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Research Article

STUDY OF THE BIOLOGICAL ACTIVITIES OF ORANGE PIGMENTS, PRODUCED BY BACTERIA *PSEUDOMONAS GENICULATA*, AND *DEINOCOCCUS SOLI*

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ABSTRACT

Two orange pigments have been produced by two different bacterial strains. They have been isolated from soil of West Bengal, India. Upon 16s rRNA analysis, one strain named as PNSMO has been identified as *Pseudomonas geniculata* deposited at NCBI with GenBank accession number, KU726551. Another one named as PNSLO has been identified as *Deinococcus soli* with GenBank accession number, KU726552. Optimum temperature and pH have been adjusted for maximum pigment production. Moreover, pigment productions by the two bacteria have also been increased by using Magnesium Chloride ($MgCl_2$) as the catalytic agent. The two orange pigments have been extracted from two different bacteria by methanol extraction procedure. Those pigments have been identified as Carotene, when passed them through HPLC and UV-Vis spectrometer. The Rf values of those pigments have been found to be around 0.88. It is probable that the carotene pigments, produced by those two bacterial strains, have been reported for the first time by us. In the present investigation, an attempt has been made to find the characteristics of the two orange pigments produced by the different bacterial strains isolated from soil. The results have been tabulated, shown graphically, and discussed.

INTRODUCTION

Plants, animals, and microorganisms have been utilized to find out remedies for diseases of human beings. It has been reported that at least 50% of existing drugs have been derived from terrestrial organisms (Fenical, 1993, Bruckner, 2002). Various microorganisms and their derivative isolates have been representing major sources of medical applications (Montaner and R. P´erez-Tom´as, 2003, Williamson *et al*., 2007). There are many pigments produced by microorganisms which have been reported to have various bioactivities such as prodigiosin, violacein, carotenoids, pyocyanin, melanin, and others (Soliev *et al*., 2011). Prodigiosin and violacein have been reported to have antibiotic, immunosuppressive and anticancer activities (Gerber and Gauthier, 1979, Kawauchi *et al*., 1997, Yamamoto *et al*., 1999, Kim *et al*., 1999). Melanin has the ability to protect living organisms from UV light (Ivanova *et al*., 1996, Kahng *et al*., 2009).

Carotenes are polyunsaturated hydrocarbons containing 40 carbon atoms. They are generally orange, yellow, or pink in colour. Carotenes are generally found in plants. There are some bacterial species such as *Pseudomonas* and *Agrobacterium* which have been reported to produce carotenes. They have antioxidant (Misawa *et al*., 1995) and anticancer activities (Temple and Basu). In addition, carotenes have been used as food colorant, light energy absorber, oxygen transporter, provitamin A producer, and in-vitro antibody production enhancer (Krinsky, Mathews-Roch and Bauernfeind, 1979, Palozza *et al*., 1979, Tomita, 1983, Tee, 1992).

MATERIALS AND METHODS

Isolation of the Bacterial Strain

The two orange pigments producing bacterial strains have been isolated from surface water and soil from Midnapore, India. Both the water and the soil have been serially diluted and spread on nutrient agar plates containing agar, peptone, and beef extract. After three days of incubation at 30°C, orange coloured bacterial colonies have been found on the plates. Single colony has been transferred on fresh nutrient agar plates. Fresh single colony has been transferred from that sample into the liquid Luria Bertani (LB) broth medium containing tryptone, yeast extract, and NaCl supplemented with peptone. The temperature of the culture has been maintained at 30°C and the incubation time has been fixed for 5-7 days.

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Culture conditions and growth curve determination

The culture media have been mixed with different types of inorganic salts, such as Sodium Chloride (NaCl), Potassium Chloride (KCl), Magnesium Chloride ($MgCl_2$), and Calcium Chloride ($CaCl_2$) for the maximum production of orange pigments and bacteria. Temperature has also been optimized by maintaining it from 15°C to 40°C.

Growth curves of the bacteria have been determined by transferring 1 ml bacterial cultures in two separate nefaloflask containing 20 ml nutrient broths. The Optical Density (O.D) values of the cultures have been measured from 0 hour to 36th hour at 600 nm and curves have been plotted.

Biochemical characterization of the bacteria

Gram staining and biochemical tests of the two bacteria have been performed by the following appropriate methods.

Molecular analysis of the bacteria

The genomic DNA of the bacteria have been isolated by following proper protocol and subjected to polymerase chain reaction followed by 16s rRNA sequencing from Xcelris, Ahmadabad, India.

Antibiotic profiling assay of the bacteria

Antibiotic profiling assay of the two bacteria have been performed to find out the antibiotics that can inhibit their growth. First of all, the bacteria have been spread on nutrient agar plates followed by various antibiotic disks of same concentration have been put on the plates. Zone of inhibition have been measured after 24 hours of incubation.

Extraction and purification of the orange pigments

These orange pigments are partially extracellular. Pigments bounded with intracellular part need to be extracted. The culture broths have been mixed with 60% methanol (1:1 v/v) to extract the pigments from the bacteria. It has been then kept at 4°C for 30 minutes for the bacterial cell burst and the release of pigments. Centrifugation has been done at 10,000 rpm for 15 minutes for the separation of pigments from the cells. After centrifugation, pigment containing supernatant has been subjected to evaporate methanol and obtain dry pigments for further experiments.

UV-Vis spectrometry of the pigments has been performed by the UV-3600 Shimadzu and the UV-Vis NIR spectrophotometer; model TCC-240A has been used to find the absorbance peaks of the pigments and have been compared with the expected standards (Miller, 1934).

High performance liquid chromatography has been used to purify the orange pigments. 60% methanol has been used as mobile phase. C18 reverse phase column has been used for both pigments and the temperature has been maintained at 25°C with flow a rate of 1 ml/minute.

Antioxidant activity of the orange pigments

Primarily, the presence of antioxidant activity has been performed with commercially available TLC plates. Pigments have been spotted on the plates and developed in a solvent mixture of methanol, chloroform, and hexane (7:2:1 v/v). After completion of solvent run, 0.05% DPPH mixed with methanol has been sprayed on the dry TLC plate. Development of yellow spot on dark purple background indicates the presence of antioxidant. Vitamin C or ascorbic acid has been taken as standard. The Rf values of both the pigments and the standard one has been measured.

Various concentrations (10-100 ppm) of the standard and the pigments have been made for the measurement of the quantity of antioxidant. 2 ml of each concentration has been mixed with 0.05% methanolic solution of DPPH, incubate at room temperature for 30 minutes, and O.D has been measured at 517 nm. The O.D values of the pigments have also been measured using same protocol. The percent of inhibition values of the standard and the pigments have been calculated using the following formula:

$$\% \text{ Inhibition} = \left(\frac{Blank\ OD - Sample\ OD}{Blank\ OD}\right) * 100$$

RESULT AND DISCUSSION

Culture conditions and growth curve determination

Some of the inorganic salts, such as Sodium Chloride, Potassium Chloride, Magnesium Chloride, and Calcium Chloride have been used to study the catalytic effects of the salts for pigment and bacterial mass production.

Table 1 Pigment production of PNSMO

Days	Media without salts	Media with $CaCl_2$	Media with NaCl	Media with KCl	Media with $MgCl_2$
1	0g/100ml	0g/100ml	0g/100ml	0g/100ml	0g/100ml
2	0.027g/100ml	0.022g/100ml	0.031g/100ml	0.007g/100ml	0.249g/100ml
3	0.087g/100ml	0.080g/100ml	0.093g/100ml	0.047g/100ml	0.391g/100ml
4	0.147g/100ml	0.137g/100ml	0.155g/100ml	0.125g/100ml	0.560g/100ml
5	0.194g/100ml	0.186g/100ml	0.202g/100ml	0.145g/100ml	0.670g/100ml
6	0.178g/100ml	0.171g/100ml	0.185g/100ml	0.138g/100ml	0.575g/100ml
7	0.151g/100ml	0.148g/100ml	0.169g/100ml	0.116/100ml	0.482g/100ml

Table 2 Pigment production of PNSLO

Days	Media without salts	Media with $CaCl_2$	Media with NaCl	Media with KCl	Media with $MgCl_2$
1	0 g/100ml	0 g/100ml	0 g/100ml	0 g/100ml	0 g/100ml
2	0.025 g/100ml	0.019 g/100ml	0.028 g/100ml	0.005 g/100ml	0.244 g/100ml
3	0.083 g/100ml	0.075 g/100ml	0.090 g/100ml	0.042 g/100ml	0.387 g/100ml
4	0.141 g/100ml	0.133 g/100ml	0.150 g/100ml	0.121 g/100ml	0.555 g/100ml
5	0.188 g/100ml	0.180 g/100ml	0.197 g/100ml	0.140 g/100ml	0.662 g/100ml
6	0.168 g/100ml	0.164 g/100ml	0.178 g/100ml	0.128 g/100ml	0.567 g/100ml
7	0.141 g/100ml	0.139 g/100ml	0.161 g/100ml	0.105 g/100ml	0.478 g/100ml

The concentrations of the salts have been selected on the basis of World Health Organization (WHO) data. The data has been shown in Table 1 and Table 2 and Fig. 1. It can be said that the pigment production effects of Sodium Chloride, Potassium Chloride, and Calcium Chloride are not very effective. On the other hand, Magnesium Chloride is very effective for the production of pigment and bacteria. So we can use Magnesium Chloride as the catalytic agent for the production of the orange pigment which is used in various biological fields as essential item.

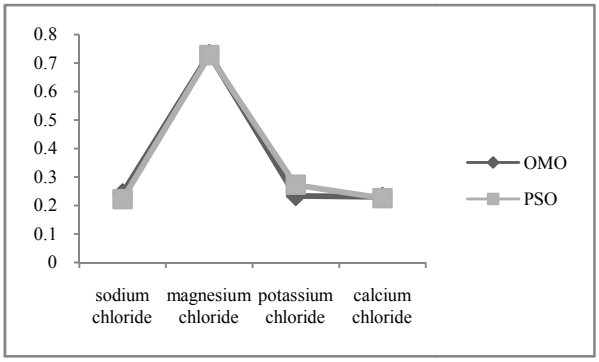

Fig.1 Comparison of two pigment production using salts like: sodium chloride, magnesium chloride, potassium chloride, and calcium chloride.

Biochemical characterization of the bacteria

The Gram staining of PNSMO has revealed that the bacterium is Gram negative and small rods in nature. On the other hand, PNSLO has been found to be Gram positive and small cocci.

Both bacteria can produce catalase and oxidase enzymes. In addition, they can reduce nitrate radicals. PNSMO can utilize citric acid as nutrient from the media and PNSLO can break down starch molecule present in the media. The Biochemical characteristics of the PNSMO and the PNSLO have been tabulated in the Table 3:

Fig 3 and Fig 4 represent the phylogenic tree analysis of PNSMO and PNSLO respectively. The bacteria named as PNSMO is *Pseudomonas geniculata* strain with GenBank ID KU726551. Another one named as PNSLO is *Deinococcus soli* strain with GenBank ID KU726552.

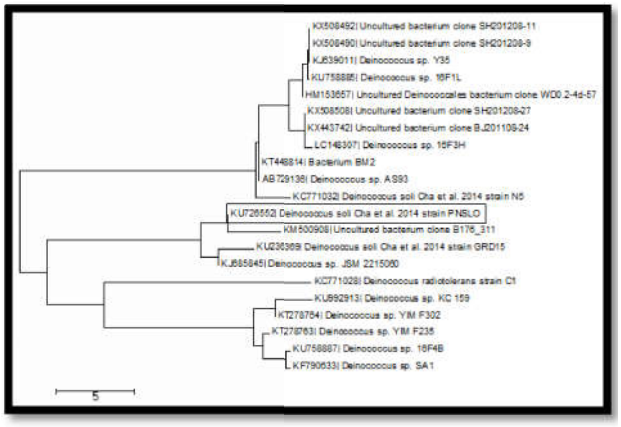

Fig.3 The phylogenetic tree of PNSLO, submitted in GenBank with ID KU726552.

Antibiotic profiling assay of the bacteria

Some of the commercially available broad spectrum antibiotics have been used against PNSMO and PNSLO. Every antibiotic has been taken in the same concentration, 30µg/100 ml. Chloramphenicol has been shown to be most effective one for killing both bacteria shown in Fig. 4.

Extraction and purification of the pigment

Each sample of the orange pigment solution has been injected in the HPLC chromatogram column. Highest peaks have been observed after two minutes for both the pigments. It has been found that the retention times of the two pigments are similar. The chromatograms have been shown in Fig. 5 and Fig. 6.

Table.3 Biochemical characteristics of PNSMO and PNSLO

	IAA	MR	VP	Citrate	Catalase	Oxidase	Nitrate	Gelatin	Urease	Starch	Ammonia	Phosphate
PNSMO	-	-	+	+	+	+	+	-	+	-	+	-
PNSLO	-	+	-	-	+	+	+	-	+	+	+	-

Molecular analysis of the bacteria

After sequencing the 16s rRNA genes of PNSMO and PNSLO, their phylogenic analysis have been done making phylogenetic tree using the Mega6 software.

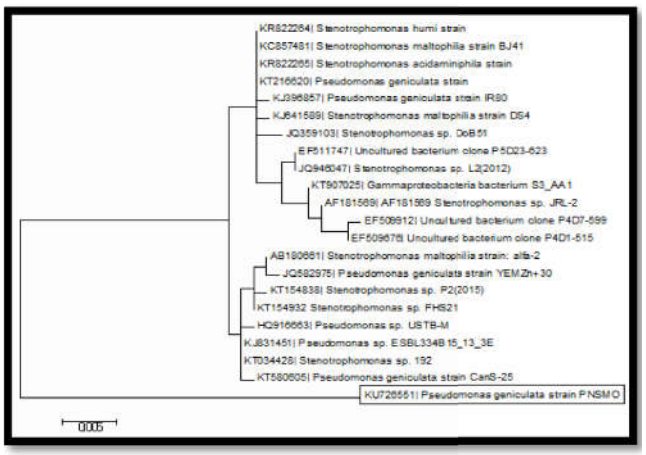

Fig. 2 Phylogenetic tree of PNSMO, submitted in GenBank with the ID KU726551.

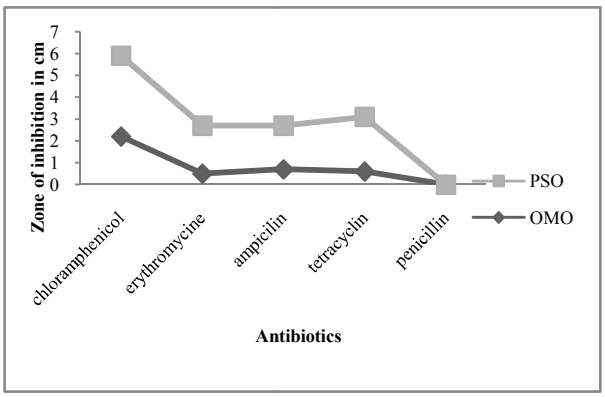

Fig. 4 Antibiotic assay of PNSMO and PNSLO

The two pigment solutions, PNSMO and PNSLO, have been run in 200-800 nm range of wavelength of light in the UV-Vis spectrometer. Two absorption peaks at 467nm and 535nm have been seen in the pigment, PNSMO, and one peak at 470nm in the pigment, PNSLO.

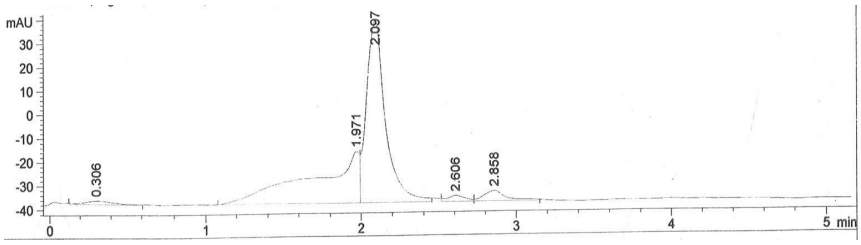

Fig.5 HPLC chromatogram of orange pigment of PNSMO

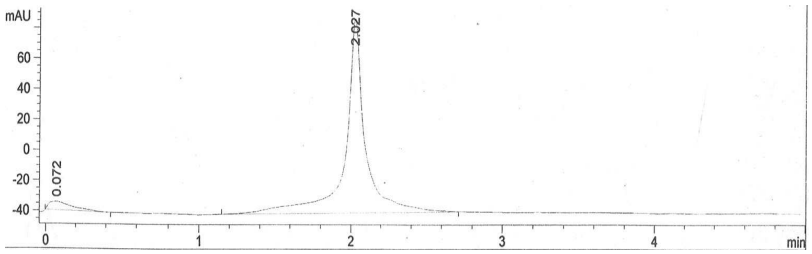

Fig.6 HPLC chromatogram of orange pigment of PNSLO

According to Miller (Miller, 1934), the absorption spectrum of beta-carotene is 470 nm. Hence, it can be said that the two orange pigments extracted from PNSMO and PNSLO are beta carotene. The spectra of both pigments have been shown in Fig. 7 and Fig 8.

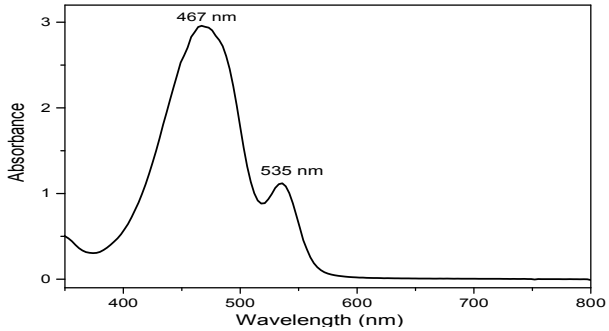

Fig.7 UV-Vis absorption spectra of pigment of PNSMO

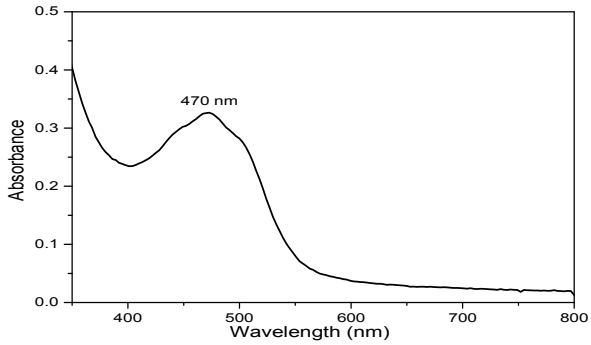

Fig.8 UV-Vis absorption spectra of pigment of PNSLO

Antioxidant activity of the orange pigments

A standard curve of vitamin C has been drawn along with two pigment curves. In the qualitative analysis, the Rf values of both pigments are found to be 1.22, which is similar to the standard one, vitamin C. Hence, it can be concluded that the pigments have antioxidant capabilities.

In the quantitative estimation of antioxidant present in the orange pigments, a standard curve of percentage of inhibition has been plotted against the concentrations (0-100ppm) of

The IC_{50} value of the standard is 12.63. IC_{50} values of PNSMO and PNSLO are 86.74 and 87.17 respectively. The DPPH free radical scavenging activities of the pigments have been shown in Fig. 9 and Fig 10:

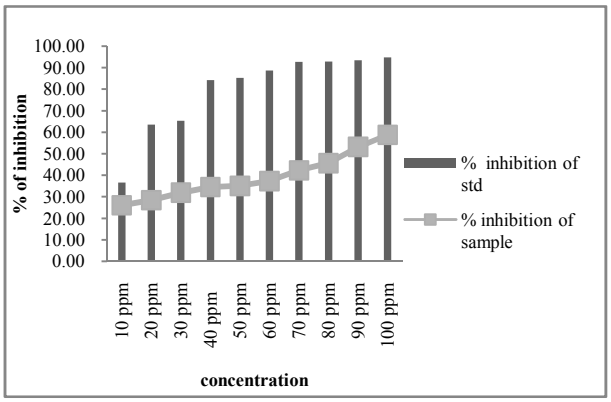

Fig. 9 Antioxidant activity of the orange pigment of PNSMO

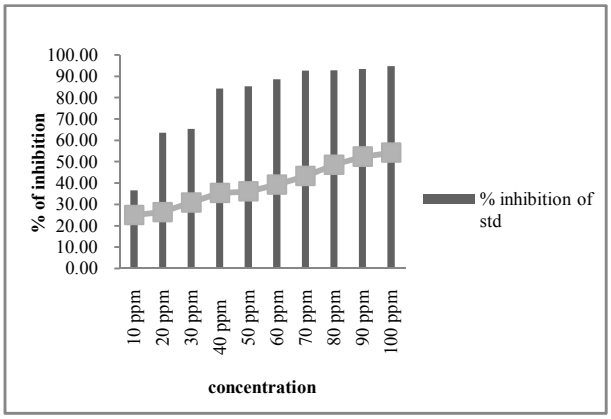

Fig. 10 Antioxidant activity of the orange pigment of PNSLO

CONCLUSION

Carotenes are well known sources of provitamin A, an essential food supplement for human being. In addition, they have roles as food colorant, oxygen transporter, antioxidant, and others. Though carotenes are mainly found in plants, our two bacterial

strains can produce carotene cheaply and sufficiently in the laboratory. Food supplement and medicine can be made from carotene in the future because such pigments have good antioxidant properties. Also, we have to find out the gene responsible for the pigment production.

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