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Studies on Bio-color Production by *Pseudomonas aeruginosa* Isolated from Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author MUH designed the study. Author AN performed the statistical analysis. Authors AAF and MUD wrote the protocol and first draft of the manuscript. Authors MKN and GM managed the analyses of the study. Author ASB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study aimed at producing biocolor from soil inhabiting bacteria. Soil samples were screened for isolation of green pigment-producing bacteria and identified molecularly using the standard method. The effect of medium, pH, temperature, incubation time, shaking and static conditions on color production were determined on the isolate, and the pigment was extracted by using chloroform. It was observed that green pigment was produced by *Pseudomonas aeruginosa* in Nutrient broth at pH 7, after 72 hours incubation and at the temperature of 37°C under shaking condition at 4,000 rpm for 15 minutes. The pigments were characterized and identified as pyocyanin using Thin Layer Chromotography (TLC), Fourier Transformed Infrared Spectroscopy (FTIR) and UV-Visible spectroscopy (UV). The stability of the pigment was tested based on pH and temperature. It was found that the green pigment showed stability at 160°C, 200°C and pH 13.

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1. INTRODUCTION

Natural Colours are generally extracted from fruits, vegetables, roots and microorganisms and often called "Biocolors" because of their biological origin. The natural pigments are comparatively often used as an antibacterial in food preservation because of their low toxicity compared with synthetic pigment [1]. Today, pigments are used in many areas such as medicine, animal feed, paper, ink, food, and textile. Pigment production is very useful for bacteria. There are many reasons for microorganisms to produce the pigments such as photosynthesis, UV protection and defense mechanism [2].

Pseudomonas aeruginosa is a versatile gram-negative bacterium that grows in soil, marshes and coastal marine habitats, as well as on plant and animal tissues [3]. It forms biofilms on wet surfaces such as those of rocks and soil [4]. The emergence of *Pseudomonas aeruginosa* as a major opportunistic human pathogen during the past century may be a consequence of its resistance to the antibiotics and disinfectants that eliminate other environmental bacteria. *Pseudomonas aeruginosa* secrete numerous proteins and secondary metabolites and many of which have biological effects against pathogens which cause these diseases [5]. The metabolism of *Pseudomonas aeruginosa* was significantly controlled in diverse ecological niches by the degree of salinity and other environmental factors. It is also known to produce pyocyanin (5-N-methyl-1-hydroxy phenazine (PCN) which is the major phenazine compound in this species [6,7].

One of the characteristics of *P. aeruginosa* is the production of soluble pyocyanin pigment, a water soluble blue green compound produced in large quantities. Pyocyanin has antibiotic activity against bacteria and fungi [8,9]. *P. aeruginosa* was found to produce phenazine pigment identified as pyocyanin. The aim of this study is to produce green pigment from pigment-producing bacteria and also to characterize the pigment produced.

2. MATERIALS AND METHODS

2.1 Isolation of Green Pigment-Producing Bacterium

Garden soil samples were collected from Gardner plantation in sterile plastic bags then transported to the laboratory. The soil samples collected were serially diluted and plated on Nutrient agar and incubated at 35°C for 48 hours. Following the incubation only blue green colonies were selected and propagated on the same medium to obtained pure cultures used for further studies.

2.2 Morphological and Biochemical Characterization of the Isolates

Gram staining reaction and microscopic studies were performed for the isolates after 48hours incubation. The biochemical tests performed were Simmon's Citrate test, Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase and Catalase tests, Coagulase test, Urease test and TSI for Identification according to Bergey's Manual of Determinative Bacteriology [10].

2.3 Molecular Characterization of the Bacterial Isolate

2.3.1 DNA extraction using boiling method

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic tip and resuspended in 100µl of sterile molecular grade water in a microcentrifuge tube and vortex for 1 minute. Then, the washing of the pellet was done with 200µl of TBE buffer and suspension was incubated at 95°C for 20 min. After this incubation, another centrifugation at 4°C was performed 10000rpm for 2 min. Following the last centrifugation, a supernatant was collected and used for PCR amplifications [11].

2.3.2 DNA extraction using phenol-chloroform method

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic tip and resuspended in 100µl of sterile molecular grade water in a microcentrifuge tube and vortex for 1 minute. 100µl of chloroform-isoamyl alcohol were added to the suspensions and, after briefly vortexing for 30 second, the mixture was centrifuged at 16.000xg for 5 min at 4ºC. 10 µl of the upper aqueous phase were used as a source of DNA template for the PCR applications. The rest of the mixture was stored at 4ºC until use [12].

2.3.3 DNA extraction using kit method

For DNA extraction, single colonies growing on solid media were removed with a sterile plastic

tip and resuspended in 100 µl of sterile molecular grade water in a microcentrifuge tube and vortex for 1 minute. 2 µL of proteinase K solution was added mixed by vortexing to obtained uniformed solution and incubated at 56°C for 30 minutes. 20 µL of RNase A solution was added vortexed for 15 minutes and 100 µL of ethanol was added and mixed thoroughly. The preparation was then transferred into column inserted in a collection tube and centrifuge for 1 minute at 6000xg and discarded the collection tube. 500 µL of washing buffer was added and centrifuge at 8000 xg for 1 minute. The 200 µL of Elution buffer was added and incubated for 2 minutes at 30°C. After incubation, the preparation was centrifuged for 1 minute at 8000 xg. After which the DNA was collected for amplifications [13].

2.4 PCR Amplification of 16SrDNA

PCR reaction was carried out in 200 µl reaction containing template DNA, primers, dNTPs, and Taq polymerase. The reaction were cycled 35 times as 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min 30 sec. followed by final extension at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel in 1x TBE buffer, run at 100V for 45 hr. Gels were stained with ethidium bromide and photographed [14].

2.5 Sequencing and Phylogenetic Analysis

Sequencing was done as per manufacturer's instructions. The sequence was aligned with corresponding sequences of 16S rDNA from the database using BLAST from the website http://www.ncbi.nlm.nih.gov/blast [15]. Multiple alignments were generated by the CLUSTAL W program and phylogenetic tree was constructed by neighbor-joining algorithm using MEGA 6 Software [14].

2.6 Optimization Studies

2.6.1 Influence of various media on pigment production

The 250ml conical flasks containing 50ml of nutrient broth, Mueller Hinton broth and Lactose broth were prepared and sterilized separately. A 48 hour – old inoculum was inoculated in each of the flasks and incubated at 37°C in a sterile condition. After 48 hours incubation, the broth was taken for pigment extraction and estimation. The media that showed highest pigmentation was observed and chose for subsequent studies [16].

2.6.2 Influence of incubation period for pigment production

Fifty milliliters (50 ml) of the medium that showed high pigment production were prepared and 48 hours old inoculum (5%) was inoculated in each of the flasks and incubated at 37°C in a sterile condition. The pigment production was estimated at intervals of 24, 48, 72 and 96 hours. The incubation time that showed maximum production of pigment was chose and maintained in the subsequent studies [16].

2.6.3 Influence of pH on pigment production

Fifty milliliters (50ml) of the medium that showed high pigment production was prepared at varying pH i.e. 3, 4, 5, 6, 7, 8, 9 and 10 respectively. A 48 hour – old inoculums were inoculated in each of the flasks and incubated at 37°C in a sterile condition. The pigment production was estimated after 24 hours. The pH that showed maximum production of pigment was chose and maintained for subsequent studies [16].

2.6.4 Influence of temperature on pigment production

Fifty milliliters (50ml) of the medium that showed high pigment production were prepared. A 48 hour – old inoculum was inoculated in each of the flasks and incubated at different temperature as 25°C, 30°C, 35°C, 40°C and 45°C in a sterile condition. The pigment production was estimated at intervals of 24, 48, 72 and 96 hours. The temperature at which the maximum production of pigments was observed, chosen and maintained in the following studies [16].

2.6.5 Production and extraction of pigment

The isolate was grown in Elemlayer flask containing 250ml nutrient broth at 37°C for 72 hours. The observation of blue-green pigmentation in a broth indicated pyocyanin production. The extraction of pigment was done by centrifuging the culture broth at 4,000rpm for 15 minutes, the cell pallets were discarded. The supernatant containing the pyocyanin was extracted using by addition of equal volume of

chloroform and the preparation was shakes gently. Two layers were observed and the bottom layer was filtered and collected in sterile container.

2.7 Characterization of Blue Green Pigment

2.7.1 Identification by Thin Layer Chromatography (TLC)

The Thin Layer Chromotography was carried out in accordance with the method described by Ranganna (1986) with slight modification. The pigment extracted was drop on the TLC plate using a capillary. The chromatogram was run in glass chamber with n-hexane, methanol and chloroform in a ratio of 12:4:4. TLC plate was kept in the chromatography chamber till the solvent moved to the end of the TLC plate. The TLC plate was kept for 5 min. for drying of the spots. The Rf (resolution front) value of spots were calculated [17].

2.7.2 UV-visible spectrophotometric analysis

The extracted pigments were subjected to Uv-visible spectrophotometric analysis. The extracted color was analyzed by scanning in a UV-Visible spectrophotometer for determining the maximum absorbance. The scanning range was selected from 200-800 nm and absorbance at an interval of 40nm was measured [18].

2.7.3 Fourier Transform Infrared Spectroscopy (FTIR)

For the structural characterization, Fourier Transform Infrared Spectroscopy (FTIR) was performed. The pigment obtained were concentrated using a vacuum concentrator for 24 hours and the powder was mixed with small amount of KBr and mixed thoroughly. The preparation was then pressed in a sample holder and analysed by computerized Fourier Transform Infrared Spectroscopy system which generates the absorbance spectra showing the unique chemical bonds and the molecular structure of the sample material [19].

2.7.4 Determination of pigment stability

The stability of the green-pigment was determined according to the method described by Ahmad et al. [19] with modification. Two milliliters (2 ml) of the pigments contained in a test tube were placed in hot air oven at a temperature 160°C and 200°C and allowed to stand for 10 minutes. After heating, the pigments were analyzed physically for change of color and their absorbance were obtained through UV-Visible spectrophotometer. For determination of stability of pigment on pH, the H_2SO_4 and NaOH were used to adjust the pH of the pigments at pH 2 and 13 and followed by observation of any changing color.

3. RESULTS AND DISCUSSION

The green pigment-producing bacterium was isolated from Garden soil. The isolate was subjected to morphological and biochemical identification (Table 1 and Plate 1). The gel electrophoresis of isolate using different methods and sequence were presented in (Figs. 1 and 2). This result is similar to that of [20] who isolated green pigment-producing bacteria from soil loosely adhering to the root of plants.

Table 1. Morphological and biochemical characteristics of the isolates

S/n	Biochemical tests	SP1
1	Gram reaction	Negative
2	Shape	Rod
3	Pigment	Blue green
4	Motility	Motile
5	Catalase	+
6	Coagulase	-
7	Methyl red test	-
8	Voges proskauer test	-
9	Indole test	-
10	Citrate test	+
11	Urease test	-
12	H_2S	-
13	Gas production	+
14	Glucose	-
15	Fructose	-
16	Lactose	-

3.1 Molecular Identification of Pyocyanin-Producing Bacterium

The Molecular identification of the pigment producing bacterial isolate was done by sequencing part of the 16SrDNA. The sequence of the isolate was subjected to BLAST analysis. As 16SrDNA gene sequence provide accurate grouping of organism even at subspecies level it is considered as powerful tool for the rapid identification of bacterial species [21]. The phylogenetic analysis by neighbor joining tree of

Pseudomonas species (Fig. 3) isolated from garden soil. The sequence showed 100% identity to the 16SrDNA gene sequence of *Pseudomonas aeruginosa* (ATCC 10145) when the sequence was blasted against NCBI database.

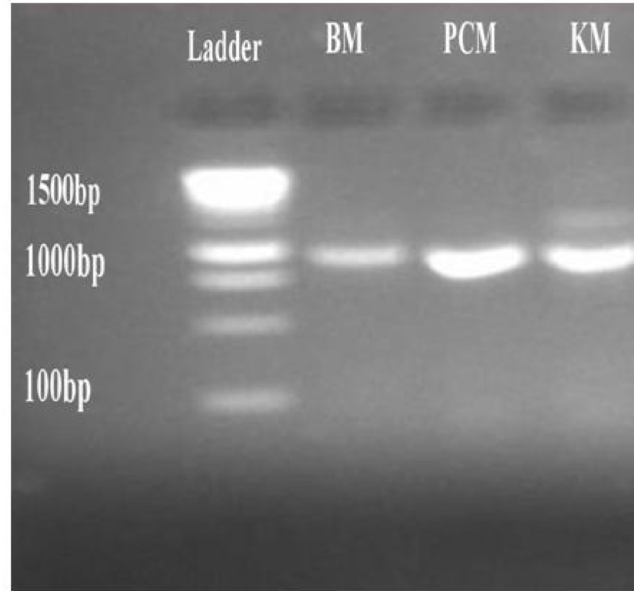

Fig. 1. Agarose gel electrophoresis of the PCR amplified 16SrRNA gene of the Isolate for different method of DNA extraction

Key: BM = DNA Extraction by boiling Method; PCM = DNA Extraction by Phenol-chloroform KM = DNA Extraction Using DNA Kit Method

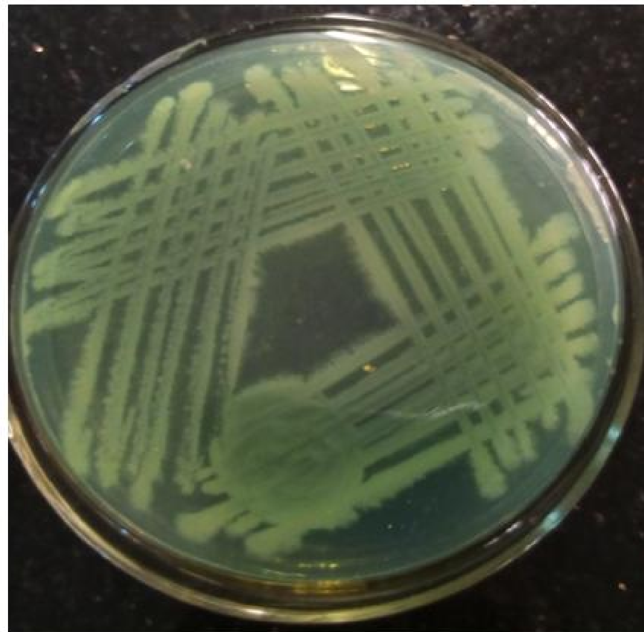

Plate 1. Isolated blue-green pigmented bacteria from organic waste soil

GAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGA
GCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACG
TCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT
CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
ACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT
ACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG
AACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGT
GGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGA
CATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCAC
CTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGG
AGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGC
TAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGAC
TGGGGTGAAGTCGTAACAG

Fig. 2. DNA sequences of *Pseudomonas aeruginosa*

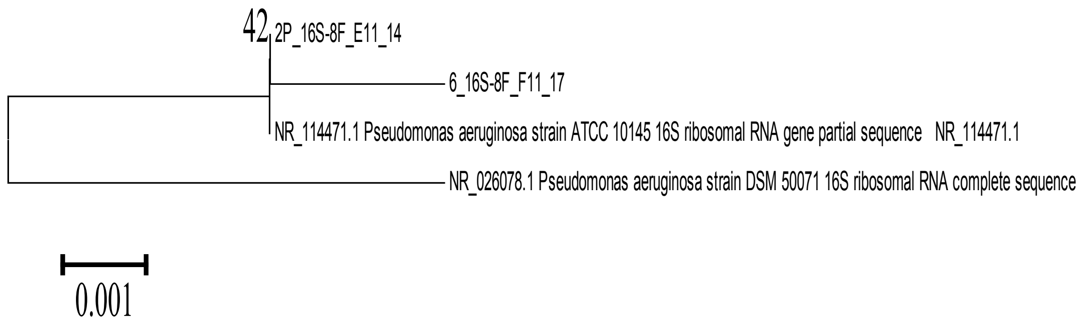

Fig. 3. The phylogenetic analysis by neighbor joining tree of *Pseudomonas specie* producing biocolor Isolated from garden soil

Pseudomonas aeruginosa showed considerable amount of pyocyanin production, the pigment got diffused throughout the medium. It was observed that the Nutrient broth favors maximum pigment production by *Pseudomonas aeruginosa,* follow by Lactose broth while no pigment production was observed in Mueller-Hinton Broth. In a Nutrient broth the *Pseudomonas aeruginosa* showed 56% absorbance (Fig. 4). The highest green pigmentation was observed after 72 hours with 49% absorbance and gradually decline to 15% absorbance after 96 hours incubation (Fig. 5). The rate of pigmentation by the *Pseudomonas aeruginosa* was higher around neutrality, at pH 7 the isolate showed highest pigmentation with 36% absorbance which gradually decline toward alkaline pH (Fig. 6). The influence of incubation temperature on pigment production was determined on *Pseudomonas aeruginosa* and it was observed that the isolate showed maximum pigmentation at 37°C with 28% absorbance (Fig. 7). The influence of static and shaking condition were determined on pigment production by the *Pseudomonas aeruginosa*. It was observed that the pigmentation was favor under shaking condition

while at static condition *Pseudomonas aeruginosa* showed minimum pigmentation (Fig. 8). This result is in agreement with the finding of Laqaa [22] reported that the *Pseudomonas aeruginosa* produced green pigment on nutrient broth and Chandran et al. [23] who observed the pyocyanin production by *Pseudomonas aeruginosa* produces maximum pigmentation at temperature of 37°C, pH 7, after 72 hours incubation. The finding is slightly contrary to finding of Abraham et al. [24] who observed the highest pyocyanin production by *Pseudomonas aeruginosa* to at 35°C, at pH 7.5 after eight (8) days incubation.

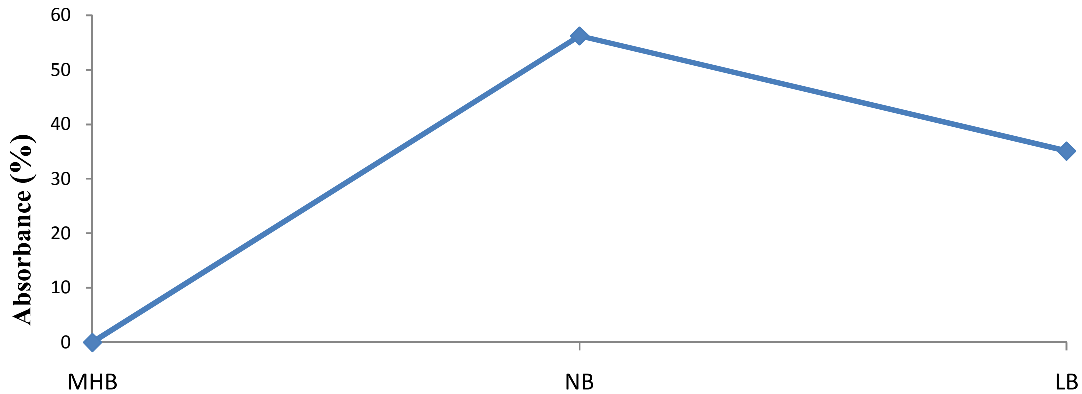

Fig. 4. Influence of media on pigment production by *Pseudomonas aeruginosa*

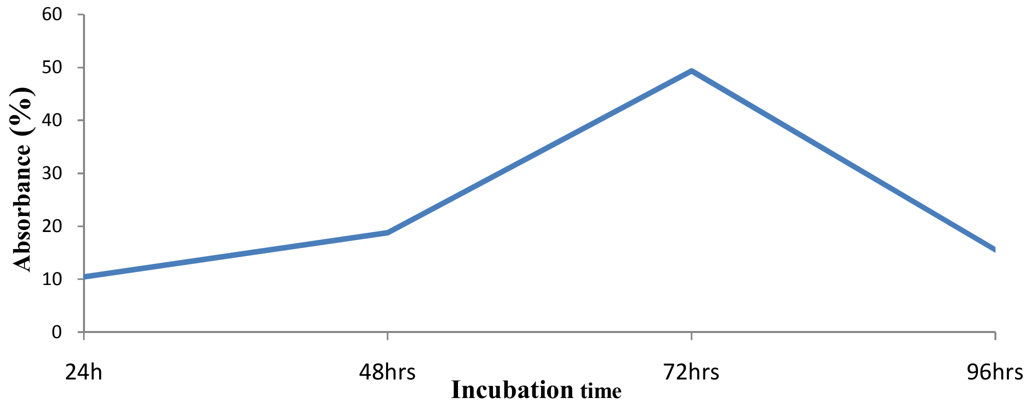

Fig. 5. Influence of incubation time on pigment production by *Pseudomonas aeruginosa*

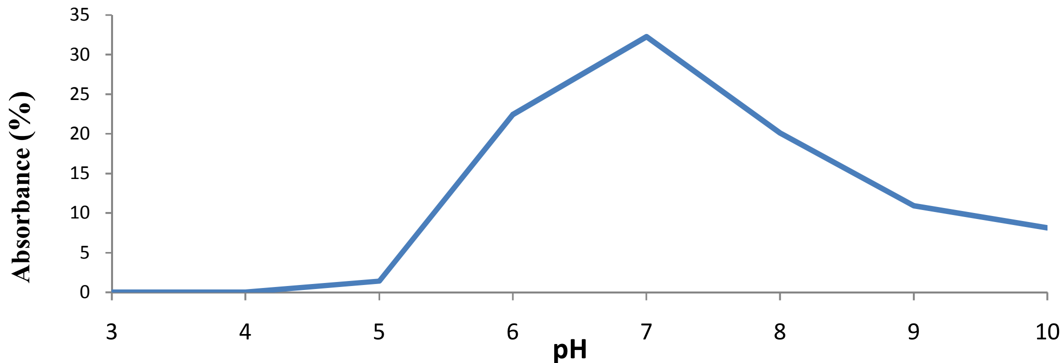

Fig. 6. Influence of pH on pigment production by *Pseudomonas aeruginosa*

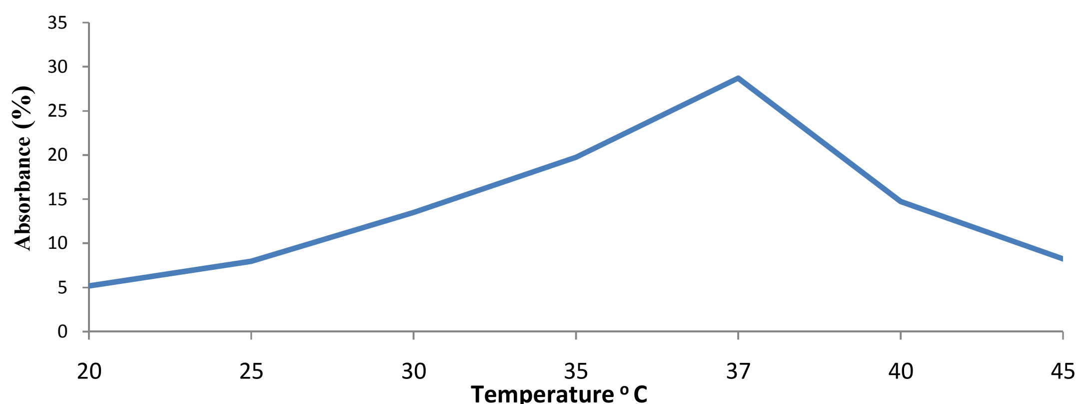

Fig. 7. Influence of temperature on pigment production by *Pseudomonas aeruginosa*

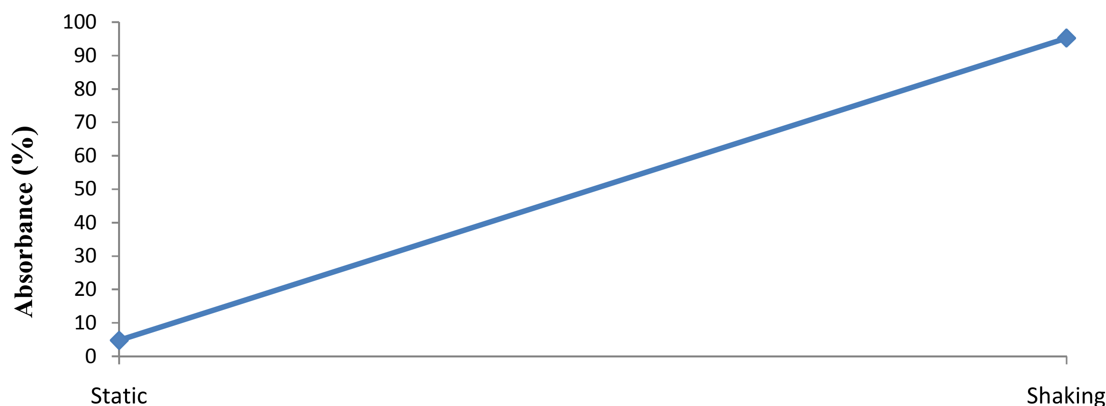

Fig. 8. Influence of static and shaking condition on pigment production by *Pseudomonas aeruginosa*

3.2 Identification and Characterization of Pigment

The chloroform extracted pigment showing bluish coloration was then concentrated and analyzed using UV-Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) and Thin Layer Chromotography. The UV-Visible spectrum of the extracted pigment was generated at a wavelength region between 200-800nm. The UV-Visible spectrophotometric analysis of extracted green pigment showed maximum peak of 270 nm. This finding is congruent with the finding of [25] who observed the UV–Visible spectra of green pigment produced by *Pseudomonas aeruginosa* was 278 nm. Similarly, Masi et al. [26] extracted the pigment from *Pseudomonas aeruginosa* using chloroform extraction method and analyzed by Uv spectrophotometer and maximum absorbance of the pigment was obtained at 270 nm and 278 nm. They reported that the pigment belonged to the chlorophyll family and pyocyanin. Sudhakar et al. [27] reported the maximum absorption of green pigment extracted from ten *Pseudomonas aeruginosa* were observed in a range between 270 nm to 280 nm. The results is in disagreement with the finding of Abdul-hussein and Atia [28] who reported the maximum wavelength of pyocyanin produced by *Pseudomonas aeruginosa* to be at 520 nm.

The Thin Layer Chromotographic analysis of green pigment produced by the isolate exhibited one spot with Rf value 0.75 which is similar to pyocyanin. This is in agreement with the finding of [29] extracted, purified and characterized the green pigment produced by *Pseudomonas aeruginosa* and reported Rf values of the green pigment range between 0.70 – 0.81 and identified as pyocyanin. Similar result was reported by Abdul-Hussein and Atia [28] observed the green pigment produced by *Pseudomonas aeruginosa* identified as pyocyanin with Rf value 0.81.

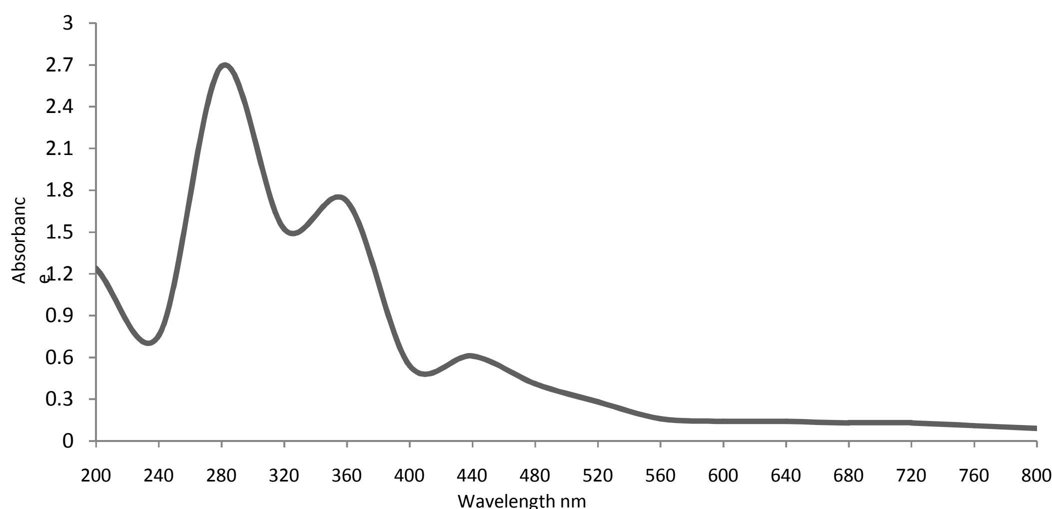

Fig. 9. UV-visible spectrum of green pigment produced by *Pseudomonas aeruginosa*

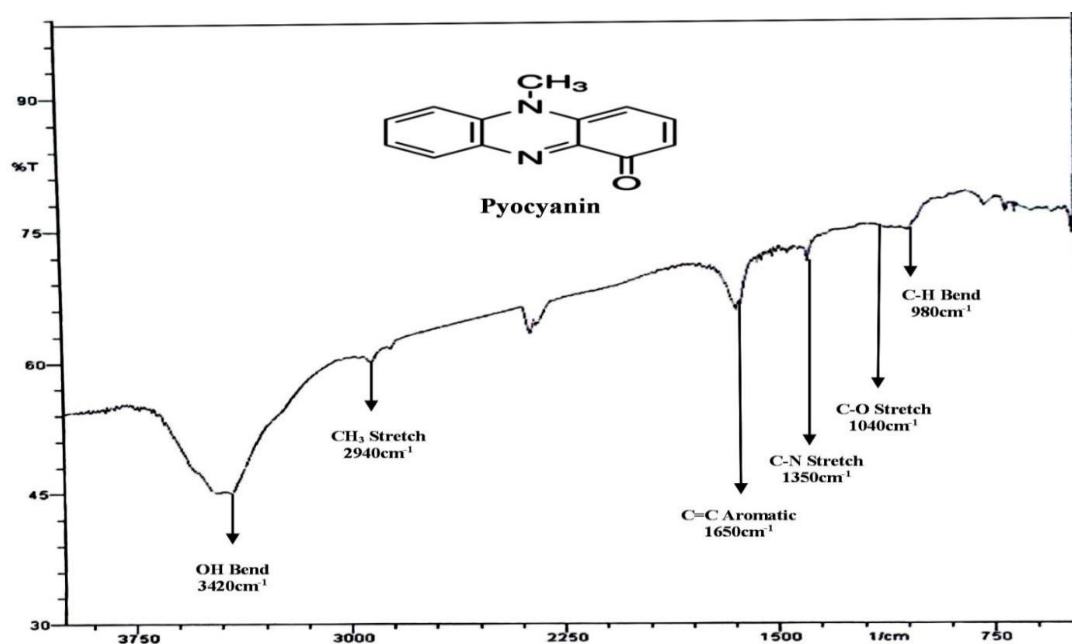

Fig. 10. FTIR spectrum of green pigment produced by *Pseudomonas aeruginosa*

The result of FTIR analysis of green pigment produced by *Pseudomonas aeruginosa* revealed the presence of the following functional groups and their absorption frequencies CH_3 (2940 cm^{-1}), C=C (1650 cm^{-1}), C-N (1350 cm^{-1}), C-O (1040 cm^{-1}) and C-H (980 cm^{-1}). The majority of the peaks appear in the spectrum 750 with 3750 cm^{-1} and absorption bands correspond to those of pyocyanin. An intense band located at 3433 cm^{-1}, corresponding respectively to the asymmetrical elongation of grouping OH of the water molecule. The result of this study agrees with the previous report [20].

The pyocyanin turned to dark red at pH 2 and remained dark red after 24 hours while at pH 13 the pyocyanin changed to light green and after 24 hours exposure it changed to yellow. In alkaline condition, the excess OH^- ions from NaOH deprotonates the phenolic group causing the formation of an anion and destruction in the conjugated structure of the pigment [19]. The

Table 2. Effect of pH toward the stability of pigments

Pigment	pH condition	Maximum wavelength (ʎmax)	Instant color changed	Color changed after 24 hours
Green pigment	Control	280 nm	Green	Green
	pH 2	460 nm	Dark red	Dark red
	pH 13	280 nm	Light green	Yellow

Table 3. Effect of temperature toward stability of the pigments

Pigments	Temperature condition	Maximum wavelength (ʎmax.)	Color changed
Green pigment	Control	280 nm	Dark green
	160°C	280 nm	Green
	200°C	280 nm	Green

pyocyanin pigment produced by *Pseudomonas aeruginosa* showed good stability when heated for Ten (10) minutes at 160°C and 200°C. This may indicate its suitability to be applied in various industrial applications such as dying industries. This result agrees with the finding of Ahmad et al. [19] which reported that the pigment from bacteria showed good stability toward temperature ranging from 45°C-120°C.

4. CONCLUSION

The finding of the present study revealed that *Pseudomonas aeruginosa* produced maximum pigmentation when grown in nutrient broth at 37°C, pH 7, after 72 hours and under shaking condition. It was observed that pigment was produced by operationalizing parameter that showed good stability toward temperature (200°C) and fairly stable toward pH especially lower pH (2). Further studies should be conducted to determine the nature of pycocyanin pigment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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