



INORGANIC CARBON ACQUISITION BY *Gracilaria edulis* AND ITS EFFECT ON GROWTH

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Abstract

The utilization of inorganic carbon by the red macroalgae, *Gracilaria edulis* (*G. edulis*) was investigated in this study. *G. edulis* were grown in brackish water aerated with varying concentration of carbon dioxide - 350 ppm (actual atmospheric carbon dioxide, CO₂), 700 ppm and 1400 ppm CO₂ gas. Higher growth rate was observed in culture medium aerated with 1400 ppm CO₂ and the lowest growth rate was observed at actual atmospheric carbon concentration. Besides that, significant pH drift was observed in the culture medium with and without enriched carbon dioxide. In the control flask, the pH was higher with a mean of 8.65 ± 0.14, and it greatly differed with carbon dioxide enriched air that exhibited lower pH of a 0.8 unit difference. Photosynthetic rate increased with an increment of inorganic carbon concentration and the highest oxygen evolution were 45 μmol g⁻¹FW h⁻¹ and 35 μmol g⁻¹FW h⁻¹ at 1400 ppm and 350 ppm, respectively. Therefore, elevated concentration of CO₂ significantly decreased the pH of the medium, enhanced growth of macroalgae and evolved more O₂.

Keywords: carbon dioxide, *Gracilaria edulis*, growth, pH, photosynthesis

Received: November, 2013; Revised final: May, 2015; Accepted: May, 2015; Published in final edited form: February, 2019

1. Introduction

Carbon dioxide (CO₂) is one of the most prevalent greenhouse gases in the atmosphere and anthropogenic emissions are primarily caused by rapid industrial revolution, combustion of fossil fuels and deforestation. Global warming has gained public attention due to a rise of temperature and air pollution (Andersen, 2005). CO₂ emission has increased by 35% since 1990 (Kaladharan et al., 2009), with these at levels classified as "dangerously high". According to Kyoto Protocol, the present amount of carbon dioxide emission has exceeded the stipulated standard (Bastianoni et al., 2008). Therefore, to meet the proposed Kyoto level, countries around the globe have ratified and implemented the use of renewable energy to alleviate CO₂ emission.

With respect to the increasing atmospheric CO₂ concentration, it is anticipated a proportional increase

in the amount of dissolved CO₂ at coastal surface, due to rapid gaseous exchange rate between air and seawater medium (Israel and Hophy, 2002; Zou, 2005). Macroalgae are a major component of coastal primary productivity with a natural mechanism in reducing pollution loads from water and can be used for controlling CO₂ emissions from the atmosphere. Therefore, macroalgae plays a key role in the coastal carbon cycle with a significant reduction of CO₂ in coastal areas which also function as bioindicators (Zou, 2014; Zou and Gao, 2009a). The presence of macroalgae are prevalent and abundant in the coastline and classified into three distinct group according to pigmentation such as red, green and brown macroalgae. In addition, macroalgae presents an easy adaptability to grow in a wide range of salinities and temperatures condition.

CO₂ is the primary carbon source for macroalgae, which subsequently produces oxygen via

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photosynthesis processes. As reported by Andersen (2005), macroalgae could produce around 73% to 87% of the net global production of oxygen. Aresta et al., (2005) noted that photosynthetic efficiency of macroalgae results are much higher on average of 4 times higher than that of terrestrial biomass. The ocean pool of carbon contains dissolved inorganic carbon (DIC), in the form of CO₂, HCO₃⁻ and CO₃²⁻ (Andria et al., 1999b; Rosenberg et al., 1995). In ordinary seawater, the pH is in the range of 8.2 ± 1.0, where 90% of DIC occurs in the form of HCO₃⁻ and less than 1% is as dissolved gaseous CO₂, with approximately 2 mM of HCO₃⁻ and only 10 µM of CO₂ (Gao and McKinley, 1994; Smith and Bidwell, 1989). In macroalgae, carbon is a fundamental resource for photosynthesis, and most macroalgae are 'HCO₃⁻ users' as tested in several studies. For example, *G. tikvahiae*, *G. foliifera*, *G. chilensis* and *G. secundata* have been reported to be capable of using bicarbonates as a carbon source (Bidwell and McLachlan, 1985; Israel and Beer, 1992; Johnston and Raven, 1990; Zou, 2005).

Macroalgae probably exhibit an increase in growth, retardation or not affected by enrichment of CO₂ concentrations that associated with its photosynthetic capacity and inorganic carbon acquisition mechanism. Gao et al., (1993) demonstrated that elevated CO₂ concentrations up to 5 times of ambient levels, enhanced the growth of the red macroalgae *Porphyra yezoensis* under optimal light and nutrient conditions. Whereas, *G. lemaneiformis* cultured in the presence of CO₂ had enhanced the growth rate by 78% at intermediate irradiance level with a saturated concentration of inorganic carbon at 2.2 mM (Zou and Gao, 2009b). On the contrary, *G. tenuistipitata* and *P. Leucosticte* also known as red macroalgae, showed a negative response to elevated CO₂ concentrations (García-Sánchez et al., 1994; Xu and Gao, 2012; Zou and Gao, 2009a). The response of macroalgae to elevated CO₂ concentrations depends on the species and its physiological adaptation.

Consequently, there is extensive interest in the influences of increasing atmospheric CO₂ concentrations on macroalgae. Cultivation of the red macroalgae, Gracilaria, are common owing to its superior properties such as rapid growth, easy for cultivation, and high commercial value for agar industry (Huo et al., 2012; Troell et al., 1999). However, screening of a species that has a high affinity towards inorganic carbon should be taken into consideration which added its value for massive cultivation. A species that meets all these requirement was selected and known as *Gracilaria edulis*, as it is anticipated to utilize inorganic carbon for enhanced growth. Thus, the aim of the present study was to assess the growth performance of *G. edulis* in the presence of atmospheric and enhanced carbon dioxide concentrations. In addition, the interaction between elevated CO₂ concentrations and pH was also studied. A profound understanding of the use of CO₂ concentration by macroalgae is important for

predicting the effects of CO₂ on biomass production. The utilization of this species is also useful for wastewater treatment and recycling of nutrients. The outcome from this study will be vital for future production of *G. edulis* in aquaculture sector with respect to increasing inorganic carbon in the atmosphere and also potential solution for the coastal eutrophication problem.

2. Material and methods

2.1. Experimental design

Healthy thalli of *G. edulis* were collected at the Brackishwater Culture Research Centre, Gelang Patah, Johor, Malaysia (1°26' 21.5"N and 103°34' 55.2"E). After collection, *G. edulis* were transported to the laboratory in a polystyrene box filled with brackish water (salinity ca. 20-25%). In the laboratory, the macroalgae was washed under running water and then cleaned of epiphytes. Healthy thalli were cut with a razor blade into few fragments (approximately 0.5-1.0 cm length) with an initial biomass of 0.5g. The samples were then incubated in natural brackish water that was brought from study location for 5 days in order to recover from cutting stress (wound respiration). The culture brackish water enriched with 100µM NaNO₃ and 10µM NaH₂PO₄ supplied with vigorous aeration.

Then, in a 500 ml flask filled with brackish water, the recovered thalli was incubated and sparged with three carbon dioxide concentration conditions-actual atmospheric carbon dioxide (350 ppm), enhanced with 700 ppm and 1400 ppm CO₂. The culture flasks were placed on an orbital shaker and supplied with continuous aeration with CO₂ gas at input flow of 300 mlmin⁻¹. The cultures were maintained at 100 µmolphotons m⁻² s⁻¹, which was measured using a quantum spherical PAR sensor, and incubated with a 12L:12D photoperiod and at 28-30 °C. The experiments were conducted over a period of two weeks. The culture medium was renewed daily throughout the experiment. Fresh weight of cultured *G. edulis* was monitored once a week. The thalli were blotted on a paper towel to remove excess water, then weighed (fresh weight), and returned to the flask (Marinho-Soriano et al., 2009). The specific growth rate (SGR) was calculated using Eq. (1):

$$SGR = \left[\ln \frac{W_f}{W_i} / (T_f - T_i) \right] 100 \quad (1)$$

where: W_i is the initial and W_f is final fresh weight, respectively. T_f and T_i were the time between the termination and initiation of the experiment, respectively (Zhou et al., 2006).

In addition, a series of different concentrations (0 to 6mM) of dissolved inorganic carbon were prepared by dissolving stock NaHCO₃ solution and the pH was adjusted (8.0) with NaOH and HCl. The response of net photosynthesis rates (NPS) to inorganic carbon concentrations were measured using

oxygen electrode probe (YSI, model 55) at saturating irradiance level of $600 \mu\text{molphotons m}^{-2} \text{s}^{-1}$. In order to obtain this rate, inorganic carbon free brackish water was prepared by acidifying to pH less than 4.0 with HCl and sparging for at least 2 days with pure N₂ gas to remove fully the inorganic carbon. Followed by adjustment of the pH to 8.0 using NaOH solution. Then, approximately 0.5 g of fresh weight thalli was incubated in the chamber containing 8ml seawater with TRIS buffer (2.1 mM inorganic carbon concentration) that was magnetically stirred. The prepared samples were left to photosynthesize to utilize the remaining inorganic carbon in the medium until a zero value of net oxygen evolution was achieved. The prepared stock solution of NaHCO₃ solution were injected into the chamber and oxygen evolution rates were recorded for the respective range of inorganic carbon concentrations up to 6mM. Photosynthetic rates for actual atmospheric and enhanced with 1400 ppm CO₂ were measured.

The experimental data were fitted to the Michealis-Menten kinetic model using Graphpad Prism software by an iterative least square procedure. The maximum photosynthetic rates (V_{\max}) and half saturation constant (K_m) were derived from this model. The inorganic carbon concentrations were measured using total organic carbon analyzer instrument (TOC-5000A, Shimadzu, Japan), pH of the water was measured by using Multi-Parameter Analyzer-Consort C35 model and salinity with refractometer (model REF 211T).

2.2. Statistical analysis

For all experiments in the laboratory, an average of three replicates were analyzed and from

this, means and standard deviations were calculated. Student's t test was applied for comparisons between means of the group. In all cases, the null hypothesis was rejected at 5% significance level.

3. Results

The present study exhibits the effect of elevated CO₂ on *G. edulis* growth rate, cell physiology, pH fluctuations, and the mechanisms involved for carbon uptake. Among the three treatments for varying carbon concentrations, the highest biomass was observed in treatment of CO₂ enriched air at 1400 ppm with SGR of 9.0% compared to 7.7% at 700 ppm and only 7.0% at normal atmospheric air (control) (Fig. 1). At 700 ppm and 1400 ppm of CO₂ enriched air, it presented significant growth ($P<0.05$) of 10.0% and 28.0%, occurred respectively, compared to the control.

The growth pattern of *G. edulis* demonstrated logistic curve where the growth increased steadily and as soon as the carrying capacity of the macroalgae was attained, it approached a constant growth rate. Thereby, the maximum biomass achieved in a two weeks period was $1.0 \pm 0.15\text{g}$ at high carbon dioxide concentration (Fig. 1).

Fig. 2 illustrates the diurnal variation in pH during dark and light period, which showed that the treatment with CO₂ enriched air has a lower pH of 7.99 ± 0.02 and 7.86 ± 0.03 at 700 ppm and 1400 ppm, respectively. While at 350ppm, the pH was higher with a mean of 8.65 ± 0.14 and this value was significantly different with carbon dioxide enriched air ($P<0.01$). In addition, CO₂ enriched culture medium exhibited lower pH, between the ranges of 0.5 to 0.8 unit compared to the actual atmospheric air.

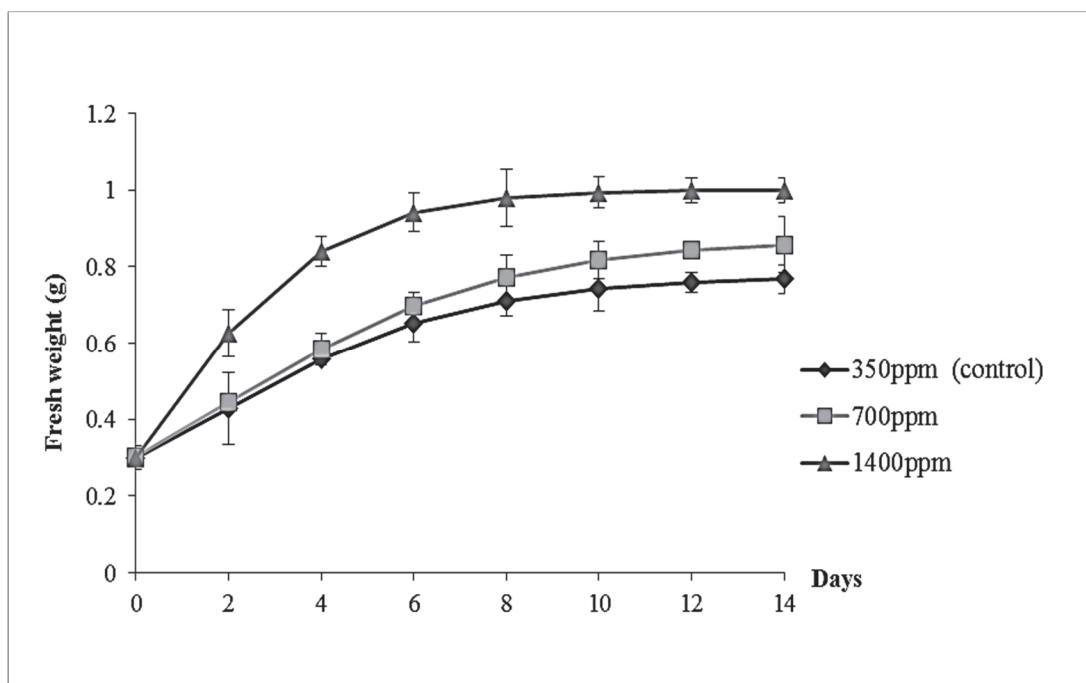


Fig. 1. Macroalgae growth in culture media aerated with normal air, and enriched carbon dioxide air

Net photosynthetic oxygen rate (NPS) was measured at the respective inorganic carbon concentrations (0-6mM) as shown in Fig. 3. Observation was made at 350ppm and enriched carbon dioxide air of 1400 ppm. The highest photosynthetic oxygen evolution in this experiment were $45\mu\text{molg}^{-1}\text{FWh}^{-1}$ and $35\mu\text{molg}^{-1}\text{FWh}^{-1}$ at 1400 ppm and 350 ppm, respectively. These values significantly differed ($P<0.05$) between control and at 1400 ppm CO_2 concentration, and these demonstrated that photosynthetic rate was dependent on the inorganic carbon concentration and semi-saturation, K_m , was achieved at about 2.5mM. Generally, the saturation point attained by macroalgae for DIC uptake at more than 2.0mM when the seawater is in equilibrium with the air (Bidwell and McLachlan, 1985). *G. edulis* has presented a higher affinity to inorganic carbon concentrations since the K_m value is higher than usual

in seawater. Furthermore, the maximum photosynthetic O_2 rates (V_{max}) at 1400ppm and 350ppm were $67\mu\text{molg}^{-1}\text{FWh}^{-1}$ and $48\mu\text{molg}^{-1}\text{FWh}^{-1}$ can be achieved by this species as predicted by the Michaelis-Menten kinetic model. The fitting of experimental data into this model has shown high goodness of fit with R^2 value of 0.9925 at high and 0.9589 at low CO_2 concentration.

4. Discussions

The photosynthetic utilization of inorganic carbon by the red macroalgae are relatively sparse compared to brown macroalgae. The results for the red macroalgae growth demonstrated in the present study showed that a higher biomass was achieved at highest CO_2 enriched air and lowest growth at normal atmospheric air.

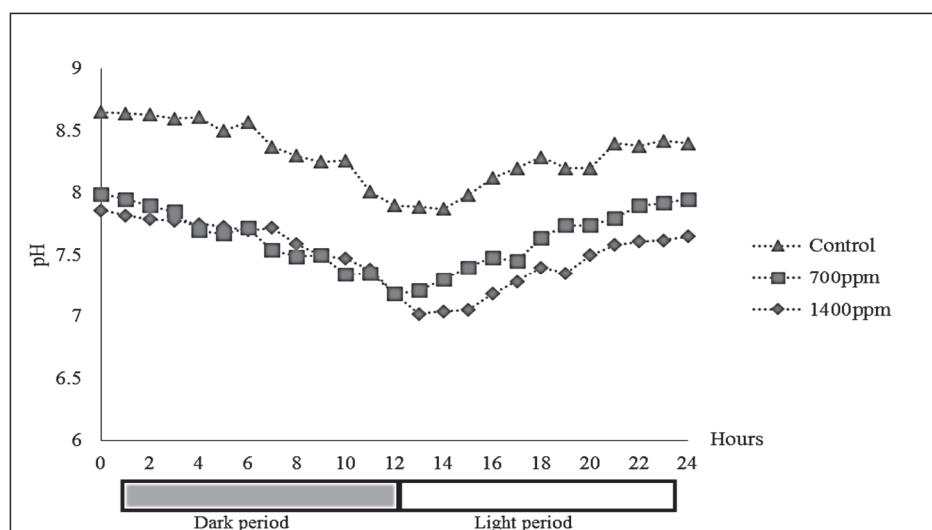


Fig. 2. Diurnal variations of pH during light and dark period at normal atmospheric carbon dioxide air, and enriched carbon dioxide air at 700 ppm and 1400 ppm, respectively

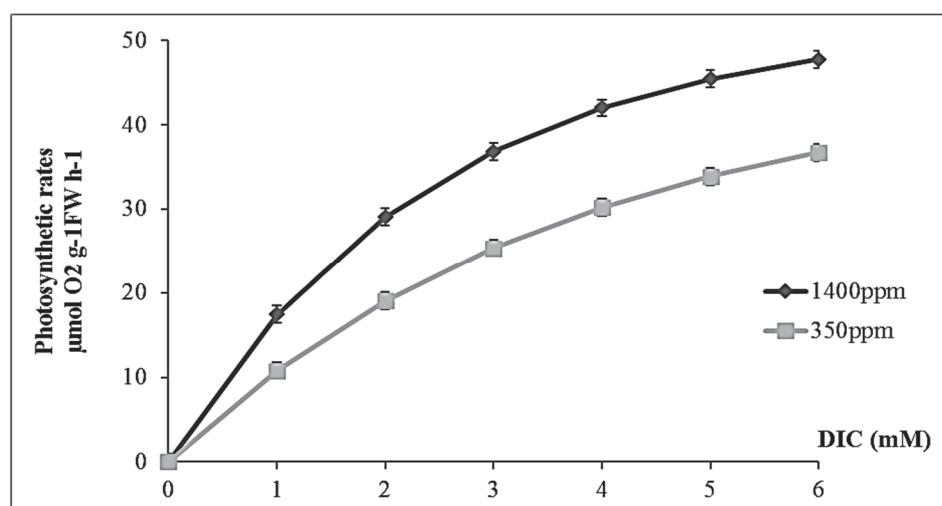


Fig. 3. The response of photosynthetic O_2 evolution to inorganic carbon concentration.
Means \pm SD (n=3). Data were fitted into Michaelis- Menten kinetic model

The inclined in biomass with respect to increase in CO₂ concentrations exhibited a positive correlation and enhanced growth. In contrast, a red macroalgae *Chondrus crispus* presented inhibition of carbon uptake at elevated CO₂ concentration (Bjork, 1992). *G. edulis* grown well in CO₂ enriched air able to utilize the inorganic carbon during photosynthesis reaction without any inhibition effect at high concentration.

The pH fluctuations between 0.5 to 0.8 unit during dark and light period were found to be similar with the work done by Zou (2005) with differences of 0.84 unit. Furthermore, a doubling of CO₂ in the atmosphere under equilibrium conditions would result in lowering of the pH by 0.279 unit in the mixed surface of the sea (Gao and McKinley, 1994). However, in this study lower pH was recorded during the light period, which was only 0.13 unit lower and increased in darkness to about 0.3 unit. The results also depicted an incline in pH during light period and a subsequent reduction as approaching dark period following 12 hours of exposure to light. Variations of pH during light and dark period were independent of CO₂ concentrations and showed similar patterns at varying concentrations as well. During the light period, CO₂ in the form of bicarbonate (HCO₃⁻) is actively utilized in the photosynthesis and produces a high rate of OH⁻ compared to a low rate of deprotonation of hydrated CO₂, thus it increases the pH of the medium (Zou, 2005). At night, reversed chemical reaction occurred, the pH values declined. The rate of replacement of CO₂ occurs at a slower rate compared to consumption rate, by means of respiration, fermentation, diffusion, and hydration of dissolved CO₂. In addition, after long hours of exposure to light, the pH became constant gradually, because if pH keeps on elevating, carbon acquisition mechanism becomes pH sensitive and may affect intercellular enzyme activity, cell photosynthesis and physiology. To counteract this effect, less fluctuation in pH was observed, at the end of light period. However, when the pH rose above 8.4, affinity for inorganic carbon decreased and photosynthesis became inorganic carbon limited.

The photosynthetic response of *G. edulis* to varying DIC concentrations exhibited a biphasic pattern, this condition can be supported by carbon-concentrating mechanism (CCM) involving two different DIC acquisition pathways. The first is the indirect use by an external carbonic anhydrase activity (CA) and the second, is the direct uptake of HCO₃⁻ across the plasma membrane (Andria et al., 1999a). The bulk of HCO₃⁻ is converted to CO₂ by the intracellular carbonic anhydrase (CA) enzyme. Macroalgae that possesses CA are capable of using HCO₃⁻ and catalyzed interconversion to CO₂ before transporting occurs across the membranes (Gao and McKinley, 1994). Consequently, this enzyme involves in active carbon transporting and accumulating mechanism for photosynthetic utilization by macroalgae. An equilibrium is maintained between

intracellular and extracellular CO₂ concentrations and achieved a constant CO₂.

The maximum rate of photosynthesis (NPS_{max}) is frequently related to Rubisco activity. The use of HCO₃⁻ can allow an increase in the CO₂ concentration around Rubisco and results in the decrease of the photorespiration, whereby it acts as a carbon-concentrating mechanisms (CCM) (Raven, 1997). Species that have an active CCM will show little response to rising levels of atmospheric CO₂, whereas those that exhibit carbon-limited photosynthesis may respond positively by higher carbon assimilation to the CO₂ enrichment. Despite the evolution of oxygen from photosynthesis, oxygen acts as a competitive inhibitor of the carboxylase activity of Rubisco and as a substrate for the oxygenase activity, leads to photorespiration as well (Lobban and Harrison, 1994).

The fact that macroalgae have a low photorespiratory rate indicates that the CO₂ to O₂ ratio may be high enough to suppress photorespiration (Bjork et al., 1992) and may increase photosynthetic efficiency and enhanced growth. According to Gao et al., (1993) higher growth enhancement in *Gracilaria* sp. was attributed to a greater depression of photorespiration by the enrichment of CO₂ in the culture. Rosernberg et al., (1995) stated that the ratio of net photosynthetic O₂ release to CO₂ uptake, (photosynthetic quotient, PQ) is equivalent to 1 and unaffected by taxonomic division, functional-form group, and nutrients. With respect to the PQ value, it can be said that under natural concentrations of DIC and saturating photon flux densities, macroalgae photosynthesis is faster in water than in air.

Furthermore, the rate of O₂ evolution is also dependent on the pH of culture medium. *Gracilaria lemaneiformis* has demonstrated fully saturated photosynthesis rate at pH 8.2 and DIC of 2.2 mM. However, when the pH of the seawater increases, the affinity for DIC is reduced and photosynthesis reaction has become carbon limited (Zou and Gao, 2009b). The photosynthetic response is also affected by the irradiance level. In *Porphyra yezoensis*, the oxygen uptake in the light was higher than that in the dark and it increases with a rise in light intensity (Gao et al., 1991). The result was also supported with the statement made by Ramus and Rosernberg (1980) that at low light density, rate of photosynthesis is linearly related to incident light and at high density, it is independent of the light density. Changes in fluorescence associated with the induction of the carbon-concentrating mechanism (CCM) in *Chlamydomonas reinhardtii* found to affect the rate of photosynthesis (Mercado et al., 1999). In addition, *G. vermiculophylla* shows a rapid increase of photosynthesis rates with increased irradiance up to 200 μmol photons m⁻² s⁻¹ while it dropped significantly beyond this point up to 710 μmol photons m⁻² s⁻¹, where saturation was attained.

Besides that, photosynthetic reaction that take place during light period is believed to consume and fix larger quantity of carbon dioxide that contribute to

rise in pH during the day. In addition, Axelsson and Uusitalo (1988) state that the removal of NO_3^- by algae increases the alkalinity which could also attribute to rise in pH, that causes elevated pH with time. Thereby, these two factors affect the rise on pH and the effect of nitrate was negligible since photosynthetic utilization of inorganic carbon are predominant.

This study has revealed the requirement of inorganic carbon and optimum pH for growth of the commercially interesting, agar-producing, red macroalgae *G. edulis* under controlled laboratory conditions. In addition, this species also able to utilize the available carbon source at elevated carbon concentrations and assimilated the carbon source for physiological activity and incorporated into its biomass at a rapid rate compared to normal condition. The high growth rate at high DIC concentration provides useful information for massive *G. edulis* biomass production.

The mechanisms for carbon acquisition has been described that strongly concludes that inorganic carbon is an important controlling factor for *G. edulis* production. Moreover, cultivation of this species can be considered at carbon emitting power plant such as flue gas that capable to alleviate impact of high CO_2 on the environment notably greenhouse effect, and enhanced biomass production at cheaper cost. Likewise, also provides useful information on ecophysiology of this species at elevated CO_2 concentration, besides its potential for bioremediation to control eutrophication problem in coastal waters. In future, the information from this study can be used for massive production of this species for enhanced its production in aquaculture sector, besides controlling the carbon emission for sustainability purposes.

5. Conclusions

The response of *G. edulis* to different inorganic carbon concentrations indicates that carbon is an important factor controlling the photosynthetic physiology in macroalgae.

The enrichment of CO_2 in seawater has increases the growth rate of *G. edulis* and also leads to higher biomass productivity of biomass. The presence of inorganic carbon also influences the pH of the culture medium and these two factors greatly influence the dissolution of carbon and the uptake rate by the respective enzymes that are involves in carbon assimilation. Thus, elevated CO_2 in the atmosphere could be fixed by macroalgae in photosynthetic reaction and results in higher growth rate.

Acknowledgements

The authors are very grateful to Ministry of Higher Education of Malaysia (MOHE) for financial support, under grant No. 4L060. This work has been supported by the Brackishwater Research Centre of Gelang Patah, Johor, Malaysia. We also thank the three anonymous reviewers for their valuable comments and suggestions.

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