Isolation and characterization of a Prodigiosin pigment producing bacterial strain from Himalayan region

Dissertation submitted in partial fulfillment of the requirement for the degree of

Master of Science

in

Microbiology

By

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Under the supervision of

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to

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this report entitled **"Isolation and characterization of a Prodigiosin pigment producing bacterial strain from Himalayan region"** in partial fulfillment of the requirements for the award of the degree of **Master of Science** in **Microbiology** submitted to the Department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from August 2023 to May 2024 under the supervision of **Dr. Ashok Kumar Nadda, Department of Biotechnology and Bioinformatics JUIT Waknaghat Solan H.P.**

I also authenticate that I have carried out the above-mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Ashish Kumar

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SUPERVISOR'S CERTIFICATE

This is to certify that the project work titled "**Isolation and characterization of a Prodigiosin pigment producing bacterial strain from Himalayan region**" by **Ashish Kumar (225112003)** during their end semester in fulfillment for the award of degree of Masters of Science in Microbiology from Jaypee University of Information Technology, Solan 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)

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Date:

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Ashish Kumar

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LIST OF ABBREVIATIONS

S.No.	Abbr.	Abbreviation(s)
1.	°C	Degree Celsius
2.	NA	Nutrient Agar
3.	NB	Nutrient broth
4.	min	Minute
5.	Gm	Gram
6.	%	Percentage
8.	ml	Milliliter
9.	mg	Milligram
10.	H_2O_2	Hydrogen peroxide
11.	HCl	Hydrochloric acid
12.	EtOH	Ethanol
13.	PG	Prodigiosin
14.	LAF	Laminar airflow
15.	SMA	Skim milk agar
16.	DMSO	Dimethyl sulfoxide
17.	nm	Nanometer
18.	Rpm	Rotation per minute

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ABSTRACT

The Himalayan region, known for its high-altitude environment, is home to a diverse array of microorganisms. The objective of this research is to identify and characterize a bacterial strain that produces prodigiosin pigment from a soil sample. The strain was characterized for its pigment production, growth optimization, and biochemical properties. The optimal conditions for prodigiosin production were determined, including temperature, pH, and nutrient sources. Biochemical tests confirmed the strain's ability to utilize carbon and nitrogen sources. Antibiotic susceptibility testing revealed the strain's resistance to several antibiotics. The isolate was identified as a Gram-negative, cocci shaped bacterium. After being removed from the microbial culture, the prodigiosin pigment was detected through UV-Vis spectroscopy. The potential of the Himalayan area as an origin of bacteria that produce pigment and the importance of prodigiosin in biotechnology are highlighted by this study. Prodigiosin shows antibiotic, anticancer and antioxidant properties which can be used in medicines and development of materials.

Keywords: *Himalayan region, Prodigiosin pigment, Growth optimization, Temperature, pH, UV-Vis spectroscopy*

CHAPTER 1

INTRODUCTION

Pigments are defined as finely separated, mostly water-insoluble colorants that reflect and absorb visible light to produce a range of colors. Compared to dyes, they have a higher molecular weight, are less soluble in water, and are less transparent[1].The use of pigments dates back thousands of years, to the early periods of ancient civilizations (Egypt, China, and India). Natural vegetation, insects, and minerals were utilized for a variety of purposes, including painting, food coloring, textile dying, body painting, and coloring religious rituals. William Henry Perkin, an English chemist, created the first organic dye in 1856 using coal tar distillate, which paved the way for the manufacture of artificial colors. But because they are the result of chemical interactions, they severely stress the environment [2]. Microbial colorants, or food coloring agents, are easy to make and have a smooth down streaming process. They are among the primary naturally occurring colorants found in microorganisms, plants, insects, and metals. While many natural pigments exist, only a few number are readily available in large enough amounts for commercial use. Microorganisms are a better source of pigment than other sources because of their quick growth, which might result in a high product productivity. While some bacteria create colors that are soluble in fat, others make pigments that are soluble in water and diffuse throughout the media in which they thrive [3]. Numerous colors, including water-soluble ones like carotenoids, prodigiosin, and violacein, are produced by microorganisms. Because synthetic dyes are damaging to the environment and cause cancer, it is better to use natural beginning materials when producing green technologies. A rising interest in natural color sources has resulted from environmental safety and conservation efforts. Microbial pigments are a viable substitute for plant pigments, providing year-round, fast, and infinite output [4].

The generation of microbial pigments is simple, needs less expensive growth media, and is not affected by the weather. The objective of this research is to separate microorganisms that produce pigments from different sources and enhance physical and chemical characteristics for the larger-scale synthesis, extraction, and identification of these pigments. This strategy is founded on the expanding understanding of environmental preservation and safety[5].

A wide variety of microbes, including bacteria, yeasts, fungi, and algae, possess the remarkable ability to synthesize natural pigments. These pigments encompass a diverse range of categories, including carotenoids, flavins, phenazines, violaceins, and melanins[3]. Moreover, these pigments are fundamental to the ability of bacteria to naturally adapt to harsh environments and carry out vital biological processes, such as photosynthesis with photosynthetic microorganisms[6]. Furthermore, due to their unique properties and beneficial characteristics, these naturally produced pigments have garnered significant interest in various industries. Their uses are beneficial to both humans and the environment in a variety of industries, including food, cosmetics, pharmaceuticals, and textiles [7]. Pigments, the naturally occurring colored chemicals, are essential to the survival and adaption of a wide range of animals, from microorganisms to humans. Their capacity to reflect or absorb particular light wavelengths contributes to the planet's visual diversity and serves vital physiological purposes, including UV protection and photosynthesis support[8], [9].With its harsh climate, the Himalayan region provides an exceptional, as-yet-largely unexplored storehouse of microbial diversity that contains important pigment-producing microorganisms such as those that produce the vivid prodigiosin [9]. Promising results have been obtained from isolating and analysing prodigiosin pigment-producing bacterial strains from Himalayan area, which will aid in the discovery of new pigments as well as our knowledge of their functions in microbial survival.

This study highlights the tremendous potential of using the diversity of Himalayan microbes for biotechnological uses, which can produce colors with remarkable qualities for use in industry. Researchers can find new uses for these pigments, such as antibacterial drugs or anti-cancer treatments, by isolating and characterizing them, which highlights the significance of these organic substances in the advancement of biotechnological domains. Prodigiosin is a bright red pigment that is quite rare in the microbial diversity of the Himalayan area. Prodigiosin is distinguished from more widely distributed pigments like melanin, violacein, and carotenoids by its special qualities and restricted availability [10]. These include acting as an energy storage medium, providing as a metabolic precursor for vital biochemicals like proline or NADPH, and acting as an air diaspora for bacteria [11].

Furthermore, its versatility and usefulness within microbial communities are demonstrated by its capacity to promote ion exchange and its energy-spilling role in *Serratia marcescens*. Pigments play a crucial role in the survival and adaptation of various organisms, with microbial pigments standing out for their natural origin and broad spectrum of biological activities [12]. The Himalayan region is a unique reservoir of microbial diversity, holding valuable pigment-producing microbes like those generating the vibrant prodigiosin. Isolating and characterizing prodigiosin pigment-producing bacterial strains from the Himalayan region has shown promising outcomes for the exploration of new pigments and understanding their roles in microbial survival [13]. Prodigiosin is notably rare in the microbial diversity of the Himalayan region and stands out due to its unique properties and limited presence. Its proposed eco-physiological roles include functioning as an air diaspora for bacteria, serving as a metabolic precursor for essential biochemicals, and facilitating ion exchange and energy spilling functions [14]. Additionally, prodigiosin has potential biotechnological applications, such as being used as a natural dye in food colorants, staining agent, and antimicrobial agent. The extraction and application of prodigiosin from Himalayan strains face significant challenges due to its rarity and the complexity of biosynthesis controlled by quorum sensing. Future research is directed towards understanding these biosynthetic pathways better and manipulating them for enhanced production of prodigiosin.

Genetic integration techniques have been employed to enhance prodigiosin production. The isolation process involves sample collection and initial processing, preparation and dilution of soil samples, microscopic examination and strain identification, and genetic integration techniques. Screening and selection of prodigiosin-producing clones, optimization of culture conditions, and characterization methods for prodigiosin pigment are also essential steps in the isolation and characterization process [15]. Prodigiosin plays a vital role in environmental significance, including photoprotection, bioremediation, waste management, antimicrobial activity, biocontrol, sustainable agriculture, and supporting biodiversity. In terms of biotechnological implications, prodigiosin has potential applications in various fields and is recognized for its extensive range of biological activities, including antibacterial, antifungal, and anticancer properties [11]. Prodigiosin, a vibrant red pigment with a unique pyrrolyl pyrromethane skeleton, has

garnered attention due to its extensive potential applications across diverse fields, including medicine, agriculture, industry, and environmental management [16]. Its cost-effective production strategies and broad spectrum of biological activities make prodigiosin a promising candidate for different industrial, environmental, and health-related applications [17]. Prodigiosin has shown promising anticancer activity and synergistic effects with other antibiotics, which could significantly advance antimicrobial therapies [18]. However, there are challenges in culturing and producing prodigiosin at scale, including optimizing culture conditions, utilizing byproducts and wastes, genetic and microbial strain challenges, scale-up and industrial application, and regulatory and clinical challenges.

Objectives of the study

- Isolation and screening of pigment producing bacterial strain
- Optimization of growth conditions for bacterial isolate
- Extraction and characterization of Prodigiosin pigment

CHAPTER 2

REVIEW OF LITERATURE

Growing interest has been seen in the production of biopowders as an alternative to synthetic pigments; 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 Pigment classification

Pigments are divided into three categories: natural, synthetic, and organic. Pigments found in nature are generated by living things, including microbes, plants, and mammals. Pigments are organic substances, both natural and artificial. Furthermore, natural pigments can be divided into the following groups based on their structural characteristics: N-heterocyclic molecules that are not related to benzopyran derivatives (anthocyanins and other flavonoid pigments), benzopyran derivatives (purines, pterins, flavins, phenazines, phenoxazines & betalains), or quinones (benzoquinone, naphthoquinone, anthraquinone, and melanins).

Pigments are classified into two categories by Food and Drug Administration (FDA) based on how they are utilized as food additives.

(i) Synthetic pigments & lakes are certified;

(ii) Pigments which are originating from natural sources, such as plants, minerals, or animals, as well as synthetic versions of natural derivatives, are exempt from certification.

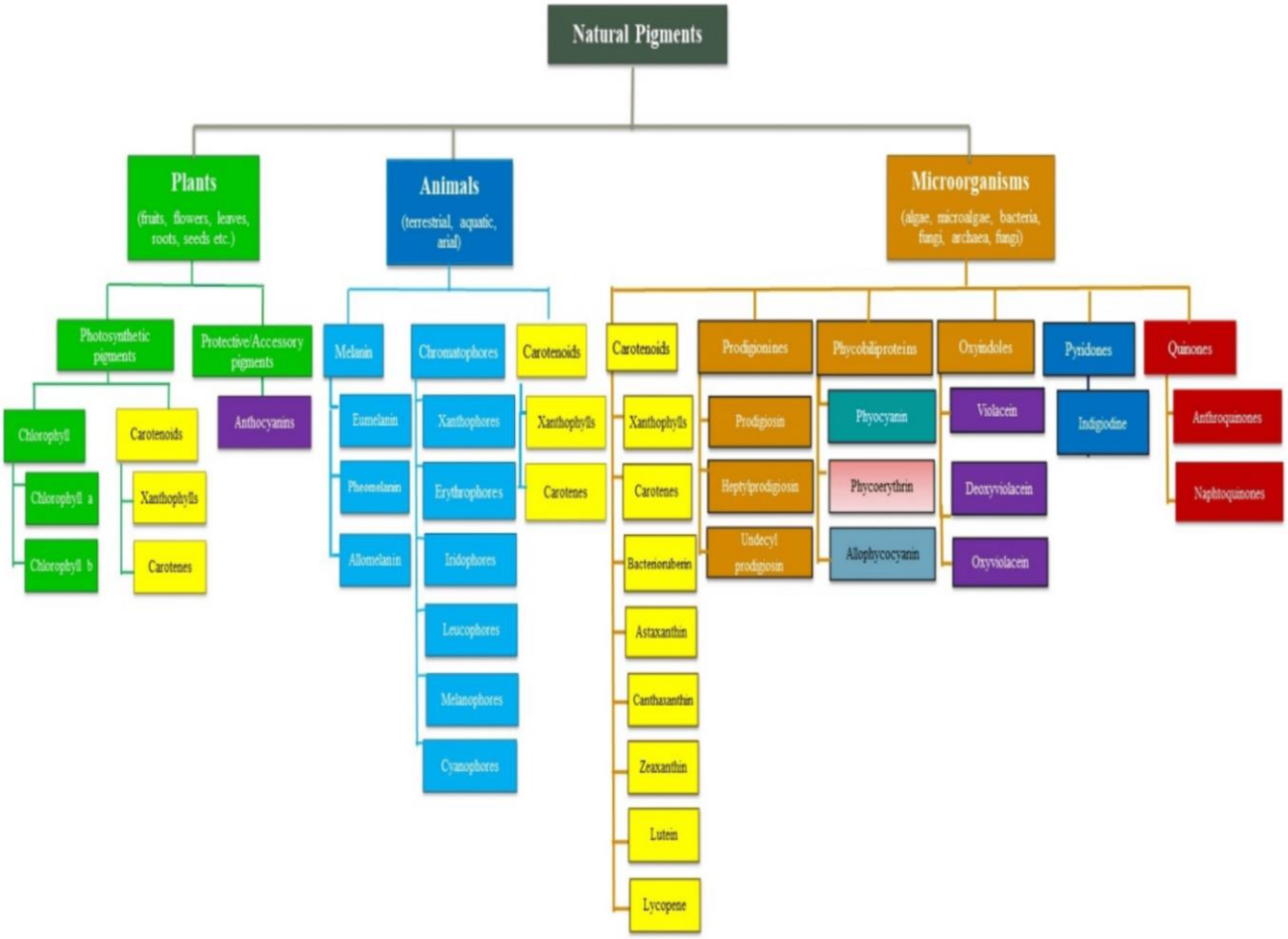

Fig2.1: Natural pigments categorized according to living things [7].

Some of pigments that are found in nature naturally are Riboflavin, Beta- carotene, Canthaxanthin, Caratenoids, Prodigiosin, Phycocyanin, Asthaxanthin.

2.2 Prodigiosin

Red pigment known as prodigiosin is generated by number of bacteria, including as *Hahellachejuensis, Vibrio psychroerythrus, and Serratia marcescens*. It is a member of the prodiginine family of substances and possesses bioactivities that include antibacterial, anticancer, and antimalarial. With characteristics including antifungal, immunosuppressive, and antibiotic qualities, this adaptable pigment has been thoroughly researched for its uses

in biotechnology and medicine [19]. Prodigiosin is a *Serratia marcescens* secondary metabolite that has been connected to a number of biological processes, including cancer cell death. Prodigiosin is a secondary metabolite, meaning it's not essential for the bacteria's basic survival but offers certain advantages. It belongs to a class of molecules called **tripyrroles** due to its core structure containing three pyrrole rings. Its creation is attributed to a complicated process involving numerous genes in its biosynthesis. With further study concentrating on its synthesis and its applications in many sectors, prodigiosin has demonstrated promise in therapeutic applications as well as a dye [20].

2.2.1Chemistry of Prodigiosin

Prodigiosin is a tripyrrole red pigment with a unique chemical structure and intriguing physicochemical properties that have captured the interest of researchers across multiple disciplines, including chemistry, microbiology, and pharmacology. Its molecular formula is $C_{20}H_{25}N_3O$, and it possesses a distinctive linear tripyrrolic structure that contributes to its vibrant red color and its range of biological activities[19]

The chemical structure of prodigiosin consists of a tripyrrole ring system with a long alkyl chain substituent. The molecular formula of prodigiosin is $C_{20}H_{25}N_3O$.

The three pyrrole rings are connected by methine bridges, forming the characteristic tripyrrole structure that is responsible for the compound's distinctive red pigmentation. Prodigiosin belongs to the prodiginine family of natural products, which are characterised by the presence of tripyrrole moiety. The alkyl chain substituent on prodigiosin can vary in length and composition, leading to the production of different prodigiosin analogs by various microorganisms [20].

The chemical properties of prodigiosin, such as its solubility, stability, and reactivity, have been extensively studied. Prodigiosin is soluble in solvents, such as methanol, ethanol, & chloroform, but it is insoluble in water. The compound is relatively stable under acidic conditions but can be degraded under alkaline conditions.

2.2.2Chemical structure of Prodigiosin

Three pyrrole rings make up prodigiosin's chemical structure. The way these rings are joined together creates a linear tripyrrolic molecule. Prodigiosin, in particular, is a 4-methoxy-2,2'-bipyrrole-5-carbaldehyde that has been condensed with a 2-methyl-3-amyloxy pyrrole.

"The 4-methoxy-2,2'-bipyrrole-5-carbaldehyde moiety & the 2-methyl-3-amyloxy pyrrole moiety are connected to opposite sides of the core pyrrole ring. Its red color and capacity to intercalate into DNA, which underpins many of its biological actions, are caused by this special structure [21]

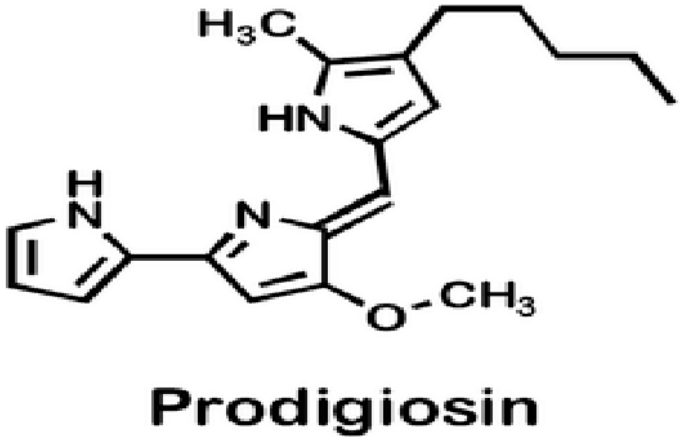

Fig2.2: Chemical structure of Prodigiosin [21]

2.2.3 Physiochemical properties

Prodigiosin is soluble in methanol, ethanol, chloroform, and other organic solvents but basically insoluble in water. Because of its employment in multiple sectors and the significance of its extraction and purification operations, this solubility profile is important [21]. Prodigiosin can break down after extended exposure to light and extremely high or low pH values, but it shows significant stability over a wide range of temperatures and pH levels. Its durability makes it useful for a variety of uses, such as biological stains and fabrics [15]. Prodigiosin's large system of conjugated double bonds gives it a vivid red color. The pH of the solution and the solvent can affect the color, which can range from red to deep purple [15].

2.3 Source of prodigiosin

S. marcescens is the major producer of prodigiosin, this pigment is also produced by other bacteria, such as *Streptomyces coelicolor, S. lividans, Hahellachejuensi, Pseudovibriodenitriccans, Pseudoalteromonas rubra, P. denitrificans, Vibrio gazogenes.*

2.4 General properties of Prodigiosin

A blood-red pigment group known as the prodiginin family was originally discovered in 1929 and is a type of secondary metabolite produced by a broad spectrum of bacteria.

The majority of bacteria that make prodiginine are Gram-negative ones. Some (i.e., *S. marcescens, S. plymuthica, and S. rubidaea), Vibrio gazogenes, Vibrio psychroerythrous*, but also in members of the Gram-positive actinomycetes, such as *Streptomyces coelicolor*, *Streptomyces longisporusruber*, *and Streptoverticillium rubrireticuli, Zooshikelarubidus.* In chemical terms, PG is redcolorant with a methane bridge joining the third ring system to the 4-methoxy-2, 2'-bipyrrole ring system. Nature contains a variety of PG isoforms with different chains of carbon lengths or molecular weights; all of them maintain their bioactive qualities for use in clinical and medical contexts [22].

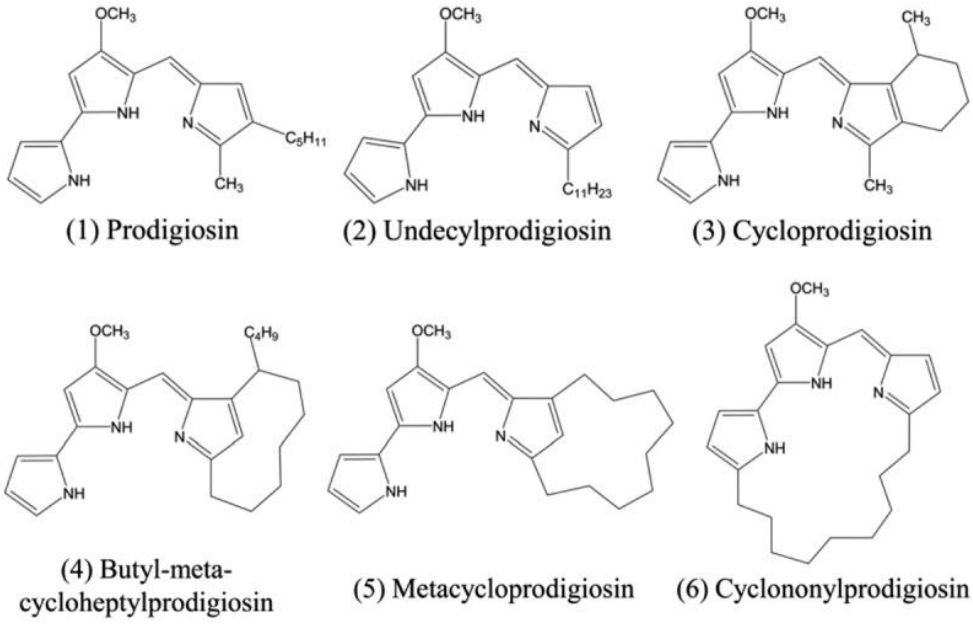

Fig 2.3: Prodigiosin's many isoforms and their comparative chemical structure [22]

2.5 Significance in microbial ecology

The significance of microbial ecology in prodigiosin lies in the understanding of the ecological roles that these bacteria play in their environments and how these roles are influenced by the production of prodigiosin. It has been discovered that prodigiosin, a red pigment generated by a variety of microorganisms, benefits the producing bacterium ecologically. This pigment is found in many taxa in both marine and terrestrial habitats, indicating that it could be important for these bacteria's ecological interactions. The ecological significance of prodigiosin is multifaceted. For instance, it has been observed to have antibacterial, antifungal, and antiprotozoal activities, which could contribute to the competitive advantage of prodiginine-producing bacteria in their environments [23]

The significance of microbial ecology in prodigiosin lies in its potential ecological roles, which are likely to have driven the evolution of prodigiosin biosynthesis and regulation in these microorganisms. This ecological context is crucial for understanding the functional significance of prodigiosin and for developing effective strategies for its biotechnological applications.

2.5.1 Natural sources and optimizing prodigiosin production

In the 1970s, PG was initially isolated from the soil bacterium *Serratia marcescens*. Following forty years of research, bacteria from the ocean to the subgingiva have been widely studied as natural sources of PG. Initially, *Hahellachejuensis, Streptomyces coelicolor,* and *Serratia sp.*—particularly *S. marcescens*were the primary species used in the synthesis of PG [24], [25]

PG may have originated from other Gram-ve bacteria, such as *Jantinobacterium sp.* and *Vibrio sp*. Two methods are employed to improve PG production: genetic engineering and culture optimization. Through genetic engineering, *E. coli, Pseudomonas putida, and Streptomyces coelicolor* were given access to a plasmid containing the entire PG production pathway. Notably, *S. marcescens* is the primary testing bacterium species in culture media optimization[25].

2.5.2 Production of pigment

Serratia marcescens, and bacterium, produces prodigiosin, a red pigment, when grown in various media. It is typically produced in NB or in peptone glycerol broth. The maximum prodigiosin production is found in maltose-containing medium. Other substrates used for prodigiosin production include triolein and trilobein[26]. The production of prodigiosin is affected by pH and temperature. The organism produces more prodigiosin at 30°Cat pH 7, while the rate decreases as pH and temperature increase above 30°C. There is a diversity in *S. marcescens* strains & their conditions optimal for prodigiosin production. These findings suggest that the antibacterial red pigment production is growth-dependent and slight fluctuations in growth parameters influence prodigiosin production [27].

2.5.3 Environment Factors influencing prodigiosin production

Environmental conditions influence the development of prodigiosin, a red pigment generated by various bacterial species, including *S. marcescens*. These factors can have a substantial impact on the yield and efficiency of prodigiosin synthesis in both natural and controlled environments (industrial or laboratory). Here are some of the main environmental elements that influence prodigiosin production:

i. Temperature

Prodigiosin production is strongly temperature sensitive. *Serratia marcescens* commonly produces prodigiosin at temperatures ranging between 20°C to 30°C.

Temperatures outside of this range can result in diminished pigment production. temperature changes can stress bacterial colonies, affecting metabolic activity and, as a result, pigment production[28]

ii. pH

The pH affects the pigmentation production. For prodigiosin its slightly alkaline ranges between 7.0 – 8.0. It may vary according to the bacterial strain.

Maintaining a constant pH throughout cultivation is critical because changes can alter enzyme activity involved in the biosynthesis pathway of prodigiosin.

iii. Aeration

The level of oxygen in the culture influences the development of prodigiosin. High amounts of aeration can boost prodigiosin synthesis in aerobic bacteria such as *Serratia marcescens*.

iv. Nutrient Concentration

The availability of carbon and nitrogen sources in the growth medium has a considerable impact on bacterial growth and pigment production. Excessive or insufficient levels of these nutrients can cause decreased prodigiosin production.
Trace Elements: Trace elements such as iron, magnesium, and phosphate influence prodigiosin synthesis.

v. Light

Light Exposure: Light exposure can affect prodigiosin synthesis, however the effect varies by bacterial strain. Some research indicate that light can suppress prodigiosin production in some *Serratia marcescens* strains, while others find no meaningful effect.

vi. Salinity

The salt content in the growth medium can influence osmotic pressure, which affects bacterial cell physiology and metabolism, including prodigiosin synthesis. Moderate salt stress can occasionally increase pigment synthesis as part of the bacterial stress response.

Genetic Factors

While not a direct environmental effect, genetic variations across bacterial strains can cause differences in how environmental factors affect prodigiosin production. Under certain environmental conditions, prodigiosin yield is increased by genetic engineering and strain modification procedures. Optimizing these environmental conditions is critical to increasing prodigiosin production. To generate high quantities of prodigiosin, researchers and biotechnologists frequently use controlled fermentation procedures that carefully regulate temperature, pH, oxygen level, and nutrient content.

The factors which influence the growth of prodigiosin production for pigment (Temperature, pH, Aeration, Light, Salinity, Genetic factors)[29]

2.6 Biosynthesis pathway of prodigiosin

Early studies have shown that prodigiosin, a red pigment, is synthesized through a pathway involving the condensation of two intermediate products, MAP and MBC. 2-methyl-3-pentyl-pyrrole is technically called MAP, but in history it has been referred to as n-amyl. In the bacterium *S. coelicolor*, at least eighteen genes are required for biosynthesis of undecylprodigiosin[30]

A separate bacteria called *Streptomyces parvulus* has been used to clone and express the full undecylprodigiosin biosynthesis cluster.

Similarly, the biosynthetic genes for prodigiosin in Serratia strains and *S. marcescens* have also been cloned and expressed in different hosts. Shared genes have been observed among the three gene clusters, as well as specific genes unique to either the Serratia or Streptomyces clusters [31].

2.7 Regulation of prodigiosin synthesis

The regulation of prodiginine synthesis is a complex process that involves both physiological and environmental factors. Small molecules and extracellular signaling play important roles in coordinating pigment production. Small-molecule signals that control prodiginine production are generated by *Streptomyces species* & *Serratia species* [32].Quorum sensing, is a mechanism used to study bacterial behavior in response to density of population, also plays a significant role in prodiginine production. The cell density increases by the chemical signals, leading to changes in gene expression and the activation of pigment biosynthesis [34]On the other hand, when cell density rises, N-AHL molecules build up and block SmaR's ability to bind DNA, which activates color manufacturing. *S.marcescens*and other Serratia strains exhibit comparable mechanisms.[33]

S. coelicolor is one of the streptomycetes that produces the γ-butyrolactone signalling molecules that regulate secondary metabolism. These compounds regulate the growth-rate of undecylprodigiosin production in conjunction with ppGpp (guanosine tetraphosphate). Nitrogen shortage starts the synthesis of ppGpp, which activates regulators unique to that

pathway and produces undecylprodigiosin. Mutations may prevent RelA from interacting, which would prevent the production of undecylprodigiosin and ppGpp[34].Overall, the regulation of prodiginine synthesis is a highly controlled process that involves multiple signaling molecules and mechanisms. Quorum sensing, small-molecule signals, and the stringent response all contribute to the coordination of pigment production in bacteria [35]

2.8 Environmental significance of prodigiosin production

- Prodigiosin's relevance is attributed to various postulated eco-physiological roles, despite its scarcity. These include acting as an energy storage medium, providing as a metabolic precursor for vital biochemicals like proline or NAD(P)H, and acting as an air diaspora for bacteria. Furthermore, its versatility and usefulness within microbial communities are demonstrated by its capacity to promote ion exchange and its energy-spilling role in *Serratia marcescens*[35].
- Prodigiosin has uses in the environmental field that include bioremediation. Prodigiosin has been linked to the bioremediation of heavy metals and the decolorization of dyes, according to studies. It is therefore an important instrument for managing and cleaning up the environment. Furthermore, by minimizing the environmental impact of industrial operations, the use of agricultural and fishery leftovers as substrates in the synthesis of prodigiosin not only minimizes waste but also conforms with sustainable practices.
- Prodigiosin is proven to have strong antibacterial properties, making it useful for biocontrol applications. Prodigiosin can enhance the health of natural environments and ecological balance by regulating unfavorable bacteria populations [36].

2.9 Purification techniques of prodigiosin

Extracting and First Purifying: Using aqueous and extracting solvents, prodigiosin is extracted from the solid-state fermentation material. The liquids are then concentrated, combined, and centrifuged to obtain the precipitate and supernatant.

Column Chromatography with Silica Gel: Following the first extraction, the crude prodigiosin product is separated and purified using a gradient elution of ethyl acetate and Harwood oil in a silica gel column chromatography.

HPLC: The prodigiosin pure product is further purified by HPLC partitioning with a C18 column and particular elution conditions comprising glacial acetic acid and methyl alcohol [37].

2.10 Biotechnological implication of prodigiosin

Prodigiosin (PG), a vibrant red pigment with a unique pyrrolyl pyrromethane skeleton, has been widely studied due to its potential applications in medicine, agriculture, industry, and environmental management.

Research on prodigiosin has led to an increase in studies evaluating its production and exploring novel uses. High-level production of prodigiosin and its applications in medicine are particularly emphasized, but there is a gap in research concerning its scaling-up for agricultural use and detailed action mechanisms[31].Cost-effective production strategies involve microbial fermentation using low-cost substrates, with byproducts and wastes from agro-industrial processes, food, and kitchen activities being increasingly used as alternative substrate sources[36]. Prodigiosin has broad spectrum of biological activities by including antibacterial, antifungal, and anticancer properties. However, the primary production strain, *Serratia marcescens*, poses risks due to its association with pathogenic effects in mammals[38].Despite its potential, the systematic production and scale-up of prodigiosin using cost-effective substrates remain challenging. The need for comprehensive pre-clinical and clinical studies and regulatory approvals is also lacking [36].Innovative production techniques, such as 2 stage fermenter optimized through Taguchi's experimental statistical design, have significantly enhanced prodigiosin production by up to 70 times. The integration of prodigiosin into bionanocomposites is a promising area of research, as these composites exhibit antimicrobial, antioxidant, and anticancer activities, broadening prodigiosin's applications in medical and biotechnological fields [36].

2.11. Applications of prodigiosin

1. **Food industry**

- **Natural food colorant:** Prodigiosin can be used as a natural food colorant, offering a safer alternative to synthetic colorants
- **Antimicrobial packing:** Prodigiosin is a natural food coloring that is safer to use than artificial colorants. It has been investigated for possible application in antimicrobial food packaging which can help food goods last longer.
- **Staining properties:** Prodigiosin staining properties can be utilized in food products, such as in development of novel food dyes or for enhancing the appearance of certain food items.
- **Food preservatives:** The antimicrobial properties of prodigiosin can be leveraged to develop novel food preservation methods, enhancing food safety and extending shelf lift [39].

2. **Biomedical applications**

   A new field of research is provided by the possible use of prodigiosin in films, microcarriers, and nanoparticles for biological applications. By overcoming the difficulties caused by prodigiosin's hydrophobic properties, these systems can improve the drug's usefulness in biotechnological and medicinal applications [22].

3. **Agriculture applications**

   Field trials, greenhouse testing, and in vitro experiments all show that further study is needed to thoroughly explore the novel and potential applications of prodigiosin in agriculture. To assure safety and efficacy, it is necessary to comprehend the mechanisms underlying the bioactivities of prodigiosin and carry out thorough toxicological investigations [35].

CHAPTER 3

MATERIALS AND METHODS

The topic of this study is "Isolation and characterization of a Prodigiosin pigment producing bacterial strain from Himalayan region ". A bacterial isolate obtained from the soil in Waknaghat, Solan, was employed for this particular instance. In the lab, the solubility, manufacturing, and applications were examined.

Experimental site

Isolation and characterization of a Prodigiosin pigment producing bacterial strain from Himalayan region was conducted at Jaypee University of Information Technology's genomics laboratory.

During the experiments all safety measures are taken.

Instrument used: Autoclave, weighing balance, laminar air flow (LAF), centrifuge, water-bath, incubator, Rota evaporator, microscope, and spectrophotometer.

Materials used: Petri plates, Nutrient agar, Nutrient broth, Flasks, falcons, Bunsen burner, round bottom flask

3.1 Isolation of bacterial strain from soil

Soil sample was taken from the area of waknaghat. Soil sample was serially diluted in the test tubes up to 6 tubes. Then 1gm of soil sample was added into 9ml distilled water. Dilution was made upto10^6. Then the NA plates were spread with the dilutions .After spreading the plates were put under incubation at 37°C for 24 h. Different colony of bacteria were obtain. We isolated the red colony from the plate. The red color colony was inoculated into the petri plates by streaking method under LAF. Then it was incubated at 25°C for 72 h after that growth was checked. Then the Red colonies were obtained.

3.2 Gram staining of culture

The Gram Staining technique is used to see how the gram +ve and gram -ve bacteria vary from one another. The culture smear was created for this purpose on a clean glass slide. Heat was then used to repair the smear. The smear was heat fixed, then crystal violet

stain was applied and left on for 60 seconds before being removed with water. Following that, gram's iodine was applied drop-by-drop for 60 seconds before being rinsed with water. After 15 seconds of ethanol washing, the smear was rinsed with water. Safranin dye was subsequently applied to the smear and left for 60 seconds. The smear was then cleaned. After air drying, the glass slide was examined under a microscope.

3.3 Biochemical testing

3.3.1 Catalase test

Procedure: smear was created on the clean glass slide for this test with the inoculating loop under LAF. Added few drops of Hydrogen peroxide onto the smear. Observed the formation of bubbles as the Oxygen releases.

3.3.2 Casein hydrolysis

Procedure: Prepared Skim milk agar media plates and then inoculated the bacterial strain into the Skim milk agar plates. Incubated the plates for 72 h at 25°C. Observed the clear zone formation around the bacterial colony.

3.3.3 Methyl red test

Two clean glass tubes were taken and MR- VP broth was prepared and autoclaved. Then the bacterial strain was inoculated into one tube and other is taken as control. Then the test tubes were incubated at 37°C. Then added few drops of methyl red indicator. Observed for color change.

3.4 Temperature optimization

Prepared NB media in the flask and autoclaved. Inoculated the bacterial strain into the flasks and incubated at different temperatures (20, 25, 30, 37 and 60°C) and the growth was observed

3.5 Optimization of pH

Prepared NA plates of different pH from pH 4.0 to pH 10.0. Inoculated the bacterial strain into these plates and incubated at 37°C.And the growth of the bacterial strain was observed.

3.6 Antibiotic Testing

MHA media was prepared in the flask and autoclaved. Plates were poured with the media and plates are incubated for 24 h at 37°C. Stock of antibiotics (Kanamycin, Ampicillin, Tetracycline and Cephalexin) was prepared at conc. 1mg/ml. DMSO was taken as control. Bacterial strain was spread into the plates and antibiotics were added and control was taken as DMSO. After incubation the susceptibility of the bacterial was absorbed.

3.7 Prodigiosin extraction from the bacterial strain

The bacterial strain was inoculated into 2L NB media and incubated at 25°C for 72 h. observed the production of pigment after incubation. The broth was centrifuged at 10000 rpm for 10 min. the supernatant was discarded wans the pellet was taken and mixed with Acidified ethanol. Then again centrifuged the media at 10000 rpm for 10 min at 4°C. The pigment was extracted into the acidified ethanol and then it was extracted with the help of Rota evaporator. After the extraction the absorbance peak was measured in the spectrum in UV spectrophotometer.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Bacteria from soil

The isolation of bacterial strain was done by taking the soil sample from the area of Waknaghat. Serial dilution of the soil sample was done. The dilutions were spreaded on the NA plates. Then plates incubated at 37°C for 24 h and then the red pigmented bacterial strain was isolated from it to a new NA plate by streaking method and incubated at 30°C for 3 days for the pigment produced by the bacteria. The steps followed for the isolation of bacterial strain was shown in Fig 4.1.

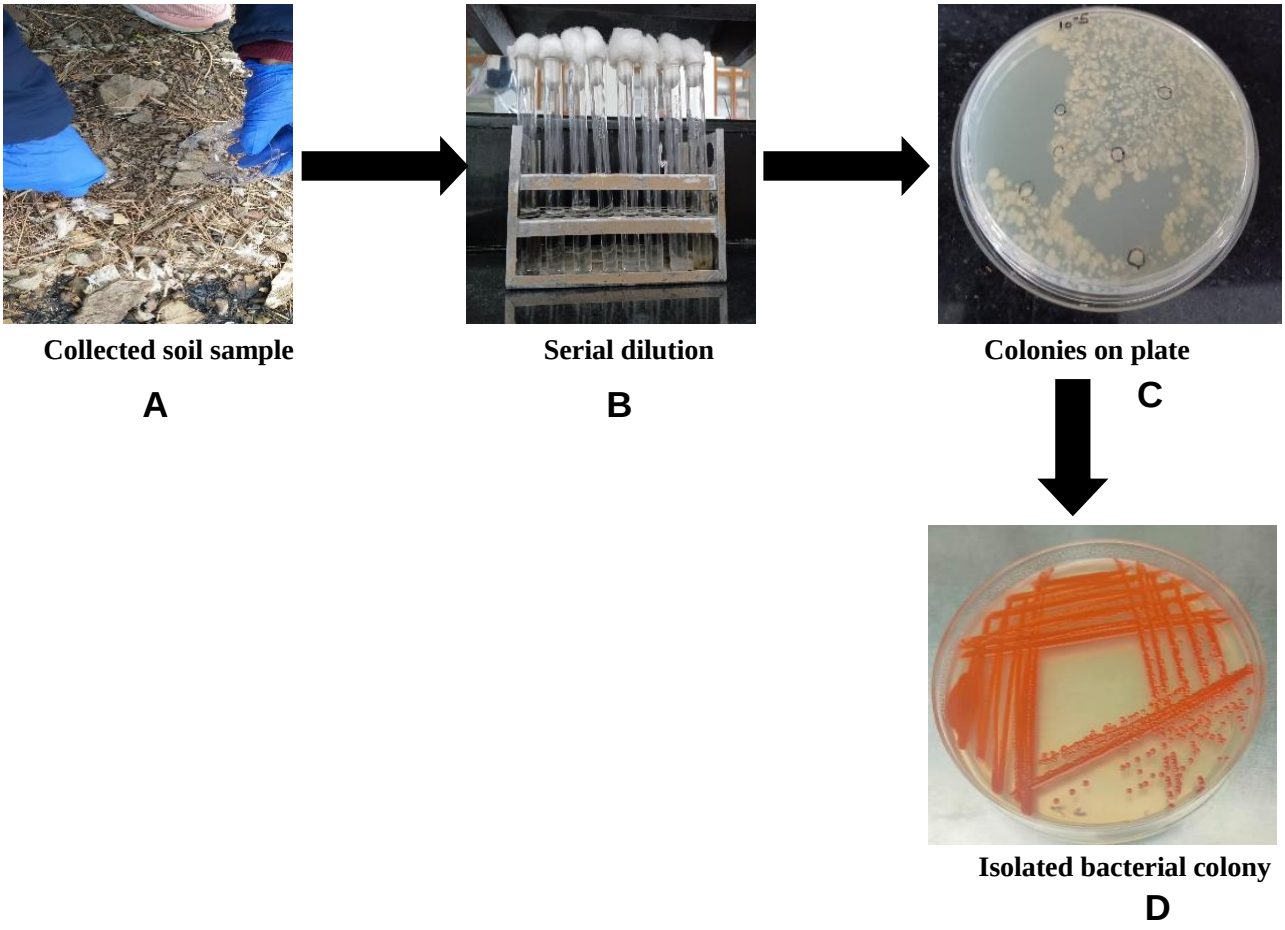

Fig 4.1 Isolation of bacterial strain from soil sample

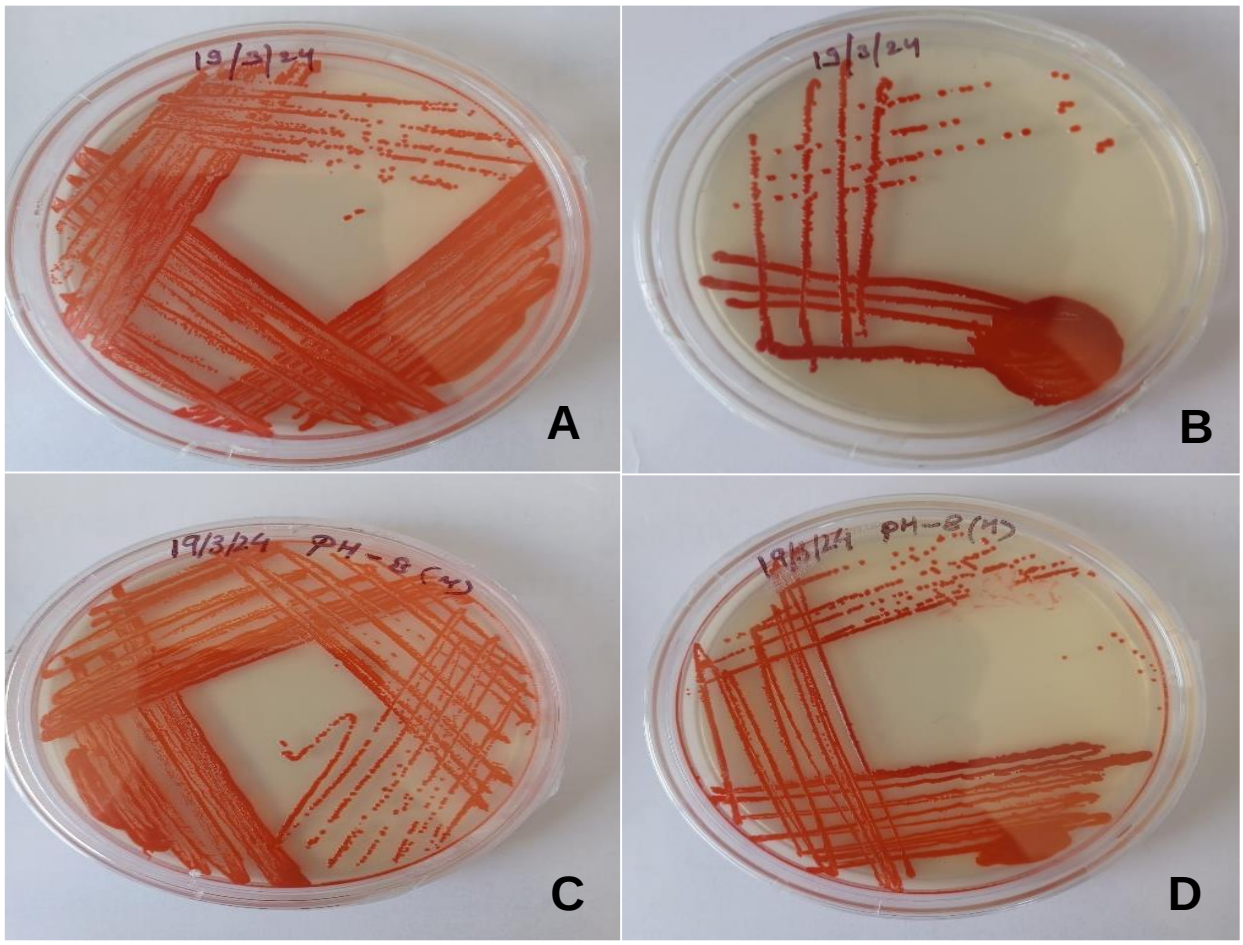

Fig 4.2. Pigmented colony isolated on the nutrient agar plates

The red pigmented bacterial strain was isolated into the NA plates and quadrant streaking was done to obtain colonies of the pigmented strain. The quadrant streaking has been done to get clear bacterial colonies.

4.2 Gram staining

Gram staining of strain was used to analyze morphological characteristics and classify the bacteria. After mounting the culture on a glass slide, various dyes were applied to it. The slide was air dried and examined at 100X magnification under a microscope. There were coci shaped, pink-colored creatures visible. The cultivated strain is gram negative when it is pink in color.

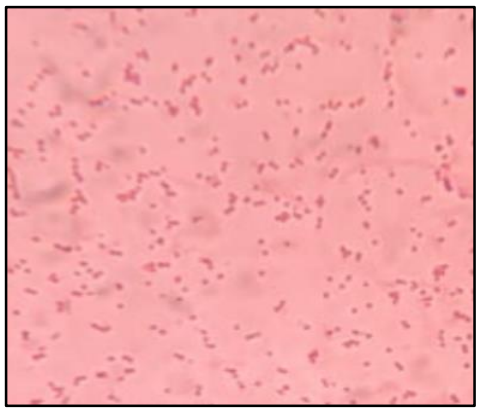

Fig 4.3. Gram staining. Pink color and coci shaped organism were observed at 100X under microscope

4.3. Biochemical test

Biochemical test has been done for the bacterial strain catalase test, casein hydrolysis test, gram staining, and methyl red test as shown in Table 4.1

Table 4.1: Biochemical tests of bacterial strain

	Biochemical test	**Result**
1.	Gram staining	Negative
2.	Catalase	Positive
3.	Casein hydrolysis	Positive
4.	Methyl Red	Negative

4.3.1 Catalase test

Catalase test has been done for the bacterial strain by putting drops of H2O2 on the smear of the bacterial strain on slide. It shows the formation of bubbles which means it was catalase positive. It was shown in figure 4.3.

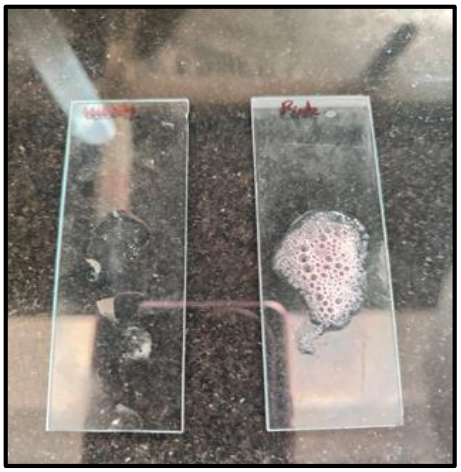

Fig 4.4. Catalase test formation of bubbles by putting H_2O_2 on the smear

4.3.2 Casein hydrolysis

Skim milk agar plates were streaked with quadrant streaking methods the bacterial strain and incubated at 37°C for 24 h and after incubation the clear zones were observed around the colony which means the positive result. The result is shown in Fig 4.4.

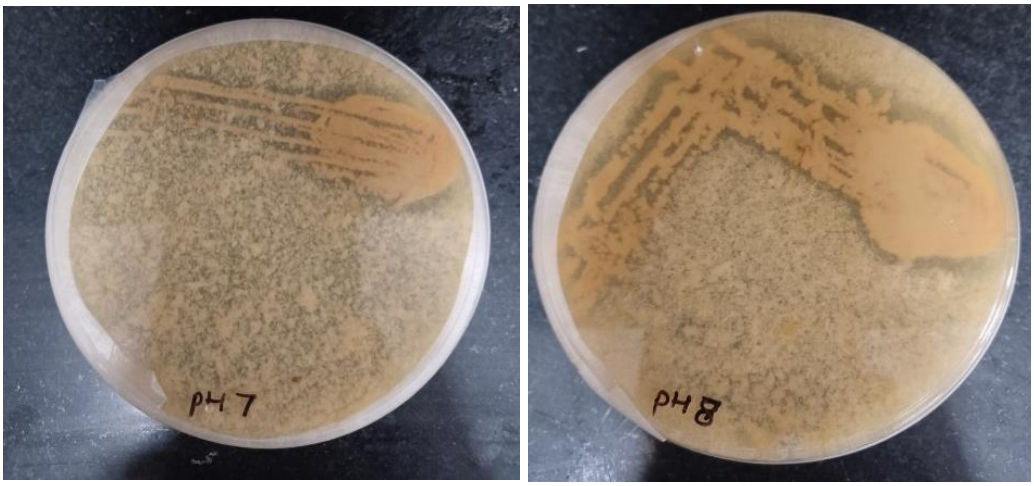

Fig 4.5. Clear zone forming around the bacteria strain which shows +ve result

4.3.3 Methyl red test

Methyl red test was done in MR-VP broth in test tube and the methyl red indicator was added into the broth. There is no red color ring formation which shows negative result. The result was shown in Fig 4.6.

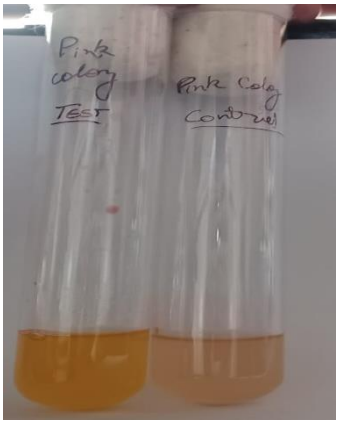

Fig 4.6. Methyl red test negative

4.4 Growth curve optimization of bacterial strain

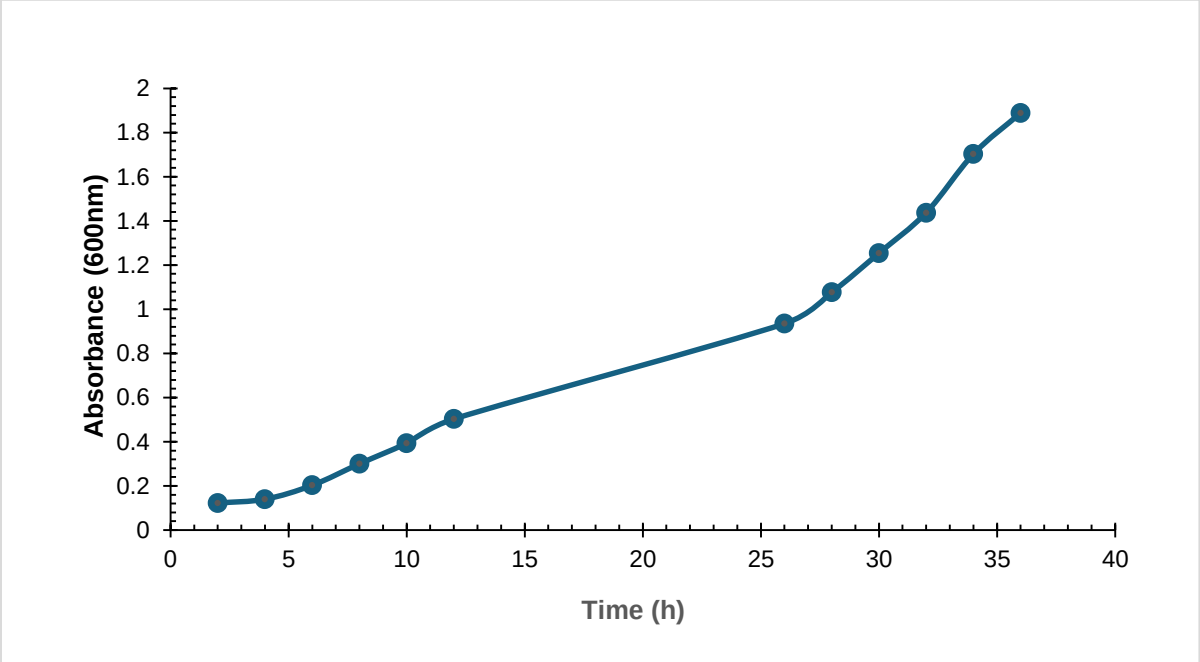

Fig 4.7 Growth curve optimization of bacterial strain

The growth curve of the bacterial strain was measured at 600nm.

4.6 Temperature Optimization of Bacterial strain

Optimization at different temperature to see the pigment production. NB media was inoculated then incubated at different temperatures (25, 30, 37, 40 & 60°C) these temperatures were observed for the pigment production of the bacterial strain.

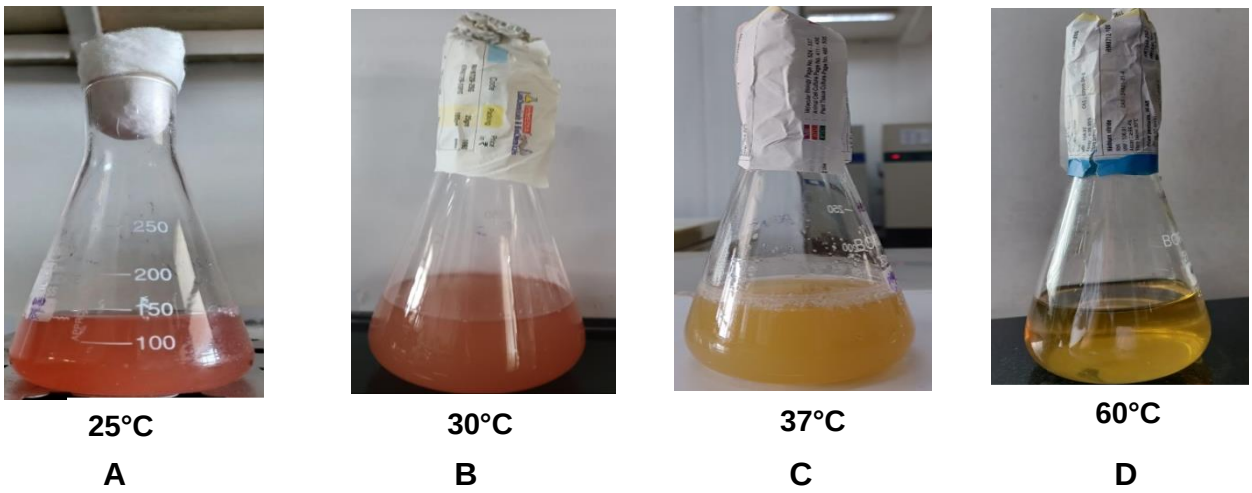

Fig 4.8. Growth of the bacterial strain under different temperatures. The pigment production was observed on in 25°C and 30°C

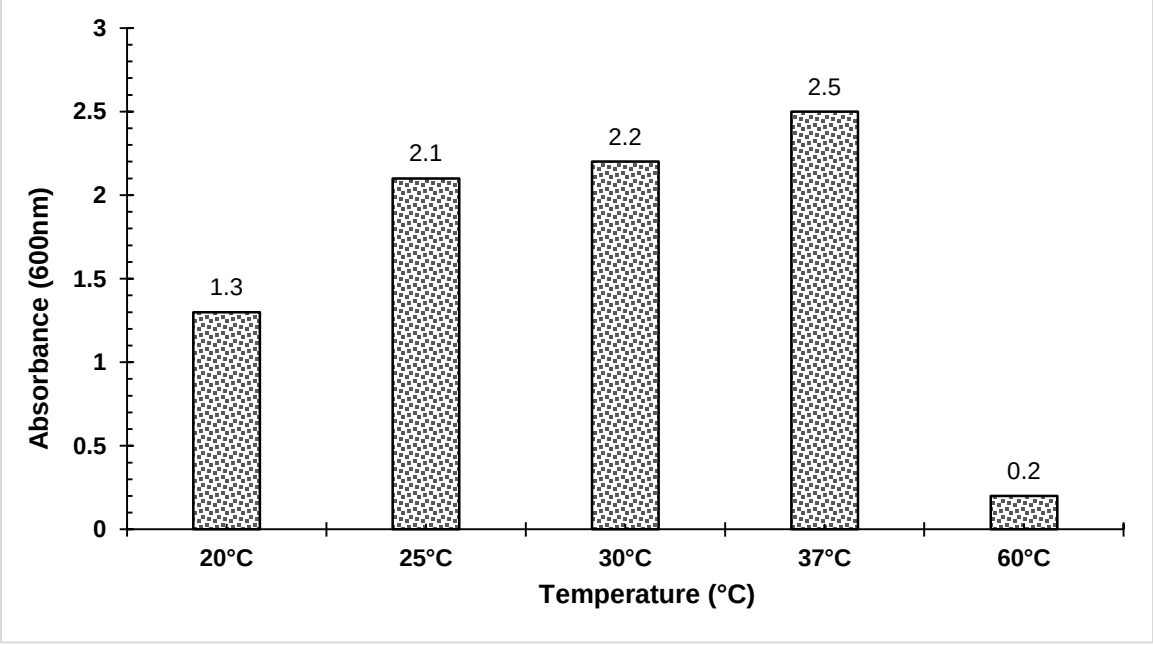

Fig 4.9: Temperature optimization for pigment production

Optimization of the bacterial strain was done at different temperatures to know at which temperature the pigment is produced by this bacterial strain. The bacterial strain shows growth at various temperature 20, 25, 30 and 37°C. But at high temperature the bacterial strain doesn't grow. After the incubation completed at different temperature the absorbance of the bacterial strain was taken at 600nm to know the growth of the strain. The pigmented growth was shown at temperature 25°C and 30°C as shown in Fig 4.8. But the bacterial growth most shown at temperature 37°C.

4.6 pH optimization of Bacterial strain

Optimization of bacterial strain at different pH (5, 6, 7, 8, 9, 10)

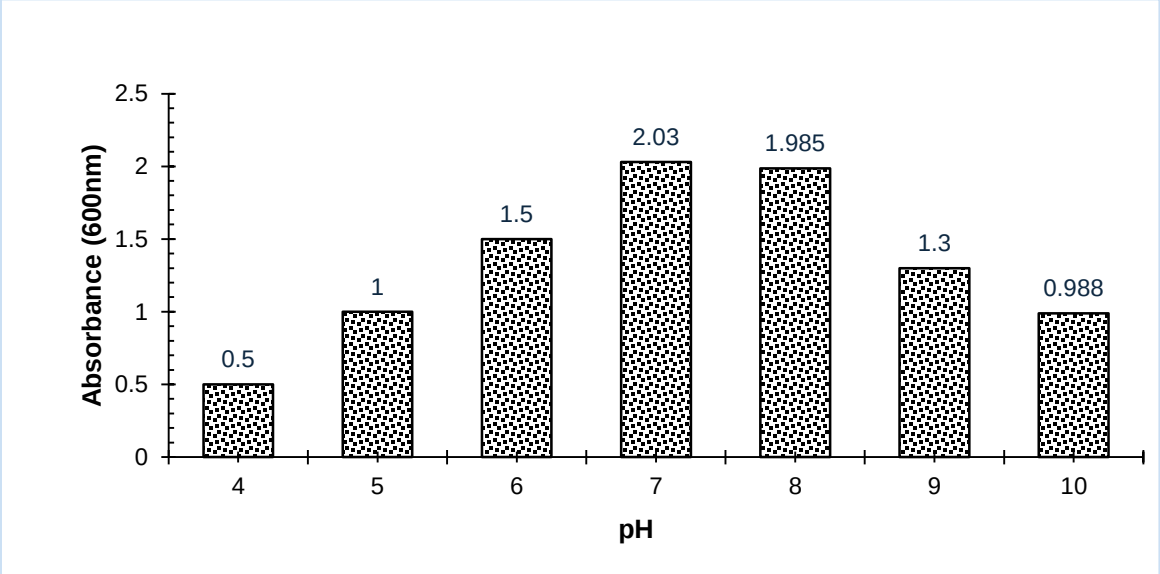

Fig4.10. pH optimization of bacterial strain at different pH (5, 6, 7, 8, 9&10) in which pH 7 and pH 8 show good growth.

To determine the growth condition of the bacterial strain the bacteria is grown at different pH (4,5,6,7,8,9,10) for three days to observe the growth in spectrophotometer at 600 nm. After the incubation at different pH levels the best growth shown in pH 7 and the lowest growth was shown in pH 4. This indicates this bacterial strain grows in neutral pH as shown in Fig 4.10.

4.7 Antibiotic Susceptibility Test for the Bacterial Strain

Antibiotic test was done using antibiotics such as Kanamycin, Ampicillin, cephalexin, Tetracycline. Bacterial strain was inoculated onto the MHA plates and then the antibiotics are inserted by making a punch hole in it. DMSO was used as control. The antibiotic stock was make of 1mg/ml.

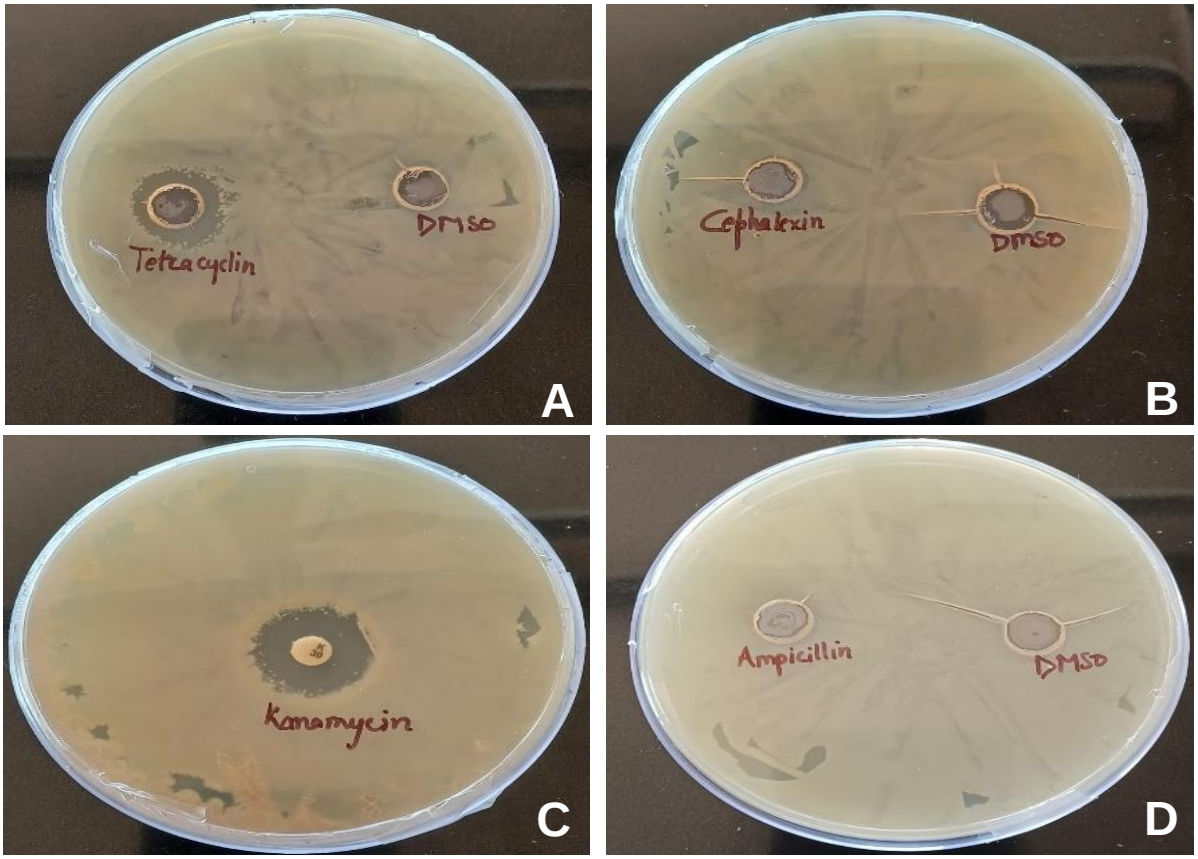

Fig 4.11. Antibiotic susceptibility test for bacterial strain

A- This bacterial strain is susceptible to tetracycline

B- It is resistant to Cephalexin

C- Its susceptible to Kanamycin

D- It is resistant to Ampicillin

4.8. Prodigiosin pigment production

The production the prodigiosin pigment was done by make more amount of bacterial broth in NB media. Then it was incubated at 25°C for 72 h. After the incubation period the red color pigment was observed in the broth culture.

Then the broth culture was centrifuged at 10000 rpm for 10 minutes and after centrifugation the supernatant was discarded and pellet was taken.[22]

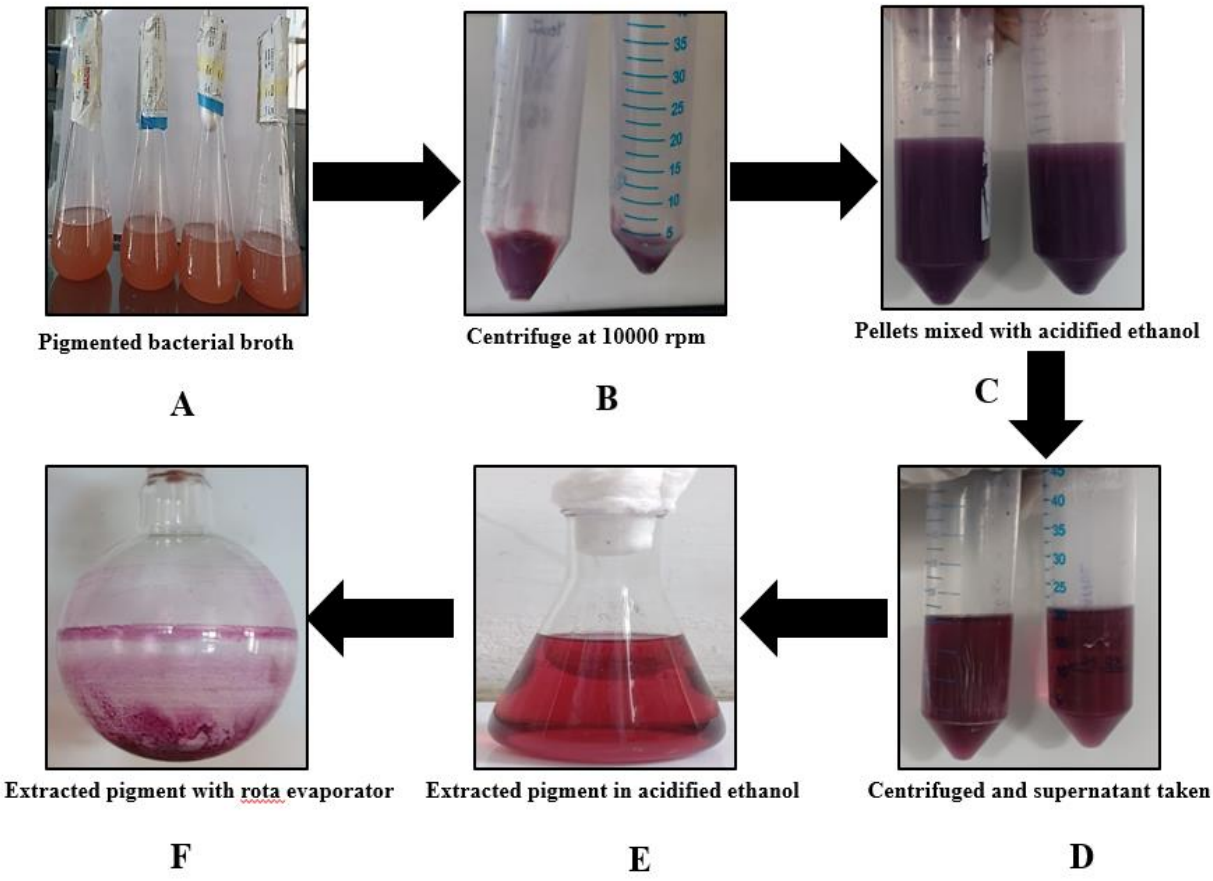

Fig 4.12. Solvent extraction technique

Then the pellets were mixed with acidified ethanol with vortex. And after mixing the pellets with acidified ethanol it was again centrifuged at 10000 rpm for 10 min at 4°C

The supernatant was collected. The centrifugation is done until the the pellet loose its color by adding acidified ethanol.

After this the solution in which the pigment is extracted is extracted by using rota evaporator. Then the extract is removed through 4:1 water ethanol mixture.

Then the dry extract is obtained through lyophillisation.[22]

4.9 UV-vis spectroscopy

The absorbance spectrum of the pigment was taken in Uv spectrophotometer. The spectrum shows the pigment peak at 540 nm. The spectrum was run against the control. The control was taken as NA media in which the prodigiosin pigment was cultured.

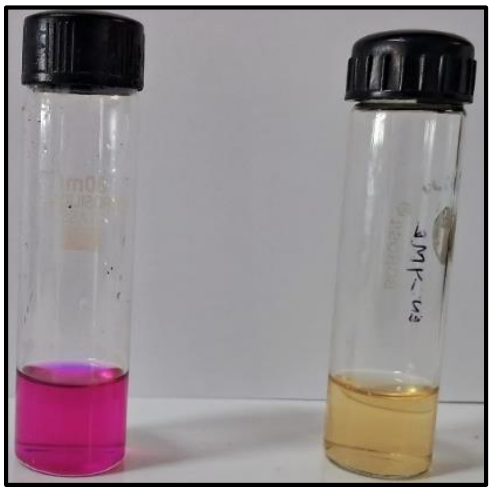

Fig 4.13. Pigment against its control media

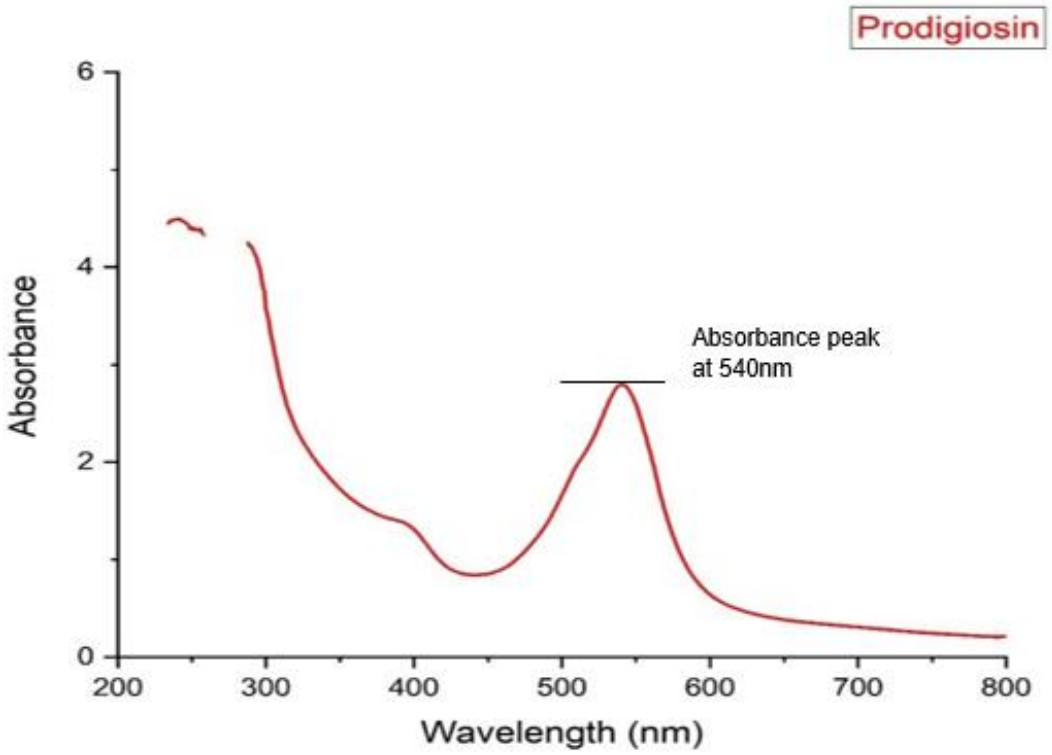

Fig 4.14. Spectrum of pigment

The spectrum shows absorbance of the pigment at 540nm which is identifies as Prodigiosin pigment. Prodigiosin pigment was identified using UV-Vis spectroscopy, which demonstrated a distinctive absorbance peak and verified the pigment's existence and purity.

CHAPTER 5

CONCLUSION

The isolation and characterization of a prodigiosin pigment-producing bacterial strain from the Himalayan region has revealed a rich world of microbial diversity and biotechnological potential. The study demonstrates the adaptational strategies of bacteria in extreme environments, showcasing the Himalayas as a rich source of pigment-producing microbes with unique properties and applications. The Bacterial isolate strain exhibits antibiotic resistant properties, highlighting the potential of prodigiosin in combating pathogenic bacteria, opening avenues for its application in biomedicine and food preservation. The research underscores the importance of exploring and preserving the microbial diversity of high-altitude regions like the Himalayas, contributing to our understanding of microbial ecosystems in extreme environments and the potential biotechnological applications of pigment-producing bacteria in various industries.

Future prospective:

- It can be used as antibacterial, antifungal, and antiviral for developing antibiotics
- It exhibits strong anticancer activity
- 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

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