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Isolation of pigmented bacteria for various applications

KEYWORDS

Pigmented bacteria, dye degradation, hydrocarbon degradation, extracellular enzymes, antioxidant property, phenolic property and antibacterial property

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ABSTRACT *Use of microorganisms for obtaining various natural products has rapidly increased over last few years, as they are important source of many industrially important products. In the present study, pigmented bacteria were isolated from samples collected from Marigold flowers, carrots, garden soil and tomatoes. The six bacterial strains were isolated, showed intense pigment producing capacity. These bacterial isolates were studied for Gram nature and colony characters. The isolates were subjected to various biochemical tests. These bacterial isolates were tested for their ability to degrade biological and textile dyes. The bacterial isolates were also tested for hydrocarbon degrading capacity and production of extracellular enzymes like amylase, beta-galactosidase, lipase , pectinase and cellulase.The extracted pigment showed properties similar to the carotenoids which were confirmed by spectrophotometric analysis and TLC. The pigments also showed phenolic activity, antioxidant capacity and antibacterial activity.*

Introduction

Nature is rich in colours obtained from fruits, vegetables, roots, minerals, plants, microalgae, and so forth. As they originate from biological material they are often called "biocolours" (Pattnaik, et al., 1997). A number of different kinds of pigments are produced by microorganisms. The pigments vary in colour from red, yellow, orange, purple etc. These pigments have been subjected to a lot of research not only for their colourful nature, but also because they play important roles in photosynthesis, photoprotection, pathogenesis etc.

Humans have always preferred natural sources for colouring of food, clothing, cosmetics, and medicines. The coloured pigments produced by microorganisms are carotenoids, melanins, flavins, phenazines, quinones, and bacteriochlorophylls, and more specifically monascins, violacein, and indigo (Nelis and deLeenheer, 1991; Dufosse, 2009). A variety of natural and synthetic pigments are available. Naturally derived pigments are represented by carotenoids, flavonoids (anthocyanins), and some tetrapyrroles (chlorophylls and phycobiliproteins). Nowadays, interest in synthetic pigments has decreased as they are toxic, carcinogenic, and teratogenic in nature and attention towards microbial sources has increased as a safe alternative (Nelis and deLeenheer, 1991, Babu and Shenolikar, 1995; Johnson and Schroeder, 1996; Canizares et al., 1998; Babitha, 2009).Microbial pigments have many advantages over artificial and inorganic colours. Besides, pigment production from microorganisms is independent of weather conditions, which produce different colour shades and grow on cheap substrates (Babitha, 2009). Moreover, pigment production from microbial sources has gained attention owing to public sensitivity regarding "synthetic food additives."

In this work, we are dealing with isolation of bacteria from fruits and flowers which are showing pigment producing capacity and characterising those isolated pigments isolated from the bacteria.

Material and methods

Sample collection:

The sample sources for isolation of pigmented bacteria were Carrot, Tomato, Garden soil, Marigold flowers. Carrots, tomatoes and marigold flowers were procured from the local Kalyan market. Garden soil was obtained from the vicinity of Birla College, Kalyan.

Isolation of bacteria:

The carrots, tomatoes and marigold flowers were crushed in distilled water using a mortar and pestle. The garden soil was mixed in distilled water. All the samples were mixed with distilled water in the ratio of 1 gm of sample in 10 ml distilled water. The samples were streaked on sterile nutrient agar plates and incubated at room temperature for 48 hours. The isolated coloured bacterial colonies were selected. The colony characters and Gram nature of isolated bacteria were studied (Harley and Prescott, 2002).

Study of growth curve:

The growth curves for all six bacterial isolates were studied. 2ml of 24 hrs old grown bacterial suspension was inoculated in 25ml of nutrient broth in side arm flask. Absorbance was measured at 540 nm using colorimeter [CL 157 (ELICO)] at an interval of 30 mins. A graph of time in mins. versus absorbance at 540 was plotted and the growth rate was calculated for each bacterial isolate (Harley and Prescott, 2002).

Identification of bacteria:

The six morphologically distinct bacterial isolates were characterized using biochemical test: motility test, MR-VP test, TSI test, nitrate test, indole test, sugar fermentation test (Glucose, Sucrose, Lactose, Mannitol and Maltose), oxidase test, catalase test, sodium thioglycollate broth test. The results obtained were compared with Bergey's manual of bacterial identification.

Hydrocarbon degrading ability

The bacterial isolates were tested for oil degrading capacity using Bushnell Hass agar plates. 24 hrs old culture suspensions of the bacterial isolates were spot inoculated. Petrol was poured in the lid of the agar plate aseptically and was kept inverted for incubation at room temperature for 48 hrs. The same procedure was repeated with kerosene. The results were observed and utilization of hydrocarbon by bacteria was interpreted (Durve et al., 2016).

Production of extracellular enzymes

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All bacterial isolates were screened for production of extracellular enzymes namely Pectinase, Cellulase, Lipase, Amylase and Beta galactosidase using simple plate assay.

For testing production of pectinase, yeast extract pectate agar medium was used. The bacterial isolates were spot inoculated and incubated at 37°C for 24 hrs. After the incubation, the plates were flooded with 1% solution of hexadecyltrimethylammonium bromide. Clear zone around colonies indicated pectinolytic activity (Vijayan et al., 2012).

For identifying lipase producing bacteria, nutrient agar containing ethidium bromide (10mg/ml) and oil was used. The cultures were spot inoculated on agar plate and the plates were incubated for 24 hrs at room temperature. After 24 hrs incubation the plates were observed in a UV transilluminator for a clear zone around the colony (Vijayan et al., 2012).

For cellulose activities, Carboxymethylcellulose (CMC) agar was used. The cultures were spot inoculated and the plates were incubated for 24 hours at room temperature.After 24hrs incubation, the plates were flooded with 10% NaCl for 10 min. Clear zone around the reddish background indicates the production of cellulase by the bacteria (Vijayan et al., 2012).

For amylase production, the bacterial isolates were spot inoculated on Starch agar plates. The plates were incubated at 37°C for 24 hrs. A clear zone of hydrolysis after Lugol's Iodine solution addition gave an indication of amylolytic bacteria (Vijayan et al., 2012).

For beta galactosidase production, Luria Bertani agar was used. X-gal and IPTG were added to the prepared LB agar. The isolates were spot inoculated on LB plate and incubated at 37°C for 24 hrs. Blue colour colonies indicated the production of beta Galactosidase (Vijayan et al., 2012).

Dye degrading ability

All six bacterial isolates were tested for their ability to degrade dyes using Bushnell Hass broth. Biological dyes namely Congo red, Malachite green, Methylene blue and textile industrial dyes namely Yellow SNR and Pink SNR were used. The 24 hr old grown isolates (0.2 OD units) were inoculated in Bushnell Hass broth containing 100 ppm of the respective dye and incubated at 37° C for 48 hrs. After the respective incubation period, the tubes were centrifuged and the supernatant was subjected to colorimetric analysis [CL 157 (ELICO)]. Percentage decolourization was calculated by the formula (Raju et al., 2007).

Decolourization (%) = Initial absorbance - Final absorbance X 100 / Initial absorbance

Extraction of pigment from isolated bacteria

The pigment producing isolates were inoculated in sterile nutrient broth and incubated at room temperature for one week. The culture suspension was centrifuged at 2500 rpm for 15 min. To the Pellet, 5ml methanol was added and kept in boiling water bath at 60°C for 20 min until all visible pigment got extracted. The supernatant was collected by centrifugation at 2500rpm and filtered through Whatman's no.1 filter paper. The methanol was allowed to evaporate and resulted pigment was collected (Vora et al., 2014; Indra et al., 2014).

Characterisation of pigment

UV-Visible absorption spectrophotometer

Spectral analysis was made on a UV-Visible spectrophotometer (Jasco V-630 Spectrophotometer) and the extract was scanned in the range of 400 to 700 nm to find out the maximum absorption spectra. Methanol was used as a blank (Vor aet al., 2014).

Pigment characterisation by TLC:

The purified pigment was analysed by thin layer chromatography using activated TLC plates. The samples were spotted, air dried and dipped in solvent system (Chloroform:Methanol (95:5;v/v) and kept for 30 mins (Vora et al., 2014). The plates were removed and the 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:

The nature of the extracted pigment was tested. The sample was treated with few drops of 85% Sulphuric acid and results were observed. The appearance of blue colour indicated the presence of carotenoids (Vora et al., 2014).

Antibacterial activity (Disc diffusion)

The extracted pigment was tested for antibacterial activity by disc diffusion method. Sterile Whatman's filter paper discs were soaked in the extracted pigment and placed on sterile nutrient agar plates swabbed with 24 hour old culture of E.coli and S.aureus resp. The plates were incubated at 37°C for 24 hrs. After incubation period, the plates were checked for zones of inhibition. The presence of inhibition zone around the discs soaked with pigment indicated antimicrobial activity. DMSO was kept as a control (Balraj et al., 2014).

Antioxidant activity:

Total Phenolic Content (TPC) was estimated using Folin-Ciocalteau phenol reagent and the colorimetric reading was taken at 670 nm (Ahmad et al., 2012; Balraj et al., 2014).

Total Antioxidant Capacity (TAC) of extracted pigment was carried out using Ascorbic acid assay. Ascorbic Acid (1mg/ml) was used as standard and the colorimetric estimation was done at 670 nm (Ahmad et al., 2012; Balrajet al., 2014).

Application of bacterial pigment in Dyeing

A pre washed cloth was 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 mins. The cloth was kept for drying overnight (Venil et al., 2013)

Results and discussion

Isolation of bacteria

Six pigment producing bacteria were isolated from samples collected from Marigold flowers, carrots, garden soil and tomatoessamples isolated on Nutrient agar (Figure 1). The colony characters were studied and the isolates were found to belong to the *Enterobacter* sp., *Proteus* sp., *Citrobacter* sp. and *Erwinia* sp. Resp. (Table 1, Figure 1)

Name of Bacteria	Pigment extracted	Probable Genus
T1	MFR	*Enterobacter* sp.
T2	MFO	*Enterobacter* sp.
V1	MFY	*Erwinia*sp.
V2	CR	*Erwinia* sp.
P1	TY	*Citrobacter*sp.
P2	SO	*Proteus* sp.

Table 1: Bacterial isolates along with the extracted pigment

Figure 1: Isolated pigment producing bacteria.

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Study of growth curve

The generation time and growth rate for all six bacterial isolates were studied. It was seen that T2 showed the least generation time while V1 and P2 showed a highest generation time (Figure 2).

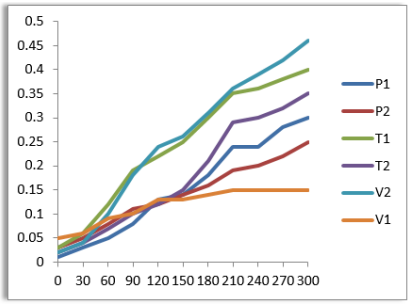

Figure 2: Growth Curves for the bacterial isolates.

Properties of bacteria

Hydrocarbon degrading ability

Hydrocarbon degrading ability of isolated bacteria was tested by spot inoculating on Bushnell Haas media. Some bacterial strains have grown on Bushnell Haas media in the presence of petrol (V2, P1) and kerosene (T1, T2, V1) indicating that they can utilize these oils as a source of carbon (Table2).

Name of isolate	Kerosene	Petrol
V2	+	+
P1	-	+
T2	+	-
T1	+	-
V1	+	-
P2	-	-

Key: + growth
- no growth

Table 2: Hydrocarbon degradation ability of the bacterial isolates

Production of extracellular enzymes

All six bacterial isolates were tested for their ability to produce extracellular enzymes. It was found that bacterial isolates T1 and V2 showed positive results for lipase activity (Figure 3) and isolate T2 showed positive result for amylase activity. None of the bacteria showed production of cellulase, pectinase and β galactosidase.

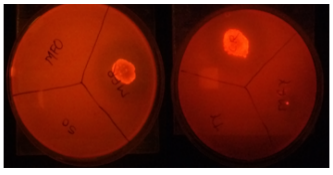

Figure 3: Bacterial isolates showing Lipase activity

Dye degrading ability

All six bacteria showed ability to degrade biological and industrial dyes to some extent. The degradation of dyes indicated that the bacterial isolates used the dyes as a source of carbon which is studied using colorimetric analysis (Fig 4 and 5). It was seen that the percentage degradation of malachite green was higher in compared to other biological stains. Maximum decolourization was seen by isolate MFY for malachite green(58.82 %) and congo red (6.90%) whereas for methylene blue (7.00%) isolateCR showed maximum decolourization.

Textile dyes like Yellow SRN and Pink SRN were used. Maximum decolourization was seen by isolate TY for the textile dyes.

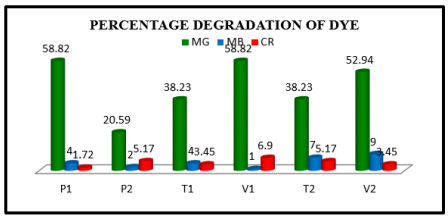

Fig 4: Percentage degradation of Biological stains (MG- Malachite green; MB- Methylene blue; CR- Congo red)

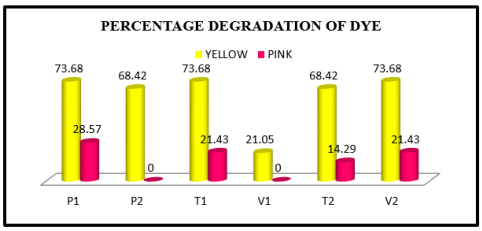

Fig 5: Percentage degradation of textile dyes (Yellow SNR and Pink SNR)

Characterization of bacterial pigment

All bacterial pigment showed characteristic maximum absorption between 400-700nm indicating a carotenoid nature. TLC showed that the pigments showed an Rf value of 0.99 which is similar to that of carotenoids indicating their similarity to carotenoids. Rodriguez-Amaya (1999) and Kaiser et al., (2007) have also suggested the absorption spectra of β -carotene to be around 450nm.

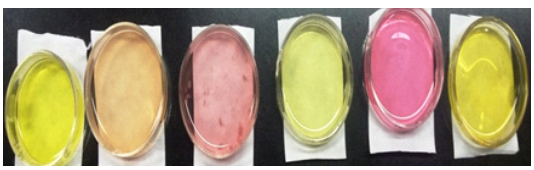

Fig 6: Extracted bacterial pigment

Antibacterial activity:

Antibacterial activity of the pigments was determined and was seen that MFR, SO and CR showed antibacterial activity against *E.coli* (Gram negative) and *S.aureus* (Gram positive). MFO showed activity against S. aureus only.

Total Antioxidant Capacity (TAC) of extracted pigment was carried out using Ascorbic acid assay. Pigment SO (0.082 mg/ml) showed maximum Antioxidant Capacity followed by CR (0.059 mg/ml) (Fig 7).

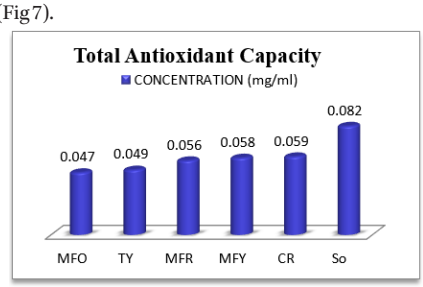

Fig 7: Total antioxidant capacity of the bacterial pigments

Total Phenolic Content (TPC) was estimated using Folin-Ciocalteau phenol reagent and the colorimetric reading was taken at 670 nm (Ahmad et al., 2012; Balraj et al., 2014). Pigment TY (0.046 mg/ml) showed maximum phenolic activity followed by SO (0.0235 mg/ml) (Fig 8). Carotenoids are very efficient physical quenchers of singlet oxygen and scavengers of other reactive oxygen species. The antioxidant potential of carotenoids is of particular significance to human health, due to the fact that losing antioxidant-reactive oxygen species balance results in "oxidative stress", a critical factor of the pathogenic processes of various chronic disorders (Fiedor and

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Burda, 2014).However, carotenoid compounds have a significant role in anti-oxidant and anti- carcinogenic characteristics (Bendich, 1989).

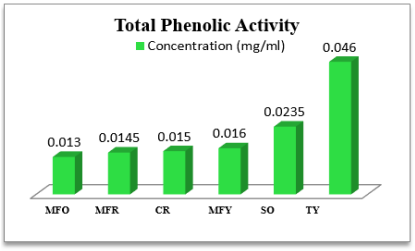

Fig 8: Total Phenolic Activity of the bacterial pigments

The isolated bacterial pigments (TY and MFR) were used to dye cotton fabric. The dye was applied to the cotton fabric fixed in NaCl solution and kept for drying overnight. The fabric retained the respective yellow and pink colour. These pigments can be utilised in the textile industries replacing synthetic dyes hence being more eco friendly (Fig 9).

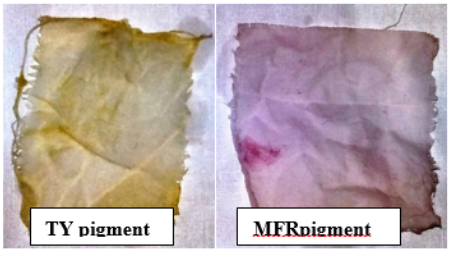

Fig 9 : Fixed cotton fabrics coloured using bacterial pigments

CONCLUSION:

Microbial pigments are not only used as food colorant, flavoring agent and dying agents they are widely applied in medicinal aspects. Apart from food and textile coloring they have been used in clinical therapy to lower the blood cholesterol concentration, Anti-Diabetic Activity, Anti-Inflammation etc. Six pigments producing bacteria were isolated from carrot, tomato, soil and marigold flowers. The pigments which were extracted from them showed colours varying from yellow, oranges and reds. Most of the isolates showed oil degrading ability and dye degrading capacity. The pigments were found to be carotenoids in nature and showed antibacterial activity against *E.coli* and *S.aureus*. All pigments showed antioxidant property to different extents. Bacterial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications (Venil and Lakshmanaperumalsamy, 2009). Most of the bacterial pigment production is still at the R&D stage. Hence, work on the bacterial pigments should be intensified especially in finding cheap and suitable growth medium which can reduce the cost and increase its applicability for industrial production (Ahmad et al., 2012).It can be used as a potential source for pharmaceutical and other cosmetic industries.

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