Identification, characterization of carotenoid pigment producing bacteria from vegetable, fruits and mini-survey on perception of urban population towards bacterial pigments

Chinmayee Mahadik, Neha Manoti, Aafra Zuzar Mujawar, Meera Nambidas Konar, Peenal Arvind Mistry, Sejal Rathod

ABSTRACT

Pigments are largely exploited compounds due to their chromophoric variations and enticing properties in several applications such as food, dairy, printing, textile and pharmaceutical industries etc. While synthetic pigments are widely used, toxic reactions and effects of the pigments has led to the prompt diversion of consumers towards natural pigment sources such as microbial pigments (MP). MP hold great promise against existent synthetic counterparts with additional properties such as biodegradability, non-toxicity, therapeutics (anti-inflammatory, antioxidant, anticancer etc. Isolation and production of unique microbial biopigments can therefore open multiple avenues into interdisciplinary diligences. Thus, aim of the current study was isolation of bacterial pigment from vegetable, fruit and soil bacteria. On the basis of Taxonomic characterization , pigment producing isolates were identified to be *Acinetobacter lwoffii*, *Micrococcus luteus* (yellow pigments) and *Salmonella enterica subspecies enterica* (orange pigment).Pigment extraction efficiencies observed be ranging between 39.8% -71 % with methanol as solvent. Establishment of the absorption spectrum from the pigments reveals the close resembles of the extracted pigments with carotenoids. Evaluation of the textile staining properties of the pigments revealed that the micrococcus derived pigment can be effectively used to stain the paper than the fabric material in absence of a mordant. For effective application development public awareness towards the microbial pigments was studied with a mini-survey which revealed statistically significant understanding of respondents towards toxic effects of synthetic pigments. Respondents showed neutral to positive approach towards usage bacterial pigments. Current work focuses on the exploration of bio pigments as an alternative to synthetic pigments for diverse applications.

Keywords: Bacterial pigment, Bioactive Pigment, carotenoid bacterial pigments , Microbial Biopigments, Microbial Pigments; Survey on biopigments,

1. INTRODUCTION

Pigments are compounds with significant characteristics in multiple natural and industrial applications. They are used in the food industry as additives, colour enhancers, antioxidants, etc. Pigments are available in a variety of colours. In recent years there has been increased interest in the production of dye from biological agents for food and textiles[1]. Nature produces a wide variety of biocolourants, including plants and microorganisms, which are possible alternatives to currently used synthetic dyes and pigments. The recent awareness of human safety and conservation has rekindled the interest in natural colour sources. Due to their non-toxic, non-carcinogenic and biodegradable nature, natural pigments or dyes derived from vegetation, animal or microbes are thought to be safe. As the current trend across the globe changes to the use of green and biodegradable products, the demand for natural colours is rising rapidly[2].

Ores, insects, plants and microbes are used for natural pigments. Biopigments made from microorganisms are preferred to plant ones due to their stability and year-round availability for cultivation. Bacteria have a great potential among microbes to produce diverse bioproducts and pigments are one of these bioproducts. Different researchers have investigated the production and application of bacterial pigments as natural colours. The production of bacterial pigments is now a new field of research which demonstrates its potential for different industrial applications. However, Most of the production of bacterial pigments is still in R&D phases[3].

The accessible and regulation authorized natural pigments from plants have numerous drawbacks such as instability against light, heat, or adverse pH and low water solubility[4]. The paramount interest in using microbial pigments is because of their natural character, safety, medicinal properties, and their ability to provide controllable and predictable yields. Consumers are relieved that these pigments do not produce any

side effects unlike synthetic pigments which when used in excessive amounts may result in adverse health issues. Industrial production of natural food colorants by microbial fermentation has several advantages such as cheaper production, easier extraction, higher yields through strain improvement, no lack of raw materials and no seasonal variations[5].

The bacterial pigment molecules such as bacteriochlorophylls, , phenazines, carotenoids, flavins, indigoides, melanins, pheomelanins, , quinone precursors,violacein, glaukothalin, xanthomonadin, phenazine, canthaxanthinephenazines, phenazanthin D, phenazins are produced as bi-products from several bacterial species [6]. Many of these compounds and their derivatives show a broad range of biological activities expressed in terms of effective dosage form/inhibitory concentration/lethal and sub-lethal concentration levels .The microbial pigments are known to have anti-oxidant, immune-modulatory and probiotic properties thereby alleviating chronic conditions such as cancer, cardiovascular, digestive disorders, diabetes and auto-immune diseases[7].

Additionally, in comparison with synthetic colours, the annual growth rate with a low growth rate of 3 to 5% of naturally derived colours was also predicted to be between 5% and 10%. Because of their natural character and safety, medicinal properties, nutrients like vitamins, season and geographic production, controllable and predictable output there is an increasing interest in microbial pigments. The bacterial pigments can be produced by utilization of various waste materials as substrate media thus proving to be sustainable options for reducing environmental pollution.

Elaborated research has been published which the isolation of pigmented microorganisms like bacteria, fungi, and yeast from terrestrial as well as marine milieus. Out of these the bacteria is one of the largest class of microbes to be exploited for the pigment production.

Their geographical distribution, diversity and abilities to thrive in extreme conditions leads to the production of plethora of pigments from same species. Therefore the exploration and manipulation of the bacterial species from distinct environments and conditions have shown a huge premise for obtaining unique bio-pigments[8]. The identification and evaluation of the isolated pigments provides a framework for developing sustainable applications. In this direction the current work involved isolation and identification of pigment producing bacteria from coloured samples such as vegetable waste materials from pomegranate and beetroot. The extraction and crude characterization of the microbial pigments from the isolates was performed as a stride to establish chemical identity of the pigments. The prospective applicability of the extracted pigments was studies by assessment of pigment to stain the cellulosic materials (paper and cotton fabric)[9].

Although the microbial pigments have shown the promise as an effective alternative to the synthetic pigments, the humans hold a prejudice against these biopigments. Therefore, in order to understand the perspective of the urban Indian population towards the bacterial pigments a mini-survey was conducted . Combinatorial studies of the aforementioned parameters of bio prospecting will provide a great foundation to establish a successful bio-enterprise

2. MATERIALS AND METHOD

2.1 Test chemicals: The media constituents (Nutrient agar and Nutrient broth) used in this work were purchased from Himedia, India. Other laboratory chemicals were prepared in distilled water. Organic solvent ethanol was obtained from SRL.LTD. India.

2.2 Isolation of pigment producing bacteria: The pigmented bacteria were isolated from three pigmented sources – Fruit and vegetable peel (Pomogranate and Beetroot) and soil .

2.3 Sample collection: Fruit and vegetables were collected from local market of Mumbai and were used for the isolation of pigment-producing bacteria[10]. From the collected samples of beetroot peel and pomegranate peel, swabs of the outer skins were taken and spread on sterile Nutrient agar plates and the plates were incubated at 37°C for 24 hours. The soil sample was collected from the potting soil of a rose plant from a nursery in Ulhasnagar, Maharashtra .The soil was collected from the depth of 5 cm from the surface. Soil suspensions were prepared using sterile distilled water and 0.1 mL was spread on sterile Nutrient agar plates and kept for incubation at 37C for 24 hours. The pigment-producing, morphologically distinct bacterial colonies growing on the nutrient agar plates were picked, and further isolated on sterile nutrient agar plates to obtain a pure bacterial culture. The colonies were further sub-cultured on the sterile Nutrient agar slants and preserved as mother cultures for additional studies. Cultures were maintained on Nutrient agar slants and stored at 4°C in the refrigerator[11].

2.4 Characterization of isolated pigmented bacteria: The bacterial isolates were studied for their primary characters and Gram nature using monochrome and Gram staining technique. The colony characters such as size, shape, colour, texture, opacity, elevation and margin were noted to identify the isolates on the basis of their morphology. The selected isolates were coded as PO, PS, RS and BT on the basis of the sample.

2.4.1 Identification of potential pigment producing bacterial isolates: Identification of the selected bacterial isolate was done based on the basis of MALDI-TOFF MS analysis (Matrix Assisted Laser Desorption Ionization-Time of flight Mass Spectroscopy) performed at SRL Diagnostics, Mahim[12].

2.4.2 Evaluation of temperature effect on pigment production: 1mL of the isolate inoculum was inoculated in 5mL of sterile nutrient broth and incubated at different temperatures i.e. 28 °C ,37°C,and 4°C for 72 hrs for assessment of the pigment formation. The isolates were examined visually for maximum colour intensity into the nutrient broth against appropriate controls.

2.7 Extraction of bacterial pigments

The isolates PO, PS, RS, BT primary inoculums of OD 0.1 were inoculated into PTC tubes containing 100 ml Nutrient broth media at optimized temperature conditions and maintained in rotary shaker for 72 hours. The culture broths were subjected to centrifugation at 10000 rpm for 15 min, to separate cell pellet. The pellets were visually evaluated for intracellular or extracellular pigment production. The pigment was extracted by rigorous mixing and vortexing of the cell pellet with 10mL methanol as solvent[13]. The homogenized mixture was subjected to centrifugation (10000rpm for 15 min) to obtain organic phase containing pigment and to separate cell debris. The supernatant was subjected for drying using rotary evaporator while the solvent was recovered for subsequent use.

2.7.1 Extraction efficiency calculation:

The extraction efficiency of the pigment using methanol as a solvent was calculated using formula 1. Here the dry cell weight was obtained by gradual drying of the cell pellet at 60 °C in vacuum oven overnight.

$$Extraction\ efficiency = \frac{Dry\ weight\ of\ the\ pigment * 100}{Total\ dry\ cell\ weight}$$

2.8 Characterization of extracted pigments :

2.8.1Estimation of absorption maxima: The extracted pigments were subjected to spectrophotometric analysis to calculate their absorption maxima. 1% solutions of the extracted pigments were scanned using UV-visible Spectrophotometer between the

wavelengths of 200-500 nm in uniform intervals. The graph of absorbance was established to determine absorption maxima (λ_{max}).

2.8.2 Staining properties: Staining properties of the extracted pigments were evaluated by treatment with cellulosic materials such as paper and cloth[14].

2.8.2.1Dyeing of paper: A white paper of 5cm * 5 cm area was soaked into 1% methanolic solution of pigment for 15 minutes and dried.

2.8.2.1 Dyeing of cotton fabric: A non-dyed cotton fabric piece of 5cm * 5 cm area was soaked into 1% methanolic solution of pigment for 15 minutes and dried.

The dried materials were visually evaluated for pigments staining abilities when compared with the unstained material controls.

2.9 Survey on microbial pigment awareness: Among the major challenges to overcome for the commercialization and widespread applications of bacterial-based pigments, is the awareness of the fact that the bacterial-derived pigments are relatively safe to their synthetic counterparts. Despite of many applications, bacterial pigments face several challenges in their way into commercialization. A survey was performed to understand the awareness and perception of public towards microbial bio-pigments among the urban Indian population. The participants answered these questions about how likely they were to try natural and bacterial pigments on 5-point Likert scale, ranging from 1 (not at all likely) to 5 (extremely likely). The sample size of 189 individuals were provided with a Google form based questionnaire as given in supplementary material-1. The results were analysed with MS excel and free statistical software Jamovi by performing Chi Square test of association and McNemar tests.

3. Results and discussion

3.1 Isolation and characterization of pigment-producing bacteria:

The collected samples of fruit, vegetable and soil obtained were spread on Sterile Nutrient agar plates and incubated at 37°C for 24 hours. A mixture of pigment producers and non-pigment producers were obtained on the crowded plate. From the individual sample plates , an intense pigment producing , morphologically different colony was selected and named as PS , PO , RS and BT. (Fig.4). The colony characteristics of the isolates are provided in the Table 1.

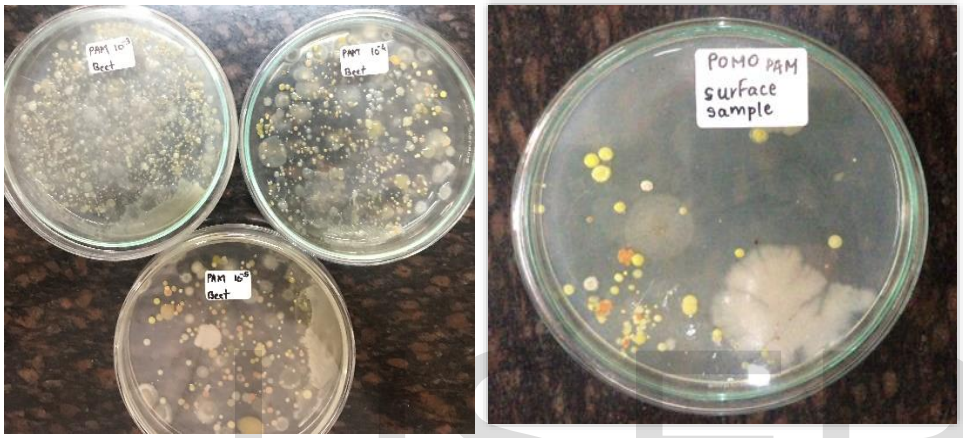

Figure 1 Crowded plates obtained from beetroot soil and pomegranate samples

Table 1 Colony characteristics of BT, RS, PS, and PO

Characteristics	**BT**	**RS**	**PS**	**PO**
Shape	Cocci	Coccobacilli	Rod	Cocci
Size	0.5-3 mm	1-2mm	2-3mm	0.5-3mm
Arrangement	Tetrads/pair	Pair/Single	Pair/Single	Tetrads/pair
Gram nature	Gram positive	Gram negative	Gram-negative	Gram positive
Surface	Non-butyrous	Butyrous	Butyrous	Non-butyrous
Opacity	Opaque	Opaque	Opaque	Opaque
Pigment	Beige to	Yellow	Orange	Beige to

	Yellow			Yellow
Margin	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex

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Pigment	Beige to Yellow	Yellow	Orange	Beige to Yellow
Margin	Entire	Entire	Entire	Entire
Elevation	Convex	Convex	Convex	Convex

3.3 Identification of the bacterial isolates

The isolates were identified using MALDI-TOFF MS (supplementary data) facility at SRL Diagnostics, Mahim. The identified species are enlisted in Table 2.

Table 2 Identification of pigment producing isolates

Sr. No.	Sample Code	Organism
1	PO	*Micrococcus luteus*
2	PS	*Salmonella enterica subspecies enterica*
3	RS	*Acinetobacter lwoffii*
4	BT	*Micrococcus luteus*

3.4 Optimization of temperature for maximum pigment production

The pigment production is initiated in bacteria on exposure to stressful conditions such as elevated temperatures, osmotic pressures, metabolic inhibition, etc. Increment in pigment yield is therefore achieved due to the restrained bacterial growth and metabolic modifications towards adaption of stress conditions. The effect of temperature variation on the growth and pigment production of the 4 bacterial isolates was evaluated. Upon incubation at 4°C ,28°C and 37°C for 24-48 hours.

Maximum growth and pigment production of all the bacterial isolates was visually observed at RT and 37°C after 48 hours of incubation, whereas minimal growth with very low or no pigment was observed for all the isolates at 4°C (Fig.3 A-D) [15].

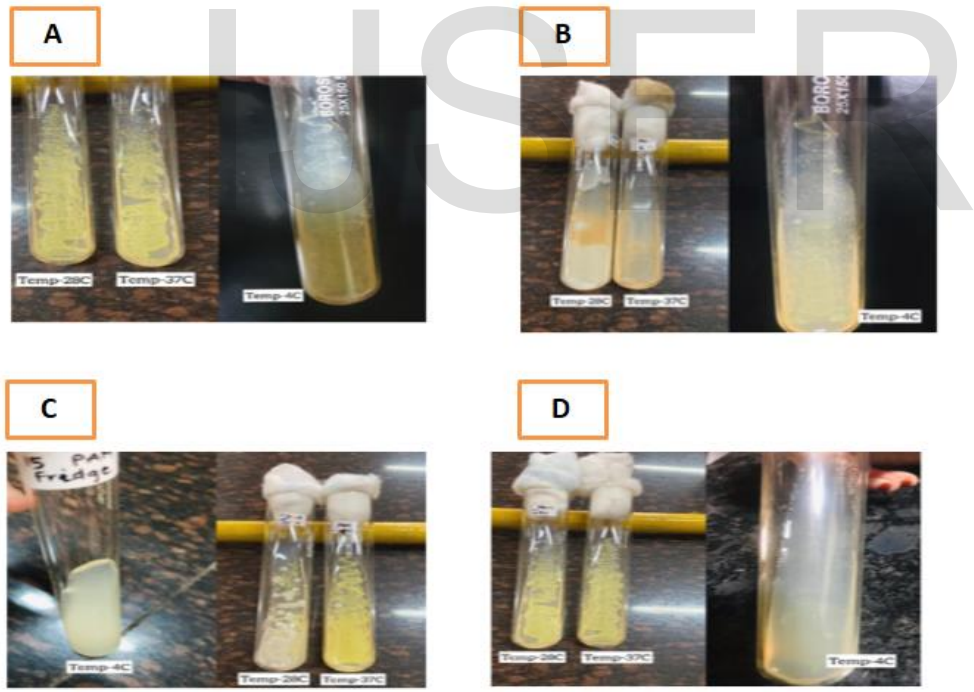

Figure 2 Effect of temperature on growth of BT, PS, PO, and RS

Extraction of bacterial pigment:

The visual evaluation of the pigments was performed after centrifugation. The pigments were majorly confined within the cell pellet and the spent broth was relatively devoid of pigment, thereby confirming the intracellular nature of the bacterial pigment. The intracellular pigments were further extracted by cell membrane disruption and by methanolic extraction[16]. Calculation of extraction efficiency (table 4) revealed the suitability of methanol as solvent for pigment extraction. However the maximal extraction efficiency can be evaluated using a wide range of solvents on the basis of their polarity[17]. The pigments were observed to be falling with the range of 0.21-0.53 µg/g of dry cell weight. The purified and dried pigments were subjected to presumptive test for structural confirmation using spectrophotometric analysis.

Table 4 Concentration of extracted pigment and the efficiency of pigment extraction

Sample	Concentration of extracted pigment (µg/g of dry cell weight)	Efficiency (%)
PS	0.34	68.6
BT	0.53	71
PO	0.21	39.8
RS	0.35	70

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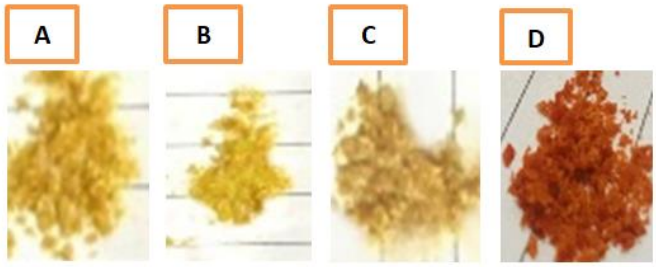

Figure 3 Extracted pigment from PO , BT, RS, PS

Evaluation of absorption maximum of extracted pigment

The absorption spectrum of the extracted pigments exhibit distinctive spectral characteristics of carotenoids with absorption bands in the region between 400 and 500 nm.. As indicated in Fig __A,B, the *Micrococcus luteus* pigment and *Acinetobacter lwoffii* pigment showed typical 3-peak-absorption curve of a carotenoid backbone in the visible spectral region. Similar observation have been reported , when studied carotenoids in polar solutions [18,19].A minor shift in the absorption maxima of the *Acinetobacter* pigment and *Salmonella enterica* pigment can be attributed to the probable addition other functional groups such as carbonyl(C=O) to the carotenoids[20] . Further FTIR and NMR analysis of the extracted pigments can aid in the functional group confirmation.

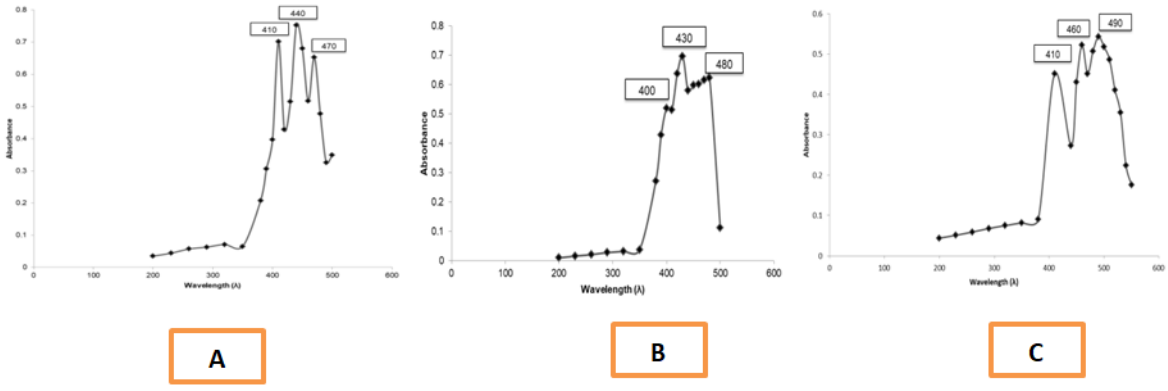

Figure 4 Absorption maxima estimation of A Micrococcus luteus ; B Acinetobacter lwoffii ; C Salmonella enterica

Evaluation of staining property of extracted pigment

The high intensity pigments were selected further for evaluating the staining properties of the extracted pigments. The dyeability of cellulosic materials with microbial pigments obtained from the *Micrococcus* and *Salmonella sp* was evaluated. The substrates showed successful binding of the pigment on single treatment without mordant. The concentrated solutions of pigments can be used in the paper dyeing and printing applications, while the fabrics can be dyed with appropriate mordants [21].

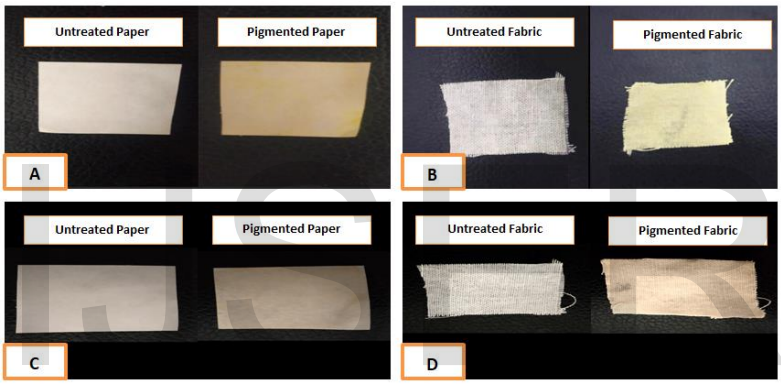

Figure 5 A,B White paper and fabric dyed with Micrococcus luteus pigment C,D White paper and fabric dyed with Salmonella enterica pigment

Analysis of survey results

The demographic of the data obtained are Mumbai dominant with several responses from other cities in Maharashtra. The survey was conducted within the urban region of Mumbai, Maharashtra,India. Out of 189 respondents, 53.96% were females. Majority of the population of the respondents belonged to the age group 18-25 showing the major respondents in the current studies are part of the youth.

Table 2 Age profile of the respondents

	Age group			
gender	**18-25**	**>25**	**18>**	**Total**
Female	82	18	2	102
Male	52	30	5	87
Total	134	48	7	189

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Eighty-two percent of respondents preferred plant-based sources over any other. Herbal and natural products have been used in India for thousands of years, and they're generally considered safe to consume.

According to the data obtained, majority of the respondents (96%) preferred natural pigments over the chemical dyes.Under the premise of majorly used industrially pigments belong to the class of chemically derived substances, the broad perception of subjects towards toxicity was evaluated using Chi-squared test and McNemars test[22,23]. High p-value indicates, subjects understanding of toxicity associated with the industrially used pigments.

Table 3 Awareness of respondents about industrial pigments and the toxicity of synthetic colorants

	Awareness about the toxic effects of synthetic colorants		**McNemar Test**		
Knowledge of industrial pigments being synthetic	Yes	No	**χ^2 Tests Value**	**Degree of Freedom (df)**	**p-value**
Yes	139	20	1.48	1	0.223
No	13	17			
Total	152	37			

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Establishment of the proportionality tables indicated that 96.83% respondents preferred usage of natural pigments over the currently available synthetic pigment which highlights about the awareness and preference of the young generation towards the illeffects of the chemical compounds. The chi square test revealed the positive relationship between willingness of subjects(N=189) to try bacteria pigments and their selective preference towards the natural pigments(table X).The overall approach was observed to dominate towards neutral to positive degrees of acceptance .

Table 4 Respondents willingness to try the bacterial pigments

	Preference of pigments			χ^2 Tests Value	**Degree of freedom**	**p-value**
Willingness to try Bacterial pigments	Natural pigments	Synthetic pigment	Total	9.63	4	0.05
1 (Strongly Agree)	54	1	55			
2 (Agree)	46	2	48			
3 (Neutral)	46	1	47			
4 (Against)	28	0	28			
5 (Strongly Against)	9	2	11			

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The respondents were asked about the familiar applications of microbial pigments. As indicated in Table 4. Chi square test of association revealed the strong awareness of respondent population in the order Therapeutics>Pharmaceuticals>Food additive>Ink for printing>Leather dyeing>Paints>Cosmetics. The ascending p values revealed the strong association of pigment application with the respondent's knowledge of the same. This gives us a valuable insight that people often are aware about applications which are associated with remedies and foodstuffs.

Table 5 Awareness of respondents about applications of bacterial pigments

Application of bacterial pigments	χ^2 Tests Value	p-value
Therapeutic agent	32	>0.01
Pharmaceutical application	4.26	0.119
Food additive	4.26	0.119
Ink	3.49	0.175
Leather dyeing	3.36	0.186
Paints	2.75	0.253
Cosmetics	1.82	0.402
Textile	1.4	0.496
Soap colorant	0.5	0.777

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Soap colorant	0.5	0.777

Women are generally thought to be more health-conscious than men, according to popular belief. Gender did not appear to be a major factor in this study when it came to the awareness of bacterial pigments. A slight gender gap existed despite the fact that there were more female than male users. Women and students, on the whole, were more aware of and willing

to use bio pigments, according to the study. However, a large portion of the respondents were not aware of this. Because they are unaware of the benefits of bio-pigments, they are disinclined to use them. Some ways to boost awareness could be through hosting educational events, seminars, webinars, news jack, social media, rally's, distributing brochures, identify target audience and tailor awareness-raising initiative appropriately, empowering and connecting others. In addition, hosting of social events, invitations to social media influencers, and testing of bio-pigmented cosmetics and foods.

Conclusion:

The results obtained from this study serve as an important insight for production of bio-pigment from soil inhabiting bacteria. The bacteria were identified as *Micrococcus luteus*, Salmonella *enterica subspecies enterica* and, *Acinetobacter lwoffii* and were able to produce yellow, orange, red pigments. The UV-Visible spectrophotometric analysis revealed that extracted pigments show carotenoid structural properties. Future studies should focus on elucidating the nature using advanced methodologies such as FTIR, HPLC and NMR. The temperature optimization studies revealed that bacteria produced highest pigment at 37degree C under shaking condition. The pigments showed cellulose staining properties showing promise to be used in textile and paper industry. The bio-pigment survey revealed largely that urban young people have a positive approach to natural pigments rather than synthetic dyes. The study showed that respondents were well aware of the therapeutic use of bacterial pigments while less aware of other industrial applications.

Authors' contributions: This work was carried out in collaboration between all authors. Authors CM and NM designed, managed the analysis of the study. CM and NM wrote the protocols and first draft of the manuscript. Author AM, MK and PM performed the designed

experiments. Author CM performed the statistical analysis. Authors AM,MK and PM managed the literature searches. SR guided and managed the overall research work. All authors read and approved the final manuscript.

Conflict of Interest:

Authors declare that there are no actual or potential conflicts of interest.

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