Production and characterization of Prodigiosin from a bacterial strain and its applications

Dissertation submitted in fulfilment of the requirement for the degree of

Master of Science

in

Biotechnology

By

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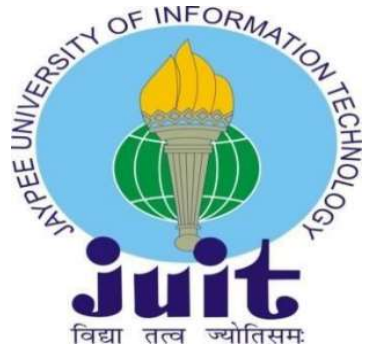
Under the supervision of

Dr. Ashok Kumar Nadda

to



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MAY,2025

SUPERVISOR'S CERTIFICATE

This is to certify that the project work titled "**Production and characterization of Prodigiosin from a bacterial strain and its applications "** by Apurva Jain(235111005)during their end semester in fulfilment for the award of degree of Masters of Science in Biotechnology from Jaypee University of Information Technology, Solan, H.P has been carried out under my supervision. This work has not been submitted partially to any other University or Institute for the award of any degree or appreciation.

(Signature of Supervisor)

Dr. Ashok Kumar Nadda,

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Date:

CANDIDATE'S DECLARATION

I hereby declare that the project work titled *"***Production and characterization of Prodigiosin from a bacterial strain and its applications***"*, submitted in partial fulfilment of the requirements for the degree of Master of Science in Biotechnology to the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, is a genuine record of my original work conducted from August 2024 to May 2025. This work was carried out under the guidance of **Dr. Ashok Kumar Nadda**, **Department of Biotechnology and Bioinformatics, JUIT, Waknaghat, Solan, Himachal Pradesh**

I further confirm that the project was undertaken as part of the Proficiency Stream of the program.

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LIST OF ABBREVIATION

S.No	Abbr.	Abbreviation (s)
1.	°C	Degree Celsius
2.	NA	Nutrient Agar
3.	NB	Nutrient Broth
4.	min	Minute
5.	G	Gram
6.	%	Percentage
7.	ml	Millilitre
8.	mg	Milligram
9.	H2O2	Hydrogen Peroxide
10.	HCl	Hydrochloric acid
11.	EtOH	Ethanol
12.	PG	Prodigiosin
13.	LAF	Laminar airflow
14.	SMA	Skim milk agar
15.	DMSO	Dimethyl sulfoxide
16.	nm	Nanometre

ABSTRACT

The Himalayan region , with high-altitude habitat and high biodiversity, provide an extremely beneficial environment to discover new microorganisms with industrial and pharmaceutical potential and therapeutic applications. Throughout this study , a strain of bacteria capable of producing the crimson red pigment prodigiosin was isolated from soil collected in this region. Prodigiosin is reported for its variety of bioactivities including antimicrobial, anticancer, immunosuppressive activity, anticancer properties and antioxidant properties, making it a compound of multiple applications in biotechnology field . The strain was identified to assess its pigment-producing ability, and growth conditions such as temperature, pH, and nutrient availability of sugar, micronutrients etc. were optimized to obtain the maximum yield of pigment output.. Tests for antibiotic resistance exhibited resistance of the strain to several antibiotics, reflecting the adaptability of the strain under severe environmental conditions. Morphological and staining methods confirmed the bacterium to be Gram-negative cocci. The red pigment was isolated and confirmed to be prodigiosin by UV-Visible spectrophotometry, owing to its characteristic absorbance patterns. This study highlights the promise of the microbial ecosystem in the Himalayas as a rich source of bioactive pigment-producing bacteria. The research not only contributes to the knowledge of microbial diversity in extreme conditions but also highlights prodigiosin as a potential molecule for future pharmaceutical and industrial use. The work provides the foundation for further research into green production techniques and the application of microbial colorants in practical applications.

Keywords :*Prodigiosin, characterization, Antibiotic resistance, Microbial diversity.*

CHAPTER 1

INTRODUCTION

1.1 Introduction

Microbial colourants, or food colouring agents, are simple to create and have a smooth downstre aming process. These natural colourants can be found in microbes, plants, insects, and metals. Th ere. Microorganisms are a more efficient source of pigment due to their rapid development, perh aps leading to higher product production[1]. Some bacteria produce fatsoluble colours, while oth ersproduce watersoluble pigments that spread throughout the environment [2].Microorganisms pr oduce a variety of colours, including watersoluble carotenoids, prodigiosin, and violacein. . Natu ral colour sources are becoming more popular due to environmental concerns and conservation e fforts. Microbial pigments offer yearround, quick, and unlimited output, making them a feasible alternative to plant pigments [3].Microbial pigment production is easy, requires lowcost growth media, and is weatherindependent. The goal of this research is to isolate microorganisms that cre ate pigments from various sources and improve their physical and chemical properties enabling l argerscale synthesis, extraction, and identification of pigment [4][5].

Since long , artists have been employing pigments to produce wonderful pieces of art , such as synthetic pigments like phthalocyanine blue and natural colours like ochre and ultramarine.Toen hancebeautypigments are frequently utilised in paintings, textiles, plastics, cosmetics, and food g oods. They are also useful in scientific domains, where they are used as markers in imaging and s tudies[6][7].

The Himalayan region provides a distinct biological niche characterised by extreme environment al circumstances such as low temperatures, strong UV radiation, and nutrient scarcity, which enc ourage the evolution of microbes with novel metabolic pathways.[8] Microorganisms isolated fro m the Himalayas have been shown to produce bioactive chemicals with increased potency and st ability, including prodigiosin[9].Its prospective involvement in treating multidrugresistant bacteri a, cancer therapy, and environmental applications such as dyes and biosensors increase its import ance. The study of Himalayan prodigiosin allows researchers to investigate the relationship betw een extremophilic microbial diversity and secondary metabolite production, potentially leading t o longterm and creative solutions in health, industry, and ecology.Prodigiosin, a vivid red pigme

nt, is rare in the Himalayan microbial community. Prodigiosin is distinguished from common pig ments such as melanin, violacein, and carotenoids by its distinct characteristics and limited avail ability [10]. It functions as an energy storage medium, a precursor for essential biochemicals like as proline and NADPH, and an air diaspora for bacteria and facilitating ion exchange and energyspilling functions [11].Prodigiosin has environmentally significant in manyways includingbioremediation, waste management, antibacterial activity, biocontrol, sustainable agricu lture, contains numerous biological activitiesnamely

antibacterial, antifungal, and anticancer characteristics, making it a promising candidate for biot echnological applications[12].Microbial pigments, particularly those found naturally, play a crucial role in the survival and adaptation of organisms, providing extreme range of biological activities [12]. The Himalayan region, with its rich microbial diversity, is home to several valuable pigment-producing microbes, including those that generate the vibrant prodigiosin. Studies focused on isolating and characterizing prodigiosin-producing strains from this region have shown great promise in discovering new pigments and understanding their role in microbial survival[13]. Although prodigiosin is scarce in Himalayan microbial communities, it is distinguished by its unique properties and limited presence [14]. Aside from its biological significance, prodigiosin has many biotechnological applications, including as a natural food dye, a staining agent, and an antimicrobial compound. Extraction and use of prodigiosin from Himalayan strains presents challenges due to its scarcity and the complexity of its biosynthesis, which is monitored by quorum sensing. Prodigiosin works as a significant promising bacteria in the field biotechnology , serving as a natural food colorant and antimicrobial agent. Current research is focused on unscrambling and understanding these pathways and increasing pigment production through methods such as genetic engineering. The process of isolating prodigiosin-producing bacteria involves several steps: collections of soil samples, conducting serial dilutions, examining samples microscopically, identifying bacterial strains, and applying genetic tools to increase pigment output. Additional essential stages include screening for productive colonies , optimizing growth parameters, and characterization of pigment using analytical methods [15].

Beyond its industrial applications, prodigiosin plays an significant role in ecological processes. It contributes to UV protection, waste degradation, microbial control, bioremediation, and supports sustainable agricultural practices as a biocontrol agent , thereby enhancing biodiversity. From a biotechnological perspective, prodigiosin is valued for its wide-ranging properties namely strong

antibacterial, antifungal, and anticancer activities, immunosuppressive effects and anticancer characteristics[11]. Chemically, it is known for its crimson red colour and distinctive pyrromethene structure feature ,which is responsible for its unique properties and biological efficacy and attracted considerable attention for its broad applications in medicine, agriculture, industry, and environmental management and in agriculture[16][17].

1.2 Research Gap

Albeit thepromising uses ofprodigiosin, there are stillsome gaps inresearch, little is known abo ut the genetic and biochemical processes controlling prodigiosin biosynthesis under varied environmentalconditions, making it difficult to maximize production.Strain impro vement for higher yieldand upscaling productionprocesses are also areas that need addressing, as these are not yet cost-efficient or sustainable. In addition, there is a lack of data on the toxicity and safety ofprodigiosin, particularly concerning its chronic usein animals and hum an beings

1.3 Objectives of the study

- Optimization of growth conditions for bacterial isolate
- Extraction and characterization of Prodigiosin pigment
- Applications of prodigiosin as a natural dye

CHAPTER 2

REVIEW OF LITERATURE

2 Review of Literature

Bacterial pigments provide intriguing prospects for a range of applications because of their better biodegradability and environmental friendliness. Certain bacterial pigments are presently synthesized by the industry and used in textiles, food, medicines, and cosmetics.

2.1 Pigments

Pigments are divided into three types:

Natural, Synthetic, and Organic Pigments that are manufactured by living things like microorganisms, plants, and animals arereferred to as naturalpigments. There are three categories of natural pigments depending on the structural characteristics. The first type has Heterocyclic compounds such as anthocyanins and other flavonoid pigments, which are known for their vivid colours in fruits and plants. The second group consists of benzopyran derivatives like purines, pterins, flavins, phenazines, phenoxazines, and betalains, all of which have shown to possess diverse biological activities and colours. The third group consists of quinones, i.e., benzoquinone, naphthoquinone, anthraquinone, and melanins, which are pigments as well as other biochemical processes.Synthetic pigments, on the other hand, are manufactured by chemical means.Azo dyes and phthalocyanine pigments are some examples that are widely used manufacturing. Finally, organic pigments comprise both natural and synthetic forms. Thesecarbonbased materials are known for their intense and vivid colours, and they find extensive application in diverse industries such as textiles, cosmetics, food and medicines[9].

2.2 Prodigiosin

Red pigment known as prodigiosin is generated by number of bacteria, including as prodigiosin is a vibrant crimson red pigment produced by definite microbe*like Hahellachejuensis*, Vibrio *psychroerythrus*, and Serratiamarcescens. It belongs to a group of compound called tripyrrole

compounds and has captured attention for its great range of biological activities. This pigment has shown potential in fighting bacteria and fungi and pathogen , combating cancer, and even suppressing immune responses. Its versatility makes it an exciting subject of research in medicine and biotechnology areas [19] [20].

2.2.1Chemistry of prodigiosin

Prodigiosin is a red-crimson pigment with a distinct chemical structure and composition, its molecular formula, **C20H25N3O**, represents a tripyrrole compound consist of three pyrrole rings arranged linearly and connected by methine bridges. This unique tripyrrole system is responsible for its vibrant red colour and its diverse biological and biotechnological properties.[19]. The structure also includes a long alkyl chain substituent, which can vary in length and composition, and lead to the production of different analogs . [20]

2.2.2 Chemical structure of prodigiosin pigment

Prodigiosin is a tripyrrole compound ,consisting of three pyrrole rings linked by methine bridges, forming a linear tripyrrole structure. This distinctive molecular arrangement gives the molecule its bright crimson red colour and notable biological features and properties. The structure also includes a long alkyl chain substituent, which can vary in length and composition, leading to the formation of different isoforms by various microorganisms[21].

2.2.3 Physicochemical properties of prodigiosin

Prodigiosin has a number of significant physicochemical characteristicsand properties that affect its behaviour and potential applications in field of biotechnology and industrial roles. It doesn't dissolve in organic solvents like methanol, ethanol, and chloroform which restricts its application in aqueous media The molecule remains stable in acidic environments, but lead to structural breakdown in the alkaline conditions [21].The tripyrrole structure of prodigiosin also contribute for its vibrant red colour, which is due to the electronic properties of the pyrrole rings[15] .

2.3 Sources of prodigiosin

Serratiamarcescens is the major source of prodigiosin; however, other bacterial species also produce this pigment. Microbes such are *Streptomyces coelicolor*, *Streptomyces lividans*, *Hahellachejuensi*, *Pseudovibriodenitrificans*, *Pseudoalteromonasrubra*, and *Vibrio* gazogenes. Notable microorganisms that's synthesize different prodigiosin derivatives is shown in table below.

Table 2.1 list of organisms that synthesis different prodigiosin derivatives

Prodigiosin Type	**Organisms**	**Reference**
Cycloprodigiosin and analogues	*Vibrio spartinae*	[22]
Prodigiosin	*Streptomyces coelicolor*	[23]
14 derivatives of prodigiosin	*Pseudomonas putida*	[24]
Cycloprodigisin ,2- (p-hydroxybenzyl) prodigiosin	*Pseudoalteromonasrubra*	[25]
Prodigiosin	*Serratianematodiphila*	[26]
Prodigiosin with 25 carbons atoms	*Achromobacterdenitrificans*	[27]
Prodigiosin	*Serratiarubidaea RAM_ALEX*	[28]
Undecylprodigiosin	*Streptoverticillium*	[29]
Prodigiosin	*Zooshikella*	[30]
Cyclic prodigiosin	*Beneckeagazogenes*	[29]
Metacycloprodgiosin	*Streptomyces longisporusruber*	[29]

Undecylprodigiosin	*Saccharopolyspora sp.*	[29]

2.4 General properties of prodigiosin

Prodigiosin is a red pigment with a peculiar tripyrroleskeleton ,which is account for vibrant crimson red colour and its unique chemical reactivity. It is readily soluble in organic solvents like methanol, ethanol, and chloroform, but its insolubility in water limits its applicationin aqueous media solutions. The compound has varied biological activities like antibacterial, antifungal, anticancer, and immunosuppressive activity, hence it is a promising therapeutic agent. But, it can be toxic at high concentrations, and its cytotoxicity has sparked interest in its application as a chemotherapeutic agent in cancer treatment[30].

2.4.1Isoforms of prodigiosin

Prodigiosin exists in various isoforms, which are structural variants of the compound, that differs primarily in the length and conformation of the alkyl chain attached to the tripyrrole ring system. These isoforms are generated by different microbial[30].The main isoform of prodigiosin, typically produced by *Serratiamarcescens*, features a straight-chain alkyl group, while other microorganisms may produce prodigiosin with variations in the alkyl chain, leading to different structural forms. For example, *Streptomyces coelicolor* and *Pseudovibrio* species can produce prodigiosinanalogs with branched or differently sized alkyl chains, which can influence properties such as solubility, stability, and biological activity[31][32].

2.4.2 Production of prodigiosin

Prodigiosin production is influenced by several environmental factors that can be optimized to enhance its yield. Nutrient availability is a key factor, as limiting certain nutrients like nitrogen, carbon, or phosphorus can trigger the bacteria to produce more pigment. Temperature also plays a crucial role, with different bacterial strains producing the highest amounts of prodigiosin at specific temperatures, generally between 20°C and 30°C[33]. The pH also plays a vital role , with slightly acidic to neutral conditions (around pH 6–7) being most favourable for prodigiosin synthesis [34]].Oxygen availability impacts production as well, with some bacteria generating

more pigment under low-oxygen or anaerobic conditions, while others require higher oxygen levels for optimal growth. Salinity also affects prodigiosin synthesis, as moderate to low salt concentrations can enhance production, though too much salt may inhibit its production [35][36].

2.4.3 Environmental factors influencing prodigiosin production

Various factors are essential in regulating the production of Prodigiosin. The main factors include:

- **Nutrient Availability**: Limiting key nutrients like nitrogen, carbon, or phosphorus can stimulate the production of prodigiosin. Therefore, the nutrient composition and balance in the growth medium are vital for boosting pigment production.
- **Temperature**: Most bacteria produce the highest amounts of prodigiosin at temperatures between 20°C and 30°C. Extreme temperatures, whether too high or too low, can negatively affect pigment sunlight [37].
- **pH Level**: Most prodigiosin-producing bacteria thrive in slightly acidic to neutral pH conditions (around pH 6–7). Significant deviations from this pH range can reduce pigment synthesis or completely inhibit it.
- **Oxygen Availability**: Some bacteria produce more pigment in low-oxygen or anaerobic conditions, while others need higher oxygen levels to maximize growth and pigment synthesis. Proper oxygen control is essential for optimizing production.
- **Salinity**: Moderate salt concentrations can enhance pigment production, whereas excessive salinity can inhibit bacterial growth and reduce pigment synthesis.
- **Environmental Stress**: Stress conditions like oxidative stress, UV radiation, or changes in osmotic pressure can trigger prodigiosin production.

2.4.4 Biosynthesis pathway of prodigiosin

Early studies have revealed that prodigiosin, the crimson red pigment, is synthesized through a pathway involving the condensation of two intermediate compounds, MAP and MBC. In *S. coelicolor*, at least eighteen genes are required for the synthesis of undecylprodigiosin[38][39]. Similarly, the biosynthetic genes for prodigiosin in *Serratia* strains, including *S. marcescens*,

have been cloned and expressed in various host organisms. The gene clusters of these bacteria show shared genes, as well as specific genes unique to either the Serratia or Streptomyces species[40].

2.5 Regulation of prodigiosin pigment

Prodigiosin biosynthesis is controlled functionally through complex interconnection of genetic, environmental, and physiological factors .There is a genetic regulation of Prodigiosin biosynthesis involving several genes classified into biosynthetic clusters , with key genes like *pigA*, *pigB*, *pigC*, and *pigD* controlling the expression of enzymes required for the pigment's production .Factors such as nutrient availability of sugars, micronutrients,NaCl, temperature, pH, and availability of oxygen levels also play a crucial role in regulate prodigiosin biosynthesis. For instance, nutrient deprivation, especially of nitrogen and phosphorus, has a tendency to triggers prodigiosin production as a secondary metabolite, while conditions like oxidative stress or low oxygen can also induce its biosynthesis[41].Also, quorum sensing, isa bacterial cell to cell communication mechanism , Assists in coordinating pigment 's production when bacterial populations reach a certain density. Global regulatory systems like the cAMP-Crp system, which responds to changes in carbon sources, and the GacS/GacA system, which stimulate secondary metabolic pathways, further modulate the biosynthesis of pigment production[42][43].

2.6 Environmental significance of prodigiosin production

Prodigiosin production plays an important role in the environment .They are :

- **Competitors Deterrence** :When bacteria produce this pigment, it may inhibit the growth of other microorganisms present in the environment , thus providing a competitive advantage in a situation where resources or limited or in stressful conditions [43].
- **Stress Protection**: The pigment also acts as an protective coat to the bacteria, protecting them from environmental stress, such as UV radiation, high salt tolerance, or oxidative damage. Bydamaging harmful molecules known as reactive oxygen species (ROS.

- **Microbial Communities shaping**: Prodigiosin not only protect bacteria from death ;but it also helps with the interaction of bacteria with each other. It can affect how they form biofilms or communicate through quorum sensing, shaping the structure and dynamics of microbial communities.
- **Helping with Pollution Clean-up**: Because of its antimicrobial and antioxidant properties, prodigiosin might act as cleaning agent of polluted environments.
- **Supporting Nutrient Cycles**: This helps regulate in nutrient cycling, especially in ecosystems where bacteria can be employed in processing carbon and other key elements decomposition and recycling [44].

2.7 Purification techniques of prodigiosin

Several techniques are commonly used to isolate and purify and extract prodigiosin efficiently while maintaining its chemical integrity:

- **Solvent Extraction**: This is the most widespread method for extracting prodigiosin. Organic solvents like methanol, ethanol, or chloroform are used to extract the pigment, by dissolving it in the culture medium .
- **Liquid-Liquid Extraction**: Following initial extraction, liquid-liquid extraction can further purify and extract the pigment by transferring it between solvents based on its solubility, helping to eliminate impurities along with pigment.
- **Column Chromatography**: Methods like as silica gel chromatography or high-performance liquid chromatography (HPLC) are used to extract prodigiosin and purify it from other compounds on the basis of polarity, molecular weight, or chemical nature [45].

2.8Biotechnological implications of prodigiosin

Prodigiosin, with its distinctive molecular structure and biological properties, has immense potential in field of biotechnology, including medicine, agriculture, and environmental science and industrial role as a natural dye . Its strong antimicrobial activity against bacteria, fungi, and

certain parasites makes it an best substitute for conventional antibiotics and therapeutics , and it may offer promising solution to the problem of antibiotic resistance [40].

Additionally, the selective action of prodigiosin to induce apoptosis in cancer cells but not normal cells has generated interest in its potential use as a anticancer agent for cancer therapy. The pigment also provide immunosuppressive activity, that could be useful in against autoimmune conditions as well as organ failure or rejections in organ transplant recipients[46].

For agriculture, the antimicrobial potential of prodigiosin can be harnessed as a natural pesticide and fertilizer or bio-control agent, which is a green solution to synthetic pesticides. Its antioxidant nature also make it useful for applications in the environment and in cosmetics , such as bioremediation, where it could employed to assist in neutralizing toxins and control microbial communities within polluted surroundings. Besides , its vibrant red hue and distinctive chemical properties in textiles and cosmetics field as biosensor technology as a biodegradable dye or natural dye , offering alternative options to synthetic dyes [44].

2.9 Applications of prodigiosin[49]

- **Medical Applications**:
  - **Anticancer Agent**: Selectively attacks cancer cells with minimal impact on healthy cells.
  - **Antimicrobial Agent**: It kills a broad spectrum of bacteria, fungi, and parasites, and provides potential alternatives to treating antibiotic resistance.
  - **Immunosuppressant**: Holds promise for treat autoimmune diseases and prevent organ rejection in transplant patients.
- **Agriculture**:
  - **Biological Pesticide**: A natural, eco-friendly alternative to chemical pesticides for controlling plant diseases.[43]

- **Soil Microbiome Management**: Enhances plant health by regulating soil microbial populations.

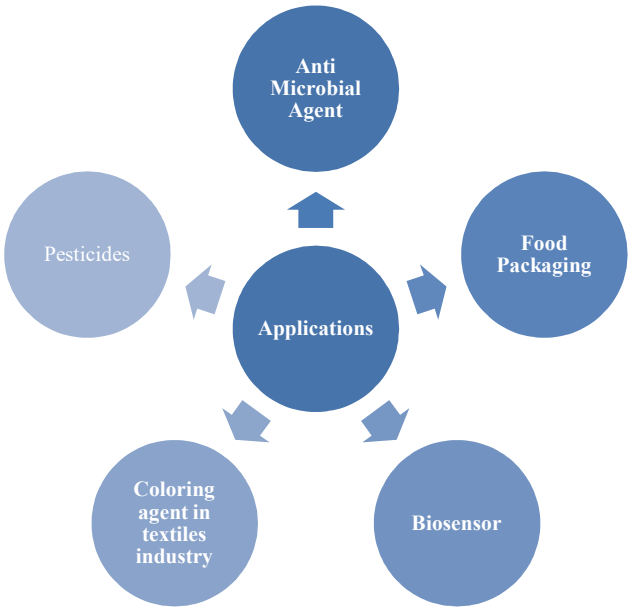
❖ **Environmental Applications**:

- **Pollution Clean-up**: Assists in breaking down contaminants and managing microorganisms in polluted environments.
- **Water Treatment**: Effective in reducing harmful microbes in water systems.

❖ **Industrial Uses**:

- **Sustainable Dye**: A natural, biodegradable option for use in textiles and cosmetics.
- **Biosensor Material**: Useful for detecting environmental changes or specific chemical molecules.

❖ **Food and Feed Industry**: Potential as a natural, safe colorant for food and animal and



can be used in food packaging.

Fig 2.3: Applications of prodigiosin pigment

CHAPTER 3

MATERIALS AND METHODS

3Materials

3.1.1Microbial strains

Strain used was procured from Jaypee University of Information Technology, Solan ,H.Pfrom genomics laboratory

3.1.2Chemical and materials

The chemicals such as Nutrient Broth, Nutrient Agar ,Luria Broth ,Skim Milk Agar ,Yeast Extract Broth ,Gram's Iodine,Peptone, Sucrose ,Safarnin,(HIMEDIA) and Glycerol, cysteine (Merck)Crystal Violet, Hydrochloric acid (Loba Chemie) and Glycerol, Lysine, Galactose (Sisco Research laboratory) and Lactose, Maltose (Fisher Scientific)were used directly as received without any further purification.

3.1.4 Instrument

Weighing machine(Citizon Model :CG203) ,Autoclave(RELITECH ,Model No: VPS75), Incubator(EppendorfAG ,22331 Hamburg Model No :5805), Centrifuge(EffendorfNo.DST, Laminar air flow(Rescholar Equipment),Rotaevaporator (Heidolph) , Light Microscope ,Vortex (REMI ,CM 101 CYCLE MIXER) ,pH meter (Eutech)

3.2 Reviving of bacteria

For the revival of bacteria ,Nutrient agar (NA) plates were prepared and then streaked with the old culture of bacterial strain ,after 72 hours when plates kept at 37 ° , red colonies were appeared. For Reviving bacteria strain in liquid culture Nutrient Broth (100ml) was prepared and further inoculated with bacterial culture using a inoculation loop [3][47].

3.3 Gram staining of bacterial culture:

Gram staining was conducted to distinguish bacterial types based on cell wall characteristics. A clean glass slide was used to prepare a thin smear of the bacterial culture ,which was then heat-fixed to secure the cells in place. Subsequently, crystal violet was applied for one minute to uniformly stain the bacterial cells. Gram's Iodine was applied for one minute to serve as a mordant ,binding the primary stain within the cells. The slide was then treated with ethanol for approximately 15 seconds to decolorize, followed by an immediate water rinse to halt the decolourization process. A counterstain, safarnin ,was applied for one minute to impart colour to cells that had lost the primary stain[47][49].

3.4 Biochemical testing:

3.4.1 Catalase test:

Procedure: A clean glass slide was used to prepare a bacterial smear with the help of a sterile inoculating loop under a laminar airflow cabinet. A few drops of hydrogen peroxide was put to the smear. The enzymatic breakdown of hydrogen peroxide is indicated by the appearance of bubbles and confirmed a positive catalase reaction due to the release of oxygen[50]

3.4.2 Casein hydrolysis test:

Procedure: Skim milk agar plates were inoculated with the bacterial culture . The inoculated plates were kept at 25°-30 °C for a duration of 72 hours (3days). The clear zone proved the breakdown of casein protein and confirmed protease activity of the organism[50]

3.4.3 KOH string test:

Procedure:2-3 drop of 3% KOH solution was put on a glass slide, and a minute portion of the bacterial culture was mixed. After mixing for 60–90 seconds, the presence of a viscous string when the loop was lifted indicated a positive result (Gram-negative). The absence of a string suggested a negative result (Gram-positive)[50]

3.4.4 Phenylalanine deaminase test :

Procedure: A culture of bacterial was streaked on phenylalanine agar slant and kept at 37°C for 24 to 48 hours.Subsequent to the incubation time , the slant was subjected to 10% ferric chloride solution. A greenish colour indicates a positive test , which signifies the presence of phenylalanine deaminase, whereas no change in colour indicates a negative result[50].

3.5 Antibiotic testing :

Antibiotic susceptibility was assessed with a variety of antibiotics, including Kanamycin, Gentamycin, Cephatoxime, and Rifampicin. The bacterial suspension was evenly distributed across Mueller-Hinton Agar (MHA) plates and antibiotic discs were carefully positioned onto the surface after creating small wells using a sterile punch. Stock solutions of the antibiotics were made at a concentration of 1 mg/ml[54].

3.6 Prodigiosin extraction from the bacterial strain:

The bacterial isolate was grown in 2 L of nutrient broth (NB) and kept at 25°C for 72 hours, where pigment production was observed. Following incubation, the culture was centrifuged at 10,000 rpm for 10 minutes to pellets the bacterial cells, and the supernatant was discarded. The resulting pellet was then acidified with ethanol and again subjected to second round of centrifugation at 10,000 rpm for 10 minutes at 4°C. After extraction, the solvent i.e ethanol was evaporated under vacuum with a rotary evaporator. Lastly , the absorbance spectrum of the isolated pigment was measured using a UV spectrophotometer.

3.7 Characterization

3.7.1Prodigiosin preliminary identification& UV-vis -vis spectral analysis :

To first detect prodigiosin, the colour of pigment's was examined under both acidic and basic conditions. Two identical samples were prepared, with one exposed to hydrochloric acid and the other to sodium hydroxide. Using a spectrophotometer, the UV-Vis absorbance of prodigiosin in

basic and acidic solutions was scanned using a spectrophotometer n a wavelength of 200 to 800 nm, with pure ethanol as the reference [47][51]

3.7.2 Thin layer chromatography:

Prodigiosin was analysed and purified using thin layer chromatography (TLC). The pigment was applied to a silica gel plate, and Diethyl Ether (5 mL) was employed as the mobile phase. The plate was positioned in a developing chamber, permitting the solvent to rise through capillary action After drying, the pigment was observed under UV light. The Rf value can be found :[48]

$$Rf = distance\ travelled\ by\ compound \div distance\ travelled\ by\ solvent$$

3.8 Effect of different media on pigment :

1. **Nutrient Broth**: A total of 1.3 g of nutrient broth powder was dissolved in 100 ml of ultra-pure water in sterile conditions.
2. **Luria Broth**: 2.5 g of Luria broth powder was fully dissolved in 100 ml of ultra-pure water.
3. **Peptone Glycerol Broth**: Mixture containing 1 to 2 g of peptone and 10 ml of glycerol were thoroughly combined with 100 ml of ultra-pure water to create a uniform solution.
4. **Yeast Extract Broth**: Under sterile environment, 1 g of yeast extract, 2 g of peptone, and 0.5 g of sodium chloride were mixed and dissolved in 100 ml of ultra-pure water.
5. **Skim Milk Broth**: To prepare this media , 5 g of skim milk powder and 1.3 g of nutrient broth powder were dissolve in 100 ml of ultra-pure water.

3.9 Yield calculation[48]:

$$\text{Yield} = \text{Mass of final pigment} \div \text{Mass of biomass} \times 100$$

3.10 Media Optimization:

3.10.1 Effect of sugar source on prodigiosinpigment production:

Firstly 1.3 g of nutrient broth was mixed in 50 ml of distilled water and sterilized by autoclaving at 121°C. Further 0.5% (w/v) sugar source was weighed and mixed with autoclaved distilled water (50ml) and filtered in sterilized nutrient broth using whatman filter paper under aseptic conditions in a laminar airflow (LAF) cabinet. Subsequently the medium was inoculated with a bacterial strain under aseptic conditions. The inoculated flasks were kept at 20°C for 72 hours. After the growth period, the development of a crimson red pigment was observed in the culture broth. To extract the pigment, the culture broth initially to centrifuged at 10,000 rpm for 10 minutes to separate bacterial cells and debris. The resulting pellet was then acidified with ethanol and centrifuged again under the same conditions ab above , then the solvent was evaporated using a rota evaporator to obtain the pigment in powder form. The yield of the pigment was subsequently calculated [53].

- Different sugar sources used were sucrose, fructose, maltose , glucose, galactose, mannitol, lactose

3.10.2 Effect of maltose concentration on prodigiosin pigment production

Initially 1.3 g of nutrient broth was dissolved in 50 ml of distilled water and sterilized by autoclaving at 121°C. Different amounts of maltose (0.5 %,1%, 1.5% ,2% w/v of reaction culture) were weighed and mixed with autoclaved distilled water (50ml) and filtered in sterilized nutrient broth using whatman filter paper under aseptic conditions in a laminar airflow (LAF) cabinet. Subsequently the medium was inoculated with a bacterial strain under aseptic conditions. The inoculated flasks were kept for growth at 20°C for 72 hours. After the growth period, the development of a red pigment was observed in the culture broth. To extract the pigment, the culture broth was initially subjected to centrifugation at 10,000 rpm for 10 minutes to remove bacterial cells and debris. After The pellet was then acidified with ethanol i.e 2ml and again subjected to second round of centrifugation at 10,000 rpm for 10 minutes at 4°C, then the solvent was evaporated using a rota evaporator to obtain the pigment in powder form. The yield of the pigment was subsequently calculated [50].

3.10.3 Effect of NaCl concentration on prodigiosin pigment production

First of all 1.3 g of nutrient broth powder was mixed in 100 ml of distilled water and different amount of NaCl (0.5 %,1%, 1.5% ,2% w/v) were weighed and mixed with nutrient broth solution and further sterilized by autoclaving at 121°C . Subsequently the medium was inoculated with a bacterial strain under aseptic conditions. The inoculated flasks were kept for growth at 20°C for 72 hours. After the growth interval , the development of a red pigment was observed in the culture broth. To extract the pigment, the culture broth was initially subjected to centrifugation at 10,000 rpm for 10 minutes to remove bacterial cells and debris. The resulting pellet was then treated with ethanol for acidification and again subjected to second centrifugation at 10,000 rpm for 10 minutes at 4°C, then the solvent was evaporated using a rota evaporator to obtain the pigment in powder form. The yield of the pigment was subsequently calculated [54].

3.10.4 Effect of amino acid on prodigiosinpigment production:

Firstly 1.3 g of nutrient broth was mixed in 50 ml of distilled water and sterilized by autoclaving at 121°C. Further 0.5% (w/v) amino acids were weighed and mixed with autoclaved distilled water (50ml) and filtered in sterilized nutrient broth using whatman filter paper under aseptic conditions in a laminar airflow (LAF) cabinet. Subsequently the medium was inoculated with a bacterial strain under aseptic conditions. The inoculated flasks were kept at 20°C for 72 hours. After the growth interval, the development of a red pigment was observed in the culture broth. To extract the pigment, the culture broth was initially subjected to centrifugation at 10,000 rpm for 10 minutes to remove bacterial cells and debris. The resulting pellet was then acidified with ethanol and again subjected to second round of centrifugation at 10,000 rpm for 10 minutes at 4°C, then the solvent was evaporated using a rota evaporator to obtain the pigment in powder form. The yield of the pigment was subsequently calculated [53]

- Different amino acid used are methionine, cysteine, glycine, lysine, glutamic acid , arginine

3.11 Antioxidants assay :

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is a standard spectrophotometric method used to determine the antioxidant potential of prodigiosin. It works by assessing the compound's ability to donate hydrogen atoms or electrons, this lead to the reduction DPPH free radicals,accompanied by a noticeable colour change from deep purple to yellow. For the assay, a 0.1 mM DPPH solution was then mixed with varying concentrations of prodigiosin and incubated in the dark at room temperature for 30 minutes. Following incubation, the absorbance was measured at 517 nm[48].

$$\text{Scavenging Unit \%} = (\text{A 517 of control} - \text{A517 of sample}) \div \text{A517 of control}) \times 100$$

3.12 Applications of pigment:

Seven different types of paper—Whatman filter paper, blotting sheet, A4 sheet, filter paper, filter paper cover sheet, tissue paper, and regular classmate register paper—were cut into 2 cm squares and labelled. A solution of prodigiosin in ethanol was applied to the papers, which were then put to maintain at 25°C for 15 minutes. Control samples, without the pigment, were also prepared. To test the pigment fastness, the samples were immersed in hot (60-70°C) and cold (room temperature) water for 30 minutes, as well as in alkaline (NaOH) and acidic (HCl) solutions for 30 minutes . Afterward, the papers were air-dried, and any loss or leaching of the pigment was observed visually[48]

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Reviving of bacteria: Reviving of bacteria from the previously culture present in Jaypee Institute of Information Technology , Solan present in genomic laboratory.

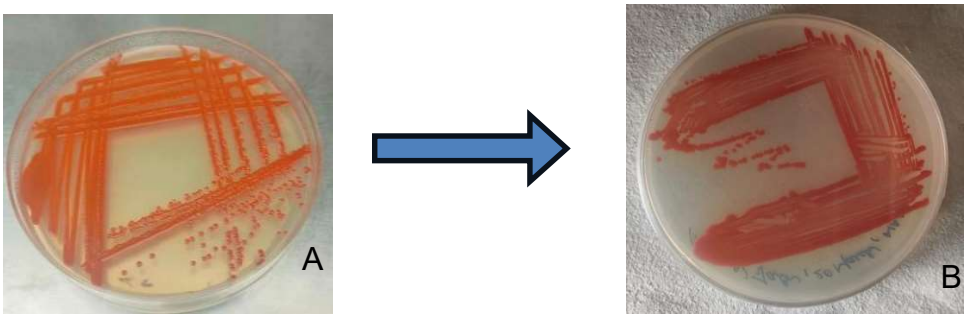
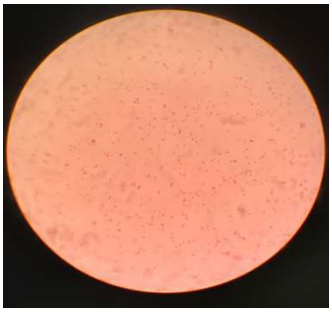


Fig 4.1 Pigmented colony isolated on the nutrient agar plate where A= old bacterial culture B=New revived culture

Reviving of strain from previously present in the genomic laboratory was done, then bacteria as streaked on the nutrient agar plate after 3 days that is 72 hours deep red colonies were appeared Kulandaisamyet al. found that after streaking the bacteria ,it was found that colonies appear crimson red in colouration and the growth of colonies appeared after 72 hour when kept at 20°C .

Gram staining: Gram staining was carried out to assess the morphological characteristics and determine the Gram nature of the bacterial strain. After air drying, The slide was analysed under a microscope at 100X magnification. The observed cells were pink in colour and cocci in shape,



indicating that the strain is Gram-negative.

Fig 4.2 Gram Staining*:* Microscopic observation at 100X revealed pink, cocci-shaped cells, confirming the strain as Gram-negative.

After Gram Straining is done bacteria, it was observed as that the bacteria has cocci – shaped cells when final strained with safarnin and revealed pink cells which further indicated that the bacterial strain is Gram-negative in nature .Kulandaisamy et al. [49] study suggested that the bacteria was Gram negative in nature after performing the procedure of Gram staining. C Ghosh study suggested the same biochemical study of strain[52].

4.3 Biochemical Tests

Table 4.1 Biochemical analysis of bacterial strain

Test	**Result**	**Output**
Gram Straining	Negative	Bacterial strain is gram negative
KOH String Test	Positive	Bacterial strain becomes vicious and stringy
Catalase Test	Positive	Bubbles appear indicates the presence of catalase
Casein Hydrolysis	Positive	Clear zones forming around the bacteria strain which shows +ve result
Phenylalanine Deaminase Test	Negative	Yellow colour indicating negative result of phenylalanine Deaminase Test

The biochemical test was done to understand the characterization of bacteria in which grams Straining result indicates hats the bacteria belongs to grams negative family. When catalase test was done itsFor the catalase test, hydrogen peroxide (H_2O_2) was applied to a bacterial smear on a microscope slide. The rapid appearance of bubbles signified a positive catalase reaction. The bacterial strain was inoculated onto skim milk agar plates using the quadrant streaking technique

and After incubating at 37°C for 24 hours, clear zones were formed around the colonies, indicating a positive result for casein hydrolysis. The KOH string test yielded a positive result, as the bacterial strain turned viscous and formed a string when lifted. This indicates a Gram-negative bacterial reaction. The phenylalanine deaminase test resulted in a negative reaction, as shown by the yellow colour of the medium. This indicates that the bacterial strain does not possess phenylalanine deaminase. The lack of green coloration upon adding ferric chloride further confirmed the negative result.Ghosh et al[50] did the same work on the strain and attain the same result as discussed above [50].

4.4 Antibiotic susceptibility test :

Antibiotic susceptibility was tested using various antibiotics, such as Kanamycin, Gentamycin, Cephatoxime, and Rifampicin.The bacterial suspension was evenly distributed across Mueller-Hinton Agar (MHA) plates and antibiotic discs were carefully positioned onto the surface after creating small wells using a sterile punch. Dimethyl sulfoxide (DMSO) was used as a negative control. The stock solutions of the antibiotics were prepared.

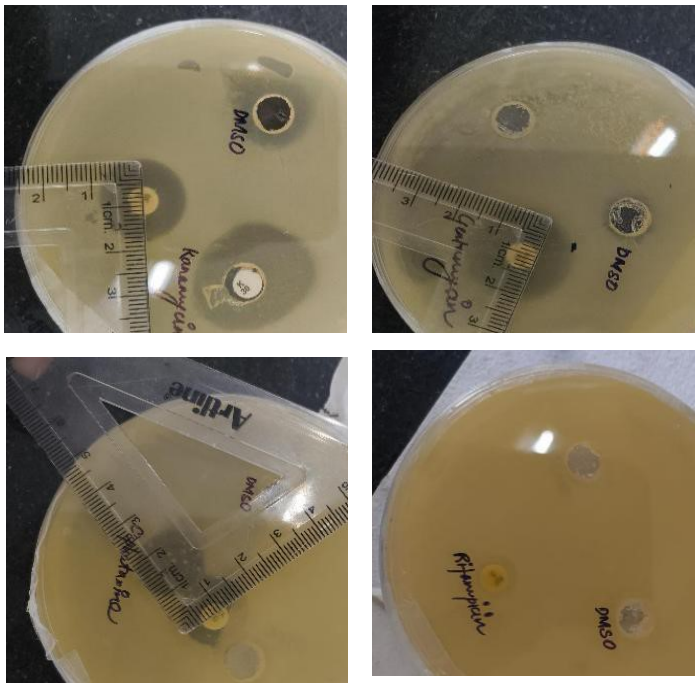


Fig 4.3 Antibiotic susceptibility test for bacterial strain

Table 4.2 Antibiotic susceptibility test results

Antibiotic	**Zone of inhibition diameter (cm)**	**Resistant**
Kanamycin	1.9	Susceptible
Gentamycin	2.2	Susceptible
Cephatoxine	1	Susceptible
Rifampicin	Nil	Resistant

Prodigiosin bacteria[100 ul] was put in the wells and then disc were placed above it to check its antibiotic susceptibility for the same antibiotic [50]where Kanamycin, Gentamycin, Cephatoxime are Susceptible where as Rifampicin is Resistant for the pigment [54]

4.5 Extraction of pigment:

The bacterial isolate was grown in 2 L of nutrient broth (NB) and kept for growth at 25°C for 72 hours, where pigment production was observed. Following incubation, the culture was centrifuged at 10,000 rpm for 10 minutes to pellets the bacterial cells, and the supernatant was discarded. The resulting pellet was then acidified with ethanol and again subjected to second round of centrifugation at 10,000 rpm for 10 minutes at 4°C. After extraction, the solvent i.e ethanol was evaporated under vacuum with a rotary evaporator. Lastly , the absorbance spectrum of the isolated pigment was measured using a UV spectrophotometer[49]

4.6 Characterization

4.6.1 Prodigiosin preliminary identification test :

The pigment obtained in acidified ethanol displayed a reddish-pink coloration in acidic medium, indicating the possible presence of prodigiosin. This was further validated by UV-Vis spectrophotometric analysis, which revealed an absorption peak in the range of 535–540 nm,

matching the typical spectrum associated with Prodigiosin alkaline pH, prodigiosin undergoes a noticeable colour change, typically fading from deep red to yellow or colourless, as a result of structural modifications. This pH-responsive property is a key indicator used in the identification of prodigiosin.

4.6.2 Thin layer chromatography :

Prodigiosin was analysed and purified using thin layer chromatography (TLC). The pigment was applied to a silica gel plate, and Diethyl Ether (5 mL) was employed as the mobile phase. The Rf value given below.

$$Rf = distance\ travelled\ by\ compound \div distance\ travelled\ by\ solvent7$$

Result of TLC,Rf value was found to be 0.61 its ground lie between 0.6-0.81[53]**.**Study revealed that on the characterization of prodigiosin from *Serratiamarcescens* NJZT-1 , when using a solvent system of dichloromethane: ethyl acetate (1:5) on silica gel Gf254 plates where found to 0.61[52].Another study involving *Serratiamarcescens* KH1R found Rf values ranging from 0.64 to 0.96, depends on the specific strain used for study[55]

4.7 Effect of different media on Prodigiosin pigment production :

- Diverse media like Luria Broth, Skim Milk Broth, Yeast Extract Broth, Nutrient Broth, Peptone Glycerol Broth were used to calculate prodigiosin yield.

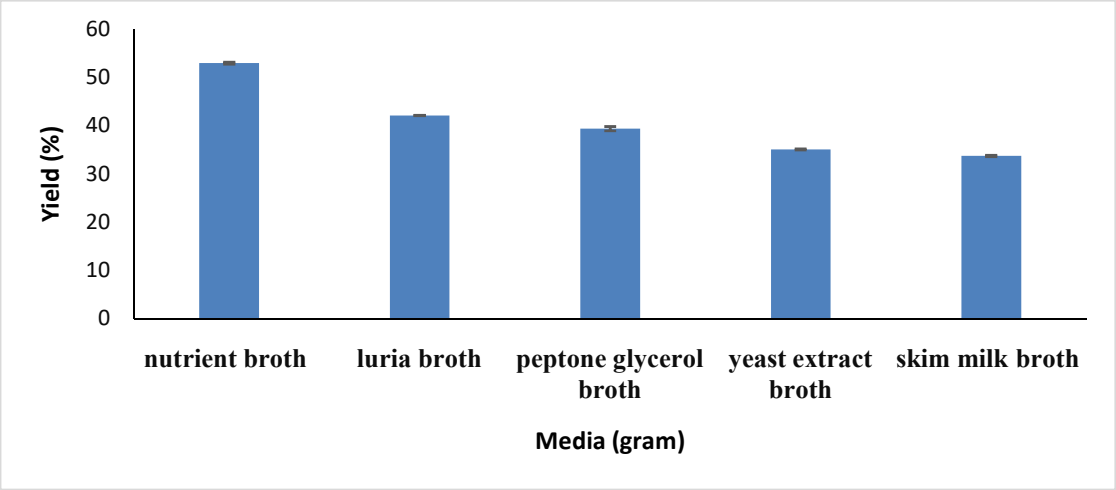


Fig 4.4 Effect of different types of media on prodigiosin pigment production

The highest prodigiosin yield among the above media used were found to be with nutrient broth ,because of its useful composition which can be effective for pigment synthesis. Similar study by Tania Paul et al.[51]the highest production of pigment (15 mg/L) was estimated in the nutrient broth medium when compared to other media,followed by peptone glycerol, then tryptone soy broth, Luria–Bertani broth, and glycerol beef broth. Study conducted byA.V Giri et al.[54] suggested the same result. The nutrient broth that is most favourable in the present study for the production of pigment may contain the necessary nutrients. Beef extracts, yeast extracts, NaCl, and peptone are key components of nutrient broth media[55] Peptone is found to be a good nitrogen source and has fatty acids and amino acids[55]. Yeast and beef extracts provide additional nutrients like carbohydrates, vitamins, salt, and other organic nitrogen compounds in it [55].So NB has all the major nutrients or components which is required for the production of pigment prodgiosin.

4.8.1 Effect of sugar source on prodigiosin pigment production

0.5 % of different sugar source were dissolved in 1.3 g of nutrient broth powder (100 ml) in ultra-pure water under sterile conditions and then calculated its yield

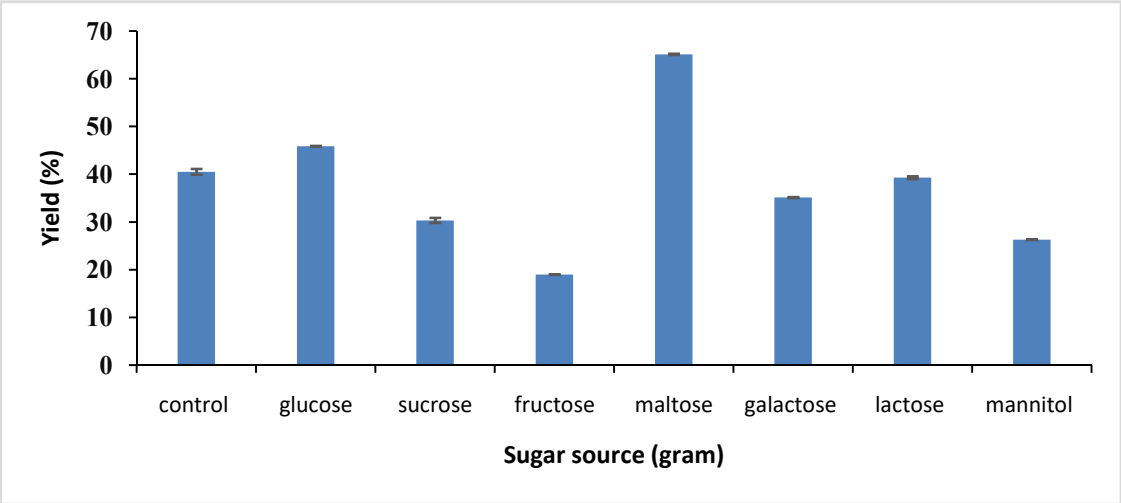


Fig 4.5 Effect of sugar source on prodigiosin pigment production

During the early growth phase, bacteria utilize carbon sources primarily for building cell structure and generate ATP.However, as they pass to into the stationary phase—when growth slows due to nutrient exhaustion,they begin producing secondary metabolites like prodigiosin pigments .This shift allows the organism to use available carbon resources efficiently and may confer competitive advantages in the surroundings. Among the various sugars tested above , maltose led to the highest prodigiosin production asit slow release helps maintain steady bacterial metabolism and synthesis of pigment . Not like fast-acting sugars such as glucose, maltose doesn't induce catabolite repressioni.ea regulatory mechanism employed by certain bacteria to use preferred carbon sources, such as glucose, over other carbon sources, allowing efficient pigment production[50]. Therefore, it proves to be the most effective,efficient and reliable carbon source for boosting prodigiosin yield. For instance similar study by C Ghosh and S Patel[51] demonstrated adding maltose(0.5%) to nutrient broth doubled prodigiosin yieldat 28°C and 30°C and with nutrient broth added with glucose showed a twofold increase only at 28°C. According to Tania Paul et al.[52] Various carbon sources such as dextrose, lactose, sucrose, maltose, mannitol, and fructose were added in nutrient broth media with a concentration of 0.05% (w/v). The maximum prodigiosin production (17.8 mg/L) was obtained when lactose

was added to the nutrition broth . Maltose is the second most yield providing (13.5mg/L) supplement after lactose, followed by other carbon sources such as mannitol, fructose, glucose, and sucrose,since other carbon sources may inhibit prodigiosin yield by lowering the pH of the medium or suppressing catabolites[52][53]. The presence of maltose,being a disaccharide sugar as an additional carbon source in nutrient broth hasbeen shown to enhance prodigiosin production compared to other carbon sources such as glucose as it maintain a steady slow release of carbon [53].To contradict above result ,for examplePereira et .al[56]found sucrose was optimal for their *Serratiarubidaea*strain. Study byNguyen et al.[53] showed lactose and sucrose significantly boost prodigiosin (e.g. lactose +18%, sucrose +12%), whereas glucose often inhibit prodigiosin yield due to catabolite repression [57][53].

4.8.2 Effect of Maltose concentration on prodigiosin pigment production

Different concentrations of Maltose was added in nutrient broth to observe its effect on pigment production and calculate its yield

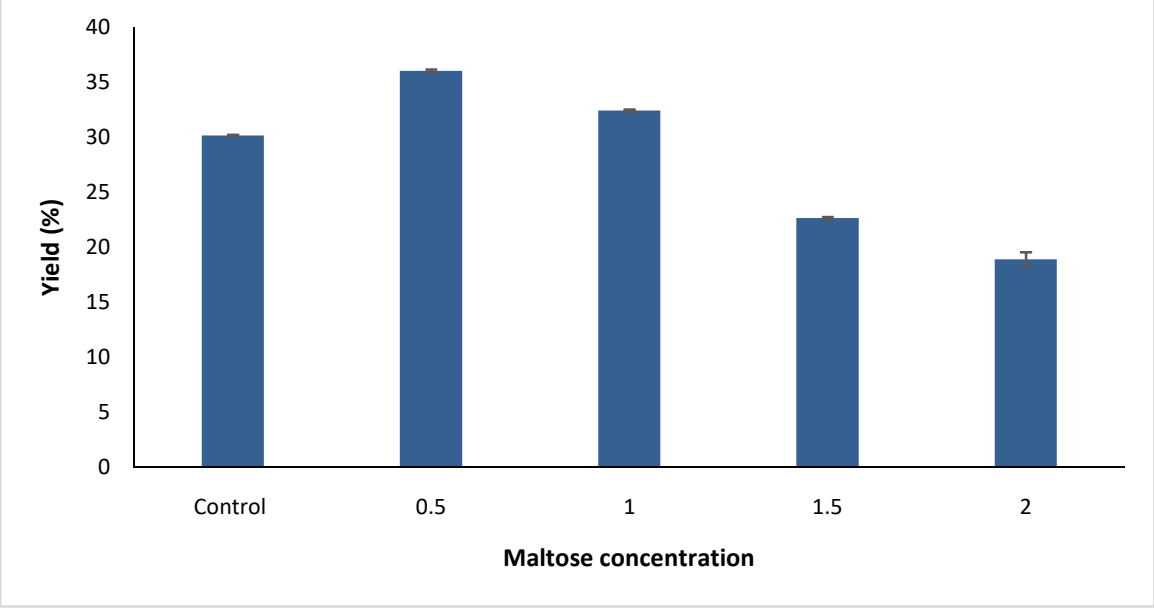


Fig 4.6 Effect of Maltose concentration on prodigiosin pigment production

Maltose (being a disaccharide sugar source)concentration has a significant impact on prodigiosin production. At 0.5%, it provides the ideal ATP for bacterial growth and pigment biosynthesis, without overwhelming the system with excess carbon source that could lead to catabolite repression. This concentration proves to be the most efficient for enhancing prodigiosin yield . According to study suggested by A.V Giri et al.[58] In nutrient broth at 28 °C, *S. Marcescens* produced 0.52 mg/mL prodigiosin with no sugar, but adding 0.5% maltose raised this to 1.836 mg/mL (a ~3.5× increase), by contrast 0.5% glucose gave 1.689 mg/[54]. Thus 0.5% maltose gave the highest yield [58]. These findings suggests that 0.5% maltose is optimal under the examined conditions, whereas higher maltose levels do not further enhance and may even inhibit pigment production. This optimization study confirms that maltose is a best carbon source for prodigiosin in nutrient broth media[59]. A recent review suggested that yield found to be 1836 mg/L prodigiosin at 0.5% maltose (vs. 1689 mg/L at 0.5% glucose), again imply that 0.5% maltose gave the highest reported yield among tested sugars[54].Similar study by , Sundaramoorthy et al.[58]found that maltose additionaled to the highest yield of 425 ± 40 mg/L under submerged fermentation conditions study . 0.5% maltose amount in nutrient broth at 28 °C has been shown to significantly enhance pigment synthesis without stimulating catabolite repression.Understanding the regulatory mechanism, including the role of cAMP, is essential for developing efficient and effective steps for pigment production. In *S. marcescens* this secondary messenger works as a negative regulator of pigment synthesis.The intracellular concentration of cAMP is dependent on the availability of carbon sources,hence , the presence of glucose can increase cAMP levels, triggering catabolite repression of secondary metabolite pathways of pigment biosynthesis[59][62].

4.8.3 Effect of NaCl concentration on prodigiosinpigment production

Different concentrations of NaCl was added in nutrient broth to observe its effect on pigment production and calculate its yield

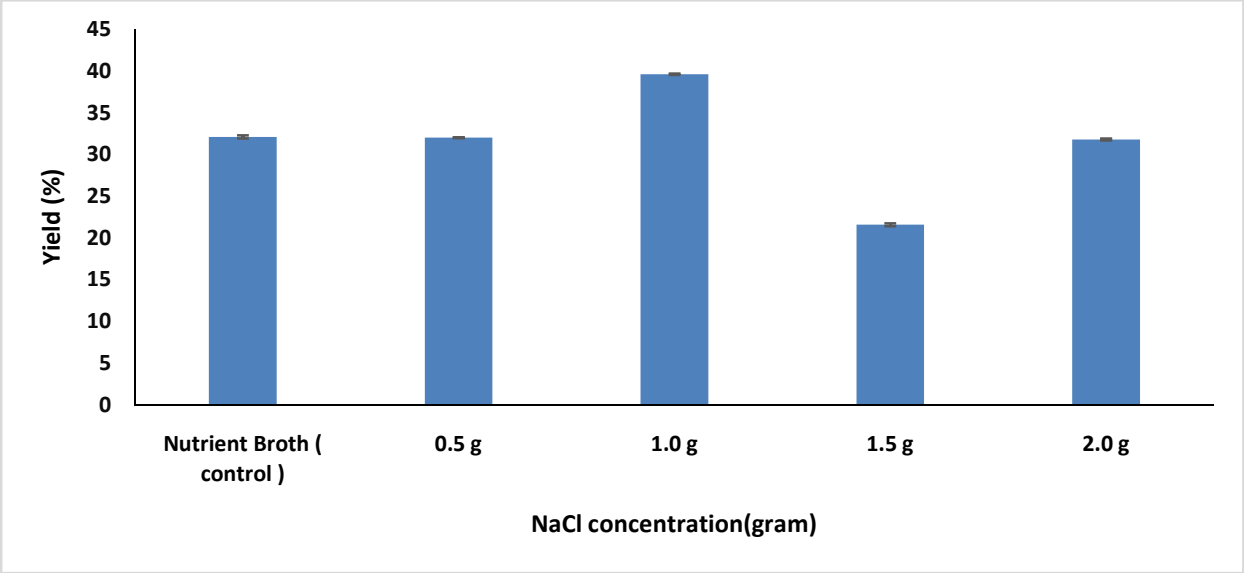


Fig 4.7 Effect of NaCl concentration on pigment production

NaCl concentration affects osmotic pressure and bacterial biosynthesis, influencing pigment production. Its concentration can affect quorum sensing mechanisms, which are critical for mechanisms of secondary metabolite pigments, including prodigiosin.At 1%, it creates ideal conditions to promote effective pigment synthesis. Higher concentrations may stimulate stress, inhibit both growth and pigment production. Thus, 1% NaCl achieves the best results for pigment production. The current study by C Ghosh and S Patel[51] indicated optimal pigment production by S. *nematodiphila* in NB (pH 8) has 1% NaCl, on growth period at 25 °C for 48 h under stationary conditions[50].Moderate salt tolerance is common factor . For example, prodigiosin was produced by *Vibrio gazogenes*had1–5% NaCl**.** Many studies find ~1–2% NaCl is optimal for growthover a 3-day period at 28°C in minimal media and NaCl amount ranging from 1 to 5% (w/v) were added to media [61].High-salt media would inhibit the growth of secondary metabolite during a scale-up production[62].To contradict above study , According to Tejada et al. [63] evaluated *S. marcescens* QSC23, tested NaCl from 0.5% to 2.5% and found best prodigiosin(516.0 ± 15.8 units/cell) at 1.5% NaCl. In that experiment, 1% NaClyielded noticeably less pigment than 1.5%.According to Pereira at al.[56] studied a marine *Serratiarubidaea*strain and using varying NaCl amount up to 7% ,they observed prodigiosin peaked at 2% NaCl (20 g/L)[59]. For example, Silverman & Muñoz , noted that very high salt (3% NaCl) inhibitedprodigiosin synthesis, whereas very low salt (0.1%) did not inhibit pigment

production[64].Another study by Rasha et al.[63] focused on optimization of pigment growth by *S. rubidaea*RAM_Alex, a marine strain extracted from bivalve samples in Egypt.Using statistical methods, the researchers found that a medium containing 10 g/L NaCl, with other nutrients, produce the high yield and pigment production was (~1600.511 mg/L).This further supports the fact that moderate NaCl amounts are preferred for pigment biosynthesis in *S. Rubidaea*[65].

4.10.4 Effect of Amino Acid on prodigiosin pigment production

0.5 % of different amino acid were added in nutrient broth to observe its effect on pigment production and calculate its yield .Amino Acid used were Methionine, cysteine, lysine, Glycine, Arginine, glutamine

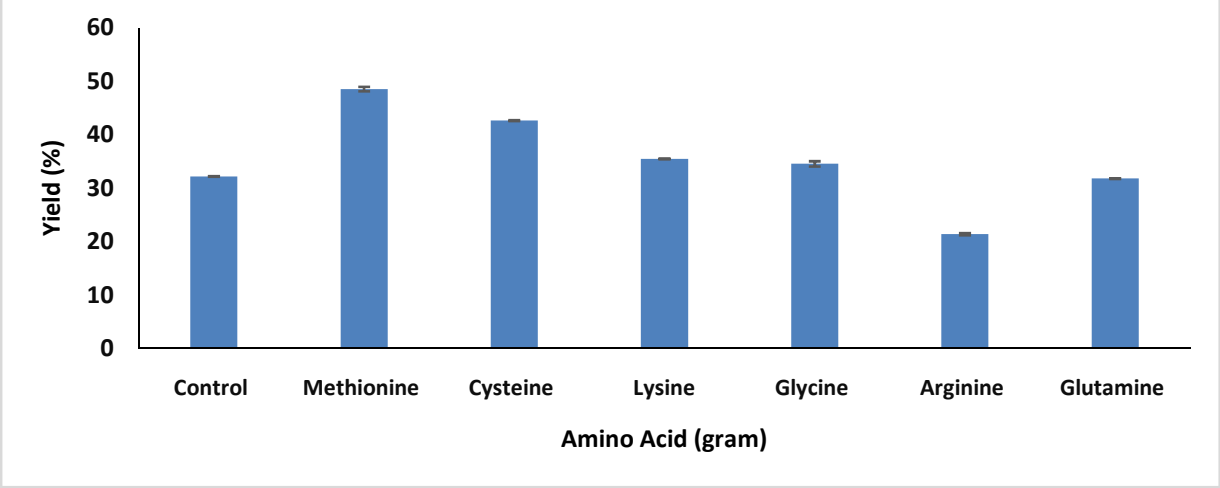


Fig 4.8 Effect of Amino Acid on prodigiosin pigment production

Methionine and cysteine are effective because of their sulphur content is vital for pigment mechanismof prodigiosin (PG), a red pigment produced by *Serratia marcescens*, involves the process of production of precursors, notably 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC).The biosynthesis of MBC requires the presence of notable amino acidswhich are proline, serine, and methionine.These amino acids serve as building blocks in the mechanism f MBC, which subsequently condenses with another precursors to form pigment[66]. These amino acids support the enzymatic processes involved in prodigiosin synthesis and help sustain cellular

redox balance, increases pigment production[68].Thus, methionine and cysteine provide the most optimum conditions for maximizing prodigiosin yield.Qadri and Williams [67] observed that methionine amino acid, when combined with other amino acids, significantly increased prodigiosin synthesis.This suggests a synergistic effect methionine, works well when other amino acids are added along with it , contributes to the efficient synthesis of pigment.Moreover, methionine was found to shorten the lag phase in pigment production, leading to increased yield.[67] According to study conducted by C Ghosh and S Patel [50] among all the amino acids tested , methionine and cysteine did not inhibit pigment at the concentrationof 0.1%. In fact ,it was observed that that lower concentration specifically 0.006% cysteine and 0.02% methionine significantly enhanced prodigiosin production[50]. Another study similarly reported that methionine and cysteine effectively promoted prodigiosin synthesis at concentration of 0.01 and 0.03% respectively[66].To contradict above study , Nguyen reported that supplements such as glutamic acid, proline, etc. gave a 1.6–1.7× increase in prodigiosin synthesis and proline addition as most beneficial for Serratia's pigment production [53].

4.9 Antioxidants Assay

In the assay, a 0.1 mM DPPH solution dissolved in ethanol is mixed with varying concentrations of prodigiosin and kept to incubate in the dark for 30 minutes at RT . Absorbance is then measured at 517 nm, with a larger decrease in absorbance reflecting greater antioxidant activity.

$$\text{Scavenging Unit \%} = (\text{A 517 of control} - \text{A517 of sample}) \div \text{A517 of control}) \times 100$$

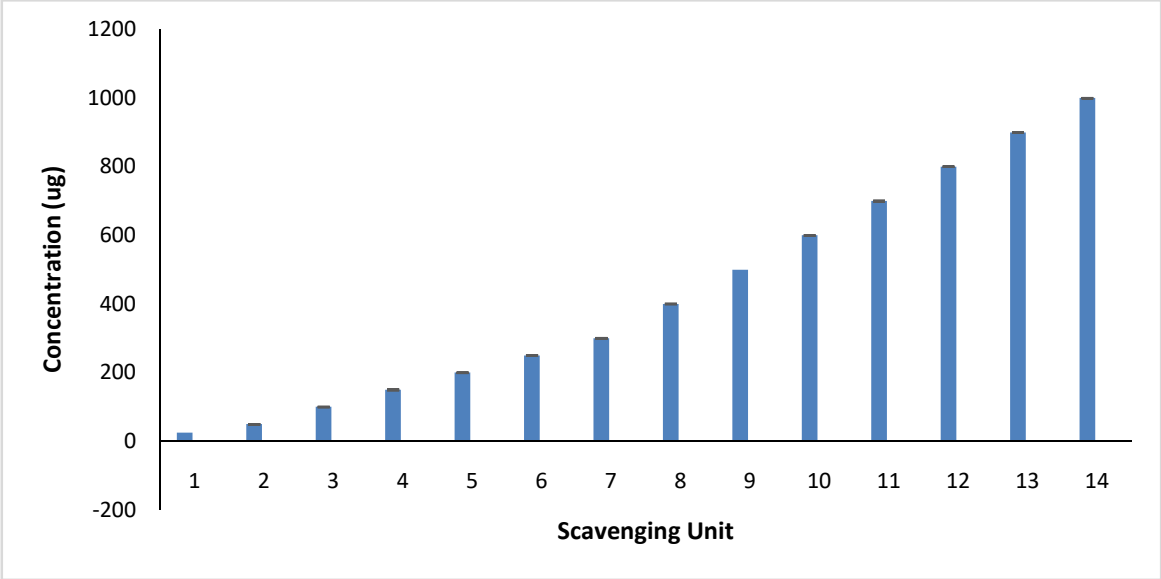


Fig 4.9 Antioxidant Assay

The Scavenging Unit is coming in negatives in above study for the concentrations of 400-1000 ml. To contradict the above study Arivizhivendhan et al. found that prodigiosin completely scavenged DPPH radicals at a concentration of 10 mg/L .which is not found in the above study as in the above study DPPH is not properly reduced no colour was observed above[48][68].

4.10 Applications of Pigment

Seven different types of paper—Whatman filter paper, blotting sheet, A4 sheet, filter paper, filter paper cover sheet, tissue paper, and regular classmate register paper—were cut into 2 cm squares and labelled. A solution of prodigiosin in ethanol was applied to the papers, which were then put dry at 25°C for 15 minutes. Control samples, without the pigment, were also prepared. To test the pigment fastness, the samples were immersed in hot (60-70°C) and cold (room temperature) water for 30 minutes, as well as in alkaline (NaOH) and acidic (HCl) solutions for 30 minutes . Afterward, the papers were air-dried, and any loss or leaching of the pigment was observed visually[40].

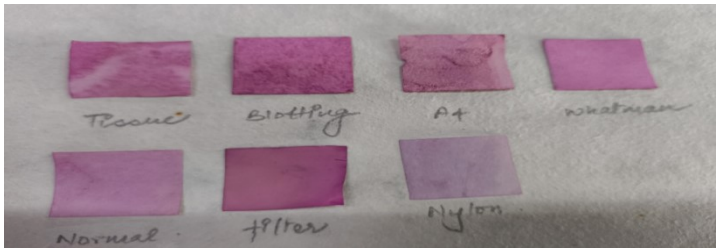


Fig 4.10 Different types of paper samples were dyed with prodigiosin pigment

A.Pigmentfastness test with hot and cold water:

This test was conducted to determine the stability and retention of prodigiosin on dyed paper when exposed to water at different temperatures. Upon immersion in both hot and cold water, no dye was released, indicating strong adherence of the pigment to the paper. These results reflect high water resistance and thermal stability of prodigiosin.

4.12.1 Hot Water: To evaluate thermal stability, prodigiosin-dyed paper was immersed in hot water

When paper dyed with prodigiosin was exposed to hot water treatment , no colour was detected in the water, suggesting that the pigment was not readily released or solubilized under these conditions. This indicates that prodigiosin remains stable and does not easily leach into water when heated. Hence , can be used as a biodegradable dye .According to Olukannietal. [73]found that prodigiosin (propylprodigiosin) is stable under heat, salt, and acidic conditions, but changes to yellowin alkaline conditions (structural break down)[69].Olukanni also reported0% dye fade and after strong wash [73] and Mouro et al.[74] noted "very good fastness properties" for prodigiosin-dyed textiles[73][75].

4.12.2 Cold Water: To evaluate thermal stability, prodigiosin-dyed paper was immersed in hot water

No dye was observed to leach into cold water, indicating that prodigiosin remains stable and is not easily soluble at lower temperatures or in cold water [62].According to Shen and Yang[71]study they observed that prodigiosin has limited water solubility because of its

hydrophobic nature , which correlates with findings that the pigment does not readily leach into water under hot or cold condition[71].Gulani and colleagues explored the application of prodigiosin as a fabric dye and subjected the dyed materials to various treatments ,including exposure to acid ,alkali ,cold water and hot water .They observed that the colour of the dye was completely intacted in cases of cold water treatments[72].

B. Pigment fastness with NaOH(alkaline solution) and HCl (acidic solutions):

The dyed paper was treated with NaOH (alkaline) and HCl (acidic) solutions to assess prodigiosin's stability. No noticeable dye leaching occurred in either solution, suggesting that prodigiosin is stable and resistant to both acidic and alkaline conditions.

4.12.3 Alkaline solution： The dyed paper was subjected to an alkaline NaOH solution to test the stability of prodigiosin

Upon treatment with NaOH solution, the paper exhibited a yellow coloration, indicating a reaction with prodigiosin. However, no pigment leaching was observed, implying that the prodigiosin remained attached to the paper without diffusing into the solution. According to Olukanni et al.[73]found that prodigiosin (propylprodigiosin) is stable under heat, salt, and acidic conditions, but changes to yellowin alkaline conditions (structure break down)[71].Gulani and colleagues explored the application of prodigiosin as a fabric dye and subjected the dyed materials to various treatments ,including exposure to acid ,alkali ,cold water and hot water .They observed that the colour of the dye was completely intacted in cases of cold water treatments[76].

They observed that the colour of the dye was completely intacted in cases of acid and cold-water treatments to reject the study above , slight discoloration occurred after alkali treatment[69].

4.12.4 Acidic solution： The dyed paper was treated with an acidic HCl solution to test the stability of prodigiosin

Treatment with HCl resulted in no dye leaching, suggesting that prodigiosin remained stable and did not diffuse into the solution under acidic. According to Olukannietal.[73] found that prodigiosin (propylprodigiosin) is stable under heat, salt, and acidic conditions, but changes to yellowin alkaline conditions (structure break down).Gulani and colleagues[76] investigated the dyeing of fabrics with prodigiosin and subjected them to varying treatments including acid, alkali, cold water, hot water . They observed that the colour of the dye was completely intacted in cases of acid treatments[75][76].

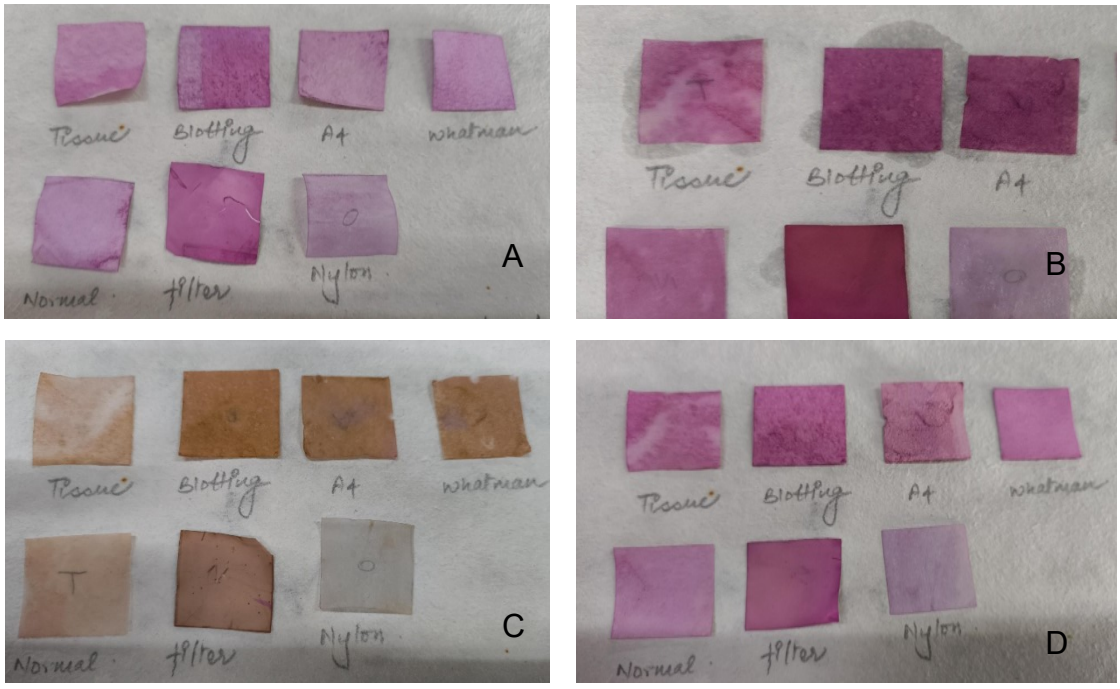


Fig4.11[A] After Hot water treatment

[B] After cold water treatment

[C] After alkaline solution treatment

[D] After acidic solution treatment

CONCLUSION

The purification and characterization of a prodigiosin pigment-producing bacterial strain from the Himalayan region and its applications, highlight the vast microbial diversity and biotechnological,industrial potential of this unique pigment . This study suggests the adaptive strategies employed by bacteria in extreme harsh conditions, emphasizing the Himalayas as a valuable source of pigment-producing microorganisms with unique properties and applications and characteristics. The bacterial isolate exhibits antibiotic resistance,UV protection, and biodegradable dye , demonstrating Prodigiosin potential in combating pathogenic bacteria and paving the way for its use in biomedicine and food preservation, industrial uses . This research is focused on the significance of exploring and conserving the microbial diversity of high-altitude regions like the Himalayas, enhancing our understanding of microbial ecosystems in extreme harsh environments and their potential applications in various industries and fields.

FUTURE PERSPECTIVE

- It can be used as antibacterial, antifungal, and antiviral for developing antibiotics. It exhibits strong anticancer activity, immunosuppressive effects, because of its algicidal qualities, prodigiosin is useful in reducing toxic algal blooms and promoting environmental preservation.
  It can be used as alternative to synthetic dye in dyeing industries, it can used as bio control agent or bio pesticides, in the field of agriculture

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