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Characterization and application of prodigiosin produced by *Serratia rubidaea* KAP (LC201792)

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Abstract
Bacterial pigments are emerging as sustainable and biocompatible alternatives to synthetic colorants. Prodigiosin, a red secondary metabolite, was extracted from *Serratia rubidaea* KAP (LC201792), isolated from oil-contaminated soil and characterized. Ethanol was identified as the optimal solvent for pigment extraction. Characterization through UV-Visible spectrophotometry, FTIR, GC-MS, and HPLC confirmed the molecular identity of prodigiosin. A maximum absorbance peak at 534 nm was observed via UV-Visible spectrophotometry, while FTIR analysis identified key functional groups, including -NH, C=C, and CH stretches. The antimicrobial assay revealed selective activity against Gram-positive bacteria. Prodigiosin demonstrated a higher antioxidant efficacy of 96.12%, highlighting potential as a natural antioxidant. Furthermore, this study highlights its application as a dye that showed excellent color fastness on diverse substrates, including nylon, silk, polyester, candles, paper, and plastic, reinforcing its viability as an eco-friendly alternative to synthetic dyes.

Keywords: Prodigiosin, *Serratia rubidaea* KAP, bacterial pigments, antimicrobial activity, antioxidant activity

Introduction
Colorants are used in different industries such as in the food, pharmaceutical, and textile industries. Synthetic colorants are often preferred by industries due to their varied color shades, low-cost production, and high availability throughout the year. But these synthetic colorants have prone to cause detrimental effects on the environment as well as on human health. Most of the colorants used by the industries are not biodegradable. Apart from these, they are carcinogenic (e.g., azo dyes) and are not easily treated by the waste treatment process [1]. A long-term solution to the problems caused by synthetic colorants is green synthesis and application of natural colorants. This will also minimize the expense of remediation and the pollution burden brought by the chemically derived colorants.

Nature has become a ubiquitous source of various bioactive compounds such as Bio-pigments produced by various genres of microorganisms [2]. Microbial pigments are a developing area of study and of significant interest for many applications due to certain characteristics. These include accessibility to cultivation technology, stability, quick and simple growth on culture media, independence to weather conditions, availability of a wide range of environmentally friendly and biodegradable shades. Different color tints are in demand due to the growing demand. As a result, there is a large scope, economic need, and preference for biological pigment sources [3].

Microbial pigments are reported to be a substitute for natural food-grade pigments and have a lot of promise for their use in food applications because of their inherent therapeutic benefits, ability to be produced without regard to time constraints, and anticipated yield. Among the gram-negative bacteria, the *Serratia* species has gained attention due to its common appearance as an environmental isolate, ease of cultivation, and ability to produce attractive light pink to deep red pigment and its inherent bioactive properties [4]. It is proven to inherent various biological properties like immunomodulatory, antibacterial, antimycotic, and antimalarial activities [5]. Due to these properties, they have been widely used as food additives, textile colorants, paper production, and agricultural practices [6].

In view of the flourishing market status of industries with respect to associated health risks and environmental problems, a sustainable approach that uses natural alternatives to harmful chemicals is highly desirable in the current situation. The objective of the study is to work on the use of natural pigments obtained from microbial origin and implement them in industries.

Materials and Methods

1. Growth Condition of Microorganisms

Serratia rubideae KAP, bacteria were isolated from oil-contaminated soil and maintained at 4ºC on Sterile Nutrient Agar slants supplemented with 1% glycerol. Regular subculturing of the stock cultures were done to maintain the functional capacity of the isolated bacteria.

2. Extraction of pigment using standardized procedure

2.1.1 Selection of optimum solvent for extraction of pigment

To analyze the optimum solvent option to obtain maximum pigment extraction, solvents like methanol, absolute ethanol, acetic acid, acetone, ethyl acetate, chloroform, petroleum ether, and n-hexane were selected for the study. Further experiments used the solvent which yielded the highest amount of extracted pigments from this selection process.

2.1.2 Procedure for Extraction of Pigment

Serratia rubidaea KAP was inoculated into a Sterile Nutrient broth and incubated at room temperature under static conditions for 5 days. After incubation, 25ml of culture broth was subjected to centrifugation at 10,000rpm for 10min. The cell pellet was resuspended in the respective solvents and vortexed for 1 min to ensure proper mixing, followed by a centrifugation at 10,000rpm for 10min. The supernatants were then spectroscopic analyzed for the maximum absorbance (of pigment) at 535nm [7].

2.1.3 Quantification of red pigment

To quantify the pigment produced by the *Serratia rubidaea* KAP *strain*, firstly pigment is extracted according to section 2.2.2. The pigment produced was estimated using the formula given by Ivanchenko, D. A. Balasubramaniam B, (2019) [14]. The pigment absorbance was taken at 534 nm, and the bacterial cell optical density data was measured at 620nm.

$$\text{Prodigiosin (unit/cell)} = (OD534 - (1.381 \times OD620)) \times 1000 \div OD620$$

OD534 = refers to the absorbance of pigment at 534nm.
OD620 = refers to the optical density of the culture broth.
1.381 = Constant
Ethanol was used as a blank for taking pigment absorbance at 534nm. An uninoculated nutrient broth was maintained as both a control and a blank in all experiments to record the optical density of the culture broth.

3. Characterization of pigment produced by Serratia rubidaea KAP

3.1.1 Presumptive test for identification of Prodigiosin pigment.

The pigmented cell culture broth was centrifuged at 10,000rpm for 10min. The supernatant was discarded, and pellet was collected. The cell pellet was then mixed with 95%methanol and was centrifuged again at same condition. The supernatant was collected and subjected to acidic and alkaline solutions to note the change in colour. Conc. HCl (35%,0.1ml) was used as an acid solution and Ammonia solution(25%,0.1ml) was used as an alkaline solution. A change in colour from orange to pink in acidic environments and from orange to yellow in alkaline ones indicates the presence of prodigiosin [8, 9]

3.1.2 Determination of wavelength for maximum absorption of prodigiosin.

To determine the absorption maxima of the red pigment UV- Visible Spectrophotometry was employed. The ethanolic extract of pigment was analyzed using ethanol as blank. A spectrum scan from 400nm to 800nm was selected and absorbance at lambda max was recorded.

3.1.3 Thin Layer Chromatography

The extracted red pigment was subjected to Thin Layer Chromatography. The volatile of the pigment molecule was separated on a TLC plate (14cm x 9cm) coated with silica gel (Mereck, 60 F254), a standardized solvent system of n-butanol: hexane (2:1) was prepared and left overnight to saturate the chromatographic tank. The Rf value of the chromatogram developed on the TLC plate was calculated using the following formula given below [10, 11]

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

3.1.4 Fourier Transform Infrared Spectroscopy Analysis

The Functional group analysis was carried out using FT-IR spectroscopy with a frequency range of 4,000-500 cm^{-1}. The procedure included mixing the KBr powder with the extracted pigment and then firmly pressing it into a pellet.

3.1.5 HPLC analysis of the pigment

The chromatographic separation of red pigment was carried out using the Shimadzu HPLC system. The separation was performed using a B-C18 Sepax column with a flow rate of 0.6mL/min and an injection volume of 20uL at 35℃ temperature. The mobile phase consisted of acetonitrile water in a 60:40 v/v ratio. Prodigiosin was detected at its maximum absorbance (534nm) [12].

3.1.6 GC-MS analysis of the pigment

The ethanolic extract of pigment was subjected to GC-MS analysis for determining the molecular weight of the compound. The condition maintained for the analysis included a flow rate of 1ml/min with helium as carrier gas. The oven temperature was set initially at 70°C for 1min and 15min at 280 °C. Other instrument specifications were electron energy of 70 eV, mass range scanned of 35-500 m/z, oven temperature of 70 °C, injector temperature of 280°C, and detector gain of 1.03 kV +0.00 kV [13, 14]

4. Application of Prodigiosin pigment extracted from *Serratia rubidaea* KAP

4.1.1 Antimicrobial activity of pigment

The antimicrobial potency of the prodigiosin pigment extracted from *Serratia rubidaea* KAP was anaylsed using agar well diffusion method. The bacterial culture suspensions examined for the study were *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus aureus* 6538p, *Streptococcus pyrogens*, *Bacillus subtillis*, as well as fungal cultures of *Candida albicans* and *Saccharomyces cerevisiae*. Sterile Muller-Hinton agar butts for bacterial cultures and Sterile Sabouraud's agar butts for fungal culture were inoculated with 0.2ml of each culture and pour plated into sterile empty plates. After solidification of plates, it was divided in half and wells were created using a sterile cork borer. The culture density of each organism was adjusted to 0.1 OD at 540nm. In one well 50µL of ethanolic extracted pigment

was added, while in another well 50µL of absolute ethanol was added as a control. The plates were incubated upright at 37°C for 24hrs for bacteria and room temperature for 48hrs for fungi. After incubation, the zone of inhibition around the well was measured in millimeters to assess the pigment antimicrobial activity [15-, 20].

4.1.2 Antioxidant activity of prodigiosin pigment estimated using 1,1-diphenyl- picrylhydrazyl (DPPH) method.

- **Qualitative Assay**

The antioxidant activity of the extracted pigment was assessed using the DPPH method. A 0.1mM DPPH solution was mixed with equal volume of methanolic extract (2ml each) and methanol served as blank. All the tubes were wrapped with silver foil and incubated in dark for 30 minutes. Absorbance was measured at 517nm before incubation and after incubation. A decrease in the absorbance indicated DPPH free radical scavenging activity [21].

- **Quantitative method**

The extracted pigment was quantitatively evaluated for antioxidant activity using the DPPH method. An equal volume of 0.1mM DPPH solution was added to the methanolic extract of red pigment (2ml). The tube containing the reaction mixture was wrapped with silver foil and subjected to incubation in dark conditions for 30min. Methanol served as blank. After 30min, the absorbance at 517 nm was measured and compared to the suitable standard. A fresh Vitamin C tablet (500mg) was used as the standard. To compare the antioxidant activity of the pigment against the Vitamin C standard, a standard concentration range (10ug to 20ug with an interval of 2ug) was prepared from 50mg of Vitamin C stock solution. All the concentrations were prepared in methanol solvent. 2ml of each concentration mixture was added to 2ml of 0.1mM DPPH solution and the antioxidant activity was accessed by incubation in dark conditions for 30min. A reaction mixture's lower absorbance denotes a higher level of DPPH free radical scavenging activity. To calculate the percentage inhibition of DPPH activity the following formula was used [22-27].

$$\% \text{ inhibition of DPPH activity} = (\text{Abs control} - \text{Abs test}) \times 100 \div (\text{Abs control})$$

4.1.3 Application of prodigiosin as Bio colorant:

- **Textile dyeing**

The dyeing potential of prodigiosin pigment from *Serratia rubidaea* KAP was examined on different types of textile fabrics (cotton, silk, nylon, and polyester) [27-30]. All the fabrics were cut into 2cm by 2cm sized and then used.

Step 1. Pre-treatment of cloth by Scouring

- The Scouring of the Cloths were carried out by using 5 gm per L Surf Excel and 2gm/L Sodium Carbonate, boiling for 20min using the material to liquor ratio of 1:50.
- After Scouring, a cold wash with water was given for 5min followed by a hot wash by boiling for 10min.

Step 2. Dyeing

Without Salt

- The cloths were introduced in culture broth in a 1:30 material-to-broth ratio.
- The Culture broth pH was adjusted to 4.
- The temperature of the water bath was raised to 85ºC by heating and dyeing carried out for 1h.
- After dyeing, the cloth was given a cold wash with D/W.
- The cloth was rinsed and air-dried.

With Salt

- The cloths were introduced in culture broth in a 1:30 material-to-broth ratio. The Culture broth pH was adjusted to 4.
- 2% Glauber salt was added to the culture broth containing the cloths. (Half the amount added)
- The temperature of the water bath was raised to 85 degrees by heating and kept for 15min. After 15min the remaining amount of Glauber salt (5ml) and 2% sodium carbonate was added.
- Further the dyeing process was carried out for 45min.
- After dyeing, the cloth was given a cold wash with D/W.
- The cloth was rinsed and air-dried.

Fastness Testing

After Dyeing the color fastness properties of the cloth were determined w.r.t. light and washing. To check the light fastness property all the dyed fabrics were placed in Petri plates and exposed to sunlight and indoor light for 24hs. To check the colour fastness, all the fabrics were subjected to 2g/L detergent washing. The commercial detergent used was Surf Excel [31-32].

Thermal stability of Dyed cloth

After dyeing, the 7cm by 2cm dyed fabrics were subjected to Autoclaving at 121ºC at 15lbs for 20min for checking the thermal stability of the dyed cloth.

- **Dyeing of Plastics**

To check the dyeing potential of extracted pigment plastic material like centrifuge tubes were used. The prodigiosin extracted in ethanol solvent was poured into centrifuges tubes for colouring the plastics. The fastness property of the plastic was determined by excessive washing with detergent solution [33-37].

- **Dyeing of Paper**

To check the colouring properties of extracted pigment, Whatman paper was used. The Whatman filter paper was dipped into the ethanolic extract of the pigment extracted from *Serratia rubidaea* KAP [29, 30, 36].

- **Dyeing of Candles**

Prodigiosin extracted from *Serratia rubidaea* KAP was used to dye colourless wax candles. The wax was melted and poured into Eppendorf tubes. To the melted candle the pigment extract was added, and the wax was allowed to solidify. The light fastness property of the dyed candle was estimated by exposing the candle to indoor light for 24h [37, 38, 39].

- **Prodigiosin as a colorant in paints**

The extracted pigment was mixed with commercially availed distemper in the market. The pigment was added until a visible pink coloured was obtained after mixing. The bio-paint formulated was further applied on the wall. The color fastness property of the paint was determined was exposing it indoor light and sunlight for 24h [40-43]. All media and reagents used were from Hi-media and chemicals used were of analytical Grades

Results and Discussion

In this study, the bright red pigment prodigiosin produced by the bacterial strain *Serratia rubidaea* KAP was studied. The strain was isolated from oil contaminated soil and was identified to be a good biosurfactant producer [44]. Biochemical tests, VITEK analysis, 16S rRNA sequencing, and genetic characterization were done to determine the strain, that was then contributed to the National Center for Biotechnology Information (NCBI) under accession number LC201792.

The purpose of this study is to characterize the metabolite produced by *Serratia rubidaea* KAP, which was then evaluated for antibacterial activity, antioxidant, and dyeing properties for future application.

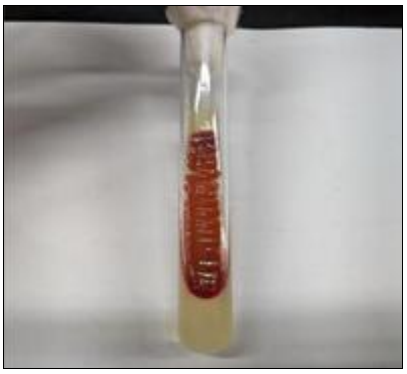

Fig3.1: *Serratia rubidaea* KAP on Nutrient Agar Slant

4.2 Growth Condition and colony characteristic of Bacterial strain *Serratia rubidaea* KAP

Serratia rubidaea KAP was isolated from oil contamination and subculturing was done on Nutrient agar slant supplemented with 1% glycerol which retained the pigment-producing capability of *Serratia rubidaea* KAP.

Macroscopic and Microscopic colony characteristics were studied to obtain large, dark red pigmented colonies which were found to be gram-negative coccobacilli in clusters and chains when observed under 100X.

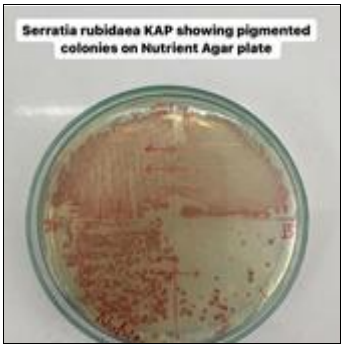

Fig 3.1.a

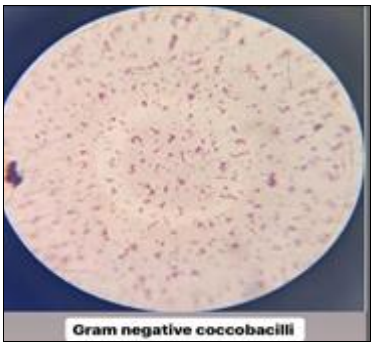

Fig 3.1.b

4.3 Characterization of pigment produced by *Serratia rubidaea* KAP

4.3.1 Presumptive test for identification of Prodigiosin pigment.

The presumptive test for prodigiosin was conducted by centrifuging the nutrient broth containing the culture at 10,000 rpm for 10 minutes. After centrifugation, the cell pellet was dissolved in 95% methanol and centrifuged again. The methanolic extract of the pigment was then divided into two portions: one for the addition of 35% hydrochloric acid (HCl) and the other for the addition of 25% ammonia solution.

Upon mixing the methanolic extract of the red pigment produced by *Serratia rubidaea* KAP (Fig. 3.2.1) with 35% HCl there was a shift in color from orange to pink. The addition of 25% ammonia solution resulted in a color change from orange to tan, and yellow (Fig. 3.2.1). The change in color indicated positive prodigiosin production and it was found that this result is in alignment with the findings of Othman *et al*. (2019) [45], Maurya *et al*. (2020) [13], Sruthy *et al*. (2014) [4], and Shaikh (2016) [38].

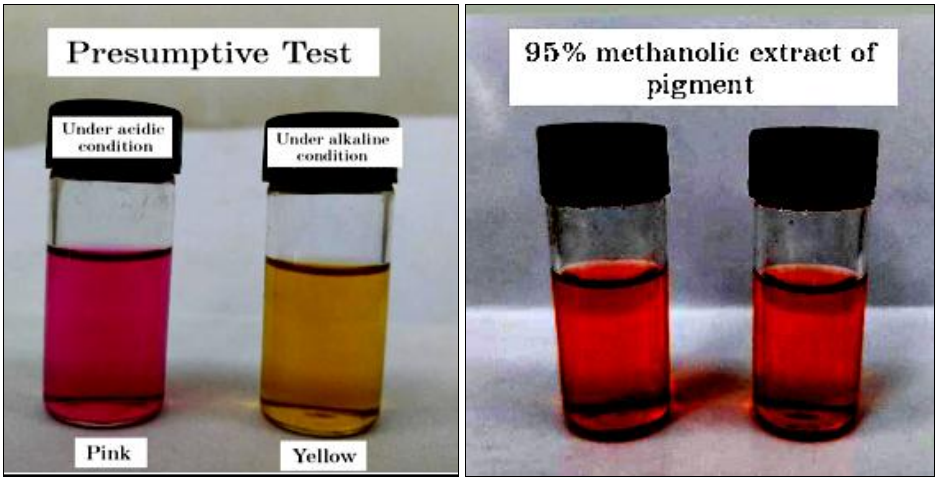

Fig 3.2.1: Presumptive test of red pigment extracted from *Serratia r ubidaea*KAP

4.3.2 Determination of wavelength for maximum absorption of prodigiosin.

A spectrophotometer was used to investigate the ethanolic extract of the red pigment generated by *Serratia rubidaea* KAP, as well as determining its maximum absorbance (λmax). It was then

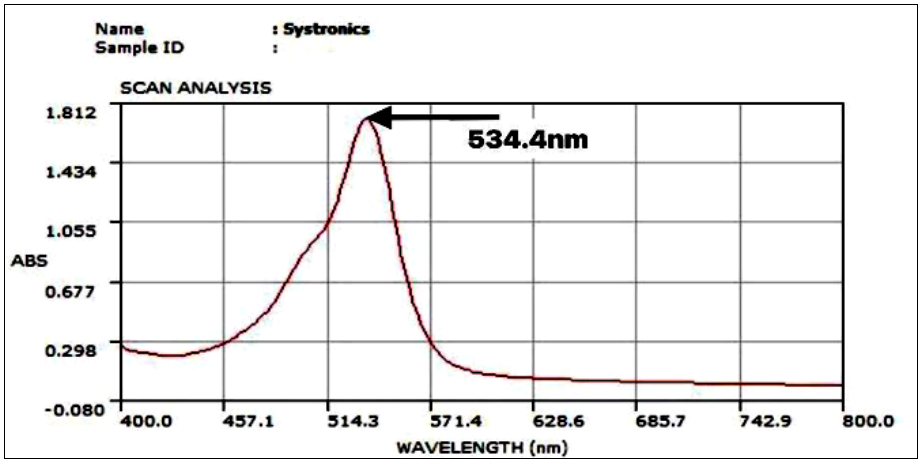

Fig: 3.2.2 Spectrum scan of red pigment extracted from *Serratia rubidaea* KAP

observed across visible wavelengths between 400-700 nm, by recording its absorption spectrum. λmax for the red pigment was 534.4 nm (Figs. 3.3.2), as in other studies reported by Dalili *et al*. (2012), Lapenda*et al*. (2015), Andreyeva *et al*. (2015), Veni *et al*. (2009), and Gerber *et al*. (1976) [9, 20, 47, 48].

4.3.3 Thin Layer Chromatography

Thin layer chromatography (TLC) is a separation method especially well-suited for the separating of microbial secondary metabolites, as the method is simple, accurate, and of high resolution [49]. In this study, the TLC analysis of the ethanolic extract of the red pigment produced by *Serratia rubidaea* KAP was undertaken. Fractionation of the red pigment was carried out with a solvent system consisting of n-butanol and hexane in a 2:1 ratio. A distinct pink spot was observed after purification at an RF value of 0.79 (Figure 3.2.3). The RF value obtained was similar to those reported for prodigiosin in earlier studies by Srimathi *et al*. (2017), Elkenawy *et al*. (2017) and Song *et al*. (2000). [8, 50, 51].

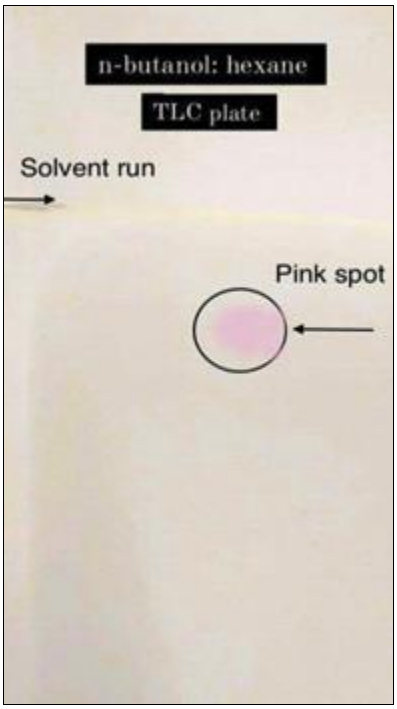

Fig 3.2.3: Thin Layer Chromatography Separation of red pigment.

4.3.4 Fourier Transform Infrared Spectroscopy Analysis

Fourier transform infrared (FTIR) spectroscopy has become a widely used analytical method that measures the absorption of infrared light by a sample, yielding much information on the molecular composition and architecture of the sample. FTIR spectroscopy of the extracted pigment was performed and analyzed

The FTIR analysis in multiple peaks of different intensity was studied. Strong absorption in this region was observed at 796.6 cm^{-1} (48.142) and 889.18 cm^{-1} (42.018) and peaks.

Extra peaks were found at 1060.85 cm^{-1} (32.978), 1097.5 cm^{-1} (34.405), 1421.54 cm^{-1} (35.371), and 1454.33 cm^{-1} (34.277). The absorption at 1653 cm^{-1} (29.951) and 1674.21 cm^{-1} (28.984) were moderate, though the absolute strongest peak appeared at 1921.1 cm^{-1} (80.672). The other prominent peak came at 2133.27 cm^{-1} (61.914). Further peaks were found at 20.529 (2902.87cm−1), 17.757 (2985.81cm−1), 18.484 (3321.42cm−1), 12.604 (3477.66cm−1) and 11.706 (3662.82cm−1). These Peaks serve to denote the varied molecular bonds, and functional groups, which comprise the sample.

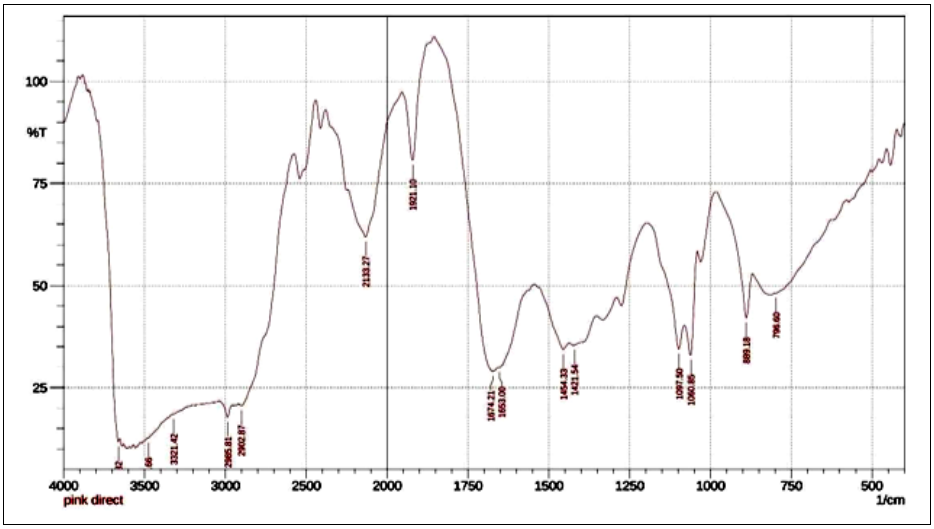

Fig 3.2.4: FTIR analysis peak report

The FTIR peak observed at 2985.81 cm^{-1} is due to stretching of the methylene group(s) (-CH), in the case of aromatic compounds is the sp^3 CH stretch (as in the structure of prodigiosin). The FTIR spectrum of the red pigment in KBr showed strong absorption bands at 1921.1 cm^{-1} and 2133.2 cm^{-1}, which dominate the FTIR spectrum. The peaks at 3477.66 cm^{-1} and 3321.42 cm^{-1} evidence for N - H stretching, the peak at 1653 cm^{-1} associated C=C stretching and C - N stretching. The CH bending is associated with absorptions at 1454.33 cm^{-1} and 1421.54 cm^{-1}. Further, the peaks at 1060.85 cm^{-1} and 1097.5 cm^{-1} are assigned for C-O stretching, and 796.6 cm^{-1} and 889.18 cm^{-1} for C-H bending. The chemical structure identified and the characteristic peaks of prodigiosin at 2985.81 cm^{-1}, 1653, 3477.66 cm^{-1}, coincide with that reported in Namazkar *et al*. (2013), Faraag *et al*. (2017), Song *et al*. (2006) Sumathi *et al*. (2014), and Jimtha *et al*. (2021) [52-56].

4.3.5 HPLC analysis of red pigment from *Serratia rubidaea* KAP

High performance liquid chromatography (HPLC) is an analytical technique used to separate, recognise and quantify components in a mixture. HPLC analysis of the pigment extracted from S*erratia rubidaea KAP* produced multiple peaks in its chromatographic profile (Figure 5.6.5). Retention times of 3.069 and 4.595 were assigned to the solvent used in the pigment extraction. The HPLC profile was compared to a standard prodigiosin profile published in the literature to verify purity of the pigment. According to Jeong *et al*. (2021), [57] the observed retention time for the degradation of prodigiosin from *Serratia marcsescens* was 2.734 minutes.

In this study there was a characteristic peak at a R.T. of 2.128 confirming prodigiosin presence corresponding to the findings of Gohil *et al*. (2020) and Jeong *et al*. (2021) [57, 58]. Earlier, Song *et al*. (2006) [51]. Have previously reported a single peak with 95% purity of prodigiosin produced by *Serratia sp*. KH-95. Although, total yield during purification was relatively low. Previous investigation has suggested that instability and decomposition of prodigiosin molecules as well as a role for light exposure in interaction of prodigiosin molecules with other cellular components leads to multiple peaks on the HPLC profile [59, 60]. We propose these factors could be responsible for the complexity observed in the current HPLC analysis.

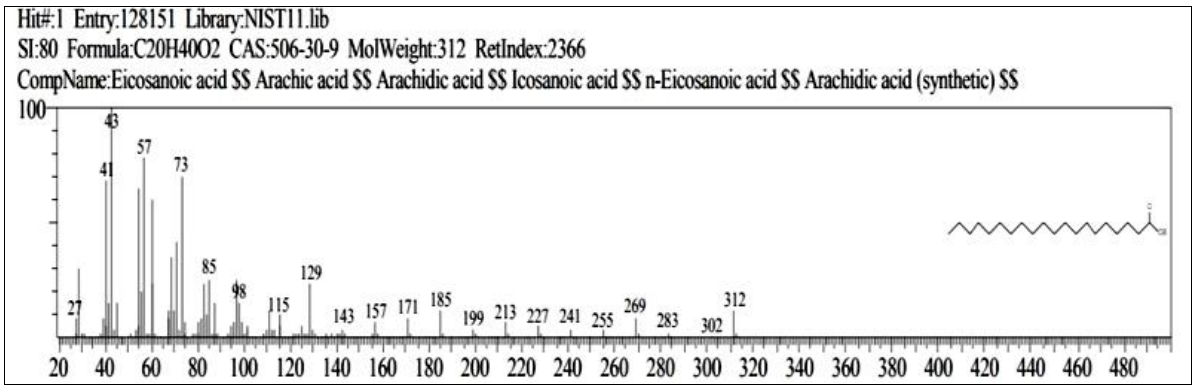

Fig 3.2.5: HPLC profile of prodigiosin pigment from *Serratia rubidaea* KAP.

4.3.6 GC-MS analysis

Gas chromatography-mass spectrometry (GCMS) is a comprehensive technique employed in identifying and quantifying many chemical components in the sample. Figure 5.6.6 shows the GC MS peak report. The peak at a molecular weight of 312, very close to the value reported in the literature, [61] authenticated it. Based on the GC-MS profile of the pigment, we posit that *Serratia rubidaea* KAP produces a variant of the prodigiosin pigment, that correlates closely with previously reported data.

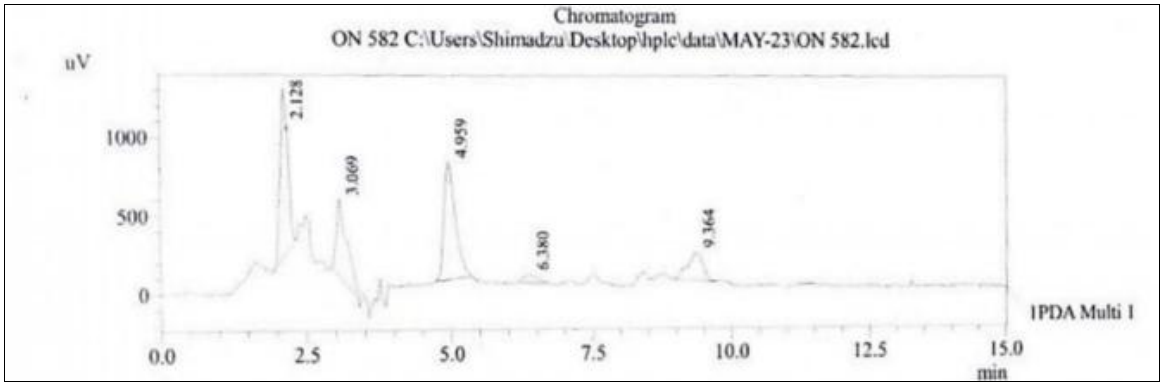

Fig 3.2.6: GC-MS profile of pigment

4.4 Application of Prodigiosin pigment extracted from *Serratia rubidaea* KAP

4.4.1 Antimicrobial activity of pigment

Although the physiological role of the pigment is not entirely known, it has been shown to possess antibacterial properties [11]. This experiment was performed as agar well diffusion assay with several Gram-positive and Gram-negative bacteria including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus aureus 6538p, Streptococcus pyogenes, and Bacillus subtilis*. Furthermore, fungal cultures of *Candida albicans* and *Saccharomyces cerevisiae* were tested because these microorganisms are often implicated in nosocomial infections. Table 3.3.1 gives the zones of inhibition in millimeters caused by the pigment extract.

The highest zone of inhibition was found against *Streptococcus pyogenes* where the average diameter was 37.3 mm, then *Staphylococcus aureus* 31.6 mm and then *Staphylococcus aureus* 6538p 25 mm. Inhibition against *Bacillus subtilis* (15 mm) was obtained. Red pigment extraction from *Serratia rubidaea* KAP exhibited strong antibacterial activity against gram-positive bacteria but not against gram-negative bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Chromobacterium violaceum*.

As per Suryawanshi *et al*. (2014), [63] microbial pigments, including prodigiosin, are more effective against Gram-positive than Gram-negative bacteria. Prodigiosin is antibacterial because it is able to penetrate the outer membrane and inhibit DNA gyrase and topoisomerase IV. It is a mechanism that produces reactive oxygen species (ROS) which damage important cellular components and inhibit bacterial growth [63]. In this case, however, the lipopolysaccharide (LPS) shell of Gram-negative bacteria prevents prodigiosin from entering the cell and thereby making it ineffective [64]. Results from this study confirm that *Serratia rubidaea* KAP prodigiosin is a potent antibacterial agent selective for Gram-positive bacteria. These finding agrees with what was reported by Li *et al*. (2018), Araújo *et al*. (2022), Hazarika *et al*. (2022), Mnif *et al*. [64-68]

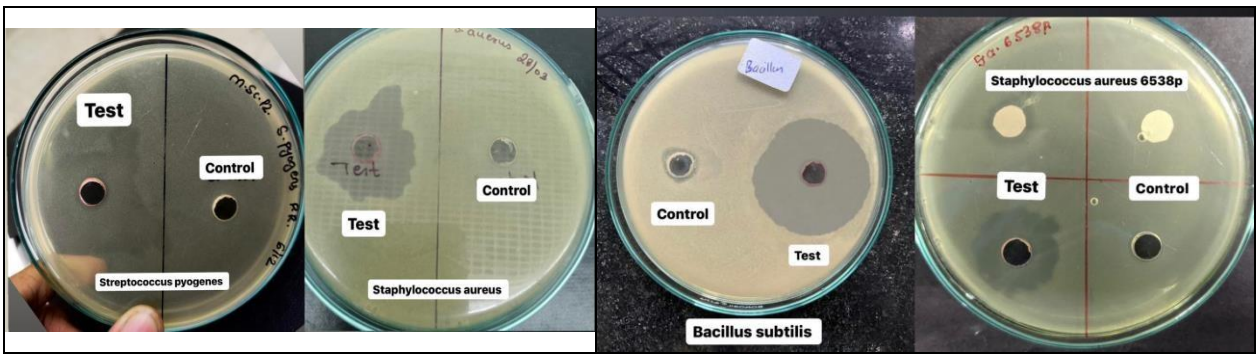

Fig 3.3.1: Antimicrobial activity of prodigiosin against Gram-positive organisms

Table 3.3.1: Zone of inhibition values exhibited by the pigment produced by *Serratia rubidaea* KAP against test organisms.

Sr. no.	Test organism	Zone of Inhibition (mm)
1.	*Escherichia coli*	No zone of Inhibition
2.	*Pseudomonas aeruginosa*	No zone of Inhibition
3.	*Chromobacterium violaceum*	No zone of Inhibition
4.	*Klebsiella pneumonia*	No zone of Inhibition
5.	*Staphylococcus aureus*	31.6mm

6.	*Staphylococcus aureus 6538p*	25 mm
7.	*Streptococcus pyogenes*	37.3mm
8.	*Bacillus subtills*	30mm
9.	*Saccharomyces cerevisiae*	No zone of Inhibition
10.	*Candida albicans*	No zone of Inhibition

4.4.2 Antioxidant activity of prodigiosin pigment estimated using 1, 1-diphenyl- picrylhydrazyl (DPPH) method.

The extracted pigment was evaluated for antioxidant activity using the DPPH method. In DPPH, absolute maximum absorbance is at 517 nm. The absorbance is reduced when DPPH radicals interact with a substance that donates a proton (namely, an antioxidant to neutralize the radicals) Scalzo. The effectiveness of the radical scavenging activity is measured by this reduction in absorbance. The pigment was analyzed qualitatively and quantitatively, in terms of its DPPH radical scavenging activity.

4.4.2.1 Qualitative Assay

Methanol was used as the blank and a methanolic extract of the pigment was added to a tube containing the DPPH solution. In the dark they were wrapped in aluminum foil and left to incubate for 30 minutes. Incubation was performed with the absorbance measured at 517 nm before and after. A decrease in absorbance during incubation indicated a positive qualitative result suggesting a DPPH free radical scavenging activity (Figure 5.7.2.a).

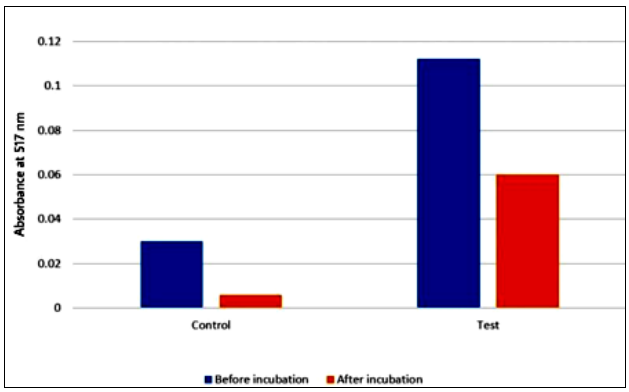

Fig 3.3.2.1: Qualitative Assay

4.4.2.2. Quantitative Assay

The extracted pigment was assessed quantitatively in terms of its antioxidant activity with the DPPH method. The methanolic extract of the red pigment was mixed with a solution of DPPH in equal volume, a 0.1 mM solution. The absorbance was recorded at a wavelength of 517 nm after incubating for 30 minutes and compared with a Vitamin C standard. A standard concentration range of 10 µg to 20 µg, with 2 µg intervals was prepared to evaluate the pigment's antioxidant activity compared to vitamin C. DPPH activity inhibition percentage was calculated by the following formula,

$$\%\ \text{inhibition of DPPH activity} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}) \times 100}{\text{Abs}_{\text{control}}}$$

Table 3.3.2.2: Quantitative Assay

The standard concentration of Vitamin C(ug/ml)	Absorbance at 517nm before incubation	Absorbance at 517nmafter 30min
10	1.919	1.814
12	2.761	1.651
14	2.091	1.639
16	2.958	1.568
18	3.059	1.511
20	3.059	1.504
Control (DPPH Solution)	-	2.941
Test (pigment extract)	0.515	0.114
Blank (Methanol)	-	-

Table 3.3.2.2: Percentage inhibition of DPPH activity of Standard and Test

Standard Vitamin C concentration (ug/ml)	Scavenging activity percentage (%)
10	38.32
12	43.86
14	44.27
16	46.68
18	48.62
20	48.86
Test	96.12

The DPPH free radical scavenging activity was proven through the incidence of a notable reduction in absorbance following incubation (Figure 5.7.2c). Prodigiosin scavenging was measured to be 96.12% (Figure 5.7.2). The extracted pigment had greater antioxidant activity than that of standard ascorbic acid, which implies that this result. The present findings are concordant with Arivizhivendhan *et al*. 2018, Gulani *et al*. 2012, Vora *et al*. 2014, Renukadevi *et al*. 2017 [23, 69-71].

4.4.3Application of prodigiosin as Bio colorant

4.4.3.1 Textile dyeing

The dyeing potential of the prodigiosin pigment from *Serratia rubidaea* KAP was evaluated on four types of textile fabrics: These are silk, cotton, nylon and polyester. The results were similar to Alihosseini *et al*. (2008) [72] in that a light shade was dyed onto cotton fabric (Figure 5.7.3.1). However, contrary to what we observed with these natural fabrics, polyester, silk and nylon delivered a pink shade with nylon being the most dyeable (Figure 5.7.3.1) similar to what Shete *et al*., 2021 [73] report. The cationic dye nature of the prodigiosin molecule has been shown to relate to its ability to function as dye. Under acidic conditions the cationic dyes exhibit strong bonding properties by forming salt bonds within the fibers which help in efficient dyeing of fabrics like silk, nylon and polyester [65]. The hue of the dyed fabric was influenced by the pigment concentration in the broth and dependence of the dyeing process on the kind of fibers in the material [65]

For both light and washing resistance, the color fastness properties of dyed fabrics were evaluated. Prodigiosin degrades quickly when exposed to sunlight (Figure 3.3.3.1.a), indicating that the energy supplied by ultraviolet light is sufficient to break prodigiosin apart, while natural light does not break prodigiosin down very much. Testing of the dyed fabrics for washing fastness using Surf Excel shemes revealed that prodigiosin color retention is high. These findings are consistent with a previous study by Shalinimol, C. R. (2019), Venil *et al*. (2019), Chauhan *et al*. (2015) and Gong *et al*. (2017) [74-77]. Despite the potential for prodigiosin in textile dyeing, more work is needed to maximize dyeing performance and the scale up of the production, for the commercial use of prodigiosin in different sectors [78].

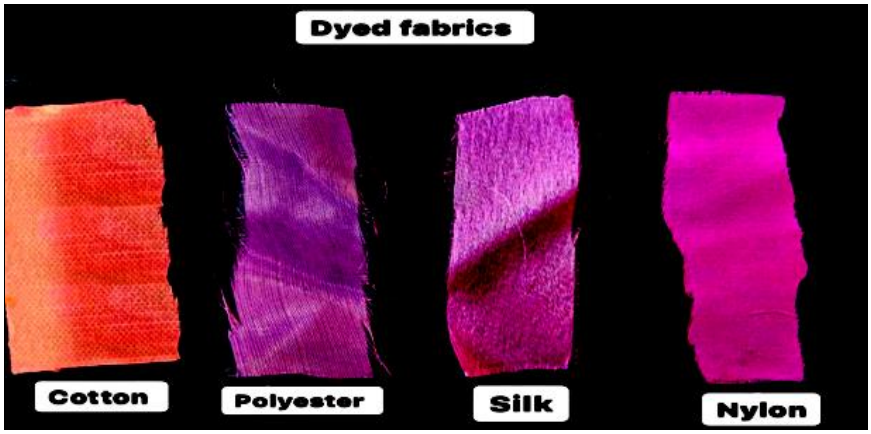

Fig 3.3.3.1 a: Fabrics dyed with *Serratia rubidaea* KAP prodigiosin

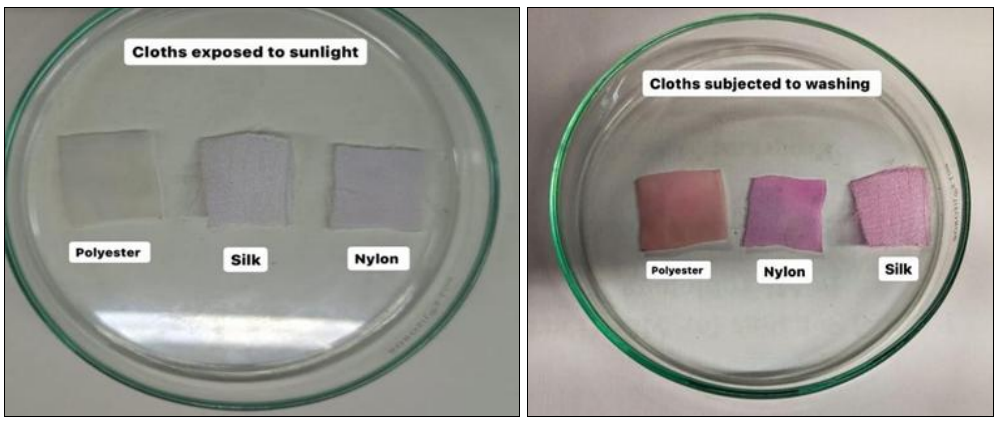

Fig 3.3.3.1: Color fastness property w.r.t to light and washing**.**

Fig 3.3.3.1.c: Thermal stability of dyed cloths.

4.4.3.2 Plastic dyeing

The ability of the pigment from *Serratia rubidaea* KAP to dye plastic centrifuge tubes was also tested. They left the extracted pigment in suspension in the tubes for 24 hours, during which the plastic surfaces were successfully dyed with the extracted pigment (Figure 3.3.3.2). To check the fastness property, the dyed tubes were thoroughly washed up with detergent. However, the tubes still kept their colour even after being washed with extreme rigor. Shalinimol, C. R. (2019), Siva *et al*. (2012) [74, 79] have previously shown successful staining of plastics with prodigiosin. In addition, Mandal *et al*. (2017) [80] showed strong binding capabilities of the red pigment extracted from *Serratia marcescens* strains to plastic materials.

Fig 3.3.3.2: Plastic centrifuged tubes dyed with pigment extracted from *Serratia rubidaea* KAP

4.4.3.3 Dyeing of Paper

Serratia species produces the red pigment prodigiosin, which has several applications, including the paper dyestuff industry. Pigment extracted from *Serratia rubidaea* KAP showed effective dyeing capability on paper in this study. Likewise, Krishna *et al*. (2013) [36] dyed paper and polymethyl methacrylate sheets using a marine strain, Serratia sp. BTWJ8. The authors also emphasized the possibility of using prodigiosin as an indicator in pH indicator strips on dyed paper. Shaikh, Z. (2016) and Stankovic *et al*. (2014) [38, 81] record additional research on using prodigiosin dyed paper as a pH indicator.

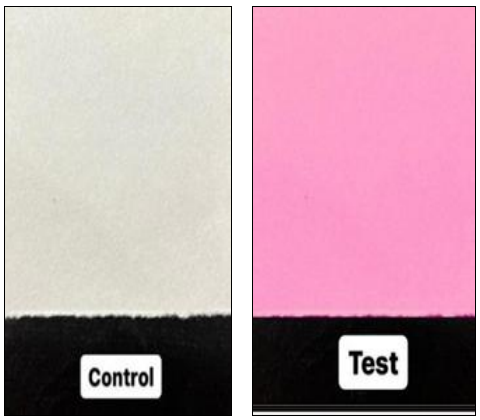

Fig 3.3.3.3: Dyeing of paper with pigment extracted from *Serratia rubidaea*KAP

3.3.3.4 Dyeing Candles

Candles can be made more appealing and environmentally friendly by incorporating natural ingredients during production. One such ingredient is natural dyes derived from microbial sources. Prodigiosin is a well-known pigment recognized for its ability to produce various shades of red. It has been widely studied as a coloring agent for candles, paper, plastics, and textiles [82]. In this study, prodigiosin extracted from *Serratia rubidaea* KAP was tested for its effectiveness in coloring wax candles. The results showed that the pigment successfully dyed the candles (Figure 5.7.3.4). However, when the dyed candles were evaluated for color fastness under exposure to light, it was observed that the color faded completely within two days, indicating poor light fastness of the prodigiosin pigment.

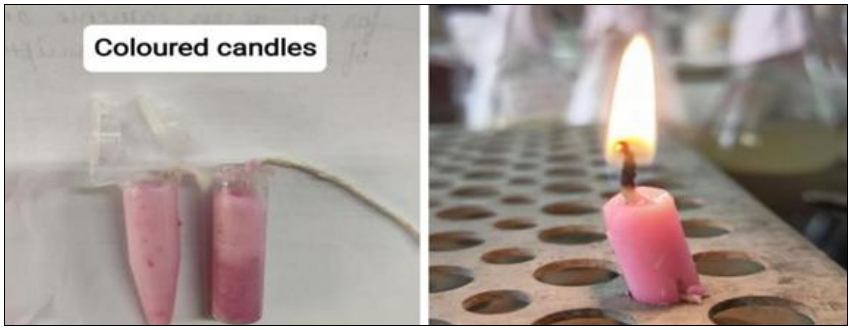

Fig 3.3.3.4: Dyed candles with pigment extracted from *Serratia rubidaea* KAP

3.3.3.5 Prodigiosin as a colorant in paints:

Additionally, studies of prodigiosin's potent antibacterial properties have led to studies regarding its use as a dye as a paint formulation to combat biofouling [83]. The extracted prodigiosin was mixed with commercially available distemper until the hue became so noticeable that a pink color could be seen. When applied to a wall, the resulting bio-paint showed the pigment dissolved well in the distemper and created a pinkish color (Figure 3.3.3.5a). Later the paint's color fastness under different lighting conditions was assessed. The pigment had poor light fastness and changed color when exposed to sunlight and retained its color in the presence of indoor lighting (Figure 3.3.3.5 b). The observation of this study is consistent with earlier work by [84] reporting on the light sensitivity of the prodigiosin pigment extracted from *Serratia marcescens* strain B2.

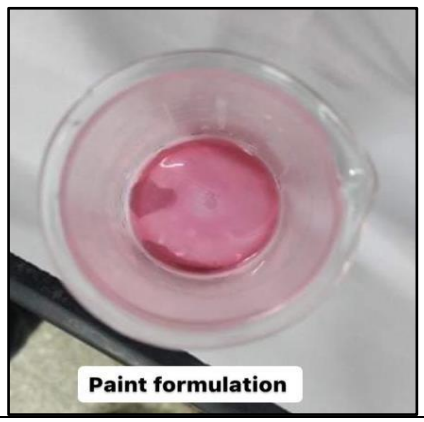

Fig 3.3.3.5 a): Pigment mixed with Distemper

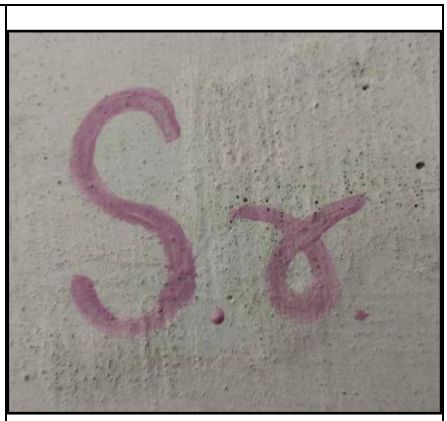

Fig 3.3.3.5 b): Pigment applied on an indoor wall

Fig 3.3.3.5 c): Paint applied on the outdoor wall and exposed to sunlight.

Summary and Conclusion

This present work focused on the red pigment "prodigiosin," produced by *Serratia rubidaea* KAP, a bacterium isolated from oil-contaminated soil. The bacteria were grown on nutrient agar with glycerol, and ethanol was found to be the most efficient solvent for extracting the prodigiosin pigment. Furthermore, prodigiosin was identified using multiple characterization methods, including UV-Visible spectrophotometry, Thin-Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), High-Performance Liquid Chromatography (HPLC), and Gas Chromatography-Mass Spectrometry (GC-MS). These techniques confirmed the presence of prodigiosin, with TLC showing a retention factor (Rf) of 0.79 and HPLC displaying a peak at a retention time of 2.128.

The antimicrobial properties of the pigment demonstrated its capacity to inhibit Gram-positive bacteria including *Streptococcus pyogenes* yet it was ineffective against both Gram-negative bacteria and fungal species. The pigment demonstrated a substantial antioxidant potential because it scavenged 96% of the DPPH in the analysis exceeding the antioxidant action of vitamin C.

The study further explored the potential of prodigiosin as a natural dye for textiles. Interestingly, nylon fabric showed the best color uptake, followed by polyester and silk. The dyed fabrics were resistant to sunlight but had lower wash fastness. The evaluation of prodigiosin as a component in biopaints revealed poor light-fastness properties of the painted surface.

In conclusion, ethanol was identified as the best solvent for prodigiosin extraction, with optimal production achieved in nutrient broth at pH 8.0, 28°C, under static conditions after 120 hours of incubation. This study successfully demonstrated the potential of prodigiosin for applications in antimicrobial, antioxidant, and dyeing industries.

Conflict of Interest

Not available

Financial Support

Not available

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