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Bio prospecting Halo alkalotolerent Bacteria from Lonar Crater for Production of Carotenoids.

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ABSTRACT

Carotenoids are yellow, orange and red pigments distributed in variety of bacteria. Carotenoids are currently produced as constituents in the vitamins and dietary supplements for human and animal consumption. Total 40 red, yellow and orange pigmented bacteria were primarily screened from 20 different samples of soil, mud, sediment and water collected from different sites of a natural extreme source of Lonar Crator. All bacteria were isolated at pH 8.5 using Nutrient agar. Based on intensity of pigmentation 34 bacterial isolates were selected for secondary screening. Screening was done on the basis of pH (6-12) and salt (NaCl) tolerance (2-6%). From selected isolates, 12 bacteria have potential to grow and produced intense pigments up to pH 12 and 6% salt. The methanol extract of pigment gave characteristics lambda max at different wave length viz. 472 nm,443 nm,475 nm, 449 nm and 468 nm which corresponds to absorption maxima (λmax) near to known carotenoids viz. Astaxanthin (472nm), Zeaxanthin (443nm), B-Carotene (450nm) and Canthaxanthin (470nm-474nm). A potential SL_2* bacterium was selected having ability to produce Astaxanthin, with 2.8 degree of pigmentation/g wet weight and 53% free radical scavenging activity. Screened strain of SL_2* will be optimised to produce high yield of carotenoids for application studies in food and feed as source of pro- vitamin A and antioxidant.

KEY WORDS-Carotenoids, Haloalkalotolerent, antioxidant, DPPH assay

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1. INTRODUCTION

The isolation and screening of microorganisms from natural sources is always been an important aspects of research. So far human and animal health is concern;carotenoids are gaining important consideration for their preventive role for diseases generated by free radicals. In present study we have used various samples from Lonar crator to isolate and screen out certain Carotenoids producing bacteria. Carotenoids are terpenoid pigment produced as primary metabolite by plants,fungi,yeast and bacteria.Both photosynthetic and non-photosynthetic bacteria synthesizecarotenoids which play an important role in protection of bacterial cells from photodynamic action and free radical damage.The carotenoids are capable of quenching photo-sensitizers[1]and, interacting with singlet oxygen and scavenging proxy radical β-carotene[2] Astaxanthin, Lutein, Canthaxanthin and Lycopene are some examples of the carotenoids[3.]Many carotenoid producing bacterial species are known till date includes *Halococcus, Halobacterium*[4], *Micrococcus, Staphylococcus, Flavobacteria*[5], *Paracoccus*[6].

From commercial point of view there is an increased demand of carotenoids as a natural color.It also play an important role in inhibiting many, “life style related” diseases such as cardiovascular diseases, “age related muscular degeneration”and it acts as a source of pro-vitamin A and has antioxidant potential[7] and exhibits significant anti-carcinogenic activities[8].

Carotenoid from microbial source is an alternative to decrease hypersensitivity due to inactive isomers present in synthetic colorants.Recently carotenoids are used commercially as feed additives,animal feed supplement and more recentlyasnutraceuticals for cosmetics,and pharmaceutical purposes[9]

In the present study an attempt was made to isolate halo-alkalotolerant carotenoid producing bacteria having good ability to produce carotenoid and better antioxidant activities from Lonar crater. The Lonar crater is hyper-saline,hyper-alkaline water body formed by meteoroid impact at Buldhana district of Maharashtra state in India.To the best of our knowledge there are very few carotenoid producing bacteria isolated combine from the hyper saline and hyper alkaline environment.

2. MATERIALS AND METHODS

2.1 Sample collection, enrichment and Isolation of carotenoid producing bacteria

Around 20 different samplesi.e. soil(05), mud(05), sediment(05) and water(05) were collected from various locations of Lonar crater in sterile container. The enrichment of samples was carried out in100ml Nutrient Broth(Himedia) pH 8.5 by inoculating 1ml of 1% soil solution of respective samples volume by volume (v/v) and water sampleswhich were incubated for 48 hours at room temperature (RT) near window inpresence of day light.

2.2 Primary screening

Each enriched sample was streaked on the Nutrient Agar with pH 8.5 and incubated at RTfor 48 hoursnear window in presence of day light. After incubation red, yellow and orange pigmented bacterial colonies were primarily screened and transferred on the nutrient agar slants for further studies

2.3 Secondary screening

The screening was done on the basis of pH and salt tolerance. The pH tolerances of all isolatedbacteriawere checked qualitatively on Nutrient gar plates with varying pH (5-12). The isolates showingbright pigmentation and broad range pH tolerance were screened for further studies.Similarly, salttolerance of all isolated bacteria was checked qualitatively on nutrient agarplates with varying concentration of NaCl (1-6%). The isolate showing bright pigmentationand broad range salt tolerance wasscreened for further studies.

2.4 Tertiary Screening

Tertiary screening was done on the basis of Spectrophotometric analysis of crude pigmented extract and evaluating their degree of pigmentation (DOP). For extraction of pigment from different bacteria procedure given by Sasidharan P. *et al.*[10] with some minor modifications was adopted. The secondarily screened bacteria were inoculated in 100 ml of Nutrient Broth with pH 9.5 and were incubated at RT on rotary shaker at 120rpm for 48 hours. After incubation liquid culture was centrifuged at 8000 rpm for15 minutes at 4°C and washed with sterile distilled water at 6000rpm for 10 minutes. Pigment extraction was done by its further incubationby adding 5ml methanol by using 60^0 C water bath for 30 minutes until all visible pigments are extracted and centrifuged at 6000rpm for 10 minutes.

Colored supernatant was carefully separated from pellet and stored in amber colored tubes for analysis.The pigment extracts were analyzed by-

2.4.1. Spectrophotometric characterization (400-600nm)

Extracted pigments were separatelyanalyzed by scanning the absorbance in the wavelength region of 400-600nmby using spectrophotometer. The total carotenoid content in organic solvent extract was estimated by measuring the absorbance at λ_{max}.

2.4.2 *Evaluation of degree of pigmentation.*

The degree of pigmentation (DOP) was estimated by slight modification of previously used method by Sasidharan P. *et al.*[10]where ratio of cell mass to pigmentproduced was estimated,cultures giving higher values for this ratio were selected for further screening which was calculated by using formula-

$$DOP = \frac{\textbf{Absorbance at } \lambda\textbf{max}}{\textbf{Absorbance at 660}}$$

2.5 Quaternary screening

Quaternary screening was done by evaluating antioxidant potential of tertiaryly screened isolates. The antioxidant potential was estimated by evaluating free radical scavenging activity by procedure given bySasidharan P. *et al.*[10] with some modifications. The bacterial isolate showing highest %RSA was selected as a potential carotenoid producer.Free radical scavenging activity (%RSA) of the fraction of methanol extract of carotenoids was measured by 1,1-diphenyl-2-Picryl -hydrazil (DPPH) (Himedia) Solution of DPPH (0.1mM) in methanol was prepared; 1ml of DPPH solution was added to 3 ml of fraction in methanol and shaken vigorously and allowed to stand at room temperature for 2 hours in dark. Control was prepared by taking 1ml of DPPH and 2ml of methanol. Absorbance was measured at 517nm and %RSA was calculated by-

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

1.6. Characterization of selected potential carotenoid producing bacteria.

Among the quaternary screened bacteria, the isolate showing better value for DOP and %RSA was selected as potential carotenoid producer. Morphological and colony characterization of selected bacteria was done.

3.RESULTS AND DISCUSSIONS

3.1 Sample collection, enrichment and Isolation of carotenoid producing bacteria

Different samples were collected from different locations of Lonar Crater of Buldhana district, Maharashtra state, India. A total 20 samples i.e. soil, mud, sediment and water were collected and subjected for isolation. From 20 different samples 120 bacterial isolated were obtained, among these soil samples given highest i.e. 60% isolates (Figure-1). Out of 120 different isolates 40 pigmented i.e. yellow, red and orange pigmented colonies were selected (Figure-2).

3.2 Primary screening

All bacteria producing bright pigment on Nutrient Agar with pH 8.5 at RT for 48 hrs. incubation in day light condition near window were screened primarily from pigmented and non-pigmented isolates. Figure-2 indicates the distribution of different pigment producing bacteria among isolates.A Godinho and S. Bhosle[5] isolated alkaliphilic orange pigmented strain of *Microbacterium arborescens* – AGBS from costal sand dunes. Janani Balraj *et al*[11] isolated yellow pigmented marine bacteria from Peninsular Region of India. There are very few references showing both alkali tolerance as well as salt tolerance carotenoid producing bacteria. The present study was focused on isolation of Haloalkalotolerent carotenoid producing bacteria from Lonar Crater.

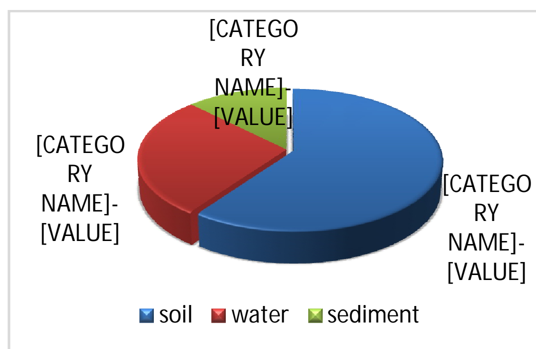

Figure No.1 "Distribution of pigment Bacteria"

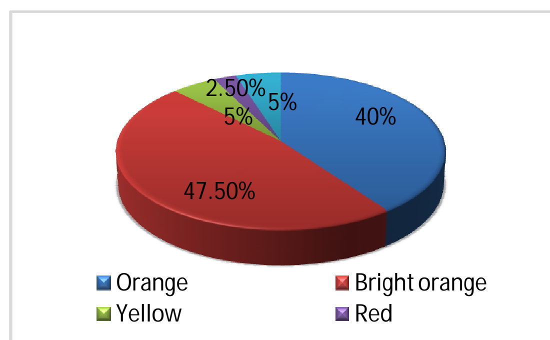

Figure No.2 "Distribution Pigment Producers"

3.3 Secondary Screening of carotenoid producing Bacteria

In secondary screening pH and NaCl tolerance of 40 pigmented i.e., yellow, red and orange pigmented bacteria was evaluated qualitatively. All isolates were grown on Nutrient Agar with varying pH(6-12) and NaCl (1-6%) separately by streak plate method where out of 40isolated bacteria 12 pigmented isolates were found most tolerant to pH (6-12) and 6% NaCl with good pigmentation. Above pH 11.0 and 4% NaCl there is growth but no pigment formation by most of the isolates. Similar studies were carried out by Jae Hyung Lee *et al*[12] where Astaxanthin producing *Paracoccus haeundaensis sp*. show pH tolerance ranges from (6-10.5) and NaCl tolerance up to 6%.

3.4. TertiaryScreening of carotenoid producing Bacteria

Broad range pH and NaClwas tolerated by12 isolated Bacteria which were subjected for tertiary screening on the basis of extraction of pigment and its Spectrophotometric characterization. According to the Spectrophotometric analysis $\lambda_{max}$of extracted pigmentsfrom different isolates was different varying between 400-600nm (Table -1). The degree of pigmentation is a ratio of biomass to pigment produced andit is considered as efficiency of bacteria to produce carotenoids.These isolates viz., SL_2*, SL_4*, SL_2y, SL_{10} and SL_4-1 were selected on the basis of its Spectrophotometric characters and degree of pigmentation for further study (Figure -1).

Table No.1. "Tertiary Screening on the basis of extraction and Spectrophotometric characterization"

Sr. No.	**Bacterial Isolates**	λ_{max} (nm)	**Predicted PIGMENT**	**Absorption at 660nm**	**Absorption at λ_{max}**	**Degree of pigmentation**
1	**SL_2***	**473**	**ASTAXANTHIN**	**1.35**	**3.797**	**2.81**
2	SL_2**	466	RHODOXANTHIN	0.968	0.2726	0.56
3	**SL_4-1**	**443.5**	**ZEAXANTHIN**	**1.446**	**2.1036**	**1.60**
4	SL_6	440.5	ZEAXANTHIN	1.655	1.022	0.63
5	SL_9	475	--	1.363	1.3152	0.96
6	**SL_{10}**	**475**	**--**	**1.162**	**1.6204**	**1.67**
7	IC	536.5	PRODIGIOSIN	0.391	2.5496	13.03
8	$LBSL_4$	411	LEUTIN	1.167	0.3904	0.64
9	LW_1	449	β-CAROTENE	0.779	0.2663	0.68
10	**SL_4***	**449**	**β-CAROTENE**	**1.829**	**1.576**	**0.86**
11	SD_4	446	ZEAXANTHIN	1.235	0.2159	0.17
12	**SL_2y**	**468**	**--**	**1.993**	**2.8712**	**1.44**

3.5. Quaternary screening on the basis of antioxidant activity performed by DPPH assay.

In present study all the isolates were found to produce carotenoids. Their degree of pigmentation was different. The isolates showing good degree of pigmentation were selected for evaluation of its antioxidant potential where the isolates SL_2*, SL_4*, SL_2y, SL_{10} and SL_4-1 evaluated for antioxidant activity or free radical scavenging activity (%RSA). DPPH is the stable radical and is frequently used for of natural colorant product. Out of 05 different selected isolates SL_4* and SL_2* showed highest free radical scavenging activity i.e. 60% and 53% respectively. The isolate SL_2* shown good degree of pigmentation as well as % RSA i.e. 2.8/g and 53% (Table-2) was selected for further studies. Similar studies on degree of pigmentation of carotenoid producing bacteria were carried out by Sasidharan *et al*[10]where highest degree of pigmentation was shown by bacterial isolate RS7 i.e. 8.31found.RSA were carried out by the Indra Arulselvi P. *et al*[13]by DPPH method showed 70%activity. NishinoT.*et al*[14]suggested that DPPH free radical scavenging activity of methanol extract depends on concentration of carotenoids and according to Snandesh K.[15]generally carotenoid potential for antioxidants vary many times in vivo due to pro-oxidant effect.

Figure No. 3. "Crude Carotenoid extracts in Methanol from screened carotenoid producing Haloalkalotolerent bacteria."

Table No.2. "Selection of potential carotenoid producing bacteria."

Sr.No.	Bacterial culture	Acidic pH	Alkaline pH	NaCl tolerance (%)	λmax (nm)	DOP	%RSA
1	**SL_2***	**6**	**12**	**6**	**472**	**2.81**	**53**
2	SL_4-1	6	12	6	443	1.60	19
3	SL_{10}	6	12	6	475	1.67	21
4	SL_4*	6	12	6	449	0.94	60
5	SL_2y	6	12	6	468	1.12	11

3.5 Characterization of selected potential carotenoid producing bacteria.

Among the quaternary screened bacteria viz.SL_2*, SL_4-1, SL_{10}, SL_4* and SL_2ythe isolate showing better value for DOP and % RSA Out of quaternary screened bacteria SL_2*was selected as potential carotenoid producer which show λ max at 472nm which shows similarities with an important carotenoid i.e. Astaxanthin with DOP 2.81 and RSA 53% which is highest among all isolated and screened bacterial isolates it also shows tolerance to pH in alkaline range and NaCl (Table-2). Morphological and colony characterization of bacterial isolate SL_2* was done (Table-3, Figure-4)

Table No.3 "Colony characters of SL_2

Sr.No	Colony characters	Observations
1	Size	0.1mm
2	Shape	Round
3	Color	Bright orange
4	Margin	Entire
5	Surface	Smooth
6	Elevation	Convex
7	Consistancy	Mucoid
8	Opacity	Opeque
9	Grams Nature	Gram Negative
10	Morphology	Coccoid rods
11	Motility	Non motile

Figure No. 4. "Carotenoid producing Haloalkalotolerent bacteria SL_2*"

4. CONCLUSION

A potential Haloalkalotolerent SL_2* bacterium was selected having ability to produce carotenoid having λ_{max}472 nm which is near to Astaxanthin, with 2.8 DOP and 53% RSA estimated by DPPH method. Screened strain of SL_2* will be further optimized to produce high yield of carotenoids and its application will be studied.

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