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Extraction, purification and characterization of pyocyanin produced by *Pseudomonas aeruginosa* and evaluation for its antimicrobial activity

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Abstract

Pseudomonas aeruginosa, a gram negative bacteria, which exerts broad antagonistic activity against other bacterial and fungal pathogens through the production of a secondary metabolite-pyocyanin. In the present study, various clinical samples were taken for P. aeruginosa isolation. From sixteen primary P. aeruginosa isolates, five isolates (PS_1, PU_5, PU_8, PU_{10} and PP_3) were selected, on the basis of pigmentation in cetrimide agar. The screening for antimicrobial activity of P aeruginosain cross streak method showed that Staphylococcus aureus (ATCC 6538), Salmonella enterica (NCTC 6017), and Bacillus cereus (ATCC 14579) were sensitive, Escherichia coli (ATCC 8739) was intermediate, and K. pneumoniae (ATCC 43816) was resistant to the inhibitory action of the selected P. aeruginosa isolates. The antimicrobial pigment pyocyanin was extracted from culture broth following solvent extraction method. Purification of the pigment was done by column and thin layer chromatography. The R_f value was found around 0.81 for all the extracted pigment solution. Confirmation of the pigment as pyocyanin was done through FTIR, and UV-visible spectrophotometric analysis. FTIR analysis revealed different functional groups (-OH, -C=N, -CH3 etc.) which belongs to the aromatic structure of pyocyanin. In UV-Vis spectrophotometric analysis, a maximum absorption was observed at 270-275nm.The modification of media composition enabled to increase pyocyanin production and the highest amount was produced by the isolate PU_{10} in Medium-B having a concentration of 9.45 µg/mL. The antimicrobial activity of the purified pyocyanin pigment against different test organism showed that highest concentration of pyocyanin (25 µg/mL) was needed to produce zone of inhibition in case of E. coli, and the lowest (5 µg/mL) was for S. aureus.

Keywords: Pyocyanin, Extraction, Purification, Antimicrobial activity, Characterization.

Introduction

Pseudomonas aeruginosa is a Gram negative aerobic rod and an opportunistic pathogen. It is widespread in the terrestrial and aquatic environment and can cause infection in a wide range of organisms[1]. Chronic infections in cystic fibrosis patients and wound infections especially of burns are also associated with this pathogen[2]. It is now obvious that some phenazine like bioactive compounds have antimicrobial activity and *P. aeruginosa* frequently produce such compounds. Among the recognized phenazine pigments, pyocyanin, 1-hydroxy phenazine, phenazine-1-carboxamide and phenazine-1-carboxylic acid are notable[3]. It has been found that phenazine compounds are associated with biofilm development, such as Phenazine-1-Carboxylic acid, which is secreted by some pseudomonads, promote bacterial biofilm formation via acquisition of ferrous iron[4]. Pyocyanin also has significance as quorum sensing (QS) signaling molecule as well as a virulence factor for *P. aeruginosa*. Although pseudomonads are frequently reported for their pathogenicity, the ability of this microorganism to produce antimicrobial pigment opened the door of using this as biological control agent. Researchers invented an appreciable number of new antimicrobial agents in the last three decades, bacterial resistance to antimicrobial agents has also increased simultaneously. Common antimicrobial agents are not working properly against those infectious organisms[5]. The present study was designed to isolate some high pyocyanin yielding *P. aeruginosa* isolates to extract antimicrobial pigment from its culture, purify the pigment, augment its production and test its antimicrobial activity.

Materials and methods

Isolation and identification of *P. aeruginosa* from clinical samples: To isolate *P. aeruginosa* from various clinical samples (burned skin swab, urine and pus) Pseudomonas Cetrimide Agar (Oxoid™) was used as selective media[6]. At first, samples were enriched in Brain-Heart infusion (BHI) broth medium (Oxoid™). Then the enriched samples were cultured on cetrimide agar by streak plate and pour plate method, observed for distinguishing pigmentation and 5 isolates (PS_1, PU_5, PU_8, PU_{10} and PP_3) were selected from 16 initial isolates on the basis of visual vigorous pigmentation for further study. By comparing the microscopic features, physiological and biochemical characteristics of the isolates with the standard description given in "Bergey's Manual of Determinative Bacteriology", these were identified as *P. aeruginosa*[7].

Primary screening for the isolates having antimicrobial activity: Cross streak method was primarily followed to investigate the antimicrobial activity of the selected isolates[8]. Here, the following organisms - *S. aureus* (ATCC 6538), *B. cereus* (ATCC 14579), *E. coli* (ATCC 8739), *S. enterica* (NCTC 6017), *K. pneumoniae* (ATCC 43816) and *P. aeruginosa* (ATCC 9027) - were used as test organism. A clear zone of growth inhibition near the adjoining culture line of *P. aeruginosa* and test organisms would be indicative of pseumonads' antimicrobial activity.

Extraction, purification and characterization of the pigment produced by *Pseudomonas* isolates: The selected *Pseudomonas* isolates were grown first in the Pseudomonas broth at 37°C for 48 hours for pigment production. Pigment rich broth culture was then centrifuged at 10000 rpm for 15 min and the supernatant was collected. It was then filtered through 0.45 µm pore sized membrane filter and used as crude extract.

Extraction of pigment from crude extract was done using chloroform and HCl as Ingledew and Campbell, 1969 with some modification[9]. Chloroform was added in the culture broth at the ratio of (2:1) and after vortexing a blue solvent layer was produced. The blue layer was collected, then 0.1N HCl solution (20% of the blue layer's volume), was added and vortexed, which produced an acidified pink upper layer. The pink layer was then neutralized with Tris-Base and the neutralized layer was again treated with chloroform. The whole procedure was repeated several times to make it pure.

Purification of the extracted pigment was done by column chromatography (column size 45 x 3.5 cm) using silica gel G, as stationary phase and a solution of methanol and chloroform at the ratio of 1:1 as mobile phase[10]. The eluted sample was further analyzed by TLC using methanol and chloroform at the ratio of 1:1 as mobile phase to check its purity and the R_f value of the partially purified pigment was checked. The fraction of the eluted sample was also treated with 0.1 N NaOH and kept for 2 -3 hours for crystallization[11].

The crystals were separated by membrane filtration, dried and observed under microscope. The crystals was stored and suspended in water when necessary. Extracted pigment solution from the 5 efficient isolates of *P. aeruginosa* were subjected to UV-visible spectrophotometer[12,13] and a maximum absorbance was recorded by UV-1800 UV-VIS Spectrophotometer (Shimadzu). Fourier Transform Infrared (FTIR) spectroscopic analysis of the pigment was also done using potassium bromide (KBr) as a window material[14].

Augmentation of pigment production: Pigment production was primarily done by using Pseudomonas broth. Then it was augmented using a modified Pseudomonas broth (Glycerol 1ml, $MgCl_2$ 0.14g, K_2SO_4 1g, Asparagine 0.1%, Peptone 2g, Distilled water upto100ml) which we named Medium-A and Glycerol-alanine broth (Glycerol 1ml, $MgCl_2$ 0. 14g, K_2SO_4 1g, D-alanine 0.1%, Peptone 2g, distilled water 100ml) which we named Medium-B. 48 hours of incubation period was needed for vigorous pigmentation. After pigmentation, following the extraction procedure, the concentration (µg/mL) of the pigment in extracted solution was determined by measuring the optical density (absorbance) at 520 nm wavelength using a UV-visible spectrophotometer (Shimadzu) and multiplying the optical density value (OD_{520}) with 17.072[15,16].

Determination of antimicrobial activity of the purified pigment: The antibacterial activity of the purified pigment produced by the selected isolates against different bacterial strains - *S. aureus* (ATCC 6538), *S. enterica* (NCTC 6017), *E. coli* (ATCC 8739) and *B. cereus* (ATCC 14579) - was investigated following agar well diffusion method[17]. Mueller-Hinton Agar was used as agar medium and different concentration of the purified pigment – 5, 10, 15, 20, 25, 30, 35, 40 and 45 µg/ml in water was prepared to test the antibacterial activity. First the prepared media was seeded with the test organism (OD equivalent to 0.5 McFarland), poured in the petriplate, solidified and wells were made in the agar medium. Then 100 µL of each prepared pigment solutions were poured in different wells of the agar plate and incubated for 24 hours at 37ºC. After incubation the diameter of zone of growth inhibition was measured to determine the antimicrobial activity of the test agent. Each experiment was replicated trice.

Results and discussion

Isolation and identification of *P. aeruginosa* from clinical samples: Three types of colony morphology was observed on the selective medium Pseudomonas Cetrimide Agar–colonies with vigorous yellow-green pigmentation (31%), colonies with scanty pigmentation (38%) and no pigmentation (31%). The selective media contain a quaternary ammonium compound, cetrimide, which has broad spectrum bactericidal activity against a wide range of Gram-positive and some Gram-negative microorganisms. A number of water soluble iron chelators, like the yellow-green or yellow-brown fluorescent pyoverdin are also produced by *P. aeruginosa*.

The characteristic bright green colour of *P. aeruginosa* broth culture is developed when water-soluble blue pyocyanin combines with pyoverdin[6]. We have found 5 isolates having such pigmentation characteristics and initially recognized as *P. aeruginosa*. Among these isolates 3 were from urine and remaining each isolate from burned skin swab and pus. *P. aeruginosa* is an opportunistic pathogen and responsible for nosocomial infection of urinary tract and burn patients[18].

Their identification was confirmed by microscopic observation and biochemical tests as noted in Table-1 and it was also found that all the isolates had the same characteristics. The organisms have diverse metabolic and physiological ability like catalase activity, sugar fermentation, growing at different temperature and pH.

Table-1: Morphological, cultural, biochemical and physiological characteristics of the selected isolates.

Characteristics	Isolates				
	PS_1	PU_{10}	PU_8	PU_5	PP_3
Gram staining	negative	negative	negative	negative	negative
Oxidase test	positive	positive	positive	positive	positive
Catalase test	positive	positive	positive	positive	positive
Motility test	motile	motile	motile	motile	motile
Citrate utilization	positive	positive	positive	positive	positive
Gelatin hydrolysis	positive	positive	positive	positive	positive
Deep glucose agar test	positive	positive	positive	positive	positive
Casein hydrolysate test	positive	positive	positive	positive	positive
Voges-Proskauer test	negative	negative	negative	negative	negative
Methyl red test	negative	negative	negative	negative	negative
Nitrate reduction test	negative	negative	negative	negative	negative
H2S production test	negative	negative	negative	negative	negative
Indole test	negative	negative	negative	negative	negative
Glucose fermentation	Acid	Acid	Acid	Acid	Acid
Fructose fermentation	Acid	Acid	Acid	Acid	Acid
Mannitol fermentation	Acid	Acid	Acid	Acid	Acid
Arabinose fermentation	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas
Sucrose fermentation	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas	No acid, no gas
Growth response at different pH					
pH-5	+	+	+	+	+
pH-7	++	++	++	++	++
pH-8	+++	+++	+++	+++	+++
pH-9	+++	+++	+++	+++	+++
Growth response at different temperature (ºC)					
4 ºC	-	-	-	-	-
30 ºC	+++	+++	+++	+++	+++
37 ºC	+++	+++	+++	+++	+++
42 ºC	++	++	++	++	++

Note: - = no growth, + = scanty growth, ++ = moderate growth, and +++ = heavy growth.

Primary screening for the isolates having antimicrobial activity: *P. aeruginosa* synthesizes various bioactive substances among these pyocyanin is the best known for its antimicrobial activity against *S. aureus, S. epidermis, Clostridium botulinum, Micrococcus luteus, B. subtilis*, as well as *B. licheniformis*[11,19,20]. In the present study of primary screening for antimicrobial activity of *P. aeruginosa* against different test organism following cross streak method we found, *S. aureus* (ATCC 6538), *B. cereus* (ATCC 14579), and *Salmonella enterica* (NCTC 6017) showed visually maximum sensitivity as shown in Figure-1. *E. coli* (ATCC 8739) showed intermediate sensitivity, *K. pneumonia* (ATCC 43816) was weakly sensitive and *P. aeruginosa* (ATCC 9027) was not sensitive at all. In a previous study, it was found that *P. aeruginosa* showed antagonistic activity against *S. aureus, E. coli, P. vulgaris, Bacillus* sp. in cross streak method[13]. Machan also found anti-staphylococcal activity of *P. aeruginosa* with cross streak test. Prevention of Gram negative bacterial growth by this molecule was also recorded[21]. For example, *E. coli Enterobacter cloacae*, *Serratia marcescens*, *Salmonella typhi, Vibrio parahaemolyticus, Proteus mirabilis,* Proteus *vulgaris, Paracoccus denitrificans*, *Citrobacter* sp. and *Erwinia carotovora*[22,23]. This metabolite exhibits not only antibacterial effect but also anti-parasitic activity against the nematode *Caenorhabditis elegans*[24,25].

Extraction, purification and characterization of the pigment produced by *Pseudomonas* isolates: The inoculated pseudomonas broth turned into bluish green or yellowish green after vigorous pigmentation within the given incubation period. *Pseudomonas aeruginosa* produce different pigments, including pyocyanin (blue green), pyomelanin (light-brown), pyoverdin (yellow, green and fluorescent) and pyorubrin (red-brown)[26] which are responsible for this colour change elaborated by pseudomonads[27]. Following the extraction procedure we found a blue colour solution in chloroform that turned into pink-red upon addition of 0.1 N HCl. Pyocyanin is soluble in chloroform producing blue colour[10]. It is considered as a resonance hybrid of the mesomeric forms of N-methyl-1-hydroxyphenazine and is capable of undergoing a two-electron reduction to a colourless product, leukopyocyanin. It can exist either in reduced or oxidized form. The reduced form of pyocyanin was unstable and rapidly reacts with molecular oxygen[28]. The pigment is wine-red at acid condition because of the basic property of one of the nitrogen atoms and blue at alkaline reaction[29].

In the column chromatographic analysis we found only a single blue colour band during separation indicating the presence of no other pigment in the extracted solution. The TLC analysis of the extract also substantiates the findings in column chromatography. A blue spot with R_f value ranging from 0.70 to 0.81 was observed for the pigment extracted from all the isolates which was characterized as phenazine compound such as pyocyanin[13]. Following crystallization process, the pigment produced crystal which was needle like when observed under microscope (Figure-2). In 2011, El-Shouny reported that 0.1M NaOH treatment for 2 hours in the chloroform extract of pyocyanin forms needle like crystals[11].

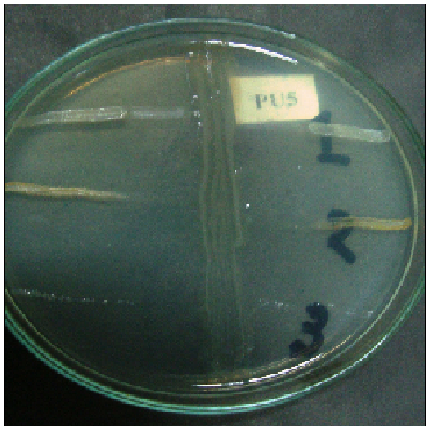

Figure-1: Antimicrobial activity of *P. aeruginosa* (PU_5) against (1) *S. enterica* NCTC 6017 (2) *S. aureus* ATCC 6538 (3) *B. cereus* ATCC 14579.

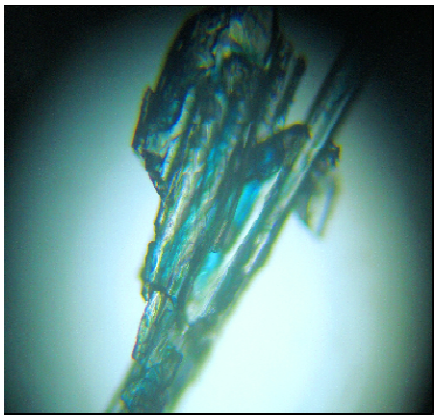

Figure-2: Microscopic observation of needle like crystals of the pigment pyocyanin formed in the crystallization process (10X).

The UV-visible spectrophotometric analysis of extracted purified pigment dissolved in 0.1 N HCl showed that peak was observed at a maximum range of 270-275 nm (Figure-3). These results are almost congruent with those of Ohfuji *et al.* who found that UV-visible spectra of pyocyanin was around 278 nm[13,14].

The FTIR spectra, as in Figure-4, of the extracted compound in different selected isolates shows bands between 3400-3300 cm^{-1} which indicate the presence of –OH group and the appearance of bands between 3000-2900 is an indication of C-H stretch for aromatic compound[30]. As an Aromatic hydrocarbon the compound shows absorptions in the regions 1500-1400 cm^{-1}and1600-1585 cm^{-1} due to vibrations of carbon- carbon stretching in the aromatic ring. Absorption between 1590-1600 cm^{-1} and 1280- 1250 cm^{-1} are indicative of C=N bonds and C-N bonds respectively in aromatic stretching. In case of PP_3 sample a hump appeared at 1630 cm^{-1} which can be assigned for C=N bond. The presence of $–CH_3$ group is confirmed with the –C-H

stretches of the alkyl (methyl) group in the 1380-1400 cm^{-1} range. It shows no peak at 1690-1760 cm-1 that means the compound has no chance to have a C=O group in its structure. Comparing the spectra, presented at Table-2, it was averred that the purified pigment was pyocyanin since most of the functional groups present in pyocyanin structure were found here.

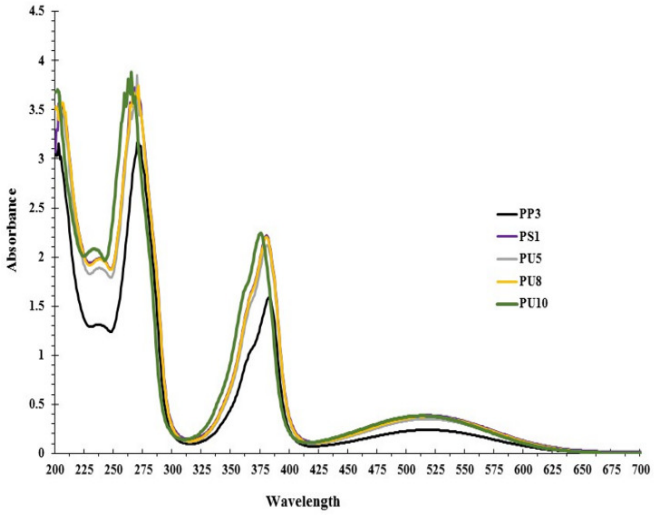

Figure-3: UV absorption spectra of the extracted pigment dissolved in 0.1N HCl.

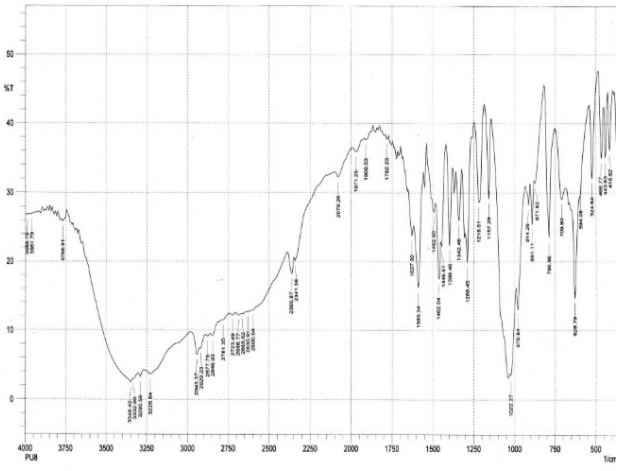

Figure-4: FTIR spectra of the pyocyanin pigment extracted from the isolate *P. aeruginosa* PU_8.

Augmentation of pigment production: Carbon and nitrogen sources highly affect the production of pyocyanin. Production of pyocyanin was once carried out in King's Medium[27]. It was later modified by combining different amino acids, like asparagine, alanine, tyrosine and leucine, in the culture medium which resulted in a more vigorous pigment production based on King's Medium[26,31]. At present study, Pseudomonas broth was modified by adding 0.1% asparagine (Medium-A) for pyocyanin production. The addition of both glyceroland alanine as substrates in Medium-B augmented more pyocyanin production than Medium-A with asparagine. Inorganic ion such as K^+, Mg^{2+}, PO_4^{3-}, SO_4^{2-}, Fe^{2+} were essential for pyocyanin production. So in the Medium-B, K^+, Mg^{2+}, PO_4^{3-}, SO_4^{2-}, Fe^{2+} ions were included and it was seen that it gives better pigmentation than Medium A. Both medium contained $MgCl_2$ which also increase pigment formation[32]. It was found that all the isolates had better pigmentation in Medium-B than Medium-A. The isolate PU_{10} produced highest amount of pyocyanin (9.45 µg/mL) in Medium-B whereas the lowest (2.79 µg/mL) is produced by PS_1 in Medium-A as shown in Figure-5. Carbon and nitrogen sources in the growth media affect the production of pyocyanin but most nutrients support pyocyanin production as long as the phosphate ion concentration is low; high phosphate concentration had been shown to inhibit pyocyanin production[33]. The presence of Sulfate (SO_4) increased the pyocyanin synthesis, while sulfite (SO_3) inhibited its synthesis; the presence of Ca^{2+} ion increases the synthesis from three to five folds. Production of pyocyanin was encouraged by the addition of 1% (weight/volume) of sodium citrate[34]. Synthesis of this pigment also appears to be under the control of iron concentration since addition of iron to a medium containing low phosphate stimulates the synthesis of pyocyanin and related phenazine pigments by other species of bacteria. Tyrosine, alanine, leucine, amino acids stimulate the production of pyocyanine[31]. It is also noticeable that all the *P. aeruginosa* isolates (PU_5, PU_8 and PU_{10}) isolated from urine sample produced more pyocyanin than those isolates like PS1and PP3 isolated from burned skin swab and pus respectively. There might be some relationship among the pseudomonads' hosting environment and their pyocyanin productivity.

Table-2: FTIR spectroscopic analysis of the pigment extracted from different isolates.

Functional groups	FTIR Spectra of pigment extracted from				
	PU_{10}	PS_1	PU_8	PP_3	PU_5
-OH	3429.43	3429.43	3348.42	3348.42	3348.42
-C=N	1620.21	1627.92	1627.92	1630[h]	1627.92
-C=C	1604.7	1562.34	1589.34	1589.34	1550.77
-C-H	2924.09	2924.09	2920.23	2920.23	2943.37
C-O	1319.31	1354.03	1342.46	1342.46	1342.46
-C-N	1265.30	1261.45	1288.45	1288.45	1261.45
Methyl groups	1384.89	1384.89	1396.46	1400.32	1396.46

Note: h = hump

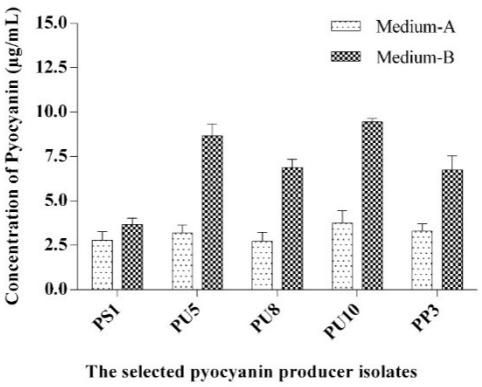

Figure-5: Rate of pyocyanin production by the selected isolates (PS_1, PU_5, PU_8, PU_{10} and PP_3) in Medium-A and Medium B.

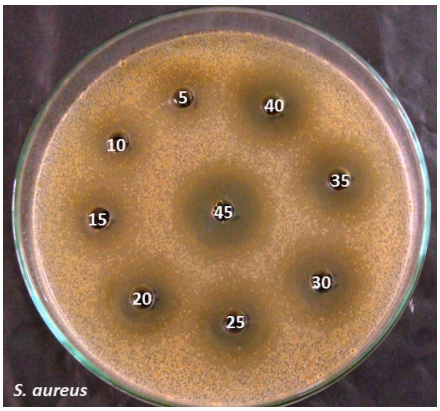

Figure-6: Effect of different concentration (µg/mL) of pyocyanin on the growth of *S. aureus* ATCC 6538.

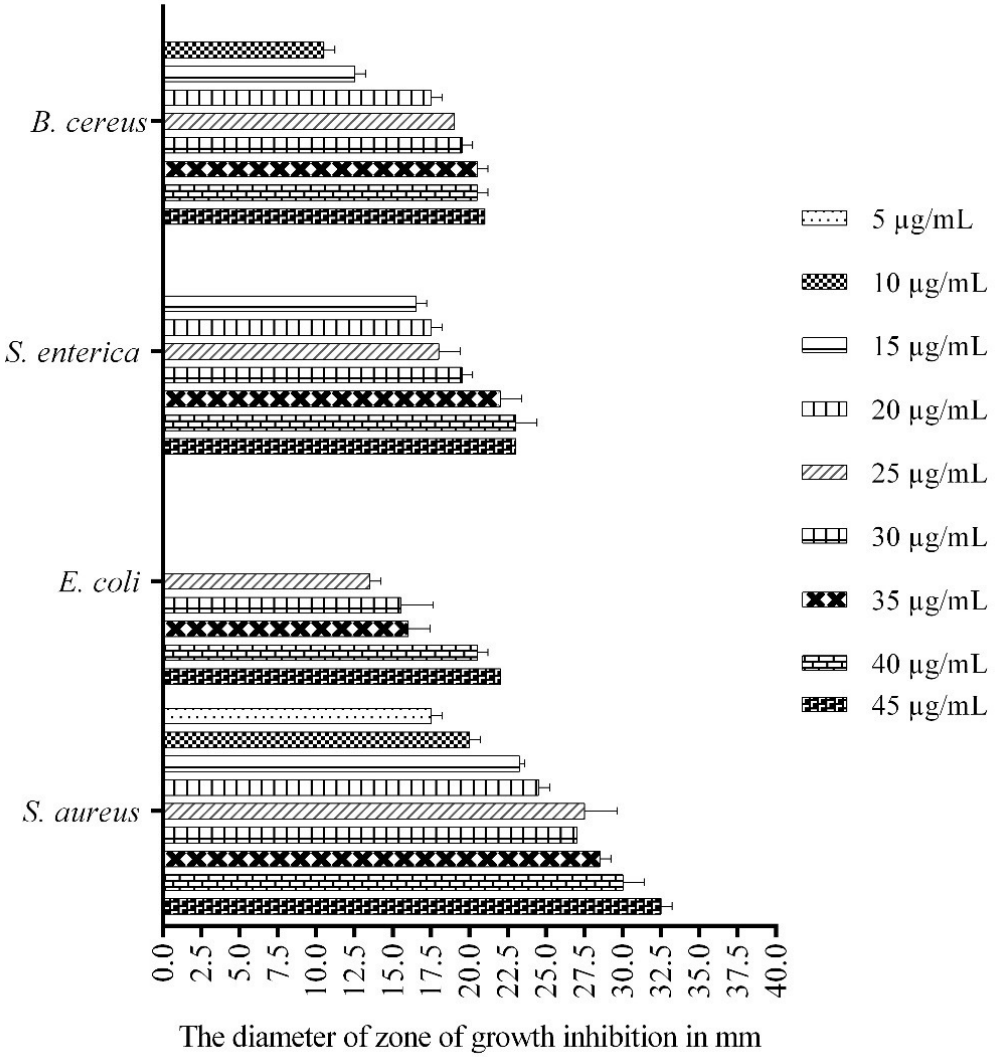

Figure-7: The antimicrobial activity of different concentration (µg/mL) of pyocyanin against different test microorganisms. Here, S. aureus was most sensitive to pyocyanin and produced zone of inhibition at lowest concentration (5 µg/mL) of pyocyanin and *E. coli r*equired highest concentration (25 µg/mL) of pyocyanin.

Antimicrobial activity of the purified pyocyanin: Previously in the primary screening process, we have found that *P. aeruginosa* have good antimicrobial activity against gram positive and gram negative bacteria. The subsequent antimicrobial activities of purified pigment corroborate the initial findings in the primary screening. Here we found that the bactericidal effect of pyocyanin that we observed was concentration dependent in all cases. The Figure-6 and 7 showed that among the test organisms *S. aureus* was the most sensitive to pyocyanin and it was inhibited at 5 µg/mL of pyocyanin producing 17.5 mm of growth inhibition zone. *B. cereus, Salmonella enteric* and *E. coli* showed zone of growth inhibition at 10, 15 and 25 µg/mL of pyocyanin respectively. From the results it is clear that gram negative bacteria were less sensitive than gram positive bacteria. It was reported earlier that purified pyocyanin showed good antimicrobial property against *S. aureus, E. coli, Klebsiella sp, S. typhi, Shigella sp. C. albicans*[14]. The lowest MIC of purified pyocyanin was reported as 20 µg/ml whereas the highest MIC was 50 µg/ml was against *E. coli*[12]. In 1981, Baron and Rowe proposed that around 2.9 µg of purified pyocyanin is sufficient for inhibiting bacterial growth where gram positive *Micrococcus luteus, S. aureus* and *Bacillus licheniformnis* showed maximum zone of inhibition and *P. aeruginosa* remained resistant to pyocyanin action[10].

The present study depicts the antimicrobial activity exerted by purified pyocyanin which is dependent on concentration. It was revealed in earlier reports that pyocyanin inhibits bacterial growth by interrupting active metabolic transport in bacterial cells through the interaction with respiratory chain[10]. Pyocyanin upon up-taking an electron, produce a relatively stable anion radical and readily undergo a redox cycle which is responsible for its antagonistic action. Pyocyanin itself becomes reduced and reduces oxygen univalently to toxic superoxide radical which are actually responsible for its antimicrobial activity[35]. The bacteria which are resistant to pyocyanin have the ability to produce catalase and superoxide dismutase and the resistance is also contingent on presence of oxygen. It is surprising that although *P. aeruginosa*is a strictaerobe, it is resistant to pyocyanin and readily evade the injury caused by toxic free-radical produced during pyocyanin synthesis by itself or exposed in the environment[28].

Pyocyanin has enormous antimicrobial potentiality to be used as antibacterial, antifungal, antiprotozoal agent as well as nitric oxide antagonist. The inhibitory action of pyocyanin against different fungi like *Aspergillussp, Candida albicans,* some pathogenic yeast and phytopathogens was reported in earlier[36]. The antiprotozoal activities of pyocyanin was studied by Dive found that pyocyanin inhibited the growth and division of *Colpidiumcampylum*[37]. In various pharmacological preparations, it is used as a nitric oxide (NO) antagonist and has various pharmacological effects[38]. It has also been reported that it has anticancer activity on growth of cancer cells such as Leukemic cells that was inhibited when treated with pyocyanin, more over induce apoptosis on neutrophils[39].

Conclusion

In this study, it has been found that pyocyanin pigment is a good antimicrobial agent, as it can inhibit both gram positive and gram negative bacteria, such as *S. aureus, B. cereus E.coli* and *S. enterica.* Antimicrobial resistance is an alarming issue to deal with, as most of the pathogen becoming resistant to their common treatable antibiotic. Recent studies report that *S. aureus* and *E. coli* are multidrug resistant bacteria. Inhibition of these and others like pathogens by pyocyanin, expresses its importance and potentiality as an antimicrobial agent. It can be used as therapeutic agent to treat infections caused by these pathogenic bacteria.

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