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Isolation of *Pseudomonas aeruginosa* for bacterial pigment production and its application on synthetic knitted fabric

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This study concentrates on the isolation of bio colour producing bacteria from goat droppings and application of its colour pigment in the textile dyeing process. The isolated strain is subjected to biochemical as well as molecular characterization and is identified as *Pseudomonas aeruginosa* using thin layer chromatography. An optimum condition for production of pigment 'Pyocyanin (blue-green)' has been studied by one-factor at a time approach. The pigment (Pyocyanin) is extracted using chloroform, and 0.2N hydrochloric acid. The application of this extracted bio color as coloring agent on polyester fabric has also been studied, and it is found that the color retained on the fabric is yellow, which is different from the extracted pigment colour. Color quality evaluation of dyed polyester fabric has been performed with references to international standard protocols. The color producing is found to be 1-hydroxyphenazine, obtained by the hydrolysis of Pyocyanin at high temperature (130^{o}C). This 1-hydroxyphenazine imparts the yellow shade to polyester fabric on dyeing.

Keywords: Bio colorant, Bacterial pigment, Dyeing, *Pseudomonas aeruginosa*, Pyocyanin pigment, Polyester fabric

1 Introduction

Synthetic dyes became popular because of their lasting color pay-off and wide range of color choices. However, synthetic dyes have harmful effects on the environment and human beings[1,2]. Synthetic dyes are made up of chemical compounds that can be harmful to humans. Some of the chemicals found in synthetic dyes are mercury, lead, chromium, copper, sodium chloride, toluene, and benzene[3]. Exposure to large doses of these substances can be toxic and can have severe effects in the human body. Water pollution can also result from manufacturing of synthetic dyes when untreated dye effluent is dumped directly on bodies of water[4,5]. Dye manufacturers are minimizing the use of harmful chemicals in their products and are more focused on creating dyes with the use of environment-friendly ingredients[6].

Bio pigments play a major role in food and textile industries as colorants. Many studies focus on replacing the synthetic pigments with eco-friendly natural and bio pigments[7]. Pigments are naturally found in plants, microorganisms, ores and insects. Among those, microbial pigments are highly favored due to availability throughout the year and for the level of control as well as reproducibility of the pigment's quantity and quality[8,9]. Among those pigment-producing microbes, *Pseudomonas* species is a highly researched and explored microbe. Different species of *Pseudomonas* has been found to produce different pigments, such as Pyocyanin (Bluish-green), pyorubin (red), pyoverdine (Yellow-fluorescent), pyomelanin (brown) and many more[10,11]. But all these pigments are very low in quantity and their extraction is high resource-consuming process[12].

In short, *Pseudomonas* is a microbe that can produce phenazine and their derivatives. Most of *Pseudomonas* species has a characteristic yellowish-green colour due to two compounds, phenazine-1-carboxylic acid and chlororaphin which are an amide of phenazine-1-carboxylic acid[13]. Though these pigments have been explored and exploited for their biological activities, they have not been studied as a colorant for industry. This study focuses on how the pigments of *Pseudomonas* can be used for textile application particularly synthetic textiles.

2 Materials and Methods

2.1 Media and Chemicals

Pseudomonas aeruginosa strain is isolated from goat droppings, and 16srDNA sequence is done to confirm the isolated strain. Polyester filament knitted single jersey fabric (160 g/m^2) was sourced from the knitting division Eastman Exports Global Clothing

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Private Limited Knitting Division. Nutrient broth, glycerol, lactose, maltose, sucrose and glucose, were used for culture strain isolation as well as pigment fermentation and Cultivation. Sodium hydroxide and fatty alcohol ethoxylated surfactant used for dyeing of polyester were purchased from Merck Life Science Private Limited, India. Demineralized water used for media preparation and quality drinking water was used in dyeing experiments.

2.2 Isolation and Identification

The goat dropping was serially diluted and then pour plated on nutrient agar plates to obtain isolated colonies. These colonies were streaked on Cetrimide agar plates for screening *Pseudomonas* from the colonies. The colonies were selected based on the characteristics of diffusible green pigment production. The selected isolate was subjected to biochemical tests and gene sequencing followed by BLAST analysis for species confirmation. Biochemical characterization was carried out based on Bergey's manual of determinative bacteriology.

The DNA of the isolate was extracted using high pure PCR template kit (Roche). The DNA is cut short and amplified using PCR primer set, 27F-5'AGAGTTTGATCMTGGCTCAG3'and 1492R-5'TACGGYTACCTTGTTACGACTT3'. This amplified product was sequenced using automated sequencer with the primer set 518F-5'CCAGCAGCCGCGG TAATACG3' and 800R-5'TACCAGGGTATCTAA TCC3'. This sequence obtained is aligned and ran through BLAST to identify the organism, only sequence obtained with 1200 base pairs or above is considered to give assured result in BLAST.

2.3 Fermentation and Extraction

Four types of medium including nutrient broth, nutrient broth supplemented with glycerol (2% w/v), and mineral salt media supplemented with lactose, maltose, sucrose, glycerol and glucose (2% w/v) were tested for the biosynthesis of pyocyanin for 3 days at 35 °C and 110 rpm. The purpose of the study on mineral salt media (MSM) with different carbon source supplements is to find the carbon source that best influences the pyocyanin production. The *p*H of the culture medium, inoculation quantity, and fermentation were also optimized.

The parameter for screening growth media and deciding optimum growth pyocyanin quantity produced in media. The bacterial cultures were centrifuged to obtain cell-free supernatant. To the 5 mL supernatant 2.5 mL of chloroform was added. The pigment dissolves in chloroform making it blue. To the 2.5 mL of chloroform, 0.5 mL of 0.2 N HCL was added and vortexed. The HCL layer turns pink. To 200 µL of this pink solution, 2.3 mL of 0.2 N HCL was added and absorbance was measured at 520 nm. The concentration of the pigment is found by multiplying absorbance and dilution factor with 17.072, which is the extinction coefficient of pyocyanin, as shown below:

Conc. of pyocyanin pigment (µg/ mL) =

O.D @ 520 nm × Dilution factor × 17.072

Dilution factor = Final volume/ Initial volume

= 2.5/0.2

= 12.5

2.4 Polyester Dyeing

Polyester fabric dyeing process consumes less water, as it can retain 0.4% moisture, and also negates the use of salts and other chemicals used in the dyeing of cotton fabric. For polyester dyeing, *p*H of the acidified bacterial pigment is adjusted at 9 using NaOH and the dyeing is carried out at 130^{o}C. The material: liquor ratio is 1:6. Dyeing process of polyester is shown in Fig. 1.

The bacterial pigment solution is used as the dyeing liquor. After treatment, the dyed fabric was subjected to washing using fatty alcohol ethoxylated surfactant using the concentration of 4 g/L (*p*H 7) for 20 min at 80^{o}C temperature. After treatment, the fabric was washed with hot and cold water and dried using hot air oven. The unfixed dyes were removed from the fabric and this process was repeated number of times necessary until the bath recovered after treatment is void of any colour.

2.5 Characterization

The extracted pigment in chloroform was run in a solvent system of methanol: chloroform (1:1). If the retention factor value is around 0.71, then the

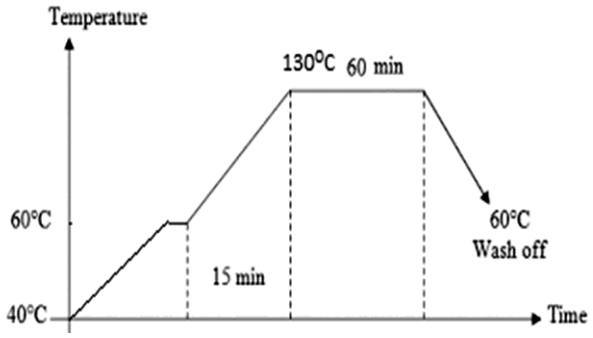

Fig. 1– Dyeing process of polyester

extracted pigment is conformed as pyocyanin. The dyed fabric is also extracted with a suitable solvent and is analyzed in GCMS to cross verify the pigment taken up by the fabric. This also helps in deducing the modification done to the pigment by dyeing process parameters of polyester fabric. CIE chromaticity color space encompasses the color as tristimulus value. The color tristimulus values are denoted in L^* for the lightness and a^* and b^* for the green–red and blue-yellow color components. This color measurement will guide to find pyocyanin color map after application. The colour fastness to rubbing, washing and light fastness were tested based on ISO 105-X12, ISO 105-C10 and ISO 105-B02, respectively.

3 Results and Discussion

3.1 Identification

The green pigment colonies were selected for biochemical characterization and subjected to gene sequencing for molecular identification. Table 1 shows the list of biochemical tests results for the isolate.

In gene sequencing, a sequence of 1493 base pairs have been obtained. On running through BLAST, it is observed that for 100% query sequence, a 99% identity is observed for *Pseudomonas aeruginosa*. On cross verification with the results obtained from biochemical tests, the microbe is affirmatively identified as *Pseudomonas aeruginosa*.

3.2 Effects of Different Growth Media

From all growth medium, the pigment is extracted using chloroform, and it is separated using acidified HCl quantified at λ520 nm. The medium that shows the highest absorbance is found to have high pyocyanin content. Table 2 shows the absorbance of pigment at λ520 nm for respective media. In media screening, no pigments are observed in MSM supplemented with glucose, sucrose, and maltose. A very low quantity of pigment is observed in MSM supplemented with lactose and glycerol.

Only nutrient broth and nutrient broth supplemented with glycerol is shown to contain high pigment concentration. Hence, nutrient broth supplemented with glycerol is considered for further studies as it has the highest pigment yield.

Table 1 — Biochemical test result of the isolate

Biochemical / morphology test	Triplicate	Result
Gram staining	-ve	Gram-negative
Capsule staining	-ve	Non- capsulated
Shape	-	Rod shaped bacteria
Pigment production	-	Green pigment diffusible in agar
Litmus milk	(-)(-)(-)	Negative
Lactose	(-)(-)(+)	Negative
Methyl red	(-)(-)(-)	Negative
Indole	(-)(-)(-)	Negative
Citrate test	(+)(+)(+)	Positive
Catalase test	(+)(+)(+)	Positive
Salt tolerance test	(+)(+)(+)	Positive
Urease test	(-)(-)(-)	Negative
Starch Hydrolysis	(+)(+)(+)	Positive
Gelatin Hydrolysis	(+)(+)(+)	Negative
Casein Hydrolysis	(+)(+)(+)	Positive
Voges Proskauer	(-)(-)(-)	Negative
Oxidase	(+)(+)(+)	Positive

3.3 Chromatographic Analysis of Pigments

The pigment extracted using chloroform is run in TLC sheet using chloroform and methanol (Fig. 2). It is verified that the extracted pigment is Pyocyanin as the retention factor is 0.74 (solvent front = 5 cm and solute front = 3.7 cm). From the GCMS result of the fabric extract, it is found that the structure,

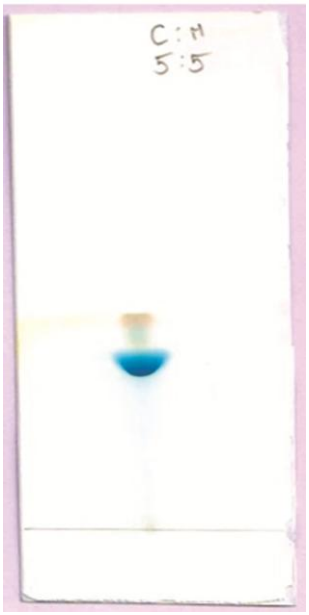

Fig. 2 — TLC analysis of pyocyanin

Table 2 — Absorbance at λ520 nm for the respective media

Media	Absorbance at λ520nm	Pigment concentration µg/ mL
Nutrient broth	0.242	51.6428
Nutrient broth + glycerol	0.246	52.4964
MSM + lactose	0.013	2.7742
MSM+ glycerol	0.027	5.7611
Pyocyanin production media	0.132	28.1655

(1-Hydroxy phenazine) imparts the yellow shade to the fabric (Fig. 3).

3.4 Effect of Different *p*H and Temperature

Nutrient broth supplemented with glycerol is prepared with 6, 7, 8, and 9 *p*H. After inoculation, the culture broths are incubated at 35°C with 110 rpm. Figure 4 shows the pigment yield for the cultures grown at the respective *p*H. From the observation of the effect of *p*H, it is evident that *p*H of range 7-8 shows high pyocyanin production. Thus, *p*H 8 is fixed as the optimum *p*H for pyocyanin production for further studies.

Similarly, nutrient broth supplemented with glycerol is prepared at *p*H 8 and is incubated for a day at different temperatures (28, 35, 40, and 50°C), Since the incubation period is only one day, 1 mL inoculum is taken for 100 ml media. Figure 5 shows the effect of different temperatures in pyocyanin production.

On observing the effect of temperature, it is evident that the isolated *Pseudomonas* is able to produce pigment at high temperature (40°C), but the highest yield is recorded at 35°C. Hence, for further production of pigment, nutrient broth supplemented with glycerol at *p*H 8 is used and the culture broths are incubated at 35°C for 3 days.

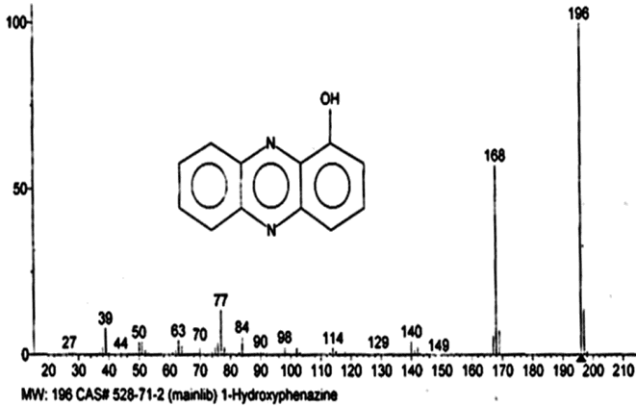

Fig. 3 — Structure prediction of pyocyanin using GCMS

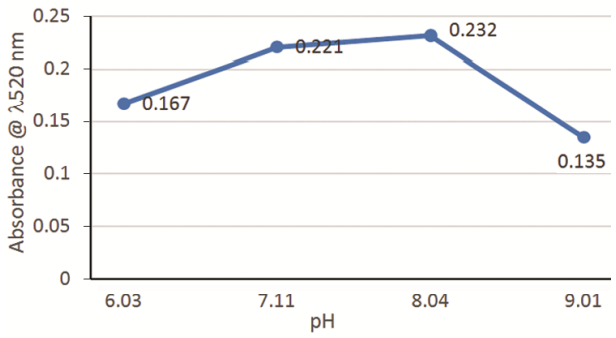

Fig. 4 — Effect of different *p*H in pyocyanin production

3.5 Dyeing of Polyester Fabric

The dyed fabric is found yellow in shade. The bacterial pigment in acid *p*H is pink in colour, and when adjusted to alkaline *p*H 9 of the pigment colour changes to blue. This blue colour solution of the pigment is taken as bath liquor for dyeing. But the resulting fabric is yellow in colour and statement of chemical reaction for color change is shown by application stability. So, for further investigation, the color giving compound from the dyed fabric is extracted and analyzed using analytical techniques.

Color on fabric is directly related to application concentration of pigments. Table 3 denotes the color in CIE chromaticity based on concentration. CIE values are measured under D65 light source and standard observer of 10 degree.

Tristimulus values show that with the increase in concentration of pyocyanin, shade on fabric becomes darker and redder with obvious changes in color chroma and hue. Initially, i.e at low concentration, it shows shade in brighter tone with lighter effect. This trend is changed when the concentration is increased, and shade becomes darker, duller, and redder.

3.6 Colour Fastness

Color quality is more important in textile application to promote the fabric to the next stage of garmenting. Generally, color quality assessed in two ways i.e. multi-fibre staining and color change after washing. Table 4 shows that pyocyanin on polyester has excellent property to withstand the wash fastness i.e.in higher concentration also it has excellent color fastness properties on textile application.

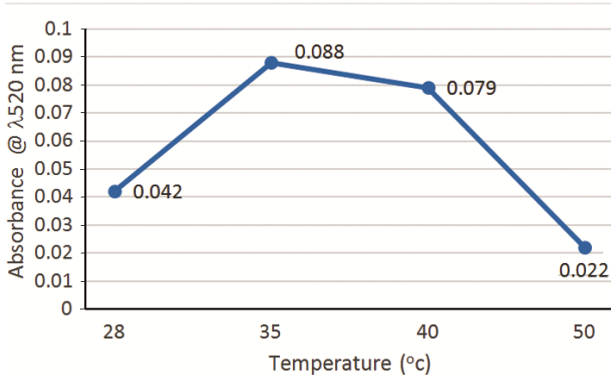

Fig. 5 — Effect of different temperatures in pyocyanin production

Table 3 — Color space values

Concentration, % (owf)	Shade/Color	L*	a*	b*	C*	H*	*K/S* value
0.5		86.40	-2.95	19.55	19.77	98.59	48.30
1		74.18	0.24	19.28	19.28	89.28	43.64
1.5		70.35	0.35	23.79	23.79	89.16	43.58
2		66.16	1.48	25.27	25.32	86.66	42.36
2.5		62.97	2.22	23.99	24.10	84.71	41.36
3		61.97	2.30	24.21	24.31	84.58	41.29

Table 4 — Color fastness results (Staining results for overall grade)

Shade depth %	Color fastness							
	Washing		Water		Perspiration (acid)		Perspiration (alkali)	
	Staining	Color change	Staining	Color change	Staining	Color change	Staining	Color change
0.5	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5
1	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5
1.5	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5
2	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5
2.5	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5
3	4-5	4-5	4-5	4-5	4-5	4-5	4-5	4-5

4 Conclusion

The green diffusible pigment-producing isolate is found to be *Pseudomonas aeruginosa*. The isolate's optimized growth condition is found to be *p*H 8, and temperature 35°C. The cultures are incubated for 3 days at 110 rpm for better results. Also, in analyzing different medium, nutrient broth supplemented with glycerol is found to show enhanced pyocyanin pigment production. The extracted pigment is verified to be pyocyanin by GCMS analysis. The extracted pigment is found pink in acid *p*H, and blue in alkaline *p*H. The alkaline pigment solution is used as a bath in dyeing of polyester, but the resulting fabric is yellow. To study the fixed pigment, the fabric is extracted using chloroform. In higher concentration, pyocyanin has excellent colour fastness (CF 4-5) properties on polyester. With the help of GCMS, the structure in the extract is found to be 1-Hydroxyphenazine. Considering the change of structure from pyocyanin to 1-Hydroxyphenazine, it is understood that pyocyanin undergo hydrolysis at 130°C during polyester fabric dyeing. This 1-Hydroxyphenazine imparts the yellow shade to polyester fabric on dyeing. Pyocyanin pigment is an eco-friendly and natural colorant in synthetic textile without any chemicals.

References

1 Frank L H & Demoss R D, *On the Biosynthesis of Pyocyanin* (McCollum-Pratt Institute, Johns Hopkins University and Department of Bacteriology, University of Illinois), 1959.

2 Raoof W M & Latif I A R, *Eur J Sci Res*, (2010) 47.

3 Garber A Abo-zaid, Elsayed E Wagih, Saleh M Matar, Nader A Ashmawy & Elsayed E Hafez, *Chem Tech Res*, 8(9) (2015) 137.

4 Khouloud M Barakat, Magdy Z Mattar, Shawky Z Sabae, Osama M Darwesh & Sahar H Hassan, *Res Pharmaceut Bio Chem Sci* , 6(5) (2015) 933.

5 Sudhakar T, Karpagam S & Sabapathyshiyama, *Int J Chem Technol Res*, 5(3) (2013) 1101.

6 Popy Devnath, Kamal Uddin, forkan Ahamed, Towhid Hossain & Mohammed Abul Manchur, *Int R J Biol Sci*, 6(5) (2017).

7 Mavrodi D V, Bonsall R F, Delaney S M, Soule M J, Phillips G & Thomashow L S, *J Bacteriol*, 183(21) (2001) 6454.

8 Brown V I & Lowbury E J L, *J Clin Pathol*, 18(6) (1965) 752.

9 Ingledew W M & Campbell J R, *Can J Microbiol*, 15(6) (1969) 595.

10 El-Fouly M Z, Sharaf A M, Shahin A A M, El-Bialy H A & Omara A M A, *J Radiat Res Appl Sci*, 8(1) (215) 36.

11 Saosoong K, Tongtumma S, Chanthai S, Wongphathanakul W, Bunyatratchata W & Ruangviriyachai C, *KKU Res J*, 12(1) (2007) 24.

12 Saha S, Thavasi R & Jayalakshmi S, *Res J Microbiol*, 3(3) (2008) 122. DOI:10.3923/jm.2008.122.128.

13 Jayaseelan S, Ramaswamy D & Dharmaraj S, *World J Microbiol Biotechnol*, 30(4) (2014). DOI: 10.1007/s11274-013-1552-5.

14 Kerr J R, Taylor G W, Rutman A, Høiby N, Cole P J & Wilson R, *J Clin Pathol*, 52(5) (1999) 385.

15 King E O, Ward M K & Raney D E, *J Lab Clin Med*, 44(2) (1954) 301.

16 Ahmed A S, Ahmed W Y W, Zakaria Z K & Yosof N Z, *Applications of Bacterial Pigments as Colorant: The Malaysian Perspective* (Springer Briefs in Molecular Science, New York), 2012.

17 Mahmood & Ibraheem Abudl Rahman Latif, *J Sci Res*, 47(3) (2010) 405.

18 Shirata A, Tsukamoto T, Yasui H, Hata T, Hayasaka S & Kojima A, *Japan Agricult Res Quarterly*, 34 (2000) 131.

19 Young G, *J Bacterioll*, 54 (1947) 109.

20 Taylor G W, Machan Z A & Mehmet S, *J Chromatogr B Biomed Appl*, 664 (1995) 458.