Antibacterial Actions of Algal Extract against Food Spoilage Bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens*

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Modes of antibacterial action of the extract from edible brown algae *Ascophyllum nodosum* against food spoilage bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens* were investigated. The colony forming units in *P. fluorescens* and *S. putrefaciens* were 0.70 and 1.03 log cfu/mL less than the negative control in the time-kill assay, indicating the inhibitory effect of the extract on the two bacteria at high cell density. The extract also inhibited the extracellular protease activity of the two bacteria, where the exopolysaccharide (EPS) content was reduced by 44.8% in *P. fluorescens* and 64.7% in *S. putrefaciens* after treating them with the extract of the minimum inhibition concentration (MIC). Reduction in live bacteria cells in the biofilm formed by the two bacteria strains exposed to 1×MIC and 2×MIC levels was also observed. Significant increase in 260-nm absorbing material, protein content, and electrical conductivity of the culture media was observed after 6-h incubation with the extract in both bacteria strains, suggesting a leakage of nucleic material, protein, and other intracellular constitutes. It was concluded that the extract of *A. nodosum* could exert antibacterial activity against *P. fluorescens* and *S. putrefaciens* by reducing their extracellular enzymatic activity, inhibiting EPS production and biofilm formation, and increasing cell permeability.

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INTRODUCTION

Food spoilage is a serious problem that adversely affects the quality of life and wellbeing of people worldwide. It results in the loss of quality of food and beverage products and contributes to starvation. According to the World Health Organization, spoiled food causes illness of at least 2 billion people annually worldwide (Hintz *et al*. 2015). Many physical, chemical, and biological factors induce the spoilage or deterioration of foods. Among all the factors, microbial deterioration is the major cause. More than 250 foodborne diseases have been confirmed worldwide and bacteria were found to be the main

cause of these diseases (Liu *et al*. 2020). Preservatives, thermal processing, and appropriate packaging are the major methods to preserve foods and beverages and prevent spoilage (Zheng 2014). However, as consumers' concern about the side effects of synthetic preservatives and desire for minimally processed foods increase, natural antimicrobials and antioxidants derived from plants become an attractive alternative to synthetic preservatives (Liu *et al*. 2020). Terrestrial plants such as cinnamon, mustard, vanillin, clove, oregano, rosemary, thyme, sage, and basil have been used as herbs and spices for a very long time and their extracts have been studied as natural food preservatives in recent years. Among the phytochemicals naturally found in plants, phenolic compounds are the major source of antioxidants and antimicrobial agents (Hintz *et al*. 2015; Martinez-Gracia *et al*. 2015). These natural chemicals could reduce microbial growth through inhibition of extracellular enzymatic 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, are an under-exploited source of natural antimicrobial agent due to the harsh environments they live in (Fayzi *et al*. 2020). The major bioactive components identified in seaweeds include phenolic compounds such as phlorotannins, sulphated polysaccharides, fatty acids, proteins, and peptides (Gupta and Abu-Ghannam 2011; Cabral *et al*. 2021). The antimicrobial activity and mechanism of seaweed extract are still not well understood compared to terrestrial plants (Gupta and Abu-Ghannam 2011). As the major bioactive compounds in seaweed, phlorotannins and polysaccharides might contribute to the antibacterial activity in the following modes: inhibition of extracellular microbial enzymes, disruption of microbial metabolism and biofilm formation, deprivation of the substrates necessary for microbial development, affecting cell membrane permeability and causing leakage of cellular components (Hierholtzer *et al*. 2012). 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 their nitrogen and energy source (Zhu *et al*. 2015). Biofilm is a self-produced matrix of exopolysaccharide (EPS) that could provide protection to the microorganisms against antimicrobials (Yan and Xie 2021). EPS is responsible for binding cells with other particulate materials together or binding cells to surfaces, which is essential in biofilm architecture and maturation. Measuring the EPS content in the broth is a method to evaluate biofilm formation (Zhang *et al*. 2018). 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 a key regulatory factor for various cellular functions such as cell metabolism maintenance, solute transport, and energy transduction process (Patra and Baek 2016a). The phenolic compounds of macroalgae could alter cell membrane permeability and once the cell membrane is damaged, leakage of intracellular constituents, *e.g.*, potassium, 260-nm absorbing materials (mainly nucleic acids), proteins, and phosphates would occur and serve as a key step in bactericidal action (Hierholtzer *et al*. 2012; Cabral *et al.* 2021).

This study focused on two typical spoilers of seafood, *P. fluorescens* and *S. putrefaciens* that are also rare opportunistic human pathogens (Kumar *et al*. 2019; Müller *et al*. 2023; Remenant *et al*. 2015). Both bacteria could produce extracellular protease and lipase, and form biofilms on the surface of food and food processing equipment, which make them common food spoilers that require special attention (Kumar *et al*. 2019; Wang *et al*. 2020). *Pseudomonas* are resistant to plant antimicrobials due to their exopolysaccharide layers that offer protection and delay penetration of antimicrobial

agents (Hintz *et al*. 2015). However, *Pseudomonas* produce heat-stable extracellular enzymes and form surface-adhesion biofilms, which aid in their survival in food systems (Kumar *et al*. 2019; Myszka *et al*. 2016; Remenant *et al*. 2015). *Shewanella* spp. are the typical and native microorganisms in fresh water and seawater, and the biogenic amine they produce are responsible for the fishy, ammonia-like off odors in fish (Remenant *et al*. 2015). Various antimicrobials have been investigated for their inhibitory mechanisms on *Pseudomonas* and *Shewanella*, including the extract from *Origanum vulgare*, *Avicennia marina*, *Sedum aizoon*, and *Ginkgo biloba*, and antimicrobial compounds such as linalool, alkyl gallates, dimethyl phthalate, and cinnamaldehyde (Chen *et al.* 2022; Guo *et al.* 2021a; Ibrahim *et al.* 2022; Lan *et al.* 2019; Li *et al.* 2018; Liu *et al.* 2020a; Qian *et al.* 2021; Rossi *et al.* 2018; Wang *et al.* 2020; Zhang *et al.* 2021). However, the antimicrobial mechanisms of macroalgal extracts on the two bacteria have not been fully investigated.

This study explored the antimicrobial mechanisms of the extract from edible brown algae *Ascophyllum nodosum* against *P. fluorescens* and *S. putrefaciens*. To the authors' knowledge and based on recent literature reviews (Silva *et al*. 2020; Cabral *et al*. 2021), the antimicrobial mechanism of *A. nodosum* extract against *P. fluorescens* or *S. putrefaciens* has not been reported. Three major modes of action including extracellular enzyme activity, biofilm and EPS formation, and cell membrane permeability were investigated.

EXPERIMENTAL

Bacteria Strains

P. fluorescens (ATCC 13525) and *S. putrefaciens* (ATCC 8071) were purchased from American Type Culture Collection (Manassas, VA, USA). The antibacterial effect of *A. nodosum* extract has been demonstrated in the authors' previous research (Liu *et al*. 2023). Bacteria strains were stored in 80% LB broth/20% glycerol under -80 °C. Working cultures were prepared by sub-culturing 0.1 mL stock culture in 100 mL LB broth and incubating at 30 °C for 24 h.

Extraction

The extraction procedure was performed and investigated in the authors' previous research to obtain a phlorotannin-enriched extract (Liu *et al*. 2019, 2021). Briefly, whole leaves of dried *A. nodosum* (Fig. 1) were ground into powder and filtered using a 1-mm sieve.

Fig. 1. The whole leaves of *Ascophyllum nodosum*

The powder was collected, dried in a vacuum oven at 50 °C, and extracted with 70% ethanol at a liquid-solid ratio of 30 mL solvent/g dry algae. The extraction was performed in a 30 °C water bath for 30 min. Then the supernatant was rotary evaporated and filtered using a 0.2-µm sterile syringe filter to obtain the concentrated extract (4.0 mg dry mass/mL, pH 6.85), which was used immediately in the antibacterial assay.

Extracellular Enzyme Activity

The proteolytic activity assay was performed using the method of Zhu *et al*. (2015) with minor modifications. Briefly, 50-µL concentrated seaweed extract was added at the center of the bottom of bored wells in skim milk agar plates (an equal volume of sterilized deionized water was used 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 the diameter of clear zones around wells were recorded as proteolytic activity. All experiments were performed in triplicate.

The lipolytic activity was determined on nutrient agar plates supplemented with 1% polysorbate 20 (Tween 20) and 1% polysorbate 80 (Tween 80). A droplet of 50 µL extract was added at the center of the bottom of bored wells in nutrient agar plates (an equal volume of sterilized deionized water was used as a negative control). After water was evaporated, 30 µL of overnight cultured bacterial cells were added. The plates were incubated at 30 °C for 24 h. The diameter of the precipitated zone was measured as the lipolytic activity. All experiments were performed in triplicate.

Time-kill Assay

After incubation at 30 °C overnight, bacterial cells cultured in 10-mL culture media were collected by centrifugation for 15 min at 3,000 × g. Precipitated cells were resuspended in 10 mL fresh LB broth containing the seaweed extract (concentration = 1 × MIC) for 6 h. The MICs were 0.95 mg/mL for *S. putrefaciens* and 1.15 mg/mL for *P. fluorescens*, which were confirmed in the authors' previous research (Liu *et al*. 2023). The colony forming units per mL broth (cfu/mL) was determined on LB agar every 2 h. Cells resuspended in LB broth without the extract were applied as the negative control. The results were expressed as log cfu/mL broth.

Exopolysaccharide (EPS) Production and Biofilm Formation

LB broth was supplemented with the seaweed extract at a concentration of 1×MIC and 2×MIC for *P. fluorescens* and *S. putrefaciens*, respectively. The broth was inoculated with 1% of test bacterial cultures and incubated at 30 °C overnight. Broth without the extract was applied as a negative control. After incubation, culture tubes were centrifuged at 5,000 g for 30 min. The supernatant was added to three volumes of chilled ethanol and incubated overnight at 2 °C to precipitate the dislodged 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 the 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 seaweed extract. The EPS content was expressed as mg rhamnose per mL deionized water (mg RHA/mL). All experiments were performed in triplicate.

LB broth with and without adding the 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. The plates were shaken to remove loosely attached cells, and the broth containing free bacteria cells were discarded. The biofilm attached to the bottom of the petri dish was rinsed with 3.5 mL PBS buffer, and 0.1 mL of the rinsed biofilm suspension was spread on LB agar. All experiments were performed in triplicate.

Leakage of 260-nm Absorbing Material

Bacterial cells cultured overnight were collected by centrifugation for 15 min at 3,000 × g. Cells were washed three times and resuspended with PBS buffer (0.1 M, pH 7.4). The cells collected were incubated in LB broth containing the seaweed extract (1 × MIC and 2 × 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 260 nm was measured every two h. All experiments were performed in triplicate.

Leakage of Proteins

Bacterial cells cultured overnight were collected by centrifugation at 3,000 × g for 15 min. Cells were washed three times and resuspended with PBS buffer. The cells collected were then incubated in LB broth containing the seaweed extract (1 × MIC and 2 × 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 the OD was measured at 595 nm. Correction was made by measuring the OD of LB broth containing the seaweed extract. Results were expressed as protein content in diluted supernatant (mg BSA/mL) in 1-h time interval. All experiments were performed in triplicate.

Conductance of the Cell-free Supernatant

After incubation at 30 °C overnight, bacterial cells were collected by centrifugation at 3,000 × g for 15 min. Cells were washed three times and resuspended with PBS, then cells collected were incubated in LB broth containing the seaweed extract (0.5×MIC and 1 × 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 measured using a conductivity meter during 6-h incubation. Correction was made by measuring the conductivity of LB broth containing the seaweed extract. Results were expressed as conductance (mS) of the diluted supernatant. All experiments were performed in triplicate.

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.

RESULTS AND DISCUSSION

Extracellular Enzyme Activity

Adding the seaweed extract to bacteria grown on LB agar could inhibit their extracellular proteolytic and lipolytic activities (Table 1). The proteolytic activity of both bacteria strains was significantly hindered. The lipolytic activity of *S. putrefaciens* was

more sensitive to the extract than *P. fluorescens*, since no significant difference was observed in the precipitated zones between the treatment and control groups in *P. fluorescens*. The extracellular enzymes produced by *Pseudomonas* spp. were robust and stable even during thermal treatment (Remenant *et al*. 2015), which might explain the negative result in the lipolytic activity of *P. fluorescens* treated with the seaweed extract. Algal phlorotannins were reported to have strong interaction with proteins due to their rich hydroxyl groups (Stern *et al*. 1996; Wang *et al*. 2009), which contribute to the inhibition effects of phlorotannins on microbial enzymes and proteins (Nagayama *et al*. 2002). 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. Even the oxidized compounds of phenolics could serve as inhibitors on microbial enzymes (Hintz *et al*. 2015), which also explains the strong inhibitory effects of the phlorotannin-enriched seaweed extract on the enzymatic activities.

Table 1. Inhibition Effects against the Extracellular Proteolytic and Lipolytic Activity of *P. fluorescens* and *S. putrefaciens*

	Proteolytic activity (mm)		Lipolytic activity (mm)	
	P. fluorescens	*S. putrefaciens*	*P. fluorescens*	*S. putrefaciens*
Extract treated group	*11.17±0.76$_b$	12.33±0.58$_b$	21.67±0.58$_a$	18.67±1.15$_b$
Control group	14.17±0.77$_a$	16.00±1.00$_a$	22.33±3.21$_a$	22.00±0.50$_a$
* Different letters indicate statistical difference between extract and control groups ($p<0.05$)				

Time-kill assay

The seaweed extract exerted inhibitory effects on cell reproduction of both bacteria strains after incubating for 2 h (Fig. 2).

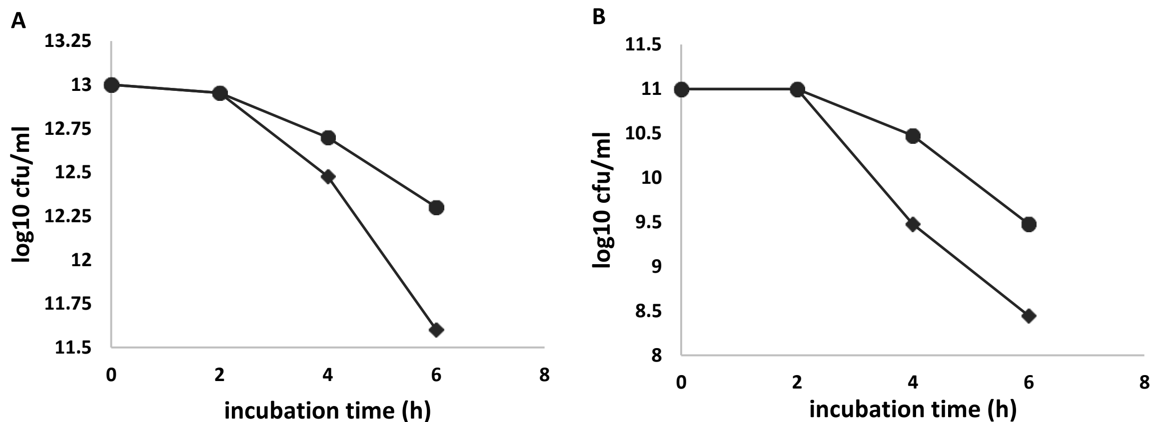

Fig. 2. Time-kill assay of *P. fluorescens* (graph A) and *S. putrefaciens* (graph B) against seaweed extract. Different symbols indicate different treatment groups (●: control group; ◆: extract treated group)

A reduction of 1.40 log unit was observed in *P. fluorescens* and 2.56 log unit in *S. putrefaciens* treated at 1×MIC after 6-h of incubation, while in the control group, only 0.70 and 1.53 log unit decreased, respectively, for *P. fluorescens* and *S. putrefaciens*. The results suggested that the extract could effectively inhibit and eliminate the bacteria at the stationary phase, in which the bacteria culture stabilized and had the maximum colonies.

The bactericidal effects of ethanol extract of brown algae *Laminaria japonica* was also found to be dependent on 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 h using 500 μg/mL extract, respectively. Water extract of green algae *Enteromorpha linza* killed *Bacillus cereus* after incubating at 1×MIC for 6 h, and reduction in bacterial count occurred after 4 h (Patra and Baek 2016a). However, the initial concentration of bacteria applied in the research mentioned above (10^3 to 10^6 cfu) was much lower than the present research. The essential oil of *E. linza* applied at MIC of 12.5 mg/mL showed complete bactericidal effect on 10^8 cfu/mL *Listeria monocytogenes* after incubation for 4 h (Patra and Baek 2016b), while the concentration of the essential oil was higher but initial bacteria concentration was lower than the present study.

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. One possible mode of the bactericidal effect is the permeation of the extract into bacterial cells and cell lysis induced (Patra and Baek 2016b). The extract of *A. nodosum* may not only cause a bacteriostatic but also a bacteriolytic mode of action. It has been reported that 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 a 24-h incubation (Wang *et al*. 2009). However, their initial cell concentration was much lower than in the present study. In the present study, the mature bacteria culture harvested at stationary phase was tested for their sensitivity against seaweed extract. Although natural death occurred in both bacteria strains, the colony forming units of the extract-treated *P. fluorescens* and *S. putrefaciens* were 0.70 and 1.03 log cfu/mL, respectively, which was less than the negative control, suggesting the inhibition effect of *A. nodosum* extract.

Exopolysaccharide (EPS) production and biofilm formation

The EPS content in the cell-free supernatant was significantly reduced after co-culturing with seaweed extract (Fig. 3). EPS production in both *P. fluorescens* and *S. putrefaciens* decreased significantly at 1×MIC, with a decrease of about 44.82% in *P. fluorescens* and 64.67% in *S. putrefaciens*.

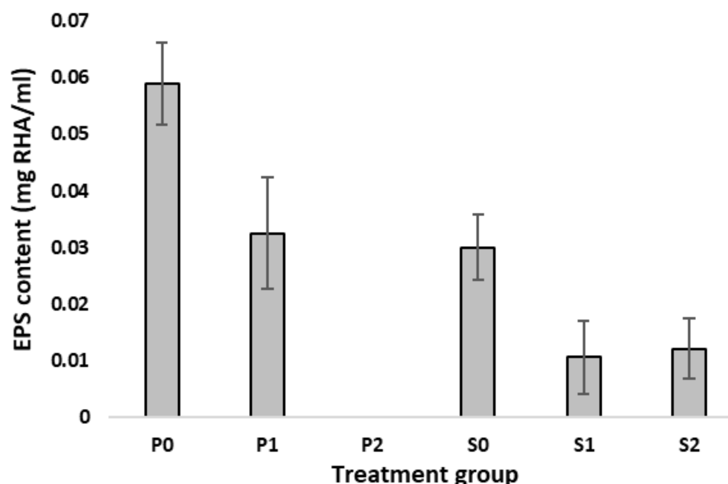

Fig. 3. EPS content in the cell free supernatant (P0: control group of *P. fluorescens*; P1: 1×MIC of algae extract; P2: 2×MIC of algae extract; S0: control group of *S. putrefaciens*; S1: 1×MIC of algae extract; S2: 2×MIC of algae extract)

The biofilm rinsed from the bottom of petri dishes was spread on LB agar and the results are shown in Fig. 4. More live cells were observed in the negative controls of *P. fluorescens* and *S. putrefaciens*, indicating that the seaweed extract reduced the number of cells in the biofilm and inhibited biofilm formation. *P. fluorescens* and *S. putrefaciens* are widely known to be good biofilm producers, whose biofilms are difficult to remove from abiotic surfaces (Myszka *et al*. 2016; Yan *et al*. 2021). Various methods and antimicrobial agents have been explored for their anti-biofilm effects against *S. putrefaciens* and *P. fluorescens* in the literature. Acidic electrolyzed water at a concentration of 3 g/L could remove biofilms formed by *S. putrefaciens* from seafood processing-related contact surfaces (Yan *et al*. 2021). However, complete removal of *P. fluorescens* biofilm is difficult. Reduced *P. fluorescens* KM121 adhesion on stainless steel surface was observed by Myszka *et al* (2016) using *Thymus vulgare* essential oil, carvacrol, or thymol as an anti-biofilm agent. Treating *P. fluorescens* with 0.1 mg/mL hexanal was reported to prevent the bacteria from forming mature three-dimensional biofilms, with a thin monolayer biofilm observed under scanning electron microscopy (Zhang *et al*. 2018). Li *et al*. (2018) observed a biofilm inhibition extent of 35.7 to 54.5% against *P. fluorescens* by cinnamaldehyde of 0.025 to 0.1 μL/mL.

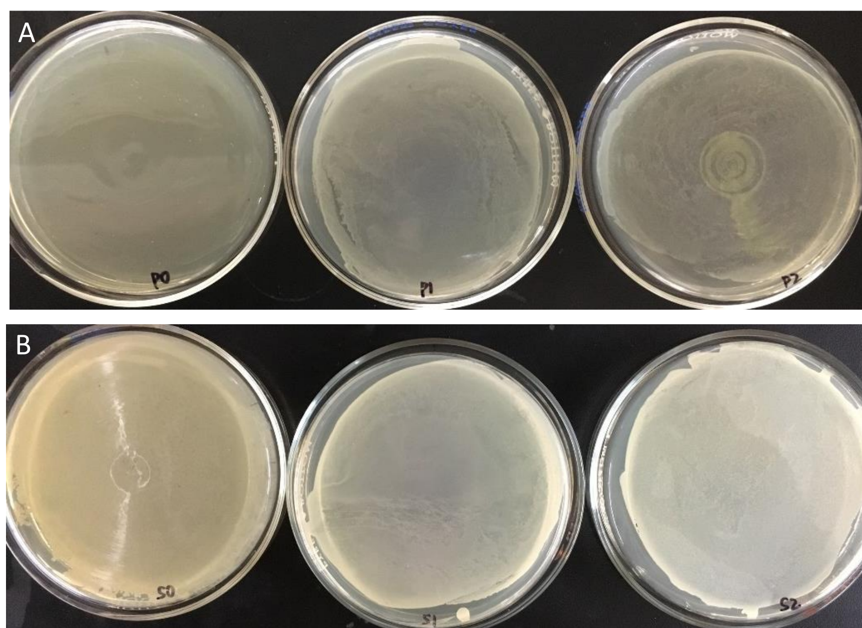

Fig. 4. Live cells in the biofilm of *P. fluorescens* (graph A from left to right: negative control, cells cultured with 1×MIC of seaweed extract, and 2×MIC of seaweed extract) and *S. putrefaciens* (graph B from left to right: negative control, cells cultured with 1×MIC of seaweed extract, and 2×MIC of seaweed extract)

Cell membrane permeability

Alteration of cell membrane permeability is an important aspect of antibacterial action of inhibitors. When the cell membrane is damaged, small ions flow out first, followed by proteins and nucleic acids (Liu *et al.* 2020a), which could be reflected in increasing conductivity, protein, and 260-nm absorbing material. Compared to the negative control, a greater increase in 260-nm absorbing material in the extract treated group was observed, indicating more leakage of nucleic acids in both bacteria strains (Fig. 5). The maximum leakage of 260-nm material was observed at 6 h and 2 h in *P. fluorescens* and *S.*

putrefaciens, respectively. Similar findings have been reported that a longer incubation time or higher dose resulted in the release of more nucleic acids from *P. fluorescens* and *S. putrefaciens* in response to linanool (Guo *et al*. 2021a and 2021b) and daphnetin (Liu *et al*. 2020a). The flavonoids of *Sedum aizoon* also exerted a promoting effect on releasing 260-nm absorbing materials from *S. putrefaciens* (Wang *et al*. 2020). The higher OD of the supernatant containing seaweed extract should not be due to cell coagulation, since none has been observed in the present study. Nucleic acid leakage due to natural death of bacteria cells was considered non-significant in *P. fluorescens* since no significant increase was observed between 0 and 6 h. The nucleic acid released from *S. putrefaciens* was found to be greater than *P. fluorescens* without the stimulation of the seaweed extract. This agreed with the time-kill assay that the colony forming unit of both bacteria reduced as the incubation time increased, and *S. putrefaciens* was more sensitive to incubation time and the extract (Fig. 2).

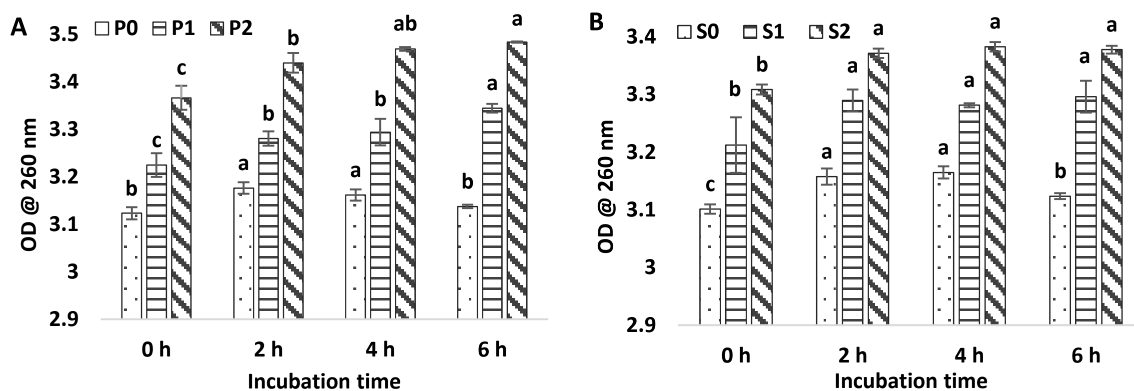

Fig. 5. Leakage of nucleotide at 260 nm (graph A: *P. fluorescens*; graph B: *S. putrefaciens*). Different bars indicate different treatment groups (P0: control group; P1: 1×MIC of seaweed extract; P2: 2×MIC of seaweed extract; S0: control group; S1: 1×MIC of seaweed extract; S2: 2×MIC of seaweed extract). Different letters indicate statistical differences in the OD at a different time in each treatment group ($p<0.05$)

The protein content of the cell free supernatant indicates the amount of proteins that leaked from bacteria cells (Guo *et al*. 2021b). Coculturing *P. fluorescens* and *S. putrefaciens* with 2×MIC extract significantly increased the protein content. *S. putrefaciens* was more sensitive to the seaweed extract, as 1×MIC extract induced increase in protein in the media but not in *P. fluorescens* (Fig. 6). Wang *et al*. (2020) also reported an immediate increase in the protein concentration of *S. putrefaciens* with the effects of flavonoids from *S. aizoon*. The extracellular protein concentration of *S. putrefaciens* was increased by 72.3% and 74.2%, respectively, following treatment with 1×MIC and 2×MIC linanool for 2 h (Guo *et al*. 2021b). No significant change in protein content was observed in *P. fluorescens* in the first h but was identified after 2 h. A similar observation was made by Guo *et al*. (2021a) that a significant increase in protein concentration occurred after treatment with linalool for 2 h. When the seaweed extract concentration reached 2 × MIC, significant increase in protein content was recorded in both bacteria strains.

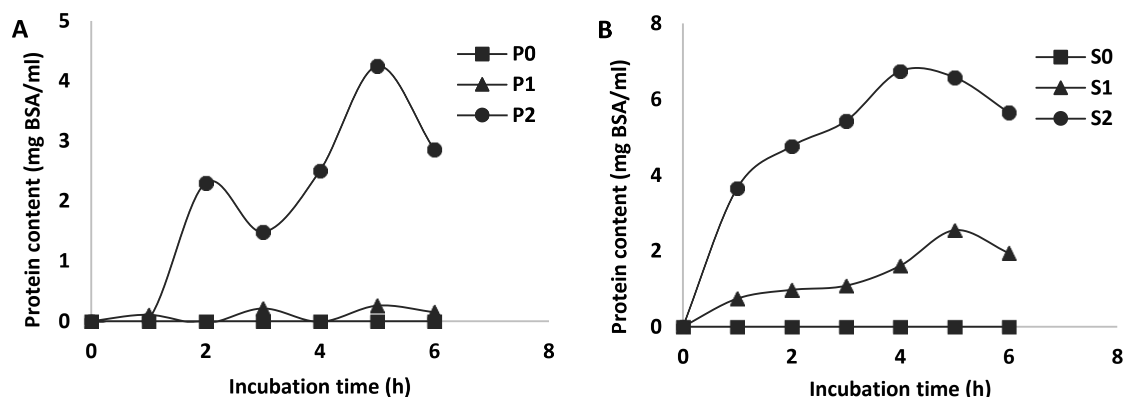

Fig. 6. Leakage of protein into the medium by Bradford method. Graph A is *P. fluorescens* (P0: control group; P1: 1×MIC of algae extract; P2: 2×MIC of algae extract), and graph B is *S. putrefaciens* (S0: control group; S1: 1×MIC of algae extract; S2: 2×MIC of algae extract)

Electrical conductivity of the supernatant was mainly contributed by ions and small molecules released to the media by bacteria. Treating *P. fluorescens* and *S. putrefaciens* with the seaweed extract at 0.5×MIC or 1×MIC significantly increased the electrical conductivity (Fig. 7), suggesting that treating *P. fluorescens* and *S. putrefaciens* with the extract could modify cell membrane permeability and cause leakage of ions and small molecules. It has been reported that the electrical conductivity of *S. putrefaciens* increased after 1 × MIC and 2 × MIC treatments of *Ginkgo biloba* leaf extract after 2-h, and the 2 × MIC extract was more effective than 1 × MIC (Zhang *et al*. 2018). Similar results were also reported by Lan *et al*. (2019) using ε-polylysine as an inhibitor.

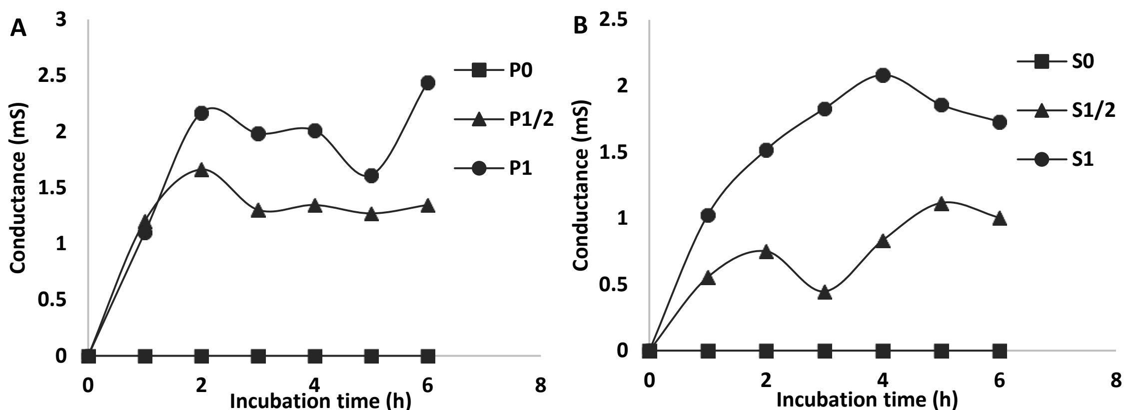

Fig. 7. Conductance of the cell free supernatant of *P. fluorescens* (graph A, P0: control group; P1/2: 0.5×MIC of algae extract; P1: 1×MIC of algae extract) and *S. putrefaciens* (graph B, S0: control group; S1/2: 0.5×MIC of algae extract; S1: 1×MIC of algae extract applied)

In the present study, leakage of proteins and small molecules was observed soon after adding the seaweed extract to the medium, while significant cell death was recorded after 2 h of co-incubation with the extract. It may suggest that the seaweed extract induced a loss of membrane integrity and leakage of cellular compounds and resulted in cell lysis. Cell surface texture of oral microorganisms was found to be modified by brown algae *L. japonica* extract, with rougher surface and small bumps observed by scanning electron microscopy (Kim *et al*. 2013). The transmission electron micrographs revealed that the

extract of brown algae *L. digitata* could disturb membrane and cell wall structure, cause coagulation of EPS, and separate 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 compounds due to the hydrophilic surface of their outer membrane. However, hydrophilic compounds such as phlorotannins in the seaweed extract might be able to penetrate the barrier through abundant porin proteins on the bacteria cell membrane (Kalemba and Kunicka 2003). This could explain alteration of membrane permeability and inhibition effects in the present study.

CONCLUSIONS

1. Modes of antibacterial actions of the extract from brown algae *A. nodosum* against food spoilage microorganism *P. fluorescens* and *S. putrefaciens* were studied in their enzyme inhibition, biofilm inhibition, and modification of cell membrane permeability.
2. Extracellular proteolytic and lipolytic activity of *S. putrefaciens* were reduced while only proteolytic activity in *P. fluorescens* was negatively affected by the extract.
3. Formation of biofilm was inhibited and the content of EPS in the cell free supernatant reduced by 44.82% in *P. fluorescens* and 64.67% in *S. putrefaciens*.
4. Leakage of cellular material was observed from the increasement of electrical conductivity, protein content, and 260-nm absorbing material in cell free supernatant collected, which suggested modified cell membrane permeability induced by the extract.
5. Significant inhibition effects were observed in concentrated bacteria culture (≥1011 cfu/mL) in a 6-h time-kill assay, with the cell density decreased by 1.40 log unit in *P. fluorescens* and 2.56 log unit in *S. putrefaciens* treated at 1×MIC, respectively.
6. The observation above indicated that the seaweed extract enhanced the cell permeability and induced leakage of intracellular components, which resulted in the antibacterial effects against *P. fluorescens* and *S. putrefaciens*. Due to the significant inhibitory influence of the extract, the authors believe in the necessity in further research and development of *A. nodosum* extract as a food preservative.

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