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Yellow Pigment from Fast-growing Marine Soil Bacteria *Citricoccus* sp. for Dyeing Cotton Fabrics

D. Ezhil [a++], S. Meignanalakshmi [b#*] and B. Haritha [c†]

[a] Department of Epidemiology, Cancer Institute, Chennai, Tamil Nadu, India.
[b] Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author ED has done the literature searches, wrote the protocol, performed experiments, wrote the first draft of the manuscript, performed data analysis and interpretation. Author MS has designed the study, data analysis, critical revision of the article and data interpretation and author HB performed experiments and technical support. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The study's objective is to isolate, characterize pigment-producing bacteria from marine soil and to optimize culture parameters such as pH, temperature, carbon, nitrogen sources to enhance the pigment production. The obtained pigment was used to dye cotton fabrics and woollen thread.
Place and Duration of Study: The samples were collected from the Marina beach, Chennai, Tamil Nadu and the study was carried out at the Department of Animal Biotechnology, Madras Veterinary College, Chennai, between January 2021- April 2021.
Methodology: From the soil sample fast-growing yellow pigmented bacteria was isolated and the

[++]*Scientific Assistant,*
[#]*Professor,*
[†]*Senior Research Fellow,*
**Corresponding author: E-mail: smeignanalakshmi@gmail.com;*

morphological characteristics were observed. Gram staining, biochemical test, sugar fermentation test, motility test was performed and 16S rDNA sequencing was done. Pigment was extracted through solvent extraction method, characterized through UV-Spec and FTIR. The phytochemicals test was done and the culture conditions were optimized to bring out maximum pigment yield. The pigment was then used to dye cotton fabrics and woollen threads.

Results: The isolated yellow-pigmented bacteria was identified as *Citricoccus* sp. and the pigment is confirmed as carotenoid compound and through optimizing the culture conditions, the pigment production was increased significantly. The woollen thread and cotton fabrics were dyed effectively and uniformly with the use of copper sulphate(CuSO4) as mordant.

Conclusion: The result of this study reveals that *Citricoccus* sp. is a fast-growing bacterium capable of producing a higher amount of pigment in 24-hour period. Since it is confirmed to be a carotenoid compound, it is safe to be used as a dye. The extraction process is cost-effective and economical, this study can be expanded to explore the potential applications of the pigment in the textile and dyeing industries.

Keywords: Carotenoid; dyeing; fabrics; marine; yellow-pigment; 16S rDNA.

1. INTRODUCTION

Color is the main characteristic perceived by the most human senses and it also determines consumer acceptance in various industries like textiles, food, and cosmetics. To meet these needs, industries started using synthetic dyes which are totally carcinogenic and toxic to the environment [1,2]. In order to replace chemical dyes, natural pigments from insects, bacteria, fungi, plants, and animals are used. The growing interest in microbial pigments is due to their natural character, properties like anti-bacterial, anti-inflammatory, anti-cancerous, anti-fungal, biodegradable, and non-toxic [3-6]. Moreover, microbial pigments are preferable rather than plant pigments for being independent of weather conditions, fast growth, cost-effective, and the percentage of pigment production can be enhanced by medium optimization and addition of low-cost substrates like rice bran, husk, whey, urea, and molasses [7-9].

There are many bacteria like *Serratia marcescens*, *Pseudomonas aeruginosa*, *Janthinobacterium lividum*, *Methylobacterium* sp, *Cyanobacteria* sp, *Micrococcus roseus*, which occur in a variety of colors like red, orange, green, blue, purple, yellow, and pink, composed of carotenoids, quinones, monascins, violacein, melanin, prodigiosin, etc [10-12]. The composition of each pigment varies depending on the type of nutrient availability. Some pigmented bacteria change their colors with respect to different pH and temperatures. The tremendous growth of textile industries has increased the demand for dyes. Microbial dyes are the major alternative to synthetic dye, the production of microbial dye is a fast process and many such dyes possess antibacterial properties [13-15].

The present study was undertaken to isolate and characterize pigment from marine bacteria from the natural habitat and use them for dyeing cotton fabrics and woollen thread.

2. MATERIALS AND METHODS

2.1 Sample Collection

The soil sample was collected from Marina beach, Chennai, Tamil Nadu in a sterile container, taken to the laboratory and stored at 4°C until use.

2.2 Isolation of Pigment-Producing Bacteria

Bacteria were isolated from marine soil by a 10-fold serial dilution method,100µl of sample from 10^{-6}-10^{-10} dilution was taken and plated on the nutrient agar medium, incubated at 37°C for 24 hours.

2.3 Characterization of Pigment-Producing Bacteria

Isolated bacteria were identified based on Colony morphology, Motility test, Gram staining, and Biochemical test.

2.4 Isolation of Genomic DNA and 16S rDNA Amplification

Genomic DNA was isolated from 24-hour culture, quantified, and checked on 1% agarose gel electrophoresis. The DNA was amplified using 16S rDNA primers. The forward and the reverse primer used were 799F AND 1193R respectively (799F – AACMGGATTAGATACCCKG; 1193R – ACGTCATCCCCACCTTCC).

For PCR, the following conditions were used:

Initial Denaturation	: 94^{0}C for 4 minute	
Denaturation cycles	: 94^{0}C for 4 minute	35Cycles
Annealing	: 60^{0}C for 1 minute	
Extension	: 72^{0}C for 1 minute	
Storage	:4^{0}C	

The PCR product was analyzed on 1.5% Agarose gel electrophoresis. PCR product was sent for sequencing at Eurofins Scientific India Pvt. Ltd., Bangalore, Karnataka, India. The assembled sequence was subjected to NCBI-BLAST analysis. The result was analyzed with the top ten similar sequences and retrieved in FASTA format.

2.5 Extraction of Pigment

The colored bacterial pellet was harvested from 24-hour culture nutrient broth through centrifugation at 8,000 rpm for 10 minutes and washed by adding twice the amount of sterile distilled water and centrifuged at 5000 rpm for 5 minutes [Fig.1]. The pellet alone was retained and mixed with twice the amount of methanol, incubated for 15 minutes at 60°C water bath to allow the cell to get lysed. The pellet was totally de-colorized when absolute methanol was used. Then the released pigment in the supernatant was collected separately by centrifugation at 2000 rpm for 10 min. The supernatant was then filtered through Whatman no. 1 filter paper and dried under a Hot air oven at 45°-50°C [16].

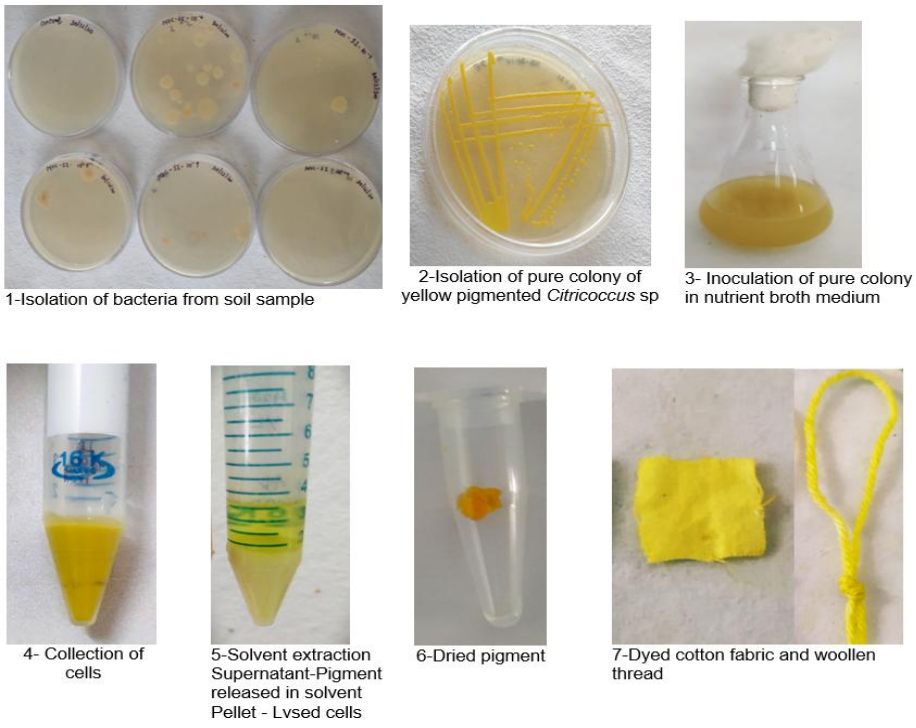

Fig. 1. Diagrammatic representation of the extraction procedure

2.6 Optimization of Parameters for Pigment Production

2.6.1 pH optimization

50 ml nutrient broth was prepared and the pH of the medium was adjusted to 4, 5, 6, 7, and 8 individually using 1N HCl and 1N NaOH and sterilized. 10µl of inoculum (5×10^6 CFU/ml) was added to the media and incubated at 37°C for 24 hours in a shaker at 120 rpm.

2.6.2 Temperature optimization

The culture flask with the test organism was incubated at varying temperatures of 20°C, 25°C, and 37°C respectively.

2.6.3 Incubation period optimization

The test organism was inoculated into four independent culture flasks, each of which was incubated at 37°C. The pigment synthesis was estimated at 24, 48, 72, and 96-hour intervals.

2.6.4 Media optimization

2.6.4.1 Nitrogen source

To the 50 ml nutrient broth, 0.5% of nitrogen supplements like urea, beef extract, peptone, and tryptone were added individually and sterilized. 10µl of inoculum (5×10^6 CFU/ml) was inoculated and incubated overnight at 37°C in the shaker. After 24- hours of incubation, the quantity of the pigment was extracted and quantified.

2.6.4.2 Carbon source

To the 50 ml nutrient broth, 1% of carbon supplements like lactose, maltose, dextrose, sucrose, and glycerol were added individually and sterilized. 10µl of inoculum (5×10^6 CFU/ml) was inoculated in each of the culture flasks and incubated overnight at 37°C in the shaker. After 24- hours of incubation, the pigment was extracted and quantified.

2.7 Phytochemical Test

2.7.1 Carotenoid test

To the 1ml of extracted pigment, 2 ml of chloroform was added, shaken vigorously, and filtered through Whatman no.1 filter paper. A few drops of 85% sulphuric were added to the filtrate and observed for the color change. The presence of carotenoids will be indicated by the emergence of the blue color [17].

2.8 Characterization of Pigment

2.8.1 UV-Visible spectroscopy

UV-Visible spectroscopy (UV-Vis) analysis was carried out and the absorbance range of the extracted pigment was scanned between 400nm-700nm. Methanol was used as blank [18].

2.8.2 Fourier-transform infrared spectroscopy (FTIR)

The methanolic extract of pigment was analyzed by liquid FTIR in the range of 4000-5000cm^{-1} [19].

2.9 Dyeing of Cotton Fabrics Using Extracted Pigment

For the dyeing process, the cotton fabric of 2×2 diameter and the woollen thread was pre-washed to eliminate any wax and grime, then steeped into the extracted pigment and left overnight. The next day, the fabric was immersed in the mordant, copper sulphate (CuSO4) at the concentration of 0.5 gram/L and kept in the water bath (60°C for 20 minutes) to increase the binding capacity. Finally, the fabric was washed with normal tap water, dried, and tested against acid, alkali, detergent, cold water and hot water to inspect whether the fabric retains its color strongly [20,21].

3. RESULTS AND DISCUSSION

3.1 Isolation of Bacteria

From a group of bacterial colonies, intracellular yellow-pigmented bacteria were isolated (Table 1).

3.2 Characteristics of the Bacteria

Table 1. Shows the Morphological characteristics of the bacteria

Characteristics	Observation
Gram nature	Gram-Positive cocci
Motility	Non-motile
Colony characteristics	
Colony shape	Circular
Colony opacity	Opaque
Elevation	Convex
Margin	Entire
Surface	Smooth
Color	Yellow

3.3 Biochemical Tests

Biochemical tests were performed to identify the name of the bacterium based on the difference in their biochemical activity. The bacteria were tested negative for Methyl red, Voges-Proskauer, oxidase, citrate, triple sugar iron test, indole test, and positive for catalase test.

3.4 Sugar Fermentation Test

A sugar fermentation test was carried out to detect the ability of the bacteria to ferment both the simple and complex sugar molecules by following the glycolysis pathway, along with the production of gas. The following Table 2 shows the observation.

3.5 Molecular characterization

Through 16S rDNA gene sequencing, the name of yellow-pigmented bacteria was identified as *Citricoccus* sp.

3.6 Pigment extraction

The Pigment from the bacteria was tried extracting using different solvents like ethyl acetate, methanol, hexane, ethanol, acetone, and chloroform (Fig. 2). Compared with other solvents, methanol was found to be the best in solubilizing the pigment from the bacteria. The methanol extract of the pigment was collected in a petri dish and dried under a hot air oven at 45°-50°C.

3.7 Media Optimization

3.7.1 pH optimization

The *Citricoccus* sp showed growth in the acid and alkaline environment, but the pigment production was high at pH 7(9mg/50ml). At pH 4, the quantity of pigment was 1mg/50ml, at pH 5, it was 2mg/50ml. At pH 6 and 8, the yield was 7mg/50ml [Fig. 3].

3.7.2 Temperature optimization

When the temperature was held at 37°C, the pigment production was 8mg/50ml. At 27°C the pigment production was 6mg/50ml and at 24°C it was 3mg/ml [Fig. 4].

Table 2. Sugar fermentation test

Sl. No	Name of the sugar	Sugar utilization	Gas production
1	Sucrose	–	–
2	Dextrose	+	–
3	Maltose	–	–
4	Lactose	–	–

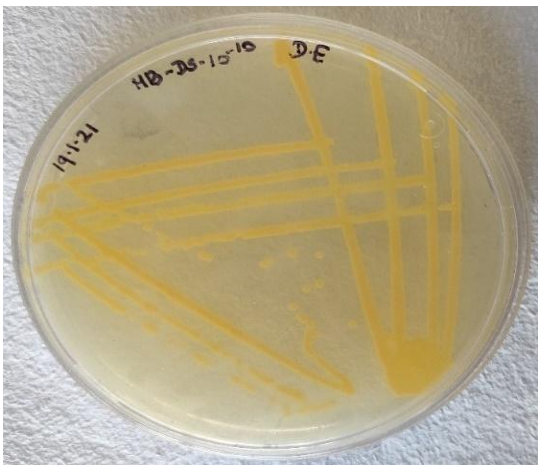

Fig. 2. Yellow pigmented bacteria plated on nutrient agar medium

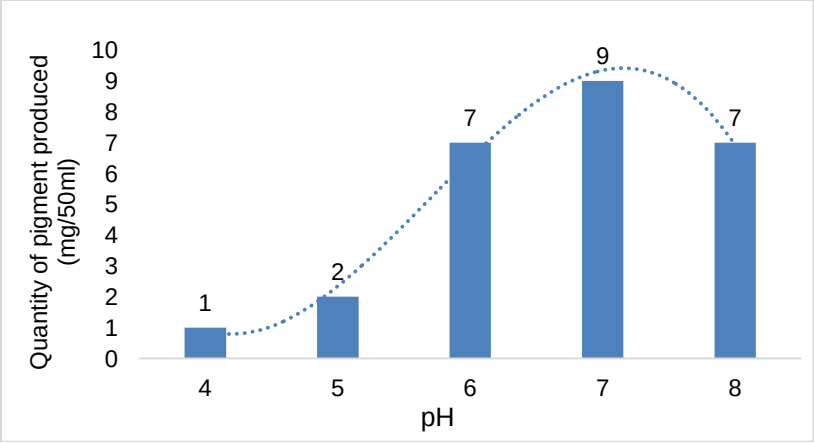

Fig. 3. Effect of different pH on the production of yellow pigment by *Citricoccus* sp.

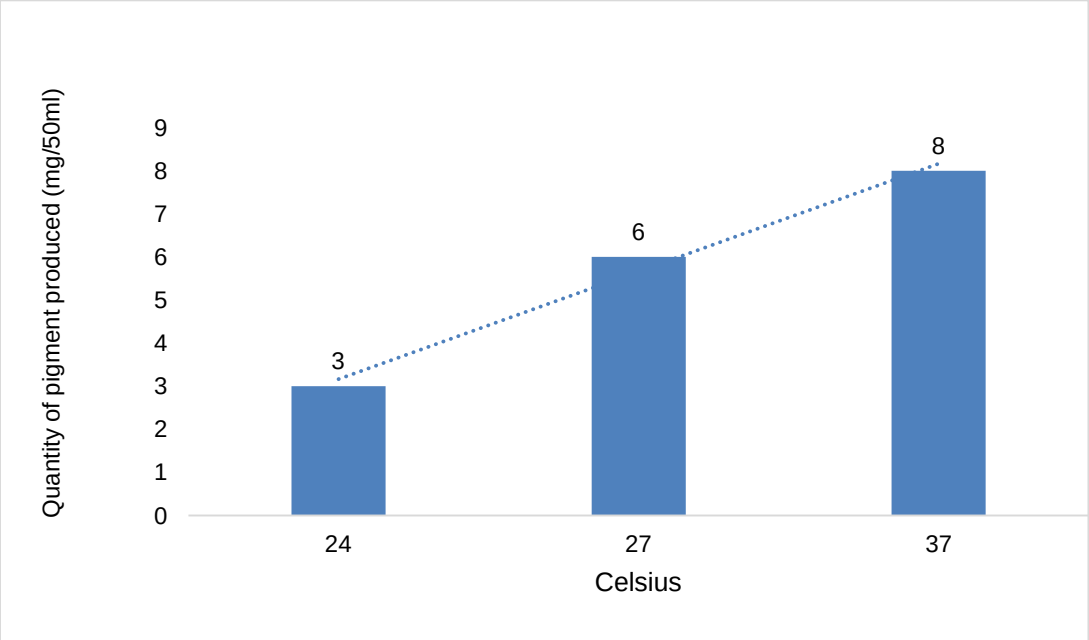

Fig. 4. Effect of different temperatures on the production of yellow pigment by *Citricoccus* sp

3.7.3 Incubation period optimization

The highest yield of yellow pigment production was noted at the 24th hour of incubation(10mg/50ml). The cells begin to die as the incubation time increases, resulting in reduced pigment output [Fig. 5].

3.7.4 Media optimization for carbon source

Citricoccus sp generated 8mg/50ml of pigment in regular nutrient broth medium [peptone (5g/L), beef extract (1g/L), NaCl (5g/L), yeast extract (2g/L)]. Cell biomass significantly high [27mg/50ml] when the culture media was supplied with 1% of glycerol a carbon source, indicating greater pigment formation [sucrose 14mg/50ml, dextrose 16mg/50ml, lactose 20mg/50ml, maltose 26mg/50ml] [Fig. 6].

3.7.5 Media optimization for nitrogen source

Citricoccus sp generated 8mg/50ml of pigment in regular nutrient broth medium [peptone (5g/L), beef extract (1g/L), NaCl (5g/L), yeast extract (2g/L)]. The amount of pigment generated in each substrate increased when the growth media was supplied with 0.5% of a specific nitrogen source [Fig.7]. The pigment production was highest when beef extract [21mg/50ml] was used, [urea 10mg/50ml, peptone 13mg/50ml, tryptone 17mg/50ml].

3.8 Phytochemical Test

3.8.1 Carotenoid test

The appearance of blue color confirms the presence of carotenoid in the pigment.

3.9 Characterization

3.9.1 UV-visible spectroscopy

The yellow pigment extracted from *Citricoccus* sp showed the highest peak at 437nm. This result implies that pigment belongs to the carotenoid group because carotenoids usually absorb UV at 400-500 nm [22].

3.9.2 Fourier transmission infrared spectroscopy

The FTIR analysis showed a unique functional group and chemical bond [Figs. 8-10, Table 3]. Yellow pigment showed the following functional group of =C-H(900.70cm^{-1}), NO_2 (1339.47 cm^{-1}), O-H (2719.44cm^{-1}), C-H (2843.84cm^{-1}), N-H (3448.49cm^{-1}) and their absorption frequencies corresponds to that of beta-carotene.

3.10 Dyeing of Cotton Fabrics using Extracted Pigment

Cotton fabrics and woollen threads were successfully dyed using the pigment extracted

from *Citricoccus* sp. The use of copper sulphate in the concentration of 0.5 gram/L as mordant has fixed the pigment with the fabric so well, that hasn't vanished even after washing several times in water and detergent. Fig. 11 depicts the outcomes.

Table 3. FTIR spectrum of yellow pigment extracted from *Citricoccus* sp with corresponding functional groups

Yellow pigment				
Sl. No	**Wavenumber (cm^{-1})**	**Group**	**Functional group**	**Vibration**
1	3448.49	N-H	Amide	Medium
2	2949.92	C-H	Alkanes	Medium To Strong
3	2843.84	C-H	Aldehyde	Strong
4	2719.44	O-H	Carboxylic Acid	Broad, Medium to Weak
5	1637	C=C	Alkenes	Weak To Medium
6	1339.47	NO 2	Nitro Compound	Strong
7	900.70	=C-H	Alkenes	Strong

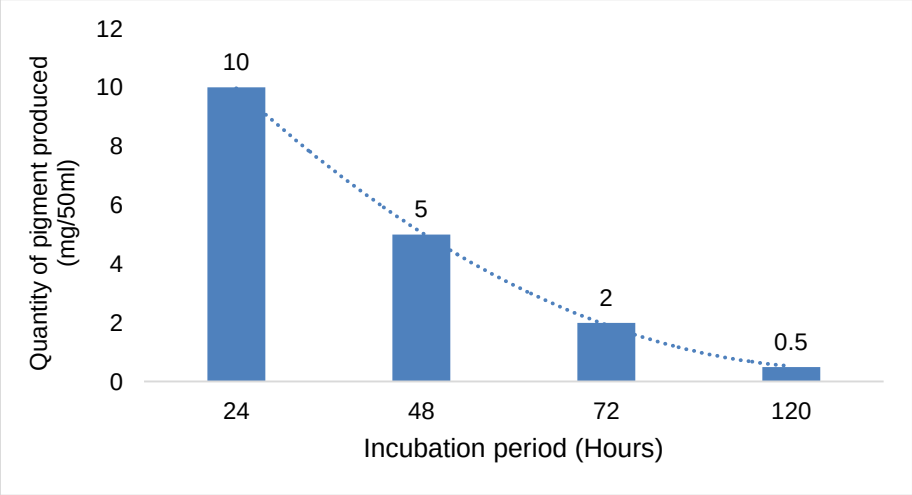

Fig. 5. Effect of different incubation periods on the production of yellow pigment by *Citricoccus* sp.

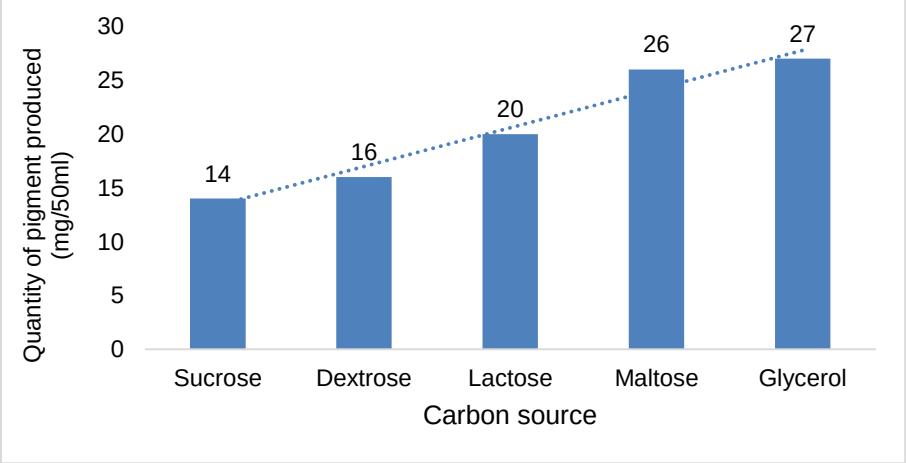

Fig. 6. Effect of various carbon sources on the production of yellow pigment by *Citricoccus* sp.

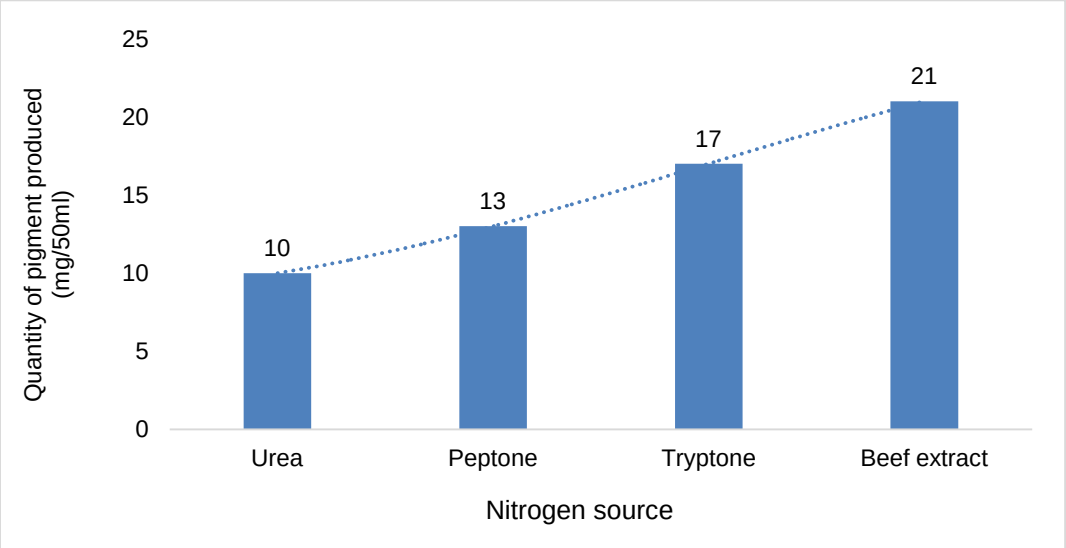

Fig. 7. Effect of various nitrogen sources on the production of yellow pigment by *Citricoccus* sp.

Fig. 8. Yellow pigment extracted from *Citricoccus* sp showing a positive result for carotenoids

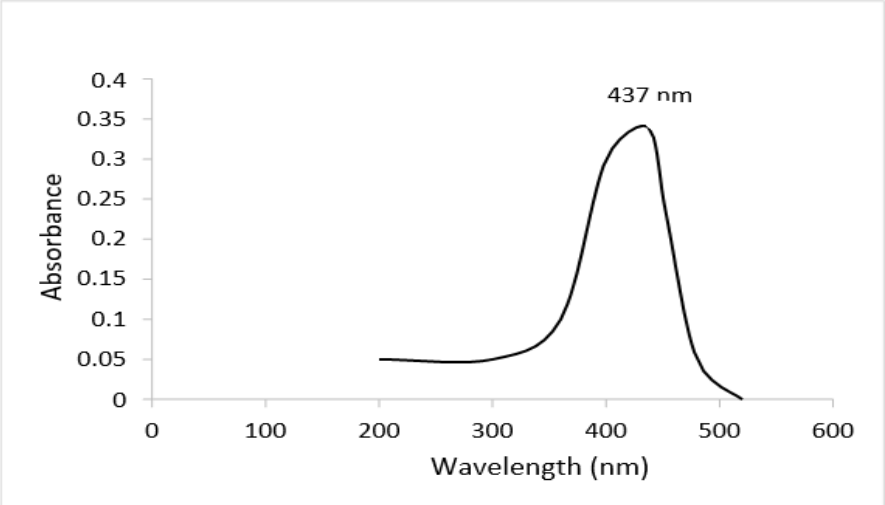

Fig. 9. UV - visible spectrum of yellow pigment isolated from *Citricoccus* sp

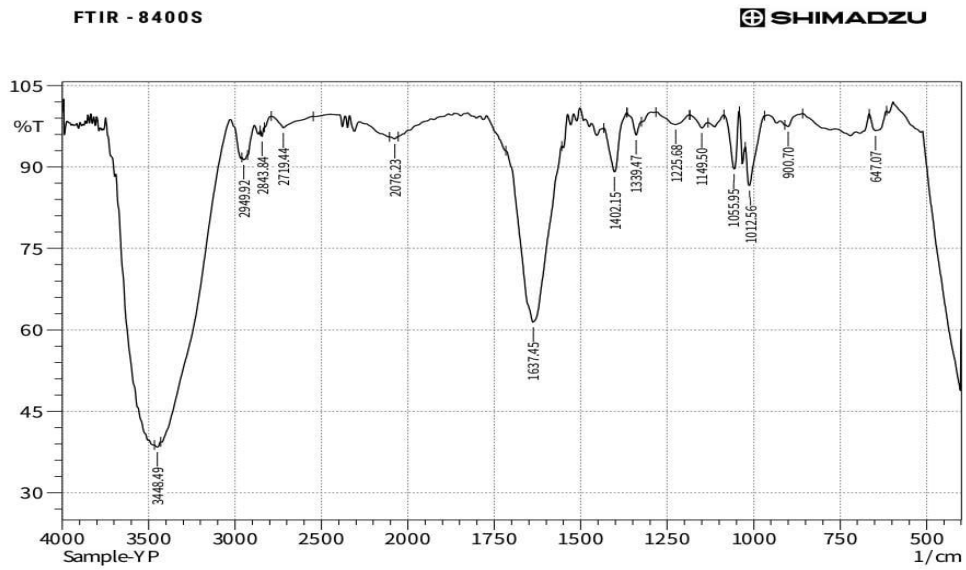

Fig. 10. FT-IR spectrum of the yellow pigment isolated from *Citricoccus* sp

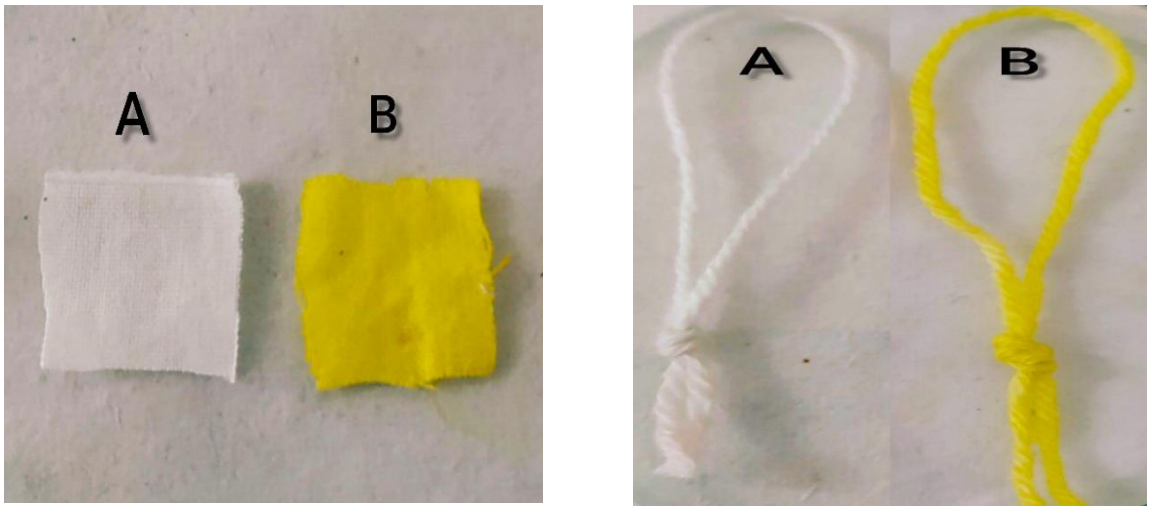

Fig. 11. Cotton fabric and woolen thread dyed using yellow pigment extracted from *Citricoccus* sp (A-control, B- Dyed)

4. CONCLUSION

This study deals with the successful Isolation of Fast- growing yellow pigmented bacterium and optimization of the culture parameters that facilitated the enhanced production and extraction of the pigment produced by *Citricoccus* sp. Sterile nutrient broth medium was found to be good choice of media for production of yellow pigment. This study reveals that the addition of 0.5% beef extract as nitrogen source and 1% glycerol as carbon source increases the pigment production. The optimum growth and pigment production of *Citricoccus* sp. was achieved at pH 7 in 37°C. Through UV-Spec and FTIR characterization, the pigment is confirmed to be a carotenoid compound that can be used to dye cotton fabrics and a wool effectively and uniformly. Since the extraction is cost-effective and economical, this study can be further taken up for finding the possibilities to use them in the textile industry and dyeing industry so that they could be of great use to mankind [23,24].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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