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Characterization of a Red Bacterium Strain Isolated from Root Nodule of Faba Bean (*Vicia faba* L.) for Growth and Pigment Production

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Abstract: An unidentified red bacterium strains isolated as endophyte of root nodule of faba bean, when characterized for morphological, microscopic, biochemical and molecular (16SrDNA sequence) features were identified as a novel strain of *Serratia marcescens*. The strain formed small red colony size measuring 2-3mm in diameter on Glucose Mannitol Tryptone Yeast Extract (GMTYE) agar medium. Under scanning electron microscopy, the single cell rods measured 3.55 - 4.18µm x 0.48µm - 0.68µm. The bacterium strain could efficiently utilize a wide range of carbohydrates (monosaccharide, disaccharide and polysaccharide) both for growth and pigmentation. It's preferred simple form of organic nitrogen for growth and pigmentation but was unable to utilize inorganic sources of nitrogen. The bacterium could grow over a wide range of medium pH (4.00-11.00) and temperature (20-37°C) and NaCl concentration (0.5-2.0%) but optimal growth and pigmentation were observed in the GMTYE medium of pH (6.00-7.00) containing NaCl at 0.5 percent level and at 28°C of incubation temperature.

Keywords: Characterization of red bacterium, Identification, Growth, Pigmentation.

1. Introduction

As against widespread use of synthetic dyes not known to be environmentally friendly, demand for natural pigments for coloring fabrics, foods/feeds, cosmetics and printing inks are increasing. A number of natural carotenoids pigments produced by plants also contribute to an enhanced immune system and reduced risk of degenerative diseases, such as cancer, cardiovascular diseases, macular degeneration and cataract by scavenging reactive oxygen radicals and acting as anti-aging agent (Camera *et al*., 2009; Gurine *et al*., 2003; Helmerson *et al*., 2009; Hussein *et al*., 2006; Wertz *et al*., 2005). Astaxanthin, a xanthophylls widely distributed in nature functions as chemo-protective by acting as nutraceuticals that prevent carcinogenesis through anti-oxidative, anti-free radical (Fassett and Coombes, 2011).

Of the various microbial pigments, prodigiosin and violacein are types of red and blue bacterial pigments that have found application in medical areas due to their activities as immunosuppressive, anticancer, antibacterial and antifungal agents. The red pigment prodigiosin is a member of prodiginines- produced as a typical secondary metabolite by some *Serratia* species, actinomycetes, and few other bacteria (Williamson *et al*., 2006; Williams *et al*., 1971). Prodigiosin is known to potently trigger apoptosis in haematopoietic cancer cell lines (Williamson *et al*., 2007). Due to high specificity for human cancer cells and no toxicity in nonmalignant cell lines (Williamson *et al*., 2007; Raj *et al*., 2009), prodigiosin is a strong candidate for developing antitumor drugs (Perez-Thomas *et al*., 2003).

During our investigations on the diversity of endophytic bacteria, a red bacterial strain was a chance isolate from surface sterilized nodules of faba bean (*Vicia faba* L.). In view of the significance of microbial pigment, in this paper, we report on the characterization of this strain of red pigmented bacterium both for its identification and production of an intense red pigment.

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2. Materials and Methods

2.1 Red bacterium isolates

The pure culture of the bacterium was maintained by subculturing on GMTYE-Agar (Tryptone, 10g/l; yeast extract, 3g/l; $MgSO_4.7H_2O$, 2g/l; mannitol, 5g/l; glucose, 5g/l; NaCl, 5g/l ; bacteriological agar 16g/l, pH 7.0) slants followed by storage at 4°C. When needed, culture was inconsistently derived from a master culture by streaking on GMTYE-Agar in order to maintain its genetic stability. The bacterial isolates when cultured on this medium at 28±2°C formed intense red colored colonies after an incubation period of 3-4 days.

2.2 Colony morphology and cell characterization

The bacterium isolate was plated on GMTYE-Agar and allowed to grow at 28±2°C for 3-5 days and studied for different cultural and cell morphological parameters, such as colony size, colony elevations, colony margin and colony pigmentation. Motility (hanging drop method) and Gram's reaction of the bacterial cells were performed using standard methods.

2.3 Bacterial cell morphology

The bacterial cell size, cell shape and cell arrangement were observed through bright field, phase contrast and Scanning Electron Microscopy (SEM). Whereas, wet preparations were used for bright field and phase contrast microscopy, for SEM bacterial cell pellet (from one ml of the 48 hrs grown culture broth) was fixed in 2.5 percent glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 30 minutes followed by three washings in the same buffer for 15 minutes each at 4°C. After secondary fixation with 1% osmium tetroxide (OsO_4) for 1 hour at 4°C, the fixed cell mass was dehydrated in graded concentrations of ethanol (15, 30, 50, 70, 80, 90 and 100% ethanol in order) for 15 minutes in each grade at 4°C. The dehydrated cell mass was transferred onto a microscopic cover slide, dried under vacuum, soaked in isoamyl acetate and then dried to a critical point in 'Hitachi HCP-2 vacuum drier'. The cell mass was mounted on carbon tape coated 'aluminum stub of SEM' and 'gold sputtered (100-300°A)' in 'sputter coater' (Hitachi E-1010 Ion Sputter). The gold coated cell mass on the stub was examined under 'scanning electron microscope', (SEM-HITACHI S-3400N, Thermo Electron Corporation) set at pre-standardized conditions (accelerating voltage- 15,000V, decelerating voltage- 0V, magnification- 14000- 18000x, working distance- 9900µm, emission current- 100,000nA and calibration scan speed- 25). The length and width (diameter) of the individual bacterial cells were measured with the SEM micromarker (photo-size 1000 micromarker 4000) and observations recorded in a photograph.

2.4 Biochemical test

Various biochemical tests as per 'Bergey's Manual of Determinative Bacteriology' (Holt *et al.*, 1994) were performed on 48 hrs grown bacterial culture using standard protocols, such as;

i. Catalase, lipase and oxidase production (Cowan, 1974),
ii. Oxidative and fermentative metabolism of glucose, mannitol, maltose and sucrose (Hugh and Leifson, 1953),
iii. Koser's citrate utilization and nitrate reduction (Cowan, 1974), and
iv. Hydrolysis of starch, cellulose, chitin and gelatin (Gordon, 1967).

2.5 Molecular identification of bacterial isolates based upon 16S ribosomal DNA (16SrDNA) sequence

The total DNA isolated from the bacterial cell mass as per Cubero *et al.*, (1999) was PCR amplified using 16SrDNA specific primers (PigB16S-F 5`-cggcaggcttaacacatgca-3` & PigB16S-R 5`-tctacgaatttcacctctacact-3`). The 25µL of PCR reaction mixture contained 20ng template (bacterial) DNA solution, 1mM dNTPs mix, 10µM of each primers, 2.0 U Taq Polymerase (MBI, Fermentas) and 1.5mM $MgCl_2$ in 1X Taq reaction buffer. The PCR amplification program was consisted of 95°C for 5 min (preheating), 95°C for 1 min, 53°C for 1 min, 72°C for 2 min (36 cycles), 72°C for 10 min (final extension) and stored at 4°C until used. The amplified DNA product along with a DNA size marker (100 bp ladder plus, MBI Fermentas) was separated by electrophoresis using 1.0% agarose gel in TAE. The gel was stained with ethidium bromide and the banding profiles recorded using UV-Gel Documentation system (UltraLum). Amplified product (665 bp) was purified from the gel band (using 'QIAquick Gel Extraction Kit' of Qiagen), cloned in the PCR cloning vector pTZ57R/T using 'InsTAclone PCR Product cloning kit' (MBI Fermentas, USA) and transformed into *E. coli* DH5- alpha competent cells. The recombinant clone was submitted to M/S Xcelris, Ahmedabad, India for custom sequencing of the 16SrDNA using M_{13} primers. Identification of the bacterial isolate was ascertained based on nucleotide sequence homology of this 16SrDNA with the sequences available for different organisms in the GenBank database.

2.6 Effect of medium pH and NaCl concentration on growth and pigmentation

In order to determine optimum conditions of medium pH or NaCl, the GMTYE broth medium was prepared (75ml lots in 250ml Erlenmeyer flasks) with different initial pH (3.0-11.0) values or NaCl concentrations (0.1-8.0 percent). The sterilized medium (15psi, 20 min) was inoculated with 0.1ml of bacterial inoculum (24 hrs growth of a single isolated clone in GMTYE) and incubated at 28±2°C under stationary conditions. After five days, the relative growth and extent of pigmentation was observed visually and recorded on a zero to ++++ Scale.

2.7 Effect of incubation temperature

Effect of incubation temperature (20 to 37°C) on the bacterial cell growth and pigmentation was observed after growing the inoculated complete GMTYE broth medium at different temperatures. After five days incubation period, the growth of the bacterial isolate was observed visually for relative growth and pigmentation.

2.8 Effect of carbon and nitrogen sources

The effect of different carbon sources on the growth and pigmentation of the bacterial isolate was studied by supplying different carbohydrates as sole sources of carbon and energy (at 0.5 percent level) in GMTYE basal medium (less glucose and mannitol). Alternatively, the effect of different organic and inorganic nitrogen sources as sole sources of nitrogen was studied by supplying a respective nitrogen source (at the one percent level) in GMTYE basal medium (less yeast extract and tryptone). All the different GMTYE broths containing appropriate carbon or nitrogen sources were inoculated with standard inoculum and incubated at 28±2°C. After five days of incubation period, the growth and pigmentation of the bacterial isolate was visually determined as described earlier.

3. Results and Discussion

3.1 Colony morphology and cell characteristics

3.1.1 Colony morphology

The colony morphology and cell characteristics of the bacterial isolate in GMTYE-Agar showed that within 3-5 days of incubation the bacterial isolate grew to form small round red colonies (2-3mm in diameter) having an entire margin and smooth and shiny surface with centrally raised colony elevation (Plate 1A).

3.1.2 Cell characteristics

Gram staining of bacterial cells established it to be gram negative. Observations on the wet preparation of the bacterial isolate under bright field microscope revealed that the bacterial cells were solitary short rod (single cell bacilli). Although few cells appeared joined end to end in pairs, the red pigment was observed as small red granule occupying a central position in the cell cytoplasm (Plate 2A). The finer details on cell shape and cell arrangements were however obtained under scanning electron microscope (SEM), wherein the bacterial cells were observed as thin long rods (Plate 2B). The cell length ranged between 3.55µm and 4.18µm with cell diameter/width ranging between 0.48µm and 0.69µm (Plate 2B). The larger cells (length and width) having grown to maximum size showed a prominent cell constriction (septum) in the middle suggesting that these mature cells were preparing to divide into daughter cells (Plate 2B). However, though the cells had earlier shown active motility (hanging drop method), flagella were not seen under the SEM, which might have been extricated (lost) during the vigorous pretreatments given for processing cell mass for SEM.

3.2 Biochemical characteristics

The results on various biochemical tests revealed that the red bacterium was able to utilize/ hydrolyze starch, cellulose, chitin, gelatin and lipids, which established its ability to produce amylase, cellulase, chitinase, gelatinase and lipase enzymes. Though the bacterium expressed a positive catalase reaction, it showed only a negative reaction for oxidase, urease and H_2S.

Following the identification key based upon various morphological (colony morphology and cell characteristics) and biochemical characteristics as given by Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994), the bacterium under study could be identified as *Serratia marcescens*. The colony characteristics and other features recorded for this bacterium also matched with those reported for different *S. marcescens* strains isolated from other diverse sources (Li *et al*., 2011; Holt *et al*., 1994; Langsrud *et al*., 2003; Tariq and Prabakaran, 2010), thus establishing the above identity of the red bacterial strain as *S. marcescens*.

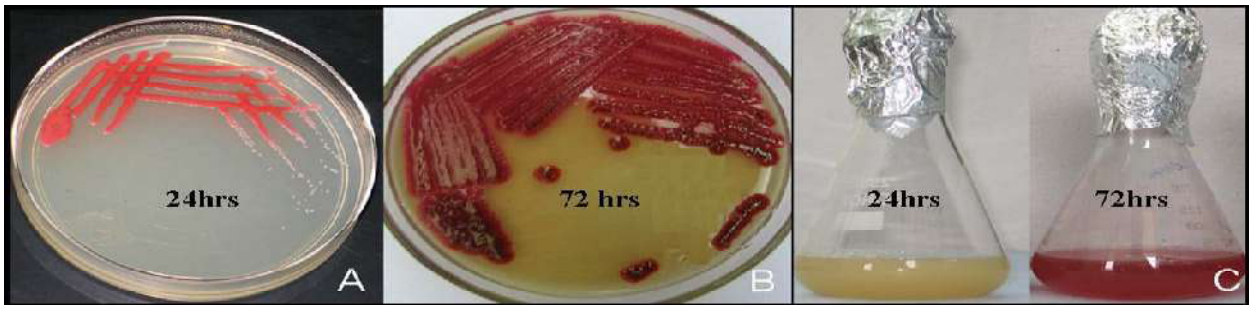

Plate 1. Growth and pigment production by the bacterial isolate in A & B: GMTYE-agar medium and C: GMTYE- liquid media at different incubation periods.

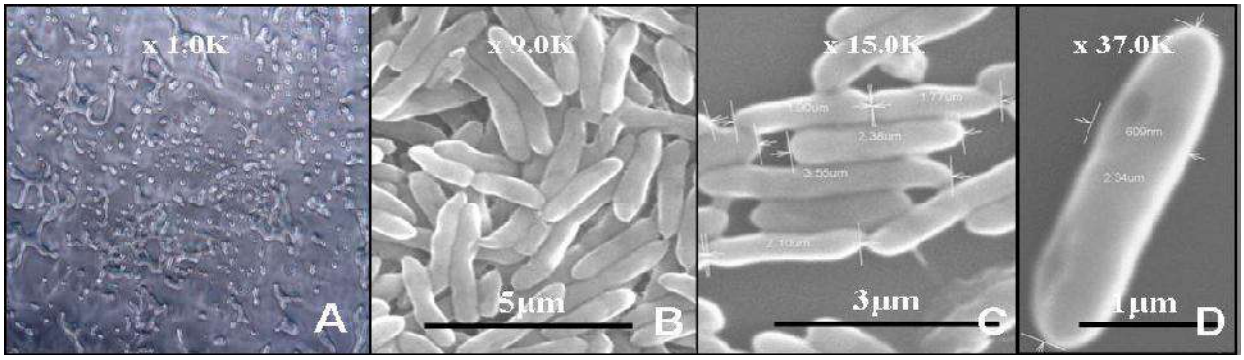

Plate 2. Cell characteristics of red bacterial Strain A- Bright field microscope at 1.0K- showing single celled rods with centrally located red pigment granule, and B -SEM at x9.0K; C-SEM at x15K; and D- SEM at x37 K- showing single cells and dividing cells with prominent cell constriction or septum.

3.3 Molecular identification of bacterial isolates based upon 16S rDNA sequence

Using the total DNA of the red bacterium isolate as a template, the PCR amplification with PigB16S primers resulted in amplification of 665 bp DNA fragment (Fig. 1). The determined sequence of this 16SrDNA fragment was submitted to GenBank and is available as GenBank Accession #JF798453 (www.ncbi.nlm.nih.gov/Blast). This sequence was blasted into Nucleotide Blast Tool' of 'National Center for Biotechnology Information' (available at www.ncbi.nlm.nih.gov/Blast) for nucleotide homology. The maximum homology report (Taxonomy Blast Report) identified a high nucleotide homology of the 16SrDNA (99% maximum identity in 100% query coverage) with 16SrDNA/ 16SrRNA sequences from 19 different *Serratia marcescens* strains (Table 1).

From the analysis of the generated taxonomy report of the 16SrDNA gene sequence, this bacterial strain with the highest score of (1212), and lowest E-value (0.0) was identified to be *Serratia marcescens.* However, the bacterial strain under study showed a maximum of 99 percent homology with the previously reported sequences. This established that the bacterial isolate now identified as *Serratia marcescens* is a novel strain (*S. marcescens*, faba bean) that has not been reported earlier.

It is to be noted that observed colony morphology, cell characteristics and biochemical characteristics of the isolate under study pointing to the above identity of the bacterial isolate also matched with those reported for other strains of *S. marcescens* (Li *et al*., 2011; Holt *et al*., 1994; Langsrud *et al*., 2003; Tariq and Prabakaran, 2010; de Araujo *et al*., 2010).

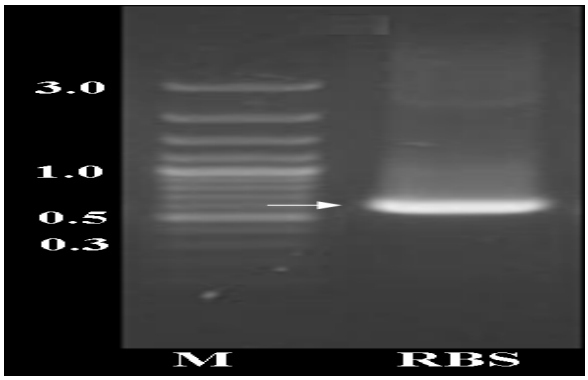

Fig. 1. PCR amplification of 665 bp 16s rDNA from total DNA of red bacterial strain. M is 100 bp DNA ladder (MBI Fermentas).

Table 1. Taxonomy Blast Report on 16S rDNA sequence with GenBank Database (http://www.ncbi.nlm.nih.gov/blast).

Accession No.	*Serratia marcescens* [enterobacteria] taxid	Score	E-Value	Query coverage, %	Maximum Identity, %
dbj|AB244453.1|	*Serratia marcescens* strain An17-1 16S rRNA	1212	0.0	100	99
dbj|AB244433.1|	*Serratia marcescens* strain A19-1 16S rRNA	1212	0.0	100	99
dbj|AB244291.1|	*Serratia marcescens* gene for 16S rRNA	1212	0.0	100	99
gb|EU048327.1|	*Serratia marcescens* strain CMG3090 16S rRNA	1212	0.0	100	99
gb|AF124040.1|	*Serratia marcescens* 16S rRNA	1212	0.0	100	99
gb|HM047514.1|	*Serratia marcescens* strain DHU-35 16S rRNA	1210	0.0	100	99
gb|HQ260324.1|	*Serratia marcescens* strain JNB5-1 16S rRNA	1206	0.0	100	99
gb|FJ608004.1|	*Serratia marcescens* strain LM8 16S rRNA	1206	0.0	100	99
gb|FJ462701.1|	*Serratia marcescens* strain AKL1 16S rRNA	1206	0.0	100	99
emb|FM207962.1|	*Serratia marcescens*strain CWS25 16S rRNA	1206	0.0	100	99
gb|EU221361.1|	*Serratia marcescens strain* J2P3 16S rRNA	1206	0.0	100	99
gb|EF627046.1|	*Serratia marcescens* strain cocoon-1 16S rRNA	1206	0.0	100	99
gb|DQ501957.1|	*Serratia marcescens* 16S rRNA	1206	0.0	100	99
gb|EF208031.1|	*Serratia marcescens* strain L1 16S rRNA	1206	0.0	100	99

3.4 Red Pigment development

The now identified *S. marcescens* faba bean produced red pigment both on solid GMTYE-Agar and in liquid GMTYE medium, but the pigment production was rapid and more intense on solid than in the liquid medium (Plate 1). On solid medium, the colony remained white up to 16 hrs of incubation period. Thereafter, colony color changed to light red to intense red by 24h and turning dark intense red by 72 hrs. However, in liquid medium, the appearance of pigment was observed only after 36 hrs with growth and pigmentation reaching maximum by 72 hours (Plate 1, Table 2). In the subsequent period, both growth and pigmentation showed only a slow decline.

3.5 Effect of medium pH, NaCl and incubation temperature on growth and pigmentation by the bacterial isolate

3.5.1 Medium pH

The effect of pH value of the liquid medium on growth and pigment production by *S. marcescens* faba bean was studied by allowing the bacterial culture to grow in GMTYE media prepared with different initial pH values (3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 8.0, 9.0 and 11.0). The bacterial isolate showed remarkable ability to grow and produce red pigment over a wide range of medium pH of 4.0-11.0. However, it showed its maximum growth and pigmentation efficiency at pH values of 6.0-7.0 (Table 3). It suggested that pigmentation was directly related with growth and that in spite of its capacity to grow over a wide pH range, the bacterial isolate was neutrophilicin its nature.

3.5.2 NaCl concentration

In order to determine the effect of different concentration of salt, the bacterial isolate was grown in GMTYE medium containing different concentrations of sodium chloride (0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 8.0 percent). The *S. marcescens* faba bean showed its ability to actively grow and produce red pigment in the range of 0.1-2.0 percent NaCl concentration but optimum growth and pigmentation was observed at only at lower salt concentrations of 0.5-1.0 percent (Table 3). Higher concentrations than this had inhibitory influence with only a scanty growth at a salt concentration of 2.0 percent with concentrations higher than this being inhibitory for growth and pigment formation. Requirement of NaCl for growth and pigmentation by different pigmented bacteria including *Serratia marcescens* have already been reported by Allen *et al.*, (1983) and Silverman and Munoz (1973).

3.5.3 Temperature

In order to determine the optimum temperature, for the growth and pigmentation of the bacterial isolate in GMTYE medium was observed at five different incubation temperatures (20, 25, 28, 32 and 37°C). All the incubation temperature allowed the growth of the bacterial isolate. However, the optimum growth of the bacterial isolate was observed in the culture incubated at 28°C, followed by 25°C and 32°C (Table 3). However, as against growth over wider range of incubation temperatures, the cell pigmentation was observed at a narrow range of temperature 25-32°C. Though the observations are in accordance with those reported for other strains of *S. marcescens*, (Williams *et al*., 1971; Giri *et al.*, 2004), the results suggest that the extent of pigmentation is somehow a function of active growth and the same is inhibited in poorly growing bacterial cultures of this bacterial species.

3.6 Effect of carbon and nitrogen sources on growth and pigmentation by the bacterial isolate

3.6.1 Carbon sources

The red bacterial strain *Serratia marcescens* faba bean, showed its ability to utilize all the different carbon sources for its growth and pigment production but to different extents (Table 4). Whereas, glucose and mannitol were the most efficiently utilized monosaccharide, maltose and sucrose as disaccharides were also metabolized by the bacterial strain with equal efficiency. The other mono- and disaccharides served only as a poor source of carbon and energy for the bacterial strain. The bacterial strain utilized all the three polysaccharides (cellulose, chitin and starch) though with reduced efficacy, which suggested the ability of the bacterium to metabolize the same for growth and pigment production. Most likely, this bacterial strain did not produce hydrolytic enzymes in enough concentration to efficiently degrade these polysaccharides for its efficient growth and hence pigment production. In this study, sugar alcohols (mannitol followed by erythrose) have found to be metabolized with efficiency equal or slightly lower than glucose. However, some other *S. marcescens* strains have also been reported which could utilize ethanol as sole carbon source for growth and pigment production better than glucose and could be attributed to differences amongst strains (Cang *et al*., 2000). Pigment production by *Serratia marcescens* has also been reported to be highly variable among its strains and is dependent on several cultural and nutritional parameters (Giri *et al*., 2004).

3.6.2 Nitrogen sources

Of the five different organic sources, beef extract, peptone and tryptone supported maximum growth and pigmentation, while casein and gelatin were only poorly utilized for the purpose. This suggested that though bacterial strain could efficiently utilize the relatively pre-hydrolyzed proteins (beef extract, peptone and tryptone), this species possessed only a reduced ability to hydrolyze intact proteins (Cang *et al*., 2000 and Wei and Chen, 2005). The bacterial isolate did not utilize any of the three inorganic nitrogen sources (Urea, KNO_3, $(NH_4)_2SO_4$) tested for growth and pigment accumulation.

Table 2. Relative growth and cell pigmentation of bacterium isolate at different incubation time.

Parameter	Incubation period (hrs)											
	12	24	36	48	60	72	84	96	108	120	132	144
Growth	+	++	++	+++	+++	++++	+++	+++	+++	+++	+++	+++
Cell Pigmentation	-	-	+	++	+++	++++	+++	+++	+++	+++	+++	+++

'-' or '+' sign represent no or positive visual growth and pigmentation. Number of '+' signs stand for relative extent of visual growth and pigmentation.

Table 3. Effect medium pH, NaCl concentration and incubation temperature on the growth and pigmentation of the red bacterial isolate

Parameter	Medium pH								
	3.0	4.0	5.0	6.0	6.5	7.0	8.0	9.0	11.0
Growth	-	+	++	++++	++++	++++	+++	++	++
Pigmentation	-	+	++	++++	++++	++++	+++	++	++
	NaCl (%)								
	0.1	0.5	1.0	1.5	2	3	4	5	8
Growth	++	+++	+++	++	++	+	+	-	-
Pigmentation	++	+++	+++	++	+	+	+	-	-
	Incubation Temperature °C								
	20		25		28		32		37
Growth	++		++		+++		++		+
Pigmentation	-		+		++		+		-

'-' or '+' sign represents no or positive visual growth and pigmentation. Number of '+' signs stand for relative extent of visual growth and pigmentation.

Table 4. Relative growth and pigment production by the bacterial isolate on media supplied with different carbon and nitrogen sources

Parameter	Carbon source								
	Eryt	Glu	Man	Lac	Mal	Suc	Cell	Chi	Star
Growth	++	+++	+++	++	+++	+++	++	++	++
Cell Pigmentation	++	+++	+++	++	+++	+++	++	++	++
	Nitrogen source								
	BE	Cas	Gela	Pep	Tryp	YE	KNO_3	Urea	$(NH_4)_2SO_4$
Growth	+++	++	++	+++	+++	++	-	-	-
Cell Pigmentation	+++	++	++	+++	+++	++	-	-	-

'-' or '+' sign represents no or positive visual growth and pigmentation. Number of '+' signs stand for relative extent of visual growth and pigmentation.

Cell- Cellulose, Chi- Chitin, Eryt- Erythrose, Glu- Glucose, Lac- Lactose, Mal- Maltose, Man- Mannitol, Star- Starch, Suc- Sucrose, BE- Beef Extract, Cas- Casein, Gela- Gelatin , Pep- Peptone, Tryp- Tryptone, YE- Yeast extract.

4. Conclusions

Based upon different morphological microscopic, biochemical and molecular parameters, the red pigmented bacterial strain isolated as an endophyte of faba bean root nodule was identified as a novel strain of *Serratia marcescens.* This strain could actively grow and produce red pigment in medium containing 0.5-1.0% NaCl and have an initial pH of 6.0-7.0 and at an incubation temperature of 28°C. The bacterial strain could efficiently utilize only simple organic nitrogen sources in the presence of simple sugars such as glucose, mannitol, maltose and sucrose with additional ability to use starch, chitin and cellulose though with slightly reduced efficiency.

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