Stimulation of the Biofilms of a marine bacterium *Pseudoalteromonas agarivorans* by Fucoidan extracted from macroalgae

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Abstract
Bacterial biofilm growth in aquatic environments can be promoted by natural compounds such as polysaccharides and proteins. Fucoidan is a group of marine sulfated polysaccharides found in the cell wall matrix of brown algae. In the present study, extraction of fucoidan was studied by using two alternate methods comparatively and efficiencies of the fucoidan extracts from Cystoseira sp. and Padina pavonica were investigated for stimulation of biofilm development of Pseudoalteromonas agarivorans.

The fucoidan rich extracts were tested for adhesion of biofilm bacterium in black polystyrene flat-bottom microplates. In the biofilm experiments, the extracts were found to stimulate biofilm development. The maximum mean for the biofilm stimulation efficiencies was 67.52% for SG1 - Cystoseira sp. and 67.50% for SG1 - P. pavonica. The results highlighted that Pseudoalteromonas agarivorans was able to adhere to the plates and the growth rates were increased with the addition of fucoidan extracts. This research indicated that fucoidan can be effective in biofilm development strategies in aquatic environments.

Keywords: *Cystoseira* sp., Fucoidan, Biofilm, *Padina pavonica, Pseudoalteromonas agarivorans*

Introduction

Biofilm is a special matrix of bacteria that live as a community in close contact, enabling several advantages like cell to cell communication, digestion of nutrients and protection for vital activities including growth, reproduction and functional activity in the marine environment[13,36]. The microbial cells inside the biofilm structure are supported by the secretion of extracellular enzymes and polymeric substances to digest and take nutrients respectively from the marine environment[18].

Bacteria frequently need insoluble substances or layer structures for growth processes, protection from infection and biomass reduction[1,4,19]. They secrete enzymes that provide an essential function for growth. These enzymes degrade polymers into dissolved constituents that can be catabolised and imported into a cell[1]. These dissolved and nutrient-rich substances can also be taken up by other microbial cells[11,21,31.] The strains of *Pseudoalteromonas* are dominant biofilm-forming bacteria that are commonly found in association with abiotic and biotic surfaces in the marine environment. The research on biofilms provides precious knowledge for ecological and other processes mediated by marine bacteria[36].

The brown macroalgae support biodiversity and reproductive ecosystems in marine environments. Most of them settle on rocky or sandy surfaces[33]. The cell wall of brown algae consists of different polysaccharides, proteins, phenolic compounds and minerals in the structure[12]. Fucoidans are sulfated polysaccharides, commonly found in the structure of brown macroalgae. The fucoidans include a large ratio of sulfate and L-fucose linked by α-(1→2)-, α-(1→3)- and/or α-(1→4)- glycosidic bonds. Also, they have minor amounts of glucose, xylose, mannose, galactose, rhamnose and uronic acid[9,26].

Marine derived natural compounds are composite materials of cells, debris, carbohydrates and proteins and these can be released into the environment by decomposition of algal cells, which are frequently rich in rhamnose and fucose[15,24,37]. Owing to the diversity of marine compounds with connected origins, particulate organic matter possibly includes a wide range of algae-originated polysaccharides in different structures[33]. These compounds provide an extracellular matrix that supports stimulation of biofilms and these compounds facilitate the growth of biofilm bacteria. During the hydrolysis of polysaccharides such as fucoidan, the biofilm bacteria use their digestive enzymes like fucoidanase[13]. Also, interactions inside a microbial group might possibly control the development of biofilms as well as the cycle of fucoidans in the marine environment[33].

This study is focused on the extraction of fucoidan and its purification from two species of brown algae *Cystoseira* sp. and *Padina pavonica* with two alternate methods and a marine biofilm bacterium *Pseudoalteromonas agarivorans* is investigated for stimulation of bacterial adhesion with these fucoidan fractions.

Material and Methods

Sampling and Pre-Treatment of Brown Algae: Marine brown algae *Cystoseira* sp. and *Padina pavonica* (Fig. 1), were collected during September and October 2020 from the coastal area of the Aegean Sea (Türkiye). The seaweeds were washed with distilled water to remove the extraneous matter, epiphytes, adhering sand and salt. The samples were dried for seven days at room temperature (25°C) and

powdered with a blender apparatus before the extraction process.

Fucoidan extraction and purification: Alternate extraction methods were studied for the isolation of fucoidan from *Cystoseira* sp. and *Padina pavonica* as shown in fig. 2.

Extraction of Fucoidan: Marine brown algae species were extracted for fucoidan with some modifications according to the methods proposed by Manikandan et al[27] and Ganapathy et al[20]. Each brown alga powder (50 g) was mixed with 1000 mL of teksoll 96% (Ethyl alcohol 96% + 2-propanol mixture) (Tekkim, Türkiye) and magnetically stirred for 18 hours at room temperature. The extract was then centrifuged at 6000 rpm for 30 min. The supernatant was removed to separate proteins and pigments. The obtained pellet was washed with acetone and dried overnight at room temperature. The dried pellet (5 g) was dissolved in 100 mL distilled water and stirred with a magnetic plate at 65 °C for 90 min. After centrifugation at 6000 rpm for 30 min, the residue was removed and the supernatant was collected. $CaCl_2$ (1%, 100 mL) was added to the supernatant to precipitate the alginate and the solution was stored overnight at 4 °C.

The resultant solution was centrifuged at 6000 rpm for 30 min. The supernatant was separated and subsequently, added to ethanol (96%) to obtain a final ethanol concentration of 30% (v/v). Then, the solution was stored at 4 °C for 4 h. This solution was centrifuged at 6000 rpm for 30 min and the supernatant was collected. After the centrifugation, ethanol (96%) was added again to reach 70% total ethanol concentration (v/v) and the final mixture was stored overnight at 4°C. Following overnight incubation, this solution was filtered with a cellulose acetate filter (0.45 µm pore size) to obtain the fucoidan. Finally, the fucoidan fraction retained on the filter was lyophilised with a freeze-dryer (Labconco FreeZone 4.5).

Figure 1: (a) *Cystoseira* sp. and (b) *Padina pavonica*

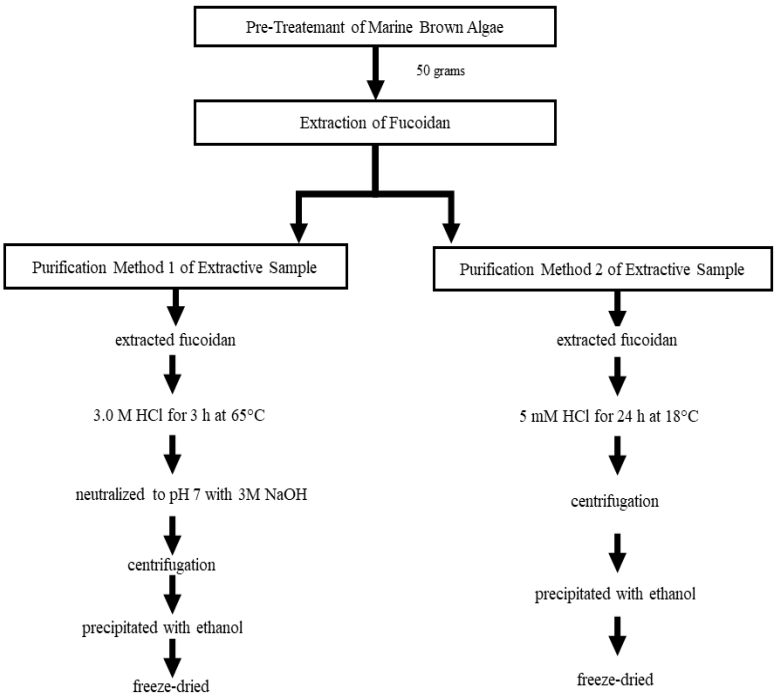

Figure 2: Schematic diagram of methods used for isolation of fucoidan from *Cystoseira* sp. and *Padina pavonica*

Fucoidan Purification Method 1: In the first purification method, freeze-dried fractions of fucoidan from *Cystoseira* sp. and *Padina pavonica* were purified according to Manikandan et al[27] with some modifications. Dried fucoidan extract samples from each marine brown algae were dissolved in distilled water at 65°C and mixed with 3.0 M HCl for 3 h using a magnetic stirrer. Following the cooling of the mixture at room temperature, the extract samples were centrifuged at 5000 rpm for 10 min. The collected supernatant was neutralised to pH 7 with 3M NaOH and precipitated overnight with 4 volumes of ethanol with respect to total volume. The mixture was centrifuged at 6000 rpm for 45 min and the supernatant was discarded. The pellet of purified fucoidan was washed with 10 mL distilled water and freeze-dried. The dried sample was coded as sample group 1 (SG1) and stored airtight in 1.5 mL tubes at room temperature.

Fucoidan Purification Method 2: In the second method, the purification of freeze-dried fucoidan fractions from *Cystoseira* sp. and *Padina pavonica* was carried out according to Yamazaki et al[35] with some modifications. Dried fucoidan pellets of *Cystoseira sp*. and *Padina pavonica* were suspended in 5 mM HCl. Then the mixtures were shaken at 200 rpm for 24 h at 18°C. The samples were centrifuged at 5000 rpm for 10 min. The collected supernatant was precipitated overnight with four volumes of ethanol to total volume at 4°C. The mixture was centrifuged at 6000 rpm for 45 min and the supernatant was discarded. The pellet of pure fucoidan was washed with 2 mL distilled water and freeze-dried. The dried sample was coded as sample group 2 (SG2) and stored airtight in 1.5 mL tubes at room temperature.

Stimulation of Biofilm Formation: The purified fucoidan extract samples and commercial fucoidan (Santa Cruz Biotechnology, Inc.) were tested on biofilm development capability of a marine biofilm bacterium. The biofilm forming bacterium had been isolated from a marine biofilm on panel systems immersed in the Izmir Bay (Türkiye, Aegean Sea). A relative 16S rRNA gene sequence analysis demonstrated that the isolate pertained to the species *Pseudoalteromonas agarivorans* FJ040188[22]. The bacterial suspension was produced from the marine biofilm bacterium which was grown for 48 hours on Zobell Marine Agar (Difco) at 26ºC to reach an OD600 of 1.5.

Adhesion of the biofilm bacterium was performed according to modified methods proposed by Aykin et al[2,3] and Leroy et al[25] using sterile seawater in flat bottom polystyrene microplates (Greiner Bio One, Austria) at 20ºC. The four dried extracts of fucoidan and commercial fucoidan (Santa Cruz Biotechnology, Inc.) were tested for the stimulation of bacterial adhesion. The extract samples (50 µl) or commercial fucoidan (50 µl) at different concentrations (5 mg/1.5 mL, 2.5 mg/1.5 mL, 1.25 mg/1.5 mL, 0.625 mg/1.5 mL and 0.3125 mg/1.5 mL) were placed into the wells 1 h after the addition of bacterial suspension (200 µL per well) and incubated for 24 h at 20ºC. Prior to fixation (1.5 h incubation at 4ºC with 200 µL of 36 g L^{-1} sterile NaCl containing 2.5% formaldehyde) and DAPI staining (20 min incubation with 4 µg mL^{-1} dye) steps, wells were washed three times with 36 g L^{-1} NaCl.

Following the DAPI incubation, the wells were washed again three times to remove excess DAPI. After that, the residuary bound DAPI was solubilised into 200 µL of 95% ethanol for 15 min. Fluorescence was quantified at 350 nm excitation and 510 nm emission wavelengths handling a Synergy HTX multi-mode reader (Biotek, USA). A blank with seawater (sterile) besides a control with bacterial suspension without extract or commercial fucoidan was included in each column of the experimental microplate (Fig. 3).

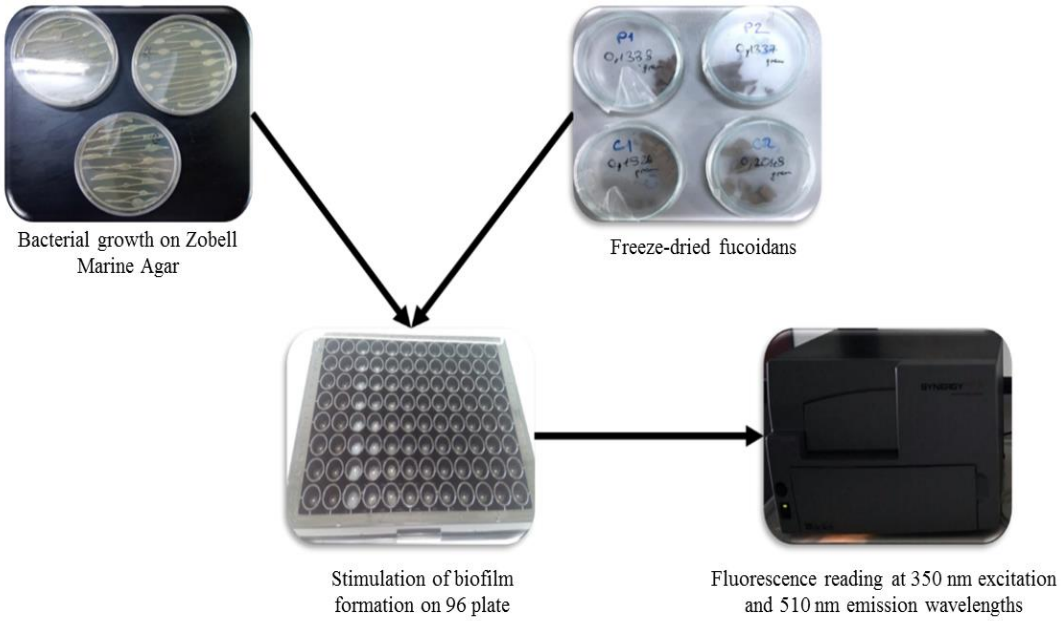

Figure 3: Schematic representation of the biofilm stimulation experiment

The stimulation of bacterial adhesion was calculated as:

$$Stimulation\ of\ Bacterial\ Adhesion\ (\%) = \frac{(F_S - F_B) - (F_C - F_B)}{F_C - F_B} \times 100$$

where F_S is fluorescence of the sample (with fucoidan), F_C is fluorescence of the control (without fucoidan) and F_B is fluorescence of the blank (without bacterium)

Results and Discussion

The yields of fucoidans obtained with the first and second purification methods from *Cystoseira* sp. and *P. pavonica* ranged from 31% and 67% respectively on a dry weight basis (table 1). In the present study, it was found that the second purification method for fucoidan was more efficient in achieving higher yields from both algae samples. It is noteworthy that the second purification method proceeded at mild conditions like lower amounts of HCl and lower incubation temperature, which may provide better stability for fucoidan over the first method. Among the two species of brown algae, the highest amount of fucoidan was obtained from *Cystoseira* sp. at a yield of 67% and from *P. pavonica* with a yield of 65%. While there were no significant differences between the fucoidan yields of the two species, the product yield varied significantly depending on the purification methods.

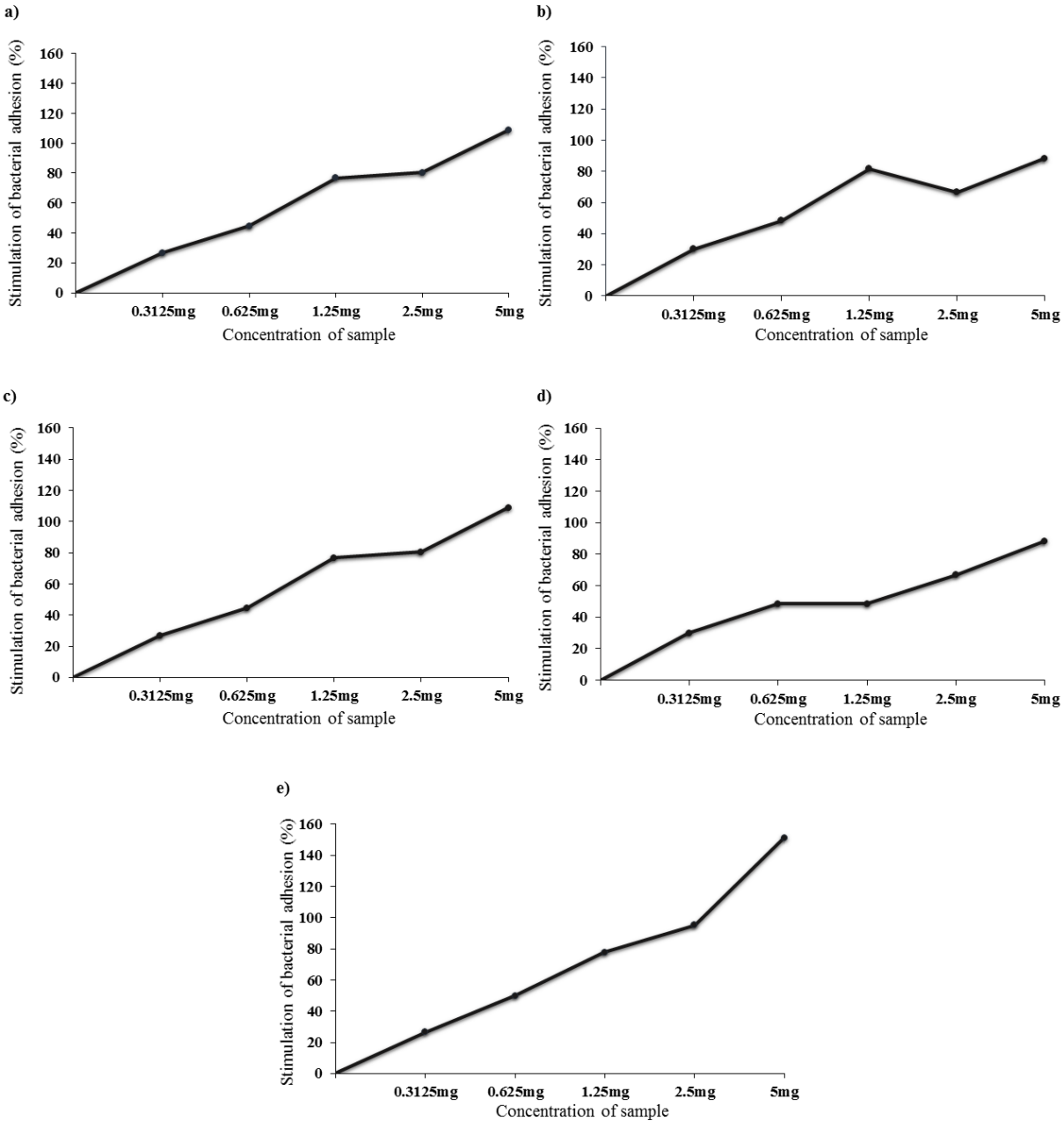

Figure 4: The percentage of stimulation of bacterial adhesion. a) SG1 - *Cystoseira* sp., b) SG2 - *Cystoseira* sp., c) SG1-*Padina pavonica*, d) SG2 - *Padina pavonica*, e) Commercial Fucoidan

Table 1
The yields of fucoidan fractions

Sample Groups	Pre-treatment	Extraction	Purification	Yield (%)
SG1 – *Cystoseira* sp.	50 g	250 mg	77 mg	31 %
SG2 – *Cystoseira* sp.		150 mg	100 mg	67 %
SG1 – *Padina pavonica*	50 g	250 mg	104 mg	42 %
SG2 – *Padina pavonica*		35 mg	22.9 mg	65 %

Table 2
The efficiency of fucoidan to stimulation of biofilm bacterium

Sample Groups	Max.	Min.	Mean
SG1 – *Cystoseira* sp.	108.97 %	26.79 %	67.52 %
SG2 – *Cystoseira* sp.	88.33 %	30 %	62.99 %
SG1 – *Padina pavonica*	108.93 %	26.79 %	67.50 %
SG2 – *Padina pavonica*	88.33 %	30 %	56.33 %
Commercial Fucoidan	151.16 %	26.74 %	80.23 %

In order to study the biofilm formation mechanisms behind potential bioactivity of fucoidan, its ability to stimulate biofilm formation was tested with bacterial adhesion experiments on 96 well plates. In the tests, a strong synergy was observed between biofilm formation and fucoidan amount and the addition of fucoidan increased the adhesion of biofilm bacteria on the plates (Fig. 4). Compared with the yield of the second sample group, sample group 1 was more successful in stimulation of biofilm formation.

The biofilm stimulation efficiencies of the purified fucoidans and the commercial fucoidan were as follows: the mean efficiencies for the sample group 1 were 67.52% and 67.50% for fucoidans from *Cysoseira sp* and *P. pavonica* respectively; the mean efficiencies for sample group 2 were 62.99% and 56.33% for fucoidans from *Cysoseira sp* and *P. pavonica* respectively and the mean efficiency for the commercial fucoidan was 80.23% (table 2). Among these biofilm stimulation rates, the most effective result was found for the commercial fucoidan.

Some marine bacteria can degrade specific polysaccharide classes, such as agar and chitin as well as the polysaccharides of marine brown algae like fucoidan[5-8,34]. During the decomposition and degradation of living organisms, extracellular polysaccharides, fecal pellets by heterotrophic bacteria and biopolymeric particulate substances are formed. During the degradation process, bacteria attach to surfaces of these biopolymer particles that have a significant role on their growth dynamics, community composition and their movement in the water column[17]. Colonisation of the bacterial assemblages on the particles is a dynamic process that involve two types of communities: the first type is primary degrader whose abundance is controlled by the particle's polysaccharide composition and the second type is cross-feeding consumer taxa whose dynamics is controlled by interspecific interactions[16,17]. According to these bacteria-particle interactions, biofilm growth dynamics of *P. agarivorans* is parallel to that of primary degraders and it is capable of metabolising available polysaccharides for its growth.

In the literature, findings from enzyme-based studies indicate that members of proteo and flavobacteria are capable of producing fucoidan-degrading enzymes that hydrolyse fucoidans into oligosaccharides[6,10,14,23,28,29]. For example, Sakai et al[30] characterised the three strains of the *Pseudoalteromonas citrea* (KMM 3296, KMM 3297 and KMM 3298) that contain intracellular endo-type fucoidanases. Their study showed that fucoidanase from *P. citrea* (KMM 3296) catalysed the hydrolysis of the alpha-1,3-glycosidic bond in the fucoidans from *Laminaria cichorioides* and *Fucus evanescens*.

In another study, Sasaki et al[32] determined that *Pseudoalteromonas issachenkonii* has an intracellular fucoidanase. Considering these reports on the presence of fucoidanases in different species of *Pseudoalteromonas* and the results of biofilm experiments in this study, it can be assumed that the biofilm bacterium *P. agarivorans* might have used available fucoidan in its degradation since the increase of fucoidan in the medium stimulated biofilm growth and adhesion of bacteria to the surfaces of wells.

Conclusion

The results of this study provided extraction yields and a stimulation effect on biofilm growth for fucoidan from two different species of brown algae. *Cystoseira sp.* was more productive in achieving higher yields of fucoidan. It is important to note that the product yield varied significantly between the brown algae species depending on the purification method. Also, the results highlighted the role of fucoidan in marine biofilm stimulation.

The marine biofilm bacterium *Pseudoalteromonas agarivorans* achieved higher growth rates with fucoidans, demonstrating that marine biofilm bacterium is capable of catalysing the hydrolysis of complex fucoidan compounds in

the marine environment. According to the results of the present study, it is suggested that more biofilm bacteria should be investigated for their ability to degrade complex polysaccharides.

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