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*Research Article*

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# Extraction, Characterization and Application studies of red pigment of halophile *Serratia marcescens* KH1R KM035849 isolated from Kharaghoda soil

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## ABSTRACT

*Red pigmented bacterial strain isolated from the soil sample of Kharaghoda area, Gujarat was used for the present study. This halophile, Gram negative bacteria was identified as a Serratia marcescens strain by 16s rRNA gene sequencing. This bacterium showed salt tolerance up to 12% Nacl. The gene sequence was submitted to NCBI and it is released by named Serratia marcescens KH1R KM035849. The highest extraction of this pigment was yielded in 2:1= 85% Methanol: Acetone mixture among different concentration of different solvents. The Rf value of the extracted pigment was 0.64-0.96 in TLC method. The maximum absorption spectrum was observed at 535 nm by UV-Visible Spectroscopy. This pigment exhibited antibacterial activity against B.cerus, S.aureus and E.coli with inhibition zone 12 mm, 07 mm and 06 mm respectively. This pigment was showing antioxidant activity confirmed by DPPH reduction method. The Serratia marcescens KH1R KM035849 strain is also siderophore producer.*

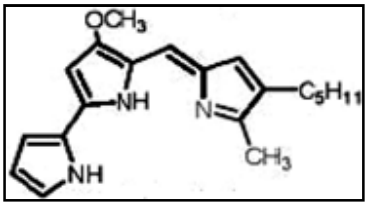
***Keywords:*** *Halophile, Serratia marcescens, Prodigiosin, Pigment extraction.*

## INTRODUCTION

Halotolerant organisms are known to tolerate extreme conditions of salt although they require normal concentrations of salt. High salt tolerant bacteria are known as Halophiles. In India, Gujarat has wide coastal areas including salt producing areas of Kharaghoda in Surendranagar district. These areas are full of biological diversity and also having halotolerant organisms including halophytes and halophiles.

*Serratia* spp are motile, short-rod shaped, Gram negative bacteria, classified in the large family of *Enterobacteriaceae. Serratia* spp. can be distinguished from other genera by its production of three special enzymes DNAase, lipase and gelatinase[1]. Another characteristic feature of the *Serratia* among the *Klebsiella* is the production of cell associated red color pigment called **prodigiosin**. Pigment production is highly variable among species and is dependent on many factors such as species type and incubation time. *Serratia marcescens* secretes a variety of extracellular enzymes including chitinases[2]. *S. marcescens* is one of the most effective bacteria for degradation of chitin[3].

Prodigiosin is a multipurpose red pigment, produced by various microorganisms such as *Serratia marcescens, Vibrio psychoerythrus, Rugamonas rubra,* actinomycetes, such as *Streptoverticillium rubrireticuli* and other eubacteria[4]. In vitro anticancer activity has also been reported for different prodigiosin analogs and synthetic indole derivative of prodigiosin[5]. It has antifungal, antibacterial and antiprotozoal activities, and thus may have potential clinical utility. The antiproliferative and cytotoxic effects of prodigiosin have been observed not only in cultured tumor cell lines but also in human primary cancer cells from B-cell chronic lymphocytic leukemia patients[6]. The use of prodigiosin for treating diabetes mellitus has also been reported where prodigiosin was found to be an active component for preventing and treating diabetes mellitus[7].




***Structure of Prodigiosin*[8]**

The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications. Hence, in this study focus was made only on prodigiosin pigment producing *Serratia* spp. for pigment production and evaluation of the same for some application towards ultimate industrial application.

## MATERIALS AND METHODS

### Selection of the Bacterial Strain

The red pigment producing halophilic isolate (shows up to 12% salt tolerance) was previously isolated from the soil sample of Kharaghodha area, Gujarat and selected for the further characterization study[9].

### Identification of the Strain

The preliminary identification of the strain was confirmed by Cultural characteristics and Morphological characteristics such as Gram Staining, Cell size, shape, arrangement etc.

### Molecular Characterization

Further confirmation of the strain was done by molecular characterization (ribo typing) using 16s rRNA gene sequencing.

### 1. Preparation of template DNA

It is important to use a pure cultivated bacterium for identification. Colonies are picked up with a sterilized toothpick, and suspended in 0.5 µl of sterile saline in a 1.5 ml centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5 µl of InstaGene Matrix (Bio-Rad, USA) and incubated 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant is used for PCR.

### 2. PCR

Add 1 µl of template DNA in 20 µl of PCR reaction solution. Use 27F/1492R primers for bacteria, and then perform 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. DNA fragments are amplified about 1,400 bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

### 3. Purification of PCR products

Remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

### 4. Sequencing

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described (Table 1). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

**Table 1. Primers of amplification and sequencing**

| Primer Name & Sequences | Amplification | Sequencing | Reference |
|---|---|---|---|
| 27F: AgA gTT TgA TCM TGG CTC Ag | ● | | Nucleic Acids Research, Vol. 18, Supplement |
| 1492R: TAC ggY TAC CTT gTT ACg ACTT | ● | | |
| 518F: CCA gCA gCC gCg gTA ATA Cg | | ● | |
| 800R: TAC CAg ggT ATC TAA TCC | | ● | |

This sequence was run on NCBI and constructed phylogenetic tree.

**Extraction and Purification of Red pigment from the isolate**

The different solvents like Chloroform, acetone, ethyal acetate and methanol were screened out for extraction of pigment but a modified procedure for the isolation of pigment was carried out according Wei *et al* (2005)[10]; a 24 h old culture broth (1.5 liters), incubated at 25°C and 200 rpm, was mixed with 4 liters of 95 % (v/v) methanol and mixed vigorously via vortex mixing. The mixture was then centrifuged at 6854 rpm, 0°C for 10 min. The resulting supernatant was collected and filtered through a 0.2 µm Whatman filter paper. The filtrate was concentrated using a rotary evaporator followed by extraction using 5.0 ml chloroform. The chloroform extract was reconcentrated using rotary evaporator until minimal volume was obtained. This minimal volume of chloroform extract was then transferred into a glass petri dish prior to drying in a vacuum drying oven. Dried pigment was dissolved in methanol or chloroform for 2-3 times and then drying it again so, pure pigment was obtained which is confirmed by TLC procedure.

**Characterization of Red Pigment**

**1. UV-Vis. Spectra absorption:**

Spectral analysis was made on dried pigment extracted by the above method by dissolving in methanol[11]. Spectral analysis was made on a UV- Visible spectrophotometer and the extract was scanned in the range of 400 to 700 nm to find out the maximum absorption spectra. Methanol was used as a blank.

**2. Pigment characterization by TLC**

The purified pigment was analysed by thin-layer chromatography with silica gel G-60 F25~ (Merck, Mumbai, India). The solvent system consists of chloroform: methanol (95:5; v/v). The chromatography chamber with the solvent was kept for 20 min. for the equilibration. The sample was spotted on the silica gel sheet using a capillary tube and air dried. The TLC sheet was then dipped in the solvent system. After 45 min. the TLC sheet was carefully removed and the Retention factor *(Rf)* value was calculated according to the following equation from the chromatogram.

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

**Application studies of Pigment**

- **Evaluation of in vitro antibacterial activity of Pigments**

The antimicrobial activities of pigment was studied on Nutrient agar by the disc diffusion technique against clinical isolates of Gram positive bacteria *Bacillus cereus*, *Staphylococcus aureus*, and Gram negative bacteria *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*. Sterile filter paper discs (6 mm) were individually impregnated with 20 µl of methanolic extract of this pigment. 95 % methanol was taken as a negative control and 1 mg/ml streptomycin was taken as a positive control. All the discs were dried and placed on the surface of the test bacteria. Following 18 to 24 h of incubation at 37 °C the plates were examined for the zones of inhibition[12]. Clear inhibition zones that formed around the discs indicated the presence of antibacterial activity.

- **Evaluation of Antioxidant activity by Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH).**

A 0.05 mM solution of DPPH in methanol was prepared. This solution was added to an equal volume (2 ml) of the solution of the tested compound (dissolved in methanol). Methanol was used as control solution. After 20 and 60 min at room temperature, the absorbance was recorded at 517 nm and compared with the appropriate standard, namely Ascorbic acid[13]. Lower absorbance of the reaction mixture indicates higher DPPH free radical scavenging activity. The percentage of scavenging activity of compound on DPPH radical was calculated as percentage (%) inhibition of DPPH (I%) using the following formula:

$$I\ \% = \frac{(Ao-As) \times 100}{Ao}$$

Ao= Absorption of control
As= Absorption of tested compound

- **Siderophore detection by use of Blue agar CAS Assay**

Siderophores are classified by the ligands used to chelate the ferric iron. These include the catecholates, hydroxamates, and carboxylates. Many bacteria utilize siderophores to help the process of ferric iron uptake in the environment. Various assays have been developed to detect different phenotypes of siderophores. Schwyn and Neiland (1987)[14] developed a universal siderophore assay using chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators. HDTMA has a moderate health and contact rating. Appropriate personal protective equipment should be used. The CAS/ HDTMA complexes tightly bind with ferric iron to produce a blue color. When a strong iron chelator such as a siderophore removes iron from the dye complex, the color changes from blue to orange.

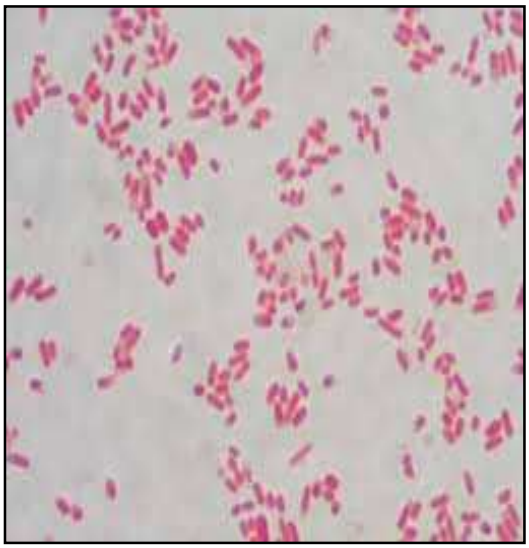
## RESULTS AND DISCUSSION

### Identification of Strain

The strain shows red pigmented growth on Nutrient Agar Plate (Fig. 1). This strain is Gram negative, short rods, mostly single forms which is shown in Figure 2. In a study done by Giri *et al*., 2004[1], shows same characteristics of *Serratia* strain. In a study done by Suryanto and Suvanto, 2003[15], results show Gram negative, mesophilic, non halophilic *Serratia marcescens* but in this study the *Serratia* strain was halophilic as tolerable up to 12% salt concentration because it was isolated from salty soil of selected site.

**Fig 1: *Serratia marcesecens* growth on Nutrient agar plate**


**Fig 2: Microscopic view of Gram Stained Bacteria**



**Molecular Characterization**

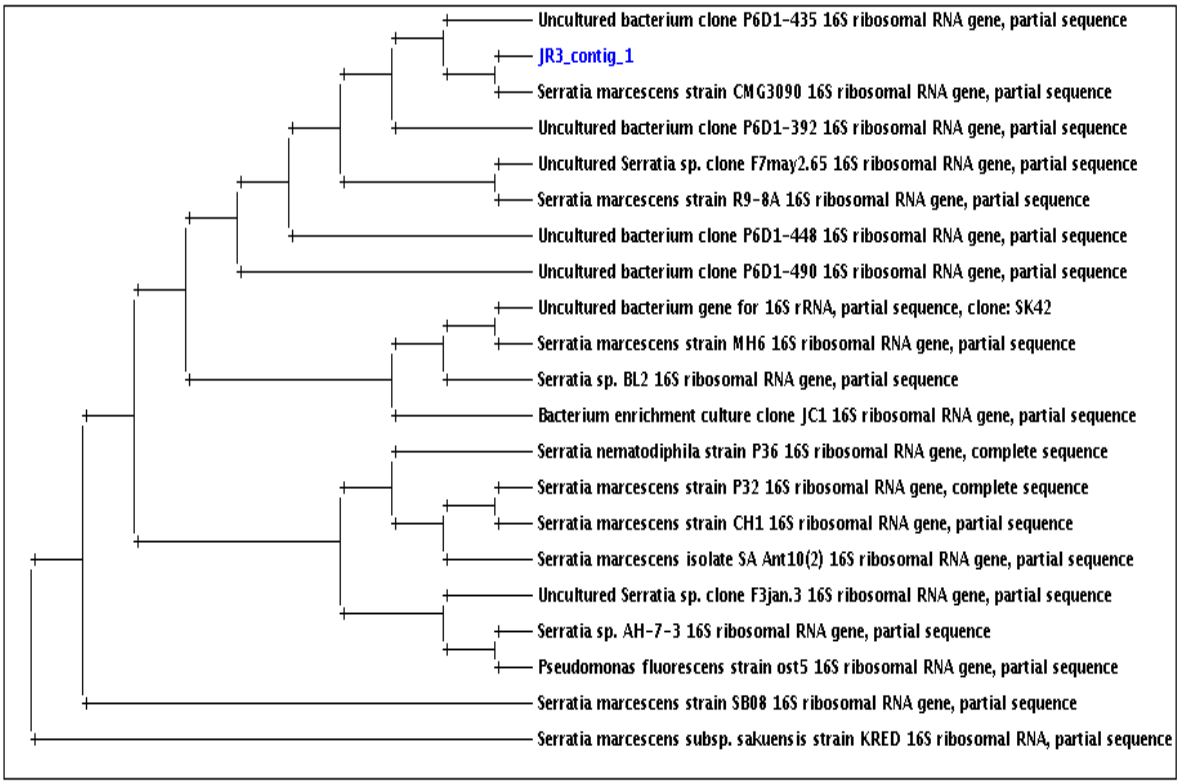
The Reverse (791 bp) and forward (1002 bp) sequence was used for conting sequence which was as below.

CAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGTAGCACAGGGG
AGCTTGCTCCCTGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGA
CCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCT
AGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATG
ACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG
CCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCA
TCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGGCGTAAAGCGCACGCAGG
CGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAA
CTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGAC
GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
TGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACG
CGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA
CTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCA
AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
GCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAAAGAGAAGCGAC
CTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCA
ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCAAA
AGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATCATGATGGGT
GACAAAAAAAAACCCCCCAAAAAACACACAAAAA

**Phylogenetic Tree**

The conting sequence was run on NCBI software and prepared phylogenetic tree as shown in figure No.3. The JR3 strain was matched with *Serratia marcescens* CMG3090 and uncultured Bacterium clone P6D1-435 but, the JR3 bacterium is culturable strain. It also produced red colored Prodigiosin pigment so, it was confirmed as *Serratia* spp.

**Fig 3: Phylogenetic Tree**




**Extraction of Pigments from cultured broth**

The pigment was extracted by different solvents with their different concentrations. Acetone, Ethyl acetate, Chloroform and Methanol were used. There was no pigment extraction observed in Chloroform and Ethyl acetate solvent. The Acetone and Methanol are the solvents, which have capacity to extract the pigment from the cell. But the highest extraction of pigment was shown in Methanol. There were different concentrations of Methanol and Acetone used for the extraction which are shown in Table 2. The highest extraction of pigment was shown in 2:1 = 85 % Methanol: Acetone.

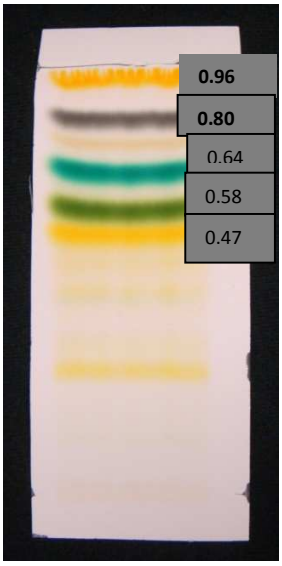
**Table 2: Results of Extraction with different concentration of Solvents**

| Solvent Concentration | | Absorbance at 535 nm |
|---|---|---|
| **(75 % Methanol : Acetone)** | 1:1 | 0.640 |
| | 2:1 | 0.596 |
| | 3:1 | 0.601 |
| | 5:1 | 0.805 |
| **(85 % Methanol : Acetone)** | 1:1 | 0.598 |
| | **2:1** | **0.989** |
| | 3:1 | 0.765 |
| | 5:1 | 0.657 |
| **(95 % Methanol : Acetone)** | 1:1 | 0.876 |
| | 2:1 | 0.734 |
| | 3:1 | 0.802 |
| | 5:1 | 0.899 |

Red pigment was extracted from the *Serratia* strain which is shown in figure No. 4

**Fig 4: Extraction of Pigment**


**Fig 5: TLC image for Pigment**




### 4.9. Characterization of Pigment

In Uv-Vis spectroscopy, maximum absorbance of Red pigment was obtained as 535nm. The maximum absorbance of red pigment was matched with Ahmad et al., 2012[16] result. In a study done by Hines *et al*, 1988[2], the maximum absorbance spectra for prodigiosin pigment was also at 535 nm. In another study done by Monreal *et al*, 1969[3], the maximum absorbance spectrum was at 534.6 nm for prodigiosin.

The TLC profile for the KH1 showed Dark orange (Rf 0.96),puple (Rf 0.80), Blue (Rf 0.64) and greenish yellow bands with Rf value is 0.58-0.47 (Fig. 5). In the Husain *et al*, 2012[17], study the Rf value of prodigiosin was 0.73 which is near to our results.

**Evaluation of in vitro antibacterial activity of Pigments:**

Pigment Prodigiosin extracted from isolate was showing clear inhibition zone against *Bacillus cereus* (12 mm), *Staphylococcus aureus* (7 mm), *Escherichia coli* (6 mm) (Table-3). In Gulani *et al*, 2012[12] result shows prodigiosin pigment activity against *Staphylococcus aureus* (17.5 mm), *Bacillus cereus* (10.5 mm), *Escherichia coli* (00 mm), but this report shows antibacterial activity of prodigiosin against *B.cereus* more and also shows activity against *E.coli*.

**Table 3: Results of Antibacterial activity**

| Name of Pathogens | Antibacterial activity-inhibition zone (mm) | | |
|---|---|---|---|
| | **Positive Control (Streptomycin-1mg/ml)** | **Negative Control (Methanol)** | **Red Pigment (in Methanol)** |
| *Bacillus cereus* (MTCC-430) | 18 mm | 00 mm | 12 mm |
| *Staphylococcus aureus* (MTCC-96) | 15 mm | 00 mm | 07 mm |
| *Escherichia coli* (MTCC-1687) | 12 mm | 00 mm | 06 mm |
| *Salmonella typhi* (MTCC-733) | 08 mm | 00 mm | 00 mm |
| *Klebsiella pneumoniae* (MTCC-3384) | 19 mm | 00 mm | 00 mm |

**Evaluation of Antioxidant activity by Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH).**

Prodigiosin pigment extracted from selected strain shows antioxidant activity by reducing the DPPH radical. DPPH is a stable radical (Violet colored) and when it reacts with an antioxidant compound which can donate hydrogen or electron, it is reduced to yellow colored diphenylpicrylhyfrazine (Fig.6). The % value of inhibition of DPPH (I%) for Pigment Prodigiosin is 30.98 %. In a study done by Gulani *et al*, 2012[12], Prodigiosin pigment also showed antioxidant activity.

**Siderophore detection by use of Blue agar CAS Assay**

The siderophore level produced by the isolate was recorded as the diameter of the orange halo produced by the colony. This strain shows orange halo in the plate which indicated this strain produced siderophore (Fig.7). In a study done by Tian *et al*, 2009[18], *Serratia* spp. also showed siderophore production. In another study done by Angerer *et al*., 1990[19], the *Serratia marcescenes* produced siderophores which were useful for plant growth in iron limitation condition.

**Fig 6: Antioxidant activity**

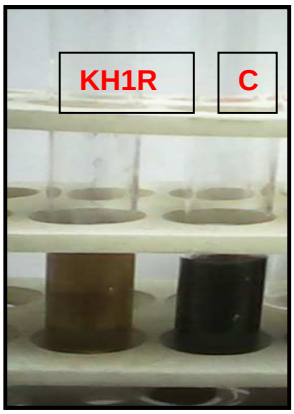
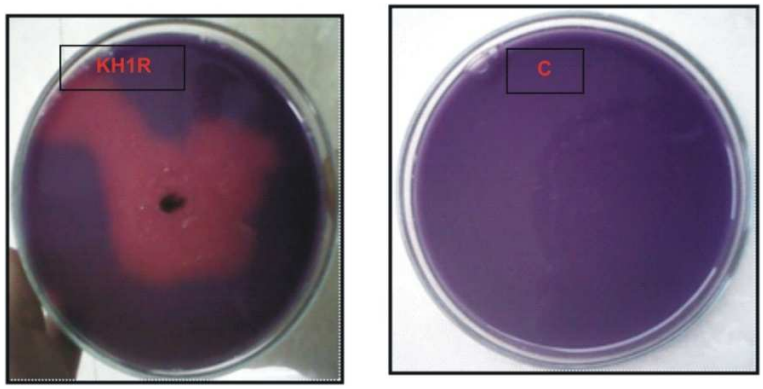



**Fig 7: Siderophore activity assay**




## CONCLUSIONS

From this study, it can be concluded that the selected halophilic strain is ***Serratia marcescens* KH1R (KM035849)** which produces red colored prodigiosin pigment which possess many useful properties like antibacterial, antioxidant and siderophore production.

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