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Antimicrobial and Antioxidant potentials of Carotenoid Pigment Produced by Indigenous Novel Soil Isolate *Rhodococcus* k*roppenstedtii*

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ABSTRACT

The present research study evaluated the antioxidant and antimicrobial potentials of intense red carotenoid pigment produced by novel soil isolate. A novel isolate from the rhizospheric soil of the soya-bean field was found to produce prominent red carotenoid pigment. Based on phenotype, biochemical and molecular features (16S rRNA sequencing), the isolate was identified as Rhodococcus kroppenstedtii. Maximum intense red pigment production appeared in yeast extract mannitol broth. Among the various solvents, 80% methanol was the best solvent for the extraction of pigment. The methanol extracted pigment exhibited efficient antioxidant and antimicrobial activity. Phosphomolybdenum and ferric analysed the total antioxidant potential of pigment, hence, reducing antioxidant power assay and the results represented in ascorbic acid equivalent. The total antioxidant activity of pigment was investigated to be 67.0 ± 0.2 mg AA/g of sample and ferric reducing antioxidant activity was observed to be 80.2 ± 0.31 µg of ascorbic acid equivalent per milligram of the sample. Likewise, another striking applicability of the pigment was investigated and found to exhibit strong antibacterial activity against four target bacterial pathogens of health significance by in vitro techniques as well as antifungal activity against Aspergillus niger, Penicillin chrysogenum, Fusarium oxysporum species. In conclusion, methanolic extract of the pigment has excellent antioxidant and strong antagonistic activity; this implies the application of pigment as an antioxidant agent in food, pharmaceutical, and cosmetics.

Keywords: *Rhodococcus kroppenstedtii, carotenoid pigment, Antioxidant and antibacterial activity, Novel soil isolate*

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INTRODUCTION

Pigments are coloring compounds produced either naturally or synthetically (Koyyati, *et al.,* 2019). Human life is covered with different colors having vital significance in all aspects of life. The pigments have many applications in the coloration of food, textile, paper, cosmetic, plastic, paint industries, and in leather processing (Mansour and Kairouan, 2018), agriculture, biology (antitumor agents (Numan, *et al*., 2018; Vishnu and Palaniswamy, 2016), anticancer agents (Afra, *et al*., 2017), and antimicrobials (Ravikumar, *et al*., 2016), *etc*. Additives usage in food has a vital role in enhancing its quality, improve nutritional value, consistency and provide color to make food more attractive (Chinaza, *et al.,* 2020). Hence, biocolorants can be one of the alternatives to artificial color for addition into any food material (Heer and Sharma, 2017). Natural pigments produced by bacteria, fungi, plants have better bio-degradability and environment acceptability than synthetic pigments (Mansy and Rathod, 2020; Choudhary and Mallya, 2019). Now a day's pigment produced from living organisms gained more importance because *synthetic* colorants are toxic and show harmful side effects like hyper-activity in children, allergenicity, mutagenecity, carcinogenicity (Sen, *et al.,* 2019; Oplatowska-Stachowiak and Elliott Christopher, 2015), neurotoxicity (Akintunde, *et al.,* 2020), genotoxicity (Khana, et al., 2020) and hurts the ecosystem. Considering the demands of bio-pigments in various applications, better quality natural colorant with higher stability is the need of the hour. Using microorganisms for the production of pigment has several advantages, which includes availability of the cultivation technology, stability of the pigment produced, easy downstream processing, and more cost-effectiveness (Azman, *et al.,* 2018). Thus, there is growing intrest in microbial pigment due to their natural character and safety to use (Parmar and Phutela, 2015). Microorganisms provide a readily available alternative source of naturally derived pigments (Rao, *et al.*, 2017). Various types of pigment such as violacein, carotenoids, quinones, indigo and melanin are reported from microbes (Caro, *et al.*, 2017). A carotenoid group of pigments is more widely studied with respect to their applications and verified to show different beneficial effects on human health through serving as precursors of vitamin A, anti-inflammatory effect, antimicrobial and antioxidant activity (Yolmeh, *et al.*, 2016), etc. Pigment-producing bacteria can be isolated from many different sources but soil source being the most common one. *Rhodococcus rhodochrous* is one of the antioxidative, antimicrobial carotenoid-producing species as previously reported (Haddad, *et al*., 2017). The present research work revealed the isolation of novel pigment-producing strains from the rhizosphere of soyabean and the investigation of antioxidant and antibacterial activity of carotenoid pigment produced by isolate. It was found that pigment exhibited excellent antioxidant and antimicrobial activity having potential applications in therapeutics.

MATERIALS AND METHODS

Materials

Bacterial strains

The bacterial isolate capable of producing an intense red carotenoid pigment, identified as *R. kroppenstedtii* was isolated from rhizosphere soil of soyabean fields of the Shahada region . Morphological and biochemical identification of isolate was determined from MTCC, Chandigarh. The isolate was found to be producing prominent red color pigment on yeast extract mannitol agar plate (YEMA) within 48 hours. The stock culture of the isolate was maintained on a YEMA slant at 4^0C. Bacterial cultures like *Staphylococcus aureus* NCIM 2079, *Bacilus subtilis* NCIM 2063, *E. coli* NCIM 2065, *Proteus* vulgaris NCIM 4175 were procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. Fungal strains like *Aspergillus niger* NCIM 1025, *Penicilium chrysogenum* NCIM 709, *Fusarium oxysporum* NCIM 1281 were also obtained from NCIM, Pune.

Analysis of isolate by 16S rRNA gene sequencing

16S r RNA nucleotide gene sequencing was performed for taxonomic characterization of the isolate from the National Centre for Cell Science (NCCS), Pune.

Sequence assembly and phylogenetic characterization

The 16S rRNA gene sequence data obtained were searched as nucleotide queries in the nucleotide database of the NCBI server (http://www.ncbi.nim.nib.gov/ Blast) to find the related sequences. Using the neighbor-joining method in MEGA V 5.04 software (Saitou and Nei, 1987; Tamura, *et al.*, 2007) and according to the Kimura two-parameter model (Kimura, 1980), the phylogenetic tree was constructed after aligning all acquired and related sequences

Biomass and pigment production

For the biomass and pigment production, the isolate was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of sterile yeast extract mannitol broth (YEMB) with 1ml of 24-48 h inoculum. Flask was incubated in a rotary shaker at 120 rpm at 30^0C for 48 to 72 h until the dark red color of the medium was obtained. After incubation cells were harvested and washed twice with distilled water by repeated centrifugation and allowed to dry to remove complete moisture till constant weight was obtained (Govindaswamy, *et al.,* 1999).

Extraction of pigment

The pigment was extracted from dry cell mass by 10,000 rpm centrifugation for 10 min by the use of 80% HPLC grade methanol (Chaudhari, 2013). The pigment was completely extracted from biomass till the cell pellet appeared colorless.

Ultraviolet-visible spectroscopic studies

UV-VIS spectrophotometer is an important tool for the identification of pigments and dyes. UV-Visible Spectrophotometric analysis (UV Mini 1240 Shimadzu, Japan). was carried out for the red colored pigment sample extracted in methanol. The scanning range selected was 200-800 nm and maximum absorption **λ**max was measured. Methanol was used as a blank and β-carotene as a Standard.

Antioxidant activity of pigment extract

Reducing power assay

Reducing power assay is simple and more effective in the analysis of the antioxidant nature of any unknown compounds. This method is based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}), in the presence of antioxidants (Dontha, 2016). The reducing power of methanol extract of the pigment sample was determined by the slight modification of the method described by Xiao, *et al.,* (2020). Various concentrations ((20 - 100 mg/mL)) of the test pigment sample were mixed with 2.5 mL of 50 µM phosphate buffer (pH 6.6). Followed by addition of 2.5 mL 0.1% (w/v) potassium ferricyanide, all the tubes of reactants were placed in a water bath at 50°C for 20 min. 2.5mL of 1% (w/v) trichloroacetic acid solution was added to the mixture with centrifugation at 3000 rpm for 10 minutes. 2.5mL of the upper layer was removed properly and combined with 0.5 mL of 5 mM ferric chloride solution and the absorbance was measured at 700 nm. Similarly, the blank was run in parallel with all mixture without the pigment sample. Ascorbic acid at various concentrations (20-100µg/mL) was used as an antioxidant standard. The ferric reducing antioxidant activity was expressed as a microgram of ascorbic acid equivalent per mg dry weight of the test sample (Al-Laith, *et al.*, 2019). Gradual increased in the absorbance values of reactants indicates an increase in the reducing activity of the pigment sample. (Yao and Qi, 2016). The experimental procedure was conducted in triplicates and the results were expressed as the mean of triplicates.

Total antioxidant activity by Phophomolybdenum assay

The phosphomolybdenum assay was used for the quantitative measurement of the total antioxidation capacity of the pigment sample. It is based on the reduction of Mo (VI) - Mo (V) by the antioxidant and conversion into green phosphate/Mo (V) complex at acid pH according to the previously reported method of Prieto, *et al.,* 1999; and Mukherjee, *et al.,* (2017). 0.1 ml-test pigment sample (100 µg) was added with a 3 ml of reagent (solution containing 0.6 m/L^{-1} sulphuric acid, 28mM/L sodium phosphate, and 4 mM/L ammonium molybdate) (Murugan, *et al*., 2016) and the tubes were kept in boiling water bath at 95^0C for 90 min. After cooling the absorbance was measured at 695 nm in a UV spectrophotometer with methanol as a blank. The experimental reaction was conducted in triplicate and the mean was expressed in results. Ascorbic acid in a varying concentration of 100mg/ml was utilized as the reference standard. (Sowndhararajan, *et al.*, 2010). The total antioxidant activity of the test pigment sample was expressed as (mean values) number of mg equivalent of ascorbic acid per gram of the dry weight of the extracted pigment.

Antibacterial activity of pigment

Determination of the antimicrobial activity of extracted pigment was carried out by agar disk diffusion technique (Rashid, *et al.,* 2014) against four targets pathogenic bacteria reported affecting health significantly. The pathogenic Gram-positive bacteria like *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063, *and Gram-negative*

pathogens like E. coli NCIM 2065, *Proteus vulgaris* NCIM 4175 were used for the antibacterial activity (0.1mL) by in vitro techniques using nutrient agar plates at 37^{0}C for 24-48 h. Antibiotic streptomycin (100 µg/mL) was utilized as positive control and methanol (100 µL) was utilized as a negative control for comparing activity (Dawoud, *et al.*, 2020) The paper disk of size 6 mm soaked into pigment (50 and 100 µg/mL) concentrations was used for antibacterial activity. A zone of growth inhibition around the disc was indicative of antimicrobial activity. The zone diameter was measured in millimeters. The experiment was repeated thrice for each pathogenic bacterium and results were expressed as mean ± Std. deviation.

Antifungal activity of pigment

Similarly antifungal activity of pigment against some common phytopathogenic fungi namely *Aspergillus niger* NCIM 1025, *Penicillium chrysogenum* NCIM 709 and *Fusarium oxysporum* NCIM 1281 was tested. Two different concentrations of 50 µL and 100 µL pigment sample soaked in filter paper discs were applied to the PDA agar plate which was inoculated separately with the fungal spore suspension 6 x 106 mL-1 spore. Commercially available antibiotic griseofulvin (100 µg/mL) was utilized as positive control and methanol (100µL) as a negative control. Results were expressed as mean ± Std. deviation.

RESULTS AND DISCUSSION

The intense red carotenoid pigment-producing organism was isolated from the rhizosphere of a soybean field and identified based on the phenotypic and biochemical characteristics from MTCC, Chandigarh as a novel strain ***Rhodococcus kroppenstedtii.***

Identification of isolate based on 16S rRNA sequence

Identification of isolate was confirmed by 16S rRNA gene amplification and sequencing from NCCS, Pune. PCR amplification of 16S rRNA gene sequences using the universal primers yielded an amplicon with a size of approximately 1415 bp. The comparison of BLAST search of 16S rRNA gene sequences of pigment-producing isolate with 16srRNA gene sequences of NCBI GenBank database revealed the highest identity of the isolate with *Rhodococcus* species. 16S r RNA sequences were submitted to NCBI GenBank database (http://www.ncbi.nim.nib.gov/Blast) under the name, ***Rhodococcus kroppenstedtii*** with accession number **JN873342.** The phylogenetic tree (**Figure 1**) based on the 16S r RNA sequences was constructed showing the relationship of the pigment-producing organism with sequences of related genera.

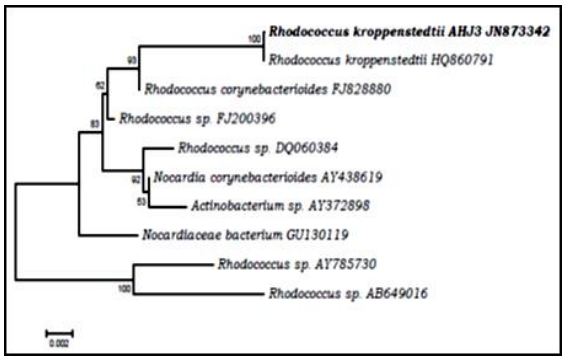

Figure 1. Phylogenetic tree of *Rhodococcus kroppenstedtii*

Ultraviolet–Visible spectroscopy

UV-VIS Spectrophotometer with 200-800 nm range was used for scanning the pigment extracted in methanol. Pigment exhibited the presence of a single major peak with absorption maxima at 472 nm. The maximum absorption of the pigment between wavelengths of 300–600 nm indicates the presence of carotenoids (Dawoud, *et al.,* 2020). Moreover, the earlier research of Ichiyama *et al,* 1989 reported about the carotenoid pigment in the genus of *Rhodococcus* the maximum absorption of pigment production in nanometers was (430-425), (440-435), (470-489). However, according to the literature (Rodriguez-Amaya, 2001; Finkel'shtein, 2016), most of the carotenoid pigments have a maximum absorption range between 375 -505 nm.

Antioxidant activity of pigment extract

Reducing power assay

The reducing capacity of the pigment was observed to be remarkable. The pigment produced by isolate *R. kroppenstedtii* exhibited stronger antioxidant capacity. The reducing power was expressed in terms of a microgram of ascorbate equivalent per mg dry weight of the test sample. Reducing power of the pigment sample was found to increase with gradually increasing (**Table 1**) the concentration of sample from 20 to 100 mg.mL^{-1} which indicated the higher reductive potential of the pigments. The experimental results revealed that the red color of the test solution changes to various shades of blue color because of the reducing power of the pigment sample. Presence of reducer radical causes the conversion of the ferricyanide complex to the ferrous form with the formation of pearls Prussian blue measured at 700 nm. The maximum antioxidant activity was found to be 80.2 ± 0.31 µg of ascorbic acid equivalent per milligram of the sample. The ferric-reducing capacity pigment serves as a significant potential antioxidant activity indicator of the pigment (Mogadem, *et al.,* 2021).

Table 1. Reductive capacity of pigment

Pigment concentration mg.mL^{-1}	OD values at 700 nm	Antioxidant activity (µg of ascorbic acid)
20	0.131	25.2 ± 0.6
40	0.219	39.5 ± 0.12
60	0.324	58.5 ± 0.53
80	0.467	62.4 ±0.71
100	0.5	80.2± 0.31

Values are means ± Std. dev.(n=3)

Total antioxidant activity by phosphomolybdenum assay

Phospomolybdenum assay was used for evaluation of the total antioxidant capacity of the pigment and expressed as the number of equivalents of ascorbic acid (AA) (Soulef, *et al.,* 2020; Fidrianny and Rika, 2016). Experimental results revealed that pigment exhibited stronger total antioxidant activity of 67.0 ± 0.2 mg AA/g of the sample.

Antibacterial activity of pigment

Experimental results of the present study revealed that (**Table 2)** the pigment extracted from a novel strain of *R. kroppenstedtii* exhibited effective antibacterial activity as compared with standard antibiotic streptomycin. Among the four pathogenic strains used to study antibacterial activity, organisms showed variation in the growth inhibition by test pigment sample. The highest zone of growth inhibition was observed against the pathogens *Staphylococcus aureus* (21.0 ± 0.2 mm). This was compared with the activity of streptomycin (19.3 ± 0.5 mm) against S. *aureus*. Similarly bacterial pathogens *Bacillus subtilis* (12.0 ± 0.21mm) *and E.coli* (10.0 ± 0.42mm) were inhibited at higher concentration (100 µg/mL) of pigment while a minor zone of growth inhibition was observed against the *Proteus* species (3.0 ± 0.2mm). This data obtained revealed that the *Staphylococcus and Bacillus*, species were found to be highly susceptible to *Rhodococcus* pigment and more effectively inhibited, followed by *E.coli* and *Proteus*. The antimicrobial activity of the pathogen may vary due to difference in cell wall composition and susceptibility of each pathogen to pigment extract.

Table 2. Antibacterial activity of pigment against human pathogens

Bacterial pathogens	Zone Diameter (mm)			
	Concentration of pigment		Streptomycin (PC) (100 µg/mL)	Methanol (NC) (100 µL)
	100 (µg/mL)	50 (µg/mL)		
B.subtilis	12.0 ± 0.21	8.0 ± 0.4	10.7 ± 0.1	0.5 ± 0.2
S.aureus	21.0 ± 0.2	11.0 ± 0.12	19.3 ± 0.5	0.4 ± 0.6
E. coli	10.0 ±0.42	7.0 ± 0.3	11.1 ± 0.2	0.4 ± 0.3
P. vulgaris	3.0 ±0.2	2.0 ± 0.5	10.0 ± 0.5	0.3 ± 0.4

PC=Positive control, NC=Negative control Values are the mean ± Std. deviation (n=3)

The literature survey reported that the carotenoid type of pigment produced by *Sporobolomyces* sp. was found to be highly inhibitory for *E. coli* with an inhibition zone of 28 mm.

The carotenoid pigment possessed good activity against *S. aureus* 26 mm zone (Manimala and Murugesan, 2014). The antimicrobial activity of *Rhodococcus rhodochrous* pigment against *Staphylococcus aureus* was with a 21 mm inhibition zone (Haddad, *et al*., 2017). According to the literature, the pigment extracted from *Micrococcus roseus* has antimicrobial activity higher against Gram-positive bacteria than Gram-negative bacteria (Yolmesh, *et al.,* 2018). Thus, this result of *Rhodococcus* pigment demonstrates the potential application of pigment in pharmaceutical and cosmetic products.

Antifungal activity of pigment

After incubation, inhibition of mycelial growth in fungus was observed but very minor zones of inhibitions were observed against the fungal pathogens after 3-7 days. The data obtained (**Table 3**) indicate that pigment extracted from *Rhodococcus* has negligible antifungal activity as compared to antibacterial activity.

Table 3. Antifungal activity of pigment against fungal pathogens

Fungal Pathogens	Zone Diameter (mm)			
	Pigment Concentratios		Griseofulvin (PC) 100 (µg/mL)	Methanol (NC) (100 µL)
	100 (µL)	50 (µL)		
A. niger	0.5 ± 0.12	2.7 ± 0.5	2.7 ± 0.5	0.1 ± 0.5
P.chrysogenum,	0.2 ± 0.5	2.2 ± 0.3	2.2 ± 0.3	0.15 ± 0.3
F.oxysporum	0.43 ±0.1	2.8 ± 0.6	2.8 ± 0.6	0.18 ± 0.2

PC=Positive control, NC=Negative control Values are the mean ± Std. deviation (n=3)

At higher pigment concentration inhibition of mycelial growth and reduction in length of mycelia was observed in *A.niger* and *F.oxysporum* followed by inhibition of *P.chrysogenum* as compared to that in control. According to the literature survey (Ferdes, *et al.*, 2009). Sometimes fungal strains are less or more inhibited by natural dyes

CONCLUSION

The bacterial isolate producing prominent red pigment was newly isolated from a rhizospheric soil sample collected from the soyabean field of the surrounding local area of Shahada. Morphological, biochemical, and molecular identification revealed the unknown isolate as *R. kroppenstedtii* with accession number **JN873342.** The maximum pigment production was observed in yeast extract mannitol broth and agar plates. Methanol extract of pigment has absorption maxima recorded at 472 nm, which revealed the presence of carotenoids. The pigment had strong total antioxidant activity determined by phosphomolybdenum assay and reducing power assay. In addition, pigments have excellent antimicrobial activity against pathogenic bacteria and minor antifungal activity. It can be concluded that pigment has the

potential use in the food, cosmetic and pharmaceutical industry as a natural antioxidant colorant. Studies in the future will be conducted on the large-scale production of pigment for industrial applications.

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