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Effect of sodium chloride and temperature on biofilm formation and virulence of *Flavobacterium columnare* isolated from striped catfish (*Pangasianodon hypophthalmus*)

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ABSTRACT

This research was conducted to investigate the biofilm formation ability at various salt concentrations and temperatures of Flavobacterium columnare isolated from striped catfish (Pangasianodon hypophthalmus) at Can Tho University. Microtiter plate assay and the in vivo challenge were used to test the virulence of this strain of F. columnare for 10 days by immersion method at different salt concentrations (0, 3, 6, 9, 12 and 15 ppt). Results showed that biofilm formation of F. columnare was inhibited at 3 and 6 ppt, and stronger reductions were recorded at 9, 12 and 15 ppt. In the same trend, the higher temperature, the lower biofilm formation, the highest biofilm formation was at 25 ℃ treatment, then it was reduced at 28 and 31 ℃, and at 35 ℃ the formed biofilm was greatly reduced. Interestingly, there were no statistically significant differences between 28 and 31 ℃ (P>0.05). The virulent study found that 100% fish died after 1- day post challenge at 0 ppt. There were 10% and 25% of fish died at 3 and 6 ppt, respectively. No dead fish was found at 9 and 12 ppt. In conclusion, biofilm formation was inhibited at 3 ppt, was almost controlled at 9, 12 and 15 ppt, and was also mostly reduced at 31 ℃ at least in the in-vitro study. Furthermore, the virulence of this bacterial strain was controlled 90% at 3 ppt and completely controlled (100%) at 9, 12 and 15 ppt.

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1 INTRODUCTION

Freshwater fish consumption is recently increasing around the world, in which *Pangasianodon hypophthalmus* distributed a value of US$ 2.2 billion for export earnings in 2018 in Vietnam according to the Ministry of Agriculture and Rural Development (mard.gov.vn). Aquaculture production is expected to produce more fish for human consumption directly rather than capture fisheries (Subasinghe *et al.*, 2009). It is necessary to find solutions for a sustainable development in such an important food-producing sector. Although striped catfish is perhaps the most widely traded fish over the world, it is now facing with many infectious pathogens such as *Edwardsiella ictaluri* and *Aeromonas hydrophila* (Crumlish *et al.*, 2010) or a serious pathogen *Flavobacterium columnare* (Panangala *et al.*, 2007). *F. columnare* is an agent causing disease on freshwater

fish worldwide including striped catfish with the clinical signs of skin lesions, fin erosion and gill necrosis (Declercq *et al.*, 2013). The first isolates of *F. columnare* were isolated from aquarium fish such as Koi (*Cyprinus carpio*), black molly (*Poecilia sphenops*) and platy (*Xiphophorus maculatus*) by Decostere *et al.*, (1998). The emergence of columnaris disease on striped catfish currently has leaded high economic loss due to high mortality within commercial hatchery ponds (Tien *et al.*, 2012). The adhesion of bacteria to tissues has been considered as a crucial step in pathogenesis of many infections in animals and human beings (Magarinos *et al.*, 1996). Interestingly, there is an evidence that resilience of biofilm posited in the closed aquaculture systems can act as a source of contagion for farmed fish (Cai *et al.*, 2013).

Biofilms can make up a single or multiple species to colonize biotic or abiotic surfaces, their architecture provides a defense and offers the microbes the spatial proximity and internal homeostasis needed for their growth and differentiation. This makes the bacterial cells within the biofilm much stronger resistant than their planktonic cells to many factors such as antimicrobial treatment, poisons, protozoans and host immunity (Long *et al.*, 2020). It is also considered as the most considerable problem of the biofilms (Mah and O'Toole, 2001). The key advantage of biofilms is their positive influence of solid surfaces on the bacterial activity. This advantage of biofilm has been taken the attention of many researchers in different fields such as water and wastewater treatment and many other biotechnology areas (Lazarova *et al.*, 1995). The effects of temperature have been tested to inhibit the adhesion of *Vibrio parahaemolyticus* and *salmonella enterica* at 37°C (Song *et al.*, 2016). Moreover, high concentration of salt (10.5% NaCl) was significantly inhibited by the adherence of bacterial cells of *salmonella enterica* (Giaouris *et al.*, 2005).

The objective of this study was to determine an appropriate sodium chloride concentration and temperature level to reduce the biofilm formation of *F. columnare* in order to control columnaris disease outbreaks in striped catfish ponds.

2 METHODOLOGY

2.1 Bacterial strain and culture condition

Flavobaterium columnare strain was isolated from striped catfish *(Pangasianodon hypophthalmus)* in Can Tho, Vietnam. Previously, this bacterial strain had been identified as *F. columnare* by PCR method (Dong *et al.*, 2014). The bacteria were proliferated in Anacker and Ordal (AO) broth for 48 hours at 28°C with gentle shaking and stock suspensions were stored in AO broth supplemented with 20% glycerol at -80°C.

2.2 Bacterial density testing

Density of the bacteria was measured by plate count method. For more details, a 1/10 dilution had been performed with 1.0 mL of the bacterial stock and 9.0 mL of the AO broth in a 150 mm screw-capped tube with label. Then a pipette had been used to transfer 1.0 mL of the first sample into new first tube with label of 10^{-1}, capped and vortexed the tube, then the dilution series was continued by using new pipette and pipet tips for each step until reaching 10^{-8}. Moreover, 100 µL of the bacteria from the last three tubes (10^{-6}, 10^{-7}, 10^{-8}) were spread onto the surface of AO agar plates by using sterile pipette and pipet tips, the agar plates were incubated for 2 days at 28ºC and colonies were counted in each agar plate. Plates with colony number at the range of 30-300 were used only (Arana *et al*., 2013).

2.3 Salinity testing by microtiter plate assay

This test was performed by using the microtiter plate assay from O'Toole (2011) to form biofilms at different salinities, the bacterial stock was incubated for 48 hours in shaker at 28°C and microtiter dish was used to produce bacterial biofilm during 48 hours. In details, 90 µL of medium broth and 10µL of the bacterial stocks ($1.8x10^8$ cfu/ml) were put into each well of the microtiter 96-well plate and was incubated for 2 days at 28°C (the optimal temperature of *F. columnare*). Sodium chloride concentrations were tested in this study were of 0, 3, 6, 9, 12, 15 ppt with 7 replications. The negative control was used 100 µL of medium broth and was incubated for 48 hours at 28°C.

2.4 Temperature testing by microtiter plate assay

The assay was prepared as described by O'Toole (2011) and the bacterial biofilm was tested at 25, 28, 31 and 35°C with 7 replications. The test was performed by putting 90µL of AO broth and 10µl of the bacterial stocks ($1.8x10^8$ cfu/ml) into each well of the microtiter 96-well plate and the negative control was used 100µL of AO media broth, and was incubated for 48 hours before staining and quantification.

2.5 Biofilm detection by staining method

The formed biofilms had been countinuing with biofilm staining with 0.1% crystal violet as described by O'Toole (2011). The plate after incubation was turned over and was shaked out all of the liquid. Unattached cells and media components were removed by gently submerging the plate into small tub of water. Then, 125 µL of 0.1% of crystal violet solution was added into each well and the plate was incubated at room temperature for 10-15 mins. After that, the plate was rinsed 3-4 times by submerging the plate into small tub of water and shaking it out. The plate was turned upside down and was dried for few hours or overnight. Finally, the plate was photographed when it dried.

2.6 Biofilm quantification

The biofilm were quantified by acetic acid 30%, all of those steps were followed by as in previous study (O'Toole, 2011). Each well of the microtiter plate was added 125µL of 30% acetic acid to solubilize the crystal violet and the plate was incubated at room temperature for 10-15 mins, then the solubilized CV was transferred to a new microtiter plate for quantification in a plate reader at 570 nm using 30% acetic acid in water as the blank.

2.7 Virulence study by immersion challenge

The virulence study was conducted with striped catfish (*P. hypophthalmus)* fingerlings bought from a catfish hatchery in Bangkok, Thailand. The fishes were treated with 1% NaCl for around 30 minutes before transfer to acclimation tank to minimize the effects from opportunistic pathogens. Catfish fingerlings (6-10g) were acclimated for 3 weeks before experimental challenge, and *F. columnare* strain used in the in-vitro test was used for challenge experiment. Bacterial isolate of *F. columnare* was cultured in AO broth at 28ºC with shaking (150 rpm) until reaching the optical density (OD) ~1.0 at 600 nm to get expected density of ~10^8 cfu/mL (Dong *et al.*, 2015). Then traditional plate count method was performed to identify cfu/mL. Designed dose for immersion challenge was $6.93x10^6$ cfu/mL according to the median lethal concentration (LC_{50}) tested in striped catfish fingerlings (3-6g) previously (Tien *et al.*, 2012). Fishes were divided into 7 groups: 0, 3, 6, 9, 12, 15 ppt (by gradually increased 3 ppt per day) and the control group. In the control treatment, the fishes were immersed with AO broth. Each group was had 3 replicates and immersion duration was 1 hour. After immersion, 10 fishes were transferred into each 100-liter culture tank. The fishes were fed twice per day with commercial feed on the demand, the temperature was maintained around 28-29ºC during the experiment. Fish mortality had been regularly checked and was recorded for 3 weeks. Fresh dead fish and moribund fish were necropsied and the bacteria were isolated from gills, skin and kidney on Anacker and Ordal (AO) agar plates.

2.8 Statistics

One-way analysis of variance (ANOVA) and Duncan test used to determine the significant difference ($P<0.05$) in different treatments. Mean and standard error was calculated by Microsoft Excel version 2016.

3 RESULTS AND DISCUSSION

3.1 Bacterial isolation

The bacterial stock stored in refrigerator at -80ºC was isolated from striped catfish with clinical signs of columnaris disease such as gill necrosis, fin erosion, or skin lesions and had been identified by PCR method (Dong *et al.*, 2014). *Flavobacterium columnare* was recovered in Anacker and Ordal (AO) medium agar plate. The bacterial colonies were recognized easily by naked eyes due to their typical characteristics of yellow rhizoid colonies and adherent deeply into the agar (Welker *et al*., 2005).

A separated colony was picked up to transfer into AO medium broth and was cultured for 2 days in shaking incubator at 28ºC. A ring of bacteria has been found to adhere strongly to the glass bottle upper layer after several hours of incubation, this phenomenon was showed the strong adherent ability of *F. columnare* (Decostere *et al*., 1999). Finally, the bacterial stock was used for biofilm formation.

3.2 Biofilm production ability at various salinities

Different biofilm formation of *F. columnare* at various salinities could be observed in Figure 1. The OD_{570} value was recorded before removing planktonic phase to measure bacterial cell growth and was recorded after removing the planktonic phase to measure formed biofilms.

The formed biofilm was highest at 0 ppt with OD_{570} value at 0.217. At treatments 3 ppt and 6 ppt, biofilm formation was significantly reduced but no statistically significant differences between two treatments ($P>0.05$) with OD_{570} value at 0.106 at both two treatments. The OD_{570} value was 0.075 at the control treatment. It could be seen that there was a big gap

between 0 ppt and 3 ppt, there is a possibility to detect a lower NaCl concentration to inhibit *F. columnare* biofilms in this gap, a previous study was identified that the adherence of *F. columnare* was inhibited at 1 ppt and the significant decrease of the biofilms was identified from 5 to 14 ppt (Altinok *et al*., 2001; Cai *et al*., 2013). In the previous study, the bacterial cell growth was found to be inhibited and only a little amount of biofilm was formed at 0 ppt (Cai *et al*., 2013) and bacteria were not grown at higher than 0.5% NaCl (Bernardet, 2007), this was different with the results of this study. It is maybe because of different bacterial strains.

In the study groups of 9, 12 and 15 ppt, cell growth and biofilm were low and there were no significant differences with control group (P>0.05). Their OD_{570} value was 0.088; 0.073 and 0.077 respectively. These results are similar to those from Welker *et al*. (2005) that the growth and adherent ability of *F. columnare* were controlled at 9 ppt.

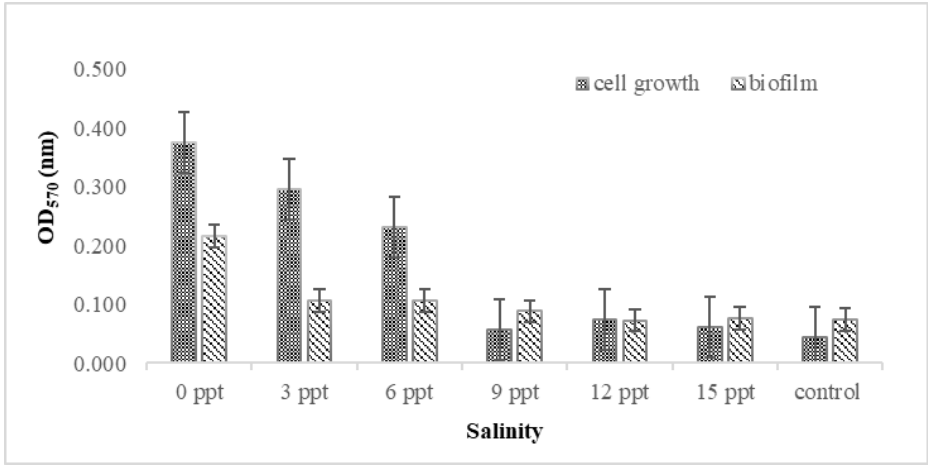

Fig. 1: Biofilm formation and cell growth on microtiter plate for 48-hour incubation at different salinities

Bacteria recovered at 0, 3, 6 ppt groups had been confirmed by streak plate technique but the bacteria were not found at 9, 12 and 15 ppt groups (Figure 2). This was confirmed that *F. columnare* was not grown at 9, 12 and 15 ppt in the in-vitro test.

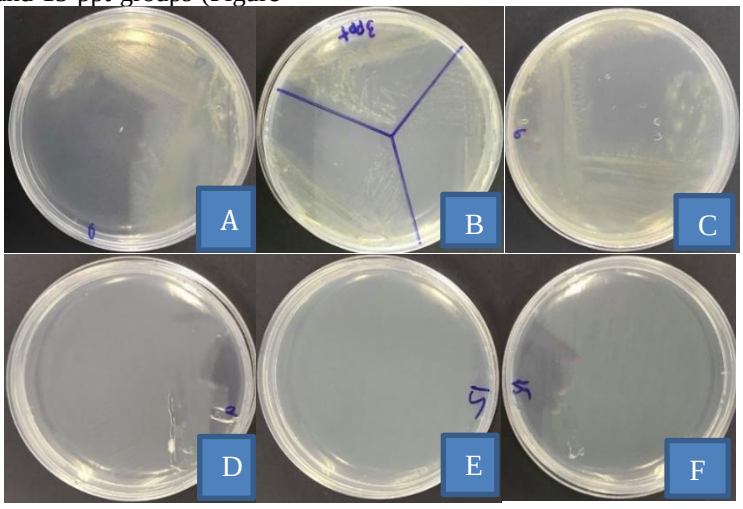

Fig. 2: Cell growth of *F. columnare* in different salinities; A. in 0 ppt; B. in 3 ppt; C. in 6 ppt; D. in 9 ppt; E. in 12 ppt; F. in 15 ppt

3.3 Biofilm production ability on various temperature levels

The biofilm formation and cell growth of *F. columnare* had been identified after incubated for 2 days (Fig. 3). The biofilm formation and cell growth were inhibited when the temperature was increased. Higher cell growth was observed at 25 and 28ºC than that at 31 and 35ºC. Based on the OD_{570} value, there was not significantly different on the bacterial cell growth between 25 and 28ºC at 0.360 and 0.351, respectively. Both two temperature levels were in the optimal range of *F. columnare* (Thomas *et al*., 2004; Cain *et al*., 2007). At 31ºC, the cell growth was slightly decreased with OD_{570} value at 0.31 and the cell growth was strongly inhibited in the treatment of 35ºC with the OD_{570} value at 0.214. We were had similar findings with those from the previous study that there was a greatly inhibition of biofilm formation at 35°C (Cai *et al*., 2013).

Biofilm formation was greatly promoted at 25ºC but it was started to inhibit at 28ºC with OD value at 0.352 and 0.261 respectively, no significant difference had been recorded between 28ºC and 31ºC (P>0.05) while biofilm formation at 35ºC was greatly inhibited with the optical density at 0.139.

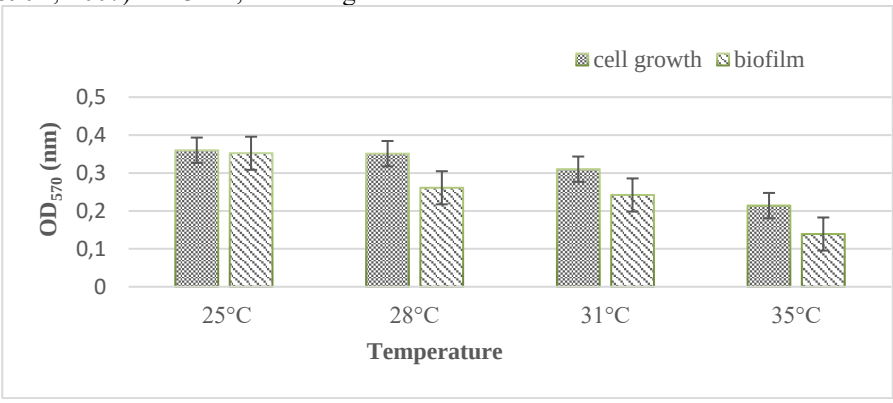

Figure 3. Biofilm formation and cell growth on microtiter plate for 48 h incubation at different temperatures

The bacterial cells in all treatments were recovered by streak plate technique, this could be said that *F. columnare* could grow at these temperatures. Although the bacterial growth and biofilm production of *F. columnare* were strongly inhibited at 35ºC, it still was not the temperature level to kill the bacteria.

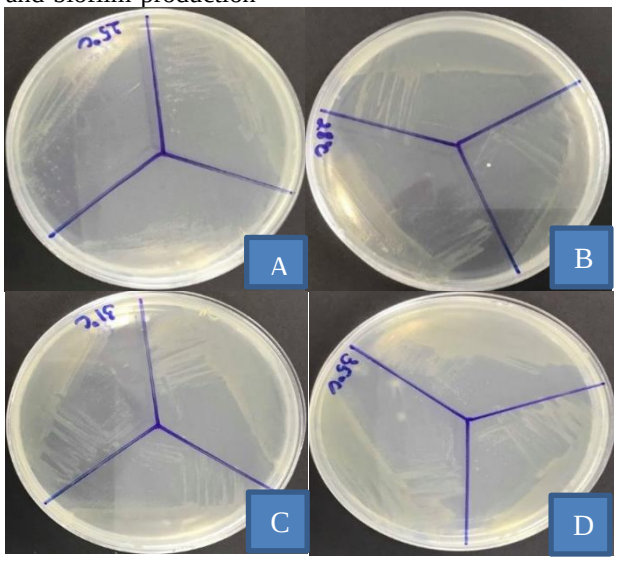

Fig. 4: The cell growth of *F. columnare* in different temperatures; A. in 25ºC; B. in 28ºC; C. in 31ºC; D. in 35ºC

3.4 Bacterial virulence

All of the fish, prior to challenge, were appeared normal without any sign of disease and no bacteria was recovered from internal organs and external organs of those fish. The *F. columnare* isolate used in the in-vitro test had been challenged with striped catfish (*Pangasianodon hypophthalmus*) to identify the virulence. The results of fish challenge shown that 100% of experimental fishes were died after one day post-challenge at 0 ppt treatment. The fishes were died 10% and 25% within 4 days of challenge at 3 ppt and 6 ppt treatments, respectively (Table 1). At the end of 14-day challenge period, *F. columnare* was not isolated from survival fishes in all treatments.

There was a correlation between virulence of *F. columnare* and its adherent ability (Zaldivar, 1985; Decostere *et al.,* 1999). Reduction of fish mortality at 3 and 6 ppt could be related to the inhibition of biofilm formation at these salinities. No fish mortality observed at 9, 12 and 15 ppt treatments was also correlated to the greatly inhibition of biofilms in the in-vitro test. The main outcome of this study is that *F. columnare* was quite sensitive to high salinities (Bernardet, 2007). High salinities (≥3 ppt) was highly reduced fish mortality, and this could be considered as a prophylactic measure.

Table 1: Percentage of fish mortality at different salinity treatments

Salinity (ppt)	Treatment	Time of mortality (day post challenge)	Mortality (%)
0	Challenged	1	100
	Control	3	10
3	Challenged	3, 4	10
	Control	*	*
6	Challenged	2, 3, 4	25
	Control	*	*
9	Challenged	*	*
	Control	*	*
12	Challenged	*	*
	Control	*	*

** No mortality**

4 CONCLUSIONS

The biofilm formation of *F. columnare* was inhibited at 3 ppt and 6 ppt. The bacterial biofilm formation was highest at 25ºC and was reduced at 28 and 31ºC. Cell growth of the bacteria was not recovered at 9, 12 and 15 ppt. Fish mortality was highest at 0 ppt treatment with 100%, while there were lower fish mortalities at 3 and 6 ppt treatments with 10% and 25% dead fishes, respectively.

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