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Biological characterization of marine fish pathogen, *Acinetobacter* sp. strain An 2 producing antibacterial metabolites

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This study presents antibacterial activity of several organic metabolites produced by a fish pathogen, *Acinetobacter* sp. strain An 2, from marine ecosystem of Goa. Cell extract demonstrated stronger inhibitory effects on Gram negative bacteria as compared to common antibiotics. GC-MS analysis of crude cell extract revealed presence of potential antimicrobials (butylated hydroxytoluene, phenol, pyrrolo-phenol, benzo-quinone, pyrrolo-pyrazine, phthalic acid butyl octyl ester and penta-fluoro-propionic acid hepta-decyl ester). FTIR analysis of its exopolysaccharide (EPS) revealed presence of amine, amide, carboxylic and phenyl groups. Therefore, this strain can be exploited as a potential candidate for several antibacterial drugs to combat bacterial pathogens causing serious fish and human diseases.

Keywords: *Acinetobacter* sp., Antibacterial activity, Crude cell extract, EPS, Indicator

Introduction

Although ocean covers more than 70% of earth's surface, microbial bioactive compounds of marine origin have been largely unexplored[1]. Marine microorganisms produce biologically active compounds to adapt to particular environmental condition[2-4]. Earlier studies reported[5-7] many organic biologically useful compounds including anticancer drugs and potential contribution of marine organisms including bacteria to the discovery of novel bioactive molecules. Several bacteria present in aquatic ecosystems inhibit growth of other microorganisms by producing antimicrobial substances (antibiotics and bacteriocins). Other inhibitors include siderophores, lysozymes, proteases, and organic acids[8]. Several biologically active substances, isolated from marine bacteria (*Pseudomonas, Yersinia, Aeromonas, Brevibacterium, Bacillus,* and *Alteromonas*), have demonstrated antibacterial activity[6,9-13]. Endophytic (*Acinetobacter baumannii*) secrete an antifungal compound, which inhibit growth of some fungal plant pathogens[14]. Microbial exopolysaccharide (EPS) plays an important role in interaction between bacteria and their environment[15] and is reported useful in adhesion, nutrient aquisition, heavy metal sequestration, detoxification of toxic compounds and protection against osmotic shock[16].

This study presents isolation of a fish pathogenic strain of *Acinetobacter,* which inhibits growth of three Gram negative pathogenic indicator bacterial isolates (*Vibrio parahaemolyticus* strain An 3, *Aeromonas hydrophila* strain An 4 and *Alteromonas aurentia* strain SE 3). This study also demonstrated its antibacterial activity by agar well diffusion assay and detected antibacterial organic metabolites in crude cell extract (CCE) by GC-MS and in EPS by FTIR.

Experimental Section

Bacterial Strain

Isolation and Screening of Marine Bacteria

Marine fishes with visible hemorrhagic symptoms and lesions on their body were screened from Goa, and infected body parts (mouth, fins and gills) were washed with sterile deionized double distilled water and swabbed with sterile cotton wool. A suspension of swab was prepared in saline and used for isolation of pathogenic bacteria on nutrient agar plates by serial dilutions. Discrete colonies were picked up for further characterization.

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Morphological Characterization and Identification of Test Bacterium

Colony characteristics of selected bacterial isolate (size, shape, colour, margin, elevation, consistency and opacity) were observed and recorded. Selected bacterial isolate was Gram stained and observed under light microscope at 100 x magnification to study cell morphology. Hi-Media (India) kit for biochemical tests was used to tentatively identify selected bacterial isolate as per Bergey's Manual of Systematic Bacteriology[17].

Molecular Identification

Genus/species of test bacterium were identified by 16S rDNA sequencing and NCBI–BLAST search[18]. Genomic DNA, extracted from bacterial isolates, was used as template for PCR amplification of 16S rDNA fragment (1400 bps) as per standard procedure[19]. Eubacterial primers [27 f (5'-AGAGTTTGATCCTGGCTCAG -3') and 1492 r (5'- GGTTACCTTGTTACGACTT -3')] from MWG Biotech India Pvt Ltd, Bangalore, India were used for PCR amplification.

Preparation of Crude Cell Extract (CCE)

Ethyl acetate extraction procedure with a slight modification was followed to extract antimicrobial metabolites from test bacterium[20]. *Acinetobacter* sp. was grown on SYEP agar (sea water based yeast extract peptone agar). After 48 h, agar along with cells was cut into pieces and suspended into ethyl acetate to extract 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[9]. Simultaneously, 48 h old culture suspension of test bacterium was centrifuged to get cell free supernatant.

Antimicrobial Bioassay (Agar Well Diffusion Assay)

To check antibacterial activity of cell extract, SYEP agar (1.2%) was poured in plates, small wells (diam, 6 mm) were made in agar plates and bottom of wells were sealed by 0.7% molten SYEP agar[1]. CCE and cell free supernatant (100 µl) were poured in wells separately and allowed to diffuse in agar media for 4 h. Indicator bacterial strains were spread plated on separate SYEP agar plates. Ethyl acetate (100 µl) was used as a control to check inhibitory effect.

Antibiotic Susceptibility Test

Overnight grown bacterial suspension (0.1 ml) of indicator pathogenic bacteria *V. parahaemolyticus* strain An 3 (accession no. FJ386958), *A. hydrophila* strain An 4 (accession no. FJ386959), *S. arlettae* strain An1 (accession no. FJ386956) and *A. aurentia* strain SE 3 was spread plated on Mueller Hinton agar plates, octadiscs (OD-007 and 014 from Hi Media, India) containing multiple antibiotics were carefully placed in the centre of agar plates and incubated at room temperature (27°C) for 24 h. Sensitivity of individual indicator bacterial isolate to a particular antibiotic was determined according to the performance standards of antibiotic disc susceptibility test approved by national committee for clinical laboratory standards[21].

GC-MS Analysis of Crude Cell Extract

Antibacterial metabolites were identified by GC-MS analysis by injecting sample (1 µl) into a RTX-5 column (7m x 0.32mm) of GC-MS (model GC-MS-QP-2010 plus) from Shimadzu, Japan and Helium (3 ml/min) was used as a carrier gas. Temperature gradient program was used at 75°C for 2 min followed by an increase from 75° to 175°C at a rate of 50°C per min and finally 7 min at 175°C. The m/z peaks (mass to charge ratio) characteristic of antimicrobial fractions were compared with those in mass spectrum library of corresponding organic compounds.

Extraction and Purification of Exopolysaccharides (EPS)

Bacterial culture (100 ml) grown for 48 h was centrifuged at 8000 rpm for 20 min. Pellet was resuspended in 300 µl EDTA solution (10 mM EDTA + 1.5 mM NaCl) and heated at 50°C for 3 min 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 filtrate and incubating overnight at 4°C. EPS was recovered by centrifugation and impurities were removed by dialysis (molecular weight cut-off - 8 kDa; Sigma- Aldrich Chemic GmbH, Seelz, Germany) against distilled water at 4°C for 24 h. EPS was lyophilised and stored until needed for FTIR analysis.

Fourier - Transformed Infrared Spectroscopy (FTIR) of EPS

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

Results and Discussion

Identification of Marine Bacteria

Test bacterial isolate was identified based on morphological, biochemical and molecular characteristics. Gram (-ve) coccobacillus formed tiny, circular, white, opaque, raised, glistening colonies with entire edge. This nitrate reducing isolate was also positive for oxidase, MR, catalase, citrate and lipase, whereas negative for VP, indole, urease, gelatinase and amylase. These characteristics tentatively established identity of this isolate as *Acinetobacter* sp., which was designated as strain An 2. PCR amplification of ribosomal gene encoding 16S rDNA (1126 bps) followed by DNA sequencing and BLAST search further confirmed identity of this bacterial isolate as *Acinetobacter sp*. strain An 2 (Accession no. FJ38695). Interestingly, 96% DNA sequence homology was found with *Acinetobacter* sp. WW21 (Accession no. EF433555.1).

Antibacterial Activity of Crude Cell Extract of Test Organism

Marine epiphytic bacteria of intertidal seaweeds (17%) showed antibacterial activity[23] against *Staphylococcus aureus.* In present study, CCE of *Acinetobacter* sp. strain An 2 prepared in ethyl acetate showed antagonistic characteristic by inhibiting growth of Gram (-ve) pathogenic bacterial indicators (*A. hydrophila* strain An 4, *V. parahaemolyticus* strain An 3 and *A. aurentia* strain SE 3), whereas it could not inhibit growth of Gram (+ve) *S. arlettae* strain An 1, possibly due to different cell wall constituents and less suceptibility to antimicrobial metabolites produced by test organism (Table 1). Thus ethyl acetate cell extract of *Acinetobacter* sp. caused remarkably bigger inhibitory zones as compared to common antibiotics tested. Antibacterial metabolites synthesized by test organism may serve as valuable drugs to control pathogenic bacterial strains causing fish and human diseases. Cell free supernatant as well as ethyl acetate (control) was unable to show any inhibitory effect on growth of indicator bacteria, indicating that antibacterial metabolites of *Acinetobacter* sp. are intracellular and not secreted out in growth medium (Fig. 1).

Antibiotic Susceptibility of Indicator Bacteria

All indicator bacterial strains exhibited significant susceptibility to majority of common antibiotics tested. Few indicator bacterial isolates were resistant to common antibiotics tested (Table 1). Treatment of infections is compromised worldwide by emergence of bacteria resistant to multiple antibiotics. Although drug resistance is mainly attributed to chromosomal genes of pathogenic bacteria, it is most commonly associated with extra-

Table 1—Comparison of antibacterial activity of *Acinetobacter* sp. strain An 2 with commonly used antibiotics on indicator bacterial isolates

Antibiotics conc., µg/ml	Indicator bacteria Zone of clearance, mm (diameter)			
	Vibrio parahaemolyticus strain An 3	*Aeromanas hydrophila* strain An 4	*Alteromonas aurentia* strain SE 3	*Staphylococcu arlettae* strain An 1
Amicacin- (Ak) 10	7	7	3	0
Carbenicillin- (Cb)100	7	5	4.5	3
Ciprofloxacin- (Cf) 10	3	4	0	0
Co-Trimazine-(Cm) 25	12	9	6	6
Kanamycine – (K) 30	4	6	10	11
Nitrofurontoin-(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
Sulphatriad- (SI) 200	9	7	6	3
Tetracycline- (T) 25	8	9	3	3
Co-Trimoxazol- (Co) 25	8	12	7	7
Cell free supernatant, 100 µl	0	0	0	0
Bacterial crude extract, 100 µl	12	11	14	0
Ethyl- acetate, 100 µl	0	0	0	0

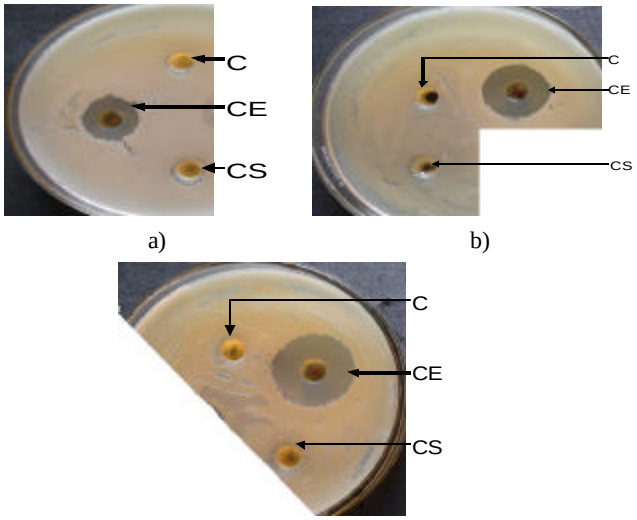

Fig. 1—Antibacterial activity of crude cell extract of *Acinetobacter* sp. An 2 on indicator bacteria: a) *Aeromonas hydrophila* strain An 4; b) *Vibrio parahaemolyticus* strain An3; c) *Alteromonas aurentia* strain SE 3 [C- Control (Ethyl acetate); CE- Cell extract; CS- Culture supernatant]

chromosomal elements acquired from other bacteria in the environment. These include different types of mobile DNA segments (plasmids, transposons and integrons), which genetically govern efflux pumps for multiple drug resistance in environmental bacterial isolates[24]. Thus drug and antibiotic susceptible indicator bacterial isolates may possibly lack these drug efflux pumps.

GC-MS analysis of Crude Cell Extract

GC-MS analysis of crude ethyl acetate cell extract of *Acinetobacter* sp. strain An 2 revealed presence of several important organic metabolites, which exhibit antimicrobial activity against bacteria, viruses and fungi[9,10,25-27]. Butylated hydroxytoluene (BHT) is one of the major fractions of cell extract (10%) followed by Pyrrolopyrazine (8.6%), Benzoquinone (8.5%), Benzene dicarboxylic acid (6.2%), and Penta -fluoropropionic acid (6.0%) (Fig. 2).

Compared with terrestrial microorganisms, secondary metabolites produced by marine organisms have novel and unique structures owing to their complex living circumstances and species diversity, thus their bioactive potentials are stronger[25,28]. Competition among microbes for space and nutrients in marine environment is a powerful selection pressure, which endows marine microorganisms to produce many natural products of medical and industrial value[29]. Several antimicrobial substances have been reported from these marine microorganisms due to their specialized role in respective hosts[30,31]. Presence of (BHT) in cell extract of *Acinetobacter* sp. as a major constituent demonstrated that this antiviral metabolite can be purified in large quantities and used against lipid containing human and bacterial viruses (Herpes simplex virus and Ô 6)[27,32]. Besides BHT, other metabolites (Pyrrolopyrazine, Benzoquinone, Benzene dicarboxylic acid, Penta-fluoropropionic acid) and a phenol derivative were also present in cell extract of test organism, which are well known potential antimicrobials[33,34].

Fourier-Transformed Infrared Spectroscopy (FTIR) of EPS

FTIR spectrum of EPS revealed characteristic functional groups (C-H stretching peak of methylene group at 2924.09 cm^{-1} and stretching peak of alkene group at 3278.99 cm^{-1}). Absorption peaks (667.37 - 881.47 cm^{-1}) are assigned to phenyl rings and one broad OH stretch at 3562.00 cm^{-1}. Amides showed absorptions at: Amide I, 1560.19 cm^{-1} and 1643.35 cm^{-1} [1560.19 (N-H bend)]; amide III (primary amine CN stretch), 1643.35 cm^{-1}; and amide IV, 1020.34 cm^{-1} and 466.77 cm^{-1}. Conspicuous absorption (2542.18 - 2632.83 cm^{-1}) corresponds to carboxylic acids and aromatic primary amine and showed CN stretching peak at 1232.51 cm^{-1} respectively (Fig. 3). Presence of several amine, amide, phenol and carboxylic groups in FTIR spectrum of EPS further confirm antibacterial activity of test bacterium.

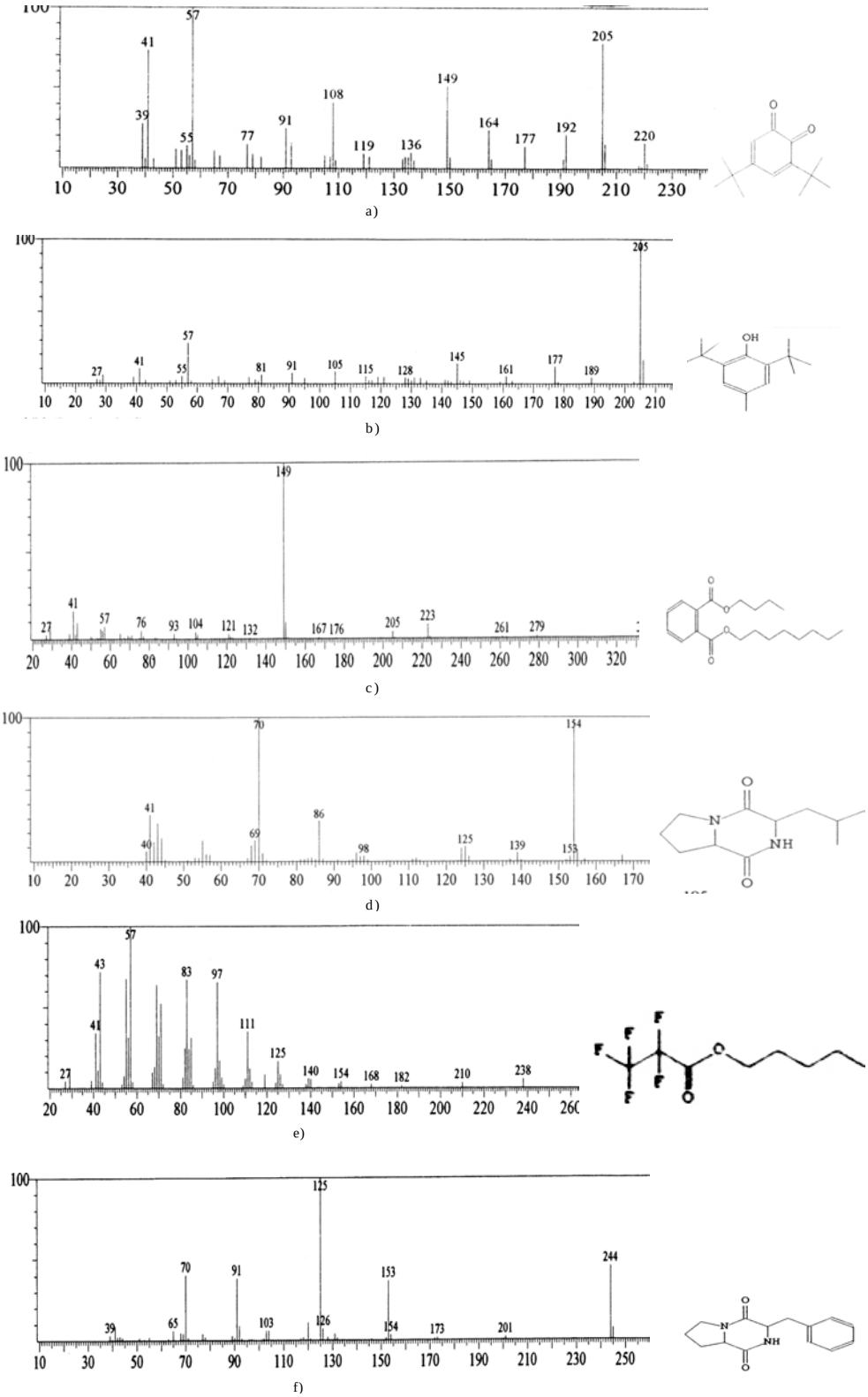

Fig. 2—Structure and GC-MS Spectrum of major antibacterial metabolites present in the crude cell extract of *Acinetobacter* sp. An 2: a) O-Benzoquinone, 3, 5 – di tert butyl- [Peak 41]; b) Butylated Hydroxytoluene (BHT) - [Peak 205]; c) 1, 2- Benzenedicarboxylic acid ,butyloctyl ester -[Peak 149]; d) Pyrrolo(1, 2-a) pyrazine-1, 4-dione, hexahydro-3 (-2- methyl propyl)- [Peak 70]; e) Pentafluoro propionic acid heptadecyl ester -[Peak 57]; and f) Pyrrolo(1, 2-a) pyrazine-1, 4-dione, hexahydro-3 (-2- phenyl methyl)- [Peak 125]

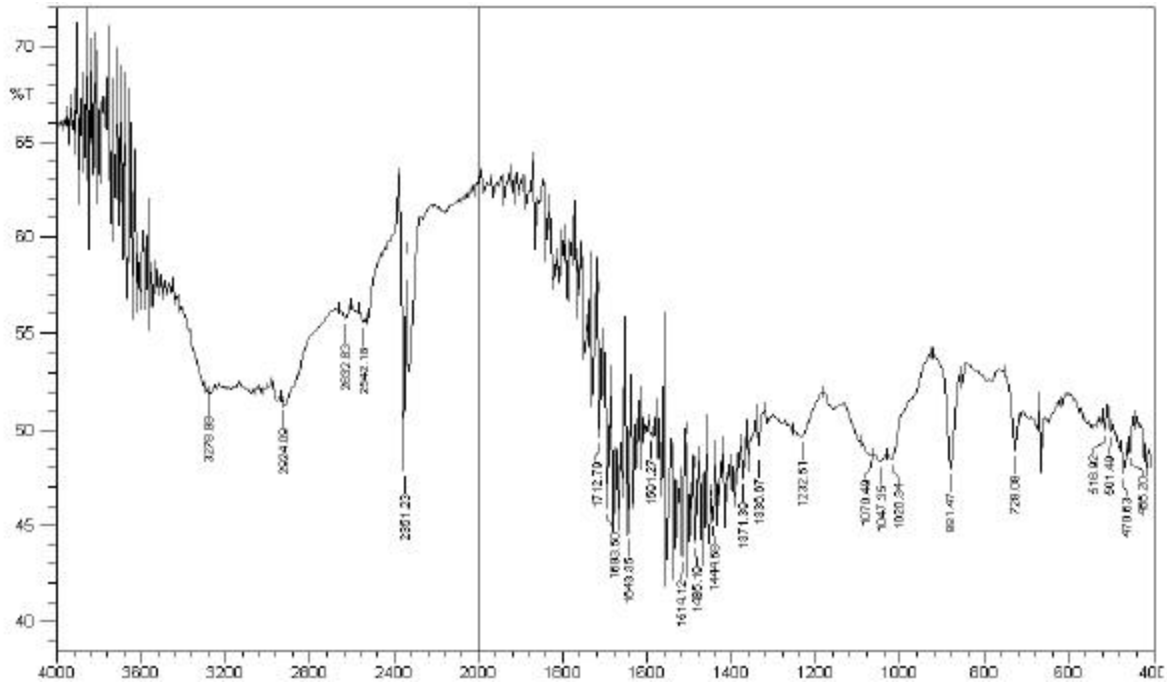

Fig. 3—FTIR spectrum of EPS of *Acinetobacter* sp. An 2

Conclusions

Agar well diffusion assay of CCE of test organism demonstrated stronger inhibitory effects on Gram negative indicator pathogenic bacteria as compared to inhibitory action of many commercially used antibiotics. GC-MS analysis of CCE revealed presence of several potential antimicrobials [BHT (major fraction, 10%), phenol, pyrrolo-phenol, benzo-quinone, pyrrolo-pyrazine, phthalic acid butyl octyl ester and penta-fluoro- propionic acid hepta-decyl ester]. FTIR analysis of EPS revealed several protein related antimicrobial amine and amide groups along with peaks corresponding to carboxylic and phenyl rings. Thus fish pathogen appears to be a potential candidate to produce several antibacterial metabolites along with unique antiviral organic compound to control pathogenic bacterial strains causing serious fish and human diseases.

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