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Archives of Applied Science Research, 2011, 3 (3):85-93

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ISSN 0975-508X
CODEN (USA) AASRC9

Biochemical characterization and cytotoxicity of the *Aeromonas Hydrophila* isolated from Catfish

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ABSTRACT

The present study deals with the isolation and characterization of protease producing Aeromonas hydrophila from diseased catfish. To isolate Aeromonas hydrophila from diseased catfish, Confirmation using biochemical tests. The effect of pH, temperature on the growth of Aeromonas hydrophila was conducted and checked the cytotoxicity of the enzyme protease responsible for pathogenicity in fishes. It was screened for resistance against ampicillin, amoxicillin, clindamycin, erythromycin, oxytetracycline, cloaxicillin and streptomycin. Protease producing Aeromonas hydrophila from a diseased catfish isolate was multidrug resistant. Enzyme protease reported as the major virulence factor was extracted and partial purification was done. A cytotoxicity test in mice DLA cell lines of this enzyme in the culture supernatant was carried out. Partially purified enzyme samples revealed its lethal characteristics. After exposure to culture supernatant, the cell death was observed after 12 hours. After exposure to the partially purified enzyme cells were detached from the monolayer (12 hours) indicating the cytotoxicity of the enzyme.

Keywords: Catfish, Pathogenicity, Antibiotic treatment, Cytotoxic test.

INTRODUCTION

Aeromonas hydrophila and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world and these bacteria frequently cause disease among cultured and feral fishes. From the description of fish diseases in early scientific literature Otte[15]. Speculated that septicaemic infections in fish caused by motile aeromonads were common throughout Europe during the Middle Ages. Although the bacterial etiology of these early reports was inconclusive, the pathology was similar to that observed with red leg disease in frogs in which *Aeromonas hydrophila* was identified as the casual organism. Because many

bacteria isolated from fish with hemorrhagic septicemias were often misidentified, it is now recognized that certain isolations of bacteria ascribed to the genera *Pseudomonas, Proteus, Bacillus, Aerobacter* and *Achromobacter* actually belonged to the genus *Aeromonas*. The Motile Aeromonas group occurs widely in fresh and estuarine waters (Hazen [4] and are also recognized as pathogens of a variety of animals and man (Trust [26][27] and Motile aeromonads have also been isolated from a wide variety of fresh foods (Palumbo[17]

Aeromonas hydrophila hydrolyses esculin and ferments both salicin and arabinose whereas *A.sobria* doesnot utilize these compounds. (Lallier[11]. Hsu[8] noted that all of the isolates of motile aeromonads (n=164) that they studied produced acid from fructose, galactose, maltose, mannitol, trehalose, dextrin and glycogen; 99.4% of the strains produced acid from glucose 98.8% from mannose and 98.2% from glycerol, Shotts[19, 20] also found that all *Aeromonas hydrophila* complex strains hydrolysed albumin, casein and fibrinogen; most strains also digested gelatin (99.9%), hemoglobin (94.3%) and elastin (73.2%) but none of the strains hydrolysed collagen, Austin & Austin [1] have shown that *A.hydrophila. A.sobria and A.caviae* comprise the most predominant clinical isolates that are typically associated with fish.

Motile aeromonads are adapted to environments that have a wide range of any conductivity, turbidity, PH, salinity and temperature. Hazen [5]. Temperature optimums may depend upon the particular strain under investigation. Consequently more epizootics among warm water fishes in the south eastern United States are generally reported in spring and early summer (Meyer[13]. Pond water, diseased fish and diseased frogs as well as convalescent frogs and fishes may become reservoirs of infection. Certain algae (Kawakami and Hashimoto [10] and other protozoa (Chang and Huang [3] that are grazed upon by fish can harbor motile aeromonads. Osborne *et al.* found high densities of motile aeromonads within the environment during midsummer when sedimentary chlorophyll and water temperature were highest. This also correlated temporally with the highest prevalence of dermally ulcerated striped mullet (Mugil cephalus) that also contain large concentration of the bacteria within stomach and on their skin. The author suggests that the mullet graze on bacteria – laden sediment for algae and consequently bioaccumulate the pathogen within their guts and on their skin which in turn, enhances disease. Thus, the intestinal tract or epidermal abrasions are likely portals of bacterial entry. Under conditions of stress, it is even likely that some strains of motile aeromonads that are ordinarily part of the normal gut flora become pathogenic. It is believed that infection occurs in winter, when fishes are relatively inactive and that the disease breaks out in spring. Aquarium fish which are usually maintained at a constant water temperature can develop this disease at any time. In other pathogenic organisms such as *Pseudomonas aeroginosa Aeromonas salmonicida*, and a fluorescent *Pseudomonad*, toxic activities of protease were examined, and they were suspected to be one of the important virulence factors. On the other hand, though *A.hydrophila* is known to produce a large quantity of extracellular protease, the role of the enzyme in disease process has not been investigated. Wakabayashi *et al.,* showed that strains of the high virulent group (*A. hydrophila biovar. hydrophila*) were also powerful producers of the enzyme and that their culture filtrate caused hemorrhages and mortalities in carp.

MATERIALS AND METHODS

Isolation of *Aeromonas Hydrophila* from Diseased Catfish

A diseased catfish showing symptoms of septicaemia (A superficial reddening of the surface of a large area of the body and hemorrhagic ulceration at the base of the fins) captured at early hours was brought to lab. It was dissected under sterile conditions and the organs like spleen and kidney seemed to be affected (The spleen was enlarged in size and cherry red in colour). These affected organs were taken in a sterile petriplate and used as inoculums to streak in to the isolation agar (Rimler and Schotts medium)and incubated at 37°C for 24 to 48 hours to ensure optimal differentiation of bacteria .The diseased tissues were laid directly on the isolation agar. The organism *Aeromonas hydrophila* grew as yellow colonies over the isolation agar. . IMViC test was carried out for the identification of the organism.

Antibiotic Susceptibility Testing

The antibiotic susceptibility of aeromonad was determined by the disk diffusion method. Aeromonad strains were streaked on Mueller-Hinton agar plates, and the various antibiotic disks were applied on the streaked cultures. Disks of ampicillin (10mcg), erythromycin (15mcg), clindamycin (3mcg), cloxacillin (3mcg), oxytetracycline (30mcg), streptomycin (10mcg), amoxicillin (10mcg). Chloramphenicol (10mcg), sulphamethazole (300mcg), ciprofloxacin (30mcg), rifampicin neomycin (30 mcg) and pefloxacin (14.5) were used. After 18 h of incubation at 37°C, the zones of inhibition were measured. Characterization of strains as sensitive, intermediate or resistant was based on the size of inhibition zones around each disc according to the manufacturer's recommendations.

RESULTS AND DISCUSSION

The present study deals with the isolation and characterization of protease producing *Aeromonas hydrophila* from a diseased catfish. The organism *Aeromonas hydrophila* grew as yellow colonies over the isolation agar. Preliminary aeromonads were identified to genus and species after checking the morphological characteristics of the culture, Gram staining, and biochemical characteristics based on the identification of *Aeromonas.* The colonies are positive for Indole, Catalase test, Oxidase test, Voges prosakur, Citrate utilization, Carbohydrate utilisation lactose, glucose, trehalose, starch, gelatine hydrolysis and for Triple sugar iron agar medium. The colonies are observed to be negative for methyl red and urease test (Table 1). Thus the organism was considered as *A. hydrophila.*

Cultural kinetic studies were done to reveal the ability of the organism to grow at low temperature. The isolate has the ability to emerge as a food borne pathogen as it survives at refrigeration temperatures. Generation of a growth curve is useful in evaluating the growth characteristics of a cell line. In the present study a significant increase in numbers till 12 hours was observed (Fig.1)

The influence of pH and temperature over the growth of the isolate was checked. The organism was able to grow well at the optimum temperature of 37ºC, but it also had the ability to grow at 4ºC. The growth at temperature range of 41ºC was found to be minimum when compared to other ranges (Fig. 2). The influence of pH the organism was able to grow well at the optimum pH

of 9, but it also had the ability to grow at pH 5. The growth at pH range of 5 was found to be minimum when compared to other ranges (Fig. 3). The isolate was not able to grow at a temperature of 50ºC indicating that the thermal death point ranges from 40º to 50ºC.

Antibiotic sensitivity test done by Kirby Bauer's method revealed that the fish isolate was multidrug resistant. It was screened for resistance against ampicillin, amoxicillin, clindamycin, erythromycin, oxytetracycline cloaxicillin and streptomycin (Fig. 4). The multidrug resistant organism harboured a plasmid which was Isolated by alkali lysis method. Plasmid curing experiment revealed to be susceptible to ampicillin. Confirming that ampicillin resistance was encoded in plasmid DNA.

Enzyme activity of different pH and temperature has been shown in Table 2 and 3. The maximum enzyme activity is at 37 ºC and pH 7 with protease activity of 2.75 and 3.33 respectively. The diameter of the zone (Z) is 1.2 cm and the diameter of the colony (G) is 4.0 cm. Protease activity of 3.3 was calculated to be highest at pH 7. The minimum enzyme activity was at pH 6 with a calculated value of 2.8.

Enzyme protease reported as the major virulence factor was extracted and partial purification was done. These enzyme cytotoxicity tests in mice DLA cell lines with the culture supernatant and partially purified enzyme samples revealed the lethality in both. After exposure to culture supernatant, the cell death was observed after 12 hours. After exposure to the partially purified enzyme cells were detached from the monolayer (12 hours) indicating the cytotoxicity of the enzyme (Table 4).

The present study deals with the isolation and characterization of protease producing *Aeromonas hydrophila* from diseased catfish. The infected tissues, liver, spleen and kidney samples of catfish grew in to yellow colonies on the Rimler Schotts isolation agar.

The organism from the isolation agar was checked for oxidase test and it was found to be oxidase negative,but after streaking on to nutrient agar it was found to be oxidase positive. This kind of observation was made by Overman [16].He reported that reactions inconsistent for the species can occur if one uses colonies picked directly from the dfferential Rimler Schotts agar. Overman [16]found 8% of the isolates that they examined were cytochrome oxidase negative when taken from differential media.

Biochemical characterization of the organism confirmed that the strain was *Aeromonas hydrophila* as per Sniesko[21], Popoff and Vernon[18]On Eosin methylene blue agar the isolate grew with a green metallic sheen, indicating that it utilizes lactose. The influence of pH and temperature over the growth of the isolate was checked. The organism was able to grow well at the optimum temperature of 37ºC, but it also had the ability to grow at 4ºC. The growth at temperature range of 41ºC was found to be minimum, when compared to other ranges. The isolate was not able to grow at a temperature of 50ºC indicating that the thermal death point ranges from 40º to 50ºC. This is also supported by the observation by palumbo [17]that *Aeromonas hydrophila* is found in refrigerated foods at spoilage.

A small increase in temperature would allow rapid growth of these strains. Temperature abuse of food even for shorter periods would permit rapid growth of this organism. These finding is of importance since refrigeration at 4ºC is generally considered to be an adequate means of preventing the growth of food poisoning bacteria.

Antibiotic sensitivity test for the isolate was performed. The isolate screened was resistant to ampicillin (10mcg), erythromycin (15mcg), clindamycin (3mcg), cloxacillin (3mcg), oxytetracycline (30mcg), streptomycin (10mcg), amoxicillin (10mcg). The organism was found to be sensitive to chloramphenicol (10mcg), sulphamethazole (300mcg), ciprofloxacin (30mcg), rifampicin and neomycin (30 mcg), pefloxacin (14.5 mcg). Oxytetracycline has been used for treatment of motile aeromonad septicaemia in fishes (Meyer[12]. In contrast the isolate has been found resistant to oxytetracycline. Chloramphenicol is the other drug used in treatment and the isolate was susceptible to Chloramphenicol. Substances used for treating fish diseases (in general) include antibiotics,drugs and chemicals. While each therapeutant is atleast particularly effective in treatment of a particular disease,problems arise with the accumulation of these substances in the environment which have led to the emergence of resistant strains. The extracted plasmid were electrophoresed on 0.8% agarose gel in 1X TAE buffer.Plasmid DNA was detected under UV illumination. They had also reported that only one third of the isolate harboured plasmids ranging from 3 to 63.4kb.Despite various studies on R plasmids in *Aeromonas hydrophila* range from 85.6 to 150kb.A much smaller plasmid was detected indicating that R factors mediating resistance are of variable size. This finding confirms the presence of R plasmid in the fish isolate and is similar to results of Sujoy saha [22]. They reported that after plasmid curing with SDS, a resistant strain of *Pseudomonas* Spp turned susceptible to ampicillin whereas wild strain was naturally resistant to it. Animals reared in aquaculture facilities are susceptible to numerous bacterial diseases, which can be treated with a variety of antimicrobial compounds (Austin and Austin [1]

The emergence of bacterial isolates that are resistant to an antimicrobial agent represents a continuing ecological battle to achieve a natural host-parasite balance. As new antibiotics are developed and used, resistant strains may develop. In addition, the paucity of reports in which physical evidence of the transmissible R plasmids possessed by fish bacteria, is noteworthy. These questions are of particular importance in the investigation of the water-borne dissemination of R plasmids, since untreated effluents from hatcheries are known to receive large numbers of bacteria (Austin 1983) which are pathogenic either for fish or homoeothermic animals .Restriction of the use of drugs in agriculture to control fish disease will aid in minimizing the development and spread of R^+ factor-carrying micro organisms that may confer drug resistance to otherwise susceptible bacteria species.

The enzyme protease was isolated from the organism and partial purification has been carried out as per the method of protein quantification was done by Lowry's method. SDS PAGE analysis of the crude and partially purified protease was carried out in 12% gel. A distinct band along with other protein bands was observed. The molecular weight of the protein bands were detected using a standard molecular weight marker (14 to 100k.da range from Genei). Purified protease bands were of 55k.da molecular weight.

Thune[24] found that hemolysin is not an important virulence for channel catfish in *in vivo* experiment. Three strains of protease but without hemolysin are virulent, yet those who cannot secrete protease but are hemolysin-producing strains are avirulent (Thune [24].If a major virulence factor of a bacteria is determined, it can be applied as a quick method of detecting the pathogenic *Aeromonas hydrophila* in aquatic food products or culture ponds by using specific DNA fragment coding for the enzyme.

Figure 1. Growth curve of *Aeromonas hydrophila*

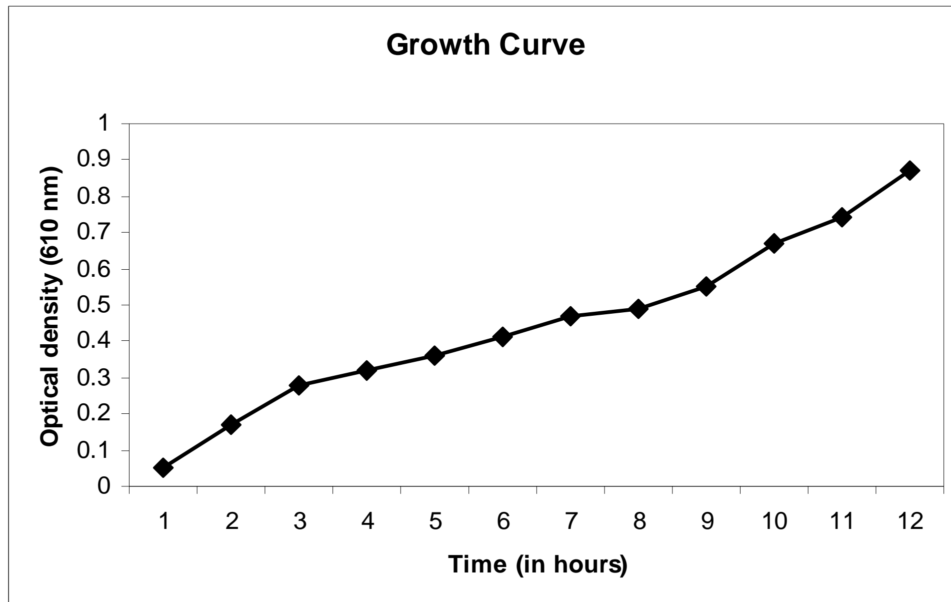

Figure 2. Growth of *Aeromonas hydrophila* at different temperature

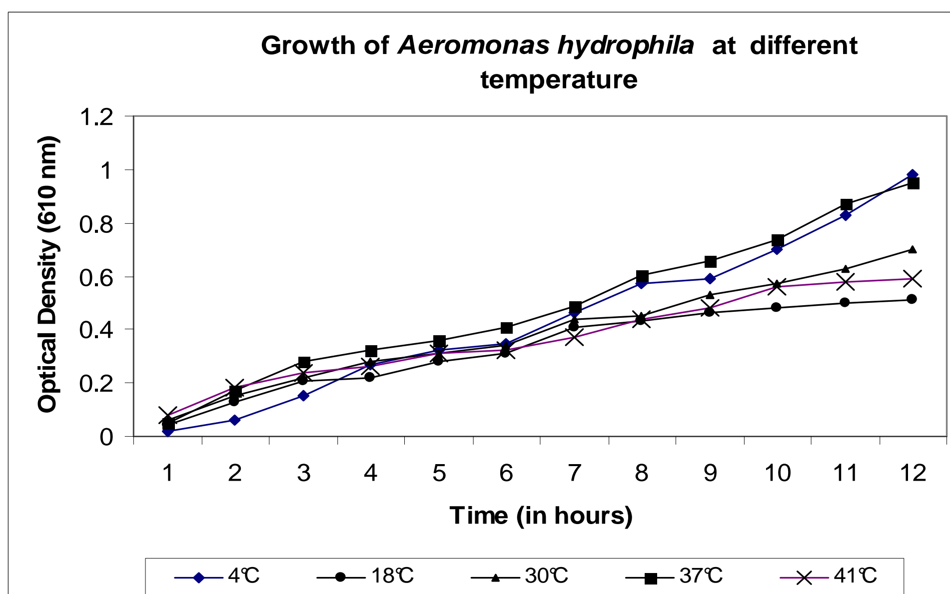

Figure 3. Growth of *Aeromonas hydrophila* at different pH

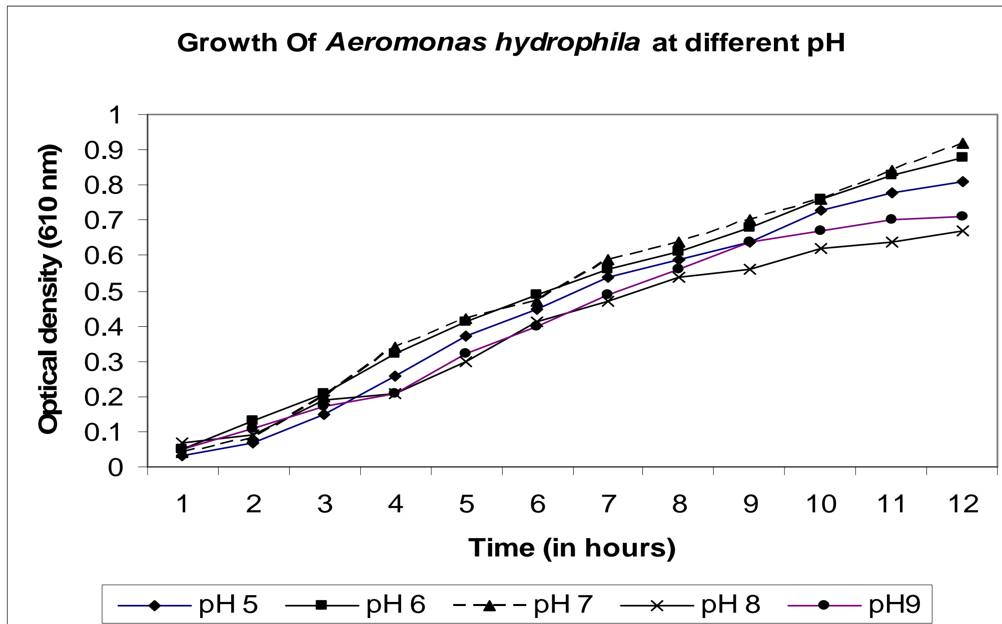

Figure 4. Antibiotic Sensitivity Test for *Aeromonas hydrophila*

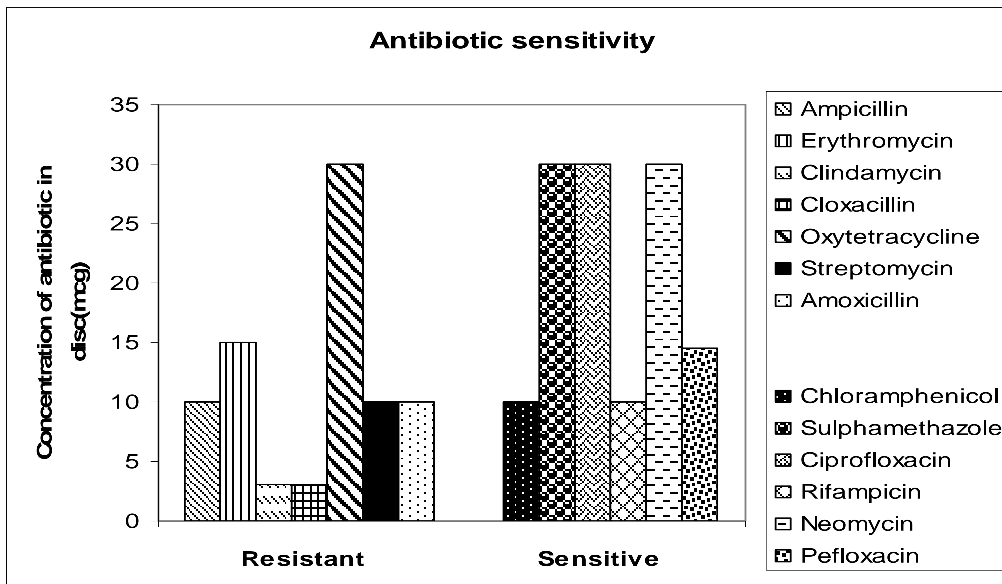

Table 1. Biochemical characteristics of *Aeromonas hydrophila*

TEST	RESULT
Shape	Rod
Motility	Positive
Gram staining	Negative
Indole	Positive
MR	Negative
VP	Positive
Citrate utilization	Positive
Catalase	Positive
Triple Sugar Iron	k/k
Urease	Negative
Oxidase	Positive
Carbohydrate utilization Lactose	Positive
Glucose	Positive
Trehalose	Positive
Starch Hydrolysis	Positive
Gelatin Hydrolysis	Positive

Table 2. Enzyme activity at different temperature

Temperature (°C)	Colony diameter (G) in cm	Zone of clearance (Z) in cm	Protease activity Z/G
4	0.2	0.4	2
30	1.1	3.0	2.30.
33	1.3	3.9	3
37	1.4	3.6	2.57
41	2.4	4.2	1.75

Table 3. Enzyme activity at different pH

pH	Colony diameter (G) in cm	Zone of clearance (Z) in cm	Protease activity Z/G
5	1.4	4.2	3
6	1.6	4.6	2.8
7	**1.2**	**4.0**	**3.3**
8	1.3	3.8	2.9
9	1.1	3.3	3.0

Table 4. Protease activity of *Aeromonas hydrophila*

	Total protein (mg/ml)	Total enzyme (U/ml)	Specific activity (IU/mg)	Fold purification
Crude	36	700	19.44	1
Dialysed	2.08	600	29.03	1.49

CONCLUSION

The present investigation showed that major virulence factor of a bacteria is determined, it can be applied as a quick method of detecting the pathogenic *Aeromonas hydrophila* in aquatic food products or culture ponds by using specific DNA fragment coding for the enzyme.

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