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Assessment of Pyocyanin and Pyorubin Pigments as Coloring Agents on Unmordanted Denim Fabric

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Abstract
The second most polluting industry in the world is considered to be the textile industry. The non-biodegradable synthetic dyes and toxic mordants wastes play a major part in this pollution. The textile dyeing practices causes almost 20% of global water pollution. These controversies with the current environmental pollutions, lead to high demand for natural colors in food, pharmaceuticals, cosmetics, textiles and in the printing dye industry. Recently, microbial pigments have been shown to be a propitious alternative not only to synthetic dyes, but also to other natural pigments derived from plants and animals as they are viewed as natural, non-toxic, have no seasonal production issues, offer excellent productivity, economical and most important they are eco-friendly. Pseudomonas aeruginosa PI21 strain isolated from lake water sample and screened based on yield of pigment was selected for current investigated based on earlier published manuscript. The pigment was extracted from the selected isolate and used for dyeing unmordanted denim fabric. Furthermore, stability of dyes following treatment with acid, alkaline, hot and cold water with liquid detergent was studied to investigate the retention of the dyes. Pyocyanin pigment in unmordanted denim fabric retained in cases of acid, alkaline and cold water with detergent treatments, while a small amount of discoloration was observed when subjected to hot water with detergent treatment. Whereas, the pyorubin pigment in unmordanted denim fabric retained in case of acid treatment, while great amount of discoloration was observed when subjected to alkali, hot and cold water with detergent treatments. The current study demonstrated that coloring ability of the natural dyes can be compared to that of the synthetic dyes. Test samples of PYC and PYR were treated with different concentrations on Human Dermal Fibroblasts (HDF) for 48 hours. The MTT assay resulted in cytotoxicity up to 59.36% and 64.48% at 320µg/mL was observed in HDF cells with PYC and PYR respectively. IC_{50} value of 207.6µg/mL for PYC and 255.2µg/mL for PYR were obtained.

Keywords: *Pseudomonas aeruginosa PI21,phenazines, pyocyanin (PYC), pyorubin (RYR), dyeing, textiles.*

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I. INTRODUCTION:

Microbial pigments are of great structural diversity. They may be derivatives of the material classes of carotenoids, phenazine dyes, pyrrole dyes, azaquinones etc. Moreover, the yield of pigment may yet be improved by optimizing the parameters at the various method steps such as dwell time, solvent ratios etc., in particular during cultivation of the bacteria (nutrients, shaking frequency, oxygen charge, pH value, salt content, temperature etc.). In general, large scale technical production can be achieved in relatively short time with results of great economic advantages [1].

The use of bacteria to permanently dye or stain cloth is not new. Fermentation processes use bacteria and enzymes to digest, transform and synthesize natural materials from one form to another which is essential in few cases of natural dyeing. These processes have been dated back to over 5,000 BC. With the advent of synthetic dyes and industrial manufacturing, many of these processes became obsolete [2].

The natural colors have proved their versatility by way of application in foods, pharmaceuticals, cosmetics and in textiles. The concept of total eco-friendly apparels can be thus introduced giving technological inputs to the textile small scale processors in rural areas which will have potential of providing employment to the rural youths strengthening their economy [3].

Natural and eco-friendly organic dyes are very useful as coloring agents. The natural dyes are much better in quality so that these dyes can to be used for food, medicines, and cosmetics, hence there is now high demand for natural organic dyes from national and international markets. One can face many problems in

dyeing with natural colors but there is no guarantee of consistency of shade and also can vary in depth in tone and hues. Currently, the application part of these natural colorantson different fabrics are required to be carried out on the lines of ease of application, shorter dyeing cycles, repetitive color yield, and pattern of synthetic dyes application, which may lead to commercialization of natural colorants. From the economic perspective, bacterial dye has best potential as commercial sources of dyes, since bacteria has high growth rate [4].

*Pseudomonas spp. a*re well known for producing several pigments such as pyocyanin, pyorubin and pyoverdines [6], [7], [8]. All three pigments have various applicational properties such as antimicrobial, biocontrol [9], textile dyes [10]and siderophore activity [11]. Pyocyanin is a blue colored water-soluble pigment, synthesized by 90–95% *Pseudomonas aeruginosa* under the control of quorum sensing coordination [12].

Most of the pigments produced by microorganisms are toxic to animals, plants, micro-organisms and humans. The cytotoxicity of the bio-pigments can be measured by MTT assay. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material [12], [13, [14], [15], [16],[17], [18].

II.MATERIALS AND METHODS

2. 1 Bacterial isolate

Pseudomonas aeruginosa PI21 strain isolated from lake water sample and screened based on yield of pigment was selected for current investigated based on earlier published manuscript [19].

2.2 Pigment extraction

The strain PI21 was used to produce phenazines (pyocyanin and pyorubin). This strain was streaked on nutrient agar plates and incubated at room temperature (25°C) for 24 hours. A single colony was picked up, inoculated into 500 mL conical flask containing 100 mL nutrient broth and incubated at 25°C, 150 rpm for 48 hours. The bacterial cells were removed from the growth medium by centrifugation at 7000 rpm for 10 minutes. The supernatant was further used to extract the pigments. The cell-free supernatant was extracted twice with equal volume of chloroform in a separating funnel. The pyocyanin pigment in chloroform layer was separated and re-extracted using 0.1 N HCl. The acidic fraction was separated, neutralized using 0.1 N NaOH, and again re-extracted using chloroform, whereas pyorubin was extracted from the aqueous fraction. The chloroform and aqueous fractions of containing PYC and PYR pigments were air dried at room temperature and used as a dye. The pigments were dried and dissolved in methanol to get160 µg/mL concentration for further studies [20].

2.3 Fabric dyeing process

Dyeing with bacterial pigments was conducted on unmordanted denim fabric. White pieces (1 cm^2) of fabric were placed in sterile petri dishes. Methanol pigment extracts of pyocyanin and pyorubin (2 mL) were spotted on the fabrics separately and subjected to incubation for 48 hrs at room temperature. Each piece of fabric was dried and then divided into four smaller sections. These sections of dyed fabrics were then treated with acid, alkali, hot and cold water with liquid detergent for 1 hr.Acid solution of pH 5 was prepared by using 0.1M HCl and an alkaline solution with pH 8 was adjusted with 0.1M NaOH. Soap solution was prepared by adding liquid soap to distilled water (1:1) [21].

2.4 MTT assay

2.4.1 Cell lines and culture medium

Human Dermal fibroblast cell lines were cultured in fibroblast growth medium (FGM) supplemented with growth factors, penicillin (100IU/mL), streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37^oC until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02 % EDTA, 0.05% glucose in PBS). The viability of the cells are checked and centrifuged. Further, 30,000cells/well was seeded in a 96 well plate and incubated for 24 hrs at 37^oC, 5 % CO_2 incubator [12], [13], [14], [15], [16], [17], [18].

2.4.2 Procedure

The monolayer cell culture (Human Dermal fibroblast) was trypsinized and the cell count was adjusted to 3 x 10^5 cells/mL using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µL of the diluted cell suspension (30,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was removed, washed the monolayer once with fibroblast growth medium and 100 µL of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37^{o}C for 48hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100µL of MTT (5mg/10mL of MTT in PBS) was added to each well. The plates were incubated for 4h at 37^{o}C in 5% CO_2 atmosphere. The supernatant was removed and 100µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590nm. The percentage growth proliferation was calculated using the following formula and concentration of test drug needed to proliferate / inhibit cell growth by 50% values is generated from the dose-response curves for each cell line [12], [13], [14], [15], [16], [17], [18].

2.4.3 Calculating Inhibition [12], [13], [14], [15], [16], [17], [18]

% Inhibition = [(OD of sample - OD of Control) / OD of Control] x 100

III. RESULTS

3.1 Effectiveness of dyeing agent

The isolate *Pseudomonas aeruginosa* PI21 producing pyocyanin and pyorubin was chosen for pigments extraction and used for dyeing unmordanted denim fabric at different conditions. The stability of dyes following treatment with acid, alkaline, hot and cold water with liquid detergent treatments was studied to investigate the retention of the dyes. Pyocyanin pigment in unmordanted denim fabric retained in cases of acid, alkaline and cold water with detergent treatments, while a small amount of discoloration was observed when subjected to hot water with detergent treatment. Whereas, the pyorubin pigment in unmordanted denim fabric retained in case of acid treatment, while great amount of discoloration was observed when subjected to alkali, hot and cold water with detergent treatments as shown in Table 1.

Table 1: Stability of pyocyanin and pyorubin dyes following treatment with concentrated alkaline, acid, and detergent solution. (+) retained, (-) destained dye.

Textile for dyeing	**Denim**			
Isolates	**Acid**	**Alkaline**	**Hot water with Detergent**	**Cold water with Detergent**
PYC	+	+	-	+
PYR	+	-	-	-

Therefore, pyocyanin pigment on denim fabric resulted positive to withhold harsh washing treatments such as in cases of acid, alkaline and cold water with detergent and showed negative results to hot water with detergent treatment. However, the pyocyanin is a color changing pigment by nature, resulting in changing form greenish blue to reddish brown color after the acid treatment. Whereas, pyorubin pigment on denim fabric resulted positive to withhold harsh acid wash treatment, and showed negative results to alkaline, hot and cold water with detergent treatments shown in Fig. 2(A), 2(B) and 3(A), 3(B).

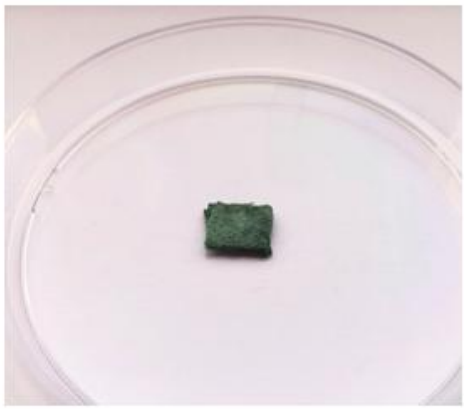

Figure 2(A): Denim fabric dyed with Pyocynin before washing treatments as control

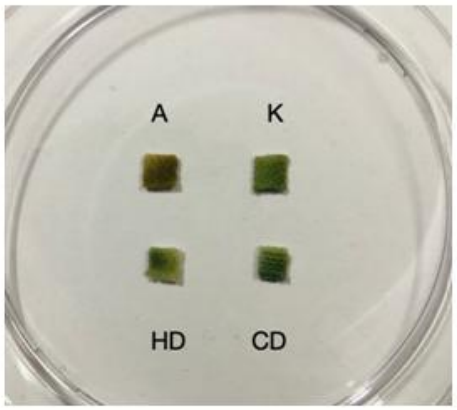

Figure 2(B): Susceptibility of dyed fabrics following treatment with acid A, alkaline K, hot water plus detergent HD and cold water plus detergents CD

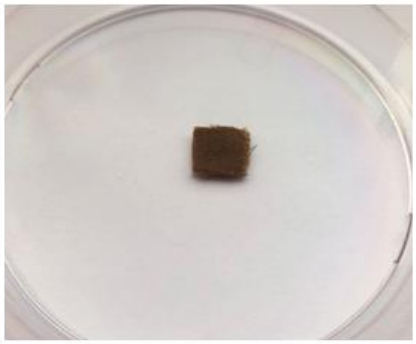

Figure 3(A): Denim fabric dyed with Pyorubin before washing treatments as control

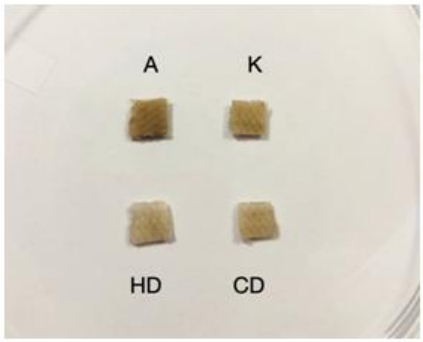

Figure 3(B): Susceptibility of dyed fabrics following treatment with acid A, alkaline K, hot water plus detergent HD and cold water plus detergents CD

3.2 MTT assay

Human Dermal Fibroblasts (HDF) were subjected to treatment with different concentrations of the pigments PYC and PYR for 48 hours. The HDF cells showed different cell morphology as compared to the control (Fig. 4(A)) after treatment with both pigments at various concentrations (10, 20, 40, 80, 160, 320 µg/mL).

The Human Dermal Fibroblast cells after treatment with PYC and PYR at 10 µg/mL concentration, were shiny, elongated and equally distributed. Whereas, most of the cells were dull, circular and unevenly distributed after treatment with PYC and PYR at 320 µg/mL concentration, respectively as shown in Fig. 4(B), 4(C), 4(D), 4(E).

The percentage of inhibition of HDF cells treated with PYC were observed as 6.40, 14.56, 21.76, 26.08, 45.60, 59.36% at different concentrations of PYC (10, 20, 40, 80, 160, 320 µg/mL). Whereas, the percentage of inhibition of HDF cells treated with PYC were observed as 4.16, 10.08, 22.08, 30.08, 39.52, 64.48% at different concentrations of PYR (0, 10, 20, 40, 80, 160, 320 µg/mL). IC_{50} value of 207.6 µg/mL for PYC and 255.2 µg/mL for PYR were obtained, as shown in Table 2.

Table 2: IC_{50} values of pyocyanin and pyorubin pigments

Human Dermal Fibroblast				
Compound Name	**Conc. µg/mL**	**OD @ 590nm**	**% Inhibition**	**IC_{50} µg/mL**
Control	0	0.625	0.00	
PYC	10	0.585	6.40	**207.6**
	20	0.534	14.56	
	40	0.489	21.76	
	80	0.462	26.08	
	160	0.340	45.60	
	320	0.254	59.36	
PYR	10	0.599	4.16	**255.2**
	20	0.562	10.08	
	40	0.487	22.08	
	80	0.437	30.08	
	160	0.378	39.52	
	320	0.222	64.48	

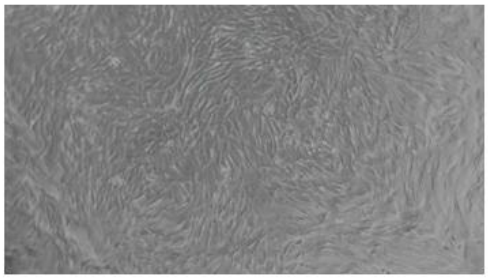

Figure 4(A): Human Dermal Fibroblast cells (Control)

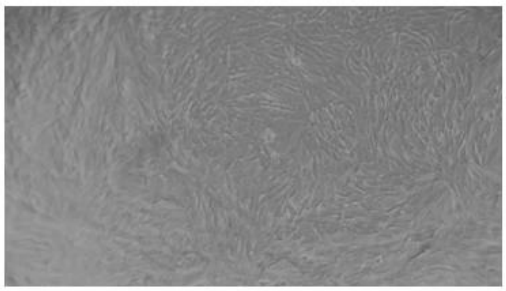

Figure 4(B):Human Dermal Fibroblast cells after treatment with PYC (10 µg/mL)

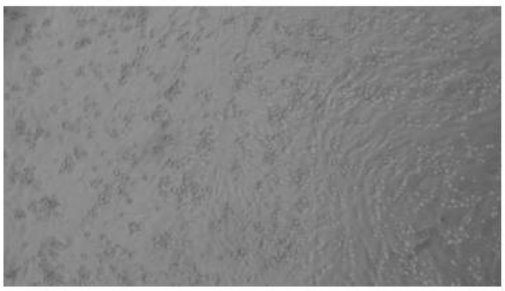

Figure 4(C): Human Dermal Fibroblast cells after treatment with PYC (320 µg/mL)

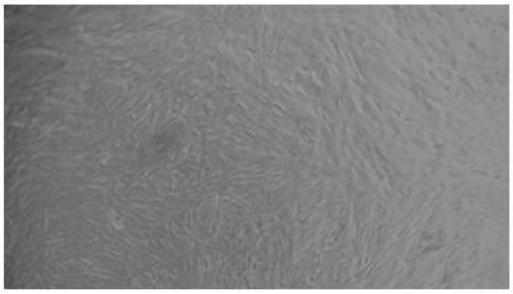

Figure 4(D):Human Dermal Fibroblast cells after treatment with PYR (10 µg/mL)

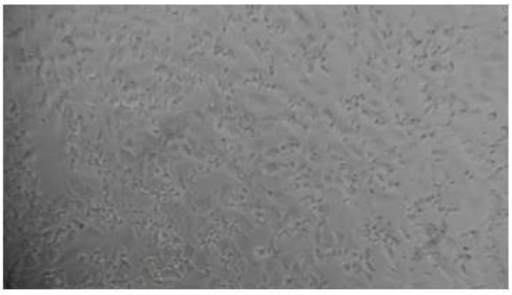

Figure 4(E): Human Dermal Fibroblast cells after treatment with PYR (320 µg/mL)

IV. DISCUSSION

According Agha et al. [22] the bacterial strain *Serratia marcescens* was considered as the promising strains for pigment production in the current, polyester and woolen fibers were vibrantly stained after dyeing with red Serratia pigment. It was reported that some fabrics such as nylon, wool, and polyester retained the dye, after the treatment with acid, alkali, cold water and detergent for 1 hr. Whereas, the others were destained. It was also found that the bacterial red dye was found to be stable, when the fabrics such as nylon, wool and polyester were treated with acid, alkali, and detergent and hot water and detergent for 1 hour.

Mishra [3] reported, the isolates of *Pseudomonas fluorescens* can be used to dye natural fibers i.e., silk, wool and cotton in cottage industries. But for reproducing similar shades and for easy transportation and storage of the dye materials, industrial application extraction and purification is required. Also reported, the use of mordants can improve the color fastness to light, washing, crocking and perspiration of the dyes. These bacterial dyes can tolerate the ultraviolet radiation to certain extent and control the growth of microbes. Hence can be used as alternative for the harmful synthetic dyes.

Pseudomonas spp. can be used to extract dyes using relatively inexpensive methods and can easily be produced in laboratory with certain specific equipment and conditions in a very short time [9].

V. CONCLUSION

Pseudomonas aeruginosa PI21 isolate was chosen for pigment extraction and used for dyeing unmordanted denim fabric. The stability of dyes following treatment with acid, alkaline, hot and cold water with liquid detergent was studied to investigate the retention of the dyes. Pyocyanin pigment in unmordanted denim fabric retained 100% in cases of acid, alkaline and cold water with detergent treatments, while a small amount of discoloration was observed when subjected to hot water with detergent treatments. Whereas, the pyorubin pigment in unmordanted denim fabric retained in case of acid treatment, while great amount of discoloration was observed when subjected to alkali, hot and cold water with detergent treatments. The current study demonstrated that coloring ability of the natural dyes can be compared to that of the synthetic dyes. However, studies to improve the fastness properties performance should be carried out. Test Samples PYC and PYR were treated with different concentrations in Human Dermal Fibroblasts for 48 hours. The MTT assay resulted in cytotoxicity up to 59.36% and 64.48% at 320 µg/mL was observed in HDF cells with PYC and PYR respectively. IC_{50} value of 207.6 µg/mL for PYC and 255.2 µg/mL for PYR were obtained.

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