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# ISOLATION, CHARACTERIZATION AND POTENTIAL OF BACTERIA AS BIOCOLORANT AGENTS IN TEXTILE INDUSTRY

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## ABSTRACT
The substantial volume of synthetic dyes used in textile industry poses a high risk of chemical hazard to human health and environment. Bacterial biopigments offer a greener alternate by virtue of their biodegradable and eco-friendly nature. The current study was designed to isolate, characterize and use bacterial biopigments as dyeing agents. Two pigmented bacteria [Pseudomonas aeruginosa (Tp10) and Pseudomonas putida (Tr6)] were purified. These strains showed optimal growth and pigment production in peptone broth media at 37ºC, pH=7, 2-4% salination of the growth media, shaking and dark conditions. Colorimetric analysis revealed maximum absorbance by the biopigments extracted in diethyl ether and chloroform at 440-450nm. The dyeing potential and coloring stability of these pigments were assessed in fabrics, cotton, tissue paper and soap, which showed satisfactory washing fastness after three washing cycles. Anti-bacterial assay of extracted pigments indicated anti-bacterial agents. This study concluded that microbial pigments can be used for dyeing in textile industry as a natural alternate to the synthetic dyes.

**KEYWORDS:** Antibacterial activity; Bioindicators; Extraction; Pigment; TLC

## INTRODUCTION
Production of synthetic dyes and their continuous use in many industries exacerbate environmental challenges [1]. Specifically, production of synthetic dyes increases air pollution through carbon emissions, while the toxic by-products contaminate soil and water resources [2,3]. Moreover, the increasing use of synthetic dyes in food, textile, feed, cosmetic and pharmaceutical industries significantly contribute to degrade underground aquatic systems, harmful residue accumulation in soil and microorganism toxicity [4,5]. Prevailing ecological concerns made it necessary to develop safe and harmless techniques for dyes production in order to reduce the adverse effects on the human health and environment [6].

An eco-friendly and greener approach is to replace synthetic dyes with bio-colorants through biotechnological production of bacterial pigments [7,8]. In addition to environmental sustainability, these biotechnologically produced dyes offer a number of advantages including ease of production of bio-dyes without seasonal distinctions, low cost, high sustainability, and high production yield [9–11]. Biopigments are biodegradable compounds derived from plants and microorganisms such as bacteria [12–15], fungi [16,17] and virus [11]. Microbial pigments are preferred over their plant counterparts because of fast growth, need of low cost culture medium, flexible control over fermentation process and proof against weather conditions [18–20]. Studies have documented that such naturally produced dyes are effective colorants, thereby provide environmental sustainability [21,22].

Pseudomonadota is a diverse phylum whose microorganisms play an important role in the production of biopigments and other natural products [23]. Within the phylum Pseudomonadota, microorganisms belonging to the genus Pseudomonas stand out because of their morphological and physiological versatility. The Pseudomonas aeruginosa and Pseudomonas putida are gram-negative bacteria endowed with significant potential for industrial biotechnology, while preserving environmental sustainability [24,25]. In particular, P. putida exhibit attractive characteristics (e.g., versatile metabolic activities with diverse enzymes, biosynthesis of pigments, potential to tolerate challenging redox reactions) which enable it as a pivotal specie for industrial applications and operational stresses, among others [26–28]. Likewise, P. aeruginosa synthesizes diverse natural products including the pyocyanin, a blue green phenazine pigment with applications in agriculture, textile and medicine [29–31]. The P. aeruginosa synthesized pyocyanin is also used as a dyeing agent for linen and cotton cloths [32,33].

Due to the increasing demand for naturally-derived pigments [34–36], the current study focuses on the collection, identification, screening and characterization of pigment-producing bacteria from diverse sources (i.e., soil, industrial run-offs, damping sites, compost, spoiled fruits and vegetables, restaurants discard water and hospitals), followed by the

isolation and growth of the selected bacteria and optimizing the biopigment production. Finally, the extracted biopigments were characterized and used as colorant agents for different organic fabrics to assess the dyeing potential and stability.

## MATERIALS AND METHODS

### Chemicals and culture media

All chemicals of reagent grade were purchased from Lab Chem Scientific, Lahore, Pakistan. These include diethyl ether (Et2O), chloroform (Chl), ethanol (EtOH) and methanol (MeOH). Moreover, silica gel with plates 60 for thin layer chromatography (TLC) were also purchased from Lab Chem Scientific, Lahore, Pakistan. The agar plates and culture medium were obtained from National Institute of Health (NIH), Islamabad, Pakistan. The L-broth medium was composed of Tryptone-10g, yeast extract-5g, NaCl-5g, Agar-20g/1L distilled water with a pH-7.

### Sample collection

Soil samples were collected from Punjab University Bridge 4 (31°30'N, 74°16'E), Botanical Garden of Punjab University (31°30'N, 74°18'E), River Ravi Lahore (31°34'N, 74°48'E) and agricultural fields of Kasur (31°10'N, 74°28'E) under sterile conditions. The uppermost layer of soil (up to 15 cm) was discarded so as to eliminate surface contaminants. Subsurface soil (20-50 grams) was procured from each core, placed in sterile vessels, sealed and transferred into zip-lock bags for transportation to the laboratory where the samples were stored at 20 °C for further processing.

### Isolation of bacteria from soil samples

Initially, subconfluent bacterial colonies were grown to promote single-species growth. Subsequently, agar plates were employed to evenly distribute and grow individual colonies. Bacterial colonies demonstrating distinct morphological features were selected for purification using streak plate techniques. Finally, 140 distinct bacterial species were confirmed through biochemical and molecular identification methods.

### Biochemical identification of bacterial Isolates

Bacterial isolates were biochemically characterized using Quick Test Strip 12 (QTS-12) bacterial identification kit (DESTO Laboratories, Karachi, Pakistan) [37]. The identification kit exploited standard biochemical methods and has the potential to record 14 different reactions, including ONPG, citrate, lysine decarboxylase, ornithine decarboxylase, $H_2S$, urease, indole, glucose, $NO_3$ reduction, TDA, oxidase, gelatin, ADH, VP and sucrose. The micro-tubes of QTS-12 containing dehydrated enzymatic substrate were inoculated with pure saline bacterial cultures and results were recorded after 18-24 hours of incubation.

### Screening of pigmented bacteria

Among the 140 screened isolates, a total of 25 isolates were identified to endow pigment production. Specifically, the 25 isolates were cultured in liquid media and pigment production was accessed through observation and spectrophotometric analysis. Next, color stability of the 25 isolates was analyzed, where 23 isolates illustrated little change in color stability after 48 hours of growth in liquid media. Thus, only two pigmented bacteria [Pseudomonas aeruginosa (Tp10) and Pseudomonas putida (Tr6)] were selected for further analysis.

### Morphological analysis of the two pigmented bacterial strains

The two pigmented bacterial isolates were streaked on L-agar plates to get single colonies of each isolate. Diverse features of the bacterial colonies (i.e., colony color, shape, elevation, size and transparency) were observed. Gram-staining was carried out to enable study of the cellular morphology for the bacterial isolates. Motility test was performed using wet mount slide depression method.

### Physiological analysis of the two pigmented bacterial strains

All bacterial isolates were incubated in L-broth medium at 37$^o$C and the bacterial growth was assessed at 24, 48, 72, 96 and 120 hours after incubation. Growth of the isolates was also observed under same incubation conditions but with constant shaking and light of 10000 lux. Moreover, the impact of pH (= 3, 5, 7, 9 and 11), temperature (= 20, 37 and 45 $^o$C) and salination (NaCl concentration = 2, 4, 6 and 8 percent) was also investigated for optimal bacterial growth. Pigment production by the two bacterial isolates was also explored under the abovementioned variables (i.e., light, pH, temperature and salinations).

### Optimization of pigment production

Various growth conditions for pigment production i.e., static, shaking, light and dark and physiological conditions i.e., varying temperatures (20$^o$C, 37$^o$C and 45$^o$C), pHs (3, 5, 7, 9 and 11) and NaCl concentrations (2, 4, 6 and 8%) were selected to observe the bacterial growth and pigment production after different time intervals i.e., 24, 48, 72, 96 and 120 hours of incubation.

**Pigment extraction, characterization and application in dyeing**

After optimization of pigment production from the selected bacterial isolates (P. aeruginosa and P. putida), their pigments were extracted using organic solvents including Et2O, Chl, EtOH and MtOH. Briefly, bacterial isolates were inoculated in flasks containing peptone broth (100ml) and incubated until the prominent production of biocolors (at one week). Pigmented broth cultures were centrifuged (9000 rpm for 20 min) and the supernatants were mixed with organic solvents (in ratio of 1:1) for pigment extraction. The mixtures were left until the complete evaporation of solvents.

The extracted pigments were examined calorimetrically through UV/Vis spectrophotometer [Shimadzu UV-1601PC UV-Visible, Scanning Spectrophotometer] in the wavelength range of 350-750 nm to determine the absorption spectra. Moreover, qualitative analysis was performed with the use of thin layer chromatography exploiting petroleum ether and acetone (v/v 2:1) as mobile solvents. Five distinct bacterial strains across different genera were employed to evaluate the antibacterial activities of the P. aeruginosa and P. putida. Specifically, antibacterial assessment of the extracted bacterial isolates were conducted using the disc diffusion methodology, against Clostridium butyricum, Bacillus safenesis, Bacillus pumilus, Pseudomonas sp. and Bacillus cereus. The resulting inhibition zones were carefully observed, and the precise diameter of each zone (expressed in mm) was recorded.

**Extracted pigments as dyeing agents**

To assess the stability and dyeing capability, the extracted pigments were dissolved in water and applied to small pieces (5 x 5 $cm^2$) of fabrics (cotton, nylon, silk and lawn), tissue paper and soap [38]. Dyeing potential and stability was evaluated by exposing the dyed specimen to high temperature, using mixtures of distilled water (20 ml containing 3% agar) and various concentrations of the extracted pigments (100, 200, 300, 400 and 500 µg/ml); the color degradation was assessed at boiling temperature [39]. Likewise, the dyeing stability of the extracted pigments were also assessed against washing and rubbing.

**RESULTS**

**Characterization of the pigmented bacterial strains**

Out of the total 140 bacterial colonies, two showed brown (Tp10) and dark green (Tr6) appearances and were selected for detailed morphological and biochemical studies. Morphological characterization through the gram staining technique revealed that the two pigmented bacterial isolates of P. aeruginosa (Tp10) and P. putida (Tr6) were orange-brown and dark green in color, respectively (Figure 1). Moreover, both these strains exhibited bacillus (rod-like) shapes and were gram negative. Biochemical studies of the two pigment producing bacterial isolates were tested positive for O-Nitrophenyl-beta-D-galactopyranoside (ONPG), sodium citrate, cytochrome oxidase and nitrate reduction, while negative for lysine decarboxylase, ornithine decarboxylase, $H_2S$ production, and Indole. Complete results of biochemical enzymatic activities are presented in Table 1. Overall, biochemical assessment of the isolated strains- using QTS-12 bacterial identification kit- revealed that the Tp10 was Pseudomonas aeruginosa while the Tr6 was Pseudomonas putida.

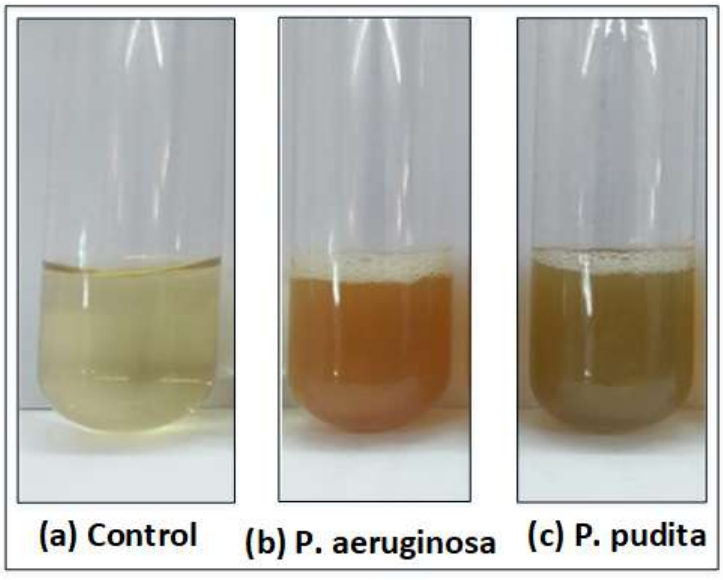


**Fig 1 Pigment production by bacterial isolates compared to control. (a) Control, (b) P. aeruginosa (Tp10) showing brown color, (c) P. putida (Tr 6) showing dark green color**

**Table: Details for Biochemical Characterization of Bacterial Isolates**

| Reaction | Tp10 | Tr6 |
|---|---|---|
| ONPG (O-Nitrophenyl-beta-D-galactopyranoside) | + | + |
| Sodium Citrate | + | + |

| | | |
|---|---|---|
| Gelatin Hydrolysis | + | - |
| Lysine Decarboxylase | - | - |
| Arginine Dehydratase | - | + |
| Ornithine Decarboxylase | - | - |
| $H_2S$ Production | - | - |
| Urea Hydrolysis | - | + |
| Indole | - | - |
| Acetoin | + | - |
| Acid from Glucose | - | + |
| Nitrate Reduction | + | + |
| Acid from Sucrose | - | + |
| Cytochrome Oxidase | + | + |

+ : positive, - : negative

**Optimization of pigment production from the bacterial strains**

Bacterial growth was investigated at 37ºC for 120 hours (Figure 2). Isolate P. aeruginosa (Tp10) exhibited log (i.e., exponential) growth after 96 hours of incubation, while P. putida (Tr 6) exhibited log growth at 72 hours of incubation. For pigment content, these isolates were incubated for 120 hours. Under static conditions, isolate P. aerginosa exhibited maximum pigment production after 72 hours, while isolate P. putida showed pigmentation after 96 hours of incubation. Similarly, in shaken broth cultures, isolate P. aeruginosa exhibited maximum pigment production after 96 hours of incubation, whereas P. putida showed moderate pigment even after 120 hours of incubation. Overall, pigment production was more pronounced in shaken broth cultures as compared to static ones. Similarly, in the presence and absence of light, both isolates exhibited moderate pigment content after 96 hours and maximum pigment production 120 hours, respectively (Figure 3).

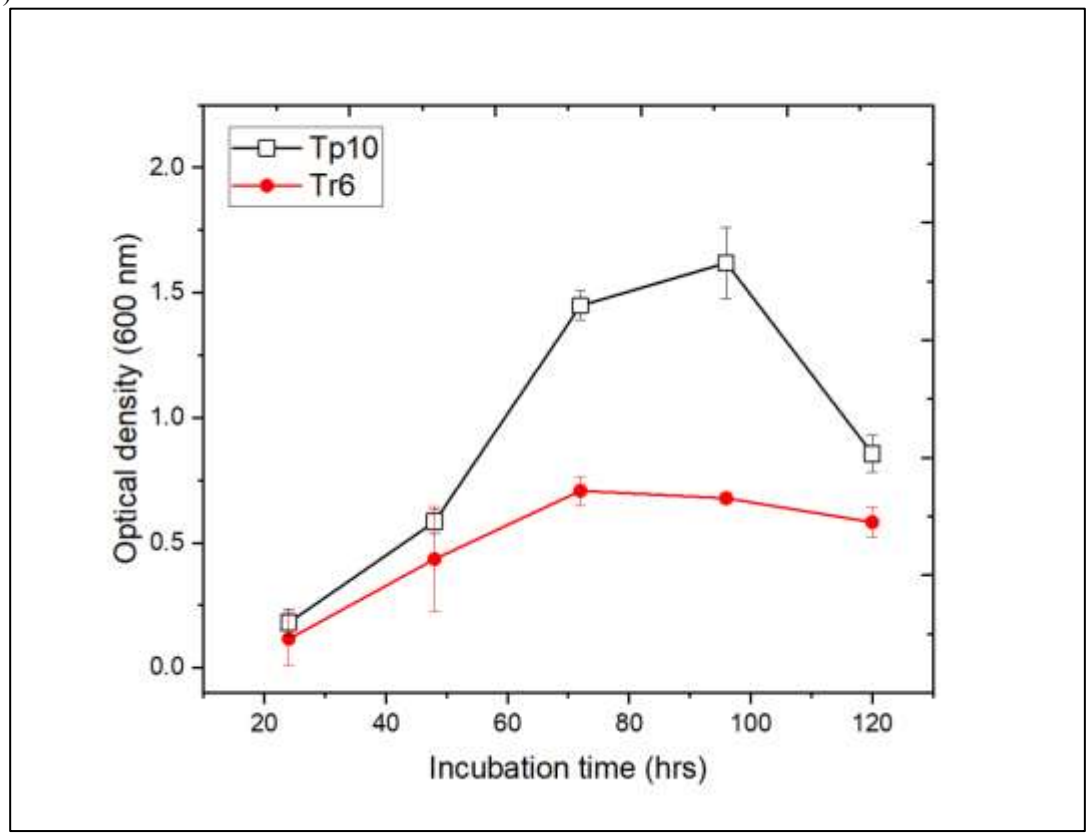


**Fig 2 Growth of P. aeruginosa (Tp10) and P. putida (Tr 6) at 37ºC for 120 hours**

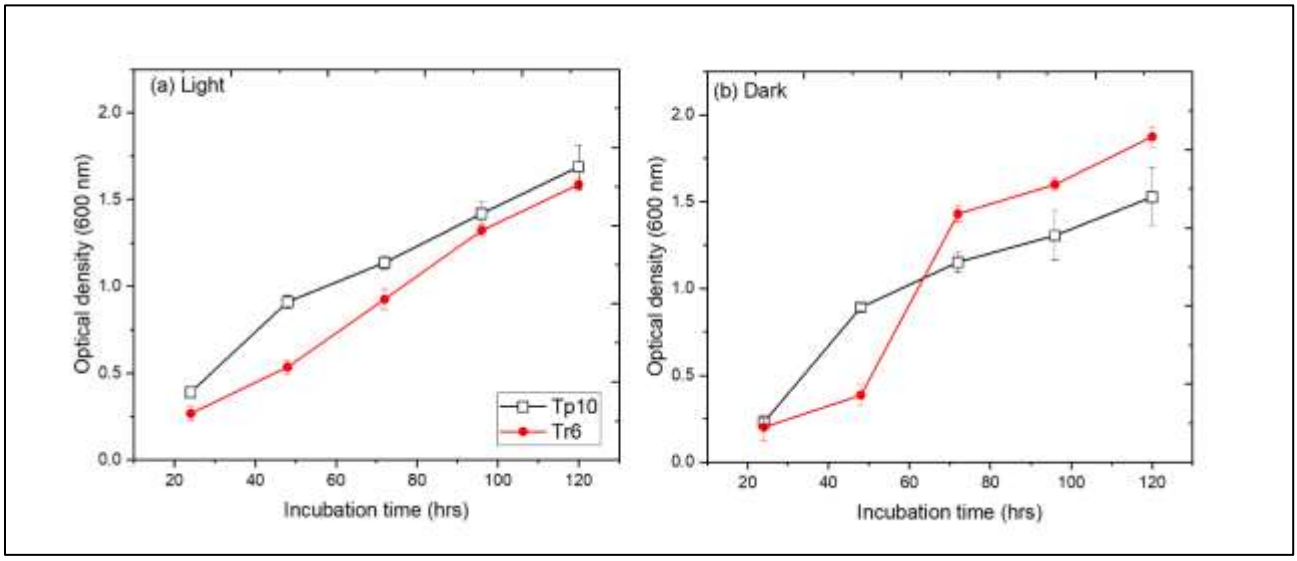


**Fig 3 Growth of P. aeruginosa (Tp10) and P. putida (Tr 6) at 37ºC for 120 hours under (a) light and (b) dark conditions**

The impact of different physiological conditions on bacterial growth and pigment production was also evaluated. The results showed highest bacterial growth (after 24 hours of incubation) at 37ºC followed by growth at 20ºC whilst no growth at 45ºC (Figure 4). It was observed that incubation temperature also have a profound effect on the pigment production. Specifically, at 20ºC, P. aeruginosa exhibited slight and moderate pigment content after 72 and 120 hours of incubation, respectively whilst P. putida exhibited slight pigment content after 120 hours of incubation. Moreover, at 37ºC, maximum pigment content was observed after 120 hours of incubation for both P. aeruginosa and P. putida. No pigment production was observed at 45ºC for both isolates. These results indicate that the optimal temperature for growth and pigment production for both bacterial isolates is 37ºC.

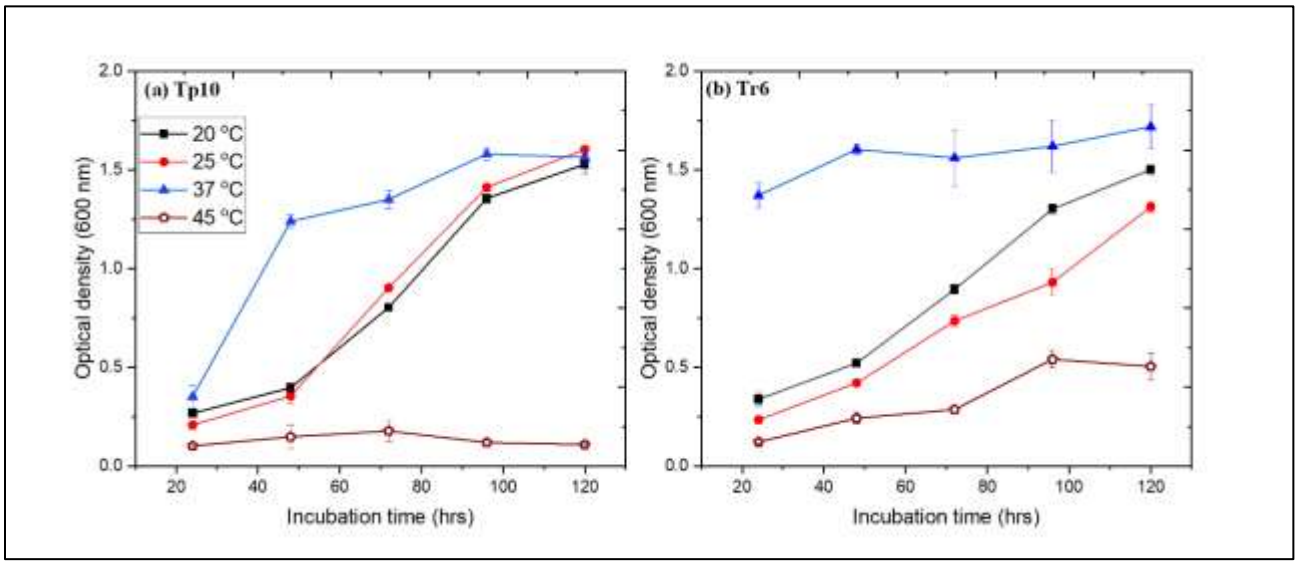


**Fig 4 Growth of (a) P. aeruginosa (Tp10) and (b) P. putida (Tr 6) at different temperatures**

The effect of pH on the growth and pigment producing potential of the isolates was also explored. In acidic conditions (i.e., pH =3 and 5), both bacterial strains showed a minor growth. However, both isolates showed exponential growth at pH = 7 and 9, whilst moderate growth at pH =11 (Figure 5). Likewise, no pigment production was observed in acidic conditions (i.e., pH = 3 and 5), whereas maximum pigment production was seen at pH = 7 for both isolates. At pH =9 and 11, both bacterial isolates showed moderate pigment production after 96 and 120 hours of incubation, respectively. Overall, these results indicate that the bacterial isolates were neutrophilse, illustrating optimal growth and pigment production at pH = 7.

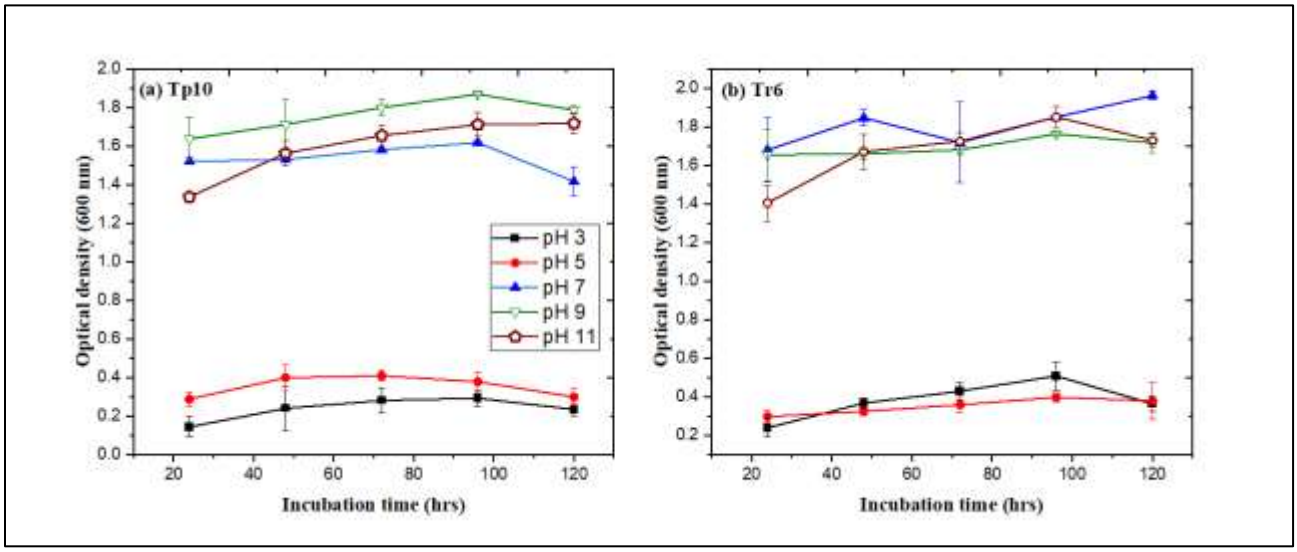


**Fig 5 Growth of (a) P. aeruginosa (Tp10) and (b) P. putida (Tr 6) at different pH**

The effect of salination in growth medium on the bacterial growth and pigment production was also investigated. In particular, maximum bacterial growth was observed for low salt (i.e., NaCl) concentrations in the growth media (i.e., 2 and 4%), as compared to high salt concentrations (Figure 6 and 7). A similar pattern was observed for pigment production. In particular, P. aeruginosa and P. putida yielded peak pigment production in 2% and 4% salinated medium after 96 and 120 hours, respectively. The pigment production for both bacterial isolates was minimal at higher salt concentration (6 and 8%) in the culture medium. In summary, the results revealed that low salination (i.e., 2 and 4% concentrations of NaCl) are more suitable for bacterial growth and production of higher pigment.

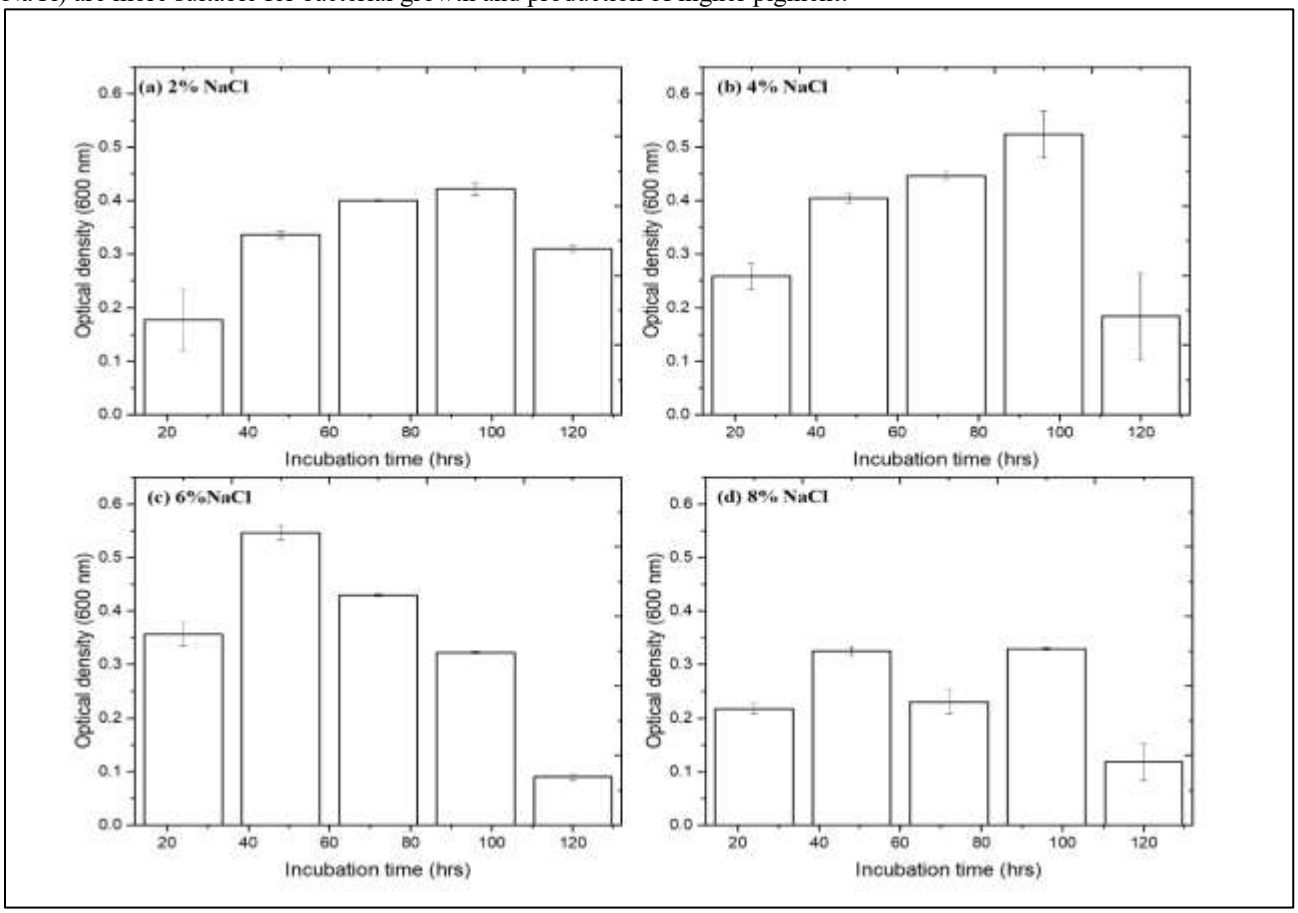


**Fig 6 Growth of P. aeruginosa (Tp10) at different salination levels. (a) 2% NaCl, (b) 4% NaCl, (c) 6% NaCl and (d) 8% NaCl**

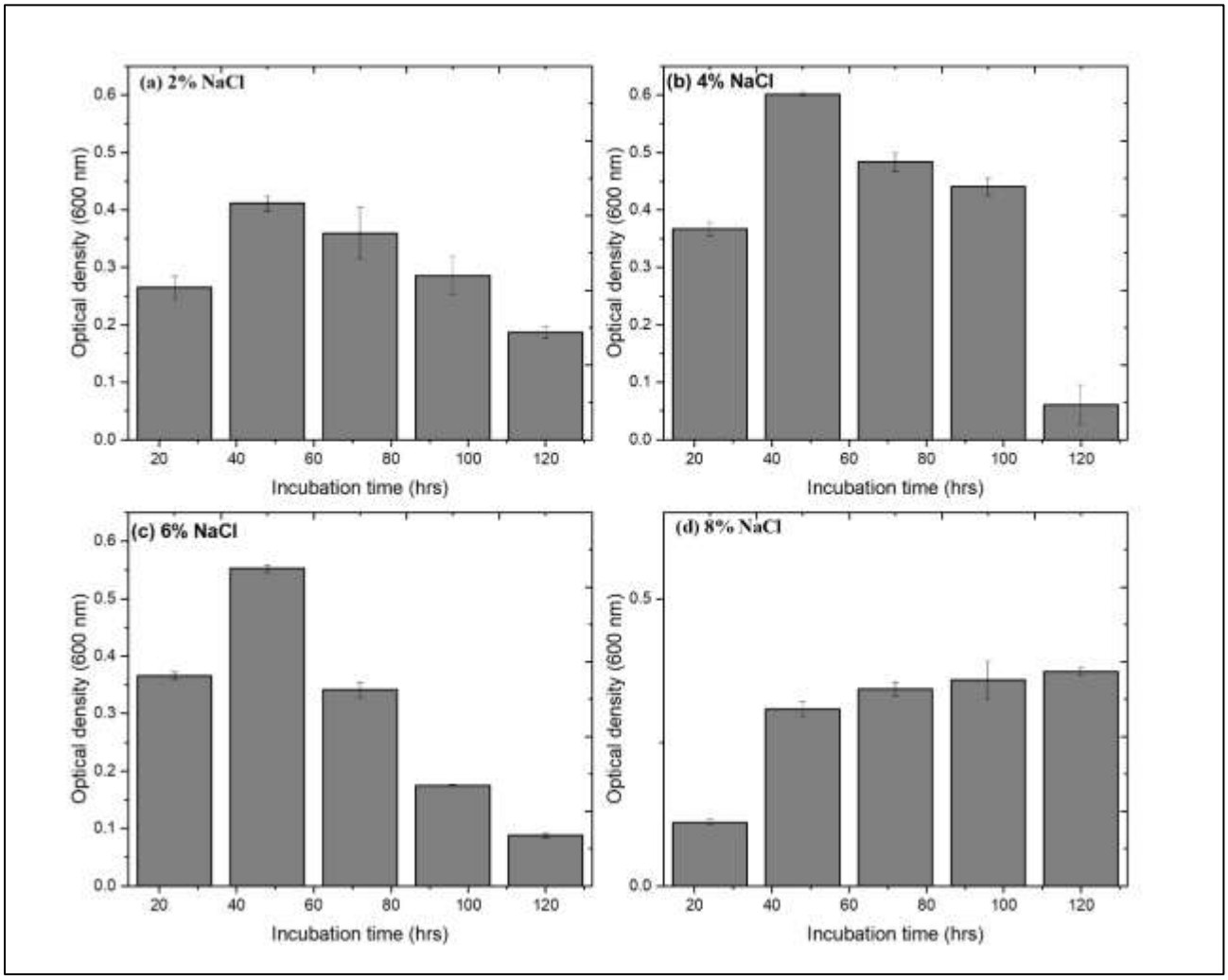


**Fig 7 Growth of P. putida (Tr6) at different salination levels. (a) 2% NaCl, (b) 4% NaCl, (c) 6% NaCl and (d) 8% NaCl**

**Characterization of extracted pigments**

UV-Vis spectrophotometry of the extracted pigments revealed that maximum pigment was produced using Et2O and Chl as extracting solvents. Both isolates exhibited moderate pigment content when extracted in EtOH and MtOH. Isolate P. aeruginosa showed a peak absorption at 450 nm for all extracted solvents except Chl (peak absorption ~ 350nm). Similarly, the P. putida showed peak absorption at 440-450 nm for all extraction solvents except in Et2O (absorption peak ~ 550nm).

Thin layer chromatographic analysis demonstrated the presence of pigmented compounds. Rf values for P. aeruginosa and P. putida were 0.14 and 0.10, respectively. To demonstrate antimicrobial activity of the isolates, antibacterial assay was performed against five bacterial strains. Isolate P. aeruginosa exhibited antibacterial activity against Pseudomonas sp. and Bacillus cerecus having inhibition zone of 0.3, and 0.9 mm, respectively. Isolate P. putida exhibited anti-bacterial activity against Clostridium butyricum, Bacillus safenesis and Pseudomonas sp. showing diameter of inhibition zone as 0.3, 0.7 and 0.5 mm, respectively.

**Dyeing efficiency of the biopigments**

The extracted pigments were used as dyeing agents and applied on fabrics, cotton, tissue paper and soap to examine their dyeing potential and reliability. The pigments extracted in Chl and Et2O demonstrated higher dyeing potential and stability compared to extracts in EtOH and MtOH. Stability of extracted pigments was assessed by exposing them to high temperature, washing and rubbing after dyeing. The results demonstrated that pigment from P. putida have maximum dyeing potential and stability for cotton, tissue paper and fabrics (lawn, silk and wool), as no color loss was noted after washing and rubbing after three cycles of washing. Pigment extracted from P. aeruginosa showed slight degrading after washing (Figure 8).

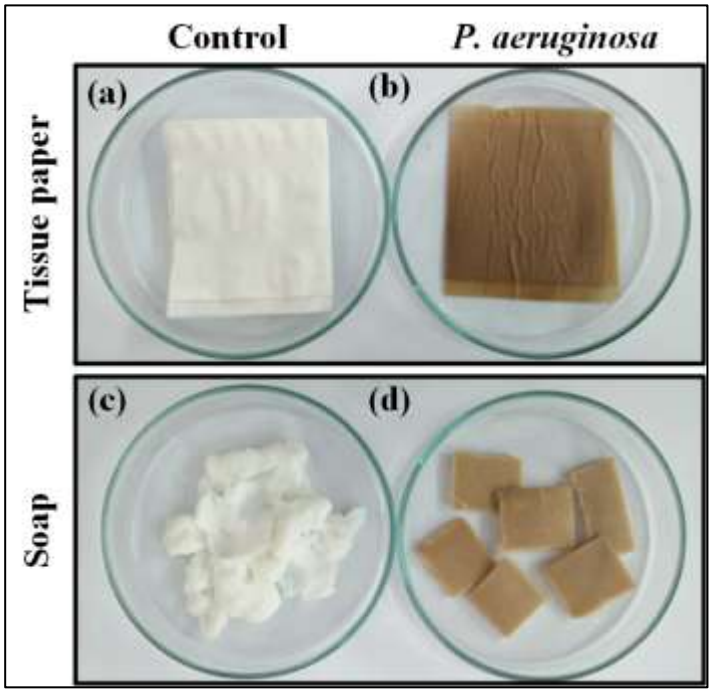


**Fig 8 Dyeing potential of P. aeruginosa (Tp10) using (a, b) tissue paper and (c, d) soap**

**DISCUSSION**

The chromogenicity and associated characteristics of P. aeruginosa and P. putida have remained relatively unexplored. Consequently, the objective of this study was to isolate these bacterial strains, investigate their pigment-producing capabilities and evaluate the practical utility of these pigments in the context of textile dyeing processes.

Two pigmented bacteria [Pseudomonas aeruginosa (Tp10) and Pseudomonas putida (Tr6)] were identified, isolated and analyzed for the ability to produce pigments. For growth and pigment production of the two bacterial strains, the optimized culture and media variables were explored, including peptone broth media at 37ºC, pH=7, 2-4% concentration of NaCl in the growth media, shaking and dark conditions. Overall, the two bacterial isolates showed the four distinct growth phases, including lag, log (i.e., exponential), stationary and decline (i.e., death) phases.

Incubation temperature have a profound effect on the growth of microorganisms and in determining microbial strains [40]. Generally, bacteria are primarily mesophilic where the optimal growth temperature belongs to 20-45 ºC [41]. We therefore explored this temperature range and found that the optimal temperature for the growth of P. (Tp10) and P. putida (Tr6) was 37 ºC, which may be correlated to the prevailing soil temperatures of the urban regions from where the samples were collected. For example, the average surface soil temperatures in Lahore, Pakistan vary from 35 to 37 ºC. Although P. aeruginosa can survive in the temperature range of 4-42°C [42], its rigorous growth is reported at the ambient temperature of 37°C [43]. This may be governed by the optimal metabolic activities of this stain at 37°C. Moreover, the temperature may also affect the cellular enzyme activity of the bacteria [44,45].

Being a critical environmental stress that could affect the bacterial growth and pigment production, the two bacterial strains were exposed to changes in the pH. The results showed that the best incubation pH for the growth of the pigment producing bacterial isolates was pH = 7 in term of maximum bacterial growth and intense pigment production (Figure 4). This pattern is consistent with most studies reporting bacteria as neutrophils and exhibiting best growth near-neutral pH. [44,46,47]. Other studies have described the effect of pH of the media on the growth and metabolites production for various bacterial stains, including Pseudomonas putida, Escherichia coli, and Pseudomonas pseudoalcaligenes [48,49].

Exploring the impact of growth medium salinity showed a decreased growth of the P. putida and P. aeruginosa isolates at hypersaline concentrations. Growth medium salinity have a profound influence on the osmotic potential, which, in turn, influences growth of the bacteria [50,51]. These results are in agreement with previous studies, which document reduced bacterial growth at high salt stress [52].

We also investigated pigment production by the two bacterial strains and its dying potential and stability. Production of bacterial pigments, being secondary metabolites, need longer times as the process occurs after the production of primary metabolites [53]. Specifically, two types of metabolites are synthesized during bacterial fermentation. First, the primary metabolites (e.g., amino acids, vitamins, polysaccharides, nucleotides, etc.) are synthesized during the log phase of bacterial growth. Second, the secondary metabolites (e.g., pigments, antibiotics, toxins, etc.) are usually derived from the primary metabolites during the late log phases of the bacterial growth [54,55]. In this study, maximum pigment production was observed after 72 hours of incubation for P. aerginosa and after 96 hours of incubation for P. putida.

After characterization of extracted pigments, these pigments were applied on various textile materials to evaluate their dyeing potential and coloring stability. Profound stability was revealed by pigments extracted in Chl and Et2O, as assessed

by subjecting the dyed material to rubbing and washing in the presence of sunlight. Prolongation of dyeing time enabled intense color. The peak absorbance of the biopigments at 440-450 nm might be indicative for presence of carotenoid in the isolates. These pigments may be used as natural coloring agent in textile industry, as an alternative source to that of synthetic pigments. Previously, studies have explored diverse pigment producing bacterial strains as a surrogate sources for synthetic dyes [56–58]. For example, the red pigment (called prodigiosin) from Vibrio spp. has shown potential applications as textiles colorant [59]. The promising results for using pigment from Serratia marcescens to color different fabrics has also been reported [18]. Nevertheless, the dyeing efficiency has been reported to vary with the type of fabrics. The dyeing efficiency of prodigiosin in different fabrics, including pure cotton, pure rayon, pure silk, cotton, polyester and silk satin demonstrated intense colorations for only pure rayon and silk satin [60].

## CONCLUSIONS

The two isolated bacterial strains Pseudomonas aeruginosa (Tp10) and Pseudomonas putida (Tr6) exhibited interesting chromogenitic properties and biopigment production. The results from this study illustrate that the two bacterial strains can act as a potential source for greener production of biopigments. Highest biopigment yields from the two bacterial isolates were obtained at 37ºC, pH 7, 2-4% NaCl concentrations and absence of light. These biopigments hold the potential for utilization within the textile industry as natural colorants, thereby mitigating the environmental burden associated with the prevailing use of synthetic dyes.

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