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ORIGINAL ARTICLE

Isolation, identification and antibacterial study of pigmented bacteria

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Abstract
Pigments obtained from natural sources gain worldwide interest in recent year as synthetic pigments have many environmental hazards because chemicals present in them are very toxic and has many disadvantages. Pigments produced by bacteria play an important role in food, pharmaceutical and textile industries. These are isolated from different sources like soil, water, vegetables and fruits. In current study these pigmented bacteria are isolated from the soil of different regions with different geographical and climatic conditions. Main objective was to isolate and identify pigmented bacteria from soil and to find their antibacterial activity.04 pigmented bacterial isolates were obtained from the soil and they were further used for pigment extraction and study. They were characterized by the Bergey's Manual of Determinative Bacteriology. The extracts were prepared using chloroform as solvent and the maximum absorption of each sample was determined by UV spectrometer. The pigments were further analyzed using paper chromatography. Antibacterial activity of pigments revealed that they had some inhibitory action against pathogens like *E. coli*, *Pseudomonas* and *Staphylococcus* sps. Thus the current study can be a useful step for large-scale production of pigments, their purification and application in various industries especially from soil, which has diverse organisms which produce different pigments with antibacterial and antifungal activity.

Keywords Antibacterial · Pigments · Soil · UV spectrometer · Pigmented bacteria

Introduction

World's beauty depends on colors and without colors we cannot imagine the world. Pigments are considered to be chemical molecules or substances that can absorb light of visible region. Chemically they are pyrrole, phenazine, quinine and xanthophylls. Also it has been proved that bacteria that have pigment-producing ability are only aerobic and facultative aerobic bacteria because molecular oxygen is important for pigment production and bacteria that are anaerobic are non-pigmented. The colors they produce is due to chromophore material and these molecules have specific structure that captures the sun energy and allows electrons to be excited from external orbit to higher orbital level, where the energy that cannot absorbed is refracted or reflected to be visible to the eye (Delgado-Vargas et al. 2000).

Natural pigments have been obtained since a long time ago and because synthetic pigments have some toxicity problems and having carcinogenic effects both for products and workers (Parekh et al. 2000). That's why the interest in natural pigments has been increased. Currently, the microbial sources are considered good and alternative as compared to synthetic pigments. In recent years, for the food coloring, pharmaceutical products and textiles products pigments produced from natural sources gain worldwide and global interest. Because of their safe and healthy use these pigments are used as natural food dye as synthetic dye has undesirable market value (Lordick et al. 2007).

The ability of bacteria to produce pigments is for different reasons. Some bacteria like *cyanobacteria* produce

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phycobilin pigment to carry out photosynthesis (Song et al. 2006). Other reasons of producing pigments UV protection, defense mechanisms, secondary metabolites for storage of energy, in stress conditions to prevent them from harsh conditions. It varies with environment differs in marine, terrestrial and space. *Serratia marcescens* produce prodigiosin, *Streptomyces coelicolor* produce actinorhodin along with prodigiosin, similarly *Chromobacterium* produce violacien and *Thalkalivibrio versutus* produce natochrome and chloronatochrome. These bacteria can be isolated and cultured (Browning et al. 2003; Woodall et al. 1997).

Differential Medias such as macConkey agar, EMB agar, Macleod agar and TSB agar are very useful in indication and characterization of pigmented bacteria (Bhawsar and Cameotra 2011). Microorganisms produce different pigments such as quinines, carotenoids, flavins, melanins, prodigiosins and mostly monascins, voilacien (Bhawsar and Cameotra 2011; Mortensen 2006; Pantanella et al. 2007; Venil et al. 2013). The classification and identification of bacterial isolates are mostly through morphological and biochemical characteristics. Researchers are trying to use statistical analysis such as Response surface method (RSM) and Taguchi method (McClean et al. 1997).

To enhance the efficacy of bacterial pigments current technologies, have different potentials besides its challenges. Bacterial pigments that are produced by bioprocess market are difficult to estimate, due to lack of statistical regional method (Simova et al. 2003). Global demand for dyes and pigments is hoped to reach almost 10 million tons by 2017 according to the analysts of Global industry. The largest consumers of organic and natural pigments are textiles industries (Joshi and Pandey 1999).

The major objective of the work was to select the effective pigment-producing microbes from the natural source like soil. Then the selection of cheaper substrates that is economically productive and important and can be used commercially. And characterization of these pigments was made on the basis of morphology and biochemical tests.

Materials and methods

Sample collection

Soil samples were collected from Reerh, Garhi Habibullah and Balakot area of Mansehra- Pakistan. These samples were collected from gardens, lawn, river, side, stream, side, and dye industry. Some samples were collected from waste of dye industry from similar regions of Mansehra–Pakistan.

Initial tests

Soil texture, Soil pH.

Chemicals and media

Chemicals like NaCl, Ethanol, NaOH, HCl and some other analytical grade chemicals were used throughout the study. Distilled water used for the experiments and aseptic conditions were maintained during the whole study. Bacteriological media such as nutrient agar, nutrient broth, lactose broth, and blood agar and MacConkey agar were used. Gram staining kit and reagents were used for biochemical tests.

Screening of pigment-producing microorganisms

Eight different soil samples from different regions (Mansehra–Pakistan) were collected. These samples were thoroughly washed with distilled water to isolate the dust and debris. Soil suspension was formed from the collected soil samples and these samples were then serially diluted (10^{-7} and 10^{-5} times) and 1 ml solvent was taken and inoculated on nutrient agar plates and observed for different pigmented colonies. All the isolates were cultivated at optimum conditions i.e. temperature 30 °C and pH 7.

Isolation and identification of pigmented bacteria

Different methods and media were used for isolation of pigmented bacteria from soil.

Serial dilutions

At first serial dilution method was used for the isolation of bacteria. Serial dilution is a method for obtaining the pure single colonies of bacteria from the sample in which the number of microbes were high. A serial dilution of each sample was made up to 10^7 and 1 ml of each 10^5 and 10^7dilution were added in sterilized petri plates in duplicate. Concentration factor for each test tube will remain the same i.e., 10 ml. In this experiment, seven dilutions were made of 10 ml in each test tube. Among them 5th and 7th dilutions were taken and sub cultured on media plates. By the process of serial dilution different colonies were obtained. These colonies were picked and sub cultured on media plates like nutrient agar, M.H.A, EMB, blood agar and Macconkey agar.

Isolation of bacteria by nutrient broth media

Nutrient broth media was prepared and autoclaved then 1 g of soil was added in 100 ml distilled water. Three sets of soil suspensions were prepared by taking three different samples of soil and incubated for 5–6 days at 37 °C. Color change was observed after 5–6 days' incubation then these samples

were inoculated on nutrient agar plates. These plates were then incubated for 24 h. Pigmented colonies were observed after 24 h incubation. These pigmented colonies were taken and sub cultured on different media such as macconkey agar, blood agar, and M.H.A, on the basis of colony morphology, shape and size as different bacteria produce different types of pigments to check that on which media most intense pigmentation produced. Gram staining was performed to study morphological characteristics like shape, size, colour, texture, opacity, elevation, margin and mobility. Further biochemical tests were performed like indole, methyl Red, citrate, urease, TSI slants, etc. for characterization.

Isolation of bacteria by lactose broth

Lactose broth solution was prepared and autoclaved. Soil was added in the broth media. This media wasthen incubated for 2–4 days. Positive and negative controls were also placed along with them. After 2 days incubation color change was observed. Inoculums were streaked on different media such as nutrient agar, M.H.A and MacConkey agar and these samples were incubated for 24 h. Pigments were observed after incubation.

Purification of cultures

Pigmented bacterial isolates were purified by streaking onto nutrient agar plate and it was incubated for 24 h at 30 °C.

Maintenance of culture

Pigmented bacterial cultures were grown on different media and were maintained at 2–4 °C temperature in refrigerator and sub cultured into the respective medium.

Biochemical characterization of isolated strains

Gram staining and biochemical test were performed which included urease, citrate, catalase, nitrate reductase, TSI, indole, methyl red.

Extraction of pigments

For extraction of pigments liquid–liquid chromatography was used. Nutrient broth solution was prepared in 100 ml nutrient flask. Broth solution was autoclaved and cultures were inoculated in sterile broth flasks. Two sets were prepared; one flask was kept at static condition. The color change was observed after 3–4 days. These pigments were then extracted by centrifugation at 6000 rpm/120 °C/15 min. Then supernatant was collected and pellets were discarded. The pigmented supernatants were mixed with an equal amount of chloroform and then they were separated using separating funnel. These colored supernatants were filtered through Whattman filter paper (Sasidharan et al. 2013).

Antibacterial activity

To check if the extracted pigments have any antibacterial property, well diffusion method was performed using different lab cultures e.g. *Staphylococus aureus*, *Pseudomonas*, *E.coli*, and *Salmonella* using Mueller Hinton agar against the extracted pigments. These cultures were plated on MHA plates and incubated for 24 h at 37 °C. After 24 h incubation, wells were bored in the plates and these wells were filled with the extracted pigments. Around 20 µl amount of pigment was taken and poured in wells. Then it was incubated again for 24 h and results were observed by measuring the zone of inhibition.

Spectrometric analysis

The pigments were analyzed for the detection of λ_{max} using spectrometer (Sasidharan et al. 2013) (Fig. 1).

Results and discussion

The collected soil samples were purified and then initial tests were performed e.g. pH, texture (sand, silt and clay percentage) etc. An ideal soil is considered to be a loam which is a mixture of clay silt and sand. Pigment production is affected by all these factors like temperature, pH, and texture etc. (Table 1).

Isolation and screening of pigmented bacteria

Different methods were used to isolate pigment-producing bacteria from soil which are as follows:

By serial dilution

After 2–10 days incubation no pigments were observed during this attempt (Fig. 2).

Serial dilution using glucose medium

By adding glucose in medium serial dilution method after 2 days showed light pink and yellow colonies on plates. On this plate stream sample was inoculated (Fig. 3a, b, c).

Isolation by nutrient broth

Nutrient broth media was prepared by adding 2.9 g nutrient broth into 100 ml water and autoclaved then 1 g of soil was added in 100 ml distilled water. Three sets of

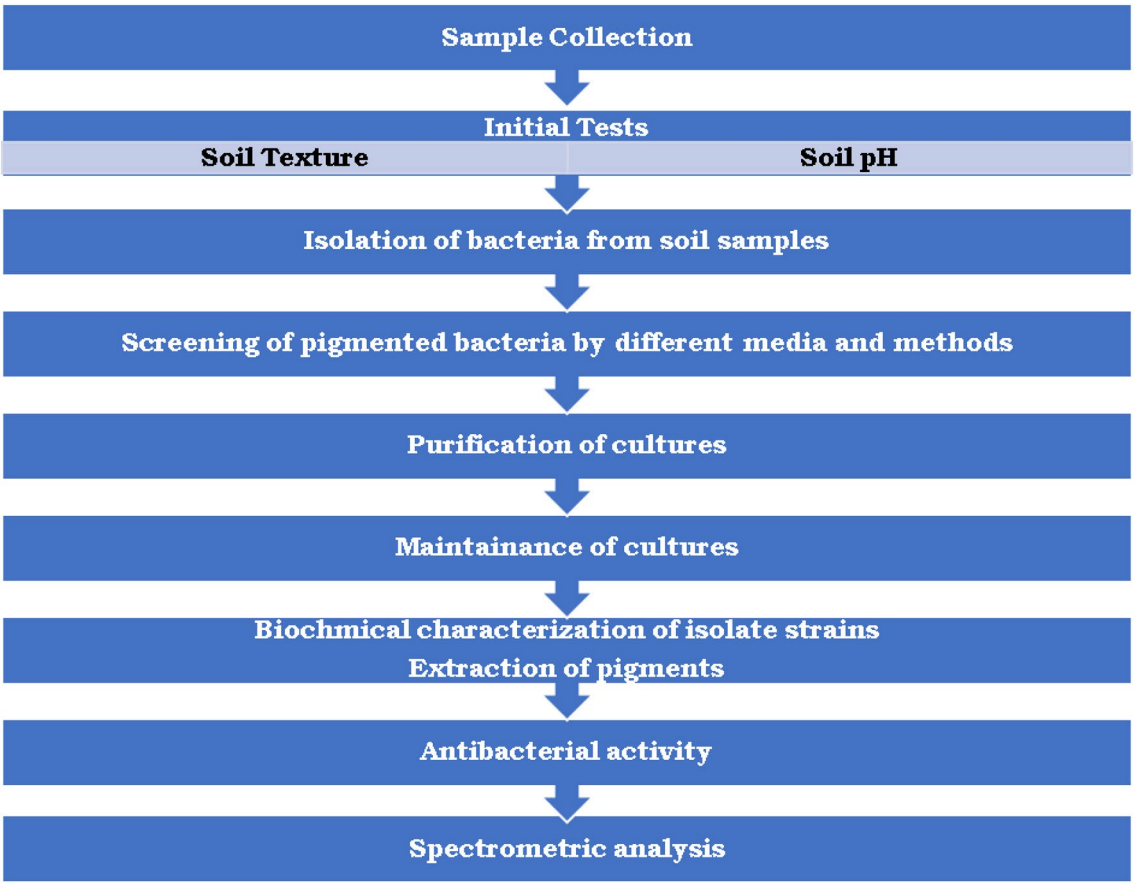


Fig. 1 Flow chart for experimental work

Table 1 Physiochemical characters of soil and pigment production analysis

Sampling area	Soil color	Soil texture	Soil pH	Pigment production
Reerh	–	–	–	-
(i) Gardens	Dark brown	Silty soil	7.5	None
(ii) Lawn	Dark brown	Clay loam	7.5–7.8	Yellow
(iii) Streams	Light brown	Sandy soil	7.8	Light-pink, orange Yellow
Balakot river side	Grey	Sandy soil	7.3–7.5	None
Garhi Habibullah				
(i) Forests	Pinkish Red	Silty soil	8.3	None
(ii) Gardens	Pinkish red	Silty soil	7.5	None
Dye-industry waste	Red	Clay soil	7.2–7.5	Reddish pink and orange
Iron containing soil	Brown	Clay soil	7.0–7.5	Blue green

soil suspensions were prepared by taking three different samples of soil and incubated for 5–6 days at 37 °C. Color change was observed after 5–6 days incubation then these samples were inoculated on nutrient agar plates. These plates were then incubated for 24 h (Fig. 4). Pigmented colonies were observed after 24 h incubation. These pigmented colonies were taken and subcultured on different media like macconkey agar, blood agar, and mueller hinton agar, on the basis of colony morphology, shape and size as different bacteria produce different types of pigments to check that on which media most intense pigmentation produced (Fig. 5; Tables 2, 3).

Fig. 2 None pigmented colonies after serial dilution on nutrient agar medium

Isolation by lactose broth

Lactose broth solution was prepared by adding 5 g media into 200 ml water. Media was then autoclaved and soil was added in the broth media. This media was then incubated for 2–4 days. Positive and negative controls were also placed along with them. After 2 days incubation, color change was observed. Inoculums were streaked on different media like nutrient agar, Mueller hinton agar and MacConkey agar and incubated for 24 h. Pigments were observed after incubation (Fig. 6).

Extraction of pigments

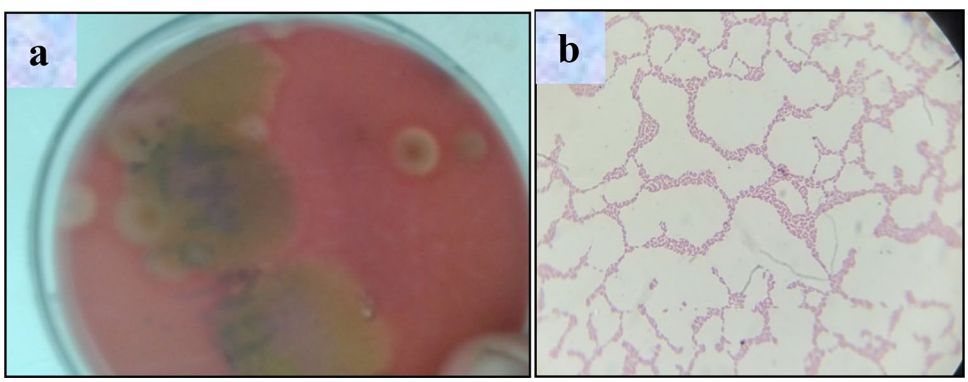
For the extraction of pigments centrifugation, filtration and addition of ethanol were done. So that cells lysis occurs and pigments can be extracted easily. Red, orange, yellow and blue-green pigments were extracted (Fig. 7).

Antibacterial activity of extracted pigments

Extracted pigments were checked for their antibacterial activity. Well diffusion method was used and results were observed by measuring zone of inhibition. Antimicrobial activity was checked by well diffusion method against different lab cultures. Results were observed by measuring zone of inhibition. Different pigments show activity against specific microbes like pigments produced by *Serratia marcesens* show antibacterial activity against *Staphylococcus aureus* and *E.coli*. Yellow pigments which were produced by Actinomycets specie show antibacterial activity against *Salmonella* and *Staphylococcus aureus*. Similarly, Orange pigments show activity against *Staphylococcusaureus* only while on the plates no zone of inhibition was observed. Blue-green pigments show

Fig. 3 **a**, **b**, **c** Serial dilution using glucose medium. **a** Light pink pigmented colonies on nutrient agar. **b** Yellow pigment on nutrient agar. **c** Gram stain image of yellow colony

Fig. 4 **a**, **b** Sub culturing of soil sample on blood agar media shows green colonies. **b** Gram stain images of green pigmented bacteria



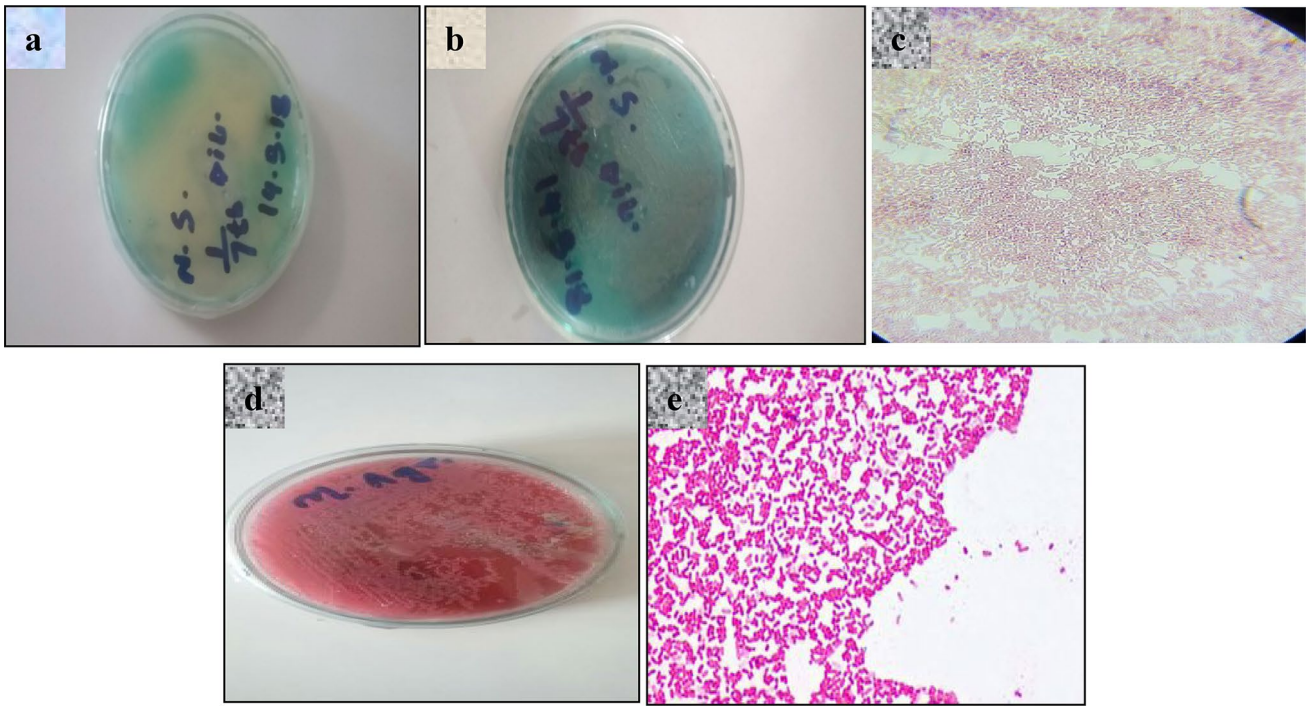


Fig. 5 **a** Green pigmented colonies on M.H.A. **b** Green pigments become intense at room temperature. **c** Gram stain image of bluish green pigmented colony. **d** Reddish pink colonies on macConkey agar. **e** Gram stain image of reddish pink colony

Table 2 Colony characteristics of isolated strains

Colony appearance	Colony characteristics		
	Form	Elevation	Margin
Reddish pink	Circular smooth	Convex	Entire
Yellow	Circular opaque	Convex	Irregular
Blue green	circular	Convex	Irregular edges
Orange	Circular opaque	Convex	Entire

activity against gram-positive bacteria and species while no antibacterial activity was observed against gram-negative bacteria such as *Pseudomonas*, *E. coli*, *Salmonella* etc.(Table 4).

Spectrometric analysis

Absorbance of extracted pigments was checked by UV spectrometer (Figs. 8, 9, 10, 11). Each pigment was checked from 450 to 600 and maximum absorbance was observed at 550 (Bhawsar and Cameotra 2011).

Current research shows that pigment production ability of microorganism depends on different environmental conditions, soil condition, texture, pH and also the medium in which they grow and also on incubation time. But the optimum values were found different for different isolates. Optimum temperature was set at 25–30 °C for different isolates. Some bacteria had show pigmentation at room temperature while some showed growth and pigmentation above 30 °C. The optimum time of incubation was 48 to 96 h butdepends on the type of isolate (Browning et al. 2003; Woodall et al. 1997).

Table 3 Biochemical characterization of isolated strains

Pigment color	Gram staining	Catalase test	Urease test	Nitrate reductase test	Citrate utilization	Indole	Methyl red
Pinkish red	Gram negative rods	+	+	+	+	−	−
Blue green	Gram-negative rods	+	−	+	+	−	−
Yellow	Gram positive	+	−	+	+	−	−
Orange	Gram negative	+	+	−	−	−	−

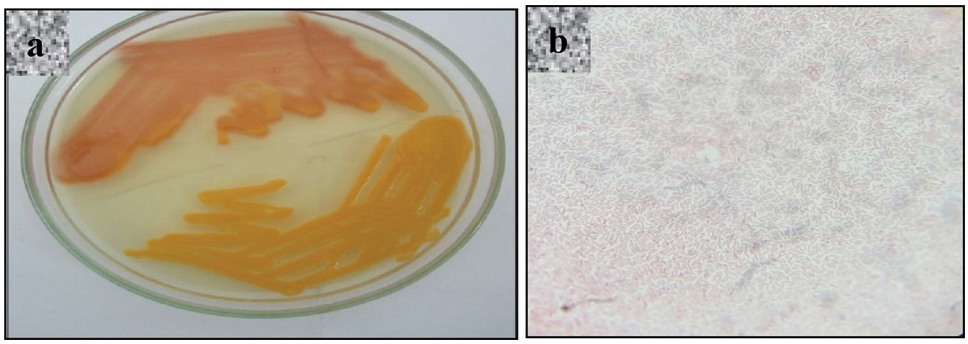


Fig. 6 Isolation of bacteria by lactose broth medium, **a** Orange pigment on NA plate, **b** Gram-stain image

Fig. 7 Extraction of isolated pigments. **a** Extracted red pigment. **b** Extracted orange pigment. **c** Extracted yellow pigment. **d** Extracted green pigment

Table 4 Antibacterial activity of isolated strains

Microorganism	Red	Yellow	Orange	Green
Salmonella	–	12 mm	–	–
Staphylococcus aureus	10 mm	22 mm	8 mm	–
Pseudomonas aeruginosa	–	–	–	–
E.coli	12 mm	–	–	–

It was also observed that pigment production ability is varies greatly with environmental conditions and medium in which they are growing. Some microbes show pigmentation in the presence of glucose in the medium while some are non glucose and lactose fermenters. Some bacteria in the presence of iron medium or bacteria containing iron also have ability to produce red or blue-green pigments. Some shows pigmentation with dye-containing soil (Figs. 8, 9, 10, 11).

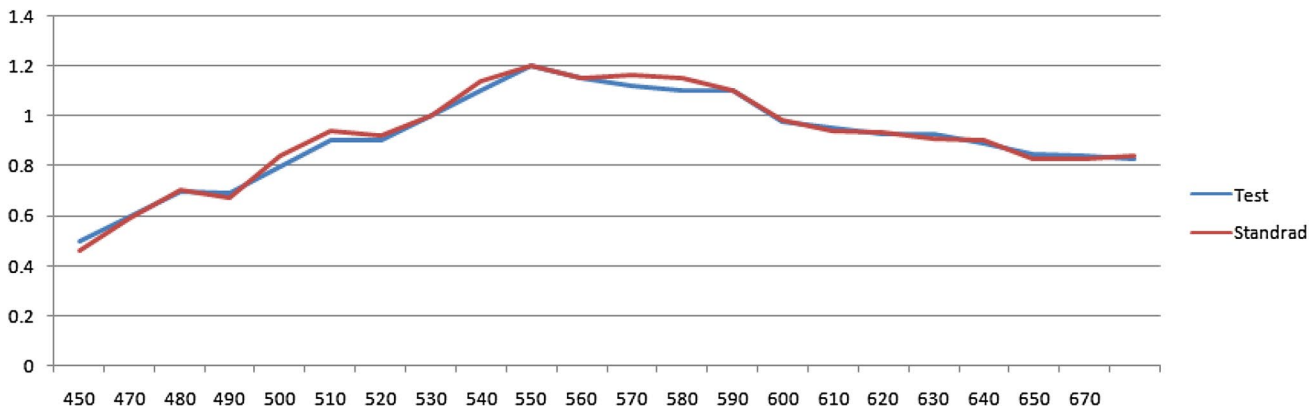


Fig. 8 Spectrometric analysis of extracted red pigment shows maximum absorbance at 540 nm

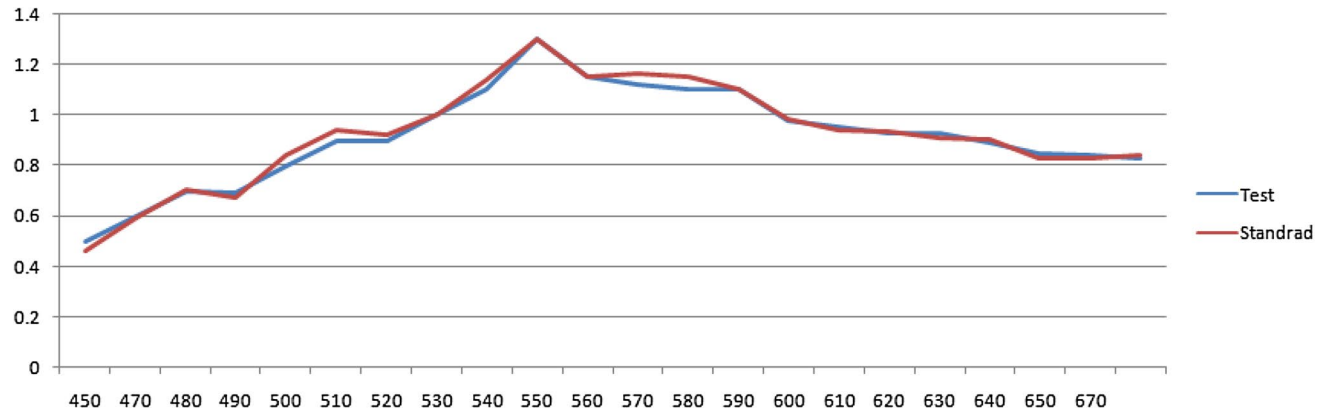


Fig. 9 Spectrometric analysis of extracted blue green pigment shows maximum absorbance 550 nm

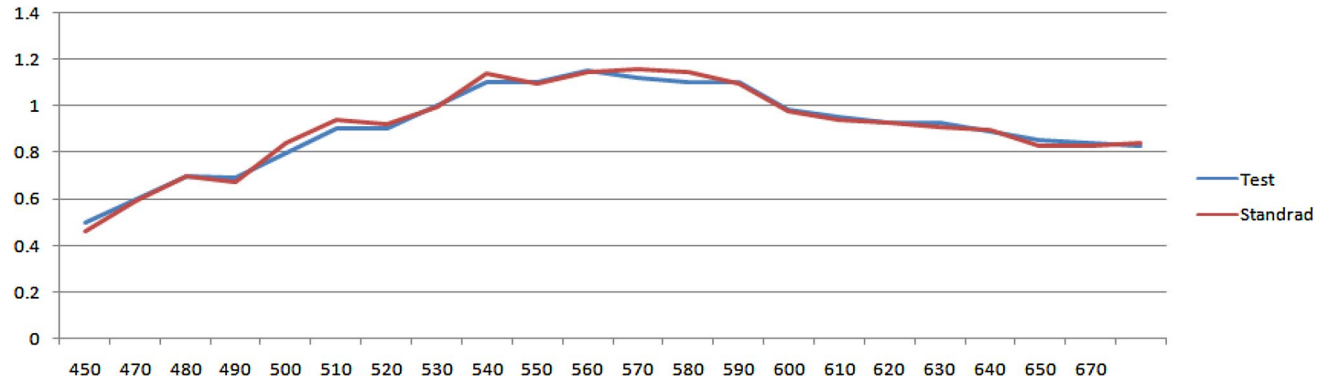


Fig. 10 Spectrometric analysis of extracted yellow pigment shows maximum absorbance at 560 nm

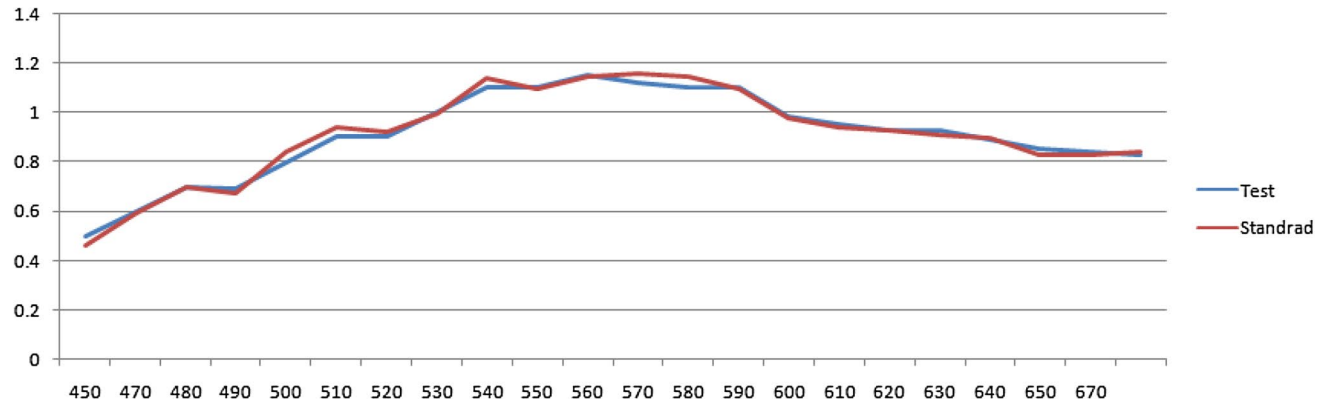


Fig. 11 Spectrometric analysis of extracted orange pigment shows maximum absorbance at 540

Conclusion

Present study was conducted to indicate the presence of different pigments producing bacteria in soil. Results suggested that soil contains a wide range of microorganisms of diverse nature. It is concluded from the current research that different factors are involved in pigment production like temperature, pH, texture, and nutrient medium that were used for the isolation. Means that pigment production is highly influenced by physical factors. Dye soil was observed to be good for pigmented bacterial growth,

similarly soil containing iron oxidizing bacteria and soil which consist of mostly clay and silt was good in pigment production while no desired results were observed from the samples collected from river and forest side. Reddish pink pigments were produced by *Serratia marcescens* which produce pigment known as prodigiosin. Similarly, yellow pigment was produced by *Actinomyces* species and orange pigment producing bacteria was *erythrobacter*. Except pigment produce by *Pseudomonas* all pigments shows antibiotic activity against different lab cultures.

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