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Research Article

Extraction of Bacterial Pigments and Analysing Their Potential as Natural Food Colourants

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Abstract

Pigments isolated from natural sources like bacteria have garnered attention over the last few decades because they are non-carcinogenic, environmentally friendly, and non-toxic as compared to their synthetic counterparts. They have immense potential to be employed for industrial purposes including food and feed industry. The aim of this study was to isolate pigmented bacteria from soil, extract their pigments and to analyze their potential to be used as natural food colorants. Three pigmented bacteria were isolated (green, orange, and yellow). These isolated bacterial cells were characterized using standard biochemical assays like Gram's staining, catalase, oxidase, citrate, MR-VP, urease, indole and starch hydrolysis tests. Following this, the bacterial pigments were extracted using submerged fermentation technique. Different techniques of bacterial cell lysis were tested (like detergent lysis, alkaline lysis and ultrasonication based) and pigments were extracted from these cells after fermentation. The extracted pigments were added to sooji (porridge) and were also tested for their effects on cell-membrane integrity (through LDH leakage assay) and for their biological properties (like trypan blue dye-exclusion, antioxidant and antibacterial assays). The pigments gave good and stable coloration when added to sooji (porridge). The effect of extracted and purified pigments on cell viability of hepatocytes upon exposure to pigments was evaluated by Trypan blue dye exclusion assay and also on cell integrity was assessed by Lactate Dehydrogenase (LDH) assay. Upon exposure to hepatocytes, orange pigment preserved the membrane integrity of hepatocytes and had high percentage of viable cells (93.8%), followed by yellow pigment which had 87.6% of viable cells. The blue pigment (suspected as Pyocyanin) was found to be moderately cytotoxic to cells with decreased cell viability. Dark green pigment was highly cytotoxic and not suitable for use in food items. All the four pigments had good antioxidant activity. The pigments did not show antibacterial effects against *Escherichia coli* and *Staphylococcus aureus*. Orange and yellow pigments showed promising results and can be used as food pigments. However, further research on toxicology and organoleptic properties of these pigments is crucial to procure regulatory approval for use in human food.

Keywords: Pigments; Bacteria; Cell Viability; Hepatocytes; Antibacterial

Abbreviations

LDH: Lactate Dehydrogenase; UAE: Ultrasound Assisted Extraction; PLE: Pressurized Liquid Extraction; MAE: Microwave Assisted Extraction; PFE: Pulsed Electric Field Assisted Extraction; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; FRAP: Ferric Reducing/Antioxidant Power Assay; ABTS: 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid); FOX: Ferrous Oxidation-Xylenol Orange (FOX) Assay; FTS: Ferric Thiocyanate (FTC) Assay; P1: Green Isolate; P2: Orange Isolate; P3: Yellow Isolate; MRVP: Methyl Red and Voges-Proskauer; HCl: Hydrochloric Acid; NaOH: Sodium Hydroxide; PBS: Phosphate Buffered Saline; SDS: Sodium Dodecyl Sulfate; EDTA: Ethylenediaminetetraacetic Acid; HBSS: Hanks Balanced Salt Solution; NBT: Nitroblue Tetrazolium

Introduction

Colors play a very significant role in our day-to-day life. One of the first things which draw the customer's attention towards any commodity is its color. Pigments can be divided into synthetic and natural pigments. Consider how plants would undergo photosynthesis without chlorophyll or how living organisms would be able

to transport oxygen in their bodies without hemoglobin; which cannot be feasible. It demonstrates how pigments are essential to life on Earth. Since the Paleolithic era, pigments have been utilized as coloring agents. In 1856, Sir William Henry Perkin created mauvine, the first synthetic dye. A historical revolution in synthetic dyes was sparked by the invention of mauvine [1]. Due to their high productivity, high stability, high intensity, low manufacturing cost, and wide range of uses, the market for synthetic pigments significantly increased throughout the post-industrial revolution era [2].

However, because these pigments have a direct impact on people's health and wellbeing as well as the environment, scientists are meticulously investigating them. Many governments have banned or restricted the use of synthetic pigments. There are recent reports suggesting that carmoisine has carcinogenic activity and that Tartrazine, Allura Red, Sunset Yellow, and Brilliant Blue have allergenic, mutagenic, and hyperactive properties [3,4]. In addition to this, pigment and dye waste from a variety of industries, including textile, leather, and pharmaceuticals, builds up and pollutes aquatic habitats. For instance, industrial effluents contain around 10,000 different synthetic textile colors that are deposited as micro-pollutants in various biospheres [5].

Pigments are used in a variety of industries including food and feed, textile, pharmaceutical and cosmetic. Modern consumers are increasingly becoming aware of adverse effects of synthetic pigments and are willing to adopt natural colorants. Natural colorants' nature as biodegradable, nontoxic, and non-carcinogenic substances is thought to make their use generally safe. The demand for natural colorants is rising as the global trend shifts towards the use of biodegradable and environmentally friendly goods [1,6].

Natural pigments can be derived from plants and microorganisms. Due to the soluble nature, stability and ease of culturing of bacteria, natural pigments derived from microbes are preferable to those derived from plants. Microbes have benefits over plants as a source of pigment production, including quick growth in inexpensive media, simple downstream processing, enhanced yield via strain improvement and consistent cultivation irrespective of seasonal fluctuations [7].

Microorganisms including fungi, bacteria, algae and Actinomycetes are a reliable and readily available alternative source of natural pigments. Foods are already colored by microorganisms in the food business, including Monascus, Arpink Red (natural red-industrial name) from *Penicillium oxalicum*, carotene from *Blakeslea trispora* and Astaxanthin from diverse bacteria. For natural colors to compete with the usage of synthetic colors, a lot of research has been done to reduce the costs of manufacturing and processing, to boost stability and shelf life. In addition to serving as coloring agents, several of these pigments also have beneficial effects on health. Large amounts of physiologically and pharmacologically active molecules which serve as antioxidants, antimicrobials, cancer-fighting agents, immuno-regulators, and anti-inflammatory substances, are produced by microorganisms [8].

According to the literary works, the production of intracellular or extracellular pigments is dependent on certain factors like light, pH, temperature and numerous medium components. Pigments produced by bacteria can be broadly classified into two types intracellular pigments and extracellular pigments [9]. While extracellular pigments are released into the broth, Intracellular pigments are held inside the bacterial cell. They require additional methods of cell lysis to release the pigment in the broth. Since intracellular bacterial pigments are also produced by bacteria, the net yield of the pigment during the extraction and purification procedure is significantly lower than for other extracellular bacterial pigments or other plant pigments. In order to get intracellular bacterial pigment, it is essential to use a suitable extraction technique. Intracellular pigment can be extracted using a variety of techniques, including the use of organic solvents, solid-phase extraction, homogenization, the freeze-thaw method, ultrasonication, inorganic acids, the Soxhlet method, and others [10]. Most commonly organic solvents are used to extract the bacterial pigments. Use of organic solvents brings along with it an array of problems such as health risks and environmental concerns. Many other green protocols for extraction are being researched. Some of them include Ultrasound assisted extraction (UAE), Pressurized liquid extraction (PLE), Microwave assisted extraction (MAE) and Pulsed electric field assisted extraction (PFE). The most common methods for purifying pigments are preparative thin layer chromatography and column chromatography. For the separation and purification of microbial pigments, polymeric resins and non-ionic adsorption resins have been utilized [11]. In order to use the bacterial pigments as food colorants, after extraction and purification they need to undergo extensive toxicology studies. There are several in-vitro cytotoxic assays which can be used to assess the effect of pigments on cultured cell lines. Lactate Dehydrogenase (LDH) assay and Trypan blue dye exclusion test are some of the common assays employed. They are robust, replicable, cost effective and a reliable measure of cell integrity. There are several assays used to measure antioxidant

capacity of pigments. These assays are associated with electron or radical scavenging, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, ferric reducing/antioxidant power (FRAP) assay, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [12], ferrous oxidation-xylenol orange (FOX) assay and ferric thiocyanate (FTC) assay.

Bacteria can produce copious amounts of pigments which can be used commercially by optimizing the parameters for bacterial growth such as temperature, pH, incubation time and suitable pigment isolation methods [13]. The objective of the study was to isolate pigmented bacteria from soil and followed by extraction and purification of their respective pigments in order to assess the pigments potential to be used as food colorant. The following parameters were examined in order to reach a conclusion:

- Addition of pigments to food item to observe their coloration.
- Lactate Dehydrogenase (LDH) leakage assay for assessing the effect of pigments on cell membrane integrity.
- Trypan blue dye exclusion test to evaluate cell viability.
- 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay to evaluate antioxidant activity of pigments.
- Agar well diffusion method to evaluate antibacterial activity of pigments.

Materials and Methods

Collection of soil sample

10 different soil samples were collected of which 3 samples were taken from the garden soil from Mount Carmel College, Autonomous and 7 samples were collected from different plots in Vasanthnagar, Bengaluru.

Isolation of pigmented bacteria from soil samples

To isolate suitable microbial population, serial dilution was performed on nutrient agar medium. 1 gram of each soil sample was suspended in 10 ml of 1% saline. Serial dilution was performed by adding 1 ml of soil suspension into 9 ml of saline to get dilution of 10^{-1} [14]. The procedure was repeated to get dilutions up to 10^{-5}. Spread plate technique was performed. 100 µl of 10^{-5} dilution was added on top of solidified nutrient agar. The culture was spread uniformly using a sterile bent glass rod. The plates were incubated at 37° C for 48 hours [15].

Screening of pigment producing microorganisms and preparation of pure culture

After 48 hours the nutrient agar plates were observed and the colored colonies which were morphologically distinct were identified. The pigmented colonies were selected and sub cultured on sterile nutrient agar plates. They were further sub cultured until pure culture of microorganisms was obtained. The pure cultures were stored at 4° C until further use [16]. The three colored colonies isolated were designated as follows: Green isolate (P1), Orange isolate (P2), Yellow isolate (P3).

Cryopreservation of pure cultures of bacteria in glycerol stock solution

A homogenous mixture of sterile nutrient broth and glycerol was prepared in 1:1 ratio. 1.5 ml of this mixture was taken in Eppendorf tubes and 2 loopfuls of bacterial culture was added to the tube. The procedure was repeated for all isolated bacterial cultures [17,18]. Triplicates were made for each culture. The vials were stored at -20° C until further use.

Biochemical characterization of isolated bacteria [19]

Gram's staining

A clean, grease free glass slide was taken. A drop of distilled water was put on glass slide. Loopful of bacterial culture was taken and a thin smear was prepared. The slide was heat fixed. Crystal violet was added to the slide and allowed to stand for 60 s. Gram's iodine was added and allowed to stand for 60 s. The slide was washed with alcohol. Safranin was added and allowed to stand for 60 s. The slide was washed with distilled water between each consecutive step. The slide was air dried and observed under 40X objective of microscope.

Catalase test

1-2 drops of hydrogen peroxide solution was put on a glass slide. A sterile loop was taken and 24-hour old test organism was introduced into the solution. It was observed for immediate bubbling.

Oxidase test

Oxidase discs were taken and aseptically placed on a square glass slide. Using a sterile loop, a colony was picked up from fresh culture of the organism (24 hour old). The loop was rubbed on the discs. It was observed for color change to purple.

Citrate test

Simmons citrate agar was prepared and autoclaved. 5 ml of agar was taken in test tube and slants were made. A loopful of bacterial culture was taken from an isolated colony and streaked back and forth on the slants. The test tubes were incubated at 37°C for 48 hours. The tubes were observed for color change from green to blue.

Methyl red (MR) test

MRVP (Methyl Red and Voges-Proskauer) broth was prepared and autoclaved. 5 ml of broth was added to test tubes. The culture was inoculated into the broth and incubated at 37°C for 24 hours. 10-12 drops of methyl red indicator was added to the test tube. The tubes were observed for red color immediately.

Voges proskauer (VP) test

5 ml of MRVP broth was taken in a test tube and it was inoculated with an 18–24-hour old culture. 15 drops of Barrit's reagent-A and 5 drops of Barrit's reagent-B were added to the tube and mixed well. The tube was shaken continuously for 30 minutes and observed for pink red color at the surface.

Urease test

Urea broth was prepared and autoclaved. 5 ml of urea broth was taken in a test tube and test organism was inoculated in it using a sterile loop. The tubes were incubated at 37°C for 24 hours and observed for color change to pink red color.

Indole test

5 ml of tryptophan broth was taken in a sterile test tube. The tube was aseptically inoculated from an 18–24-hour old culture of test organism. The tube was incubated at 37°C for 24 hours. 0.5 ml of Kovacs reagent was added to the broth. It was observed for the presence or absence of cherry red ring.

Starch hydrolysis test

Sterilized starch agar was poured in petri plates and allowed to solidify. The test organism was streaked in the center of the plate and incubated for 48 hours at 37°C. After the incubation the plate was flooded with iodine solution using a dropper. The plate was left undisturbed for 30 seconds. Excess iodine was poured off and the plates were examined for clear zone around the bacterial line of growth.

Submerged fermentation for pigment production

Submerged fermentation for production of green pigment

Kings-A broth was prepared and autoclaved using the following composition for 1L volume: peptone 20g, potassium sulphate 10g, magnesium chloride 1.64g, agar 15g and glycerol 10mL.

150 ml of Kings-A Broth was taken in 250 ml conical flask in triplicates. 2 loopful of isolate P1 were aseptically inoculated into the broth. The flasks were incubated in shaker incubator for 3-5 days. Pigment production was observed on each day.

Submerged fermentation for production of orange and yellow pigment

250 ml conical flasks were taken and 150 ml of sterile nutrient broth was added to it. 2 loopful of bacterial isolates of P2 and P3 were inoculated aseptically in separate conical flaks. 3 flasks were inoculated for each pigment. The flasks were incubated at 37°C in shaker incubator for 3-5 days. The flasks were observed for pigment production each day.

Extraction of green pigment

Kings-A broth medium was used for extraction of pigment. The broth turned green after 72 hours of incubation. The broth was centrifuged at 5,000 rpm for 20 minutes to obtain a cell free supernatant. The supernatant was taken for solvent extraction and the colorless cell pellet was discarded. Equal volume of chloroform was added to the supernatant containing pyocyanin. It was vortexed and allowed to stand for 5 minutes. Blue layer appeared on the bottom and dark green layer on top. The dark green layer was separated. The blue layer was separated and acidified with 0.1N HCl. To 10 ml of blue layer 6 ml of 0.1 N HCl was added. To the resulting pink layer 0.1 N NaOH was added drop by drop until blue color reappears. The solution was filtered to obtain a clear blue solution. The chloroform extraction procedure was repeated once again to purify the pigment. The resulting solution was kept in hot air oven at 50°C for 2 hours and chloroform was evaporated. The dried flakes of pigment were obtained which was scraped using a sterile scalpel, weighed, and resuspended in water [20]. The dark green color liquid was also kept in hot air oven at 50°C for 1 hour.

Extraction of yellow and orange pigment

Cell lysis for orange and yellow isolates

The yellow and orange broth were centrifuged at 5000 rpm for 20 minutes to obtain colored pellet and colorless supernatant.

Detergent based cell-lysis

The supernatant was discarded and the pellet was resuspended in 3 ml Phosphate Buffered Saline (PBS). To this 1ml of 0.1% Sodium Dodecyl Sulfate (SDS) [21] was added and the mixture was incubated at room temperature for 1 hour. The solution was centrifuged at 5000 rpm for 10 mins. Following the same procedure detergent lysis was performed with increasing concentrations of SDS *i.e.* 1% and 2% respectively.

Alkali based cell-lysis

Three different solutions were prepared. The composition is as follows: solution 1 resuspension buffer (50mM glucose, 25mM

EDTA and 10mM EDTA), solution 2 lysis buffer (0.2N NaOH and 1% SDS) and solution 3 (5M potassium acetate).

The pellet was resuspended in 2 ml resuspension buffer and 1.5 ml of lysis buffer was added. The tubes were incubated in water bath at different temperatures 60°C, 80°C and 100° C respectively for 10 minutes. After incubation 0.5 ml of potassium acetate was added and the suspension was centrifuged at 5000 rpm for 10 minutes.

Following alkali lysis, the suspensions were subjected to heat treatment. They were incubated in water bath at 60°C, 80°C, 100°C respectively for 10 minutes [22].

Ultrasonication

The pellet was resuspended in resuspension buffer and lysis buffer was added to it. The suspension was incubated at room temperature for 15 minutes. 50 ml of the solution was taken in a beaker and subjected to probe ultrasonication [23].

- The orange solution was ultrasonicated. The parameters were as follows: Amplitude 40, Pulse On – 30 seconds, Pulse Off- 50 seconds, Temperature 50°C, 7 times
- Yellow solution: Amplitude- 50, Pulse on- 30 seconds, Pulse off- 50 seconds, Temperature- 50°C, 10 times
- After this the mixture was centrifuged at 5000 rpm for 10 minutes.

Extraction of orange pigment

Colored supernatant was obtained after centrifugation. The pigment was further extracted by adding methanol (To 10 ml of pigment 7.5 ml of methanol was added). The extracted pigment was kept in open air for evaporation to obtain pure pigment. It was weighed, resuspended in water, and stored at 4° C in vials for further analysis [10].

Extraction of yellow pigment

The slightly yellow colored supernatant was extracted using methanol, air dried for evaporation. The solid crystals were weighed, resuspended in water, and stored at 4°C until further analysis [24].

Addition of pigments to food

40 g of sooji was taken and poured into 500 ml of boiling milk. 2 teaspoons of sugar were added and it was cooked for about 10 minutes until sooji has become soft. The mixture was added to small bowls and 4 ml of 1:1(pigment: water) was added to it. The concentration of all pigments was 5mg/ml.

LDH leakage assay for assessing the effect of pigments on cell membrane integrity

Preparation of HBSS (Hanks Balanced Salt Solution) media

HBSS is used for variety of cell culture applications, such as washing cells before dissociation, transporting cells, or diluting cells. Formulation of HBSS with calcium and magnesium are generally used as transport media or for reagent preparation as given in Table 1 [25]. The solution is buffered with phosphate and contains salts, Glucose and maintains physiological pH and osmotic balance for cells.

Components	Amount	Concentration
NaCl	8g	0.14M
KCl	400mg	0.05M
$CaCl_2$	140mg	0.001M
$MgSO_4.7H_2O$	100mg	0.0004M
$MgCl_2.6H_20$	100mg	0.0005M
$Na_2HPO_4.2H_2O$	60mg	0.0003M
KH_2PO_4	60mg	0.0004M
D-Glucose	1g	0.006M
$NaHCO_3$	350mg	0.004M
Phenol red 0.01mg is added as pH indicator		

Table 1: Required Components for 1000 ml of HBSS.

Fresh goat liver was bought and stored at 0°C for use the next day. The liver was thawed by placing in water at room temperature. Four pigments were obtained i.e., pyocyanin and dark green from *Pseudomonas aeruginosa* and orange and yellow pigment from other two bacterial isolates. The effect of all four pigments on membrane integrity of liver parenchyma cells was analyzed using LDH assay performed according to method performed by Cox., *et al.* [26] with some modifications.

Liver was cut into small pieces of approximately 1 cm and placed in a petri dish. 2 ml of HBSS was added to all plates and 2 ml of pigment was added. The pigments were added in 1:1 and 1:10 dilutions with water. The liver was incubated at room temperature for 4 hours with occasional swirling after every 15 minutes. After 4 hours the liver pieces were taken in a glass homogenizer and homogenized mechanically by adding 5 ml of 1:1 HBSS glycerol mixture. The remaining media in petri dish is taken for LDH leakage

assay. The homogenate is centrifuged at 1000 rpm for 10 minutes. The supernatant is taken for analysis of intracellular LDH.

1 ml of supernatant was taken in a test tube and 1ml of 0.1 mM sodium pyruvate, 1 ml of 0.1 mM $NADH_2$ and 1 ml of 0.01% NBT (Nitroblue tetrazolium) was added. The tubes were incubated at room temperature for 15 minutes and absorbance was measured at 570 nm. This was used to find intracellular LDH. Liver cells with only HBSS without the addition of pigments were taken as control.

1 ml of media in which the liver pieces were incubated after addition of all 4 pigments of two different dilutions is taken and same procedure is performed. This is used to assess LDH leakage.

The percentage of cytotoxicity is calculated using the formula:

Trypan blue dye exclusion test for analysis of cell viability

Liver was cut into small pieces of approximately 0.5 cm and placed in a petri dish. 2 ml of HBSS was added to all plates and 2 ml of pigment was added. The pigments were added in 1:1 dilution. The liver pieces were incubated at room temperature for 4 hours with occasional swirling after every 15 minutes. After 4 hours the liver pieces were taken in a glass homogenizer and homogenized mechanically by adding 5 ml of 1:1 HBSS glycerol mixture. The homogenate is centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in HBSS. 1 drop of this suspension was taken on a clean grease free glass slide.1 drop of trypan blue dye was added. It was incubated for 5 minutes at room temperature and observed under 40X objective of the microscope [27].

The number of viable and non-viable cells was counted. The cell viability was calculated using the following formula:

DPPH free radical scavenging assay to analyze antioxidant potential of isolated pigments

The antioxidant property of pigments was evaluated by α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging assay according to the method of Kedare and Singh [28] with some modifications. 0.1mM DPPH was prepared by adding 4mg of DPPH in 100ml of methanol. 1 ml aliquot of pigment with different concentrations of each pigment (12.5, 25, 50, 100 and 200 µg/ml) was pipetted into test tubes. 1 ml of 0.1 mM DPPH was added to the pigments. The tubes were incubated in dark for 30 minutes. Absorbance was measured at 517 nm using a UV spectrophotometer. Ascorbic acid was used as a standard in the same range of concentrations. Methanol was taken as blank. 2 ml of DPPH without the pigment or ascorbic acid served as control.

The percentage scavenging activity is calculated by using the following formula:

Where, Ac is the absorbance of the control and As is the absorbance of the test sample.

Evaluation of antibacterial activity of isolated pigments

Antibacterial activity of the different pigments was evaluated by agar well diffusion method [29]. Pure culture agar slants of Gram-negative *E. Coli* and Gram positive *Staphylococcus aureus* was obtained. Both the cultures were inoculated in sterile nutrient broth and incubated for 24 hours. 100µl of the respective cultures was added to solidified Muller Hinton agar plates and spread plating was performed. The plates were incubated for 30 minutes. Wells were made and 100 and 300 µl of pigment was added in wells respectively. The plates were incubated at 37°C for 24 hours.

Results and Discussion

Isolation of pigment producing bacteria

The different soil samples which were obtained were used for isolation of microorganisms. The soil samples were spread plated on nutrient agar and Bennet agar and incubated at 37°C for 48 hours. Bacterial colonies were obtained which included both pigmented as well as non-pigmented isolates. Three pigmented colonies were obtained. Among them one isolate was green which was isolated from garden soil in Mount Carmel College, Autonomous, in nutrient agar. Two other isolates were orange and yellow respectively. These were obtained from soil from a plot in Vasanthnagar. Orange colony was isolated in bennet's agar. Yellow and green bacterial colonies were isolated on nutrient agar. All the three colonies were sub cultured repeatedly until pure culture was obtained as depicted in Figures 1 and 2. These pure cultures were stored at 4°C and used for further analysis. Glycerol stocks of the three cultures was also made and cryopreserved.

Biochemical characterization of selected isolates

The bacterial colonies were observed for their morphological characteristics (as depicted in Table 2) and it was inferred that P1 colonies were green in color, slightly raised and irregular; P2

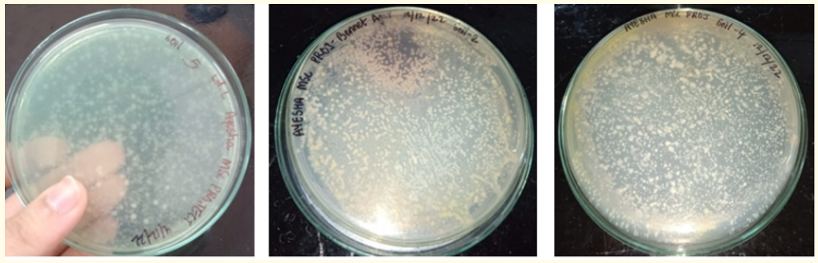

Figure 1: Isolated pigment producing bacteria: green pigment producing P1 isolate (left), orange pigment producing P2 isolate (middle) and yellow pigment producing P3 isolate (right).

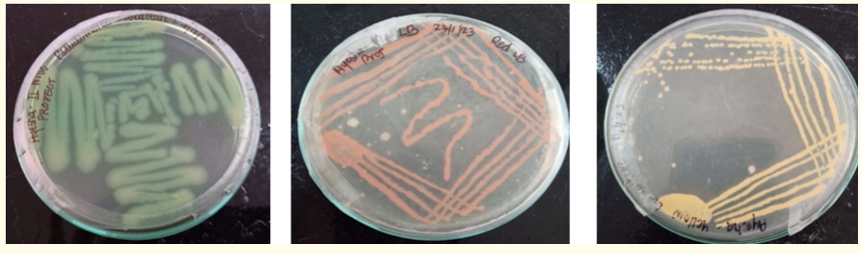

Figure 2: Pure cultures of pigment producing bacteria: P1 (left), P2 (middle) and P3 (right) isolates.

Morphological character	Isolated strain P1	Isolated strain P2	Isolated strain P3
Colour	Green	Orange	Yellow
Size	1mm	1mm	1.5mm
Margin	Irregular	Regular	Regular
Opacity	Opaque	Opaque	Opaque
Elevation	Flat	Convex	Convex
Gram's character	Gram negative rods	Gram negative long rods	Gram positive cocci

Table 2: Morphological characteristics of the isolated strains.

colonies were reddish orange in color, smooth, regular, convex and raised and P3 colonies were yellow in color, convex, smooth, regular and raised.

Gram's staining

TAs depicted in Figure 3 upon Grams staining P1 was found to be Gram negative bacilli, P2 was found to be Gram negative. It had long rods. P3 was found to be gram positive cocci in clusters.

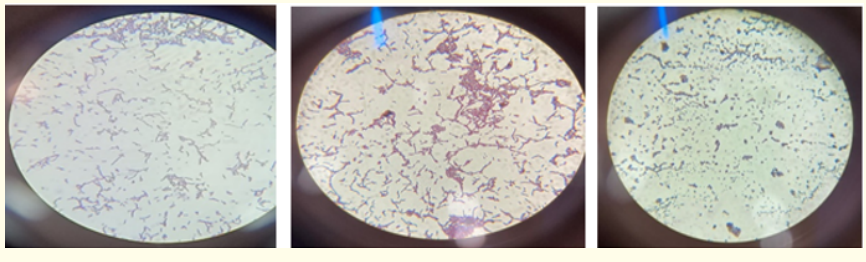

Figure 3: Gram's staining of isolated bacteria: P1 Gram negative bacilli (left), P2 Gram positive long rods (middle) and P3 Gram positive cocci in clusters (right).

Citrate test

As depicted in Figure 4. P1 changed the color of Simmons Citrate Agar from green to deep blue after 24 hours of incubation. Hence it was concluded to be Citrate positive. Upon incubation of P2 and P3 isolates on Simmons citrate agar the color remained unchanged (*i.e.,* green). Hence they were concluded to be Citrate negative.

Figure 4: Citrate test of isolated bacteria: P1 citrate positive (left), P2 and P3 citrate negative (middle and right).

Catalase test

Immediate bubbling was observed when P1 and P3 isolates were rubbed on hydrogen peroxide solution, hence these were concluded to be catalase positive. Moderate bubbling was observed in P2 which was hence concluded to be slightly positive for citrate (as depicted in Figure 5).

Figure 5: Catalase test of isolated bacteria: P1 and P3 catalase positive (left and middle) and P2 catalase slightly positive (right).

Oxidase test

A dark purple color was observed when P1 and P2 isolates were rubbed on oxidase discs. Hence, they were concluded to be oxidase positive. There was no development of purple color when P3 was rubbed on oxidase discs indicating that P3 was oxidase negative.

Methyl red (MR) test

There was no development of red color upon addition of methyl red indicator in any of the tubes indicating these isolates to be negative for MR test.

Voges Proskauer (VP) test

After 30 minutes of shaking, the tubes were observed for pink color at the surface. No pink-colored ring was seen in any of the isolates indicating that all the three isolates were negative for VP test.

Urease test

After incubating the tubes for 24 hours, the tubes were observed for development of pink color. No pink color was developed in tubes with P1 and P2 isolate. Hence P1 and P2 were found to be urease negative. Pink color was developed in tube with P3 isolate which was hence considered to be urease positive.

Indole test

Kovacs reagent was added to 24-hour old culture broth and observed for the presence of cherry red ring. No color change was observed in the tubes. Hence all the three isolates were concluded to be negative for indole test.

Starch hydrolysis

No clear zone was observed around the line of bacterial growth. All the three strains were tested negative for starch hydrolysis as depicted in Figure 6.

Morphological studies and biochemical characterization of the three selected isolates was performed as depicted in Table 3. Characterization and identification of bacteria at the genus level was done with the help of Bergey's Manual of Determinative Bacteriology. Green pigment producing isolate P1 was found to be *Pseudomonas sp.* orange pigment producing Isolate P2 was identified as *Erythrobacter sp.* Yellow pigment producing isolate P3 was suspected to belong to *Micrococcus sp.*

Pigment production and extraction

Pigment production and extraction from P1 isolate (*Pseudomonas* sp.)

Pigment production started within 12-16 hours of incubation in shaker incubator. However, marked difference in color of the broth

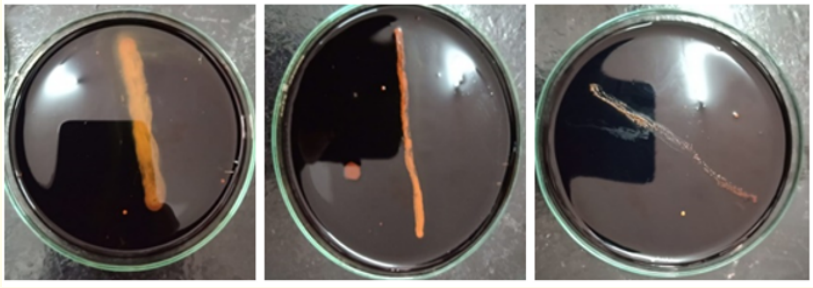

Figure 6: Starch hydrolysis: P1, P2 and P3 isolates tested negative for starch hydrolysis (left, middle and right, respectively).

Table 3: Biochemical characterization of the isolated strains.

Biochemical Test	Isolated strain P1	Isolated strain P2	Isolated strain P3
Catalase	+	+	+
Oxidase	+	+	-
Citrate	+	-	-
Starch Hydrolysis	-	-	-
MR test	-	-	-
VP test	-	-	-
Urease	-	-	+
Indole	-	-	-
Starch hydrolysis	-	-	-
Isolate identified	*Pseudomonas sp.*	*Erythrobacter sp.*	*Micrococcus sp.*

was visible after 48 hours, when broth turned to green color. The production of pyocyanin in Kings A nutrient medium was time dependent. The pigment production kept on increasing steadily with increasing hours of incubation as the color changed from light green to dark bluish green. Maximum pigment production was seen after 120 hours.

90% to 95% of *Pseudomonas aeruginosa* strains generate pyocyanin, a vibrant blue phenazine. Pyocyanin, an electron carrier for *P. aeruginosa*, is made up of two N-methyl-1-hydroxyphenazine subunits and is rapidly translatable across biological membranes. The pigment ideally gives an electron to molecule oxygen to produce a shade of blue. Different pigments are produced by *Pseudomonas aeruginosa* like the blue-green pyocyanin, pyoverdine (yellow, green and light brown), pyomelanin (dark brown) and pyorubin (reddish brown). The color change in *Pseudomonas* broth can be attributed to the production of these pigments [30]. The broth was not incubated for prolonged periods of time, because longer retention time induces the production of pyomelanin and pyoverdin. The production of pyocyanin would be hindered in such cases and also leads to difficulties in extraction.

After extraction was performed, the blue-colored solution pyocyanin which was soluble in chloroform was obtained. The pigment was blue in color at its physiological pH and neutral pH. After addition 0.1 N HCl the solution turned pink in acidic environment. Upon addition of 0.1 N NaOH, the pigment returned to its neutral pH [20]. After extraction of pyocyanin dark green colored pigment (P1b) was left.

The purified blue colored pigment, pyocyanin (P1a) was kept in hot air oven at 50°C for 2 hours for evaporation of chloroform. After evaporation of chloroform light blue-colored flakes were present which were adherent to the plates. So, they were scraped and weighed. 5 mg of pigment was suspended in 1ml of water. P1 After evaporation of P1b a dark olive green colored sticky mass was obtained. 5mg of it was suspended in 1ml of water (as depicted in Figures 7, 8 and 9).

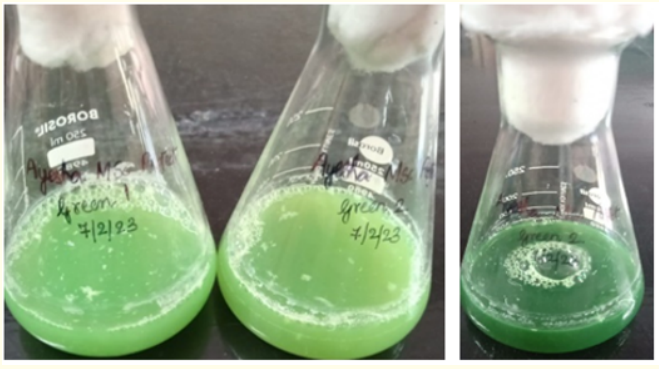

Figure 7: Time course of pigment production by P1 isolate (Pseudomonas sp.): P1 pigment production after 48 hr (left) and after 120 hr (right).

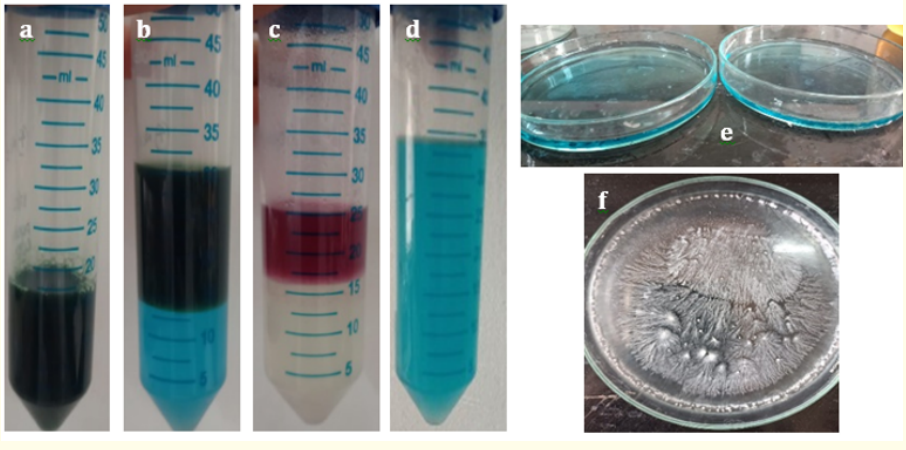

Figure 8: Extraction procedure of pigments P1a (pyocyanin): Dark green colored supernatant (a), chloroform extraction (b), acidified with 0.1N HCl (c), neutralized with 0.1 N NaOH (d), pigment P1a (e) and dried pigment P1a (f).

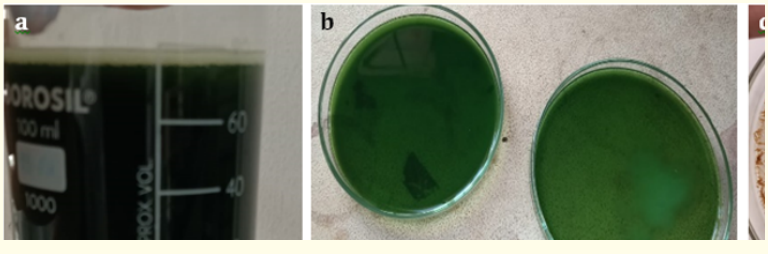

Figure 9: Extraction procedure of dark green pigments P1b: dark green supernatant left after pyocyanin extraction(a), pigment P1b (b) and dried pigment P1b (c).

Pigment production and extraction from P2 isolate (*Erythrobacter* sp.)

The nutrient broth which was inoculated with P2 isolate (*Erythrobacter*), was kept in shaker incubator at 37°C for incubation. The nutrient broth depicted color change to light orange after 48 hours. After 144 hours of incubation, pigment production was maximum and the color of the broth was orange. After centrifugation the pellet was found to be orange color and the supernatant was colorless indicating that is an intracellular pigment.

Different methods of cell lysis were employed for release of intracellular pigment. After each method centrifugation was performed to analyze the pigment release in supernatant. Detergent lysis with 0.1%, 1% and 2% SDS (Sodium Dodecyl Sulfate) was not effective in lysing the bacterial cells. Alkali lysis was performed and the pellet suspension was subjected to heat treatment. At 60 °C cell lysis did not occur. At 80°C, the pigment release was moderate, at 100°C the pigment release was good. However complete pigment release was not achieved. So, probe Ultrasonicator was used. Alkali lysis was performed and the pellet suspension was sonicated. Complete cell lysis and release of pigment was achieved with this method. The colored supernatant was extracted with 90% methanol (as depicted in Figure 10).

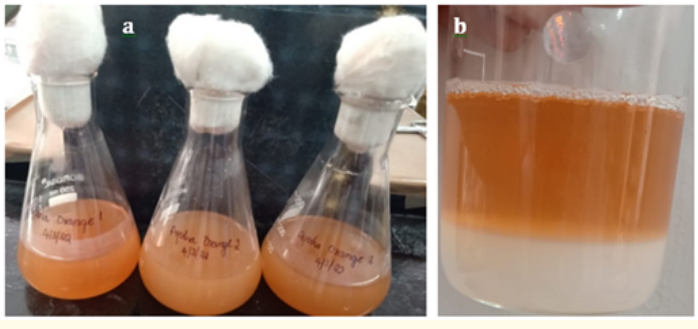

Figure 10: Pigment production by P2 isolate (Erythrobacter sp.) and extraction: P2 pigment production after 144 hours (a) and extraction in methanol (b).

Pigment production and extraction from P3 isolate (*Micrococcus* sp.)

The nutrient broth inoculated with P3 isolate started showing light yellow color after 72 hours of incubation at 37°C in shaker incubator. Maximum pigment production was seen after 144 hours (as depicted in Figures 11, 12 and 13). The bacteria formed soft jelly like mass in the flasks and were settled at the bottom. After centrifugation, it was observed that pellet was yellow colored and supernatant was colorless. The pigment was intracellular. The methods of cell lysis employed were detergent lysis, alkali lysis along with heat treatment and ultrasonication. Detergent lysis and alkali lysis were inefficient and could not lyse the cells. Alkali lysis coupled with heat treatment at 100°C could lyse the cells to some extent as some amount of pigment was released in supernatant. The pigment release in ultrasonication was better compared to other methods. However, the pellet still retained the pigment and complete release of the pigment was not possible. Other methods of cell lysis need to be further tested and employed for P3 isolate. Efficiency of cell lysis and release of pigment for P2 and P3 isolates in each method is summarized in Table 4.

Addition of pigments to food to evaluate their coloration

P2 (Orange) and P1b (dark green) gave good coloration when added to sooji (porridge). P3 (yellow) gave light coloration as the complete pigment could not be extracted from cells. P1a (Pyocyanin) also gave coloration. All the four pigments were stable while cooking when added to sooji (porridge) as depicted in Figure 14.

LDH leakage assay for assessing the effect of pigments on membrane integrity

It was found that the dark green pigment caused the highest cytotoxicity (100%) towards liver cells tested through its action of causing maximum LDH leakage into the extracellular medium hence resulting in cellular damage to a greater extent. This pigment was hence concluded to be not suitable for use as a food grade pigment. Orange pigment causes the least amount of LDH leakage. At

Figure 11: Pigment production by P3 isolate (Micrococcus sp.) and extraction: P3 pigment production after 144 hours (a) and extraction in methanol (b).

Figure 12: Pigment production by all three isolates.

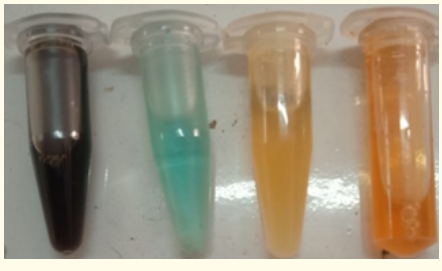

Figure 13: Pigment production by all three isolates.

Parameters	Isolated strain P2 (Orange pigment)	Isolated strain P3 (Yellow pigment)
Parameter: Detergent lysis		
0.1% SDS	-	-
1% SDS	-	-
2% SDS	-	-
Parameter: Alkali lysis and heat treatment		
60°C	-	-
80°C	+	-
100°C	++	+
Ultrasonication	+++	++
Pigment release: no release (-), little release (+), moderate release (++) and complete release (+++)		

Table 4: Assessment of suitable extraction methods for isolated strains P2 and P3.

1:1 concentration the percentage of cytotoxicity was found to be 14.50%. Hence it depicts that this pigment maintains the integrity of plasma membrane. As the pigment was diluted, the cytotoxicity was reported to decease further. At 1:10 concentration the cytotoxicity decreased to 6.90%. Hence, it was inferred that the orange pigment may be suitable to be used as a food grade pigment. Followed by orange, yellow pigment had the least percentage of cytotoxic activity on liver cells. At 1:1 concentration the percentage of LDH leakage was 21.50% which decreased to 11.8% upon increasing the dilution of the pigment to 1:10. Hence, it was inferred that the yellow pigment may be used as food colorant. The results of cytotoxicity caused by different pigments and assessed by LDH assay, have been depicted in Figure 15.

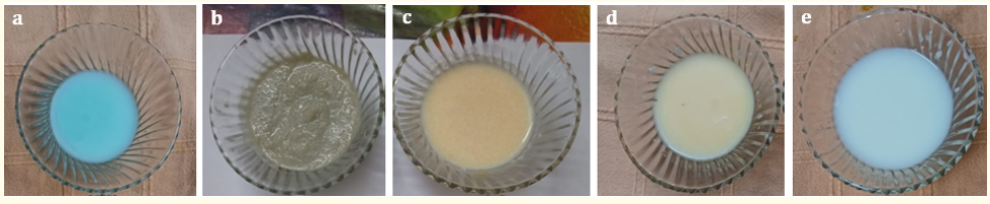

Figure 14: Pigments added to sooji (porridge): pyocyanin (a), dark green (b), orange (c), yellow (d) control no pigment (e).

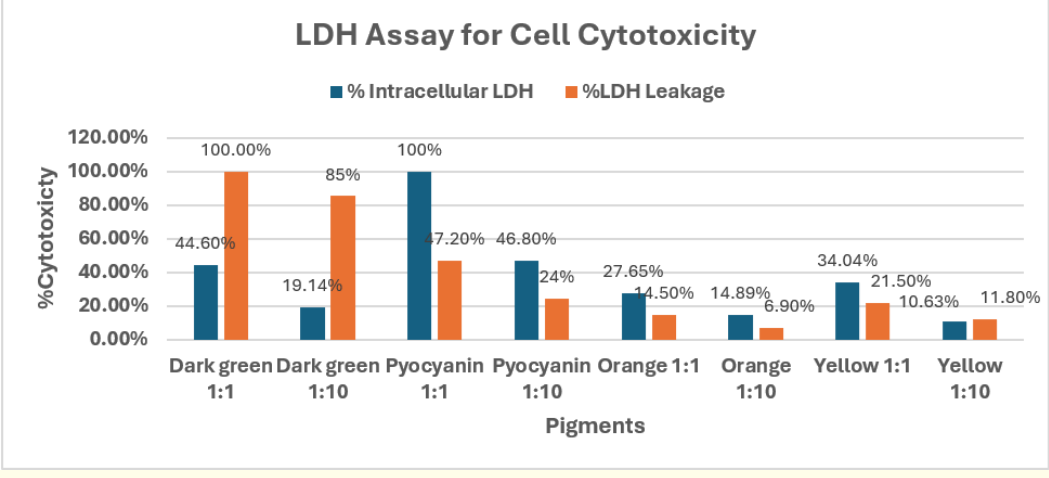

Figure 15: Percentage of cytotoxicity of hepatocytes upon exposure to different pigments at two different concentrations.

Liver cells incubated in pyocyanin had the maximum amount of intracellular LDH present at concentration of 1:1. The percentage cytotoxicity decreased when the pigment was diluted to 1:10 ratio. Pyocyanin had high amount of intracellular LDH but it did not leak out proportionally when compared to its intracellular value. Due to cellular stress or anaerobic conditions, the LDH levels were considered to be elevated to meet the increasing cellular energy demand. Due to metabolic stress exerted by the pigment on the liver cells, the LDH levels may have been elevated to meet the increasing energy demand, but have not leaked out more into the medium, indicating that the plasma membrane had not been damaged to a greater extent. Pyocyanin could be diluted further to a ratio of 1:50 or 1:100, to reduce its cytotoxic affect. Further studies need to be done to reduce its cytotoxicity and to employ it as food colorant.

Trypan blue dye exclusion test for determining cell viability

This test was employed to calculate the number of viable cells in a suspension of cells. The cells were observed under 40X objective of the microscope. Dead cells had taken up the dye and their cytoplasm appeared blue. Viable cells were intact and colorless. The cells were counted and the percentage of cell viability was calculated (as depicted in Figures 16 and 17).

93.8% cells were viable when incubated with orange pigment indicating that this pigment was not cytotoxic to cells. 87.6 % cells were viable when incubated in yellow pigment for four hours indicating that this pigment was also a good candidate for food colorant as it was quite less cytotoxic to cells. Cell viability decreased

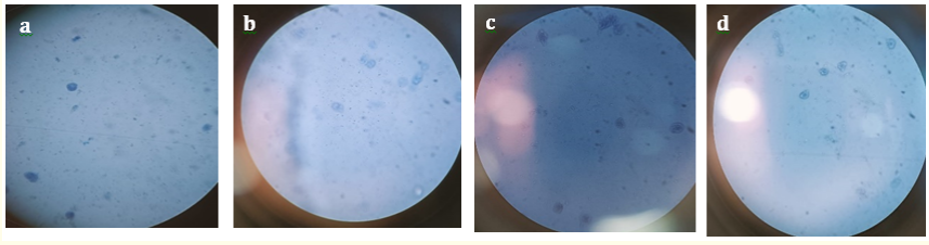

Figure 16: Microscopic observation of liver cells after exposure to pigments: after exposure to dark green pigment (a), pyocyanin (b), orange pigment (c) and yellow pigment (d).

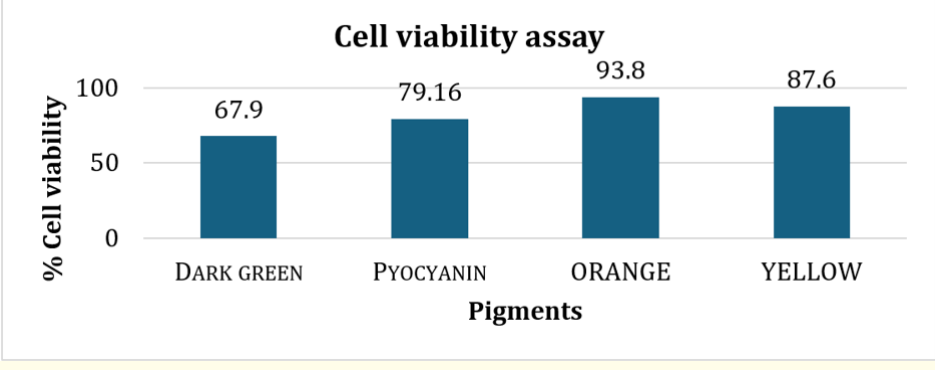

Figure 17: Percentage of cell viability of hepatocytes upon exposure to different pigments.

to 79.16% in case of pyocyanin indicating that it was moderately cytotoxic to cells. Least cell viability of 67.9% was reported in dark green pigment. These results are also in accordance with LDH assay performed. From these results, the following remarks were made: a) the dark green pigment was reported to be cytotoxic to cells, hence making it unsuitable for use in food industry; b) pyocyanin was reported to be only moderately cytotoxic to cells. Pyocyanin has been studied upon by many researchers and has been found to be toxic to *Pseudomonas sp.* [30]. There have been previously performed studies which support the introduction of pyocyanin as food colorant. Pyocyanin has been reported to be used as a feed additive for broilers because it acts as a growth promoter, is natural and functional. It was found to enhance maturation, making the meat quality better, and was also reported to have antimicrobial effects against the pathogenic gut bacteria [31]. Promising results about the use of pyocyanin as a food pigment have been widely reported [20,32].

Evaluation of antioxidant potential of isolated pigments by DPPH free radical scavenging assay

DPPH assay was performed with an aim of assessing the antioxidant potential of the isolated bacterial pigments. This test is based on the evaluation of the antioxidants' ability to scavenge free radicals. By obtaining a hydrogen atom from antioxidants and converting it to the equivalent hydrazine, the odd electron of the nitrogen atom in DPPH is lowered [28]. When exposed to a hydrogen donor, the DPPH radical's purple color turns to yellow. An antioxidant is added to a DPPH solution in ethanol or methanol to evaluate the discoloration and the reduction in absorbance is measured at 517 nm [33].

The percentage of free radical scavenging was calculated for all pigments. Different concentrations of the pigments were tested for free radical scavenging against DPPH. The DPPH free radical scavenging activity of yellow pigment (as depicted in Figure 18) was found to be 8.90%, 29.6%, 54.9%, 68.4% and 66.3% at 12.5, 25, 50, 100 and 200 µg/ml of yellow pigment concentrations, respectively. Yellow pigment showed an increase in free radical scavenging activity upon increase in concentration from 12.5 µg/ml to 100 µg/ml. The yellow pigment showed highest antioxidant value at a concentration of 100 µg/ml.

The DPPH free radical scavenging activity of dark green pigment (as depicted in Figure 19) was found to be 51.3%, 78.5%, 79.6%,

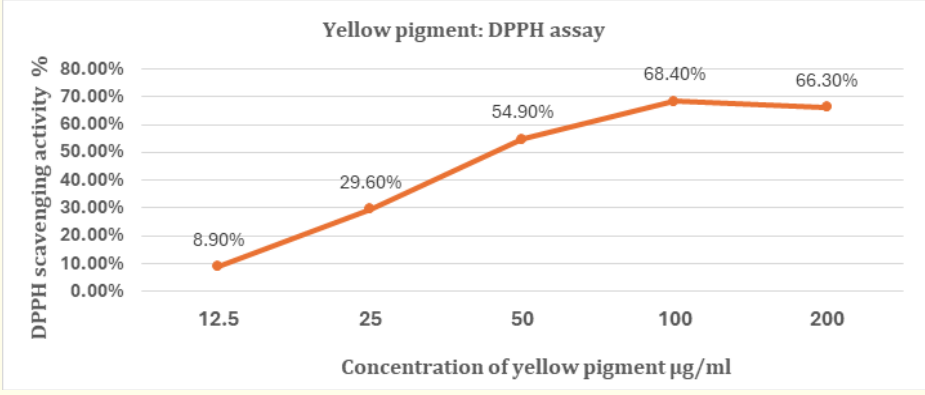

Figure 18: DPPH radical scavenging activity of yellow pigment.

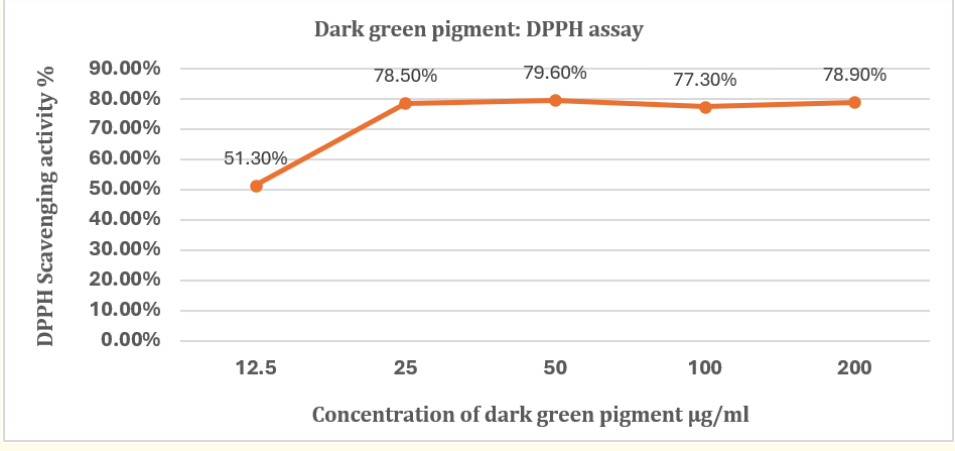

Figure 19: DPPH radical scavenging activity of dark green pigment.

77.3% and 78.9% at 12.5, 25, 50, 100 and 200 µg/ml of dark green pigment concentrations, respectively. The antioxidant value increased from 51.3%, 78.5% at dark green pigment concentrations of 12.5 µg/ml to 25 µg/ml, respectively. However further increase in concentration of dark green pigment did not have any impact on antioxidant activity and the graph was almost linear. The highest scavenging activity (79.60%) was recorded at a dark green pigment concentration of 50 µg/ml.

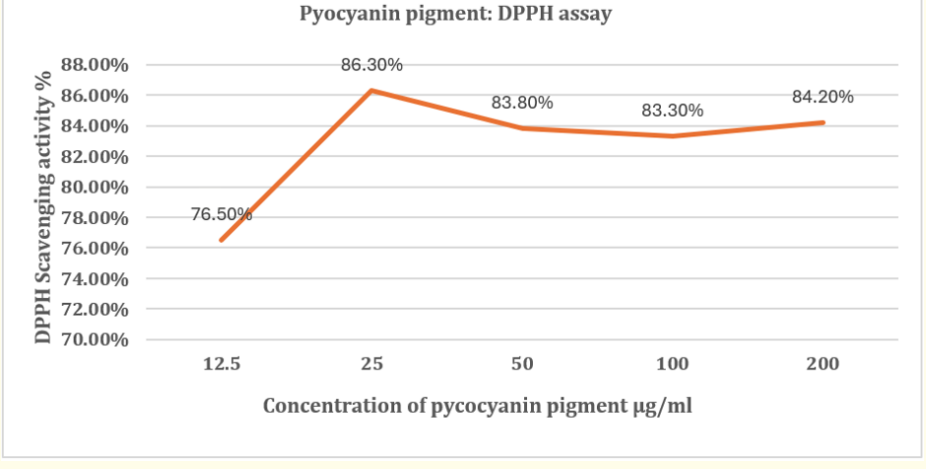

Figure 20: DPPH radical scavenging activity of pyocyanin pigment.

The DPPH free radical scavenging activity of pyocyanin (as depicted in Figure 20) was found to be 76.5%, 86.3%, 83.8%, 83.3% and 84.2% at 12.5, 25, 50, 100 and 200 µg/ml of pyocyanin concentrations, respectively. The highest scavenging activity (86.3%) was recorded at 25 µg/ml concentration of pyocyanin. However, further increase in concentration led to slight decrease in antioxidant value of pyocyanin.

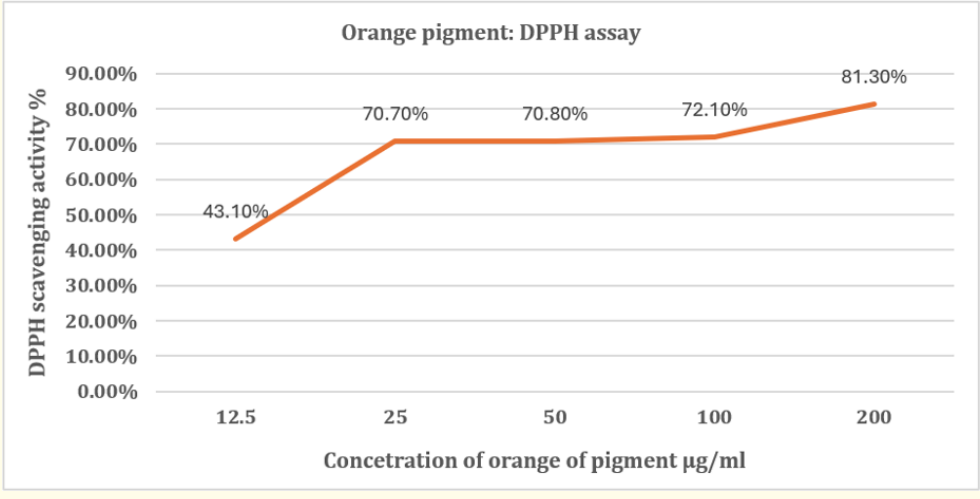

Figure 21: DPPH radical scavenging activity of orange pigment.

The DPPH free radical scavenging activity of orange pigment (as depicted in Figure 21) was found to be 43.1%, 70.7%, 70.8%, 72.1% and 81.3% at 12.5, 25, 50, 100 and 200 µg/ml of orange pigment concentrations, respectively. The highest scavenging activity (81.3%) was recorded at 200µg/ml concentration of orange pigment. The antioxidant activity of orange pigment increased upon increase in orange pigment concentration.

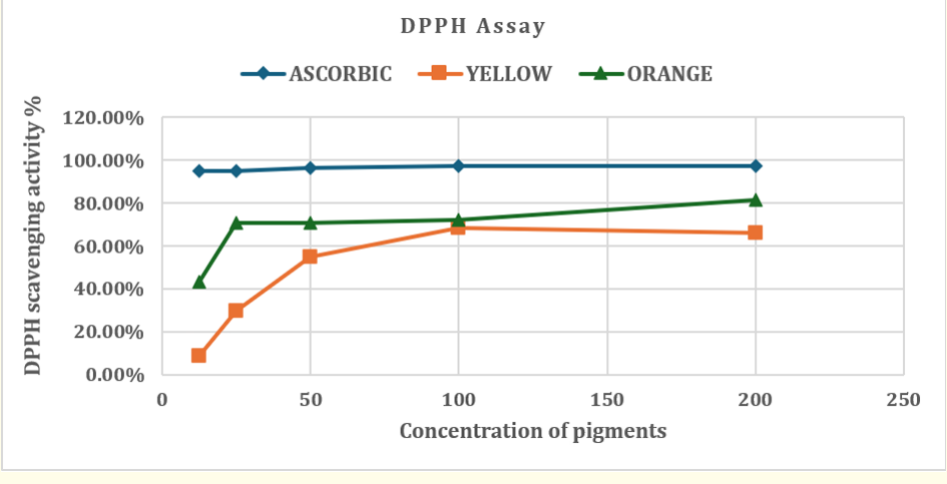

Figure 22: Comparative analysis between concentration dependent DPPH radical scavenging activities of yellow pigment, orange pigment and control ascorbic acid.

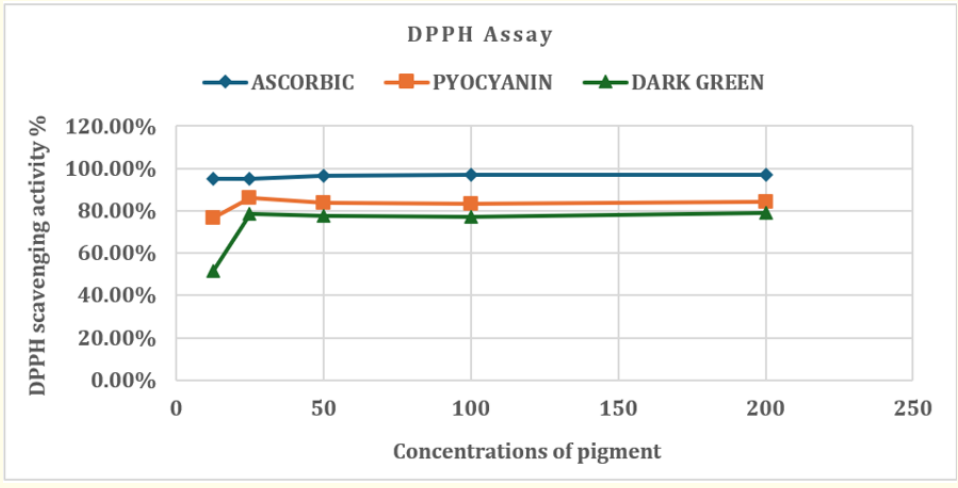

Figure 23: Comparative analysis between concentration dependent DPPH radical scavenging activities of pyocyanin, dark green pigment and control ascorbic acid.

The order of DPPH scavenging activity shown by pigments from highest to lowest (as depicted in Figures 22 and 23) was inferred as follows: pyocyanin, orange, dark green and yellow pigment. Antioxidant activity of pyocyanin has been reported in many studies. Laxmi and Bhat [34] reported that strain BTRY1 of *P. aeruginosa* produced pyocyanin which had a higher antioxidant value at a concentration lower than that of ascorbic acid. The scavenging activity was reported to be 80% at 0.2µg/mL concentration of pyocyanin.

Orange pigment has also been shown to have good antioxidant activity. These pigments when added to food along with pleasant coloration will hence also confer antioxidant property to the food material.

Evaluation of antibacterial activity of isolated pigments

The pigments P1a, P1b, P2 and P3, were tested for their antibacterial activity. After 24 hours the *E. coli* and *S. aureus* plates were observed for zone of inhibition. There was no zone of inhibition observed in case of all four pigments. Saha., *et al.* [32] showed that pyocyanin has antibacterial effects against different bacteria like *Salmonella paratyphi, E. coli, Klebsiella pneumonia.* However, our results were not in accordance with such previous studies. This could be attributed to a possible variance in antibiotic potential of different strains of *Pseudomonas* when tested against different microorganisms.

Conclusion

In this study our analysis has shown positive signs for two pigments to be used as food colorants. Orange pigment extracted from *Erythrobacter sp.*, is a good candidate for use as a natural food colorant. It is not cytotoxic to cells and preserves the integrity of cell membrane. It has good antioxidant scavenging activity. Yellow pigment extracted from *Micrococcus sp.* can also be introduced as a food colorant as it induces very meagre amount of cytotoxicity in cells. Dilutions of this pigment can be increased to increase cell viability.

Pyocyanin extracted from *Pseudomonas sp*. has moderate cytotoxicity and decreased cell viability when compared to yellow and orange pigments. However, it has highest antioxidant capacity. Further toxicological studies with varying dilutions of this pigment need to be performed to understand its cytotoxic effects. Another pigment isolated from *Pseudomonas sp*. which was dark green in color was seen to be highly cytotoxic to liver cells which showed decreased cell viability upon incubation with this pigment. Hence this pigment cannot be recommended to be used as a food colorant. The pigments pyocyanin, orange and dark green gave good coloration when added to sooji (porridge). Yellow pigment gave moderate coloration since complete pigment extraction was not achieved.

Research on organoleptic properties of pigments must be done. Further, extensive research and toxicology studies of all four pigments are crucial in order to facilitate their regulatory approval to be used as pigments in human food. Complete extraction of intracellular yellow pigment from *Micrococcus sp*. was not possible despite several methods performed. Lysis and extraction methods can be an additional area of interest for research. With regard to

industrial production of pigments, research should be emphasized on alternative cost-effective substrates.

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Conflict of Interest

The authors declare that there exist no conflicts of interest, whether internal, financial or of any other kinds.

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