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Growth rate analysis and protein identification of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) under pH induced stress culture

Mian Zi Tee [a], Yoong Soon Yong [b], Kenneth Francis Rodrigues [b], Wilson Thau Lym Yong [b,*]

[a] *Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia*
[b] *Biotechnology Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia*

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ABSTRACT

Environmental pH is one of the factors contributing to abiotic stress which in turn influences the growth and development of macroalgae. This study was conducted in order to assess the growth and physiological changes in *Kappaphycus alvarezii* under different pH conditions: pHs 6, ~8.4 (control) and 9. *K. alvarezii* explants exhibited a difference in the daily growth rate (DGR) among the different pH treatments ($p \leq 0.05$). The highest DGR was observed in control culture with pH ~8.4 followed by alkaline (pH 9) and acidic (pH 6) induced stress cultures. Protein expression profile was generated from different pH induced *K. alvarezii* cultures using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein identification and analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) and Mascot software. Ribulose bisphosphate carboxylase (Rubisco) large chain was identified to be up-regulated under acidic (pH 6) condition during the second and fourth week of culture. The findings indicated that Rubisco can be employed as a biomarker for pH induced abiotic stress. Further study on the association between the expression levels of Rubisco large chain and their underlying mechanisms under pH stress conditions is recommended.

1. Introduction

Kappaphycus alvarezii is one of the largest reported tropical red macroalgae with relatively higher growth rate among *Kappaphycus* seaweeds (Patterson-Edward and Bhatt, 2012). This species is commercially important as it is a source of kappa carrageenan, a phycocolloid that is widely applied as a thickening and stabilizing agent in food, pharmaceutical and cosmetic industries (Hayashi et al., 2011). Due to the increased demand of seaweed based-products and limited supply of raw material from natural stock, mass production of seaweed materials via tissue culture technology is highly recommended (Reddy et al., 2008; Yong et al., 2011; Yeong et al., 2014). The efficacy of seaweed tissue culture is depends on the effective manipulation of endogenous (such as age, source, developmental stage and physiological state of explants) and exogenous (including pH, salinity, light, temperature and media composition) factors (Yokoya and Yoneshigue-Valentin, 2011; Jong et al., 2015). Menéndez et al. (2001) reported that the pH of seawater is 8.2 when HCO_3^-/CO_2 ratio is approximately 150 under conditions of atmospheric equilibrium. Perturbation of this environmental situation may affect the biochemical processes of marine organisms including seaweeds and subsequently affect their growth. Ocean acidification, resulting from higher concentrations of atmospheric carbon, can lead to physiological stress and reduction in growth rates of macroalgae.

Proteins are the primary effector molecules potentially influenced by environmental, physiological and pathological conditions (El-Gamal, 2008). They are known to be associated with the response to various environmental stresses by being newly synthesized, accumulating or decreasing in levels. The ability of proteins to maintain their functional conformations and prevent the accumulation of non-native proteins is crucial for the cells to overcome environmental stress (Timperio et al., 2008). Proteomics is the study of cellular process through identification and quantification of expressed protein complement. The discipline was introduced in mids-1990s and has been instrumental understanding the cellular response to abiotic stress in terms of protein expression levels (Mitulović and Mechtler, 2006). Proteomics is intended to catalogue all expressed protein to facilitate the comparison of protein expression in different cellular states and physiological levels. It has become a powerful tool to provide better understanding of cellular processes of organisms survived under stress conditions by identifying the changes in their proteomes. Moreover, the function

* Corresponding author. Fax: +60 88 320993.
E-mail addresses: wilsonyg@ums.edu.my, wilsonyong80@yahoo.com (W.T.L. Yong).

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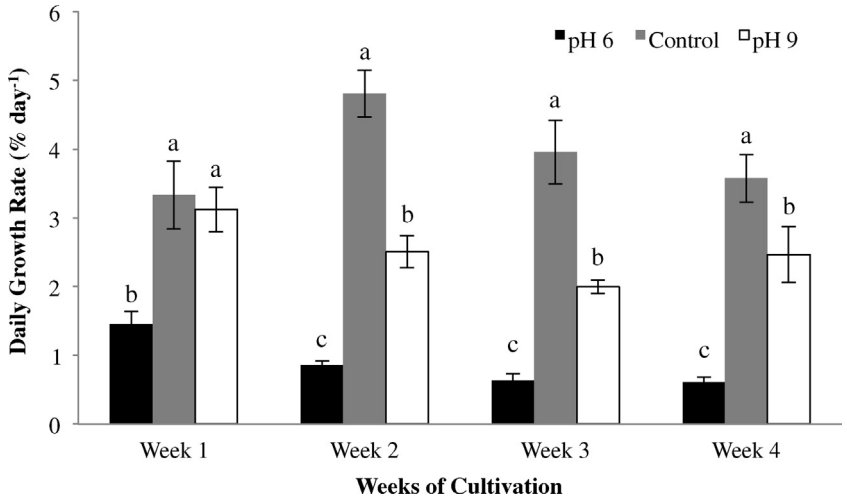

Fig. 1. Daily growth rate (% day^{-1}) of *K. alvarezii* explants under different pH treatments: pH 6, ~8.4 (control) and 9. Error bar indicates standard deviation. Alphabetical letters indicate the value on the basis of significance according to Turkeys test ($p \leq 0.05$).

and role of targeted proteins involved in the stress survival can be ascertained (Carr, 2002; Mitulović and Mechtler, 2006).

Previously, studies have been carried out to determine the novel proteins produced by seaweeds in response to the environmental stress, including study of brown macroalgae, *Ecklonia cava* under temperature stress for development of biomarkers to evaluate the health of colonies and the maintenance of marine forests (Yotsukura et al., 2012). In the case of other phaeophytes, *Saccharina japonica* has been studied for their proteome responses toward seasonal variation and pH conditions (Yotsukura et al., 2010; Kim et al., 2011). Besides, *Porphyra columbina* (Rhodophyta) has been monitored for their protein expression in response to desiccation stress (Contreras-Porcia and López-Cristoffanini, 2012). To date, part of the ongoing research on algal environmental stress induced protein analyses are being conducted in brown algae while the rest are mostly demonstrated in microalgae. The changes of protein expression under varying pH conditions have not yet been extensively studied in *K. alvarezii*. Thus, this study attempts to determine the growth response of *K. alvarezii* under different pH conditions and to identify the associated protein for better understanding of proteomic response of *K. alvarezii* during environmental pH fluctuations. The outcome of this study will be of benefit to the understanding and manipulation of culture conditions in vitro for the efficient production of seaweeds.

2. Materials and methods

2.1. pH induced stress culture

K. alvarezii explants were obtained from the stock culture regenerated via tissue culture considering previous work carried out by Yong et al. (2014). The tissue cultured seaweed stocks were maintained in the laboratory of Biotechnology Research Institute, Universiti Malaysia Sabah. The explants were rinsed with sterilized seawater, excised into 3–5 cm in length, surface sterilized and cultivated in optimized PES media (Yong et al., 2014). The culture media were adjusted to pHs 6 and 9, respectively for pH induced stress experiments using 0.1 M hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH), except for control media with pH ~8.4. The cultures were maintained at 21 ± 1 °C with 12:12 light and dark photoperiod under cool white fluorescent tube light (75 μmol photons m^{-2} s^{-1}) with continuous aeration throughout the four weeks of study (Yong et al., 2014). The experiments were carried out in triplicate and the pH of each treatment was readjusted daily, except for control.

2.2. Growth rate analysis of culture

Daily growth rate (DGR) of explants was calculated using formula recommended by Yong et al. (2013) as follows: DGR (%) = $[(W_t/W_0)^{1/t} - 1] \times 100\%$ where W_0 is the initial fresh weight, and W_t is the final fresh weight of the explants after t days of culture. The data obtained were analyzed with one-way analysis of variance (ANOVA) and Turkey multiple comparison test using SPSS version 20 (SPSS, Chicago, IL).

2.3. Total protein extraction

Total proteins were extracted from different growth stages (second and fourth weeks of cultivation) of pH induced stress and control *K. alvarezii* cultures using total protein extraction buffer as recommended by Shewry et al. (1995). The procedures of extraction and analysis were performed in duplicate according to Jong et al. (2015) with minor modification. Explants were ground and homogenized with buffer to sample ratio at 1:1. The homogenized samples were vortexed prior centrifuged at 10,000 × *g* for 2 min. The pellet was discarded while the supernatant containing the protein was collected. The supernatant was then heated at 95 °C for 5 min and kept in ice until further analysis.

2.4. One-dimensional SDS-PAGE

Sodium dodecyl sulfate (SDS) polyacrylamide gel was prepared with minor modification from Laemmli (1970). A 12% resolving gel was made with 1.5 M Tris–HCl (pH 8.8), 20% SDS, 10% ammonium persulphate and tetramethylethylenediamine (TEMED) while the 4.5% stacking gel was prepared with 0.5 M Tris–HCl (pH 6.8), 20% SDS, 10% ammonium persulphate and TEMED. Electrophoresis was performed using Hoefer SE600 Ruby Standard Vertical Electrophoresis Unit (Amersham Bioscience) at 100 V for 2 h through the stacking gel, followed by 150 V until the dye front reached the bottom edge of the gel cassette. The gel was stained overnight with Coomassie Brilliant Blue (CBB) G-250 staining solution (10% ammonium sulphate, 0.1% CBB G-250, 3% orthophosphoric acid, and 20% ethanol) in continuous agitation according to Dyballa and Metzger (2009) with minor modification. The gel was then washed with distilled water until clear bands were observed.

2.5. Protein identification

The protein gel was viewed with Alpha Innotech Alphaimager HP MultiImage II and the band of interest was cut and sent for protein identification (Proteomics International Pty Ltd). The protein identification was carried out by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) and analyzed with Mascot sequence matching software (Matrix Science) with Ludwig NR database.

3. Results and discussion

3.1. Growth performance of pH induced stress K. alvarezii

Daily growth rate of *K. alvarezii* explants showed significant difference ($p \leq 0.05$) in response to different pH conditions: pHs 6, ~8.4 (control) and 9 with other culture parameters maintained constant (Fig. 1). Among the three different pH treatments, culture without pH induced stress (control) achieved the highest daily growth rate (3.57 ± 0.34%), followed by extreme alkaline condition (pH 9; DGR = 2.44 ± 0.42%) and extreme acidified medium (pH 6; DGF = 0.61 ± 0.07%) at the fourth week of cultivation. Throughout the experiment, the highest daily growth rate (4.81 ± 0.34%) was recorded in the second week of control culture before it declined to 3.57 ± 0.34% in the last week of cultivation. Daily growth rate of explants in pH 6 induced stress culture showed a decreasing trend throughout the study and observed to be the lowest (0.61 ± 0.07%) at the end of experiment.

Lavens and Sorgeloos (1996) reported that pH range for most of the seaweed cultures is between 7 and 9, but the optimum range is between 8.2 and 8.7. The ordinary seawater is slightly alkaline (pH ~8.07) with bicarbonate ions (HCO_3^-) made up about 91% of the total dissolved inorganic carbon (DIC), followed by 8% of carbonate ions (CO_3^{2-}) and 1% of dissolved CO_2 (Roleda et al., 2012). Any changes in seawater pH may alter the equilibrium of carbonate system and change the concentration of inorganic carbon species (Cornwall et al., 2012), which in turn affect the growth of seaweeds as they are dependent on the supply of inorganic carbon for photosynthesis.

In the control medium of this study with pH resembles the ordinary seawater, HCO_3^- made up the largest proportion of DIC. Due to the low availability and diffusion rate of CO_2 in seawater, together with the bulk of HCO_3^-, adaptive mechanism termed carbon dioxide concentrating mechanism has been developed in seaweeds (Menéndez et al., 2001; Harley et al., 2012). Photosynthesis of most seaweed is fully or nearly saturated with the current ambient DIC composition as they are capable of using the HCO_3^- pool in seawater as photosynthetic carbon source (Zou and Gao, 2009). Macroalgae species with the carbon dioxide concentrating capability have an adaptive advantage over those obligate carbon dioxide users provided other essential nutrients are not limiting (Wu et al., 2008). *K. alvarezii* is expected to exhibit this adaptive mechanism by using HCO_3^- as photosynthetic carbon source and hence promoted growth rate was observed in the control medium of this study. However, Menéndez et al. (2001) has reported the rapidly declined of macroalgal photosynthetic rate at pH above 8.5 due to the diminution of dissolved CO_2 and HCO_3^- concentration. According to Cornwall et al. (2012), no photosynthesis will take place at pH 9 in obligate CO_2 macroalgae user. This further supports the observation of *K. alvarezii* to utilize HCO_3^- as photosynthetic carbon source in control medium (pH ~8.4) and do not depend solely on CO_2.

On the contrary, addition of HCl to the culture medium caused an increase of hydrogen ions (H^+) concentration together with a comparatively low concentration of HCO_3^- (Cornwall et al., 2012). The low availability of photosynthetic carbon source under the acidified medium may severely limit the photosynthesis process of explants and resulted in a decrease of their growth rate. Roleda et al. (2012) reported that inhibition of meiospore germination and gametophyte development in giant kelp *Macrocystis pyrifera* (Laminariales, Phaeophyceae) under acidified medium may due to the increased of H^+ ion effect. This further explains the minimum growth rate of *K. alvarezii* observed under the acidified medium in this study. However, another study conducted by Yunque et al. (2011) has revealed better growth of purple morphotype of *K. alvarezii* in acidic culture medium (pH 6.7) and this observation indicates different *Kappaphycus* varieties may respond differently to pH conditions.

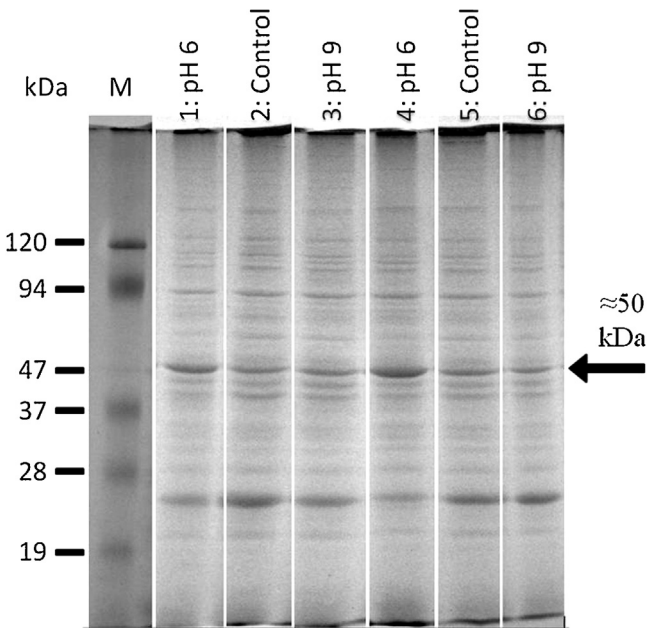

Fig. 2. Protein expression profile generated from SDS-PAGE for different growth stages of *K. alvarezii* under different pH conditions. Lane 1 loaded with Nacalai Tesque Pre-stained protein marker; Lanes 2–4 loaded with samples from second week of culture; Lanes 5–7 loaded with samples from fourth week of culture.

3.2. Protein identification of pH induces stress culture

The protein profile generated by SDS-PAGE from cultivated *K. alvarezii* under pH induced stress and control conditions (Fig. 2) revealed more than 20 bands under each of the experimental conditions. A protein with a size of approximately 50 kDa was expressed at notably higher level at pH 6 as compared to pH 9 and the controls. The highly expressed protein was identified as ribulose bisphosphate carboxylase (Rubisco) large chain based on MALDI-TOF-MS analysis followed by Mascot search for homology-based searching. The protein coverage of peptide matching is recommended to achieve minimum of 14% with at least 4 peptides matched for a best match result (Wong et al., 2006). The lower protein coverage obtained from the Mascot analysis in the present study (11% as indicated in Table 1) may due to unavailable of targeted protein homologues in the database (Lubec and Afjehi-Sadat, 2007), insufficient protein concentration or contamination of sample. However, protein similarity searches demonstrated that the best match of targeted protein belonged to the algal family with total of six matched peptides as reported in Table 1.

Rubisco is an enzyme assemblage of eight plastid-encoded large subunits and eight nuclear-encoded small subunits (Chen et al., 1988). It is a bifunctional enzyme known to catalyze carbon dioxide fixation and oxygenation (Wong et al., 2006) to create competitive metabolic pathway between photosynthesis and pho-

Table 1
Protein identification and analysis based on Mascot Search (Matrix Science).

Properties	Sample result
Protein hit	Ribulose bisphosphate carboxylase large chain (*Ceramium pacificum*)
Protein score	246
Peptide matched	6
Protein coverage	11%

Protein score >58 indicates identity or extensive homology ($p < 0.05$). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits (Mascot Search, Matrix Science).

torespiration (Chen et al., 1988; Raines, 2011). The competition between oxygen and carbon dioxide takes place at the active site of Rubisco large subunits and the enzyme is readily interconverted between activated and inactivated form (Mott and Berry, 1986). In Rhodophyta and Phaeophyta, both large and small Rubisco subunits were reported to be encoded in the chloroplast genome in an operon and the large subunits with catalytically-essential residues are critical to the activity of mature Rubisco (Wang et al., 2011).

The lowest growth rate of *K. alvarezii* explants observed in pH 6 induced stress culture in the present study can be attributed to the comparative low photosynthetic carbon source available in acidified medium which in turn is likely to have led to a disruption of the process of photosynthesis. The up-regulation of Rubisco large chain under acidic condition has suggested less efficient of CO_2 accumulation in *K. alvarezii* which in turn results in a lower growth rate. Wang et al. (2011) reported higher abundance and activity of Rubisco in parallel with the higher photochemical activities in gametophytes of some macroalgae as compared to their sporophytes. Besides, the up-regulation of Rubisco large chain may also play an important role in supporting the survival of macroalgae under harsh environments. Gylle et al. (2013) evaluated the differences in relative amount of Rubisco in *Fucus* sp. subjected to different salinity and found greater amount of Rubisco together with chlorophyll proteins in hyposaline treated algae. This may due to the demands of more ATP is required for the algae to overcome the salinity stress and greater amount of chlorophyll proteins may increase the flow of electron transport for higher rate of CO_2 concentrating mechanisms and higher rate of CO_2 fixation by Rubisco. Dubnovitsky et al. (2005) has suggested that alteration of negatively and positively charge ratio on the molecular surface of proteins may serve as a mechanism for pH stress tolerance. Hence, the charge ratio on the active site of Rubisco large chain may involve in facilitating the carbon uptake and promotion of survival and growth under stress conditions. Nevertheless, there is still limited study on the expression of Rubisco in macroalgae to date and their underlying mechanisms in response to stress conditions will need additional research.

4. Conclusion

In conclusion, *K. alvarezii* was found demonstrating higher growth rate under alkaline condition as compared to acidic condition. The protein which constituted the large chain of Rubisco was found to be differentially upregulated and over-expressed under conditions of acidic stress and this points to its likely role in ameliorating the detrimental effects of acidification. These findings support the need for a deeper understanding of the mechanism of abiotic stress response in macroalgae.

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