Original Research Article

Yellow pigment from Fast-Growing marine soil bacteria *Citricoccussp* for dyeing cotton fabrics

Abstract

Microbial pigment production is one of the burgeoning fields of research, with the potential to be used in the textile, cosmetics, and food industries. *Citricoccus*spisfast-growing intracellular yellow-pigmented bacteria isolated from Marina beach, Chennai, Tamil Nadu. Through a 10-fold serial dilution method, the bacteria were isolated on a nutrient agar medium and screened for biochemical test and pigment production. The current study focuses on the evaluation of microbial pigment for dyeing cotton fabrics and woolen thread, also finding the economical way to extract the pigment. The rate of pigment production was increased by optimizing the pH and temperature along with the addition of 1% carbon and 0.5% nitrogen sources. The pigment was extracted using methanol as solvent and was characterized by UV-Visible spectroscopy and Fourier Transformed Infrared (FTIR) spectroscopy whose values provethat the pigment belongs tothe carotenoid group. Through phytochemical analysis, the presence of carotenoid was confirmed.

Keywords: Biochemical test, Carotenoid, *Citricoccus*sp, Dyeing, Fabrics, FTIR, Marine, UV-spec

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. 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. 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.

There are many bacteria like *Serratia marcescens*, *Pseudomonas aeruginosa*, *Janthinobacteriumlividum*, *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. 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.

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 woolen thread.

2. Materials and Methods

2.1 Sample collection

The soil sample was collected in a sterile container from Marina beach, Chennai, Tamil Nadu. Taken to the laboratory within one hour 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 16SrRNA amplification

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

For PCR, the following conditions were utilized:

Initial Denaturation : 94°C for 4 minutes

Denaturation cycles : 94°C for 4 minute

Annealing : 60°C for1minutes

Extension : 72°C for 1minute

(Denaturation, Annealing, Extension: 35Cycles)

Storage :4°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. The pellet alone was retained and mixed with twice the amount of methanol, maintained for 15 minutes in a 60°C water bath to allow the cell to get lysed. The pellet was totally de-colorized when absolute methanol is utilized. 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 paperand dried undera Hot air oven under 45°-50°C [1].

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.

2.6.3 Media optimization

2.6.3.1 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.

2.6.3.2 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.

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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 [2].

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 [3].

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} [4].

2.9 Dyeing of cotton fabrics using extracted pigment

For the dyeing process,the cotton fabric of 2×2 diameter 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) in the concentration of 0.5 gram/L and kept in the water bath (60°C for20 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 [5].

3. Results

3.1 Isolation of bacteria

From a group of bacterial colonies, intracellular yellow-pigmented bacteria were isolated.

Figure 1 Yellow pigmented bacteria plated on nutrient agarmedium

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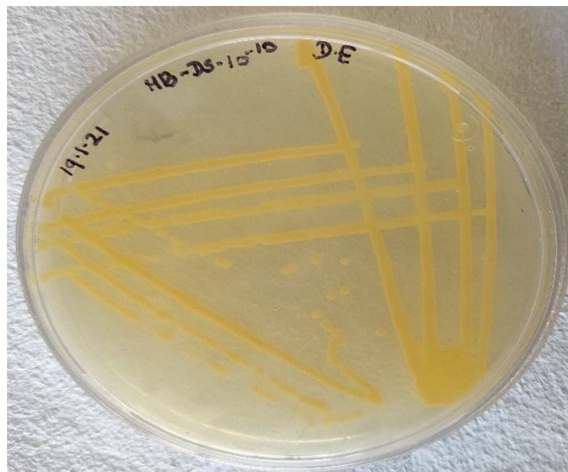


3.2 Characteristics of the bacteria

It is a non-motile, Gram-positive coccus that appears as a yellow, circular, opaque, convex colony with a smooth surface on an agar plate.

3.3 Biochemical tests

Biochemical tests are 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, indole test, and positive for oxidase test.

3.4 Sugar fermentation test

A sugar fermentation test is done 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 bacterium was able to utilize dextrose, but not the sucrose, maltose, dextrose and no gas production was observed.

3.5 Molecular characterization

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

3.6 Pigmentextraction

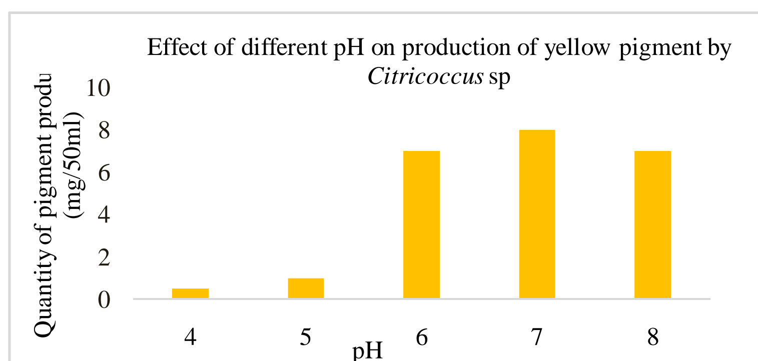
The Pigment from the bacteria was tried extracting using different solvents like ethyl acetate, methanol, hexane, ethanol, acetone, and chloroform. Compared with other solvents, methanol was found to be the best in solubilizing the pigment from the bacteria. The methanol extract of the pigment was collected 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(8mg/50ml). At pH 4, the quantity of pigment was 0.2mg/50ml, at pH 5, it was 1mg/50ml. At pH 6 and 8, the yield was 7mg/50ml.

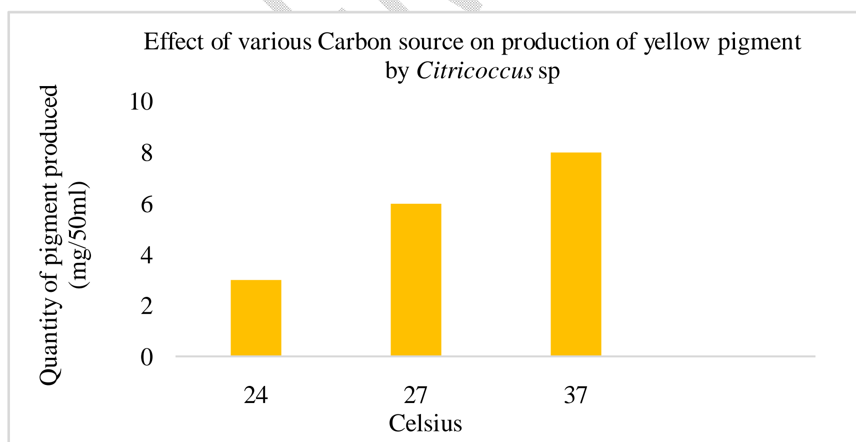
Graph 1 Effect of different pH on the production of yellow pigment by *Citricoccus*sp



3.7.2 Temperature optimization

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

Graph 2 Effect of different temperatures on the production of yellow pigment by *Citricoccus*sp



3.7.3 Incubation period optimization

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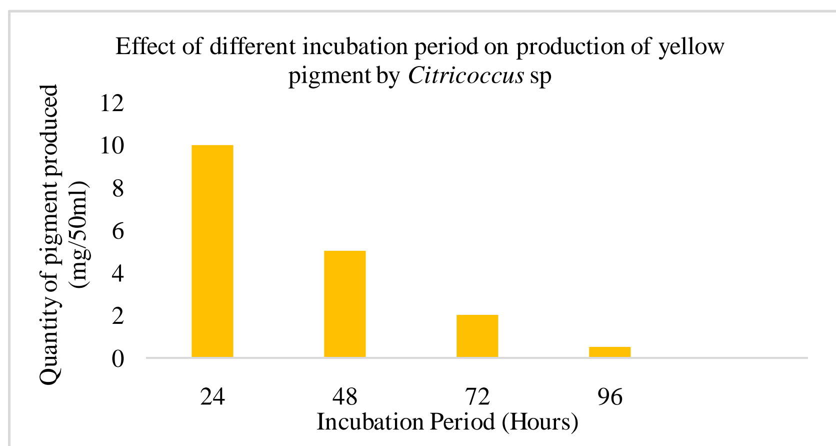
The highest yield of yellow pigment production was noted at the 24th hour of incubation(10mg/50ml). The cells begin to die as the incubation time increases, resulting in reduced pigment output.

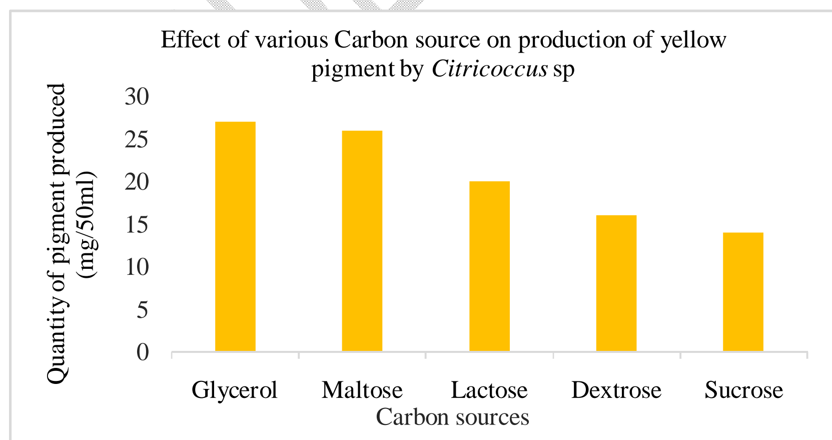
Graph 3 Effect ofdifferent incubation periodson the production of yellow pigment by *Citricoccus*sp



3.7.3 Media optimization for carbonsource

*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 increased when the culture media was supplied with 1% of distinct carbon source, indicating greater pigment formation in each substrate. [sucrose 13mg/50ml, dextrose 16mg/50ml, lactose 20mg/50ml, maltose 26mg/50ml, glycerol 27mg/50ml]

Graph4 Effect of various carbon sources on the production of yellow pigment by *Citricoccus*sp



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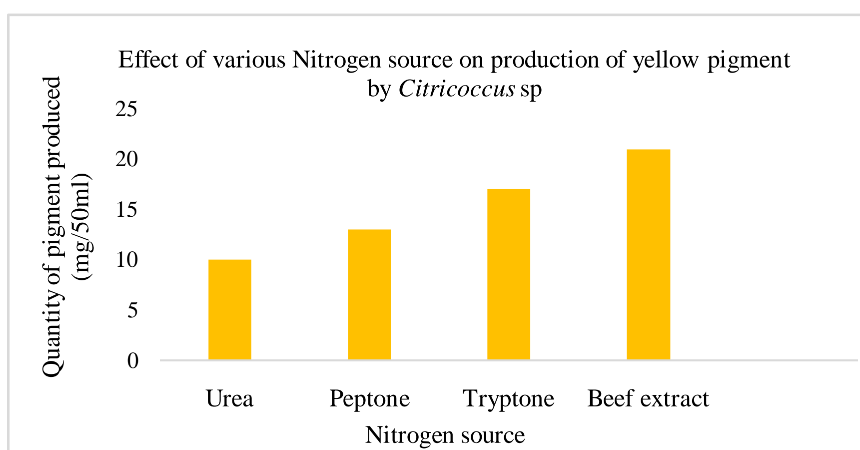
3.7.4 Media optimization for nitrogensource

*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. [urea 10mg/50ml, peptone 13mg/50ml, tryptone 17mg/50ml, beef extract 21mg/50ml,]

Graph 5 Effect of various nitrogen sources on the production of yellow pigment by *Citricoccus*sp

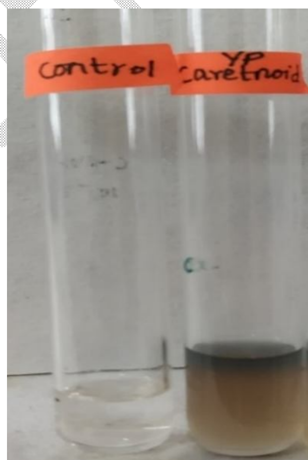


3.8 Phytochemical test

3.8.1 Carotenoid test

The yellow pigment extracted from *Citricoccus*sp showed the presence of carotenoids.

Figure 2 Yellow pigment extracted from *Citricoccus*sp showing a positive result for carotenoids



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3.9 Characterization

3.9.1 UV-Visiblespectroscopy

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 [6].

Figure 3 UV - Visible spectrum of yellowpigment isolated from *Citricoccus*sp

3.9.2 Fourier transmission infraredspectroscopy

The FTIR analysis showed a unique functional group and chemical bond. 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.

Figure 4 - FT-IR spectrum of the yellow pigment isolated from *Citricoccus*sp.

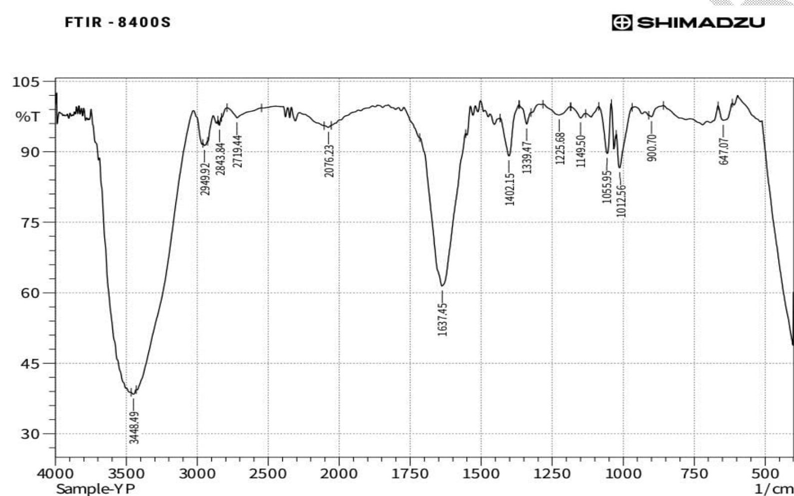

Table 1FTIR spectrum of yellow pigment extracted from Citricoccussp with corresponding functional groups

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3.10 Dyeing of cotton fabrics using extracted pigment

Cotton fabrics and woolen threads were successfully dyed using the pigment extracted from *citricoccus*sp. The use ofcopper 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. Figure 5 and 6 depicts the outcomes.

Figure 5 Cotton fabricdyed using yellow pigment extracted from *Citricoccus*sp (A-control, B- Dyed fabric)

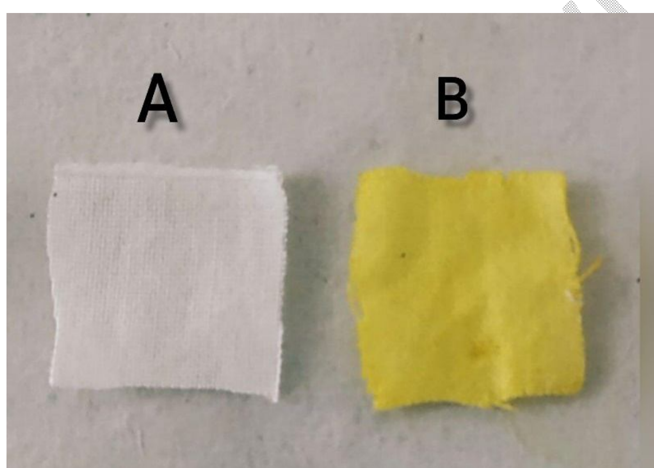

Figure 6 woolen thread dyed using yellow pigment extracted from *Citricoccus*sp (A-control, B- Dyed woolen thread)

YELLOW PIGMENT				
SI.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

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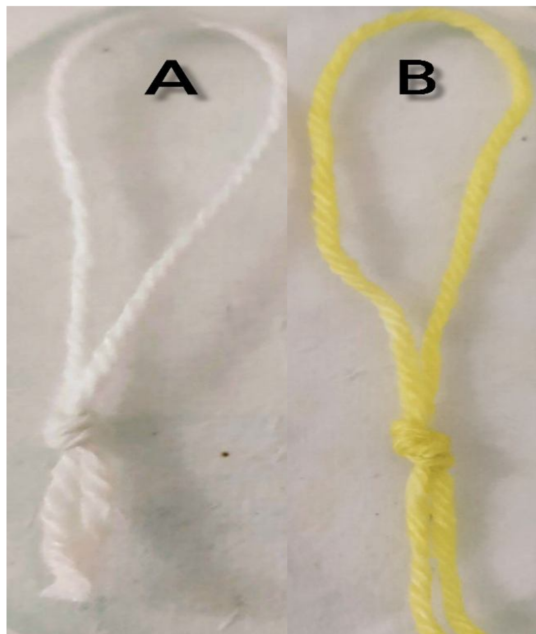

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4. Discussion

In this study, *Citricoccus*sp was isolated from the marine soil. It is an aerobic microorganism that showed optimum growth in nutrient agar medium when the temperature is maintained at 37°C in pH 7. Some pigmented bacteria take more than 5 days to produce a considerable quantity of pigment [6]. To highlight, the bacteria isolated in this study showed pigment production within 24 hours. Pigmentsare secondary metabolites with a variety of biological roles, their production can be markedly influenced by manipulating the formulation of nutrient sources. The preference of any nitrogen or carbon source totally depends upon the demand for bacteria for vitamins, amino acids, purines, and pyrimidines. The addition of 1% of carbon sources like glycerol increased yellow pigment yield from 8mg/50ml to 27 mg/50ml.Similarly, the addition of 0.5% beef extract as a Nitrogen source has uplifted the pigment yield from 8mg/50ml to18mg/50ml. This demonstrates that the addition of nutrient sources causes a significant alteration in cell metabolism.

Through phytochemical test and by characterization of the pigment, it was found to be beta-carotene belongs to the carotenoid group. Carotenoids are lipophilic compounds and soluble in the organic solvent [7]. In this study, methanol was used to extract the pigment because it is more effective as it interacts with the pigments through non-

covalent interactions and promotes a rapid diffusion of the pigments into the solution [8]. It was reported that any solvent individually or a combination of any two solvents in different concentrations can also be used to extract pigment from various microorganisms [3].When compared to previous research findings, the extraction process in this study is cost-effective since it uses a very minimal amount of methanol [9].Cotton fabric and woolen thread were dyed using the pigment derived from *Citricoccus*sputilizing copper sulphate as mordant in the concentration of 0.5gram/L. Bacterial pigments containing substantial phytochemicals have been shown to have antimicrobial and anti-cancer properties in diverse studies [10]. In addition to it,researchers have also tested dye papers, soaps, and candles using bacterial pigment.

5. Conclusion

According to the findings of this study, *Citricoccus*sp is a fast-growing bacterium capable of producing a higher amount of pigment under optimal conditions. Through UV-Spec and FTIR characterization, the pigment is confirmed to be a carotenoid compoundthat 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.

8. References

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