LIU, XIN. Extraction and Anti-Bacterial Effects of Edible Brown Algae Extracts. (Under the direction of Dr. Wenqiao Yuan).

The desire to obtain natural phytochemicals with promising bioactivity and few or no side effects has been the motivation for investigating extraction processes for plants. These bioactivities make phytochemicals as potential food-preservation agents due to their effects of inhibiting lipid oxidation and spoilage microorganism growth, thus preventing food deterioration. In this study, extraction optimization of bioactive compounds from edible brown algae and their food preservation effects were investigated via the five objectives below.

The first objective was to study the effects of extraction temperature (30 to 70°C), liquid-to-solid ratio (10 to 90 mL/g), and ethanol concentration (20 to 100%) of an ethanol-water binary solvent system on extracts from edible brown algae *Ascophyllum nodosum*. Most extracts showed higher total phenol content (TPC), total carbohydrate content (TCC) and antioxidant activity before rotary evaporation. The strongest antioxidant activity was observed at low temperature (30 and 40°C), low liquid-to-solid ratio (30 mL/g), and high ethanol concentration (80 and 100%), suggesting that the antioxidants of *A. nodosum* were thermal sensitive and had low polarity.

The second objective was to optimize the extraction process using Box-Benhken Design (BBD) and Response Surface Methodology (RSM). The conditions for maximum antioxidant activity of the crude extract were found at 70 mL/g, 80% ethanol, and 20°C, while the conditions for the highest crude extract yield were at 60°C, 50.02 mL/g, and 45.65% ethanol. The model-predicted antioxidant activity and yield were 72.75 mL/mg and 55.68 mg/g, respectively, which were in close agreement with the experimental results of 74.05±0.51 mL/mg and 56.41±2.59 mg/g, respectively, suggesting that the models could accurately predict and improve the extraction of antioxidants from *A. nodosum*.

The third objective was to evaluate stability of *A. nodosum* extract. The TPC and antioxidant activity decreased by approximately 5% and 10%, respectively, after 6 hours of thermal treatment (40-90°C). Extract exposed to air and 25°C for 108 h showed lower TPC than those stored in closed containers and 0°C. Antioxidant activity of extracts remained constant at
both 0 and 25°C, regardless of exposure to air. The results suggested that the extract of *A. nodosum* was stable under various conditions.

**The fourth objective** was to evaluate the *in vitro* anti-bacterial effects of the crude extract against *Pseudomonas fluorescens* and *Shewanella putrefaciens*, specific spoilage organisms (SSOs) of tilapia. The Lambert-Pearson model was applied to determine minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC). The best-fit MIC and NIC for *P. fluorescens* was 1.145 and 0.036 mg/mL, and 0.947 and 0.106 mg/mL for *S. putrefaciens*, respectively. Algae extract (10µg/disc) showed larger inhibition zones than penicillin (42 µg/disc) against both bacteria strains. All results suggested that crude extract of *A. nodosum* has a promising anti-bacteria effect and could be applied as potential food preservation agent.

**The fifth objective** was to investigate possible anti-bacterial mechanisms of *A. nodosum* extract against *P. fluorescens* and *S. putrefaciens*. Extracellular enzyme activity was inhibited and exopolysaccharide content was reduced by 44.82 and 64.67% in *P. fluorescens* and *S. putrefaciens*, respectively, treated with algae extract. Number of live cells in biofilms attached to petri dishes decreased after co-culturing with the extract. Significant increase of 280-nm observing material, protein content and conductance in the cell-free media was observed during co-culturing with the algae extract, suggesting enhanced membrane permeability.

This project was the first to optimize the extraction process to obtain extracts from *A. nodosum*, an underexplored seaweed, and evaluated stability of the extract. It was also the first to evaluate the *in-vitro* antibacterial effects against the food spoilage bacteria *P. fluorescens* and *S. putrefaciens*, and to illustrate the anti-bacterial mechanisms of the extract. Findings of this project suggested that the extract could be used as novel food preservation agents.
Extraction and Anti-Bacterial Effects of Edible Brown Algae Extracts

by

Xin Liu

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APPROVED BY:

Dr. Wenqiao Yuan
Dr. John van Zanten
Committee Chair

Dr. Sophia Kathariou
Dr. Otto “Chip” D. Simmons, III

Dr. Jay Cheng
DEDICATION

This work is dedicated to my parents, Mr. Xiaonan Liu and Mrs. Tongxiu Zhang, for their love, encourage, and support. And to my grandparents on my father’s side and mother’s side in heaven, Mr. Jingtang Liu and Mrs. Binghua Zhang, Mr. Yude Zhang and Mrs. Wanhua Dai, who gave me the most precious love and encouraged me to pursue my dream of studying abroad.
**BIOGRAPHY**

Xin Liu was born in Zibo, Shandong, China in 1990. She received her bachelor's degree from Harbin Institute of Technology at Weihai in 2012, majored in Biotechnology. In August 2013 she joined the research group of Dr. Wenqiao Yuan in Department of Biological and Agricultural Engineering, North Carolina State University, and obtained her MS degree in Oct 2015. In Jan 2016, she enrolled in the Ph. D program in the same department under the direction of Dr. Yuan.
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CHAPTER 1. Introduction

It has been discovered that consuming leafy green vegetables, fruits, and herbs, aids to enhance the health and well-being of human. This fact brought about a great concern for searching of bioactive compounds in foods with health benefiting effects (Rajauria et al., 2013). Macroalgae, also called seaweeds, are underexploited sources of protein, dietary fiber, micronutrients, unsaturated fatty acids, and bioactive compounds. Seaweeds can be divided into three groups, red algae (Rhodophyceae), brown algae (Phaeophyceae), and green algae (Chlorophyceae), based on the major pigments they contain (Wifesekara et al., 2011). Brown algae contain up to about 20% phlorotannins per dry mass (Steevensz et al., 2012). Phlorotannins are a subgroup of phenolic compounds that can only be found in brown algae with various bioactivities, including antioxidant (Lopez et al., 2011), anti-microbial (Kim et al., 2013), anti-diabetes (Kang et al., 2013), anti-inflammatory (Kim and Bae, 2010), etc. Phenolic compounds play an important role in plant self-defense against viruses, bacteria, and stresses such as wounding and excessive light or ultraviolet radiation (Cox et al., 2010). The harsh habitats in the ocean, e.g. marine grazers, epiphytes, and UV radiation, are driving forces for accumulation of phlorotannins in brown algae (Audibert et al., 2010). Polysaccharides are the other group of bioactive compounds that have been found in marine algae. Some of the algal polysaccharides have been found to have health-benefiting effects and potential applications as food, pharmaceuticals, and in agriculture and chemistry industry (Wifesekara et al., 2011).

One important process of obtaining these health-benefiting compounds is extraction. Yet a universal extraction protocol is not applicable, and a specific extraction process is required for each biological material to be extracted. Yield and bioactivity of the extracts harvested are highly dependent on extraction method used (Lopez et al., 2011). Ideally, optimizing extraction process resulted in decreased solvent consumption, shorter extraction time, improved yield and bioactivities of extracts obtained. The type of solvent, solvent concentration, solid/liquid ratio, extraction time, and temperature all showed great influence on extraction process efficiency (He et al., 2013). Multiple solvents should be applied to separate phenolic compounds from the plant matrix due to their nature to bind to other plant components, e.g. carbohydrates and proteins (Ajila et al., 2013). However, using too much toxic organic solvents is an obstruction for the application of the extracts in the food industry. Ethanol is a good choice for polyphenol extraction and is relatively safer for human consumption than other solvents (Dai and Mumper,
A binary solvent system containing ethanol and water has been used to obtain phenolic extracts from citrus (Li et al., 2010), plum (Li et al., 2016), mushroom (Mao et al., 2013), and spruce wood bark (Ghitescu et al., 2015).

It is globally admitted that foods are perishable, some highly nutritional foods with abundant proteins and lipids tend to be spoiled within days under an inappropriate storage condition. Synthetic preservatives have been widely used to reduce microbial spoilage, retard lipid oxidation, and extend the shelf life of foods. However, concerns about the safety, side effects, and microbial resistance of synthetic preservatives have been increased and people are looking for natural preservatives.

1.1. Problem Statement

This project was aimed to optimize the extraction process of obtaining natural antioxidants and anti-bacterial agents from edible brown algae *Ascophyllum nodosum*, and to evaluate their potential as food preservation agent and understand the mechanisms against food spoilage bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens*. The hypothesis of this project was that using ethanol-water binary solvent system could obtain algae extracts with strong bioactivities that could be used as potential food preservation agent for tilapia fillets preservation. Although investigations have been conducted on antioxidant/antibacterial effects of plant extracts, little was known about seaweed phytochemicals, especially the mechanism of their antibacterial actions and food preservation effects. Five specific research needs were listed below:

1. The influence of different extraction conditions on bioactivities of algae extract has not been studied.
2. Although process optimization has been reported in obtaining extracts of many edible materials, none has been investigated on *A. nodosum* before;
3. Thermal stability and influence of storage conditions on bioactivities of *A. nodosum* extract have not been reported;
4. The *in-vitro* antibacterial effect of *A. nodosum* extract has not been studied yet;
5. The mechanisms of antibacterial effects of *A. nodosum* extract has not been fully illustrated.
1.2. Research Objectives

This project was aimed to develop an effective extraction process to obtain algae extract with high yield and bioactivities, to evaluate the potential of applying algae extract as food preservation agent, and to understand the mechanisms of antibacterial actions of the extracts.

This work was divided into five main objectives:

1. Understand the influence of extraction temperature, liquid-to-solid ratio, and ethanol concentration of an ethanol-water binary solvent system, and study the influence of thermal treatment on TPC, TCC, and antioxidant activity of edible brown algae A. nodosum extracts;

2. Optimize the extraction process with Box-Behnken design (BBD) and response surface methodology (RSM) for the purpose of obtaining extracts with strong antioxidant activity and high yield;

3. Evaluate the stability and content of antioxidants of algae extract during thermal treatment and storage in room temperature and in a refrigerator.

4. Study the in-vitro anti-bacterial effects of A. nodosum extracted against food spoilage bacteria P. fluorescens and S. putrefaciens. Anti-bacterial effect was also evaluated on tilapia fillets stored in a refrigerator at 8°C.

5. Investigate the antibacterial mechanisms of A. nodosum extract against P. fluorescens and S. putrefaciens.
CHAPTER 2. Review of Literature

2.1. Bioactivities of edible brown algae extracts

More than 21,800 structurally different, bioactive natural compounds with astounding biological activities have been found from marine microorganisms, algae, and invertebrates (Eom et al., 2012). One important reason that marine organisms host such rich source of bioactive components is that they are living in harsh habitats with extreme conditions such as extreme variations of temperature and salinity levels, low light intensities, and nutrient-deficient (Samarakoon and Jeon, 2012). To survive in such environments, marine organisms produce a diverse group of secondary metabolites which cannot be found elsewhere. Among marine organisms, marine algae, which are also called seaweeds, have been identified as an under-exploited source of nutraceuticals, cosmetics, and pharmaceuticals. The importance of marine macroalgae as functional foods and medicine has been recognized and researchers have revealed that their active compounds exhibit various biological activities (Balboa et al, 2013; Eom et al., 2012; Wifesekara et al., 2011). Table 2.1 shows a list of literature reviews summarizing algae originated active compounds (phlorotannins, polysaccharides, and proteins) and their bioactivities.
2.2. Current research on *Ascophyllum nodosum* extracts

Also known as rockweed, *A. nodosum* is an edible brown algae growing in the northern Atlantic Ocean. It is common on the northwestern coast of Europe and the northeastern coast of North America. This algae species has been investigated in this study because it is rich in phlorotannins and easily available in North America. However, there are only a few publications reporting extracts of *A. nodosum*, suggesting this algae was not fully studied or developed.

Audibert et al. (2010) reported that total phenol content of *A. nodosum* was 2.5% of dry algae and 3.5 g/kg of fresh algae. A slightly higher phlorotannin content in *A. nodosum* was found to be 4.8±0.5% dry weight (Connan et al., 2006). Ultrasound-assisted extraction was optimized using response surface methodology for maximum total phenol, fucose, and uronic acids. The highest yields of total phenolics, fucose, and uronic acids were obtained using 0.03 M HCl at an ultrasound amplitude of 114 μm for an extraction time of 25 min (Kadam et al., 2015b). Water extracts from ultrasonic assisted extraction was analyzed using LC-DAD-ESI-MS/MS by Agregan et al. (2017) and the results showed that phlorotannins were tentatively identified as the main phenolic compounds, followed by phenolic acids, flavonoids, and fuhalols.
Laminarin was extracted using 60% ultrasonic power amplitude and 0.1 M hydrochloric acid from *A. nodosum*, showed DPPH radical inhibition level of up to 93.23% (Kadam et al., 2015a). Sulfated polysaccharides were extracted first with 80% ethanol then with hot water, and the identified components included mannose, ribose, glucuronic acid, glucose, and fucose (Chen et al., 2018).

Bioactivities of *A. nodosum* extracts reported included anti-hyperglycemic (Kim et al., 2014; Nwosu et al., 2011; Pantidos et al., 2014; Zhang et al., 2007), antioxidant (Dutot et al., 2012), anti-inflammatory (Bahar et al., 2016; Dutot et al., 2012), and anti-proliferative (Nwosu et al., 2011). They have also been investigated as potential biostimulants that target the modulation of crop stress to increase productivity (Goni et al., 2018) and reduce fungal disease (Jayaraman et al., 2011). It was also reported that feeding tilapia (*Oreochromis niloticus*) with *A. nodosum* meal could reduce the number of lesions in fish (Oliveira et al., 2014).

### 2.3. Extraction process optimization in marine algae

Although extraction process optimization has been widely reported in terrestrial plants, studies on improving efficiency of extracting bioactive compounds from seaweed are limited. Several extraction methods and tests have been performed to study the influence of various factors on the efficiency of extraction processes from seaweeds.

Solvent type has been the factor that has been studied the most. Polar solvents are more efficient at extracting phenols than water and non-polar solvents (Wang et al., 2012). It was reported by Lopez et al. that extracting solvent significantly affected total phenol content and antioxidant activity of brown algae *Stypocaulon scoparium* extracts and ethanol might dissolve more radical-scavenging active polyphenols than water and methanol (Lopez et al., 2011). Similar results were found that although water extract yield (5.2%) of brown algae *Ecklonia stolonifera* was three times higher than that of methanol extract (1.5%), the total polyphenol content in methanol extract was 3.8 times greater than water extract (Iwai, 2008). A study conducted to study the influence of methanol concentration (20, 40, 60, and 80%) on free radical scavenging activity of brown seaweed *Himanthalia elongate* confirmed that 60% methanol extract showed the highest total phenol content and yield. However, the strongest DPPH radical scavenging activity was found in the 80% methanol extract (Rajauria et al., 2013). Kuda et al.
(2006) reported that the water extract showed higher DPPH radical scavenging activity and total phenol content than ethanol extract of brown algae *Petalia binghamiae*.

Extraction temperature has also been investigated for its effects on the extraction process. It was reported that water extracts of edible brown algae *P. binghamiae* obtained at high temperature, 85 and 121°C, showed an enhanced DPPH radical scavenging activity. However, they also indicated that activity of ethanol extracts was slightly decreased after heat treatment (Kuda et al., 2006). A study using response surface methodology revealed that the optimum extraction conditions for maximum antioxidant activity from the red algae *Laurencia obtuse* was as follows: solvent:seaweed ratio 24.3:1; extraction temperature, 45.3°C; and extraction time, 58 min, while the optimum conditions for phenolic content were a combination of solvent:seaweed ratio 30:1, temperature 50°C, and extraction time 42.8 min (Topuz et al., 2016). Ultrasound assisted extraction from brown algae *A. nodosum* was optimized using response surface methodology for maximum total phenol, fucose, and uronic acids. The highest yields of total phenolics, fucose, and uronic acids were obtained using 0.03 M HCl at an ultrasound amplitude of 114μm for an extraction time of 25 min (Kadam et al., 2015b). Ultrasound assisted extraction and response surface methodology were also utilized to optimize sulfated polysaccharides extraction from *A. nodosum* by Chen et al. (2018), The components identified from the purified sample included mannose, ribose, glucuronic acid, glucose, and fucose.

2.4. **Statistical and experimental design to optimize extraction process**

Response surface methodology (RSM) have been used widely as an affective statistical technique to optimize extraction processes (Zou et al., 2015). It not only defines the effects of independent variables, but also takes their interaction into model prediction. This methodology was usually used together with Box-Behnken design (BBD) (Zou et al., 2015), central composite design (CCD) (Wani et al., 2017; Zhu et al., 2015), or full factor design (Ghitescu et al., 2015) to optimize the process variables. BBD was preferred in most studies because it requires fewer runs than CCD and full factor design (Zhu et al., 2015). Single factor experiments were often carried out to determine the range of variables for RSM (Yuan et al., 2015; Zhu et al., 2015). The software Design-Expert (State-Ease Inc., Minneapolis, USA) (Yuan et al., 2015; Zou et al., 2015), SPSS (Chicago, IL, USA) (Mao et al., 2013; Yuan et al., 2015), and Matlab (Mathworks, USA) (Chabeaud et al., 2009) have been used in literature to design or analyze the data obtained.
Experimental results were analyzed usually by quadratic stepwise regression to fit the second-order equation as follows:

\[
Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j
\]

where \( Y \) is the response function, \( \beta_0 \) is the intercept, \( \beta_i \), \( \beta_{ii} \), and \( \beta_{ij} \) are the coefficients of the linear, quadratic and interactive terms, respectively. \( X_i \) and \( X_j \) represent the coded independent variables. Finally, a validation of the predicted optimal conditions obtained from the model needs to be performed through experiments.

2.5. Stability and degradation of plant extracts during thermal treatment and cold storage

Fruits and vegetables are excellent sources of phenolic compounds, including phenolic acids, anthocyanins, and flavonoids. These phenolic components are readily oxidized and thus prone to degradation (Srivastava et al., 2007). Many chemical reactions could be induced by thermal processes, including Maillard reaction, caramelization, chemical oxidations of phenols, and degradation of polysaccharides. Reducing sugar and total phenol content are two typical variables reflecting polysaccharide and polyphenol degradation (Lu et al., 2018; Maskat and Tan, 2011). The kinetics of thermal degradation of polyphenol compounds have been studied on several terrestrial plant extracts, including elderberry (Oancea et al., 2018), grape pomace (Solyom et al., 2014), sea buckthorn (Ursache et al., 2017), plum (Turturica et al., 2016), and sour cherry (Oancea et al., 2017). Most phenolic extracts were reported to follow a first-order degradation kinetics, with higher reaction rate constant \( (k) \) and lower half live \( (t_{1/2}) \) observed at higher thermal treatment temperature and longer treatment period. Reduction in TPC was also found to be associated with decreasing antioxidant activity in plant extract because phenolics are the major plant compounds that exhibit antioxidant activity (Ursache et al., 2017). Stabilities of extracts during low temperature storage at 4/20℃ were reported in wine lee (Tao et al., 2014), grape marc (Amendola et al., 2010), and roselle (Sinela et al., 2017). TPC in the extracts studied above remained relatively stable (degradation ranging from 12 to 17%) after storage from 30 days to up to one year. However, the studies on stability and degradation of seaweed extract during thermal treatment or storage, or their stability as preservation agents during real food storage were very limited according to the author’s knowledge.
2.6. Antibacterial effects and mechanisms of seaweed extracts

The bactericidal or bacteriolytic effects of seaweeds extracts, especially brown algae extracts, have been reported in very limited studies. However, the antibacterial mechanisms of seaweed extracts were not fully understood. It has been suggested that possible modes of antibacterial actions include attacking bacteria cell wall and membrane, disruption of electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity, and altering cell membrane (Gupta and Abu-Ghannam, 2011). The MBCs (taken as the lowest concentration that could kill 99.9% of the initial bacteria inoculum within 24 h) of crude phlorotannins extracted from the brown algae *Ecklonia kurome* against nine strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were reported to be ranging from 100 to 400 mg/L. The purified phlorotannins, dieckol and bieckol especially, showed the highest bactericidal effects among other purified compounds, suggesting that the bactericidal effects of phlorotannins tend to increase with polymerization of phloroglucinol (Nagayama et al., 2002). The interaction of phlorotannins and other phenolic compounds with bacterial enzymes and proteins may play an important role in their bactericidal actions (Nagayama et al., 2002). Treating oral microorganisms with *Laminaria japonica* extract could modify the cell surface texture and inhibit the growth of the bacteria (Kim et al., 2013). Similarly, disruption of cell membranes was observed in mixed anaerobic microbial cultures treated with *Laminaria digitata* extract using transmission electron microscopy (TEM). Cell membrane leakage assessed by measuring 260-nm absorbing material (nucleotide mainly) was reported and higher energy consumption was required under the stress of algae extract (Hierholtzer et al., 2012). Leakage of 260-nm absorbing material, potassium, and increase of conductivity in medium induced by *Enteromorpha linza* L. extract was reported in foodborne pathogenic bacteria *Bacillus cereus* and *Listeria monocytogenes* (Patra and Baek, 2016a; Patra and Baek, 2016b), which indicated that algae extract might alter cell membrane permeability of both Gram positive and negative bacterium.

2.7. Food preservation effects of algae extracts

Marine algae-originated compounds and extracts have been studied for their antimicrobial and antioxidant effects for potential application as food preservatives. Brown algae *H. elongate* extracts have been confirmed to reduce growth of food spoilage and pathogenic bacteria *Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella abony, and Enterococcus*
faecalis (Rajauria et al., 2013). The extract of these algae was also investigated for its antioxidant potential and antimicrobial efficacy in carbohydrate and protein model food systems containing BSA or glucose. The extract provided up to 100% inhibition against L. monocytogenes and Salmonella abony in carbohydrate model food system and a bacteriostatic effect in protein model food systems (Cox et al., 2014). Spray-dried Laminaria digitate extract was added directly to minced pork, and 0.5% extract significantly decreased lipid oxidation in cooked patties (Moroney et al., 2013). Phlorotannin extracts of Fucus vesiculosus showed inhibition effects on lipid oxidation in washed cod muscle and cod protein (Wang et al., 2010). A. nodosum extract incorporated into a gelatin/ casein film impacted antioxidant activity and increased the level of total phenolic compounds of the film (Kadam et al., 2015c). Another study using unrefined A. nosodum extracts confirmed that Na₂CO₃ extract was adequate to develop transparent, flexible and edible films while NaOH extracts rendered films with the highest antioxidant activity, which could be used to improve food preservation (Blanco-Pascual et al., 2014). Addition of seaweed extracts from the edible seaweeds A. nodosum, F. vesiculuous, and Bifurcaria bifurcata could slightly protect pork liver pate from lipid oxidation at the end of storage. However, the pate without seaweed extracts also showed a strong lipid oxidation stability so it was not possible to assess the antioxidant potential of algae extracts (Aregan et al., 2018). Extracts from four edible seaweeds, cochayuyo, sea lettuce, ulte, and red luche were used as covering liquids in canned Atlantic salmon. After storage at 40°C for 170 days, all fish samples had acceptable oxidized odor and flavor scores, suggesting potential usage of seaweed extracts as covering liquids in canned Atlantic salmon (Ortiz et al., 2014).

2.8. Phenolic extracts from seafood as natural food preservatives

Seafoods are highly perishable due to high amount of protein and polyunsaturated fatty acids (PUFAs). The presence of myoglobin and hemoglobin and trace amounts of iron and copper ions also make fish more susceptible to lipid oxidation (Maqsood et al., 2014). Besides lipid oxidation, autolytic enzymatic spoilage and microbial spoilage are also the principle causes of quality loss and spoilage in fish (Ghaly et al., 2010). Fish muscle is sterile at the time of slaughter/catch but becomes quickly contaminated by microorganisms from equipment and humans during handling and processing (Sivertsvik et al., 2002). Such deterioration would not only cause off-flavor and decrease in nutrient value, but also posed a risk to human health.
Synthetic antioxidants such as ethoxyquin and butylated hydroxytoluene have been widely used in the past to retard lipid oxidation and extend shelf life of seafood in the past (Lundebye et al., 2010), however, concerns about their safety and side effects have been growing and people are seeking natural antioxidants as replacement.

It has been suggested that polyphenols can bind iron thereby reducing the pro-oxidative activity of metal ions in fish. Polyphenols interact with microbial membrane proteins, proteins, and lipids, thereby altering cell permeability and permitting the loss of protons, ions, and macromolecules (Maqsood et al., 2013). Plant extracts from bayberry leaf (Su et al., 2013), thyme (Alcicek, 2011), cinnamon (Rattanachaikunsopon and Phumkhachorn, 2010), green tea (Golvardzadeh et al., 2016; Song et al., 2011), grape seed (Golvardzadeh et al., 2016), cumin seed, mint leaf (Raeisi et al., 2016), and bioactive compounds like chitosan, carvacrol (Chaparro-Hernandez et al., 2015), and tocopherols (Barbosa-Pereira et al., 2013) have been investigated for their preservation effects against fish spoilage bacteria or on fish fillets. Though algae extracts have been used as food preservative in other foods, they have not been applied much on seafoods (Gupta and Abu-Ghannam, 2011; Maqsood et al., 2013). Phlorotannin extracts of *F. vesiculosus* showed inhibition effects on lipid oxidation in washed cod muscle and cod protein (Wang et al., 2010). Nori and Hijiki seaweed extracts were studied as natural additives for their preservation effects on minced tilapia. The algae extract had no effect on the composition of minced tilapia but showed an inhibitory effect on the total volatile base nitrogen (TVB-N) level (Riberio et al., 2014). Application of four seaweed extracts in canned Atlantic salmon could prevent lipid oxidation after storage at 40°C for 170 days (Ortiz et al., 2014).

**2.9. Edible coating as a method of active packaging**

Natural polymers are increasingly used as package material due to their eco-friendly nature, ability to extend shelf life, and preserve quality of perishable foods which are susceptible to oxidative and microbial deterioration (Kadam et al., 2015a). Biodegradable edible coatings serve as selective barriers to moisture transfer, oxygen uptake, lipid oxidation, and loss of aroma volatiles, thereby extending shelf life and improving food quality (Maqsood et al., 2013). Recent research has been focused on applying natural polymers with bioactive compounds and phytochemicals as edible films or edible coating (Table 2.2). Natural antioxidants and antimicrobials can be incorporated in edible coating to enhance their food preservation function.
Bioactive edible coatings are usually applied by dipping foods in film solution containing natural compounds or spraying solution on food. Protein could serve as a film-forming agent with good barrier characteristics against gas, organic vapors, and oils, but they are also reported to have poor mechanical characteristics and high water permeability (Maqsood et al., 2013). Alginate is the other natural polymer used as film-forming agent. It is a hydrophilic polysaccharide extracted from brown algae (Sakar and Shetty, 2014). Chitosan could be used as both antimicrobial and film-forming agent against spoilage bacteria *Aeromonas* and *Micrococcus* (Cao et al., 2012).

<table>
<thead>
<tr>
<th>Coating material-preservatives</th>
<th>Food studied</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alginate-thyme oil</td>
<td>fresh chicken breast fillets</td>
<td>Sakar and Shetty, 2014</td>
</tr>
<tr>
<td>alginate-grape seed extract</td>
<td>grapes</td>
<td>Aloui et al., 2014</td>
</tr>
<tr>
<td>chitosan-green tea extract</td>
<td>pork suasages</td>
<td>Siripatrawan et al., 2012</td>
</tr>
<tr>
<td>alginate-tea polyphenol/Vc</td>
<td>bream</td>
<td>Song et al., 2011</td>
</tr>
<tr>
<td>tapioca starch-green tea extract</td>
<td>fruit-based salads, romaine hearts, and pork slices</td>
<td>Chiu and Lai, 2010</td>
</tr>
<tr>
<td>chitosan-grape seed extract</td>
<td>table grapes</td>
<td>Wu et al., 2007</td>
</tr>
<tr>
<td><em>Gelidium corneum</em> powder/gelatin-grapefruit seed extract</td>
<td>pork loin</td>
<td>Hong et al., 2009</td>
</tr>
<tr>
<td>red algae/grapefruit seed extract</td>
<td>cheese and bacon</td>
<td>Shin et al., 2012</td>
</tr>
<tr>
<td>fungal chitosan/pomegranate peel extract</td>
<td>tilapia fillets</td>
<td>Alsaggaf et al., 2017</td>
</tr>
<tr>
<td>chitosan-carvacrol</td>
<td>tilapia fillets</td>
<td>Chaparro-Hernandez et al., 2015</td>
</tr>
<tr>
<td>agar-green tea extract/probiotic bacteria</td>
<td>hake fillets</td>
<td>Lopez de Lacey et al., 2014</td>
</tr>
</tbody>
</table>
REFERENCES


Thoo, Y.Y., Ho, S.K., Liang, J.Y., Ho, C.W., Tan, C.P., 2010. Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (Morinda citrifolia). Food Chemistry. 120: 290-295.


CHAPTER 3. Extraction of Antioxidants from Brown Algae \textit{Ascophyllum nodosum} Using a Binary Solvent Extraction System

\textbf{Abstract}

This research was aimed to understand the effects of extraction temperature, liquid-to-solid ratio, and ethanol concentration of an ethanol-water solvent system on crude extracts obtained from the edible seaweed \textit{Ascophyllum nodosum}. The total phenol content (TPC), total carbohydrate content (TCC), and DPPH radical scavenging assays were measured for both fresh extract (FE) and dried extract (DE). The highest value of TPC in FE was observed at 30\(^\circ\)C, 90 mL/g, and 100\% ethanol. The TCC of FE tended to increase with increasing temperature and decreasing liquid-to-solid ratio, while moderate ethanol concentration (40\%) resulted in the highest TCC. The strongest antioxidant activity of the FE was observed at low temperatures (30 or 40\(^\circ\)C), relatively low liquid-to-solid ratio (30 mL/g), and high ethanol concentration (80 or 100\%). All DEs, except those obtained at 60 or 70\(^\circ\)C, showed lower TPC, TCC and DPPH radical scavenging activity than the FEs.

\textbf{Keywords} antioxidant, polyphenols, polysaccharides, thermal treatment, brown algae, \textit{Ascophyllum nodosum}

\textbf{3.1 Introduction}

Macroalgae, or seaweeds, have a long history of being a human food or medicine in Asia due to their abundant dietary fiber, proteins, minerals, and health-benefiting secondary metabolites. Japan has the largest population of seaweed consumers, with average consumption of 1.6 kg (dry weight) per person per year. Seaweeds are also processed in Europe and America as food additives in food preparation (Fu et al., 2016). Today, the global seaweed industry is worth more than 6 billion dollars per year, of which 85\% comprises food products for human consumption (FAO, 2018). The total production of seaweed doubled to 30.4 million tons in 2015 compared to 14.7 million tons in 2005.

The brown algae \textit{Ascophyllum nodosum} grow in the cold water of intertidal zone on the coast of Northern Atlantic, usually in large swathes (McCarthy et al., 1999). \textit{A. nodosum} has demonstrated strong antioxidant activity and high total phenol contents in previous studies (Liu
et al., 2017a; Liu et al., 2017b). Antioxidants can delay or prevent the oxidation damage by reactive oxygen species (ROS) generated during normal cellular metabolic processes. These ROS are known to be related to carcinogenesis, atherosclerosis, and aging (Hu et al., 2010). As the side effects of chemical additives being revealed (Sharma 2015), the desire for natural antioxidants and antimicrobial agents as the substitute for chemical additives becomes stronger. Naturally derived antioxidants from plants or animals are thus considered superior to chemical antioxidants for consumers (Fu et al., 2016).

Extraction is the initial and the most important step in obtaining antioxidative compounds from plant or animal materials. During solvent extraction, the damaged plant materials are swelled by the solvents followed by diffusive phenomena first inside the plant matrix and secondly through the outer layer surrounding the plant cells (Silva et al., 2007). A universal extraction procedure is not applicable considering the diversity in structure, physico-chemical properties, and composition of natural components. Several factors, e.g. liquid-to-solid ratio, extraction time, temperature, and solvent type have important effects on the yield, antioxidant activity, and content of the extracts (Zou et al., 2015; Karacabey et al., 2010). Single factor design has been applied to study the influence and determine the preliminary range of extraction factors of several terrestrial plants such as mulberry, pomegranate, and plantago (Chen et al., 2015; Ye et al., 2011; Zhu et al., 2015). Only few studies, to the author’s knowledge, have been reported on single factor experiments on extraction conditions of tropical seaweeds grown in Sabah (Fu et al., 2016), while seaweeds harvested from cold waters have not been studied yet. Removing the organic solvent is the next step after extraction and very important in assuring the quality of the extract. Generally, solvents can be removed by freeze-drying or thermal treatment such as rotary evaporation. Rotary evaporation is preferred when large amounts of organic solvents need to be removed. Although thermal treatment has been widely used (Mao et al., 2013; Chen et al., 2015; Zhu et al., 2015; Zhang et al., 2015; Ye et al., 2011; Li et al., 2010) to remove solvents in extracts, its influence on the bioactivity and content of extracted compounds has not been fully studied.

The present study was aimed to investigate the influence of extraction temperature, liquid-to-solid ratio, and ethanol (EtOH) concentration of a binary solvent system, as well as thermal separation process on total phenol content (TPC), total carbohydrate content (TCC), and 2,2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of A. nodosum extracts. It
was hypothesized that all three factors, i.e., the extraction temperature, liquid-to-solid ratio, and ethanol concentration would influence the yield and bioactivity of *A. nodosum* extract, and the following thermal drying process would negatively affect the bioactivity of the extract further.

3.2. Materials and Methods

3.2.1. Materials

Dried whole leaves of *A. nodosum* sealed in airtight bags were purchased from Maine Coast Sea Vegetables (Hancock, Maine, USA). Before experiments, the whole leaves were washed, dried in a 50°C oven, ground into powder and sieved with a 1-mm sieve; the powder was then collected, sealed in air-tight containers, and stored in a -4°C refrigerator until extraction.

3.2.2. Extraction and sample preparation

*Extraction at different temperatures:* Algae powder (3 g) was placed in 250-mL flasks, added with 70% EtOH at a liquid-to-solid ratio of 40 mL/g, and sealed with parafilm to avoid contact with oxygen. Temperature was kept using a water bath with Julabo heating immersion circulator (Julabo, Germany). A magnetic stirring bar was added to each flask and a 15-place magnetic stirrer with detachable control panel (Thermo Fisher Scientific, US) was placed under the water bath to agitate solvents with solids. The temperature was set at 30, 40, 50, or 60°C. Assays at 70°C were performed with a C-MAG HS 7 magnetic stirrer (IKA, Staufen im Breisgau, Germany). All experiments were carried out in triplicate for 30 min.

*Extraction with different solvent-to-solid ratios:* Algae powder (3 g) was placed in 250-mL flasks, added with 70% EtOH at a liquid-to-solid ratio of 10, 30, 50, 70, or 90 mL/g, and sealed with parafilm to avoid contact with oxygen. The flasks were placed on a Thermo Scientific MaxQ shaker (Thermo Scientific, US) shaking at 100, 150, or 200 rpm to make sure the solvent and algae powder were fully mixed. All experiments were carried out in triplicate for 30 min.

*Extraction using solvent with different EtOH concentrations:* Algae powder (3 g) was placed in 250-mL flasks, added with 20%, 40%, 60%, 80%, or 100% EtOH at a liquid-to-solid ratio of 40 mL/g, and sealed with parafilm to avoid contact with oxygen. The flasks were placed
on a Thermo Scientific MaxQ shaker (Thermo Scientific, US) shaking at 150 rpm. All experiments were carried out in triplicate for 30 min.

Supernatants obtained after filtration were kept as the fresh extract (FE) in a refrigerator (-4°C) overnight till the TPC, TCC, and antioxidant activity tests. After the test, the FEIs were evaporated using a rotatory evaporator (Buchi Type V-850, Switzerland) with 60°C water bath to remove water and ethanol. The leftover was then placed in a 50°C vacuum oven to further remove the residual liquid from the extract until mass of the extract was constant. Then the dried extract (DE) was dissolved with corresponding concentration of EtOH that was used for extraction to assure that they were sufficiently solubilized. The TPC, TCC, and antioxidant activity of the DE were then measured.

3.2.3. Total phenol content (TPC) assay

TPC was determined using a modified version of the Folin-Ciocalteu method, using phloroglucinol (PHG) as the standard (Eom et al., 2012). The volumes of solvents used were changed to reflect different sensitivity of the device in the authors’ lab. Experiment was carried out with the following procedures: A 40-μl aliquot of the sample was mixed with 0.4-mL 1 N Folin-Ciocalteu reagent and 0.8-mL 20% Na₂CO₃ in a 1.5-mL Eppendorf tube. After standing for 3 min, the sample was incubated in the dark at room temperature for 45 min and centrifuged at 1,600 g for 8 min. Then the optical density (OD) of the supernatants was measured at 730 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The calibration equation obtained was Y = 0.472*X + 0.1124 (r² = 0.99), where X is the OD at 730 nm and Y is the concentration of phloroglucinol (mg PHG/mL). TPC test was performed in triplicate and the result was expressed as mg phloroglucinol equivalent per mg dried extract (mg PHG/mg extract).

3.2.4. Total carbohydrate content (TCC) assay

TCC was determined using the phenol-surfuric acid method in microplate format described in a previous research (Masuko et al., 2005). L-rhamnose was applied as the standard and the calibration obtained was: Y = 1.0399*X - 0.0644 (r² = 0.99), where X is the OD at 490 nm and Y is the concentration of L-rhamnose as the standard (mg RHA/mL). TCC assay was performed in triplicate and the result was expressed as mg L-rhamnose equivalent per mg dried extract (mg RHA/mg extract).
3.2.5. Antioxidant activity test

DPPH radical scavenging activity was applied to evaluate the antioxidant activity using the method of Cox et al. (2010) with minor modifications. A 152-µM DPPH radical solution was made by dissolving DPPH radicals in methanol and stored in a -20°C refrigerator until use. Then 100-µl of 152 µM DPPH radical solution was added to 100-µl extract solution. The reaction mixtures were incubated in the dark for 30 min at room temperature, and the optical density (OD) was measured at 517 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The DPPH test was performed in triplicate and the result was expressed as half maximum inhibitory concentration (IC\text{50}) value, which was the concentration of extract (mg/mL) whose radical scavenging capacity was 50%. The lower IC\text{50} value indicated the stronger antioxidant activity of the extract. The ability to scavenge the DPPH radical was calculated with the following equations:

\[
\text{Scavenging capacity (\%)} = 1 - \frac{A_{\text{sample}} - A_{\text{sampleblank}}}{A_{\text{control}}}
\]

where \(A_{\text{control}}\) is the OD of the DPPH solution only, \(A_{\text{sample}}\) is the OD of DPPH solution with sample, \(A_{\text{sampleblank}}\) is the OD value of the sample only. The DPPH radicals were purchased from Sigma-Aldrich CO. LLC. (St. Louis, MO, USA). Folin-Cioclatue reagent, concentrated sulfuric acid, phenol, ethanol, L-rhamnose, butylated hydroxytoluene (BHT), and ascorbic acid (\(V_c\)) were purchased from Thermo Fisher Scientific (Hampton, NH, US).

3.2.6. Statistical Analysis

The results were expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA and Tukey test using SAS (Cary, NC, USA). A p-value of 0.05 or less was considered statistically significant. The letters a, b, c, d, e in the tables indicate significant differences among FE data, and different numbers of the symbol * indicate significant difference among DE data.

3.3. Results

3.3.1. Effects of temperature
It can be seen from Figure 3.1 that when extraction temperature increased from 30 to 70°C, TPC of both FE and DE showed a declining trend. It suggested that phenolic compounds in A. nodosum extract are thermal sensitive and may degrade at high temperatures (Renard et al., 2017). Similar negative effects were reported that TPC of peach fruit extract decreased when extraction temperature increased from 25 to 75°C (Mokrani et al., 2016). Flavonoid yield in Citrus aurantium L. var. amara Engl was reported to decrease when temperature increased from 70 to 90°C (Li et al., 2010). Heat treatment has been performed on mengkudu extract and higher temperatures showed lower TPC (Maskat and Tan, 2011). Thermal degradation of anthocyanin in elderberry extract was reported by Oancea et al. (2018) that heating caused a decrease in fluorescence intensity and significant redshifts in λ\textsubscript{max}, which indicated structural changes and degradation of anthocyanin. An opposite result was found that the yield of phenolic compounds from Orthosiphon stamineus was increased with increasing extraction temperature (Chew et al., 2011). A study of four seaweeds grown in warm water in Malaysia suggested that their TPC increased with higher extraction temperatures (Fu et al., 2016). This result was probably because of the relatively low temperature range (25 to 65°C) applied and that antioxidants of tropical seaweeds might be more thermally stable than the cold water seaweed investigated in the present research. The authors also indicated that a higher temperature of 75°C caused a significant decline in phenolic compounds and their antioxidant activities in the seaweed extracts studied (Fu et al., 2016).

Figure 3.1. Total phenol content (Figure A) and total carbohydrate content (Figure B) of fresh and dried extract at different extraction temperatures (◆ fresh extract; ■ dried extract)
When extracted at lower temperatures (30 or 40°C), the TPC of the FE was either higher than or had no significant difference to that of the DE (Figure 3.1 A). The TPC of high-temperature (>=50°C) extracts were higher in the DE than in the FE. It can be explained by the fact that thermal treatment during rotary evaporation induced the release of polyphenol bound with other compounds, such as polysaccharides (Bachir et al., 2014; Renard et al., 2017; Thoo et al., 2010) thus increased TPC in DE. Increases in TPC of elderberry extract treated at 100 and 110°C were also observed by Oancea et al. (2018) and the authors explained that this increasing trend could be due to breakage of esterified or glycosylated linkages or Maillard reaction.

In contrast to TPC, TCC in FE was enhanced as extraction temperature increased (Figure 3.1 B). Similar observation was also observed in TCC of DE. Polysaccharides might be released from binding with phenolic compounds during thermal separation process (Bachir et al., 2014; Renard et al., 2017; Thoo et al., 2010) thus resulted in higher TCC in DE than in FE. FE obtained at high temperatures, e.g., 60 or 70°C, were significantly higher than those obtained at 30, 40, or 50°C. This can be explained by the fact that higher temperatures accelerate diffusion of polysaccharides from algae cells to the solvent and enhance solubility of polysaccharides in solvents (Chen et al., 2015). They also observed enhancement in mulberry fruit polysaccharide content as extraction temperature increased from 30 to 70°C. Zou et al. (2015) found that increasing extraction temperature from 60 to 100°C improved polysaccharide yield from *Auricularia auricula*, at various liquid-solid ratios and extraction times. The other reason for the lower TCC of FE observed at lower extraction temperature in the present study might be the enzymatic degradation of polysaccharides at lower temperature. Chang et al. (2006) found that heating aloe vera juice at 90°C to inactivate the enzymes could prohibit decrease of polysaccharide content.

Table 3.1. DPPH radical scavenging activity of fresh and dried extracts at different extraction temperatures

<table>
<thead>
<tr>
<th>Temperature (℃)</th>
<th>Fresh extract</th>
<th>Dried extract</th>
<th>Significant difference between fresh and dried extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH IC50 (mg/mL)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td>0.0468±0.0029d</td>
<td>0.0551±0.0029***</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>0.0459±0.0042d</td>
<td>0.0646±0.0020****</td>
<td>Yes</td>
</tr>
<tr>
<td>50</td>
<td>0.0565±0.0005b</td>
<td>0.0668±0.0042****</td>
<td>Yes</td>
</tr>
<tr>
<td>60</td>
<td>0.0567±0.0019b</td>
<td>0.0576±0.0015***</td>
<td>No</td>
</tr>
</tbody>
</table>
As shown in Table 3.1, DPPH radical scavenging capacity of FE declined with increasing temperature, suggesting that most of the effective antioxidants in *A. nodosum* could be thermal sensitive and degraded or lost their bioactivities during heating (Turturica et al., 2016). It might be because *A. nodosum* grows in cold water of north Atlantic Ocean (Kadam et al., 2017) thus the bioactive compounds are more stable at lower temperature. Similar findings were reported by Kuda et al. (2006) that ethanol extract of edible brown algae *Petalonia binghamiae* obtained at higher extraction temperature, 85 and 121°C, showed lower DPPH radical scavenging activity than the extract obtained at room temperature. The antioxidant activity of FE obtained at 30 and 40°C were significantly higher than commercial antioxidant BHT but no higher than vitamin C. Since TCC in FE increased with raising temperature but TPC decreased as temperature raised, it may suggest that most antioxidants in the extract were phenolic components, as it has been reported that the amount of DPPH scavenging activity is dependent on the phenolic components in algae extract (Jimenez-Sscrig et al., 2001; Kuda et al., 2006). The other reason that antioxidant activity leveled off with increasing temperature might be that more impurities were also extracted at high temperatures (Zou et al., 2015).

Antioxidant compounds in edible seaweeds were reported to be decreased after drying and storage (Araki, 1983). Antioxidant activity of most FEs were stronger than their DEs except for 60 and 70°C extracts. No significant difference was observed between FE and DE of 60°C extract. The high TPC could be responsible for the enhanced antioxidant activity of DE at 70°C. The phenolic antioxidants of *Morinda citrifolia*, a tropical plant grown in Malaysia, was reported to possess stronger DPPH radical scavenging activity at high temperature (Thoo et al., 2010). It was reported that bound polyphenols could be released at high temperatures from binding polysaccharides and proteins (Bachir et al., 2014; Renard et al., 2017; Thoo et al., 2010). Similar results were observed that DPPH scavenging activity of water extract of *Petalonia binghamiae* was enhanced after heat treatment at 85 and 121°C. In which they concluded that brown compounds generated from Maillard reaction possessed radical scavenging activity and enhanced the antioxidant activity (Kuda et al., 2006).

### 3.3.2. Effects of liquid-to-solid ratio
It can be concluded from Figure 3.2 A that larger amounts of solvents improved the TPC of FE. As the liquid-to-solid ratio increased, contact area between algae powder and fresh solvent was enlarged, making more phenolic components dissolved in solvent (Li et al., 2010). This agreed well with the observation of Durling et al. (2007) that increasing solvent to sage ratio enhanced TPC in sage extract. While the TPC of DE first inclined as liquid-to-solid ratio increased from 10 to 50 mL/g, then a decline in TPC was observed as liquid-to-solid ratio went up to 90 mL/g. TPC of FE obtained with high liquid-to-solid ratios (>=30 mL/g) were higher than DE. Interestingly, TPC of DE extracted at 10 mL/g was significantly higher than FE, indicating that phenolic compounds were unbounded from their binding polysaccharides during short time thermal process (Bachir et al., 2014; Renard et al., 2017; Thoo et al., 2010) thus enhanced TPC of DE. However, prolonged rotary evaporation due to larger liquid-to-solid ratio may cause degradation of phenols in the extract, and level down the TPC of DE.

Decrease in TCC was observed in FE as liquid-to-solid ratio increased from 10 to 90 mL/g (Figure 3.2 B). This could possibly be explained by the fact that more impurities were extracted by larger volume of solvents and the binary solvent system with high concentration of ethanol doesn’t promote solubility of carbohydrates. Zou et al. (2015) reported that polysaccharide yield from A. auricula fruit bodies increased as liquid-solid ratio increased from 20 to 40 mL/g, and then leveled off from 40 to 60 mL/g. However, water was used as the solvent and high temperature (60-100°C) was applied. The other research that used water as solvent also reported maximum polysaccharides yield from mulberry fruits at 40 mL/g using ultrasound extraction (Chen et al., 2015). Similar with TPC, the TCC of DE also began to decrease when the liquid-to-solid ratio reached above 50 mL/g, suggesting that prolonged thermal process may also cause degradation of polysaccharides in the extract. The increase in TCC of DE may be explained by the fact that polysaccharides were released from binding polyphenols during the thermal treatment (Renard et al., 2017). However, the TCC of DE began do decrease when the liquid-to-solid ratio became greater than 50 mL/g, suggesting longer time of thermal treatment caused degradation of polysaccharides. It was reported by Lu et al. (2018) that the reducing sugar content increased as the time of thermal treatment increased during black garlic processing, as reducing sugars are products of polysaccharide degradation. TCC of all FE were higher than their DE, indicating thermal treatment negatively influenced the TCC.
Figure 3.2. Total phenol content (Figure A) and total carbohydrate content (Figure B) of fresh and dried extract at different liquid to solid ratio (◆ fresh extract; ■ dried extract)

The lowest IC₅₀ values for FE and DE shown in Table 3.2 were all observed at lower liquid-to-solid ratios, 30 and 10 mL/g, respectively. It suggested that lower volume of solvent was sufficient to obtain extracts with prominent antioxidant activity without using too much solvents. Almost all extracts showed stronger antioxidant activity before rotary evaporation except for liquid-to-solid ratio of 10 mL/g, which might be due to the high TPC in the DE. Although TPC of FE increased with liquid-to-solid ratio, a decrease in DPPH radical scavenging activity was observed. Similar observation was made by Fu et al. (2016) that increasing solvent to solid ratio from 1:10 to 1:30 caused a decrease in DPPH radical scavenging activity of four seaweed extracts. As larger amount of solvent was applied, more impurities may be extracted (Li et al., 2010) thus leveled down antioxidant activity of the extracts by binding to polyphenols (Renard et al., 2017; Thoo et al., 2010). High TPC doesn’t necessarily accompany with strong antioxidant activity because of the structure and the synergistic effects of polyphenols with other compounds such as polysaccharides (Chew et al., 2011; Renard et al., 2017; Wong et al., 2014). Extracts obtained at lower liquid-to-solid ratios showed similar antioxidant activity with BHT but no stronger than ascorbic acid.
Table 3. 2. DPPH radical scavenging activity of fresh and dried extract at different liquid-to-solid ratio

<table>
<thead>
<tr>
<th>Solvent-to-solid ratio (mL/g)</th>
<th>Fresh extract</th>
<th>Dried extract</th>
<th>Significant difference between fresh and dried extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH IC$_{50}$ (mg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.0596±0.0012c</td>
<td>0.0502±0.0021**</td>
<td>Yes</td>
</tr>
<tr>
<td>30</td>
<td>0.0508±0.0009d</td>
<td>0.0909±0.0039*****</td>
<td>Yes</td>
</tr>
<tr>
<td>50</td>
<td>0.0636±0.0016b</td>
<td>0.0734±0.0027****</td>
<td>Yes</td>
</tr>
<tr>
<td>70</td>
<td>0.0609±0.0051c</td>
<td>0.0680±0.0037***</td>
<td>Yes</td>
</tr>
<tr>
<td>90</td>
<td>0.0724±0.0011a</td>
<td>0.0825±0.0044*****</td>
<td>Yes</td>
</tr>
<tr>
<td>BHT</td>
<td>0.0510±0.0050d</td>
<td>0.0510±0.0050**</td>
<td></td>
</tr>
<tr>
<td>Vc</td>
<td>0.0063±0.0002e</td>
<td>0.0063±0.0002*</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3. Effects of EtOH concentration

It can be seen from Figure 3.3 A that increasing EtOH concentration in the binary solvent system caused increases in TPC in both FE and DE, owing to the fact that phenolic components in A. nodosum are more ethanol-soluble. It was confirmed in the present study that the solubility of phloroglucinol in 20% and 100% EtOH were 0.0485 and 0.4797 g/mL, respectively, indicating that high EtOH concentration increases solubility of phenolic compounds. Fu et al. (2016) found that the TPC of four seaweeds increased with increasing EtOH concentration. Gong et al. (2012) also reported increased TPC in marigold extract as EtOH concentration went up from 0 to 70%. Wang et al. (2012) found that polar solvents were more efficient at extracting phlorotannins than water and apolar solvents from brown algae Fucus vesiculosus.
The TPC of DE obtained with higher EtOH concentrations, 60, 80 or 100%, were not significantly different from their FE (Figure 3.3 A). It suggested that high EtOH concentration in the ethanol-water binary solvent system helped to protect phenolic compounds because of reduced thermal treatment time. Maskat and Tan (2011) reported similar results that as residence time of heat treatment increased from 5 to 15 min, the TPC of mengkudu extract dropped from 42.26 to 32.35 at 50℃ and to 28.49 at 70℃. Decrease in TPC was also found in elderberry extract as the heating time increased from 60 to 120 min (Oancea et al., 2018).

The highest TCC of FE in Figure 3.3 B was observed with 40 and 60% EtOH but dropped when EtOH increased to 100% due to decreased solubility of carbohydrates in EtOH. It was confirmed in the author’s experiment that solubility of rhamnose dropped from 0.336 g/mL to 0.016 g/mL as EtOH concentration increased from 20% to 100%. Soluble saccharide content of brown algae *P. binghamiae* was reported to be higher in the water extract (78.1 mg/g dry sample) than in ethanol extract (not detected) (Kuda et al., 2006). All DE showed lower TCC than their FE after evaporation process, indicating thermal treatment of the extracts negatively affected their TCC.
The strongest DPPH radical scavenging capacity for both FE and DE were observed at 80% EtOH. Thermal separation process showed a negative effect on the antioxidant activity of the extracts as shown in Table 3.3 that all FE had better DPPH radical scavenging activity than DE. All extracts, except for DE obtained with 100% ethanol, had stronger (or at least similar) antioxidant activity than BHT, but still no stronger than vitamin C. Similar observations were made on EtOH-water extracts of *Orthosiphon stamineus* and *Hibiscus cannabinus* L. that DPPH radical scavenging capacity was enhanced with increasing EtOH concentration from 60 to 80% but decreased as EtOH went up to 100% (Chew et al., 2011; Wong et al., 2014). Low ethanol concentration was reported to favor impurity extraction as higher DPPH radical scavenging activity was observed in high EtOH concentration extracts of the seaweeds *Sargassum polycystrum*, *Kappaphycus alvarezii*, and *Eucheuma denticulatum* (Fu et al., 2016). The impurities extracted with low EtOH concentration solvents, e.g. 40 and 60%, may bind to the polyphenols (Thoo et al., 2010) and decrease their antioxidant activity. Although FE of 100% EtOH showed a strong DPPH radical scavenging activity, the IC$_{50}$ value of the DE was the highest among all DE, indicating great loss of antioxidants during the thermal treatment. DPPH radicals were reported to be more favorable to react with antioxidants with low molecular weight (Chew et al., 2011; Thoo et al., 2010). Kuda et al. reported that the <5 kDa fraction of the water extract of brown algae *P. binghamiae* possessed the strongest DPPH scavenging capacity and largest amount in the phenolic compounds extracted (Kuda et al., 2006). It may be concluded

Table 3.3. DPPH radical scavenging activity of fresh and dried extract at different EtOH concentration

<table>
<thead>
<tr>
<th>EtOH concentration (%)</th>
<th>Fresh extract</th>
<th>Dried extract</th>
<th>Significant difference between fresh and dried extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH IC$_{50}$ (mg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.0411±0.0010b</td>
<td>0.0456±0.0027****</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>0.0366±0.0022c</td>
<td>0.0421±0.0024***</td>
<td>Yes</td>
</tr>
<tr>
<td>60</td>
<td>0.0421±0.0024b</td>
<td>0.0520±0.0014*****</td>
<td>Yes</td>
</tr>
<tr>
<td>80</td>
<td>0.0245±0.0019d</td>
<td>0.0309±0.0010**</td>
<td>Yes</td>
</tr>
<tr>
<td>100</td>
<td>0.0264±0.0011d</td>
<td>0.1231±0.0015******</td>
<td>Yes</td>
</tr>
<tr>
<td>BHT</td>
<td>0.0510±0.0050a</td>
<td>0.0510±0.0050*****</td>
<td></td>
</tr>
<tr>
<td>Vc</td>
<td>0.0063±0.000e</td>
<td>0.0063±0.0002*</td>
<td></td>
</tr>
</tbody>
</table>
that using 100% EtOH as the solvent might obtain low molecular weight, thermal sensitive phenolic compounds that were more reactive with DPPH radicals (Thoo et al., 2010).

3.3.4. Correlation between tested variables and TPC, TCC, DPPH radical scavenging activity

Pearson correlation analysis was performed to reveal the relationship between assays and different extraction factors (Table 3.4). The correlation coefficients (r) between TPC, TCC, IC\textsubscript{50} values and the three tested factors, temperature, liquid-to-solid ratio, and EtOH concentration are shown in the supplementary table. All three factors showed a strong effect on TPC of FE (|r| > 0.95), indicating that phenolic compounds in FE were sensitive to extraction conditions. However, only ethanol concentration possessed strong influence (r = 0.997) on TPC of DE, while temperature had a moderate effect (r = -0.757) and liquid-to-solid ratio had weak influence (r = 0.268). EtOH concentration had no effect on TCC of DE nor FE. Liquid-to-solid ratio had a strong negative influence on TCC of FE (r = -0.974), however, it didn’t significantly affect TCC of DE. Increasing temperature had a positive effect on TCC of both FE and DE. The antioxidant activity of FE was negatively affected by increasing temperature, liquid-to-solid ratio, and decline in EtOH concentration. Because most antioxidants in \textit{A. nodosum} are thermal sensitive and more EtOH-soluble, and since lower temperature and higher EtOH concentration favored the extraction of phenolic compounds from \textit{A. nodosum}, it could be speculated that most antioxidants in \textit{A. nodosum} extract were phenolic components, which agrees with the conclusion of Turturica et al. (2016) that the DPPH radical scavenging activity was strongly dependent on the TPC of plum extract. Strong correlation was reported between TPC and DPPH radical scavenging activity of extract of \textit{Morinda citrifolia} (Thoo et al., 2010). Strong correlation between different antioxidant assays was found under influence of extraction temperature while no significant correlation was observed with regards to extraction time for \textit{Orthosiphon stamineus} extract (Chew et al., 2011). Acetone concentration also showed a positive correlation with TPC and DPPH radical scavenging activity of peach fruit extract, with high Pearson correlation coefficients of 0.96 and 0.82, respectively (Mokrani and Madani, 2016).
Table 3.4. Correlation coefficient (r) between tested variables and total phenol content, total carbohydrate content, and DPPH IC50 values of both fresh and dried extract with Pearson correlation analysis.

<table>
<thead>
<tr>
<th>Tested variables</th>
<th>Bioactivity assay</th>
<th>Fresh extract</th>
<th>Dried extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (℃)</td>
<td>TPC (mg PHG/mg extract)</td>
<td>-0.996</td>
<td>-0.757</td>
</tr>
<tr>
<td></td>
<td>TCC (mg RHA/mg extract)</td>
<td>0.927</td>
<td>0.697</td>
</tr>
<tr>
<td></td>
<td>DPPH IC50 (mg/mL)</td>
<td>0.928</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liquid-to-solid ratio (mL/g)</td>
<td>TPC (mg PHG/mg extract)</td>
<td>0.998</td>
<td>0.268</td>
</tr>
<tr>
<td></td>
<td>TCC (mg RHA/mg extract)</td>
<td>-0.974</td>
<td>-0.632</td>
</tr>
<tr>
<td></td>
<td>DPPH IC50 (mg/mL)</td>
<td>0.725</td>
<td>0.427</td>
</tr>
<tr>
<td>EtOH concentration (%)</td>
<td>TPC (mg PHG/mg extract)</td>
<td>0.990</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>TCC (mg RHA/mg extract)</td>
<td>0.124</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>DPPH IC50 (mg/mL)</td>
<td>-0.799</td>
<td>0.618</td>
</tr>
</tbody>
</table>

### 3.4. Discussion

Most polyphenols are stored inside the vacuole in plant cells, while during plant tissue disruption, they bind spontaneously to the polysaccharides by a combination of hydrogen bonds and hydrophobic interactions, which is favored by decreasing temperature and increased ion strength. This polyphenol-polysaccharide interaction limits bioavailability of polyphenols and results in the major part of “unextractable polyphenols” (Renard et al., 2017). Thermal process induces chemical reactions such as oxidation of phenols and polysaccharide degradation (Lu et al., 2018) thus reduces the antioxidant activity of the extracts (Oancea et al., 2018). It was reported by Ursache et al. (2017) that heating sea buckthorn extracts resulted in structural changes that led to reduction in TPC and DPPH radical scavenging activity. Structural changes and reduction in TPC and DPPH scavenging activity were also reported in plum extracts after heat treatment (Turturica et al., 2016). However, thermal treatment may have positive effects by inactivating the enzymes in the plant material that are responsible for hydrolysis and preventing enzymatic degradation of bioactive compounds (Chang et al., 2006). Only materials with higher proportions of thermal-stable compounds are more appropriate for extraction under higher temperatures (Thoo et al., 2010). The TPC and antioxidant activity of grape marc extract was significantly improved after heat treatment at 150°C for at least 50 min (Solyom et al., 2014). Brown compounds having radical scavenging activity were also reported to be generated by the Maillard reaction during heating (Kuda et al., 2006).
Polar solvents are more efficient in extracting phlorotannins than water and nonpolar solvents due to the “like dissolve like” principle. Water could be helpful in swelling plant cells, and ethanol is believed to disrupt the bonding between bioactive compounds and cell matrix (Ghitescu et al., 2015). The antioxidant activities of different extracts were observed to be linearly correlated with their total phenol content level (Wang et al., 2012). However, Kuda et al. (2006) found that the water extract of brown algae *P. binghamiae* had higher TPC than the ethanol extract. They also reported that the DPPH radical scavenging activity was correlated with the content of phenolic compounds, with the highest activity shown in the <5 kDa fraction, followed by <100 kDa and 10-30 kDa fractions, which were found to be phenolic compounds. This conclusion agrees with the present study that most of the extracts with high TPC also showed strong DPPH radical scavenging activity. It was reported that extraction with 60% ethanol from wheat bran induced the highest TPC in the extract since lipid components were extracted under high ethanol concentration (95%), which may limit the extraction of phenolics (Wang et al., 2008). The polarity of solvent not only affects the content of phenolics extracted, but also their composition. Antioxidant activity of the phenolic compounds is usually determined by their structure and degree of polymerization (Karacabey and Mazza, 2010).

Temperature is an important parameter that influences the yield and bioactivity of extracts by affecting their viscosity, diffusivity, solubility, and surface tension. Higher temperatures may increase the diffusivity of solvents into the material matrix, enhance desorption and solubility of solutes from cells, however, too high temperature can cause degradation or oxidation of polyphenols resulted from hydrolysis or internal redox reactions (Ghitescu et al., 2015), which agrees with our observation that rising extraction temperature resulted in decreasing TPC in both FE and DE. Yang et al. (2010) found that the yield of flavonoids dropped from 1.9 to 1.7% when the temperature increased from 70 to 90°C while an increasing trend was observed as temperature increased from 30 to 70°C. The structure of polysaccharides also tended to be destroyed at high temperatures and resulted in degradation (Chen et al., 2015). The yield of pomegranate peel polysaccharides increased as extraction temperature was raised up to 60°C but began to decrease when the temperature reached above 60°C (Zhu et al., 2015). Similar observations were reported in polysaccharides from fruits and leaves of *Rubus chinii* Hu that the peak of yield was found at 80°C, while the yield decreased as temperature increased to 90°C (Zhang et al., 2015). However, high extraction temperature (e.g. 95°C) may limit the extraction of phenolics.
60 or 70°C) in the present study might aid in enhancing polysaccharide contents in the extract when ethanol-water was applied as the solvent (Figure 3.1 B).

A greater liquid-solid ratio usually helps to improve the yield of extracts due to the greater concentration difference between interior plant cells and exterior solvent, accelerated diffusion of solutes (Zhu et al., 2015), lower concentration of solutes in the liquid, and decreased viscosity of the solvent (Chen et al., 2015). However, a too large ratio of liquid to material is a waste of solvents and requires more energy to fully mix the material with the solvent, and often causes insufficient stirring and contact between the material and solvent. Besides, too much solvent in the supernatant makes it difficult to separate and causes dilution of compounds. It was reported that a ratio greater than 20 mL water/g raw material didn’t result in significant increase in polysaccharide yield from pomegranate peel (Zhu et al., 2015) and Plantago asiatica L. (Ye and Jiang, 2011). It was also observed in the present study that the polysaccharide content in the extract did not increase as liquid-solid ratio increased from 10 to 90 mL/g, suggesting that extra amounts of solvent did not aid in enhancing polysaccharide yield when ethanol (which does not favor polysaccharide solubility) was applied. A large liquid-solid ratio of 50 mL/g resulted in decrease in flavonoid yield to 1.38%, while the highest yield was 1.44% at 40 mL/g, which was attributed to the impurities extracted that hindered the dissolution of flavonoids (Yang et al., 2010). The highest ethanol concentration applied by Yang et al. (2010) was 50%, which was lower than the present study (70%).

3.5. Conclusions

All three factors, the extraction temperature, liquid-to-solid ratio, and ethanol concentration showed significant influences on TPC, TCC, and antioxidant activity of the FE and DE. The TPC of FE was enhanced by lower temperature, higher liquid-to-solid ratio, and higher ethanol concentration, while TCC of FE tended to increase with increasing temperature and decreasing liquid-to-solid ratio at moderate ethanol concentration (40%). The strongest DPPH scavenging activity of FE was observed at relatively low temperature (30 or 40°C), low liquid-to-solid ratio (30 mL/g), and high ethanol concentration (80 or 100%). Most of the FE showed higher TPC, TCC, and DPPH radical scavenging activity than their DE, suggesting that thermal treatment negatively affected the bioactivity of the extract.
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**Abstract**

Brown algae are valuable sources of health-benefiting compounds, such as polyphenols, proteins, and polysaccharides. In the present study, a binary solvent system of ethanol and water was used to obtain crude extracts from edible brown algae *Ascophyllum nodosum*. The extraction process was optimized using Box-Behnken design and response surface methodology to obtain crude extracts with strong antioxidant activity and high yield. Three variables including solvent-to-solid ratio (30 to 70 mL/g), ethanol concentration in the solvent system (40 to 80%), and extraction temperature (20 to 60°C) were investigated to optimize the extraction process. The condition for maximum antioxidant activity of the crude extract was found at 70 mL/g solvent-to-solid ratio, 80% ethanol concentration, and 20°C extraction temperature, while the condition for the highest crude extract yield was 50 mL/g solvent-solid-ratio, 45.65% ethanol concentration and 60°C extraction temperature. Under the model-predicted optimal conditions, the predicted antioxidant activity and yield of the crude extract were 72.75 mL/mg and 55.68 mg extract/g algae, which were in close agreement with the experimental results of 74.05± 0.51 mL/g and 56.41±2.59 mg extract/g algae, respectively, suggesting that the models could accurately predict and improve the extraction of antioxidants from *A. nodosum*.

**Keywords** antioxidant, brown algae, polyphenol, phlorotannin, RSM, *Ascophyllum nodosum*

### 4.1. Introduction

As consumers’ desire for natural and organic foods with fewer additives grows, antioxidant ingredients from plants have drawn great attention due to their potential usage as food preservation agents or nutraceuticals. Antioxidants may serve as food preservatives in places of chemical additives (Hintz et al., 2015). Natural antioxidants are also recommended to be used alone or in combination with other non-thermal technologies to protect food from deterioration (Tiwari et al., 2009). Many edible and medicinal plants have been studied for their...
potential usage as antimicrobial and antioxidant agents, such as sage, thyme, cinnamon, oregano, rosemary, and mint (Martinez-Gracia et al., 2015). However, most of the subjects studied are terrestrial plants, while organisms in marine ecosystems have not been thoroughly investigated. More than 21,000 structurally diverse compounds with various bioactivities have been discovered from marine microorganisms, macroalgae and invertebrates (Eom et al., 2012). Among marine organisms, marine macroalgae have been identified as an under-exploited source of natural antioxidants and antimicrobial agent. Marine macroalgae, sometimes called seaweeds, can be divided into three groups, *Chorophyta* (green algae), *Rhodophyta* (red algae), and *Phaeophyta* (brown algae), based on their pigment composition (Li et al., 2011). Brown algae are rich in phenols, polysaccharides, proteins, and other compounds with various bioactivities (Blboa et al., 2013). The extracts of the algae studied in this research, *A. nodosum*, has been found to possess strong antioxidant activity in a previous study (Liu et al., 2017).

Extraction is the initial step to obtain bioactive compounds from plant or animal materials. Several variables, such as extraction time, temperature, solid-to-solvent ratio, and solvent type can affect the bioactivity and yield of extracts (Yang et al., 2010). Due to the complexity of structures and properties of bioactive compounds and the structure of the extracted materials, there is no universal extraction protocol available (Thoo et al., 2010). A specific extraction process must be established for each biological material extracted to obtain extracts with desirable bioactivity and high yield. Response surface methodology (RSM) has been widely used in many optimization processes for extracting phenols (Bachir bey et al., 2014; Thoo et al., 2010; Prasad et al., 2011; Topuz et al., 2016), polysaccharides (Yu et al., 2015; Yuan et al., 2015; Ye et al., 2011), and flavonoids (Yang et al., 2010) from various plant and animal materials. The main advantage of RSM is the reduced labor and time in experimental trials and its capability to simultaneously evaluate the effect of several influencing variables and their interactions on the process (Ye et al., 2011; Wani et al., 2017). The methodology includes three major steps: (1) experimental design, in which the levels and number of independent variables are set using statistical experimental design such as Box-Behken design (BBD), Placket-Burman design, or Central Composite design; (2) modeling the response surface using regression analysis; (3) model validation.

In the present study, a binary solvent system of ethanol and water was applied with a consideration of using the extracts as a diet supplement or food preservative agent in the future.
Although optimization of antioxidant activity has been extensively studied elsewhere, only a few researchers have reported optimizing the yield of extracts (Li et al., 2016; Zhu et al., 2015; Ye et al., 2011; Durling et al., 2007), yet they all focused on terrestrial plants. To the best knowledge of the authors, optimization of antioxidant extraction from *A. nodosum* or other brown algae has not been fully investigated. The objective of this study was to optimize the extraction process to obtain crude extracts with strong antioxidant activity or higher yield from brown algae *A. nodosum* using BBD and RSM. Three variables, including extraction temperature, solvent-to-solid ratio, and ethanol concentration in the solvent system were investigated for their influence on antioxidant activity and yield of the extracts. DPPH radical scavenging activity test was applied to evaluate the antioxidant activity of the extracts.

**4.2. Materials and Methods**

**4.2.1. Materials**

Dried whole leaves of *A. nodosum* were purchased from Maine Coast Sea Vegetables (Hancock, Maine, US). The samples had a greenish brown color and were sealed in airtight bags before use. Before extraction, the whole leaves were washed with tap water to remove salt and dirt. The algae materials were then dried at room temperature for three days to remove moisture on surface. After being placed in a 50°C vacuum oven for one to two days, the dried leaves of *A. nodosum* were ground and sieved with a 1-mm sieve, then the powder was collected, sealed in air-tight containers, and stored under -20°C until extraction.

**4.2.2. Extraction**

All extraction trials were performed for 30 min in triplicate. The extraction duration was pre-determined in a single-factor experiment for the strongest antioxidant activity (data to be published elsewhere and not shown here). Five grams of algae powder was placed in a 500-mL glass bottle with solvents of required volume and ethanol concentration. Temperature was kept using a water bath. A magnetic stirring bar was added to each bottle on a 15-place magnetic stirrer. After filtration, the supernatant was retained as the crude extract in a refrigerator (-4°C) overnight before the antioxidant activity test. Concentrations of extracts were determined by drying the crude extracts in a 50°C vacuum oven until the mass was constant. Yield of extracts
was calculated as the product of concentration and volume of the supernatant divided by the mass of algae powder extracted.

4.2.3. Antioxidant activity test

DPPH free radical scavenging activity was measured using the method of Cox et al. (2010) with minor modifications. A 152-µM DPPH radical solution was made by dissolving DPPH radicals in methanol. Then 0.1-mL extract solution was added to 0.1-mL of 152-µM DPPH radical solution. The reaction mixtures were incubated in the dark for 30 min at room temperature, and the optical density (OD) was measured at 517 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The DPPH test was performed in triplicate and the result was expressed as half maximum inhibitory concentration (IC$_{50}$) value (mg dry extract/mL supernatant), which was the required concentration of the crude extract to scavenge 50% of the DPPH radicals of 152-µM with the same volume. The lower the IC$_{50}$ value, the stronger antioxidant activity that the extract possesses. The ability to scavenge the DPPH radical was calculated with the following equation:

\[
\text{Scavenging activity (\%)} = 1 - \frac{A_{\text{sample}} - A_{\text{sampleblank}}}{A_{\text{control}}}
\]

where $A_{\text{control}}$ is the OD of the DPPH solution, $A_{\text{sample}}$ is the OD of DPPH solution with sample, $A_{\text{sampleblank}}$ is the OD value of the sample alone. The DPPH radicals were purchased from Sigma-Aldrich CO. LLC. (St. Louis, MO, USA). The solvent system (water + ethanol) without crude extract showed no scavenging activity at all.

4.2.4. Response surface methodology design and analysis

Design Expert (Educational version 8.0.7, Stat-Ease, Minneapolis, MN) was used for model building and data analysis. A three-level, three-factor Box-Behnken design was applied. Extraction temperature (X1), solvent-to-solid ratio (X2), and ethanol concentration (X3) were selected according to practical conditions. The levels of all three variables and the experiment trials and results are shown in Table 1. The reciprocal of IC$_{50}$ (1/IC$_{50}$) was taken as the response variable so the model can predict a maximum for antioxidant activity. All experiments were performed in triplicate. Data in Table 4.1 were analyzed to fit the following quadratic polynomial model:
\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \]

where \( Y \) is the response function, \( \beta_0 \) is the intercept, \( \beta_i \), \( \beta_{ii} \), and \( \beta_{ij} \) are the coefficients of the linear, quadratic and interactive terms, respectively. \( X_i \) and \( X_j \) represent the coded independent variables, respectively. Two models were developed in this study, one for optimizing antioxidant activity (model I) and the other for optimizing yield (model II). All plots in the present study were made when the third parameter was fixed to the center point.

Table 4.1. Box-Behnken design and experimental results of the antioxidant activity and yield of crude extract

<table>
<thead>
<tr>
<th>Run</th>
<th>X1 Temperature °C</th>
<th>X2 SL ratio mL/g</th>
<th>X3 EtOH %</th>
<th>Response 1 1/IC(_{50}) mg extract/g algae</th>
<th>Response 2 yield mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>30</td>
<td>60</td>
<td>38.706</td>
<td>21.189</td>
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<tr>
<td>2</td>
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<td>80</td>
<td>24.145</td>
<td>20.042</td>
</tr>
<tr>
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<td>40</td>
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<td>60</td>
<td>26.827</td>
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</tr>
<tr>
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<td>40</td>
<td>32.269</td>
<td>41.984</td>
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<tr>
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<td>70</td>
<td>60</td>
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</tr>
<tr>
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<td>60</td>
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</tr>
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<td>37.456</td>
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<td>80</td>
<td>60.219</td>
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<tr>
<td>17</td>
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<td>50</td>
<td>80</td>
<td>25.994</td>
<td>31.261</td>
</tr>
</tbody>
</table>

4.3. Results

4.3.1. Optimization for higher antioxidant activity

Figure 4.1. shows the interacted influence of ethanol concentration and solvent-to-solid ratio on 1/IC\(_{50}\) value. Generally, the antioxidant activity of the crude extracts increased as the
solvent-to-solid ratio and ethanol concentration increased. This observation agrees with that of Durling et al. (2007) who found that increasing solvent to solid ratio would result in recovery of more bioactive compounds such as rosmarinic acid and carnosic type of compounds. The reason may be that more bioactive compounds can be transformed from algae powder to the solvent due to increased contact surface (Yang et al., 2010). Stronger antioxidant activity observed at higher ethanol concentration also suggests that the antioxidants in A. nodosum might have low polarity based on the “like dissolves like” principle (Mokrani and Madani, 2016). Interestingly, when the solvent-to-solid ratio was relatively low, e.g. between 30 and 40 mL/g, increasing ethanol concentration would not help with enhancing antioxidant activity. When ethanol concentration was high, e.g. higher than 65%, increasing solvent-to-solid ratio could certainly raise the antioxidant activity. Similar results were also reported by Prasad et al. (2011) that increasing ethanol concentration and liquid/solid ratio could both enhance antioxidant activity of Mangifera pajang Kosterm peel extract. Wani et al. (2017) found that the strongest antioxidant activity was observed with the highest methanol concentration but the lowest solvent/sample ratio.
In Figure 4.2 it can be seen that ethanol concentration had a positive effect while temperature had a negative effect on antioxidant activity of the extracts. The strongest antioxidant activity was obtained at 20°C with a solvent-to-solid ratio of 70 mL/g. It suggests that *A. nodosum* may contain some thermal-sensitive and low polarity antioxidative compounds. When the extraction temperature was high, e.g. 44 to 60 °C, lower ethanol concentration was preferred for higher antioxidant activity of the extracts. Gong et al. (2012) reported that increasing ethanol concentration from 40 to 80% led to an increase in DPPH radical scavenging activity of *Tagetes erecta* L. extract at various temperatures (45-75°C). The authors believed that
higher ethanol concentration favored phenol and flavonoid extractions that had strong antioxidant activity. It was also reported by Prasad et al. (2011) that extracts of *Mangifera pajang* Kosterm peel showed stronger antioxidant activity when extracted with higher ethanol concentration and higher temperature due to increased solubility of phenolic compounds. While extracted at lower ethanol concentration, e.g. below 41%, increasing temperature would cause a decline in the antioxidant activity because further increase in temperature can degrade phenolic compounds (Prasad et al. 2011).

Figure 4. 2. Response surface plots showing effects of temperature (X1) and EtOH concentration (X3) on the antioxidant activity of crude extracts (1/IC50, mL/mg; X2 = 50 mL/g)
Solvent-to-solid ratio and temperature played a relatively weaker interaction influence on antioxidant activity as Figure 4.3 shows. The strongest antioxidant activity was observed at the highest EtOH concentration but lowest temperature. This agrees with the result shown in Figure 1 that increasing the volume of solvent could lead to stronger antioxidant activity. However, temperature had a negative effect on the antioxidant activity in the present study, which was opposite to some observations (Bachir bey et al., 2014; Thoo et al., 2010; Gong et al., 2012; Topuz et al., 2016). In those studies, increasing temperature and solvent-to-solid ratio could both increase the antioxidant activity of the extracts. It may be because A. nodosum is grown in and harvested from cold-water habitat, and the bioactive compounds are more thermal sensitive than plants grown in warmer habitats. However, similar to the findings of the present study, not all extracts showed an enhanced antioxidant activity at higher temperatures. A study on M. pajang peel extract showed that temperature had little effect on the antioxidant activity, but the higher liquid/solid ratio could enhance the antioxidant activity (Prasad et al., 2011). One study about water-extracted Pleurotus citrinopileatus also showed that the cold-water extract had stronger DPPH radical scavenging activity than that of the hot water extract (Chen et al., 2016).
Figure 4. 3 Response surface plots showing effects of temperature (X1) and solvent-to-solid ratio (X2) on the antioxidant activity of crude extracts (1/IC50, mL/g; X3 = 60%)

The observations were verified by ANOVA analysis in Table 4.2. The p-value was applied to estimate the significance of each factor (p<0.05 was considered statistically significant). Two variables including temperature and solvent-to-solid ratio were confirmed to have significant influence on antioxidant activity (p<0.05). This agreed well with the observation in Figure 1 that temperature and solvent-to-solid ratio had major influence while the effects of ethanol concentration were affected by temperature and solvent-to-solid ratio. However, only temperature had significant effects among all squared variables (p<0.05). All three interactions showed no significant effects with p-value greater than 0.05.
4.3.2. Optimization for higher yield

Lower ethanol concentration enhanced the yield of the extracts (Figure 4.4), suggesting that most of the extractable compounds may have high-polarity. However, solvent-to-solid ratio had no significant influence in this scenario, indicating that extra solvents are not necessary for better yield although they are helpful in obtaining higher antioxidant activity (Figure 4.1).
Figure 4.4. Response surface plots showing effects of solvent-to-solid ratio (X2) and EtOH concentration (X3) on crude extract yield (mg extract/g algae; X1 = 40 °C)

Figure 4.5. shows that the highest yield was obtained with the highest temperature but lowest ethanol concentration. This observation agrees with Figure 4.4 that lower EtOH concentration helped with extraction yield. High temperature can enhance the solubility of extractable compounds and degrade cell structure of materials extracted (Li et al., 2016). As temperature increases, movement of molecules is accelerated, and diffusivity of bioactive compounds is improved, leading to higher yield of extracts (Yang et al., 2010). Sage extract yield was reported to increase with extraction temperature rising from 22 to 63°C but slightly
decreased as ethanol content in solvent increased from 20 to 100% (Durling et al., 2007), which was similar to the result of the present study. Li et al. (2016) found that increasing extraction temperature enhanced polyphenol yield from *Prunus salicina* Lindl while ethanol concentration didn’t show significant effect during ultrasonic extraction.

![Response Surface Plots](image)

**Figure 4.5.** Response surface plots showing effects of temperature (X1) and EtOH concentration (X3) on crude extract yield (mg extract/g algae; X2 = 50 mL/g)

As Figure 4.5. shows, increasing extraction temperature could enhance extract yield but solvent-to-solid ratio didn’t show any significant influence. Ye et al. (2011) reported that increased temperature and water to material ratio both could enhance crude polysaccharide yield.
from *Plantago asiatica*. However, the water to material ratio they applied was 10-30 mL/g, which was lower than present study (30-70 mL/g). Durling et al. (2007) also observed a rise in the yield of sage extract as solvent to sage ratio increased from 6:1 to 18:1. Yuan et al. (2015) found that increasing the ratio of water to material could only affect the yield of polysaccharides of mulberry leaves within temperatures between 89 and 93°C. The highest mulberry leaf polysaccharides yield was obtained when extracted with moderate ratio of water to material (35mL/g) at high temperature (91°C) (Yuan et al., 2015). The highest polysaccharide yield of pomegranate peel was reported to be extracted with moderate ratio of water to raw material (23 mL/g) and temperature (60°C) in an ultrasound-assisted extraction (Zhu et al., 2015). An increase in yield of crude polysaccharide from *Rubus chingii* Hu was observed when liquid/solid ratio increased from 10 to 30 mL/g and temperature from 50 to 80°C. However, as the liquid/solid ratio increased to 35 mL/g, no significant increase in yield was observed, suggesting that a greater solvent-to-solid ratio may not be helpful to the extraction process (Zhang et al., 2015).
It can be concluded from Table 4.3 that temperature and ethanol concentration had a significant influence on the yield of extract (p<0.05). The solvent-to-solid ratio showed a weak influence both by itself and by interactions with the other two factors. This conclusion agreed well with the observation from Figure 4.5 that temperature and ethanol concentration showed the dominant influence and affected the effects of solvent-to-solid ratio. Although temperature by itself showed significant influence, the squared term didn’t have significant effects on yield, while ethanol concentration had significant influence both by itself and squared.
### Table 4.3. ANOVA analysis of the response surface quadratic model for optimizing the yield of crude extract

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>9</td>
<td>241.26</td>
<td>19.67</td>
<td>0.0004</td>
</tr>
<tr>
<td>X1</td>
<td>1219.66</td>
<td>1</td>
<td>1219.66</td>
<td>99.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>X2</td>
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<td>1</td>
<td>0.039</td>
<td>&lt;0.01</td>
<td>0.9565</td>
</tr>
<tr>
<td>X3</td>
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<td>1</td>
<td>573.94</td>
<td>46.80</td>
<td>0.0002</td>
</tr>
<tr>
<td>X1X2</td>
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<td>1</td>
<td>2.65</td>
<td>0.22</td>
<td>0.6559</td>
</tr>
<tr>
<td>X1X3</td>
<td>49.10</td>
<td>1</td>
<td>49.10</td>
<td>4.00</td>
<td>0.0855</td>
</tr>
<tr>
<td>X2X3</td>
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<td>1</td>
<td>6.17</td>
<td>0.50</td>
<td>0.5012</td>
</tr>
<tr>
<td>X1$^2$</td>
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<td>6.50</td>
<td>0.20</td>
<td>0.6654</td>
</tr>
<tr>
<td>X2$^2$</td>
<td>14.15</td>
<td>1</td>
<td>14.15</td>
<td>1.15</td>
<td>0.3183</td>
</tr>
<tr>
<td>X3$^2$</td>
<td>290.52</td>
<td>1</td>
<td>290.52</td>
<td>23.69</td>
<td>0.0018</td>
</tr>
</tbody>
</table>

#### 4.3.3. Establishment of quadratic regression models

The quadratic regression models in correlation of antioxidant activity and yield with three variables are shown as follows:

\[
\frac{1}{IC_{50}} = 36.21859 - 0.42914 \times X_1 + 0.25190 \times X_2 - 0.00946 \times X_1 \times X_2 - 0.015201 \times X_1 \times X_3 + 0.00729 \times X_2 \times X_3 + 0.016298 \times X_1^2 + 0.00251 \times X_3^2
\]

\[\text{(R}^2 = 0.8958, \text{ lack of fit}=0.3265)\] Model I

\[
\text{Yield} = -57.88413 + 1.14291 \times X_1 + 2.46133 \times X_3 - 0.00876 \times X_1 \times X_3 - 0.000131 \times X_2^2 - 0.021121 \times X_3^2
\]

\[\text{(R}^2 = 0.9569, \text{ lack of fit}=0.6889)\] Model II

The optimum condition for obtaining extracts with the strongest antioxidant activity was at 20°C, 70 mL/g, and using 80% EtOH according to model I. Differently, the best extraction condition to obtain the highest yield was at 60°C, 50.02 mL/g, and with 45.65% ethanol based on model II. It suggested that the optimal condition for obtaining high yield is not necessarily best for high antioxidant activity. Similar conclusions were also found by Durling et al. (Durling et al., 2007) that although higher temperature could increase the yield of extracts from sage (*Salvia officinalis*), it also extracted more non-active compounds. Ra et al. (2017) reported that 60% ethanol could harvest extracts with both highest yield and strongest DPPH and ABTS radical scavenging activities from *Sasa quelpaertensis* Nakai. However, the time applied in their...
extraction was 90 min, which was much longer than the 30 min extraction duration in the present study. Both models show high correlation coefficient ($R^2$) and low p-value, indicating that both models were successful fitting of the experimental data. The lack of fit was not significant ($p>0.05$) for the two models, suggesting that few unknown factors may contribute to the response (Yang et al., 2010). However, the models were not designed to predict extraction conditions for overall performance, which would be somewhere between the optimum conditions from the two models. A separate model is needed for that purpose to balance crude extract yield and activity.

4.3.4. Model validation

The predicted highest $1/IC50$ and yield were 72.75 mL/mg and 55.68 mg extract/g algae, respectively. In order to verify the suitability of the models, two validation experiments were carried out at the following conditions: one at 20°C, 70 mL/g, and using 80% ethanol, the other at 60°C, 50 mL/g, and with 45% EtOH. Each experiment was carried out in triplicate. The $1/IC50$ value and yield obtained from the two experiments were 74.05±0.51 mL/g and 56.41±2.59 mg extract/g algae, respectively, suggesting that the two models were suitable for optimizing the extraction process.

4.4. Discussion

Brown algae extracts have been known to contain strong antioxidative compounds such as phlorotannins and sulphated polysaccharides (Gupta and Abu-Ghannam, 2011). *A. nodosum* is a brown algae belonging to the Phaeophyceae class and is a good source of phlorotannins and carbohydrates (Agregan and Munekata et al., 2017). Phlorotannins are a group of phenolic secondary metabolites that are only found in brown algae (Eom et al., 2012), which are involved in cell wall structure, signaling, defense, or responses to environmental stress (Balboa et al., 2013). Polysaccharides are the main component of algae cell wall. They can provide strength and flexibility, maintain ionic equilibrium and prevent desiccation of the cell. Both phlorotannins and polysaccharides have been confirmed to possess strong bioactivities such as antioxidant activity (Balboa et al., 2013).

Solvent extraction is a convenient and conventional method for obtaining extracts from plant materials, especially those sensitive to heat (Tongnuanchan and Benjakul, 2014). Various solvents such as ethanol, methanol, hexane, and acetone have been applied in their individual or
mixed forms in extraction processes. Extracts obtained with a binary solvent system containing ethanol and water could be a mixture of carbohydrates and polyphenols. Yuan et al. (2015) reported that carbohydrates were the major components in the water extract of mulberry leaves. Dehydrated ethanol was even used to wash the extracts to remove non-carbohydrate impurities (Ye et al., 2011). Ruperz et al. (2002) reported that only small amounts (less than 3 g/100 g dry weight) of polyphenols were detected in the water extract of brown algae *Fucus vesiculosus* while most compounds extracted were carbohydrates. Low polarity solvent systems, such as water-ethanol and water-methanol systems, were usually applied to extract phenolic compounds from terrestrial plant materials (Li et al., 2016; Ghitescu et al., 2015; Thoo et al., 2010) as well as seaweeds (Fu et al., 2015; He et al., 2013). Several phenolic compounds were identified in the 80% methanol extracts from brown seaweed *Fucus distichus* and *Alaria marginata* (Kellogg et al., 2014; Kellogg et al., 2015). The pool of phlorotannins from *A. nodosum* can be separated into two sub-pools of different polarity, and high-polarity phlorotannins extracted with 100% methanol were significantly higher (p<0.00001) in amount than low-polarity phlorotannins extracted with 50% methanol (Breton et al., 2011), which agreed with observations in the present study that higher ethanol concentration resulted in stronger antioxidant activity of the extracts, since polyphenols are the major antioxidants in plant materials. They also reported that most of the phlorotannins extracted had a molecular weight over 50 kDa, in both phlorotannin sub-pools. Water was believed to swell plant cells while ethanol was helpful to disrupt the bond between the solutes and plant matrices (Ghitescu et al., 2015). Adding solvent with lower polarity, e.g. acetone, was reported to promote breakdown of cell structure and enhance permeability of the solvent to cell matrix (Bachir bey et al., 2014). Other components such as proteins and pigments were not considered in the present study because proteins lose their bioactivities in most organic solvents (Samarakoon and Jeon, 2012) and pigments are usually extracted using chloroform, not by water or ethanol, thus polyphenols and polysaccharides were the expected main compounds in the crude extract.

High temperature, long extraction time, and large ratio of water to raw material were reported to enhance polysaccharide yield from *A. nodosum* (Chen et al., 2018). Release of polysaccharides and other materials from brown algae can be promoted with high temperature due to accelerated molecule diffusion and higher solubility of solutes (Zhang et al., 2015; Bachir bey et al., 2014), however, degradation of the extracted bioactive compounds can also be induced
by heat. High temperature may also lead to solvent volatilization, higher energy cost, and releasing of impurities from plant materials to the supernatant (Zou et al., 2015). The alga studied in the present study usually grows in the cold water of Maine and Ireland, thus the bioactive components could be sensitive to heat. This might explain why high temperature caused decrease in the antioxidant activity of the extract. Large solvent-to-solid ratio in the present study promoted the antioxidant activity but had no significant influence on the yield of the crude extract probably because the lower end (30 mL/g) was already sufficient. Similarly, Zhu et al. (2015) found no apparent increase in polysaccharide yield from pomegranate peel above a ratio of water to raw material of 20 mL/g in a single factor design. The yield of pomegranate peel polysaccharides remained constant within the ratio range of 20 to 30 mL/g. Similar results were observed by Ye and Jiang (2011) that a ratio higher than 20 mL/g would not increase polysaccharide yield from *Plantago asiatica* L..

### 4.5. Conclusions

The extraction process of brown algae *A. nodosum* was optimized using three-level, three-factor Box-Behnken design and response surface methodology to obtain extracts with strong antioxidant activity or high yield. Two quadratic models were developed and validated to optimize the extraction process. The combination of extraction temperature (20°C), solvent-to-solid ratio (70 mL/g), and ethanol concentration (80%) was determined as the optimal condition to obtain extract with the strongest antioxidant activity (1/IC$_{50}$=74.01 mL/mg). However, a different combination of extraction temperature (60°C), solvent-to-solid ratio (40.01 mL/g), and ethanol concentration (44.83%) was determined to obtain the highest extract yield (55.60 mg extract/g algae). Under the two optimal extraction conditions, the experimental antioxidant activity and yield were 74.05±0.51 mL/g and 53.80±1.65 mg extract/g algae, respectively, demonstrating that the two models could accurately predict and improve the extraction of antioxidants from *A. nodosum*. 
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Thoo, Y.Y., Ho, S.K., Liang, J.Y., Ho, C.W., Tan, C.P., 2010. Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (Morinda citrifolia). Food Chemistry. 120: 290-295.


CHAPTER 5. Anti-Bacterial Effects of Brown Algae Extract Against Food Spoilage Bacteria *Pseudomonas Fluorescens* and *Shewanella Putrefaciens*

**Abstract**

The crude extract of the edible brown algae *Ascophyllum nodosum* was evaluated for its inhibitory effects against the food spoilage bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens*. Prolonged lag phase for both bacteria strains was observed in extract-treated cultures, and complete inhibition (no cell growth) occurred when the concentration of extract in the broth reached 1.05 mg/mL. The Lambert-Person model was applied to predict the minimum inhibitory concentration (MIC) and non-inhibition concentration (NIC). The best-fit MIC and NIC for *P. fluorescens* was 1.145 and 0.036 mg/mL, and 0.947 and 0.106 mg/mL for *S. putrefaciens*, respectively. Bactericidal assay on agar plates showed that the algae extract applied at concentration higher than MIC could cause significant bactericidal effects, especially in *S. putrefaciens*. Algae extract (42μg/disc) showed larger inhibition zones than penicillin (10μg/disc) against both bacteria strains. Disc volatilization method was applied to study the inhibition effects of extract and penicillin without direct contact, in which both the extract and penicillin showed some inhibition influence in sealed plates. Treating tilapia fillets with algae extract could significantly reduce the total bacterial viable counts during storage under refrigeration (8 ℃). All those results suggested that crude extract of *Ascophyllum nodosum* has anti-bacteria effect and could be applied as a potential natural food preservation agent.

**Keywords** edible brown algae, *Ascophyllum nodosum*, food spoilage, *Pseudomonas fluorescens*, *Shewanella putrefaciens*, food preservation

**5.1. Introduction**

It was estimated by the Food and Agriculture Organization (FAO) that 1.3 billion tons of food are lost every year due to spoilage worldwide (Miks-Krajnik et al., 2016), which leaves 1 billion people at risk for starvation (Sarkar and Shetty, 2014). Food deterioration causes quality loss and undesirable texture, flavor, or color change of foods. It can be attributed to three major factors, physical, chemical, and biological. The biological factors include enzymatic reactions, presence or growth of insects, parasites, and microorganisms. Among them, microbial spoilage is
the most important reason for food deterioration (Zheng, 2014). Sometimes spoilage can even cause food safety issues if the spoilage microorganisms are also involved in foodborne diseases such as listeriosis. According to the Centers of Disease Control (CDC), foodborne illness accounts for about 48 million cases, 3,000 deaths, and 128,000 hospitalizations in the US every year, which costs $77.7 billion annually and becomes a heavy economic burden (Sanchez-Ortega et al., 2014).

Among all food categories, seafoods are especially perishable due to their high nutrient content, e.g. protein, unsaturated fatty acids, lipids, which are easily utilized by spoilage microorganisms (Maqsood et al., 2014). Chemical and microbial spoilage are responsible for loss of 25% of fish and fish products every year (Ghaly et al., 2010). The use of synthetic and natural antioxidants to prevent seafood spoilage has been widely studied. Extracts of marine algae have recently become a focus of research due to their superior bioactivities and abundance in nature. However, most research concerning seaweed extracts mainly focused on their pharmaceutical activities, while their anti-microbial and food preservation effects have not been thoroughly investigated (Ortiz et al., 2014). Among all the seafoods, tilapia (Oreochromis sp.) is a freshwater fish with a large global yearly production of around 2.53 million tons (Kulawik et al., 2015). Pseudomonas and Shewanella were reported as the main spoilage organisms in tilapia fillets during chilled storage conditions (Cyprian et al., 2013; Ghaly et al., 2010). These two bacteria species are not only responsible for spoilage of tilapia, but also other iced fish regardless of the origin of the fish (Gram and Huss, 1996).

Thermal treatment, radiation, high-pressure processing, pulse electric field processing, and other techniques are commonly utilized in the food industry to process foods. Besides the processing techniques above, various synthetic preservatives, such as sorbic acid, sorbates, benzoic acid, benzoates, propionic acid, propionates, and sodium nitrite have been used to preserve foods and extend their shelf lives (Silva and Lidon, 2016). As consumers’ demands for minimally processed foods and natural preservatives increase, a growing number of natural antioxidants and antimicrobial agents have been developed for use, such as lactic acid, lactate, acetic acid, nisin, natamycin, as well as microbial fermentates, lysozymes, and protective cultures. The essential oils and extracts of plants such as cinnamon, mustard, black pepper, clove, vanillin, oregano, rosemary, thyme, sage, and basil, have also been confirmed to have strong antimicrobial activity (Hintz et al., 2015; Martinez-Gracia et al., 2015). These natural
antimicrobials can extend the shelf life of unprocessed foods by reducing microbial growth or killing the spoilage microorganisms (Tiwari et al., 2009). Application of natural antibacterials can also help to prevent antimicrobial resistance of microorganisms due to the complex components in the extract (Eom et al., 2012).

Determining NIC and MIC of natural antibacterials against spoilage microorganisms is an important premise of applying them as food preservation agents and evaluating antibacterial activity. If the concentration applied is less than NIC, the preservation agent may have no effect at all. Concentration higher than MIC could be a waste of inhibitor and may pose negative influence on flavor and texture. Two major methods have been used to determine MIC and NIC, one is the traditional semiquantitative method and the other is dose-response curve modelling. In the semiquantitative method, a range of inhibitor concentrations are tested, and the concentration at which no microbial growth is observed is determined as the MIC. This method can only give a rough estimation of MIC, an upper/lower limit rather than an accurate value, and information below the MIC is usually discarded. One advantage of modelling over the semiquantitative method is that the modelling approach can determine MIC, even if none of the tested concentrations could completely inhibit microorganism growth in experiments (Guillier et al., 2007). Semiquantitative method usually requires microorganisms to be cultured with a putative inhibitory compound for at least 24 h while modelling may allow rapid determination of MIC and NIC at any time. Though MIC and NIC can be calculated from the curve modelling at any time, the accuracy of the model as a change of incubation time has not been reported and the incubation time for best-fit model needs to be illustrated for more precise application of dose-response modelling. Two models have been developed to study the dose-response of microorganisms against inhibitors, the Lambert-Pearson model (Lambert and Pearson, 2000) and Guillier model (Guillier et al., 2007). The Lambert-Pearson model has been applied more widely than the Guillier model because it is simpler, and the maximum growth rate needs not to be involved. In the Lambert-Pearson model, the MIC and NIC can be calculated as the intercept of the concentration axis to the tangent at the maximum gradient of the fractional area/inhibitor concentration curve and the intercept of the tangent at the maximum gradient of the curve to the fractional area = 1 contour (Tiwari et al., 2009). However, this model has only been applied to estimate the inhibition effect of single compound, no reports have studied the accuracy of the model on crude extracts, which contain various compounds.
Marine macroalgae-based compounds and extracts have been investigated for their various bioactivities, e.g. anti-bacterial and antioxidant activity, which make them potential food preservation agents. There is a long history of seaweed utilization as food and medicine in Asian and European countries. Seaweeds, also named marine macroalgae, are divided into three groups based on their pigments: brown, red, and green algae. Most of the edible and medicinal seaweeds are brown algae due to their high contents of protein, dietary fiber, EPA, mannitol, and bioactive compounds such as phlorotannins and polysaccharides (Eom et al., 2012; Ortiz et al., 2014; Wifesekara et al., 2011). Brown algae extracts have been proven to exhibit antimicrobial effects against food-borne pathogenic microorganisms (Cox et al., 2014; Dubber and Harder, 2008) and antibiotic-resistant bacteria (Eom et al., 2012). Although seaweeds have been studied as under-exploited sources of functional foods and medicine, the food preservation effects of their extracts have not been fully investigated.

The objective of this study was to evaluate the potential of *A. nodosum* extracts as an antibacterial agent against the spoilage bacteria *Pseudomonas fluorescens* and *Shewanella putrifaciens*, both of which are gram-negative bacteria and major spoilers of tilapia (Ghaly et al., 2010; Cao et al., 2012). A binary solvent extraction system containing ethanol and water was applied to obtain crude extracts from edible brown algae *Ascophyllum nodosum*, which exhibited high phlorotannin yield and strong antioxidant activity in previous studies of the authors (Liu et al., 2017a; Liu et al., 2017b). The antibacterial effects of *A. nodosum* extract on fresh tilapia fillets were also studied.

### 5.2. Materials and Methods

#### 5.2.1. Brown algae extract

Brown algae *A. nodosum* was extracted using 70% ethanol-water at 30°C for 30 min with a solvent-to-solid ratio of 30 mL/g. Then the extracts were filtered, collected, and rotary evaporated to obtain the crude extract. The concentration of the crude extract was determined by drying the extract in a 50°C vacuum oven until the mass was constant. Finally, the crude extract was filtered using sterile syringe filter, stored in a -20°C refrigerator, and used for antibacterial assay within one week. The concentration and pH of the final crude extract was 4.20 mg dry mass/mL and 6.85, respectively.
5.2.2. Bacterial strains

*Pseudomonas fluorescens* (ATCC 13525) and *Shewanella putrefaciens* (ATCC 8071) were obtained from American Type Culture Collection (Manassas, Virginia, USA). Bacteria strains were stored using 80% LB broth culture medium with 20% glycerol at -80°C. Prior to experiments, working cultures were prepared by sub-culturing 100μL of the stock culture in 100 mL LB broth and incubating at 30°C for 24 hours. Total cell counts were obtained using spread plate method. If needed, the culture was diluted with LB broth to adjust cell density to approximately 10^6 cfu/mL.

5.2.3. Determination of bacterial growth curve, minimum inhibitory concentration (MIC), and non-inhibitory concentration (NIC)

Crude algae extract was added to sterilized LB broth and its concentration was adjusted to 0.21, 0.42, 0.63, 0.84, 1.05, or 1.26 mg dry extract/mL broth. Then 100μL working culture was added to 100 mL broth and cultured in a New Brunswick innova 42 incubator (New Brunswick, NJ, USA) at 37°C with shaking speed of 150 rpm. The optical density (OD) was measured at 600 nm with a microplate reader every one or two hours based on the growth condition of the bacteria. All assays were performed in triplicate.

The Lambert-Pearson model (Lambert and Pearson, 2000) was used to determine MIC, the lowest concentration of extract at which no growth was observed, and NIC, the concentration of extract below which the inhibitors have no effect on the growth of microorganism (Tiwari et al., 2009). Briefly, the fractional area (Fa) was calculated as follows: which the inhibitors have no effect on the growth of microorganism (Tiwari et al., 2009). Briefly, the fractional area (Fa) was calculated as follows:

\[
Fa(c) = \frac{A_c}{A_{c0}} \quad (1)
\]

where \( c \) is the concentration of the extract, \( A_c \) and \( A_{c0} \) are the area under the OD versus time curves with and without inhibitor in broth. The areas were computed by means of trapezoidal integration (TRAPZ subroutine of MATLAB software R2015a, MathWorks, Natick, USA). According to Lambert-Pearson model, the observed fractional areas were modeled as follows:

\[
Fa(c) = \exp \left[ -\left( \frac{c}{p_1} \right)^{p_2} \right] \quad (2)
\]
where $p_1$ and $p_2$ are model parameters. The MIC and NIC can be retrieved from the $p_1$ and $p_2$ values of model (2):

\[
\text{MIC} = p_1 \cdot \exp\left(\frac{1}{p_2}\right) \\
\text{NIC} = p_1 \cdot \exp\left(\frac{1-e}{p_2}\right)
\]

where the value of $e$ is the exponential of 1.

5.2.4. Bactericidal assay

Bacteria cultured at high extract concentrations whose growth was not observed after 24 h were then spread on extract-free LB agar plates with sterile cotton swab. A negative control was made with bacteria growing in LB broth for 24 hours. Plates were incubated at 30°C for 24 h and 72 h, and the growth of bacteria was compared. All assays were performed in duplicate.

5.2.5. Determination of the diameter of inhibition zone

The inhibition zone was determined using agar diffusion method. Approximately 20 mL sterilized LB agar was poured into a petri dish. After drying, the bacterial suspension (100μL, $10^6$ cfu/mL) was inoculated on the agar and spread with a spreader. Then sterile filter paper discs (6 mm diameter) were impregnated with 10μL of crude extracts (42μg/disc) using capillary micro-pipette and placed on the LB agar. Penicillin G sodium salt (10μg/disc) was used as positive control. The plates were then incubated at 30°C for 24 hours. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition. Each assay was replicated three times.

5.2.6. Disc volatilization method

A 100μL portion of each bacteria suspension containing approximately $10^6$ cfu/mL was spread over the surface of LB agar plate and allowed to dry. A paper disc (6 mm diameter) or oxford cup was laid on the inside surface of the upper lid as container of penicillin or extract. The volume of extract applied in oxford cup was 200μL (0.84 mg). Penicillin G sodium salt (1 mg/mL) was applied as positive control while sterilized water applied as negative control. The plates were sealed with parafilm to prevent leakage of vapor. Plates were incubated at 30°C for 24 hours. All tests were performed in duplicate.
5.2.7. Preservation test of tilapia fillets

Fresh tilapia fillets were purchased from a local retail store and cut into 5-g pieces using a sterilized knife and chopping board. The tilapia pieces were then dipped in concentrated algae extract (algae extract group) or deionized water (control group) for one hour. Treated pieces were placed in sterilized petri dishes and stored in an 8℃ refrigerator. Before the test, tilapia pieces were homogenized with 95 mL sterilized saline (8 g NaCl/mL) for at least 30 seconds, then the supernatant was collected. The supernatant was serially diluted with sterilized saline, and 0.1 mL aliquot of diluted supernatant was spread on LB agar, incubated at 30℃ for 24 hours to determine total viable count (TVC) and the result was expressed as log colony forming unit per gram fish tissue (log_{10} cfu/g). The TVC was determined every day during the 14-day storage.

5.3. Results and Discussion

5.3.1. Determination of bacterial growth curve, minimum inhibitory concentration (MIC), and non-inhibitory concentration (NIC)

Figure 5.1A shows that algae extract (0.21, 0.42, and 0.63 mg/mL) caused prolonged lag phase of *P. fluorescens*. As the concentration of algae extract continued to increase to 1.05 mg/mL, growth was completely inhibited, suggesting that the crude extract of *A. nodosum* could effectively inhibit the growth of *P. fluorescens*. Prolonged lag phase in the growth curve was also observed in *Pseudomonas aeruginosa* against bayberry leaf extract (Su et al., 2013). Similar with *P. fluorescens*, prolonged lag phases were observed (0.21, 0.42, and 0.63 mg/mL) in *S. putrefaciens* (Figure 5.1B). The extract (0.21, 0.42, 0.63, and 0.84 mg/mL) could also cause decrease of cell density at stationary phase. No growth was observed when the extract concentration increased above 1.05 mg/mL after 30 hours of incubation.
The MICs and NICs of *P. fluorescens* and *S. putrefaciens* retrieved at varying incubation times are shown in Table 5.1. The Lambert-Pearson model adequately described and predicted the effects of extract concentration on *P. fluorescens* and *S. putrefaciens* growth. The NICs increased with incubation time in both bacteria strains. The MIC of *P. fluorescens* was observed to decline before incubation time reached 16 hours, then increased steadily to 1.313 mg/mL at 24 hours. Similar observation was made in *S. putrefaciens*, in which the MIC decreased from 1.419 to 0.670 mg/mL as incubation time rise from 10 to 18 hours, and then began to increase after 20 hours. Lambert and Pearson (2000) reported rising MIC and NIC as incubation time increased, and large fractional area was obtained before 8 h due to low signal to noise ratio of the control group. This observation agrees with the present study in that early stage fractional areas were larger in the experimental groups, which resulted in low accuracy model. The best-fit MICs were obtained at incubation time of 20 hours for *P. fluorescens* ($r^2=0.9892$) and 32 h for *S. putrefaciens* ($r^2=0.9841$), both at the beginning of the stationary phase of the control group (0 mg/mL extract added). Low $r^2$ values (less than 0.90) were observed before 14 hours of both bacteria, which was within early log phase. Slight decrease of $r^2$ value was observed in both bacteria after the very beginning of the stationary phase (20 hours for *P. fluorescens* and 32 hours for *S. putrefaciens*), suggesting that the best-fit NIC and MIC should be derived at least after the bacteria reached late log phase or early stationary phase in the inhibitor-free culture media. To the author’s knowledge, no research has been reported on both the

![Figure 5.1. Growth curve of *P. fluorescens* (A) and *S. putrefaciens* (B). Different symbols in the figure indicate different concentration of extract (●: 0 mg/mL; ■: 0.21 mg/mL; ◆: 0.42 mg/mL; ＿: 0.63 mg/mL; +: 0.84 mg/mL; ▲: 1.05 mg/mL; —: 1.26 mg/mL).](image)
rapidity and accuracy of models developed at different incubation time. Besides, the MIC observed in present study was significantly lower than the MIC of *Eucalyptus globulus* oil against *P. fluorescens*, which was 9 mg/mL (Tyagi and Malik, 2011), suggesting *A. nodosum* extract had more prominent antibacterial effects.

Table 5.1. Minimum inhibition concentration, non-inhibition concentration, and $r^2$ of model predicted of *P. fluorescens* and *S. putrefaciens* at different incubation time

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>NIC (mg/mL)</th>
<th>MIC (mg/mL)</th>
<th>$r^2$</th>
<th>Time (h)</th>
<th>NIC (mg/mL)</th>
<th>MIC (mg/mL)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0.012</td>
<td>0.851</td>
<td>0.9392</td>
<td>10</td>
<td>0.002</td>
<td>1.420</td>
<td>0.8501</td>
</tr>
<tr>
<td>16</td>
<td>0.024</td>
<td>0.793</td>
<td>0.9650</td>
<td>12</td>
<td>0.008</td>
<td>0.856</td>
<td>0.8952</td>
</tr>
<tr>
<td>18</td>
<td>0.026</td>
<td>1.017</td>
<td>0.9820</td>
<td>14</td>
<td>0.018</td>
<td>0.718</td>
<td>0.9226</td>
</tr>
<tr>
<td>20</td>
<td>0.036</td>
<td>1.145</td>
<td>0.9892</td>
<td>16</td>
<td>0.028</td>
<td>0.716</td>
<td>0.9408</td>
</tr>
<tr>
<td>22</td>
<td>0.049</td>
<td>1.264</td>
<td>0.9865</td>
<td>18</td>
<td>0.041</td>
<td>0.670</td>
<td>0.9567</td>
</tr>
<tr>
<td>24</td>
<td>0.071</td>
<td>1.313</td>
<td>0.9796</td>
<td>20</td>
<td>0.053</td>
<td>0.674</td>
<td>0.9696</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td>0.057</td>
<td>0.727</td>
<td>0.9778</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>0.070</td>
<td>0.783</td>
<td>0.9814</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28</td>
<td>0.087</td>
<td>0.892</td>
<td>0.9832</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>32</td>
<td>0.106</td>
<td>0.947</td>
<td>0.9841</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>40</td>
<td>0.136</td>
<td>0.990</td>
<td>0.9841</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>48</td>
<td>0.149</td>
<td>1.005</td>
<td>0.9815</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52</td>
<td>0.139</td>
<td>1.033</td>
<td>0.9803</td>
</tr>
</tbody>
</table>

### 5.3.2. Bactericidal assay

Bacteria cultured in medium containing high concentration of extracts in which no growth observed after 24 hours were spread on extract-free agar plates, and the results are shown in Figures 5.2 and 5.3. By comparing Figure 5.2.a, 5.2.b, 5.2.c with 5.2.d it can be observed that culturing *P. fluorescens* in broth with high concentrations of algae extract (from 2.52 to 3.36 mg/mL) could significantly reduce live bacteria cells. Culturing for longer time (72 hours) could result in even lower counts of living cells compared with shorter incubation time (24 hours). Similar observation can be made from Figure 3 on *S. putrefaciens* but the inhibition effects were much stronger than *P. fluorescens*. As lower concentration of extract (1.26 and 1.68 mg/mL)
could significantly reduce bacteria counts and longer incubation time (72 hours) showed much stronger inhibition effects than shorter incubation time (24 hours), which indicated that *S. putrefaciens* was more sensitive to the extract than *P. fluorescens*.

![Image of bacterial growth](image1.png)

**Figure 5.2** Bactericidal assay of *P. fluorescens* with a. 3.36 mg extract/mL broth for 24 h (left) and 72 h (right); b. 2.94 mg extract/mL broth for 24 h (left) and 72 h (right); c. 2.52 mg extract/mL broth for 24 h (left) and 72 h (right); d. 0 mg extract/mL broth at 30°C for 24 h (left) and 72 h (right).

![Image of bacterial growth](image2.png)

**Figure 5.3** Bactericidal assay of *S. putrefaciens* with a. 2.94 mg extract/mL broth for 24 h (left) and 72 h (right); b. 2.52 mg extract/mL broth for 24 h (left) and 72 h (right); c. 2.10 mg extract/mL broth for 24 h (left) and 72 h (right); d. 1.68 mg extract/mL broth for 24 h (left) and 72 h (right); e. 1.26 mg extract/mL broth for 24 h (left) and 72 h (right); f. 0 mg extract/mL broth at 30°C for 24 h (left) and 72 h (right);

The minimum bactericidal concentration (MBC) test of the extracts has been applied to determine whether the microorganisms were killed or their growth was inhibited. The MBCs were found to be one to four times higher than MICs of several spoilage bacteria against microalgae extract (Ugoala et al., 2016). The MIC and MBC of several leafy green vegetable
extracts were tested against *Staphylococcus aureus* and *Bacillus subtilis*, in which *B. subtilis* was more susceptible than *S. aureus*. Among the vegetable extract, Fatsia extract exhibited inhibition on *S. aureus* but showed no effect on *B. subtilis*, which suggested that the antibacterial effect of natural extracts depends on the composition of extracts and the microorganisms (Kim et al., 2013). *Pseudomonas* has been reported to exhibit strong resistance to natural extracts and essential oils. Prominent antibacterial activity of *Veronica montana* L. extract was observed against *S. aureus* with equal MIC and MBC (7.5 mg/mL), indicating that the extract could kill *S. aureus* at concentration of 7.5 mg/mL. *P. aeruginosa* was the least susceptible, with MIC of 22.5 mg/mL and MBC of 45 mg/mL (Stojkovic et al., 2013). The MBCs of *P. fluorescens* and *P. aeruginosa* were 18 mg/mL *Eucalyptus globulus* essential oil, which were two times of their MIC (Tyagi and Malik, 2011). All MBCs reported in literature, regardless of the origin of extracts or essentials, were much higher than the highest concentration applied in the present study (3.36 mg/mL).

5.3.3. Diameters of inhibition zone

The diameters of inhibition zones of algae extract and penicillin are shown in Table 5.2. All results were expressed as mean ± standard deviation. It can be seen that algae extract (4.20 mg/mL, 10μL per disc) showed stronger inhibition than penicillin (1 mg/mL, 10μL per disc) on agar plates for both *P. fluorescens* and *S. putrefaciens*. No significant difference was observed between the inhibition zone of extract in the two bacteria strains (p>0.05). All results indicated that *A. nodosum* extract could effectively inhibit *P. fluorescens* and *S. putrefaciens*.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Inhibitor</th>
<th>Algae extract (cm)</th>
<th>Penicillin (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td></td>
<td>1.72±0.32</td>
<td>1.42±0.09</td>
</tr>
<tr>
<td><em>S. putrefaciens</em></td>
<td></td>
<td>1.58±0.31</td>
<td>1.00±0.00</td>
</tr>
</tbody>
</table>

The bacterium *Pseudomonas* has shown resistance to most essential oils, extracts, and even synthetic drugs due to their restrictive outer membrane barrier (Rahman and Kang, 2009). A selection of natural extracts has been confirmed with their antimicrobial activity against *P.*
Among which citrus extract and thyme essential oil had the largest inhibition zone. *P. fluorescens* was more resistant to natural extracts than *Listeria innocua* and *Aeromonas hydrophila* against all essential oils and extracts (Iтурриага et al., 2012). Similar ethanolic extract of *Lonicera japonica* Thunb. showed no inhibition zone against *P. aeruginosa* on LB agar plates in disc diffusion assay (400μg extract per disc) (Rahman and Kang, 2009). However, the inoculum applied in that research was $10^7$ cfu/mL and 100μL was spread on the plate, which was 100 times higher than the present study. The inhibition zone observed of *E. globulus* oil against *P. fluorescens* was around 1.4 cm in diameter, which was smaller than the present study, yet the concentration of the essential oil used was not illustrated in that article (Tyagi and Malik, 2011). *S. putrefaciens* was inhibited by R(+) limonene in agar diffusion tests, with an inhibition zone of 11.2 mm in diameter (Giarratana et al., 2016). However, not all natural extracts could exhibit inhibition zones against *S. putrefaciens*. It was reported by Wright et al. (2016) that only four plant extracts (muntries, lemon aspen, desert lime, bush tomato, and plum fruit) out of 28 Australian plant extracts showed inhibition zones against *S. putrefaciens*.

### 5.3.4. Disc volatilization method

In the disc volatilization assay, color, number, and density of colonies formed were evaluated to estimate the effect of inhibitors without direct contact with microorganisms. From Figure 5.4 and 5.5 it can be observed that volatile components in algae extracts had some inhibition effect on *P. fluorescens* and *S. putrefaciens*, especially in *S. putrefaciens*. The activity showed in the present study was not as strong as the essential oils of a series of herbs studied by Iтурриага et al. (2012), in which inhibition zones were observed while no zones of inhibition were found in the present assay. *P. fluorescens* was found to be the most resistant to the essential oil with only growth reduction underneath paper discs observed. This could be explained that water soluble extracts tested in the present study had lower amounts of volatile components than essential oils. The vapor diffusion method is considered more suitable for essential oils since they contain more hydrophobic and more volatile substances (Goni et al., 2009).
Figure 5. Disc volatilization assay of *P. fluorescens* with 10µL 1 mg/mL penicillin (up left); 30µL 1 mg/mL penicillin (up right); negative control (down left); 200µL 4.20 mg/ML algae extract (down right) incubated at 30°C for 24 hours.
In the agar diffusion assay, the antibacterial effect of the extracts could be tested in direct contact and without direct contact in the vapor phase. In the present study, the antibacterial effect of algae extract in the vapor phase was relatively weaker comparing to their disc diffusion assay, even when larger amount of extracts were applied. Essential oils of rosemary and oregano showed larger inhibition zone than their water-soluble extracts against *Listeria innocua*, *P. fluorescens*, and *Aeromonas hydrophila*. The differences shown between essential oils and their corresponding water soluble extracts could be due to different compound profile as essential oil contains more low polarity volatile components. The inhibition effect of compounds of the extract or essential oil in a vapor diffusion essay was usually not stronger than in agar diffusion assay (Iturriaga et al., 2012). Similar results were observed by Edwards-Jones et al. (2004) that essential oils showing significant inhibition effects in direct contact with *S. aureus* may not be able to reduce bacteria growth in vapor diffusion assay. Meanwhile, combinations of essential oils enhanced inhibition influence than the individual components. However, some essential oils showed stronger antibacterial effects in the vapor phase than in direct contact against...
Escherichia coli, Yersinia enterocolitica, and S. aureus, while P. aeruginosa was the only microorganism better inhibited in direct contact (Goni et al., 2009). It was observed that P. fluorescens was more sensitive to the vapor phase of E. globulus oil than its liquid phase in well diffusion assays (Tyagi and Malik, 2011). The different results observed in the literature could be contributed to the variance of antibacterial agents in volatile components and sensitivity of different bacteria. More research needs to be done to illustrate the mechanisms of antibacterial effects of volatile antibacterial components in essential oils and extracts.

5.3.5. Preservation test of tilapia fillets

TVC of algae extract treated tilapia group and control group is shown in Figure 5.6. It can be observed that TVC of the algae extract group was significantly lower than the control group (p<0.05). Besides, treating tilapia with algae extract could also prevent “bad fish” odor. The off-flavor odor was detected in the control group at day 2 but day 9 in the algae extract group, while slime began to cover the surface of tilapia pieces of the control group at day 5 yet none was observed in the algae group, suggesting that A. nodosum extract could delay spoilage of tilapia fillets during refrigerated (8°C) storage. The off-odor is usually contributed by production of total volatile base nitrogen (TVB-N) and H₂S by metabolic activity of microorganisms, and lipid peroxidation, especially deterioration of polyunsaturated fatty acids (PUFAs) in fish (Ortiz et al., 2014). The delay of off-odor observed in the algae extract-treated group could be a result of the extract acting as a protective coating deterring contact between fish tissue and oxygen in the air (He et al., 2017), inhibition of SSO growth and production of volatile compounds. Similar off-odor was observed in sterile gilt-head seabream inoculated with Pseudomonas spp. and S. putrefaciens when the bacteria count reached 10⁷ and 10⁸ cfu/g, respectively (Koutsoumanis and Nychas, 2000), while Pseudomonas spp. and S. putrefaciens were the specific spoilage organism (SSO) of iced fresh fish regardless of the origin of the fish (Gram and Huss, 1996). The majority of H₂S producing bacteria isolated from ice-packed fish were found to be Shewanella spp. (Garcia et al., 2015). The off-odor observed in the control group in the present study could be caused by metabolic products of Shewanella spp. and Pseudomonas spp. since SSO produce typical off-odor and grow faster than other microorganisms (Koutsoumanis and Nychas, 2000; Miks-Krajnik et al., 2016). Plant extracts were reported to inhibit SSO growth in chill storage meat. For example, Staphylococcus aureus
was not detected in the chilled chicken samples treated with pomegranate peel extract until 12 days of storage, but the bacteria were detected in the untreated chicken samples as early at day 7 (Kanatt et al., 2010).

![Graph showing microbial growth over days of storage](image)

Figure 5.6. Total viable count after incubation at 30°C for 24 hours of algae extract treated group (◆) and control group (●) of tilapia stored in 8°C refrigerator

Inhibition of microbial growth of natural plant extracts against fish spoilage has been reported in several studies. Antimicrobial effect of bayberry leaf extract against large yellow croaker has been studied in a 16-day storage period. TVC of control group reached 8.8 log_{10} cfu/g while the extract treatment group was only 7 log_{10} cfu/g (Su et al., 2013). In a 150-day storage study of liquid smoked rainbow trout, adding thyme oil could significantly reduce TVC, TVB-N, psychrotrophic viable counts, and lactic acid bacteria (Alcicek, 2011). Aerobic plate counts of tilapia fillets showed that immersing tilapia fillets in chitosan could significantly reduce aerobic microbial growth during 12-day refrigerated storage (Cao et al., 2012).

Combining natural plant extracts with film material is an effective way of applying natural preservatives and preserving their bioactivities. Antibacterial effects of sodium alginate-based edible film containing tea polyphenol and vitamin C have been reported to involve reductions of TVC of refrigerated bream (Song et al., 2011). During a 30-day refrigerated storage of tilapia fillets, the group treated with chitosan-pomegranate peel extract had significantly lower TVB-N, peroxide value, and TBARS compared to the control group (Alsaggaf et al., 2017).
5.4. Conclusions

The water-soluble extracts of the edible brown algae *A. nodosum* exhibited significant antibacterial effects against *P. fluorescens* and *S. putrefaciens*, which are SSOs of tilapia. *S. putrefaciens* was observed to be more sensitive than *P. fluorescens* against algae extract. Prominent zones of inhibitions were observed in the disc diffusion assay and algae extract (42 μg/disc) showed larger zones than penicillin (10 μg/disc). No inhibition zones were observed in the vapor phase antibacterial assay, suggesting that the antibacterial effects of the volatile components in the extracts were not strong, though some inhibition effects were observed.

Treating tilapia fillets with algae extract could significantly reduce microorganisms and delay off-odor and slime development. These results indicated that *A. nodosum* extract could be used as potential antibacterial agent against *P. fluorescens* and *S. putrefaciens*, and serve as natural food preservative to extend shelf life of tilapia fillets.
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**Abstract**

Stability of edible brown algae *Ascophyllum nodosum* extract was evaluated under thermal treatment (40-90°C). The total phenol content (TPC) and antioxidant activity decreased by approximately 5% and 10%, respectively, after 6 hours of thermal treatment, while the reducing sugar content (RSC) increased at high temperature. Experiments were also performed to evaluate general stability of the extract stored in closed or open containers at room temperature (25°C) and low temperature (0°C). It was found that extracts exposed to air and room temperature for 108 h showed lower TPC than those stored in closed containers and low temperature. The RSC increased during storage at room and low temperatures, with extracts exposure to air showed higher RSC. Antioxidant activity of extracts remained constant at 25°C and 0°C, regardless of exposure to air. Results of the present study suggested that the extract of *A. nodosum* was stable at various conditions and could be used as a stable antioxidant.

**Keywords** Brown algae, *Ascophyllum nodosum*, thermal treatment, storage condition, extract degradation

**6.1. Introduction**

Seaweeds, also known as macroalgae, have been consumed as food since ancient times in Asian countries, and to a lesser extent in Europe and America. They are often used for extraction of phytocolloids such as agar and carrageenan, which are used as thickening agents in the food industry (Agregan et al., 2017). Edible seaweeds are good sources of proteins, dietary fibers, and bioactive compounds, e.g. polysaccharides, polyphenols, fatty acids, vitamins (Kadam et al., 2017). *Ascophyllum nodosum* belongs to class Phaeophyceae and mainly grows in the rocky intertidal zones of North Atlantic Ocean. It has a wide use in food and agricultural applications (Kadam et al., 2017). Major components of brown algae extracts are polysaccharides, phenolic compounds, proteins, pigments, peptides, polyunsaturated fatty acids and terpenoids (Chen et al., 2018). The total carbohydrate and polyphenol contents of *A. nodosum* extract were found to be 63 g glucose/100g extract and 0.96 g phloroglucinol/100g
extract, respectively. Addition of the extract could effectively inhibit the oxidation of canola oil and its antioxidant effect was significantly higher than butylatedhydroxytoluene (BHT) (Agregan et al., 2017). Extract of A. nodosum has been utilized as biostimulants to enhance lettuce growth under potassium deficiency (Chrysargyris et al., 2018). Research has also been performed on the pharmaceutical activities of A. nodosum extract, such as anti-hyperglycemic (Pantidos et al., 2014), anti-inflammatory, and anti-senescence (Dutot et al., 2012). Because of its various active components and pharmaceutical activities, the extract of A. nodosum could potentially be applied as a food ingredient and preservation agents.

The production and separation of extracts from plant materials usually involve thermal treatment. In the food industry, thermal processes are also necessary to extend the shelf life of fresh and processed foods (Oancea et al., 2018). However, thermal treatment of extracts induce many undesired chemical reactions, such as Maillard reaction, oxidation of phenolic compounds, and degradation of polysaccharides (Lu et al., 2018), which is a loss of health benefiting compounds and antioxidants. Among all components in plant extracts, polyphenols are suffering from poorer stability towards high temperature, depending on phenolic component content, pH, temperature, light intensity, and oxygen, among other active compounds (Oancea et al., 2018). Polyphenol contents are usually negatively affected by thermal process, but the antioxidant activity of plant food and extract is mainly related to their polyphenol content (Turturica et al., 2016a; Ursache et al., 2017). Heating also resulted in loss of other bioactive compounds, such as anthocyanins (Oancea et al., 2018; Turturica et al., 2016a), flavonoids, and carotenoids (Ursache et al., 2017). Decrement of these bioactive components would not only reduce the antioxidant activity of the extract but also results in great loss of health-benefiting components. Research has been conducted to investigate the influence of thermal process on physico-chemical properties of extracts, such as antioxidant activity, total phenol content, pH, viscosity of extract from Mengkudu (Maskat and Tan, 2011), elderberry (Oancea et al., 2018), Aloe vera (Chang et al., 2006), and grape marc (Solyom et al., 2014). Thermal degradation of phenolic components in elderberry extract (Oancea et al., 2018) and grape marc extract (Solyom et al., 2014) was found to follow first-order reaction kinetics, while sea buckthorn extract was found to follow a fractional conversion kinetic model (Ursache et al., 2017). The studies mentioned above were performed on fruit extracts and higher range of treatment temperature (80 to 160°C) was applied.
to analyze degradation kinetics. However, to the author’s knowledge, no research has been performed on the chemical stability of seaweed extract yet.

The objective of this study was to evaluate the phytochemical stability of the extract from edible brown algae *Ascophyllum nodosum*, under the influence of temperature and exposure to oxygen. The total phenol content (TPC) and reducing sugar content (RSC) of the extract were measured, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was analyze to determine the antioxidant activity of algae extract.

6.2. Materials and Methods

6.2.1. Brown algae extract preparation

Dried powders of *A. nodosum* was extracted using a 70% ethanol-water binary solvent system at 30°C at a liquid-to-solid ratio of 30 mL/g for half an hour. Then the extract was filtered and the supernatnat collected as unconcentrated extract. The unconcentrated extract was then rotary evaporated under vacuum at 58°C. Both unconcentrated and concentrated extracts were stored in sealed containers in an -8°C refrigerator, and tested within 48 h.

6.2.2. Total phenol content (TPC) test

TPC was determined using a modified version of Folin-Ciocalteu method, using phlorogluconol (PHG) as the standard (Eom et al., 2012). A 0.04 mL aliquot of the sample was mixed in a 1.5 mL Eppendorf tube with 0.4 mL 1 N Folin-Ciocalteu reagent and 0.8 mL 20% Na₂CO₃. After standing for 3 min, the sample was incubated in the dark at room temperature for 45 min and centrifuged at 1600 g for 8 min. Optical density (OD) of the supernatant was measured at 730 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The calibration equation obtained was $Y = 0.472X + 0.1124$ ($r^2 = 0.99$), where $X$ is the OD at 730 nm and $Y$ is the concentration of phlorogluconol (mg PHG/mL) as the standard. The TPC test was performed in triplicate.

6.2.3. Reducing sugar content (RSC) test

RSC is a typical variable reflecting polysaccharide degradation. The RSC was determined by DNS (3,5-dinitrosalicylic acid) method (Garriga et al., 2017) with L-rhamnose
(RHA) as the standard. The calibration equation obtained was \( Y = 4.529X - 0.160 \) \((r^2 = 0.99)\), where \( X \) is the OD at 540 nm and \( Y \) is the concentration of L-rhamnose (mg RHA/mL).

6.2.4. DPPH radical scavenging activity test

DPPH radical scavenging activity was applied to evaluate the antioxidant activity using the method of Cox et al. (2010) with minor modifications. An aliquot of 0.1 mL of 152 μM DPPH radical solution was added to 0.1 mL treated extract. For the extracts obtained after rotary evaporation, the treated extracts were diluted 10 times before test. The reaction mixtures were incubated in the dark for 30 min at room temperature, and the optical density (OD) was measured at 517 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The DPPH test was performed in triplicate and the result was expressed as scavenging activity (percentage radical scavenged), which was calculated with the following equations:

\[
\text{Scavenging activity (\%) = 1 - \frac{A_{\text{sample}} - A_{\text{sampleblank}}}{A_{\text{control}}}}
\]

where \( A_{\text{control}} \) is the OD of the DPPH solution only, \( A_{\text{sample}} \) is the OD of DPPH solution with sample, \( A_{\text{sampleblank}} \) is the OD value of the sample only. The DPPH radicals were purchased from Sigma-Aldrich CO. LLC. (St. Louis, MO, USA). Folin-Ciocalteu reagent, ethanol, 3,5-dinitrosalicylic acid, sodium and potassium tartrate tetrahydrate, sodium hydroxide, and L-rhamnose were purchased from Thermo Fisher Scientific (Hampton, NH, US).

6.2.5. Treatment of extract

A 0.1 mL aliquot of concentrated extract was placed in sealed Eppendorf tubes to prevent evaporation. Sealed tubes were incubated in a water bath of 40, 50, 60, 70, 80, or 90 °C for 6 h. The total phenol content, reducing sugar content, and DPPH radical scavenging activity were tested every hour. The unconcentrated extract was also placed in sealed Eppendorf tubes and incubated in water bath of 50 or 60°C for 6 h to evaluate the thermal stability. Temperatures higher than 60°C were not considered because the ethanol-water solvent system applied in the present study is very unstable at temperatures higher than 60°C. After thermal treatment, the tubes were cooled immediately in an ice-water mixture to prevent further degradation.

The influence of storage condition on concentrated extract stability was also investigated. Four groups of concentrated extracts were placed in sealed Eppendorf tubes to avoid contact with oxygen in the air, stored in a 0°C refrigerator (closed freeze, CF), at room
temperature (closed room temperature, CRT), or placed in open Eppendorf tubes, stored in a 0°C refrigerator (open freeze, OF) and at room temperature (open room temperature, ORT) for 108 hours. The volume of group OF and ORT was adjusted by adding deionized water to compensate for liquid evaporation during storage.

Results were expressed as $A_t/A_0$, where $A_t$ is the bioassay (TPC, RSC, or DPPH scavenging capacity) at treatment time $t$ and $A_0$ is the bioassay at the very beginning of thermal treatment.

6.2.6. Statistical analysis

All results were expressed as mean±standard deviation. Statistical analysis was performed using one-way ANOVA and Tukey test using SAS (Cary, NC, USA). A p-value of 0.05 or less was considered statistically significant.

6.3. Results

6.3.1. Influence of thermal treatment on concentrated extract

The influences of thermal treatment on the total phenol content (TPC), reducing sugar content (RSC), and DPPH radical scavenging activity are shown in Figure 6.1. As treatment time and temperature increased, the TPC and antioxidant activity showed a declining trend while the RSC was observed to increase. At the highest treatment temperature, 90°C, the TPC decreased by about 5%, suggesting that phenolic compounds might be lost or degraded during thermal treatment (Turturica et al., 2016a). The RSCs of samples treated at a relatively lower temperature (<80°C) increased by approximately 7%-15%, while the RSCs of samples treated with higher temperature (80 and 90°C) increased by 35%, indicating that more polysaccharides were degraded under higher heating temperature. Although reducing sugar increased during thermal process, the antioxidant activity decreased by 0.5~10%, which suggests that the antioxidants in *A. nodosum* extract are thermal sensitive and their activity might depend on phenolic content (Ursache et al., 2017; Zeng et al., 2019).
Figure 6. 1. Influence of thermal treatment on total phenol content (graph A), reducing sugar content (graph B), and antioxidant activity (graph C). Different symbols indicate different treatment temperature (○: 40°C; ◊: 50°C; ∆: 60°C; □: 70°C; —: 80°C; ╳: 90°C)
Thermal degradation of bioactive components has been studied in various plant materials and extracts. It has been reported that during thermal treatment of black garlic processing, the higher treatment temperature would cause polysaccharides in garlic to degrade into reducing sugars sooner than lower treatment temperature (Lu et al., 2018), which agrees with the observation of the present study that higher treatment temperature resulted in enhanced RSC of algae extract. Similarly, Maskat and Tan (2011) reported that increasing temperature (30 to 90°C) caused the TPC of Mengkudu extract to decrease, which is in agreement with the findings of the present study that the TPC of *A. nodosum* extract also decreased during thermal treatment. Reduced TPC or total anthocyanin content was also observed during thermal treatment of elderberry extract (Oancea et al., 2018), grape marc extract (Solyom et al., 2014), roselle extract (Mourtzinos et al., 2008), and sea buckthorn extract (Ursache et al., 2017). The DPPH scavenging activity of elderberry extract was reported to decrease from 21 to 49% as treatment temperature raised from 100 to 150°C up to 90 min of heating (Oancea et al., 2018). The highest treatment temperature in the present study was 90°C but the treatment time was longer (up to 6 hours), yet the algae extract showed relatively higher stability, with only 10% loss of DPPH radical scavenging activity at 6 h. Heat treatment ranging from 70 to 110°C induced a reduction in DPPH radical scavenging activity with a loss (3-12%) observed when heating plum extract for 5 min. The authors also found that TPC of plum extract decreased between 4 and 23°C under the same treatment conditions and concluded that loss or degradation of certain phenolic components in plum extracts was responsible for reduction of DPPH radical scavenging activity (Turturica et al., 2016a). As heating temperature raised from 50 to 120°C, the TPC and DPPH radical scavenging activity of sweet cherry extract were also reported to decrease and higher temperature induced greater loss in both TPC and DPPH radical scavenging capacity (Turturica et al., 2016b).

**6.3.2. Influence of thermal treatment on unconcentrated extract**

Supernatants obtained without rotary evaporation were tested for their chemical stability to investigate the influence of rotary evaporation on the composition and thermal stability of extracts. Similar with the extracts obtained with rotary evaporation, the RSC of supernatants treated at 50 and 60°C was increased (Figure 6.2), suggesting degradation of polysaccharides under thermal treatment. The antioxidant activity of samples treated at both 50 and 60°C didn’t
decrease during thermal treatment, indicating that the antioxidants were stable at medium treatment temperatures. However, unlike the extracts obtained using rotary evaporation, the TPC of supernatant increased during treatment, which was probably because polyphenols were released from their bindings with other components such as polysaccharides (Solyom et al., 2014) during thermal treatment, thus resulted in higher TPC. The results indicate that the supernatant showed higher thermal stability than the concentrated extracts when treated at medium temperature.

![Figure 6. 2. Influence of thermal treatment (graph A: treatment at 50°C; graph B: treatment at 60°C) on the unconcentrated extract obtained without rotary evaporation. Different symbols indicate total phenol content (○), reducing sugar content (△), and antioxidant activity (□)](image)

Only a few studies investigated the thermal stability of supernatant obtained without drying process. The phenolic compounds in grape marc extract obtained after filtration showed no further degradation at 80°C and an increase in TPC was observed when the extraction temperature reached 100°C. The antioxidant activity didn’t change at 80°C but an increment was observed at 100°C (Solyom et al., 2014). However, the polyphenolic compounds in red grape skin extract were very stable during heating at 40°C but activity was found to decrease at 60 and 80°C (Tomaz et al., 2019). A similar result was reported in sea buckthorn extract when heated at 60°C. Significant decreasement in TPC and DPPH radical scavenging activity was observed during a thermal treatment period of 25 min (Ursache et al., 2017).
6.3.3. Influence of different storage conditions on concentrated extract

General stability of concentrated extract under different storage condition was studied and the results were shown in Figure 6.3. A longer period of observation (0-108 h) was applied, and as time goes on, decrement in TPC and increment in RSC was observed, while the antioxidant activity did not show significant change for all four groups tested. Freezing could inhibit oxidation of phenolic compounds but exposure to oxygen did not show influence on TPC at room temperature and freezing, suggesting that storage temperature played an important role in pohenicolic oxidation. Exposure to oxygen could significantly increase RSC in the extract, as the RSC contents in group OF and ORT were significantly higher than group CF and CRT. However, lower storage temperature might aid in preventing reducing sugars from oxidation or degradation since the RSC of group OF was greater than ORT. The DPPH radical scavenging activity did not change significantly in all four groups, suggesting that the antioxidants in the algae extract was stable under storage conditions applied in the present research.

Phenolic compounds in plant extracts could remain stable after long-period storage at low and room temperature in previous studies. As reported by Tao et al. (2014), after 30-day storage, the TPC in wine lees extract stored at 4 and 20°C decreased by 12.5 and 12.1%, respectively, while anthocyanin showed better stability at 4°C. An up to 400 or 115 days of storage experiment was performed on grape marc phenolics in darkness at 4 or 25°C. It was revealed that the TPC of grape marc phenolic extracts remained stable during long-period storage (Amendola et al., 2010). Two major anthocyanins in roselle extract, delphinidin 3-O-sambubioside and cyaniding 3-O-sambubioside, decreased by 11% and 17% after storage at 4°C for 60 days, respectively. The degradation of the two anthocyanin compounds were found to be more rapidly at higher temperature of 20, 30, and 37 °C (Sinela et al., 2017), which agrees with the findings of the present study that TPC of algae extract stored at room temperature decreased more rapidly than those stored in the refrigerator (0°C).
Figure 6.3. Influence of freezing and exposure to oxygen on the total phenol content (graph A), reducing sugar content (graph B), and antioxidant activity (graph C). Different symbols indicate different treatment groups (○: closed freeze; □: closed room temperature; ∆: opened freeze; ◊: opened room temperature)
6.4. Conclusions

The extract obtained from edible brown algae *A. nodosum* remained stable under thermal treatment and storage conditions. Increasement of RSC was observed in all treated extracts, indicating degradation of polysaccharides during treatment. The TPC and antioxidant activity of concentrated extracts decreased by 1~5% and 0.5~10% after thermal treatment (40, 50, 60, 70, 80, and 90°C), respectively, while the antioxidant activity of supernatant didn’t change at medium heating temperature, 50 and 60°C. After long-period storage at 0 and 25°C with/without exposure to oxygen, the antioxidant activity of the algae extract remained stable. Decrease of phenolic components and antioxidant activity was the lowest in the extract stored in sealed tubes at 0°C. Results of the present study show that bioactive components in *A. nodosum* extract were stable under various treatment conditions.
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CHAPTER 7. Mechanisms of Anti-Bacterial Actions of Ascophyllum Nodosum Extract Against the Food Spoilage Bacteria Pseudomonas Fluorescens and Shewanella Putrefaciens

Abstract

Mechanisms of antibacterial actions of edible brown algae Ascophyllum nodosum extract against the food spoilage bacteria Pseudomonas fluorescens and Shewanella putrefaciens were investigated. Treating the two bacteria with algae extract could inhibit extracellular protease activity, with smaller clear zones observed on skim milk agar. The lipase activity of Shewanella putrefaciens measured on nutrient agar was more sensitive to the extract than Pseudomonas fluorescens. Measurements of exopolysaccharide (EPS) content in the culture media by phenolic-sulfuric acid method indicated that EPS was reduced by 44.82% in Pseudomonas fluorescens and 64.67% in Shewanella putrefaciens treated with algae extract at their minimum inhibitory concentration (MIC). The number of live bacteria cells in the biofilms formed by both Pseudomonas and Shewanella exposed to 1 and 2 times their MIC were reduced. Leakage of nucleic material, proteins, and small molecules from bacterial cells was evaluated. Significant increases of nucleic acid material was observed after 6-h incubation with algae extract in both bacterial strains. The protein content and conductance in the media were also observed to increase during co-culturing of the bacteria with algae extract.

Keywords edible brown algae, Ascophyllum nodosum, antibacterial mechanism, food spoilage, Pseudomonas fluorescens, Shewanella putrefaciens

7.1. Introduction

Food spoilage is a serious problem that negatively affects life quality and wellbeing of people worldwide. It causes loss of quality and safety of food and beverage products and leads to starvation. According to the reports of World Health Organization, unsafe food results in the illness of at least 2 billion people worldwide annually (Hintz et al., 2015). Many physical, chemical, and biological factors induce the spoilage or deterioration of food. Among all the factors, microbial deterioration is the major cause. Using preservatives, thermal processing, and appropriate packaging are the major ways to preserve foods and beverages (Zheng, 2014) and
prevent spoilage. However, as consumers’ concern about side effects of synthetic preservatives and desire for minimally processed foods increased, natural antimicrobials and antioxidants derived from plants become a possible alternative for synthetic preservatives. Terrestrial plant species, such as cinnamon, mustard, vanillin, clove, oregano, rosemary, thyme, sage, and basil have been used as herbs for a very long time and their extracts have been studied as natural food preservatives in recent years. Among all phytochemicals in the plant extracts, phenolic components are the most effective antioxidants and antimicrobial agents (Hintz et al., 2015; Martinez-Gracia et al., 2015). Possible antimicrobial mechanisms of polyphenols include inhibition of extracellular microbial enzyme activities, inhibition of microbial metabolism, deprivation of the substrates necessary for microbial development, increasing cell membrane permeability, and disruption of quorum sensing (Hierholtzer et al., 2012; Martinez-Gracia et al., 2015).

Macroalgae, also known as seaweed, is an under-exploited source of natural antioxidants and antimicrobial agent due to the harsh environments they live in. The major bioactive components identified in seaweeds include sulphated polysaccharides (laminarins and fucoidans), polyphenols such as phloroglucinols, and carotenoid pigments such as fucoxanthin (Gupta and Abu-Ghannam, 2011). Antimicrobial activity and mechanism of seaweed extract has been reported in only a few studies. Treatment of microorganisms with seaweed extract could induce modification of cell surface texture of the bacteria (Hierholtzer et al., 2012; Kim et al., 2013), decrease methane production of anaerobic microbial cultures (Hierholtzer et al., 2012), disrupt electron transport, nutrient uptake, protein and nucleic acid syntheses (Gupta and Abu-Ghannam, 2011), and induce cell membrane leakage (Hierholtzer et al., 2012). However, the antimicrobial activity and mechanism of seaweed extracts are still not well understood as with the terrestrial plants (Gupta and Abu-Ghannam, 2011).

*Pseudomonas putrefaciens* and *Shewanella putrefaciens* are both Gram negative and typical spoilers of seafood (Remenant et al., 2015). Generally, plant extracts and essentials oils are more effective against Gram positive than Gram negative bacteria (Martinez-Gracia et al., 2015). *Pseudomonas* spp. are resistant to plant antimicrobials, possibly due to their exopolysaccharide layers that offer protection and minimize penetration of antimicrobial agents (Hintz et al., 2015). Besides, *Pseudomonas* produce heat-stable extracellular enzymes and forms surface-adhesion biofilms, which aid in their survive in foods (Myszka et al., 2016; Remenant et
al., 2015). *Shewanella* spp. are typical and native microorganisms in fresh and seawater, and the biogenic amines they produce are responsible for the fishy, ammonia-like off-odors in spoiled fish (Remenant et al., 2015). Antimicrobial activity and mechanisms of plant extracts against the two bacteria have not been fully studied compared to other common foodborne pathogens, e.g. *Listeria monocytogenes* (Yuan et al., 2017; Zhao et al., 2015), *Bacillus cereus* (Ultee et al., 1999), *Staphylococcus arreus* (Cui et al., 2015; Tagousop et al., 2018; Yuan et al., 2018; Zhao et al., 2015), and *Escherichia coli* (Cui et al., 2015; Yuan et al., 2017; Zhao et al., 2015). Current research concerning antimicrobial mechanisms against these two bacteria were focused on quorum sensing disruption and biofilm formation (Myszka et al., 2016; Truchado et al., 2015; Zhang et al., 2018; Zhu et al., 2015), while the extracellular enzyme activity and membrane permeability are less studied. To the authors’ knowledge, no studies have been reported on the antimicrobial mechanism of *A. nodosum* extract against *P. putrefaciens* or *S. putrefaciens* yet.

The objective of this study was to investigate the possible antimicrobial mechanisms of edible brown algae *A. nodosum* extract against *P. fluorescens* and *S. putrefaciens*. Three major aspects of mechanisms were evaluated, including extracellular enzyme activity, biofilm and exopolysaccharide formation, and cell membrane permeability.

### 7.2. Materials and Methods

#### 7.2.1. Extraction

Whole leaves of dried *Ascophyllum nodosum* were grounded into powder and filtered using a 1-mm sieve. Then the powder was collected, dried in a vacuum oven at 50°C until the mass was constant, and extracted with 70% ethanol-water at a liquid to solid ratio of 30 mL/g. The extraction procedure was performed in a 30°C water bath and the process lasted 30 min. Then the supernatant was filtered and rotary evaporated to get the concentrated extract (approximately 4.0 mg dry mass/mL extract). Concentrated extract was used immediately for antibacterial assay.

#### 7.2.2. Extracellular enzyme activity

A droplet of 50-μL concentrated algae extract was added at the center of the bottom of bored wells in skim milk agar plates, and equal volume of deionized water was applied as a negative control. After water was evaporated, 30-μL of overnight cultured bacterial cells were
added. The plates were then incubated at 30°C for 24 h and clear zones around wells were recorded as proteolytic activity. The lipolytic activity was determined on nutrient agar plates supplemented with 1 % Tween 20 and 1 % Tween 80, with the same experimental procedure of proteolytic activity assay. Lipolytic activity was determined as precipitated zone around and under each colony in plates containing Tween. All experiments were performed in triplicate.

7.2.3. Time-kill assay

After incubation at 30°C overnight, bacterial cells cultured in 10 mL of culture media were collected by centrifugation for 15 min at 3,000 g. Precipitated cells were resuspended in 10 mL fresh LB broth containing algae extract (concentration applied as the MIC) for 6 hours. The MICs were determined in a previous study of the authors, which were 0.95 mg/mL for *S. putrefaciens* and 1.15 mg/mL for *P. fluorescens*. The colony forming units per mL broth (cfu/mL) was determined on LB agar every 2 hours. Cells resuspended in LB broth were applied as negative control. The results were expressed as log 10 cfu/mL broth (log cfu/mL).

7.2.4. Exopolysaccharide production

Measurement of exopolysaccharide (EPS) content was performed used a modified version of method described by Zhang et al (2018b). LB broth was supplemented with algae extract at a concentration of 1 and 2 times of its MIC against *P. fluorescens* and *S. putrefaciens*. The broth was inoculated with 1% of test bacterial cultures and incubated at 30°C overnight. Broth without algae extract was applied as a negative control. After incubation, culture tubes were centrifuged at 5,000 g for 30 min. Supernatant was added to three volumes of chilled ethanol and incubated overnight at 2°C to precipitate the EPS. Precipitated EPS was collected by centrifugation at 5,000 g for 30 min at 2°C, and the pellet was dissolved in 1 mL deionized water. Total carbohydrate content of dissolved EPS was quantified by the phenol–sulfuric acid method using rhamnose as the standard. Correction was made by measuring the carbohydrate content of LB broth containing the same concentration of algae extract. The result was expressed as mg rhamnose per mL deionized water (mg RHA/mL). All experiments were performed in triplicate.

7.2.5. Biofilm formation assay

LB broth with and without algae extract was inoculated with 1% of test bacterial
cultures, and 2.5-mL aliquot was added to a 6-cm petri dish and incubated at 30°C overnight. Then the plates were gently shaken to remove loosely attached cells and the broth containing free bacteria cells were discarded, and the biofilm attached to the bottom of the petri dish was rinsed with 3.5 mL PBS buffer with agitation with a pipette. The 0.1 mL rinsed biofilm suspension was spread on LB agar. All experiments were performed in triplicate.

7.2.6. Leakage of 280-nm absorbing material

The leakage of nucleotides from bacterial cells was determined by measuring the 280-nm absorbing material in cell-free supernatant. Bacterial cells cultured overnight were collected by centrifugation for 15 min at 3,000 g. Cells were washed three times and resuspended with PBS (0.1 M, pH 7.4). The cells collected were then incubated in LB broth containing algae extract (1 and 2 times of MIC) at 37°C for 6 h. Then the broth was centrifuged at 6,000 g for 5 min. After that, the supernatants were collected, diluted ten times with PBS, and the absorption at 280 nm was measured. This wavelength was determined to avoid false positive as phlorotannin and phloroglucinol have been found to have absorbance peaks at 261 and 266 nm (Hierholtzer et al., 2012). Results were expressed as OD of 280 nm absorbing materials in 2-hour time interval. All experiments were performed in triplicate.

7.2.7. Leakage of protein

Bacterial cells cultured overnight were collected by centrifugation for 15 min at 3,000 g. Cells were washed three times and resuspended with PBS. The cells collected were then incubated in LB broth containing algae extract (1 and 2 times of MIC) at 30°C for 6 h. Then the broth was centrifuged at 6,000 g for 5 min. After that, the supernatants were collected, diluted ten times with PBS, and 140 μL suspension was mixed with 140 μL Bradford reagent, shaken for 30 s, incubated for 10 min at room temperature, and OD measured at 595 nm. Correction was made by measuring the OD of LB broth containing the same concentration of algae extract. Results were expressed as protein content in diluted supernatant (mg BSA/mL) in 1-hour time interval. All experiments were performed in triplicate.

7.2.8. Conductance of the cell-free supernatant

After incubation at 30°C overnight, bacterial cells were collected by centrifugation for
15 min at 3,000 g. Cells were washed three times and resuspended with PBS, then cells collected were incubated in LB broth containing algae extract (0.5 and 1 times of MIC) at 30°C for 6 h. Then the broth was centrifuged at 6,000 g for 5 min. A 1-mL supernatant was taken and diluted ten times with PBS buffer, and the conductance of the diluted supernatant was recorded using a conductivity meter every 1 hour during 6-hour incubation. Correction was made by measuring the OD of LB broth containing the same concentration of algae extract. Results were expressed as conductance (mS) of diluted supernatant in 1-hour time interval. All experiments were performed in triplicate.

7.2.9. Statistical analysis

Statistical analysis was performed using one-way ANOVA and Tukey test using SAS (Cary, NC, USA). A p-value of 0.05 or less was considered statistically significant. The results were expressed as mean ± standard deviation.

7.3. Results

7.3.1. Extracellular enzyme activity

The extracellular enzymes, especially protease and lipase, are of vital importance to spoilage bacteria in the process of breaking down food proteins and lipids, which are then used as nitrogen and energy source. Thus, extracellular enzyme activity is an essential part of spoilage activities of many spoilage microorganisms, especially those in proteinaceous foods such as fish (Zhu et al., 2015). Adding algae extract to bacteria grown on LB agar could inhibit their extracellular proteolytic and lipolytic activity (Table 7.1). The proteolytic activity of both bacteria strains was significantly reduced. The lipolytic activity of S. putrefaciens was more sensitive to the algae extract than P. fluorescens, as significant difference was observed in the precipitated zones between extract and control group in S. putrefaciens but not in P. fluorescens. The extracellular enzymes produced by Pseudomonas spp. are robust and could survive pasteurization or other heat treatments (Remenant et al., 2015), which might explain the negative result came from the lipolytic activity in P. fluorescens treated with algae extract in this study.
Table 7.1. Inhibition effect against the extracellular proteolytic and lipolytic activity of *P. fluorescens* and *S. putrefaciens*

<table>
<thead>
<tr>
<th></th>
<th>Proteolytic activity (mm)</th>
<th>Lipolytic activity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. fluorescens</em></td>
<td><em>S. putrefaciens</em></td>
</tr>
<tr>
<td>Extract group</td>
<td>11.17±0.76b</td>
<td>12.33±0.58b</td>
</tr>
<tr>
<td>Control group</td>
<td>14.17±0.77a</td>
<td>16.00±1.00a</td>
</tr>
</tbody>
</table>

*Different letters indicate statistical difference between extract and control groups (p<0.05).

7.3.2. Time-kill assay

The algae extract showed inhibition on cell reproduction of both bacteria strains after incubating for 2 hours (Figure 7.1). Approximately 1.40 log unit was observed to decrease in *P. fluorescens* and 2.56 log unit decreased in *S. putrefaciens* treated at their MIC after 6-hour incubation. In the control group, only 0.70 and 1.53 log unit decreased, respectively, for *P. fluorescens* and *S. putrefaciens*. The results suggested that adding algae extract to the medium could efficiently inhibit growth and reproduction of concentrated bacteria culture. The bactericidal effect of ethanol extract of brown algae *Laminaria japonica* was also found to be depending on extract concentration and incubation time (Kim et al., 2013). The time required to kill the three bacteria reported, *Streptococcus mutans*, *Actinomyces odontolyticus*, and *Porphyromonas gingivalis*, were approximately 2, 2, and 1 hour, respectively. Water extract of green algae *Enteromorpha linza* killed *Bacillus cereus* after incubating at 1*MIC for 6 hours, and reduction in bacterial count occurred after 4 hours (Patra and Baek, 2016a). However, the initial concentration of bacteria applied in the research mentioned above (10^3~10^6 cfu) was much lower than the present one. *E. linza* essential oil (applied at MIC of 12.5 mg/mL) showed complete killing effect on *Listeria monocytogenes* at an initial concentration of 10^8 cfu/mL after incubation for 4 hours (Patra, J.K., Baek, K.H., 2016b), yet the concentration of that algae extract was much higher and bacteria concentration was much lower than in the present study.
7.3.3. Exopolysaccharide production

Measuring the exopolysaccharide (EPS) content is a method to evaluate biofilm formation. EPS is responsible for binding cells with other particulate materials together or binding cells to surfaces, which is essential in biofilm architecture and maturation (Zhang et al., 2018b). As biofilms mature and EPS accumulates, entry and activity of antimicrobial agents is reduced (Myszka et al., 2016). The carbohydrate content in the cell-free supernatant was significantly reduced when co-cultured with algae extract (Figure 7.2). EPS production in both *P. fluorescens* and *S. putrefaciens* reduced significantly when the extract was applied at the MIC. The EPS content in *P. fluorescens* decreased by about 44.82% and in *S. putrefaciens* by 64.67%. The best inhibitory effect observed by Zhang et al. (2018b) against EPS of *P. fluorescens* was salicylic acid, whose inhibition ratio was 37.61%.
7.3.4. Inhibition on live cells in the biofilm

The biofilm rinsed from the bottom of petri dish was spread on LB agar and the result is shown in Figure 7.3. As it can be seen, more live cells were observed in the negative controls of \textit{P. fluorescens} and \textit{S. putrefaciens}, indicating that algae extract could inhibit biofilm formation and reduce the cells attached to surfaces. \textit{Psedomonas} species have been well known of their abilities to form biofilms on food and food-processing equipment (Remenant et al., 2015). Phytochemicals might be able to prevent cell adhesion by switching off the generation of quorum sensing autoinductors and down regulating the flagella gene expression in \textit{P. fluorescens} (Myszka et al., 2016). Treating \textit{P. fluorescens} with 0.1 mg/mL hexanal was reported to prevent the bacteria from forming mature three-dimensional structure biofilm, yet a thin monolayer biofilm was still observed under scanning electron microscopy (Zhang et al., 2018b).
7.3.5. Leakage of 280-nm absorbing material

Leakage of 280 nm material reached the maximum at 6 hour and 2 hour in *P. fluorescens* and *S. putrefaciens* treated with 1* and 2* MIC, respectively, as shown in Figure 7.4. Nucleic acid leakage due to natural death of bacteria cells was considered not significant in *P. fluorescens* since no significant increase was observed during the 6-hour incubation. The nucleic acid released from *S. putrefaciens* due to natural decease was found to be greater than *P. fluorescens* under no stress pressed by algae extract. This agreed with our observation that the colony forming unit of both bacteria cells reduced as incubation time increased, and *S. putrefaciens* was more sensitive to incubation time (Figure 7.1). Compared to the negative control, greater increase of 280-nm absorbing material in the algae extract treated group was observed, indicating promoted leakage of nucleic acids. The higher absorption of the supernatant containing algae extract should not be due to cell coagulation since none has been observed in the present study. Plant extract has been known to increase cytoplasmic membrane permeability and break down bacteria cell wall structure, thus leading to cytolysis, leakage of cellular material, and finally cause death of bacteria cells (Stojkovic et al., 2013). Similar observation
was reported in *Bacillus cereus* in response to *Enteromorpha linza* L. essential oil that the longer incubation time, the more release of nucleic acids (Patra and Baek, 2016a).

![Figure 7.4. Leakage of nucleotide at 280 nm (graph A: *P. fluorescens*; graph B: *S. putrefaciens*). Different bars indicate different treatment groups (P0: control group; P1: 1*MIC of algae extract; P2: 2*MIC of algae extract; S0: control group; S1: 1*MIC of algae extract; S2: 2*MIC of algae extract). Different letters indicate statistical difference in the OD at different time in each treatment group (p<0.05).](image)

### 7.3.6. Leakage of protein

Protein content of the cell-free supernatant indicates the amount of proteins that were released from bacteria cells. Coculturing *P. fluorescens* and *S. putrefaciens* with 2 times of their MIC could significantly increase the protein content. *S. putrefaciens* was more sensitive to the algae extract as 1 times of MIC caused increase in protein in the media but not in *P. fluorescens* (Figure 7.5). When the algae extract concentration reached 2*MIC, significant increase in protein content was recorded in both bacteria strains.
Figure 7.5. Leakage of protein into the medium by Bradford method. Graph A is *P. fluorescens* (P0: control group; P1: 1*MIC of algae extract; P2: 2*MIC of algae extract), and graph B is *S. putrefaciens* (S0: control group; S1: 1*MIC of algae extract; S2: 2*MIC of algae extract)

### 7.3.7. Conductance of the cell-free supernatant

Conductivity of the supernatant was mainly contributed by ions and small molecules released to the media by bacteria. Treating both *P. fluorescens* and *S. putrefaciens* with algae extract at relatively lower concentration (0.5 and 1*MIC) could cause significantly increase in conductivity (Figure 7.6). Leakage occurred almost at the beginning of incubation as there was an increase at the first hour. The result suggests that treating *P. fluorescens* and *S. putrefaciens* with algae extract might modify cell membrane permeability and cause leakage of ions and small molecules. Extract of green algae *E. linza*, after co-incubating with *B. cereus* (Patra and Baek, 2016a) and *L. monocytogenes* (Patra and Baek, 2016b) for 6 hour at a MIC of 12.5 mg/mL, caused great increase in relative conductivity in both bacteria strains.
Figure 7.6. Conductance of the cell free supernatant of *P. fluorescens* (graph A, P0: control group; P1/2: 0.5*MIC of algae extract; P1: 1*MIC of algae extract) and *S. putrefaciens* (graph B, S0: control group; S1/2: 0.5*MIC of algae extract; S1: 1*MIC of algae extract applied)

### 7.4. Discussion

Mechanisms of anti-bacteria actions of seaweed extract and essential oil have not been fully investigated compared to those obtained from terrestrial plants. Phlorotannins and polysaccharides, being the major bioactive compounds in seaweed extract, might contribute to the antibacterial activity in the following modes: inhibition of extracellular microbial enzymes, inhibition of microbial metabolism, deprivation of the substrates necessary for microbial development, affect cell membrane permeability including leakage of intracellular constituents, and triggers survival mechanisms such as endosporulation (Hierholtzer et al., 2012).

Phlorotannins in seaweed extracts are readily oxidized upon exposure to air and contain more hydroxyl groups than condensed tannins and hydrolysable tannins. Oxidation of tannins and liberation of hydrogen peroxide has been identified as the factor responsible for the antimicrobial properties of these phenolics (Wang et al., 2009). Toxicity of phloroglucinol, which is the monomer of phlorotannins and found in most seaweed extract, decreased through its degradation by tolerant microorganisms in mixed anaerobic bacteria (Hierholtzer et al., 2012). Degradation of phloroglucinol was not reported in pure bacteria culture, nor were seaweed extracts or essential oils, whose components were complex thus difficult to develop a resistance of. It has been proven that phlorotannins from *L. digitata* included a stronger inhibition on microorganisms than phloroglucinol, whose effect was overcome after a few days. It also suggested that bactericidal activity of phlorotannins could also be function of the level of polymerization of the compounds.
Extracellular microbial enzymes are essential for food spoilage microorganisms because they need those enzymes to digest the proteins and lipids to provide the energy and nutrients essential for survival. Production of extracellular protease by *Pseudomonas* accelerates the degradation of the food matrix. Moreover, the production of extracellular protease by *P. fluorescens* is also associated with the high cell density that is typically encountered towards the end of the exponential phase of growth. This cell density-dependent protease synthesis could suggest the involvement of a quorum sensing mechanism (Liu et al., 2007). Algal phlorotannins were reported to have strong interaction with proteins (Stern et al., 1996) due to their rich hydroxyl groups (Wang et al., 2009), which could explain the inhibition of phlorotannins on microbial enzymes and proteins (Nagayama et al., 2002). Besides, the binding of phlorotannin-protein and phlorotannin-carbohydrate (Wang et al., 2009) in food matrix could also slow down enzymatic digestion and contribute to the bactericidal action of phlorotannins. The oxidized compounds of phenolics also serve as inhibitors of microbial enzymes (Hintz et al., 2015). It has been reported that protease production was regulated by the expression of aprX gene in *P. fluorescens* isolated from milk (Liu et al., 2007), which is regulated by quorum sensing. Therefore, inhibiting quorum sensing could also reduce extracellular enzyme secretion and activity (Zhang et al., 2018a).

The cytoplasmic membrane of bacteria cells serves as a selective permeability barrier and bed for many metabolically active enzymes. The control of permeability of cell membrane is the key regulatory factor for various cellular functions such as cell metabolism maintenance, solute transport, and energy transduction processed (Patra and Baek, 2016). Once the cell membrane was damaged by the external pressure, leakage of intracellular constituents, e.g. potassium, 260- or 280-nm absorbing materials (mainly nucleic acids) and phosphates would occur and serve as a key step in bactericidal action (Hierholtzer et al., 2012). In the present study, leakage of proteins and small molecules (notified as increase in conductance) were observed almost immediately after cells came in contact with algae extract, while significant cell death was seen after 2 hours of co-incubation with algae extract. It may suggest that the algae extract first caused the cells to lose their membrane integrity and leakage of cellular compounds occurred as a result of the damaged membrane integrity. Losing cellular components finally killed the bacteria co-cultured with algae extract. Cell surface texture of oral microorganisms was found to be modified by brown algae *Laminaria japonica* extract. The surface of the bacteria
was rougher and small bumps were observed by scanning electron microscopy (Kim et al., 2013). Further observations were made on the cell surface of mixed anaerobic microbial cultures exposed to extract of brown algae *L. digitata*. As the transmission electron micrographs revealed the disturbed membrane and cell wall structure, coagulation of exopolysaccharides, and separation of the cytoplasmic membrane from the cell envelope (Hierholtzer et al., 2012). Gram negative bacteria display an intrinsic resistance to a wide variety of hydrophobic essential oil components due to its hydrophilic surface of their out membrane, rich in lipopolysaccharide molecules. However, the hydrophilic components, e.g. small phlorotannin molecules, in the ethanol-water algae extract obtained in the present study might be able to penetrate the barrier through abundant porin proteins on the bacteria cell membrane (Kalemba and Kunicka, 2003).

Treating bacteria with plant extract or essential may also alter the lipid and protein profile on cell membrane. Cinnamon oil was reported to increase the saturated/unsaturated fatty acids (SFA/UFA) ratio and affect the secondary and tertiary structure of membrane proteins in *S. putrefaciens*. The *Shewanella* spp. naturally reside the deep sea environment with low temperature, the mechanism for their survival lies in that the high UFA percentage in the bacterial membrane, which confers the better membrane fluidity and enhances its capacity of cold adaptation (Lyu et al., 2017).

Exposing bacteria cells to seaweed extract or essential oil could induce cell death and the bactericidal effect was found to be dependent on extract concentration and incubation time. As the concentration of *L. japonica* extract applied reached 125 µg/mL, the living cells decreased by approximately 50% in the oral microorganisms (Kim et al., 2013). One possibility for the decrease in cell abundance is the permeation of algae components and cell lysis induced (Patra and Baek, 2016). The extract may not only cause a bacteriostatic but also a bacteriolytic mode of action (Dubber and Harder, 2008). A concentration of 50 µg/mL of *A. nodosum* extract could kill four *Escherichia coli* strains and a bacteriostatic action was observed at a concentration of >=25 µg/mL, during 24 incubation. Yet the initial cell concentration applied in other research was much lower than the present study. In this study, the mature cell inoculum at late log phase (*S. putrefaciens*) and early stationary phase (*P. fluorescens*) was tested for their sensitivity against algae extract. Although natural death occurred in both bacteria strains, with presence of algae extract in the medium, decrease of *P. fluorescens* and *S. putrefaciens* were 0.70 and 1.03 log cfu/mL more than negative control. Cell death could be a direct cause of leakage of protein,
small molecules, and nucleic acids into the medium.

Biofilm formation on abiotic surfaces is one of the most serious concerns of food manufactures worldwide. Bacteria from the Pseudomonas genera are known to be good biofilm producers, whose biofilm are difficult to be removed from abiotic surfaces (Myszka et al., 2016). Final biofilm inhibitory activity depended on a variety of factors, such as inhibition of bacterial motility, EPS production, and related gene expression (Zhang et al., 2018b). The flagella in P. fluorescens is likely to be the part of bacteria that first contacts the surface where the biofilm settles. Production of flagella in Pseudomonas spp. is controlled by quorum sensing (QS) autoinducors (acyl-homoserine lactones; AHLs). AHLs bind to a cognate quorum sensing receptor (LuxR family) to from a ligand-receptor complex that activates the expression of the gene required for flagella production (Myszka et al., 2016). Reduced P. fluorescens KM121 adhesion on stainless steel surface was observed by Myszka et al (2016) using Thymus vulgare essential oil, carvacrol and thymol as anti-biofilm agents. EPS constitutes the most extensive part of biofilm, and as its production and content in the biofilm is regulated under QS, inhibition of QS results in reduced EPS synthesis and biofilm adhesion (Zhang et al., 2018b). Extracts of Cuminum cyminum were reported to present inhibition on EPS synthesis and swimming/swarming activity of Pseudomonas aeruginosa PAO1 (Packiavathy et al., 2012). The authors also concluded that C. cyminum extract loosened the architecture of the matured biofilm by inhibiting EPS production and cell motility. The damaged cell wall and membrane might also make cells lose their ability to attach to the surface and form biofilms (Kerekes et al., 2013).

7.5. Conclusions

Mechanisms of antibacterial actions of brown algae A. nodosum extract against food spoilage microorganism P. fluorescens and S. putrefaciens were studied. Extracellular proteolytic and lipolytic activity of S. putrefaciens were reduced while only the proteolytic activity in P. fluorescens was inhibited by algae extract. Adhesion of cells and formation of biofilm were inhibited and the content of exopolysaccharides in cell free supernatant was decreased in both bacteria strains treated with algae extract. Leakage of cellular material was observed by increases in conductance, protein content, and 280-nm absorbing material in cell free supernatant collected, which suggested enhanced cell membrane permeability induced by algae extract.
Significant bactericidal effects were observed in concentrated bacteria culture ($\geq 10^{11}$ cfu/mL) in a 6-hour incubation period.
REFERENCES


CHAPTER 8. Conclusions and Future Work

8.1. Conclusions

The extraction process of natural antioxidants from edible brown algae *Ascophyllum nodosum* was investigated, and the stability and antibacterial effects of the extracts were evaluated. The following conclusions are summarized:

During extraction, all three factors, extraction temperature, liquid-to-solid ratio, and ethanol concentration showed influences on TPC, TCC, and antioxidant activity of FE and DE. The TPC of FE was enhanced at lower temperature, higher liquid-to-solid ratio, and higher ethanol concentration, while TCC of FE tended to increase with increasing temperature and decreasing liquid-to-solid ratio, and moderate ethanol concentration (40%) resulted in the highest TCC value. The strongest DPPH scavenging activity of FE was observed at relatively low temperature (30 or 40℃), low liquid-to-solid ratio (30 mL/g), and high ethanol concentration (80 or 100%). Most of the FE showed higher TPC, TCC, and DPPH radical scavenging activity than their DE, suggesting that thermal treatment negatively affected the bioactivities of the extracts in most cases. However, the FEs obtained at higher extraction temperature, 60 or 70℃, didn’t possess stronger antioxidant activity than their DEs, which might be explained by the unbounding of phenolic compounds caused by thermal process.

The extraction process of brown algae *A. nodosum* was optimized using three-level, three-factor Box-Behnken design and response surface methodology to obtain extracts with strong antioxidant activity or high yield. Two quadratic models were developed and validated to optimize the extraction process. The combination of extraction temperature (20℃), solvent-to-solid ratio (70 mL/g), and ethanol concentration (80%) was determined as the optimal condition to obtain extract with the strongest antioxidant activity (1/IC50=74.01 mL/mg). However, a different combination of extraction temperature (60℃), solvent-to-solid ratio (40.01 mL/g), and ethanol concentration (44.83%) was determined to obtain the highest extract yield (55.60 mg extract/g algae). Under the two optimal extraction conditions, the experimental antioxidant activity and yield were 74.05±0.51 mL/g and 53.80±1.65 mg extract/g algae, respectively, demonstrating that the two models could accurately predict and improve the extraction of antioxidants from *A. nodosum*.

The extract obtained from edible brown algae *A. nodosum* remained stable under thermal treatment and storage conditions. Increment of RSC was observed in all treated extracts,
indicating degradation of polysaccharides during treatment. The TPC and antioxidant activity of concentrated extracts decreased by 1~5% and 0.5~10% after thermal treatment (40, 50, 60, 70, 80, and 90℃), respectively, while the antioxidant activity of supernatant did not change at medium heating temperature, 50 and 60℃. After long-period storage at 0 and 25℃ with/without exposure to oxygen, the antioxidant activity of algae extract remained stable. Reduction of phenolic components and antioxidant activity was the lowest in the extract stored in sealed tubes at 0℃. Stability tests in the present study indicated that A. nodosum extract was stable at various treatment conditions and could be used as a stable antioxidative agent.

The water-soluble extracts of edible brown algae A. nodosum exhibited significant antibacterial effects against P. fluorescens and S. putrefaciens. S. putrefaciens was observed to be more sensitive to P. fluorescens. Prominent zones of inhibitions were observed in the disc diffusion assay and algae extract (42 µg/disc) showed larger zones than penicillin (10 µg/disc). No inhibition zones were observed in the vapor phase antibacterial assay, suggesting that the antibacterial effects of the volatile components in the extracts were not strong. Treating tilapia fillets with algae extract could significantly reduce microorganisms and postpone unpleasant smell and slime from occurring. All results indicated that A. nodosum extract could be used as potential antibacterial agent against P. fluorescens and S. putrefaciens, and natural food preservative to extend shelf life of tilapia fillets.

Mechanism of antibacterial actions of brown algae A. nodosum extract was studied against food spoilage microorganism P. fluorescens and S. putrefaciens. Three major aspects that might contribute to the antibacterial activity, including extracellular enzyme activity, biofilm formation, and permeability of cell membrane, were evaluated. Extracellular proteolytic and lipolytic activity of S. putrefaciens were reduced while only proteolytic activity in P. fluorescens was affected by algae extract. Adhesion of cells and formation of biofilm was inhibited and the content of exopolysaccharides in cell free supernatant was decreased in both bacteria strains treated with algae extract. Leakage of cellular material was observed in increase of conductance, protein content, and 280-nm absorbing material in cell free supernatant, which suggested increased cell membrane permeability induced by algae extract. Significant bactericidal effects and inhibition on cell reproduction were observed in concentrated bacteria culture (>=10^{11} cfu/mL) in an 6-hour incubation period.
8.2. Contributions

In this research, the extraction process to obtain a natural antioxidant and antibacterial agent from edible brown algae *A. nodosum*, a novel source of food and nutraceutical agent, has been optimized. The extract obtained has shown strong stability and antibacterial effects against food borne spoilage bacteria during storage of tilapia fillets. To the author’s knowledge, no research has been conducted to investigate the extraction process optimization and stability of the extract of *A. nodosum* yet, nor to evaluate its anti-bacterial effects or the mechanisms behind the anti-bacterial actions. The findings of this research can be used to further understand the food preservation effect of phytochemicals, especially to evaluate the potential of using natural antioxidants as food preservation agents to extend shelf life of seafood, which has high nutrition value but is very perishable.

8.3. Future Work

Characterization and identification of bioactive components in the extract needs to be considered. The most active components in the extract could be identified and selected as major antioxidants/antibacterial agents in the preservation agent. Besides, considering that the extract could be applied as nutraceutical or food preservation agent, identification of major components in the extract would be a necessary step to introduce algae extract to market.

The preservation effect of algae extract needs to be further studied on other foods under different storage or processing conditions. Stability and bioactivities of natural extracts would likely be reduced or even damaged during processing, e.g. thermal process and irradiation. This will help to understand the real food preservation effects of algae extracts in the processed foods.

Besides the antioxidant/antibacterial effects studied in the present project, the extract of *A. nodosum* could have other potential pharmaceutical activities, e.g. anti-HIV, antidiabetes, and anti-cancer. Investigating the pharmaceutical activities would be an essential step for the application of the extract as a potential nutraceutical and pharmaceutical agent.