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Isolation and characterization of yellow pigment producing *Exiguobacterium* sps.

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Abstract

Thirteen yellow pigment producing bacterial strains were isolated by air and soil sampling method and screened for carotenoid production, as many of them exhibited a deep orange pigmentation when cultured on NA-plates. We found that most of them produced trace amount of carotenoids. The isolates RS7, RSS3, RS13 and RS14 had significant amounts of the yellow pigment producing ability (Degree of pigmentation 8.31, 2.72, 6.66 and 5.5). Free Radical Scavenging Activity of the extracts from the isolates were analyzed by DPPH (1, 1-Diphenly-2-picryl-hydrazil) method**.** Isolate RS7 showed maximum free radical scavenging activity (72%). HPLC result revealed that the isolated strains are the natural producer of Astaxanthin. Based on the Biochemical characterization and 16S rDNA gene sequencing analysis, out of the four isolates two of them (RS7 and RS13), were identified as *Exiguobacterium aurantiacum* and the other two isolates (RSS3 and RS14) as *Exiguobacterium profundum.*

Keywords: Yellow pigment; DPPH; *Exiguobacterium sp.;* Phylogenetic tree

Introduction

Colours are one of the significant visual properties of food and colouring of foods has been an age old practice. This practice has amplified many folds with the invention of synthetic colourants principally due to their physical properties of good stability and colouring ability (Pattnaik *et al*., 1997). Despite the fact that the commercial market is ruled by the synthetic, even among the permitted synthetic pigments, some of them may be toxic, carcinogenic or may cause severe damage to vital organs (Duran *et al*., 2002). Because of this, a strong interest in natural colouring alternatives is needed. As compared to other available synthetic pigments, natural pigments from microbial sources are potentially good alternative ones.

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Carotenoids are a group of coloured terpnoids with antioxidant properties which are widespread in the plant and animal kingdoms, as well in fungi and in photosynthetic and non-photosynthetic microorganisms (Phadwal, 2005). Recently carotenoids are used commercially as food colourants, animal feed supplement and, more recently, for nutraceuticals, cosmetic and Pharmaceutical purposes (Klein-Marcuschemer *et al*., 2007). In addition, carotenoids have attracted superior attention as compared to synthetic pigments due to the beneficial role on human health. Carotenoids can inhibit various types of cancer and it enhances the immune response (Guerin *et al*., 2003). These pigments are capable of quenching photo sensitizers; interacting with singlet oxygen (Krinskyet al., 1994) and scavenging proxy radicals (Conn *et al*., 1992). It also protects "life style – related" diseases such as cardiovascular disease and age related macular degeneration, due to their antioxidant activity and pro-vitamin A function (Steven *et al*., 2000). They play an important role in protection of macular region of the retina and hence prevents of cataracts and increases levels of iron absorption (Mares *et al*., 2002).

In the present study, an attempt was made to isolate and characterize yellow pigment producing bacterial strains for carotenoid production and antioxidant activity. The 16s rDNA analysis showed that the isolated strains are *Exiguobacterium aurantiacum* and *Exiguobacterium profundum*. To the best of our knowledge, this is the first report representing the carotenoid production from *Exiguobacterium aurantiacum* and *Exiguobacterium profundum.*

Materials and methods

Isolation of yellow pigment producing microbes

The yellow pigment producing bacterial isolates were isolated from soil samples (11.717629°N, 78.077687°E and 11.756643°N, 78.040488°E) and air samples (11.719079ºN, 78.078007ºE) by soil and air sampling method (Arunkumar *et al.,* 2006). The isolates were purified by pure culture method. The isolated strains were characterized by colony morphology and biochemical characteristics to confirm the identity of the culture.

Extraction of yellow pigment from bacterial isolates

The yellow pigment producing bacterial isolates were grown in LB broth in rotary shaker incubated for three days at 28±2°C. After three days, cells were harvested by centrifugation (3575g) for 15 min. Then the pellet was washed with sterile distilled water and spin for 15 min (894g). The pellet was suspended with 5 ml of methanol. Then it was incubated in water bath at 60^0C for 15 min until all visible pigments were extracted and centrifuged (894g) for 15 min. The coloured supernatant was separated and filtered through Whatman no.1 filter paper. The yellow coloured extracts were analyzed by scanning the absorbance in the wavelength region of 400-600 nm using the spectrophotometer. The total coloured content in the methanol extract was estimated by measuring the absorbance at *λmax* (490nm). The highest yellow pigment producing isolates were selected for further analysis.

DPPH (1, 1-Diphenly-2-picryl-hydrazil) Free Radical Scavenging Activity

The free radical scavenging activity of the fractions of methanol extract of Astaxanthin, a carotenoid family compound was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH), solution of DPPH (0.1 mM) in methanol was prepared; 1 ml of the solution was added to 3 ml of the fraction in methanol at different concentrations (25-500 mg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer (Schimadzu UV-Vis 1700). The percentage DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) = [(A0-A1)/A0] x 100

Where A^0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the standard sample or fraction.

HPLC analysis of the pigment

The yellow coloured extracts from the selected isolates were analyzed by HPLC equipped with a UV-Vis detector. The samples were analyzed on a Waters C-18 reverse-phase column (4.6 by 250 mm; Dupont, Wilmington, Del.) which was eluted with a gradient of 80 to 100% methanol at a flow rate of 1ml/min. The run lasted for 30 min, and the samples were monitored at 470 nm. The absorption spectra of all relevant peaks were recorded with the help of the on-line photo diode array detector.

Phylogenetic Analysis

Total bacterial genomic DNA was isolated by phenol chloroform method. The 1.45 kb of 16SrDNA fragment was amplified using 16S rDNA bacterial forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse (5'-GGC TAC CTT GTT ACG ACT T-3') primer set. PCR amplified product was purified by Gene Jet PCR purification kit. The sequence data were aligned and analyzed to identify bacterium and its closest neighbors using the NCBI web-based BLAST program (http:// www.ncbi.nlm.nih.gov/BLAST/). Closest known species were compared with percentages of identity. Sequences were aligned using the Clustal W program. Phylogenetic trees were constructed using MEGA5.1 software.

Statistical Analysis

All analysis was carried out in triplicates and statistical analysis was performed with the help of mean and standard derivation (SD).

Result and discussion

In this study, thirteen numbers of yellow pigmented bacterial strains were isolated from Soil and Air samples (Table 1). Yassin *et al.,* (2010) also suggested "open plate technique" for microbial isolation. Bacterial colonies on plates of NA medium were studied under a light microscope at a magnification of 10X to 15X for size, pigmentation, form, elevation, and margin. The isolation of carotenoid producing microbes from some abnormal environmental condition was also reported by Arunkumar *et al.,* (2006).

Table 1: Sample collection from different environmental sites

S. No	Sites	Sampling type	No. of isolates (%)
1	11.717629°N, 78.077687°E	Soil	55
2	11.719079°N,78.078007°E	Air	33
3	11.756643°N,78.040488°E	Soil	12

Sampling site: 1) 11.717629°N, 78.077687°E Periyar University Garden;
2) 11.719079°N,78.078007°E Periyar University Campus;
3) 11.756643°N,78.040488°E Agricultural site Omalur.

Based on the morphological characterization and degree of pigmentation four isolates (RS7, RSS3, RS13 and RS14) were selected for further studies. These isolates are showed bright orange/ orange colour colonies, rod shaped gram positive cells and it can grow at the temperature of 28°C to 48°C and the optimum temperature is 37°C. They can grow at pH 6 to 10 (optimum growth pH 7) and showed no fluorescence emission under the Ultra Violet light (UV). The biochemical characterizations of the four yellow pigment producing bacterial strains were discussed in Table 2.

Table 2: Morphological and Biochemical characterization of yellow pigment producing isolates

S. No	Characteristic	Yellow pigment producing bacterial isolates			
		RS7	RSS3	RS13	RS14
1.	Sampling source	Soil	Air	Soil	Air
2.	Morphological characteristic				
	Gram reaction	+	+	+	+
	Cell shape	Rod	Rod	Rod	Rod
	Colony morphology on NA medium	Convex and Bright orange	Convex and Bright orange	Flat and Orange	Flat and Orange
	Optimum growth temperature (°C)	37	37	37	37
	Optimum growth pH	7	7	7	7
	Fluorescent under the UV light	-	-	-	-
3.	Biochemical characteristic				
	Catalase	+	+	+	+
	Indole	-	-	-	-
	MR	+	+	-	-
	VP	+	+	-	-
	Citrate	-	-	-	-
	TSIA	+	+	+	+
	Oxidase	+	+	+	+

Extraction of yellow coloured pigments extraction from the isolated strains was carried out by methanol as a solvent. Methanol showed to be better than acetone, which is not very efficient in the extraction and quantification of pigments from autotrophic cell cultures (Henriques *et al.,* 2007). In our study, all the extract containing

yellow pigment from the isolated strains gave identical absorption spectra of carotenoid. The spectra are characterized by maximum

Table 3: Yellow pigment production ability of bacterial isolates

Strain name	OD at 490	OD at 660	Degree of pigmentation 490/660
RSS1	0.050	0.090	0.55
RS7	**0.064**	**0.532**	**8.31***
RSS4	0.024	0.146	0.60
RSS5	0.060	0.110	0.54
RSS3	**0.021**	**0.572**	**2.72***
RS12	0.140	0.370	2.64
RS13	**0.030**	**0.200**	**6.66***
RS14	**0.020**	**0.110**	**5.5***
RS15	0.040	0.080	2.0
RS17	0.76	0.846	1.11
RS18	0.080	0.120	0.15
RS20	0.020	0.200	1.0
RS21	0.040	0.050	1.25

* High yellow pigment producing isolates

peaks at 493 and 527 nm with a broad shoulder at 467 nm, indicating that the main carotenoid is w-bacterioruberin. Arunkumar *et al.,* (2006) also reported the same kind of spectral range as our absorption spectra result. The primary diagnostic tool for the identification of carotenoids is to study the spectrum of the organic solvent extract (Rodriguez-Amaya *et al.,* 1999). Table 3 summarizes the degree of pigment production by yellow pigmented bacterial isolates. From this table we can observe that only four isolates have highest carotenoid producing ability *viz* RS7, RSS3, RS13 and RS14. Interestingly, Moss (2002) earlier reported that all pigmented bacterial isolates need not be carotenogenic. With very few exceptions, carotenoids are lipophilic. They are insoluble in water and are soluble in organic solvents.

DPPH is a stable radical and is frequently used for evaluating the antioxidant activity of natural colourant products. Nishino *et al.,* (2000) reported that DPPH radical is known to be stoichiometrically decolorized by potent reducing substance and antioxidants such as cysteine, glutathione, ascorbic acid and tocopherols. Out of this four methanol extracts, only RS7 methanol extract showed highest amount of DPPH free radical scavenging activity (Fig 1). Generally carotenoids are potential antioxidants, many a times in *in vivo* but they lack such properties because of pro-oxidant effect (Sandeshkamath *et al.,* 2007). However, carotenoid compounds have significant role in antioxidant and anti- carcinogenic characteristics (Bendich, 2004). Further, carotenoid esterification does not pose impediment for bio availability in human (Bowen *et al.,* 2002), hence carotenoid can be a major factor in ulcer prevention.

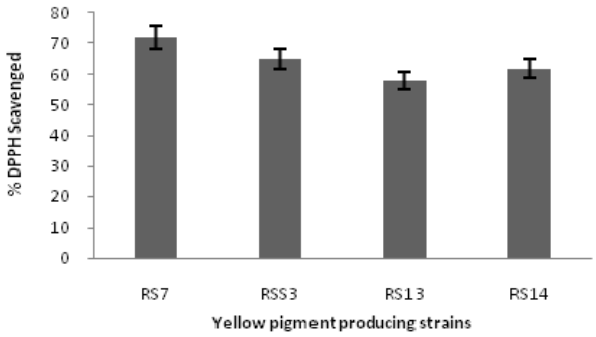

Figure 1: DPPH free radical scavenging activity of methanolic extract of yellow pigment producing bacterial isolates. All the values are mean of three replicates. *Error bars* represent ± standard deviation (SD)

HPLC analysis of the yellow pigment from the strains (RS7, RSS3, RS13 and RS14) showed various retention times out of which retention times of 3.28, 3.27, 3.28, 3.27 respectively corresponded to the retention time value (3.3) of a carotenoid known as Astaxanthin (Fig 2). The HPLC analysis of the carotenoids mixtures by Razavi and Sayed Hadi (2006) also reported retention time of 3.3 for Astaxanthin in their technical characteristics methods used for the identification of carotenoids mixtures.

These four isolates, the 16S rDNA gene sequences of strains (RS7, RSS3, RS13 and RS14) were compared with sequence to determine the relatedness of strains at the genetic level the entire 16S rDNA gene was sequenced from each isolate. The phylogenetic trees showed the isolates RS 7 (NCBI Gen Bank accession No. KC131544) and RS13 (NCBI GenBank accession No. KC131547) were closely related to *Exiguobacterium aurantiacum*. However, isolates RSS3 (NCBI GenBank accession No. KC131545) and RS14 (NCBI GenBank accession No. KC131546) belonged to the *Exiguobacterium profundum* (Fig. 3). To assertion the phylogenetic position of obtained from GenBank (National Center for Biotechnological Information; http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequence were performed by CLUSTAL_W software (Ryan M. Potter., 2008). A phylogenetic tree was constructed with the evolutionary distances using the Neighbor-joining method. Tree topologies were evaluated by performing bootstrap analysis of 100 data sets with the MEGA5.1 software (Koichiro Tamura *et al.,* 2011).

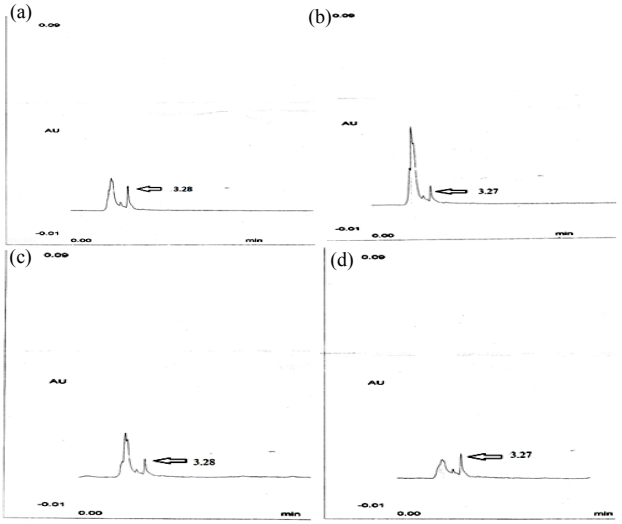

Figure 2: HPLC chromatogram of the carotenoid from *Exiguobacterium Sps.,* (*viz* RS7, RSS3, RS13 and RS14). Peak identification: Astaxanthin.

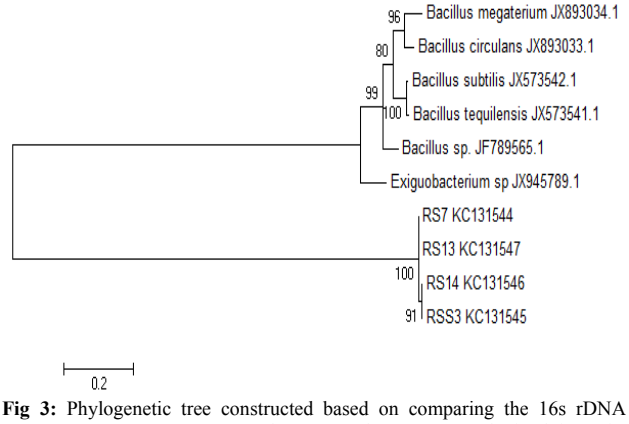

Fig 3: Phylogenetic tree constructed based on comparing the 16s rDNA sequence (RS7, RS13, RSS3 and RS14). Other sequence obtained from the gene bank database with accession number. The tree topology shown is a

rooted tree obtained using a neighbour joining algorithm. Algorithm with bootstrap values expressed as percentage of 100% replication.

Conclusion

Exiguobacterium Sp. can be used as a potential source of Astaxanthin for food, pharmaceutical and other cosmetic industries. Recent developments in the molecular biology of carotenoids biosynthesis from organisms that accumulate different carotenoid product have provided a variety of genes that can be employed as tools for a new strategy of heterologous expression in different host organisms. Engineering of microbial pathway enzyme can produce high quantity of carotenoids in industrial process from these strains.

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