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Research Article

Hemolysin, Protease, and EPS Producing Pathogenic *Aeromonas hydrophila* Strain An4 Shows Antibacterial Activity against Marine Bacterial Fish Pathogens

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A pathogenic *Aeromonas hydrophila* strain An4 was isolated from marine catfish and characterized with reference to its proteolytic and hemolytic activity along with SDS-PAGE profile (sodium dodecyl sulphate-Polyacrylamide gel electrophoresis) of ECPs (extracellular proteins) showing hemolysin (approximately 50 kDa). Agar well diffusion assay using crude cell extract of the bacterial isolate clearly demonstrated antibacterial activity against indicator pathogenic bacteria, *Staphylococcus arlettae* strain An1, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3, and *Alteromonas aurentia* SE3 showing inhibitory zone >10 mm well comparable to common antibiotics. Further GC-MS analysis of crude cell extract revealed several metabolites, namely, phenolics, pyrrolo-pyrazines, pyrrolo-pyridine, and butylated hydroxytoluene (well-known antimicrobials). Characterization of EPS using FTIR indicated presence of several protein-related amine and amide groups along with peaks corresponding to carboxylic and phenyl rings which may be attributed to its virulent and antibacterial properties, respectively. Besides hemolysin, EPS, and protease, *Aeromonas hydrophila* strain An4 also produced several antibacterial metabolites.

1. Introduction

Aeromonas infections are one of the most common bacterial diseases diagnosed in marine and cultured freshwater fish. *Aeromonas hydrophila* is found in diverse habitats, including soil, water, and is pathogenic to warm and cold-blooded animals [1]. Aquatic environment along with seafood is thus important potential source for the transmission of *Aeromonas hydrophila* resulting in human infections. *Aeromonas* spp. have been involved in wound infections, sepsis, outbreaks of water, and food-borne gastroenteritis [2]. Virulence in *Aeromonas hydrophila* is multifactorial which consists of aerolysins, hemolysins, enterotoxins, and proteolytic enzymes which play significant role in pathogenesis.

EPS, (exopolysaccharides) also play very important role in the interaction between bacteria and their environment as they are organic molecules formed by polymerization of organic fractions, carbohydrates, proteins, and humic substances [3]. In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock [4]. Despite their importance, very few studies have been done on chemical characterization of EPS produced by *Aeromonas hydrophila*.

Over 120 of modern drugs have been isolated from terrestrial microorganisms, which includes important antibiotics and other drugs [5]. Although the ocean covers more than 70% of the earth's surface, microbial bioactive compounds of marine origin have been largely unexplored [6]. Marine environment is a special niche for many unique microorganisms, which produce bioactive compounds to adapt to particular environmental conditions [7]. There is an increasing demand of therapeutic drugs from diverse natural resources. Earlier studies focused on terrestrial plants and microbes proved extremely fruitful, yielding many organic

bioactive compounds including anticancer drugs [8]. But the potential contribution of marine organisms to the discovery of new bioactive molecules is very challenging [9]. Several bacteria present in aquatic ecosystems possess the ability to inhibit the growth of other microorganisms by producing antimicrobials and other molecules, namely, antibiotics, bacteriocins, siderophores, lysozymes, and proteases [10]. Many bacterial strains cause major diseases in fish and marine invertebrates resulting in mass mortalities worldwide and are equally responsible for human infections as well. Recently several novel bioactive substances such as Vibrindole, zafrin, nigrospoxydon pyrroles, pyrazines, and phenolic compounds have been isolated from marine bacteria. Several marine antagonistic bacteria including *Pseudoalteromonas* sp.A1-J 11, *Vibrio alginolyticus,* and *Pseudomonas* sp. 55b-11 were isolated from Japan which produced low molecular weight compounds inhibitory to the growth of *Vibrio* strains [11]. There are many reports about antibacterial activity shown by marine bacteria, namely, *Pseudomonas, Yersinia, Brevibacterium, Bacillus*, and *Alteromonas* [9, 12–15]. Recent studies however have targeted bacteria from an increasing assortment of nutrient rich and nutrient poor marine habitats including the surfaces or interior spaces of marine invertebrates in order to fully assess the potential of marine bacteria as a natural drug source.

In the present investigation, we have isolated and characterized *Aeromonas hydrophila* strain An4 from marine catfish with reference to its proteolytic, hemolytic activity and SDS-PAGE profile of ECPs showing hemolysin along with inhibitory activity against three-gram negative pathogenic bacterial isolates, namely, *Acinetobacter sp.* strain An2, *Vibrio parahaemolyticus* strain An3, and *Alteromonas aurentia* strain *SE3* and one-gram positive *Staphylococcus arlettae* strain An1. We have also identified and characterized its several antibacterial organic metabolites by GC-MS and EPS by FTIR.

2. Material and Methods

2.1. Bacterial Strain

2.1.1. Screening and Identification of Marine Bacteria. The test bacterial strain was isolated by serial dilutions of the swab suspension from infected regions of mouth, fins, and gills of a catfish (*Ictalurus punctatus*) with visible symptoms of hemorrhage and lesions. Determination of colony characteristics and gram staining of the test bacterial isolate was followed by biochemical tests using Hi-Media (India) kit to tentatively identify as per Bergey's Manual of Systematic Bacteriology [16]. Further confirmation of genus and species of the test bacterium was done by 16S rDNA sequencing and NCBI-BLAST search [17]. PCR amplification of the 16S rDNA was done using eubacterial forward and reverse primers: 27 f (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492 r (5′-GGTTACCTTGTTACGACTT-3′) (MWG Biotech India Pvt. Ltd., Bangalore, India), according to the standard procedure [18].

2.2. Detection of Proteolytic Activity and Assay. The bacterial isolate was spot inoculated on nutrient agar plate supplemented with 2% skimmed milk and stab inoculated in 10% gelatine agar in test tube, and proteolytic activities were observed in the form of clearance zone and liquefaction, respectively, after 24 hours and 48 hours of incubation. Protease production assay was done in broth, following Kunitz method [19].

2.3. Hemolytic Activity. Hemolytic activity of the pathogenic isolate was detected by streaking the isolate on trypticase soy agar plate supplemented with human blood [20].

2.4. Isolation of Extracellular Proteins (ECPs) and SDS-PAGE Analysis. The bacterial isolate was grown overnight in 5 mL LB broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl) for preculturing.100 μL of this culture suspension (inoculum) was added to 50 mL LB broth and incubated overnight at 37°C at shaker speed of 200 rpm. Culture suspension was harvested at 5000 rpm at 4°C for 15 minutes. Supernatant was precipitated by addition of 10% (w/v) trichloroacetic acid with overnight incubation at 4°C. Further centrifugation at 11000 rpm for 20 minutes resulted in pellet containing extracellular proteins which was suspended in 50 μL of 1 M Tris-HCl buffer (pH 8). Protein sample mixed with sample solubilizing buffer (10 μL) was analyzed by SDS-PAGE using 12% acrylamide followed by Coomassie blue staining [21].

2.5. Extraction and Purification of Exopolysaccharide (EPS). 48-hour grown bacterial culture (100 mL) was centrifuged at 8000 rpm for 20 minutes. Pellet was resuspended in 300 μL EDTA solution (10 mm EDTA + 1.5 mM NaCl) and heated at 50°C for 3 minutes in order to extract cell-bound EPS. Suspension was centrifuged, and supernatant was decanted and mixed with previous supernatant and pressure filtered through cellulose nitrate filters. EPS was precipitated by adding three volumes of chilled ethanol to the filtrate and incubating overnight at 4°C. EPS was recovered by centrifugation, and to remove impurities, it was dialysed (molecular weight cutoff of 8 kDa; Sigma-Aldrich Chemic GmbH, Seelz, Germany) against distilled water at 4°C for 24 hours. EPS was lyophilised and stored.

2.6. Fourier-Transformed Infrared Spectroscopy. Major functional groups of purified EPS were detected by FTIR [22]. Pellets for infrared analysis were obtained by grinding 2 mg of EPS with 200 mg of dry KBr the mixture was pressed into a 16 mm diameter mould. The FTIR spectra was recorded on a SHIMADZU-FTIR 8201PC instrument (Shimadzu, Japan) in the 4000–400 cm^{-1} region and spectra traced with a Hewlett Packard plotter.

2.7. Antibiotic Susceptibility Test. Overnight grown bacterial suspension (0.1 mL) of indicator pathogenic bacteria *Staphylococcus arlettae* strain An1 (accession no. FJ386956), *Acinetobacter sp.* strain An2 (accession no. FJ38695), *Vibrio parahaemolyticus* strain An3 (accession no. FJ386958), and

Alteromonas aurentia strain SE 3 was spread plated on Mueller Hinton agar plates; octadiscs (Hi Media, India) were placed in the center of the agar plates and incubated at room temperature (27°C) for 24 hours. Sensitivity of the individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic disc susceptibility test approved by NCCLS [23].

2.8. Preparation of Crude Cell Extract. Modified ethyl acetate extraction procedure was followed to extract antimicrobial metabolites from the test bacterium [24]. Cells were grown on SYEP agar (seawater-based yeast extract peptone agar). After 48 hours, agar along with the cells was cut into pieces and suspended into ethyl acetate to extract the antibacterial metabolites. Overnight suspension was decanted followed by centrifugation to get cell-free solvent. Solvent was subjected to evaporation at 40°C for final recovery of crude extract [6]. Simultaneously 48-hour old culture suspension of the test bacterium was centrifuged to get cell-free supernatant.

2.9. Antimicrobial Bioassay (Agar Well Diffusion Test). In order to check the antibacterial activity of the cell extract, SYEP agar (1.2%) was poured in the plates, small wells of about 6 mm diameter were made in the agar plates, and bottom of the wells was sealed by 0.7% molten SYEP agar [6]. 100 μL crude cell extract and cell-free supernatant were poured in the wells separately and allowed to diffuse in the agar for 4 hours. Different indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 μL) was used as control.

2.10. GC-MS Analysis of Crude Cell Extract. Identification of the antibacterial metabolites was done by GC-MS analysis, injecting 1ul of sample into an RTX-5 column (7 m × 0.32 mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan, and Helium (3 mL/min) was used as a carrier gas. The following temperature gradient program was used: 75°C for 2 minutes followed by an increase from 75°C to 175°C at a rate of 50°C per min and finally 7 minutes at 175°C. The m/z peaks representing mass to charge ratio characteristic of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compound.

3. Results

3.1. Identification of Marine Bacteria. The test bacterium was gram (−ve) rod which formed tiny, circular, white, opaque, raised, glistening colonies with entire edge.This isolate hydrolysed, starch, esculin casein, and gelatin exhibited positive reactions for oxidase, catalase, MR, indole, citrate, and lipase, and negative once for VP and urease.These morphological and biochemical characteristics tentatively established the identity of this isolate as *Aeromonas* sp. which was designated as strain An4. 16S rDNA sequencing and BLAST search further confirmed the identity of this bacterial isolate as *Aeromonas hydrophila* strain An4 (accession no. FJ386959). This strain exhibited 98% homology with *Aeromonas hydrophila* (accession no. AB 368776.1).

Figure 1: Protease activity shown by the *Aeromonas hydrophila* strain An4.

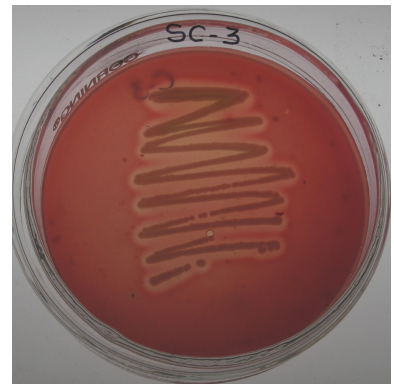

Figure 2: β Hemolytic activity shown by *Aeromonas hydrophila* strain An4. On blood agar.

3.2. Protease Activity and Assay. *Aeromonas hydrophila* strain An4 exhibited significant protease production in the form of clearance zone and liquifaction (Figure 1). This pathogenic bacterial isolate interestingly revealed commencement of protease production during late log phase (18 hours) with maximum level of production around 22 hours of incubation which is stationary growth phase of bacterial pathogen (Figure 3).

3.3. β Hemolysis. *Aeromonas hydrophila* strain An4 interestingly demonstrated a very clear β hemolysis in the form of clearance zone along the streak on blood agar plate within 24 hours of incubation at room temperature (Figure 2).

3.4. SDS-Page Analysis of Extracellular Proteins. The protein profile of the extracellular fraction clearly demonstrated the presence of protein bands ranging between 35 to 97 kDa with a significantly prominent band of 50 kDa corresponding to the virulence protein, hemolysin of *Aeromonas hydrophila.* (Figure 4).

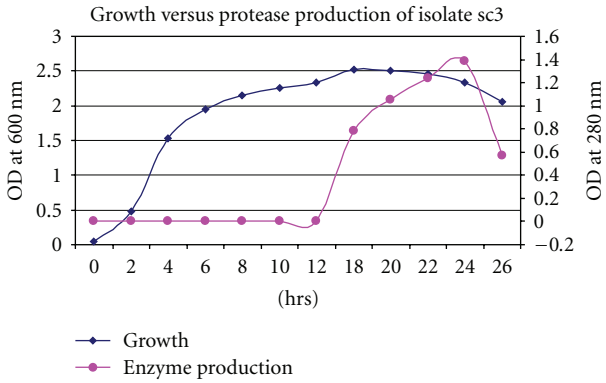

Figure 3: Growth versus Protease production in *Aeromonas hydrophila* strain An4.

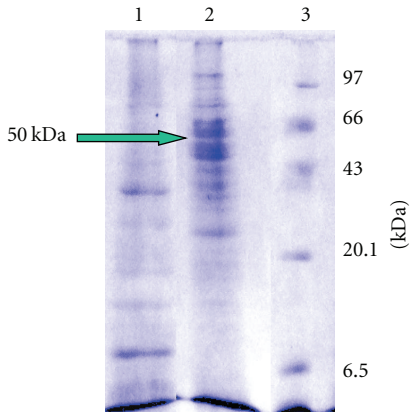

Figure 4: SDS-PAGE analysis of extracellular protein fraction. (1) Control. (2) Extracellular protein fraction from *Aeromonas hydrophila* strain An4 showing hemolysin (50 kDa). (3) Protein molecular weight marker.

3.5. Fourier-Transformed Infrared Spectroscopy. The FTIR spectrum of the EPS revealed characteristic functional groups such as C–H stretching peak of methyl group at 2918.30 cm^{-1} and stretching peak of alkene group at 3024.38 cm^{-1}. Absorption peaks ranging between 667.37 cm^{-1}–881.47 cm^{-1} are assigned to phenyl rings. Amide I, amide III (primary amine CN stretch), and amide IV showed absorption peak at 1643.35 cm^{-1}, 1020.34 cm^{-1}, and 466.77 cm^{-1}, respectively. Conspicuous absorption centered between 2524.05 cm^{-1} and 2627.82 cm^{-1} corresponded to carboxylic acids, and aliphatic primary amine showed NH stretching peak at 3302.13 cm^{-1} (Figure 5).

3.6. Antibiotic Susceptibility Test for the Indicator Bacteria. All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested and were also resistant to few antibiotics (Table 1).

3.7. Antibacterial Activity of the Crude Cell Extract of the Test Organism. Agar well diffusion experiment clearly demonstrated the antagonistic characteristic of crude cell extract of *Aeromonas hydrophila* strain An4 as manifested by growth inhibition of gram (−ve) indicator bacteria, namely, *Acinetobacter* sp. strain An2, *Vibrio parahaemolyticus* strain An3, *Alteromonas aurentia* strain SE 3, and gram (+ve) *Staphylococcus arlettae* strain An1. Interestingly, ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 caused remarkably wider inhibitory zones as compared to most of the common antibiotics tested. Interestingly, cell-free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on the growth of indicator bacteria. This clearly revealed that intracellular antibacterial metabolites are present inside the cells of *Aeromonas hydrophila* strain An4 without being secreted out (Figure 6 and Table 1).

3.8. GC-MS Analysis of the Crude Cell Extract. GC-MS analysis of crude ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 clearly revealed the presence of several important organic metabolites which includes Butylated hydroxytoluene (BHT) as major constituent (7.25%) followed by pyrrolopyrazines (6.5%), pyrrolo-pyridine (0.65%), and other phenolic compounds (Figure 7; Table 2).

4. Discussion

Two haemolytic toxins, haemolysin and aerolysin, have been reported in *A. hydrophila*. Hemolytic proteins are commonly isolated from pathogenic bacteria, and β-hemolysins are one of the important bacterial virulence factors. Hemolysins and related proteins containing cystathionine β synthase (CBS) domains are bacterial toxins that function by assembling identical subunits into a membrane-spanning pore [25]. The molecular mass of bacterial hemolysins ranges from 49 to 60 kDa [25–29]. We have demonstrated presence of a prominent protein band of approximately 50 kDa in extracellular protein fraction of this pathogenic isolate which indicates the possible presence of the bacterial toxin, hemolysin [26].

It has been suggested that proteolytic enzymes of fish pathogen, *Aeromonas hydrophila,* play an important role in causing massive tissue damage in the host which may facilitate establishment of infection [30]. Conspicuous protease activity along with hemolytic activity shown by *Aeromonas hydrophila* strain An4 further strengthens its pathogenic potential as it has already been proved that presence of both the proteins enhances the intensity of virulence in this fish pathogen [31]. EPSs also play a very important role in virulence of pathogenic bacteria as they mediate the interaction between pathogenic bacteria and their environment through adhesion to the host [32, 33]. FTIR analysis of EPS of *Aeromonas hydrophila* strain An4 clearly revealed presence of many protein-related amine and amide groups indicating the possible presence of bacterial toxins. Therefore we can assume that EPS may also contribute significantly in pathogenesis. EPS could also account for antibacterial activity against indicator organisms due to presence of several phenolic and carboxylic groups which is evident from corresponding stretching peaks in FTIR spectrum of the EPS. Competition among microbes for space and nutrients in

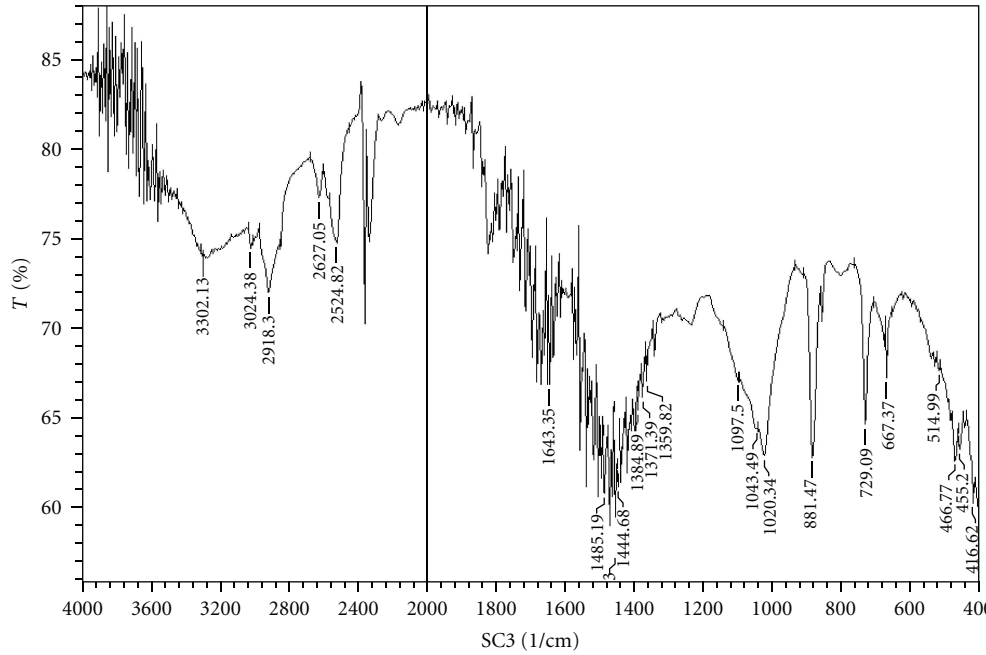

Figure 5: FTIR Spectrum of EPS of *Aeromonas hydrophila* strain An4.

Table 1: Comparison of antibacterial activity of *Aeromonas hydrophila* strain An4 with commonly used antibiotics on indicator bacterial isolates.

Antibiotics	Concent-ration (μg/mL)	Zone of inhibition (mm) Indicator bacteria			
		Vibrio parahaemolyticus strain An3	*Acinetobacter* sp. strain An2	*Alteromonas aurentia* strain *SE3*	*Staphylococcus arlettae* strain An1
Ciprofloxacin-(Cf)	10	3	4	0	0
Cotrimazine-(Cm)	25	12	9	6	6
Kanamycin-(K)	30	4	6	10	11
Nitrofurantoin-(Nf)	300	2	7	0	0
Streptomycin-(S)	10	8	14	6	10
Tetracycline-(T)	30	6	14	2	1
Ampicillin-(A)	10	0	0	14	0
Cephalothin-(Ch)	5	0	0	13	3
Colistin methane sulphonate-(Cl)	25	1	1	7	9
Gentamycin-(G)	10	4	7	0	0
Streptomycin-(S)	10	3	5	2	3.5
Cotrimoxazol-(Co)	25	8	12	7	7
Cell-free supernatant	100 μL	0	0	0	0
Bacterial crude extract	100 μL	10	11	12	10.5
Ethyl acetate	100 μL	0	0	0	0

marine environment is a powerful selection pressure which endows marine microorganisms to produce many natural products of medical and industrial value. Compared with the terrestrial microorganisms, the secondary metabolites produced by marine organisms have more novel and unique structures, owing to their complex living circumstances and species diversity [34]. Several antimicrobials have been reported from marine microorganisms associated with animals due to their specialized role in their respective hosts [35].

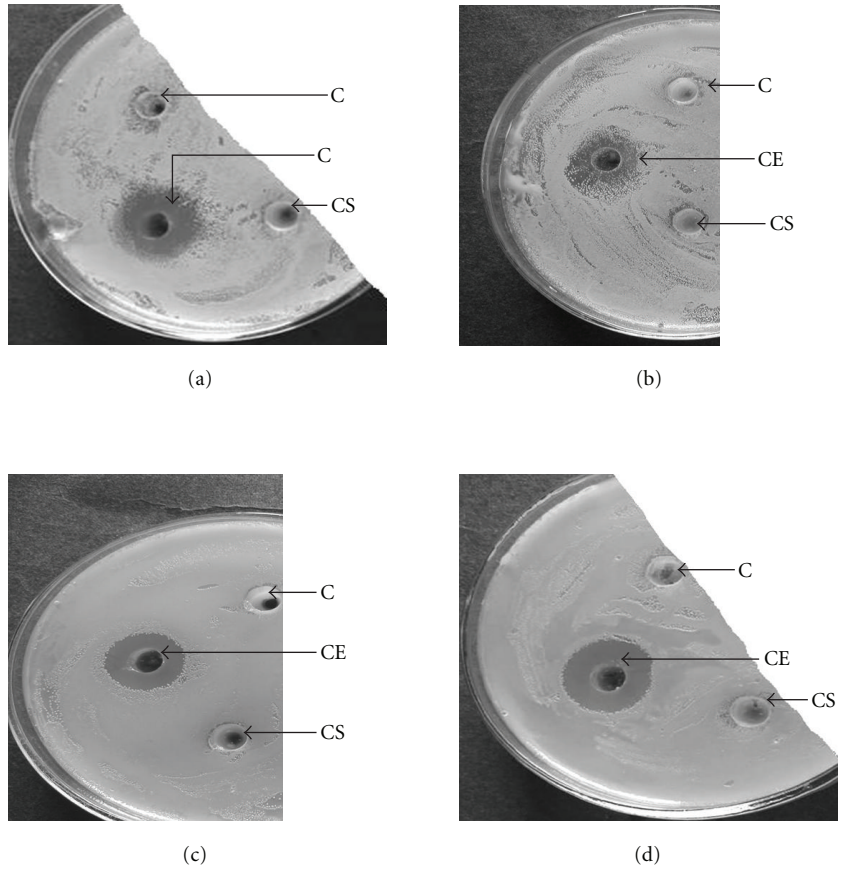

Figure 6: Antibacterial activity of the crude cell extract of the *Aeromonas hydrophila* strain An4 on indicator bacteria. (a) *Staphylococcus arlettae* strain An1, (b) *Acinetobacter* sp. strain An2, (c) *Vibrio parahaemolyticus* strain An3, and (d) *Alteromonas aurentia* strain SE 3. C: control (Ethyl acetate); CE: cell extract; CS: culture supernatant.

Table 2: Major antibacterial metabolites present in ethyl acetate cell extract *Aeromonas hydrophila* strain An4.

Antibacterial metabolites	Retention time	% in test volume
Butylated hydroxytoluene	12.625	7.25
Pyrrolo-(1,2-a) pyrazine-1,4- dione, hexahydro-3 (2-methylpropyl)	20.033	4.6
Pyrrolo-(1,2-a) pyrazine-1,4- dione, hexahydro-3 (2-methyl phenyl)	24.417	1.9
Phenol, 4-(1,1,3,3-tetramethyl butyl)	15.425	0.65
Pyrrolo(1,2-a) pyridine	10.042	0.44

Presence of Butylated hydroxytoluene (BHT) in the cell extract of this test bacterium as a major constituent demonstrated that this important antiviral compound can be used against Herpes simplex virus and Φ 6 [36, 37]. In addition, other metabolites, namely, pyrrolopyrazines and phenolics, were also present in the cell extract of the test organism which are also well-known antimicrobials [38]. Since cell-free supernatant of the test organism could not inhibit the growth of indicator bacteria, it was assumed that these antimicrobial compounds were present inside the cell as well as in the cell exudates. Treatment of infections is compromised worldwide by the emergence of bacteria resistant to multiple antibiotics. In general bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents. One way to prevent antibiotic resistance is by using new compounds which are not based on existing synthetic antimicrobial compounds. GC-MS analysis of crude ethyl acetate cell extract of *Aeromonas hydrophila* strain An4 clearly demonstrated the presence of several important organic metabolites which are known antagonist to bacteria, viruses, and fungi [9, 12, 36, 39, 40]. It is interesting to note that even pathogenic strains can be used as biocontrol agents against other pathogens due to inherent presence of various virulence factors, namely hemolysin, proteases, and EPS along with several antibacterial organic compounds.Thus

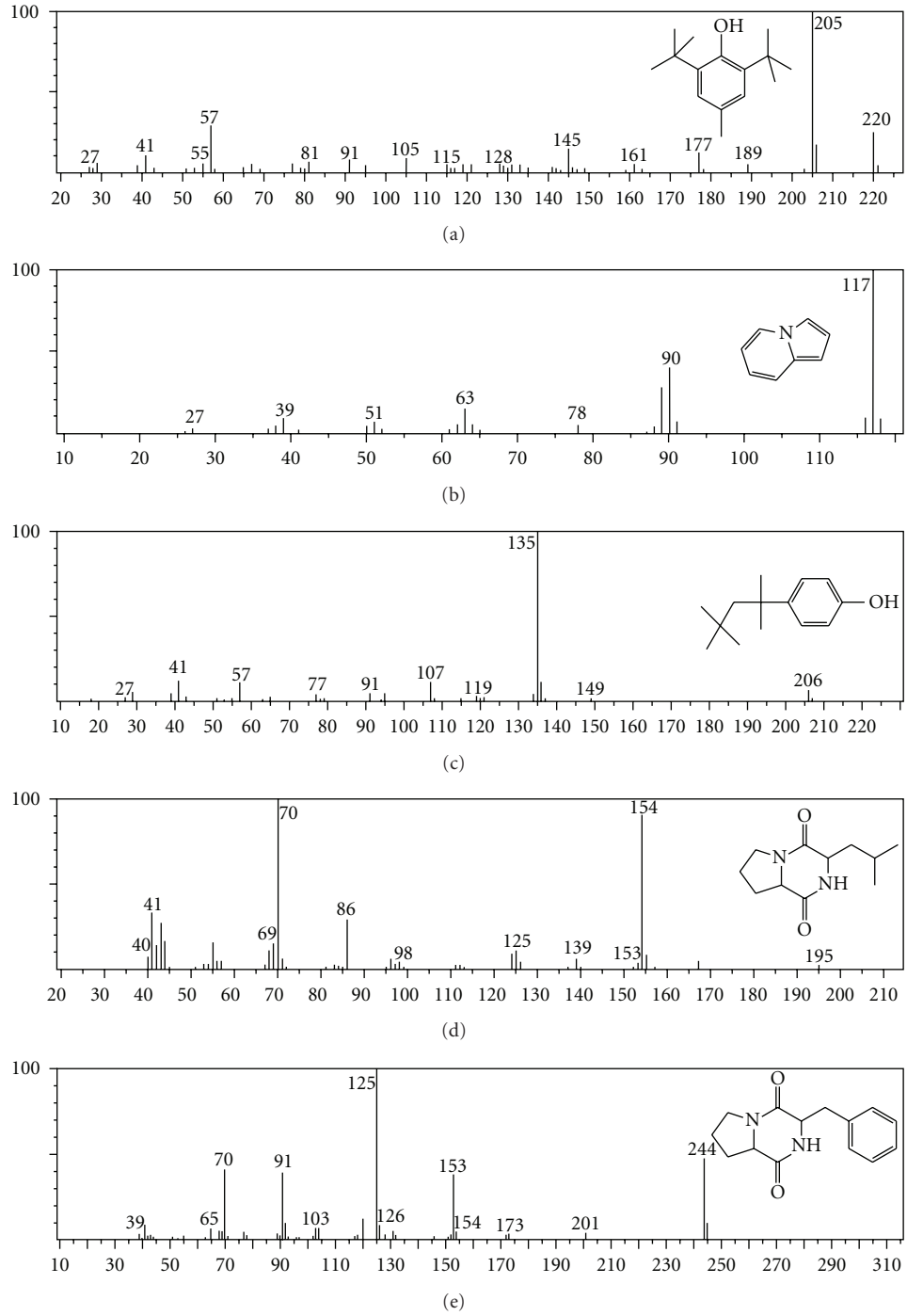

Figure 7: Molecular structure and corresponding GC-MS peaks of major antibacterial metabolites from crude cell extract of *Aeromonas hydrophila* strain An4. (a) Butylated Hydroxytoluene(BHT)—[Peak 205], (b) Pyrrolo[1,2-a] pyridine [peak 117.15], (c) (1,1,3,3-tetramehyl butyl)-Phenol [Peak 135], (d) Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2-methyl propyl)—[Peak 70.1], and (e) Pyrrolo(1,2-a) pyrazine-1,4-dione, hexahydro-3 (-2-phenyl methyl)—[Peak 125].

antibacterial metabolites synthesized by the test organism may serve as valuable drugs to control pathogenic bacterial strains causing fish and human diseases, and isolation of novel bacterial strains with antimicrobial activity suggests that marine ecosystem is a valuable source of antimicrobials.

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