

FACTORS IN CULTURE MEDIA AFFECTING THE GROWTH, AND PIGMENT CONTENTS OF ALGA *TRENTEPOHLIA MONILIA*

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Abstract. A genus *Trentepohlia* is the major type of filamentous subaerial green alga that grows in tropical zones. Its species have a yellow to red colour because of a large amount of total carotenoids. *Trentepohlia monilia*, a dominant *Trentepohlia* species, was investigated during the winter season at 720 metres above sea level in the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, in northern Thailand. Growth of the species in different liquid culture media was measured to find the best such medium, and various factors in the media were measured to determine which ones resulted in the highest amount of total carotenoids. The results showed that the enriched seawater medium (ESM) liquid culture produced the maximum growth of the species in week 9 of the study and also produced the highest total carotenoid content. A pH of 7.0, with an added peptone of 1%, culture strength of 50%, nitrogen source of –0.50 times and vitamin B₁₂ source of –0.50 times the usual concentration in the ESM generated the optimum conditions for growing *T. monilia* and also produced a total carotenoid content that was higher than the total chlorophyll content. This level of growth could make the species a future source of carotenoids for industrial products.

Keywords: *Chiang Dao Wildlife Sanctuary, subaerial green algae, enriched seawater medium, chlorophyll content, total carotenoid content*

Introduction

The genus *Trentepohlia* Martius, a subaerial or terrestrial alga, is the largest genus in the Trentepohliaceae family, belonging to the Trentepohliales order, Ulvophyceae class and Chlorophyta division (John, 2002; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009; Guiry and Guiry, 2015; Lemes-da-Silva et al., 2017). The alga grows on a wide range of substrata, from soils, rocks, bark, stems, and the leaves of trees to various manmade constructions (John, 2002, 2003; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009, 2018; Guiry and Guiry, 2015; Kharkongor and Ramanujam, 2017; Binoy et al., 2019). It is the most diverse and dominant of the subaerial algae that are abundant in tropical and subtropical regions (John, 2002; López-Bautista et al., 2002; Rindi, 2007; Rindi et al., 2009, 2018; Allali, 2011; Binoy et al., 2019). In addition, all *Trentepohlia* species produce a large amount of total carotenoids, which protects them from ultraviolet light or high irradiance. The pigment of the carotenoids gives the algae a yellow, orange or red color that is easily recognizable in natural habitats (López-Bautista, 2008; Rindi et al., 2018). Of the carotenoids, β-carotene is the one most abundantly found in this genus (Czeczuga and Maximov, 1996; Abe et al., 1998, 1999; Mukherjee et al., 2010; Aburai et al., 2013; Chen et al., 2015; Rindi et al., 2018; Binoy et al., 2019). Many scientists have suggested, therefore, that these algae could be used as a rich source of foods or supplementary foods for humans, natural food colorants, animal feeds, cosmetics and medicines (e.g., as a source of vitamin A, an enhancer of the immune response, an antioxidant or an anticancer agent) (Mortensen, 2006; Rao and Rao, 2007; Cazzonelli, 2011; Guedes et al., 2011; Takaichi, 2011; Priyadarshani and Rath,

2012; Eldahshan and Singab, 2013; Kharkongor and Ramanujam, 2017). Many scientists around the world have been sampling *Trentepohlia* species to culture and isolate their total carotenoid content (López-Bautista, 2008; Rindi et al., 2009; Aburai et al., 2013; Chen et al., 2015), but no one has done it yet in Thailand. Therefore we collected *Trentepohlia monilia* De Wildeman, a dominant species, from the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, in northern Thailand, which has the highest limestone mountain in the country and the third highest overall.

Our study aimed to examine the optimum factors that would enhance the growth of *T. monilia* by collecting samples of the alga from natural sites. Samples were taken to the laboratory, and various liquid culture media were used for growing the algae. We measured the growth of the algae to find which liquid culture medium was the best one. When we found the best medium, we attempted to determine which of its factors were involved in producing the highest total carotenoid content. This study of *T. monilia* could be the first step in gathering data that could stimulate the development of algal carotenoids as a marketable product in Thailand.

Materials and methods

Sampling area

The filamentous subaerial green alga *T. monilia* was collected from a curved steel barrier at points along the Ban Yang Thung Pong and Sop Hui Pha Tang Na Lao trails in the Chiang Dao Wildlife Sanctuary, Chiang Mai Province, northern Thailand, at 720 metres above sea level (latitude 19° 22.588' North and longitude 98° 45.080' East). Most of the samples were found in a dry evergreen forest. The algae were collected during the winter (dry season).

Algae materials

The algal samples were collected by following Saraphol et al. (2020), with a sterile scraper and placed into plastic boxes for further identification and culturing. Some environmental factors, such as the type of substrate on which the algae were found and their colour and preliminary morphologic characteristics, were observed and noted to help with identifying the samples correctly.

Algae identification

The algal samples were freeze-dried at -4 °C in the laboratory. Identification of *T. monilia* was done under the Olympus SZ30 stereomicroscope, Olympus CH30 light compound microscope (both from the Olympus Corp., Tokyo, Japan) and scanning electron microscope (Quanta 450 FEI, Thermo Fisher Scientific, Inc., Hillsboro, Oregon, United States) using the floristic keys and algae base website of López-Bautista et al. (2002), John (2002, 2003) and Guiry and Guiry (2015).

Algae sampling and culture

Once the algal samples had been collected from their habitats, they were kept in plastic boxes until used for further study. They were then cleaned by submersion in 70% ethanol and 1.2% sodium hypochlorite for 5 minutes. Each sample was transferred to a 1.5 ml Eppendorf tube, which contained one of seven liquid media: enriched seawater medium (ESM), BG-11 medium, Jaworski's medium (JM), Bold's basal (BB) medium with

NaNO₃ or NH₄Cl source, Bristol medium (BM), high-salt medium (HSM), and tris-acetate-phosphate (TAP) medium in the field. Upon arrival at the Department of Botany, Kasetsart University, each sample was transferred from its Eppendorf tube to a sterile 250 ml Erlenmeyer flask containing one of the seven media.

The subaerial green algal cells of *T. monilia* were grown on a shelf at room temperature (25 °C) under continuous illumination by cool-white fluorescent lamps (3,000 lux) with a light-to-dark ratio of 12:12 for 3 months. During this time, the algae were subcultured for purification to an axenic culture. The cells grew and increased by large amounts. The subaerial green algal cells were identified by microscope again as being *T. monilia*.

Measurement of algae growth

T. monilia cells grown for six months were shaken, and then 10 mL of a minimum of about 0.1 g algal fresh weight were transferred to a sterile 100 ml Erlenmeyer flask, which contained a liquid medium on the shaker. The optical density of algal growth was set as 0.1 at 550 nm. The growing cells were shaken at 100 rpm, at 25 °C, under continuous illumination by cool-white fluorescent lamps (3,000 lux) with a light-to-dark ratio of 12:12. The growth of cells in each 10 ml was measured every week from week 1 to week 11. The photosynthetic pigments in the total chlorophyll (a and b) and the total carotenoid content were measured with the spectrophotometer methods of Pompelli et al. (2013) and Chen et al. (2015, 2016). The pigment contents were calculated as follows:

$$\text{Chlorophyll a content (g/l)} = (12.19A665) - (3.45A649) \quad (\text{Eq.1})$$

$$\text{Chlorophyll b content (g/l)} = (21.99A649) - (5.32A665) \quad (\text{Eq.2})$$

$$\text{Total carotenoid content (g/l)} = \frac{[(1000A480) - (2.14 \text{ Chlorophyll a} - (70.16 \text{ Chlorophyll b}))]}{220} \quad (\text{Eq.3})$$

$$\text{Car/Chl ratio} = \frac{\text{Total carotenoid content}}{\text{Chlorophyll a content} + \text{Chlorophyll b content}} \quad (\text{Eq.4})$$

The algae in different media were analyzed to find the best medium for optimal growth so this medium could be used in the next experiment.

Analysis of various liquid culture factors

The best strain of algae in the best liquid culture medium was chosen based on previous studies to evaluate the various liquid culture factors (Table 1) that might have affected it.

The first *T. monilia* cells were evaluated every week from week 1 to week 6 and the total chlorophyll (a and b) and total carotenoid contents were measured with the spectrophotometer methods from the above method.

Table 1. The summaries of liquid culture factors using in the experiment

pH	Medium strength	NaNO ₃ or nitrogen source	Vitamin B ₁₂ or thiamine HCl solution
3.0	25%	-0.50 times	-0.50 times
5.0	50%	-0.25 times	-0.25 times
7.0	75%	0 times	0 times
9.0	100%	0.25 times	0.25 times
11.0	200%	0.50 times	0.50 times

Statistical analysis

These measurements were carried out with three replicates, and the results presented were the means of the three replicated experiments. Duncan's new multiple range test (DMRT) (ANOVA, $\alpha=0.05$) was used to determine the significant differences. The Jamovi statistic programming version 21.0 (The jamovi project, Sydney, Australia) was performed.

Results

Morphologic characteristics of the algae

The colonies of *T. monilia* that were sampled were crustose algae in thallus form found on a steel barrier along a trail in a dry evergreen forest at 700 metres above sea level. They had dark-green to yellow-greenish filaments. The thallus consisted of a dense mat with marked separations between the dense prostrate parts and slightly erect parts. The prostrate parts had a spreading form and produced a pseudoparenchymatous layer, which consisted of several layers of globular cells. The erect part had short filaments arising from the upper prostrate parts; they were 37.47 to 58.68 μm tall, with a thin cell wall. The cells of the erect filaments had a globular, swollen or inflated shape and were 2.67 to 3.59 μm wide and 4.21 to 3.60 μm long. The lateral filaments branching in the central region and the apical cells were often slightly pointed, usually with a small pectic cap at the tip. Neither zoosporangia nor gametangia were observed in this habitat (*Fig. 1*).

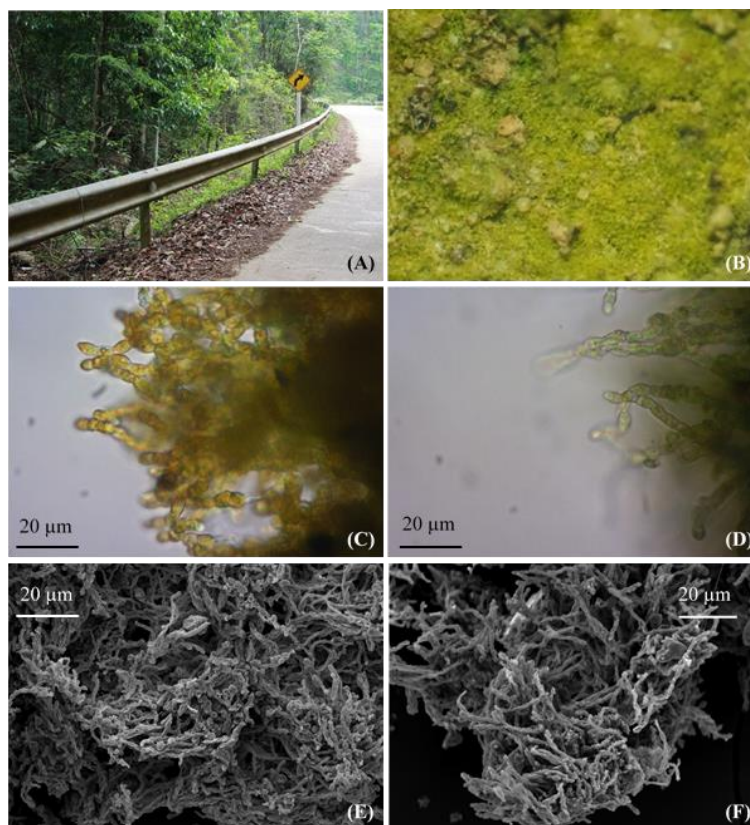


Figure 1. Characteristics of *T. monilia*. (A) Habitat from which algae were taken. (B) Cell morphologic characteristics as seen by the naked eye, (C to D) under the light compound microscope and (E to F) by scanning electron microscopy

Growth of algae

T. monilia samples from the Chiang Dao Wildlife Sanctuary were successfully grown and isolated only in ESM for 3 months (90 days) (Fig. 2). The ESM was used in culture for the analysis of the growth. After the cells had been placed in a fresh ESM culture for 3 months, the growth of *T. monilia* was measured by photosynthetic pigment methods to confirm the growth curves.



Figure 2. *T. monilia* cells culture at week 6 in 7 liquid media (liquid enrich seawater medium (ESM), liquid BG-11 medium, liquid Jawoski's medium (JM), liquid Bold's basal (BB) medium with NaNO_3 or NH_4Cl source, liquid Bristol medium (BM), liquid high salt medium (HSM) and liquid Tris acetate-phosphate (TAP) medium, respectively)

The growth of the *T. monilia* cells was measured by the photosynthetic pigments of the total chlorophyll (a and b) and total carotenoid content. In the media, the growth curve was the same growth pattern from week 1 to week 11, but we found that in the ESM, the growth was the highest in week 9 ($p\text{-value} \leq 0.05$) (Table A1). We found that the amounts of total chlorophyll were highest in week 9, at 0.1793 ± 0.0852 g/l, but the total carotenoid content was highest in week 11, at 0.0530 ± 0.0005 g/l. The ratio of total carotenoids to total chlorophyll did not vary much over the time of the study; it was 0.1800 ± 0.0088 to 0.4660 ± 0.0852 (see Fig. 3 and Fig. 4).

Total carotenoid content of algae in various culture conditions

The influence of different features of the cultures on the total carotenoid content accumulation of *T. monilia* varied. The effect of the pH on *T. monilia* growth, as shown in Fig. 5 (A), and on the total carotenoid content was similar and changed quite a lot from week 1 to week 6. The total carotenoid content at a pH of 5.0, 7.0, 9.0 or 11.0 were un-pattern changed in all of the weeks, except in the final week, when it was very high (Table A2). It was highest at a pH of 7.0, at 0.0273 ± 0.0140 g/l in week 6. The effect of peptone on the *T. monilia* growth is shown in Fig. 5 (C). The total carotenoid content with various amounts of peptone was similar in pattern and was also quite high from week 1 to week 6 (Table A3). The total carotenoid content with peptone of 0.5% and 1.0% changed the most from week 5 to week 6, and peptone of 1.0% caused the most accumulation of total carotenoids (0.0301 ± 0.0000 g/l). The effect of the culture strength

on *T. monilia* growth was shown in *Table A4* and *Fig. 6 (A)*. The total carotenoid content with various culture strengths had a similar pattern and changed quite a bit from week 1 to week 6, similar to the peptone growth curve. At a culture strength of 50%, the total carotenoid content had a positive pattern of accumulation from week 1 to week 6. It was highest in week 6, at 0.0256 ± 0.0000 g/l. The effect of the nitrogen source on *T. monilia* growth was statistically significant (*Table A5*) and shown in *Fig. 6 (C)*. The total carotenoid content with various nitrogen sources had quite a similar pattern and was quite changed from week 1 to week 6, just like the peptone growth and culture strength curves. The total carotenoid content had a positive pattern of accumulation from week 1 to week 6 when only NaNO_3 was used at -0.50 and 0.50 times. NaNO_3 of -0.50 times led to the highest accumulation of total carotenoids in the final week, at 0.0484 ± 0.0004 g/l. Finally, the effect of vitamin B_{12} on the *T. monilia* growth was statistically significant (*Table A6*) and shown in *Fig. 7 (A)*. The total carotenoid content with various vitamin B_{12} sources was quite similar and quite changed from week 1 to week 6, like the peptone growth, culture strength and nitrogen source curves. With vitamin B_{12} of -0.50 times, the total carotenoid content was found to be high from week 1 and highest in week 6, at 0.0423 ± 0.0000 g/l.

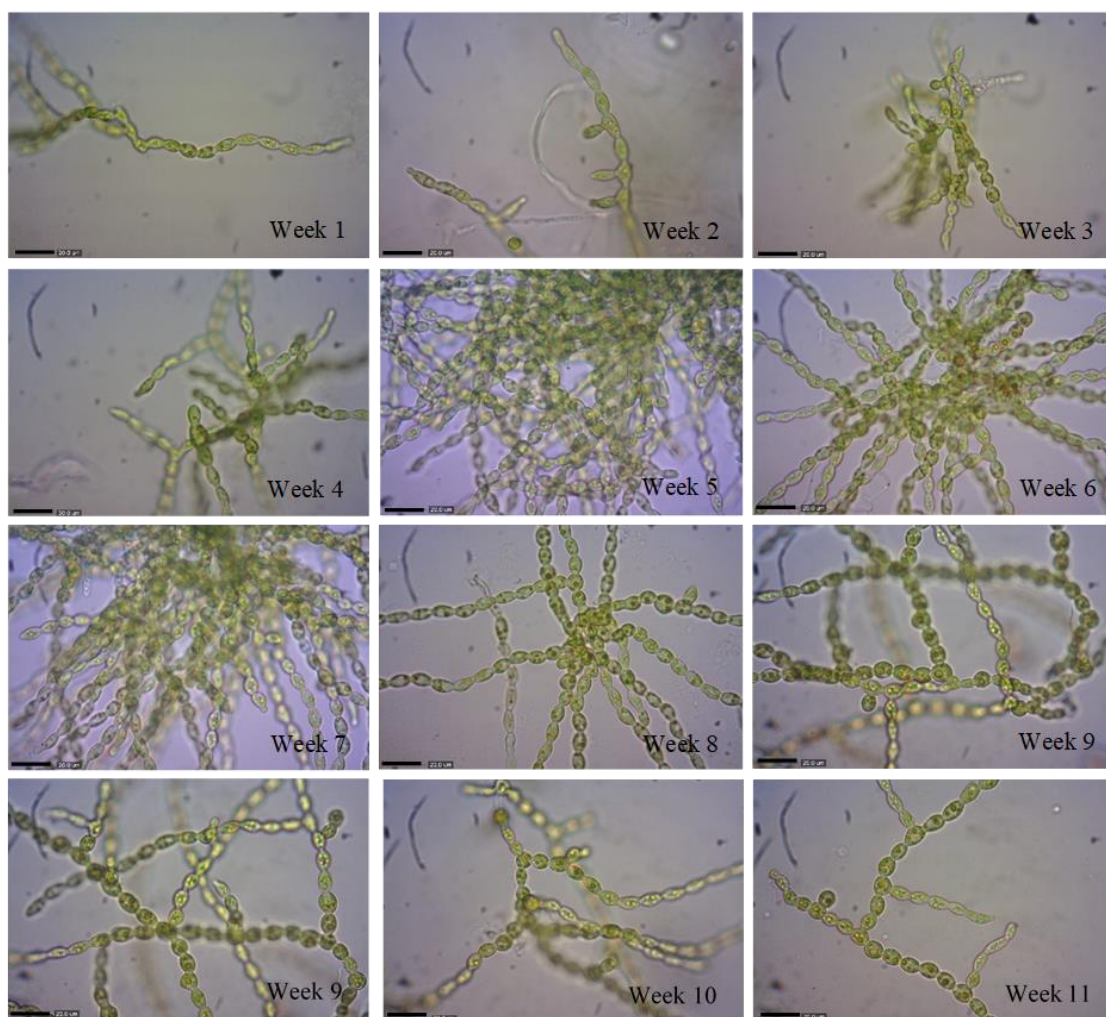


Figure 3. Characteristic cells of *T. monilia* in liquid ESM from week 1 to week 11 (scale bars=20 μm)

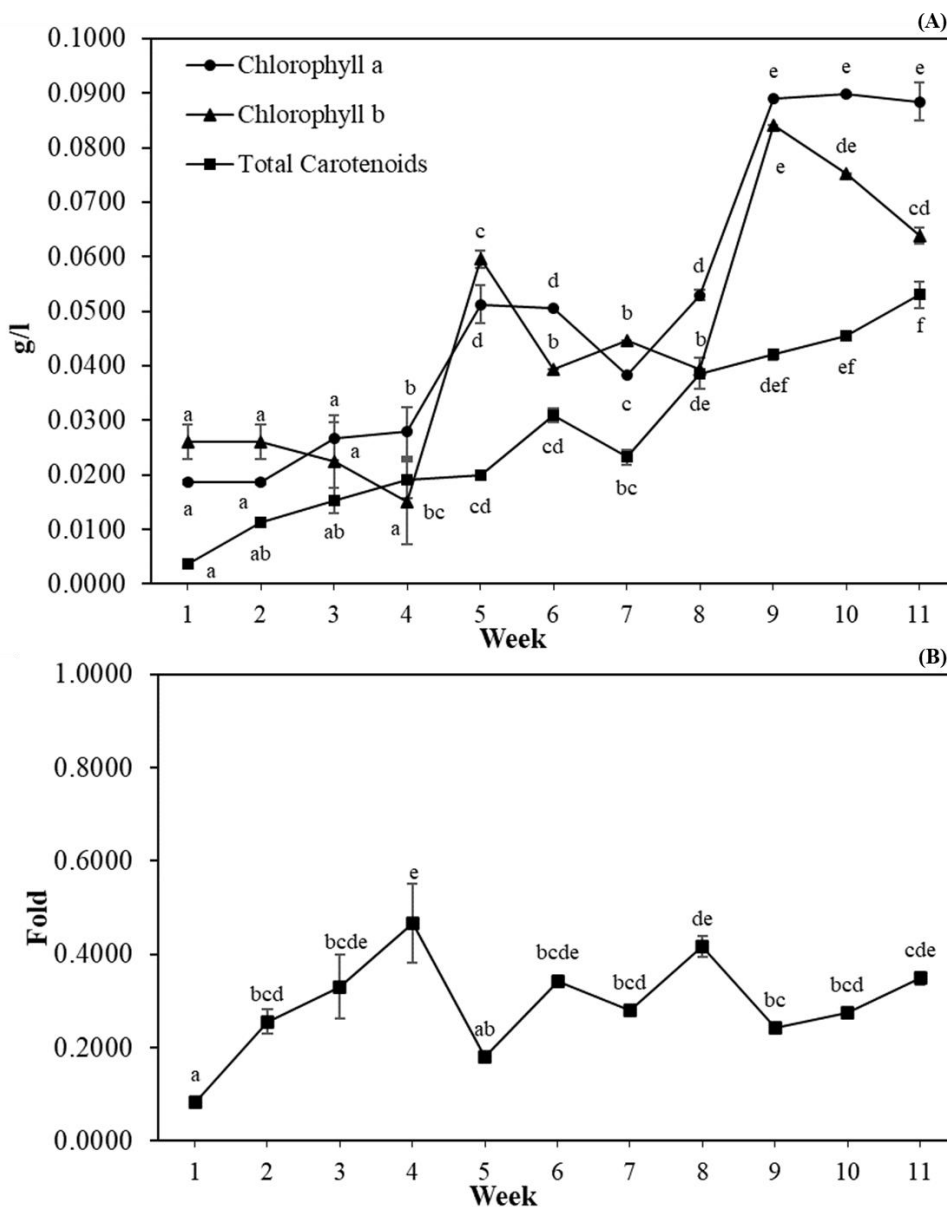


Figure 4. The growth of *T. monilia* in liquid ESM as measured by photosynthetic pigment methods. (A) Pigment accumulation and (B) total carotenoids/chlorophyll content ratios. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT

All of the measurements of the total carotenoids/total chlorophyll ratios for all of the culture factors are shown in Fig. 5 (B, D), Fig. 6 (B, D) and Fig. 7 (B). The results were clear because the ratios changed in all of the weeks from week 1 to week 6.

In the condition of pH7, added peptone 1%, culture strength 50%, nitrogen source -0.50 times and vitamin B₁₂ source -0.50 times which nitrogen source -0.50 times from general concentration in enriched seawater medium (ESM) were the optimum of *T. monilia* growth to produce the increased of total carotenoids content more than total chlorophyll content accumulation in algae.

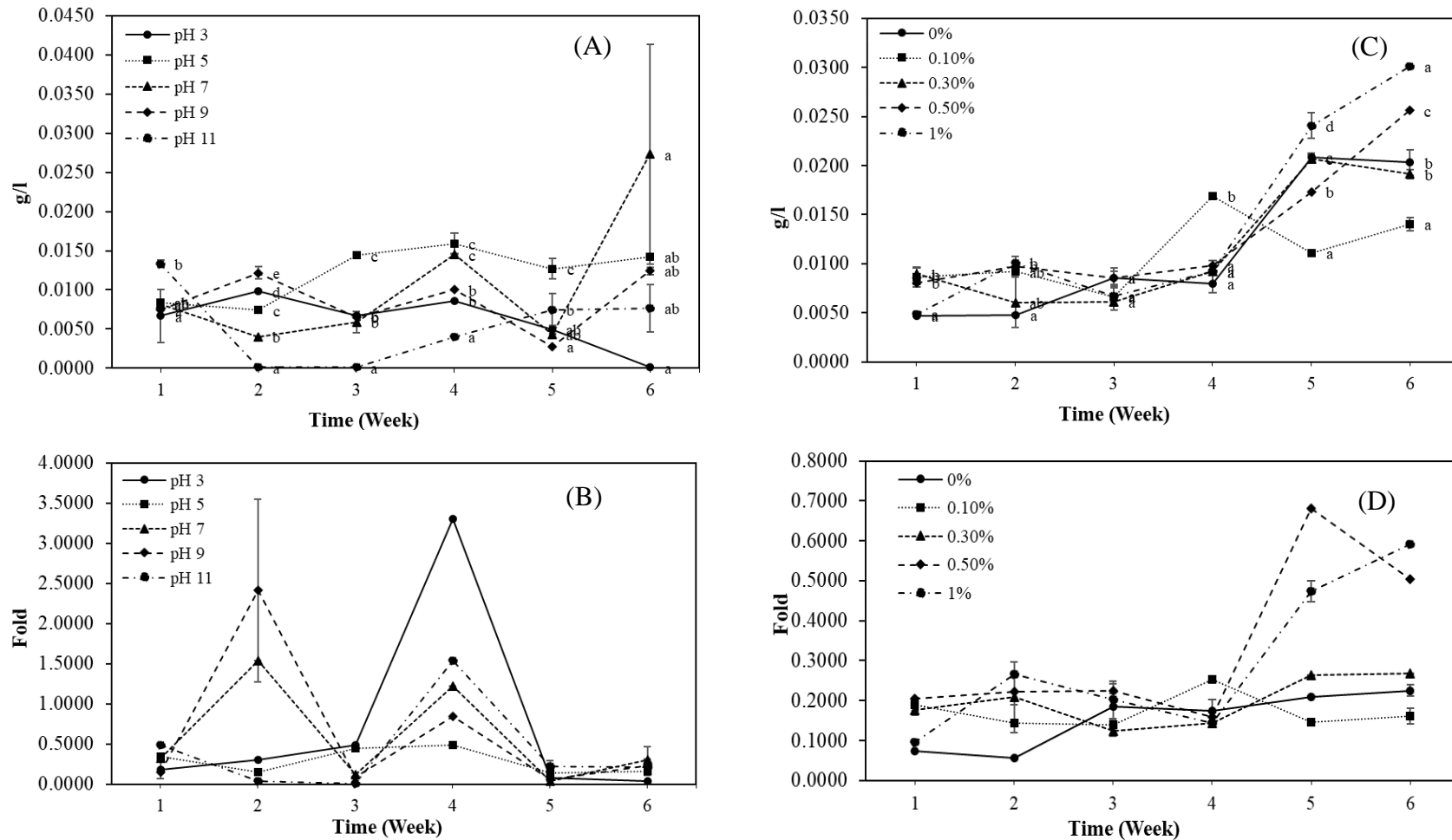


Figure 5. Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of *T. monilia* in liquid ESM with various culture factors: (A and B) pH, and (C and D) peptone. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT

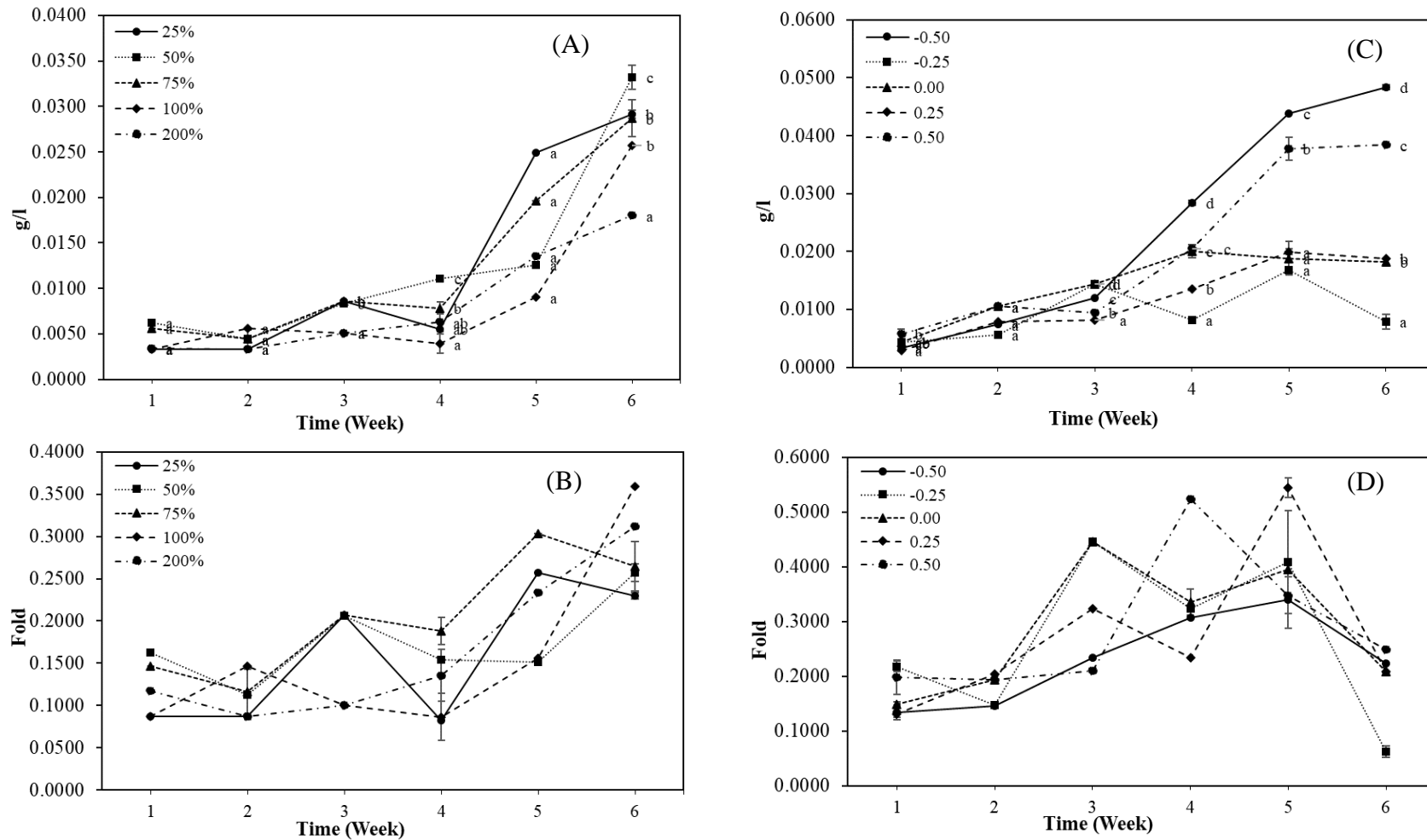


Figure 6. Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of *T. monilia* in liquid ESM with various culture factors: (A and B) culture strength, and (C and D) nitrogen source. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT

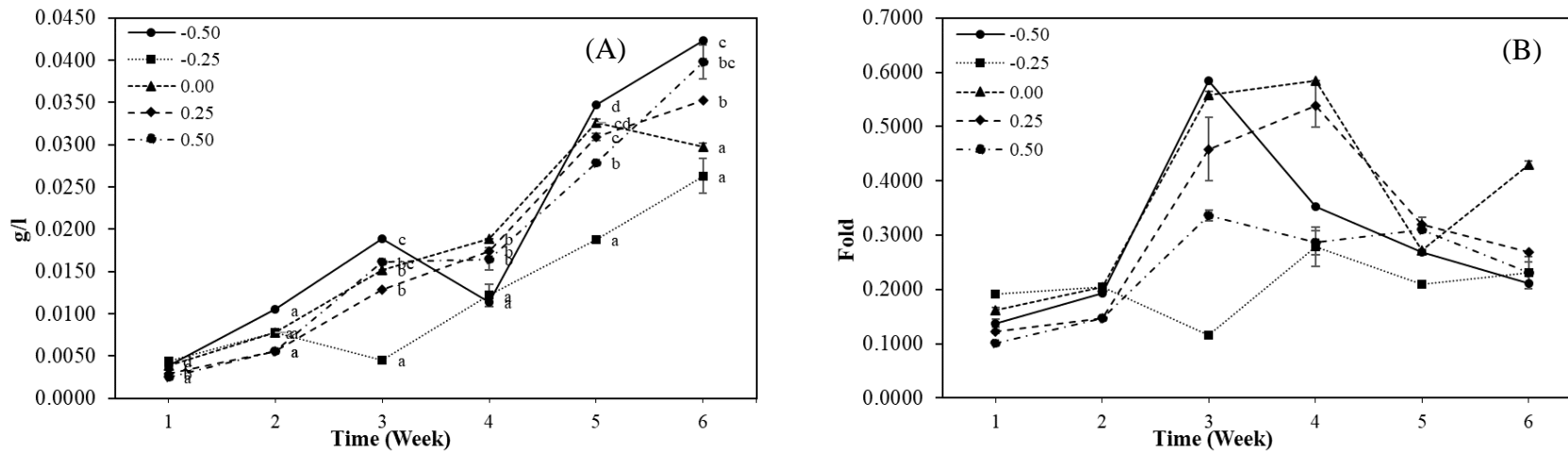


Figure 7. Evolution over time of the total carotenoid content and total carotenoid/total chlorophyll content of *T. monilia* in liquid ESM with various culture factors: (A and B) vitamin B₁₂ source. Data are mean values of three replications and SE are indicated by the bar. The different letters indicate significant differences ($p \leq 0.05$) at each time by DMRT

Discussion

Trentepohlia spp. grow mainly in tropical and subtropical zones of the world. They grow on tree barks, soils and rocks and also on manmade structures exposed to full sunlight (John, 2002; López-Bautista et al., 2002; Kharkongor and Ramanujam, 2017; Rindi et al., 2018). These algae are thought to have a high tolerance for and adaptability to extreme conditions such as desiccation and high temperatures (Rindi, 2007; Rindi et al., 2009; Bartoli et al., 2019; Binoy et al., 2019). As a source of useful substances, *T. monilia* has the negative characteristics of a longer lag phase and a lower growth rate than other microalgae grown in liquid cultures. This study showed that *T. monilia* grew the most and produced the most total carotenoid content only in ESM. According to Abe et al. (1998), who studied *Trentepohlia aurea*, the best growth also occurred in liquid ESM with 3,000 lux. The ESM has nutrients and properties of soil extraction similar to those of the natural habitats of *Trentepohlia* spp.

In terms of various growth factors, a pH of 6.0 to 8.0 is optimal for the growth of *Trentepohlia* spp. (Abe et al., 1999; Lemes-da-Silva et al., 2017). The optimum pH was 7.0 in a liquid culture, based on colony size, the relative abundance of new colonies formed and the dimensions of apical cells. The algae preferred a slightly alkaline (pH 7.5) environment, although they could be grown in a wide range of pH conditions. The preference for an alkaline environment is not surprising. In nature, the species colonizes whitewashed building walls, as well as painted surfaces or manmade constructions, where the pH is distinctly alkaline (Lee et al., 1990; Lemes-da-Silva et al., 2017; Bartoli et al., 2019; Binoy et al., 2019). In the presence of peptone, the growth rate was even greater. Peptone supplementation activated nitrogen metabolism in the cells of *T. monilia*, resulting in an acceleration of the algal growth rate (Abe et al., 1998). An earlier study had shown that the growth rate and total chlorophyll content increased markedly with the addition of peptone as a nitrogen source. The culture strength, at nutrient strengths of 75%, 100% and 200%, resulted in a normal-appearing growth rate and the cells remained green. Transferring the algae from a diluted 25% or 50% culture medium changed the color to yellow or orange (Lee et al., 1990), which was optimal for producing total carotenoids. With a nitrogen source of -0.50 times, the chlorophyll a content was the lowest. The carotenoid content was higher than the chlorophyll a content with the lowest nitrogen source (Chen et al., 2016). Nitrogen deficiency had a significantly positive effect on carotenoid accumulation in *Trentepohlia arborum* and other species in the same genus (Tan et al., 1993; Abe et al., 1998); this might have depended on high expression levels of enzymes involved in β -carotene synthesis, resembling that for *Haematococcus pluvialis*, when grown under nitrogen deficiency (Recht et al., 2014). The effect of a vitamin B₁₂ source or thiamine was negligible. It has been reported that vitamins are necessary for the growth of various groups of algae, but the growth of *T. monilia* in their presence was slow and have high total carotenoid content accumulation (Lee et al., 1990).

In the present study, the growth and accumulation of total carotenoids in the subaerial green alga *T. monilia* in liquid ESM were shown. It was thus possible to demonstrate the simultaneous production of useful materials such as β -carotene by *T. monilia*. The algae could be used to provide a rich source of foods or supplementary foods, natural food colourants, animal feeds, cosmetics and medicines. *T. monilia* could also be utilized as a biofunctional material in the future. This report provides new information on the nature of different carotenoids biosynthesized by *T. monilia* collected from natural sources without culturing the alga in an artificial medium.

Conclusion

T. monilia grows the best in liquid ESM culture. The maximum growth occurred in week 9 of the study. The total carotenoids accumulated at the highest rate when the species was being grown in this culture. In ESM, the best liquid culture, the optimal conditions were a pH of 7.0, peptone of 1%, culture strength of 50%, nitrogen source -0.50 times and vitamin B₁₂ source -0.50 times the normal concentration. This culture produced the optimum growth of *T. monilia*, the highest amount of total carotenoids and situations in which the total carotenoids were higher than the total chlorophyll in a liquid culture. In further studies, we will focus on modifying more specialized ESM mediums to optimize for a short period to cultivate *T. monilia* less than three weeks, which should be promising for future carotenoid-producing industries.

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APPENDIX

Table A1. ANOVA analysis results of growth of *T. monilia* in liquid ESM medium which measured by chlorophyll-a, chlorophyll-b and total carotenoids content from week 1 to week 11

Source	df	Mean square	F	Sig.
Intercept	1	0.083	3612.965	.000
Chlorophyll a	10	0.002	100.561	.000
Error	22	0.00002302		
Total	33			
Intercept	1	0.067	1322.438	.000
Chlorophyll b	10	0.002	30.756	.000
Error	22	0.00005071		
Total	33			
Intercept	1	0.027	499.133	.000
Total Carotenoids	10	0.001	13.330	.000
Error	22	0.00005392		
Total	33			

Table A2. ANOVA analysis results of total carotenoids content in *T. monilia* on ESM medium with a different pH on week 1 to week 6

Source	df	Mean square	F	Sig.
Intercept	1	0.001	126.811	.000
pH (Week 1)	4	0.00002067	2.253	.136
Error	10	0.000009174		
Total	15			
Intercept	1	0.001	1285.735	.000
pH (Week 2)	4	0.00006813	130.342	.000
Error	10	0.0000005227		
Total	15			
Intercept	1	0.001	464.237	.000
pH (Week 3)	4	0.00007769	53.456	.000
Error	10	0.000001453		
Total	15			
Intercept	1	0.002	1248.444	.000
pH (Week 4)	4	0.00006841	50.678	.000
Error	10	0.000001350		
Total	15			
Intercept	1	0.001	129.948	.000
pH (Week 5)	4	0.00004526	9.453	.002
Error	10	0.000004787		
Total	15			
Intercept	1	0.002	13.860	.004
pH (Week 6)	4	0.000	1.818	.202
Error	10	0.000		
Total	15			

Table A3. ANOVA analysis results of total carotenoids content in *T. monilia* on ESM medium with a different peptone (%) on week 1 to week 6

Source	df	Mean square	F	Sig.
Intercept	1	0.001	576.697	.000
peptone (Week 1)	4	0.00001314	10.214	.001
Error	10	0.000001287		
Total	15			
Intercept	1	0.001	156.244	.000
peptone (Week 2)	4	0.00001722	2.821	.084
Error	10	0.000006103		
Total	15			
Intercept	1	0.001	326.392	.000
peptone (Week 3)	4	0.000004094	1.666	.233
Error	10	0.0000024658		
Total	15			
Intercept	1	0.002	1901.323	.000
peptone (Week 4)	4	0.00003839	43.197	.000
Error	10	0.0000008887		
Total	15			
Intercept	1	0.005	3062.149	.000
peptone (Week 5)	4	0.00007277	42.029	.000
Error	10	0.000001731		
Total	15			
Intercept	1	0.007	3751.864	.000
peptone (Week 6)	4	0.000	60.454	.000
Error	10	0.000001905		
Total	15			

Table A4. ANOVA analysis results of total carotenoids content in *T. monilia* on ESM medium with a different culture strength (%) on week 1 to week 6

Source	df	Mean square	F	Sig.
Intercept	1	0.000	.	.
Culture strength (Week 1)	4	0.000006069	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.000	163.030	.000
Culture strength (Week 2)	4	0.000002766	1.693	.227
Error	10	0.000001633		
Total	15			
Intercept	1	0.001	17956.000	.000
Culture strength (Week 3)	4	0.00001051	246.273	.000
Error	10	0.00000004267		
Total	15			
Intercept	1	0.001	344.008	.000
Culture strength (Week 4)	4	0.00002219	10.650	.001
Error	10	0.000002084		
Total	15			
Intercept	1	0.004	.	.
Culture strength (Week 5)	4	0.000	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.011	2274.012	.000
Culture strength (Week 6)	4	0.00009633	20.122	.000
Error	10	0.000004787		
Total	15			

Table A5. ANOVA analysis results of total carotenoids content in *T. monilia* on ESM medium with a different nitrogen source (times) on week 1 to week 6

Source	df	Mean square	F	Sig.
Intercept	1	0.000	339.381	0.000
Nitrogen source (Week 1)	4	0.000003656	4.857	0.019
Error	10	0.0000007527		
Total	15			
Intercept	1	0.001	.	.
Nitrogen source (Week 2)	4	0.00001351	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.002	37765.444	.000
Nitrogen source (Week 3)	4	0.00002411	446.556	.000
Error	10	0.00000005400		
Total	15			
Intercept	1	0.005	3975.497	.000
Nitrogen source (Week 4)	4	0.000	141.990	.000
Error	10	0.000001241		
Total	15			
Intercept	1	0.011	1111.761	0.000
Nitrogen source (Week 5)	4	0.000	45.605	0.000
Error	10	0.00001015		
Total	15			
Intercept	1	0.010	6013.979	.000
Nitrogen source (Week 6)	4	0.001	474.511	.000
Error	10	0.000001731		
Total	15			

Table A6. ANOVA analysis results of total carotenoids content in *T. monilia* on ESM medium with a different vitamin B₁₂ source (times) on week 1 to week 6

Source	df	Mean square	F	Sig.
Intercept	1	0.000	3555.696	0.000
vitamin B ₁₂ source (Week 1)	4	0.000001882	35.728	0.000
Error	10	0.00000005267		
Total	15			
Intercept	1	0.001	.	.
vitamin B ₁₂ source (Week 2)	4	0.00001229	.	.
Error	10	0.000		
Total	15			
Intercept	1	0.003	838.516	.000
vitamin B ₁₂ source (Week 3)	4	0.00009016	27.599	.000
Error	10	0.000003267		
Total	15			
Intercept	1	0.004	864.735	.000
vitamin B ₁₂ source (Week 4)	4	0.00003272	8.079	.004
Error	10	0.000004050		
Total	15			
Intercept	1	0.013	8075.653	0.000
vitamin B ₁₂ source (Week 5)	4	0.000	74.217	0.000
Error	10	0.000001561		
Total	15			
Intercept	1	0.018	2626.223	.000
vitamin B ₁₂ source (Week 6)	4	0.000	19.499	.000
Error	10	0.000006875		
Total	15			