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Antibacterial Activities of Striped Snakehead Murrel Fish *Channa striata* Autochthonous Gut Bacterium *Achromobacter xylosixidans* against Bacterial Fish Pathogens

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Abstract: ***In the present study, a total of 25 healthy and infected striped snakehead murrel fishes (Channa striata) were collected from Veeranam Lake of Cuddalore district. Eight morphologically distinct autochthonous bacteria (VLFAu1-8) were isolated from the healthy fish gut and ten different bacterial fish pathogens were isolated from the infected fish samples. Screening of autochthonous isolates for antibacterial activity against bacterial fish pathogens revealed VLFAu6 as the potential strain and it was identified as Achromobacter xylosixidans using 16S rRNA sequencing. The observed antibacterial activities were Vibrio cholerae (2.2cm), Vibrio parahaemolyticus (2.5cm), Vibrio flavius (2.2cm), Salmonella typhi (1.9cm), Salmonella paratyphi (2.3cm), Vibrio fischeri (2.3cm), E.coli (2.4cm), Aeromonas hydrophila (3.1cm), Pseudomonasaeruginosa (2.7 cm) andPseudomonas fluorescens (2.9 cm).The ideal conditions for the maximum growth of A. xylosixidans were 36 h incubation, 160 rpm agitation, pH 7, 37°C, 2.5% salinity, 2% glucose as carbon source and 1.5% yeast extract as nitrogen source and the antimicrobial activity was found to be growth dependent. Hence this study proved that the autochthonous bacterial isolates of fish gut are the capable of producing of antimicrobial compound against fish pathogens tested.***

Keywords: Striped murrel, snakehead fish, *Channa striata*, *Achromobacter xylosixidans*, autochthonous bacteria, antibacterial activity

1. Introduction

Aquaculture is the farming of aquatic organisms such as fish, crustaceans, mollusks, and aquatic plants. This involves cultivating freshwater and saltwater populations under controlled conditions. Under these conditions fishes are continuously facing wide range of pathogenic and nonpathogenic microbial exposures (Shephard, 1994). Pathogens spread from one to other fishes cause infection to them; also cause some serious illness when consumed by human. The most important aquaculture species of murrel in India is the striped murrel (*Channa striata*), the great snakehead murrel (*Channamarulius*) and the spotted snakehead (*Channapunctata*).

Channa striata also called the striped snakehead murrel, is a species of snakehead fish belongs to Channidae family. These species are mostly found in the inland water bodies, freshwater plains, in bottom mud of lakes, canals and swamps and generally referred as the mudfish. They are in great demand in the domestic market of tropical Africa, South East region predominantly in India, Pakistan, Afghanistan, Nepal, Sri Lanka, Thailand and China for its protein content with important economic value as food, medicine and ornamental purposes (Jais, 2007 and Achmad*et al.*, 2019). They have widely been used in traditional medicines for healing surgical wounds, curing kidney disease and impotency (Atif *et al*., 2015 and Rahayu *et al*., 2016).

Microbes collected from natural sources have gained much attraction for their broad range of bioactive properties like antibiotics, antiviral, antitumor, antioxidant and anti-inflammatory. In the fish gut microbiota, bacteria are the major constituent (Spanggaard *et al*., 2000; Pond *et al*., 2006). Autochthonous (associated forms of the intestinal mucosa) bacteria plays significant role in the enteric antagonism, colonization resistance and provide defense in gut mucosa against the fish pathogenic bacteria (Ringø *et al.*, 2007). The present study aims for the isolation and screening of gut associated autochthonous bacterial isolate for the production of antibacterial compound against the fish pathogenic bacteria.

2. Materials and Methods

2.1. Collection of fish samples

A total of 25 fishes (*Channa striata*)including both infected and healthy in live condition were fished out at 10 to 15 feet depth using fishing net in the Veeranam Lake of Cuddalore district. Samples (i.e.) healthy and infected were kept in separate fish collection boxes, immediately transported to the laboratory and processed within 2 h of arrival of the sample for microbial analysis.

2.2 Isolation of autochthonous microbiota from *C. striata* gut

The gut was aseptically dissected out, the mid gut portion alone selected and thoroughly washed three times with PBS (phosphate buffer saline, pH 7.2) to remove the undigested food materials and the allochthonous bacterial flora as described by Ringø (1993). Samples were homogenised 1 ml from each homogenate was mixed with 0.9% NaCl solution and were serially diluted up to 10^{-6} dilution (Das and Tripathi, 1991 and Beveridge *et al.*, 1991). 100µl of sample from each dilution was spread on tryptone soy agar (Hi-

media, Mumbai) and incubated at 37°C for 24-48 h. After incubation, the well isolated colonies with distinct morphologies were selected for further study.

2.3 Isolation of bacterial pathogens from infected fish samples

For theisolation of bacterial pathogens, the infected *C. striata* fishes collected used. The gut and tissue homogenate was prepared as above and 0.1 ml of the serially diluted samples were plated on different selective agar mediums such as **TCBS, SS agar, Mannitol salt agar, EMB agar, Pseudomonas agar, PALCAM agar (Fraser medium), Bismuth sulphate agar and Kenner faecal agar** using spread plate technique. These pathogens were used for testing antibacterial activity screening.

2.4 Screening for antibacterial activityof autochthonous isolates against bacterial fish pathogens

Eight different autochthonous bacterial isolates (ABI)isolated from the healthy *C striata* gut was screened for aantimicrobial activity of the against different fish bacterial pathogens isolated from the infected fish samples using agar well diffusion method on Muller Hinton agar plates (Millette*et al*., 2007). The fish bacterial pathogen isolates were *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio flavius, Salmonella typhi, Salmonella paratyphi, Vibrio fischeri, E.coli,Aeromonas hydrophila, Pseudomonasaeruginosa* and *Pseudomonas fluorescens*. The cell free culture supernatant from each ABI inoculated in Muller Hinton broth were obtained by centrifugation and loaded into the wells made on each bacterial pathogen spread plates and incubated at 37ºC for 18-24 hrs. Based on the diameter of the zone of inhibition, the most potential autochthonous bacterial isolate with antimicrobial activity was selected for further study.

2.5 Identification of the potential strains

The potential autochthonous bacterial isolate (*VLFAu*6) displaying highest antimicrobial activity isolated from the healthy fish (*Channa striata*) gut and the bacterial pathogens from the infected fish tissue samples were identified based on biochemical methods using Bergey's manual of determinative bacteriology (Buchanan *et al*., 1974) and 16S rRNA sequencing was done using universal Eu-bacterial primers 27F (5′AGAGTTTGATCCTGGC TCAG-3′) and 1492R (5′-GGTTACCTTGTTACGA CTT-3′). Sequence similarity search was done using BLAST. Phylogenetic tree was analyzed using MEGA software version 7.0 (Kumar *et al*., 2016).

2.6 Growth optimization and mass culture of *A. xylosixidans*

Growth of the most potent *A. xylosixidans* strain was optimized for different physicochemical parameters such as incubation periods 0-48 h (with 6 h interval), agitation (0-200 rpm with 40 rpm interval), pH 4-10 (with 0.5 interval), temperatures (20, 25, 30, 35, 37, 40 and 45°C), salinity (NaCl concentration - 0.5-3% with 0.5 interval), different carbon sources (sucrose, glucose, maltose, starch and cellulose); ideal carbon source glucose (1-5%) and nitrogen sources (beef extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate and sodium nitrate); ideal nitrogen source yeast extract (0.5-2.5%) were tested. Ideal conditions from the above optimization were used for mass culturing.

The optimized growth conditions such as 36 hrs of incubation, agitation 160 rpm, pH 7, temperature 37°C, 2.5% salinity, 2% glucose as carbon source, 1.5% yeast extract as nitrogen source were maintained in the medium. Mass culture was done in 1L conical flask with 0.5 L of the medium. Growth and antimicrobial activities were evaluated at end of 36 hrs incubation.

3. Result and Discussion

Fish intestine is the major source of aerobic and anaerobic heterophilic bacterial isolation (Sugita *et al*., 1992). The density of microbial population in the gastrointestinal tract of fish is comparatively higher than its surrounding water indicates the favourable ecology of the digestive tract for the survival of both allochthonous (transient) and autochthonous (the established microbiota associated with the intestinal mucosa) bacterial forms (Ringø *et al*., 1995 and Ringø and Birkbeck, 1999). The authochthonous gut bacteria of the fishes are capable of producing many bioactive substances including antimicrobial compounds and hence presence of these native bacterial forms provide host defense and suppress the growth of pathogenic bacteria (Bruijn*et al*., 2018 and Tarnecki *et al*., 2019).

3.1 Collection of fish samples

In the present work, a total of 25 *Channa striata* fishes including both healthy and infected were collected from Veeranam Lake of Cuddalore district (Fig. 1).

3.2 Isolation of autochthonous microbiota from *C. striata* gut and bacterial fish pathogens from the infected fish samples

Eight morphologically different autochthonous isolates (*VLFAu*1-8) were isolated from the healthy *C striata* gut and ten different bacterial fish pathogens were isolated from the infected fish samples (Figs. 2 and 3).

3.3 Screening for antibacterial activity and identification of autochthonous isolates against bacterial fish pathogens

In the present study, eight morphologically different autochthonous isolates isolated from the healthy *C striata* gut were screened for antimicrobial activity against different fish bacterial pathogens isolated from the infected fish samples using agar well diffusion method. When the autochthonous isolates were screened for antibacterial activity against the pathogens showed that the most potential autochthonous isolate *VLFAu*6displayed broad spectrum of antibacterial activity against the tested bacterial fish pathogens. The pathogenic isolates were identified biochemically and the *VLFAu*6 was identified using 16S

rRNA sequencing revealed that it was *Achromobacter xylosixidans* (Fig. 4).

Microbial antagonism plays a significant role in the reduction or elimination of opportunistic pathogens occurrence in the gastrointestinal tract of aquatic animals (Balcazar *et al*., 2006). In the present study, The observed antibacterial activities were *Vibrio cholerae* (2.2cm), *Vibrio parahaemolyticus* (2.5cm), *Vibrio flavius* (2.2cm), *Salmonella typhi* (1.9cm), *Salmonella* paratyphi (2.3cm), *V. fischeri*(2.3cm), *E.*coli (2.4cm), *Aeromonas hydrophila* (3.1cm), *Pseudomonasaeruginosa* (2.7 cm) and *Pseudomonas fluorescens* (2.9 cm) (Fig. 5 and Table 1).

As in the present study, Prayitno*et al.,* 2018 reported antagonistic activity of gut bacteria isolated from *Osteochilusmelanopleurus* fish collected from Melintang Lake, East Kalimantan Province, Indonesia against *Aeromonas hydrophila* (AH-1), *A. hydrophila* ATCCR 35654 and *Pseudomonas* sp. (PS-1). Similarly, Kavitha *et al.*, 2018 reported antagonistic activity of *Bacillus amyloliquefaciens* isolated from the digestive tract of *L. calbasu* against several fish pathogens like *A. hydrophila* (KX756709), *A. veronii* (KX688046), *A. junii* (KX756708), *A. tandoii* (KX775222) *Acinetobacter* sp. (KX775221), and *P. stutzeri* (KX721473) isolated from the naturally infected fish, *Dawkinsiafilamentosa*. Sugita *et al.*, 1996 reported antagonistic activity of the intestinal bacteria isolated from freshwater fish against Aeromonas sp. and Pseudomonas sp. Araujo *et al.,* 2015 in *Oncorhynchus mykiss* gut bacteria against bacteria belonged to the Family of Aeromonadaceae, Enterobacteriaceae, Bacilaceae. Ariole and Aso (2015) reported antibacterial activity of intestinal bacteria isolated from the farmed fish *Clariasgariepinus* against three fish pathogens Vibrio sp., Salmonella sp. and *S. aureus* and three human pathogens *E. coli*, Shigella sp. and Klebsiella sp. Achmad*et al*., 2020 reported antimicrobial activity of *C. striata* extract against periodontal pathogens.

3.4 Growth optimization and mass cultivation of*A. xylosixidans*

Growth optimization revealed that the ideal conditions favored the maximum growth of the potential antimicrobial compound producing autochthonous *A. xylosixidans*isolate were 36 h incubation (2.22 OD), 160 rpm agitation (1.72 OD), pH 7 (1.81 OD), 37^{o}C (1.75 OD), 2.5% salinity (1.8 OD), 2% glucose as carbon source (1.98 OD) and 1.5% yeast extract as nitrogen source (1.93 OD) (Fig. 6).

Growth and antimicrobial activities in the mass cultivation medium at end of 36 h of incubation were 3.42 OD with enhanced antibacterial activity (data not shown). In the present study, the antimicrobial compound production was found to be growth dependent.

4. Conclusion

The present study on isolation of autochthonous bacterial isolates from the gut of the striped snakehead murrel; *Channastriata* collected from the Veeranam Lake of Cuddalore district for potential antibacterial activity the bacterial fish pathogens has proved this a worth attempt. The gut autochthonousisolate *Achromobacter xylosixidans* showed broad range of antibacterial activity against various fish pathogens tested. Hence, the autochthonous bacterial isolates of fish gut are the rich source of antimicrobial compounds.

5. Acknowledgements

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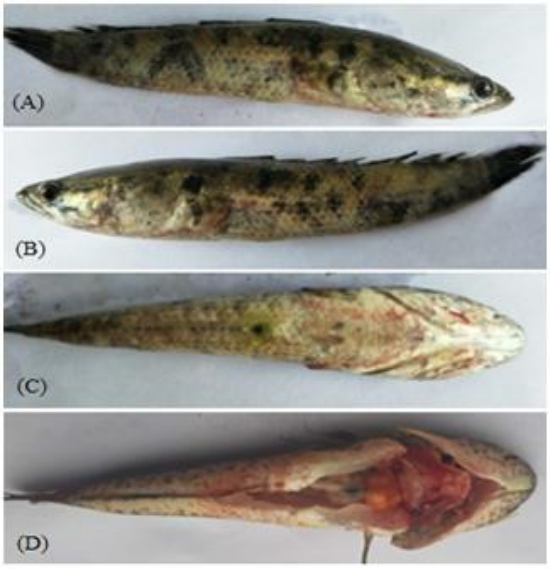

Figure 1: Collection of fish samples; anterior view of striped murrel (*Channa striata*) or Snakehead fish (A and B), posterior view (C) and after the gut dissection (D)

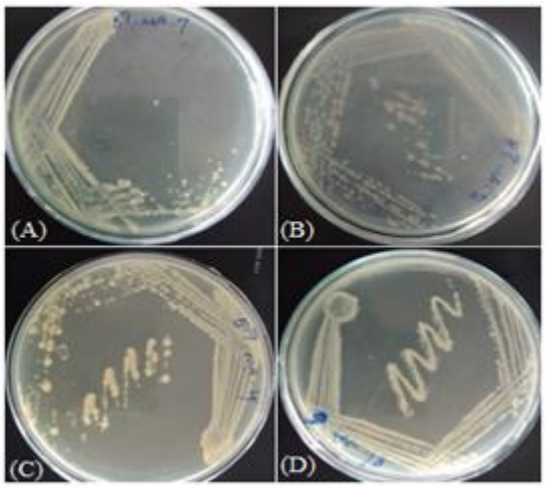

Figure 2: Isolated pure cultures of different autochthonous microbes from the gut of *Channa striata*on nutrient agar plates (A-D)

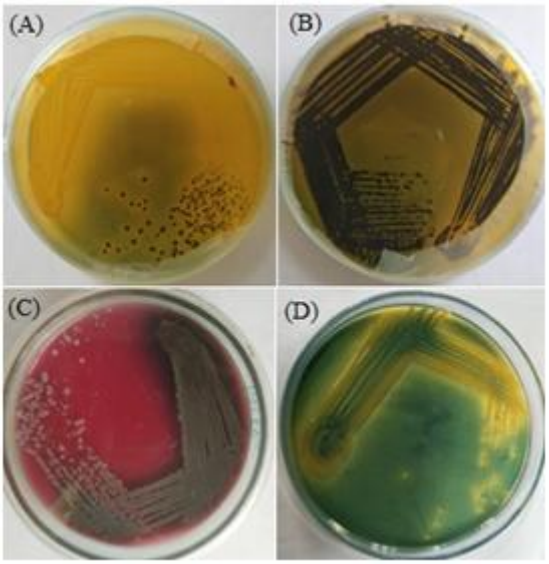

Figure 3: Isolation of different bacterial pathogens of *Channa striata* (A-D) on selective agar medium plates

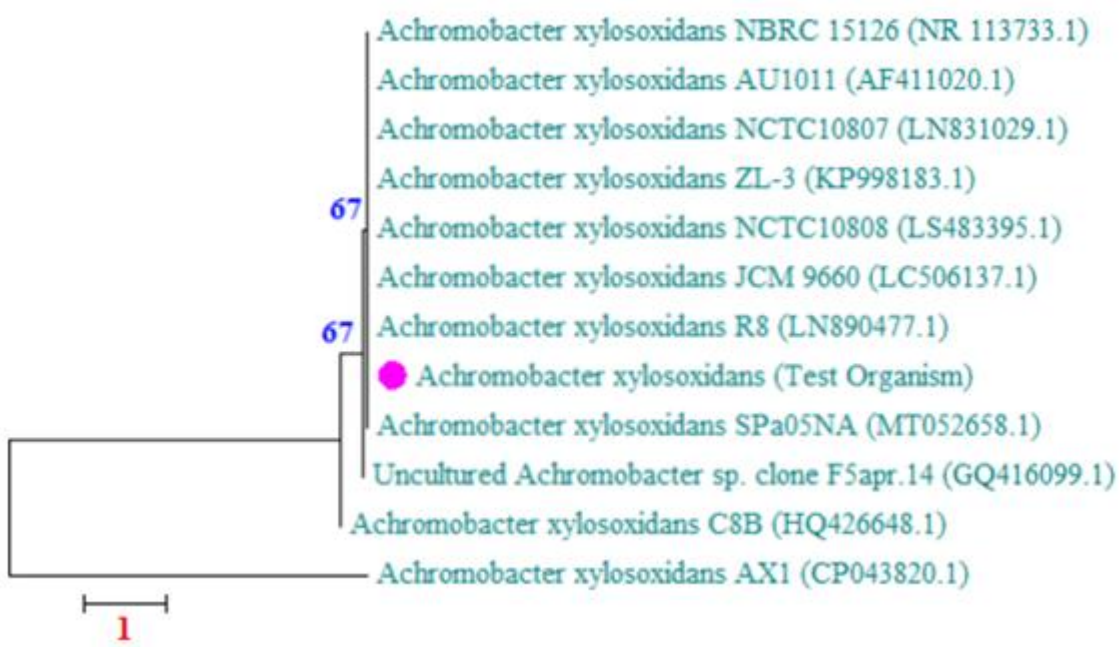

Figure 4: Evolutionary analyses of *Achromobacterxylosoxidans*and phylogenetic tree construction using neighbor-joining method

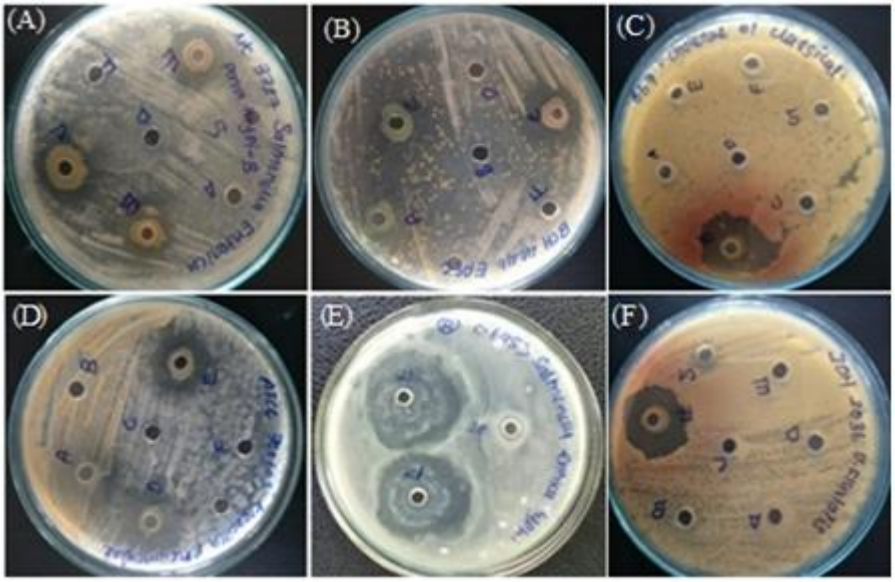

Figure 5: Antibacterial activity of antimicrobial compound produced by *A. xylosoxidans*against different bacterial pathogens (A-F)

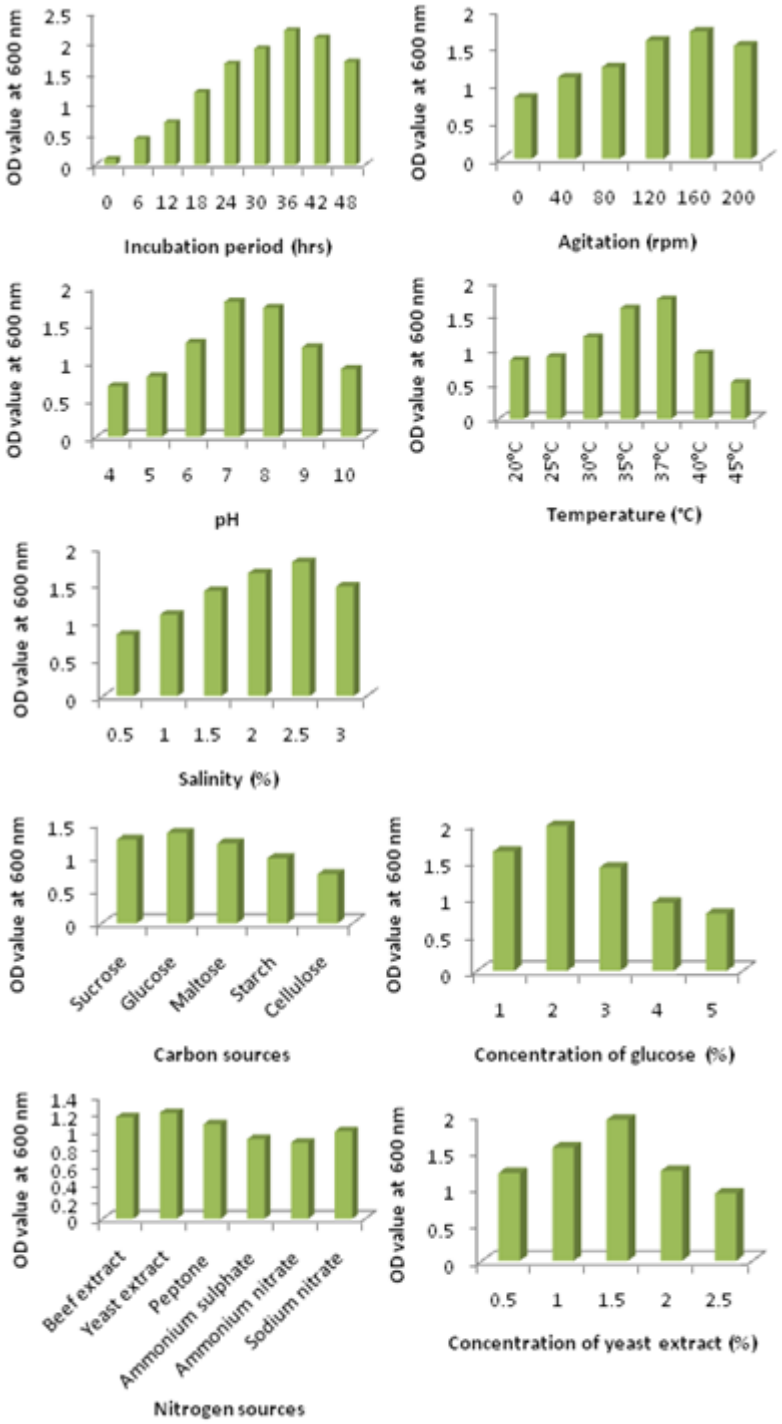

Figure 6: Effect of various physicochemical parameters on growth of potent antimicrobial compound producing isolate *A. xylosoxidans*

Table 1: Antibacterial activity of cell free supernatant of *C. striata* gut autochthonous isolate *A. xylosixidans* against bacterial fish pathogens

Pathogens tested	Zone of clearance in diameter (cm)
Vibrio cholerae	2.2
Vibrio parahaemolyticus	2.5
Vibrio flavius	2.2
Salmonella typhi	1.9
Salmonella paratyphi	2.3
Vibrio fischeri	2.3
Escherichia coli	2.4
Aeromonas hydrophila	3.1
Pseudomonas aeruginosa	2.7
Pseudomonas fluorescens	2.9