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Heavy metal accumulation in field cultured and tissue cultured *Kappaphycus alvarezii* and *Gracilaria changii*

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Abstract

Use of raw seaweed for direct consumption and its extract in food production has increased steadily throughout the world. However the ability of metal sorption in seaweed may result in accumulation of some heavy metals which could be harmful to consumers. Tissue culture has been considered as an alternative method to produce uncontaminated seaweeds as seedlings for sustainable farming and raw materials for various industries including food production. In this study, Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was used to determine the metal concentration in both field cultured and tissue cultured *Kappaphycus alvarezii* and *Gracilaria changii*. Results indicated that concentration of heavy metals with great scientific importance such as arsenic (As), cadmium (Cd) and lead (Pb) from tissue cultured samples has met the specific standard of health requirement from Joint FAO/WHO Expert Committee on Food Additive (JECFA) and Health Council and National Medicine Academy of France. Tissue cultured seaweeds also appeared to have lower As, Cd and Pb concentrations as compared to field cultured seaweeds. This may due to the stable and clean environment provided in tissue culture, contrasting with uncontrollable seasonal inflow of heavy metals in the field.

Introduction

Since the value of seaweeds have been noticed by various industries decades ago, the raw materials have been largely harvested especially in Indian and Pacific countries for animal and human foods, soil manure, salt, cosmetics, pharmaceuticals, energy production and colloid industry to produce agar, alginate, carrageenan, furcellaran and more (Caliceti *et al*., 2002). Seaweed extracts are often used as additive and intermediate for food production. In the Asian countries, it has been a tradition for several centuries to employ seaweeds as natural source of food and medicines. Fresh seaweeds in Indonesia and Malaysia are often consumed as salad components while they have been introduced into the cuisine for many European countries. Besides, processed seaweeds in the form of fine powder known as seaweed meals are often used as animal feed for sheep, cattle and horses (FAO, 2003). Direct consumption has been steadily growing since the early 1980s (Besada *et al*., 2009). Seaweeds are characterized by high concentrations of fiber and minerals, low fat content and relatively high protein levels (Ródenas de la Rocha *et al*., 2009). The nutritionally value of consuming seaweeds have been recognized to be an alternative dietary sources for macro-, trace and ultratrace elements (Larrea-Marín *et al*., 2010).

Both carrageenan and agar, extracted from *Kappaphycus alvarezii* and *Gracilaria changii* respectively, are the main components used in various industries. About 90 percent of agars are used for food application while the remaining 10 percent for bacteriological and other biotechnological uses (FAO, 2003). The ability of agar to withstand high temperature has made it a good component in baking industry as stabilizer and thickener. It has also been used in confectionery with high sugar content due to its 'sugar reactive' characteristic for able to increase the gel strength in the presence of sugar (FAO, 2003). On the other hand, protein reactivity is an important property of carrageenan which enables its utilization in several applications including as gelling, thickening, stabilizing and water-binding agent in various food products (Chan *et al*., 2013). Carrageenan molecules carrying negative charges allowed them to combine with positively charged particles such as potassium in potassium salts and protein in milk (casein) to form a three dimensional gel network (FAO, 2003).

The high minerals content in seaweeds may due to their cell wall polysaccharides and proteins with anionic carboxyl, sulphate and phosphate groups as

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excellent binding sites for metal retention (Tropin, 1995). This characteristic has made seaweeds become suitable biosorbents for removal of toxic heavy metals from industrial wastewater (Davis *et al.*, 2003; Ródenas de la Rocha *et al.*, 2009). The metal sorption activity involves binding of metals on the cell surface and intracellular ligands. Biosorption capacities were reported significantly affected by pH conditions, with higher pH favoring higher metal-ion removal (Kumar *et al*., 2007). Attributable to this capability, seaweeds are also used for environmental monitoring and restoration. However, the concentrations of contaminants in the environment do not always reflect their bioavailability. Under similar environmental conditions, mineral composition differs greatly among the different families, genera and species of seaweed, and varies according to their geographical origin and harvesting time (Larrea-Marín *et al*., 2010). The content of certain elements may also differ during different stages of seaweed life cycle.

Human and industry activities followed by the development of technology and urbanization have caused a lot of pollutions to the marine environment and destruction of marine habitats. One of the widely recognized issues is the oil related pollution caused by ship leakage and waste generated by the local shore facilities (Al-Shwafi and Rushdi, 2008). Chemical contamination has become a global concern especially heavy metal contamination in the costal environment due to its toxicity, persistence for several decades in the aquatic environment, bioaccumulation and biomagnifications in the food chain (Gochfeld, 2003; Valls and de Lorenzo, 2002). In this study, *K. alvarezii* and *G. changii* from different culture methods were examined for their heavy metal contents by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The aims of this study are to present detail information of heavy metals concentration for comparison between tissue cultured and field cultured *K. alvarezii* and *G. changii*, and to magnify the benefit of tissue culture as an alternative source to provide uncontaminated raw materials for farming, food processing and other seaweed-related industries.

Materials and Methods

Sample collection

Field cultured *K. alvarezii* and *G. changii* were collected freshly from seaweed farm at Sebangkat Island, Semporna, Sabah. Tissue cultured samples were obtained from tissue culture laboratory in Biotechnology Research Institute, University Malaysia Sabah (Yong, Ting, Yong *et al.*, 2014). Both field cultured and tissue cultured seaweeds were washed and rinsed with deionised water for several times to remove surface contaminants and salt. The wet samples were dried in oven at 60°C for 24 h prior to homogenized and ground into powder form (Yong *et al*., 2015).

Sample preparation

In order to digest and extract mineral and trace elements from seaweed samples, methods from Larrea-Marín *et al*. (2010) and Ródenas de la Rocha *et al.* (2009) were modified and applied. A 0.5 g ± 0.01 of each dried sample was weighed and introduced into a 50 mL glass vessel. Approximately 10 mL of 70% nitric acid (Merck, Germany) was added into the glass vessel and the solution was shook gently in room temperature. Heat was applied for complete digestion and extraction until the solution became stable. The solution was then cooled down before placed in water bath for 24 h with 100 rpm continuous shaking under 80°C. About 1 mL of 30% hydrogen peroxide (Merck, Germany) was added into the cooled samples with gently shaking in room temperature prior to heat application for further digestion until stable. After cooling in room temperature, the solution in glass vessel was topped up to 50 mL with deionised water produced by a Milli-Q Plus pure water generating system from Millipore (Elix, France) forming 100x dilution. Digested samples were then filtered with 0.2 µm syringe filter prior to metal content analysis.

Sample analysis

The content of metals comprising aluminium (Al), arsenic (As), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), strontium (Sr) and zinc (Zn) in seaweed samples were analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Optima 5300DV, Perkin Elmer, USA) according to standard protocol and operating procedure provided by manufacturer. Perkin Elmer NIST® traceable quality control standards were used as the stock standards for preparing working standards. The working standards were prepared on volume-by-volume dilution basis. Calibration was performed with a blank (ASTM® type I water acidified with nitric acid) followed by five standard solution samples covering the range of interest. The calibration curves were selected to match the expected concentrations of all the elements of the sample studied and the correlation coefficient (r) of the calibration curve must be ≥ 0.995. The detection limit for all the metals was 1 ppb or 0.001 mg kg^{-1}. Heavy

Table 1. Comparison of metals concentration between field cultured and tissue cultured *Kappaphycus alvarezii* (mg kg^{-1} dry weight)

Element	Field Cultured Sample (mg kg^{-1})	Tissue Cultured Sample (mg kg^{-1})	$p < 0.05$
Al	2.46 ± 2.25	1.28 ± 2.39	NS
As	4.63 ± 0.23	0.34 ± 0.49	**
Ca	2575.76 ± 150.57	3242.35 ± 212.39	**
Cd	0.69 ± 0.04	0.003 ± 0.005	**
Co	0.14 ± 0.15	24.32 ± 1.34	**
Cr	0.00	0.00	NA
Cu	0.01 ± 0.02	0.00	NS
Fe	7.27 ± 2.03	112.39 ± 4.14	*
Mg	4571.47 ± 324.53	5797.52 ± 401.19	**
Mn	1.13 ± 0.04	88.32 ± 4.30	*
Ni	0.37 ± 0.16	0.05 ± 0.07	NS
Pb	0.00	1.52 ± 0.41	**
Se	0.63 ± 0.68	2.19 ± 1.21	*
Sr	33.04 ± 2.31	34.70 ± 2.17	NS
Zn	5.41 ± 0.29	162.35 ± 5.47	**

Values are Mean ± SD of six samples.
NS: Not significant; NA: Not applicable.
* $p < 0.05$ significant differences for the same element.
** $p < 0.001$ significant differences for the same element.

metals with great scientific importance including As, Cd and Pb were compared with specific health requirement standard determined by Joint FAO/WHO Expert Committee on Food Additive (JECFA) and Health Council and National Medicine Academy of France. All the measurements were carried out with six experimental samples and the concentration data were subjected to statistical analyses by using SPSS version 16 software considering $p < 0.05$.

Results and Discussion

The relative abundance of metals analysed in field cultured *K. alvarezii* was Mg > Ca > Sr > Fe > Zn > As > Al > Mn > Cd > Se > Ni > Co > Cu, while in tissue cultured *K. alvarezii* was Mg > Ca > Zn > Fe > Mn > Sr > Co > Se > Pb > Al > As > Ni > Cd (Table 1). On the other hand, the relative abundance of metals in field cultured *G. changii* was examined to be Mg > Ca > Mn > Fe > Al > Zn > Sr > As > Co > Pb > Se > Ni > Cd, while in tissue cultured *G. changii* was Mg > Ca > Fe > Zn > Sr > Mn > Al > Se > Pb > Ni > As > Cd (Table 2). Cr was found to be absence in all the samples while the presence of Cu was determined to be 0 mg kg^{-1} in all samples except minute in field cultured *K. alvarezii*.

Heavy metals with great scientific importance such as As, Cd and Pb in all tissue cultured samples were reported fulfilled the standard of JECFA and Health Council and National Medicine Academy of France (Table 3). Meanwhile, As in field cultured samples were found exceeded both standards requirement. The presence of high concentration of As in its organic form was not represented as high toxicity as compared to its inorganic form (López *et al*., 1994; Oygard *et al*., 1999). The accumulation ratio of As is more heavily dependent on the particular seaweed instead of the environmental factors (Besada *et al.*, 2009). Generally, brown seaweeds are known to accumulate As hundred times as much as terrestrial plants (Ito and Hori, 1989). Besides, Pb concentration in field cultured *G. changii* was also discovered exceeded JECFA requirement. This may be explainable by the high gasoline combustion activity of surrounding industries in the area where the samples have been collected.

The presence of high concentration of Ca and Mg in all the samples can be attributed to the fact that they are important micronutrients for various metabolic functions in the plants. In contrast, the presence of low concentration of Cd and Cu may because of the incapability of seaweeds to incorporate such metals or the reduction of metal toxicity through certain biochemical or metabolic processes. The absence of Cr in all the samples may because of the less mobility of the metal and its existence mostly bound to the organic matter with decreased bioavailability (Kamala-Kannan *et al*., 2008). Some

Table 2. Comparison of metals concentration between field cultured and tissue cultured *Gracilaria changii* (mg kg^{-1} dry weight)

Element	Field Cultured Sample (mg kg^{-1})	Tissue Cultured Sample (mg kg^{-1})	$p < 0.05$
Al	140.55 ± 12.57	5.56 ± 4.36	**
As	4.40 ± 0.82	0.11 ± 0.18	**
Ca	2483.70 ± 240.33	2686.47 ± 156.18	NS
Cd	0.05 ± 0.04	0.03 ± 0.03	NS
Co	3.43 ± 0.21	0.00	**
Cr	0.00	0.00	NA
Cu	0.00	0.00	NA
Fe	704.92 ± 75.96	92.90 ± 5.24	**
Mg	3082.28 ± 274.06	3044.02 ± 205.24	NS
Mn	880.50 ± 83.02	8.18 ± 1.59	**
Ni	0.91 ± 0.20	0.73 ± 0.33	NS
Pb	3.09 ± 0.57	1.74 ± 0.26	**
Se	2.02 ± 0.81	2.30 ± 0.67	NS
Sr	18.53 ± 1.80	17.92 ± 1.29	NS
Zn	21.87 ± 1.93	22.46 ± 0.76	NS

Values are Mean ± SD of six samples.
NS: Not significant; NA: Not applicable.
* $p < 0.05$ significant differences for the same element.
** $p < 0.001$ significant differences for the same element.

metals may also exhibit seasonal variation and weakly bind to the suspended particulate fraction. The low concentration of chloride and decreased pH may enhance their solubility and mobility resulting to their increased bioavailability (Kamala-Kannan and Krishnamoorthy, 2006).

The two most relevant factors for seaweeds to accumulate metal are the metals bioavailability in the surrounding water and the uptake capacity of seaweeds including surface reaction in which metals are absorbed through electrostatic attraction to negative sites and active uptake in which metal ions are transported across the cell membrane into the cytoplasm (Sánchez-Rodríguez *et al*., 2001). The high concentration of metals in observed samples may therefore reflect the high bioavailability of metals and the capacity of the seaweeds to accumulate metals. By referring to metal bioavailability, higher concentration of heavy metals in field cultured samples may due to the presence of related metals in the surrounding sediments. During monsoon season, metal concentrations were found to be higher in sediment as compared to other seasons (Kamala-Kannan *et al*., 2008). The heavy rainfall during monsoon may lead to the fluvial inputs carrying excess metals. The increased level of organic compounds, nutrients, phosphate and decreased salinity may also enhance the formation of metal complexes and their deposition in sediments by reducing their mobility (Kamala-Kannan *et al*., 2008).

Some metal concentration in seaweeds depends on the growth dynamics and geochemical processes of seaweeds in different stage of life cycle. The ideas that metal concentrations decrease in macroalgae during periods of growth and increase during the dormant period in winter have long been considered (Villares *et al*., 2002). Explanation for higher metal accumulation during growth period may due to the higher rate of metabolic processes such as photosynthesis and respiration. Furthermore, increased metal concentration during monsoon and post-monsoon periods may attributable to the presence of high amount of young individual seaweeds with high adsorption rate (Kamala-Kannan *et al.*, 2008). Different seaweeds may exhibit different affinity for heavy metals as a result of different reasons including their cell wall structures with different amount and composition of polysaccharides. In some cases, the chemical elements content in seaweeds may appear to be more influenced by the environmental parameters of sampling site such as salinity, temperature, pH, light, nutrient concentration and oxygen (Zbikowski *et al*., 2006).

Current findings revealed that tissue cultured seaweeds have lower content of heavy metals thus a better source of uncontaminated raw materials for sustainable farming and various industries. However it is passionately debated if the tissue culture *(in vitro)*

Table 3. Comparison of As, Cd and Pb concentrations with determined standards

Metal	Sample	Concentration (mg kg^{-1})	JECFA*	France**
As	Field cultured *K. alvarezii*	4.63 ± 0.23	< 3 mg kg^{-1}	≤ 3 mg kg^{-1}
	Tissue cultured *K. alvarezii*	0.34 ± 0.49		
	Field cultured *G. changii*	4.40 ± 0.82		
	Tissue cultured *G. changii*	0.11 ± 0.18		
Cd	Field cultured *K. alvarezii*	0.69 ± 0.04	< 2 mg kg^{-1}	≤ 0.5 mg kg^{-1}
	Tissue cultured *K. alvarezii*	0.003 ± 0.005		
	Field cultured *G. changii*	0.05 ± 0.04		
	Tissue cultured *G. changii*	0.03 ± 0.03		
Pb	Field cultured *K. alvarezii*	0.00	< 2 mg kg^{-1}	≤ 5 mg kg^{-1}
	Tissue cultured *K. alvarezii*	1.52 ± 0.41		
	Field cultured *G. changii*	3.09 ± 0.57		
	Tissue cultured *G. changii*	1.74 ± 0.26		

* JECFA: 57th Joint FAO/WHO Expert Committee on Food Additive (2001).
** France: Health Council and National Medicine Academy of France.

method is economical and can cater for the biomass required by the industries. In the foremost place, tissue cultured seedlings have proved to have higher growth rate and reduced epiphyte after outplanting in the field compared to farm cultivated seaweeds (Dawes *et al.*, 1993, 1994; Yong, Chin, Thien *et al.*, 2014). Tissue culture environment and the enrichment of culture media in the early stage of propagation have demonstrated production of healthy seedlings with higher nutritional values (Yong *et al.*, 2015). The carrageenan derived from the tissue cultured seaweeds also exhibited the similar chemical structure of kappa-carrageenan obtained from their wild types (Thien *et al.*, 2016). The economy efficiency (cost effective) of a seaweed farming system with incorporation of production technology including tissue culture and micropropagation techniques can be measured by its amortised capital cost, per unit of seaweed production (Hurtado *et al.*, 2015). Production of seaweed relied on seawater quality, monsoons, and seaweed quality that affecting growth rate of the seaweeds and the number of cultivation cycles per year. The main advantage of seaweed tissue culture is the production of high quality and uniform planting material on a year-round basis under a controlled environment with disease-free conditions. Hence, the number of cultivation cycles per year could be increase and subsequently enhance the productivity of farming as sustainable source for various seaweed-related industries. This technology is expected to have an increasing impact on crop improvement and commercial advantages.

Conclusion

Present study revealed that both tissue cultured *K. alvarezii* and *G. changii* contain lower concentration of heavy metals especially As, Cd and Pb. The information provided in this study may be of benefit to the seaweed related industries for the appropriate source of raw materials, consumers for the better quality of seaweed demands, and seaweed growers/ farmers for the selection of improved planting materials and the alert of harmful heavy metals existence in the field.

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