IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

Isolation and Screening of Carotenoid-Producing Bacteria from Lonar Crater Water Samples.

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Abstract:

The goal of this study was to look for microorganisms that produce carotenoids in water samples. Water and mud samples were taken from the Lonar Crater in the Maharashtra district of Buldhana (India). The spread plate method was used to isolate microorganisms that produce carotenoids. 64 bacteria were identified from Lonar Crater water and mud samples, 11 of which were pigment producers. Pigment-producing bacteria were screened and biochemically characterised. 11 isolates produced pigment, with many displaying vivid orange, orange, yellow orange, lemon yellow, and red pigmentation. Following primary screening, 11 isolates that are efficient carotenoid producers were identified. Carotenoid production is also carried out employing various isolates.

Keywords: Water Sample, Pigments Producers, Lonar Crater, Screening.

1. INTRODUCTION

Humans have long preferred natural pigments as food additives to avoid the adverse effects of artificial mineral and metal-based colours. The majority of chemically manufactured colours came from aniline and hazardous petroleum compounds. Natural colours are mostly created by plants; however, their availability fluctuates depending on season and geographical dispersion. Microbial sources of pigment are now being emphasised due to their biodiversity, year-round availability, and high production capability. Carotenoid are C40 compounds that can operate as a pigment supply as well as a medicinal molecule by providing vitamin A, antioxidants, and possibly tumour suppressing agents.

Carotenoids are pigments found in all photosynthetic organisms as well as some non-photosynthetic bacteria, fungus, yeast, and algae. Non-photosynthetic bacteria produce carotenoids, which protect cells from stress by modifying membrane fluidity. Bacteria are more suitable for large-scale carotenoid production than algae and fungi because to their unicellular form, relatively high growth rate, and ease of handling and processing. Colours are one of the first characteristics that humans detect. Interaction between people and nature is essential.

Colours in food, clothing, cosmetics, and medications have traditionally been added from natural sources. Microorganisms create carotenoids, melanin, flavins, phenazines, quinines, and bacteriochlorophylls, as well as violacein, and indigo. There are numerous natural and synthetic pigments available. Carotenoids (anthocyanins) and several tetrapyrroles are examples of naturally derived colours (chlorophylls and phycobiliproteins). Several algae, fungus, and bacteria species have been economically used for pigment synthesis. Microbial pigments have numerous advantages over synthetic and inorganic colours. Carotenoid pigments now number in the thousands. Carotene is the most well-known member of this category, and it plays an important role in health and medicine as the progenitor of Vitamin A.

Carotenoids are a class of natural pigments that are utilised as colourants, food supplements, and nutraceuticals, as well as for medicinal, cosmetic, and biotechnological uses. Pigments as a biomarker. Violet coloured bacteria, as well as *Flexibacter* and *Sporocytophaga* species, were found in polluted drinking water samples. *Vogesella indigofera,* a blue pigmented bacterium, can be utilised as a bioindicator of chromium contaminated environments. Carotenoids, as provitamin A, can perform a variety of critical activities in humans. Due to photosensitivity associated with quinidine consumption, which absorbs harmful short wavelength section of the light spectrum, carotenoids have been discovered

to have a role in photoprotection against hereditary disorders, erythropoietin protoporphyria (EPP), and erythema (skin reddening). Premature fatalities in underdeveloped countries, particularly among children, have been linked to vitamin A deficiency. Lycopene, a hydrocarbon with antioxidant properties, was discovered to be effective at quenching the destructive potential of singlet oxygen and at mitigating the detrimental effect of oxidation, which significantly contributes to the risk of chronic diseases. Because of its strong antioxidant activity, astaxanthin has health benefits such as cardiovascular disease prevention, immune system strengthening, bioactivity against Helicobacter pylori, and cataract prevention. Several researchers have noted the role of carotenoids in influencing immune defences. The pigments improved both specific and nonspecific immune activities and shown the ability to boost tumour immunity. Colours abound in nature (minerals, plants, microalgae, etc.) as do pigment-producing microbes (fungi, yeast, and bacteria).

This study details the isolation and screening of carotenoid producing bacteria from various environmental niches, as well as the selection of promising carotenoid producers for further investigation. To isolate carotenoid-producing bacteria, a selective enrichment culture approach was utilised to promote the growth of bacteria from a sample that was initially low in quantity.

2. MATERIALS AND METHODS

I. Sample Collection and Enrichment

Random sampling was done in May and June to isolate carotenoid producing bacteria. Before use, different samples (water and mud) were collected in sterile containers and held at 4 ^{o}c. The hypersaline and hyperalkaline environments of Lonar Crater, Buldhana, Maharashtra, (India) were sampled. Samples were enriched in nutrient broth and cultured at room temperature for 48 hours in the presence of day light.

II. Isolation and Primary Screening

Isolation was performed through serial dilution of enriched culture in physiological saline (0.85% NaCl), with 0.1 ml of each diluent used for spreading on nutrient agar (pH 8.5) with 1.5% agar-agar. The plates were prepared in duplicate and incubated at room temperature for 48 hours under day light near a window. Following incubation, isolated colonies exhibiting pigmentation were chosen as master plates and stored for future research. Growing pigmented isolates from master plates on nutrient agar with pH 8.5 at room temperature for 48 hours in duplicates was used for first screening of all pigment generating cultures. The ability to produce yellow, orange, or red pigment, which is likely to be non-diffusible among other pigment producers and non-pigmented cultures on plates, was used to screen isolates. The isolates with yellow, orange, and red pigmentation were isolated, placed to nutrient agar slants, and stored at 4^{0}C for future research.

III. Secondary Screening

Secondary screening was carried out on the basis of qualitative tolerance to pH and salt (NaCl%). All primary screening bacterial isolates were streaked separately on nutrient agar with pH ranging from 5 to 12 and nutrient agar with NaCl (1-6%). For 48 hours, the plates were incubated at room temperature under day light near a window. Tolerant cultures were isolated from nutrient agar with a wide pH range and streaked on nutrient agar with increased NaCl (1-6%). Cultures with good pigmentation, pH and NaCl tolerance, and vivid pigmentation were screened secondary and selected for tertiary screening.

IV. Tertiary Screening

Tertiary screening was performed based on the isolate's qualitative and quantitative ability to generate pigment. In tertiary screening, actively developing (48-hour) cultures of secondary screened isolates were inoculated individually into nutrient broth (pH 8.5 and 1% NaCl) and incubated at room temperature with shaking (100 rpm) for 48 hours. The culture with strong development and bright pigmentation was subjected to pigment extraction by slightly modifying the process employed by Bhat et al., (2015), in which the enriched cultures were centrifuged separately at 8000 rpm for 15 minutes at 4 0C to separate cells. Separated cell pellets were centrifuged at 8000 rpm for 10 minutes and rinsed twice with sterile distilled water. Separate cell pellets were suspended in 5 ml of methanol and left overnight before centrifugation at 8000 rpm for 10 minutes at 4 ^{0}C. The supernatants were spectrophotometrically analysed separately between 400-600 nm to determine the max. The degree of pigmentation was also calculated during tertiary screening. The degree of pigmentation is the ratio of pigment produced to biomass produced, measured by dividing the absorbance of the pigmented extract at its respective max by the absorbance of 48 hours culture at 660 nm. The isolate with the highest degree of pigmentation and typical max of carotenoids was selected for quaternary screening.

V. Quaternary Screening

Based on antioxidant activity, quaternary screening was performed by measuring the antioxidant potential of pigment extract from tertiary screened isolates. The antioxidant potential was calculated by measuring free radical scavenging activity using the approach described by Sasidharan et al. (2013) with minor modifications in which 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was utilised. A 0.1 mM DPPH solution in methanol was produced, and 1 ml of this was mixed with 2 ml of pigment extract in methanol. The mixture was vigorously mixed and set aside at room temperature for 30 minutes. The entire DPPH assay was performed in low-light conditions. Following incubation, the absorbance of various carotenoid extracts in the experiment was measured separately at 517 nm using a U.V. visible spectrophotometer. The control reaction used 1 mL of 0.1 mM DPPH solution and 2 mL of methanol with the same treatment as the test samples. DPPH is a persistent radical that is dark violet in solution and is decolorized by strong reducing agents and antioxidants. The high rate of decolorization with pigment extract indicates increased antioxidant activity. The bacterial isolate extract with the highest free radical scavenging activity (% RSA) was evaluated as a potential antioxidant function, and the isolate as a potential carotenoid generator. The radical scavenging activity (% RSA) of carotenoids extracts in methanol was tested and calculated.

$$\% \text{ RSA}\left(1 - \frac{\text{absorbance of sample}}{absorbance\ of\ control}\right)100$$

Munsell colour chart was used to match the colour of bacterial cell pellet utilising Munsell colour code Android app as "DIC Colour Guide" as per Koll morgen Corporation, "Munsell colour charts for plant tissue" as offered by Munsell Colour division, Maryland (1972).

3. RESULTS AND DISCUSSION

I. Sample Collection and Enrichment

The biodiversity and uniqueness of the source from which the samples were collected were taken into account during the collecting process. Water and mud were taken from Lonar Creator Buldhana in Maharashtra State, (India), and subjected to enrichment in nutrient broth pH8.5, where water samples were analysed for their physiological properties before being used as an inoculum for enrichment. Zeni et al. (2011) isolated carotenoid producing microorganisms from soil, leaves (Eucalyptus and orange), fruits (orange, papaya, persimmon, peach, apple, and so on), teas, and agro-industrial wastes, whereas Brown (1963) isolated carotenoid producing bacteria from sediment samples. Arunkumar et al. (2006) and Shatila et al. (2013) collected air samples in order to isolate carotenoid-producing bacteria. Godlinho et al. (2008) identified orange-coloured bacteria from the rhizosphere of sand dune creeper-Ipomoea pes-caprae, whereas Arulselvi et al. (2014) isolated yellow pigment generating bacteria from soil samples. Pathak et al., (2012) and Oren et al., (2001) isolated carotenoid-producing haloarchaea and halophilic bacteria from brine samples, respectively. Sasidharan etal. (2013) isolated pigmented isolates from soil 67% of the time and 33% of the time. A variety of samples were studied in the current study, including water and mud (marine, hypersaline-hyperalkaline), mud from these ecosystems for the isolation of carotenoid pigment generating bacteria. There have been very few research where identical samples have been obtained and investigated for the isolation of carotenoid producing bacteria. As carotenoids are produced by bacteria in response to harsh settings and unfavourable growth conditions, it can be inferred that extreme situations can encourage the growth of carotenoid generating bacteria.

II. Isolation and Primary Screening

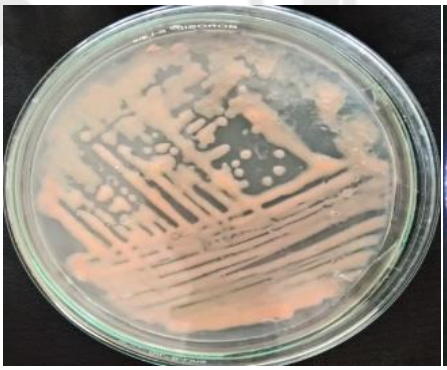

Fig:1 Spread Plate method

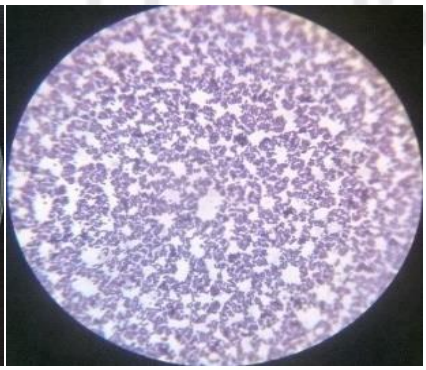

Fig: 2 Grams Staining

Colony	Characteristics
Size	0.4
Shape	Circular
Margin	Entire
Colour	Orange
Elevation	Concave
Opacity	Opaque
Surface	Smooth
Consistency	Buterous
Gram's nature	Gram positive
Motility	Motile
Morphology	Coccus

Table: 1 Colony Characteristics

Carotenoid-producing bacteria were isolated from enriched samples using a serial dilution and spread plate approach on nutrient agar pH 8.5. Following incubation, 64 bacterial isolates were obtained. Out of 64 bacterial isolates, 53 were pigment-free, whereas 11 had yellow, orange, yellow orange, lemon yellow, and red pigmentation and were subjected to screening. Because carotenoids have yellow, orange, and red pigmentation, all isolates exhibiting bright yellow, yellow orange, lemon yellow, orange, and red pigmentation on nutrient agar were screened predominantly among

Fig: 3 Substrate

Fig: 5 Crude pigments

Fig: 6 Extraction of pigments

Fig: 7 Pure form of pigments

pigmented and non-pigmented isolates. The most isolates were found with yellow orange and lemon-yellow pigmentation. Godinho and Bhosle (2008) isolated the orange pigment generating alkaliphilic bacterial strain *Microbacterium arborescens*-AGBS from the rhizosphere of the costal sand dune creeper, whereas Balraj et al. (2014) isolated yellow-coloured marine bacteria from the sea water of India's Peninsular Region. The chances of pigment-producing bacteria in this study are equivalent to current research findings, where the most yellow-coloured bacterial isolates were collected. There have been no previous publications on the isolation and screening of carotenoid producing bacteria from hyper saline hyper alkaline environments, hence this is the first study on the isolation and screening of carotenoid producing bacteria from such an extreme environment. The current study also found that samples from severe environments are a significant source of pigment-producing bacteria.

III. Secondary Screening of Carotenoid Producing Bacteria

Secondary screening was performed on 11 pigmented bacteria based on their qualitative pH and NaCl tolerance. All isolates were grown separately on nutrient agar with varying pH (6-12) and NaCl (1-6%) where most of the secondary screened isolates showed growth above pH 11.0 and 4% NaCl with reduced pigmentation while some isolates did not grow at high pH and concentration of NaCl and were eliminated for further studies during secondary screening. Lee et al. (2004) conducted similar studies in which astaxanthin generating *Paracoccus haeundaensis* sp. demonstrated pH tolerance in the range 6-10.5 and NaCl tolerance up to 6%. Zheng et al. (2011) discovered that *Paracoccus beibuensis* JLT1284T can grow in pH ranges ranging from 6.0 to 8.0 and NaCl concentrations ranging from 2 to 15%. The objective in these research studies was on screening potential carotenoid producing bacterial isolates, hence screening was done based on both pH and NaCl tolerance. There have been no analogous research investigations where combined criteria of tolerance to pH and NaCl were employed for screening of carotenoid producing bacteria, emphasising the uniqueness of the current findings.

IV. Tertiary Screening of Carotenoid Producing Bacteria

Sr. No.	Isolates	Systematic Name	Munsell colour code	Appearance of Pigment
1	DM1	Vivid Yellow Red	1.3YR6.6/17.5	
2	DM4	Vivid Yellow Red	4.0YR 6.7/16.9	
3	DM7	Vivid Reddish Yellow	1.0Y 7.9/13.4	
4	DM9	Vivid Yellow Red	4.0YR 6.7/1	
5	DM10	Vivid Greenish yellow	7.1Y8.8/15.0	
6	DBK2	Vivid Reddish Yellow	1.0Y7.9/13.4	
7	DK1	Vivid Reddish Yellow	4.0YR 6.7/16.9	
8	DK4	Vivid Greenish Yellow	1.0Y 8.8/15.0	
9	MBK5	Light Yellow Red	1.5YR 7.0/10.7	
10	DK8	Deep Yellow Red	9.0R 4.5/12.3	
11	MBK6	Vivid Yellow Red	1.3YR6.6/17.5	

Table-2 Munsell colour code of the pigment produced by screened isolate

A total of 11 pigmented isolates with a pH range of 6-12 and NaCl tolerance of up to 1-6% were subjected to tertiary screening based on pigment extraction and spectrophotometric characterization. The maximum of extracted pigments from different isolates varied between 400-600 nm, according to spectrophotometric analysis. The degree of pigmentation is defined as the ratio of biomass to pigment generated and is used to determine the efficiency of bacteria in producing carotenoids. Based on their absorption maxima, the selected isolates indicated pigments such as DM1 as astaxanthin (473 nm), DM4 as zeaxanthin (443 nm), DM7 as astaxanthin (475 nm), DM9 as -carotene (449 nm), DM10 as -carotene (450 nm), DBK2 as cryptoxanthin (463 nm), DK4 as (436nm). Using the Munsell colour android application, the Munsell colour code of each pigment from screened bacteria was determined. El-Banna et al. (2012) used the Munsell colour chart to characterise the pigmented yeast colony colour as 10R7/8. According to Rodriguez-Amaya et al. (1999), the absorption spectra of -carotene should be about 450 nm. Shatila et al. (2013) investigated orange pigment from *Exiguobacterium* within the wavelength range 400-690 nm and discovered a shoulder peak at 463 nm, which is a characteristic pattern of carotenoids' absorption spectrum. Oren et al. (2001) isolated a red carotenoid pigment from the halophilic bacteria *Salinibacter* and analysed it spectrophotometrically, finding an absorption peak at 482 nm with a shoulder at around 510 nm. In this research, tertiary screening extraction and pigment analysis were performed to determine the type of carotenoid, isolate pigment production capacity, and prospective applications. There were very few research research where spectrophotometric characterisation and pigmentation degree were analysed as a screening strategy.

V. Quaternary Screening Based on Antioxidant Activity Performed by DPPH Assay

The isolates with a high degree of pigmentation and a high concentration of carotenoid pigments were chosen for further testing of their antioxidant potential. Crude methanolic extracts from 11 tertiary screened isolates were tested for antioxidant activity or free radical scavenging activity (% RSA) using the DPPH method. The stable radical of DPPH is widely employed to assess the antioxidant activity of natural colourant products. DM1, DBK1, and SS1 had the highest free radical scavenging activity (53%, 23%, and 23%) of the 11 isolates tested. The isolate DM1 displayed a high level of pigmentation and RSA (2.81 and 53%). Sasidharan et al. (2013) employed the degree of pigmentation of carotenoid producing bacteria as a screening criterion, with bacterial isolate RS7 having the highest degree of pigmentation (8.31). Arulselvi et al., (2014) measured % RSA for yellow pigment from bacterial strains using the DPPH technique and found 70% activity. According to Nishino et al., (2000), the DPPH free radical scavenging activity of methanol extract is dependent on carotenoids concentration. According to Sandesh (2007), the antioxidant potential of carotenoid varies many times in vivo due to the pro-oxidant action. In this study, % RSA was utilised as the final

criterion for isolate selection, highlighting the usefulness of carotenoids as an antioxidant molecule. The antioxidant potential of the selected isolate increases its industrial usefulness for carotenoids production. Isolate DM1 was finally selected because it can produce bright orange pigment, tolerate a wide pH range of 6-12 and NaCl up to 6% with a good degree of pigmentation (2.81), methanolic extract of pigment showed absorption maxima at 473 nm with characteristic shoulder peak of ketocarotenoids and 53% RSA, and thus it was finally screened, selected, and preserved for further studies.

4. CONCLUSION:

Results of the studies showed that from the 64 isolates 11 isoates have a potential to produce pigments. The study involved isolation and screening of pigments producers from Lonar Crater. Characterization was performed using standard methods as salt tolerance, extraction, antioxidant and photometric. The above results are promising but still there is a need for DNA sequencing, gene identification and other bioinformatics parameters study for further establishment of value of the different isolates.

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