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Production and Characterization of Biologically Important Red Pigment (PRODIGIOSIN) Produced by *Serratia marcescens.*

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ABSTRACT
The study was aimed to formulate and evaluate cream from prodigiosin extract from *Serratia marcescens.* Gram staining technique revealed the bacterium's cell wall structure. By analysing the behaviour of *S. marcescens* in a hanging drop preparation, determined it's motility and presence and type of flagellar it possesses. Endospore staining illuminated the presence or absence of endospores within the bacterial population. From Endospore staining concluded that *S. marcescens* does not produce endospores. Biochemical tests such as:- Catalse, Urease, Citrate, Nitrate reduction, MR-VP (Methyl Red–Voges Proskauer), Indole, etc.revealed key metabolic pathways and enzymatic activities. The oxidative-fermentative test provided insights into its metabolic versatility. Antibiotic susceptibility testing (AST) elucidated its response to antibiotic. Furthermore, fermentation media preparation and growth optimization growth optimization techniques were explored to enhance culture conditions at different temperature, pH and carbon source. TLC analysis showed a single band with an Rf value of 0.62. Prodigiosin has a characteristic absorption peak in the visible region of spectrum typically around 500-550nm. Moreover, a cream formulation was developed, incorporating *S. marcescens* pigments called Prodigiosin with subsequent antibiotic susceptibility testing to evaluate its potential therapeutic efficacy. After that evaluation of cream was done. pH of formulated cream was 6, no skin irritation, homogeneity was good and AST test of cream showed zone of inhibition against *S.aureus.* This multidisciplinary approach offers a comprehensive understanding of *S. marcescens* and it provides valuable insights for clinical and pharmaceutical applications.

Keywords: *Serratia marcescens,* Gram's staining, hanging drop motility, Endospore staining, biochemical analysis, Antibiotic Susceptibility test, Fermentation media, Growth optimisation., Cream Formulation

1. INTRODUCTION

Microorganisms are known as a potential source for bio-pigment production due to their advantages over plants in terms of availability; stability; cost efficiency; labor; yield and easy downstream processing. Varieties of bio pigments have been produced by microorganisms such as carotenoids, melanins, flavins, quinines, monascins, violancein using microorganisms. Cultivation of microorganisms can be attained through solid state and submerged fermentation on natural raw material / industrial organic waste. Many of the microbial pigments not only act as coloring agents in various food processing and cosmetics industry but also possess anticancer, antioxidant, anti-inflammatory, antibacterial activities There is huge demand for coloring agents in industries like textile, plastic, paint, paper and printing (Tuli et al. 2015).

In addition to fulfilling function of giving color, many natural pigments produced by plants and microorganisms are known as interesting bioactive compounds with potential health benefits. These compounds have variety of applications. In recent times, in the food industry, there has been a spread of natural pigment application in many fields, such as biocosmatics, pharmacology and toxicology, in the textile and printing industry and in the dairy and fish industry, with almost all major natural pigment classes being used in at least one sector of the food industry. Obtaining easily usable, non-toxic, ecofriendly, sustainable, cheap and biodegradable pigments represents the future in which researchers should invest (Salvo et al. 2023).

Among bacteria, pigments production are highly variable, although usually present in Actinobacteria. Several genera, such as Streptomyces, Nocardia, Thermomonospora, Microbispora, Streptosporangium, and Rhodococcus, produce a wide variety of pigments. While the main application of these pigments is in the biocosmatics, textile, and food, pigments like melanin, quinones, , and indigoidines have been reported as good antimicrobial agents. Besides, pigments can be used as antioxidants, bioindicators, and anticancer agents, immunosuppression. Thus, their potential is becoming an most important field of biomedical application (Celedon and Diaz 2021).

Serratia marcescens is a gram-negative,non-motile, non-endospore forming, facultatively-anaerobic bacterium and opportunistic pathogen which produces the red pigment called Prodigiosin (Haddix and Shanks 2018)..

Other members of this genus includes *S. rubidaea* and *S. liquefaciens,* which have also been reported to cause hospital-acquired infections, albeit less frequently (Williams et al. 2022).

Prodigiosin (PG) are a group of hydrophobic red tripyrrole pigments produced by Serratia spp., actinomycetes, and some marine bacteria. The most known pigment of the class is prodigiosin, produced mainly by *Serratia marcescens*. Prodiginines can have a straight chain, such as prodigiosin and undecyl prodigiosin, or can bear a cyclic structure, like cyclononylprodigiosin and butyl-meta-cycloheptylprodiginine (Barreto et al. 2023)

Prodigiosin can be assigned to the group of bioactive colored molecules developed by microbial fermentation. Prodigiosin, a red pigment mainly produced by *Serratia marcescens* strains and other microorganisms, that shows many promising potential therapeutic activities. Prodigiosin displays antimicrobial, antiparasitic, insecticidal, and immunomodulatory activities. Therefore, natural pigments like Prodigiosin seem to be an attractive bioactive alternative and have been the subject of intensive research during the last decade (Islan et al. 2022).

The pigment was confirmed by UV-Vis spectrometer, thin layer chromatography (TLC) Absorbance reading was noted at 530 nm. Higher absorbance reading meaning higher pigment extraction (Bhagwat and Padalia 2020).

Prodigiosin was inactive against *Pseudomonas aeruginosa.* No activity against *Escherichia coli, Proteus vulgaris, Candida albicans, Trichoderma koningi or Penicillium notatum.* Pigments from *Serratia marcescens* and other microorganisms can serve as an alternative source to replace synthetic pigments used in the food industry, with few limitations including sensitivity, solubility, and short stability upon exposure to pH, light and high temperatures. The requirements of natural pigments used for polymer coloration, namely, wide acceptable temperature range, lightness stability, fine dispersion in the carrying material, a good migratory stability are challenging and result in high cost of organic pigments. (Darshan and Manonmani 2015).

2. MATERIALS AND METHODS

2.1 Sample collection: -

The Soil sample was collected from the Noida sector 12 at 12:15 pm on 6 January. These samples were taken in a sterile conical flask.

2.2 Isolation of Red pigment producing bacteria from soil sample:

Soil sample was collected from the Noida sector 12 and then serial dilution on 10^1 was prepared using normal saline, spread on Nutrient Agar Media and incubate it for 24 hours.

2.3 Broth inoculation

Inoculation loops or a inoculation rod are used by microbiologists to mainly transfer inoculum (some amount of sample of microbe culture). The apparatus comprises a fine handle with a tiny loop towards its terminal.

Inoculation needles are lab tools used for transferring inoculate living microbes. Inoculation needles are used to retrieve solid media, an inoculation loop can retrieve liquid media.

Basically, the nutrient broth is the nutrient agar that lack agar powder. They remain in liquid form at room temperature. Nutrient broth are usually used to maintain the stocks of microorganisms. In general, they are used to grow fastidious organisms.

2.4 Morphological Staining

2.4..1 Gram's Staining:

Gram staining involves three processes, staining with a water-soluble dye called crystal violet, decolourization, and counterstaining, usually with safranin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram-positive and Gram-negative bacteria, Gram-positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolourization process, while Gram-negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process.

2.4.2 Hanging drop Motility:

Some bacteria are motile and some are non-motile. Generally Motile bacteria use flagella as their locomotory organ. Bacteria tend to move towards or away from the various chemotactic, phototactic, aerotactic or magnetotactic stimuli. There are several ways to demonstrate the motility of bacteria. These include, a simple wet mount, hanging drop preparation, or employment of soft gels.

2.4.3 Endospore staining

The main purpose of endospore staining is to differentiate bacterial spores from other vegetative cells to differentiate spore formers from non-spore formers. A primary stain-malachite green is forced into the spore by steaming the bacterial emulsion. Malachite green is water soluble in nature and has a low affinity for cellular material, so vegetative cells may be decolorized with water. Safranin is then applied to counterstain any cells which have been decolorized. At the end of the staining process, vegetative cells will be pink or brownish-red and endospores will be dark green.

2.5 Screening of Red pigment producing bacteria by biochemical testing:

A series of biochemical tests were performed to confirm the bacteria was *Serratia marcescens*. Catalase, Urease, citrate, Nitrates reduction, Lipase, D- Mannitol test, Hydrogen sulphide test, MRVP, Carbohydrate fermentation test:- Such as Sucrose, Dextrose, Glycerol, Lactose, Maltose, Sorbitol tests were performed.

2.5.1 Carbohydrate fermentation test

The bacteria has ability to form organic compounds by metabolizing certain carbohydrates and related compounds is a widely used method for the identification of microorganisms. Different fermentation media are used to differentiate organisms based on their ability to ferment different carbohydrates incorporated into the basal medium.

Carbohydrate fermentation is the process through which microorganisms use different carbohydrate sources to produce energy. Most microorganisms convert glucose into pyruvate during glycolysis process; however, some organisms use alternate pathways. A fermentation medium consists of a basal medium containing a single carbohydrate (Dextrose, lactose, sucrose, mannitol, sorbitol, glycerol,etc.) for fermentation. D-Glucose is an excellent carbon source to promote growth of *S. marcescens*; however, as early as 1949, d-glucose was reported as a potent inhibitor of prodigiosin production. Because other useful secondary metabolites are inhibited by glucose, it would be advantageous to engineer strains for which glucose could support both growth and generation of secondary metabolites (Fender et al. 2012). Extracellular D-glucose oxidation by five enterobacterial species was studied with the purpose of selecting conditions useful for taxonomic studies. *Serratia marcescens, Yersinia frederiksenii, Erwinia cypripedii and Cedecea lapagei* oxidized D-glucose without added PQQ (Bouvet and Grimont 1988).

2.6 Oxidative Fermentative test of *Serratia marcescens.*

This test is used to determine if gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are non-sacchrolytic and therefore have no ability to use the carbohydrate in the media.

Bacteria can metabolize sugar and produce acid either via oxidation (aerobically) or via fermentation (anaerobic) or both. The anaerobic fermentation process results in the production of a mixture of different organic acids. This causes a higher concentration of acid in the medium decreasing pH of the medium. This decrease in pH causes the bromothymol blue indicator of the medium to change color from green to yellow/orange.

During aerobic respiration small amount of weak organic acid is produced during glycolysis and the Krebs cycle. There results in a decreased amount of peptone and an increased amount of glucose in the media. This weak acid in the presence of a higher glucose and lower amount of peptone can be detected by the bromothymol blue indicator in the medium. The addition of a dipotassium phosphate buffer further increases for the detection of this weak and small amount of acid developing yellow color in the media.

Table 1. Composition of Oxidative-Fermentative

Ingredients	Quantity (Standard)
Peptone	2g
NaCl	5g
Dipotassium hydrogen phosphate	0.3g
Agar	20g
Bromothymol blue	Few drops
Glycerol	10ml
Carbohydrates (Glucose, Maltose, Lactose)	10g
Distilled water	1000ml

pH should be 7.1

2.7 Preparation of Fermentation media

Fermentation media is the preparations that support the growth of microorganisms used in a fermentation process. The fermentation media used for the operation of fermentation processes in the industry are usually in the liquid state or solid state. Thus, liquid media (broth) and solid media are basically the major types of media utilized in most industrial fermentation processes. Liquid media are mostly used in fermentation processes because they require less space compared to solid media; and they are cheaper to work with – since no additional cost of procuring agar or solid agents will be accrued.

Table 2. Composition of Fermentation media

Ingredients	Standard	For 50ml
Peptone	5g	0.25g
NaCl	3g	0.15g
Yeast Extract	5g	0.25g
Distilled water	1000g	50ml

pH should be 7

2.8 Growth optimization of Prodigiosin pigment

The Optimization of bacterial growing medium composition, including the carbon and nitrogen source in different concentrations, the pH value of the medium and the temperature.

2.8.1 Procedure of temperature optimization

- Take five test tube, out of five test tube one was considered as blank
- Prepared NAM and add 5ml NB in each test tube.
- Autoclaved at 121°C at 15psi pressure for 30 min • After autoclaving, take a red hot inoculating loop and inoculate the broth with isolated colonies except blank under laminar air flow.
- Kept five test tube on five different places for 24 hours

1. First test tube (blank) kept in freeze
2. Second test tube (T1) kept in freeze at 5°C.
3. Third test tube (T2) kept at room temperature at 25°C.
4. Fourth test tube (T3) kept in incubator at 5°C.
5. Fifth test tube (T4) kept in autoclave.

- After incubation take the OD of each test tube. Here, blank was used to set the absorbance at zero. For the following sample measurement the absorbance change compared to the blank measurement is determined.

2.8.2 Procedure for pH optimisation

- Take five test tube, out of five test tube one was considered as blank
- Prepared NA media and add 5ml NB in each test tube
- Now maintain different pH in each test tube.

1. First test tube (blank), pH 7.
2. Second test tube (T1), pH 3.
3. Third test tube (T2), pH 5.
4. Fourth test tube (T3), pH 7.
5. Fifth test tube (T4), pH 9.

- Autoclaved at 121°C at 15psi pressure for 30 min.

- After autoclaving, take a red hot inoculating loop and inoculate the broth with isolated colonies except blank under laminar air flow.
- Kept blank test tube in freeze, and rest four test tube kept where result of OD was maximum in temperature optimisation for 24 hours.
- After incubation take the OD of each test tube. Here, blank was used to set the absorbance at zero. For the following sample measurement the absorbance change compared to the blank measurement is determined.

2.8.3 <u>Procedure for carbon source optimisation</u>

- Take five test tube, out of five test tube one was considered as blank
- Prepared NA media and add 5ml NB in each test tube
- Now add 1% different carbon sources in each test tube except blank.

1. First test tube (blank), no carbon source.
2. In Second test tube (T1), 1% (0.05g) lactose added.
3. In Third test tube (T2), 1% (0.05g) dextrose added.
4. In Fourth test tube (T3), 1% (0.05g) maltose added..
5. Fifth test tube (T4), 1% (0.05g) sucrose added.

- Autoclaved at 121°C at 15psi pressure for 30 min.
- After autoclaving, take a red hot inoculating loop and inoculate the broth with isolated colonies except blank under laminar air flow.
- Kept blank test tube in freeze, and rest four test tube kept where result of OD was maximum in temperature optimisation for 24 hours.
- After incubation take the OD of each test tube. Here, blank was used to set the absorbance at zero. For the following sample measurement the absorbance change compared to the blank measurement is determined.

2.9 Extraction of Red pigment from Fermentation media.

Prodigiosin is a secondary metabolite widely produced by several species including Serratia marcescens, Vibrio psychroerythrous, and other such bacteria. This multifaceted secondary metabolite is a dark red pigment with a common 4-methoxy-2,2 bipyrrole ring system and has a series of close relatives bearing the same structure with different alkyl substituents. Prodigiosin (red pigment) is known to have immunosuppressive, anti-fungal, anti-viral, anti- microbial, anti-malarial, and anti-proliferative properties. It also induces apoptosis in cancer cells (Islan et al. 2022).

<u>Procedure of extraction of Prodigiosin</u>

- •Take 8 sterile centrifuge tube and add 2ml fermented media into each tube.
- Centrifuge at 8000rpm for 10min.
- After centrifuge discard the supernatant and add lysis buffer in centrifuge tube containing pellet and vortex it.
- Again centrifuge at 8000rpm for 10min.
- After centrifuge discard the supernatant in test tube and add ethyl acetate to the test tube.
- Observe for the separation of Prodigiosin along with ethyl acetate on the top of the test tube and water remains at the bottom of the tube.

2.10 UV-Vis Scanning

Different pigments vary in the wavelength of light that they absorb. The quantitative determination of the red pigment prodigiosin was done by measuring the absorbance at 530 nm using double beam UV-Visible spectrophotometer.

Procedure of UV-Vis Scanning

The pigments were observed in the UV-Vis spectroscopy from wavelength range of 500-540nm. The blank was kept as ethyl acetate .

2.11 Thin Layer Chromatography (TLC)

TLC is a technique used to isolate non-volatile mixtures. This experiment is conducted on a sheet of aluminium foil, plastic, or glass coated with a thin layer of adsorbent material such as aluminium oxide, cellulose, or silica gel.

After separation, each component appears as spots separated vertically.

Each and every spot has a retention factor (Rf) expressed as:-

Rf = dist. travelled by the compound/ dist. travelled by the solvent front.

TLC depends on the principle of separation. Compounds of mobile phase move over the stationary phase. The movement of compounds occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the compounds which have a less affinity to the stationary phase travel fast.

TLC analysis showed a single band with an Rf value of 0.62 (Metwally et al. 2017)

Procedure of TLC

Hexane and ethyl acetate was taken in the ratio of 3:1 the solvent was prepared, the TLC sheet was taken which is coated with silica gel and acts as the stationary phase. From the 1cm above the TLC, the sheet line is drawn and the samples are spotted on the sheet. The sheetis made to run for 30 mins and RF value are calculated by using the formula.

2.12 Antibiotic Susceptibility Test (AST)

AST is a fundamental mission of the clinical microbiology laboratory. AST provides an in vitro measure of response of bacteria to an antimicrobial agent that predicts therapeutic efficacy (Smith & Kirby 2019). . The red pigments produced by *S. marcescens* exhibited absorption at 534 nm, Rf of 0.59. Antimicrobial activity was tested against oxacillin-resistant Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes, Acinetobacter sp. and oxacillin-resistant S. aureus. The standard antibiotics employed were ampicillin, chloramphenicol, gentamicin and oxacillin. The disc-diffusion tests demonstrated significant inhibition zones for S. aureus (Lapenda et al. 2015).

Procedure of AST by disc-diffusion method

Prepared NA media , autoclaved at 121°C at 15psi for 30min..Affter autoclaving take a sterile Petri plates and poured the media under laminar air flow. Preparaed standardized inoculum from a bacterial culture. Nutrient agar plate was inoculated with standardized inoculum of test microorganisms . Now, take three filter paper discs , one was considered as negative control, second was considered as positive control and dipped in antibiotic and placed on the NA plate, and third one was sample (Prodigiosin) dissolved in DMSO and place on NA plate. Incubate the plate at 30°C for 24 hours. After incubation observed for zone of inhibition. Measuring the zone of inhibition. Interpretation of AST results.

2.13 Formulation of Cream

Cosmetics such as nail polish, lipstick, and others biocosmetics have attractive colors. Most industrial pigments are made from benzene, toluene, and many other chemical reagents. Due to this, many people find them dangerous and
unacceptable for skin. In contrast, natural colors are widely used in some products because they are safe and stable. Numerous investigations on microorganisms as sources of economical, stable, novel, and safe biological pigments have been reported (Kiki 2023).
Applications of bacterial pigments are more favoured and demanding than synthetic pigments because of a number of advantages. Bacterial pigments are simple to grow and safe for use by
humans. Furthermore, their extraction methods and scaling up processes are more economical and cheap as compared to synthetic pigments. Additionally, pigments are secondary metabolites created by a living creature that support the cell in numerous ways, including photosynthesis, UV protection, defence against competing species, and even energy-storing molecules. They are a good contender for the current pigment industry because of their ease of growing, resistance to temperature and pH changes, variety, and non-toxic/eco-friendly.
For instance, bacterial pigments, such as zeaxanthin, astaxanthin, yellow and orange carotenoids, prodigiosin, violacein, pyocyanin, and actinorhodin are the subject of intense research for their possible use in modern medicine (Agarwal et al. 2023).

Table 3. Formulation of Cream

Ingredients	Quantity	Role
Liquid Paraffin	1ml	Lubricating agent
Pectin	1.5g	Emulsifying agent
Prodigiosin (Pigment of *Serratia marcescens)*	10ml	For coloration
Tween 80	7-8 drops	allowing for the blending of water and oil-based ingredients
Distilled water	7ml	Vehicle

Procedure of Formulation of cream

The oil phase consisted of 1.5g of emulsifying pectin and 1 ml of liquid paraffin was added and transferred into a 100 ml beaker and they were allowed to melt at 60°C . Using a glass rod to mix well. The aqueous phase 7ml of water was added and transferred into a 100ml beaker constituting the aqueous phase. The aqueous phase was added to the oil phase gradually and kept at 50°C along with it 10ml of Prodigiosinn extract was added. Then 5 to 7 drops of Tween 80 were added and then the cream was cooled.

2.14valuation of Cream

1. **Physical Evaluation:-** Physical parameters of prepared gels were visually analysed for clarity, color and transparency.
2. **pH determination:-** pH of Formulation wd determined by using a pH meter.
3. **Stability study:-** The stability study was carried out by storing cream at different temperature are 4°C and 25°C.
4. **Homogeneity:-** The formulation was tested for homogeneity by visual appearance. and touch.

5. **After feel:-** Slipperiness and amount of residue left after the application of the fixed amount of cream was added.
6. **Smear:-** Smear was made on the skin.
7. **Removal:-** The ease of removal of the cream applied was examined by washing with tap water.
8. **Skin irritation test:-** Skin irritation safety testing for new products, and the ingredients they contain, is a critical requirement before market introduction. In the past, much of this skin testing required the use of animals for testing. However, latest best approaches for skin corrosion and skin irritation testing and risk assessment are being defined, obviating the need for animal test methods. Many in vitro skin corrosion test methods have been endorsed after successful validation and are gaining acceptance by regulatory authorities. In vitro test methods for acute, repeat exposure, and chronic (prolonged exposure) skin irritation are under development (Robinson and Perkins 2002).

Skin irritation test was tested by applying cream on skin (epidermis), spread properly and allow it for some time until skin absorb cream completely. After that check any type of irritation on skin on not.

2.15 Antibiotic susceptibility test of formulated cream

Antibiotic susceptibility testing is used to determine which antimicrobials will inhibit the growth of the bacteria or fungi causing a specific infection. The results from this test will help a healthcare practitioners to determine which drugs are likely to be most effective in treating a person's infection (Desai et al. 2005).

Procedure of Antibiotic susceptibility test

Prepared NA media , autoclaved at 121°C at 15psi for 30min. After autoclaving take a sterile Petri plates and poured the media under laminar air flow. Preparaed standardized inoculum from a bacterial culture of *S.aureus*. Nutrient agar plate was inoculated with standardized I inoculum of test microorganisms. Now, take three filter paper discs, one was considered as negative control, second was considered as positive control and dipped in antibiotic (Azithromycin) and placed on the NA plate, and third one was sample (cream) in which filter paper discs dipped and place on NA plate. Incubate the plate at 30°C for 24 hours. After incubation observed for zone of inhibition. Measuring the zone of inhibition. Interpretation of AST results.

2.16 Shelf-life testing

Products of cosmetics with antimicrobial effect can be described as preparations with the ability to provide consumer's protection against the presence of antimicrobial compounds, having bactericidal effect. Currently, the limit between cosmetic products and drugs and with antimicrobial effect is increasingly indistinct. Sometimes the difference between a cosmetic product and a drug lies in the concentration of the active ingredient in the product. There is an unclear distinction between the definition of cosmetic and dermatological treatment (e.g., acne treatment). That's why, some modern cosmetics are in an increasingly grey zone and can almost be defined as drugs or over-the-counter (OTC). In all cases, a decision on product qualification must be made by the competent national authorities on a case-by-case basis, and taking into account all relevant factors, such as their physical parameters , the type of active ingredient, length of use, mode of action, and claims (Halla et al. 2018).

Procedure of shelf life testing

Prepared 25ml MSA media in conical flask. Check the pH with the help of pH strip and cover the mouth of conical flask with cotton plug. Autoclaved at 121°C at 15psi for 30 min. Take a sterile Petri plates, poured the media under laminar air flow and allow them to solidify. When media becomes solidify spread

sample (cream) on it to check the presence of *S.aureus* in the cream. Incubate the plate at 30°C. Take observation everyday for 5 days.

3. RESULTS AND DISCUSSION

3. 1 Isolation of Red pigment producing Bacteria

Colonial morphology – On Nutrient agar plate, red pigment producing bacteria appears as smooth, red, convex, and round colonies after 24 hours incubation at 35°C.

Figure 1. Plate of Red pigment producing Bacteria

3.2 Staining of Red pigment producing Bacteria

Red pigment producing bacteria is a Gram-negative, rod-shaped, non-motile and non-endospore forming Bacteria.

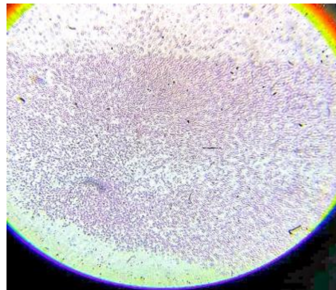


Figure 2. Gram's staining (gram negative Bacteria.)

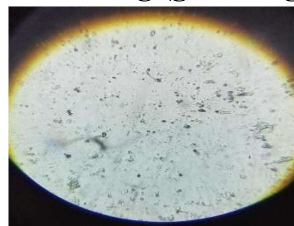


Figure 3. Endospore staining (non-spore forming)

3.3 Screening of Red pigment producing bacteria by biochemical testing

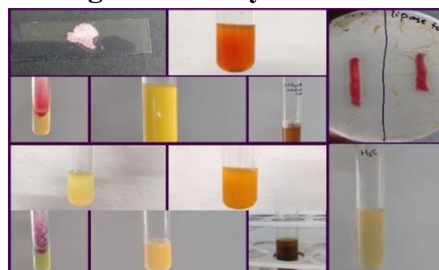


Figure 4. Biochemical analysis

Table 4. Biochemical characterization

S.No.	Biochemical Test	Result
1.	Catalse	++
2.	Urease	+
3.	Citrate	+
4.	Nitrate reduction	+
5.	Lipase	+
6.	H2S	-
7.	MR	-
8.	VP	+
9.	Mannitol	+
10.	Indole	+
11.	Sucrose	+
12.	Dextrose	+
13.	Glycerol	+
14.	Lactose	+
15.	Maltose	+
16.	Sorbitol	+

Note:- From above biochemical analysis, it was confirmed that red pigment producing bacteria is *Serratia marcescens.*

3.4 Oxidative fermentation of *Serratia marcescens*

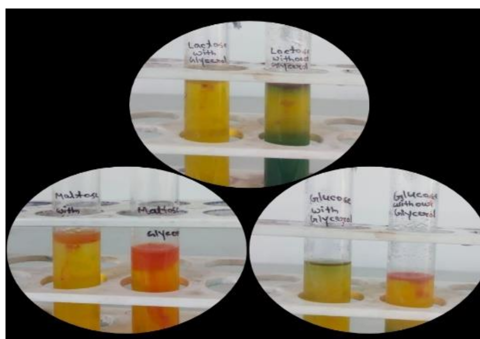


Figure 5. Oxidative-Fermentative

Table 5. Oxidative-Fermentative

S.No.	Oxidative fermentation test	Result
1.	Glucose (a)with glycerol (b)without glycerol	Oxidative
2.	Maltose (a)with glycerol (b)without glycerol	Fermentative
3.	Lactose (a)with glycerol	Oxidative

	(b)without glycerol	

Note:- From above oxidative Fermentative results ,it was confirmed that *Serratia marcescens* is a Facultative Anaerobes.

3.5 Growth Optimization of *Serratia marcescens*

Temperature Optimisation

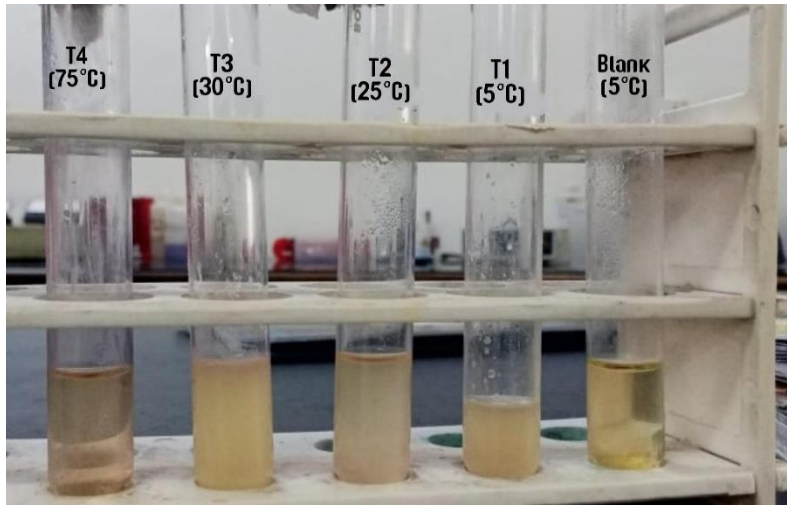


Figure 6. Temperature optimisation

Table 6. Temperature optimisation

Temperature	Absorbance at 600nm
Blank (5-8°C)	0.000abs
T1 (5°C)	0.705abs
T2 (25°C)	1.415abs
T3.(30°C)	1.625abs
T4. (65°C)	0.075abs

Note:- Maximum growth at temperature 28°C in incubator because highest absorbance observed in T3 (30°C).

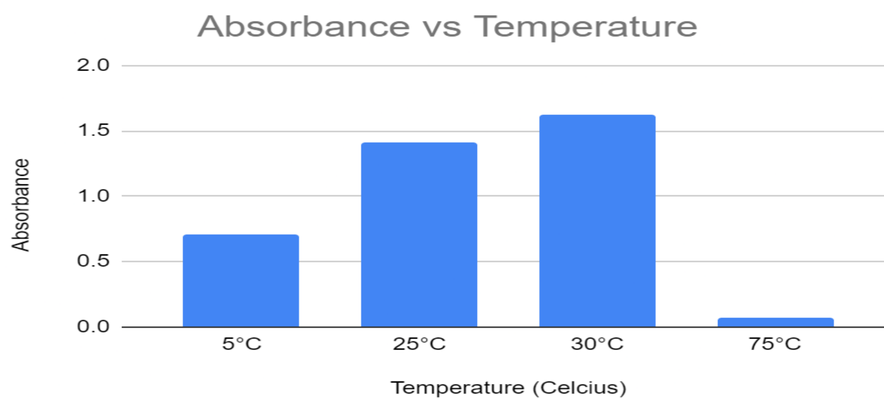


Figure 7. Graph for Temperature optimisation

pH optimisation

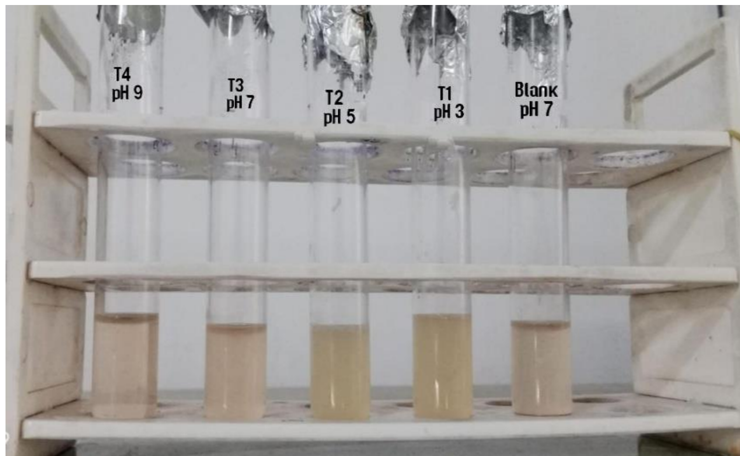


Figure 8. pH optimisation

Table 7. pH optimisation

pH	Absorbance at 600nm
Blank (pH 7)	0.000abs
T1 (pH 3)	0.170abs
T2 (pH 5)	0.160abs
T3 (pH 7)	0.803abs
T4 (pH 9)	0.811abs

Note:- Maximum growth at pH 9 because highest absorbance observed in T4 (pH 9).

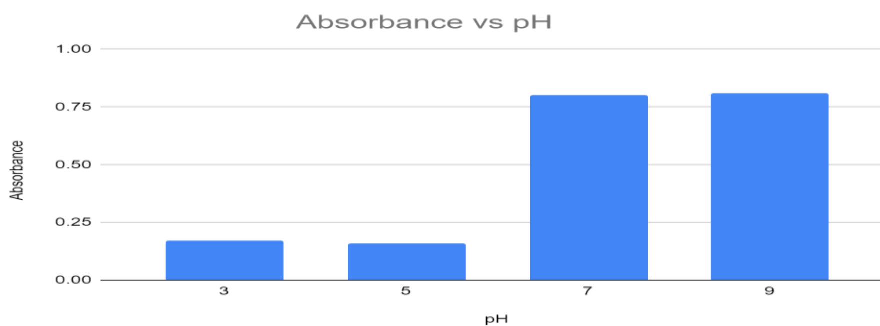


Figure 9. Graph for pH optimisation

Carbon source optimization

Figure 10. Carbon source optimisation

Table 8. Carbon source optimisation

Carbon source	Absorbance at 600nm
Blank	0.000abs
T1 (Lactose)	1.880abs
T2 (Dextrose)	1.602abs
T3 (Maltose)	1.682abs
T4 (Sucrose)	2.057abs

Note:- Maximum growth in T4 test tube containing sucrose because highest absorbance observed in T4 (sucrose).

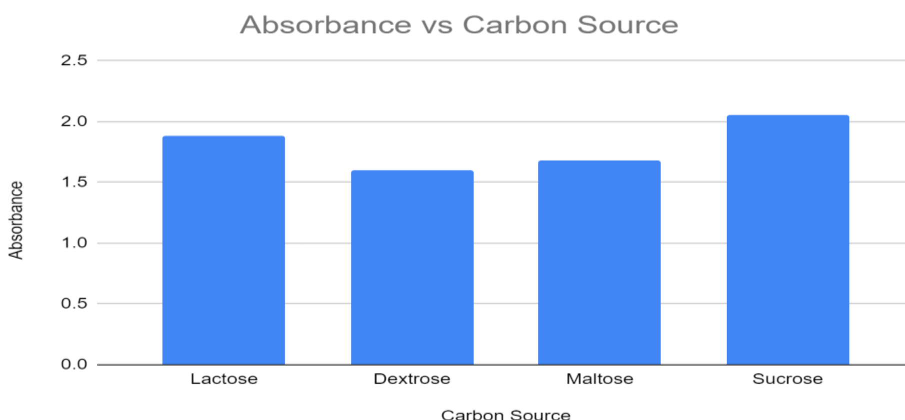


Figure 11. Graph for Carbon source optimisation

3.6 Preparation of Fermentation media for the extraction of Prodigiosin pigment.

- Presence of pink color in the media indicates presence of Prodigiosin in the fermentation media.

Figure 12. Fermentation media

3.7 Extraction of Prodigiosin pigment from fermentation media

- When ethyl acetate added in the centrifuge tube containing fermentation media, prodigiosin separated along with ethyl acetate in the upper part of the tube and water remains at the bottom.

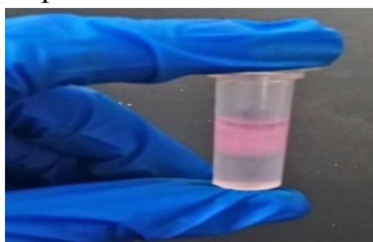
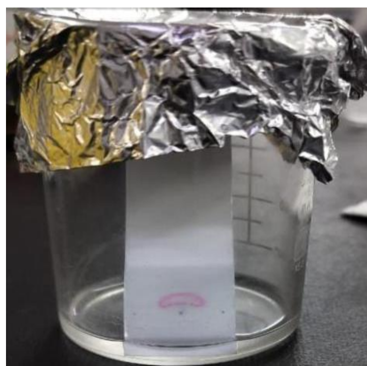


Figure 13. Prodigiosin pigment in Ethyl acetate

3.8 TLC of Prodigiosin.

Solvent system consisted of Hexane:ethyl acetate (3:1,v/v).

TLC before running.



TLC during running

TLC after running

Figure 14. TLC of Prodigiosin.

Rf value= dis. travelled by the compound/dis. travelled by the solvent front
Rf = 3.8/6.1 = 0.622

3.9 UV-Vis Scanning of Prodigiosin pigment

- Prodigiosin has a absorption peak in the visible region of spectrum typically around 500-540nm.

Table 9. UV-Vis scanning

Wavelength(nm)	Absorbance
400nm	0.026abs
425nm	0.020abs
450nm	0.017abs
475nm	0.022abs
500nm	0.07abs
525nm	0.065abs
550nm	0.048abs
575nm	0.015abs
600nm	0.008abs
625nm	0.008abs
650nm	0.007abs
675nm	0.007abs
700nm	0.007abs
725nm	0.006abs
750nm	0.005abs
775nm	0.004abs
800nm	0.003abs

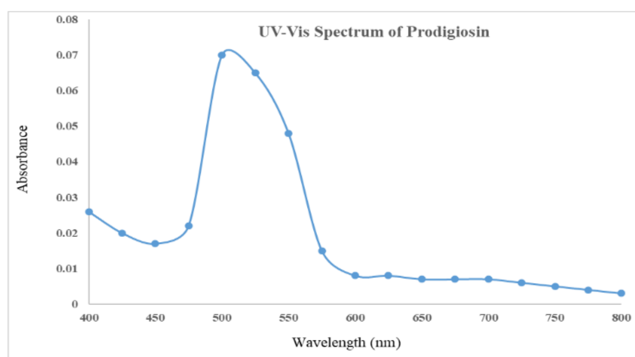


Figure 15. Graph for UV-Vis spectroscopy of Prodigiosin.

3.10 Antibiotic Susceptibility Test (AST) of Prodigiosin by Disc dffusion method.

• Prodigiosin showed zone of inhibition against *S.aureus.*

S.aureus

Figure 16. Antibiotic Susceptibility Test

Sample:- Prodigiosin in DMSO

Antibiotic:- Azithromycin

Table 10. Antibiotic Susceptibility test

Sample and Antibiotic	**Zone of inhibition**
Prodigiosin	9.5mm
Azithromycin	10mm

3.11 Formulation of Base

Figure 17. Formulation of Base

3.12 Formulation of Cream

Figure 18. Final cream formation with Prodigiosin pigment

3.13 Evaluation of Formulated cream

pH

Figure 18. pH test of Cream

Observation:- pH of the cream was 6

Homogeneity test

Figure 19. Homogeneity test of cream

Observation:- Homogeneity of cream was greasiness

(Skin irritation test

Figure 20. During Cream use.

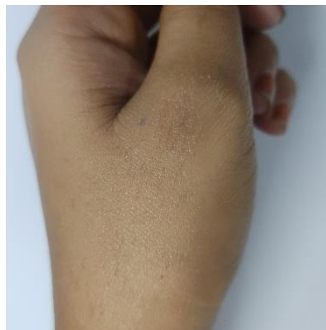


Figure 21. After use of cream

Observation:- There was no irritation on skin during implementation of cream on Epidermis.

3.14 Evaluation of Formulated Cream

Table 11. Evaluation of Formulated Cream

S.No.	Parameters	Cream
1.	Appearance	Light pink
2.	pH	6
3.	Homogeneity	Good
4.	Type of Smear	Greasiness
5.	After feel	Slipperiness
6.	Removal	Easy

3.15 Antibacterial activity

Formulated cream showed zone of inhibition against *S.aureus*.

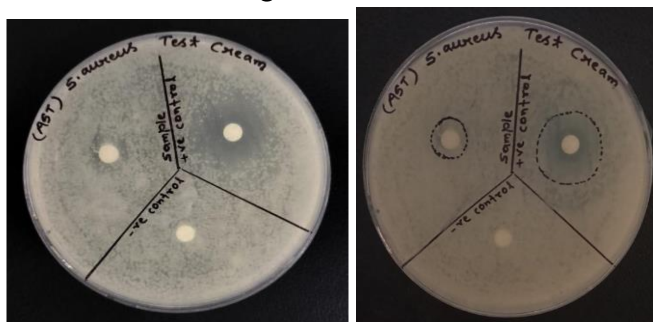


Figure 22. AST of Formulated cream

Table 12. AST test of Formulated cream

Organism	Zone of inhibition
S.aureus	9mm

3.16Shelf life testing

Spreading of formulated cream on MSA plate to check the presence of *S.aureus* bacteria which is related to skin.

(No growth of *S.aureus* colonies)

Figure 23. Day 1,
Cream spreading on MSA

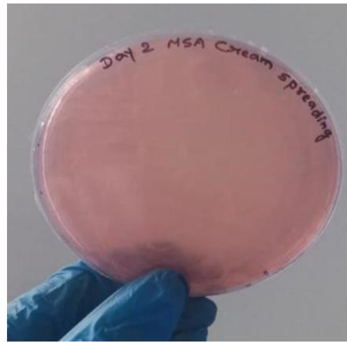
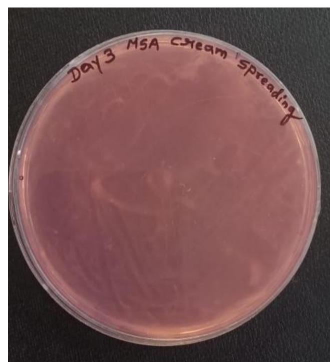
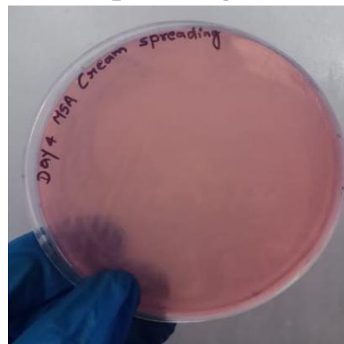


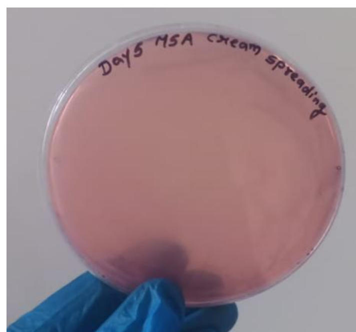
Figure 24. Day 2
Cream spreading on MSA



(No growth of *S.aureus* colonies).
Figure 25. Day 3,
Cream spreading on MSA.



(No growth of *S.aureus* colonies).
Figure 26. Day 4,
Cream spreading on MSA



(No growth of *S.aureus* colonies)

Figure 27. Day 5, Cream spreading on MSA

4. CONCLUSION

This study investigated the potential of prodigiosin pigment, extracted from *S. marcescens* bacteria, for the development of a topical cream formulation. Prodigiosin was successfully extracted from *S.marcescens* and incorporated into a cream base. The formulated cream exhibited antimicrobial activity against *S.aureus.* The result of AST provide valuable insights into the potential of the prodigiosin cream as an antimicrobial agent. Further research is warranted to elucidate its specific advantages and optimize its effectiveness for various therapeutic applications.Stability studies are necessary to determine the pH, homogeneity, Shelf life and skin irritation for the prodigiosin cream.

In vivo studies are crucial to assess the safety and efficacy of the cream formulation in a biological system.

5.FUTURE SCOPE

- •.Employing metabolic engineering techniques to optimize prodigiosin production in *S.marcescens*. This could involve manipulating the growth conditions, introducing precursor genes, or deleting genes that regulate secondary metabolite biosynthesis.
- Developing efficient extraction methods and purification techniques for prodigiosin. This might involve exploring alternative solvents, employing different chromatography techniques, or utilizing biocompatible nanoparticles for selective isolation.
- Investigating the potential of prodigiosin-based creams for wound healing and regeneration of tissue due to the pigment's well-documented antibacterial and angiogenic properties.
- Analysing the efficacy of prodigiosin in treating inflammatory skin conditions like psoriasis and eczema, leveraging its anti-inflammatory and immunomodulatory effects.
- Unveiling the potential use of prodigiosin-containing creams in sunscreens due to the pigment's UV-protective properties.
- Encapsulating prodigiosin within biocompatible nanoparticles for elucidating enhanced topical delivery, improved stability, and controlled release of the pigment within the skin.
- Utilizing targeted nanoparticles functionalized with specific ligands to deliver prodigiosin to specific cell types within the skin.

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