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# Structural Development of Marine Green Alga (*Ulva rigida* C. Agardh, 1823) during Cultivation

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**Abstract**

*Ulva rigida* C. Agardh (sea lettuce) is a marine green alga. It grows naturally near shorelines in areas with freshwater influence. It can also be cultivated easily on farms and can be developed into many healthy food products, which bring in revenue for Thailand. It is important to develop an effective way to cultivate the alga. In this research, the molecular biology of sea lettuce using the *tufA* gene for the identification of *Ulva* genus and morphological characters at different culturing periods of *U. rigida* thallus were investigated using a light microscope (LM), a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The results revealed that the *U. rigida* alga consisted of various cell shapes including elliptic, irregular triangle, irregular quadrilateral, pentagonal and polygonal. The cell size increased with increasing periods of culture. However, when the alga was cultured for 28 days, the cell sizes were smaller than the original cells due to cell division during aging, resulting in an increase in cell numbers. *U. rigida* had grown rapidly in 14 days which was a relatively short period of cultivation. The results of this study indicate that *U. rigida* has a potential to be scaled up in pilot and commercial scales.

**Keywords:** *Ulva*, Sea lettuce, Algae, Structure, Morphology, Anatomy, Molecular, Microscopy

## Introduction

*Ulva rigida* C. Agardh is a green macroalga in the division of Chlorophyta Ulvaceae family. Its common name is "sea lettuce". It can be found near seashore areas, in tidepools, on intertidal rocks, and on reef flats. It can also be found in freshwater runoff high in nutrients such as near the mouth of a stream. The thallus is a thin sheet approximately 10 - 30 cm long with shades of green that often becomes colorless sheets upon any stress [1]. There are 2 layers of cells in the transverse section of the thallus. The cells are rectangular and are arranged on the thallus surface in groups of 2 cells and 4 cells [2]. The cells can be round. They are 11 - 17 µm wide and 15 - 22 µm long. *U. rigida* can be found in Eastern Atlantic, Caribbean, India and Pacific Oceans [3]. Melton and Lopez-Bautista [4], found that *U. rigida* was distributed in the Gulf of Mexico and Atlantic side of the U.S.A. Ismail and Mohamed [5], studied the cell surface of *Ulva* spp. under a light microscope and reported that the cells were varied in sizes and shapes. The chloroplast occurred on the outer end of the cell with one or several pyrenoids. Coat *et al.* [6], reported that the cells in the surface view were round shape and in the cross-section were spindle shape. Dural and Demir [7], found that the sizes of *U. rigida* thallus were varied with their habitats. For instance, the thallus was small on rough water coast and larger in stagnant coast. Hofmann *et al*. [8], found that the cells of *U. rigida* thalli consist of a parietal chloroplast with multiple pyrenoids. Rajanaran and Kamra [9], reported that the

structure of the *Ulva* cell was like Chlamydomonas but without eyespot and cilia. The cytoplasm is covered with a single layer of the membrane. The cytoplasm contained a nucleus, mitochondria, dictyosomes (golgi bodies), endoplasmic reticulum, multivesicular bodies, a cup-shaped chloroplast with pyrenoid and starch grains. Barrett *et al.* [10], found that the pyrenoids were in the chloroplast of algae which was related to the algal biophysical $CO_2$ concentration mechanism that accelerated with photosynthesis and carbon fixation.

*Ulva* is a genus. However, it is difficult to morphological identify into species because there is phenotypic plasticity. The identification of alga species requires a combination of molecular markers and morphological structures [11]. In addition, the morphological characters of *Ulva* can change with impacting environmental factors and developmental stages of the alga [5]. *Ulva* can grow in freshwater, brackish and saline environments [12]. Kalita and Tytlianov [13], found that the temperature of water and illumination affected the growth rate of *U. fenesteata.* A temperature of 10 °C and illumination of 40 mE/($m^2$s) were the most optimum for vegetative growth. The growth rate of *U. fenesteata* was 30 % reduced when the cultivated temperature increased or decreased. Moreira *et al*. [14], found that the lipid content of *U. rigida* was also varied with temperature and light intensity.

DNA is the genetic material that characterizes an organism. The selection of short DNA strands from segments of chromosomes showed high interspecies differences but low heterogeneity between organisms of the same species. DNA barcodes are used to identify organisms (according to the Consortium for the Barcoding of Life, CBOL; http://barcoding.si.edu/) [15]. In the group of green seaweed, besides the morphological characteristics, DNA barcoding from the *tufA* gene was also used to classify molecular characteristics of both green marine and freshwater macroalgae [16-18]. Therefore, the *tufA* gene was used as the standard marker to identify green marine macroalgae [19].

Muthuvelan *et al*. [20], reported that the light color (energy) affected the cell integrity of *Ulva pertusa.* The white, blue and red light provided well developed thylakoids, starch and pyrenoid, which composed of a dense protein body. Fleurence [21], reported that *U. lactuca* contained protein of 10 - 21 % dry weight and *U. pertusa* contained higher protein of 20 - 26 % dry weight. Therefore, both could be complementary protein sources for human and animal nutrition. Moustafa and Saeed [22], reported that *Ulva* sp. had high 8.7 - 15.75 % dry weight of lipid, 20.7 - 27.6 % dry weight of protein and 42.31 - 51.37 % dry weight of carbohydrate. This alga had been used as good food and energy resources for human, animal and life in aquaculture. Thayawanichnondh [23], found that *U. rigida* had the potential to be developed as a healthy snack due to its high fiber and protein with low-fat content. Ergun *et al*. [24], found that mix of 5 % *Ulva* in the meal was successfully used as a feeding ingredient for tilapia, which was shown to increase growth performance.

In this research, DNA extraction and purification of sea lettuce were carried out to determine genetic materials. Morphological characters of *U. rigida* were investigated using a light microscope (LM), a scanning electron microscope (SEM) and a transmission electron microscope (TEM). The structures of *U. rigida* thallus at different cultured periods were determined to identify cell size and cell density. This will be basic for further research to obtain the optimum harvesting time of *U. rigida* with maximum dietary components that benefit human health.

## Materials and methods

### Preparation of *U. rigida* thalli

Samples of *U. rigida* were collected from Phetchaburi Coastal Aquaculture Research and Development Center, Sub District: Bang Kaeo, District: Ban Laem at Phetchaburi Province, Thailand. *U. rigida* was cultured in 5×4×1.5 $m^3$, wells containing 25 $m^3$ sea water with 30 - 32 ppt salinity. The wells were covered with 60 % light-intensity slant. The structural and bioactive compounds in *U. rigida* samples were determined at various culturing days: at the beginning of thallus 0, 7, 14, 21 and 28 days. The experiments were performed in September-October, 2021.

### DNA extraction and purification

DNA of sea lettuce sample was extracted according to the CTAB method, described by Doyle and Doyle [25], [using 100 mM Tris/HCl; 20 mM $Na_2EDTA$; 1.4 M NaCl; 2 % (w/v) CTAB; pH 8.0, 0.5 % 2-mercaptoethanol, 1 % PVP]. The extracted DNA was examined for quality and quantified by Nanophotometer. (Nanophotometer® NP80 (Implen, Germany).

### Polymerase chain reaction (PCR) amplification and sequencing

Total genomic DNA was diluted to 50 ng/µl for the DNA template. Primers used to amplify were a part of *tufA* gene (tufGF4 5’ GGN GCN CAA ATG GAY GG 3’, tufAR 5’ CCT TCN CGA ATM GCR

AAW CGC 3' [16]. Total volume of PCR reaction was 50 µl. The reaction consisted of 10 µl DNA template, 10 µl 5X buffer (5X Hot FIREPol® Blend Master Mix, Solis Biodyne™, Estonia), 2.5 µl 10 pmol/µl for each primer and the adjusted final volume was 50 µl. PCR amplification was performed by thermocycle machine (PCRmax® ALPHA 1, UK) with the following program: initial denaturation at 94 °C for 12 min, followed by 35 cycles of denaturation step at 94 °C for 30 s, annealing step at 55 °C for 30 s, extension step at 72 °C for 2 min, and 1 cycle of final extension step at 72 °C for 5 min. The amplified DNA fragments were separated on 1.5 % agarose gel added with 50 µg of ethidium bromide in 1X TAE, then were observed and photographed under UV light using the gel documentation system (UVITEC Imaging Systems, UK). 100 bp DNA ladder (Solis Biodyne™, Estonia) was used for detecting the size of the DNA fragments on the agarose gel.

PCR products were analyzed by automated DNA sequencing (Sanger sequencing, ABI 3730XL, BIONICS KOREA CO., LTD.) The resulting sequences were compared to nucleotide sequences in Genbank database by BLAST (Basic Local Alignment Search Tool) program. For more details on the GenBank, its related retrieval and analysis services, go to the NCBI home page at: http://www.ncbi.nlm.nih.gov [26].

**Sample preparation for microscopy analysis**

The *U. rigida* fresh thallial samples were examined by a compound light microscope (Zeiss: Axiostar Plus) for the surface view. The cells were examined and counted to determine cell size and number of cells per 1 $mm^2$. The samples were prepared in transverse sections and observed by a compound light microscope and a transmission electron microscope. The samples were cut into 1×2 $mm^2$ and prefixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for overnight at 4 °C in a refrigerator. The samples were post-fixed in 2 % osmium tetroxide in distilled water for 2 h. They were dehydrated in acetone series for 10 min/time, infiltrated and embedded in Spurr's resin [27-29]. The specimens were cut into 2 µm thick with a glass knife on an ultramicrotome (Leica: EM UC7), double stained with 1 % toluidine blue solution in 2.5 in 1 % borax at 85 °C [30], and with 1 % basic fuchsin in distilled water [31,32]. The samples were examined under a compound light microscope. The 100 nm of thin sections were double stained with 5 % uranyl acetate in distilled water and lead citrate for 15 min. Then, they were examined under a transmission electron microscope (Hitachi: HT7700) [33,34]. To prepare scanning electron microscope specimens, fresh thalli were cut into 5×5 $mm^2$. The samples were fixed with 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 for overnight at 4 °C and 1 % osmium tetroxide in distilled water for an hour. They were dehydrated in acetone series for 15 min/time [35,36], and dried in a critical point dryer (Quorum: K850) for an hour [37]. The samples were coated with a thin layer of platinum deposited with a sputter coater (Quorum: Q150R ES). The dry samples were examined by a scanning electron microscope (Hitachi: SU8020) [38].

## Results and discussion

**Molecular analysis of *U. rigida***

PCR products were amplified by *tufA* gene and were visualized by gel electrophoresis. The apparent size of the PCR product was approximately 840 base pairs (**Figure 1**). The PCR products were sequenced and compared to 10 sequences in the GenBank database (analyzed on August 30, 2022). It was found that the sequence was similar to 98 % of the *Ulva rigida* as the following accession numbers: HE600178 98.71 %, MH475484 98.70 %, MT859885 98.65 %, MH475485 98.57 %, MT859883 98.51 %, MT859889 98.50 %, MT859890 98.49 %, MH538637 98.44 % and MH538634 98.44 %. All 10 sequences of the *tufA* gene were aligned and constructed a phylogenetic tree, which was comparable with that of the *Ulva rigida* (**Figure 2**).

Sequencing analysis of *tufA* gene was used to identify sample species in this study. The sample was compared to sequence alignments in GenBank database showing a sequence of *Ulva rigida,* which was closely related to 98.71 % identity of *Ulva rigida* accession number HE600178.

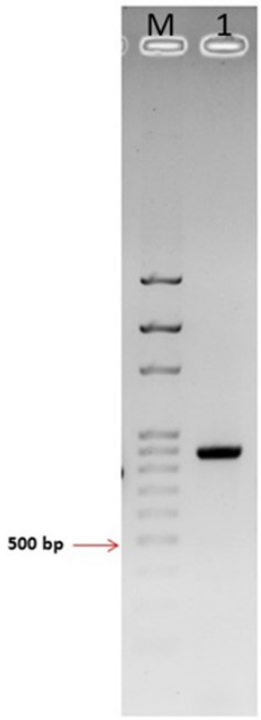


**Figure 1** Agarose gel electrophoresis (1.5 %) for PCR products using specific primers for *tufA* gene, showing a positive band at 840 bp. Lane M = marker 100 bp DNA ladder (Solis Biodyne TM, Estonia), Lane 1 = sample of sea lettuce.

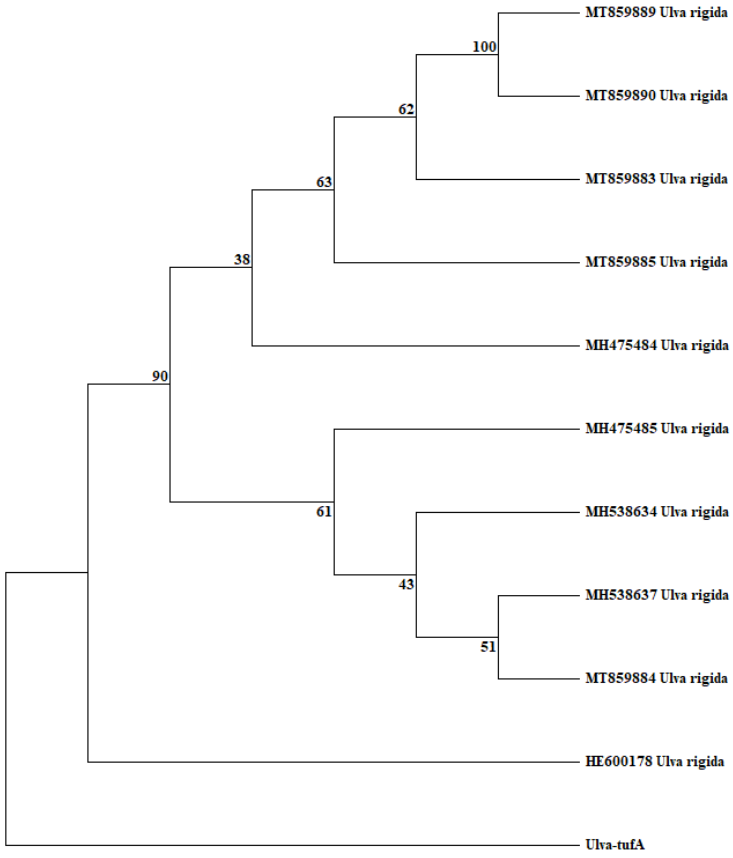


**Figure 2** Phylogenetic tree using the neighbor-joining method based on nucleotide sequences of *tufA* gene.

The molecular characteristics showed that the nucleotide analysis of *tufA* gene from sea lettuce having 98.74 % similarity to *U. rigida* Accession number HE 600178 in GenBank. Ten sequences of nucleotides from similar sea lettuce were analyzed and constructed a phylogenetic tree. The result confirmed that the alga in this study was *U. rigida*. The t*ufA* gene was identified to be green algae and genus *Ulva* using molecular markers [16,17,19]. This *tufA* gene was successfully amplified for 94 % and used for green algal identification. Twenty-one out of 22 species of them were identified [18].


**Figure 3** *U. rigida* thallus cultured in September-October, 2021 at Phetchaburi Coastal Aquaculture Research and Development Center, Thailand

**Morphological and anatomical characters of *U. rigida***

*U. rigida* had thin and green thallus. The edge of the thallus was jagged and curved (**Figure 3**). The surface view shows multicellular structure. The cells have various shapes, including oval, triangular, quadrilateral, pentagon and polygonal (**Figure 4**). A cup shaped chloroplast occurs in a cell (**Figure 5**), giving the cell greenish tint. The cell sizes were varied with the culture period (**Tables 1** and **2**). Cells elongated larger with increasing time of culture. The data in **Table 1** showed that the cells elongated until 14 days. At 21 and 28 days the cell sizes were smaller than those at 7 and 14 days due to cell divisions. The lateral growth caused the thallus to be enlarged. In terms of the cell numbers, the *U. rigida* thallus at 21 and 28 days had more cell numbers than the original due to cell division. The cell consisted of a thick cell wall and cytoplasm. There was a cup-shaped chloroplast granule in the marginal of cells (**Figure 5**). The chloroplast contained starch grains and pyrenoids, which had a core protein covered with starch.

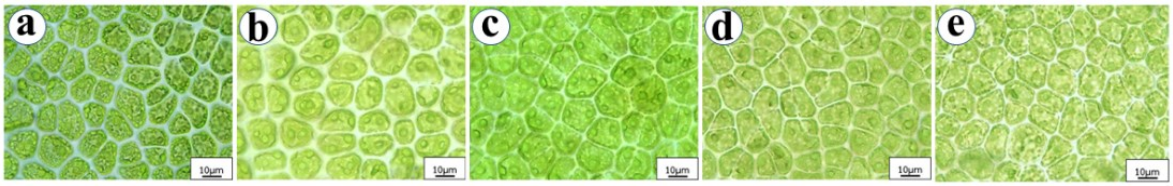


**Figure 4** Light microscopic photographs of *U. rigida* thallus cells cultured in September-October, 2021; a) at the beginning of thalloid cells, day 0, b) 7 days, c) 14 days, d) 21 days, and e) 28 days of culture.

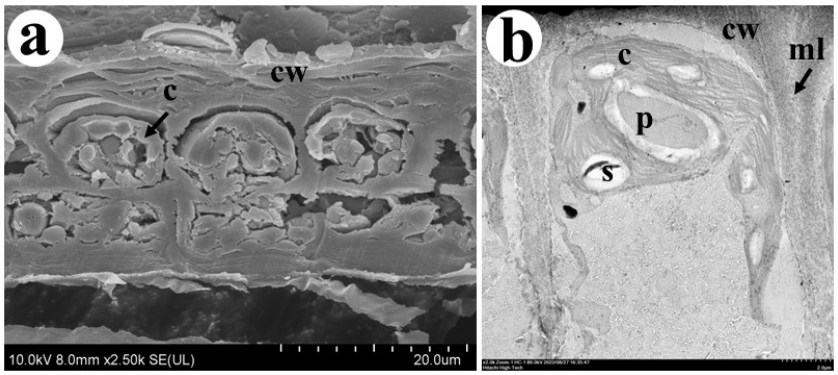


**Figure 5** Micrographs of cup shape chloroplast granules in the cell of *U. rigida*. a) Scanning electron micrograph of the transverse section of thallus shows 2 layers of cells: inner part composed of a cup shape chloroplast and outer layer of the cell wall (cw), b) Transmission electron micrograph of the transverse section of thallus shows cup shape chloroplast (c), pyrenoid (p) coated with a layer of starch (s), starch granules, cell wall (cw) and middle lamella (ml).

For the transverse section of the *U. rigida* thallus, there were 2 layers of cells. The cell wall became thicker when the culture time increased. The cell shapes in the inner layer got taller and the width of the cells was smaller (**Figure 6**). Cell divisions were observed at 21 days (white arrow in **Figure 6(d)**).

**Table 1** Cell size and cell density at the surface view of *U. rigida* thallus. The data were collected at 0, 7, 14, 21, and 28 days of culture.

| Day of culture | Cell size | | Cell density (# per mm$^2$) |
|---|---|---|---|
| | Length (µm) | Width (µm) | |
| 0 | 17.84 ± 2.53$^c$ | 12.47 ± 2.10$^c$ | 4,142 ± 4.23$^a$ |
| 7 | 20.52 ± 3.08$^a$ | 14.63 ± 2.47$^a$ | 2,838 ± 3.65$^c$ |
| 14 | 20.77 ± 3.25$^a$ | 15.00 ± 2.85$^a$ | 2,672 ± 3.25$^d$ |
| 21 | 18.91 ± 2.68$^b$ | 12.68 ± 2.29$^c$ | 3,586 ± 4.75$^b$ |
| 28 | 19.05 ± 2.60$^b$ | 13.66 ± 1.96$^b$ | 3,191 ± 4.87$^b$ |

Note: The number followed by the same letters in columns (a, b, c, d) are not significantly different based on Duncan's multiple range test ($p < 0.05$)
n = 150 for cell size, n = 50 for cell density

**Table 2** Cell size at transverse section of *U. rigida* thallus. The data were collected at 0, 7, 14, 21 and 28 days of culture.

| Day of culture | Cell size | | Thickness of thallus (µm) | Thickness of cell wall (µm) |
|---|---|---|---|---|
| | Length (µm) | Width (µm) | | |
| 0 | 31.05 ± 3.77$^d$ | 15.39 ± 3.75$^c$ | 61.14 ± 6.46$^d$ | 8.55 ± 2.02$^b$ |
| 7 | 34.81 ± 5.35$^b$ | 16.82 ± 3.60$^b$ | 68.51 ± 8.91$^b$ | 7.33 ± 1.63$^c$ |
| 14 | 38.82 ± 3.01$^a$ | 19.50 ± 4.66$^a$ | 74.90 ± 11.96$^a$ | 9.33 ± 1.61$^a$ |
| 21 | 40.00 ± 4.09$^a$ | 16.70 ± 3.25$^b$ | 75.82 ± 7.30$^a$ | 7.16 ± 2.28$^c$ |
| 28 | 32.59 ± 6.75$^c$ | 13.14 ± 2.53$^d$ | 65.36 ± 12.58$^c$ | 6.26 ± 0.95$^d$ |

Note: The number followed by the same letters in columns (a, b, c, d) are not significantly different based on Duncan's multiple range test ($p < 0.05$)
n = 100 for cell sizes, n = 150 for thickness of thallus, n = 150 for thickness of cell wall

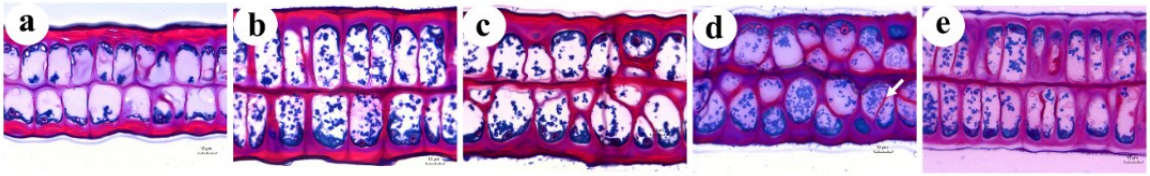


**Figure 6** Photographs of a transverse section of *U. rigida* thallus cells at various times of culture; a) beginning of thalloid cells, day 0, b) 7 days of culture, c) 14 days of culture, d) 21 days of culture (the arrow is cell division position), and e) 28 days of culture.

The morphological and anatomical characteristics of *U. rigida* were investigated. It was found that the cells in the surface view had various shapes including oval, triangular, quadrilateral, pentagon and polygonal (**Figure 4**). Similar to a report by Dural and Demir [7], the cells in the surface view were polygonal or quadrangular. Antica and Marcenko [39], showed that the cells in the surface view were rectangular or slightly polygonal. Phillips [40], found that the cells in the surface view were polygonal or quadrangular and the cells in the transverse section were round, oval or rectangle. These were different from Coat *et al.* [6], report, which indicated only round cells. The thallus of *U. rigida* was composed of a thick cell wall of 6.26 - 9.33µm and cytoplasm. The cytoplasm contained a large cup-shaped chloroplast with starch grains and pyrenoids. Dawes [41], reported that the thallus of algae in Ulvales Order had uninucleate cells, 1 parietal plastid and either one or many pyrenoids. There were many cell shapes of *U. rigida* when they were grown in different environmental locations with varied salinity of water [8].

The chloroplast was a cup shape and located in the marginal of cell. Koeman and van den Hoek [42], reported that the chloroplast was slightly tilted towards 1 side of the outer cell wall and contained many small starch grains and the pyrenoids having 1 - 4 granules in a cell. The pyrenoid had a core protein covered with starch. The cell enlargement of thallus occurred at 14 days. After that, cell divisions were observed, which caused cell numbers to increase and cell sizes to be smaller. The result agreed well with Phillips [40], study, which reported that the chloroplast position varied in the marginal of the cell. There were 1 - 3 pyrenoids in a cell. Meyer *et al*. [43], revealed that the pyrenoid of *Chlamydomonas* could be identifiable in microscopy as a dense protein coated with starch while Meyer *et al*. [44], reported that the pyrenoid peripheral structure was composed of Rubisco matrix and surrounded with starch in *Chlamydomonas*. Frikha *et al*. [45], and Paiva *et al.* [46], found that *U. rigida* from Tunisia and Portugal contained low protein and low carbohydrate. Therefore, the location and environment of cultures affected the growth and nutritional value of the algae. Oca *et al.* [47], found that light influenced the growth rate of *U. ohnoi*. The alga exposed to the highest photon irradiance (886 µmol photons $m^{-2}s^{-1}$) showed decreased growth rate, chlorophyll concentration and morphological change. Queiros *et al.* [48], found that protein and fat contents of the farmed *U. rigida* ranged between 7.6 - 25.8 % dry weight and between 0.2 - 1.3 % dry weight, respectively. Both protein and fat were at the highest levels during the autumn and winter seasons. The low temperature seems to promote nitrogen uptake and consequently high synthesized protein. From this result, the cultivation at 14 days was the optimum cultured period for *U. rigida* production when viewed from the size of cell. In practice, the cultivation of *U. rigida* can be in the period of 14 - 24 days. The short period of cultivation was 14 days which could produce the high biomass yields of *U. rigida*. This was similar with the study by de Casabianca *et al*. [49]. They reported that *U. rigida* thalli, harvested in 7 - 10 days in Thau and Venice lagoons, could grow faster than *U. fenestrate,* which took 92 days, reported by Steinhagen *et al*. [50]. Dion *et al*. [51], reported that *U. armoricana* thalli took 5 months to grow up. Our results can be used to encourage growing *U. rigida* as a commercial product for instant health food for human and animal feed. In the future, we plan to study changes of nutritional values of *U. rigida* as a function of cultivation time.

## Conclusions

The molecular characteristic of sea lettuce was verified. The nucleotide analysis of t*ufA* gene from sea lettuce showed 98.74 % similarity to *U. rigida* Accession number HE 600178 in GenBank. Ten sequences of nucleotides from similar sea lettuce were analyzed and constructed a phylogenetic tree. The result confirmed that the alga in this study was *U. rigida*. Its morphological and anatomical characteristics were investigated. It was found that the thallus of *U. rigida* was large, thin and green; and the edge was jagged and curved. The thallus of *U. rigida* was composed of a thick cell wall and cytoplasm. The cytoplasm of the cell contained a large cup-shaped chloroplast with starch grains and pyrenoids. The pyrenoid had a

core protein covered with starch. The cell of the thallus elongated until 14 days of culture. After that, cell divisions were observed, which caused cell numbers to increase and cell sizes to be smaller. The knowledge gained from this research provides basic information that can be used to determine the duration of the cultivation period and the optimum harvesting time of *U. rigida*. In addition, the methods of this study will be applied to investigations of other algae

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