Thesis for the Degree of Master of Engineering

Bioactive Compounds Produced from *Laminiaria Japonica* Powder

by

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Pukyong National University

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Bioactive Compounds Produced from Laminiaria Japonica Powder

다시마 분말에서 생산된 생리 활성 물질

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by

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A Dissertation

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요 약

최근 몇 년 동안 산업 급증으 나 여러 요인들로 인해 해조류의 폐기 량이 늘어나고 있다. 따라서, 산업 공정 과정을 통해 빌생되는 해조 폐기물들의 효과즈인 처리 빙안으 요구 된다. 폐 해조류 중 길조루는 laminarine과 alginate 등 다당류를 포힘 하고 있다. 길조류에는 antioxidant와 antibacterial으 기능으 있다고 일려져 있다. 본 연구에서는 *Laminaria japonica* 분밀을 분해하여 그 기능의 능력을 알아보았다. 하지만 *Laminaria japonica* 분말은 용해도가 낮기 때문에 균들이 잘 분해 하지 못한다. 따라서 용해도를 증가시키기 위해서 pre-treatment 공정을 선택하였다. 이 공정은 물리적인 빙법과 화학적은 빙법을 사용하였다. 그 결과 용해도는 58%에서 94%로 증가하였다. Antioxidant 결과로는 reducing power는 0.263 g/L, Hydroxyl radical scavenging activity 는 99%, DPPH radical scavenging activity는 60%, 그리고 ABTS radical scavenging activity는 90%로 나왔다. 본 연구 결과를 통해서 *Microbacterium oxydans*을 이용하여 효과즈으로 대길조류를 분해하여 생리활성물질이나 부가 가치 높은 식품 생산 등으로 재활용하여 효과가 있을 것으로 사료 된다.

Bioactive Compounds Produced from Laminiaria Japonica Powder

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Abstract

During recent years, the amount of seaweed waste has increased due to its culturing as an industrial resource and a depolluting plant for cleaning inland sea area, and eutrophication of seawater. The efficient treatment of seaweed waste is deemed necessary nowadays. Brown seaweed contains polysaccharides such as laminarin and alginate. brown seaweed is known as having antioxdant and antibacterial activitiy. In this study, we used Laminiaria japonica powder and some bacteria which have potential to degrade it. Because of its low solubility, it is difficult for bacteria to degrade brown seaweeds for producing sugars. So, for increasing solubility of the powder, pre-treatment was chosen. This procedure was divided two parts, physical and chemical treatment. As a result, the solubility was increased to 94% compared with its initial solubility (58%). Reducing power was 0.263 g/L. Hydroxyl radical scavenging activity was 99%. DPPH radical scavenging activity was 60%. ABTS radical scavenging activity was 90%.

I. INTRODUCTION

Seaweeds are suitable for consumption by human beings and animals, and known to be a favorite food particularly in Asian countries [1]. They are often used in fertilizer, fungicides, herbicides, and phycocolloids such as alginate, carrageenan, and agar [2]. Worldwide consumption of seafood including seaweeds has increased steadily due to its health benefits. During recent years, the amount of seaweed waste has increased due to its culture as an industrial resource and a depolluting plant for cleaning inland sea area, and eutrophication of seawater [3,4]. Accordingly, the disposal and reutilization of seaweed waste has been essential for preservation of the marine environment and recycling of organic substances [5].

From 2008 to 2010 in Korea, annual production of seaweed was approximately 882 thousand-tons on the average, mostly by aquaculture [6]. The major species was brown seaweed, such as *Laminaria japonica* and *Undaria pinnatifida* because they are fit for Korean weather. Approximately a quarter of the total amount of produced seaweed is assumed to be discarded annually [7]. Unused seaweeds waste is customarily disposed via landfill, incineration, or by dumping into the sea. Therefore, the greater part of seaweed waste has been utilized inefficiently so far, and their disposal rather affects the local environment. Besides, the cost for disposal of seaweed waste will remarkably increase after 2012, since dumping waste into the sea will become prohibited in Korea according to the

London Convention [8]. Under this circumstance, there is an urgent need to find ecologically acceptable means for reutilization of seaweed waste.

Seaweeds posses a high water content of approximately 70-90%, a relatively high protein content of approximately 10%, and contain varying levels of carbohydrates, compared with terrestrial plants [9]. The content of carbohydrates in brown seaweed is 30-50%, which are mostly alginate and fructose-containing polysaccharides. In the case of *U. pinnatifida,* the content of alginate reaches over 50% in dry weight [10]. In addition, laminarin is a storage foundin, the content of which reaches approximately 20 wt% in the period of maturity [9]. Alginate is a linear copolymer of β-1,4-D-mannuronic acid and α-1,4-L-guluronic acid with the residues organized in blocks of polymannuronate and polyguluronate, as well as heteropolymeric sequences of both uronic acids [11]. Laminarin is a linear polysaccharide made up of β-1,3-glucan with occasional β-1,6-linked branches and with a β-1,3:β-1,6 ratio of 3:1 [12]. Therefore, the biodegradation of brown seaweed is not easy, mainly due to its complicated molecular structure.

In recent years, marine sulfated polysaccharides have been demonstrated to have antioxidant activity [13]. Sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. Marine algae are the most important source of non-animal sulfated polysaccharides. Sulfated polysaccharides from algae possess important pharmacological

activities such as anticoagulant [14], antioxidant [15-16], anti-inflammatory, antiviral [17], antibacterial [18-27], anti-proliferative [28], antitumoral [29], anti-complementary [30], and anti-adhesive activities [31]. In recent years, algal sulfated polysaccharides, especially those extracted from brwon seaweed, have been of interest. They have been demonstrated to play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms [32,33].

Free radicals are any chemical species capable of independent existence with one or more unpaired electrons in their outermost shell, which seek out and capture electrons from other substances to achieve neutrality. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, resulting in a chain reaction. If two radicals meet they can combine their unpaired electrons, thus forming a covalent bond. Reactive oxygen species (ROS) refers to an array of metabolites derived from molecular oxygen (O_2). These cellular renegades damage DNA, proteins, and lipids, altering biochemical compounds and corroding cell membrane. Such molecular mayhem plays a major role in the development of various diseases such as cancer, atherosclerosis, and respiratory ailments. Antioxidant activity has become a issue and the subject of intensive investigation due to the ever-increasing demand by the food and pharmaceutical industries to develop natural bioactive anti-aging and anti-carcinogenic compounds that demonstrate measurable health benefits. Antioxidative substances obtained from

natural sources, such as seed oil, grains, beans, vegetables, fruits, leaf waxes, bark, roots, spices and hulls, have already been investigated [33]. Hence, the present study was undertaken to evaluate the antioxidant and antibacterial activity of the sulfated polysaccharides from *Laminaria japonica* with respect to their free radical scavenging properties.

Ⅱ. MATERIALS AND METHODS

1.microorganisms

The bacterial strain that was used for the biodegradation of laminarin was *Microbacterium oxydans*, which were isolated from silt and sandbar locations in a coastal area near Busan (Korea), where brown seaweed often drifts and accumulates. This strain formed a transparent ring (on lamnarin agar) around each colony according to the plate assay [34,35], indicating that this strain possesses high alginate lyase activities. displayed Gram-positive rods, which measured 0.5-1.0 μm in width and 0.5-1.5 μm in length. The pure culture was maintained on a 1.5% nutrient agar plate at 4 ℃ until use and transferred to a fresh agar plate every month. Periodically, the potential degrading ability of this strain was checked on alginate agar medium by the plate assay.

2. Biodegradation of Laminaria japonica.

To characterize the degradation of *Laminaria japonica* by *Microbacterium oxydans*, two experiments were performed in a 1000 ml flask (with a 300 ml working volume). The culture medium used for the biodegradation experiment was the different medium. First media solvent is distilled water. Seconde media is added pretreatment.

This procedure was divided two parts, physical and chemical treatment. Before inoculation, the previously harvested cells (0.1%, w v^{-1}) were proliferated for 9 h under the same culture conditions. After then, the proliferated cells were inoculated in to a 1 L Erlenmyer flask containing 1% *Laminaria japonica* powder culture medium (10 g l^{-1} *Laminaria japonica* powder and pH 6). The biodegradation experiment was carried out with incubation in a rotary shaking incubator at 37 °C and 180 rpm for 6d. During the incubation, samples were periodically taken from each flask to measure the changes in pH, cell density, clear zone and the concentration of reducing sugars. The samples were also analyzed to examine antioxidant activity.

3. Determination of Antioxidant Activity

3.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity Assay

The DPPH free radical scavenging ability of the culture supernatant was assayed as described by [36] with some modifications. Two milliliters of 0.1 mM DPPH solution in 80% ethanol was added to 1 ml of each culture supernatant. The mixture was maintained at 25°C in the dark for 30 min, and the absorbance was measured using an Opron-3000®UV/VIS Spectrophotometer (Hanson technology Co., Ltd., Korea) at 517 nm against a blank sample. The sample blank was prepared by replacing DPPH with 80% ethanol. The DPPH radical scavenging activity was determined using the following formula:

$$avenging\,activity = \frac{Absorvance\,of\,control - Absorbance\,of\,sample}{Absorvance\,of\,control} \times 100$$

A control sample was prepared by mixing 1 ml of 80% ethanol with 2 ml of 0.1 mM DPPH. L-Ascorbic acid (0.1 mM) was used as a positive control under the same assay conditions. The assay was conducted in triplicate.

3.2 ABTS Radical Cation Decolorization Assay

The ABTS radical cation decolorization was determined by the method prescribed by [37] with some modifications. The ABTS reagent (radical cation) was prepared by mixing 5 ml of 7 mM ABTS (2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid) with 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$). The mixture was maintained in the dark at 25°C for 16h .The absorbance of the ABTS reagent was then adjusted to 0.72 ± 0.02 at 734 nm with 80% ethanol. To determine the scavenging activity, 900 µl of the ABTS reagent was added to 100 µl of each culture supernatant, and the absorbance was measured at 734 nm after a 6-min interval. L-Ascorbic acid (0.1 mM) was used as a positive control. The percentage inhibition of the sample was calculated according to the following equation:

$$hibition\,(\%) = \frac{Absorvance\,of\,control - Absorbance\,of\,sample}{Absorvance\,of\,control} \times 100$$

The control sample was prepared by replacing the culture supernatant with distilled water (DW). The sample blank was prepared by replacing the ABTS reagent with 80% ethanol. The analysis was conducted in triplicate.

3.3. Hydroxyl Radical Scavenging Activity Assay

The hydroxyl radical scavenging activity was determined according to [38] with some modifications. One milliliter of each culture supernatant was mixed with 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide and 0.3 ml of 20 mM sodium salicylate. After incubation at 37°C for 1 h, the absorbance of the hydroxylated salicylate mixture was measured at 562 nm. L-Ascorbic acid (0.1 mM) was used as a positive control. The hydroxyl radical scavenging activity was calculated using the following formula:

$$droxyl\,radical\,scavenging\,activity\,(\%) = \frac{1-(A1-A2)}{A} \times 100$$

where A is the absorbance of the control (without sample), A1 is the absorbance in the presence of sample, and A2 is the absorbance without sodium salicylate.

3.4. Reducing Power Assay

The reducing power assay was conducted according to the method described by [39] with some modifications. One milliliter of each culture supernatant was mixed with 1.0 ml of 0.2 M phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide. The resultant mixture was incubated at 50°C for 20 min in a shaking incubator. After incubation, the reaction was stopped by adding 1.0 ml of 10% (w v^{-1}) trichloroacetic acid, and the mixture was centrifuged at 3000 rpm for 10 min. Two milliliters of solution were collected from the upper layer and mixed with 2 ml of DW and 0.4 ml of 0.1% $FeCl_3$. The mixture was incubated at 25°C for 10 min. After the 10 min incubation, the absorbance of all sample solutions was measured at 700 nm. An increase in absorbance represented an increase in reducing power. The control sample was prepared by replacing the culture supernatant with DW, and L-ascorbic acid (0.1 mM) was used as a positive control. The test was performed in triplicate.

4. Analytical methods

Cell growth of the isolate was measured by CFU(colony forming unit). The characterization of degradation ability on *Laminaria japonica* by amounts of total reducing sugars and glucose. The amount of total reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [40]. Culture broth (150 μL) was taken in a 1.5-mL Eppendorf tube and centrifuged at 9,500 rpm for 20 min. The supernatant (100 μL) was pipetted, added to 1 mL of DNS reagent and mixed well. The mixed solution was heated at 90-95 ℃ for 10 min and then subjected to measurement using a VIS/UV spectrophotometer at 550 nm after rapid cooling to room temperature. All measurements were performed in triplicate. To differentiate and identify diverse *Laminaria japonica*-decomposition products, TLC was 3 type used. First TLC work was conducted by the ascending method, using Silica Gel-60 TLC plates (E. Merck, Darmstadt, Germany). n-butanol : formic acid : water (4 : 6 : 1) solutions were used to develop alginate decomposition products on the TLC plates, respectively. After spraying with 10% ($v \cdot v^{-1}$) H_2SO_4 in ethanol, the products depolymerized from alginate were visualized by heating at 110 °C for 10 min. Second TLC was n-butanol : isopropanol : ethanol : water (2 : 3 : 3 : 2) solutions were used to develop alginate decomposition products on the TLC plates, respectively. After spraying with 10% ($v \cdot v^{-1}$) H_2SO_4 in ethanol, the products depolymerized from laminarine were visualized by heating at 110°C for 10 min. Third TLC was n-butanol : acetic acid : water (2 :

1 : 1) solutions were used to develop fucoidan decomposition products on the TLC plates, respectively. After spraying with 20% ($v \cdot v^{-1}$) H_2SO_4 in methanol, the products depolymerized from alginate were visualized by heating at110°C for 10 min.

Ⅲ.RESULTS AND DISCUSSION

1. Biodegradation of Laminaria japonica.

It is known that brown seaweed is utilized in many fields because it contains biologically active substances. The carbohydrate content in brown seaweed is approximately 30-50% and consists mostly of alginate. For this reason, the uses of carbohydrate and its degradation oligosaccharides have been extended. Thus, this study characterized *Laminaria japonica* biodegradation using *Laminaria japonica* lyase-synthesizing *Microbacterium oxydans*. The two type media used in this study, one solvent is D.W. The other media is pretreatment. The degradation of *Laminaria japonica* by *Microbacterium oxydans* was performed in a flask-scale for 6 d, and is shown in Figure 1 and Figure 2. During the 6 d cultivation, the CFU of the colony slightly decreased during the first 2 d and then decreased until the end of the culture period. The pH slightly decreased from 6.23 to 5.59 on not pretreatment media, from 6.31 to 5.52 on pretreatment media. Thus, the pH decreased as laminarine was degraded by *Microbacterium oxydans*. A similar result can be found in the study of [41]. The maximal concentrations of reducing sugars produced by *Microbacterium oxydans* were 0.079 mg l^{-1} on not pretreatment media , 0.072 mg l^{-1} on not pretreatment media. This result is low production of reducing sugars. Because brown

seaweed cell wall composition is cellulose microfivrils. [42,43]. Therefor *Microbacterium oxydans* is not well degradaion by monosaccharide. Therefor *Microbacterium oxydans* To support this theory, a TLC and clear zone analysis was conducted. The clear zone size slightly increased from 0.7 to 0.9 on not pretreatment media, from 0.7 to 0.8 on pretreatment media.

Laminaria japonica main carbohydrates are alginate, Laminarin and Fucoidan. So Performed experiments of 3 type TLC. As observed in Figure 3, the alginate-decomposition products migrated on the TLC plate as alginate was degraded over time in culture; however, the monomer was present in the lowest amount. A similar result can be found in the study of [41]. And observed in Figure 4, the Laminarine-decomposition products migrated on the TLC plate as alginate was degraded over time in culture; however, the monomer was present in the little amount. A similar result can be found in the study of [44]. The observed in Figure 5, the Fucoidan-decomposition products migrated on the TLC plate as alginate was degraded over time in culture; however, the monomer was present in the lowest amount. Therefore, it was concluded that biodegraded pretreatment *Laminaria japonica* exhibited a better reducing power than not pretreatment *Laminaria japonica*.

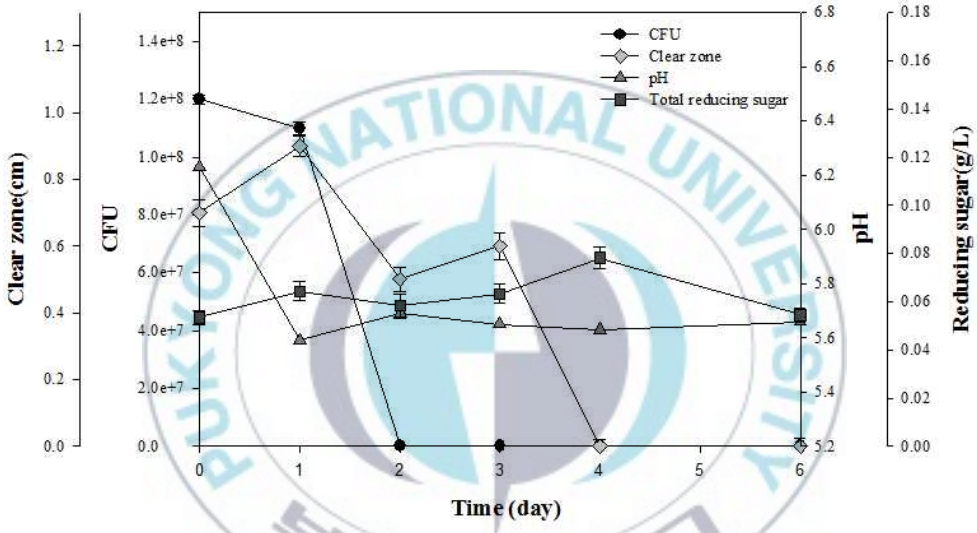

Fig. 1. Degradation of *Laminaria japonica* by seaweed powder media. Error bars: mean ± S.D. of three replicates.

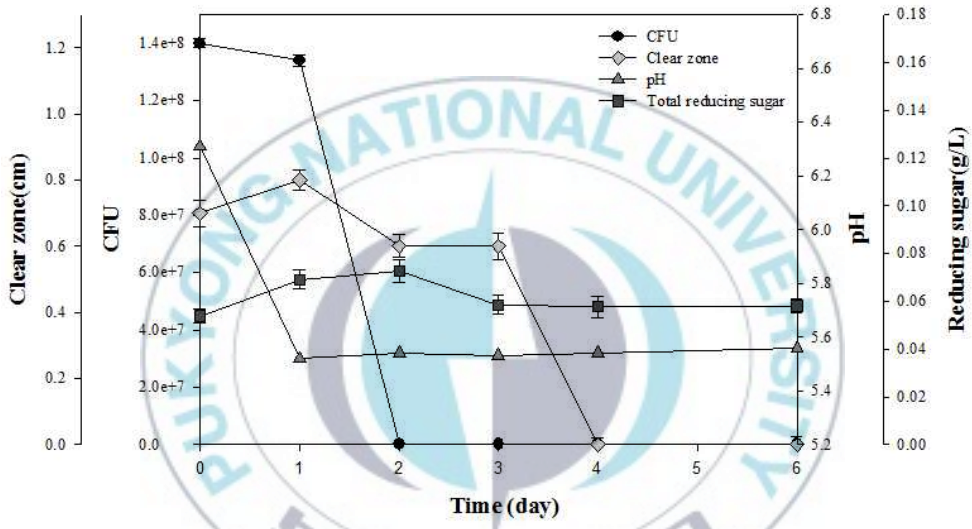

Fig. 2. Degradation of *Laminaria japonica* by pretreatment seaweed powder media. Error bars: mean ± S.D. of three replicates.

(A)

(B)

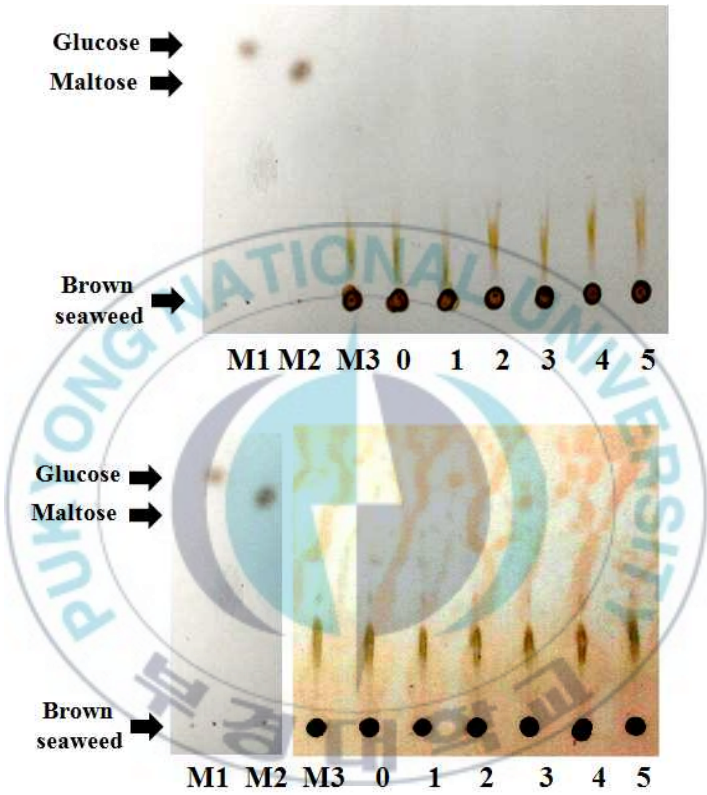

Fig. 3. TLC of the alginate degradation products (A) in the seaweed powder culture broths, prtreatment seaweed powder culture broths (B).

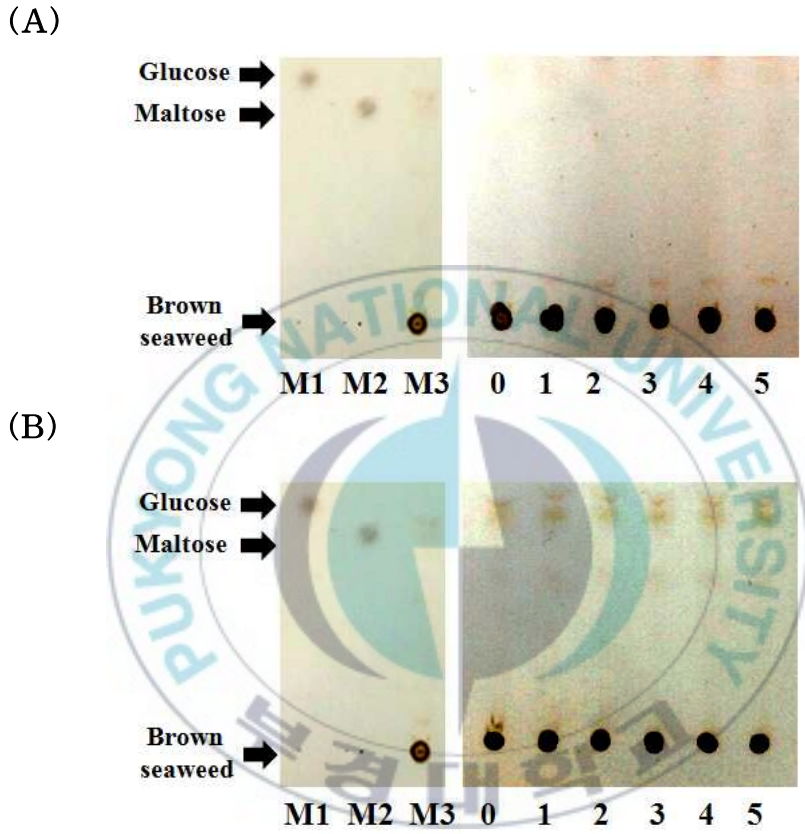

Fig. 4. TLC of the laminarin degradation products (A) in the seaweed powder culture broths, prtreatment seaweed powder culture broths (B).

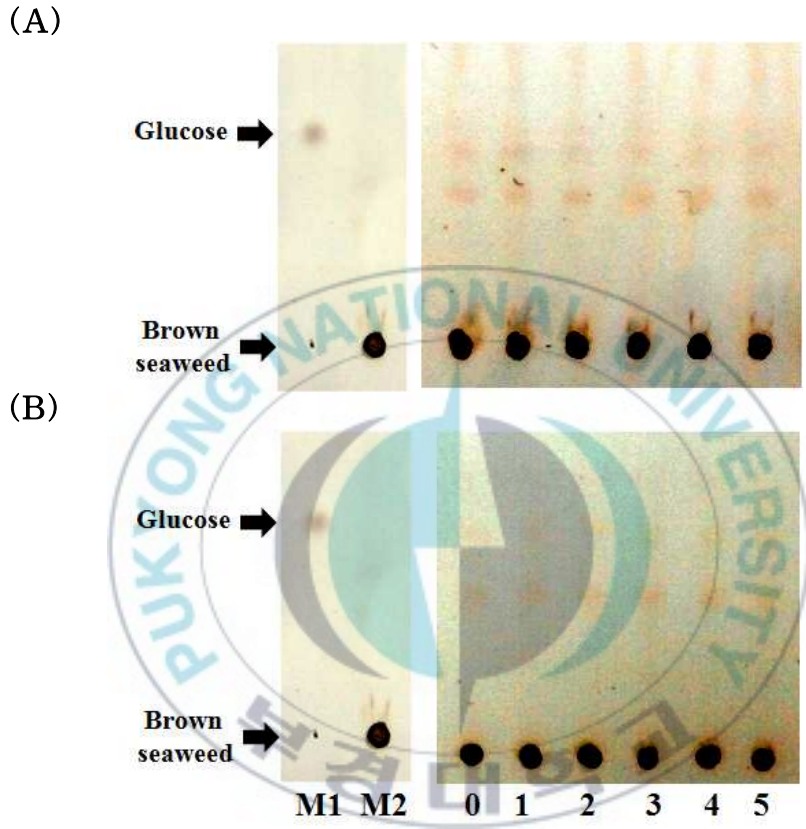

Fig. 5. TLC of the fucoidan degradation products (A) in the seaweed powder culture broths, prtreatment seaweed powder culture broths (B).

2. Biological Functions of the Biodegraded Culture Broths of Laminaria japonica.

2.1. Determination of Antioxidant Activity

2.1.1. DPPH Radical Scavenging Activity

The DPPH free radical is a stable free radical that is widely used as a tool for estimating the free-radical scavenging activities of antioxidants [45]. Free radicals are highly reactive species of atoms or molecules that are unstable because of single or unbalanced electrons. DPPH is a compound with a proton free radical that has a characteristic absorption. The absorption of a DPPH solution decreases significantly in the presence of proton radical scavengers. The scavenging of the DPPH free radical by antioxidants is due to their hydrogen-donating ability [46,47]. As shown in Figure 6, the DPPH free radical scavenging abilities of the various culture supernatants were diverse. The highest scavenging ability for DPPH (62%) were obtained from the culture supernatants collected at 3^{th} day of biodegradation using pretreatment seaweed. This value was a little lower than that (78%) of 0.1 mM L-ascorbic acid used as a positive control. A similar result can be found in the study of [48]. They reported that DPPH scavenging activity for *Sargassum tenerrimum* extract as 64.66% ± 2.08%.

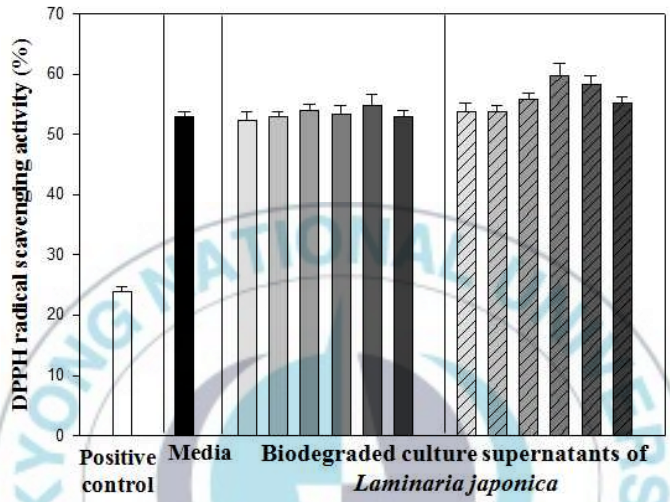

Fig. 6. DPPH radical scavenging activities of culture supernatants collected at 0^{th}(▉), 1^{st}(▉), 2^{nd}(▉), 3^{th} (▉), 4^{th} (▉) and 6^{th} (▉) days of biodegradation on *Laminaria japonica.* The biodegradations were seaweed media (▭), prtreatment seaweed media (▨). respectively. The DPPH radical scavenging activities of culture supernatantswere compared with those of positive control and *Laminaria joponica* itself. Error bars: mean ± S.D. of three replicates.

2.1.2. ABTS Radical Cation Decolorization Assay

It has been known that ABTS is considered as lipophilic and hydrophilic antioxidants [49]. As shown in Figure 7 The highest scavenging ability for DPPH (90%) were obtained from the culture supernatants collected at 3th day of biodegradation using pretreatment seaweed. The ABTS radical scavenging activity was not significantly dependent upon the media type. However, the values had an upward tendency as the biodegradation progressed. In the meantime, 0.2 mM L-ascorbic acid as a positive control exhibited 67% ABTS radical scavenging activity, while the values (between 78 and 90%) of *Laminaria japonica* itself at were little higher. There are some reports in the literature on the antioxidant capacity of algae. Alcoholic and aqueous extracts of seaweeds have been evaluated for antioxidant activity by lipoxygenase inhibition, DPPH assay and deoxyribose assays [50, 51, 52]. Recently, several marine alginate derivatives, sulfated fucoidans from the brown seaweed *Laminaria japonica,* agar-like sulfated galactans from the red seaweed Nori and sulfated polysaccharides from *Fucus vesiculosus*, have been reported to have antioxidant activity [53, 54, 55].

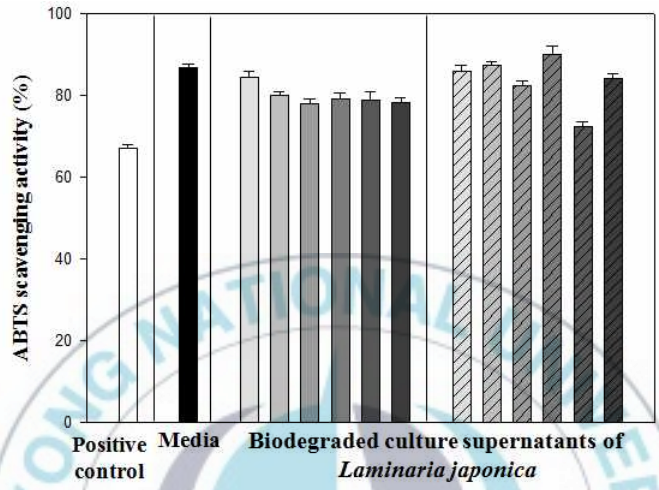

Fig. 7. ABTS radical scavenging activities of culture supernatants collected at 0^{th}(▉), 1^{st}(▉), 2^{nd}(▉), 3^{th} (▉), 4^{th} (▉) and 6^{th} (▉) days of biodegradation on Laminaria japonica. The biodegradations were seaweed media (▭), prtreatment seaweed media (▨), respectively. The ABTS radical scavenging activities of culture supernatants were compared with those of positive control and *Laminaria joponica* itself. Error bars: mean ± S.D. of three replicates.

2.1.3. Hydroxyl Radical Scavenging Activity Assay

It has been known that the hydroxyl radical is an extremely reactive oxidizing species. The hydroxyl radical can react with most biomolecules and is responsible for the formation of other radicals [56]. In this study, the hydroxyl radical scavenging activity of the culture supernatant was between 88 to 99% during 6 d of incubation (Figure 8). The hydroxyl radical scavenging activity was not significantly dependent upon the media type. However, the values had a slightly upward tendency as the biodegradation progressed. In the meantime, 0.1 mM L-ascorbic acid as a positive control exhibited 61% hydroxyl radical scavenging activity, while the values of *Laminaria japonica* at various concentrations were between 67 and 92%. [57] reported that the scavenging ability was non-linearly concentration-dependent. In this study, thus, its oligosacchirides exhibited higher hydroxyl radical scavenging activities.

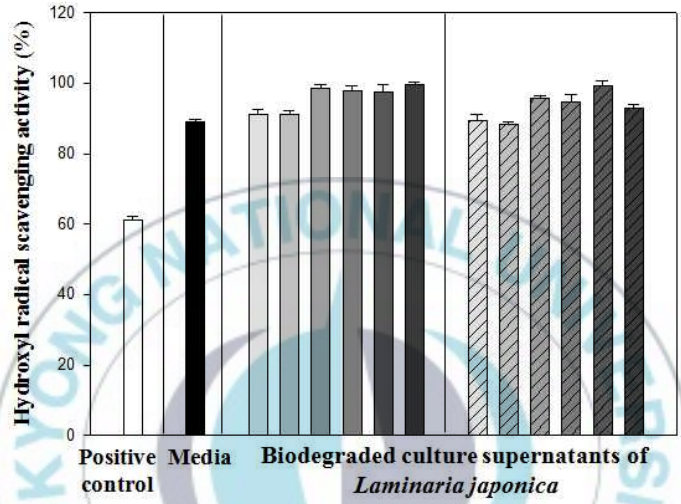

Fig. 8. Hydroxyl radical scavenging activities of culture supernatants collected at 0^{th}(▉), 1^{st}(▉), 2^{nd}(▉), 3^{th} (▉), 4^{th} (▉) and 6^{th} (▉) days of biodegradation on *Laminaria japonica*. The biodegradations were seaweed media (▭), prtreatment seaweed media (▩). respectively. The Hydroxyl radical scavenging activities of culture supernatantswere compared with those of positive control and *Laminaria joponica* itself. Error bars: mean ± S.D. of three replicates.

2.1.4. Reducing Power Assay

The reducing power assay is often used to evaluate the ability of an antioxidative compound and serves as an important indicator of potential antioxidant activity [58]. The antioxidative effect is reported to be related to the development of reductones [59] and correlated directly with the reducing power of certain bioactive compounds [60]. In the reducing power assay, the presence of reductants in the culture supernatant creates a reduction of ferric cyanide complex to a ferrous form, and the increase in the absorbance indicates increase of reducing power. During the 6 d of incubation, the reducing power of the culture supernatant was between 0.18 and 0.29 at A_{700nm}(Figure 9). The reducing power was significantly dependent upon the media type. And the reducing power had a slightly upward tendency as the biodegradation progressed. In the meantime, the reducing power of 0.1 mM L-ascorbic acid as a positive control was 0.28 at A_{700nm}, while the values (0.29) of pretreatment *Laminaria japonica* at various concentrations were little higer. Therefore, it was concluded that biodegraded pretreatment *Laminaria japonica* exhibited a better reducing power than not pretreatment *Laminaria japonica*.

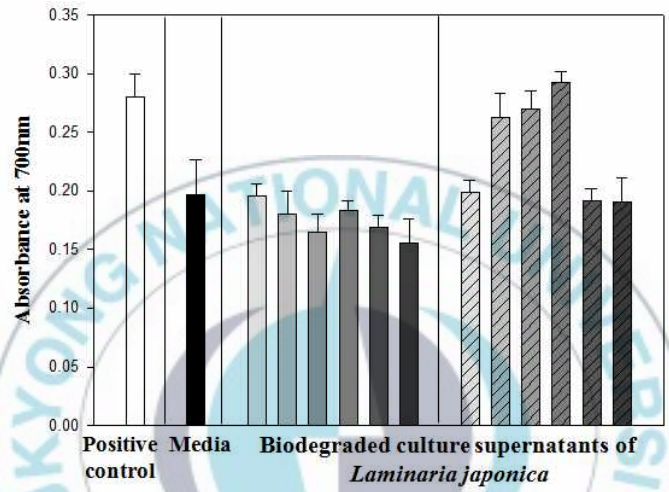

Fig. 9. Reducing powers of culture supernatants collected at 0^{th}(■), 1^{st}(■), 2^{nd}(■), 3^{th} (■), 4^{th} (■) and 6^{th} (■) days of biodegradation on *Laminaria japonica*. The biodegradations were seaweed media (▭), prtreatment seaweed media (▨). respectively. The Reducing powers of culture supernatants were compared with those of positive control and *Laminaria joponica* itself. Error bars: mean±S.D. of three replicates.

Ⅳ. CONCLUSION

The solubility was increased to 94% compared with its initial solubility (58%). *M. oxydans* was showed distinct *Laminiaria japonica* powder-degrading activity. The TLC showed alginate, laminarin and fucoidan degradation, even at low pH. Hydroxyl radical scavenging activity was 99%. Biodegraded products, various types of saccharides, would be used as bioactive substance, bioethanol and etc. To achieve higher productivity, fed-batch fermentation should be investigated.

V. ACKNOWLEDGEMENTS

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