International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 06-08-2021, Accepted: 19-08-2021, Published: 17-09-2021
Volume 6, Issue 5, 2021, Page No. 52-56

Extraction and characterization of microbial pigment

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Abstract
Pigments from natural sources have been obtained since long time ago, and their interest has increased due to the toxicity problems caused by those of synthetic origin. Many microorganisms including bacteria, fungi, yeast and mold are employed for the industrial production of various pigments by fermentation technology. These microbial pigments have broad area of application mainly in food industries, pharmaceutical and textile industries. Microbial pigments have numerous beneficial properties like anticancer, antiproliferative, immunosuppressive, antibiotic, biodegradability etc. Thus, the microbial pigments are looked upon for their safe use in food, pharmaceutical and textile industries will not only benefit human health but also preserve the biodiversity, as harmful chemicals released into the environment while producing synthetic colorants could be stopped.

Keywords: microbial pigments, pharmaceutical, anticancer, antiproliferative, immunosuppressive, antibiotic, biodegradability

Introduction
Colour has been used to enhance the aesthetic value of everyday human life [1]. Natural pigments sourced from ores, insects, plants and animals were used as colourants, since prehistoric period. In the middle of 19th century, Synthetic dyes took the place of natural pigments still rule the field to the maximum extent, in spite of its hazardous effects to humans, animals and environment. The colourant, refers to any chemical compound that imparts colour, while the pigments indicate normal constituents of cells or tissues giving colour. Plants, animals and microbes are the sources of natural pigments. The natural pigments extracted from microorganism are termed as "microbial pigments" [2], where bacteria, algae and fungi produce variety of pigments and therefore, are the promising source of food colorants [3, 4] which possess the desirable properties like stability to light heat and pH [5]. Microorganisms belongs to the species of *Monascus*, *Paecilomyces*, *Serratia*, *Cordyceps*, *Streptomyces*, and yellow-red and blue compounds produced by *Peniciilium herquei* and *Penicilliu matrovenetum*, *Rhodotorula*, *Sarcina*, *Cryptococcus*, *Monascus purpureus*, *Phaffia* sps., *Bacillus* sps., *Achromobacter*, *Yarrowia* and *Phaffia rhodozyma* have the ability to produce pigments in high yield [6]. Bacteria are a good source of pigments can be an alternative to synthetic pigments, as they have a better biodegradability and higher compatibility with the environment, offer promising avenues for various applications [7]. Thus, Natural colourants will not only be beneficial to the health of human beings, but it will be a boom for the preservation of biodiversity. Colour of a food substance is important to indicate its freshness and safety that are also indices of good aesthetic and sensorial values. Consumers are avoiding synthetic food colorants and ready to buy food products with natural colorants at premium price. The demand for natural source of such compounds is increasing day by day because of awareness of positive health benefit out of natural compounds. It is therefore, essential to explore various natural sources of food grade colorants and their use potentials. Most often, the colorants are extracted from plant material, but other sources such as insects, algae, bacteria, and fungi are used as well. Additionally, organisms other than plants can span the entire colour spectrum and thereby reducing dependence on synthetic colours. Microorganisms could be made to produce colorants in high yield by inserting genes coding for the colorant even colorants not naturally produced by microorganisms (e.g., turmeric) could be made in this way. Furthermore, natural colorants will not only be beneficial to the health of human beings, but it will be a boon for the preservation of biodiversity as harmful chemicals released into the environment while producing synthetic colorants could be stopped [8]. Novel technologies and sources could facilitate the application of natural colorants to produce a broad variety of hues, which can increase stability of food matrices, by materials that impart health-promoting properties in addition to color [9].

Materials and Methods
Black soil sample was collected in a large sterile container from Murukkankudi, Perambalur district for isolation of pigment producing bacterial species was stored at 27˚c room temperature.

Isolation of pigmented bacteria
From the collected soil sample, soil suspension was prepared using distilled water in the ratio of 1 gram of soil sample in 10 ml distilled water**.** Soil suspension was streaked on sterile nutrient agar plates and incubated at 37 c for 48 hours. Only the pigmented bacterial colonies were selected and sub-cultured on the nutrient agar plates for further studies.

Morphological identification and biochemical characterization of the pigmented isolates
The isolated coloured bacterial isolates were further analysed for their morphological characteristics. Based on

Bergey's manual of Determinative Bacteriology (1995), the following tests gram staining, motility test and biochemical characterization of the pigmented isolates were performed to identify and confirm the isolated organisms.

Physiological characterisation of pigmented isolates
The isolates were inoculated into nutrient broth of five varied pH range such as neutral, acidic ranges and alkaline, then were incubated for 24- 48 hours at 37˚c. Pigmented isolates were inoculated into nutrient broth of neutral pH and incubated at three temperature ranges such as 27˚c, 37˚c and 4˚c, respectively.

Qualitative screening of lipolytic production
The ability of the selected isolates to produce the enzyme lipase was screened using tributyrin nutrient agar medium supplemented with glyceryl tributyrate. The plates were streaked vertically and incubated for 48-72 hours at 37˚c. The organism itself produce clear zone around the colonies was recorded as positive.

Production of bacterial pigment
The pigment producing isolates were inoculated into sterile nutrient broth and incubated at room temperature in a rotatory shaker for 72 hours, until the visible of pigment colour in the medium.

Extraction of bacterial pigment from isolated strains
The pigments were isolated using liquid-liquid extraction method by inoculating into sterile nutrient broth and incubated at 37°c in a rotatory shaker for about seven days. At the end of incubation, the pigments were extracted by centrifuging at 6000 rpm for 15 minutes, until the complete extraction of pigment with solvent.

Characterization of extracted pigments
The extracted purified pigments were further characterized physically and chemically using analytical techniques by thin layer chromatography. Silica gel was used as a stationary phase and chloroform-methanol in the ratio 95:5 used as a solvent system for mobile phase. The pigment samples were spotted on the silica gel slide, air dried and dipped in solvent system. The complete set-up is allowed undisturbed for the elusion of particles for about 30 minutes. The plate was removed and retention factor (Rf) for each sample was calculated.

$$Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Test for carotenoids and flexirubin type pigment
The nature of the extracted pigment was tested by treating the samples with few drops of 85% sulphuric acid. The appearance of blue colour indicates the presence of carotenoids. A small mass of bacterial isolates collected with loop and deposited on a glass slide and flooded with 20% KOH. The colour shift of the colony changes from yellow-orange to red-brown indicates flexirubin type pigment. It is a small diagnostic test for flexirubin.

Antibacterial activity (disc diffusion method)
The extracted pigments were tested for antibacterial activity by disc diffusion method. Muller hinton agar plates were prepared and swabbed with 24 hours old cultures of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The plates were incubated at 37°c for 24 hours. The zone of inhibition around the discs indicates pigments having antimicrobial activity.

Antioxidant activity
The free radical scavenging of pigment was measured by ascorbic acid assay. Total antioxidant capacity of extracted pigment was carried out by ascorbic acid assay. Colorimetric readings were taken at 610 nm.

Application of pigment in dyeing
A pre washed cloth was first immersed in 0.5% NaCl solution for half an hour for fixation. After fixation, the cloth was placed in dye bath to absorb colour for 20 to 30 minutes. The cloth was kept for drying overnight.

Results and Discussion
In the present study, the overall aim was to isolate the microorganisms which are able to produce microbial pigments as its secondary metabolite.

Isolation of pigmented isolates
Morphological, physiological and biochemical characterization tests were carried out to identify the pigmented organisms. These isolates were screened for their ability to produce extracellular enzymes. Extraction, physical and chemical characterization of microbial pigments from the isolates were carried out to study the ability of extracted pigments. Two different isolates were identified from the black soil sample and based on the colour of the pigment produced it was designated as orange and yellow (Fig.1).

Cultural characterization of pigmented isolates
The two isolates were selected on the basis of the different colour it produces on the nutrient media. The morphological characterization of the isolates was to determine their cell shape, cell arrangement, gram nature, motility and colony morphology on the agar plates and results were presented in the Table 1 and Figure 1.

Table 1: Morphological characterization of the isolates

Characters	Orange	Yellow
Colony shape	Round	Circular
Colour	Orange	Yellow
Margin	Punctiform	Entire
Elevation	Convex	Flat
Opacity	Opaque	Transparent
Gram nature	Gram –ve	Gram –ve
Cell shape	Cocci	Cocci
Motility	Non motile	Non motile

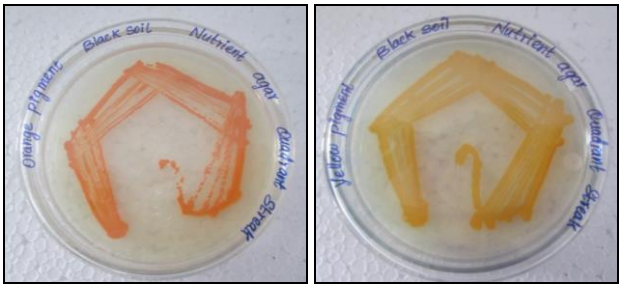

Fig 1: Isolated Pigmented Bacterial Cultures

Biochemical characterization of the isolates
The biochemical characters of the pigmented isolates were shown in the Tables 2 and Figure 2.

Table 2: Biochemical and Sugar Fermentation Tests

Biochemical tests	Orange	Yellow
Indole	-ve	-ve
Methyl red	+ve	+ve
Voges-proskaur	+ve	+ve
Citrate	-ve	+ve
Urease	+ve	+ve
TSI	+ve, red butt and slant	+ve, yellow butt, red slant, gas production
Nitrate reductase	+ve	-ve
Oxidase	+ve	+ve
Catalase	+ve	+ve
Sugars	Orange	Yellow
Glucose	-ve	+ve
Sucrose	-ve	+ve
Lactose	+ve	-ve
Fructose	-ve	-ve

Note: +ve represents positive and –ve represents negative.

Physiological characterization of the isolates
Effect of different pH range and temperature ranges on growth of the isolates were studied and tabulated in Tables 3.

Table 3: Effect of different pH and temperature range

pH	Orange	Yellow
2	-ve	-ve
4	-ve	-ve
7	+ve	+ve
9	+ve	+ve
10	-ve	-ve
Temperature (°C)	Orange	Yellow
20	+ve	+ve
37°c	+ve	+ve
4°c	-ve	-ve

Note: +ve represents positive and –ve represents negative

Qualitative screening for lipase production
Both the isolates show lipase activity. Orange sample show increased lipase activity with maximum zone around its growth in the plate supplemented with tributyrin whereas, yellow sample show very low lipase activity (Fig. 2 & 3).

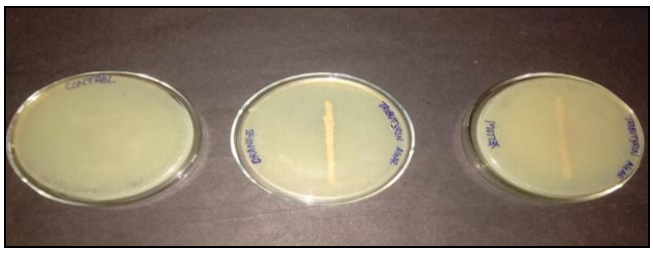

Fig 2: Lipase Activity

Fig 3: Pigment Production

Extraction of pigment from cell biomass
Different solvents like acetone, methanol, water, ethanol, chloroform and hexane were used to check for the maximum solubility of pigments where, chloroform was used to extract the pigment in partial purification (Figure 4). Finally, acetone was used as a solvent for extracted pigments.

Fig 4: Extracted Pigment

Characterization of extracted pigments
Thin layer chromatography was carried out for crude acetone extract of orange pigment sample. TLC showed that the pigment orange showed an Rf value of 0.83. The Rf range between 0.89 to 0.99 denotes the pigment be a carotene (Figure 5).

Fig 5: Thin layer Chromatography

Test for carotenoids and flexirubin type pigment

Both the pigment samples were screened for carotenoid test. The colour shift of the pigment samples was changed to yellow to deep red colour after addition of sulfuric acid. This shows that the pigment samples are not a carotenoid. Both the pigment samples were tested for type of pigment. After the addition of 20% KOH solution, both the cultures did not show any colour change. This shows that both the samples are not flexirubin type pigment.

Antibacterial activity

Antibacterial activity of pigments was carried out against 4 cultures such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae and Pseudomonas aeruginosa* by disc diffusion method. The antibiotic susceptibility test was studied against four cultures to define the antibiotic profile of the extracted pigments. Among all the cultures, orange pigment shows resistant to *Pseudomonas aeruginosa*. Yellow pigment shows increased sensitivity against *Klebsiella pneumoniae*. Orange pigment show increased sensitivity zone against *Staphylococcus aureus*. The zone of inhibition was measured in mm and tabulated in Figure-6. This antibacterial profile of the pigments shows that, they act well against both the gram positive and gram-negative species.

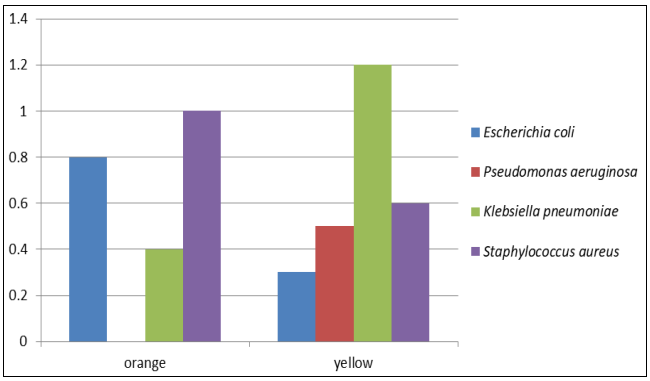

Fig 6: Antibacterial activity of extracted pigment

Antioxidant activity

The antioxidant activity was carried out by ascorbic acid assay. This test is based on the colour shift of the samples when added with reagent and incubated in water bath at 80°c for 10 minutes. Ascorbic acid is used as a standard. Calorimetric readings were taken at 610nm. The orange pigment shows increased scavenging activity at 125µl and the yellow pigment show increased scavenging activity at 25µl concentration (Figure-7)

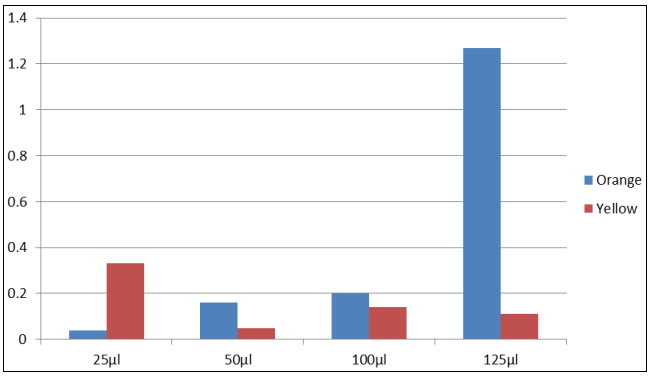

Fig 7: Antioxidant activity of extracted pigments

Application of pigment in dyeing

The extracted bacterial pigment orange was used to dye cotton cloth. The dye was applied to the cotton which is fixed in NaCl solution and kept for drying overnight. The fabric retained the orange colour of the pigment (Figure 8). Thus, orange pigment can be utilized in the textile industries replacing synthetic dyes hence being more eco-friendly.

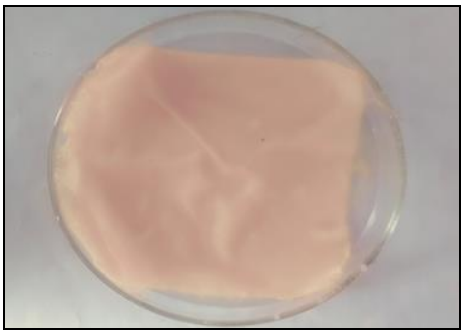

Fig 8: Application of pigment in dyeing

Discussion

Pigmentation is a common characteristic of many bacterial species. Black soil was sampled for isolation and identification of pigment producers. Both the isolates show lipase activity in the screening of enzyme production, whereas orange pigmented species showed increased lipase activity. The yellow pigment producers do not provide that much yield of pigment in submerged fermentation method. Hence, work on the production of yellow pigment can be carried on solid substrate fermentation by experimenting production on different agro industrial wastes. Low cost by products and residues of agro-industrial origin have shown their potential in production of different pigments by diverse group microorganisms. The fabric retained the orange colour of the pigment. Thus, orange pigment can be utilized in the textile industries replacing synthetic dyes hence being more ecofriendly. Both the yellow and orange pigments showed antioxidant activity to different extents, yet orange pigment showed increased scavenging activity. Both the pigments showed antibacterial activity against both gram-positive and gram-negative species. Thus, these pigments showed broad spectrum of antibacterial profile can be further studied for utilization as a antimicrobial agents.

Summary and Conclusion

Natural colourants will be a boom for the preservation of biodiversity as harmful chemicals released into the environment while producing synthetic colours could be stopped. Recombinant DNA technology for strains development is worth to increase the microbial pigment production that have increased advantages over plant and animal sourced pigments [10]. Bacterial pigments have wide applications in fluorescent probing, organic food and clothing dyes, vitamins (β-carotene), and even industrial paints [11]. The current technology status and with metabolic engineering of bacteria the production of bacterial pigments has tremendously increased, as it can be used in food industry, pharmaceutical industry, dyeing, printing, cosmetics as well as on other applications [12]. Microbial pigments act as bioindicators of environmental pollution and also widely utilized in medicinal arena as antioxidants, ant-inflammation, antibiotic, antimicrobial, anticancer, immune modulatory effect etc. Therefore, discovering cheap

substrates for pigment production is the need of the hour for alternative colourants to create pollution free ecosystem. As current inclinations towards health concerns are on rise, nontoxic resources are taken into consideration in various industrial fields.

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