Antagonistic Activity Exhibited by Crude Extracts of *Pseudomonas aeruginosa* (PIC-4) against *Aeromonas hydrophila* and *E. coli*

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Abstract*—*Pseudomonas aeruginosa* (PIC-4) (Genbank accession No: KF803248) was isolated from Coastal waters of Visakhapatnam. Cell free culture broth of the isolate exhibited antagonistic activity against pathogenic bacteria *Aeromonas hydrophila* and Ecoli in agar well diffusion method with the diameter of inhibition zones 30mm and 36 mm respectively. Optimum temperature and time for growth and antibiotic production were standardised as 28°C and 48 hrs respectively and optimum NaCl concentration was determined as 1%, in addition to the LB broth medium. Two solvent fractions, F1 and F3 obtained on 100% hexane and ethyl acetate: hexane (1:1) respectively exhibited antibacterial activity against tested pathogens. F1, a colourless with low melting crystals found to have more antibacterial activity than that of F3, colourless short needles. Minimum inhibition concentration of both F1 and F3 were recorded as 32µg/ml on *E. coli* and 64µg/ml *Aeromonas hydrophila.

***Index Terms*—*Pseudomonas aeruginosa, aeromonas hydrophila, Ecoli,* anttibacterial activity.**

I. Introduction

Genus *Pseudomonas* is found in natural habitats like soil, freshwater, marine environments and is familiar with production of a remarkable array of secondary metabolites [1]. Aquatic *Pseudomonads* are often antagonistic against other microorganisms by producing a diverse range of potent antimicrobial compounds, some *Pseudomonas* biocontrol strains have been developed into commercial products, e.g., *P. chlororaphis* MA342. (Cedomon, Cerall) [2]. *A. hydrophila* cause disease in aquatic animals, such as red leg disease which is caused by endotoxin and haemolysin produced by the bacteria [3], [4]. In humans, *Aeromonas hydrophila* causes gastrointestinal infections such as watery diarrhoea to dysenteric or bloody diarrhoea [5] and non-gastrointestinal complications include haemolytic syndrome, kidney disease, cellulitis, wound & soft-tissue infection, meningitis, and septicemia [6], [7]. *Escherichia coli* belongs to normal microflora of the gastrointestinal tract, but certain *E. coli* strains are associated with urinogenital diseases, pleuro-pneumonitis, gastroenteritis and septicaemia [8], also causes traveler's diarrhoea [9].The morbidity and mortality associated with several recent large outbreaks of gastrointestinal disease caused by *E. coli* has highlighted the threat to public health [8]. The discovery of antimicrobial compounds produced by organisms isolated from different environments might provide new or more-efficient means for the inhibition of selected microorganisms [10].

II. Materials And Methods

A. Antagonistic Activity

Pathogenic indicator bacteria *Aeromonas hydrophila* (MTCC: 1739) and Ecoli (MTCC: 1678) were obtained from Microbial type culture collection (MTCC) Chandighar India. *Pseudomonas aeruginosa* (PIC4) was isolated from Coastal water of Visakhapatnam. Antibacterial activity of the isolate was tested by agar well diffusion method against indicator bacteria *Aeromonas hydrophila and E.coli*. Ten mille letters of 18 hrs young culture of *P. Aeruginosa* (PIC4) grown in Luria Bertani (LB) broth medium (Himedia, India) was centrifuged at 5000g. The cell free supernatant broth was concentrated under reduced pressure (Buschi-210) at 30°C and diluted in 1 ml of double distilled water and stored at 4°C. 100µl of each 18hr young culture of *A. hydrophila and E.coli* cultured in LB broth were spread on LB agar plates. Wells of 1 cm diameter were made at the centre of the agar dish with sterile cork borer. The concentrated cell free culture broth (100µl) of *P. aeruginosa* PIC 4 was directly filled in the wells of agar dishes pre-inoculated with indicator bacteria. Diameter of inhibition zone was measured after 24 hrs of incubation at 28°C. Filtered cell free culture broths (through 0.22µm and 0.45µm porosity) were also tested for antagonistic activity.

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B. Optimum Temperature, Time and Sodium Chloride (NaCl) Concentration

Upon confirmation of antibacterial activity, culture conditions of the *P. aeruginosa* (PIC4) were optimised in order to maximise the production of secondary metabolites [11]. *P. aeruginosa* PI C4 was cultured in 250 ml conical flasks (5 Nos) containing 100 ml of LB broth and incubated at various temperatures viz 25, 28, 30, 35and 40^0C on orbital shaker at 100 rpm. Hundred micro litres of concentrated cell free broth samples obtained (as mentioned in II.A) at various time intervals (12, 24, 36, 48, 60 & 72hrs) were subjected to antibacterial screening by agar well diffusion method [12] against *A. hydrophila and E. coli*. Diameter of inhibition zone was measured after 24 hrs of incubation at 28°C. The bacterial count was measured by reading the optical density (OD) at 600nm and serial dilution followed by spread plate method. Optimum NaCl concentration was standardised by supplemented LB broth with 0.5, 1, 1.5, 2 and 5% NaCl. Cell free broth samples from these flasks at optimum time temperature were tested for antimicrobial activity and followed by enumeration of bacterial count (as mentioned above).

C. Preparation of Crude Extract

Bulk production of crude extract was achieved by culturing of the isolate PIC4 in 5 litre conical flask (10 No.s) containing 1.8 litre of LB broth added with 1% NaCl at 28 °C for 48 hrs on a rotary shaker moving at 100 rpm. The culture broth was filtered through muslin cloth to get the cell free media. Equal amounts of cell free culture broth and ethyl acetate taken in separating funnel, mixed well and allowed to separate organic and aqueous fractions for 10 minutes and phases were collected separately. Both aqueous and organic phases were tested for antibacterial activity. Only the organic phase was performed to concentrate extract under reduced pressure (Buschi 210) at 30 °C as it alone showed antibacterial activity on tested bacteria.

D. Screening of the Solvent Extracts for Bioactivity

The crude extract obtained was further subjected to fractionation with solvents of increasing polarity viz 100% hexane, 10% ethyl acetate in hexane, 50% ethyl acetate in hexane, 100% ethyl acetate and 100% methanol. Purity of fractions was tested by thin layer chromatography (TLC) and impurities were resolved by re-crystallization using organic solvents. Antimicrobial activity of each purified solvent fraction was further tested by agar well diffusion method briefly; well made on the agar dishes pre-inoculated with indicator bacteria. Each concentrated solvent fraction was dissolved in ethyl acetate (1mg/ml) and 50, 100, 150 & 200 µl of dissolved crude extract (at the concentration of 50, 100, 150 and 200 µg respectively) was directly filled in to the wells of agar dishes pre inoculated with indicator bacteria. The plates were incubated at 28 ℃ and the diameter of the inhibition zone was measured at 24 hrs of incubation.

E. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of purified fractions against the pathogenic bacteria was determined by micro dilution method [13]. One mille litre of the nutrient broth containing the compound (F1 or F3) at various concentrations was distributed in eight sterile test tubes (1 µl to 128 µl) besides keeping a positive and negative control (Table I). The concentration of the compound was maintained in each tube at geometric progression such as 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml. Bacterial suspension of (*A. hydrophila* or *E. coli*) was prepared by dissolving in 5 ml equivalent to a Mc Farland 0.5 standard [13]. 0.1 ml of bacterial suspension was added to 9.9ml of saline with a fresh pipette. From which 0.1 ml of diluted bacterial suspension transferred to broth tubes containing the compound with varying concentration, numbered 1 through 8 and also to the positive control tube. 0.1ml sterile saline was added to the negative control. Contents in all the tubes were mixed well and incubated for 24hrs. Test tube containing lowest concentration of the compound with no bacterial growth was considered as minimum inhibitory concentration.

TABLE I. MICRO-DILUTION TUBES CONTAINING COMPOUND CONCENTRATION IN DESCENDING ORDER

Tube No	1	2	3	4	5	6	7	8	9	10
Conc. of the compound µg/ml	128	64	32	16	8	4	2	1	positive control	Negative control

III. RESULTS

A. Optimization of Antagonistic Activity

The diameters of inhibition zones formed by concentrated cell free culture broth were obtained. There was no difference found in diameter of inhibition zone between filtered and unfiltered cell free culture broths (Fig. 1 and Fig. 2).

The optimum Temperature and time for growth and antibiotic production were determined as 28°C and 48 hrs respectively. Even though cell growth reached maximum after 36hrs at 48°C but inhibitory secondary metabolite production was very low (Fig. 3 and Fig. 4). Optimum concentration of NaCl was determined as 1% to the LB medium (Fig. 5 and Fig. 6).

B. Bioactivity of Crude Extract

Three grams of dark pungent smelling crude extract obtained on evaporation of organic phase under reduced pressure. Upon five solvent fractions obtained from crude extract, fraction1 (100% hexane) and fraction 3 (hexane:

ethyl acetate 1:1) showed antibacterial activity on both the tested bacteria, while fraction 2, 4 and 5 are devoid of activity. (Fig. 7) Fraction 1 (F1) was obtained as viscous oily liquid with unbearable smell. TLC of F1 has shown one major spot with three other minor spots which were well resolved by re crystallization using acetone and methanol. Purified F1, a colourless with low melting crystals was exhibited antibacterial activity with inhibition zone of 20 & 32 mm in dia on *E. coli* and *A. hydrophila* respectively at 200 µg concentrations (Table.2, Fig. 8). Fraction 3 (F3) showed dark major spot and several closely related spots on TLC which were resolved by re-crystallization using hexane: acetone (60:40). Purified F3, colourless short needles exhibited antibacterial activity with inhibition zone of 32 & 26mm in dia on *E. coli* & A. *hydrophila* respectively at 200 µg concentration (Fig. 9).

Figure 1. Antagonistic activity of cell free culture broth of *P. aeruginosa* on *Aeromonas hydrophila*.

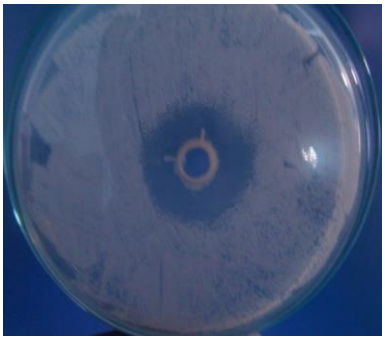

Figure 2. Antagonistic activity of cell free culture broth of *P. aeruginosa* on *Ecoli*

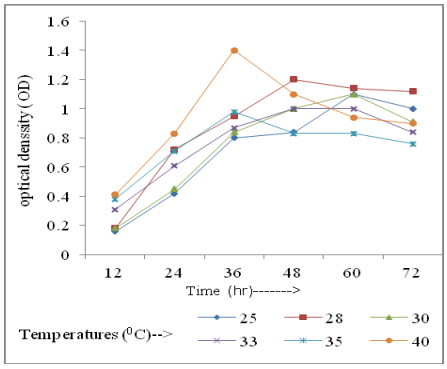

Figure 3. Optimal culture conditions of the isolate PIC 4 (Temperature & Time).

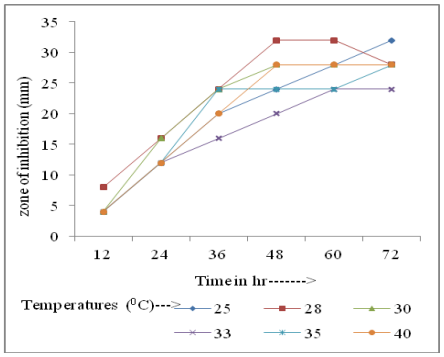

Figure 4. Inhibitory secondary metabolite production at various temperature and time intervals

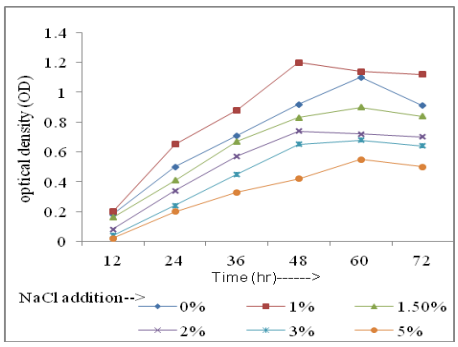

Figure 5. Growth of PIC4 culture at different NaCl concentrations.

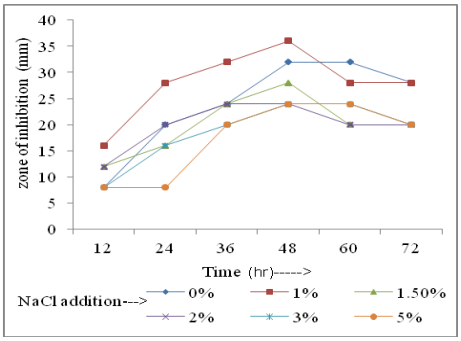

Figure 6. Inhibitory secondary metabolite production at different NaCl concentrations.

C. *Bioactivity of Crude Extract*

Three grams of dark pungent smelling crude extract obtained on evaporation of organic phase under reduced pressure. Upon five solvent fractions obtained from crude extract, fraction1 (100% hexane) and fraction 3 (hexane: ethyl acetate 1:1) showed antibacterial activity on both the tested bacteria, while fraction 2, 4 and 5 are devoid of activity (Fig. 7). Fraction 1 (F1) was obtained as viscous oily liquid with unbearable smell. TLC of F1 has shown one major spot with three other minor spots which were well resolved by re crystallization using acetone and methanol. Purified F1, a colourless with low melting crystals was exhibited antibacterial activity with inhibition zone of 20 & 32mm in dia on *E. coli* and *A. hydrophila* respectively at 200 µg concentrations (Table II, Fig. 8). Fraction 3 (F3) showed dark major spot and several closely related spots on TLC which were resolved by re-crystallization using hexane: acetone (60:40).

Purified F3, colourless short needles exhibited antibacterial activity with inhibition zone of 32 & 26mm in dia on *E. coli* & A. *hydrophila* respectively at 200 µg concentration (Fig. 9).

D. Minimal Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) of both F1 and F3 fraction were determined as32 µg/ml on E.coli and 64 µg/ml on *Aeromonas hydrophila.*

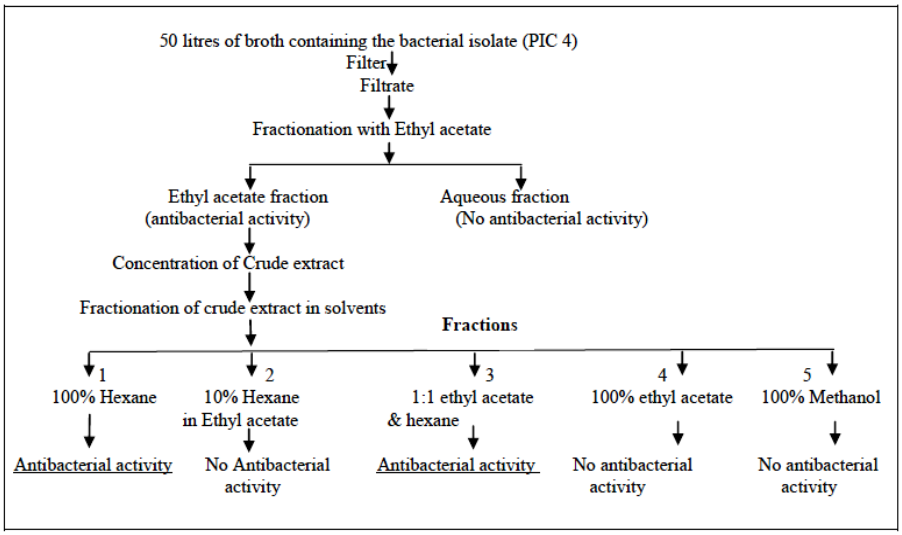

Figure 7. Flow chart showing extraction of bioactive compounds.

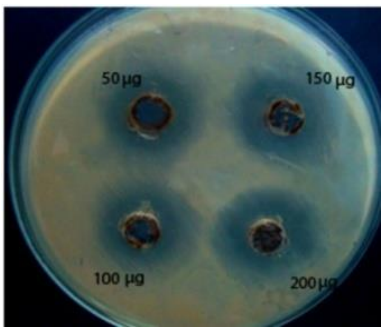

Figure 8. Agar dish showing inhibition zone formed by F1 Slovent extract on *E. coli*.

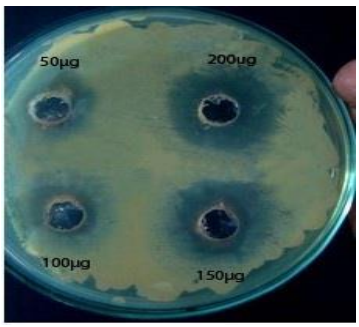

Figure 9. Agar dish sshowing inhibition zone formed by F3 Slovent extract on *E. coli*.

TABLE II. DIAMETERS OF INHIBITION ZONES FORMED BY SOLVENT FRACTIONS ON INDICATOR BACTERIA

Concentration of Compound in µg	F1		F3	
	Diameter of inhibition zone(mm) on		Diameter of Inhibition zone (mm) on	
	A. hydrophila	*E. coli*	*A. hydrophilia*	*E. coli*
50	6	10	8	10
100	10	18	16	14
150	14	26	20	18
200	20	32	22	26

IV. DISCUSSION

Inhibitory activity of Pseudomonas against a number of pathogens such as *Salmonella*, *Staphylococcus aureus* and *V. parahaemolyticus* has been reported since the year 1960 [14]-[18]. Kinetics of any bioorganic reaction depends heavily on the external conditions, where temperature plays a significant role. In our experiment 28°C is the optimum temperature for production of bioactive compound(s) it is in the optimal temperature range of 25-40°C earlier reports showed [19]-[21] and 38°C as observed by Vijayan *et al.*, (2006) [11]. Generally microbes produce secondary metabolites in the stationary phase. The time duration for this phase is usually 24-48 hours [22]. In our study, optimal production (peak production) was observed at 48 hours. Earlier reports on various *Pseudomonads* showed an optimal production after 36 hrs [23]-[25]. Vijayan *et al.* has observed peak production of bioactive metabolites

from *Pseudomonas* sp. in a 30 hrs culture. Sodium chloride concentration plays major role in the maintenance of osmotic balance [26]. In the tested range of 0.5 to 5%, optimum NaCl concentration required for our microbial isolate was found to be 1%. Similar pattern was also observed for Pseudomonas I-2 strain by Chytanya *et al.*, [27]. It is assumed that the compound may not be an enzyme or protein since no difference found between in antibacterial activity between filtered and unfiltered culture broth. MICs of our two fractions are in the good range between 32 and 64 µg/ml [13] and form clear inhibition zones in agar diffusion method. A number of earlier studies have also shown that bacteria produce inhibitory substances that inhibit the bacterial pathogens.

V. Conclusions

Pseudomonas aeruginosa isolated from Marine waters exhibited antibacterial activity. Culture conditions for the isolate were optimized in order to produce maximum zone of inhibition against the tested pathogens. Antibacterial activity was found in the crude extract obtained in the organic phase after fractionation in Ethyl acetate, hexane and Methanol. Two fractions exhibited antibacterial activity against the indicator bacteria tested *A. hydrophila* & *E. coli*. The use of such bacteria which inhibit pathogens by release of antimicrobial substances is now gaining importance in a better and more effective pathogen control.

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