EFFECTS OF TEMPERATURE ON BIOFILM FORMATION AND QUORUM SENSING OF *AEROMONAS HYDROPHILA*

M.F.R. MIZAN[a], I.K. JAHID[a, b], S.Y. PARK[c], J.L. SILVA[d], T.J. KIM[e], J. MYOUNG*[f] and S.-D. HA*[a]

[a]Department of Food Science and Technology, Advanced Food Safety Research Group, Brain Korea 21 Plus, Chung-Ang University, 72–1 Nae-Ri, Daedeok-Myun, Anseong, Gyunggi-do 456-756, South Korea

[b]Department of Microbiology, Jessore Science and Technology University, Jessore-7408, Bangladesh

[c]Department of Seafood and Aquaculture Science, Institute of Marine Industry, Gyeongsang National University, Togyeong 53024, South Korea

[d]Department of Food Science, Nutrition and Health Promotion, Mississippi State University, Mississippi State, Mississippi 39762, USA

[e]Food and Nutrition Department, College of Education, Health and Human Sciences, Wisconsin's Polytechnic University, 712 Broadway St S, Menomonie, WI 54751, United States

[f]Korea Zoonosis Research Institute, Chonbuk National University, Deokjin-dong 1ga, Deokjin-gu, Jeonju-si, Jeollabuk-do, South Korea

*E-mail addresses: jinjong.myoung@jbnu.ac.kr; sangdoha@cau.ac.kr

ABSTRACT

Aeromonas hydrophila is an emerging foodborne pathogen that causes infections more frequently in summer than in winter. This study evaluated the effects of temperature (4-37°C) on the biofilm formation and quorum sensing abilities of *A. hydrophila* on microtiter plates, stainless steel (SS), and crab surfaces. The incubation of the bacterium in Luria-Bertani broth at temperatures of 20-25°C significantly ($P < 0.05$) enhanced the biofilm formation and intra-species quorum sensing ability (via C4-AHL and C6-AHL). Field-emission electron microscopy revealed that the bacterium colonized the surface of crab and formed biofilms at 25°C. Thus, the present study demonstrates that temperature control in food processing environments may reduce *A. hydrophila* biofilm formation. Therefore, the study has significant applications in food processing plants.

1. INTRODUCTION

Aeromonas hydrophila has recently received much attention as an emerging opportunistic and foodborne pathogen and a causative agent of various human infections such as gastrointestinal tract infections, wound and soft tissue infections, and blood-borne dyscrasias (DASKALOV, 2006). The incidence of these infections is higher during summer, owing to elevated temperatures (JANDA AND ABBOTT, 2010). The importance of *A. hydrophila* in food safety (DASKALOV, 2006), fish diseases, and human infections (JANDA AND ABBOTT, 2010) as well as its role in quorum sensing and biofilm formation (CHOPRA *et al.*, 2009) have been studied. The bacterium has been isolated from various fresh and estuarine water samples and animals living in these waters, including fish, crab, shrimp, and other mollusks (OTTAVIANI *et al.*, 2011; DENG *et al.*, 2014).

Microbial biofilms are sessile microbial communities that are attached to either biotic or abiotic surfaces. Biofilm formation is a common phenomenon in nature; for instance, biofilms are formed on foods and food-contact surfaces as well as in waste treatment plants. The effect of temperature on the production of biofilms and virulence factors has been studied using various microorganisms, including *Enterococcus* spp., *Salmonella* spp., and *Listeria monocytogenes* (DI BONAVENTURA *et al.*, 2008; JAHAN AND HOLLEY, 2014). Although *Salmonella* is commonly associated with human and animals, it can also be found in the environment (CHRONICLE, 1997; GIAOURIS *et al.*, 2005). As these are environmental microorganisms, such as *A. hydrophila*, temperature is expected to modulate their survival and ability to form biofilms.

Quorum sensing is a density-dependent process by which microorganisms coordinate and control intraspecies and interspecies communication (FUQUA *et al.*, 1994). Several authors have reviewed the importance of quorum sensing in relation to food microbiology (SKANDAMIS AND NYCHAS, 2012; MIZAN *et al.*, 2015). *A. hydrophila* carries quorum-sensing genes, including *ahyI* (encoding N-3-butanoyl-DL-homoserine lactone [C4-AHL]) and *ahyR* (encoding N-3-hexanoyl homoserine lactone synthase [C6-AHL]) (SWIFT *et al.*, 1997). In addition, the bacterium produces autoinducer 2 (AI-2) for inter-species communication (KOZLOVA *et al.*, 2008). Intra-species quorum sensing in *A. hydrophila* (via C4-AHL and C6-AHL) has been reported to control biofilm formation, motility, and virulence factors (KHAJANCHI *et al.*, 2009).

Temperature is known to play an important role in governing the rate of microbial activity as well as for the propagation of biofilms and settlement of organisms in aquatic systems (RAO, 2010). Biofilm formation is influenced by several factors, available nutrients, and organic matter. MELO AND BOTT (1997) reported that the development and maturation of biofilm is dependent on temperature, nutrient availability, and water flow rate. As *A. hydrophila* is common in aquatic environments, it may face adverse environmental conditions such as fluctuations in temperature, humidity, and pH (DASKALOV, 2006). Survey studies have shown that aeromonads are found in high numbers in late summer/early autumn (temperatures are around 20–25°C) but are rarely detected during the cold months (GAVRIEL *et al.*, 1998). The present study focused on the effects of temperatures on biofilm formation and quorum sensing by *A. hydrophila* on the surface of microtiter plates, stainless steel (SS), and crab shells. In the food industry, biofilm formation is a major concern as an important source of food contamination. Therefore, the results of this study will provide potential approaches to avoid contamination, as they reveal the temperatures favorable for *A. hydrophila* biofilm formation and quorum sensing.

2. MATERIALS AND METHODS

2.1. Bacterial strains, culture media, and growth conditions

In the present study, the following strains were used: A. hydrophila KCTC 11533 (isolated from surface water) and KCCM 32586 (a clinical isolate), Vibrio harveyi strain BB120 and BB170, and *Chromobacterium violaceum* CV026. The bioreporter strain CV026 was provided by the Animal, Plant, and Fisheries Quarantine and Inspection Agency, Korea. Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) was used for the bacterial cultivation and violacein production assay. Prior to each experiment, frozen culture aliquots (100 μL) were thawed and inoculated into 5 mL of LB broth. The cultures were incubated at 30°C and 220 rpm for 24 h. These starter cultures were subsequently inoculated into fresh LB broth and cultured to a final optical density of 1.0 at 600 nm (OD600), followed by their dilution (1:50) for biofilm formation experiments and quorum sensing assay.

2.2. Quantitative biofilm formation assay in microtiter plates

The quantitative biofilm assay was performed as previously described (O'TOOLE, 2011; MIZAN et al., 2016). A. hydrophila was cultured in LB broth for 24 h without shaking, followed by its dilution at 1:50 in LB broth. A total of 100 μL aliquots were placed in the wells of 96-well polystyrene microtiter plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and the microtiter plates were incubated at 4, 10, 15, 20, 25, 30, 35, and 37°C for 72 h without shaking. Biofilm formation was normalized to planktonic growth and determined using the following equation (1), according to TEH et al. (2010):

$$\text{BFI} = \frac{(AB - CW)}{(GB - GW)}$$

(1)

Where BFI is the biofilm formation index, AB is OD_{595} of the crystal violet (CV)-stained attached microorganisms, CW is OD_{595} of the stained blank wells containing sterile (microorganism-free) medium only, GB is OD_{600} of the cells in suspended culture, and GW is OD_{600} of the blank well. Biofilm production was classified into three categories according to MARTINEZ-MEDINA et al. (2009): weak (0.1 > BFI ≤ 0.5), moderate (0.5 > BFI ≤ 1), and strong (BFI > 1).

2.3. Determination of biofilm formation on SS surfaces

Austenitic SS coupons (type 302, 2 × 2 × 0.1 cm; Chung-Ang Scientific Inc., Seoul, Korea) were processed as described by SHEN et al. (2012). Thereafter, *A. hydrophila* cell suspensions were diluted at 1:50 and inoculated into 7 mL of fresh LB in 50 mL Falcon tubes containing a completely submerged SS coupon. The tubes were incubated without shaking at 4, 10, 15, 20, 25, 30, 35, and 37°C for 24 h to allow biofilm formation on SS coupons. Following incubation, each SS coupon was transferred into a small Petri dish (55 × 12 mm) containing 1 mL of 0.1% peptone water (PW; Oxoid, Hampshire, UK) and agitated by rotation in clockwise and anticlockwise direction using sterile forceps. Agitation was always performed by the same person; thus, it was assumed that the same amount of pressure was applied to all coupons (JAHID AND HA, 2014). The cells were separated, vortexed, and diluted in PW for enumeration. Cell number was quantified using Bacto R2A agar (Difco, USA) following incubation for 24 h.

2.4. Preparation of inocula for crab surfaces

Cultures grown in LB broth were centrifuged (10,000 ×g for 10 min at 4°C) and the pellets were washed twice with Dulbecco's phosphate-buffered saline (DPBS). The pellets were re-suspended in a suitable amount of DPBS to obtain an absorbance of 1.0 at 600 nm wavelengths. To determine the cell density, serial dilutions were performed and plated on *Aeromonas* selective medium (Oxoid, Hampshire, UK). These inocula were used to inoculate the crab surfaces.

2.5. Biofilm formation on crab surfaces and detachment of cell population

Crabs (*Corystes cassivelaunus*) used in this study were purchased from a local grocery store in Anseong, Korea. A delimited area (cm²) of crab shell was dissected and processed immediately, as described by JAHID *et al.* (2015). The shells were incubated at different temperatures (4, 10, 15, 20, 25, 30, 35, and 37°C) without shaking. The detachment of microbial populations from shell surfaces was performed as described by JAHID *et al.* (2014), with minor modifications. Briefly, the crab surfaces were washed twice with PBS to free planktonic cells and placed in 10 mL of PW in a sterile stomacher bag (Nasco Whirl-Pak, USA). These were processed using a stomacher (BagMixer, Interscience, Saint Nom, France) at maximum speed for 2 min to free the biofilm-forming microbes from the crab shells. *A. hydrophila* was enumerated after serial dilution and spread plating on *Aeromonas* selective medium containing ampicillin. The plates were incubated at 30°C for 48 h. Colonies were counted and the results for biofilm production were expressed as colony-forming unit (CFU)/cm² for biofilm populations. For each of three independent experiments, two plates per dilution were assessed to obtain the final data.

2.6. Quantification of violacein production

To quantify violacein production, the procedures described by KIM *et al.* (2013) and JAHID *et al.* (2015) were followed. *A. hydrophila* was cultivated in LB broth at different temperatures for 24 h and the supernatant was collected by centrifugation at 15,000 ×g for 15 min, followed by its filter sterilization using 0.22 μm filters (Millipore Corporation, Billerica, MA, USA). LB agar was prepared, cooled, and poured using the open side of a 1 mL pipette tip to make a well. A loop full of *C. violaceum* CV026 overnight culture was spread on the wall of the well and treated with 100 μL of supernatant from each condition at 28°C for 24 h in an upside up of petri dish. Next, whole CV026 cells grown on the plate were collected and solubilized with 250 μL of dimethyl sulfoxide (DMSO; Sigma Aldrich). The mixture was vortexed to ensure the release of violacein pigment. After centrifugation at 15,000 ×g for 15 min, the absorbance of 200 μL of colored DMSO from CV026 cells was measured at 585 nm wavelength using a microplate reader (Spectra Max 190; Molecular Devices).

2.7. Autoinducer-2 (AI-2) bioassay

The secretion of AI-2 by *A. hydrophila* during its incubation with crab surfaces at different temperatures (4-37°C) was assessed with minor modifications in previously described procedures (SONI *et al.*, 2008). *A. hydrophila* was inoculated on crab surfaces in cyanobacteria BG-11 freshwater solution (Sigma Aldrich, Inc., St. Louis, MO, USA) and incubated at different temperatures without shaking. The cultures were centrifuged at 15,000 ×g for 10 min. The supernatant from the cell-free culture was passed through 0.2 μm Tuffryn syringe filters and stored at -20°C. The cell-free supernatants were tested for

the presence of AIs that induce luminescence of *V. harveyi* reporter strain BB170. This strain carries sensor 2, but not sensor 1, and is capable of sensing AI-2 but not AI-1. *V. harveyi* strain BB170 was grown overnight at 30°C with aeration in the autoinducer bioassay (AB) broth and diluted to 1:1,000 in AB medium (BASSLER *et al.*, 1993). Next, 4.5 mL of diluted *V. harveyi* strain BB170 and 500 μL of the cell-free supernatant from each sample (*A. hydrophila* supernatant incubated with crab at different temperatures) was added to 50 mL Falcon tubes and shaken for 16 h at 220 rpm to allow the reporter strain to produce luminescence. A total of 100 μL samples were transferred to white microtiter plates and the luminescence was measured using a computer-controlled microplate luminometer (GloMax® 96 Microplate Luminometer for Luminescence, Promega, Madison, WI, USA). *V. harveyi* strain BB120 that produces AI-1 and AI-2 was used as a positive control. Control *V. harveyi* strains were grown overnight at 30°C with shaking at 220 rpm in LB broth and 1 mL of cell-free supernatant from each culture was prepared as described above.

2.8. Field-emission scanning electron microscopy

A. hydrophila biofilm formation was observed on crab surfaces at 4, 25, and 37°C by field-emission scanning electron microscopy (FESEM). The inoculation and incubation procedures were the same as those described above. FESEM samples were processed according to the previously described procedures (MIZAN *et al.*, 2016). The dehydrated samples were sputter-coated with platinum and visualized with an FESEM microscope (Sigma, Carl Zeiss, Germany) at an accelerated voltage of 5 kV and a working distance of 5 mm. Digitized images of biofilms were collected for further analysis.

2.9. Statistical analysis

Biofilm formation and quorum sensing of *A. hydrophila* at different temperatures (4–37°C) were analyzed by analysis of variance (ANOVA) using SAS software (Version 9.2; SAS Institute Inc., Cary, NC, USA) for a completely randomized design to determine the significance of differences due to temperature variation. The mean separation was evaluated with Duncan's multiple-range test when the effect was significant ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Impact of temperature on biofilm formation

Temperature is one of the major factors that affect bacterial growth. Most of the clinically important pathogens are mesophiles that grow well at optimum temperatures between 25°C and 40°C (MURRAY *et al.*, 2003). The optimum growth temperature is 20°C for some *Aeromonas* species and 37°C for others (EWING *et al.*, 1961). MAALEJ *et al.* (2004) reported a decline in *A. hydrophila* population to a level below the detection level at 23°C and 5°C. The optimum temperature for *A. hydrophila* infection in goldfish (*Carassius auratus*) is 17–25°C (RAHMAN *et al.*, 2001). The results from the examination of the biofilm formation on microtiter plates at different temperatures are shown in Table 1. A significant increase in the biofilm production was observed at 20–25°C for *A. hydrophila* strains 11533 and 32586. Biofilm formation declined at temperatures over 25°C (i.e., 30-37°C) or below 20°C. RACHID *et al.* (2000) reported the formation of dense biofilms with an increase in temperature.

Studies have shown that *A. hydrophila* may attach and produce biofilm on to SS surfaces (LYNCH *et al.*, 2002), glass (WHITELEY *et al.*, 1997), and vegetables (JAHID *et al.*, 2014) in laboratory settings. LYNCH *et al.* (2002) reported that *A. hydrophila* produces a thin biofilm that may cover 40-50% of SS surface. Biofilm formation by *A. hydrophila* strains 11533 and 32586 on SS coupons is presented in Table 1. The trend observed was similar to that reported with microtiter plates. A significant increase ($P < 0.05$) in biofilm formation was observed at 20-25°C, indicative of the optimum temperature range for biofilm formation. Similar trend was observed for both *A. hydrophila* strains, although they had different origins (i.e., clinical versus environmental).

Biofilms on fish surfaces and bacteria from marine water source may contaminate seafood-processing facilities. *Vibrio*, *Aeromonas*, *Listeria*, and *Salmonella* isolated from seafood are known to cause foodborne illness and form biofilms (TAKAHASHI *et al.*, 2009; ABEROUM and JOOYANDEH, 2010; NORHANA *et al.*, 2010; JAHID *et al.*, 2015; MIZAN *et al.*, 2017). The ability of *A. hydrophila* to produce biofilms on crab surfaces at 4, 10, 15, 20, 25, 30, 35, and 37°C is shown in Table 1. Biofilm formation was significantly lower ($P < 0.05$) at 4, 10, and 15°C than at 20-37°C. A significant increase in the biofilm formation was observed for both strains at 20-30°C, while the biofilm growth gradually reduced at 37°C. No significant difference ($P > 0.05$) was observed in the biofilm formation between the two *A. hydrophila* strains.

Table 1. Viable counts of biofilm cells of *Aeromonas hydrophila* in Luria-Bertani (LB) medium with different temperature from 4°C to 37°C.

Support	Microtiter plates		Stainless steel surfaces		Crab surfaces	
Temperature (°C)	11533 (BFI±SEM**)	32586 (BFI±SEM)	11533 (log CFU/cm^2 ±SEM)	32586 (log CFU/cm^2 ±SEM)	11533 (log CFU/cm^2 ±SEM)	32586 (log CFU/cm^2 ±SEM)
4	0.29±0.02de	0.22±0.03d	4.56±0.26c	3.62±0.28d	2.05±0.10h	1.66±0.17f
10	0.38±0.03cd	0.47±0.01bc	4.89±0.15bc	5.14±0.22bc	2.76±0.09g	2.81±0.07e
15	0.79±0.02ab	0.63±0.04ab	5.26±0.11b	5.50±0.32b	4.74±0.26f	4.45±0.15d
20	0.91±0.08a	0.72±0.03a	6.23±0.07a	6.22±0.24a	5.98±0.03ab	4.77±0.14d
25	1.06±0.04a	0.70±0.07a	6.35±0.18a	6.72±0.24a	6.08±0.11a	5.55±0.09ab
30	0.34±0.05de	0.45±0.06bc	5.46±0.25b	5.29±0.12b	5.88±0.07abc	5.67±0.17a
35	0.24±0.04e	0.36±0.02cd	5.16±0.08bc	5.34±0.08b	5.43±0.24cd	4.87±0.09c
37	0.16±0.02e	0.38±0.06cd	2.65±0.26e	3.26±0.27de	4.96±0.30de	4.73±0.23d

**The values are mean ± SEM (Standard Error Mean) of 3 independent experiments. The values with same letters within a column were not significant ($p < 0.05$) according to Duncan's multiple-range test.

3.2. Violacein production

The modulation in the quorum sensing ability of *A. hydrophila* at different temperatures has been previously investigated (MEDINA-MARTINEZ *et al.*, 2006). The biosensor *C. violaceum* strain CV026 (a *cvil* transposon mutant) with quorum sensing regulation defect fails to produce violet color in the absence of AHL, which controls violacein production. However, CV026 produces color if AHL is externally supplied. The mutant CV026 produces color when grown in the presence of *A. hydrophila*, as violacein is produced in response to AHL secreted by *A. hydrophila*. The intensity of the color is proportional to AHL concentration. Violacein production was significantly lower ($P < 0.05$) at 4-15°C than at 20-25°c. AHL produced by strain 11533 was significantly higher ($P < 0.05$) than that

produced by strain 32586 (Fig. 1). JAHID *et al.* (2015) stated that the production of AHL by *A. hydrophila* is strain-dependent. PONCE-ROSSI *et al.* (2016) reported *A. hydrophila* ATCC7966 to be negative for AHL production, while *A. hydrophila* Embrapa 029 was shown to produce AHL.

Both *A. hydrophila* strains produced the highest AHL levels at 25°C (Fig. 1). MEDINA-MARTINEZ *et al.* (2006) noted that the optimum temperature for AHL production was 22°C, and no AHL production was observed at 37°C. At 12°C, however, C4-HSL production was detected after 70 h of incubation.
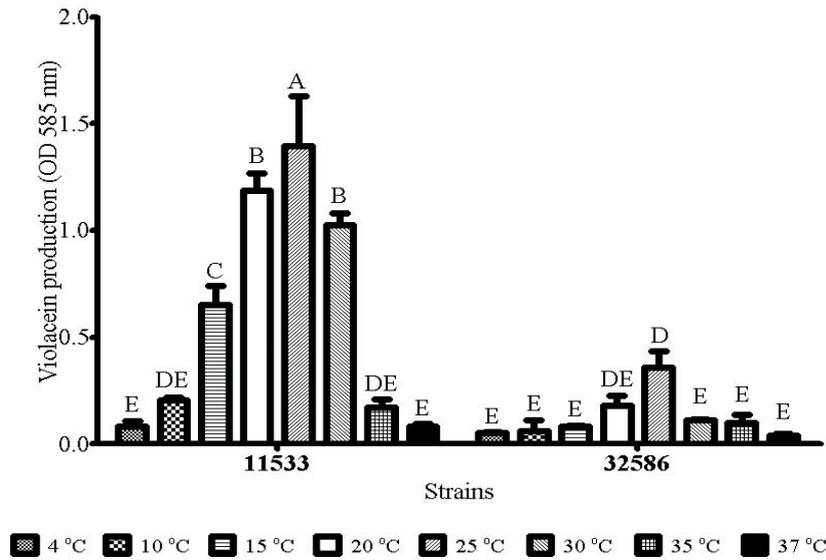
Figure 1. Violacein production in *A. hydrophila* at different temperatures. Values shown are the mean ± SEM of three independent experiments. Within each treatment, the values marked with the same letter are not significantly different according to Duncan's multiple-range test (P < 0.05).

3.3. AI-2 production

Although AI-2 production and biofilm formation may be associated with each other (MIZAN *et al.*, 2016), no strong relationship was observed between AI-2 levels and biofilm formation, as the procedure for AI-2 measurement was too sensitive and variable to compare independent experiments (Fig. 2). Temperature modulates cell density, and quorum sensing is dependent on cell density. AI-2 production by *A. hydrophila* was analyzed in crab fresh water samples incubated at different temperatures. As shown in Fig. 2, AI-2 production by *A. hydrophila* strains was origin-dependent; more strains are necessary to justify the results. The environmental strain (KCTC 11533) produced lower levels of AI-2 than the clinical strain (KCCM 32586). A significant (P < 0.05) increase in AI-2 production was observed in both *A. hydrophila* strains at 20–30°C (Fig. 2). Further studies on the role of food composition and environment in the production of AI-2 and C4-HSL may increase our understanding of the synthesis and accumulation of these signal molecules in food products.
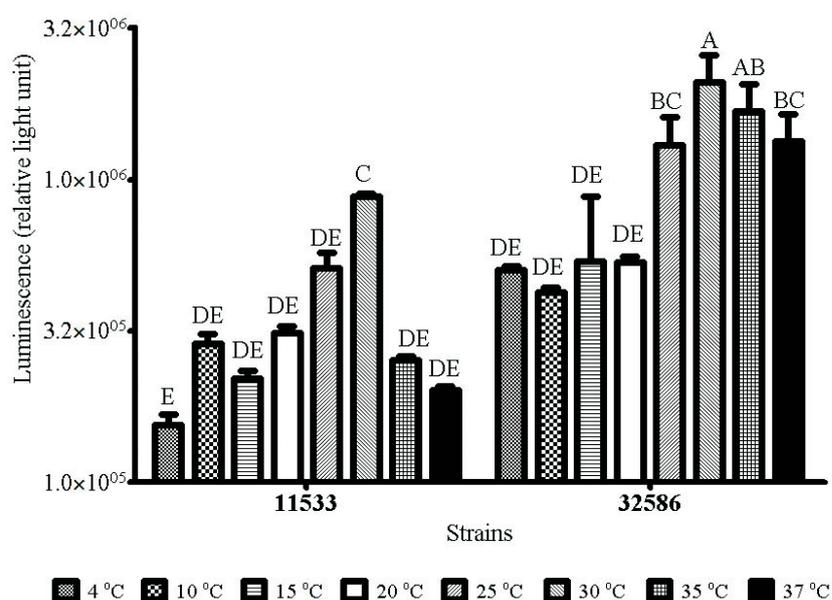
Figure 2. AI-2 production in *A. hydrophila* at different temperatures. Values shown are the mean ± SEM of three independent experiments. Within each treatment, the values marked with the same letter are not significantly different according to Duncan's multiple-range test (P < 0.05).

3.4. Analysis of crab samples with FESEM

ABEROUM AND JOOYANDEH (2010) reported that processing facilities of seafood products may act as a source of contamination and that *A. hydrophila* is commonly isolated from unprocessed and processed seafood products. TSAI AND CHEN (1996) observed that *A. hydrophila* contaminated 50% of oysters purchased from native marts of Taiwan. Biofilm formation may differ, owing to differences in the growth surface and temperature (NOORI *et al.*, 2016). JAHAN AND HOLLEY (2014) observed dense biofilm formation (*Enterococcus* spp.) at temperatures lower than the optimum growth temperature. NOORI *et al.* (2016) reported that higher temperatures induced extensive biofilm formation, whereas lower temperatures resulted in the attachment of the bacterial cells (*V. parahaemolyticus*) as monolayers on crab surface. MIZAN *et al.* (2015) observed *A. hydrophila* cell attachment on crab shell. Biofilm formation on crab samples incubated at 4, 25, and 37°C (according to biofilm formation strength) is presented in Fig. 3. *A. hydrophila* failed to form biofilms on crab surfaces at 4℃; the bacterium attached on the crab surface (Fig. 3A). In contrast, *A. hydrophila* formed a strong three-dimensional structure at 25°C, (Fig. 3B). At 37℃, the bacterium formed biofilms on the crab surface (Fig. 3C). PONCE-ROSSI *et al.* (2016) evaluated biofilm formation in two strains of *A. hydrophila* at the same temperature but different incubation times. These authors found that the biofilm formation was maximum at 48 h and decreased at 72 h. ALMEIDA *et al.* (2017) studied *Salmonella* and observed that maximum biofilm formation may occur after 36 h incubation in contrast to other times evaluated.
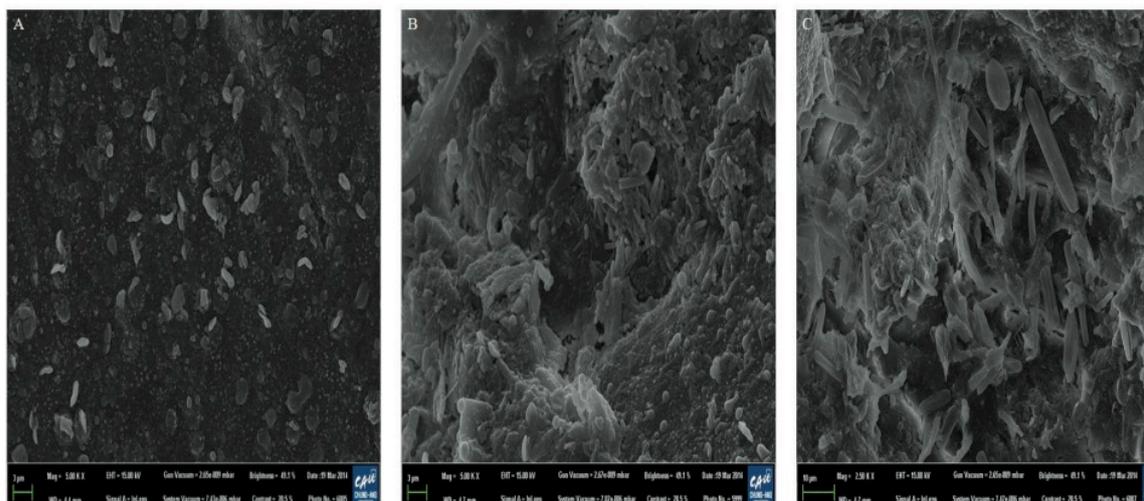
Figure 3. FESEM images of *A. hydrophila* biofilm formation on crab surfaces at different temperatures. The image shown is a representative result for strain KCTC 11533. (A) 4°C, (B) 25°C and (C) 37°C.

4. CONCLUSIONS

The two *A. hydrophila* strains, one originally isolated from surface water (strain KCTC 11532) and the other from clinical sample (strain KCCM 32586), showed significant variations in the tested phenotypes (i.e., biofilm formation and quorum sensing), indicating strain-specific regulation. Strain KCTC 11533 was found to produce high concentrations of AHL and lower concentrations of AI-2. Strain KCCM 32586, on the other hand, showed high AI-2 production and lower AHL activity. Therefore, the phenotypic properties differed between the two strains. Such studies will elucidate the effect of quorum sensing on the regulation of virulence factors produced by opportunistic pathogens such as *A. hydrophila* on microtiter plates, SS, and crab surfaces. However, the experimental scope of the study is limited to only one strain of environmental and clinical origin. Further studies are warranted to extend the application of this study in food quality and safety regulations.

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