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Original Article

Effects of culture conditions on the growth and reproduction of Gut Weed, *Ulva intestinalis* Linnaeus (Ulvales, Chlorophyta)

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Abstract

In vitro cultivation of Gut Weed, *Ulva intestinalis* Linnaeus, was experimentally studied to support its near optimal farming, with potential impact in Thailand on its direct use as human food or its co-cultures in shrimp farming.

Germling clusters (2 weeks old and 7.50±2.98 mm long) were seeded into 500 mL flasks and biomass growth rate optimized with respect to the main controllable factors; seedling density, salinity, light intensity, and temperature. These factors were assumed to each have an optimal value independent of the others, and the factors were optimized one at a time. The maximum growth at three to four weeks of cultivation was obtained at the factor levels of 0.05 gL^{-1}, 20 ppt, 80 µmol photon $m^{-2}s^{-1}$ and 25°C. Early zoosporangia were obtained from 2nd to 4th weeks. The relative growth rate ranged from 9.47 to 22.18 % day^{-1}, and only asexual reproduction of *U. intestinalis* was observed under these culture conditions.

Keywords: Gut Weed, *Ulva intestinalis*, Ulvales, Ulvaceae, *Enteromorpha intestinalis*

1. Introduction

Ulva (Enteromorpha) intestinalis Linnaeus is a green alga, known also as Gut Weed and Grass Kelp, in the division Chlorophyta, order Ulvales and family Ulvaceae, and occurs naturally worldwide. In general, this alga grows as a tube of 1-2 mm length (though it can reach up to 2 cm), composed of irregularly arranged cells as a single layer. The alga blade is smooth at the early stages, while later it is wrinkled and also changes its colour from dark green to light green or yellow-green. Branching occurs near the holdfast, which is small and narrow (1 mm) (Prud'homme van Reine and Trono, 2001; Ruangchuay *et al.*, 2007).

The life cycle of *Ulva* consists of two phases: haploid and diploid. The haploid plants produce biflagellated gametes, which may or may not fuse, whereas the sporophyte plant produces a quadriflagellated zoospore that develops into the gametophyte. Although cultivation of the genus using parthenogenic thalli has been reported (Ohno, 1993), a new method using germling clusters has been presented by Hiraoka and Oka (2008) for the production of a free-floating form suited for tank cultivation.

Ulva spp. have been cultivated in many Asian countries, including Japan, Korea, India and Indonesia (Prud'homme van Reine and Trono, 2001). In Japan, *Ulva (Enteromorpha) prolifera* is used for the production of nutritionally valuable food due to its high content of minerals and

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vitamins (Aguilera-Morales *et al.*, 2005). Furthermore, *Ulva pertusa* is used for commercial consumption in Japan, and *Ulva compressa* is used as a snack ingredient in India (Mamatha *et al.*, 2007). In addition to its use as a food source, also extracts of *Ulva* spp. are used in the cosmetics and pharmaceutical industries due to their antibiotic, antibacterial, antifungal, and antitumorigenic properties. The nutritional content of *U. intestinalis* has been reported as 19.5% protein, 0.3% fat, 58.1% soluble carbohydrates, 6.8% insoluble carbohydrates, and 15.2% ash including essential minerals. The minerals include calcium, phosphorus, iron, sodium, and potassium, at 910, 800, 35, 570, and 3500 mg Kg^{-1} dry weight, respectively (Aguilera-Morales *et al.*, 2005).

In Thailand, *U. intestinalis* is called Sarai Sai Kai. The alga has been used as a feed and a bio-filter in aquaculture, especially in earthen-pond co-cultures with giant tiger prawns (*Penaeus monodon*). However, these cultivation systems experience fluctuations in production due to the variable environmental conditions of the shrimp ponds. In attempts to implement a stably sustainable system, it is necessary to determine the optimal culture conditions of the algae and possibly maintain a controllable reservoir to supplement them into the pond. This providing the motivation, we employed laboratory conditions to observe the growth and reproduction of this species and we report at least near optimal values of main control variables for maximal biomass growth.

2. Materials and Methods

2.1 Seedling preparation

Parental plants of *U. intestinalis* were collected from a shrimp pond from Sarai Tiphaya Farm, Amphoe Yaring, Pattani Province, and the algal samples were cleared of epiphytes and other contamination. The samples were then washed several times with seawater of the same salinity level as the collection. The samples were additionally inspected with microscopy for contamination. The immature explants were cut and transferred for culture in 500-mL flasks, filled with seawater enriched with Modified von Stosch (Grund) Medium (MGM) (Andersen *et al.,* 2005) at 25 ppt salinity. The flasks were subjected to a light intensity of 50 μmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period until reproductive cells were produced and released. The clusters of zoospores were obtained following Hiraoka and Oka (2008) and were cultured continuously until the germlings were approximately 5-10 mm in length. The clusters were randomly sampled and weighed, and the number of seedlings was counted; they were then used as seedlings for the ensuing experiments to optimize the levels of seedling density, salinity, temperature and light intensity for maximal growth and reproduction. Each variable was examined in succession (in the order listed above) assuming its optimal value is independent of the other controllable factors.

2.2 Effect of seedling density on the growth and reproduction of *U. intestinalis*

The seedling density of germling clusters had 4 levels: 0.05, 0.10, 0.15, or 0.20 g wet wt L^{-1}, which approximately corresponds to 1000, 2000, 3000, or 4000 filament L^{-1} of germling at current 7.50±2.98 mm in length. Two replicates were run for each density. The growth environment was a 500-mL flask with air bubbled in through the bottom. MGM was used to enrich 25 ppt salinity water and the flasks were placed under a light intensity of 50 μmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period. Under light microscopy, 30 thalli of maximal length were checked to observe maturation. The total weight of the fresh thalli was determined every week, and the medium was renewed after the observation.

2.3 Effect of salinity on the growth and reproduction of *U. intestinalis*

The clusters of *U. intestinalis* were separated for culture under different salinity levels, at 10, 20, 30, or 40 ppt, using the empirically determined optimal seedling density from the previous experiment (0.05 g L^{-1}). Cultures were grown in 500-mL flasks containing MGM, at 25°C under 50 μmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period. Thirty thalli of maximal length were measured every week and were used to observe maturation under light microscopy. The total weight of the fresh thalli was determined every week, and the medium was renewed after the observation.

2.4 Effect of temperature on the growth and reproduction of *U. intestinalis*

The germling clusters were then cultured in 500-mL flasks at different temperatures, 20, 25, or 30°C, in incubators using the optimal seedling density 0.05 gL^{-1} and optimal salinity level 20 ppt, enriched with MGM medium. The cultures were incubated under 50 μmol photon $m^{-2}s^{-1}$ with a 12:12-hr light:dark period. The sampling for growth and reproductive parameters was as in previous stages.

2.5 Effect of light intensity on the growth and reproduction of *U. intestinalis*

To estimate the optimal light intensity, reproductive clusters were cultured at the light intensity levels, 40, 80, or 120 μmol photon $m^{-2}s^{-1}$. The empirically optimized seedling density (0.05 g L^{-1}), seawater salinity (20ppt), and temperature (25°C) were used with MGM enriched medium in 500-mL flasks. The cultures were grown under a 12:12-hr light:dark period. The sampling for growth and reproductive parameters was similar to the previous.

2.6 Wet and dry biomass of *U. intestinalis*

Each week, samples of *U. intestinalis* were weighted and returned to their appropriate experimental conditions. For each treatment, the culture medium was renewed by transfer to a new flask. After the final wet weights were determined, the algae were rinsed with distilled water to remove external salt and were dried to a constant weight at 60ºC. The algal dry weights were recorded, and the wet weight: dry weight ratios were calculated.

2.7 Data analysis

The data are reported as the mean±SD. The data on thallus length were analysed by one-way ANOVA to compare between the treatments. Tukey's test was performed at an $\alpha = 0.05$ significance level. The weekly growth rate was calculated as the relative growth rate (RGR) using the formula of Lobban and Harrison (1994) as follows:

Relative growth rate (% day^{-1}) = 100 (ln Wt- ln Wo) t^{-1} (in days), where Wt = the final weight (g), Wo = the initial weight, and t = the time interval (days).

3. Results

3.1 Characteristics of seedlings

Parental thalli of *U. intestinalis* were 8-53 cm long (Figure 1 a). After induction by stress of the explants (by keeping them dry and dark for 12 hrs), the reproductive cells were released within 8-15 days (Figure 2 b). The spores then aggregated into clusters. The germling clusters were cultured for a one week until they developed into a one-row filament of 6-18 cells (Figure 1 c). After another week, the seedlings (7.50±2.98 mm in length and 25±6 plants per cluster) were used for the experiments described below (Figure 1 d).

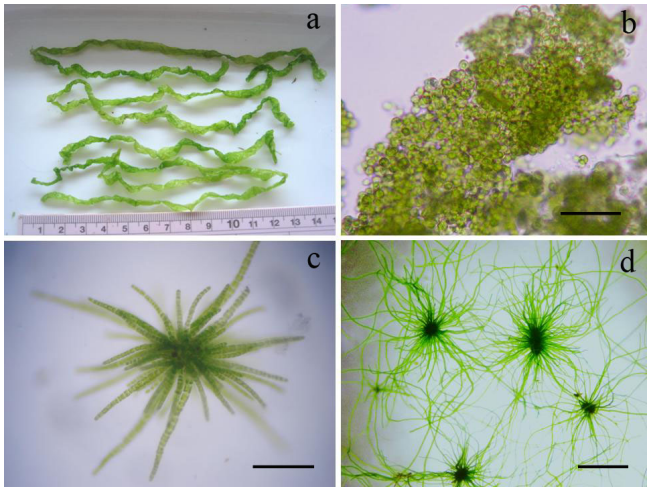

Figure 1. Characteristics of thalli and germling clusters of *Ulva intestinalis*; a. Thalli from natural conditions b. Aggregated spores c. A germling cluster at one week of age d. Germling clusters at two weeks of age. Scale bar, b. 100 µm c. 200 µm d. 2 cm

3.2 Effect of seedling density on the growth and reproduction of *U. intestinalis*

A seedling density of 0.05 g L^{-1} or 1000 filament L^{-1} provided the maximum growth each week, and the best maximum length in the 4th week was 112.87±10.21 mm, whereas the growth at 0.10 g L^{-1} or 2000 filament L^{-1} showed the second maximum length of 93.60±12.48 mm at the 4th week (Figure 2). Zoosporangia were found in some of the thalli cultured at 0.05 gL^{-1} at 4th week. The cultivation showed a significant effect ($p<0.05$) on the thallus length among the experimental densities. The mean wet weights at 4 weeks from the seedling densities 0.05, 0.10, 0.15, and 0.20gL^{-1}, were 12.44±1.78, 11.48±1.64, 8.15±0.83, and 7.58±1.21 gL^{-1} (Figure 3). The mean RGRs over 4 weeks were 22.18, 19.42, 16.74, and 15.46 % day^{-1}, in respective order.

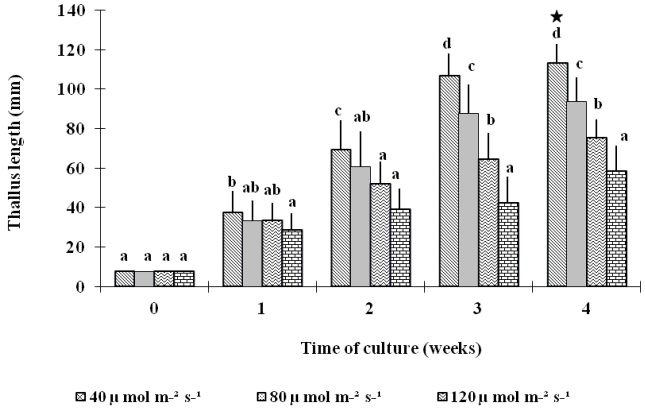

Figure 2. Thallus length (mm) of *Ulva intestinalis* cultured under different seedling densities at 20 ppt salinity, 25°C, 60 µmol photon $m^{-2}s^{-1}$ and a light:dark period for 4 weeks. Stars indicate zoosporangia formation. Different letters indicate significant differences based on Tukey's multiple comparison ($p<0.01$), Error bars = SD

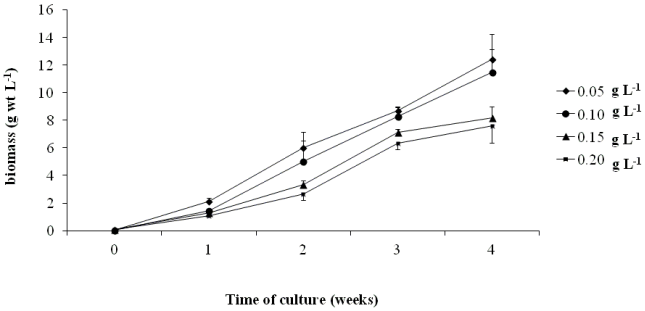

Figure 3. Biomass (g wet wt d^{-1}) of *Ulva intestinalis* cultured under different seedling densities at 20 ppt salinity, 25°C, 60 µmol photon $m^{-2}s^{-1}$ and a light:dark period for 4 weeks. Error bars=SD

The seedling density then is an important factor to keep near its optimum, as the 4 week output can be reduced by about a half by too high seedling density.

3.3 Effect of salinity on the growth and reproduction of *U. intestinalis*

Germling clusters of *U. intestinalis* grew well in all salinity levels tested. The germlings in 20 ppt showed the best maximal length of 174.26±36.37 mm at the 4th week, whereas the germlings at 30 ppt had the second greatest length of 160.93±43.98 mm at the 4th week (Figure 4). The tip of the thalli cultured in 30 and 40 ppt produced reproductive cells, and the earliest reproductive release was found in the filaments cultured at 40 ppt in the 2nd week. The next earliest release of reproductive cells was found at the 3rd week in the filaments cultured at 30 ppt. After 4 weeks, the thalli cultured at 10, 20, 30, and 40 ppt became wider, growing to 0.25-0.35, 0.30-0.52, 0.40-0.80, and 0.70-1.10 mm in width, respectively (Figure 6). The mean wet weights at 4 weeks in the salinities 10, 20, 30, and 40 ppt were 9.98±0.96, 12.08±0.17, 11.35±0.35, and 8.42±1.16 gL^{-1} (Figure 5), respectively. The mean RGRs were 16.82, 17 .50, 17.28, and 16.21 % day^{-1}, in the same order.

3.4 Effect of temperature on the growth and reproduction of *U. intestinalis*

The germlings cultured a t 25°C showed the most length growth, at 197.76±47.3 mm at the 4th week, in which also the first maturation was observed (Figure 7). The statisctical tests on the thallus length mostly showed significant effects ($p<0.05$) at 20, 25, and 30°C between 2nd-4th week. Thallus length at 25°C decreased from 5th to 8th week, while maturation was found through those weeks. The mean wet weights obtained at the temperatures 20, 25, and 30°C in 4

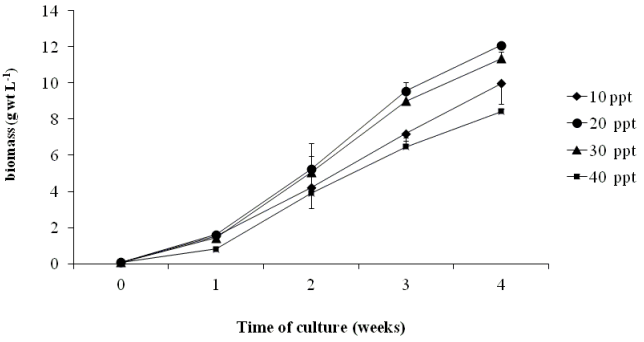

Figure 5. Biomass (g wet wt d^{-1}) of *Ulva intestinalis* cultured under different salinity levels within initial density of 0.05 g wet wt d^{-1} at 25°C, 60 µmol photon m^{-2} s^{-1} and a 12:12-hr light:dark period for 4 weeks. Error bars = SD

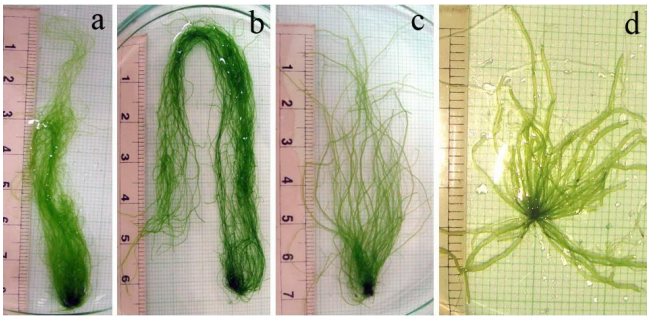

Figure 6. Characteristics of *Ulva intestinalis* thalli cultured under different salinities with a density of 0.05 g wet wtd^{-1} at 25°C, 60 µmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period at 4th week. a. 10 ppt; b. 20 ppt; c. 30 ppt; d. 40 ppt

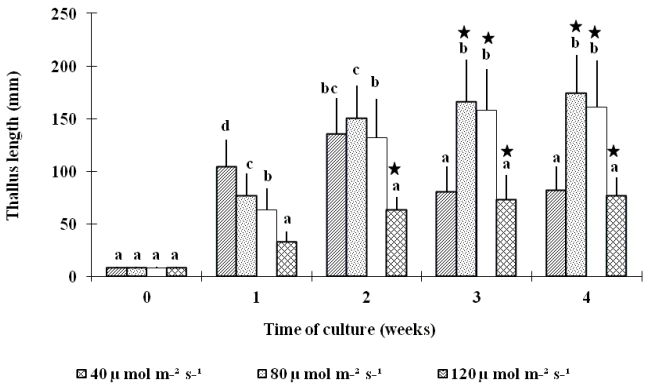

Figure 4. Thallus length (mm) of *Ulva intestinalis* cultured under different salinity levels within initial density of 0.05 g wet wt d^{-1} at 25°C, 60 µmol photon m^{-2} s^{-1} and a 12:12-hr light:dark period for 4 weeks. Stars indicate zoosporangia formation. Different letters indicate significant differences based on Tukey's multiple comparison ($p<0.01$), Error bars = SD

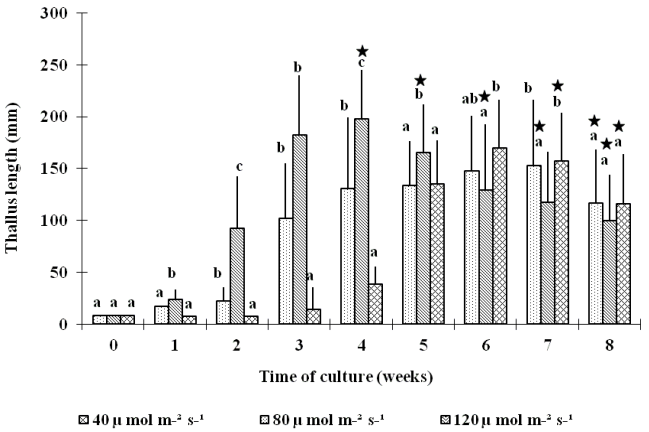

Figure 7. Thallus length (mm) of *Ulva intestinalis* cultured under different temperatures with an initial density of 0.05 g wet wtd^{-1} in 20 ppt salinity, 60 µmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period for 8 weeks. Stars indicate zoosporangia formation. Different letters indicate significant differences based on Tukey's multiple comparison ($p<0.01$), Error bars = SD

weeks were 10.08±2.43, 13.19±0.21, and 7.97±0.95 and the mean wet weights in 8 weeks were 9.49±1.23, 10.35±0.72, and 8.90±1.27 gL^{-1} (Figure 8), showing that at 4 weeks the biomass density mostly reached its saturation. The mean RGRs at 4 weeks were 18.95, 19.91, 18.11% day^{-1} while at 8 weeks they were 10.84, 10.98, and 10.60% day^{-1}, in the same order of temperatures.

3.5 Effect of light intensity on the growth and reproduction of *U. intestinalis*

The maximum length of the germlings cultured under different light intensities was found for 80 µmol photon $m^{-2}s^{-1}$, being 132.4±65.07 mm in the 3rd week, and the next longest group was obtained under 120 µmol photon $m^{-2}s^{-1}$, at 130.3±45.96 mm in 5th week (Figure 9). The characteristics of the thalli were not much affected and the effects on thallus length were mostly not statistically significant. The earliest zoosporangia occurred in the thalli cultured under 80 µmol photon $m^{-2}s^{-1}$ at the 4th week, and the next in the thalli cultured under 120 µmol photon $m^{-2}s^{-1}$ at the 6th week. The mean wet weights by week 4 were 9.72±1.13, 11.44±1.70, 9.85±1.57, and by week 8 they were 10.05±0.98, 9.13±0.69, 9.06±0.57 g wet wt L^{-1} (Figure 10), confirming the earlier observation of growth saturation. The mean RGRs through 4 weeks were 18.82, 19.40, 18.87 % day^{-1} and those through 8 weeks were 9.47, 11.02, and 10.83 % day^{-1}.

3.6 Wet and dry biomass of *U. intestinalis*

Dry weight: wet weight ratios from the experiment of seed density, salinity, temperature and light intensity were 1:7.58±0.22, 1:8.08±1.11, 1:8.09±0.59 and 1:8.29±0.52, respectively. The mean dry weight: wet weight ratio was 1: 8.01± 0.26.

3.7 Reproductive phenomena of *U. intestinalis*

Only asexual reproduction of the zoosporangium of *U. intestinalis* was observed. The zoosporangia formed at the tip of the thallus in an area of 1-3 mm (Figure 11 a) and were 8-18 mm in diameter, and there were 13-20 zoospores per cell (Figure 11 b). The zoospore release occurred approximately one week after the formation. The cells of the tips were

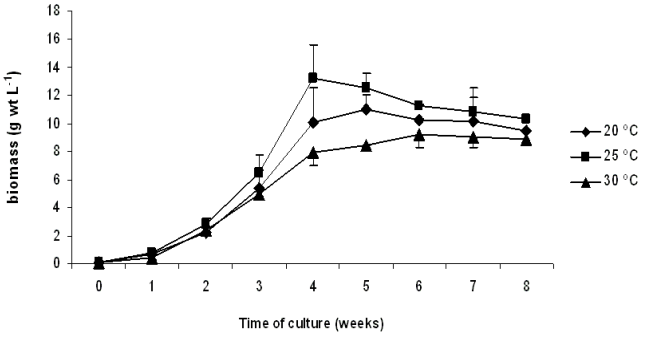

Figure 8. Biomass (g wet wt d^{-1}) of *Ulva intestinalis* cultured under different temperatures with an initial density of 0.05 g wet wtd^{-1} in 20 ppt salinity, 60 µmol photon $m^{-2}s^{-1}$ and a 12:12-hr light:dark period for 8 weeks. Error bars = SD

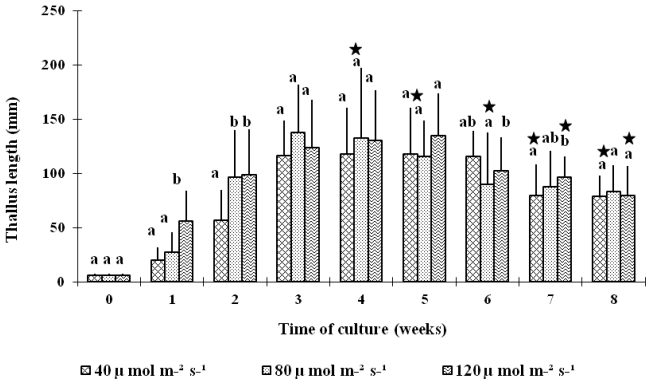

Figure 9. Thallus length (mm) of *Ulva intestinalis* cultured under different light intensities with an initial density of 0.05 g wet wtd^{-1} under 20 ppt salinity, 25°C and a 12:12-hr light:dark period for 8 weeks. Stars indicate zoosporangia formation. Different letters indicate significant differences based on Tukey's multiple comparison ($p<0.01$), Error bars = SD

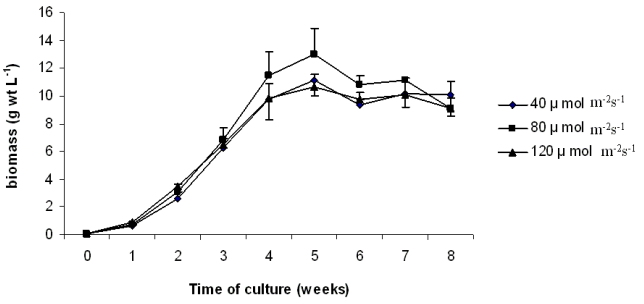

Figure 10. Biomass (g wet wt d^{-1}) of *Ulva intestinalis* cultured under different light intensities with an initial density of 0.05 g wet wtd^{-1} under 20 ppt salinity, 25°C and a 12:12-hr light:dark period for 8 weeks. Error bars = SD

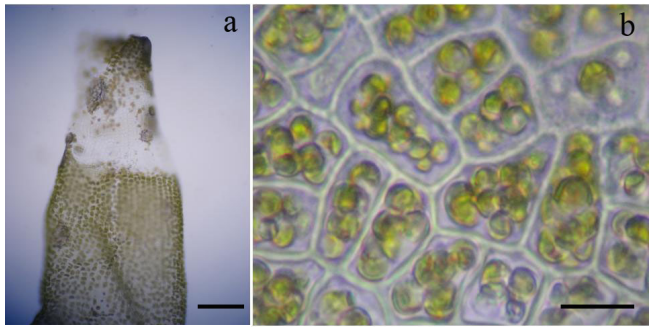

Figure 11. Zoosporangial formation in *Ulva intestinalis*; a. the tip of a thallus b. surface view. Scale bar, a. 1 mm b. 10 µm

empty then and retained only the cell walls; these decayed and caused the thalli to become shorter.

4. Discussion

The parental plants of *U. intestinalis* released reproductive cells within 8-15 days, which was observed as a green paste in the flasks. Such spore release behaviour is similar to that of *Ulva prolifera* in that after the release of its spores, they attach to each other and form clusters (Hiraoka and Oka, 2008). We found zoosporangia from the surface explants, which is in agreement with both Lin *et al.* (2008), who have reported that *U. prolifera* only reproduced asexually under culture conditions, and Millner *et al.* (1979), who have reported that stimulation of reproductive release in *U. intestinalis* can be achieved by cutting the thalli into small pieces.

Data on the seedling density for the cultivation of green algae have not been previously reported; our results indicate that the optimal density was at 0.05 g L^{-1} or 1000 filament L^{-1} of seedlings 7.50±2.98 mm in length, and within the range tested a low density is better than a high density. As the optimum was observed at the lower bound of the range tested, seedling densities under 0.05 g L^{-1} and the seed size for initial culture should be investigated further.

Prud'homme van Reine and Trono (2001) have reported that *U. intestinalis* is able to grow in a wide range of salinities. In the present study, the statistics showed a significant effect of the salinity level on the thallus length, with optimum salinity between 20 and 30 ppt. This result is similar to Martins *et al.* (1999), who have reported an optimal salinity range for Portuguese Gut Weed, *Enteromorpha intestinalis* (*U. intestinalis*), as between 15 and 20 psu (1 psu equals 1 ppt), which was lower than the optimal salinity range of Thai Gut Weed (*U. intestinalis*). In addition, this optimum salinity of Thai Gut Weed is similar to Korean Gut Weed *U. intestinalis*, which was reported to be 24 ppt (Kim and Lee 1996). However, early maturation of *U. intestinalis* in the present study occurred under high salinity (40 ppt) within two weeks, and maturation was found during the culture period until the thalli became shorter and wider.

Zhang *et al.* (2010) have reported that somatic cells of *Enteromorpha prolifera* could survive for 2 months under various temperatures, from 0 to 30°C, which was similar to Thai Gut Weed , *U. intestinalis*, grown under a wide temperature range of 20-30°C. Also Kim *et al.* (2011) and Mantri *et al.* (2011) mention that somatic cells of *Ulva* spp. grow under a wide range of temperatures and salinity. In the present study *U. intestinalis* produced zoosporangium early (4 weeks) at the optimal temperature of 25°C, while it takes a longer time (8 weeks) at the other temperature levels used.

The present study demonstrates that *U. intestinalis* is able to grow under a wide range of light intensities because our statistical tests mostly found no significant differences between the thallus lengths at 40, 80, or 120 μmol photon $m^{-2} s^{-1}$. In the present study, *U. intestinalis* was found to favour a light intensity of 80 μmol photon $m^{-2} s^{-1}$. However, we could obtain sporangia also at the high end of our temperature range when the light intensity was 120 μmol photon $m^{-2} s^{-1}$.

The relative growth rates of *U. intestinalis* were quite high and ranged between 9.47 to 22.18 % day^{-1} when compared with other algae in Class Ulvophyceae, Order Ulvales, such as *Gayralia* (approximately 6 % day^{-1} under natural and approximately 10 % day^{-1} *in vitro*, Pellizzari and Oliveira, 2007), *Ulva fenestrata* (11.2±1.16 %day^{-1} under laboratory conditions, Kalita and Tytlianov, 2003), *Ulva lactuca* (9-16 % day^{-1} under controlled cultivation, Ale *et al.*, 2011), and *Ulva fasciata* (16 % day^{-1} under controlled cultivation, Mantra *et al.*, 2011). This particular alga seems to have a high potential for cultivation. Furthermore, maturation of *U. intestinalis* in the present study was found only with zoosporangia and occurred continuously due to its sporulation cycle in high salinity (especially 40 ppt), which caused the thallus length to become shorter than in the other culture conditions. However, in early zoosporangium inducing conditions a lower biomass is produced – the optimal conditions to obtain zoospores may be different from the optimal conditions to maximize biomass. The complete asexual life cycle of *U. intestinalis* was in agreement with the description given by Pellizzari and Oliveira (2007).

Sporulation in *Ulva fenestrata* normally occurred every 10 days with both sporangia and gametangia (Kalita and Titlyanov, 2003), and the sporulation of *U. fenestrata* has been induced in just 5 days by temperature (Kalita and Titlyanov, 2011). Regarding our temperature experiment, the first zoosporangia were found at 25°C within 5 weeks, whereas the first zoosporangia found at 30°C was within 8 weeks. Therefore, a temperature increase in our experiments did not promote zoosporangia formation. Furthermore, maturation of *Enteromorpha prolifera* J. Ag. has been reported within 5 days, with mainly asexual reproduction (Fu *et al.*, 1993). Generally, the formation of gametes or spores attains a periodicity of 10, 15, or more days, and under unfavourable conditions, one or more reproduction cycles are omitted (Kalita and Titlyanov, 2011).

The average biomass range was between 7.58 to 13.19 g wet wt L^{-1} within 1 to 2 months depending on culture conditions. The release of reproductive cells from the tips of thalli caused some loss of biomass. Maximizing biomass production did not correlate with reproduction activity in specific conditions such as high salinity (40 ppt) or high temperature (30°C). The wet weight:dry weight ratio was 1:8.01 ±0.26, and the dry production of *U. intestinalis* was approximately 1000 g dry wt ton^{-1} in 2 months; thus, our study also demonstrated that the algae may potentially be used for farm cultivation.

5. Conclusions

U. intestinalis appears to achieve comparatively high biomass growth rates in *in vitro* culturing, relative to other algae – showing in that sense a high potential for such cul-

turing. Of the controlled variables used to optimize biomass production, the seedling density has the largest effect size with low densities providing highest outputs at about four weeks of culturing. Overseeding can reduce the output by about a half. The other factors studied had a lesser effect size on biomass production, and near optimal values were found and reported here for salinity, intensity of light, and temperature. The fully asexual reproduction phenomena observed were in agreement with prior studies.

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