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Anti-Bacterial Activity of Pigments Isolated From Pigment-Forming Soil Bacteria

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Authors' contributions

Authors MF and MMR designed the study. Authors MMR and FK conducted the soil analysis and pigment extraction. Authors RMM and MAC performed the characterization and anti-bacterial activity of the pigment. Author MF supervised all the works and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Search for novel antimicrobials such as bacterial pigments is an issue of priority now. This study aims to isolate pigments with anti-bacterial activity from soil bacteria.
Methodology: In this study, Pigment forming bacteria was isolated from soil samples collected from different sites of Dhaka city and its adjoining areas. Colonies of various colors such as yellow, golden yellow, red, pink, blue, green, purple and cream with both diffusible and non-diffusible pigments were isolated in pure cultures on nutrient agar plus 2 percent glycerol at pH 7.2 and 37ºC. Anti-bacterial activity of the pigments extracted from the bacteria were determined.
Results: 15 pigment forming bacteria was isolated from soil and identified to genus level as *Pseudomonas, Flavobacterium, chromobacterium, Xanthomonas, Aeromonas, Escherichia* and *Bacillus*. All the pigments showed to be broad spectrum in terms of inhibitory activity against all the pathogens included in this study. Most of the pigments

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showed better anti-bacterial activity against gram-negative bacteria. Highest zone of inhibition was resulted by pigment no 15 against *Salmonella typhi* and lowest zone of inhibition was observed for pigment 13 against *Staphylococcus aureus*. Most of the pigments except four (pigment no- 3, 10, 12, 15) were found to be bacteriostatic to the test pathogens. MIC value of the pigments ranged from 1500-4000 µg/ml and most of the pigments showed lower MIC value against gram-negative organisms.
Conclusion: On the basis of anti-bacterial activity and minimum inhibitory concentration (MIC) pigment form *Aeromonas* (no- 6), *Escherichia* (no-10) and *Pseudomonas* (no-15) can be selected as effective anti-bacterial agent. Further studies are needed to use these pigments in food, cosmetic and textile industries.

Keywords: Pigment; soil; bacteria; antibacterial activity.

1. INTRODUCTION

Soil microbial communities are among the most complex, diverse and important assemblages of organisms in the biosphere and they are an important source for the search of novel antimicrobial agents and molecules with biotechnological importance such as microbial pigments that can be used as natural colorants as well as antimicrobial agents in place of antibiotic [1]. The demand for new antibiotics continues to grow due to the rapid emergence of antibiotic resistant pathogens causing life threatening infections in spite of considerable progress in the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds [2]. This changing pattern of diseases and the emergence of resistant bacterial strains to currently used antibiotics continuously put demand on the drug discovery scientists to search for novel antibiotics such as bacterial pigments [3].

Pigments of various colors are synthesized to protect the cells of micro-organisms from injurious effect of light rays of visible and near ultraviolet range [4]. These pigments are synthesized by various types of microorganisms as secondary metabolites and not often found in all types of organisms [5]. An important group of organic constituents of bacterial protoplasm is that of pigments. Some of these, like prodigiosin, pyocyanin, violacein, phenazine, pulcherrimin, iodinin, indigoidine and melanin are metabolic by-products formed under special circumstances [6]. Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo [7].

There is growing interest in microbial pigments due to their natural character and safety to use, medicinal properties, nutrients like vitamins, production being independent of season and geographical conditions, and controllable and predictable yield [8]. Again microbial pigments can be produced from waste material reducing water and environmental pollution [9].

Inspired by these facts, the aim of this research was to evaluate the antimicrobial activity of pigments against human pathogenic bacteria.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Eight (8) Soil samples were collected from different sites and locations of Dhaka city and its adjoining areas. Different soil conditions such as submerged and marshy places, dustbin areas, garden, lake, park, pond; cultivated, plain and high lands were taken under consideration for the site selection and sample collection. Soil samples were collected at a depth of 0 to 7.5 cm from the surface. A composite soil sample was made by collecting samples from different spots. 4 different samples were collected from a single area at a distance of not less than 200 meters between the sampling sites. A portion of the soil sample was collected in 4 ounce vial for bacteriological Analysis (about 200 gm) and another portion (about 1 kg) was taken in a cleaned polythene bag for physical and chemical analysis. All the samples were immediately transported to the laboratory. Washed and sterilized containers were used for sampling.

2.2 Chemical Analysis of the Soil Samples

Soil samples were analyzed for pH, Moisture, texture, organic matter, total N_2, exchangeable potassium, C/N ratio and available phosphorus [10].

2.3 Isolation of Pigmented Bacteria

Nutrient agar plus 2% glycerol with pH 7.2 was used as isolating media. Isolation of pigment producing bacteria as well as total bacterial count was done by decimal dilution technique followed by spread plate count. The plates were then incubated at 37^{o}C for 24 to 72 hours. That plate which contains 30 to 300 colonies was selected for counting. Generally 48 to 72 hours of growth produced a good number of colonies of pigmented bacteria and those bacteria that have antibacterial Property.

2.4 Purification of Pigment Producing Bacteria

Plates with discrete pigmented colonies were isolated and their morphological characteristics were recorded. For pure culture, the morphologically-selected dissimilar isolated colonies were picked up from the plates and sub-cultured as streak plate method. 4-6 morphologically dissimilar discrete bacterial colonies were picked with the help of sterile straight wire and streaked on nutrient agar plate (with glycerol) for pure culture. After overnight incubation at 37ºC, morphological characteristics of the colonies were recorded.

2.5 Screening of Pigment Production

Bacteria were grown in nutrient broth plus 2% glycerol (pH 7.2) was used for pigment production. One ml of the standard inoculum was added into the 50 ml broth in 250 ml Erlenmeyer flask and incubated for 24 hours in a shaking water bath at 37ºC with exposure to light. The extracellular pigments were obtained as diffused in the culture broth.

2.6 Identification of Pigment Producing Bacteria

Gram test and microscopic study were performed for the isolates of 18 hour culture from nutrient agar plates. The biochemical tests performed were Simmon's Citrate Slant test, Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase and Catalase tests. Identification of isolates obtained in pure culture was based on Gram staining, morphology, growth characteristics on selective and differential media and biochemical test results recommended in the Bergey's Manual of Determinative Bacteriology [11,12].

2.7 Antibiotic Susceptibility of Pigment Producing Bacteria

All the pigment producing isolates were tested for antibiotic resistance by the standard agar disc diffusion technique described by Kirby-Bauer [13] on Mueller Hinton agar using commercial discs (Oxoid, UK). The following antibiotics with the disc strength in parentheses were used: Tetracycline (Tet, 30µg), Streptomycin (Str, 10µg), Ceftriaxone (Cef, 30µg), Ampicillin (Amp, 25 µg), Chloramphenicol (Clr, 20µg), Gentamycin (Gen, 30µg), Penicillin (Pen, 10 µg), Ceftazidime (Caz, 30µg), Polymixin B (Pol, 300 IU) and Nalidixic acid (Nal, 30µg). A control strain of *E. coli* ATCC 25922 was included in each plate. Antimicrobial breakpoints and interpretation were taken from the CLSI standards [14].

2.8 Extraction and Analysis of Pigment

Extraction of the pigment was done following the method given by Asker and Ohta [15] with modifications. The extracellular pigments were separated by centrifuging the culture broth at 14000xg for 45 min. The harvested cells were re-suspended in distilled water for cell lyses to occur. The pigment was then extracted with methanol by repeated centrifugation until the cell debris turned colorless. These supernatants containing the diffused pigment were filtered through millipore membrane filter (pore diameter 0.22 µm). The filtrates were then collected in sterilized screw cap-tubes. Visible absorption spectrum of the separated pigments in nutrient broth was analyzed with UV-Spectrophotometer between the wave-length of 350-750 nm. The maximum absorbance of the pigments was recorded.

2.9 Anti-bacterial Activity of the Pigments

The antibacterial activity of the pigments was tested by Agar-Cup Diffusion Method. Briefly 20 ml of Nutrient Agar was poured into the Petri-dish and 8 mm well bored in the agar. 100 µL of extracts (1500 µg/ml) was poured into the wells. The plates were incubated for 24 h at 37ºC and the zone of inhibition was measured in mm. Commercially available penicillin, streptomycin and chloramphenicol disc were used as positive control and the 100 µL of water was used as a negative control [16]. Nine different human pathogens belonging to both gram positive and gram negative groups such as *Bacillus subtilis* ATCC 11774*, Bacillus megaterium* ATCC 13578*, Bacillus cereus* ATCC 10876*, Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*ATCC 65154*, Shigella flexneri* ATCC 12022 and *Vibrio cholera* ATCC 15748 were used in this study as test pathogens. Antibacterial activity was further characterized by determining whether bacteriostatic or bactericidal. The test was performed by swabbing of the growth inhibition zone of the plate and then swab was streaked onto nutrient agar plate and incubated aerobically at 37ºC for 48 hours. The presence of growth in nutrient agar plate was interpreted as an inhibitory activity i.e. bacteriostatic, while no growth was interpreted as bactericidal [17].

2.10 MIC of Pigments

Minimum Inhibitory Concentrations (MIC) of pigments was performed by macro dilution method [18]. The crude pigment extract was dissolved in 30% dimethyl sulfoxide (DMSO) to obtain 10% (w/v) solution. MIC value of seven selected isolated bacteria (isolate no- 3, 4, 5, 6, 10, 12 & 15) was determined against nine different human pathogens belonging to both gram positive and gram negative groups such as *Bacillus subtilis* ATCC 11774*, Bacillus megaterium* ATCC 13578*, Bacillus cereus* ATCC 10876*, Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi* ATCC 65154*, Shigella flexneri* ATCC 12022 and *Vibrio cholera* ATCC 15748. The extract was first diluted in sterilized Mueller-Hinton broth to the highest concentration of 10,000 µg/ml and then dilution were performed at concentration of 5000 µg/ml, 4000 µg/ml, 3500 µg/ml, 3000 µg/ml, 2500 µg/ml, 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml and 250 µg/ml in screw caped tube containing broth medium. Bacterial suspensions of the test organism were prepared in sterilized Mueller-Hinton broth. Then 1ml of the dilution was added to each sterilized screw capped tube containing 1ml of compound suitably diluted in the sterilized broth medium to make final volume of 2ml. Culture medium without samples and others without microorganisms were used in the tests as control. Tubes were incubated at 37ºC for 20-24 hours and growth was indicated by turbidity.

3. RESULTS

3.1 Characteristics of Soil Samples Collected

The soil samples collected were varied widely in terms of texture, moisture content, pH, organic matter, total N, available phosphorus, exchangeable potassium and C/N ratio. Physico-chemical characteristics of the soil samples collected are summarized in Table 1.

Table 1. Physico-chemical characteristics of the soil samples

Sample no.	Texture	Moisture at field condition (%)	pH	Organic matter (%)	Total N (%)	Available Phosphorus (ppm)	Exchangeable Potassium (ppm)	C/N ratio
1	Loam	23.57	7.7	4.56	0.22	85.00	106.00	12.02
2	Sand	19.80	6.9	2.68	0.105	63.00	42.40	14.80
3	Silty loam	36.81	6.0	2.71	0.10	72.00	61.80	15.72
4	Sandy soil	13.45	5.4	1.81	0.07	42.00	39.60	14.99
5	Clay loam	10.26	7.2	1.88	0.08	14.00	41.60	27.26
6	Silty loam	29.78	7.9	5.48	0.20	76.00	99.76	15.89
7	Sandy soil	14.5	6.7	2.90	0.10	66.00	41.53	16.82
8	Silty loam	19.00	6.2	1.86	0.13	41.00	111.00	8.30

High C/N ratio of soil increases microbial activity especially bacterial activity as it indicates the presence of carbonaceous material in soil which is the source of carbon for energy production and tissue building of microorganisms. Generally, C/N ratio of 24 or greater of

freshly added organic matter increases the number of microorganisms and thus immobilization of carbon and nitrogen occurs. The C/N ratio of 24 causes stabilization and less than 24 decreases the number of microorganisms.

3.2 Isolation of Pigment Producing Bacteria

15 Pigment producing bacteria were isolated from 8 soil samples collected from different places of Dhaka city. The isolated colonies were of following colors: red, brown, pink, black, violet, blue, green, cream, golden, dark orange and light yellow (Fig. 1). Among those the yellow was most dominant. The isolates were labeled consecutively from 1 to 15.

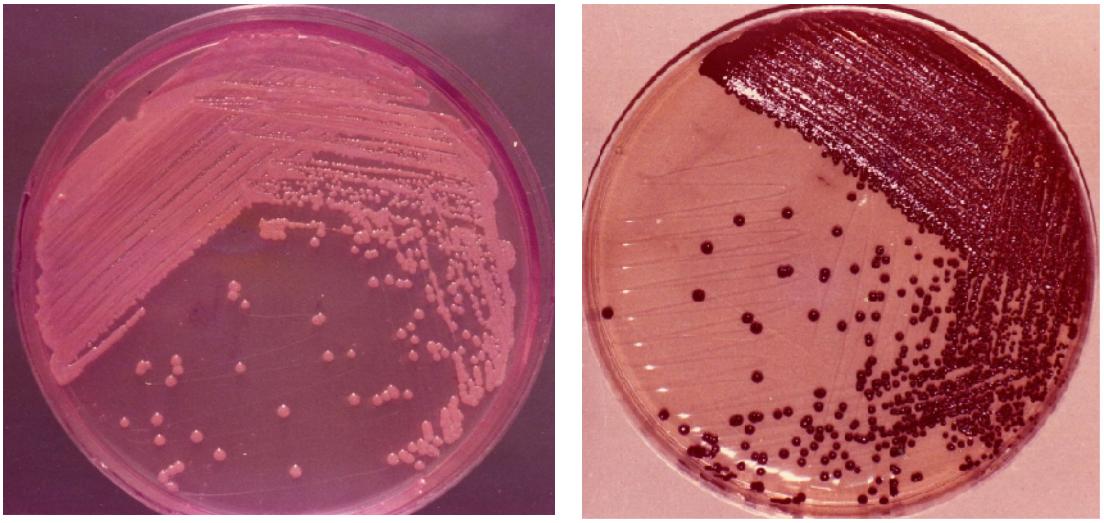

Fig. 1. Pigment producing bacteria isolated from soil samples

3.3 Identification of the Isolates

The isolates were purified by re-streaking further on Nutrient agar (NA) and incubated for 18-24 hr at 37ºC. Following overnight incubation in nutrient agar at 37ºC, the isolates were preserved in 30% glycerol at -20ºC. The pigment producer strains were then identified according to Bergey's manual of determinative bacteriology (Holt, 1994) based on Gram staining, biochemical characteristics and growth pattern on selective and differential media. The strains were found to belong to the genera *Aeromonas* (20%), *Pseudomonas* (20%), *Chromobacterium* (13.3%), *Flavobacterium* (6.7%), *Bacillus* (13.3%), *Xanthomonas* (6.7%) and *Escherichia*(20%) (Table 2).

Table 2. Genus level identification of the isolates

Sl. no	Genus identified	No and Identity of isolates	Percentage (%)
1	*Aeromonas*	3 (2, 6, 11)	20
2	*Pseudomonas*	3 (1, 9, 15)	20
3	*Chromobacterium*	2 (5, 8)	13.3
4	*Flavobacterium*	1 (3)	6.7
5	*Bacillus*	2 (7, 12)	13.3
6	*Xanthomonas*	1 (4)	6.7
7	*Escherichia*	3 (10, 13, 14)	20
	Total isolate	15	

3.4 Antibiotic Susceptibility Pattern

All the isolates were tested for antibiotic sensitivity against 10 commonly used antibiotics belonging to different groups. Out of the strains tested, most of them were resistant to at least 3 antibiotics. 66.7% isolates were resistant to Tetracycline, 53.3% resistant to Streptomycin, 23% resistant to Ceftriaxone, 73.3% resistant to Ampicillin, 66.7% resistant to Chloramphenicol, 33.3% resistant to Gentamycin, 60% resistant to Penicillin, 13.3% resistant to Ceftazidime. All the isolates were sensitive to Polymixin B and Nalidixic acid. The antibiotic resistance pattern of pigment forming isolates is shown in Fig. 2.

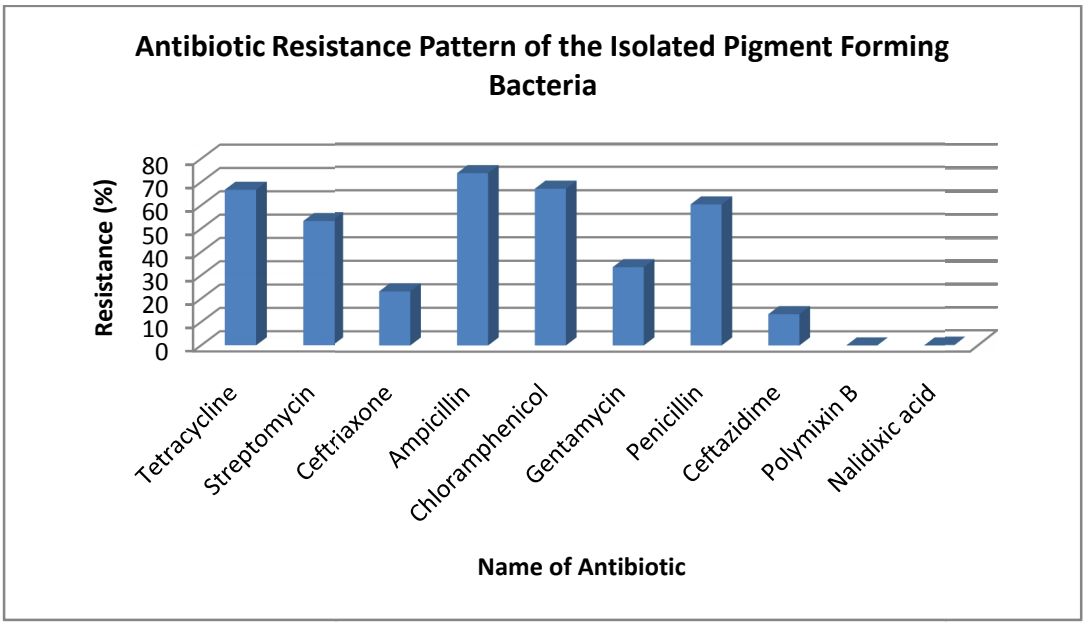

Fig. 2. Antibiotic Resistance Pattern of the Isolated Pigment Forming Bacteria

3.5 Absorption Characteristics of Pigments from Different Strains

Absorption spectra of different pigments (water soluble) from the fifteen strains were studied in the visible range between the wave lengths of 350-750 nm and spectrophotometric analysis at the respective wave lengths at which maximum absorbance (λ_{max}) were observed were determined (Table 3).

Table 3. Absorption maxima of the pigments

Isolate no	Color of pigment	Absorption maxima (λ_{max}) (nm)	Isolate no	Color of pigment	Absorption maxima (λ_{max}) (nm)
1	Violet	390	8	Pink	416
2	Black	355	9	Green	397
3	Blue	525	10	Dark orange	512
4	Golden	385	11	Yellow	412
5	Yellow	505	12	Cream	445
6	Brown	545	13	Red	566
7	Red	418	14	Violet	486
			15	Yellow	391

3.6 Anti-bacterial Activity of the Pigments

Anti-bacterial activity of the extracted pigments from the isolated pigment forming bacteria was determined by agar cup diffusion method. All the pigments isolated showed anti-bacterial activity against the test pathogens but anti-bacterial activity was found better in case of pigments from isolate 3, 4, 5, 6, 10, 12 and 15. All the pigments showed better antibacterial activity against gram negative pathogens than gram positive pathogens. Mode of anti-bacterial action of most pigments was bacteriostatic. Anti-bacterial activity of the extracted pigments against the test pathogens are shown in Table 4.

3.7 MIC of the Pigments

MIC value was determined for the seven pigments (3, 4, 5, 6, 10, 12, 15) which showed better anti-bacterial activity. The MIC value of the pigments against Gram-positive and Gram-negative bacteria ranged from 1,500 to 4,000 µg/ml (Table 5). Lowest MIC value (1500 µg/ml) was found against only gram negative bacteria. MIC value was much higher than control antibiotic, Doxycycline (30 µg/ml). Considering the impurities and complex composition of the extracted pigment, MIC value against the pathogens included in this study was promising though Doxycycline had much lower MIC value (Table 5).

Table 4. Anti-bacterial activity of the pigments extracted from isolated pigment forming bacteria

Isolate no	Zone diameter (mm)								
	Gram Positive				Gram Negative				
	Bacillus cereus	***Staph. aureus***	***Bacillus subtilis***	***Bacillus megaterium***	***Salmonella typhi***	***Shigella flexneri***	***E. coli***	***Vibrio cholerae***	***Pseudo. aeruginosa***
1	22 (Bs)	19 (Bs)	21 (Bs)	24 (Bs)	12 (Bs)	9 (Bs)	13 (Bs)	12 (Bs)	11 (Bs)
2	15 (Bc)	18 (Bs)	21 (Bc)	20 (Bc)	25 (Bs)	24 (Bs)	24 (Bc)	27 (Bs)	21 (Bc)
3	25(Bc)	28 (Bc)	24 (Bc)	28 (Bc)	32 (Bc)	30 (Bc)	34 (Bc)	31 (Bc)	32 (Bc)
4	30 (Bc)	29 (Bc)	30 (Bc)	34 (Bc)	28 (Bs)	30 (Bc)	26 (Bs)	29 (Bc)	28 (Bs)
5	33 (Bc)	25 (Bs)	27 (Bc)	31 (Bs)	21 (Bc)	23 (Bs)	19 (Bc)	22 (Bs)	21 (Bc)
6	16 (Bs)	19 (Bc)	18 (Bs)	16 (Bs)	26 (Bc)	31 (Bc)	27 (Bs)	32 (Bc)	31 (Bc)
7	12 (Bs)	12 (Bs)	14 (Bs)	15 (Bs)	16 (Bs)	18 (Bc)	13 (Bs)	17 (Bs)	14 (Bs)
8	10 (Bs)	11 (Bc)	16 (Bc)	14 (Bs)	11 (Bs)	15 (Bc)	16 (Bc)	14 (Bs)	12 (Bs)
9	15 (Bs)	17 (Bs)	12 (Bs)	9 (Bs)	11 (Bs)	10 (Bs)	9 (Bs)	13 (Bs)	12 (Bs)
10	29 (Bc)	34 (Bc)	35 (Bc)	31 (Bs)	30 (Bc)	38 (Bc)	41 (Bc)	39 (Bc)	38 (Bc)
11	15 (Bs)	14 (Bs)	15 (Bs)	16 (Bs)	13 (Bs)	12 (Bs)	11 (Bs)	12 (Bs)	9 (Bs)
12	34 (Bc)	31 (Bc)	27 (Bs)	33 (Bc)	36 (Bc)	39 (Bc)	34 (Bc)	42 (Bc)	38 (Bc)
13	14 (Bc)	8 (Bs)	11 (Bs)	12 (Bc)	23 (Bc)	21 (Bs)	25 (Bc)	20 (Bs)	19 (Bc)
14	18 (Bs)	16 (Bc)	16 (Bs)	18 (Bc)	14 (Bs)	15 (Bc)	11 (Bc)	13 (Bc)	10 (Bs)
15	35 (Bc)	39 (Bc)	41 (Bc)	44 (Bc)	46 (Bc)	45 (Bc)	38 (Bc)	36 (Bc)	32 (Bc)
Pen	45	45	48	44	49	51	50	49	47

(Bs= Bacteriostatic; Bc= Bactericidal; Pen= Penicillin, 10 µg)

Table 5. Minimum Inhibitory Concentration (MIC) of pigments

Test organism	MIC value (µg/ml)							
	3	**4**	**5**	**6**	**10**	**12**	**15**	**DX**
Gram-positive								
Bacillus subtilis	3500	4000	3000	3500	2500	3000	2500	5.0
Staph. aureus	3500	3000	3500	3000	3500	3500	3000	5.5
Bacillus cereus	3000	4000	4000	3500	3000	3500	2500	4.5
Bacillus megaterium	3500	3500	3500	3000	4000	3000	2500	5.5
Gram-negative								
Salmonella typhi	2500	2000	2500	1500	2000	2500	1500	5.5
Shigella flexneri	2500	2000	3000	2500	2000	2000	2000	6.0
E. coli	2000	2000	2500	1500	2000	2000	1500	5.0
Vibrio cholerae	2500	2000	1500	2500	2000	1500	1500	5.0
Pseudomonas aeruginosa	3000	2500	3000	1500	2000	2000	2500	4.5

(DX= Doxycycline hydrochloride)

4. DISCUSSION

Microbial pigments are a promising alternative to other color additives extracted from vegetables or animals because they are considered as natural, pose no seasonal production problems and show high productivity. Pigment producing microorganisms are yeast, fungi, bacteria, micro algae and are quite common in Nature [19].

The present study indicates that pigment production is influenced by physical factors such as temperature and pH of the culture medium. There should be many other factors, affecting pigmentation by the bacterium such as shaker speed, source and concentration of nutrient components. A thorough understanding of the regulation and pathway of pigment production will allow us to develop defined bioprocess for the enhanced production of the desired pigment [20].

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, enzymes, vitamins, texturizing agents and so on [21]. There is growing interest in the food industry in the use of natural ingredients. Ingredients, such as colors, are considered natural when derived from biological sources like plants or microorganisms [22]. Microbial colors are in use in the fish industry already, for example to enhance the pink color of farmed salmon [7]. Further, some natural colorants have commercial potential for use as antioxidants. The industry is now able to produce some microbial pigments for applications in food, cosmetics or textiles [23]. In nature, color rich and pigment producing microorganisms (fungi, yeasts, and bacteria) are quite common [24]. Microorganisms produce various pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo [25].

The increase in the frequency of multi-resistant pathogenic bacteria has created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity, which resist the inactivation processes exploited by microbial enzymes [26, 27].

The need for new, safe and effective antimicrobial agent is the major challenge to the pharmaceutical industry nowadays, especially with the obvious increase in opportunistic infections in the immune compromised host via multiple drug resistant strains [28].

The soil samples were collected from the upper surface layer (0 - 7.5 cm) as the attention of microbiologists is usually drawn to the surface soil because here the population is most dense and the nutrient supply is abundant [29]. Area or locations of sample collection were selected randomly. Eight (8) soil samples were collected from different areas of the Dhaka city. Different field conditions were considered for sample collection. It included grass land, low land, high land, marshy places, barren field, cultivated field, organic matter rich area like soils around the city dustbin, cow shade, kaccha sanitary place. These wide variations were considered to cover wider search area because it had been suggested by previous workers that search should be done in different areas of soil with different conditions and under different climatic zones.

The collected samples possess differences in their physico-chemical characteristics. From this investigation neither a definite condition of site selection to obtain maximum pigment forming bacteria could be determined nor a relationship of the pigment forming bacteria with type of soils could be established. The 15 pigment producing bacteria that produced extracellular pigments (Fig. 1) and that have antibacterial property were identified after the

Bergey's manual of determinative bacteriology. The identified genera were *Aeromonas* (isolate no- 2, 6, 11), *Pseudomonas* (isolate no- 1, 9, 15), *Chromobacterium* (isolate no- 5, 8), *Flavobacterium* (isolate no- 3) and *Bacillus* (isolate no- 7, 12), *Xanthomonas* (isolate no- 4) and *Escherichia* (isolate no- 10, 13, 14) (Table 2).

Inhibitory effect of the extracellular pigments from the 15 isolates were observed on nine common human pathogen. All the pigments from the isolates can inhibit growth of both gram-positive & gram-negative bacteria & thus could be designated as broad spectrum. Zone of inhibition formed by each pigment was different in size & nature, even in case of isolates of same genus. This may be due to differences in composition of the different pigments.

Of the 15 pigment extracted from the 15 isolated bacteria, seven (3, 4, 5, 6, 10, 12, 15) showed better anti-bacterial activity in terms of diameter of zone inhibition. All other pigments showed moderate anti-bacterial activity against the test pathogens. In general, most of the pigments showed better anti-bacterial activity against gram-negative bacteria. Four (1, 4, 5, 14) pigment showed comparatively better anti-bacterial activity against gram-positive bacteria under test conditions.

Highest zone of inhibition (46 mm in diameter) was observed for pigment no 15 against *Salmonella typhi*. Pigment 15 also showed highest inhibition against *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Shigella flexneri* & *Staphylococcus aureus*. Pigment 10 showed highest inhibition against *E. coli*, pigment 12 showed highest inhibition against *Vibrio cholerae* whereas pigment 10 & 12, both showed highest inhibition against *Pseudomonas aeruginosa*.

Mode of anti-bacterial action (bacteriostatic or bactericidal) was also determined. Most of the pigments were found to be bacteriostatic to the test pathogens. Pigment from isolate 3 & 15 were bactericidal to all the test pathogens whereas pigment 10 & 12 were bactericidal to 8 test pathogens. Pigment 1, 9 & 11 were bacteriostatic to all the nine (9) test pathogens.

The effectiveness of the pigments as anti-bacterial agent was determined by comparing the cleared zones produced by pigments & standard antibiotic, penicillin (10 µg/disc) on test organisms. Pigment from isolate 15 & 12 showed zone diameter closer to penicillin.

To determine the effectiveness of the pigments as anti-bacterial agent, MIC (Minimum Inhibitory Concentration) of the pigments against the test pathogens was determined. MIC of seven (7) pigments that showed better anti-bacterial activity was determined (Table 5). MIC value of all the pigments was lower against gram-negative bacteria than gram-positive bacteria.

Lowest MIC value (2500 µg/ml) against *Bacillus subtilis* was showed by pigment from isolate 10 (*Escherichia*) & isolate 15 (*Pseudomonas*). Lowest MIC value (3000 µg/ml) against *Staphylococcus aureus* was showed by pigment from isolate 4 (*Xanthomonas*), 6 (*Aeromonas*) & 15 (*Pseudomonas*). Lowest MIC value (2500 µg/ml) against Bacillus cereus & Bacillus megaterium by pigment from isolate 15 (*Pseudomonas)*. Lowest MIC value (1500 µg/ml) against *Salmonella typhi* was showed by pigment from isolate 6 (*Aeromonas*) & 15 (*Pseudomonas*). Lowest MIC value was 2000 µg/ml for *Shigella flexneri* by pigment from isolate 4 (*Xanthomonas*), 10 (*Escherichia*), 12 (*Bacillus*) & 15 (*Pseudomonas*). Lowest MIC value for *E. coli*, *Pseudomonas aeruginosa* & *Vibrio cholerae* was 1500 µg/ml. Lowest MIC against *E. coli* & *Vibrio cholerae* was showed by pigment from isolate 15 (*Pseudomonas*)

and against *Pseudomonas aeruginosa* by pigment from isolate 6 (*Aeromonas*). MIC value of the pigments against the test pathogens are much higher than that of Doxycycline hydrochloride which may be due to the crude nature of the pigment extracts.

On the basis of the results of anti-bacterial activity and MIC, pigment from isolate 6 (*Aeromonas*), 10 (*Escherichia*) & 15 (*Pseudomonas*) can be selected as effective anti-bacterial agent to be used in food, cosmetic or textile industries, but before selection for use in food, toxicity/allergenic studies against humans should be undertaken.

In this study soil samples isolated from a small number of spots were investigated but appreciable numbers of pigment forming bacteria having antibacterial activity were obtained. This implies that further exploration of different nature of soil under different environmental condition might prove to be reservoirs of such bacteria. It was observed that growth condition influenced formation of pigments. These results would be an important adjunct for further investigation to find out the optimal growth condition for maximal pigment formation with maximum bacterial activity. The results obtained with the pigments against pathogenic organisms suggest that further studies might provide some illuminating data in the control of these diseases.

5. CONCLUSION

The emergence of strains of bacteria resistant to common antibacterial agents has become one of the most important problems in clinical medicine. The search for new antibiotic is always on. Out of all the secondary rnetabolites having antibiotic activity, pigments are the least studied group. As the reports for pigments having antibiotic like activity are rapidly increasing, they should be studied for selective toxicity so that they can be produced commercially for human use. The present study reports local microbial isolates able to produce pigment with anti-bacterial activity. Scope for further research would be to improve antimicrobial agent production by the strains with purification and characterization of the antibiotic obtained from this isolate.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

The authors declare no competing interests exist.

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