The Effect of Marine Probionts on the Growth of Fish and Shellfish Pathogens

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Abstract: Four isolates of *Pseudomonas* spp. from brackish water were screened for antimicrobial activity against ten fish and shellfish pathogens (*Vibrio* sp., *Salmonella* spp., *Staphylococcus* spp., *Escherichia coli* and *Aeromonas* spp.) by agar well diffusion assay. Two isolates of *Pseudomonas* spp.(P2 and P3) were found active against all the bacterial strains. The challenging experiment showed that *Pseudomonas* P2 with 1.0 x 10^6 cfu/ml was enough to suppress *Salmonella* SM1 within 12 hours. The isolated strains *Pseudomonas* P2 and *Pseudomonas* P3 could have potential against *Salmonella* SM1 under *in vitro* condition and might be useful as biological control agents in fish and shellfish culture.
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1. Introduction

Fish and shellfish farming are the fastest growing food-protein producing sector in the world. (Qi *et al*., 2009). An important issue affecting production is the loss of stock through disease. When faced with disease problems the common response has been to turn to antimicrobial drugs (antibiotics). Nevertheless, the continuous use of antibiotic has contributed to the occurrence of antibiotic-resistant bacteria population (Rahman *et al*., 2009) and to an increase in more virulent pathogens. Furthermore, some chemicals used in fish and shellfish such as organotin compounds, copper compounds and other compounds, with a high affinity to sediment, leave persistent, toxic residues and are likely to have a negative impact on the environment (Graslund *et al*., 2003).

The problem of antibiotic resistance and its epidemiological consequences led to the exploration of several alternative approaches for disease management in aquaculture systems. The research of probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture. Some probiotics were designed to treat the rearing medium, like biocontrol when the treatment is antagonistic to pathogens or bioremediation when water quality is improved. Probiotics have also found use in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds (Irianto and Austin, 2002; Sahu *et al*., 2008).

In Nigeria, probiotics research is still at its infancy and data on aquatic indigenous probiotics are lacking. Therefore, the present study was aimed at isolating indigenous strains of *Pseudomonas* from brackish water and evaluating their probiotics effect on some isolated pathogenic bacteria of fish and shellfish.

2. Materials and Methods

2.1. Sample Collection

Brackish water was collected in sterile plastic bottle from Sombriero River in Buguma, Rivers State of Nigeria. Moribund shrimp (*Penaeus monodon*) and fish (*Tilapia guineensis*) were collected from the same river through the assistance of local fishermen.

2.2. Bacterial Isolation

The Brackish water sample was diluted in a range 1:10 to 1:100. Sub samples of 0.1ml of both the diluted and the undiluted brackish water samples were plated on Pseudomonas cetrimide agar (Oxoid). The moribund fish and shrimp were cleaned externally with ethanol and their gastro-intestinal tracts dissected under sterile conditions. The gut contents were weighed and placed in a physiological solution and then diluted in a range 1:10 to 1:1000. Sub samples of 0.1ml of the dilutions were plated on five different media. The media chosen were: Thiosulphate citrate bile salt sucrose (TCBS) agar (Oxoid) (for *Vibrio* species), Salmonella Shigella agar (Fluka) (for *Salmonella* species), Mannitol salt agar (Lab M) (for *Staphylococcus* species), MacConkey agar (BioTech) (for *Escherichia coli*) and Aeromonas medium with supplement (Ryan) (Oxoid) (for *Aeromonas* species). All the media were supplemented with 1.0 % sodium chloride and incubated at 37^{O}C for 24 – 48hours. Isolates with distinct colony morphology were picked and streaked repeatedly on nutrient agar plates until pure. The purified isolates were identified to generic level

based on their morphological and physiological characteristics (Holt *et al*., 1994).

2.3. Determination of Antimicrobial Activity

The antimicrobial activity was first determined by agar diffusion method (Baydar *et al*., 2004 and Dobner *et al*., 2003). Further study was made by broth assay where *Pseudomonas* P2 and *Salmonella* SM1 were mixed and survival determined by plate counting at various time intervals from 0 to 48hours (Chythanya *et al*., 2002).

2.4. Agar diffusion assay

Antimicrobial activity of four isolates of *Pseudomonas* spp. was carried out against ten target strains. Wells were punched with a cork borer (6mm, diameter) in plates of nutrient agar freshly seeded with 0.1ml of 24 hour old broth culture of each tested bacterial stains. Exactly 0.1ml of a 24 hour old broth culture of each of the *Pseudomonas* strains and the control (nutrient broth containing 1.0% sodium chloride) were put into the wells. The plates were incubated for 24hours at 37^{O}C. The diameter of clear zones surrounding the wells were measured and recorded expressing the antibacterial activity.

2.5. Effect of *Pseudomonas* P2 on growth of *Salmonella* SM1 in sterile nutrient broth.

Two 250mL flasks containing 100mL of nutrient broth containing 1.0% sodium chloride was sterilized at 121^{O}C for 15 minutes. Cell suspension of *Salmonella* SM1 was then added to all flasks to get a cell density of approximately 1.0 x 10^5 cfu/ml. Cell suspensions of *Pseudomonas* P2 adjusted to 1.0 x 10^6 cfu/ml final cell concentration were added to one flask while the other flask without *Pseudomonas* P2 added served as control. The cultures were incubated at 37^{O}C for 48 hours with manual shaking at intervals. *Pseudomonas* and *Salmonella* SM1 were enumerated at 0, 12, 24 36 and 48 hour on Pseudomonas cetrimide agar (Oxoid) and Salmonella Shigella agar (Fluka) respectively by standard spread plate method.

3. Results Analysis

The antibacterial activity of fish and shellfish pathogens by *Pseudomonas* spp. are shown in Table 1 and Figure 1.

A total of four bacterial strains identified as *Pseudomonas* P1, *Pseudomonas* P2, *Pseudomonas* P3 and *Pseudomonas* P4 were isolated from brackish water. Seven pathogenic isolates from moribund shrimp (*Penaeus monodon*) were identified as *Vibrio* sp. V2 *Salmonella* sp. SM1, *Salmonella* sp. SM2, *Staphylococcus* sp. ST1, *Staphylococcus* sp. ST2, *Escherichia coli* E1 and *Aeromonas* sp. AS1 while three pathogenic isolates from moribund fish (*Tilapia guineensis*) were identified as *Aeromonas* sp. A5, *Aeromonas* sp. A6 and *Aeromonas* sp. A8. *Pseudomonas* sp. P2 and *Pseudomonas* sp. P3 produced inhibition zones higher than 8mm and against all the pathogenic strains employed while *Pseudomonas* P1 and *Pseudomonas* P4 had no antibacterial activity against the pathogens.

The inhibition of *Salmonella* SM1 (1.0 x 10^5) cfu/ml) by *Pseudomonas* P2 (adjusted to 1.0 x 10^6 cfu/ml final cell concentration) in nutrient broth containing 1.0% sodium chloride is shown in Figure 2. The *Pseudomonas* P2 could inhibit *Salmonella* SM1 growth within 12 hours. It was found that the concentration of *Salmonella* SM1 was constant (about 10^3 cfu/ml) until 48hours. For the control, an increase of *Salmonella* SMI was observed from about 10^5 to 10^6 cfu/ml.

Table 1: Antibacterial activity of *Pseudomonas* spp. against fish and shellfish pathogens

	Inhibition zone (cm) ± S. D.							
Pseudomonas spp.	*Vibrio sp. V2*	*Salmonella sp. SM1*	*Salmonella sp. SM2*	*Staphylococcus sp. ST1*	*Staphylococcus sp. ST2*	*Escherichia coli E1*	*Aeromonas sp. AP1*	*Aeron sp. AS*
P1	-	-	-	-	-	-	-	-
P2	1.7±0.00	1.8±0.00	1.0±0.01	1.8±0.00	0.9±0.03	1.2±0.01	1.2±0.01	2.1±0
P3	1.6±0.01	1.0±0.00	1.2±0.00	1.2±0.02	1.2±0.01	1.6±0.02	1.0±0.00	2.3±0
P4	-	-	-	-	-	-	-	-

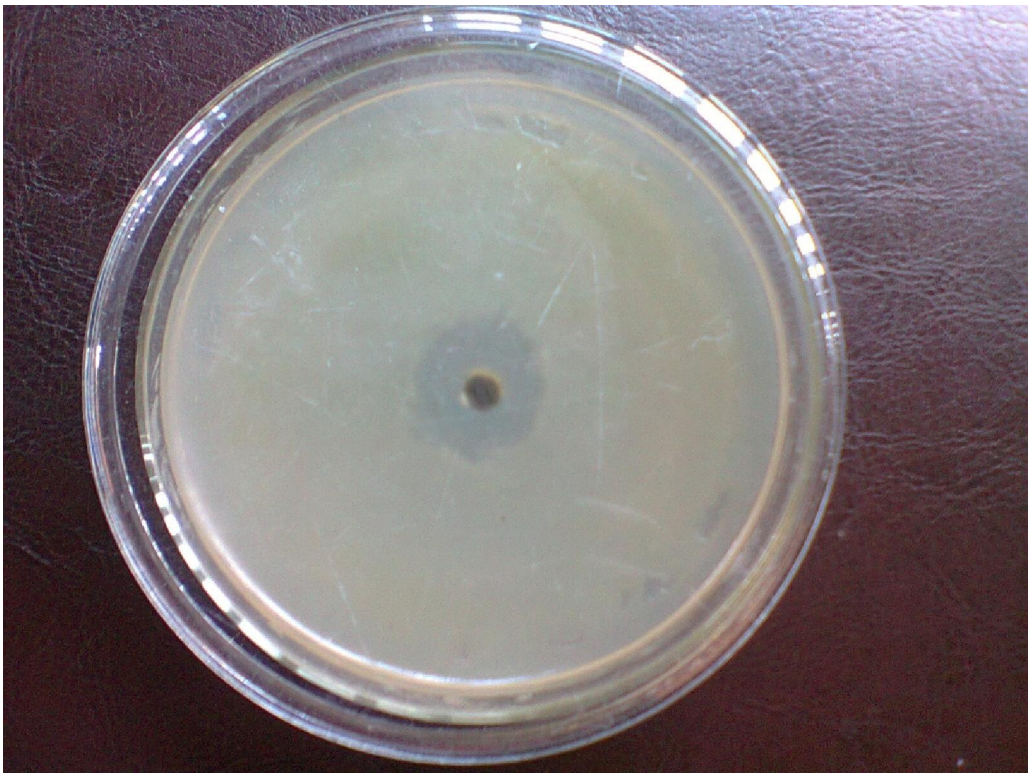

Figure 1. Inhibition zone of *Pseudomonas* sp. P2 against *Salmonella* SM1

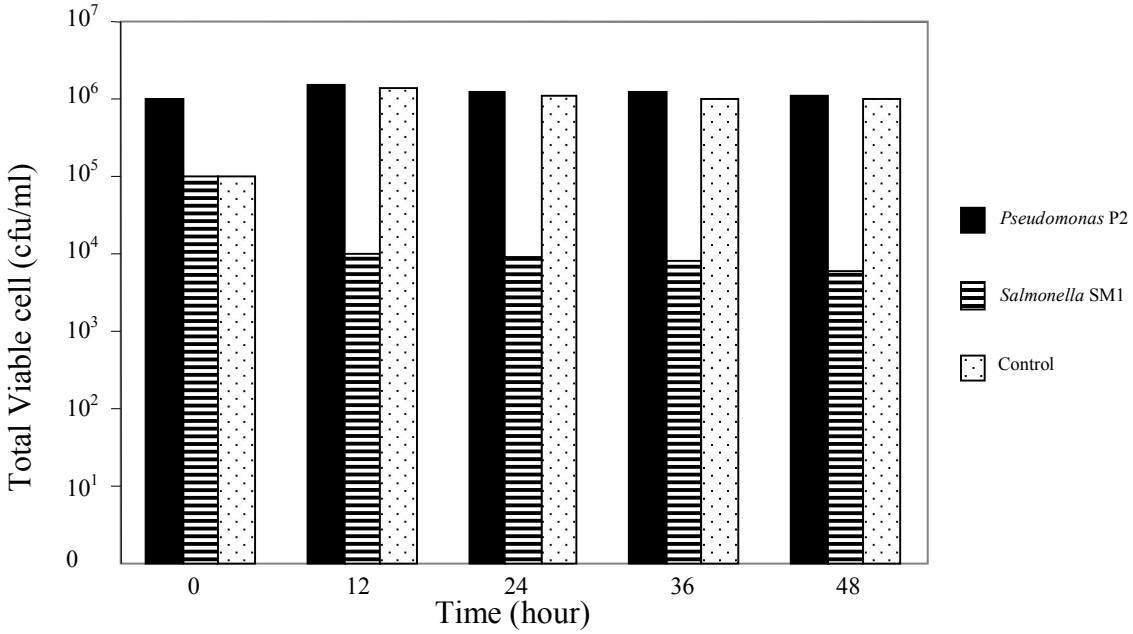

Figure 2: Effect of *Pseudomonas* P2 at 1.0 x 10^6 cfu/ml on growth of *Salmonella* SM1 in nutrient broth containing 1.0% sodium chloride

4. Discussion

The present study reports two marine probionts, *Pseudomonas* P2 and *Pseudomonas* P3, isolated from brackish water from Sombriero River in the Niger Delta which showed antimicrobial activity against a range of pathogenic bacteria isolated from moribund fish and shellfish from the same river (Table 1, Figure 1 and 2).

The growth of *Salmonella* SM1 and other fish and shellfish pathogens used in this study were inhibited by *Pseudomonas* spp. because *Pseudomonas* can secret antimicrobial compounds.

Numerous studies have implicated siderophores as bacteriostatic substances produced by *Pseudomonas* species (Guerinot, 1994; Raaijmakers *et al*., 1997; Vijayan, 2000). Various strains of *Pseudomonas* spp. have been reported as effective against *Vibrio harveyi* and other *Vibrio* species determined by using agar diffusion technique (Torrento and Torres, 1996: Chythanya *et al*., 2002; Vijayan *et al*., 2006). Chythanya *et al*. (2002) reported that *Pseudomonas* 1-2 strain displayed antimicrobial activity against shrimp pathogen, *Vibrio harveyi* (diameter 1.7cm). This result is similar to the one obtained in this study where *Pseudomonas* P2 displayed antimicrobial activity against shrimp pathogen, *Vibrio* sp, V2, with inhibition zone diameter of 1.7 cm (Table 1).

A number of earlier studies have also shown that bacteria produce inhibitory substances that inhibit the bacterial pathogens in aquaculture systems (Austin *et al.,* 1995; Rengpipat *et al*., 1998; Gram *et al*., 1999). The use of such bacteria to inhibit pathogens by release of antimicrobial substances is now gaining importance in fish and shrimp farming as a better and more effective alternative than administering antibiotics to manage the health of fish and shrimp (Verschuere *et al*., 2000; Vine *et al*., 2004). Therefore, the isolated indigenous strains of *Pseudomonas* P2 and P3 had the inhibitory property of a biocontrol agent for use in control of fish and shellfish pathogens and might be useful for replacing the commercial antibiotics. Further co-culture experiments to determine the minimum inhibitory concentration of the antagonists against the pathogenic strains, the species identification and optimization of *Pseudomonas* growth are going on in our laboratory. Furthermore, the *in vivo* effect on pathogen in fish will be a further course of work.

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