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Isolation and Screening of Carotenoid Producing Bacteria

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A B S T R A C T

This article includes isolation and screening of carotenoid producing bacteria. Samples were collected from different eco-climatic regions of Maharashtra. From soil, mud, sediment, and water samples total 620 bacterial strains were isolated out of which 105 isolates were pigment producers, many of which exhibited bright orange, orange, yellow orange, lemon yellow and red pigmentation. Out of 105 isolates efficient carotenoid producers were screened after primary, secondary, tertiary, and quaternary screening based on pigmentation, tolerance to pH and NaCl%(w/v), degree of pigmentation, absorption maxima and free radicle scavenging activity respectively. The selection of potential carotenoid producer was done from finally screened isolatesSL2, SL4*, SL4-1, SL10, SL2y, DKMIII, DABW1, DGM, SSD2, SWT3 and SS1 based on free radicle scavenging activity by DPPH (1,1 Diphenyl-2 Picryl Hydrazil) method. Isolate SL2 showed bright orange pigmentation, tolerance to pH (6-12), NaCl (1-6%), λmax at 473 nm and maximum 53% free radicle scavenging activity, so it was selected for further studies as a potential carotenoid pigment producer.

Introduction

Natural pigments are always favored by human beings as an additive in food to avoid side effects of the artificial mineral and metal-based colors. The bulk of chemically synthesized colors were derived from aniline and toxic petroleum products. Natural colors are produced mainly by the plants but their availability varies according to season and geographical distribution. Now a day's microbial sources of pigment are emphasized due to their reach biodiversity, year around availability and high production capacity. Carotenoid are C40 compounds which can act as source of pigment as well as therapeutic

molecule acting as a source of vitamin A, antioxidants or possible tumor inhibiting agent. Carotenoids pigments are present in all photosynthetic organisms as well as in some photosynthetic and non-photosynthetic bacteria, fungi, yeast, and algae. The non-photosynthetic microorganisms produce carotenoids where, carotenoids protect cell from stressed environment by altering membrane fluidity. Bacteria are more convenient for large scale production of carotenoid than algae and fungi due to their unicellular nature, relatively high growth rate, easy handling, and processing. This research article describes the isolation and screening of carotenoid producing bacteria from different environmental niche and selection of potential carotenoid producer for further studies. For isolation of carotenoid producing bacteria selective enrichment culture technique was used to favor the multiplication of bacteria from the sample which were low in number initially.

Materials and Methods

Sample collection and enrichment

For isolation of carotenoid producing bacteria, random sampling was done in the month of May and June. Different samples (water, soil, mud, and sediment) were collected in sterile container and stored at 4^0C prior to use.

The samples collected includes hypersaline-hyperalkaline environment of Lonar crater, Buldhana, Maharashtra, Marine water from Kerala, Goa, and Mumbai sea shore. Some samples were also collected from different regions of Aurangabad such as water samples from Panchakki; soil, mud, sediment, and water samples from Harsul lake and Dr. Salim Ali Lake, Aurangabad and Hatala lake, Hingoli. Enrichment of samples was done in Nutrient broth and incubated for 48h in presence of day light at room temperature.

Isolation and primary Screening

Isolation was done by serial dilution of enriched culture in physiological saline (0.85% NaCl) and 0.1 mL of each diluent was used for spreading the nutrient agar (pH 8.5) with 1.5% agar-agar. The plates were prepared in duplicates and incubated for 48 h in day light near window at room temperature. After incubation, plates with isolated colonies with pigmentation were selected as master plates and kept for further use. Primary screening of all pigment producing cultures was carried out by growing pigmented isolates from master plates on nutrient agar with pH 8.5 at room temperature for 48 h in duplicates. The primary screening of isolates was done based on ability to produce yellow, orange or red pigment which likely to be non-diffusible among other pigment producers and non-pigmented cultures on plates. The isolates showing yellow, orange and red pigmentation were separated and transferred on nutrient agar slants and preserved at 4^0C for further studies.

Secondary screening

The Secondary screening was done based on qualitative tolerance to pH and salt (NaCl %). All primarily screened bacterial isolates were streaked on nutrient agar with different pH from 5-12 and nutrient agar consisting NaCl (1-6%) separately. The plates were incubated at room temperature in day light near window for 48 h.

The isolates growing on nutrient agar with wide range of pH were considered as tolerant cultures and streaked on nutrient agar with higher NaCl i.e. 1-6%. Cultures showing good pigmentation with pH and NaCl tolerance while retaining its bright pigmentation were screened secondarily and selected for tertiary screening.

Tertiary screening

Tertiary screening was done based on qualitative and quantitative performance of the isolate to produce pigment. In tertiary screening, actively growing (48 h) cultures of secondarily screened isolates were inoculated separately into nutrient broth (pH 8.5 and 1% NaCl) and incubated with shaking condition (100 rpm) at room temperature for 48 h. The culture with vigorous growth along with bright pigmentation were subjected to extraction of pigment by slight modification of the procedure used by Bhat *et al*., (2015) where, the enriched cultures were centrifuged separately at 8000 rpm for 15 min. at 4^0C to separate cells. Separated cell pellets were washed twice with sterile distilled water by centrifugation at 8000 rpm for 10 min. The cell pellets were suspended separately in 5 mL of methanol and kept overnight, followed by centrifugation at 8000 rpm for 10 min. at 4^0C. The supernatants were separately analyzed spectrophotometrically between 400-600 nm to find the λ_{max}. During tertiary screening degree of pigmentation was also calculated. Degree of pigmentation is the ratio of pigment produced to the biomass produced where, ratio of absorbance of the pigmented extract at its respective λmax to the absorbance of 48h culture at 660nm was calculated. The isolate showing higher values for degree of pigmentation and characteristic λ_{max} of carotenoids were selected for quaternary screening.

Quaternary screening

Quaternary screening was done based antioxidant activity and hence it was done by evaluating antioxidant potential of pigment extract from tertiary screened isolates. The antioxidant potential was estimated by evaluating free radical scavenging activity as per procedure given by Sasidharan *et al*., (2013) with slight modifications where, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was used. The solution of DPPH 0.1 mM was prepared in methanol and of this 1 mL was added 2 mL of pigment extract in methanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The whole steps of DPPH assay were carried out in subdued light. After incubation absorbance of different carotenoid extracts under assay were measured separately at 517 nm by using UV-visible spectrophotometer. The percentage of DPPH scavenging activity was calculated by using the equation. The control reaction was carried out by taking 1 mL of 0.1 mM DPPH solution and 2 mL of methanol with same treatment as to test samples. DPPH is stable radical and in solution it is dark violet in color and it is decolorized by potent reducing substances and antioxidants. High rate of decolorization with pigment extract suggest better antioxidant activity. The extract from bacterial isolate showing highest free radical scavenging activity i.e. % RSA was considered as potential antioxidant function and isolate as potential carotenoid producer. The radical scavenging activity (% RSA) of the methanol fraction of carotenoids extracts were measured and calculated by-

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

The color of bacterial cell pellet was matched by Munsell color chart by using Munsell color code Android app as 'DIC Color Guide'as per Kollmorgen Corporation, "Munsell color charts for plant tissue" given by Munsell Color division, Maryland (1972).

Results and Discussion

Sample collection and enrichment

The sampling was done by considering biodiversity and uniqueness of source from which samples were collected. Samples were collected from different locations of Maharashtra state and India (Table-1a)

(Figure-1a, 1b). Total 88 samples i.e. soil, mud, sediment, and water were collected and subjected for enrichment in nutrient broth pH 8.5 where, water samples were analyzed for their physiological parameters before using as an inoculum for enrichment (Table 1b). Maximum number of samples i.e. 31% was collected from Lonar crater while least number of samples was collected from fresh water lakes. Zeni *et al*., (2011) collected soil, leaves (Eucalyptus and orange), fruits (orange, papaya, persimmon, peach, apple etc.) teas, and agro-industrial wastes for isolation of carotenoid producing microorganisms while, Brown, (1963) used sediment samples for isolation of carotenoid producing bacteria. Arunkumar *et al*., (2006) and Shatila *et al*., (2013) done air sampling for isolation of carotenoid producing bacteria. Godlinho *et al*., (2008) isolated orange pigmented bacteria from rhizosphere of sand dune creeper-*Ipomoea pes-caprae* while Arulselvi *et al*., (2014) used soil samples for isolation of yellow pigment producing bacteria. Pathak *et al*., (2012) and Oren *et al*., (2001) used brine samples for isolation of carotenoid producing haloarchaea and halophilic bacteria respectively. Sasidharan *et al*., (2013) isolated 67% pigmented isolates from soil and 33% from air. In the present studies the variety of samples were used which includes soil, water (marine, hypersaline-hyperalkaline, and fresh water), mud and sediment of these ecosystems for isolation of carotenoid pigment producing bacteria. There are very few studies reported where, similar samples were collected and studied combinedly for isolation of carotenoid producing bacteria. From the data generated from current research (Table-1a and 1b), from 88 samples, 105 yellow, orange, and red pigment producing bacterial isolates were recovered. Therefore, it can be assumed that growth carotenoid producing bacteria can be induced by extreme environments as carotenoids are produced by bacteria in response to extreme environment and adverse growth conditions.

Isolation and primary screening

The isolation of carotenoid producing bacteria was done from enriched samples by serial dilution and spread plate technique on nutrient agar pH 8.5. After incubation, total 620 bacterial isolates were recovered. Out of 620 bacterial isolates 515 isolates were not having any type of pigment and 105 isolates were with yellow, orange, yellow orange, lemon yellow and red pigmentation which were subjected for screening.

Carotenoids are pigments with yellow, orange and red pigmentation hence, all isolates producing bright yellow, yellow orange, lemon yellow, orange and red pigment on nutrient agar were screened primarily among pigmented and non-pigmented isolates. Table-2a, 2b and figure-2a, 2b shows the distribution of different pigment producing bacteria among isolates. Highest number of isolates was recovered with yellow orange and lemon-yellow pigmentation i.e. 32%, and 29% respectively while, least quantity of red pigmented isolate was recovered i.e. 4%. Bright orange and orange pigmentation was found with the 11% and 24% isolates respectively. Godinho and Bhosle, (2008) isolated orange pigment producing alkaliphilic bacterial strain *Microbacterium arborescens*–AGBS from rhizosphere of costal sand dune creeper while, Balraj *et al*., (2014)isolated yellow pigmented marine bacteria from sea water of Peninsular Region of India. They have isolated 61 different bacterial strains out of which 19 (31%) strains were pigmented and out of 19 pigmented strains 11 (58%) were yellow and 6 (32%) were orange pigment producing strains and remaining 2 (10%) strains were peach in color. The probabilities of pigment producing bacteria from this study are similar with

present research findings where maximum yellow pigmented bacterial isolates were recovered. None of reports were found on isolation and screening of carotenoid producing bacteria from hypersaline-hyperalkaline environment so it is the first report on isolation and screening of carotenoid producing bacteria from such said extreme environment. The present research finding also highlights that samples from extreme environment are rich source of pigment producing bacteria.

Table.1a Distribution of samples collected

Sr. No.	Sample source	Geographical Location	Soil	Mud	Water	Sediment	Total
1	Lonar Crater, Buldhana	19.9758° N, 76.5069° E	18	05	13	07	43
2	Marine water sample	--	--	--	23	--	23
3	Dr. Salim Ali lake, Aurangabad	19.8992° N, 75.3423° E	05	--	03	05	13
4	Harsul Lake, Aurangabad	19.9282° N, 75.3368° E	01	--	05	08	14
5	Panchakki, Aurangabad	19.8896° N, 75.3155° E	--	--	05	--	05
6	Hatala Lake, Hingoli	19.7174° N, 77.1494° E	--	--	07	--	07
		Total	24	05	56	20	105

Table.1b Physicochemical properties of the sample collected

Sr. No.	Sample source	pH	Temperature (^{0}C)	Turbidity	Odor	Color
1	Lonar Crater, Buldhana	10.8±0.2	36	P	Algal	Colorless
2	Marine sample	7.2±0.2	35	NP	NP	Colorless
3	Dr. Salim Ali lake, Aurangabad	8.8±0.2	32	P	Pungent	Turbid
4	Harsul Lake, Aurangabad	7.8±0.2	32	NP	NP	Colorless
5	Panchakki, Aurangabad	7.4±0.2	30	NP	Algal	Colorless
6	Hatala Lake, Hingoli	7.2±0.2	30	NP	Algal	Colorless

Table.2a Distribution of isolates according to sample source and pigmentation

Sr. No.	Pigmentation Sample source	Non-pigmented isolates	Bright Orange	Orange	Yellow Orange	Lemon Yellow	Red	Total
1	Lonar Crater, Buldhana.	103	09	13	10	09	02	146
2	Marine water samples.	96	01	04	06	12	0	119
3	Dr. Salim Ali lake samples.	112	01	01	07	02	02	125
4	Harsul Lake water.	87	00	05	06	03	00	101
5	Panchakki water.	49	01	02	02	00	00	54
6	Hatala Lake water	68	00	00	03	04	00	75
	Total	515	12	25	34	30	04	620

Table.2b Distribution of pigmented isolates according to ecosystem selected

Pigment produced	Number of isolates from ecosystem selected		
	Lonar Crater	Marine Water	Fresh water
Bright orange	08	01	03
Orange	14	04	08
Yellow orange	10	06	19
Lemon yellow	09	12	07
Red	02	00	02
Total	43	23	40

Table.3a Secondary screening of carotenoid producing bacteria from Lonar Crater

Sr. No.	Name of Culture	Pigment	pH tolerance	NaCl (%) tolerance	Variation in pigment (pH)						
					6	7	8.5	9.5	10.5	11	12
1	**SL2**	**Bright orange**	**6-12**	**1-6**	**O**	**BO**	**BO**	**BO**	**BO**	**BO**	**BO**
2	SL2**	Orange	7-12	1-6	W	FO	O	O	O	O	P
3	**SL4-1**	**Yellow orange**	**6-12**	**1-5**	**O**	**YO**	**YO**	**YO**	**YO**	**O**	**O**
4	SD4	Bright orange	6-12	1-3	BO	LO	LO	LO	LO	LO	LO
5	**SL9**	**Orange**	**6-12**	**1-6**	**O**	**O**	**YO**	**BO**	**BO**	**BO**	**BO**
6	**SL10**	**Bright orange**	**6-12**	**1-3**	**LO**	**BO**	**BO**	**BO**	**BO**	**BO**	**BO**
7	**IC**	**Dark Red**	**5-12**	**1-4**	**DR**	**DR**	**DR**	**DR**	**DR**	**DR**	**DR**
8	LBSL4	Yellow orange	6-12	1-4	O	FO	FO	FO	FO	FO	FO
9	**SL2y**	**Lemon yellow**	**6-12**	**1-5**	**FY**	**LY**	**LY**	**LY**	**LY**	**LY**	**LY**
10	LWT1	Orange	6-12	1-4	O	P	FO	FO	FO	P	P
11	SL6	Orange	6-12	1-4	O	BO	YO	YO	YO	O	O
12	**SL4***	**Orange**	**6-12**	**1-6**	**FO**	**O**	**O**	**FO**	**FO**	**P**	**P**

Table.3b Secondary screening of carotenoid producing bacteria from marine samples

Sr.No.	Name of Culture	Pigment	pH tolerance	NaCl (%) tolerance	Variation in pigment (pH)						
					6	7	8.5	9.5	10.5	11	12
1	**AN9**	**Lemon Yellow**	**6-8**	**3-6**	**LY**	**LY**	**LY**	**NG**	**NG**	**NG**	**NG**
2	**ANN10**	**Orange**	**6-10**	**3-6**	**O**	**O**	**O**	**O**	**O**	**FO**	**FO**
3	DKMO	Yellow orange	5-10	3-5	YO	YO	YO	YO	YO	NG	NG
4	**DKMIII**	**Yellow orange**	**6-8**	**3-4**	**YO**	**YO**	**YO**	**FO**	**NG**	**NG**	**NG**
5	ANAB5	Bright orange	6-8	3-6	BO	BO	BO	FO	FO	NG	NG
6	**DABW1**	**Orange**	**6-8**	**> 3**	**O**	**O**	**O**	**FO**	**FO**	**P**	**NG**
7	**DGM**	**Lemon yellow**	**6-10**	**3-8**	LY	LY	LY	LY	LY	NG	NG
8	ANG7	Lemon yellow	6-10	3-6	LY	LY	LY	LY	LY	NG	NG
9	**ANB6**	**Lemon yellow**	**6-10**	**3-6**	**LY**	**LY**	**LY**	**LY**	**LY**	**W**	**W**
10	ANB11	Lemon yellow	6-10	3-6	LY	LY	LY	LY	LY	NG	NG
11	**DKMY**	**Yellow orange**	**6-10**	**3-5**	**YO**	**YO**	**YO**	**YO**	**FO**	**W**	**NG**
12	**DKN11**	**Yellow orange**	**6-8**	**3-5**	**YO**	**YO**	**YO**	**FO**	**FO**	**W**	**NG**

Table.3c Secondary screening of carotenoid producing bacteria from Aurangabad and Hingoli samples

Sr. No.	Name of Culture	Pigment	pH tolerance	NaCl (%) tolerance	Variation in pigment (pH)							
					5	6	7	8.5	9.5	10.5	11	12
1	SSD1	Yellow orange	5-11	1-4	YO	YO	YO	YO	YO	FY	NG	NG
2	**SSD2**	**Orange**	**5-11**	**1-3**	**O**	**O**	**O**	**O**	**FO**	**FO**	**P**	**P**
3	SSD4	Yellow orange	5-11	1	YO	YO	YO	FO	FO	FO	FO	NG
4	SWT2	Yellow orange	5-11	1-4	YO	YO	YO	FO	FO	FO	FO	NG
5	**SWT3**	**Red**	**5-11**	**1-2**	**DR**	**DR**	**DR**	**DR**	**DR**	**DR**	**DR**	**NG**
6	**SS1**	**Bright orange**	**5-11**	**2-3**	**O**	**O**	**BO**	**BO**	**BO**	**O**	**O**	**P**
7	HSD6	Yellow orange	5-10	1-4	FY	FY	YO	YO	YO	FY	NG	NG

Legend for table-3a., 3b. and 3c.YO- yellow orange, FY- faint yellow, NG- no growth, FO- faint orange, DR-dark red, BO-bright orange, O-orange, W-white, FP- faint pink, P- pink, LY-lemon yellow and LO-light orange.

Table.4a Tertiary screening of bacteria from Lonar Crater based on extraction and characterization along with degree of pigmentation

Sr. No.	Bacterial culture	Wet weight of pellet/100ml	Absorption maxima (λmax) (nm)	O.D.at λmax (nm)	O.D. at 660nm	DOP	Predicted pigment
1	**SL2**	**0.941**	**473**	**3.796**	**1.350**	**2.81**	**Astaxanthin**
2	SL2**	0.534	466	0.272	0.968	0.28	Cryptoxanthin
3	**SL4-1**	**0.773**	**443**	**2.220**	**1.446**	**1.53**	**Zeaxanthin**
4	SD4	0.528	446	0.215	1.235	0.17	Zeaxanthin
5	SL9	1.067	475	1.345	1.363	0.98	Astaxanthin
6	**SL10**	**0.699**	**475**	**1.620**	**1.162**	**1.39**	**Astaxanthin**
7	IC	0.542	536	2.54	0.391	6.41	Prodigiosin
8	LBSL4	0.619	411	0.390	1.167	0.33	--
9	**SL2y**	**1.361**	**437**	**2.868**	**1.993**	**1.43**	**Cryptoxanthin**
10	LWT1	0.542	449	0.266	0.779	0.34	β-Carotene
11	SL6	0.873	440	1.022	1.655	0.61	Zeaxanthin
12	**SL4***	**1.100**	**449**	**1.576**	**1.829**	**0.86**	**β-Carotene**

Table.4b Tertiary screening of bacteria from marine samples based on extraction and characterization along with degree of pigmentation

Sr. No	Bacterial culture	Wet weight of pellet/100ml	Absorption maxima (λmax-nm)	O.D. at λmax-nm	O.D. at 660nm	DOP	Predicted pigment
1	AN9	0.175	437	0.474	1.375	0.34	Cryptoxanthin
2	DKN11	0.940	437	0.598	1.777	0.33	Cryptoxanthin
3	**DKMIII**	**0.237**	**450**	**1.354**	**1.347**	**1.00**	β-Carotene
4	**DABW1**	**0.234**	**463**	**0.912**	**1.363**	**0.66**	Cryptoxanthin
5	**DGM**	**0.232**	**436**	**0.702**	**1.706**	**0.41**	Cryptoxanthin
6	ANB6	0.423	438	0.281	1.880	0.14	Cryptoxanthin
7	DKMY	0.884	436	0.580	1.817	0.30	Cryptoxanthin

Table.4c Tertiary screening of bacteria from Aurangabad and Hingoli samples based on extraction and characterization along with degree of pigmentation

Sr. No.	Bacterial culture	Wet weight of pellet/100ml	Absorption maxima (λmax-nm)	OD at λmax-(nm)	O.D. 660nm	DOP	Predicted pigment
1	SSD2	0.347	468	0.771	0.834	0.93	Canthaxanthin
2	SWT3	0.921	536	4.63	1.723	2.69	Prodigiosin
3	**SS1**	**0.532**	**474**	**1.54**	**1.089**	**1.42**	**Astaxanthin**

Table.5a Quaternary screening of carotenoid producing bacteria isolated from Lonar Crater

Sr. No.	Bacterial Culture	(λmax-nm)	DOP	Biomass (g/100ml)	% RSA	Chemical nature	Predicted pigment
1	**SL2**	**473**	**2.81**	**2.89**	**53**	**Carotenoid**	**Astaxanthin**
2	SL4-1	450	1.6	3.28	19	Carotenoid	β-Carotene
3	SL10	473	3.4	1.73	21	Carotenoid	Astaxanthin
4	SL4*	463	1.1	2.62	60	Non- carotenoid	--
5	SL2y	437	0.6	2.84	11	Non- carotenoid	--

Table.5b Quaternary screening of carotenoid producing bacteria isolated from Marine water

Sr. No.	Bacterial Culture	(λmax-nm)	DOP	Biomass (g/100ml)	% RSA	Chemical nature	Predicted Pigment
1	**DKMIII**	**450**	**1.00**	**0.237**	**23**	**Carotenoid**	**β-Carotene**
2	DABW1	463	0.66	0.234	14	Carotenoid	Cryptoxanthin
3	DGM	436	0.41	0.232	12	Non-Carotenoid	--

Table.5c Quaternary screening of carotenoid producing bacteria isolated from Aurangabad and Hingoli samples

Sr. No.	Bacterial Culture	(λmax-nm)	DOP	Biomass (g/100ml)	% RSA	Chemical nature	Predicted Pigment
1	SSD2	468	0.93	0.347	18	Carotenoid	Cryptoxanthin
2	SWT3	536	2.69	0.921	56	Non-Carotenoid	Prodigiosin
3	**SS1**	**474**	**1.42**	**0.532**	**23**	**Carotenoid**	**Astaxanthin**

Table.6 Munsell color code of the pigment produced by screened isolate

Sr. No.	Isolate	Systematic Name	Munsell color code	Appearance of pigment
1	SL2	Vivid yellow Red	1.3YR6.6/17.5	
2	SL4*	Vivid yellow red	4.0YR 6.7/16.9	
3	SL4-1	Vivid reddish yellow	1.0Y 7.9/13.4	
4	SL10	Vivid yellow Red	4.0YR 6.7/1	
5	SL2y	Vivid Greenish yellow	7.1Y8.8/15.0	
6	DKMIII	Vivid reddish yellow	1.0Y7.9/13.4	
7	DABW1	Vivid reddish yellow	4.0YR 6.7/16.9	
8	DGM	Vivid Greenish Yellow	1.0Y 8.8/15.0	
9	SSD2	Light Yellow Red	1.5YR 7.0/10.7	
10	SWT3	Deep yellow Red	9.0R 4.5/12.3	
11	SS1	Vivid yellow Red	1.3YR6.6/17.5	

Figure.1a Distribution of number of samples collected according to source of sample,1b. distribution of samples according to location of samples collected

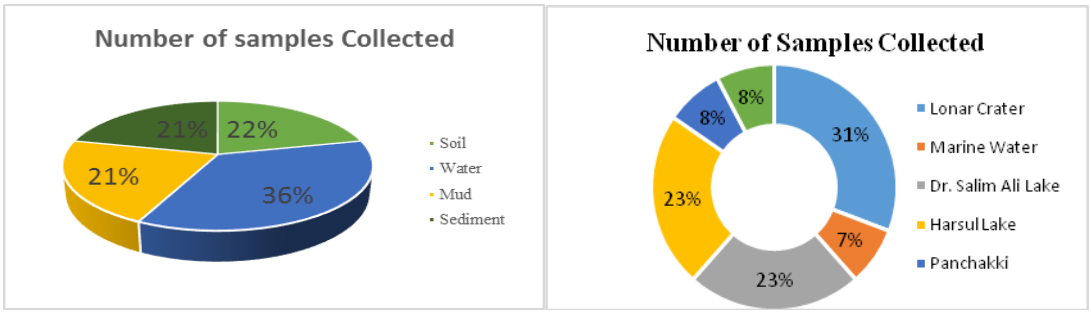
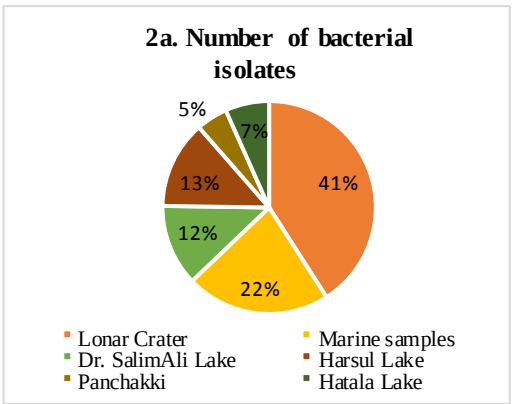
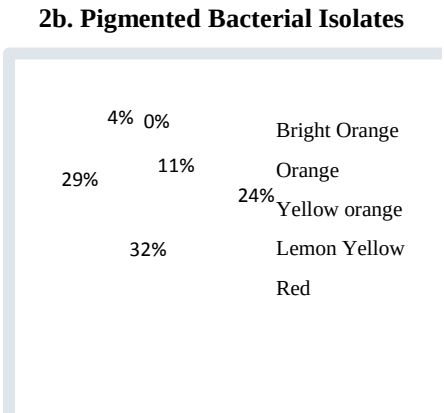


Figure.2 2a. Distribution of bacterial isolates from different sources of isolation, **2b.** Distribution of primarily screened isolates according to pigment produced





Secondary Screening of carotenoid producing Bacteria

Secondary screening was done based on qualitative pH and NaCl tolerance of 105 pigmented bacteria. All isolates were grown on nutrient agar with varying pH (6-12) and NaCl (1-6%) separately where, out of all primarily screened isolates 12 pigmented isolates from Lonar Crater, 12 pigmented isolates from marine samples and 07 from fresh water lakes isolates were screened secondarily based on bright pigmentation with broad range of pH (6-12) and salt (1-6%) tolerance). Most of secondarily screened isolates shown growth above pH 11.0 and 4% NaCl with reduced pigmentation while some isolates do not grow at high pH and concentration of NaCl, such isolates were eliminated for further studies during secondary screening(Table-3a, 3b and 3c). Similar studies were carried out by Lee *et al*., (2004) where, astaxanthin producing *Paracoccus haeundaensis* sp. nov. showed pH tolerance in the range 6-10.5 and NaCl tolerance up to 6%. Zheng *et al*., (2011) found that *Paracoccus beibuensis* JLT1284^T can grow between pH 6.0 to 8.0 and NaCl i.e. 2to 15%. In these research studies, emphasis was given to screen potential carotenoid producing bacterial isolate and hence, screening was done based on both pH and NaCl tolerance. None of similar research studies were found where, combined criteria of tolerance to pH and NaCl used for screening of carotenoid producing bacteria which highlights the uniqueness of the current studies.

Tertiary Screening of carotenoid producing Bacteria

Total 31 pigmented isolates showing broad range pH between 6-12 and NaCl up to 1-6% tolerance was subjected for tertiary screening based on extraction of pigment and its spectrophotometric characterization. According to the spectrophotometric analysis, λ_{max} of extracted pigments from different isolates were different varying between 400-600 nm (Table -4a, 4b, and 4c). The degree of pigmentation is a ratio of biomass to pigment produced and it is considered as efficiency of bacteria to produce carotenoids. Based on spectrophotometric characters and degree of pigmentation 05 isolates from Lonar crater, 03 from marine water samples and 03 isolates from fresh water lakes were selected for quaternary screening. The selected isolates showed pigments which are predicted based on their absorption maxima as SL2 as astaxanthin (473 nm), SL4-1 as zeaxanthin (443 nm), SL10 as astaxanthin (475 nm), SL4* as β-carotene (449 nm), DKMIII as β-carotene (450 nm), DABW1 as cryptoxanthin (463 nm) and DGM as cryptoxanthin (436 nm). The Munsell color code of each pigment from screened bacteria was determined by using Munsell color android application (Table-6).

El-Banna *et al*., (2012) described pigmented yeast colony color with the help of Munsell color chart as 10R7/8. Rodriguez-Amaya *et al*., (1999) suggested that absorption spectra of β-carotene are to be around 450 nm. Shatila *et al*., (2013) analyzed orange pigment from *Exiguobacterium* within wavelength region 400-690 nm and demonstrated shoulder peak at 463 nm which is a typical pattern of absorption spectrum of carotenoids. Oren *et al*., (2001) extracted red carotenoid pigment from halophilic bacteria *Salinibacter* and analyzed it spectrophotometrically and found 482 nm as absorption maxima with a shoulder at about 510 nm. In this research, for tertiary screening extraction and analysis of pigment was done to know the type of carotenoid, pigment production potential of isolate and possible applications were also considered. Very few research studies were found where spectrophotometric characterization and degree of pigmentation was evaluated as screening strategy.

Quaternary screening based on antioxidant activity performed by DPPH assay

The isolates showing good degree of pigmentation and characteristic λ_{max} of carotenoid pigments were selected for evaluation of its antioxidant potential where, crude methanolic extract from 11 tertiary screened isolates were evaluated for antioxidant activity or free radical scavenging activity (% RSA) by DPPH method. The stable radical of DPPH is used frequently for evaluation of antioxidant activity of natural colorant product. Out of 11 selected isolates SL2, DKMIII and SS1 showed highest i.e. 53%, 23% and 23% free radical scavenging activity respectively. The isolate SL2 showed good degree of pigmentation and % RSA i.e. 2.81 and 53% (Table-5a). Sasidharan *et al.*, (2013) also used degree of pigmentation of carotenoid producing bacteria as a screening criterion where, highest degree of pigmentation was shown by bacterial isolate RS7 i.e. 8.31. % RSA was evaluated by Arulselvi *et al*., (2014) for yellow pigment from bacterial strain using DPPH method and observed 70% activity. Nishino *et al.*, (2000) suggested that DPPH free radical scavenging activity of methanol extract depends on concentration of carotenoids. According to Sandesh (2007) generally carotenoid potential for antioxidants vary many times '*in vivo*' due to pro-oxidant effect. In this research studies evaluation of % RSA used as last criteria for selection of isolate which highlights importance of carotenoids as an antioxidant molecule. Antioxidant potential of selected isolate increases its applicability at industrial level for production of carotenoids.

Isolate SL2 was finally selected as it can produce bright orange pigment, tolerate wide range of pH between 6-12 and NaCl up to 6% with 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 hence, it was finally screened, selected and preserved for further studies.

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