

Original Research Article

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Production, Extraction and Characterization of Red Pigment Produced by *Serratia rubidaea* JCM 1240T isolated from Soil

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ABSTRACT

Keywords

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A red pigment producing bacteria was isolated from soil sample which was identified by 16S r RNA sequencing as *Serratia rubidaea* JCM 1204T. The red pigment produced was tested for prodigiosin by presumptive color test, adsorption spectra, and its quantification was carried out on King's B medium. For presumptive color test, the extracted pigment solution was divided into two portions. One part was acidified with a drop of concentrated HCl and the other part was alkalized with a drop of concentrated ammonia solution. A red or pink color in the acidified solution and a yellow or tan color in the alkaline solution indicated a positive, presumptive test for prodigiosin. For adsorption spectra, the ethanolic acidified and alkalized red pigment extract was tested by spectrophotometer in range between 300 to 700 nm and found that the ethanolic acidified red pigment extract absorbed maximum light at 535 nm while ethanolic alkalized red pigment extract absorbed maximum light at 470 nm which is characteristic of prodigiosin. The solid red pigment was also further characterized by IR, NMR, and Mass spectrophotometer, which confirms that the red pigment produced by *Serratia rubidaea* JCM 1204T as prodigiosin.

Introduction

Many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, comprise various hazardous effects. To counter act the ill effect of synthetic colorants, there is worldwide interest in process of development for the production of pigments from natural sources. The utilization of natural pigments in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes has

been increasing in recent years thanks to the knowledge about the harmful effects of synthetic pigments and their industrial byproducts on humans and the environment (Unagul *et al.*, 2005; Chidambaram and Lakshmanaperumalsamy, 2009).

Natural pigments can be obtained from two major sources, plants (Mizukami, *et al.*, 1978; Papageorgiou *et al.*, 1979) and micro-organisms (Cross and Edinberry, 1972; Ryu *et*

al., 1989; Parisot *et al.*, 1990; Yongsmith *et al.*, 1994; Kim *et al.*, 1998a, and Cho *et al.*, 2002). Biopigments from the micro-organisms have been preferred over those from plants because of their stability (Raisainen *et al.*, 2002) and the availability of their cultivation technology (Kim *et al.*, 1999; Parekh *et al.*, 2000) throughout the year. On the other hand, biopigments from plants have numerous drawbacks like instability against light, heat or adverse pH, low water solubility and often non-availability throughout the year (Chandni *et al.*, 2012).

One of the studied biopigments of microbial origin is the prodigiosin. Prodigiosin (PDG) is a natural red pigment characterized by a common pyrrolylpyrromethane skeleton, having low molecular weight (323.4 Dalton), appearing only in the late stages of bacterial growth as secondary metabolite (Harris *et al.*, 2004) and produced by many bacterial species, including many strains of *Serratia marcescens*, *Hahella chejuensis*, *Streptomyces variegatus*, *Colwellia (Vibrio) psychrerythraea*, "*Pseudomonas magnesorubra*, and other eubacteria (Yamamoto *et al.*, 1999). The prodiginine group, of which prodigiosin is a member, is a group of structural isomers that contain a tripyrrole core with different alkyl chains, have no defined role in the physiology of producing strains but have been reported to have antifungal, antibacterial, algicidal, antiprotozoal, antimalarial, immuno-suppressive, anticancer and antiproliferative activities (Castro, 1967; Boger and Patel, 1988; Williams and Quadri, 1980; Demain, 1995; Han *et al.*, 1998; Cerdano *et al.*, 2001; Furstner, 2003; Montaner and Pe´rez-Toma´s 2003 and Samrot *et al.*, 2011).

According to a recent report, prodigiosin or its analogues have been considered effective biological control agents against harmful algae (Jeong *et al.*, 2005; Nakashima *et al.*, 2006) have been considered cell growth

regulators (Haddix *et al.*, 2008) and can be used as a natural dye (Alihosseini, *et al.*, 2008).

The present investigation focuses on i) Identification of red pigment producing bacteria by 16 S rRNA Sequencing ii) Test the red pigment produced by the red bacteria for prodigiosin by presumptive color test and adsorption spectra, and iii) confirmation red pigment as prodigiosin by IR, NMR and Mass spectrophotometer.

Materials and Methods

Isolation of Red Pigment Producing bacteria from Soil

Soil samples, for isolation of red pigment producing bacteria, were collected from different field and brought in poly-ethylene bags to the laboratory, Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna.

The collected soil samples were serially diluted and plated on King's B agar plates. Red-pigmented colonies were isolated on the plates after incubation and preserved at 4°C. The isolated red pigmented bacteria were identified by 16S r RNA gene sequence analysis carried out commercially from Agharkar Research Institute, Pune.

Presumptive color tests for prodigiosin

Red pigment produced in suitable medium was presumptive tested (Gerber and Lechevalier, 1976) for prodigiosin. For presumptive test, pigmented broth was suspended overnight in 95% ethanol at 22 to 24°C. Debris was removed from the suspension by centrifugation at 5,000 rpm for 15 min. The clear solution was then divided into two portions. One part was acidified with a drop of concentrated HCl and the other part was alkalized with a drop of concentrated

ammonia solution. A red or pink color in the acidified solution and a yellow or tan color in the alkaline solution indicative of a positive presumptive test for prodigiosin.

Determination of adsorption spectra

For determination of absorption spectra, Gerber and Lechevalier, (1976) method was used. Pigmented bacteria were treated with ethanol, and the cell debris in the suspensions was removed by centrifugation at 5,000 rpm for 15 min.

Samples (1 ml) of the ethanol extracts were acidified or alkalinized with 0.1 ml of 1 N HCl or 1 N NaOH, respectively. The absorbance spectrum of sample was measured with a Single Beam UV – Visible Spectrophotometer (Bioera Life Science Pvt. Ltd.) in the range of 300 to 700 nm. Acidified or alkalinized 95% ethanol was used as a blank.

Production of Prodigiosin in suitable Medium and Quantification of prodigiosin

In order to determine the suitable media supporting the maximal production of prodigiosin, red pigment producing bacteria was grown in Nutrient Broth, and King's B Broth, at 25 °C. The levels of prodigiosin in these conditions were estimated after 24hr, 48hr, 72hr and 96 hr.

For quantification of prodigiosin produced, bacterial cell absorbance in the King's broth and Nutrient broth was measured at 620 nm, following which the broth suspensions were subjected to centrifugation at 5000 rpm for 15 min to collect the cell pellet. 10 ml of 95% methanol was added to the cell pellet and centrifuged under the same condition. 95% methanol was used as a blank. Methanolic extract of prodigiosin showed characteristic maxima at 499 nm. Extracted prodigiosin was estimated using the following equation (Mekhael and Yousif, 2009).

$$\text{Prodigiosin unit/cell} = \frac{[\text{OD}_{499} - (1.381 \times \text{OD}_{620})] \times 1000}{\text{OD}_{620}}$$

Where,

OD₄₉₉ – pigment absorbance;
OD₆₂₀ – bacterial cell absorbance
1.381 – constant

Extraction and Characterization of Red pigment

Serratia rubidaea grown in King's B broth was centrifuged at 10000 rpm for 20 minutes and the cell pellet was collected. The pigment from the cell pellet was extracted with acidified methanol (1N HCl 1ml: Methanol 24 ml) and the extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was concentrated in Rota evaporator at 60 °C

to obtain crude pigment. The crude concentrated pigment was recrystallized with methanol (Min – Jung – Song *et al.*, 2005; Chandrashekhar *et al.*, 2012).

The chemical structure of the purified pigment was characterized by FTIR BRUKER model RXI Spectrometer, mass spectrometry and NMR (Bruker, Germany). IR Spectra of prodigiosin was scanned on FTIR BRUKER model RXI Spectrometer.

¹HNMR spectra were recorded in DMSO on Gemini 200 MHz instrument using TMS as an internal standard (chemical shift given in δ ppm). The mass spectrum was recorded on Cintra – 15 – GCMS – Spectrophotometer.

Result and Discussion

Isolation of Red Pigment producing bacteria from Soil

Serially diluted soil samples plated on king's B agar plates produced wide range of red pigmented colony. Out of these colonies, a red pigment colony was selected whose pigment was to be tested for prodigiosin, isolated on the plates after incubation and preserved at 4°C. Later on the isolated red pigmented bacterium was identified by 16S r RNA gene sequence analysis as *Serratia rubidaea* JCM 1240T (photo plate 3.1) whose phylogenetic relation (Figure 3.2) and partial 16S r RNA gene sequence is as follows (Figure 3.1).

Presumptive color tests for prodigiosin

For this presumptive test pigmented broth was suspended overnight in 95% ethanol at 22 to 24°C. Debris was removed from the suspension by centrifugation at 5,000 rpm for 15 min. The clear solution was then divided into two portions. One part was acidified with a drop of concentrated HCl and the other part was alkalinized with a drop of concentrated ammonia solution. A red or pink color in the acidified solution was observed while a yellow or tan color in the alkaline solution indicated a positive presumptive test for prodigiosin as shown (photo plate 2).

Similar types of results were also shown in studies carried out by Ming-Jer Ding and Robert P. Williams (1983) where strain of *Serratia marcescens* isolated from clinic shows a red or pink color in the acidified solution while a yellow or tan color in the

alkaline solution. This test indicative for presumptive test for prodigiosin.

Determination of adsorption spectra

For determination of absorption spectra, pigmented ethanol extracts were acidified or alkalinized with 0.1 ml of 1 N HCl or 1 N NaOH, respectively. The absorbance spectrum of sample was measured with a Single Beam UV – Visible Spectrophotometer (Bioera Life Science Pvt. Ltd.) in the range of 300 to 700 nm. Acidified or alkalinized 95% ethanol was used as a blank.

Pigment extracted with ethanol from *Serratia rubidaea* JCM 1240T showed characteristic maxima of 535 (Figure 3.3) and 470 nm in acid and alkaline solutions, respectively, which is indicative that the pigment extracted with ethanol was prodigiosin. Similarly, Prodigiosin extracted with ethanol from *S. marcescens* Nima showed characteristic maxima of 535 and 470 nm in acid and alkaline solutions, respectively (Williams and Qadri, 1980; Ming-Jer Ding and Robert Williams, 1983).

Prodigiosin was reported to display a characteristic absorption spectrum in ethanol, with a maximum at 534 nm (Slater *et al.*, 2003) and single peak absorbance at 535 Nm (Giri *et al.*, 2004; Song *et al.*, 2006; Montaner *et al.*, 2000) extracted prodigiosin by shaking the *S. marcescens* 2170 cells with a mixture of methanol/1N HCl in the ratio 24:1.

Absorption spectrum of the pigment produced by *Serratia rubidaea* JCM was dependent on pH value similar to that reported earlier (Song *et al.*, 2006). Thus at pH 2.0, the pigment was red and showed a maximum absorption at 535 nm, which is identical to that of prodigiosin hydrochloride. Under neutral condition (pH 7.3), its absorption intensity decreased and the colour of the pigment changed to pink.

However, in alkaline condition (pH 10.0) the colour was orange and its absorption spectrum shifted to 470 nm. Pigment produced by *Serratia rubidaea* JCM 1240T recorded maximum absorption at and 470 nm in acid and alkaline solutions, respectively suggesting that this pigment is prodigiosin.

Production of Prodigiosin in suitable Medium and Quantification of prodigiosin

To determine suitable medium for production and quantification of prodigiosin, *Serratia*

rubidaea JCM 1240T was grown in Nutrient Broth, King's B Broth, at 25 °C.

For quantification of prodigiosin produced, bacterial cell absorbance in the medium was measured at 620 nm, while absorbance of methanolic extract of prodigiosin was measured at 499 nm (Table 3.1). The concentrations of prodigiosin in these conditions were estimated after 0hr, 24hr, 48hr 72hr and 96 hr. By using these absorbance, prodigiosin concentration was determined as represented in (Table 3.2).

Table.3.1 Pigment Absorbance at 499nm

Hour	Nutrient Broth (O.D.)	King's B Broth (O.D.)
24 hr	00	00
48 hr	1.603	1.673
72 hr	1.654	1.686
96 hr	1.566	1.580

Table.3.2 Total prodigiosin estimated in nutrient broth and King's B broth

Hour	Prodigiosin in Nutrient Broth mg/lit	Prodigiosin in King's B Broth mg/ lit
24 hr	00	00
48 hr	1070.33	1188.94
72 hr	1055.96	1246.10
96 hr	885.38	957.10

Fig 3.1

ATTGCCCATGGGCGCAAGCCTGCATGCNGCCATGCCGCGTGTGTGAAGAACGCCTTCGGGTTGTA
 AAGCACTTTCAGCGAGGAGGAAGGTGGTGANCTTAATACGTTCATCAATTGACGTTACTCGCAGA
 AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGTAATACGGAGGGTGCAAGCGTTAATCGGAA
 TTAAGCGTAAAGCGCACGCCAGGCGTTTGTTAAGTCANATGTGAAATCCCCGANCTTAACTT
 GGGAACTGCATTTGAACTGGCAAGCTAGAGTCTCGTATAGGGGGTGAATTCCAGGTGTACCG
 GTGGAATGCGTACAAGATCTGGAGGAATACCGGTGGCGAANGCNGCCCCCTGGGACNAAAAA
 CTGACGCTCATGTGCGAAAGCGTTGGGGAGCAAACCGGAATCACATACCCTGGTCAATCCACGCC
 CGNTAAACGATGANCCATTTTGC GGAGGTTGNNGCCCTTNGGAGGCGTGGGNTTCCGNGANCTAA
 CACTTAAAATCC

Figure 3.1: 16 S rRNA Gene Sequencing

Fig 3.2

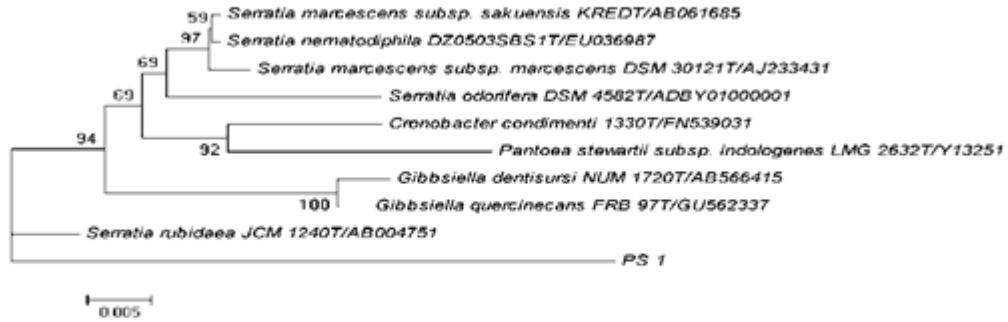


Figure 3.2: Closest Phylogenetic relation of *Serratia rubidaea* JCM 1240T

Fig 3.3

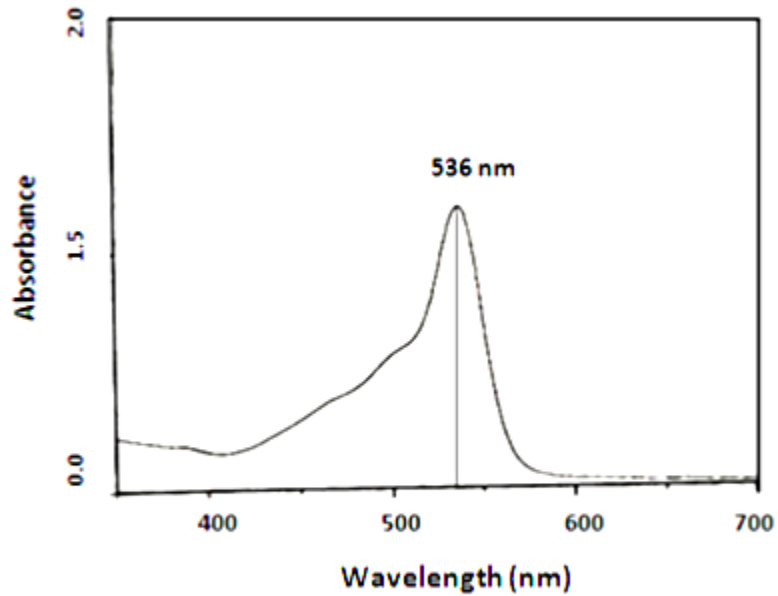


Figure 3.3: Absorption Spectra of Acidified Ethanolic Red Pigment

Fig 3.4

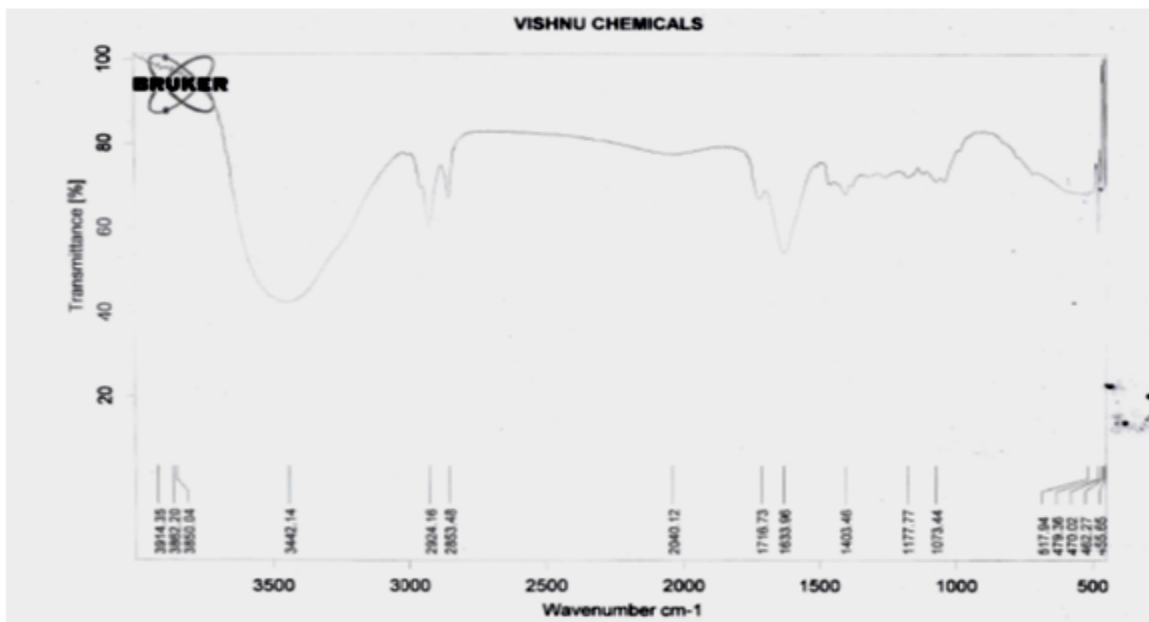


Figure 3.4: IR spectra of purified Prodigiosin from *Serratia rubidaea* JCM 1240T

Fig 3.5

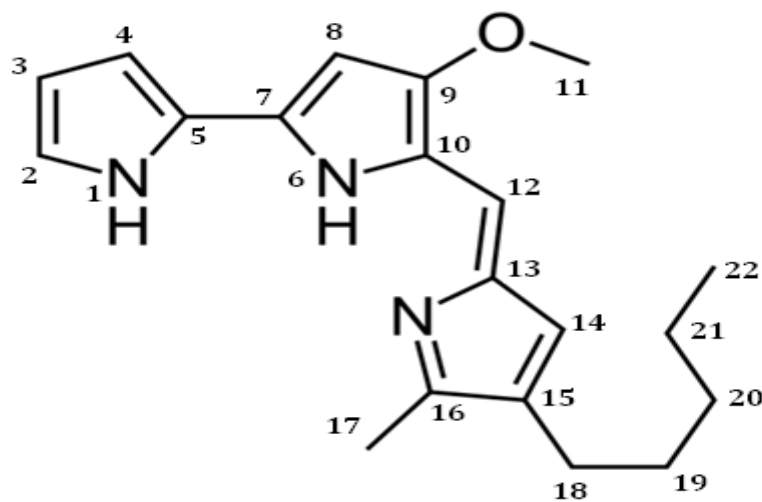


Figure 3.5: Structure of Prodigiosin determined by ¹HNMR spectra

Photo 3.1

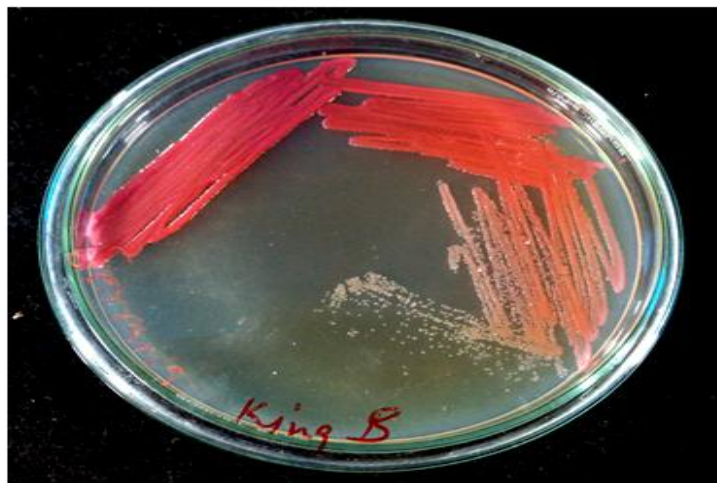


Photo plate 3.1: Isolated *Serratia rubidaea* JCM 1240T on King's B Agar Plate

Photo 3.2

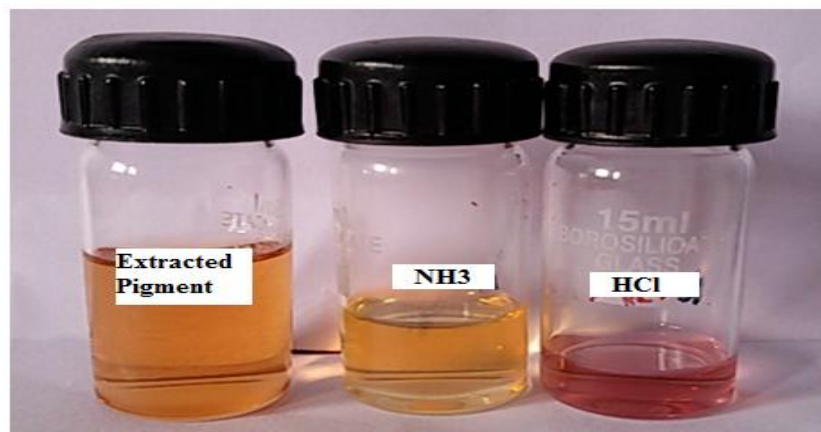


Photo plate 3.2: Presumptive Test for Prodigiosin

Prodigiosin unit per cell after 48hr was found to be 1070.33 and 1188.94 in nutrient broth and King's B broth respectively. Maximum production was reported in 72hr thereafter production ceased towards 96hr. The lowest prodigiosin unit per cell was found after 96hr i.e. in nutrient broth it was 885 and in peptone glycerol broth i.e. 957.1.

Similar results were obtained by Kamble and

Hiwarale (2012) where they compared prodigiosin production in Nutrient Broth and Peptone Glycerol Broth. In this study, they found highest prodigiosin production after 72 hr in peptone glycerol broth than nutrient broth.

Extraction and Characterization of Red pigment

The chemical structure of the purified prodigiosin was characterized by FTIR

BRUKER model RXI Spectrometer, mass spectrometry and NMR (Bruker, Germany). IR Spectra of prodigiosin was scanned on FTIR BRUKER model RXI Spectrometer. ¹HNMR spectra were recorded in DMSO on Gemini 200 MHz instrument using TMS as an internal standard (chemical shift given in δ ppm). The mass spectrum was recorded on Cintra – 15 – GCMS – Spectrophotometer.

IR Spectra

IR Spectra of prodigiosin (Figure 3.4) showed characteristic band at near region 3442.14 cm⁻¹ due to >N – H group (amide gr.), bands at 2924.16 – 2853.48 cm⁻¹ due to C – H (sp³) stretching vibrations from methyl and Methylene group, bands at near region 1633.96 cm⁻¹ is due to Pyrrole group and the band at 1716.73 cm⁻¹ is due to C = C stretching vibrations. The visible peak at 1403.46 cm⁻¹ is due to the presence of C–O group in prodigiosin.

Thus from the IR Spectrum the main functional groups that resulted in red pigment were Pyrrole, amide, Methylene, methyl and alkene.

¹HNMR spectra:

¹HNMR spectra of prodigiosin were studied in DMSO, represents the proton peaks corresponding to chemical shifts at δ5.323 ppm, δ5.053 ppm, δ3.773 ppm, δ2.354 ppm, δ1.236ppm, and δ0.853 ppm assigned to the carbon atoms C2, C12, C11, C18, C21, and C22 based on the ¹HNMR spectra structure of prodigiosin presented (Figure 3.5).

Mass Spectra

The mass spectra for the prodigiosin exhibited correct molecular ion peaks for its molecular weights.

In the present study, the data obtained for the

spectroscopic analyses of the red pigment with IR, ¹HNMR and Mass spectra very clearly testify that the pigment produced by *Serratia rubidaea* JCM 1240T is prodigiosin-like pigment. Sumathi *et al.*, 2014 used same technique to characterize the pigment produced by *Serratia marcescens* NPLR1 where they found the pigment produced was also prodigiosin.

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