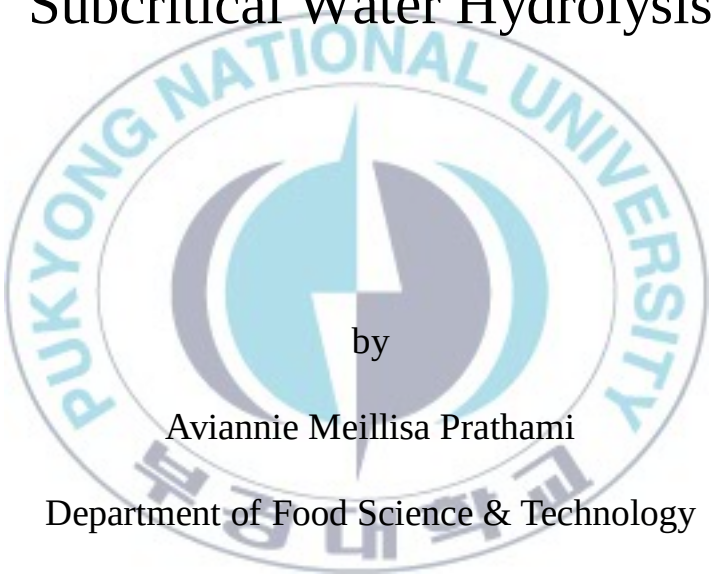
Thesis for the Degree of Master of Engineering

Production of Monosaccharides and Antioxidants from Purified Alginate and Brown Seaweed (*Saccharina japonica*) by Subcritical Water Hydrolysis



by

Aviannie Meillisa Prathami

Department of Food Science & Technology

The Graduate School

Pukyong National University

February 2014

Production of Monosaccharides and Antioxidants from Purified Alginate and Brown Seaweed (*Saccharina japonica*) by Subcritical Water Hydrolysis

(아임계 수 가수분해를 이용하여 정제된 알지네이트와 갈조류(*Saccharina japonica*)로부터 단당류 및 항산화물질 생산)

Advisor: Prof. Byung-Soo Chun

by
Aviannie Meillisa Prathami

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

in Department of Food Science and Technology, The Graduate School,
Pukyong National University

February 2014

Production of Monosaccharides and Antioxidants from Purified Alginate and Brown Seaweed (*Saccharina japonica*) by Subcritical Water Hydrolysis

A dissertation

by

Aviannie Meillisa Prathami

Approved by:

_____
Yang-Bong Lee, Ph. D.

(Chairman)

_____
Ji-Young Yang, Ph. D.

(Member)

_____
Byung-Soo Chun, Ph. D.

(Member)

February, 2014

Contents

List of Tables

List of Figures

아임계 수 가수분해를 이용하여 정제된 알지네이트와 갈조류(*Saccharina japonica*)로부터 단당류 및 항산화물질 생산

Aviannie Meillisa Prathami

부 경 대 학 교 대 학 원 식 품 공 학 과

요약

거대 해조류는 아직 탐구되지 않은 유용물질들이 많으므로 중요한 해양생물이다. 갈조류의 주요 구성성분은 탄수화물(50-65%), 단백질(10-20%), 지질(1-10%)로 종류마다 조금씩 다르다. 알지네이트는 선형 이형다당류로 갈조류에서 생성되며 건조중량의 40%를 차지한다. 알지네이트는 중요한 성분으로써 많은 연구와 여러 분야에서 사용되고 있다. 가치 있는 화합물을 얻기 위해서는 알지네이트를 단량체 분해를 해야 하는데, 예전에는 주로 유기용매나 핵산, 메탄올 또는 에탄올을 사용하는 방법으로 하였다. 그러나 최근에는, 유해한 화학 용매의 발생을 막기 위해 친환경적인 방법을 사용하고 있다. 임계가수분해법은 친환경적인 방법 중 하나로 용매를 주로 물을 이용한다. 짧은 시간에 분해된 생성물을 평가하기 위해 갈조류와 정제된 알긴산 나트륨에 아임계 수 가수분해를 사용하였다. 실험은 반응 온도를 위해 1.3-6.0MPa 와 180-260℃의 조건에서 실험하였다. 재료와 물의 비율은 1:25 이고 각 조건의 반응 시간은 3 분으로 유지하였다. 그 다음 실험과정을 위해 가수분해 공정후의 수용액을 준비하였다. 다시마나 정제된 알지네이트에서 높은 온도의 촉매를 추가할 때 수율이 더 높게 나타났다. 반면, 총 회수한 당과 환원당은 촉매를 사용하여 낮은 온도에서 실험을 했을 때, 다른 조건 하에서 실험한것 보다 더 높게 나타났다. 다시마의 항산화성이 180℃에서 촉매를 넣었을 때 정제된 알지네이트보다 더 높게 나타났으며, TPC, TFC, FRAP,DPPH 는 각각 9.63 ± 0.01 mg/L, 1.92 ± 0.05 mg/L, 3.11 ±

0.01 mg/L 와 97.17% 였다. 총 유기탄소와 분자량을 정하는데 있어 높은 회수를 위한 최적의 조건은 180℃에서 촉매와 함께 가수분해하는 것이었다. 아임계 수 처리를 통해서 탄수화물과 올리고당이 생성된 것을 MALDI-TOF 를 이용한 mass spectrum 에서 확인되었다.

180℃에서 촉매를 사용했을 때의 가수분해는 다시마와 정제된 알지네이트의 구조를 변화시키는데 있어서 효율적인 방법으로 보인다. 임계가 수 가수분해법은 복잡한 탄수화물 결합을 단순하게 분해하는데 효과적이었다. 아임계 수를 사용하여 생산된 가수분해물질은 유용한 바이오 화합물을 함유하고 있으므로 식품산업과 바이오산업에 적용될 수 있다.

Introduction

Marine macroalgae (seaweeds) are good potential sources of high biotechnological and high value interest due to production of a great diversity of their biological activities. They can be classified into three groups based on the basis of their pigmentation: green seaweed (chlorophyta), red seaweed (rhodophyta) and brown seaweed (phaeophyta). The industry of seaweed provides a wide variety of products that have an estimated total annual value of US$ 5.5-6 billion. Food products of human consumption contribute about US$ 5 billion of total annual value (McHugh, 2003).

Seaweed is an excellent source of magnesium, iron, sodium, calcium, iodine; an excellent source of vitamin A, B1, C, E, and K; low in fat and very low in calories. The main products of seaweed industry are alginates, agar and carrageenans, which have the ability to increase the viscosity of aquaeous solution, form gels, stabilize properties of water-based products and widely applied in medicine industry as wound healer (Perreira and Costa-Lotufo, 2011). The nutrient composition of seaweed varies and is affected by the species, geographic areas and seasons of the year and temperature (Manivannan et al., 2011).

Green seaweed usually appears green because of abundant chlorophyll a and b in the same proportion as the 'higher' plants. The other pigments of green seaweed are lutein and β-carotene. Red seaweed is probably well known for their economic values, for example as marine hydrocolloid sources of agars and carrageenans. Red algae contains a variety of pigments, including cholorophyll a, phycobiliproteins, red phycoerythrin, blue phycocyanin, carotenes, lutein, and zeaxanthin. Most of red seaweed is pink to deep red in color because their plastids contain large amounts of the red accessory pigment of phycoerythrin, which obscures chlorophyll a (Graham, 2009). Phycoerythrin is the most important pigment on red seaweed, this pigment reflect red light and absorb blue light. Brown seaweed has larger morphology than red and green seaweed. The brown seaweed size range from the large kelp is around 20 m. (Meinita, 2010).

Brown seaweed produces terpenoids, acetogenins and terpenoid-aromatic compounds of mixed biosynthetic origin as their most common secondary metabolites (Blunt et al., 2011). Most of brown seaweed contains fucoxanthin, which is responsible to give greenish-brown color. Moreover, it also has polyphenol i.e. phlorotannins, but in higher concentration mainly in species from temperate and polar region (Koivikko, 2008).

Alginates are quite abundant in nature science as a major structural component in brown seaweed (*Phaeophyceae*) and exist as the most abundant polysaccharide comprising up to 40% of the dry matter. Alginate is a linear hetero polysaccharide that consists of two unit monosaccharides, D-mannuronic acid (M) and L-guluronic acid (G) that occur in homo polymeric M blocks, homo polymeric G blocks and hetero polymeric random MG blocks (Haug A et al., 1967). The physical and chemical properties of alginate change with its molecular weight and M/G ratio that vary according to its origin. Pharmaceutical, food and technical applications (such as in print paste for the textile industry) are the main market areas for alginates.

Polysaccharides have been widely used as thickener agent, stabilizers in pharmaceuticals, cosmetics and as emulsifier in food products. There is also a large and growing potential for alginate in biotechnological applications (Draget KI et al., 2005). To obtain monomeric sugar from alginate, it must be prepared by breaking down the polysaccharide structure into simple monosaccharide. Therefore, to produce the appropriate monomeric sugar and other compounds, depolymerization treatment of alginate is needed.

Saccharina japonica belongs to the brown algae family phaeophyta which has abundance bioactive compounds. *S. japonica* is well known as good source of hydrocolloid agent such as alginate. Furthermore, it has lot of unexplored bioactive compounds which have high value on economical aspect and on human

live. Brown seaweed abundantly contains the polysaccharide such as alginate, mannitol and glucan (Rioux, 2007). Other important metabolites found in brown seaweeds are polyphloroglucinol phenolic compounds (Steinberg et al., 1991) and secondary metabolites such as terpenoids, acetogenins, terpenoid-aromatic compounds (Blunt et al., 2011) and carotenoids (e.g. fucoxanthin).

More recently, a growing area of inquiry has focused on seaweed substances as functional foods and possibility of renewable bioenergy production. There are several examples of pure compounds and crude extracts of seaweeds that exhibit biological activities. Therefore, we show examples of some of these activities, such as cytotoxicity, antiviral, antioxidant, antibacterial, neuromodulator, NAPDPH-dependent lipid peroxidation, antifungal, anthelmintic, anti-inflammatory, and anticancer activities (Table 1).

Polysaccharides and other metabolites in brown seaweed have been reported to have antimicrobial activities (Osman et al., 2009; Tuney et al., 2006; Nagayama et al., 2002), antifouling (Cho, 2012), antioxidant (Alstyne, 1994; Peng et al., 2012; Rajauria, 2010), anti-allergic (Samee, 2009), anticoagulant, anti-cancer (Go, 2010), antiviral activities (Pereira, 2011). Also brown seaweed is known as a new source of biofuel (Wargacki et al., 2012).

Depolymerization method of alginate has widely investigated. Several methods have been applied to adjust the molecular weight and G/M ratio. Some of those methods are acid hydrolysis (Haug et al., 1967), base hydrolysis,

enzymatic depolymerization (Rigouin et al., 2009), photolytic depolymerization (Burana-osot et al., 2009), and hydrothermal condition (Aida et al., 2010). Traditional methods usually use organic solvent such as ethanol, methanol or hexane on the extraction process (Osman et al., 2010; Demirel et al., 2009). The available traditional methods are time-consuming and remain organic solvent residue at the end of process. To solve the lack of the traditional methods, recently developed innovative and green methods have attracted more attention. The subcritical water technique is environmentally friendly and uses hot water for extraction. During subcritical water extraction, water is maintained in the subcritical state, between its boiling (100 °C and 0.10 MPa) and critical point (374 °C and 22 MPa), where it remains as a liquid due to the high pressure (King, 2000).

The purpose of this study was to obtain the optimum condition in depolymerization process of alginate and brown seaweed *S. japonica*. The parameters used in this work were total organic carbon, mannuronic and guluronic contents, total sugar, total reducing sugar, molecular weight and analysis of antioxidant activity.

Table 1. Different algae bioactive compounds

Seaweed	Bioactive compounds	Activity	Reference
Fucus evanescens	Fucoidan	• anti-tumor • anti-metastatic	Alekseyenko et al., 2007
Adenocystis utricularis	Galactofuran	inhibitory against HSV 1 and 2	Ponce et al., 2003
Laminaria japonica	Laminarin	anti-apoptotic	Kim et al., 2006
Undaria pinnatifida	Sulfated polysaccharide	anti-viral	Hemmingson et al., 2006
Ecklonia cava	Phlorotannin	• inhibitor of HIV-1 RT • whitening effect • anti-cancer	Artan et al., 2008 Ahn et al., 2004 Heo et al., 2009
Sargassum vulgare	Alginic acid	antitumor	de Sousa, Torres et al., 2007
Gracilaria edulis	Crude extract	antibacterial	Vallinayagam et al., 2009
Chondria atropupurea	Chondriamide B	anthelmintic	Davyt et al., 1998

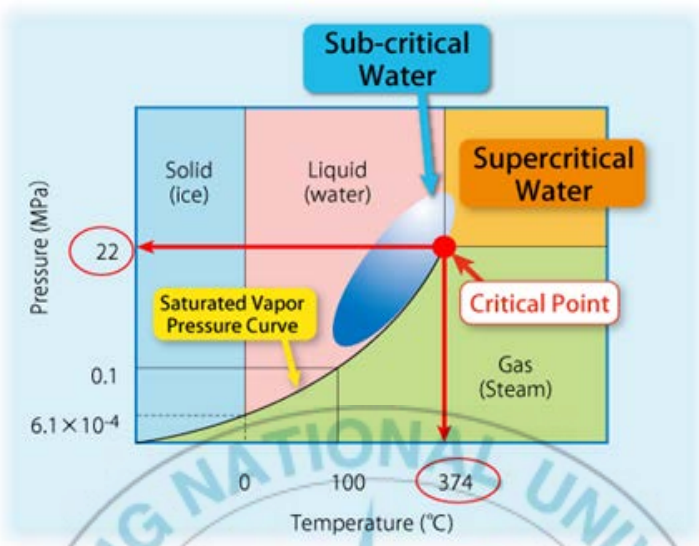


Fig. 1. The phase diagram of water as a function of temperature and pressure.

Materials and Methods

1. Materials

Brown seaweed *S. japonica* was collected from Guemil-eup, Wando-gun, Jeollanam-do, South Korea. Purified alginate from brown algae was provided by Sigma Aldrich (United Kingdom). High purity nitrogen gas (99.99%) was supplied by Kosem (Yangsan, Republic of Korea). Standards of 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, catechin, D-gulose and L-mannose high purity ≥98.0% and D-glucose were purchased from Sigma Aldrich Chemical (St. Louis, Mo. USA). Distilled water was used in this experiment. Formic acid, Folin Ciocalteu reagent, Na_2CO_3 anhydrous, $AlCl_3$, NaOH, $FeCl_3$, concentrated H_2SO_4, phenol, 3,5-dinitrosalycylic acid (DNS), potassium phosphate buffer, tricloroacetic acid and all other reagents used in this study were of analytical grade. Water for HPLC solvent was filtrated and degassed before used and purchased from Sigma Aldrich Chemical Co. (St. Louis, Mo. USA).

2. Sample preparation

Fresh *S. japonica* samples were washed with fresh water to dispose unused materials and then cut into small pieces. The small pieces of seaweeds were dried in freeze drier (Eyela FDU-2100, Tokyo Rikakikai Co., LTD, Japan) equipped by square-type drying chamber (Eyela DRC-1000, Tokyo Rikakikai Co., LTD, Japan) at temperature -80 ^{o}C for 3 days. The dried samples of *S. japonica* were collected into seal plastic bag. After then, the dried samples were finely ground by mechanical blender (PN SMKA-4000 mixer) and sieved by 710 μm stainless steel sieving mesh. The samples which pass through of the sieving mesh were stored at −20 °C for one day prior to use.

3. Subcritical Water Hydrolysis

The subcritical water hydrolysis was carried out in 200 cm^3 of a batch reactor made of 276 Hastelloy with temperature control (Fig. 2). Six grams of material samples were loaded into the reactor. The 1% formic acid which was used as a catalyst was suspended separately in 150 mL of distilled water. The reactor was then closed and heated by an electric heater to the temperature needed (180-260 ^{o}C). The pressures were estimated based on saturated steam to be between 15 to 65 bar for the temperature range studied. The temperature and pressure in the

reactor of each experiment were controlled by temperature controller and pressure gauge, respectively. The sample was stirred by 4 blades stirrer at 140 rpm. The reaction time for reaching the desired temperature took from 30 to 75 min. After rapid cooling to room temperature, the hydrolyzed samples from the reactor were collected and filtered using Whatman nylon membrane filter 0.45 μm and stored at 4 ^{o}C.

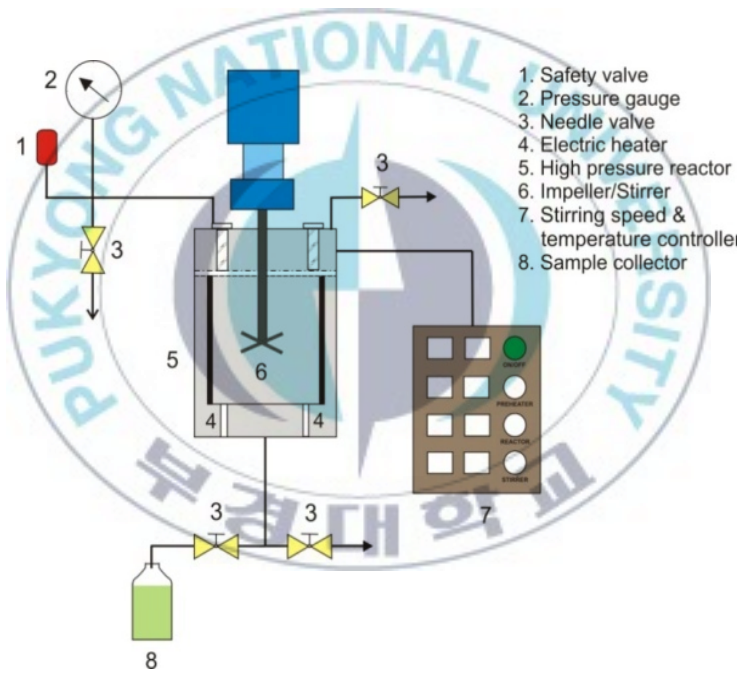


Fig.2. Schematic diagram of subcritical water hydrolysis.

4. Measurement of proximate composition

4.1. Measurement of moisture content

Proximate compositions such as the moisture content, crude lipid, crude protein and ash content of samples were measured. Moisture content was measured using AOAC (2000) methods with slight modifications. Two grams of samples was heated in oven at temperature 105 ^{o}C for an appropriate period and weighed. Weighing samples was stopped when the sample weight was constant. The moisture content was calculated using an equation:

$$\frac{w_1 - w_2}{w} x\ 100\%$$

w_1 is initial weight of samples and chamber (g), w_2 is final weight of samples and chamber (g) and w is initial weight of samples (g).

4.2. Crude lipid analysis

Crude lipids were conducted by AOAC (2000) method with slight modifications using Soxhlet system. Soxhlet boiling flask was dried for a recent time, cooled and balanced without touching it with fingers. Five grams of samples was put into a thimble and placed in the extraction apparatus. Petroleum ether was loaded into the flask at 2/3 of total volume. Heater was turned on and maintained the temperature at 105 ^{o}C for a period of times. When the measurement was finished, solvent was evaporated using a rotary evaporator and then weighed. The crude lipid content was calculated using the below equation:

$$\frac{w_1 - w_2}{w} x\ 100\%$$

w_1 is weight of flask with fat (g), w_2 is weight clean dry flask (g) and w is weight of samples (g).

4.3. Crude protein analysis

Crude protein was determined based on AOAC (2000) method using Kjedahl system. This method evaluates the total nitrogen content of the sample after it has been digested in sulphuric acid with a mercury or selenium catalyst. One grams of samples was loaded into Kjedahl's flask followed the addition of 10g potassium sulphate, 0.7 g mercuric oxide and 20 ml concentrated sulphuric acid. The flask was placed at an angle in the digester, brought to boiling point and retained until the solution was clear. The 90 mL of distilled water was added to cool the mixture followed of 25 mL sodium sulphate solution and stirred. One glass bead and 80 mL of 40% sodium hydroxide solution were added and the flask was kept tilted. The flask was rapidly connected to the distillation unit, heated and collected 50 mL of distillate containing ammonia in 50 mL of indicator solution. At the end of distillation, the receptor flask was removed, rinsed and titrated with the standard chlorhydric acid solution.

The crude protein contents were calculated using the below equation:

$$\text{Nitrogen content in sample (\%)} = \left(\frac{A \times B}{C} \times 0.014\right) \times 100\%$$

$$\text{Crude protein content in sample} = \text{nitrogen in sample (\%)} \times 6.25$$

A is chlorhydric acid used in titration (ml), B is normality of standard acid and C is weight of sample (g). 6.25 is the nitrogen conversion factor and 0.014 is the molar mass of nitrogen.

4.4. Measurement of ash content

The ash determination was done using a method described by AOAC (2000) with slight modifications. Two grams of samples was placed in a crucible which previously weighted until it reached the constant weight. The crucible was placed in a furnace of 550 ^{o}C for 10-15 hours or until the sample color changed into white. In the last of analysis, crucible with sample was weighted to obtain the ash content. The calculation of ash contents was obtained by the below equation:

$$\frac{w_1 - w_2}{w} x\ 100\%$$

w_1 is initial weight of crucible with sample (g); w_2 is final weight of crucible with sample; w is weight of sample (g).

5. Measurement of total glucose

The total glucose measurement was carried out by phenol sulphuric acid method (Dubois, 1956). D-glucose was used as standard (Fig. 3) to make calibration curve. The 0.75 mL sample was mixed with 2.25 mL of concentrated sulphuric acid. Then 0.45 mL of 40% phenol were added and heated in water bath. The mixture was cooled at room temperature and the absorbance was measured by using a spectrophotometer at 490 nm.

6. Measurement of reducing sugar

Reducing sugar analysis was conducted by 3,5-dinitrosalicylic (DNS) acid method (Miller, 1959). The reagents were made by mixing 10 g NaOH and 700 mL water until the mixture dissolved perfectly. Then, 300 g of potassium sodium tartrate added and followed by 10 g DNS acid. After all of the mixtures were fully dissolved, 0.5 g sodium sulfite was added and followed by 2 g phenol. The volume of mixture was adjusted to 1000 mL and kept away from light. The analysis of reducing sugar was done by mixing 0.5 mL of hydrolysate water and 0.5 mL of reagents. The mixture was heated for 10 minutes and 5 mL of cold water was immediately added. The absorbance was measured at 540 nm.

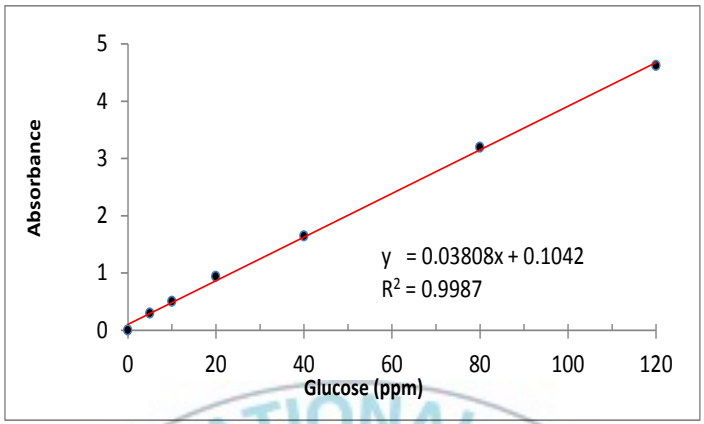


Fig. 3. Calibration curve of total glucose.

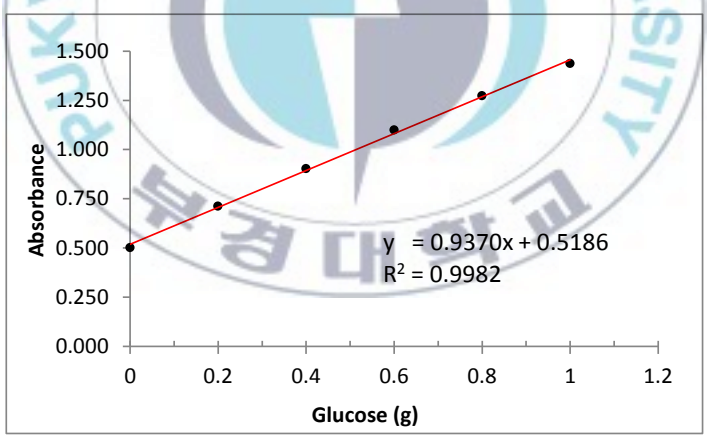


Fig. 4. Calibration curve of reducing sugar.

7. Molecular weight determination by viscometer

Molecular weight determination was analyzed using a viscometer equipped with a temperature sensor. The spindle SC4-18 with 200 rpm rotation was used to measure the viscosity. Hydrolysate sample was loaded into the viscometer chamber and waited for a second until the viscosity value was appeared. The average of hydrolysate temperature was used to determine water viscosity in the same temperature as the average.

Viscosity values of hydrolysate water were calculated using Mark-Houwink equation:

$$\eta = 0.00485 * Mw^{0.97}$$

η is apparent viscosity of the hydrolysate water (mL/g) and Mw is molecular weight (Da).

8. pH measurement

pH of hydrolysate water was measured by Mettler Toledo Five Easy Plus pH meter at 20 ^{o}C. The adjustment of pH meter was done before measurement by technical buffer solution at pH 4.01±0.02, 7.00±0.02 and 9.21±0.02.

9. Measurement of total organic carbon

Total organic carbon (TOC) of hydrolysate water was measured using TOC analyzer. The analysis was done using 80 ppm of hydrolysate water of each condition and temperature 180 ^{o}C. Total organic carbon parameters were described in Table 2.

10. MALDI-TOF MS analysis

Direct measurement of molecular weight and compound identification in the sample were confirmed with matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF MS). Samples were prepared by mixing 0.5 µL of the product and 0.5 µL of the matrix solution. The matrix solution was prepared by mixing a norharmane–acetonitrile solution and trifluoroacetic acid with ratio of 7:3. The norharmane–acetonitrile solution was prepared by mixing norharmane (10 mg) with acetonitrile (1 mL). Aliquots were placed on a stainless steel sample plate and then ionized by smart beam laser pulses. MALDI-TOF parameters conditions were described in Table 3.

Table 2. Operating conditions of total organic carbon analyzer

Parameters	Conditions
Instrument	Shimadzu (Japan), TOC-Vcph, SSM
System	TOC-V
Detector	Combustion
Catalyst	Regular sensitivity
Cell length	Long

Table 3. Operating conditions of MALDI-TOF MS

Parameters	Conditions
Instrument	Bruker Autoflex III
Ion mode	Positive
Laser	Smart beam
Nd	YAG laser
Voltage	19.98 kV
Matrix	2, 5 Dihydroxybenzoic acid (DHBA)
Solvents	Acetonitrile : water (75:25)

11. Gulose and mannose quantification by HPLC

The gulose and mannose was quantified by high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD). The HPLC analysis was carried out by a Jasco HPLC (Easton, USA) model 400 equipped of ChromNav analysis software. Nitrogen of high purity (99.99%) (KOSEM Co. Yangsan, Korea) was used as a carrier gas in this experiment. Shodex (Japan) sugar SP0810 with dimension 8x300 mm was used as column to analyze gulose and mannose. Hydrolysate water was diluted 4 times using filtrated and sonicated water HPLC grade. The column was thermostated with a Waters column heater model at 80 ^{o}C. The water used for elution in this analysis was filtrated using Whatman nylon membrane filter 0.45 µm and sonicated. The flow rate of eluent was maintained at 0.6 mL/min. Gulose and mannose standard (purity > 98%) was purchased from Sigma Aldrich (United Kingdom). The calibration curves of mannose and gulose were depicted on Fig. 5 and 6.

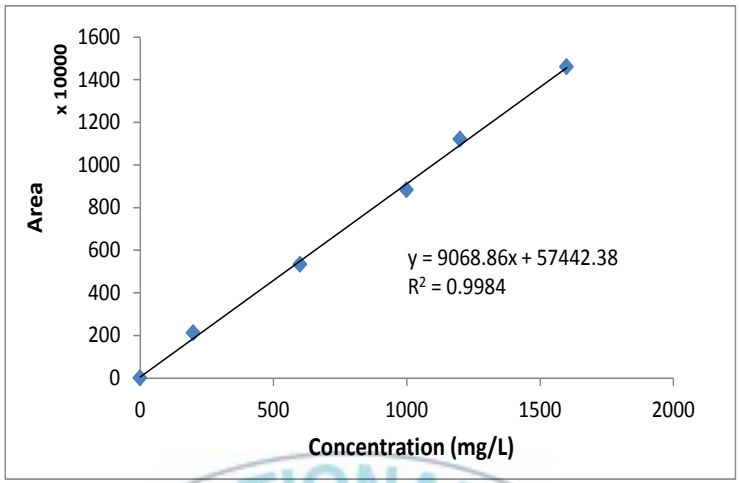


Fig. 5. Calibration curve of D-mannose standard.

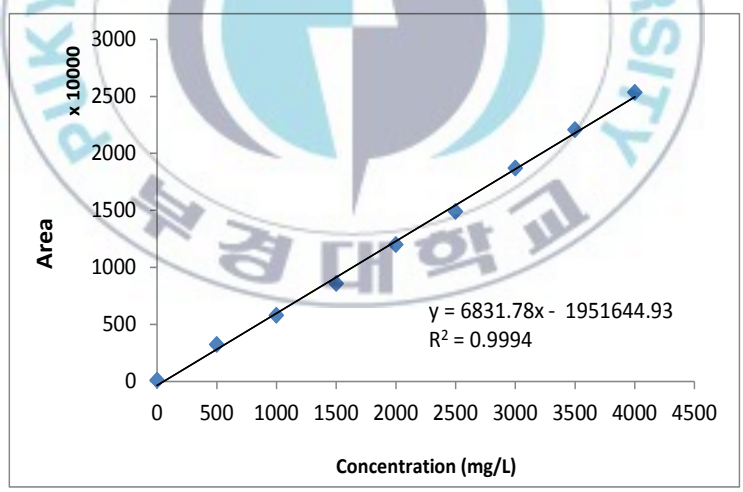


Fig. 6. Calibration curve of L-gulose standard.

12. Antioxidant properties

12.1. DPPH radical scavenging activity assay

DPPH assay was determined by using the method of Sharma et al (2009) with slight modification. The 0.1 mL of samples was mixed with 3.9 mL of methanolic DPPH 50 µM. The mixture was vortexed for 1 min, wrapped in aluminum foil and kept at 30 ^{o}C for 30 min in dark room. The absorbance of negative control was measured by replacing sample with methanol (100 µL). Spectrophotometric measurements of hydrolysate water and negative control were done at 517 nm (UVmini 1240, Shimadzu Co., Japan). All tests were carried out in triplicate. The DPPH radical-scavenging activity (%) was calculated using the below equation:

$$\text{Scavenging activity} = [1- (As/Ac)] \times 100\%$$

As is absorbance of crude extract at 517 nm and Ac is absorbance of blank at 517 nm.

12.2. Ferric-Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out using Chu et al (2000) method with slight modification. The 2.5 mL of 0.1 M potassium phosphate buffer (pH 6.6) was mixed with 2.5 mL of 1% potassium ferricyanide. Then, 1 mL of sample was mixed and incubated at 50 ^{o}C for 20 minutes. The 2.5 mL of 10% tricloroacetic acid, 2.5 mL water and 0.5 mL of 0.1% of iron (III) chloride were added into the mixture. The mixture was incubated in room temperature for 30 minutes and the absorbance was measured using a UV spectrophotometer at 700 nm. Gallic acid was used as a standard solution (Fig. 7).

12.3. Total Phenolic Content (TPC)

TPC of hydrolysate water was determined with Folin-Ciocalteu reagent, according to the method of Chew et al (2011) with slight modifications. The 1 mL of diluted samples was mixed with 1 mL of Folin-Ciocalteu reagent (1:10). The mixture was incubated at room temperature for 4 minutes. After then, 0.8 mL of 7.5% sodium carbonate anhydrous solution was added and incubated in dark room for 2 hours. The absorbance was measured at 765 nm. Gallic acid dissolved in ethanol was used as a standard (Fig. 8).

12.4. Total Flavonoid Content (TFC)

TFC of hydrolysate water was determined using a method described by Ozsoy et al (2007) with slight modifications. The 0.38 mL of hydrolysate water was added with 1.875 mL of water, followed by 112 µL of 5% sodium nitrite. The mixture was then incubated and followed by the addition of 225 µL of 10% aluminium chloride. The 0.75 mL of 1 M sodium hydroxide and 0.41 mL distilled water were added. The absorbance was measured at 510 nm. Catechin was used as a standard (Fig. 9).

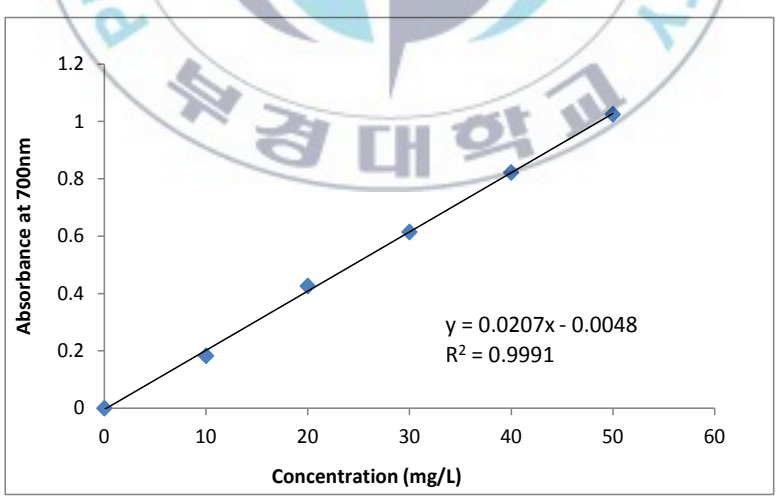


Fig. 7. Calibration curve of gallic acid standard for FRAP.

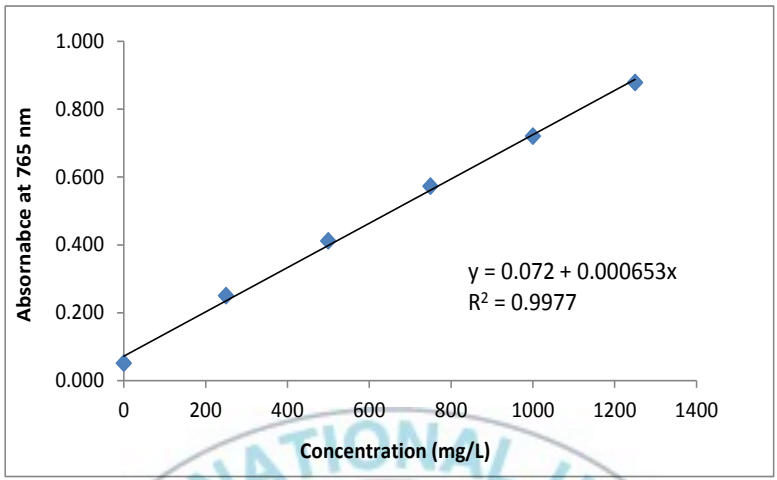


Fig. 8. Calibration curve of gallic acid standard for TPC.

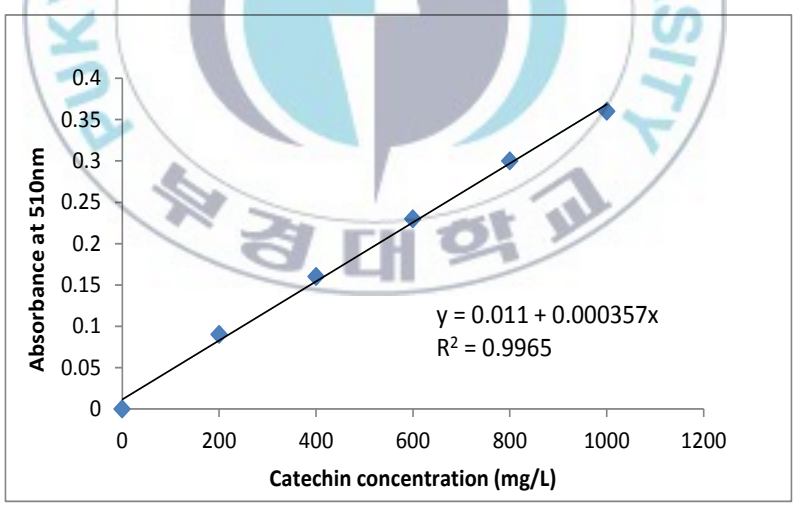


Fig. 9. Calibration curve of catechin standard for TFC.

Results and Discussion

1. Proximate analysis of *S. japonica*

Proximate analysis was done to measure the general composition of *S. japonica*. The proximate analysis is the vital information of a research to identify and analyze the relationship between proximate compositions and other compounds inside. The proximate analysis of *S. japonica* was shown in table 4. Based on the data of proximate analysis, carbohydrate was found as the highest compound in *S. japonica*, approximately 63.32%, followed by ash 20.62%.

The majority of edible marine macro algae are concentrated primarily in the ash (8.4-43.6% dry weight) and not particularly rich in lipids (0.92-5.2%). *Phaeophyceae* algae species are generally noted to have lower protein contents compared with others (Yuan, 2008). The variability affect in the proximate composition of the same algae species are not only between studies conducted on samples, but also within the same study on samples collected at different times of the year (Hagen-Rodde et al., 2004). As the photosynthetic organisms, the

proximate substituents of marine macro algae were affected by water temperature, nutrient concentration in the sea water and irradiation during growth, which are all dependent on geographical location and season (Yuan, 2007).

Table 4. Proximate analysis of *S. japonica* samples

Contents	Amount (%)
Carbohydrate	63.32
Crude lipid	1.15
Crude protein	9.12
Ash	20.62
Moisture	5.33

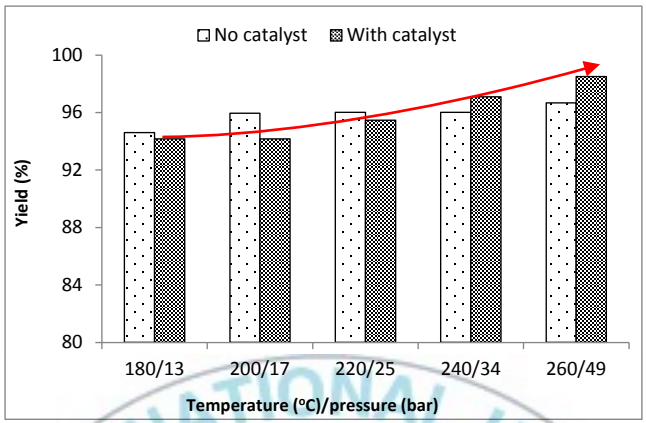
2. Hydrolysis yield

Hydrolysis yield was measured to analyze the experiments conditions which resulted high effectiveness of hydrolysate water production. In this study, higher temperature was used and higher yield was attained. The highest hydrolysis was obtained in 260 ^{o}C temperature with catalyst added. Hydrolysis rate on the

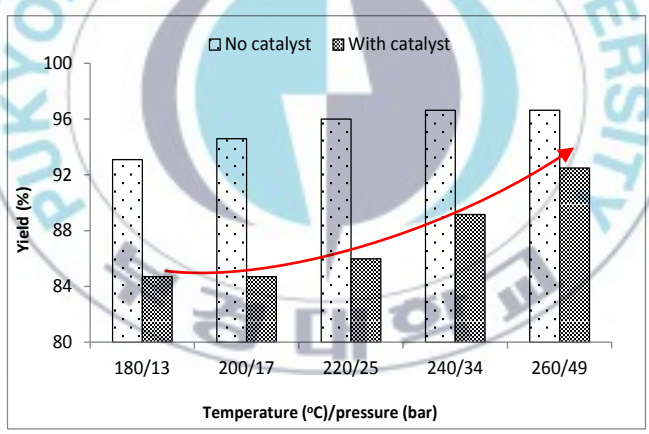
treatment with no catalyst added was bigger on early stages (180 and 200 ^{o}C), but at next stages (240 ^{o}C and so on), the hydrolysis rate was almost steady states.

The addition of catalyst in subcritical water hydrolysis system was proven to increase the hydrolysis rate. Obviously, at relatively higher temperatures, the amount of solid was reduced by increasing the hydrolysis and decomposition of the sample. The high yield produced by subcritical hydrolysis was obtained because of the difference on the density and polarity of water as a main solvent in this system. Water becomes less polar and show dramatic increase in solubility as temperature rises (King, 2000).

Higher hydrolysis yield means that smaller residue is obtained from hydrolysate water. The changing form from sample into hydrolysate water was occurred during subcritical state. Subcritical state was occurred on the temperature over than 121 ^{o}C with pressure more than 0.1 MPa. Higher temperature was applied resulted in the changes of physical properties of water, such as it behaves like non-polar organic solvent (similar with acetone), thus it can substitute for some of organic solvents, become a clean medium for chemical reactions, density fluctuation, low dielectric properties and molecular clusters, that would give the impact on decomposition of organic matter in samples (Cheng et al., 2008).



(a)



(b)

Fig. 10. Percentage of hydrolysis yield (%) on different conditions: (a) purified alginate hydrolysis yield (b) *S. japonica* hydrolysis yield.

3. Reducing sugar analysis

Reducing sugar was examined using DNS method (Miller, 1959) with slight modifications. D-glucose was used as a standard and diluted with distilled water on different concentrations. The absorbance of the mixture was measured using a spectrophotometer at 540 nm.

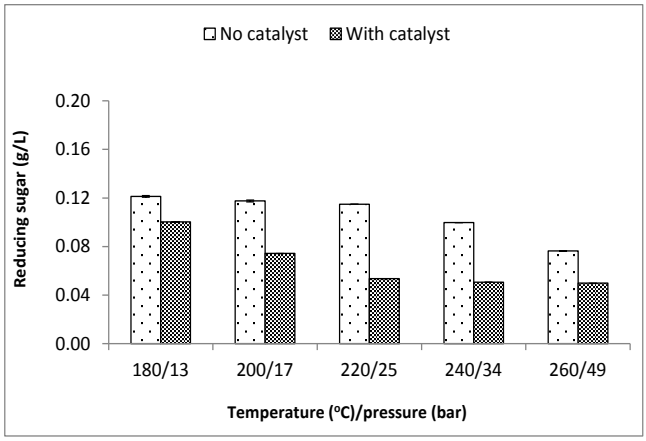
Reducing sugar contains aldehyde groups that are oxidized to carboxylic acids. It has an open chain form with an aldehyde groups or a free hemiacetal group. The kinds of sugar which has aldehyde group in the chain are glucose, mannose, galactose, arabinose, maltose and glucose. *S. japonica* comprises of 70% carbohydrates. Typical carbohydrates in *S. japonica* consist of fucoidan, laminaran (β-1.3-glucan), cellulose, sugar and alginates. The decomposition of carbohydrate into a reducing sugar, monosaccharide and polysaccharide offers economic value of the material itself.

Many works have been done to convert carbohydrate of marine macroalgae into monosaccharides and other compounds. Alnaief et al., (2011) suggested the possibility of alginate as a nanoporous biodegradable material, Meinita (2010) has been done to produce bio-ethanol by fermentation of the commercial yeast of *Saccharomyces cerevisiae* and Fitton et al., (2007) made a new invention to

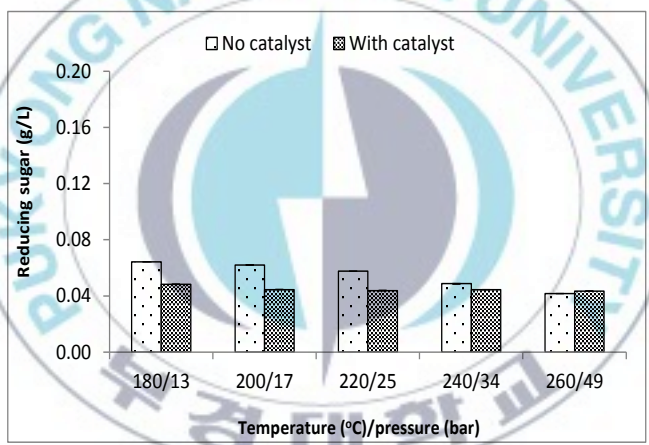
produce marine cosmetics using fucoidan fractions and phloroglucinol. Glucose, mannose and galactose in brown seaweed belong to reducing sugar groups and they are very essential compounds that can be converted into valuable intermediate products.

Reducing sugar on alginate was found higher than *S. japonica*, with approximately 0.04-0.12 g/L. Purified alginate consists of high purity (95%) of alginate and at elevated temperature, alginate will be converted into simpler chain. *S. japonica* is composes of oligosaccharide, fat, protein, mineral and other compounds inside. Moreover, the presence of other components in subcritical water hydrolysis affects the decomposition rate of carbohydrate (Kruse and Dinjus, 2007).

Reducing sugar of purified alginate at 180 ^{o}C with no catalyst showed higher values than other experiment conditions (0.12 g/L). When the higher temperature was applied in this process, the recovery of reducing sugar in hydrolysate water showed a decreasing trend. It means that the reducing sugar substances from hydrolysate water were not stable in the high temperature.



(a)



(b)

Fig. 11. Percentage of reducing sugar (g/L) on different conditions: (a) purified alginate (b) *S. japonica.*

4. Total glucose analysis

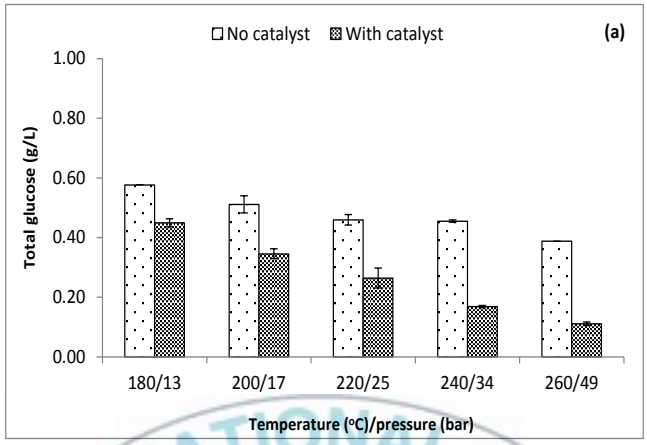
Efforts at the discovery of sustainable bioenergy are a big concern of most countries during this time. Our dependency on fossil fuels for energy production is problematic. Biomass (biofuel, biogas, bioethanol, *etc*.), one of renewable energies, is considered to be carbon-neutral and it is the only organic matter among renewable energies. In other words, fuels and chemicals can be produced from biomass in addition to electricity and heat. Bioethanol production from marine products is one of the most potential renewable energies. Bioethanol will be attained from the fermentation of monosaccharide in marine products by yeast.

In this work, total glucose of hydrolysate water was analyzed using phenol sulphuric acid method (Dubois, 1956) with slight modifications, to check the possibility of sugar which will be converted to ethanol. Total glucose in the hydrolysate water was calculated using the D-glucose as a standard on different concentrations from 0 to 120 ppm and the absorbance was measured at 490 nm.

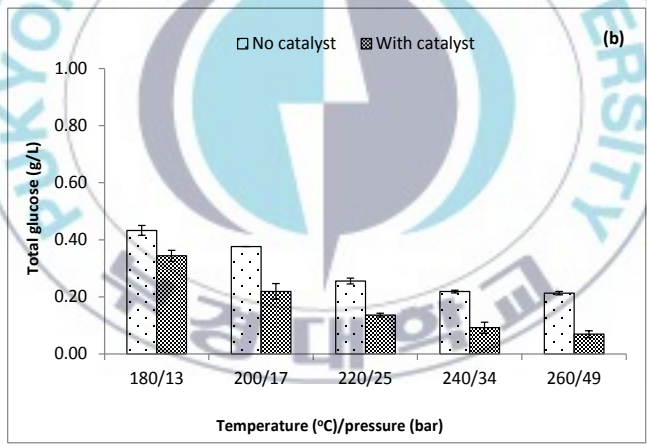
The subcritical water technique has a wide range of applications, such as extraction and wet oxidation of organic compounds (Kruse and Dinjus, 2007). This technique uses hot water with temperature ranging from 100 to 374 ^{o}C and

high pressure to maintain water in the liquid state. Under these conditions, the characteristics of water change. This leads to a lower solvent viscosity, surface tension and dielectric constant.

The carbohydrate content in samples could be converted into a wide range of water-soluble sugars (such as poly-, oligo-, and mono-saccharides) by hydrolysis under subcritical water conditions. Figure 12 shows the amount of total glucose as a function of temperature. The highest total glucose was achieved in 180 ^{o}C hydrolysis temperature without catalyst either in purified alginate or in *S. japonica*. Total glucose of purified alginate was higher yield (0.58 g/L) than that of *S. japonica* (0.43 g/L). Differentiation of total soluble sugar in hydrolysate water is caused by the change of water physical properties during subcritical state (Daneshvar et al., 2012). Higher temperature applied in this work showed the decreases of total sugar in all conditions. Obviously, water-soluble sugars are on of attractive or important factors for fermentation reactions. Therefore, subcritical water treatment could play an important role as a pretreatment of marine bioethanol production.



(a)



(b)

Fig. 12. Total glucose contents (g/L) in different conditions: (a) purified alginate (b) *S. japonica.*

5. Molecular weight determination by a viscometer

Molecular weight was determined by using apparent viscosity which was measured by a viscometer. Spindle was SC4-18 with Spindle Multiplier Constant (SMC) values (3.2) and torque constant (TK) (0.09373). The determination was calculated using the following equation: $\eta = 0.00485 * Mw^{0.97}$, where η is viscosity was measured by viscometer and Mw is molecular weight.

The raw alginate solution had an average molecular weight of 4.2×10^5 Da. Molecular weight of the products showed lower value than that of the raw alginate solution. The lowest molecular weights were found in the conditions with catalyst addition either in purified alginate or in *S. japonica*. Depolymerization rate was slowest at 180 ^oC hydrolysis temperature. After then at temperature 200 ^oC and so on, the rate increased as temperature raised. Molecular weight distribution of the products shifted towards lower values as reaction time increased. The determination of molecular weight is very important as a basic criterion to get the best condition of mass spectrometry analysis.

6. Total organic carbon analysis

Total organic carbon in the hydrolysate water was determined to evaluate carbon recovery in the liquid phase. The decrease of carbon recovery indicated that hydrothermal treatment produced carbon products that were not soluble in liquid phase. Higher temperature was used, lower total organic carbon was obtained. Based on the data described on Table 5, the addition of catalyst in this process gives the significant results on carbon recovery in liquid phase.

Amount of total carbon in each condition describe rate of decomposition of oligosaccharides in purified alginate or *S. japonica*. When the lowest total carbon was obtained in a condition, it means that the highest decomposition rate was reached in the condition. In this study, the lowest total carbon was obtained at 180 ^{o}C hydrolysis temperature, and it showed the decreasing trend when higher hydrolysis temperature was applied. Decomposition of carbohydrate into oligosaccharides and monosaccharides were found elevated in the hydrolysis condition with temperature more than 240 ^{o}C and pressure 34 bar.

Table 5. Molecular weight determination of each condition by using viscometer

(Unit: Da)

Temperature (°C)/ pressure (bar)	**Purified Alginate**		***S. japonica***	
	Catalyst	No catalyst	Catalyst	No catalyst
180/13	1735	6295	2135	2706
200/17	1695	2695	2135	2706
220/25	1615	1670	1874	2367
240/34	1299	1299	1834	2315
260/49	1086	1078	1795	2263
Before treatment	9.8×10^7		5.4×10^6	

Table 6. The total amount of organic carbon contained in hydrolysate water

(Unit: mg/L)

Temperature (°C)/ pressure (bar)	**Purified Alginate**		***S. japonica***	
	Catalyst	No catalyst	Catalyst	No catalyst
180/13	27.47	20.96	27.23	20.53
200/17	27.43	20.82	26.24	20.2
220/25	25.68	20.31	24.74	20.01
240/34	17.24	19.49	24.61	19.77
260/49	15.84	17.25	23.73	18.55

7. Hydrolysate mass spectrum

Direct measurement of molecular weight of compounds in hydrolysate water was confirmed using MALDI-TOF mass spectrometer. MALDI-TOF mass spectrometry analyses of several target compound material such as protein, lipid and carbohydrate. Result of mass spectrum in MALDI-TOF was affected by the sample preparation method and matrix was used. The function of the matrix is adsorption of energy from laser pulse, and then transfers to sample, thereby causing desorption of the target molecules in an expanding plume, to ionize the desorbed target molecules and to prevent aggregation of the target molecules. The 2,5-dihydroxybenzoic acid (DHB) was photoionized at different irradiances of a UV laser and widely used in carbohydrate analysis. The characterization of hydrolysate water by mass spectrum was explained in Table 7 and 8.

Fig. 13 and 14 shows a representative MALDI-TOF mass spectrum of the product solution after hydrolysis process. Based on the whole chart of MALDI-TOF mass spectrum, high intensity readings were obtained at 575, 643, 806, 822 and 1222 m/z. These results showed that oligosaccharides were produced during the hydrothermal process. It means that hydrothermal liquefaction succeeds to break down alginate structure from polysaccharides into oligosaccharide and also

produce new other substances such as the compounds which belong to polyphenol and flavonoids group.

Table 7. Mass spectrum of purified alginate hydrolysate water

Compounds	Chemical formula	Molecular weight
GlcNAc2Man2XylManGlcNAcFucGlcNAc	$C_{61}H_{102}N_4O_{44}$	1594.58
GalGlcNAc2Man3GlcNAcFucGlcNAc-II	$C_{62}H_{104}N_4O_{45}$	1624.59
GlcNAcMan4GlcNAcGulManGlcNAc2	$C_{62}H_{104}N_4O_{46}$	1640.59
Man7GlcNAc2-I	$C_{58}H_{98}N_2O_{46}$	1558.53
GalGlcNAc2Man2GlcNAcManGlcNAcFucGlcNAc-I	$C_{70}H_{117}N_5O_{50}$	1827.67
GalGlcNAc2Man2GlcNAcManGlcNAc2-II	$C_{64}H_{107}N_5O_{46}$	1681.61
Gal2GlcNAc2Man3GlcNAcFucGlcNAc	$C_{68}H_{114}N_4O_{50}$	1786.65

Abbreviations :
GlcNAc (N-acetylglucosamine); GalNAc (N-acetylgalactosamine); Man (Mannose); Fuc (Fucose); Gal (Galactose); Xyl (Xylose); Gul (Gulose)

Table 8. Mass spectrum of *S. japonica* hydrolysate water

Compounds	Chemical formula	Molecular weight
GalGlcNAc2Man2GlcNAcMan GlcNAc2-II	$C_{64}H_{107}N_5O_{46}$	1681.61
GlcNAc2Man2GlcNAcManGlc NAcFucGlcNAc	$C_{64}H_{107}N_5O_{45}$	1665.62
Man5GlcNAc2-I	$C_{46}H_{78}N_2O_{36}$	1234.43
4-GlcNAc3Man3GlcNAc2	$C_{58}H_{97}N_5O_{41}$	1519.56
Man6GlcNAc2-I	$C_{52}H_{88}N_2O_{41}$	1396.48
GalGlcNAcMan3GlcNAc2-I	$C_{48}H_{81}N_3O_{36}$	1275.45

Abbreviations :
GlcNAc (N-acetylglucosamine); GalNAc (N-acetylgalactosamine); Man (Mannose); Fuc (Fucose); Gal (Galactose); Xyl (Xylose); Gul (Gulose)

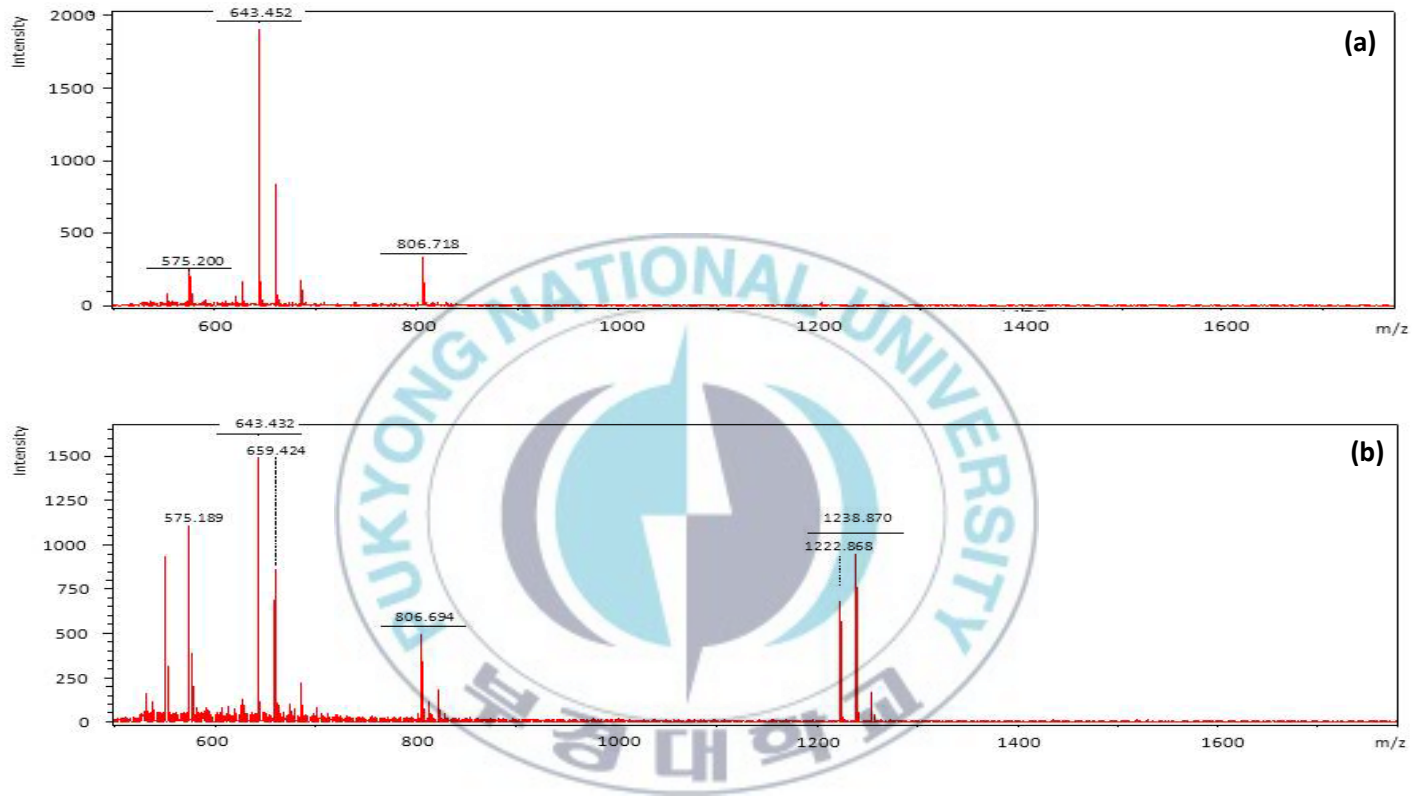


Fig. 13. MALDI-TOF mass spectrum of purified alginate hydrolysate water at 180 ^{o}C (a) without catalyst (b) with catalyst.

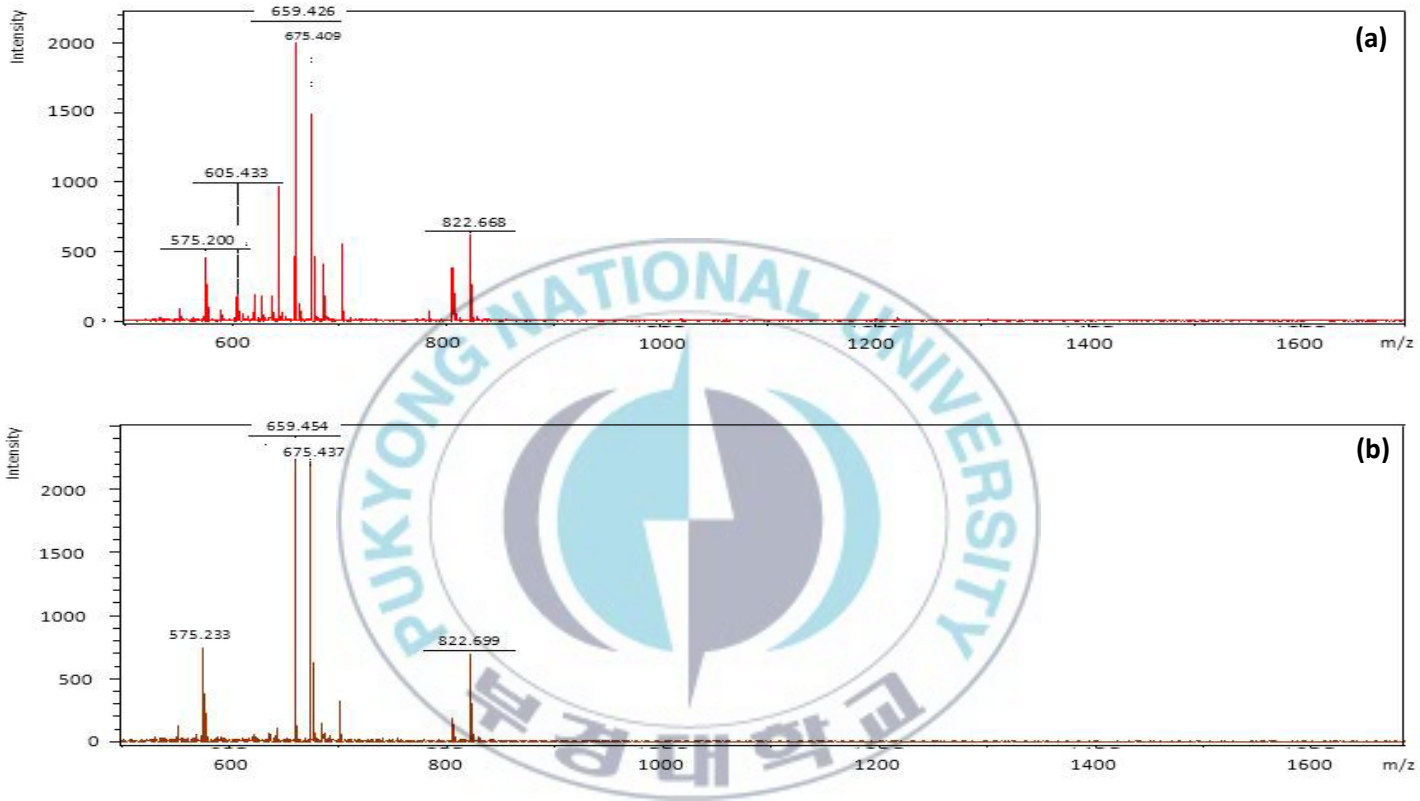


Fig. 14. MALDI-TOF mass spectrum of *S. japonica* hydrolysate water at 180 ^{o}C (a) without catalyst (b) with catalyst.

8. Quantification of gulose and mannose by HPLC

The most abundant compound found in brown seaweed was carbohydrate. Alginate was identified as a major constituent among carbohydrates present. It occurs in all brown seaweeds as a structural component of the cell wall in the form of insoluble alginate salts of mainly calcium, with lesser amounts of magnesium, sodium, potassium and it is concentrated in the intracellular space (Venugopal, 2009).

Alginate contains three kinds of polymer units consisting of D-mannuronic acid (M), L-guluronic acid (G) and alternates of the M and G units. In addition of mannuronic and guluronic acid of seaweed, it also contains monosaccharides which have been proven to give bioactive activities. The recovery of monosaccharides was needed to increase economic value of seaweed.

The depolymerization method of *S. japonica* and purified alginate was done using liquefied hydrothermal process and the contents of gulose and mannose were conducted using high performance liquid chromatography (HPLC) equipped by Evaporative Lightning Scattering Detector (ELSD). Gulose and mannose standards were used to quantify the G and M contents in hydrolysate water.

The depolymerization method using liquefied hydrothermal process succeeds to break down the polysaccharides polymer either in purified alginate or in *S. japonica*. The results of Fig. 15 described the amounts of mannose and gulose recovery in hydrolysate water in all conditions. The recovery of G and M in all conditions was enhanced with the presence of formic acid as catalyst. The highest amounts of M and G of purified alginate obtained from the experiment with catalyst added were 3.16 g/L and 6.50 g/L, respectively. Whereas mannose and gulose was obtained in *S. japonica* were 3.24 g/L and 9.07 g/L, respectively. All of the highest recovery of G and M were occurred at temperature 180 ^{o}C.

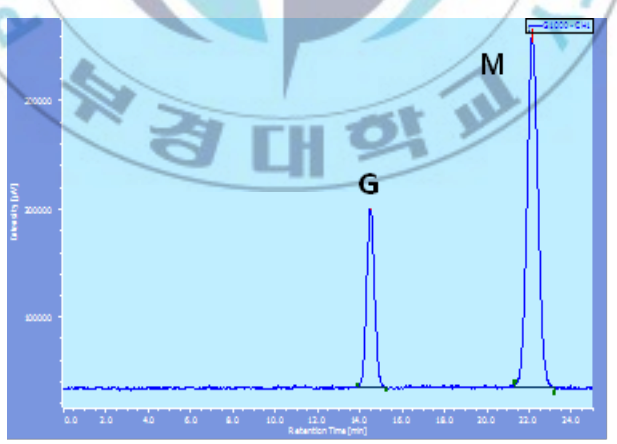
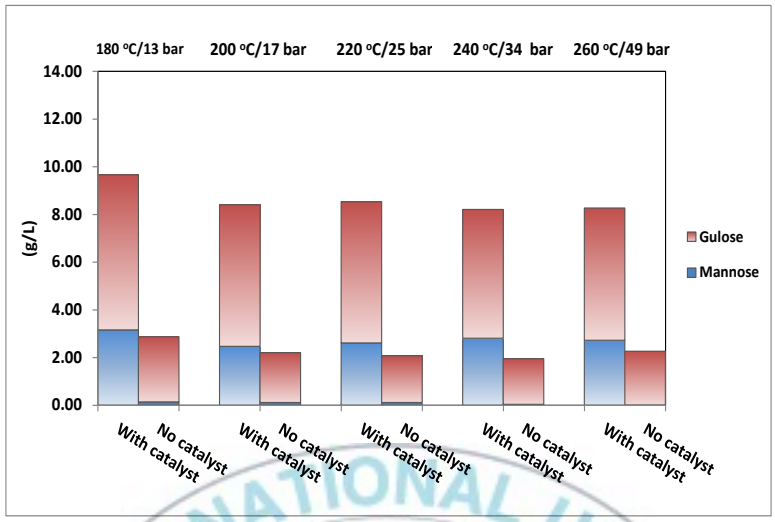
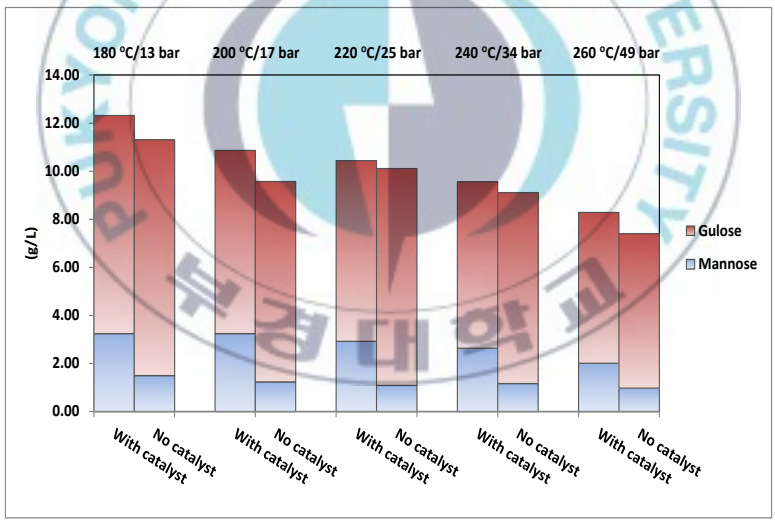


Fig. 15. Standard peak of gulose and mannose.



(a)



(b)

Fig. 16. The amounts of gulose and mannose (g/L) in (a) Purified alginate (b) *S. japonica* in different hydrolysis conditions.

9. Antioxidant properties

Antioxidant properties of hydrolysate water was conducted using some antioxidant assays, i.e. total phenolic content (TPC), total flavonoid content (TFC), DPPH radical-scavenging activity and ferric-reducing antioxidant power (FRAP). TPC and TFC describe the total amount of phenolic and flavonoid, respectively, contained in hydrolysate water. DPPH radical-scavenging activity measures the ability of a substance to prevent free radical molecules. FRAP is a test to determine total antioxidant power.

Based on Fig. 17 and 18, the antioxidant properties were higher in the experiments with catalyst added. The best temperature to gain the best antioxidant properties was found at 180 ^{o}C. In the treatment of purified alginate, the highest total phenolic, flavonoid, FRAP and DPPH were found in the treatments with catalyst addition. They were 4.06 ± 0.02 mg/L, 1.39 ± 0.00 mg/L, 6.30 ± 0.00 mg/L and 96.92%, respectively. The highest TFC (72.00 ± 0.10 g/L) was found in purified alginate with no catalyst. Whilst on *S. japonica* highest value of TPC, TFC, FRAP and DPPH were 9.63 ± 0.01 mg/L, 1.92 ± 0.05 mg/L, 3.11 ± 0.01 mg/L and 97.17%, respectively.

Antioxidant properties of hydrolysate water were affected by bioactive compounds such as phenolic, flavonoid, minerals and others. The condition of

growth environment, harvest time and storage condition also give huge effects to the amount of those compounds (Roh et al., 2008). The addition of formic acid as catalyst had proven to raise the antioxidant properties of hydrolysate water. Addition of acid as catalyst in hydrolysis process had produce new properties of water such as variation in density, polarity and solubility. Furthermore, the presences of acid give more protection of antioxidant compounds inside from oxidation and damage and also changed the solubility and density of water.

Most of antioxidant compounds found in brown seaweed belong to the group of polar compounds. As we know the general principle in solvent extraction “like dissolves like”, which means that suitable solvents only dissolve suitable substances which have similar polarity with the solvents used. Hence, those antioxidant compounds were dissolved in a presence of water as a main solvent. Higher temperature used in this process was decreased antioxidant properties of compounds. The antioxidant ability of hydrolysate water was not stable at high temperature.

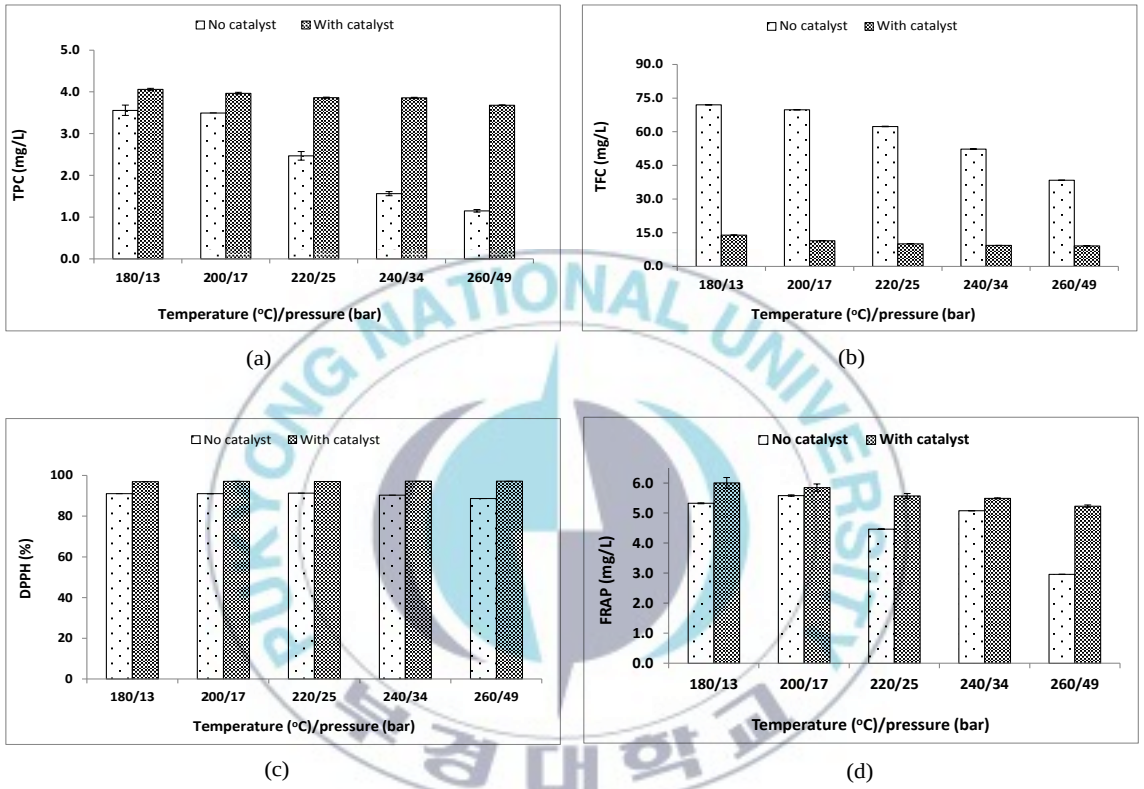


Fig. 17. Antioxidant properties of purified alginate hydrolysate samples on different conditions. Tags: (a) Total Phenolic Content (TPC); (b) Total Flavonoid Content (TFC); (c) DPPH radical scavenging activity; (d) Ferric Reducing Antioxidant Power (FRAP). Data are the mean value of three replicates ± S.D.

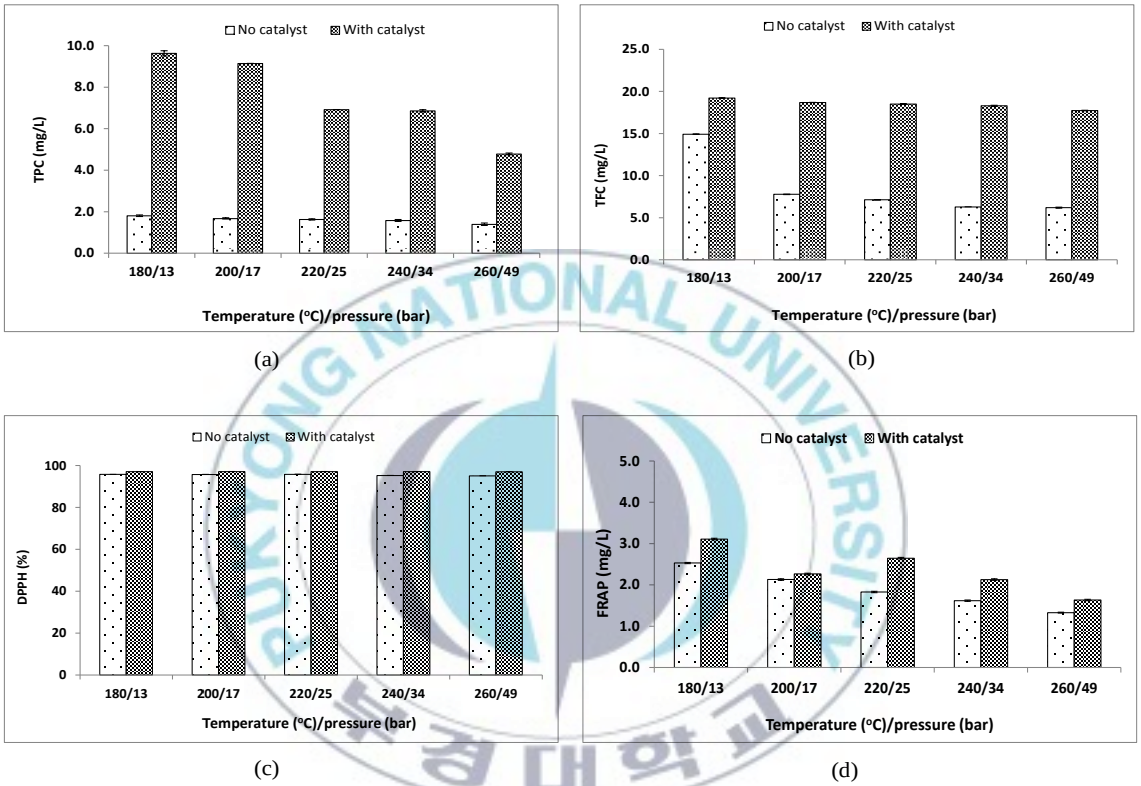


Fig. 18. Antioxidant properties of *S. japonica* hydrolysate samples on different conditions. Tags: (a) Total Phenolic Content (TPC); (b) Total Flavonoid Content (TFC); (c) DPPH radical scavenging activity; (d) Ferric Reducing Antioxidant Power (FRAP). Data are the mean value of three replicates ± S.D.

10. Effect of pH to antioxidant properties and sugar recovery

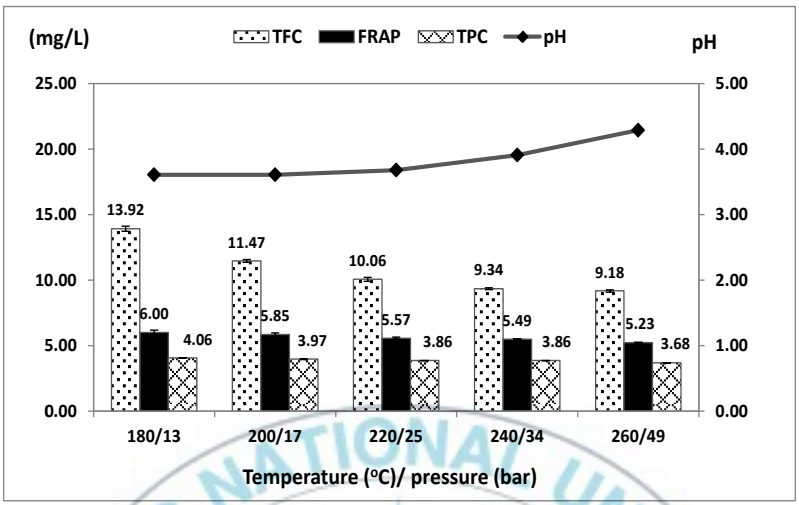
Effect of pH to antioxidant properties (TPC, TFC and FRAP) and sugar recovery (total sugar and reducing sugar) were analyzed on hydrolysate water with 1% formic acid addition. The pH values of purified alginate (3.61-4.29) and *S. japonica* (2.98-3.31) were found to increase as temperature was elevated. Antioxidant properties of both purified alginate and *S. japonica* showed reverse effect against pH values obtained. When pH value was raised, the antioxidant properties showed lower, and vice versa (Fig. 19). Lower pH values or higher acidity levels of hydrolysate water were proven to give the best protection for antioxidant compounds inside. The same things were described by Ragan and Glombitza (1986) and Santos-Buelga and Williamson (2003). They found that the addition of acid has been effectively used to prevent the oxidation of polyphenolic groups.

Temperature is one of the most important factors which gives huge influence to antioxidant activity. Pokorny (1986) described that heating causes an acceleration of the initiation reactions, and hence a decrease in the activity of the presence of antioxidants. The relationship between antioxidant activity and

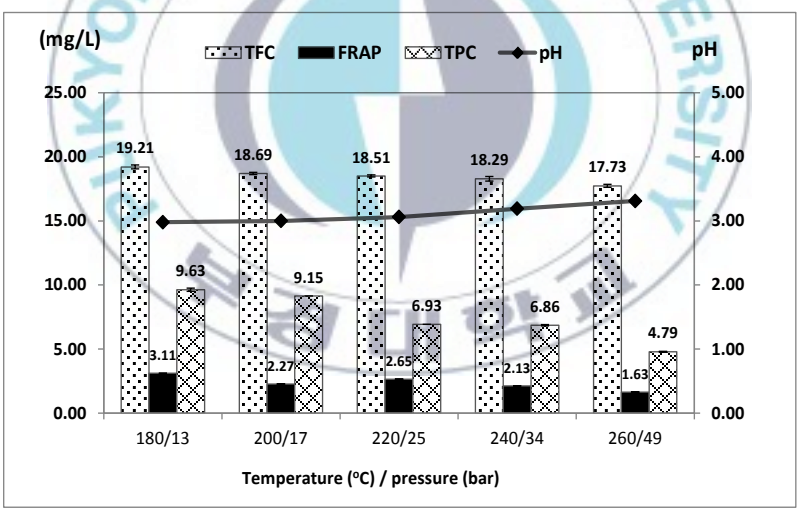
temperature could be understood by the knowledge of oxidisability. Oxidisability is the capability to undergo a chemical reaction with oxygen; the capability to lose hydrogen atoms. The reactivity of antioxidants against free radicals is characterized by bond dissociation energy of the O-H bonds in antioxidant compounds. The easily oxidisable antioxidant compounds showed a decrease pattern in antioxidant activities with increasing temperature. This phenomenon is caused by a decrease in the ability of antioxidants to react with free radicals at higher temperature. Hence, it is logical that less oxidisable antioxidants lose their antioxidant activity sooner than the easily oxidisable ones (Reblova, 2012).

Sugar recovery was monitored as a factor affected by pH and temperature. The higher temperature was applied, lower sugar was obtained. Basically, temperature and the chemical interaction between a given component and the water molecule determine the solubility of the component in water. In subcritical water state, raising the temperature and pressure causes significant changes in the properties of water. The properties of water, as a solvent, vary owing to variation of its dielectric constant, conductivity, ionic product and the structure of the H bond network (Galkin and Lunin, 2005).

The solubility of compounds in water also was affected by saturation cell. The size of saturation cell varied depending on the increase in solubility of the compounds in water as a function of temperature. King and Srinivas (2012) showed that solubilities of sugar and flavonoids compounds showed degradation value, with flavonoids above 353 K and sugar above 413 K. In the case of sugar saturation cell, there was evidence of charring inside the saturation cell, which resulted in the dark color of collected hydrolysate water. The same results were also reported by Tomasink (1989). He reported that dehydration of sugars was occurred when heated over periods of time and temperature above 373 K. Addition of acid or base catalyst in subcritical water system gives the influence to the physical properties of water. Espinoza and Morawicki (2012) examined the effect of additives on subcritical water hydrolysis process. It was concluded that the additives could change physical properties of water i.e. solubility, polarity, dielectric constant, viscosity, etc. which give the influence to solubility of compounds in water.

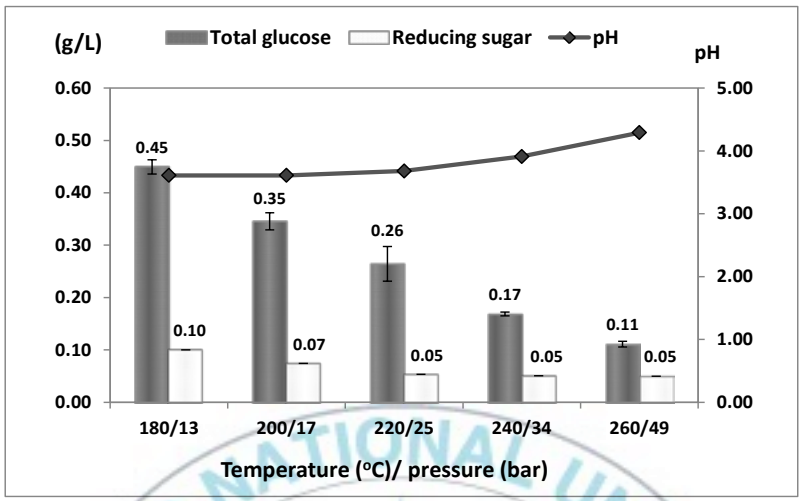


(a)

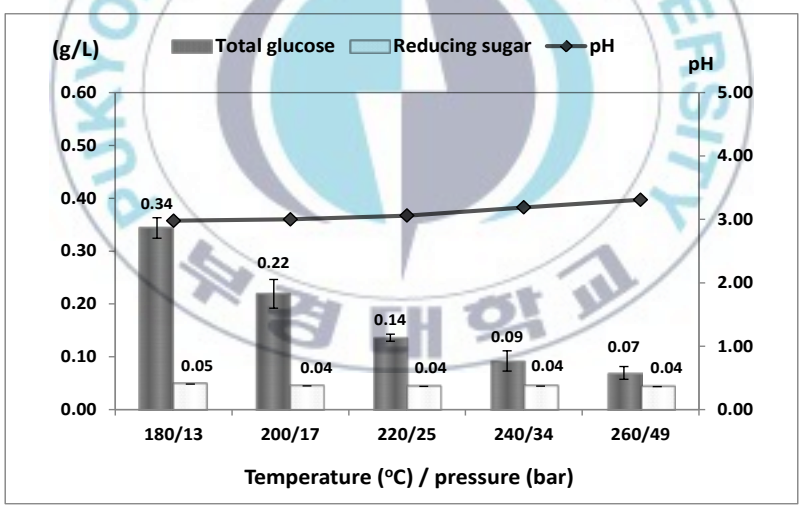


(b)

Fig. 19. Effect of pH value to antioxidant properties of (a) purified alginate (b) *S. japonica* hydrolysate water.



(a)



(b)

Fig. 20. Effect of pH value to sugar recovery of (a) purified alginate (b) *S. japonica* hydrolysate water.

Conclusions

Purified alginate and brown seaweed (*S. japonica)* consist of complex structural bonds. The utilization of both is in limited field. The certain technology is needed to modify the structure. Subcritical water hydrolysis was chosen as a new invention of eco-friendly technology to break through these limitations. The modification of structure either in *S. japonica* or in purified alginate is expected to raise bio-active compounds which have an important role in human well being.

Total glucose and reducing sugar were measured to give the information about the probability of these comodities which can be changed into renewable bioenergy resources. Sugar like glucose, fructose and galactose are important monosaccharides that can be converted to ethanol as sources for bioethanol production. Total sugar of purified alginate hydrolysate water was found higher than one of *S. japonica*. Moreover, the best condition was reached in low temperature of 180 ^{o}C without catalyst addition.

Purified alginate and *S. japonica* has valuable bioactive compounds, e.g. flavonoid, phenolic and groups of tannnins. It has been compromised to prevent free radical. Hydrolysate water of *S. japonica* was proved to give the best antioxidant activites than that of purified alginate. *S. japonica* is a natural marine

resource which still has the bioactive compounds. Purified alginate is material which structured by polysaccharide with small amounts of contaminants such as polyphenol groups, endotoxin and proteins. The presences of acid give more protection of antioxidant compounds inside from oxidation and damage. It also changed the solubility and density of water.

Total organic carbon showed the potential of hydrolysis treatment to break down complex structure into the simpler one. The decrease of carbon recovery indicated that hydrothermal treatment produced carbon products that were not soluble in liquid phase. Hydrolysis treatment at the elevated temperature was proven to give more influence in depolymerization.

Identification of compounds can be done by several methods, and one of the methods is determination by molecular weight. Molecular weight determination by viscometer was done to get gross molecular weight of hydrolysate water before MALDI-TOF analysis. In MALDI-TOF, before doing the analysis, it is important to know the range of molecular weight which wants to be analyzed. Gross molecular weight of hydrolysate water in both materials showed that the range of molecular weight was between 1078-6295 Da. MALDI-TOF MS analysis was done in the hydrolysate water with 180 ^{o}C temperature condition

and catalyst addition. Based on the reviewed and obtained data, the further analysis with mass spectrum is needed to identify the components inside. Mass spectrum showed that the range of molecular weight was between 575 to 1238 Da, whereas raw alginate molecular weight was $2x10^5$ Da. Moreover, oligosaccharide and polysaccharide were produced during hydrolysis process.

Acknowledgements

It is a pleasure to convey my deepest gratitude to Almighty God, who always gives me strength, encourage, hopes and endurance during my lifetime. He always teached me also that there are beautiful plans waiting for me. I would like to express my greatest gratitude to my advisor Professor Byung-Soo Chun for giving me opportunity to join in research work under his supervision. Without his guidance and persistent help, this thesis would not have been possible. I am very grateful to him for providing the financial assistance and for chance to have experience for study in Korea.

It is my honor to thank you to my thesis evaluation committee, Professors Yang-Bong Lee and Ji-Young Yang, for their fruitful evaluations, reviews, criticsms and suggestions to improve my thesis. I would like to express my sincere thanks to all professors in Department of Food Science and Technology for their teaching, guidance and kindness.

I would like to express my cordial thanks to all of my lab members, I have been blessed with a friendly and cheerful environment in lab: A.K.M Assaduzzaman and family, A.S.M Tanbirul Haque, Evi Amelia Siahaan, the only Indonesian student in my department, thank you for her advices, helps and times, Kim-Ryong Hee, who always help me with guidances and support, and Yin Shi Peng. Special thanks also for Veronika Pak and Lee Jae Kyung for helping me with the Korean translation. Thanks also for all

Korean students, international students and all Indonesian friends for their helping hands and comfortable atmosphere during my study period.

I extend my sincere gratitude to my beloved family, my parents, my eldest and my younger sister. They deserve special mention for their tremendous supports and prayers. I also extend my cordial thanks to GEIS JB3 family, thank you for faith, hopes and prays for me during my study period.

Finally, I would like to thank everybody who gives their help and support for me. Let me express apologies that I could not mention personally one by one.

References

Ahn M.J., Yoon K.D., Min S.Y., Lee J.S., Kim J.H., Kim T.G., Jeong H.K., Kim G.T., Kim S.H., Kim N.G., Huh H., Kim J.W. (2004) Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. Biological and Pharmaceutical Bulletin, 27, 544-547.

Alekseyenko T.V., Zhanayeva S.Y., Venediktova A.A., Zvyagintseva T.N., Kuznetsova T.A., Besednova N.N., Korolenko T.A. (2007) Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk sea *Fucus evanescens* brown alga. Bulletin of Experimental Biology and Medicine, 143, 730-732.

Alnaief M., Alzaitoun M.A., García-González C.A., Smirnova I. (2011) Preparation of biodegradable nanoporous microspherical aerogel based on alginate. Carbohydrate Polymers, 84, 1011–1018.

AOAC International (2000) *Association of Official Analytical Chemists*. Official Methods of Analysis of AOAC. Washington DC: AOAC International.

Artan M., Li Y., Karadeniz F., Lee S.H., Kim M.M., Kim S.K. (2008) Anti-HIV-

1 activity of phloroglucinol derivative, 6,6'- bieckol, from *Ecklonia cava*. Bio-organic and Medicinal Chemistry, 16, 7921-7926.

Blunt J.W., Copp B.R., Munro M.H.G., Northcote P.T., Prinsep M.R. (2011) Marine natural products. Natural Product Reports, 28, 196-268.

Cheng H., Zhu X., Zhu C., Qian J., Zhu N., Zhao L., Chen J. (2008) Hydrolysis technology of biomass waste to produce amino acids in sub-critical water. Bioresource Technology, 99, 3337–3341.

Cho J.Y. (2012) Antifouling chromanols isolated from brown alga *Sargassum horneri*. Journal of Applied Phycology, 25, 299-309.

Daneshvar S., Salak F., Ishii T., Otsuka K. (2012) Application of subcritical water for conversion of macroalgae to value-added materials. Industrial Engineering Chemistry Research, 51, 77–84.

Dawczynski C., Schubert R., Jahreis G. (2007) Amino acids, oilty acids and dietary fibre in edible seaweed products. Food Chemistry, 103, 891-99.

Demirel Z., Yilmaz-Koz F.F., Karabay-Yavagoslu U.N., Ozdemir G., Sukatar A. (2009) Antimicrobial and antioxidant activity of brown algae from the Aegean Sea. Journal of the Serbian Chemical Society, 6, 619-628.

Draget K.I., Smidsrod O., Skjak-Braek K. (2005) Alginates from algae. In Polysaccharides and Polyamides in The Food Industry. Properties, Production, and Patents., ed. A Steinbuchel, SK Rhee:1-30, Weinheim, Wiley-VCH Verlag GmbH & Co. Number of 1-30 pp.

Espinoza A.D. and Morawicki R.O. (2012) Effect of Additives on Subcritical Water Hydrolysis of Whey Protein Isolate. Journal of Agricultural and Food Chemistry, 60, 5250−5256.

Fitton J.H., Irhimeh M., Falk N. (2007) Macroalgal fucoidan extracts: new opportunity for marine cosmetics. Cosmetics and Toiletries Magazine, 122 (8), 55-64.

Galkin A.A. and Lunin V.V. (2005) Subcritical and supercritical water: a universal medium for chemical reactions. Russian Chemical Reviews, 74 (1), 21-35.

Go H., Hwang H.J., Nam T.J. (2010) A glycoprotein from *Laminaria japonica* induces apoptosis in HT-29 colon cancer cells. Toxicology in Vitro, 24, 1546–1553.

Graham L.E., Graham J.M., Wilcox L.W. (2009) Algae Second Edition. Pearson.

Hagen-rodde R.S., Varum K.M., Laren B.A., Myklestad S.M. (2004) Seasonal and geographical variation in the chemical composition of the red algae *Palmaria palmata* (L.) Kuntze. Botanical Marina, 47, 125-133.

Haug A., Myklestad S., Larsen B., Smidsrod O. (1967) Correlation between Chemical Structure and Physical Properties of Alginates. Acta Chemica Scandinavica, 21,768-778.

Hemmingson J.A., Falshaw R., Furneaux R.H., Thompson K. (2006) Structure and antiviral activity of the galactofucan sulfates extracted from *Undaria pinnatifida* (Phaeophyta). Journal of Applied Phycology, 18, 185-193.

Heo S.J., Ko S.C., Cha S.H., Kang D.H., Park H.S., Choi Y.U., Kim D.K., Jung W.K., Jeon Y.J. (2009) Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photooxidative stress induced

by UV-B radiation. Toxicology in Vitro, 23, 1123-1130.

Kim K.H., Kim Y.W., Kim H.B., Lee B.J., Lee D.S. (2006) Antiapoptotic activity of laminarin polysaccharides and their enzymatically hydrolyzed oligosaccharides from *Laminaria japonica*. Biotechnology Letters, 28, 439-446.

King J.W. (2000) Advances in critical fluid technology for food processing. Food Science and Technology Today, 14, 186-191.

King J.W. and Srinivas K. (2012) Measurement of Aqueous Solubility of Compounds at High Temperature Using a Dynamic Flow Apparatus and a Teledyne Isco Syringe Pump. University of Arkansas, Dept. of Chemical Engineering.

Koivikko R. (2008) Brown algal phlorotannins improving and applying chemical methods. Dissertation, University of Turku.

Kruse A. and Dinjus E. (2007) Hot compressed water as reaction medium and reactant: 2. Degradation reactions. Journal of Supercritical Fluids, 41 (3), 362-380.

Manivannan K., Karthikai D.G., Anantharaman P., Balasubramanian T. (2011) Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters, Gulf of Mannar. Asian Pacific Journal of Tropical Biomedicine, 114-120.

McHugh D.J. (2003) A guide to the seaweed industry. FAO Fisheries Technical Paper 441.

Meinita M.D.N. (2010) Production of bioethanol using red seaweed *Kappaphycus alvarezii* (cottonii) colected from Indonesia, Thesis for the degree of doctor engineering, Pukyong National University, South Korea.

Nagayama K., Iwamura Y., Shibata T., Hirayama I., Nakamura T. (2002) Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. Journal of Antimicrobial Chemotherapy, 50, 889-893.

Osman M.E.H., Abushady A.M., Elshobary M.E. (2010) *In vitro* screening of antimicrobial activity of extracts of some macroalgae collected from Abu-Qir bay Alexandria, Egypt. African Journal of Biotechnology, 12, 7203-7208.

Park J.N., Shin T.S., Lee J.H., Chun B.S. (2012) Production of Reducing Sugars from *Laminaria japonica* by Subcritical Water Hydrolysis. APCBEE Procedia, 2, 17-21.

Peng Z., Liu M., Fang Z., Zhang Q. (2012) In vitro antioxidant effects and cytotoxicity of polysaccharides extracted from *Laminaria japonica*. International Journal of Biological Macromolecules, 50, 1254-1259.

Pereira R.C. and Costa-Lotufo L.V. (2011) Bioprospecting for bioactives from seaweeds: potential, obstacles and alternatives. Brazilian Journal of Pharmacognosy.

Pokorny J. (1986) Addition of antioxidants for food stabilization to control oxidative rancidity. Czech Journal of Food Sciences, 4, 299–307.

Ponce N.M.A., Pujol C.A., Damonte E.B., Flores M.L., Stoerz C.A. (2003) Fucoidans from the brown seaweed Adenocystis utricularis: extraction methods, antiviral activity and structural studies. Carbohydrate Research, 338, 153-165.

Ragan M.A. and Glombitza K.W. (1986) Phlorotannins, brown algal polyphenols. In *Progress in Phycological Research*, Round FE and Chapman DJ (ed). Biopress

Ltd: Bristo, 129-241.

Rajauria G., Jaiswal A.K., Abu-Ghannam N., Gupta S. (2010) Effect of hydrothermal processing on color, antioxidant and free radical scavenging capacities of edible Irish brown seaweeds. International Journal of Food Science and Technology, 45(12), 2485-2493.

Reblova Z. (2012) Effect of temperature on the antioxidant activity of phenolic acids. Czech Journal of Food Sciences, 30 (2), 171–177.

Rioux L.-E., Turgeon S.L., Beaulieu M. (2007) Characterization of polysaccharides extracted from brown seaweeds. Carbohydrate Polymers, 69, 530-537.

Roh M.K., Salim Uddin Md., Chun B.S. (2008) Extraction of fucoxanthin and polyphenol from *Undaria pinnatifida* using supercritical carbon dioxide with co-solvent. Biotechnology and Bioprocess Engineering, 13(6), 724-729.

Samee H., Li Z.X., Lin H., Khalid J., Guo Y.C. (2009) Anti-allergic effects of ethanol extracts from brown seaweeds. Journal of Zhejiang University Science B, 10, 147-153.

Santos-Buelga C. and Williamson G. (2003) Methods in polyphenol analysis. The Royal Society of Chemistry: Cambridge, UK.

Steinberg P.D., Edyvane K., de Nys R., Birdsey R., van Altena I.A. (1991) Lack of avoidance of phenolic-rich brown algae by tropical herbivorous fishes. Marine Biology, 109, 335-343.

Sugiura Y., Matsuda K., Yamada Y., Nishikawa M., Shioya K., Katsuzaki H., Imai K., Amano H. (2007) Anti-Allergic phlorotannins from the edible brown alga, *Eisenia Arborea*. Food Science and Technology Research, 13, 54-60.

Takamatsu S., Hodges T.W., Rajbhandari I., Gerwick W.H., Hamann M.T., Nagle D.G. (2003) Marine natural products as novel antioxidant prototypes. Journal of Natural Production, 66, 605-608.

Tam S.K., Dusseault J., Polizu S., Menard M., Halle J.P., Yahia L.H. (2005) Impact of residual contamination on the biofunctional properties of purified alginates used for cell encapsulation. Biomaterials, 27, 1296–1305.

Tomasink P. (1989) Advances in Carbohydrate Chemistry and Biochemistry.

Academic Press Inc., New York, 47, 203.

Tuney, Cadirci B.H., Unal D., Sukatar A. (2006) Antimicrobial activities of the extracts of marine algae from the Coast of Urla (Izmir, Turkey). Turkey Journal Biology, 30, 171-175.

Van Alstyne K.L. (1994) Comparison of three methods for quantifying brown algal polyphenolic compounds. Journal of Chemical Ecology, 21, 45-58.

Wargacki A.J., Leonard E., Win M.N., Regitsky D.D., Santos C.N.S., Kim P.B., Cooper S.R., Raisner R.M., Herman A., Sivitz A.B., Lakshmanaswamy A., Kashiyama Y., Baker D., Yoshikuni Y. (2012) An engineered microbial platform for direct biofuel production from brown macroalgae. Science, 335, 308-313.

Yuan Y.V. (2007) Antioxidants from edible seaweeds. In: Antioxidants Measurement and Applications, Fereidoon Shahidi and Ho CT. ACS Oxford Press, pp 268-301.

Yuan Y.V. (2008) Marine Algal Constituents. In: Collin Barrow and Fereidoon Shahidi: Marine Nutraceuticals and Functional Foods. CRC Press, pp 259-265.

de Sousa A.P.A., Torres M.R., Pessoa C., de Moraes M.O., Filho F.D.R., Alves A. P.N.N., Costa-Lotufo L.V. (2007) In vivo growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed *Sargassum vulgare*. Carbohydrate Polymers, 69, 7-13.