Production and Characterization of Yellow Pigment from Pomegranate and its application

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Abstract

The pigmented microorganism can be obtained from a wide range of environmental sources which could be cultured and purified. Various bacterial growth media can be used to separate the different kinds of microorganisms producing pigments. The utilization of agricultural residues would provide a profitable means of diminishing substrate cost. Pigment synthesized by bacteria can be isolated through the aid of solvent extraction and likewise characterised by using a number of instrumental diagnostic methods such as NMR, GC-MS, TLC, UV-Vis, FTIR, HPLC and gel permeation chromatography.

INTRODUCTION

Pigments are substances with distinguishing importance in several industries and these pigments are utilized as additives, color intensifiers, antioxidants in food industries. Pigments come with a wide range of colours, some of them are water-soluble (Tibor, 2007). Natural dyes extracted from flora and fauna are believed to be eco-friendly, In view of the fact that non-toxic, non-carcinogenic and biodegradable in nature [Cristea D, Vilarem G.2006]. As a choice to man-made pigments, pigments which are produced by bacteria will have upper hand due to their higher biodegradability and greater compatibility with the environment, providing promising avenues for a number of applications. Bacterial pigment manufacturing is now one of the rising fields of lookup to display its achievable for quite a number industrial application.

Color of a food substance is necessary to point out its freshness and security that are additional indices of correct aesthetic and sensorial values. Many natural colorings are available, microbial colorants play a massive position as a meals coloring agent, due to the fact of its manufacturing and handy down steaming process. Industrial production of natural food colorants with the aid of microbial fermentation has quite a number advantages such as more cost worthwhile production, not so much as difficult extraction, greater production through pressure improvement, no lack of uncooked elements and no seasonal variations.

Over the path of the twentieth century, naturally occurring natural pigments have been nearly totally changed by way of artificial molecules such as phthalocyanines that differs from blue to green, arylides similar to yellow to greenish or reddish to yellow, quinacridones that ranging from orange to violet.

Natural pigments and man-made dyes are extensively used in wide fields of day-to-day life such as food production, textile industry, paper production and agriculture process. It is well known that some synthesized dyes manufacturing is prohibited due to the carcinogenicity of the precursor or product and additionally the impact of disposal of their industrial wastes in the ecosystem, natural pigments now not solely have the ability to make bigger the marketability of the merchandise however additionally they show the wonderful organic things to do such as antioxidants and anticancer agents.

Isoprenoid, alkaloids and flavonoids which are already used by humans as colourants, flavours and fragrances which are derived from natural products. It can contain anticancer activity, possess pro-vitamin A and have some physical properties like stability to light, heat and pH (Joshi *et al.*, 2003). Natural colours furthermore being eco-friendly, can also serve the dual need for visually attractive shades and probiotic fitness advantages in meals products.

The pigmented microorganism can be obtained from a wide range of environmental sources which could be cultured and purified. Various bacterial growth media can be used to separate the different kinds of microorganisms producing pigments. The utilization of agricultural residues would provide a profitable means of diminishing substrate cost. Pigment synthesized by bacteria can be isolated through the aid of

solvent extraction and likewise characterised by using a number of instrumental diagnostic methods such as NMR, GC-MS, TLC, UV-Vis, FTIR, HPLC and gel permeation chromatography.

Extraction of bacterial derived pigments is mostly pure and concentrated forms is the predominant technological challenge. Bacteria synthesize two kinds of pigments, those that primarily remain intact to the mycelia of bacteria and those that are released into the fermentation broth. Whereas bacterial derived pigments from the previous kind can be effectively recovered with the use of interference the filtered mycelia with help of acetone, secreted natural products are normally retrieved by way of extracting the aqueous broth with large quantities of natural solvents such as ethyl acetate.

Biosynthetic pathways can also be engineered to change the molecular structure of pigments and, as a result, their colour. Alternatively, biosynthesis of actinorhodin can additionally be manipulated to synthesize orange or yellow-red anthraquinones. Finally, the bacterial pigments should have suitable balance when exposed to environmental stresses, especially UV light. UV light initiates undesirable free-radical reactions in industry that eventually cause their degradation. A variety of UV absorbers such as free-radical scavengers such as hindered amines and triazine-based and benzotriazole molecules are previously used in the industry and are commercially available, whereas they show effectiveness in conjunction with bacterial pigments which are yet to be analysed. The current technology will have the capability to augment the utility of bacterial pigments besides its challenges.

MATERIALS AND METHODS

Isolation of yellow pigment producing bacteria

Liquid samples (2ml) aseptically transferred into a series of 250ml conical flask containing 150ml nutrient broth medium followed by incubation at 30°C 200 rpm for 24 hours. One loopful of bacterial culture was then streaked onto nutrient agar plates and incubated at 30°C for 24 hours (W. A. Ahmad *et al*., 2012).

Maintenance of pure culture

The colored colonies were picked and streaked on a nutrient agar plate and kept for incubation at 30°C for 3 days. Single colonies were maintained and sub-cultured for every 3 days (W. A. Ahmad *et al*., 2012).

Growth Optimization of bacteria in different pH, Salt and temperature

Effect of pH: Nutrient broth medium with 2% salt was prepared and transferred into 5 test tubes. Adjust the pH of the medium to 2, 4, 6, 8 and 10 respectively. Inoculate the tubes with overnight bacterial culture and incubate the tubes for 24 hours. The optical density was measured at 540nm (Goswami *et al.,* 2020).
Effect of different temperature: Nutrient broth was prepared and transferred into test tubes. The test tubes were inoculated with bacterial culture and incubated the tubes for 24 hours. The optical density was measured at 540nm (Goswami *et al.,* 2020).
Effect of salt concentration: Nutrient broth was prepared and transferred into 5 test tubes. Add NaCl at different concentrations (2, 4, 6, 8 and 10).Inoculate the tubes with bacterial culture and incubate at 24 hours. The optical density was measured at 540 nm (Goswami *et al.,* 2020).

Extraction of pigment

The following pigment extraction method was based on the protocol Sasidharan *et al.,* (2013). The bacterial isolates producing yellow pigment were grown in nutrient broth in a rotary shaker incubated for three days at 28±2°C. The cells were harvested by centrifugation 8000 rpm for 15 minutes after three days. Then the pellet was suspended in 5ml methanol. It was then incubated in a water bath for 30 minutes at 70°C until all the visible pigments were extracted. The colored supernatant was isolated and filtered through whatmann no: 1 filtered paper. With help of a spectrophotometer yellow colored extracts were examined by reading the absorbance in the 400-600 nm wavelength range.

Purification of the pigment

Thin Layer chromatography

The mixture of silica gel powder and distilled water of 2g/10 ml was mixed thoroughly and applied on a clean glass slide of 3.4cm*5cm and the oven dried. The solvent mixture contained chloroform: methanol. This method is based on the modified protocol of Shaw *et al*., (1997).

Crude extract was dissolved in chloroform and made upto a concentration of 100mg-1.2µl was submitted to TLC slide using chloroform: methanol as a mobile phase. The visualization was done under UV transilluminator chamber for UV active spots.The retention time(R_f) was measured by formula

RF=distance moved by solutedistance moved by solvent

Column chromatography

The crude extract was purified by column chromatography using acetone as a solvent. Silica gel G (230-400 mesh size) was used as a stationary phase in a glass column. The column was packed with silica gel by wet packing technique whereby a padding of cotton was placed at the bottom of the column and then it was filled with an eluting solvent. Silica gel was packed into the column to form a bed of silica with a maximum height of 30 cm. The crude extracts of red banana had been then poured onto the bed of silica separately and eluted successively with 50 mL of acetone. Seven fractions were collected from. pigment extract. The obtained fractions were evaporated and stored at -80°C for further use.

This method is based on the modified protocol of Shaw *et al*., (1997).

Antioxidant activity determination by DPPH assay

This method is based on the modified protocol of Sasidharan *et al*., (2013).

The percentage of antioxidant activity was determined by the DPPH assay method. Methanolic DPPH 0.1mM of methanolic DPPH radical solution was prepared and to 1.9ml methanol solution of DPPH was added with 0.2ml of extract. Shake the mixture and mix well, it is kept undisturbed for 30 minutes in dark at room temperature. The absorbance was measured at 517nm against the methanol as blank.

The scavenging activity (AA %) was determined according to Mensor *et al*., (2001).

$$\mathbf{AA\% = 100- [(Abs_{sample} – Abs_{blank}) X 100/Abs_{control}}$$

Antibacterial activity

This method is based on the modified protocol of August *et al*., (2000). The bacterial strains were inoculated into the nutrient broth and incubated at 37°C for 24 hours. Muller Hinton agar plates were prepared. In the plates wells were made and spread plating was done with the 24 hours grown pathogens which were to be tested such as *Escherichia coli, Serratia spp Bacillus spp., and Pseudomonas spp*. Then 100µl of pigment extract was added to the wells and incubated the plates at 30°c for 24 hours. The results were observed by the presence of a clear zone around the disc in the medium.

Antifungal activity

This method is based on the modified protocol of August *et al*., (2000). Muller Hinton agar plates were prepared and the antifungal activity was carried out by using fluconazole and voriconazole discs in the Muller hinton agar plates. The plates were swabbed with yellow pigment bacterial culture on which the discs were kept and incubated for 24 hours at 30°C. Finally the result was observed for the presence of a clear zone around the disc in the medium.

Antibiotic sensitivity test

This method is based on the modified protocol of August *et al*., (2000). The antibiotic sensitivity tests were carried out by using tetracycline, vancomycin and kanamycin disc on the Muller hinton agar plates. The plates were swabbed with yellow pigment bacterial culture on which antibiotic discs were kept and incubated the plates for 24 hours at 30°C. After incubation, the clear zone formation was seen around the antibiotic disc.

Structural Characterization of compound

UV-VIS Spectrophotometry: This method is based on the modified protocol of Song *et al*., (2000). Purified extract of the pigment was scanned from 200 to 800nm by Hitachi U-2000 UV-VIS Spectrophotometer, Japan against methanol as blank (ethanol in case of ethanol extract)

FTIR Analysis: This method is based on the modified protocol of Song *et al*., (2000). The bacterial yellow pigment (10mg) was dissolved in chloroform and poured into a thallium bromide disc. Immediately after the chloroform evaporated the disc was exposed to IR radiation in the range 400-400cm^{-1}

Quorum quenching activity of pigment

Luria Bertani agar plates were prepared and the plates were swab with overnight bacterial culture, wells are made and add 100µl of the pigment extract to the well. Incubate the plates at 30^{0}C for 24 hours. After incubation the plates were observed for formation of a clear zone around the well in the medium. This method is based on the modified protocol of Chong *et al*., (2012)

RESULTS

Isolation of pigment producing Bacteria

Among the samples collected, ten isolates were isolated from Pomegranate. From this ACPFM04 showed yellow color colony were selected for characterization and its clearly shown in Fig 1

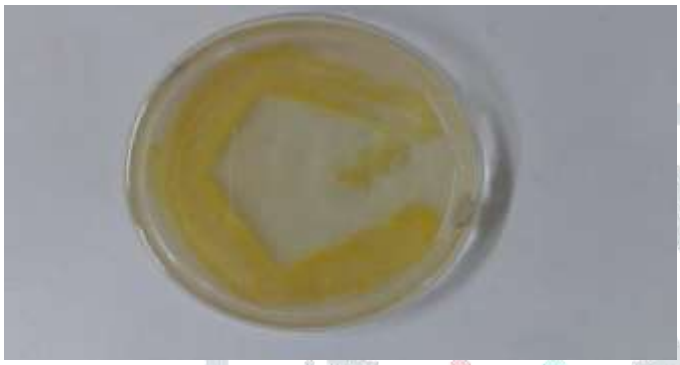

Fig 1 The plate showing the yellow color colony

Optimization of pigment production

Temperature: The bacterial isolates were cultivated at different temperatures and the maximum production of yellow pigment was obtained at temperature 30^{0}C. A standard graph was plotted with temperature in y –axis and cell density in x-axis.

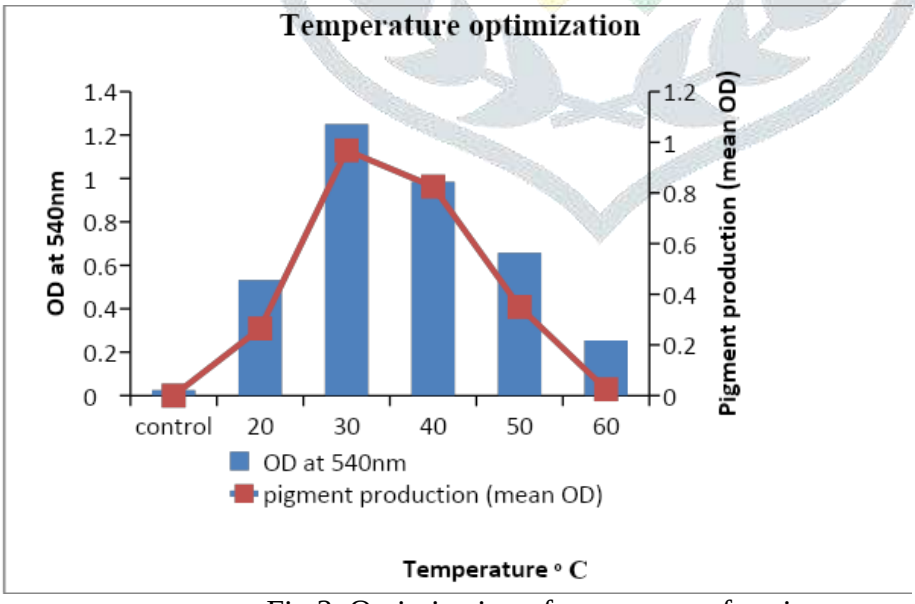

Fig.3. Optimization of temperature for pigment production

pH

The bacterial isolates were cultivated in different pH (2, 4 ,6,8 & 10) and the maximum production of yellow pigment were obtained at the pH 8.A graph was plotted with pH in the y-axis and the cell density, pigmentation in the x-axis.

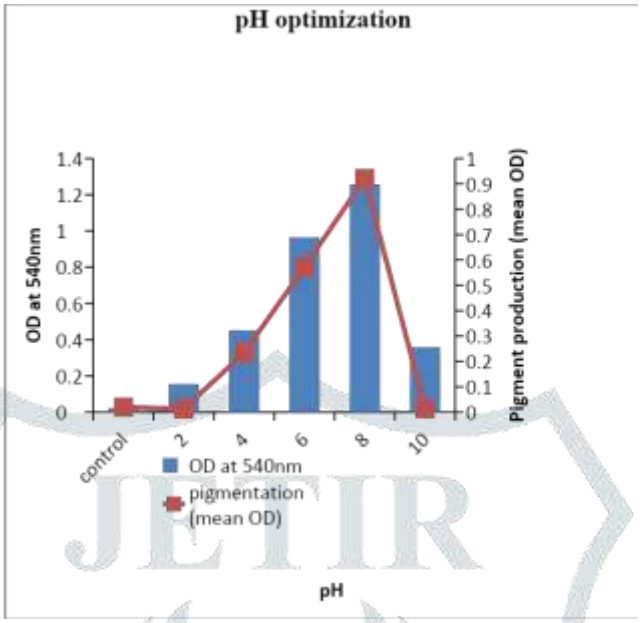

Fig.4.Optimization of pᴴ for pigment production

Salt concentration

Bacterial isolates were cultivated in different Nacl concentrations (2%, 4%, 6%, 8% &10%) and the maximum production of yellow pigment was obtained at the concentration of 2% Nacl. A standard graph was plotted .

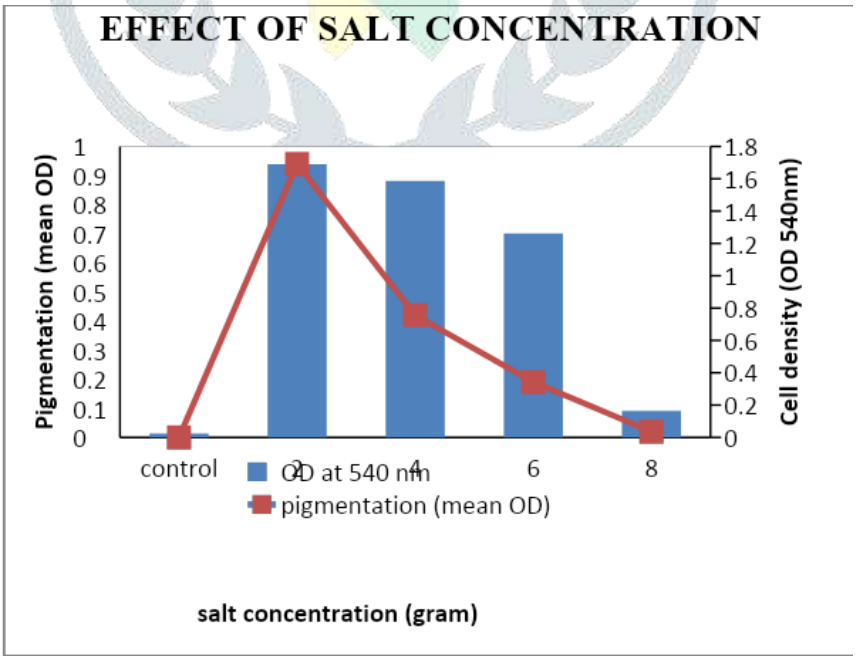

Fig.5. Optimization of salt for pigment production

Extraction of Pigment

The solvents used for the extraction of the pigment are ethanol, methanol, and cold ethanol. Among the solvents used, methanol followed by ethanol showed maximum extraction of metabolite from isolates.

Fig.6. pigment extract

Purification of the pigment

- **TLC**
  Chloroform and methanol are the solvent mixture (ratio 9:1) used in the thin layer chromatography. One purified band was separated **.** The purified fraction of yellow pigment was obtained by column chromatography

Fig.7.Purified band Column Chromatography

Characterization of the pigment

Antioxidant activity determination by DPPH assay

The DPPH scavenging activity of methanol extract of yellow pigment obtained at absorbance of 517nm is 0.129. A standard graph was plotted with values in the y-axis and absorbance in the x-axis.

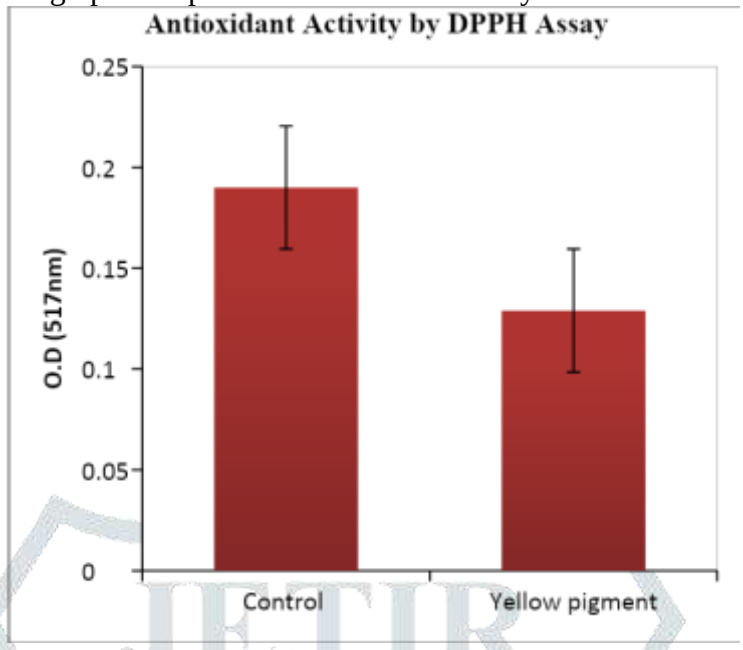

Fig.8. Antioxidant activity by DPPH assay

Structural Characterization of compound

UV-VIS Spectrophotometry

Ultraviolet (UV) spectra of yellow pigment are shown in Figure 9. The methanol extract showed UV maxima at 450nm at absorbance of 2.88 whereas the UV maxima showed by ethanol extract at 300nm at absorbance 1.6

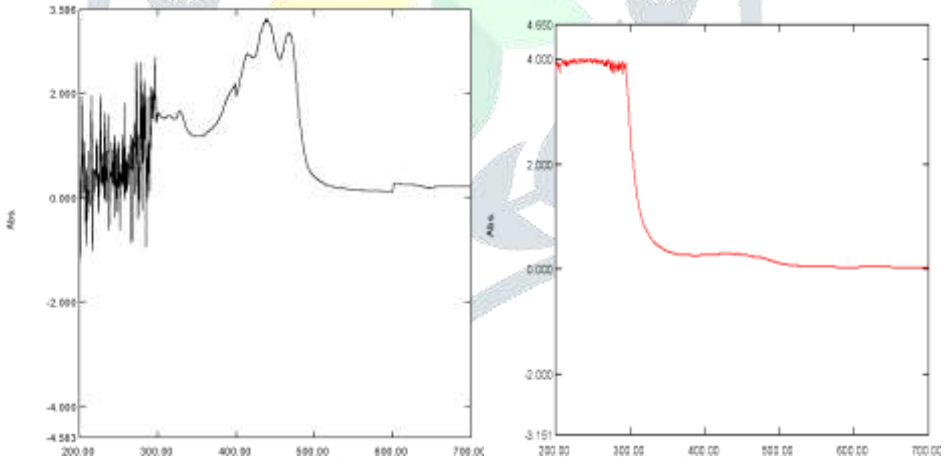

Fig.9. (a) methanol extract (b) ethanol extract

GC-MS

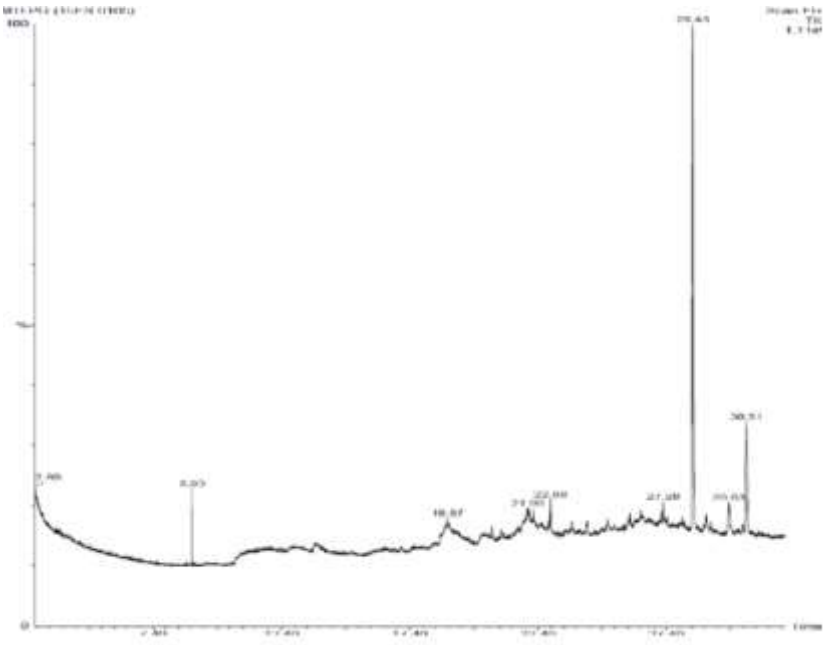

Fig.10. GC-MS chromatogram of bacterial strain ACPFM04

Quorum quenching

Quorum quenching activity of extract- violet pigment disruption in *Chromobacterium violaceum.*

Fig.11.Quroum quenching activity

DISCUSSION

In the present study 10 different strains were isolated from different sources and the isolate ACPFM04 isolated from the Pomegranate was selected because of its colony color. Gram's staining showed that the isolate is Gram negative cocci. Effect of pH, salt and temperature of bacterial isolates were studied and influence the growth of bacterial isolates can be determined. After 24 hours of incubation, the maximum production of yellow pigment was obtained at the temperature 30^{0}C and 60^{0}C, pH 8 and salt concentration 2% can be observed. A graph was plotted with temperature, pH and Nacl concentration on the x-axis and absorbance on the y-axis. Solvents chosen for the extraction of the metabolite from the isolate were ethanol, ethyl acetate, acetone and chloroform, among which methanol and ethanol completely extracted the metabolite from the isolate which is indicated by the loss of color of the isolate after extraction. The color value was colorimetrically measured at a wavelength 200-700nm and the maxima at 450nm at absorbance of 2.88 (methanol extract) and in case of ethanol extract the maxima at 300nm absorbance of 1.6. DPPH, known formally as 2, 2-diphenyl-1-picrylhydrazyl, is a cell-permeable, stable free radical that is commonly used to evaluate the ability of compounds to act as free radical scavengers or hydrogen donors and to measure the antioxidant activity of tissue extracts (Kedare, S.B. and Singh R.P., 2011). The reaction of DPPH with an antioxidant or reducing compound produces the corresponding hydrazine DPPH2, which can be followed by color change from purple (absorbance at 515-528 nm) to yellow. The decrease in absorbance of DPPH radical is caused by antioxidants through the reaction between antioxidant molecules and radical results in the scavenging of the radical by hydrogen donation. The development of foods with an appealing appearance is an important goal in the food industry. Increasingly, food producers are turning to natural food colours, since certain synthetic color additives have shown negative health issues following their consumption.

Artificial dyes have some drawbacks, primarily, their production process requires hazardous chemicals, creating worker safety concerns, they may generate effluent wastes, and these dyes are not eco friendly. Biosynthesis of colourants (natural dyes) for textile applications has drastically increased interests in recent years.Most studies suggested that bacteria have shown more advantages and potential in clinical applications and their pigment has been used in treating several diseases and pigment also have certain properties like antibiotic, anticancer, and immunosuppressive compounds. Significant progress has been achieved in this field, and analysis of bioactive compounds produced by these microbes are rapidly increasing. These bacterial colourants in addition to being eco-friendly, can also serve the dual need for visually attractive colours and probiotic health benefits in food products. Anthocyanins are involved in a wide range of biological activities such as decrease the risk of cancer and reduce inflammatory and stimulates immune response. Several natural bio colourants include anthocyanin, additionally shown antagonistic activity to certain bacteria, viruses and fungi thus it protects food from microbial spoilage. Some are also active against protozoa and insects. Sometimes, carotenoids can act as sunscreen to maintain the quality of food. These pigments prohibit the settlement of marine invertebrate larvae and prevent germination of algal spores and protect the host surface by hindering bacterial colonization and biofilm formation. They may also hinder other organisms that compete for space and nutrients.

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