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SCREENING FOR ANTIBIOTIC PRODUCING MARINE BACTERIA AGAINST FISH PATHOGENS

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ABSTRACT

The need for the development of new antibiotics to counter drug resistance in bacterial pathogens has been stressed by various researchers worldwide. As the discovery of novel chemical classes have been in decline for the past two decades, the need to exploit new resources in search for effective chemicals with novel mechanism of actions is imperative. Marine bacteria are such a resource yet to be tapped, and the potential it offers is vast. Aquaculture has developed into a prime industry to tap the enormous turnover of bio-energy for the benefit of mankind. Since aquaculture depends on self-renewable natural resources, it has infinite future for hundreds of years to come. Aquaculture is also being viewed as the only alternative to augment fish production due to over exploitation of natural fish resources. In this present study a total of 633 marine bacterial strains were isolated. In the initial antibiotic production test using *E.coli*, 170 strains were found to be antibiotic producers and out of which 101 strains were found to be active against 3 major fish pathogens namely *Vibrio harveyi, V. parahaemolyticus, Aeromonas hydrophilla.* In the genus level identification of the potential strains the genus *Alteromonas sp*. dominated followed by *Streptomyces sp., Vibrio sp., Bacillus sp., Flavobacterium sp*. and *Pseudomonas sp.*

KEYWORDS

Antibiotics, Marine bacteria, Aquaculture, antimicrobial activity

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INDRODUCTION

Aquaculture has been the world's fastest growing food production system for the past decade [1,2]. Aquaculture production increased from 7.4 million tones in 1980 to more than 42 million tones in 1999 valued over US$53 thousand million. The sector's production is growing at an average rate of more than 10% per year as compared with a growth of 3% for terrestrial livestock meat production and 1.5% for capture fisheries production. In 1997, over 30% of food consumed by humans is provided by aquaculture [3].

Global projections for future supplies from aquaculture production are 47 million tonnes for the year 2010. But with this remarkable worldwide growth has emerged problems of success, particularly catastrophic animal diseases. Since its beginning 25 years ago, for example, high production shrimp farming has been a 'boom and bust' phenomenon. Nearly all of the farmed shrimp in the world is grown in South American and Asian countries such as Taiwan, Thailand, Indonesia and Ecuador. Yet virtually every country with a major shrimp farming industry in the 1980s and early 90s suffered viral epidemics. In Asia alone, shrimp viral diseases caused farmers about $ 1 billion loss a year since 1994[4].

The impact of diseases in aquaculture is enormous and millions of dollars is lost annually worldwide. These disease outbreaks may be the result of pathogens or of erroneous husbandry practices. The diseases were initially controlled almost exclusively by the use of antimicrobial drugs. There is a growing concern about the use and particularly the abuse of antimicrobial drugs not only in human medicine and agriculture but also in aquaculture. The massive use of antimicrobials for disease control and growth promotion in animals, increases the selective pressure exerted on the natural emergence of bacterial resistance[5]. Hence the need of the hour is a search for novel antibacterial compounds with therapeutic potential for which the pathogens may not have resistance[6].

The present study was undertaken to screen for antibiotic production by marine bacteria against three major fish pathogens such as *Vibrio parahaemolyticus, Vibrio harveyi*, and *Aeromonas hydrophila* .

MATERIALS AND METHODS

Screening for antibiotic production by marine bacteria against fish pathogens. The screening for antibiotics production against fish pathogens was carried out using the methods of Spragg[7].

In the first method, antibiograms of marine strain was streaked onto TSA (Tryptone Soya Agar + 1% NaCl) and incubated at room temperature for 5 days. Test strains *Vibrio parahaemolyticus, Vibrio harveyi,* and *Aeromonas hydrophila* were streaked perpendicular to the marine strain onto the agar and incubated overnight. Inhibitory activity was indicated by inhibited growth of pathogenic strain on the agar as compared to the control plate showing healthy growth of the test strains.

In the second method, all the marine strains were inoculated into 100 ml of Zobell marine broth individually. The inoculated strains were broth cultured in a rotary shaker at 260 rpm for 5-7 days at room temperature (27°C). Then the broth culture was extracted using equal volume of ethyl acetate using a magnetic stirrer for 30 minutes. The two phases were then separated in a separatory funnel and the ethyl acetate phase was removed and concentrated by evaporation. The concentrate (crude extract) was then impregnated onto sterile Whatman No.1 disc (6 mm) and antibacterial activity was assayed following the conventional disc-diffusion assay. The inhibition zone was measured from the border of the disc to the edge of the clear zone, and the results were tabulated.

The potential strains were identified upto genus level following the biochemical methods outlined in Bergey's manual of systematic bacteriology[8,9].

RESULTS

A total of 170 strains were screened for antibacterial activity against three fish pathogens and out of that 101 strains were found to be active. In the algae associated bacteria 32 out of 49 strains exhibited activity, of which 13 strains exhibited potential activity (inhibition zone 7mm and above) and 9 strains exhibited broad spectral activity (activity against all the 3 pathogens) (Table 1).

Table 1

Antibacterial activity of algae associated bacterial against fish pathogens

Strains	Zone of Inhibition (mm)		
	V. harveyi	*V.parahaemolyticus*	*Aeromonas hydrophila*
AA2	-	-	-
AA5	7	-	3
AA7	t	2	T
AB3	-	-	-
AB6	5.5	4	t
AB9	-	-	7
AB19	t	-	-
AB21	8.5	7	3.5
AC3	-	-	-
AC6	-	-	-
AD3	4.5	2	t
AD7	-	-	t
AD9	7	8	-
AD15	1	-	-
AD16	12	7.5	-
AD18	t	1	3.5
AE2	-	-	-
AE3	2.5	3	-
AE4	5	3	1
AE7	-	-	t
AE8	2.5	5	4
AF2	7.5	t	3
AF3	1	t	7.5
AF12	-	-	-
AF15	11.5	8	-
AF16	4.5	2	1
AG1	3.5	2	4
AG2	6.5	4	t
AG4	-	-	-
AG11	1	t	-
AG12	2	3.5	1
AH4	-	-	-

AH13	-	t	-
AI9	7.2	7	-
AI11	2	t	-
AI13	9	7	2
AJ3	t	1	-
AJ10	6.5	5	-
AK4	t	1	7.5
AK6	2	3	t
AK10	5	4	2
AK14	10.5	12	7
AK17	7	6.5	4
AL3	-	-	-
AL7	t	1	-
AL11	-	t	-

In sponge associated bacteria, antibacterial activity was exhibited by 13 strains but potential activity was exhibited by 7 strains of which 2 strains showed broad spectral activity (Table 2).

Table 2
Antibacterial activity of sponge associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
SA3	-	-	-
SA6	-	8	-
SB3	7.5	-	-
SB5	-	1	t
SC1	-	-	-
SC2	3	-	-
SC3	6.5	2	9.5
SE2	-	-	t
SE3	8.5	9	-
SE5	-	-	-
SE11	1.5	2	t
SF5	-	-	1
SF6	7	-	-
SG5	-	-	-
SH2	-	-	7.5
SH3	-	-	t
SH5	1	2	-
SH6	-	-	-
SH8	3.5	5	-
SJ2	t	-	-
SJ4	-	-	-
SJ7	7.5	5	7

Fifteen strains from biofilm and sediment samples were found to be active, with 5 potential strains including one with broad spectral activity (Tab.3).

Table 3

Antibacterial activity of biofilm and sediment associated bacteria against fish pathogens.

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
BFA7	7.5	-	-
BFA8	-	-	-
BFA9	7	3.5	t
BFA18	15	10	-
BFA19	-	-	-
BFB6	t	4.5	2
BFB12	t	-	8
BFB13	2	t	-
BFC2	-	-	-
BFC3	7.5	6	2
BFC15	-	-	-
BFC16	2.5	3	t
SM1	8	7.2	-

Among the 7 strains isolated from sea cucumber, sea urchin and jellyfish 5 strains were active. Potential activity was exhibited by 4 strains including two strains with broad spectral activity (Tab.4).

Table 4

Antibacterial activity of sea cucumber, sea urchin and jellyfish associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
SCU2	-	-	-
SCU7	7.6	6	t
SUR3	3.5	2	1
SUR4	8	7	-
SUR7	6.5	7	5
JF2	-	-	-
JF4	7	8	-

Six strains from the gut microflora of gastropods exhibited antibacterial activity. Potential activity was exhibited by two strains, with one strain exhibiting broad spectral activity (Table5).

Table 5
Antibacterial activity of gut microflora of gastropods against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
GMA3	-	-	-
GMA4	t	2	-
GMA8	4.5	6	3
GMB2	9	7.5	-
GMB3	2	t	7
GMB8	-	-	-
GMC1	-	-	-
GMC4	t	2	t
GMC8	2	1	-
GMC9	-	-	-
GMC10	-	-	-

The strains isolated from ophisthobranch surface were used for the antibacterial assay against fish pathogens and among the 10 strains, 5 strains exhibited potential activity with 3 strains exhibiting broad spectral activity (Table 6).

Table 6
Antibacterial activity of ophisthobranch surface bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
OBSA1	-	-	-
OBSA2	8.2	7	t
OBSA9	t	-	7.5
OBSA12	5.5	3	1
OBSA18	7.8	-	-
OBSB2	-	-	-
0BSB3	2.5	1	t
OBSB4	7.5	6.2	5
0BSB5	8	7	4.5
OBSB9	-	-	-

Among the 11 strains isolated from crabs, two strains were found to be potential and 2 strains exhibited broad spectral activity (Table 7). From ascidians, out of 8 strains, 5 strains exhibited antibacterial activity, 3 strains were found to be potential and 1 strain exhibited broad spectral activity (Table 8).

Table 7
Antibacterial activity of crab associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
CA4	-	-	-
CA7	2	1	-
CB2	5.5	6.2	t
CB3	7	9	6
CB7	-	-	-
CB9	-	-	-
CC1	8.4	7.2	1
CC2	-	-	-
CC6	5.5	3.5	t
CC7	-	-	-
CC10	-	-	-

Table 8
Antibacterial activity of ascidian associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	V. parahaemolyticus	Aeromonas hydrophila
ASA2	2.5	3	-
ASA3	7.5	8	t
ASA4	-	-	-
ASA7	12.5	9.2	t
ASB2	1.5	3	-
ASC1	13	10.5	5
ASC3	-	-	-
ASC5	-	-	-

From the bacterial strains isolated from crab and molluscan eggs, 11 strains out of 21 were found to be active, of which six strains were found to be potential and 4 strains exhibited broad spectral activity (Table 9). Among the14 strains isolated from three species of corals, 8 exhibited activity and 4 strains were found to be potential exhibiting broad spectral activity (Table 10).

Table 9
Antibacterial activity of egg associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	*V. parahaemolyticus*	*Aeromonas hydrophila*
CE4	t	t	-
CE5	9	7	2
CE11	6.5	5	3
CE12	-	-	-
GE3	8.2	11	-
GE4	1	2.5	t
GE6	-	-	-
GE10	5	-	7.5
GE11	-	-	-
SPE2	-	5	-
SPE3	t	-	-
SPE4	3	7.5	1
SPE5	-	-	-
SPE9	13	8	7
SQE1	2	3	-
SQE2	7	6.5	t
SQE3	-	-	-
SQE4	1.5	3	t
SQE5	-	-	-
SQE6		-	-
SQE8	-	-	-

Table 10
Antibacterial activity of coral associated bacteria against fish pathogens

Strains	Pathogens (Inhibition Zone in mm)		
	V. harveyi	V. parahaemolyticus	Aeromonas hydrophila
BCL5	3	2.5	t
BCL7	13	7.6	5
SCL2	-	-	-
SCL3	14	12.5	8
SCL9	t	3	2.5
SCL10	-	-	-
SCL12	-	-	-
STCL4	5.5	6.2	3
STCL5	1	1	t
STCL9	9	7.2	6.2
STCL13	t	-	-
STCL14	7.5	7	-
STCL15	-	-	-
STCL17	-	-	-

Out of the total number of strains screened, 59.4% was active against fish pathogens. Out of the total producers 50.4% was found to be potential and 28.7% exhibited broad spectral activity.
In the genus level identification of the potential strains the genus *Alteromonas sp*. dominated followed by *Streptomyces sp., Vibrio sp., Bacillus sp., Flavobacterium sp*. and *Pseudomonas sp*. (Table 11).

Table 11
Genus level identification of potential strains exhibiting antibacterial activity against fish pathogens

Strains	Genus
AA5	*Alteromonas sp.*
AB9	*Pseudomonas sp.*
AB21	*Bacillus sp.*
AD9	*Streptomyces sp.*
AD16	*Alteromonas sp.*
AF2	*Flavobacterium sp.*
AF15	*Micrococcus sp.*
AI9	*Alteromonas sp.*
AM 3	*Alteromonas sp.*
AK4	*Alteromonas sp.*
AK14	*Streptomyces sp.*
AK17	*Streptomyces sp.*
SA6	*Alteromonas sp.*
SC3	*Vibrio sp.*
SE3	*Bacillus sp.*
SF6	*Alteromonas sp.*
SH2	*Alteromonas sp.*
SJ7	*Streptomyces sp.*
BFA7	*Flavobacterium sp.*
BFA9	*Vibrio sp.*
BFA18	*Vibrio sp.*
BFB12	*Bacillus sp.*
BFC3	*Bacillus sp.*
SM1	*Alteromonas sp.*
SCU7	*Bacillus sp.*
SUR4	*Micrococcus sp.*
JF4	*Streptomyces sp.*
GMB2	*Vibrio sp.*
GMB3	*Alteromonas sp.*
OBSA2	*Alteromonas sp.*
0BSA9	*Pseudomonas sp.*
OBSA18	*Streptomyces sp.*
0BSA5	*Vibrio sp.*

CB3	*Alteromonas sp.*
CC1	*Flavobacterium sp.*
ASA3	*Flavobacterium sp.*
ASA7	*Streptomyces sp.*
ASC1	*Vibrio sp.*
CE5	*Alcaligenes sp.*
GE3	*Micrococcus sp.*
SPE4	*Streptomyces sp.*
SQE2	*Pseudomonas sp.*
BCL7	*Alteromonas sp.*
SCL2	*Flavobacterium sp.*
STCL9	*Streptomyces sp.*
STCL14	*Bacillus sp.*

DISCUSSION

Occurrence of diseases in fish culture systems pose a great threat to fish farmers due to heavy losses. Indiscriminate use of antibacterial compounds has led to the development of resistant strains of fish bacterial pathogens[10]. Plasmid-mediated resistance to antimicrobials has been identified in a number of bacterial fish pathogens including *Aeromonas salmonicida, A.hydrophila, Vibrio anguillarum, Pseudomonas fluorescens, Pasteurella piscida* and *Edwardsiella tarda*[11]. In clinical terms there is ample evidence that antibiotic-resistant strains of fish pathogens have developed over the time in which antibiotics have been used to control fish disease. The antibiotic resistance can be transferred experimentally from a fish pathogen to a potential human pathogen. Wound infection remains a possible route by which infection may occur and resistant bacteria infecting such wounds could be difficult to treat. *Aeromonas hydrophila, Vibrio* sp. and *Mycobacterium* sp. are the organisms most likely to be involved and the persons most exposed will be fish farm staff and staff working in fish processing units. So discovery of new class of antibiotics becomes imperative to counter these single as well multiple antibiotic resistance bacterial pathogens.

In the present study 170 marine bacterial strains were screened for their antibacterial activity against 3 major fish pathogens. Out of the 170 strains 101 strains were found active against these pathogens. Lemos[12] screened 224 bacterial strains isolated from macroalgae and tested against many human and fish pathogens namely *Vibrio anguillarum*, *Virbrio harveyi, Pasteurella piscicida* and *Aeromonas hydrophila*. Patil[6] reported the control of bacterial pathogens associated with fish diseases by antagonistic marine actinomycetes isolated from marine sediments. They screened marine actinomycetes against *Aeromonas hydrophila, A.sobria* and *Edwardsiella tarda* and found that 77 isolates were active against the fish pathogens out of 104 isolates. In this present study many potential strains exhibiting 7 mm and above inhibition zone were identified. A zone of 13 mm was exhibited by 2 strains, isolated from an ascidian (ASC 1) and a coral species (BCL7). Maximum zone of 14 mm was noted against *Vibrio harveyi* by another strain (SCL3) isolated from a soft coral. James era/., 1996 purified an antibacterial protein active against *Vibrio anguillarum* isolated from an ascidian *Ciona intestinalis*. Out of the screened strains 50.4% of the strains exhibited potential activity, which is a significant result. Jayant[13] reported

37.35% exhibiting antagonism against fish pathogens. In comparison, the results seems significant because more than 50% of the strains screened were found to be potential. This may be due to the fact that the screened bacterial strains were already found to be antibiotic producers against human pathogens.

Strains exhibiting broad spectral activity against the 3 pathogens used for the assay were 28.7%, which is higher than that of human pathogens - 14% (Fig4). The result of this screening is encouraging and many active novel metabolites may be isolated and purified against fish pathogens from these strains. The possibility for using egg-derived strains as potential probiotic bacteria is also seen.

Many potential strains came under the genus *Alteromonas* followed by *Streptomyces sp., Vibrio sp., Bacillus sp., Flavobacterium sp*. and *Pseudomonas sp*. Until 1999, 63 bioactive compounds have been reported from marine *Alteromonas sp*., 140 from *Streptomyces sp*., 19 from *Vibrio sp*., 12 from Bacillus sp., 4 from *Flavobacterium sp*., 21 from *Pseudomonas sp*.[14].

According to WHO, much needs to be done to reduce the overuse and inappropriate use of antimicrobials. The emphasis in disease management should be on prevention, which is likely to be more cost effective than cure[15]. In aquaculture the overall antibiotic use was reduced due to antibiotic resistance and residue problem. The addition of probiotics is now a common practice in commercial shrimp hatcheries in Mexico[16]. According to Browdy[17] one of the most significant technologies that have evolved in response to disease control problems is the use of probiotics. In view of the above statement, it can be concluded in a positive note that many bacterial strains in this study showing antagonism against fish pathogens can be evaluated for their potential as probiotic bacteria for disease management in aquaculture.

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