Antibacterial Effects of Brown Algae Extract against Tilapia Spoilage Bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens*

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Inhibitory effects were evaluated for the extract from edible brown algae *Ascophyllum nodosum vs. Pseudomonas fluorescens* and *Shewanella putrefaciens*, which are tilapia spoilage organisms. Modified Gompertz and Logistic models were used to describe the inhibition effect of the extract, and both models indicated that the extract could inhibit bacteria growth by extending lag time and reducing maximum growth rate. The Lambert-Pearson model was applied to calculate the minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) of the extract. The best-fit MIC and NIC values for *P. fluorescens* were 1.145 and 0.036 mg/mL, and 0.947 and 0.106 mg/mL for *S. putrefaciens*, respectively. Bacteriostatic assays on agar plates showed that the extract applied at concentrations higher than the MIC caused significant bacteriostatic effects, especially in *S. putrefaciens*. Algae extract (42 μg/disc) had inhibition zones against both *P. fluorescens* (1.72 cm) and *S. putrefaciens* (1.58 cm) in a disc diffusion assay. Treating tilapia fillets with the extract significantly reduced the total viable counts of both bacterial strains and postponed spoilage odor occurrence time (day 2 for the control group *vs.* day 9 for the extract treated group) during storage at 4 °C. These findings suggest that the extract could be used as a natural anti-bacterial and preservation agent to extend the shelf life of cold storage tilapia.

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INTRODUCTION

The Food and Agriculture Organization estimates that 1.3 billion tons of food is wasted every year due to spoilage, which equals to the loss of USD $936 billion (Ishangulyyev *et al*. 2019). Food deterioration causes quality loss and undesirable texture, flavor, or color change. The loss is attributed to physical, chemical, and biological factors. The biological factors include enzymatic reactions, presence or growth of insects, parasites, and microorganisms. Among them, microbial spoilage and poisoning are the most important reasons for food deterioration and foodborne illnesses (Zheng 2014). Spoiled and contaminated food results in 600 million foodborne diseases and 420,000 deaths in the world every year, and the frequency of foodborne illness is continuously growing (Lee and

Yoon 2021). Seafoods are highly perishable due to their high nutritional value, *e.g*., proteins and unsaturated fatty acids, which are deteriorated easily by spoilage microorganisms (Maqsood *et al.* 2014). Approximately 10% (10 to 12 million tons per year) of seafood and fishery products produced globally are discarded due to spoilage (Shahidi and Hossain 2020).

Tilapia (*Oreochromis sp.*) has a large global yearly production of around 2.53 million tons and is widely consumed as whole fish and fillets (Kulawik *et al.* 2015). *Pseudomonas* and *Shewanella* are the main spoilage organisms in tilapia fillets during chilled storage conditions (Ghaly *et al.* 2010; Cyprian *et al.* 2013). Both are Gram-negative and psychrotrophic spoilage bacteria detected in seafoods (Ghaly *et al.* 2010; Cao *et al.* 2012; Ding *et al.* 2019). These species are responsible for spoilage of tilapia and other iced fish regardless of the origin of the fish (Gram and Huss 1996; Zhou *et al.* 2015). Bacteria of the *Pseudomonas* genera form biofilms and secrete heat-stable proteases and lipases during low temperature storage (Myszka *et al.* 2016). The most frequently detected species is *P. fluorescens*, which contributes predominantly to the deterioration of many refrigerated aquatic foods (Ding *et al.* 2019). *S. putrefaciens* is one of the main spoilage organisms of fish and survives cold storage. It can cause severe quality loss of fish due to its capability of generating H_2S from cysteine, reducing trimethylamine oxide (TMAO) to trimethylamine (TMA), participating in degradation of protein and lipid, and producing unpleasant off-odors during storage (Zhou *et al.* 2015; Lyu *et al.* 2017).

Various synthetic preservatives, such as sorbic acid, sorbates, benzoic acid, benzoates, and sodium nitrite have been used to preserve foods and extend their shelf lives (Silva and Lidon 2016). As consumer demands for minimally processed foods and natural preservatives increase and concerns regarding the safety and potential side effects of chemical and synthetic preservatives rise (Hassoun and Coban 2017; Gokoglu 2018), a growing number of natural antioxidants and antimicrobial agents have been developed such as lactic acid, acetic acid, nisin, natamycin, as well as microbial fermentates, lysozymes, and protective cultures. The essential oils and extracts from plants such as cinnamon, mustard, black pepper, clove, vanillin, oregano, rosemary, thyme, sage, and basil also have strong antimicrobial activity (Hintz *et al.* 2015; Martinez-Gracia *et al.* 2015). These natural antimicrobials extend the shelf life of unprocessed foods by reducing microbial growth or killing the spoilage microorganisms (Tiwari *et al.* 2009). Application of natural products also hinders antibiotic resistance (Eom *et al.* 2012).

One source of natural antibacterial agents is extracts from seaweeds, also known as macroalgae. Marine macroalgae-based compounds and extracts have been investigated for their various bioactivities, *e.g*., anti-bacterial and antioxidant activity, which make them a potential antimicrobial and food preservation agent (Moubayed *et al.* 2016; Martelli *et al.* 2020). There is a long history of seaweed utilization as food and medicine in Asian and European countries. Twenty million tons of seaweeds are harvested worldwide every year, and half of the production is used as food (Martelli *et al.* 2020). Seaweeds are divided into three groups based on their pigments: brown, red, and green algae. They are generally recognized as safe (GRAS) and allowed as safe additives in food (Penalver *et al.* 2020). Most of the edible and medicinal seaweeds are brown algae due to their high contents of protein, dietary fiber, eicosapentaenoic acid (EPA), mannitol, and bioactive compounds such as phlorotannins and polysaccharides (Wifesekara *et al.* 2011; Eom *et al.* 2012; Ortiz *et al.* 2014). Brown algae extracts exhibit antimicrobial effects against food-borne pathogenic microorganisms (Dubber and Harder 2008; Gupta *et al.* 2012; Cox *et al.* 2014; Baleta *et al.* 2017; Martelli *et al.* 2020) and antibiotic-resistance bacteria (Eom *et al.* 2012;

Moubayed *et al.* 2016). Application of seaweed extract as a preservation agent could be compatible with fish because they both have a seafood flavor (Martelli *et al.* 2020). Although brown algae have been studied as an under-exploited source of functional foods, medicine, and antimicrobial agents, the food preservation effect of their extracts against spoilage organism has not been elucidated (Ortiz *et al.* 2014).

This study evaluated the potential of *Ascophyllum nodosum* extracts as an antibacterial agent against spoilage bacteria *Pseudomonas fluorescens* and *Shewanella putrefaciens*. A binary solvent extraction system containing ethanol and water was applied to obtain extracts from *A. nodosum*, which exhibited high phlorotannin yield and strong antioxidant activity in previous studies (Liu *et al.* 2017a,b). *In vitro* antibacterial effects of the extract were studied, and the growth kinetics were investigated using the modified Gompertz and Logistic model. The minimum inhibitory and non-inhibitory concentration of the extract were determined using Lambert-Pearson model. The antibacterial effects of the extract on fresh tilapia fillets were studied to provide knowledge for future application of the extract as preservatives.

EXPERIMENTAL

Brown Algae Extract

Brown algae *A. nodosum* was purchased from Maine Coast Sea Vegetables (Franklin, ME, USA) and was extracted using 70% ethanol-water at 30 ℃ for 30 min with a solvent-to-solid ratio of 30 mL/g based on previous research (Liu *et al.* 2017a,b). 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 an oven until the mass was constant. Finally, the crude extract was filtered using a 0.2 μm sterile syringe filter, stored at -20 ℃, and used for antibacterial assay within one day. The total phenol content, dry mass concentration, and pH of the final crude extract was 12.00 ± 0.13 mg phloroglucinol/g algae, 4.20 ± 0.00 mg dw/mL extract, and 6.85, respectively.

Bacteria Strains

P. fluorescens (ATCC 13525) and *S. putrefaciens* (ATCC 8071) were obtained from American Type Culture Collection (Manassas, VA, USA). Bacteria strains were stored using 80% LB broth culture medium with 20% glycerol at -80 ℃. Prior to experiments, working cultures were prepared by sub-culturing 100 μL of the stock culture in 100 mL LB broth and incubating at 30 ℃ for 24 h. 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.

The modified Gompertz model and Logistic model (Chang *et al.* 2019) were applied to describe the inhibition of microbial growth under the influence of the algae extract. The modified Gompertz model is given in Eq. 1, and the Logistic model is given in Eq. 2,

$$logN(t) = logN_0 + log\frac{N_{max}}{N_0} \times \exp\{-\exp\left[\frac{2.718\times\mu_{max}}{\log\left(\frac{N_{max}}{N_0}\right)} \times (\lambda - t) + 1\right]\} \quad (1)$$

$$N(t) = N_0 + \frac{N_{max}-N_0}{1+\exp\left[-\mu(t-M)\right]} \quad (2)$$

where t is the time (h) and $N(t)$ is the microbial cell density at time t; N_{max} and N_0 are the maximum cell density and the initial cell density at t= 0; λ is the duration of the lag phase (h); μ_{max} and μ are the maximum growth rate (h^{-1}) and the relative maximum specific growth rate (h^{-1}), respectively; M is the time (h) at which the specific growth rate is maximum. The cell density was expressed as the OD600 of the culture medium.

Minimum Inhibitory Concentration (MIC) and Non-Inhibitory Concentration (NIC)

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 (F_a) was calculated as follows,

$$F_a(c) = \frac{A_c}{A_{c0}} \quad (3)$$

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 area was modeled as follows,

$$F_a(c) = \exp\left[-\left(\frac{c}{p_i}\right)^{p_2}\right] \quad (4)$$

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 Eq. 4,

$$\mathrm{MIC} = p_1 \cdot exp(\frac{1}{p_2})\mathrm{MIC} = p_1 \cdot exp(\frac{1}{p_2}) \quad (5)$$

$$\mathrm{NIC} = p_1 \cdot exp\,(\frac{1-e}{p_2})\mathrm{NIC} = p_1 \cdot exp\,(\frac{1-e}{p_2}) \quad (6)$$

where the value of e is the exponential of 1.

Bacteriostatic Assay of High Concentration Extract

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

Disc Diffusion Assay

The inhibition zone was determined using the disc diffusion method. Approximately 20 mL sterilized LB agar was poured into a petri dish. After drying, the bacterial suspension (100 µL, 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 10 µL of crude extracts (42 µg/disc) using capillary micro-pipette and placed on the LB agar. Penicillin (10 µg/disc) was used as positive control. The plates were then incubated at 30 °C

for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition. Each assay was replicated three times.

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 pieces were dipped in concentrated algae extract (algae extract group) or sterilized deionized water (control group) for 1 h. Treated pieces were placed in sterilized petri dishes and stored at 4 °C. Before the test, tilapia pieces were homogenized with 95 mL of sterilized saline (8 g NaCl/mL) for at least 30 seconds, and the supernatant was collected. The supernatant was serial diluted with sterilized saline, and 0.1 mL aliquot of the diluted supernatant was spread on LB agar to determine total viable count (TVC), and the result was expressed as log colony forming unit per gram fish tissue (log10 CFU/g). The TVC was determined every day during the 14-day storage period.

RESULTS AND DISCUSSION

Bacterial Growth Curve and Kinetics

Figure 1A shows that algae extract (0.21, 0.42, and 0.63 mg dw/mL) caused prolonged lag phase of *P. fluorescens*. As the concentration of algae extract continued to increase to 1.05 mg dw/mL, growth was completely inhibited. Similar prolonged lag phases were observed (0.21, 0.42, and 0.63 mg dw/mL) in *S. putrefaciens* (Fig. 1B). The extract also decreased the cell density at stationary phase. This bacterium grows slowly in the medium, so a longer incubation period was applied compared to *P. fluorescens*. No growth was observed when the extract concentration increased above 0.84 mg dw/mL after 30 h of incubation.

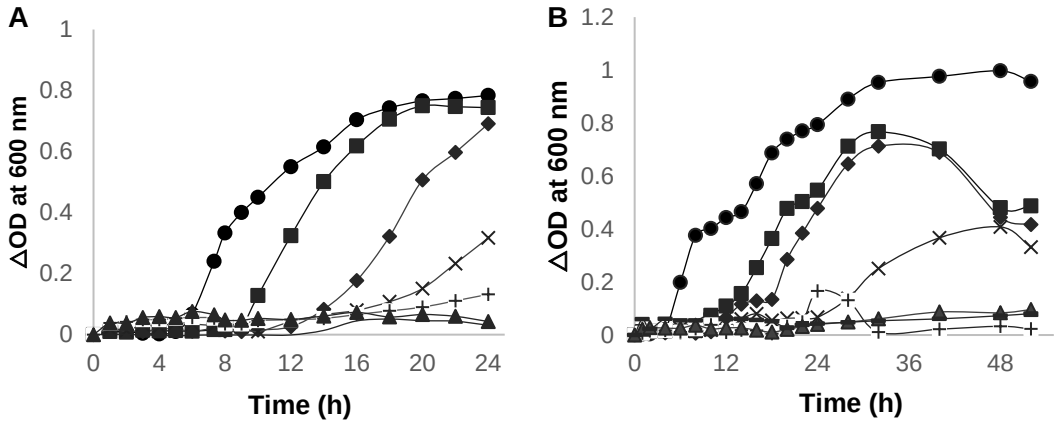

Fig. 1. Growth curve of *P. fluorescens* (A) and *S. putrefaciens* (B) showing average of triplicate measurements. Different symbols in the figure indicate different concentrations of extract applied (●: 0 mg/mL; ■: 0.21 mg/mL; ◆: 0.42 mg/mL; ╳: 0.63 mg/mL; +: 0.84 mg/mL; ▲: 1.05 mg/mL; —: 1.26 mg/mL).

The postponed lag phase and decreased microbial cell density observed in Fig. 1 was confirmed by the modified Gompertz model and the modified Logistic model (Table 1). Generally, the maximum growth rate was reduced and lag time prolonged as the extract

concentration applied increased from 0 to 0.42 mg/mL, suggesting that bacteria cells were more sensitive at higher extract concentration. The two models could successfully fit the growth curve obtained at relatively lower extract concentration (no more than 0.42 mg/mL), with R^2 values greater than 0.95. As the concentration extract became greater than 0.42 mg/mL, the R^2 values were lower than 0.90, and the growth rate generated were negative (data not shown). Similar results were summarized by Gupta *et al*. (2012); the maximum growth rate μ obtained from modified Gompertz and Logistic models were negative when the concentration of brown seaweed *Himanthalia elongata* applied was 6%. They also reported a declining trend in lag phase and an increasing trend in growth rate when the concentration of *H. elongata* applied decreased, which agreed with the observations of this study.

Table 1. Kinetic Parameters Obtained in the modified Gompertz model and the modified Logistic model

Bacteria	Extract Concentration (mg/mL)	Gompertz			Logistic		
		μ_{max} (h^{-1})	λ (h)	R^2	μ (h^{-1})	M (h)	R^2
P. fluorescens	0	0.241	5	0.99	0.598	9.6	0.98
	0.21	0.235	7.6	0.98	0.517	12.76	0.99
	0.42	0.135	10.45	0.96	0.444	18.12	0.99
S. putrefaciens	0	0.196	1.29	0.96	0.216	12.58	0.97
	0.21	0.083	5.38	0.97	0.213	18.92	0.99
	0.42	0.077	7.93	0.98	0.210	21.47	0.99

Table 2. Minimum Inhibitory Concentration (MIC), Non-Inhibitory Concentration (NIC), and r^2 of Model Predicted of *P. fluorescens* and *S. putrefaciens* at Different Incubation Time

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

Minimum Inhibitory Concentration (MIC) and Non-Inhibitory Concentration (NIC)

The MICs and NICs of *P. fluorescens* and *S. putrefaciens* retrieved determined at varying incubation times are shown in Table 2. The Lambert-Pearson model adequately described 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 h, then it increased steadily to 1.313 mg/ mL at 24 h. A similar observation was made in *S. putrefaciens*, in which the MIC decreased from 1.419 to 0.670 mg/mL as incubation time rose from 10 to 18 h and then began to increase after 20 h.

Determining NIC and MIC of spoilage microorganisms against inhibitors is an important premise of applying inhibitors as food preservation agents. 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: the traditional semiquantitative method and dose-response curve modelling. In the semiquantitative method, a range of inhibitor concentration was tested, and the concentration inhibiting all microorganism growth was determined as the MIC. This method gives 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 a semiquantitative method is that the modelling approach could determine MIC, even if none of the tested concentration could completely inhibit microorganism growth in experiments (Guillier *et al.* 2007). The semiquantitative method usually requires microorganisms to be cultured with inhibitor for at least 24 h, while modelling may allow rapid determination of MIC and NIC at any time. Only 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 does not need to be involved. Though MIC and NIC can be calculated from the curve modelling at any time, the accuracy of the model has not been reported and the incubation time for best-fit model needs to be illustrated for more precise application of dose-response modelling.

Lambert and Pearson (2000) observed 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 h 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 (no extract added). Low R^2 values (less than 0.90) were observed before 14 h of both bacteria, which was within early log phase. A slight decrease in R^2 value was observed in both bacteria after the very beginning of the stationary phase (20 h for *P. fluorescens* and 32 h 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 authors' knowledge, no research has been reported on both the rapidity and accuracy of models developed at different incubation times (Table 3). The MIC observed in the present study was lower than the MIC of *Eucalyptus globulus* oil against *P. fluorescens*, which was 9 mg/mL (Tyagi and Malik 2011), suggesting that *A. nodosum* extract had more prominent antibacterial effects.

Bacteriostatic Assay of High Concentration Extract

Bacteria cultured in media containing high concentrations of extracts in which no growth was observed were spread on extract-free agar plates, and the results are shown in Figs. 2 and 3. Figure 2 shows that culturing *P. fluorescens* in broth with high concentrations of algae extract (from 2.52 to 3.36 mg/mL) reduced the live bacteria cells. Culturing for longer time (72 h) resulted in even lower counts of living cells.

Table 3. A Brief Summary of Research Articles Using Models for Analyzing Antimicrobial Effect

Antimicrobial Agent	Microbes Studied	Important Findings	References
Phenethyl alcohol, dodecylcholine Cl, etc.	*S. aureus* and *P. aeruginosa*	Rapidity of the Lambert-Pearson model was analyzed and the values of MIC and NIC were found to be changed with incubation time.	Lambert and Pearson 2000
Phenethyl alcohol, trichloro-phenol, thymol, etc.	*S. aureus*	MIC and NIC calculated using Lambert-Pearson model could be influenced by inoculum size and inhibitor concentration.	Lambert 2000
Thymol, carvacrol, etc.	*S. aureus* and *P. aeruginosa*	Fractional inhibitory concentrations of antimicrobial combinations were analyzed using Lambert-Pearson model.	Lambert and Lambert 2003
Satureja spinosa essential oil	*S. aureus*, *E. coli* O157:H7, *L. monocytogenes*, *S. enterica*, *S. serovar*, and *B. cereus*	Optical density method and impedimetric method could both be used for investigating microbial growth using Lambert-Pearson model.	Chorianopoulos *et al*. 2006
pH adjusted using HCl	*E. coli*	Extended Lambert-Pearson model was able to model the data better than other models.	Lambert *et al*. 2010
NaCl and/or KCl	*A. hydrophila*, *C. sakazakii*, and *E. coli*	Effects of combined antimicrobials in foods were analyzed using Loewe reference models.	Anastasiadi and Lambert 2017
Gelam honey	*S. aureus, E. coli, B. cereus* and *P. aeruginosa*	MICs and NICs of the four microbials were reported and the gelam honey was found to be the most effective against *S. aureus*.	Ismail *et al*. 2022a
Allivum sativum extract	*A. hydrophila*	The MIC and NIC of *A. sativum* extract against *A. hydrophila* were calculated and reported.	Ismail *et al*. 2022b

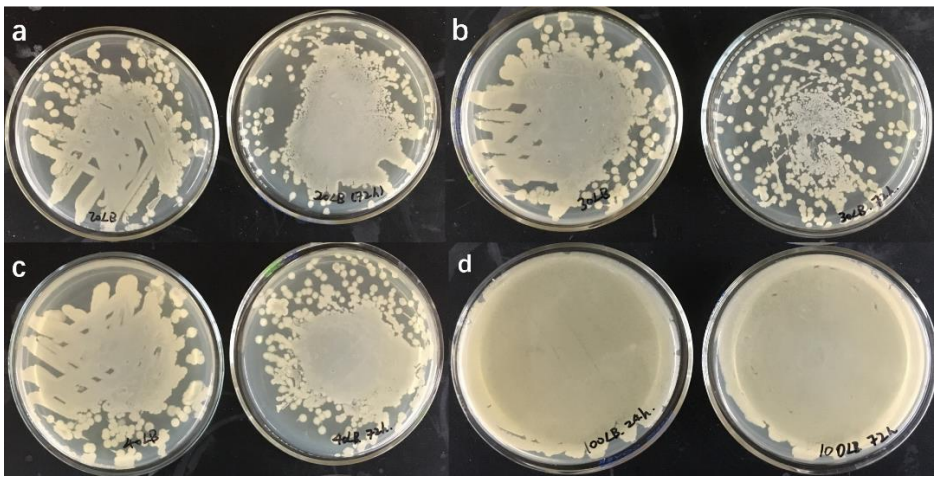

Fig. 2. Bacteriostatic assay of *P. fluorescens* with a. 3.36 mg extract/mL broth for 24 h (left) and 72 h (right); b. 2.94 mg extract/mL broth for 24 h (left) and 72 h (right); c. 2.52 mg extract/mL broth for 24 h (left) and 72 h (right); d. 0 mg extract/mL broth for 24 h (left) and 72 h (right)

Similar observations were made on *S. putrefaciens* (Fig. 3), but the bacteriostatic effects were much stronger than *P. fluorescens*. A lower concentration of extract (1.26 and 1.68 mg/mL) reduced bacteria counts, and the longer incubation time (72 h) showed much stronger inhibition effects than the shorter incubation times (24 h). This result suggested that *S. putrefaciens* was more sensitive to the extract than *P. fluorescens*.

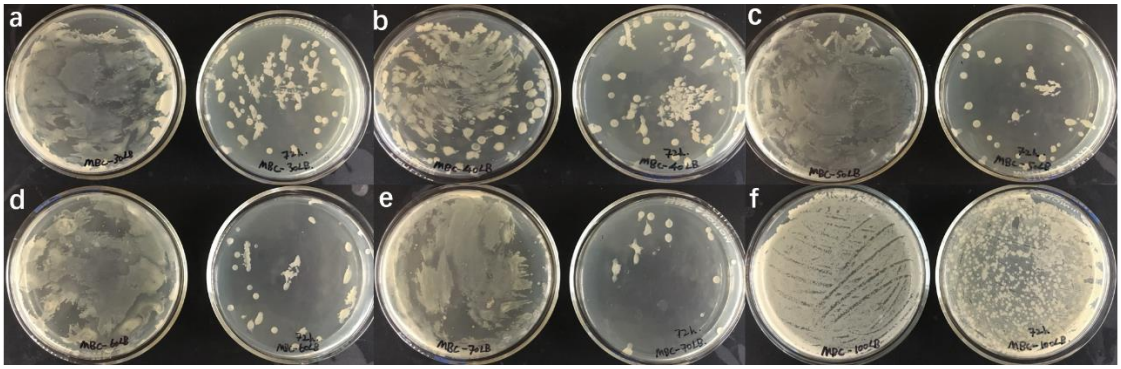

Fig. 3. Bacteriostatic assay of *S. putrefaciens* with a. 2.94 mg extract/mL broth for 24 h (left) and 72 h (right); b. 2.52 mg extract/mL broth for 24 h (left) and 72 h (right); c. 2.10 mg extract/mL broth for 24 h (left) and 72 h (right); d. 1.68 mg extract/mL broth for 24 h (left) and 72 h (right); e. 1.26 mg extract/mL broth for 24 h (left) and 72 h (right); f. 0 mg extract/mL broth for 24 h (left) and 72 h (right)

Although less common than MIC determination, the bactericidal assay or minimum bactericidal concentration (MBC) test of the extracts has been performed to determine whether the antibacterial agent is bacteriostatic or bacteriocidal (Tan and Lim 2015). The genus *Pseudomonas* exhibits strong resistance to natural extracts and essential oils. For example, *Pseudomonas aeruginosa* was the most insusceptible among the microorganisms tested against *V. monata* L. extract, 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* reported were 18 mg/mL *E. globulus* essential oil, which was two times their MIC (Tyagi and Malik 2011). Regardless of the origin of extracts or essentials, the MBCs reported in the literature were much higher than the highest concentration applied in the present study (3.36 mg/mL). The highest concentration of the extract applied in the present study did not exhibit bactericidal effects, though bacteriostatic influence was observed. Bactericidal effects might be observed if the extract concentration were higher than 3.36 mg/mL in the broth.

Disc Diffusion Assay

The diameters of inhibition zones of algae extract and penicillin were expressed as mean ± standard deviation. The extract (42 µg dw per disc) showed clear inhibition zones on agar plates against both *P. fluorescens* (1.72±0.32 cm) and *S. putrefaciens* (1.58±0.31 cm), while the inhibition zones of penicillin (10 µg dw per disc) were 1.42±0.09 and 1.00±0.00 cm, respectively. The results indicated that *A. nodosum* extract effectively inhibited the two bacteria strains.

The bacterium *Pseudomonas* has shown resistance to most essential oils, extracts, and even synthetic drugs in the diffusion assay due to its restrictive outer membrane barrier (Rahman and Kang 2009). A selection of natural extracts has been confirmed with their antimicrobial activity against *P. fluorescens*, among which only citrus extract and thyme essential oil had significant activity. *P. fluorescens* was reported to be more resistant to

natural extracts than *Listeria innocua* and *Aeromonas hydrophila* against all essential oils and extracts tested (Iturriaga *et al.* 2012). Ethanolic extract of *Lonicera japonica* Thunb. showed no inhibition zone against *P. aeruginosa* on LB agar plates in disc diffusion assay (400 μg extract per disc) (Rahman and Kang 2009). *E. globulus* essential oil was reported to show clear inhibition zones against *P. aeruginosa* in diffusion assay (Tyagi and Malik 2011). *S. putrefaciens* was inhibited by R(+) limonene in agar diffusion test, with an inhibition zone of 11.2 mm in diameter (Giarratana *et al.* 2016). However, not all natural extracts exhibit inhibition zones against *S. putrefaciens*. Wright *et al*. (2016) reported that only five plant extracts (muntries, lemon aspen, desert lime, bush tomato, and plum fruit) out of 28 Australian plant extracts showed inhibition zones against *S. putrefaciens*. The inhibition zones of various extracts/essential oils against *P. fluorescens* and *S. purefaciens* reported in the literature are listed in Table 4.

Table 4. Diameter of Inhibition Zones against *P. fluorescens* and *S. putrefaciens* in the Literature

Bacteria	Antimicrobial Agents	Diameters of Inhibition Zones (mm)	References
P. fluorescens	Bitter orange essential oil	1.3±0.6 (5 μL oil)	Iturriaga *et al*. 2012
P. fluorescens	Clove essential oil	4.3±0.6 (5 μL oil)	Iturriaga *et al*. 2012
P. fluorescens	Rosemary essential oil	1.0±0.0 (5 μL oil)	Iturriaga *et al*. 2012
P. fluorescens	Oregano essential oil	4.7±1.2 (5 μL oil)	Iturriaga *et al*. 2012
P. fluorescens	Thyme essential oil	5.3±2.5 (5 μL oil)	Iturriaga *et al*. 2012
P. fluorescens	Citrus extract	5.7±0.6 (5 μL extract)	Iturriaga *et al*. 2012
P. fluorescens	R(+) limonene	7.9 ± 0.7 (4 μL) and 11.1 ± 0.9 (8 μL)	Giarratana *et al*. 2016
S. putrefaciens	R(+) limonene	11.2 ± 1.5 (2 μL), 17.5 ± 1.2 (4 μL), and 24.1 ± 2.3 (8 μL)	Giarratana *et al*. 2016
S. putrefaciens	Methanolic muntries extract	10 ± 0.0 (10 μL extract)	Wright et al. 2016
S. putrefaciens	Aqueous muntries extract	8.3 ± 0.3 (10 μL extract)	Wright et al. 2016

Preservation Test of Tilapia Fillets

The TVC of algae extract treated tilapia group and control group is shown in Fig. 4a. The TVC of the extract treated 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 not until day 9 in the algae extract treated group. Slime began to cover the surface of tilapia pieces of the control group at day 5, while none was observed in the treatment group during the whole storage period (Fig. 4b), suggesting that *A. nodosum* extract could delay o prevent spoilage of tilapia fillets during storage.

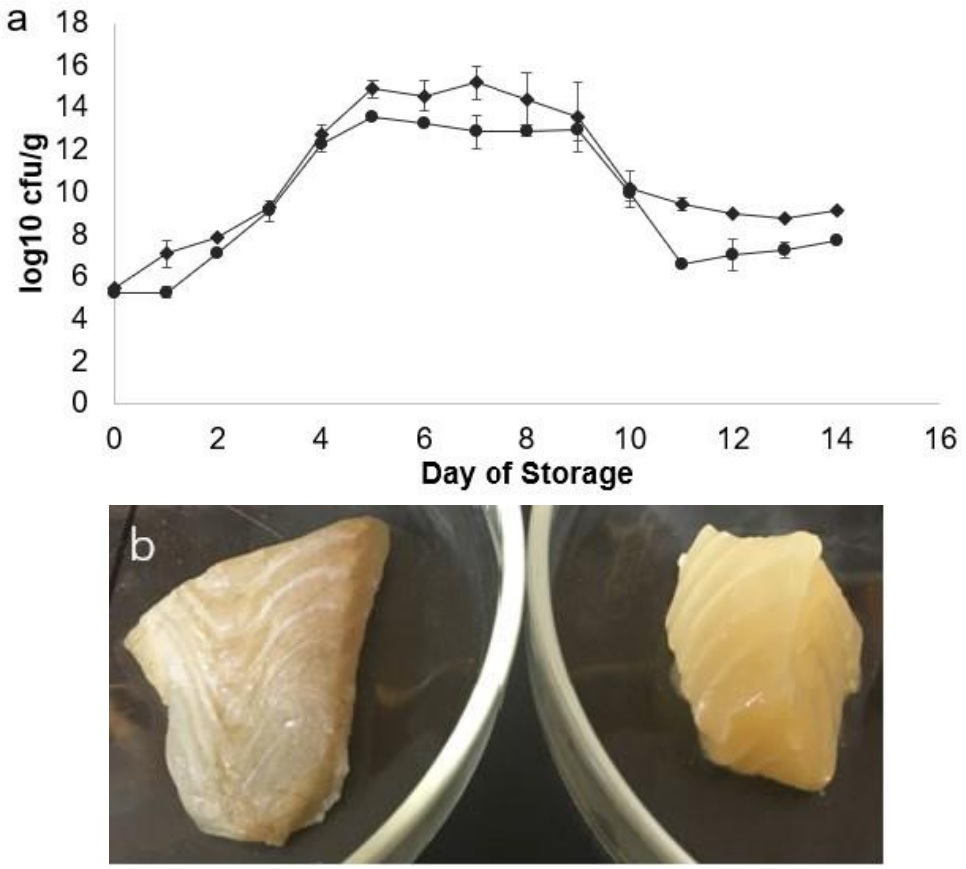

Fig. 4. Preservation test on tilapia fillets. a. Total viable count of algae extract treated group (●) and control group (◆) of tilapia. b. Storage condition of extract treated group (left) and control group (right) at day 14

Spoilage in fish leads to degradation of fish quality and other undesirable changes such as the bad fish odor. This odor is contributed by production of TVB-N and H_2S by metabolic activity of microorganisms and lipid peroxidation, especially deterioration of polyunsaturated fatty acids (PUFAs) in fish (Ortiz *et al.* 2014). *S. putrefaciens* contributes to the production of H_2S and other unpleasant odors (Lyu *et al.* 2017). The delay of off-flavor odor observed in the extract treated group could be a result of the protective barrier deterring contact between fish tissue and oxygen in the air (He *et al.* 2017), inhibiting specific spoilage organism (SSO) growth and volatile compounds production. Plant extract inhibits SSO growth in chill storage meat. The presence of *Staphylococcus aureus* was not detected in the chilled chicken samples treated with pomegranate peel extract until 12 days of storage, but this bacterium was detected in the untreated chicken samples as early as day 7 (Kanatt *et al.* 2010). Similar off-odor was observed in sterile gilt-head seabream inoculated with *Pseudomonas spp.* and *S. putrefaciens* when the bacteria count reached 10^7 and 10^8 CFU/g, respectively (Koutsoumanis and Nychas 2000), while *Pseudomonas spp.* and *S. putrefaciens* were the SSO of iced fresh fish regardless of the origin of the fish (Gram and Huss, 1996). The majority of H_2S producing bacteria isolated from ice-stored fish was found to be *Shewanella spp.* (Garcia *et al.* 2015; Lyu *et al.* 2017). The bad fish odor observed in the control group in the present study could be caused by metabolic products of *Shewanella spp.* and *Pseudomonas spp.* since SSO produces typical off-odor and grows faster than other microorganisms (Koutsoumanis and Nychas 2000; Miks-

Krajnik *et al.* 2016), while the postponement of unpleasant odor could be contributed by the antibacterial effect of the extract on SSOs growing on storage tilapia fillets.

CONCLUSIONS

1. Prolonged lag time and reduced growth rate of *Pseudomonas fluorescens* and *Shewanella putrefaciens* treated by the extracts were observed and confirmed by the modified Gompertz and Logistic model. The best-fit MIC and NIC values generated using the Lambert-Pearson model for *P. fluorescens* were 1.145 and 0.036 mg/mL, and 0.947 and 0.106 mg/mL for *S. putrefaciens*, respectively.
2. Clear inhibition zones were observed in the disc diffusion assay (42 µg extract per disc) against both *P. fluorescens* (1.72 cm diameter) and *S. putrefaciens* (1.58 cm diameter).
3. Treating tilapia fillets with algae extract significantly reduced total viable count of both bacteria and postponed unpleasant smell and slime from occurring. The results indicated that *Ascophyllum nodosum* extract could be used as potential food preservative to extend the shelf life of cold storage tilapia fillets.

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