultures via indoor bioreactors, as well as through outdoor raceway (Hemaiswarya et al. 2011). So this species is initially utilized as a live food in commercial hatcheries for developing stages of bivalves and growth stage of fry.

4.1.3 Lipids in *Isochrysis zhanjiangensis*

Isochrysis and *Dicrateria* are able to produce certain high-value components such as pigments, protein and oils (Rawat, 2011). Because of the nutritional values, *Isochrysis* have been used to feed larval organisms in the aquaculture for decades. It was used as essential diet in early stage of marine bivalves, helping their transformation from juvenile to adult stages. Molina Grima (1994) cultivated one *Isochrysis* strain to yield eicosapentaenoic acid (EPA), while Hu and Richmond (1994) enhanced biomass productivity and DHA content in *Isochrysis* cultivation. Previous research found that the main PUFAs were 18:3(ω-6) and 22:6(ω-3) in *Isochrysis sp* (Cobelas et al. 1989; Qiang Hu et al. 2008). Several research results showed that *Isochrysis* spp. was one of good DHA producers.

4.2 Materials and methods

4.2.1 Algal strain

Isochrysis zhanjiangensis was provided by Dr. Fan Lu (Hubei University of Technology, China).

4.2.2 Preparation of f/2 medium

The component of f/2 medium was referenced from formula of early f/2 medium (Guillard and Ryther 1962, Guillard 1975). The concentration of $NaNO_3$ was increased to 750 mg/L. The trace metal solution is composed of 315 mg $FeCl_3 \cdot 6H_2O$, 436 mg $Na_2EDTA \cdot 2H_2O$, 9.8 mg $CuSO_4 \cdot 5H_2O$, 6.3 mg $Na_2MoO_4 \cdot 2H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 10 mg $CoCl_2 \cdot 6H_2O$, and 180 mg $MnCl_2 \cdot 4H_2O$ dissolved in a final volume of 100 ml distilled water. The vitamin solution is composed of 20 mg thiamine HCl (vitamin B1), 10 mg biotin (vitamin H), and 10 mg cyanocobalamin (vitamin B12) dissolved in a final volume of 100 ml distilled water.

4.2.3 Algae Collection

Ten milliliters (10 ml) of *P. carterae* cells were collected at each daytime from 6:00 to 18:00 and sampling gap is two hours. Fifty milliliters (50 ml) of cells were collected once for everyday growth analysis.

4.2.4 Algae Culture

The photobioreactor which used in outdoor or indoor condition was 800ml vertical column photobioreacter (Fig 4.1 and Fig 4.4 - 4.5). All of bioreactors were sealed with suitable rubber stopper to form closed system. All of glass was split to two groups. One group was cultured in laboratory condition with constant temperature and 24 hours illumination. The room temperature was set to 25℃ and light density was kept between 55-60 $\mu mol/m^2/s$. Another group moved to

rooftop of laboratory building, to adapt variable temperature and light density. The aeration was provided by continuous gas pumping, consisted with air and CO_2 gas, meanwhile the ratio of CO_2/air was 0.02.

4.2.5 The determination of cell density

The value of light absorption at 550nm was measured by spectrophotometer. Samples were diluted with appropriate ratios to ensure that the measured OD 550nm values were in the range of 0.100~0.500 if applicable (Lu et al. 1994). Moreover, cell number was calculated everyday by Hemocytometer (Model: Bright-Line™). Each sample was counted twice.

4.2.6 The efficiency of photosynthesis

As an indicator of photosynthetic energy conversion, chlorophyll fluorescence was commonly existed in high plants, algae and cyanobacteria. Fluorescence which originates from chloroplast is created after light absorption. The energy from photon can be photochemically converted or dissipated in the form of heat. Therefore, the yield of chlorophyll fluorescence is complementary to the energy conversion and heat loss:

$$\text{fluorescence} + \text{photochemistry} + \text{heat} = 1$$

PAM Chlorophyll Fluorometers were designed to calculate chlorophyll fluorescence yield with high sensitivity and selectivity. PAM-measurements has the ability to distinguish between fluorescence quenching by photochemistry and heat dissipation while algae sample was flashily exposed in saturation pulse of light source with certain wavelength.

By analysis of PAM-CONTROL, several parameters are measured. Fluorescence yield (F) initially measured prior to saturating light pulse. Maximal fluorescence yield (M = Fm or Fm')

computed during the last saturating light pulse. With the treatment of dark-adaption, maximal yield of photochemical energy conversion approximate to ΔF/Fm = (Fm-Fo)/Fm = Fv/Fm

The yield of energy conversion can evaluate physical condition of green algae, so this value should be monitored several times during daytime. Therefore, the gap of sampling is two hour, from 6:00 to 18:00.

4.2.7 Determination of growth

The specific growth rate (μmax) in exponential phase of algal growth was calculated by using the equation below: $\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)$

The N_2 and N_1 are defined as cell quantity in 1 liter culture medium at time t_2 and t_1 in exponential growth phase.

4.3 Results

4.3.1 Indoor culture of *Isochrysis zhanjiangensis*

In order to optimize initial cell density for algae culture, the following experiment had been conducted. Four groups of algae with different initial concentrations were set up as shown in **Fig. 4.1**. They were group A (OD=0.1), group B (OD=0.2), group C (OD=0.4) and group D (OD=0.8). *I. zhanjiangensis* cells were inoculated into column photobioreactors (diameter: 5 cm; culture volume: 700ml), all initial cell concentrations were adjusted after cell inoculation. The experiment was conducted for 9 days under same light density of 60 $\mu mol.m^{-2}.s^{-1}$.

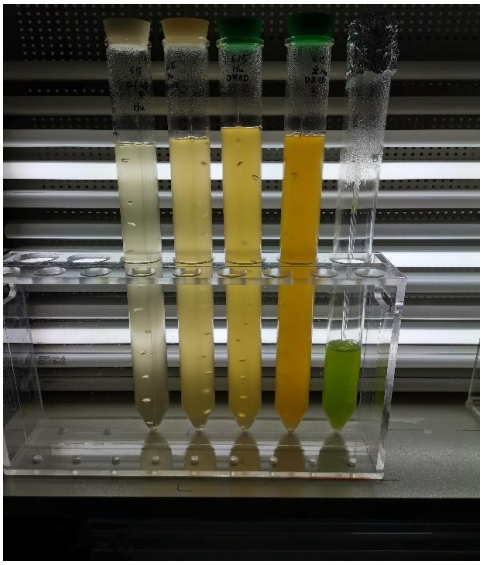

Fig 4. 1 Effects with initial cell density on growth of *I. zhanjiangensis*. Cells were cultured in vertical column photobioreactors with 24 hours illumination and aeration. Four initial cell concentrations were tested on beginning day.

Fig. 4.2(a) shows the growth curves of *I. zhanjiangensis* measured as increases in cell density. The group A and B both have two days period of lag phase, while group C and D started to grow immediately after inoculation. The final cell concentrations in each group didn't show significant difference.

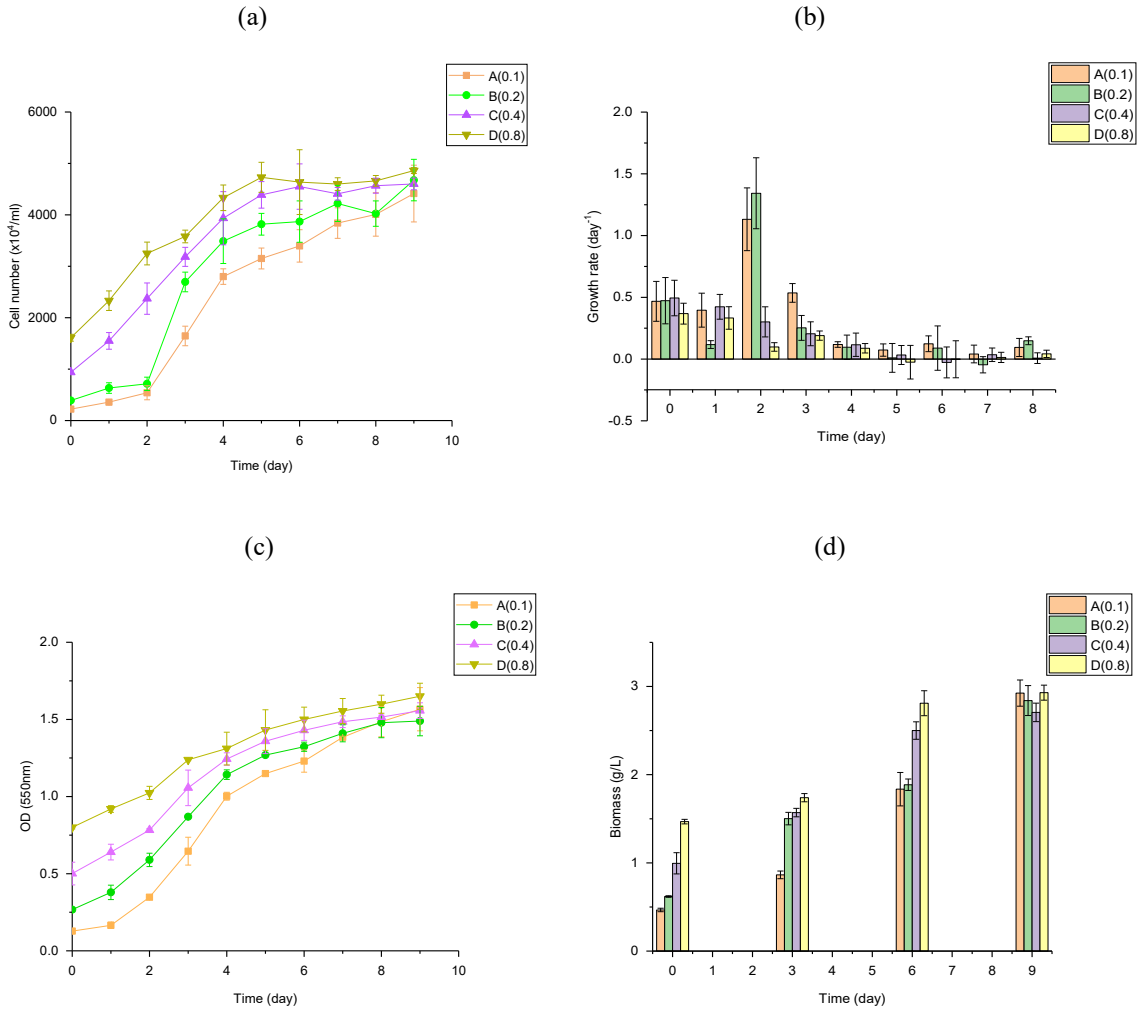

Fig 4. 3 Effect of various initial cell density on growth of *I. zhanjiangensis*. Four initial cell concentrations were tested in: A(0.1OD), B(0.2OD), C(0.4OD) and D(0.8OD). Data are reported as mean ± standard deviation of duplicates: (a) Cell density. (b) Daily growth rate. (c) Optical density. (d) Dry weight.

In order to assess the changes of growth rates during everyday cultivation, the value of specific growth rate μ was calculated based on daily cell density and charted in Figure 4.2(b). The results showed that the μmax in the group A and B were 1.13 day^{-1} and 1.342 day^{-1}, respectively higher than μmax in group C and B. Moreover, the final concentrations of each group didn't show

significant difference. The result indicated that *I. zhanjiangensis* with low initial concentration has an advantage on the growth rate, but the final biomass concentration was not affected significantly by the initial cell density.

Similar results were observed when the growth of *I. zhanjiangensis* was measured as increases in the optical density (OD at 550 nm) of the culture (Fig. 4.2(c)). The group A and B (lower initial OD) had higher growth rates compared to group C and D (higher initial OD). The final optical densities at Day 9 are similar among all groups.

The changes in cellular dry weight were measured at a 3-day intervals (Fig. 4.2(d)). At the day 0, the dry weight of group A and B (lower initial OD) were proportionally lower than group C and D (higher initial OD). However, after nine days of culture, the dry weight in each group was similar, indicating that the growth rates of group A and B were higher than group C and D. It should be pointed out that the group C and D (higher initial OD) reached stationary phase at day 6, while the group A and B (lower initial OD) continuously grew till day 9. The similar tendency was observed when the growth was measured as increases in cell number or optical density (Fig. 4.2(a) and Fig 4.2(c)).

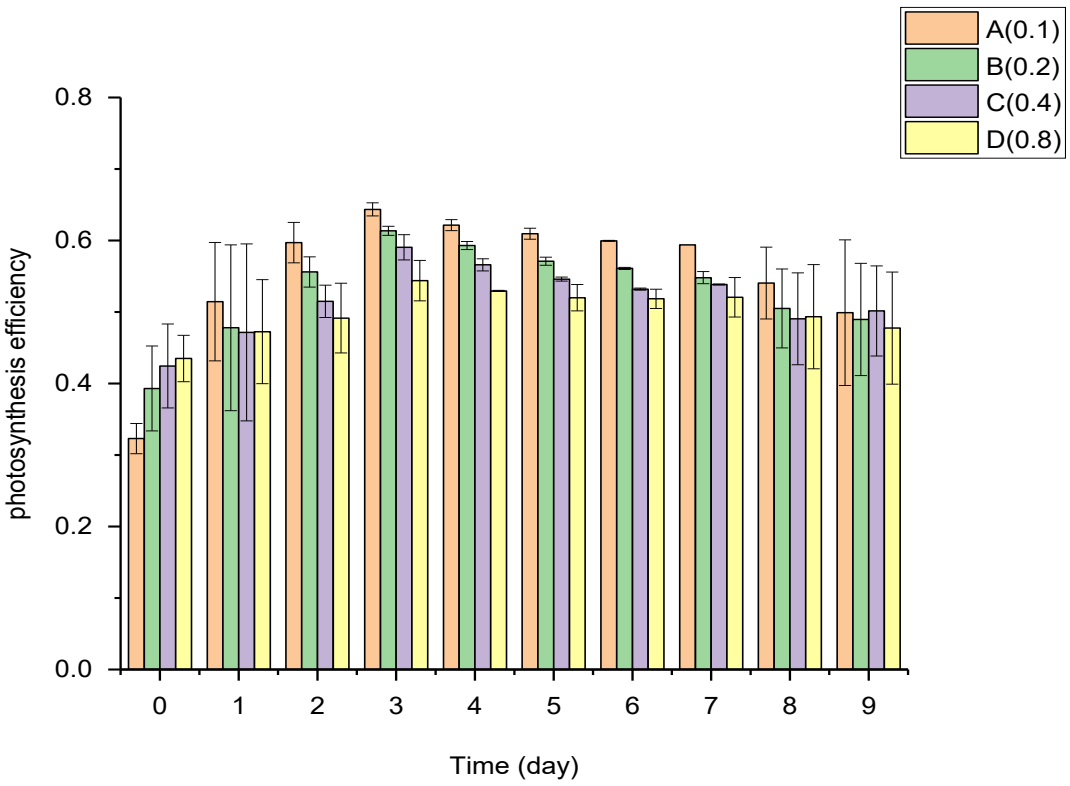

Fig 4. 5 The efficiency of photosynthesis of *I. zhanjiangensis* were obtained during cultivation in indoor. Four initial cell concentrations were tested: A(0.1OD), B(0.2OD), C(0.4OD) and D(0.8OD). Data are reported as mean± standard deviation of duplicates.

In general, photosynthesis efficiency could represent the conversion of light energy in cell chloroplast to chemical energy. The higher photosynthetic efficiencies indicate more energy accumulated by cells. Photosynthetic activity measured as Fv/Fm was monitored during the cultivation process (Fig. 4.3). At first two days' cultivation, the efficiency perform clear lag phase and don't meet 0.5. At the day-0 when cells were inoculated into the experimental settings, the values of Fv/Fm showed clear difference among all groups, indicating the cell responses to the different culture conditions. At the lower initial OD groups (A and B) cells received higher light

illumination, thus photosynthetic activity was suppressed. While at the higher initial OD groups (C and D) cells received less light illumination because of self-shading, the photosynthetic activity was relatively higher. After one day incubation, Fv/Fm values became similar in all groups, indicating that all cells have adapted to the culture condition and photosynthetic activity recovered to normal level between 0.5-0.6. After day-2, the photosynthetic activities in all groups were constantly kept at normal level. These results suggested that cells in all groups were kept in physiologically active status without significant stress, owing to constant light density and room temperature.

4.3.2 Outdoor culture of *Isochrysis zhanjiangensis*

To compare the growth characteristics and photosynthetic efficiency of *Isochrysis zhanjiangensis* in outdoor condition, the effects of initial cell concentrations on growth were studied in summer (July-August/2020). Due to strong sunlight and high temperature during the summer season, the cell growth was inhibited by excessive light and high temperature. To reduce the effects of high light and temperature, the culture site was covered with "shade-net", which could block about 70% of sunlight. The light intensity under the net was below 600 $\mu mol.m^{-2}.s^{-1}$ during the noon time (Fig. 4.4(b)). In order to enhance cell growth, the aeration was provided with compressed air enriched with CO_2 at a final concentration of ~2% (Fig. 4.4(a)).

The *I. zhanjiangensis* cells at exponential phase were inoculated into 700 ml columns with two different initial cell concentrations measured as Optical Density (OD550): 0.20 (group A), 0.8 (group B). The cell number kept growing until 8^{th} day when most cells were crashed by

excessively high temperature.

Fig 4. 7 *I. zhanjiangensis* was cultured on the roof of a ten-floor building: (a) Aeration was made up with 2% CO_2 (Sundo Hand-held pump CO_2 detector) and air. (b) All of bioreactors were covered by black net to reduce excessive sunlight. (c) One column as control at left; two different initial cell density (0.2OD at middle and 0.8 OD at right) with a duplication.

At the first several days, algal growth was not adversely influenced by outdoor temperature and sunlight (Fig. 4.5). However, high temperature at the 5th day started to inhibit the growth of cells (Fig. 4.6(a)). The cell number peaked at the 4th day, and declined afterward. By the 7th day, the cell number drastically reduced. At the 8th day, the culture changed color from yellow to light yellow, indicating the starting of cell death. At the 8th day the high temperature resulted in death of all cells and clearance of the culture. All groups of cells were crushed and bleached during daytime and didn't recover at night time (Fig. 4.5 (j)). It should be pointed out that the complete bleach of culture happened within only a few hours at the 8th day, since the culture was still light

yellow at 8:00 AM. However, when outdoor temperature of culture medium exceeded 40°C, the

color of each column quickly became white and bleached. (Fig. 4.5 (h), (i)).

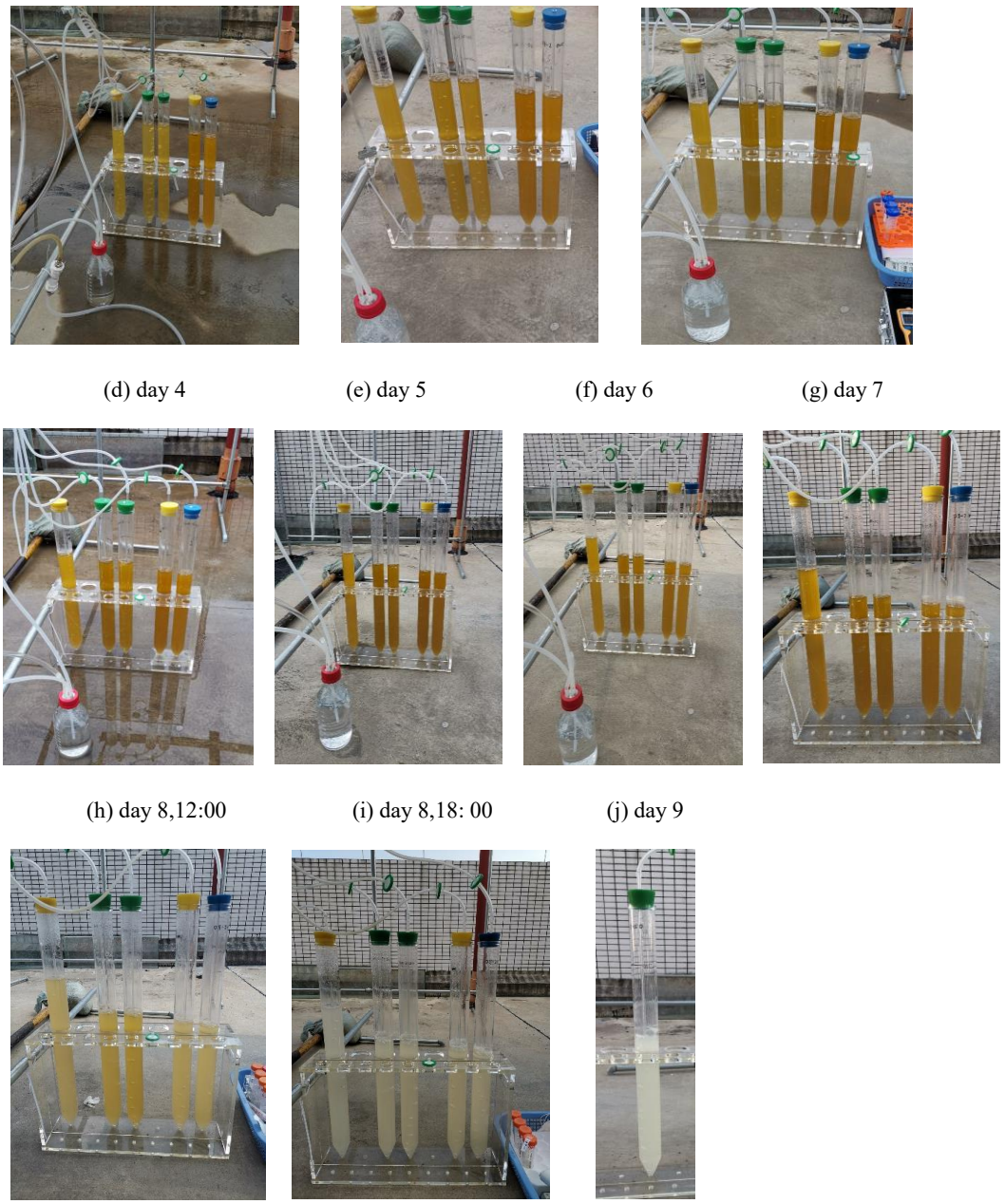

Fig 4. 9 The daily color changes of *I. zhanjiangensis* in each bioreactor from Jul.24-Aug.1. These images (a-g) showed algal density was keeping increase growth except in 8th and 9th day. Most of algae were crushed in only 10 hours, and column color changed from brown to gray (h-i). Finally column color was white in 9th day (j).

When the algal growth was measured as increases in optical density (OD550), a similar growth trend was observed as measured in cell number (Fig. 4.6(b)). The OD550 increased in the first four days and started to decline at the 5th day and after. At the 8th day, though cell number declined drastically, the OD550 only reduced about 50%.

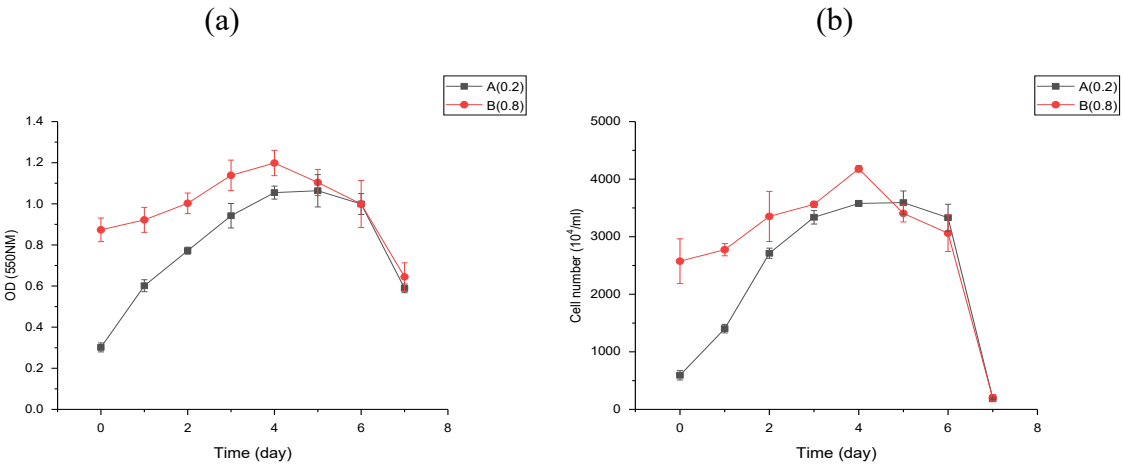

Fig 4. 11 The growth data of *I. zhanjiangensis* was obtained during cultivation A(0.2OD) and B(0.8OD) in outdoor: (a) Light absorption on wavelength of 550nm; (b) Cell numbers.

The growth of cells at the first seven days and death of cells at the 8th day were clearly controlled by high temperature. As shown in Fig. 4.7(a), the highest temperatures in the culture medium were mostly under 35°C in the first four days. The cell growth measured as increases in cell number was normal. Cells at group A (low initial OD) grew faster than cells at group B (high initial OD). However, when temperatures reached a level higher than 35°C at 6th day and afterwards, cell number started to decline, indicating the inhibition on the growth, and finally led to the death of all cells. These results suggested that this strain of *I. zhanjiangensis* did not have a strong ability to neutralize the adverse effects of high temperature.

High light intensity may also cause cell death by photobleach. However, during this experiment, the sunlight was blocked by the shade-net. As shown in Fig. 4.7(b), the highest light intensity on the column photobioreactors was below 600 μmol.m^{-2}.s^{-1}, which was safe for algal cells. The relatively low light intensities at the first several days were beneficial for the algal growth.

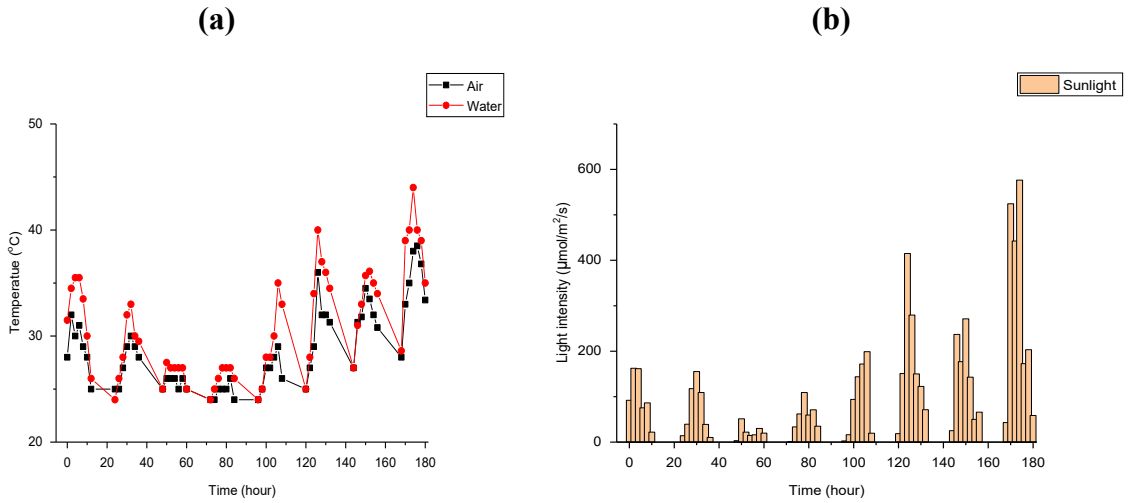

Fig 4. 13 The variation of environmental factors was tested during the cultivation in outdoor: (a) maximum and minimum air and water temperature; (b) irradiance.

The changes of photosynthetic efficiency showed an interesting pattern during the outdoor culture of *I. zhanjiangensis* (Fig. 4.8). In the first day of culture, there was reduction in the Fv/Fm values, indicating the adaptation process of cells after inoculating to the column photobioreactors. Starting at the second day, the Fv/Fm values reached a normal value of 0.4 and higher. Starting from the 5th day and after, the Fv/Fm value decreased and fluctuated significantly. At the 8th day,

the Fv/Fm dropped to near zero, indicating no photosynthetic activity was observed. The changes of Fv/Fm value corresponded to the changes in the cell growth pattern, suggesting the temperature played an important role in regulating the photosynthetic activity of *I. zhanjiangensis* in outdoor culture.

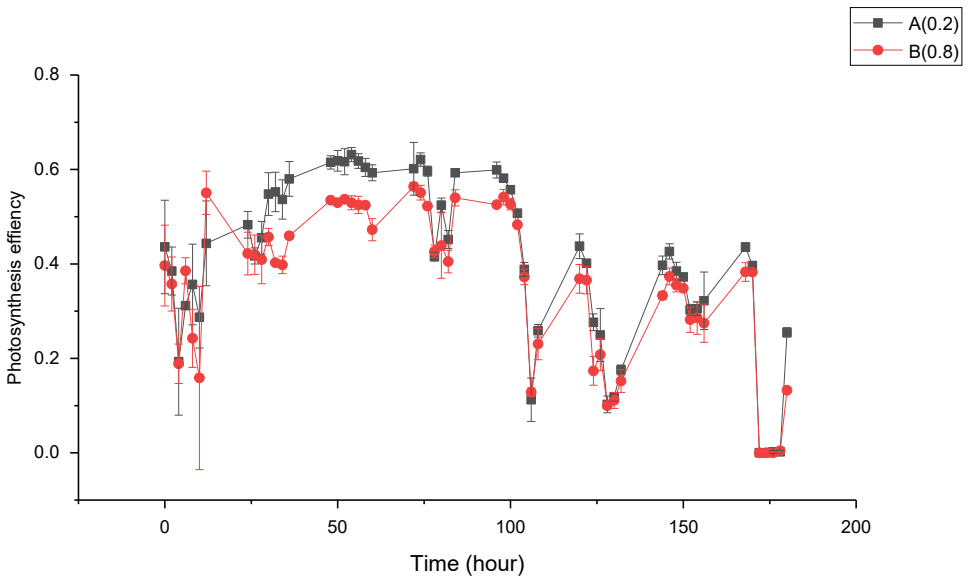

Fig 4. 15 The efficiency of photosynthesis in chloroplast of *I. zhanjiangensis* was tested during the cultivation A(0.2OD) and B(0.8OD) in outdoor.

4.4 Conclusion and Discussion

I. zhanjiangensis is a significant marine microalga for aquaculture because of its application in larval feed. The growth characteristics were defined under indoor condition. The lower initial cell density was favorable for high growth rate. However, under outdoor conditions, cell growth was controlled not only by initial cell density, but also by the fluctuating light intensity and

temperature. The cells exhibited normal growth when the temperature was below 30ºC, but cells were bleached when temperature reached 40ºC. The changes in photosynthetic activities showed that cells adapted to outdoor conditions quickly. More research are needed to optimize the culture conditions for the maximum productivity of this alga, and to elucidate the more detailed physiological changes during the cultivation under outdoor conditions.

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CHAPTER V

Conclusions and future recommendations

5.1 Conclusions

The life cycle of *Pleurochrysis carterae* consists of four stages, and coccoliths occurred in single cell stages but not in colony stages. The cell wall of *P. carterae* consists of scales with or without coccoliths. A new pathway for the formation of coccoliths and scales is proposed: the coccoliths and scales could be synthesized extracellularly.

It is the first report of the chloroplast genome analysis of *Pleurochrysis* and the results will be helpful for understanding the genetic structure and function of chloroplast in other species of *Pleurochrysis,* even in Haptophyte. For example for the molecular evolution *P. dentata* has a close lineage with other two haptophyte, *Phaeocystis antartica* and *Phaeocystis globose*.

The cultivation of *Isochrysis zhanjiangensis* were successfully carried out in indoor and outdoor conditions, lower initial cell density led to higher growth rates, however, high temperatures (>37^{o}C) resulted cell death. By cell counting, a large proportion of cell were destroyed by high temperatures and was not recovered after 12 hours, even photosynthesis efficiency returned to 0.4 values.

5.2 Future recommendations

Related cell wall development of *P. carterae* in next experiment, we are planning to obtain further research data to support our hypothesis on the scale extracellular formation pathway. We try to obtain the newer SEM or TEM images, especially for coccolith images in our near future experiment. Further experimental and ultra-structural works are needed obviously to clarify the

relationship of coccolith & scale extracellular formation pathway with coccolith & scale intracellular formation pathway in the Coccolithophyceae.

We need to do further experiments for confirming the relationship between the thick mucilage cell wall (a single cell and colony cells) (Fig. 2.7 F-H) and compact scales coat of *P. carterae* in SEM (Fig. 2.8 B. F-H) and TEM (Fig. 2.10 D.F.G.).

We would like also to clarify another kind of partial mineralized $CaCO_3$ particle on the edge of scales (alike coccolith) as below images (Fig. 5.1).

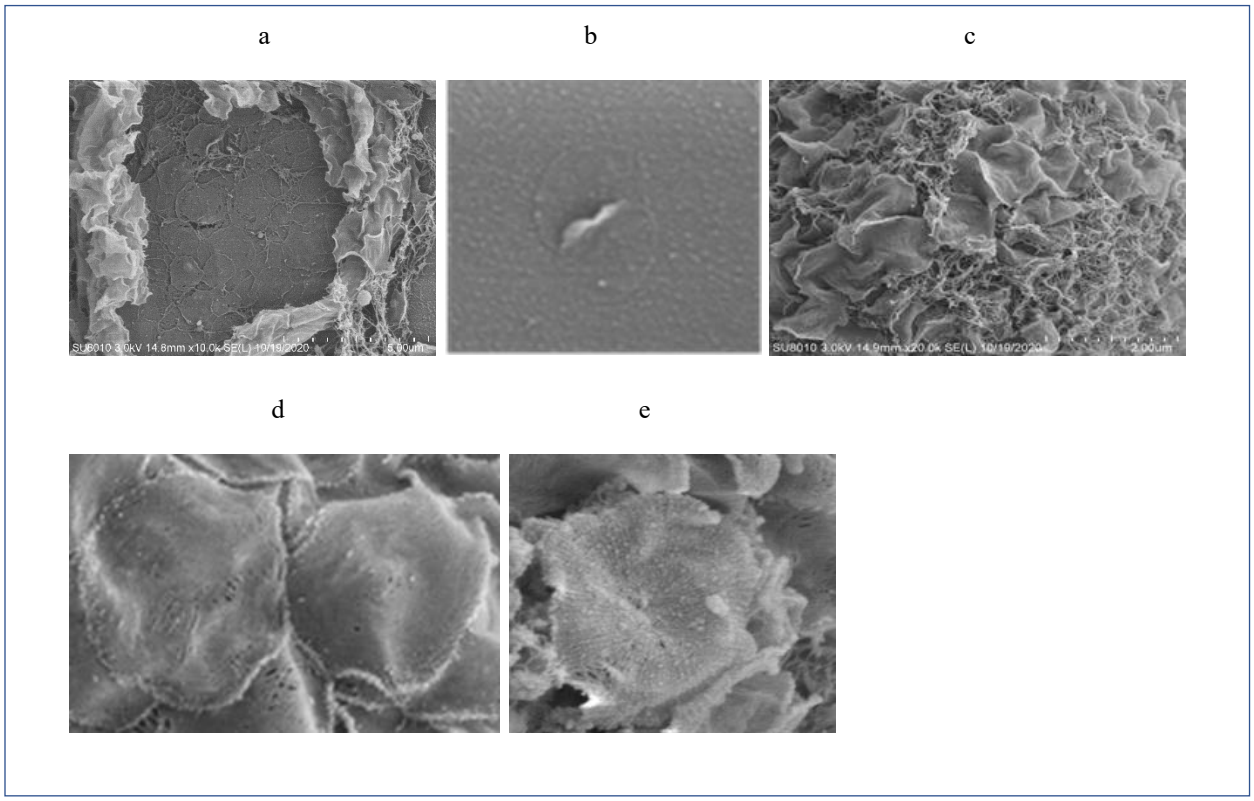

Fig 5. 1 The *Pleurochrysis carterae* images of scale in different development stages.

Pleurochrysis carterae was purchased from National Center for Marine Algae and Microbiota, East Boothbay ME, USA. (NCMA - https://ncma.bigelow.org/ccmp646). We take this species for chloroplast genome analysis, the analysis result of the species name is changed to *Pleurochrysis*

dentata. We need to make clear the all species of *Pleurochrysis* molecule systematics or relationship.

About the chloroplast genome analysis this paper obtained the complete chloroplast genome of the *Pleurochrysis dentata* preliminary showed its chloroplast genome composition and structure and laid a foundation for further analysis of the evolution of this genus. Next step, it should get more chloroplast genomes of the other representative species of genus *Pleurochrysis* and from the perspective of the comparative genomics, it will be the good solution to analysis the phylogenetic relationships and evolution within the genus *Pleurochrysis*, especially for the relationship between *Pleurochrysis carterae* and *Pleurochrysis dentata*.

As shown in Chapter II and like many other species in genus *Pleurochrysis, P. dentata* has great potential for fixing carbon dioxide and lipid producing. Photosynthesis plays an important role in this process. In the future, we are planning to clarify the key genes and improve the efficiency of photosynthesis by modifying the algae genome via genetic engineering and physiological regulation, which is conducive to more efficient carbon dioxide fixation and lipid producing.

We will try to cultivate *Isochrysis zhanjiangensis* in large scale outside looking for proper growth conditions. According to previous data from indoor and outdoor cultivation, we found the low initial density of *I. zhanjiangensis* has several advantages on algal growth, not only cell numbers and optical density, but also biomass accumulation. These data provide important information for outside mass culture of *I. zhanjiangensis* in large-scale cultivation. Moreover, the data of photosynthesis efficiency reveal the effect of high temperature on cell viability. Meanwhile, new type of bioreactors should be tested for future mass cultivation. On the other hand, component composition of PUFA in cell will be analyzed and modify culture medium or culture ways to

accumulate more Omega-3 fatty acid.

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