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Production and Characterization of Bioactive Pyocyanin Pigment by Marine *Pseudomonas aeruginosa* OSh1.

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ABSTRACT

Marine *Pseudomonas aeruginosa* OSh1 was isolated from El-Mex station, Alexandria, Egypt and identified by 16S rDNA gene sequencing. The characteristic feature of *Pseudomonas aeruginosa* is the production of soluble blue pigment (pyocyanin) as a secondary metabolite. Production and characterization of pyocyanin were studied to evaluate its antimicrobial potential against pathogenic strains. Considerable amounts of blue pigment were produced by *P. aeruginosa* OSh1 when grown on the four tested media. King's broth medium was selected for further use in pigment production. Pigment production began during the first 24 hrs of growth and maximal bioactive pigment yield was achieved at 30.3 µg/ml after 96 hrs. Neutral pH at 28 ^{o}C showed the optimum conditions for maximum pyocyanin production (76.11 µg/ml) and antimicrobial activities (30 – 45 mm) obtained from the culture supernatant of the *P. aeruginosa* OSh1. The bioactivity of pyocyanin was superior compared with some standard antibiotics. Chloroform extractable pigment was characterized using Gas Chromatography – Mass Spectrum analysis. In conclusion, the properties of pyocyanin from *P. aeruginosa* OSh1 make it an important bioactive compound with the ability to arrest the virulent pathogens and be explored as antimicrobial agents for future drug development.

Keywords: Marine *P. aeruginosa*, pyocyanin, bioactive pigment, antimicrobial agent.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacterium, ubiquitous organism in nature widespread in soil, water and many other environments [1]. *P. aeruginosa* attracts attention by producing a variety of extra-cellular phenazine pigments [2]. Pyocyanin pigment: a water soluble blue green compound has various pharmacological effects [3]. Nearly 90–95 % of all isolates of *P. aeruginosa* produce pyocyanin pigment, which is referred to as ‘‘blue pus’’ (from pyocyaneus) [4]. Pyocyanin is produced abundantly in media with low iron content and plays an important role in iron metabolism which considered as a crucial requirement for the growth of *P. aeruginosa* [5].

Pyocyanin pigment is a phenazine which is a nitrogen-containing heterocyclic compound. It is a redox active secondary metabolite and is soluble in chloroform considered for many applications. It has a characteristic feature of inhibiting many bacterial colonization and fungal growth both *in vivo* and *in vitro* condition. The antagonism of *P. aeuginosa* against pathogenic *Vibrios* in aquaculture has been identified due to its metabolite, pyocyanin [6]. Pyocyanin compounds produced from the rhizosphere of plants contribute to the biological activity of *P. aeruginosa* against *Fusarium* (wilt of chickpea) and *Pythium* (damping-off of bean) [7]. In addition, the bioprocess and downstream processing of pyocyanin have been studied for aquaculture applications [8]. Pyocyanin, also, can be used as electron shuttle in microbial fuel cells (MFC) by enabling bacterial electron transfer towards the cell anode [9]. Pyocyanin could conjugate to organic compounds and forming new complexes those used in organic light emitting devices (OLED), gaining importance of low voltage requirements, wide color range, and light weight [10].

Despite the various applications, synthesized pyocyanin was costly compound in the market (www.sigmaaldrich.com). Bio-synthesis of pyocyanin with efficient grade compensates its higher cost and makes its bio-amelioration procedures easier [11]. Pyocyanin, as a versatile and multifunctional phenazine pigment, has been applied as bio-control agents against many pathogens [12]. However, *P. aeruginosa* is not yet experimented to improve the yield of pyocyanin production. Considering the above facts, the present work attempts to make on the production of pyocyanin from marine *P. aeruginosa* as functional activity against different virulent microbes (Gram-ve, Gram +ve and fungi). Production conditions for maximal pyocyanin yield was carried out, and then compared with some commercial antibiotics used for therapeutic applications.

MATERIAL AND METHODS

Isolation and identification of pigments producing marine potent Strains

Different water and sediment samples were collected from different locations of the Egyptian coasts. Out of 25 pigments producing strains, one blue green pigment producing strain from El-Mex station, Alexandria, Egypt, was isolated on marine agar plates at 35 ^{o}C, and purified. The pure isolate was maintained on marine agar plates and slants at 4 °C as confirmed media for blue green strain. Biochemical analysis according to Bergey’s Manual of Systematic Bacteriology, was preliminary used to characterize *P. aeruginosa* [13]. The molecular identification of the strain was achieved by 16S rDNA gene sequencing using enzymatic lyses method [14]

Pathogenic microbes used in this study

Twelve tested pathogens, used in this study (Table 1), were obtained from (1) the American type culture collection (ATCC; Rockville, MD, USA), and kindly provided by (2) Microbiology Lab, Marine Environment Department, National institute of oceanography and fishers, (3) El Salam international hospital and (4) National Research centre (NRC), Egypt. Stock bacterial cultures were maintained on nutrient agar slants at 4 ˚C with monthly transfer, whereas, fungal mycelia were maintained on Sabouroud's agar slants.

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Table 1: Pathogenic Tested Microbes

Type	Microorganisms
Gram +ve	*Lesteria monocytogenes* ATCC- 35152
	Staphylococcus aureus (MRSA)
	Staphylococcus aureus ATCC-47077
	Staphylococcus lentus
Gram –ve	*Aeromonas hydrophila*
	E. Coli ATCC- 25922
	Peudomonas sp
	Vibrio cholera
Fungi and Yeast	*Aspergillus niger* ATCC- 16888
	Candida albicans ATCC- 10231
	Candida tropica
	Fusarium oxysporum

Production of pyocyanin pigment

P. aeruginosa OSh1 strain produces soluble blue green pigment named (pyocyanin). Pyocyanin production was carried out using King's broth Medium as described by King *et al*. [15]. *P. aeruginosa* was inoculated into king's broth medium (pH 7) and incubated under shaking condition (130 rpm) at 35 °C for 3 days. Coloured supernatant free bacterial cells removed by centrifugation at (10.000 rpm x 10 min) and filtration through 0.45 µm filter, was used for further applications.

Extraction and Purification of pyocyanin

Pyocyanin was separated from culture supernatants by the addition of chloroform solvent system. Extractable chloroform layer was further mixed with 1 ml of 0.2 N HCl which converted pyocyanin to the acidic (red) form. Red colour obtained pigment was separated and subjected to UV spectrophotometric analysis (Jenway UV/Visible- 2605 spectrophotometer, England) and scanned at range of 200-800 nm. Absorption was measured at 520 nm [16]. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were calculated using the following equation:

$$\text{Concentration of pyocyanin } (\mu g/ml) = O.D_{520} \times 17.072$$

Antimicrobial activity of pyocyanin

The antimicrobial activity of pyocyanin was tested by disc diffusion technique to test the antimicrobial activity of marine bacterial isolate. Agar plates of Müller Hinton for bacteria and potato dextrose for yeasts and fungi were prepared. The plates were inoculated with 0.1 ml containing 10^6 cfu/ml of fresh bacterial cultures and spore suspensions of pathogenic strain. Sterile discs of 5 mm diameter were loaded by 10 µl of pyocyanin dissolved in dimethylsulfoxide (DMSO). Negative control disc was applied for DMSO. Plates were left for two hour in the refrigerator to allow the diffusion. The plates were incubated for 24 h at 30 °C for bacteria and incubated for 72 h at 30 °C for fungi and examined for inhibition zone. All the assays were performed in duplicate [17].

Factors affecting on pyocyanin production

Pyocyanin has been used as an effective agent in killing many microorganisms that were resistant to most antibiotics. Therefore, trials to enhance the production of pyocyanin pigment using different condition as: different media, incubation period, pH and temperatures during the growth of *P. aeruginosa* OSh1, was studied.

Effect of different growth media

Four types of Media including nutrient broth, tryptone water, peptone water and King's B were testing for the biosynthesis of pyocyanin for 3 days at 35 °C at 130 rpm. Bacteria were centrifuged and filtered. Pyocyanin concentrations were determined by the dark blue supernatant of the chloroform extraction [5]. The antimicrobial effects of pyocyanin were recorded according to the diameter of the inhibition zone. The obtained pigments were stored at 4°C in the dark place.

Effect of incubation period

The selected Kings' B (KB) medium was used in 100 ml flasks which were inoculated and incubated at 35 °C at 130 rpm for time periods from zero to 120 h. Samples were collected every 24 hours and concentration of pyocyanin measured. The antimicrobial effects of pyocyanin were recorded.

Effect of different pH and temperature

The effect of four adjusted temperatures (28, 37, 40 and 50°C) and four different pH values (6, 7, 8 and 9) were carried out using the Kings' (KB) and shaking at 130 rpm. The dark blue supernatant was subjected to chloroform extraction; pyocyanin concentrations were determined [5].The antimicrobial activity of pyocyanin against the tested pathogens were determined.

Comparison using standard antibiotics

Seven formulated antibiotic discs were purchased from Sigma (Dorset, UK), and arranged according to their group (Table 2) and used to compare the inhibition of the tested organisms with pyocyanin using agar diffusion test similar to the routine laboratory.

Table 2: Antibiotics used to evaluate the antimicrobial activity of pyocyanin

Antibiotic group	Antibiotics	Abbreviation
Beta-lactams	Ampicillin	AM
Macrollides	Erythromycin	E
Quinolone	Nalidix acid	NA
Aminoglycosid.	Germaycin	GM
	Flucloxacillin	AF
Chloramphenic.	Chloramphenicol	C
Glycopeptides	Vancomycin	VA

GC-MS analysis

Gas chromatograph-mass spectrometer coupling (GC/MS) measurements were performed on an Agilent Technologies 7890A GC System with a flame ionization detector, a 5975C inert XL MSD Triple-Axis Mass Detector and Agilent 19,091S-433 Trace Analysis column. GC-conditions: 1 µL of sample was injected with an evaporation temperature of 250 °C, 1.8 bar, 2.5 mL/min, split 20:1. He carrier gas, temperature gradient 50 °C/1 min, 40 °C/min gradient 300 °C/min, 300 °C/5 min. The components were identified by comparing their retention times to those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library [18]. Quantitative data were obtained by the peak normalization technique using integrated FID response.

RESULTS

P. aeruginosa, as a potent bacterial strain, was collected from El-Mex Station, Alexandria, Egypt. The selection process was based on its ability to produce bioactive pigment (pyocyanin) against pathogenic microbes. Molecular identification was carried out at National Research centre (NRC), Egypt. Partial sequence

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of 16S rDNA was achieved using the BLAST program (National Centre for Biotechnology Information, NCBI). *P. aeruginosa* strain OSh1 was deposited in the GenBank of NCBI under accession number: KT032066. Phylogenetic trees of potent strain match with other strains available in public database (Figure 1).

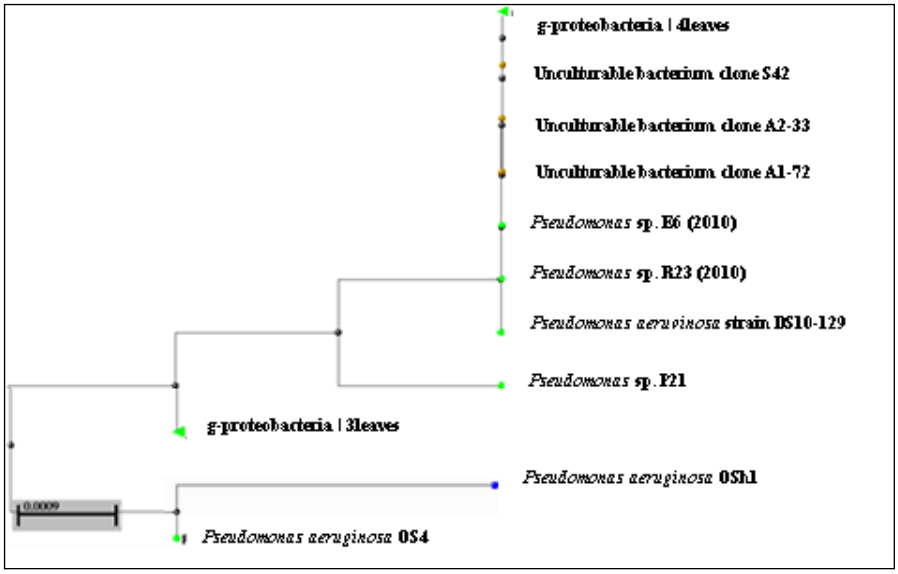

Figure 1: Phylogenetic tree based on the 16S rDNA genes of marine *P. aeruginosa* strain OSh1 with accession No. KT032066.

Production of pyocyanin

Cultures of *P. aeruginosa* produced a blue green pigment, reminiscent of pyocyanin, during growth on King's broth medium. Pigment production was indicated by change in color in King's broth to a blue green indicating the production of pyocyanin pigment (Figure 2). The appearance of pigment starts after 24h of incubation and increased with time until 72h. Pyocyanin quantification assay is based on the pyocyanin's measuring color at 520 nm in acidic phase and using 0.2 M HCl used as a blank. Absorbance was multiplied by 17.072 the extinction coefficient and the yield was expressed in micrograms (µg) of pyocyanin produced per milliliters (ml) of solution (µg/ml). Pyocyanin concentration obtained after 72 h of incubation was 26µg/ml.

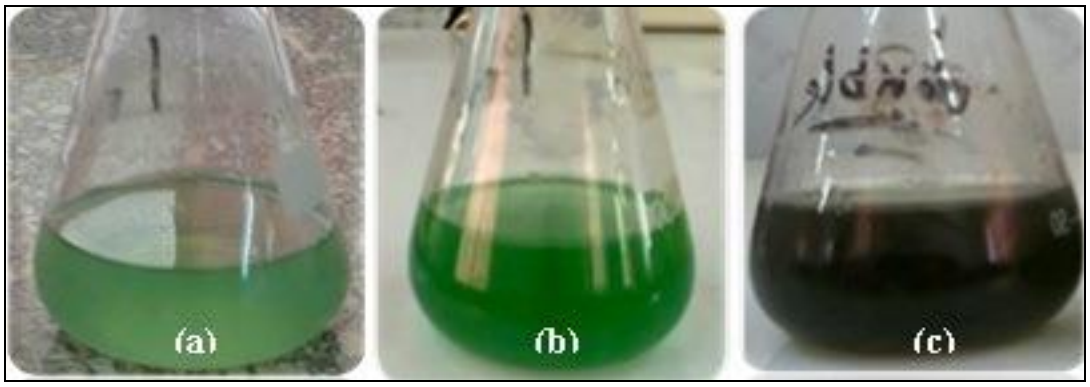

Figure 2: Gradual increase in blue green pigment (pyocyanin) produced by *P. aeroginosa* OSh1 after 24h (a), 48h (b) and 72h (c).

Growth of *P. aeruginosa* OSh1 in different culture media

As indicated from Table (3), basal King's B medium gave the maximum yield of pyocyanin production (25.5 µg/ml) when compared to nutrient broth (5.28 µg/ml) tryptone water (9.55 µg/ml) and water peptone (11.74 µg/ml) broth. Bioactivities measured in mm showed maximum average of inhibition at 20.8 mm against Gram –ve and 18.3 mm against Gram +ve and fungal strains that recorded using King B medium.

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Time for maximum Pyocyanin production

Maximum pyocyanin yield and antimicrobial activity were measured at 0 h and after 24, 48, 72, 96 and 120 hr. A gradual increase in yield and inhibition zone diameters were observed from 24 to 96 hr reaching maximum values 42 µg/ml and 30.3 mm, respectively. The loss in activity was evidently determined after 4th days of incubation (Figure 3).

Table 3: Bioactivity and yield of the produced pyocyanin pigment by *Pseudomonas aeruginosa* using different type of medium

Type of Media	Yeild (µg/ml)	Inhibition zone diameter (mm)*											
		Gram negative				Gram positive				Fungi			
		A. hydrophila	*E.Coli* ATCC-25922	*Peudomonas* sp	*V. cholera*	*St. aureus* (MRSA)	*L. monocytogenes* ATCC- 35152	*St. aureus* ATCC-47077	*St. lentus*	*C. albicans* ATCC-10231	*C. tropica*	*A. niger* ATCC-16888	*F. oxysporum*
Nutrient broth	5.28	15±0.75	0	7±0.35	10±0.5	15±0.75	13±0.65	10±0.5	15±0.75	7±0.35	0	0	0
Tryptone water	9.55	16±0.8	10±0.5	0	16±0.8	17±0.85	17±0.85	12±0.6	0	9±0.45	10±0.5	0	0
Peptone water	11.74	20±1.0	12±0.6	0	20±1.0	21±1.05	21±1.05	14±0.7	14±0.7	19±0.95	20±1.0	12±0.6	10±0.5
King'sB	25.5	30±1.25	20±1.0	10±0.5	25±1.25	33±1.65	23±1.15	27±1.35	0	28±1.4	27±1.35	7±0.35	11±0.55

*Values are means ±SEM of three replicates

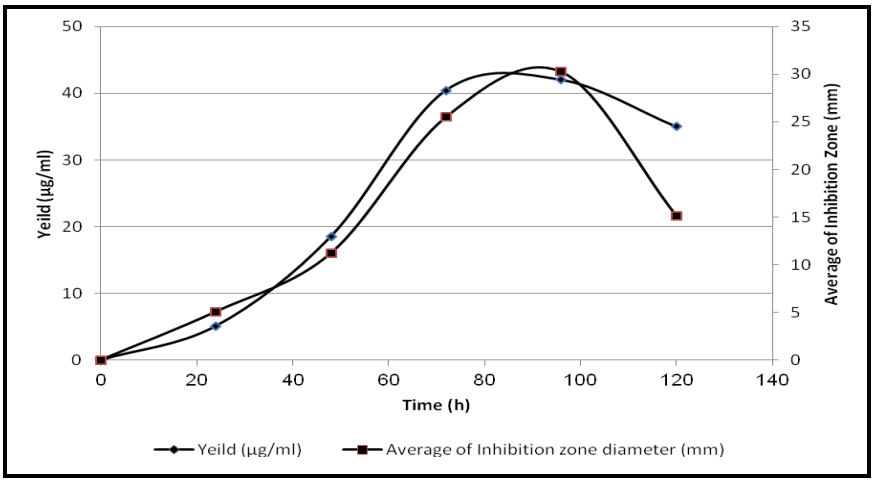

Figure 3: Consolidated view of the pyocyanin yield (µg/ml) and bioactivity (mm) by *Pseudomonas aeruginosa* OSh1 using King's B broth.

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Different pH and temperature affecting pyocyanin production

Different pH and temperature were experimentally tested against the twelve pathogens and graphically illustrated in Figures (4&5). It was found that pyocyanin bioactivities and so as its yield were increased with decrease of pH (7-6) and temperature (28°C) level. Maximum activities (37 mm) and yield (75.15µg/ml) were observed at pH 7. Optimum temperature at 28°C showed maximum pyocyanin activity at 36.3 mm and yield 76.11 µg/ml. Lower activities were recorded for pH 9 at 40 °C.

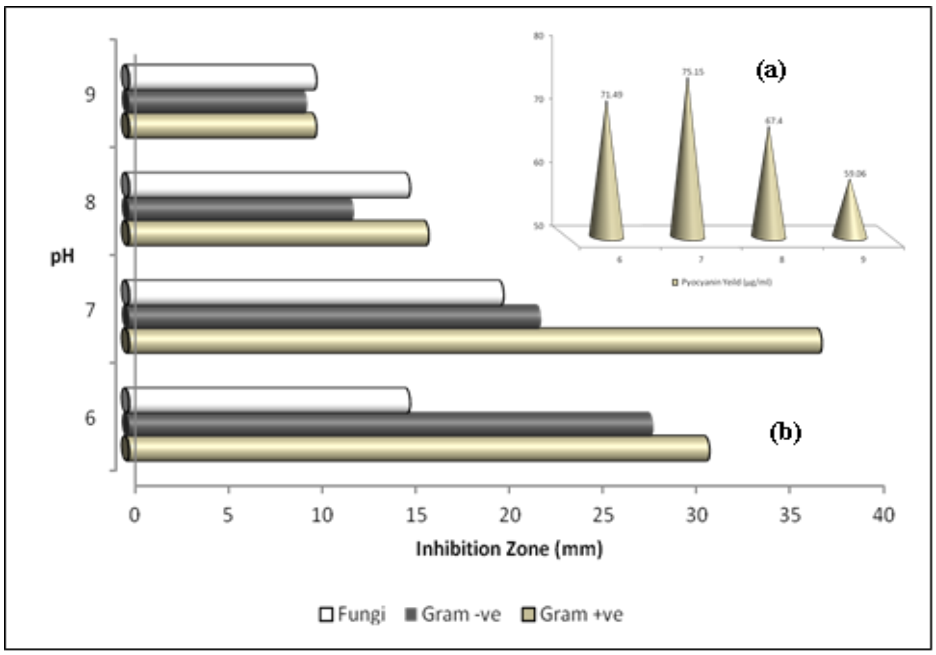

Figure 4: Effect of pH values on pyocyanin yield (a) and activity (b)

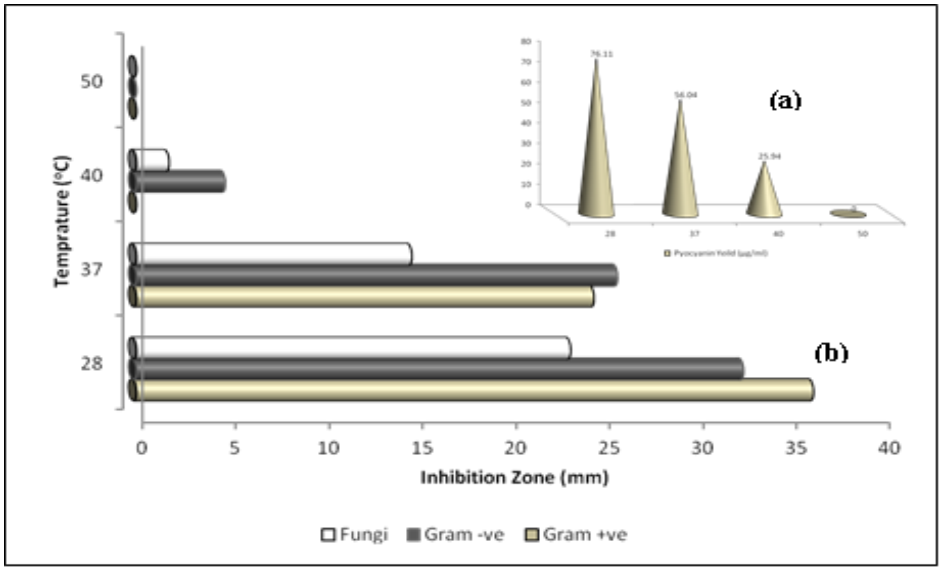

Figure 5: Effect of temperature degrees on pyocyanin yield (a) and activity (b)

Compared with formulated antibiotics

Formulate antibiotics were used to compare with the activity of pyocyanin obtained from marine *Pseudomonas aeruginosa* OSh1 against the twelve pathogens. The pathogens were sensitive to chloramphenicol by average zone of inhibition of 16.6 mm and showed high resistance towards ampicilin based on the resulted inhibition zone diameter by average 5.8 mm. Bioactive pyocyanin showed higher

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activities 28.3, 37.3 and 21 mm compared with the tested antibiotics with total inhibition zone average 13.1, 16.7 and 3.5 mm, against Gram positive, Gram negative bacteria and fungi, respectively (Table 4 and Figure 6).

GC-MS analysis of pyocyanin

A major sharp peak was observed at acquisition time 20.76 min with the highest count % 2.4 x102 on gas-chromatographic analysis. This compound was identified by mass spectrum library as 1-hydroxy-N-methyl phenazine "pyocyanin" with intense molecular ion peak at 196 m/z and its structure was shown in Figure (7).

Table 4: Comparison between antibiotics sensitivity test and bioactive pyocyanin pigment against the pathogenic strains

Group	Antibiotics (AB)	Code	Inhibition zone diameter (mm)*												Ab AVG*
			Gram negative				Gram positive				Fungi				
			A. hydrophila	*E.Coli* ATCC-25922	*Peudomonas* sp	*V. cholera*	*St. aureus* (MRSA)	*St. aureus* ATCC-47077	*St. lentus*	*L. monocytogenes* ATCC- 35152	*C. albicans* ATCC- 10231	*C. tropica*	*A. niger* ATCC-16888	*F. oxysporum*	
Beta-lactams	Ampicillin	AM	0	0	20	0	0	0	18	20	0	0	11	0	**5.8**
Macrollides	Erythromycin	E	22	0	0	17	0	26	22	23	10	0	0	12	**11.0**
Quinolone	**Nalidix acid**	NA	18	24	0	25	0	8	15	22	7	0	0	0	**9.9**
Aminoglycosid.	Germaycin	GM	23	15	11	25	11	21	18	26	13	11	0	0	**14.5**
	Flucloxacillin	AF	10	14	0	17	0	20	28	29	0	0	0	0	**9.8**
Chloramphenic.	Chloramphenicol	C	15	22	24	23	25	20	17	25	10	0	10	8	**16.6**
Glycopeptides	**Vancomycin**	VA	22	0	0	20	20	17	16	20	0	0	0	7	**10.2**
Pyocyanin (76.11µg/ml)			42	31	0	40	37	36	37	39	34	35	0	15	**28.8**

*Ab_{AVG} = Antibiotics Average

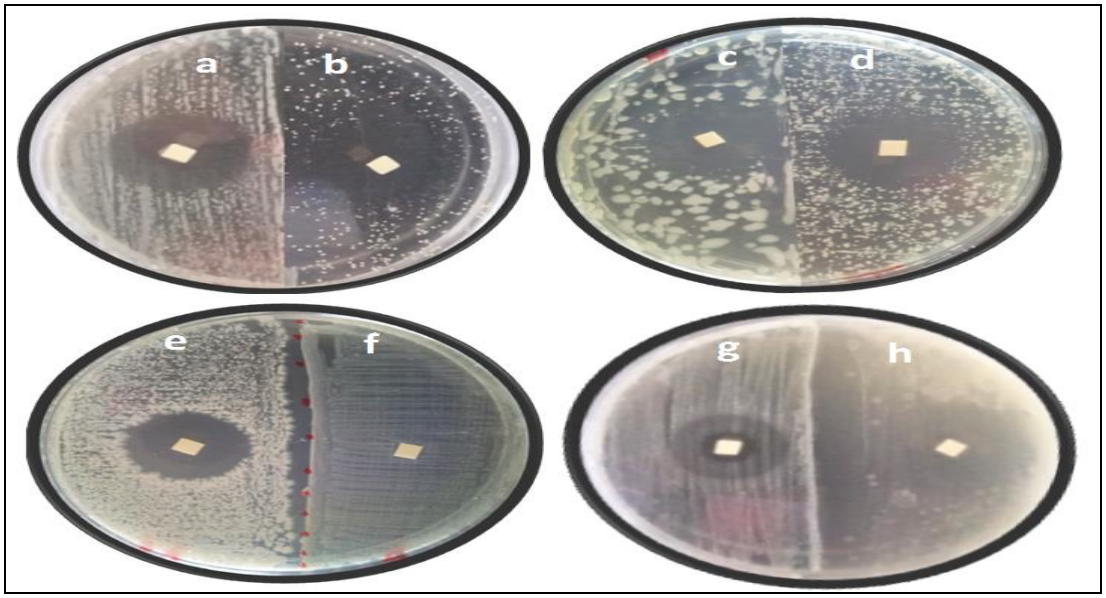

Figure 6: Antimicrobial activity of pyocyanin produced by *P. aeruginosa* strain OSh1 against pathogenic microbes;a: *A. hydrophila,* b: *V. cholera*, c: *St. aureus* ATCC-47077, d: *St. aureus* (MRSA), e: *St. lentus*, f: *Pseudomonas* sp., g: *L. monocytogenes* ATCC- 35152 and h: *E. coli* ATCC- 25922.

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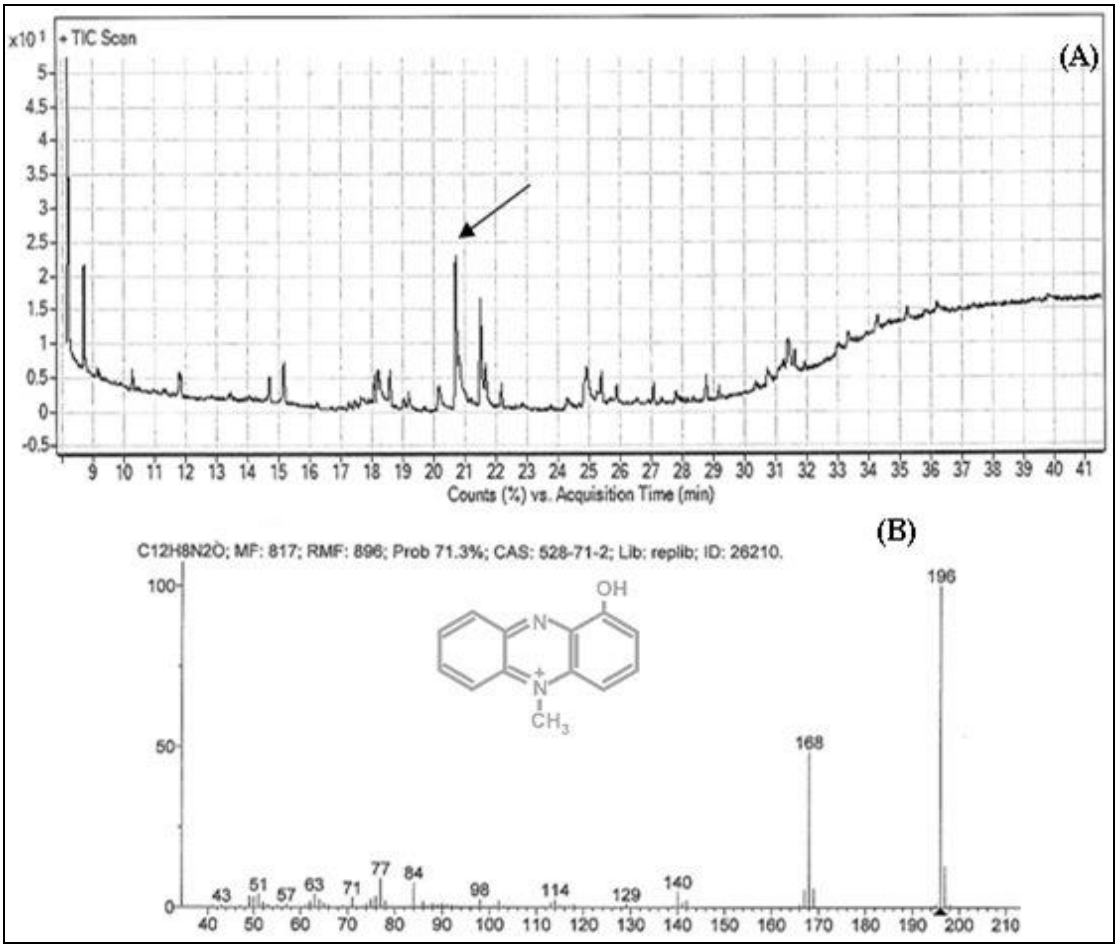

Figure 7: Gas Chromatography-Mass Spectra analysis showing major peak in GC analysis (A) and identified compound (Pyocyanin) in Mass Spectral analysis (B).

DISCUSSION

P. aeruginosa is belonging to the bacterial family *Pseudomonadaceae* which is widespread in the environment; in soil, fresh water, marine environment etc. [19]. It has received intensive attention due to its medical importance, as well as its catabolic versatility [20]. This study revealed that, the higher pyocyanin pigment producer *P. aeruginosa* OSh1 was selected and its identification confirmed by molecular tools. Purified and characterized pigment was carried out. Marine *P. aeruginosa* OSh1 has the capability of maximum production of bioactive blue-green phenazine (pyocyanin) pigment within 4 days of incubation and grow on King's B medium at 28^{o}C and pH 7. Similarly, a blue natural product metabolite pyocyanin isolated from a marine microbial population was identified as synergistic production between two distinct bacterial species [21]. Application of pyocyanin pigment produced by *P. aeruginosa* as antimicrobials and food colorant was carried out [13]. Analysis of pyocyanin compound and its antagonistic activity against phytopathogens were studied by Sudhakar *et al*. [7]. Recently, El-Fouly *et al*. [11] showed the biosynthesis of bioactive pyocyanin pigment by *P. aeruginosa*.

Pyocyanin compound was produced and extracted. A blue-green shade colour of the solution was obtained, extracted by adding chloroform which separated a blue colour compound. It was then confirmed by adding 0.2 N HCl and a pinkish red colour compound was obtained which indicated the presence of pyocyanin pigment [22].

Marine *Pseudomonas aeruginosa* OSh1 produced the highest yield of blue-green pigment at 26 µg/ml developed on King's B medium. Based on King's medium, *Pseudomonas* agar medium was formulated which is recommended for pyocyanin production by *Pseudomonas* species. This medium enhances the elaboration of pyocyanin but inhibits the formation of other pigments [23]. Detection, isolation and identification of

phenazine-1-carboxylic acid pigment produced by biocontrol strains of *Pseudomonas aeruginosa* using King's B were studied [24]. The enhancement effect of king's a medium on pyocyanin production was previously mentioned and explained the protective action of the redox-active pyocyanin production [25]. King's B medium showed the growth of pigmentous marine *Pseudomonas* [26]. Different *Pseudomonas* sp. were isolated and plated onto King's medium specific for blue-green pigment production yield a range of 1.7 - 6.3 µg /ml [11]. Despite this point of view, production of pyocyanin from hundred strains of *P. aeruginosa* using seven liquid modified medium, among these medium used, cetrimide showed maximal production of pyocyanin [23]. A report by Shouny *et al*. [27] showed *P. aeruginosa* isolates when grown on four tested media; Müller-Hinton was further used for growth and pyocyanin pigment production.

There are some factors that influence pigment production when using various pH and temperatures. Pyocyanin production has a great tendency toward acid condition pH 6-7 at marine ambient temperature 28 ^{o}C after 4 days of fermentation condition. Many reports determined the cultural characteristics and conditions which favor the production of pyocyanin. *Pseudomonas* sp. M18 grown at 28 °C and pH 4 showed pyocyanin production and antimicrobial activities against cystic fibrosis fungal mycelium [28]. Culture conditions for pyocyanin production by *P. aeruginosa* were studied at pH 7 and 30 °C with a maximum yield of 10 µg /ml [29]. Similar results reported by Das *et al*. [30] showed that *P. aeruginosa* PA14 strains could produce pyocyanin at pH 7 for 3 fermentation days at 30 °C. *P. aeruginosa* was inoculated in selected broth to optimize the maximum production of pyocyanin then incubated at 37ºC on a rotary shaker for 24h and observed for color change [30]. Maximal pyocyanin pigment production was achieved after 72 hrs by *P. aeruginosa* isolates at 37 °C and neutral pH [27].

This compound was further analyzed for its activity and found to be superior compared with some standard antibiotics. Maximum pyocyanin yield obtained from *P aeruginosa* OSh1 was recorded at 76.11 µg /ml showed higher antibacterial activities at 28.3, 37.3 and 21 mm against Gram positive, Gram negative bacteria and fungi, respectively. Pyocyanin yield obtained from culture supernatants of *P. aeruginosa* isolates (grown in peptone water liquid medium) reached about 62.8 µg/ml and showed activity ranges from 15 to 32 mm against Gram positive, Gram negative bacteria and yeast [31&32]. Phenazine-1-carboxylic acid (50 µg /ml) produced by the bio-control *P. aeruginosa* showed inhibition zone ranged from 8 to 10 mm against five fungal pathogens [24].

Extracted compound represented by *P. aeruginosa* OSh1 strain was subjected to GC-MS analysis to confirm the presence of the pyocyanin compound the results of this technique reveals the molecular ion peak of about 196 m/z and the retention time was found using GC, the eluted peak had a retention time of about 20.758 min. This peak was the major peak, while, the minors were not taken in our work since the peak was found in negligible amount. The results were correlated to Ker *et al*., [33] who showed the related hemi-pyocyanin pigment extracted from *P aeruginosa* and identified by its electron impact mass spectrum after gas chromatography at ions at m/z 196.

CONCLUSION

In conclusion, purified pyocyanin pigment produced by marine *P. aeruginosa* OSh1 had the capacity to inhibit the growth of twelve virulent microbes. Therefore, it was recommended that the bio-synthesis of pyocyanin have a huge potential to be anti-microbial agent for medicinal and pharmacological development.

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