CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD


# Towards Sustainable Colorants: Molecular Characterization and Optimization of Bacterial Pigments

by

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A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

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*I dedicate this thesis to my loving and supportive family and friends who have fully helped me in achieving my life goals.*


**CERTIFICATE OF APPROVAL**

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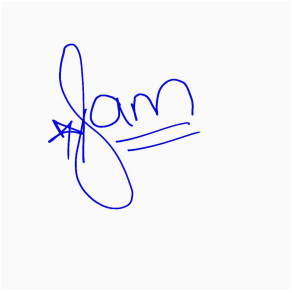

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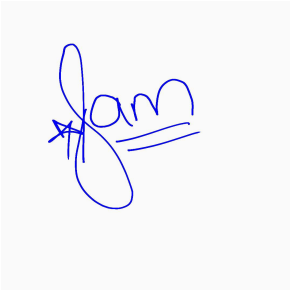

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I want to thank ALLAH, a guiding force in our lives. We may not understand everything, but ALLAH helps and guides us. My first and foremost acknowledgement is to ALLAH, the Lord and Creator of the universe. His knowledge is vast, and even though we can't understand everything, He gives us the courage to keep learning and exploring. I also want to show my respect and gratitude to Prophet Muhammad (S.A.W), who worked hard to guide us, and we can never thank him enough for that.

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# *Abstract*

The growing concerns over the environmental and health impacts of synthetic pigments, commonly used in industries such as textiles, food and cosmetics, have spurred interest in alternative, eco-friendly solutions. This study investigates the potential of microbial pigments as sustainable substitutes, particularly focusing on pigments produced by bacteria isolated from desert soil in Bahawalpur. A total of 4 pigmented bacterial strains were identified through 16S rRNA analysis, which included *Planomicrobium* sp. (PQ878368), *Pseudarthrobacter* sp. (PQ878392), *Priestia aryabhattai* (PQ661249) and *Priestia megaterium* (PQ661253). These strains produced pigments with varying colors such as orange, yellow and pink with notable pigments being carotenoids particularly, beta carotene, and prodigiosin a pink pigment with a tetrapyrrole structure. The chemical composition of these pigments was analyzed using UV- vis Spectrophotometry, High-Performance Liquid Chromatography (HPLC), and Fourier Transform Infrared Spectroscopy (FTIR) and it confirmed the presence of carotenoids a Beta-carotene, with characteristic absorption at 450 nm and prodigiosin with an absorption peak at 530 nm. Beta-Cryptoxanthin, zeaxanthin, lutein, violaxanthin, Beta-carotene, and lycopene alongside prodigiosin and cyclo prodigiosin by their distinct retention times. FTIR spectra revealed key functional groups such as C-H stretches, conjugated C=C bonds for carotenoids, and pyrrole ring vibrations for prodigiosin. Additional compounds including flavonoids and phenolic acids, were also identified as contributing to the pigments' bioactive properties. Moreover, the antibacterial and antifungal activities of four bacterial strains and pigments were assessed against *Aspergillus fumigatus*,*Rhizopus delemar*, *Staphylococcus aureus* and *Kerstersia gyiorum*. *P. aryabhattai* showed the highest antifungal activity, particularly against *R. delemar*, while *Pseudarthrobacter* sp. exhibited strong antibacterial activity against *S. aureus*. Yellow pigment from *Pseudarthrobacter* sp. demonstrated significant antimicrobial effects. The study underscores the environmental and health benefits of using microbial pigments such as carotenoids, and prodigiosin, which are biodegradable, non-toxic and capable of replacing harmful synthetic dyes. The findings highlight the potential of these microbial pigments for

use in various industries, particularly in areas where reducing the environmental footprint of dyes is a priority. Further optimization of production processes could enhance their commercial viability, offering a sustainable alternative to synthetic dyes and pigments.

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# Abbreviations

| | |
|---|---|
| **CRA** | Congo-Red Agar |
| **EMB** | Eosin Methylene-Blue Agar |
| **FTIR** | Fourier Transform Infrared Spectroscopy |
| **HPLC** | High-Performance Liquid Chromatography |
| **MSA** | Mannitol Salt Agar |
| **NA** | Nutrient Agar |
| **PDA** | Potato Dextrose Agar |
| **SCA** | Starch Casein Agar |
| **UV-vis** | Ultraviolet Visible |

# Chapter 1

# Introduction

The word pigment is derived from the Latin word *pigmentum* meaning "drug". It acts as a coloring agent when an insoluble material is suspended in it and maintains its physical properties [1]. Pigments are the molecules that absorb the specific wavelength of light within the range between 360nm-780nm while reflecting the remaining visible light [2]. Pigments can be natural (organic) or artificial (inorganic). In pigment, the colour depends on the wavelength that is absorbed, size, texture and shape of the grain [1]. Natural pigments are obtained from plants, minerals, insects and animals. They contain a color spectrum from yellow to black [3]. Artificial pigments can be synthesized in the laboratory. Some artificial pigments are iron dioxide and chromium oxide etc. They are known for their stability and durability [4].

Stability is the dye core structure's resistance to disintegration by sources. These variables include the medium's pH, temperature, and chemical incompatibility. This kind of stability data is extremely helpful when determining the dye's chemical compatibility for specific applications or its shelf-life when it is already covalently bonded to another molecule (like an antibody). The dyes' durability determines their suitability in various applications such as temperature resistance, rub fastness, wash fastness, light fastness, and chemical stability [5].

Natural pigments are obtained from natural sources such as animals, plants, minerals, and micro-organisms. Plants are the basic source of natural dyes because

natural dyes are biodegradable and renewable. The sources of natural pigments, examples, and which color they produce are mentioned in Table 1.1. The plants' natural dyes are obtained from leaves, roots, fruits, flowers, and bark. They give different colors from these parts such as the barks of the Sappon-wood tree giving brown, its pod giving red and its roots giving yellow, respectively. Animal dyes are obtained from dried dead bodies of insects such as insects that live on Cactus plants that give red dye i.e., called Cochineal. The chrome yellow, iron buff, manganese bistre, and iron black are colors that are obtained from minerals [6].

TABLE 1.1: Color produced by natural pigment molecules [6].

| **Source** | **Examples** | **Color Produced** |
|---|---|---|
| Plants | Turmeric | Yellow |
| | Annatto | Red-Orange |
| | Spinach | Green |
| Animals | Lac | Red |
| | Cochineal | Red |
| | Shellfish | Purple |
| Minerals | Iron | Black |
| | Malachite | Green |
| | Chrome | Grey |
| Micro-Organisms | *Staphylococcus* sp. | Golden |
| | *Pseudomonas* | Green |
| | *Serratia* | Red |

Natural pigments have many advantages such as they are non-toxic, renewable, derived from natural sources, less allergic, and biodegradable than synthetic pigments but they have some mutagenic effects for example elderberry color is extracted from *Sambucas Nigra* fruits and safflower yellow from *Carthamus tinctorius* can cause asthma during continuous inhalation. Natural pigments have some disadvantages. It is expensive to dye a fabric like one gram of fabric is dyed with 5 grams of synthetic dye but using natural dye would take 230 grams to dye the same material [3].

Natural pigments made from plants have some drawbacks, such as it is expensive to cultivate the plants only to extract the pigments [7]. Moreover, plant-based pigments denature when the pH changes and batches don't provide reproducibility because natural pigments are produced in plants by secondary metabolic activities or by some special animal processes that depend on age, seasonal variation and climate conditions. Thus, it isn't easy to control the reproducibility of the shades [3]. Meanwhile, synthetic pigments have some toxic concerns shown in Figure 1.1.

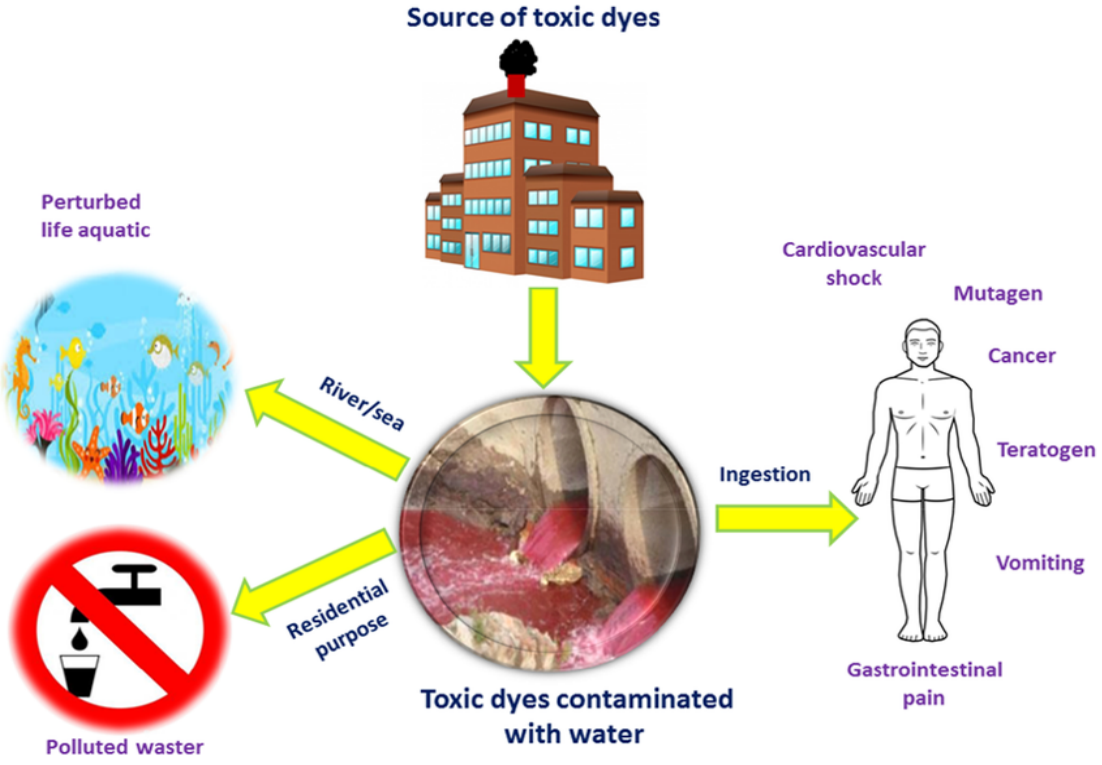


FIGURE 1.1: Adverse effect of toxic dyes [8].

They also contribute to greenhouse gases, when researchers dump the material into the ponds and streams, leading to algal bloom [7]. They are non-biodegradable so they accumulate into land and rivers, causing ecological concerns. They have a complex molecular structure and synthetic origin so they are a serious threat to water streams and the environment. Cationic dye causes tissue necrosis, jaundice, vomiting and increased heart rate in humans. Disperse dyes at low perspiration can cause allergy, skin irritation, mutation and cancer [3]. Azo dyes can cause various types of cancer (bladder, skin and liver cancers), in their cleaved aromatic amine state [9].

Due to the non-biodegradable properties of synthetic pigment, they also violate the different Sustainable Development Goals such as affecting Good health and Well-being by leeching toxic substances into water and soil causing pollution which can result in cancer and skin problems, Clean Water and Sanitation by discharging the toxic chemical in water result in unsafe for drinking, bathing and irrigation purposes, Responsible Consumption and Production by disposal of synthetic dyes without proper environmental management and disrupting the ecosystem, Life Below Water by dumping the chemical into water leads to bioaccumulation and bio-magnification of toxin in the food chain at last Life on Land adversely disrupts the terrestrial ecosystem by disrupting biodiversity, flora and fauna [10].

Organisms are used to produce pigmented compounds with potential biological applications in industry must meet the specific criteria. They need to be easily cultured, grow rapidly, be produced efficiently in limited time and space, be readily available and be non-toxic and non-pathogenic, thriving on a variety of nutrients. Microbes are often favored for these purposes over animals, plants and synthetic compounds [2].

Various microorganisms such as fungi, bacteria, yeast, and algae can produce natural colors. Some of them are shown in Table 1.1. Carotenoids, flavins, violaceins, melanins, and phenazines are among the pigments synthesized by these microbes [7]. Some other examples are *Trichoderma viren*, *Chitosan*, *Serratia* spp., and *Alternaria alternata* are used to obtain natural pigments. Phytocyanin produces blue pigments from *Spirulina plarensis* algae, and red color is obtained from *Monascus anka* and the fungus *Echinidontium tinctorium* [6].

Microbial pigments are classified as secondary metabolites, are produced under stressful conditions and are responsible for generating vibrant colors. These pigments offer both nutritional and therapeutic benefits, setting them apart from other types of pigments [7]. Sunlight plays a dual role in bacterial pigments. Bacterial pigments absorb different wavelengths of visible light and give different colors. In plants, algae and cyanobacteria chlorophyll absorbs blue and red light and gives out green color. Phototropic bacteria have photosynthetic pigments i.e., chlorophyll and carotenoids that convert light into chemical energy.

They capture the light energy and transfer it to the reaction centre that produces ATP and NADPH. Bacteria generate a variety of pigments by absorbing different wavelengths of light that also help them survive in various environments. The accessory pigments i.e., carotenoids help the photoautotrophic bacteria against reactive oxygen species generated during photosynthesis [11].

Some pigments like flavin, phenazines, melanins and quinones help the non-phototropic bacteria to adapt under stress and dark environments. These pigments are called secondary metabolites and maintain bacteria health under stress conditions, starvation and microbial competition. The blue light also plays a major role in regulating virulence, motility and metabolic pathways [11].

Phototropic and non-phototrophic microorganisms produce pigments with the help of both light-dependent and light-independent mechanisms, as shown in Figure 1.2. Each family of pigments captures the visible light of sunlight in a specific range. The microbial fitness acts like virulence factors, anti-oxidants, and anti-microbial increases in the dark [11].

Microbial pigments are cruicial in various areas, including anti-oxidant activities, anti-tumor, anti-carcinogenic, cell-signaling communication, photosynthesis, UV absorption, radiation protection, antibiotic activities, membrane stabilization and virulence. These serve as biological markers and are utilized to identify various microbes. Bacterial pigments find applications in industries such as plastics, textile dyeing, food colorants, cosmetics, painting and pharmaceuticals [2].

## 1.1 Problem Statement

Many sectors such as textile, food, and other industries depend on conventional pigments that cause pollution and environmental degradation. These pigments release toxic chemicals into the water bodies that impact the health of both aquatic animals and humans. Moreover, these synthetic pigments are resource-intensive and require more energy for their synthesis. Natural pigments from plants and animals require high cost and their longevity has some obstacles.

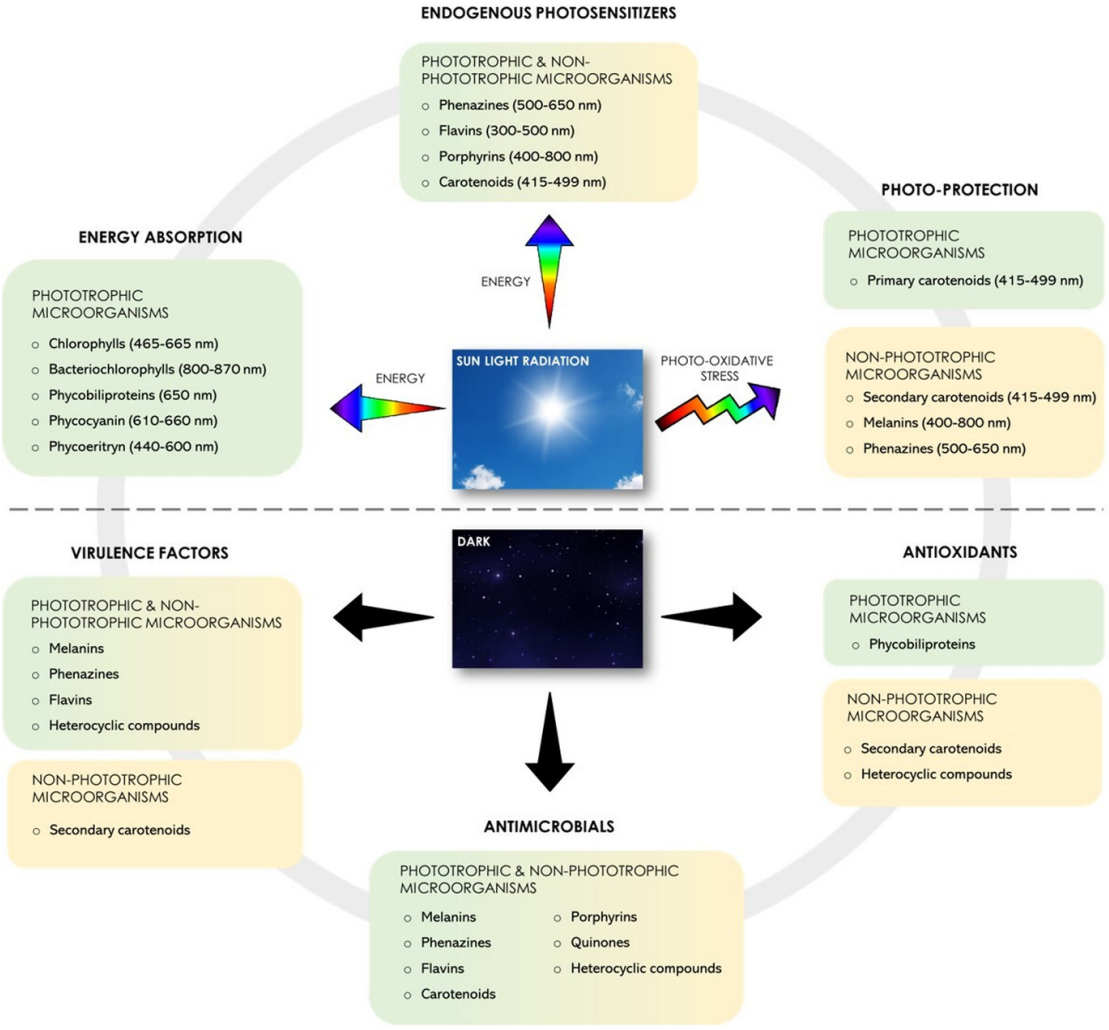


FIGURE 1.2: The physiological role of bacterial pigment [11].

## 1.2 Gap Analysis

The transfer of conventional pigments into microbial sustainable pigments has some gaps that need to be addressed to achieve benefits for the environment, health, and economy. Microbial pigments can reduce pollution and carbon footprint, conserve resources, and protect biodiversity. In terms of human health, these pigments are non-toxic and biodegradable. Economically, there is a high demand for sustainable pigments that can be less costly due to lower energy consumption and reduced waste management costs. So, microbial-derived dyes need to be developed to overcome the problems caused by synthetic dyes. Addressing the above gaps is important for achieving a cleaner environment, a more sustainable economy and a healthier population.

## 1.3 Aim and Objectives

This study aims to isolate, characterize, evaluate, and screen the pigment-producing bacterial strains through molecular identification for the safety of living organisms.

The objectives of the project are as follows:

- To isolate bacterial strains and optimize for enhanced pigment production.
- To identify the chemical composition of the pigments and their functional groups.
- 16SrRNA-based identification of pigment-producing strains.
- To study the antibiotic sensitivity of the bacterial strains as well as the antimicrobial properties of the pigment-producing bacterial strains and their respective pigments.

# Chapter 2

# Literature Review

The rising emphasis on sustainability has sparked interest in producing bio powders as a promising alternative to synthetic pigments. Bacterial pigments, in particular, are attracting attention because of their excellent biodegradability and reduced environmental impact. These pigments hold significant potential for various industrial applications. Currently, some bacterial pigments are being synthesized on an industrial scale and utilized in textiles, providing vibrant colors without the environmental issues associated with conventional synthetic dyes. Beyond textiles, these pigments are also being used in the food industry as natural colorants, for their therapeutic benefits in medicines, and their safety and compatibility in cosmetics. The adoption of bacterial pigments not only represents an eco-friendly choice but also meets the increased desire for sustainable and natural products among consumers. As research and technology continue to evolve, the use of bacterial pigments is likely to expand, offering new opportunities for innovation across multiple industries.

## 2.1 Pigments

Pigments are small-particle substances that are insoluble in their application system and act as colorants due to their properties of inhibiting corrosion and other magnetic properties. They differ from dyes, as dyes also act as a colorant but

they are soluble in their application system. Pigments play a significant role in cosmetic formulation, plastics, paints, printing inks, and industrial and automotive coatings. Some other applications of pigments are in porcelain, glass, paper, rubber, stains and glazes [12].

The term pigment is derived from the Latin word *pigmentum*, which was used for the sense of colorant. In the Middle Ages, pigment was used as the colorant that was extracted from the plant [12]. When pigments react with specific colorant absorption, it changes the color of the light [13].

### 2.1.1 Classification of Pigments

Pigments are classified into four categories: natural or synthetic and organic or inorganic pigments.

#### 2.1.1.1 Organic Pigments

Different extraction techniques extract Organic pigments from plants (fruits, vegetables) and animals. In the Middle Ages, cave artists used organic pigments to decorate the walls of caves. Biological pigments are classified based on structural affinity and natural occurrence of pigment. Examples of some naturally occurring pigments are Chlorophyll (green), carotene (yellow-red) tannins (brown-red) and anthocyanin (blue-red) [13].

Many biological molecules are composed of only seventeen elements in the periodic table. The most pigmented compounds consist of four major elements i.e., Hydrogen, Carbon, Nitrogen and Oxygen. The molecular weight of the pigmented molecules ranges from 200 nm (anthraquinones), 300 nm (anthocyanidins), 400 nm (betalains), 500 carotenoids and 800 nm (chlorophylls) [13].

Moreover, these biological pigments are classified into six major structural classes based on structural affinities shown in Figure 2.1 i.e., metalloprotein, O-heterocyclic, N-heterocyclic, tetrapyrroles, quinines and tetraterpenoids [14].

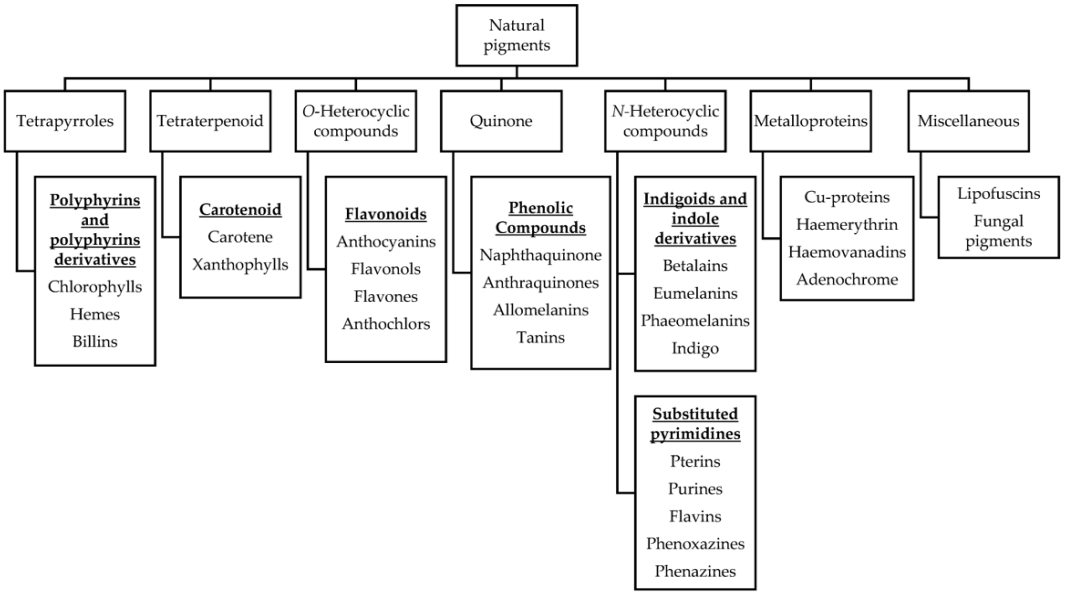


FIGURE 2.1: Classification of biological pigments based on structural affinities [14].

#### 2.1.1.2 Inorganic Pigments

The source of inorganic pigments is minerals (Figure 2.2). These pigments are more stable under the action of temperature, light, atmosphere and chemicals [12]. Through electronic transitions, the colors of inorganic pigments are produced that are differ in nature and distinct from those that produce a color from organic colorants. They show high inherent opacity that contributes to a high refractive index, leading to stronger atom arrangement in a crystal structure. Different processes are used for the manufacturing of these pigments like gas-phase processes, high-temperature solid state reaction etc. [13].

#### 2.1.1.3 Synthetic Pigments

Synthetic organic pigments are created by precipitating water-soluble dyes onto colorless inorganic bases like barium and alumina sulfate, resulting in products known as “lakes”. A key development in the industry of organic pigment in 1928 was the finding of copper phthalocyanine. Synthetic organic pigments are known for their unique color brightness and color intensity as compared to inorganic pigments. However, they typically lack the opacity seen in inorganic pigments due

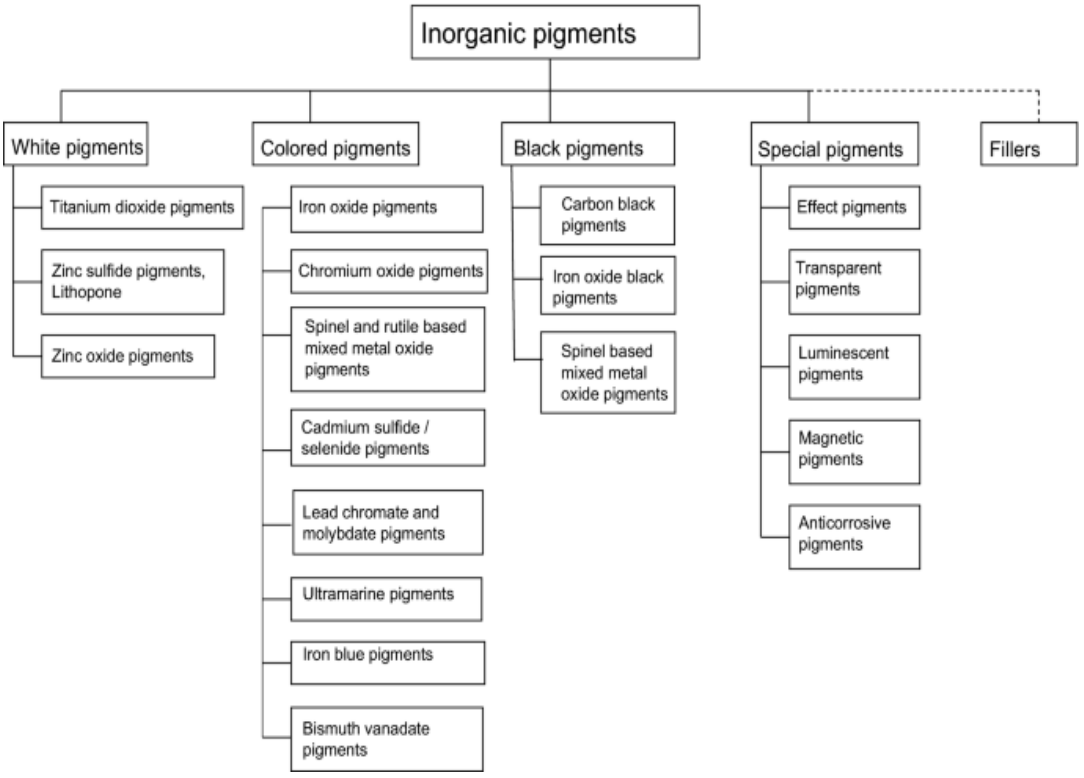


FIGURE 2.2: Classification of inorganic pigments [12].

to the reduced refractive index of organic crystals [15]. The commercial variety of inorganic pigments offers a variety of different fastnesses, which are influenced by both the characteristics of intermolecular interactions in the solid phase and their molecular structure. Since organic molecules dissolve in organic solvents. Moreover, their pigments are designed in such a way as to improve their resistance to solvents due to their structural modifications. Copper phthalocyanines and azo pigments are some examples of synthetic organic pigments (Figure 2.3) [13].

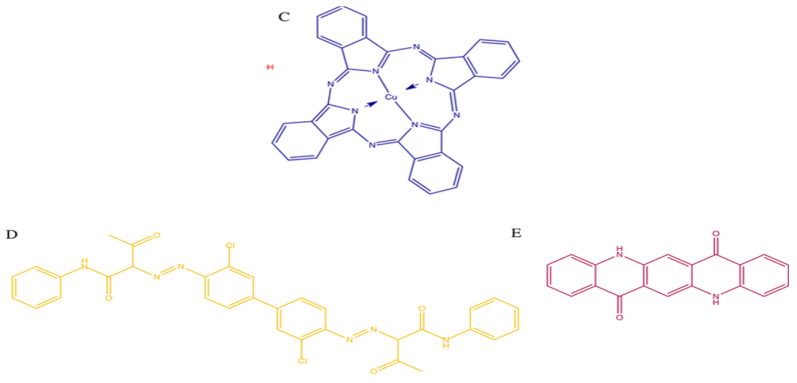


FIGURE 2.3: Structure of synthetic pigments: C is the copper phthalocyanine-blue, D is the benzidine yellow and E is the red pigment of the quinacridone family [16].

The synthetic pigment industries are ending now due to the dumping of toxic chemicals into the freshwater, lakes, rivers and ponds resulting in the production of algal blooms that contribute to greenhouse gases. These chemicals are very hazardous and carcinogenic to living things. Due to these reasons, synthetic pigments are said to be "toxic contaminants" and are often used less [7].

## 2.2 Need for the Natural Pigments

### 2.2.1 History of Colorants

Since Stone Age, use of colorants had been started after the advancement in textile industries by introducing different techniques like weaving [17]. Some common ancient pigments are blue-indigo, yellow and madder [18]. The major source of natural colorants were plants, animals and minerals. Most of the plant parts like fruits, flowers, leaves, bark and seeds were used as colorant combinations [19]. In Stone Age mostly minerals were used to extract pigments while in Ancient India, Ancient Africa, Ancient Rome, Ancient Egyptian and Phoenician minerals as well as plants and animals were used to extract the pigments (Figure 2.4) [20].

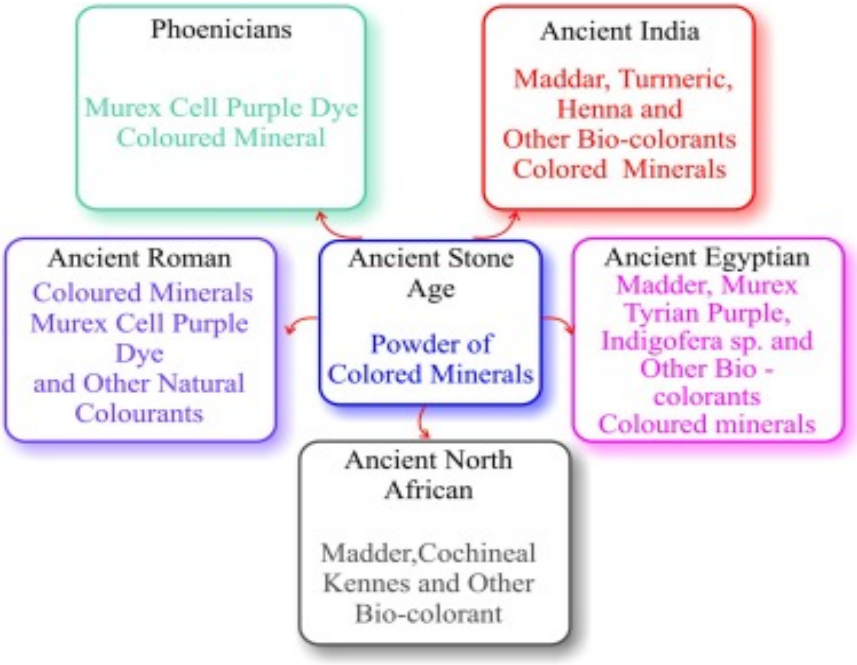


FIGURE 2.4: History of the utilization of pigments in different eras [17].

In recent times, only 1% of natural pigments have been used as compared to synthetic pigments due to the higher cost of production and limited biomaterials, so less amount of pigments are extracted and have poor binding capacity. In that case, synthetic dyes have an advantage over natural dyes but they cause many health issues during consumption and are not eco-friendly. They contribute to the climate change. In many countries, synthetic pigments are banned. Moreover, industries are looking towards the replacement of natural pigments that are extracted from plants, animals and minerals [20].

### 2.2.2 Effect of Synthetic Pigments on Humans and Environment

Pigments are coloring agents and play a crucial role in printing ink, textiles, food coloring, paper making, leather, pharmaceutical industries and in cosmetics. For many years, azo dyes have been used in large quantities compared to natural dyes because of their cost-effectiveness, sustainable life and color fastness. It was estimated that nearly every year almost $10^3$ tons of azo dyes are produced. Due to this people are very concerned about wastewater treatment because azo dyes are highly toxic and pose a threat to the environment. Various types of hazardous chemicals and carcinogens are present in the synthetic dyes that affect human beings and fauna. The colored wastewater dumped into the water bodies like lakes, streams or rivers affects the visibility in the littoral zone and that leads to the entry of sunlight into the water bodies. A huge amount of aqueous waste and effluents of dyes are being dumped from the textile industries, which increases the high biological demand (BOD) that is harmful for the environment [21].

It is estimated that around $2\times10^5$ tons are discharged into the water bodies from finishing and dyeing processes every year. Due to the inefficiency of the dyeing process, it is difficult for azo dyes to deteriorate through conventional treatment processes of wastewater. As a result, they are present in the atmosphere for a longer time because of their water, soap, detergents, bleaching, chemicals, temperature and stability to light [21].

Moreover, an antimicrobial agent that is commonly used in the textile industry inhibits the biological degradation of natural fibres like cotton. The aromatic structure and complex origin of azo dyes make them resistant to biodegradation. However, environmental regulations require industries to remove color from dye wastewater before it is discharged into water bodies, to prevent contamination of aquatic environments [21].

Textile wastewater is characterized by variations in parameters such as color, pH, chemical oxygen demand (COD), salinity and biochemical oxygen demand (BOD). The composition of the wastewater is primarily influenced by the various chemicals, organic compounds, and dyes utilized during the wet and dry processing stages. Major pollutants found in textile effluents include color, toxic substances, surfactants, chlorinated compounds, persistent organic materials, and salts [21].

## 2.3 Natural Pigments

The natural pigments can be obtained by plants, animals and micro-organisms.

### 2.3.1 Animal-Based Natural Pigment

In animals, the melanin pigments can be extracted from fur, hair, nails, iris and skin. Moreover, some animals like amphibians (frogs), reptiles (lizards, chameleons), insects (lake flies, stick and grasshoppers) and teleost (catfish, eel and minnows) have some specialized cells called chromatophores that help them to change color under certain conditions. Thus, pigment helps them to imitate and camouflage the organisms in their environment [22]. Subclasses of animal based pigments can be noted in Table 2.1.

TABLE 2.1: Subclasses of animal-based pigments [7].

| **Pigments** | **Subclass** |
|---|---|
| Melanin | Eumelanin |

Table 2.1: (Continued).

| **Pigments** | **Subclass** |
|---|---|
| | Pheomelanin |
| | Albomelanin |
| Chromatophore | Xanthophores |
| | Erythrophores |
| | Indophores |
| | Leucophores |
| | Melanophores |
| | Cynophores |
| Carotenoids | Xanthophylls |
| | Carotenes |

### 2.3.2 Plant-Based Natural Pigments

Plant-based natural pigments are found easily due to their wide distribution. These pigments are present in vacuoles or plastids. They are present in the protoplasms of vegetative cells, producing different bright colors in fruits, flowers, vegetables and leaves. These pigments give colour and serve a major part in the protection and metabolic reactions of vegetative cells [23]. Based on the chemical structure, plant pigments are divided into four categories, described in Fig 2.5 [24].

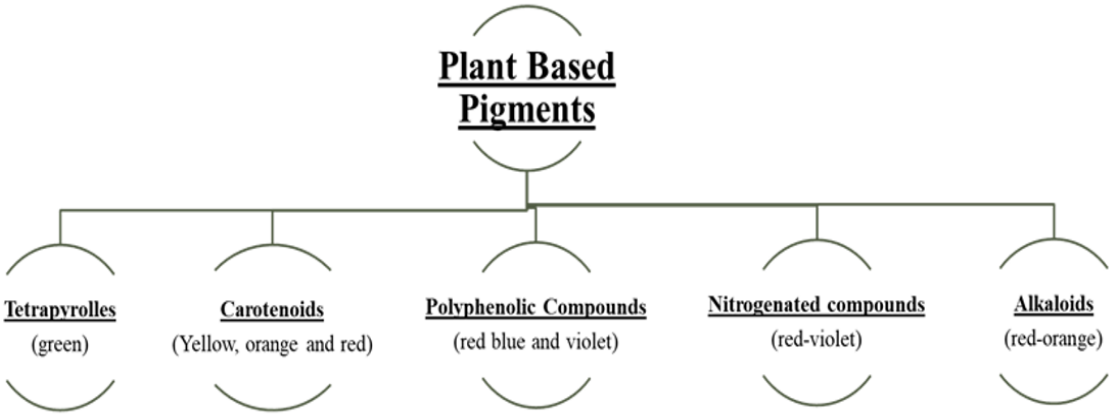


FIGURE 2.5: Classification of plant-based pigments [24].

### 2.3.3 Micro-organisms Based Natural Pigments

#### 2.3.3.1 Fungi

The filamentous fungi produce a variety of pigments that have applications in various industries like food, cosmetics and feed. The major group of fungi is carotenes consisting of different fungal classes like [25]:

1. Zygomycetes include Phycomyces, Blakeslea and Mucor.

2. Basidiomycetes include Rhodosporidium, Ustilago, Sclerotium, Sporidiobolus and Sclerotinia.

3. Ascomycetes (Penicillium, Aschersonia, Cercospora and Aspergillus).

Some pigments like flavins, quinones and melanins are produced from different fungal species like *Ashbya*, *Eurotium*, *Curvularia*, *Trichoderma*, *Eremothecium*, *Curvularia*, and *Fusarium* [26].

Many pigments can be extracted from the spore of fungi, it is less costly to focus on the screening of the extraction of the pigments and produces high yields. It produces low and non-toxic byproducts, which is why they can easily withstand extreme pH and temperature changes in industries [27].

In the textiles and tannery industries, the common pigments-producing strains of fungi are *Penicillium marneffei*, *Emericella nidulans*, *lsaria farinose*, *Monascus purpureus*, *Fusarium verticillioides*, *Thermomyces* and *Monascus ruber* [28].

Marine environments are characterized by extreme conditions such as fluctuating temperature, pressure, and salinity. These harsh conditions can prompt certain fungi, including *Microsporum*, *Halorosellinia*, and *Eurotium rubrum* species, to produce distinctive pigments that may release light or heat. Fungal genera like *Trimmatostroma*, *Hortaea*, *Aspergillus* and *Phaeotheca* are also capable of producing pigments, often mirroring their identity and having morphological associations with corals, algae or marine plants [29].

#### 2.3.3.2 Micro Algae

Micro-algae along with the prokaryotic cyanobacteria are eukaryotic photosynthetic micro-organisms that convert light energy into chemical energy through photosynthesis. Carotenoids, chlorophylls and phycobiliproteins are the three major classes of micro-algae that are responsible for cell growth and pathways in photosynthesis. Micro-algae-derived pigments have great physiological activity like antibacterial and anti-oxidants compared to other natural sources of pigments derived from fruits plants or animals [30].

Micro-Algae is the stable natural source of pigments that can easily be culture in an eco-friendly environment. It is a renewable source and can easily be found in any season [31]. Meanwhile, they are recognized for their rapid growth rate during stress conditions. They produce high pigment content and don't require any arable land to cultivate. For enhancing pigment, some cultivation strategies have been used. Many active pigments of Micro-Algae are used in pharmaceutical, food, cosmetic, aquaculture and various industries like $\beta$-carotene (*Dunaliella* produces yellow pigment), PBPs (*Spirulina* produces blue pigment) and astaxanthin (*Haematococcus* produces yellow to red color) [32].

Pigments extracted from Micro-Algae are produce either during under stress or normal growth. The quality of the pigments can be influenced by many non-living and living factors [32]. Many new methods and approaches have been introduced to enhance the efficiency of pigments [33]. Furthermore, during processing, extraction and purification, these pigments can get damaged which can affect their pigment. Thus, it is important to improve the extraction techniques and processing methods to ensure the stability and retention of the pigment [34].

#### 2.3.3.3 Bacteria

Like animals and plants, prokaryotes are also the source of producing pigments. In photosynthetic bacteria, these pigments contribute to the manufacturing of carbohydrates, while some of them play a significant role in protecting the bacteria

from UV radiation [35]. Prokaryotes can be divided into two main groups are described in the following table 2.2.

TABLE 2.2: Difference between pigment-producing Autotrophic and Heterotrophic prokaryotes [36].

| **Autotrophic Prokaryotes** | **Heterotrophic Prokaryotes** |
|---|---|
| They contain pigments that contribute to photosynthesis. Examples: xanthophylls, chlorophyll, and carotene. | They contain accessory pigments that help them to survive in harsh environments. Example: Bacterium *Xanthomonas oryzae* pv. *oryzae* secreted xanthomodin, a membrane-bound yellow pigment that helps them against photo damage. |

When species of bacteria are grown in their specific growth conditions, then bacterial pigments are synthesized from their secondary metabolites [37]. When the bacteria are exposed to sunlight they start to produce orange pigment that helps them to protect from UV radiation from the Sun. During the lag phase, when cells face the depletion of resources, at that time only a few pigments are produced. Different bacterial species require different growth needs, which determine the specific pigments they generate. The percentage of the pigment can depend on the absorption of the pigment, more the absorption, the more pigment production will be. Media optimization and maintaining the accurate incubation condition can give higher yields and good quality of pigment [38, 39].

TABLE 2.3: Pigments produced by bacteria [40].

| **Pigments / Color Produced** | **Bacteria** |
|---|---|
| **Astaxanthin** | |
| Pink-Red, Bluish-Red | *Agrobacterium aurantiocum*<br>*Paracoccus carotinifaciens*<br>*Xanthophyllomyces dendrorhous*<br>*Rhodococcus maris* |

Table 2.3: (Continued).

| **Pigments / Color Produced** | **Bacteria** |
|---|---|
| **Canthaxanthin** | |
| Dark Red | *Haloferax alexandrinums* |
| | *Bradyrhizobium* species |
| **Zeaxanthin** | |
| Greenish to creamish, Brown, | *Corynebacterium michigannise* |
| Creamy, Yellow, Orange-Yellow | *Bacillus* |
| | *Achromobacter* |
| | *Paracoccus zeaxanthinifaciens* |
| | *Brevibacterium* species |
| **Prodigiosin** | |
| Red | *Serratia marcescens* |
| | *Serratia rubidaea* |
| | *Alteromonas rubra* |
| | *Vibrio gaogenes* |
| | *Rugamonas rubra* |
| | *Streptoverticillium rubrireticuli* |
| **Violacein** | |
| Purple | *Chromobacterium violaceum* |
| | *Janthinobacterium lividum* |
| **Pyocyanin** | |
| Blue-Green | *Pseudomonas aeruginosa* |
| **Indigoidine** | |
| Blue | *Corynebacterium insidiosum* |
| **Xanthomonadin** | |
| Yellow | *Xanthomonas oryzae* |
| **Staphyloxanthin/ Zeaxanthin** | |
| Golden-Yellow | *Staphylococcus aureus* |

## 2.4 Distribution of Microbial Pigments Based on Ecology

Pigmented microorganisms, or chromogenic microbes, including bacteria, microalgae, and haloarchaea, have been discovered in a wide range of environmental and geographic settings, such as land, air, and marine areas [41]. Extensive research is taking place on these colorful microorganisms, particularly those isolated from marine environments like seawater, marine sediments, sponges, sea ice (e.g., *Algoriphagus*), salt flats, microbial mats, and other unique habitats due to their diverse and novel pigmentation [7].

Additionally, many pigment-producing microorganisms have been identified in extreme environments, including lava caves and hot springs, as well as in more typical environments. Certain geographic regions also exhibit a higher prevalence of these pigmented microorganisms compared to others [42].

Deep-sea hydrothermal vents, glaciers, salt lakes, and ice cores are also sites where high concentrations of pigmented microorganisms can be found [43]. This widespread distribution of pigment-producing microorganisms across the globe offers significant opportunities for scientists to explore their industrial applications and their potential in medicines [7].

## 2.5 Importance of Using Bacterial Pigments over Plants, Animals and Fungi

The natural pigments produced by microorganisms are put forward as compared to plants due to their stability. Bacteria can produce a wide range of pigments, among microbes. The advantage of using bacteria over, plants, animals, fungi and algae is that they can be found very easily and grow on suitable growth medium which ultimately leads to low cost and is ideal for industrial production [44]. Their production is adaptable and can be more easily regulated than plant and animal

sources [13]. Due to their non-toxic and eco-friendly nature, antimicrobial, anti-cancer and anti-oxidant activities, bacterial pigments are used in the cosmetic and pharmaceutical industries, moreover, in food flavouring [44].

The pigments that are extracted from plants denatured very easily due to any change in temperature, reproducibility and pH [45]. Additionally, plant pigments require extensive monoculture areas, pest management, and reliance on rainfall, along with other situational factors that influence the quality and yield of crops, potentially leading to losses [46]. Consequently, although this option is organic, biodegradable, and organic. It still has some environmental impact due to the need for large amounts of irrigation water and the occasional use of pesticides for pest management [47].

Pigments derived from animals, particularly mollusks and insects, were commonly used in old times. There are many concerns and production challenges related to the usage of animal-based pigment. So, over the years, the usage of animal pigments has decreased [17].

Insects provide various pigments like melanins, ommochromes, pterins, aphids, anthraquinones, papiliochromes and tetrapyrroles [48]. However, extracting these pigments involves expensive insect cultivation and purification processes [49]. Additionally, there have been reports of allergic reactions associated with these pigments [50].

Moreover, the production of pigments in higher organisms like animals, plants, and fungi can be harder to utilize because they are found in complex tissues or only produced during specific stages of the organism's life cycle. For instance, some pigments that attract mates are only produced after other parts of the life cycle are completed, making them difficult to exploit or manipulate. On the other hand, bacteria, which are abundant in nature and can be found in common water and soil, offer an alternative. They do not require genetic modification and can thrive on simple, inexpensive media. Bacteria have the remarkable ability to use low-cost carbon and nitrogen sources to produce valuable metabolites, both small and large [13].

TABLE 2.4: Advantages and disadvantages between animals, plants and microbial pigments [51–53].

| Parameters | Animals | Plants | Microbial |
|---|---|---|---|
| **Economically** | Expensive | Moderately costly | Highly cost-effective |
| **Environment Friendly** | Biodegradable | Biodegradable | Biodegradable |
| **Contaminant Free** | Potential contaminants | Potential contaminants | Contaminant-free |
| **Sustainable** | Not renewable | Renewable | Renewable |
| **Substrate Utilization** | Not applicable | Utilizes substrate | Utilizes substrate |
| **Climatic Resilience** | Sensitive to climate | Sensitive to climate | Highly resilient |
| **Genetic Engineering Potential** | Limited potential | Highly potential | Highly potential |

### 2.5.1 Advantages of Using Bacterial Pigments over Synthetic Alternatives

Pigments extracted from bacteria are increasingly favored over synthetic pigments due to several advantages. They are easy to grow and safe for human use. The extraction and scaling-up processes for bacterial pigments are also more cost-effective. These pigments, which are secondary metabolites produced by living organisms, play various roles, including aiding in photosynthesis, offering protection from UV, defending against rival species, and serving as energy storage molecules [54].

Due to their easy cultivation, resilience to temperature and pH fluctuations, diversity, and non-toxic, eco-friendly nature make them excellent candidates for the

modern pigment industry and their production doesn't cause any climate change [55].

Moreover, bacterial pigments have impressive medicinal properties, further supporting their potential to replace synthetic dyes. For instance, pigments like yellow and orange carotenoids, zeaxanthin, prodigiosins, astaxanthin, pyocyanine, violacein, and actinorhodin are being extensively researched for their possible applications in medicine. Advances in recombinant DNA technology now allow the biosynthesis of natural pigments using bacterial host cells, eliminating the need to grow large quantities of plants to obtain pigments [7]. The demand for naturally derived colors has grown significantly due to their stability and positive consumer perception. The annual growth rate for these natural colors is projected to be between 5-10%, compared to the lower growth rate of 3-5% for synthetic colors [44].

The increasing interest in microbial pigments is driven by their natural origin, safety, medicinal benefits, nutritional content like vitamins, and the fact that their production is not dependent on seasonal or geographical factors, leading to a controllable and predictable yield [44]. Different pros and cons of synthetic dyes can be noted in Fig 2.6.

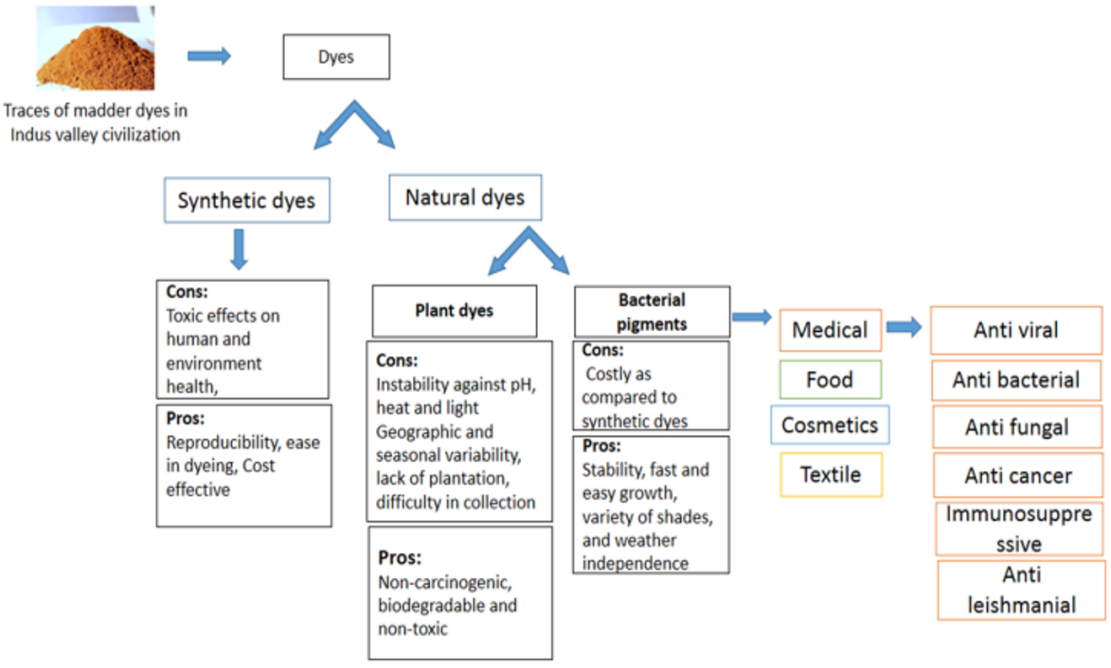


FIGURE 2.6: Pros and cons of different types of dyes [44].

## 2.6 Characteristics of Microbial Pigments

Many microorganisms such as microalgae, fungi, archea and bacteria produce primary and secondary metabolites that help them to survive in an environment. These compounds are pigments that provide protection against reactive oxygen species and UV-radiations. Moreover, these pigments have antifungal and antibacterial properties. These properties help them to survive in an environment and prevent the other species from making colonies in their areas. Certain pigments can play a significant role in photosynthesis to provide energy to the cells [56–58]. Some characteristics of microbial pigments are shown in Fig 2.7.

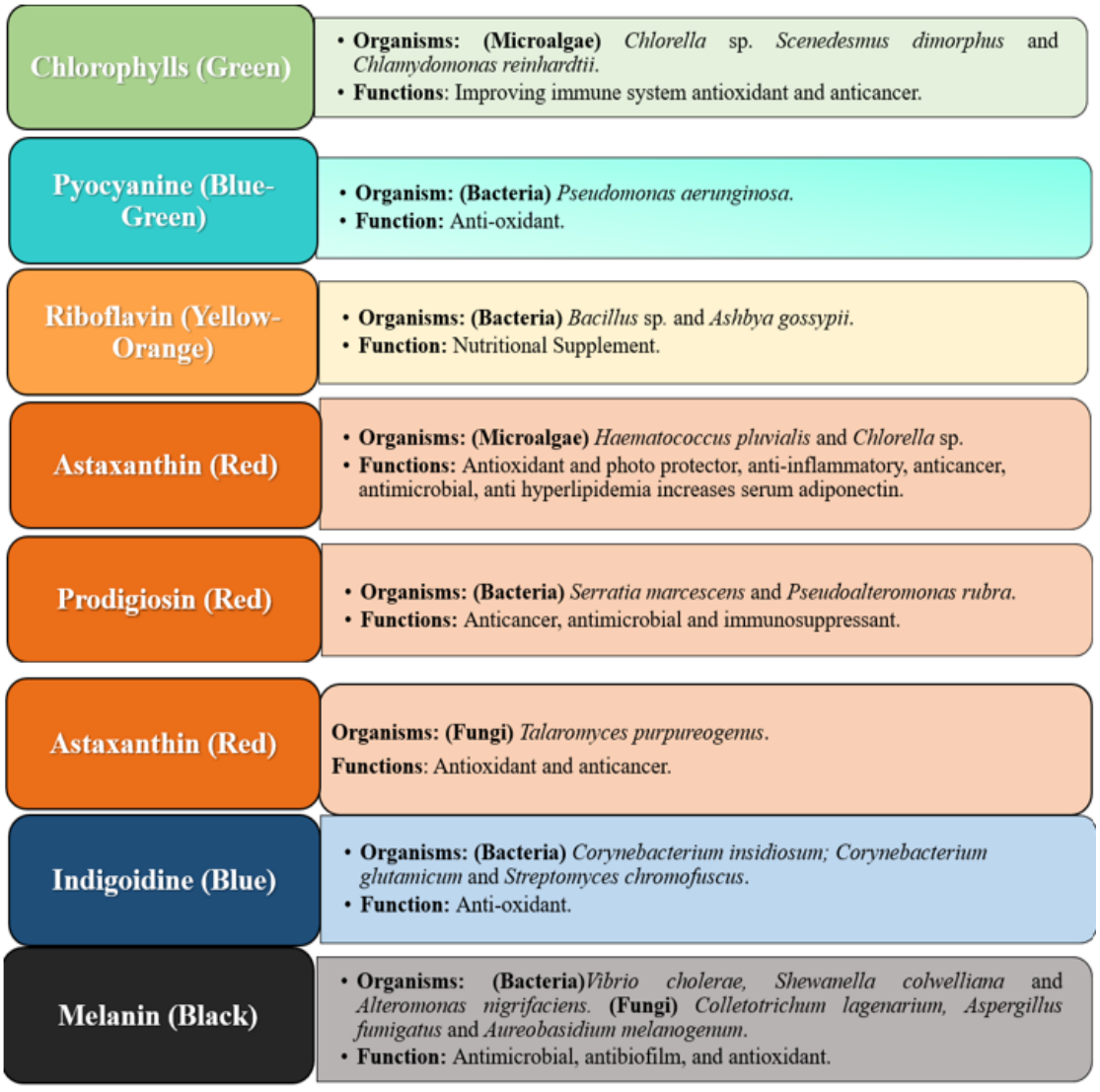


FIGURE 2.7: Characteristics of Microbial pigments along with their therapeutic functions [34, 59–74].

## 2.7 Major Chemical Groups and Functions of Microbial Pigments

### 2.7.1 Isoprenoid Pigments

Carotenoids, a broader group of isoprenoid-derived compounds, are among the most widespread pigments in nature, displaying vibrant yellow, purple, red and orange hues. These pigments are synthesized by a variety of organisms including plants, algae, fungi, eubacteria and archaea [75–77]. The vivid colors observed in some animals, such as insects, birds and crustaceans are often attributed to carotenoids acquired through their diet or from symbiotic and pathogenic microorganisms [76, 78].

Carotenoids originated from the precursor phytoene (C40), although bacterial species can produce carotenoids C50 and C30 from alternative precursors [78]. The fundamental structure of carotenoids consists of a polyene chain with nine or more conjugated double bonds, capped with end groups on both sides [77, 78]. These polyene chains absorb light in the 450–570 nm range, which reflects the absorption gap of chlorophyll. Moreover, carotenoids serve as accessory pigments in the process of photosynthesis [78]. The bright colors seen in certain animals, including crustaceans, birds, and insects, are often due to carotenoids, which these animals acquire through their diet or from symbiotic or pathogenic microorganisms [76, 77, 79].

The carotenoid biosynthetic pathway begins with the condensation of two geranyl pyrophosphate (GGPP) molecules, a reaction initiated by the phytoene synthase enzyme, resulting in the formation of phytoene. This precursor then passes through the multiple steps of isomerization and desaturation to produce red pigment i.e., lycopene. Lycopene can subsequently be cyclized by cyclases to generate compounds such as Alpha, Beta and Gamma caotenes. These carotene molecules can be further modified by ketolases, hydroxylases, or other enzymes to form xanthophylls [79, 80].

Through Mavalonic acid or MEP pathways, C-5 precursors are synthesized that vary from organism to organism. Meanwhile, GGPP itself is derived from the precursors of C5. Most bacteria, fungi and archaea depend on the MVA pathway, whereas photosynthetic organisms can employ the MEP pathway [79, 80]. The complete sketch diagram of the biosynthesis of carotenoids is shown in Figure 2.8. microbial sources of carotenoids can be noted in Fig 2.9.

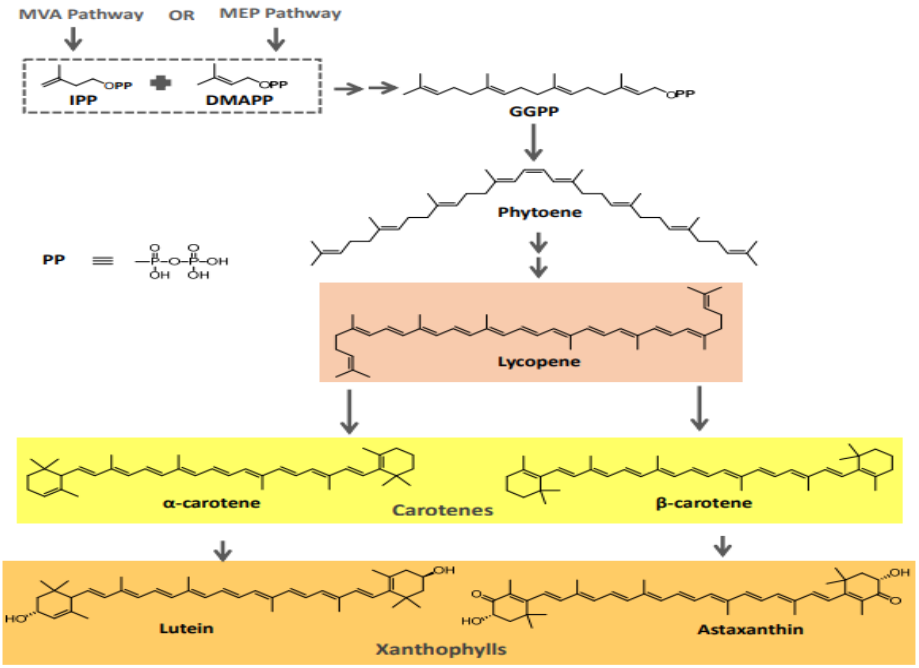


FIGURE 2.8: Pathway of biosynthesis of carotenoids [4].

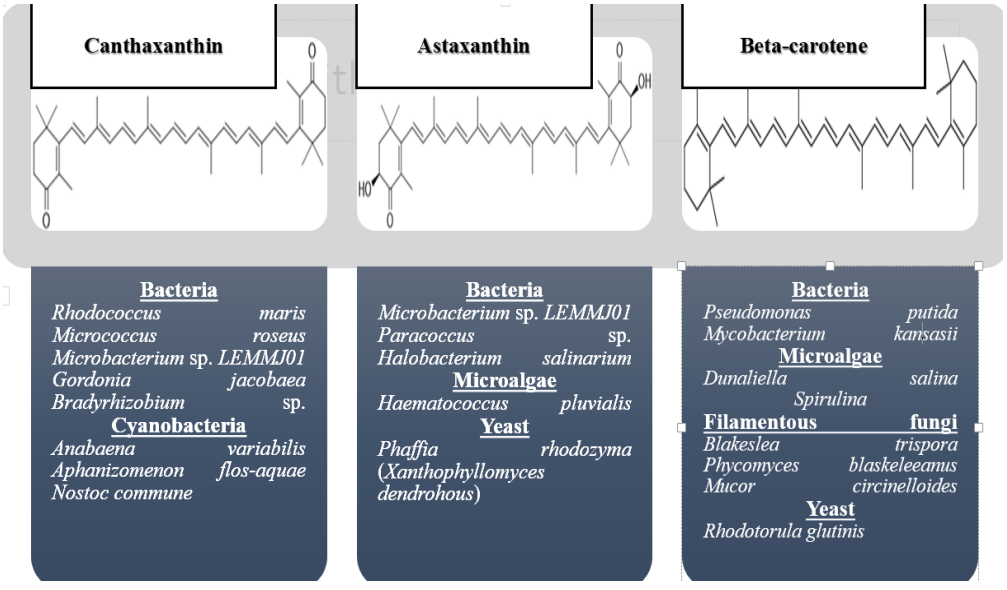


FIGURE 2.9: Microbial sources that produce carotenoids with their structure [75, 81–83].

### 2.7.2 Flavin Pigments

Flavins are yellow compounds based on pteridine structures, featuring an N-heterocyclic isoalloxazine ring (see Figure 2.10). These compounds are synthesized by plants and a wide range of microorganisms [84]. Riboflavin, also referred to as vitamin B-12 is a water-soluble substance with pigment properties and acts as the precursor for all flavins that play a significant role in biology [85].


FIGURE 2.10: Structure of riboflavin and roseoflavin [4].

Riboflavin is produced from the GTP (Guanosine triphosphate) and Ru5P which originate from the pentose phosphate pathway [85]. GTP contributes the two nitrogen atoms and the pyrimidine portion, required for the isoalloxazine ring as well as the ribityl side chain. Ru5P gives the additional carbon atoms that are necessary for the heterocyclic ring. In the last step, the enzyme riboflavin synthase is used and dismutation can be done in which four carbon atoms are exchanged with the help of 2 molecules of DrL (6,7-dimethyl-8-ribityllumazine) [86]. This process is depicted in Fig 2.11.

Flavin pigments play a crucial role in mediating both one-electron processes and two-electron processes in biological systems. Both of them absorb light which gives them their distinctive colors and results in intense absorption in both the visible and ultraviolet regions of the spectrum. Riboflavin, commonly referred to as vitamin B12, is a vital compound for living organisms and serves as a precursor to all biological important flavins [87]. Microorganisms generate a wide range of secondary metabolites that play a crucial role in their biological processes and survival [88].

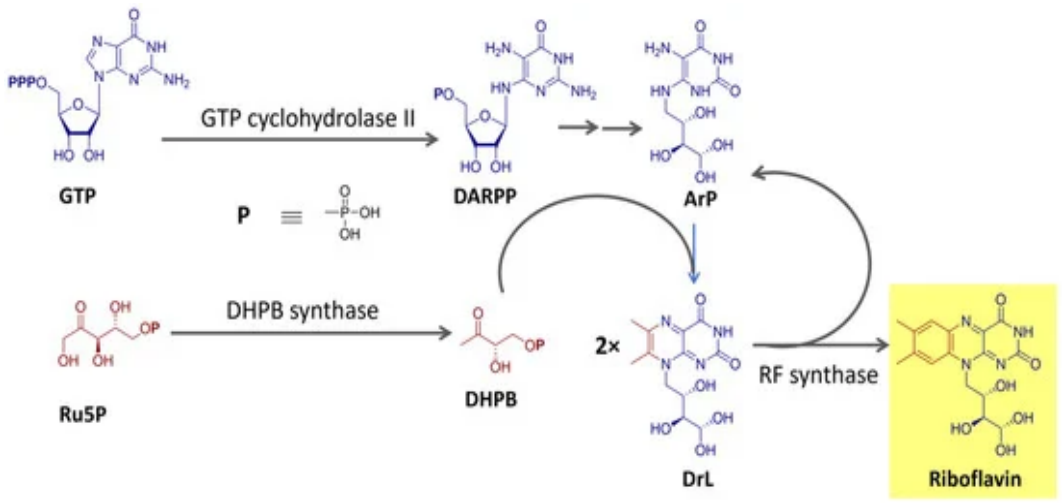


FIGURE 2.11: Biosynthesis pathway of riboflavin [4].

### 2.7.3 Tetrapyrrole Pigments

In micro-organisms, tetrapyrroles are essential for light absorption and the transfer of electrons in chemical reactions. They act as catalysts for many important enzymes and sensory proteins. Consequently, these pigments help manage oxidative stress and protect the cells by assisting in detoxifying the reactive oxygen species. Tetrapyrrole metabolism and oxidative stress have a direct link and it is alleviated in photosynthetic organisms [89].

It is produced through a five-carbon amino acid i.e. $\delta$-aminolevulinic acid (ALA). In fungi, bacteria and animals, ALA is produced by the condensation of succinyl coenzyme A and glycine i.e. C-4 pathways, while in archaea bacteria, algae and plants, it is derived from $\alpha$-ketoglutarate i.e. C-5 pathways [90, 91].

Porphobilinogen contains a pyrrole ring, which is synthesized from the condensation of two ALA molecules by the enzyme porphobilinogen synthase. The tetrapyrrole ring is created by the condensation of four PBG molecules, after the deamination of porphobilinogen. This compound is subsequently modified and combined with metal ions to produce various complex molecules, such as chlorophyll, coenzyme B-12, heme group, and others, as illustrated in Figure 2.12 [91]. This section will focus on two types of tetrapyrrole pigments: chlorophylls and phycobiliproteins [4].

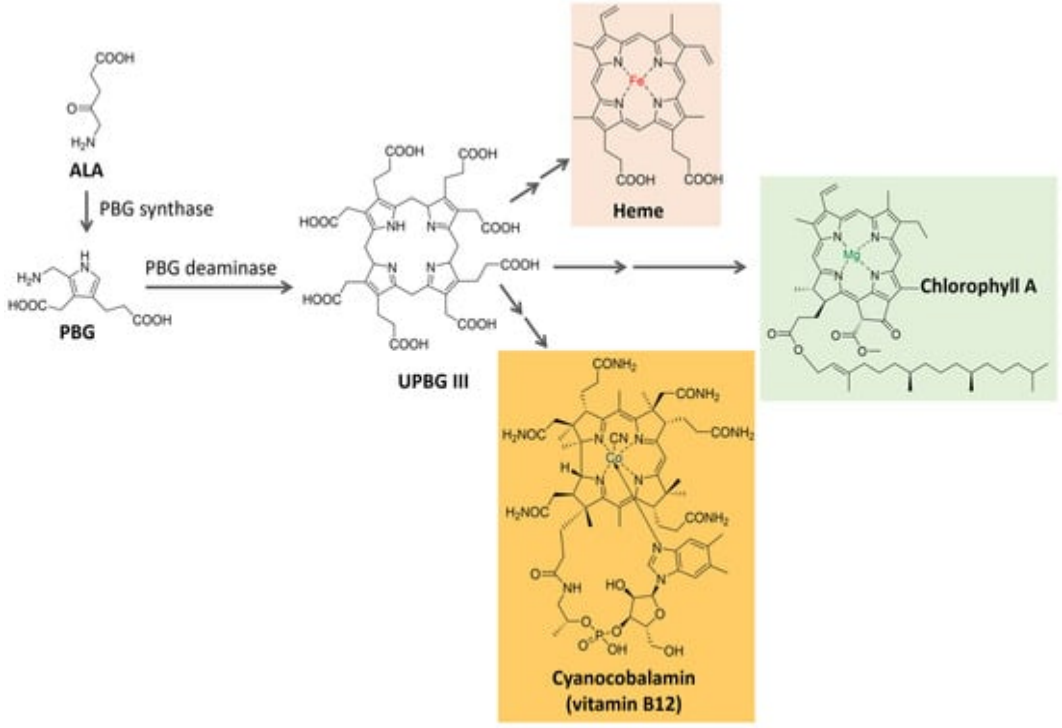


FIGURE 2.12: Simplified overview of the biosynthetic pathway for tetrapyrrole macrocyclic compounds [4].

#### 2.7.3.1 Phycobiliproteins

They are vividly colored, and easily soluble in proteins. They are covalently bonded with open-chain tetrapyrrole chromophores called phycobilins. In cyanobacteria, red algae and various other algae, phycobiliproteins are the main component of the complexes of light-harvesting. They captured the sunlight at 470-660nm range. After the absorption, chlorophyll a receives the energy. This process can be done in phycobilisomes i.e. subcellular structure [92]. PBPs are used in flow cytometry, immunohistochemistry, fluorescence microscopy, biomedical research and fluorescence immunoassays, due to their great absorption abilities. Various red microalgae, including species like Rhodella spp., Bangia spp., and Porphyridium spp., are known to produce red PBPs [93].

#### 2.7.3.2 Chlorophylls

It is the most prevalent pigment on Earth and is crucial for photosynthesis as it captures light and converts it into chemical energy [94]. Chlorophylls are formed

when tetrapyrrolic macrocyclic structure is attached to magnesium. At C-17, they are frequently esterified to a long chain of alcohol [95].

These pigments absorb the light of the visible spectrum regions like yellow-orange or red around 600-700nm and violet-blue around 400-500 nm. In light-harvesting complexes found in cyanobacteria, algae and plants, chlorophylls work with proteins and carotenoids. Chlorophylls a-d are the most common examples [95]. They vary in terms of unsaturation within the pyrrolic macrocycle of their side chains (Fig 2.13), which affects the characteristics of light absorption [4].

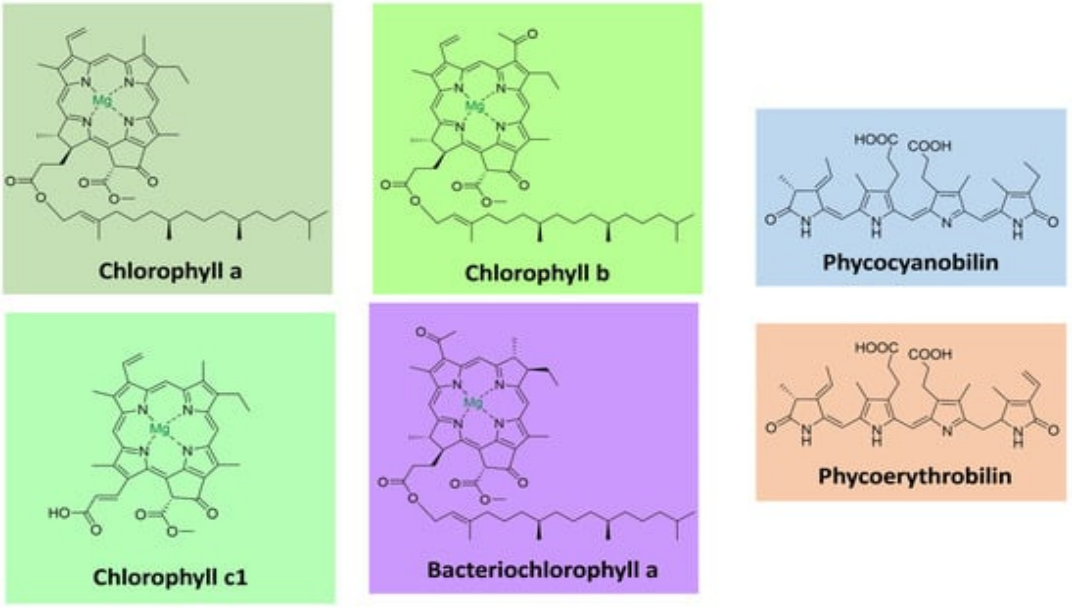


FIGURE 2.13: The structures of chlorophylls a, b, and c1, as well as bacteriochlorophyll and phycobilins [4].

#### 2.7.3.3 Phycobiliproteins

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### 2.7.4 Melanins

These pigments are unique, stable, and have high molecular weight formed through the oxidative polymerization of phenolic substances. They are brown or black [96]. They consist of three types i.e. allomelanins, eumelanins and pheomelanins. Eumelanins are found in some microorganisms, animals and fungi. They are produced from tyrosine with the action of tyrosinase enzyme. Thus, the consists of 5,6-dihydroxy indole which is their main building block. Pheomelanins are found in mammals, reptiles and birds. They are produced from tyrosine-derived units by inserting sulfur from L-cysteine, at the end they formed benzothiazole and benzothiazine as their building blocks. Allomelanins are found in plants and fungi and are a diverse group that does not contain nitrogen. It is derived from various nitrogen-free precursors, including caffeic, gallic acids, catechol, 1,8-dihydroxy naphthalene, chlorogenic, gamma-glutaminyl-3, protocatechuic, and 4-dihydroxybenzene [97]. Pathway of biosynthesis of melanin can be noted in Fig 2.14.

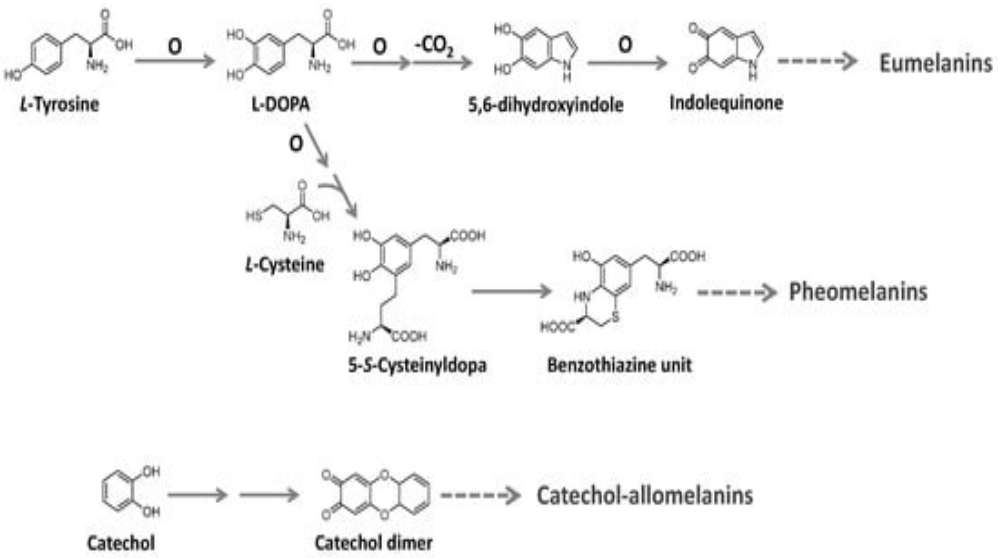



FIGURE 2.14: Schematic pathways of biosynthesis of Melanin [4].

### 2.7.5 Phenol like Pigments

Styrylpyrones are the major group of polyphenolic pigments [20]. These yellow substances are predominantly located in Ranunculaceae [98]. Fungi are belonging to the Hymenochaetaceae family, including genera such as *Inonotus* and *Phellinus*. They are also present in some plant families like Lauraceae. In 1889, styrylpyrone and hispidine were isolated from *Inonotus hispidus*. Various other styrylpyrone, such as the dimer hypholomin B and bisnoryangonin (see Figure 2.15), can be extracted from its fruiting bodies [99]. Hispidin serves as a precursor molecule for fungal luciferin in bioluminescent fungi belonging to the order Agaricales [100]. Pigments containing polyphenols, derived from genetically modified microorganisms, demonstrate significant antioxidant properties. Examples include flavonoids, nagirenim, and anthocyanins produced by *Saccharomyces cerevisiae* through metabolic engineering [101], as well as curcuminoids from engineered *E. coli* [102].

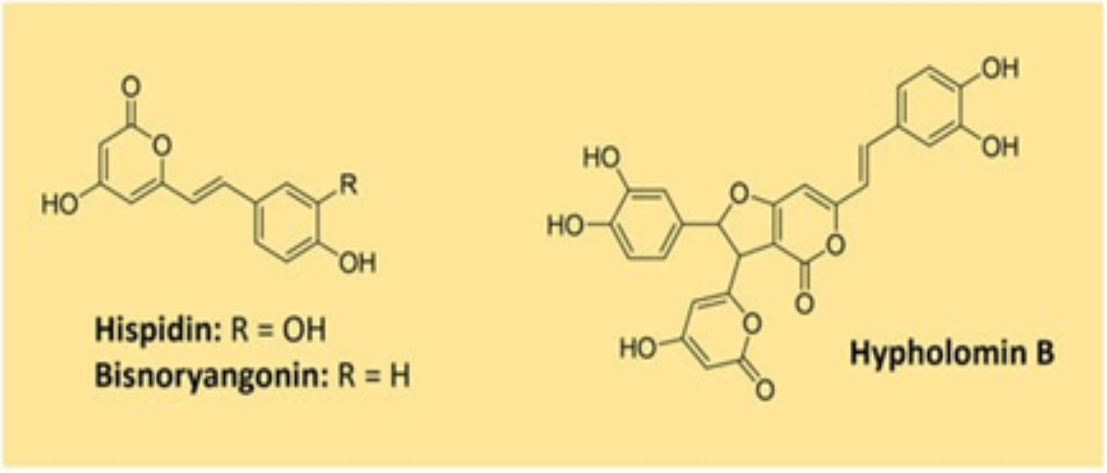



FIGURE 2.15: Styrylpyrone pigment produced by fungi [103].

### 2.7.6 Polyketides Pigments

They are a diverse group of compounds formed through the condensation of malonyl-CoA derivatives by an enzyme system known as polyketide synthases (PKSs). Several microbial pigments fall into this category, as will be discussed further below [104].

### 2.7.6.1 Quinone

Certain pigments that are extracted from natural sources have quinone in their structure. Its color spectrum ranges from yellow to red [105]. Some examples are shown in the following table 2.5 and Fig 2.16.

TABLE 2.5: Pigments producted by the fungus and having quinone in their structure [106, 107].

| S.No. | Organisms (Fungus) | Color |
|---|---|---|
| 1 | *Penicillium oxalicum* | Arpink red |
| 2 | *Nigrospora* sp. | Yellow (nigrodiquinone) |
| 3 | *Aspergillus* sp. | Red (aspergiolide) |
| 4 | *Fusarium* sp. | Red (bikaverin) |

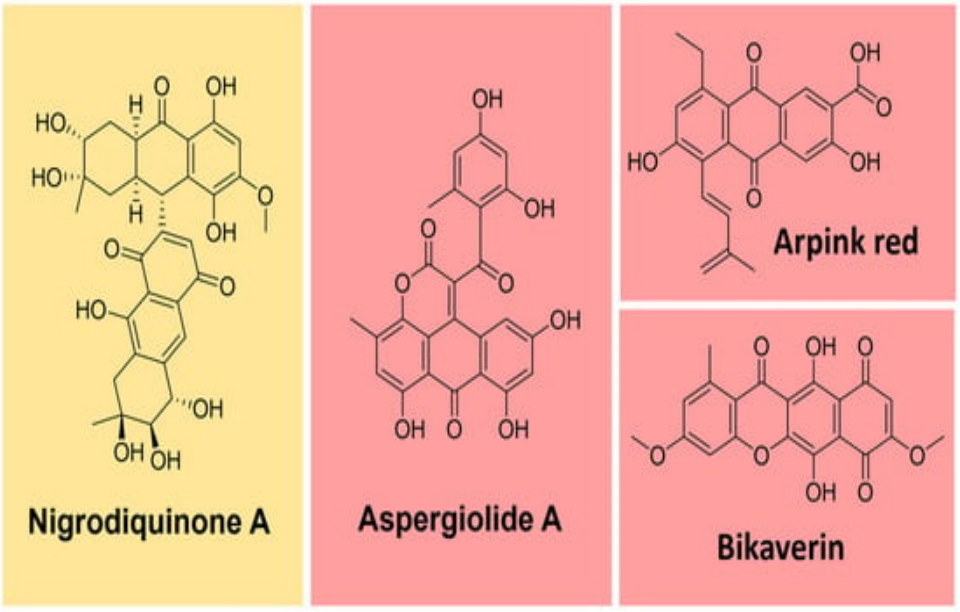



FIGURE 2.16: Pigment of Quinone extracted from Fungi [106, 107].

### 2.7.6.2 Azaphilones

These pigments are produced by fungi that have unique bicyclic ring chromophores like *Aspergillus* sp. and *Monascus* sp. Six well-known examples are shown in the following table 2.6 [108].

TABLE 2.6: Azaphilones pigments and their wavelength produced by fungal genera [108].

| S.No. | Pigment | Color | Wavelength |
|---|---|---|---|
| 1 | *Monascin & Ankaflavin* | Yellow | 330-450nm |
| 2 | *Rubropunctatin & Monascorbin* | Orange | 460-480nm |
| 3 | *Monascorubramine & Rubropunctamine* | Red | 490-530nm |

Through the acetate/malonate pathway, these pigments are synthesized. They are formed by the esterification of the chromophore of azaphilone i.e. derived by polyketide a beta-ketoacid. Through the mechanism of polyketide synthase and fatty acid synthase, the above precursors are produced as summarized in Figure 2.17. It is an aminophilic reaction in which primary amine is reacted by the orange molecules, where the nitrogen atom replaces the oxygen in the heterocyclic structure resulting in red pigment [109].

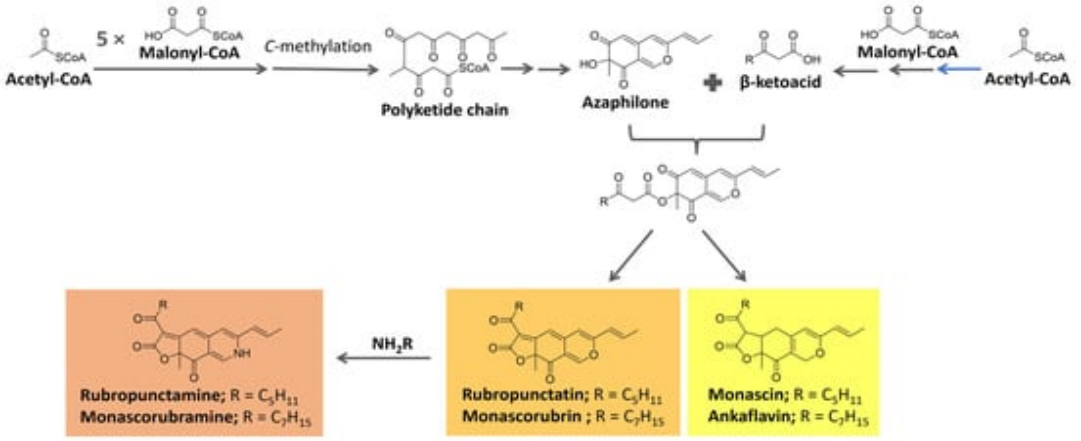



FIGURE 2.17: Biosynthesis pathway of azaphilone pigment [109].

### 2.7.7 Alkaloids Pigments

Alkaloids are formed from an amino acid. It has a low molecular weight and typically consists of those compounds that have nitrogen atoms in them [110]. This class includes various pigment groups such as betalains, prodigiosine and tambjamines. These compounds act as a reserve in micro-organisms, with secondary

metabolites frequently released into the environment. For instance, research on alkaloids derived from polyketides, such as citrinin. When *P. citrinum* started to grow, then these pigments synthesized and decomposed [111].

#### 2.7.7.1 Prodigiosin

Prodiginines are a set of hydrophobic red pigments with a tripyrrole structure. They are synthesized by marine bacteria, *Serratia spp.*, and *actinomycetes*. *S. marcescens* produced the prodigiosin pigment (see Figure 2.18). Prodigiosin have a linear chain and some have cyclic structures as seen in the below diagram [67, 112].

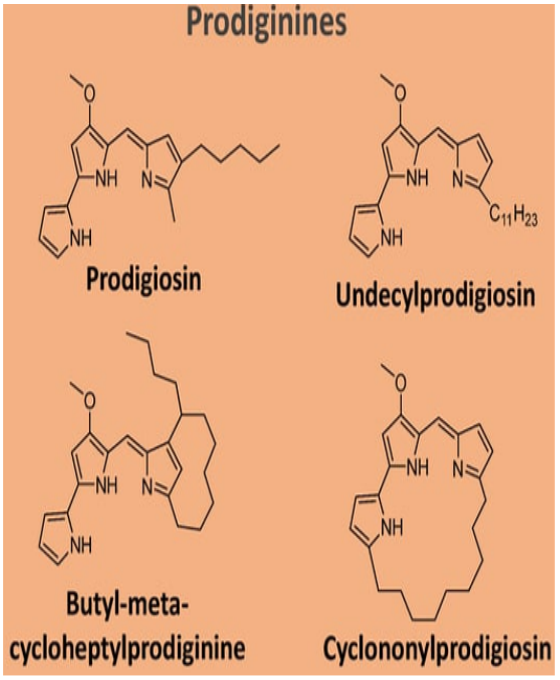



FIGURE 2.18: Structure of Prodiginines [67, 112].

#### 2.7.7.2 Tamjamines

They produce yellow pigments that resemble to prodiginines. Their bipyrrole core is reduced with a primary amine, in contrast with the structure of manopyrrole in prodiginins (Figure 2.19). Some common examples of tambjamine A and C are shown in the following diagram. They are discovered in marine invertebrates, including bryozoans, nudibranchs and ascidians as well as in bacteria like *Pseudoalteromonas* spp [113].

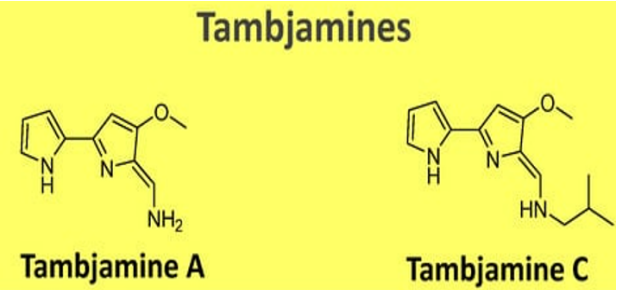



FIGURE 2.19: Structures of Tambjamines [114].

#### 2.7.7.3 Betalains

They are easily dissolved in water. They have a betalamic acid with a structure containing N-heterocyclic and act as a chromophore. They are classified into two main groups based on the light absorption properties and the structure is illustrated in the table 2.7 [115].

TABLE 2.7: Betalains pigment, their wavelength and the process of synthesis [115].

| **Pigments** | **Wavelength ($\lambda_{max}$)** | **Process** |
|---|---|---|
| Red-violet betacyanins | 535-540nm | Resulting from the reaction between cyclo-DOPA and betalamic acid. |
| Yellow betaxanthins | 460-480nm | Resulting from the combination of betalamic acid with many amino acids and other amines. |

The above pigments are relatively rare in the environment and are discovered in the species of fungi in the *Amanita* genus and the order Caryophyllales in the plant family [116]. Some common examples from *Amanita muscaria* are vulgaxanthine I and muscaurin I have orange-yellow pigment, while the purple pigment is produced by muscapurpurin (Figure 2.20) [117].

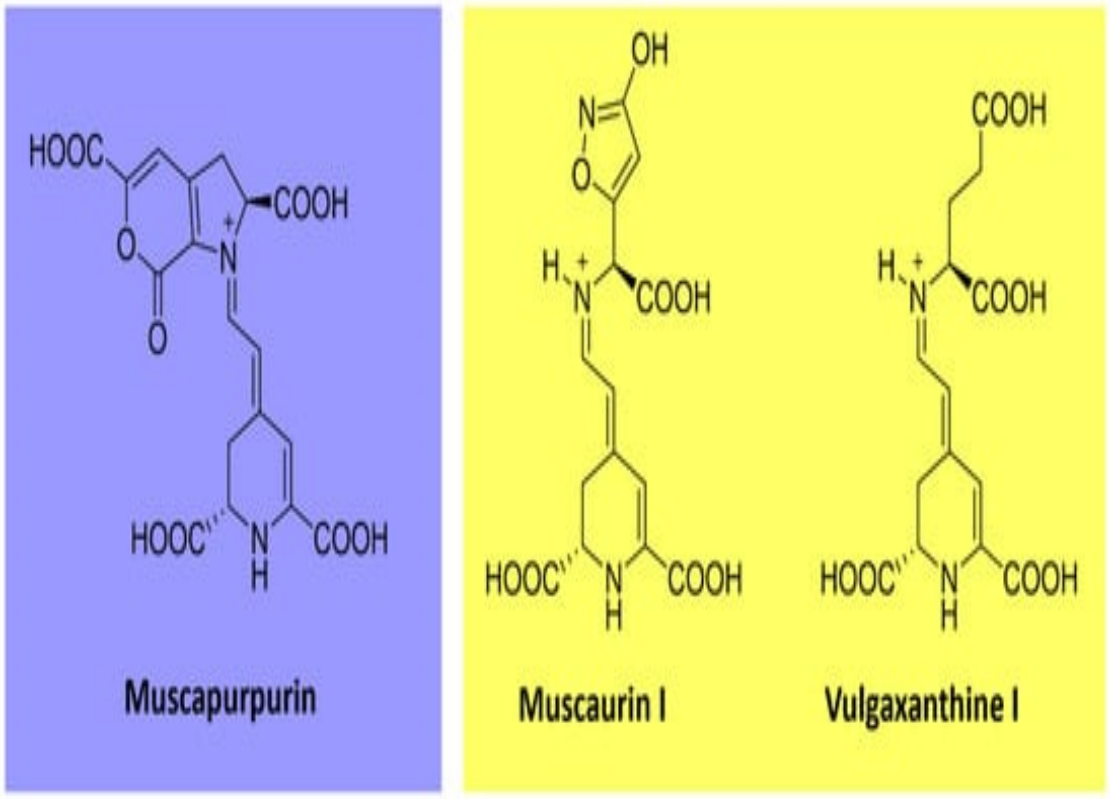



FIGURE 2.20: Structures of Betalains [117].

## 2.8 Role of Genes in the Production of Bacterial Pigments

Mostly, bacterial pigments are not soluble in water, but they can be dissolved in organic solvents like ethyl acetate, methanol, acetonitrile, acetone, hexane, ethanol or acetone. The pigment is completely dissolved in an organic solvent, leaving behind a powdered residue that is pure pigment and ready to analyze the function [118].

Various types of pigments are produced by different micro-organisms under a controlled environment like beta-carotene, phenazine, prodigiosin, canthaxanthin and violacein. This process involves multiple enzymes encoded by specific genes [7]. A list of other pigments and their corresponding genes are described in the following figure 2.21.

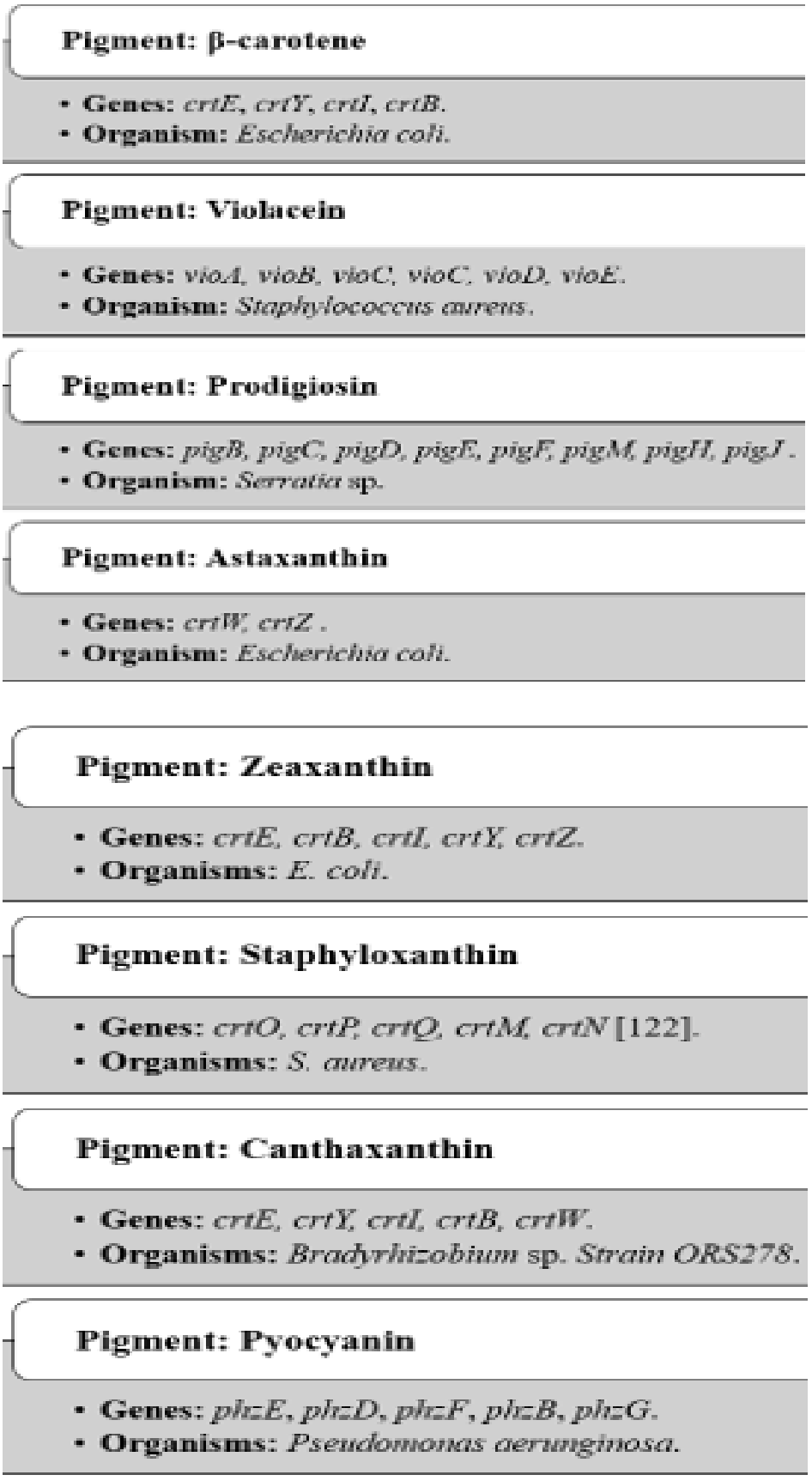



FIGURE 2.21: Genes associated with the synthesis of pigments [119–126].

### 2.8.1 Bacterial Pigment Production Enhanced through Genetic Engineering Techniques

Genetically engineered techniques are used in the micro-organisms that do not produce pigment naturally if they do, they produce it in small quantities. The pigments that are introduced in the micro-organism are lutein, zeaxanthin, carotenoids, lycopene, eumalanin etc [127].

Various approaches have been proposed for developing genetically engineered micro-organisms to produce desired pigments. These include enhancing the key enzyme expression that is involved in pigment biosynthesis and modifying the desired genes through insertion or deletion. Such genetic alterations not only enhance the strains but also address the problems related to toxicity that are linked with synthetic dyes [7].

Recent progress in genetic engineering has enabled researchers to modify micro-organisms like yeast and *E. coli* for large-scale production of carotenoids (Table 2.8).

For instance, the *E. coli* strain has been genetically engineered to make Zeaxanthin from lycopene. This process can be done by introducing intergenic regions into the substrate channels of two fusion proteins. The *crtY* and *crtZ* expression was initiated encoding Beta-carotene-hydroxylase and lycopene-beta-cyclase enzyme [128].

Through several alterations, lycopene was produced by *Rhodobacter sphaeroides* which was genetically modified. First, its *crtl3* gene was replaced with the gene *crtl4* from *Rhodospirillium rubrum*. Subsequently, the gene *zwf* was knocked out and removed the gene *crtC*, while another gene was introduced i.e. *dxs*. The competitive pentose pathway was blocked by this modification and the methyl erythritol phosphate pathway was regulated [129]. As a result, genetically engineered microorganisms can be widely used for large-scale pigment production, offering numerous therapeutic and industrial applications [7].

TABLE 2.8: Some genetically engineered techniques are used to enhance the pigments of bacteria..

| **Microorganisms** | **Modification Type** | **Targeted Gene or Pathways** | **Gene Source** | **Pigment Outcome** | **Refs** |
|---|---|---|---|---|---|
| *Corynebacterium glutamicum* | Gene deletion | Sigma B factor | - | Enhanced production of decaprenoxanthin, Beta-carotene | [130] |
| | Gene overexpression | Sigma A factor | - | Increase in Decaprenoxanthin, Lycopene | |
| *E. coli* | Pathway modification | MEP biosynthesis pathway | - | Production of lycopene | [119] |
| | Pathway insertion | MVA biosynthesis pathway | - | - | |
| | Gene insertion | ORF438 and *mel* gene | *Streptomyces antibioticus* | Eumelanin | [131] |
| | Introduction of intergenic regions | Intergenic regions of *crtY* and *crtZ* | *Pantoea ananatis* | Zeaxanthin | [132] |
| | Gene removal | *zwf* gene | - | Boost the level of Beta-carotene | [128] |
| | | *ptsHIcrr* operon | - | | |
| *R. sphaeroides* | Gene replacement | *crtI3* with *crtI4* | *R. rubrum* | Production of Lycopene | [129] |
| | Gene deletion | *crtC* gene | | | |
| | Gene knock-out | | | | |

## 2.9 Soil Bacterial Pigments

Various micro-organisms produce a range of pigments such as carotenoids, flavins, melanin, quinones, prodigiosin and more specialized ones like indigo, violacein, and monascins [133].

### 2.9.1 Applications of Bacterial Pigments of Soil

Infectious diseases rank among the leading causes of death globally, accounting for approximately 13.3 billion deaths Worldwide. The situation is worsening as infectious agents increasingly develop resistance to the drugs designed to combat them, leading to more severe and life-threatening conditions. The rise in this unstoppable infectious outbreak remains largely unexplained.

Consequently, natural sources are continually being explored and assessed for their potential in preventing and treating these diseases. The chemical applications, effectiveness, and specific properties of pigmented bacteria such as anti-fungal, anti-cancer and immune suppression are highlighted in various studies and have made them a significant focus of interest. Considerable advancement has been made in this area, with the study of bioactive compounds from pigmented bacteria overgrowing. The rate at which these compounds are isolated from bacteria is notably faster than from other organisms some therapeutic applications are discussed below [133].

#### 2.9.1.1 Anti-cancer Ability of Bacterial Pigments of Soil

Although over the last 20 decades, the cancer rate has declined, resulting in approximately 1.7 million cases being prevented and a 23% overall reduction, cancer remains a main cause of death. Ongoing preclinical and clinical research is essential to combat this deadly disease [134]. While many potential anticancer drugs from organic sources have been investigated. Moreover, in the past bacterial pigments received less attention. However, recent studies have shifted focus towards

these pigments, such as the antitumor activity of prodigiosin from *S. marcescens* [135]. The cytolytic effectiveness of prodigiosin has been studied using the standard panel of 60 human tumor cell lines. These investigations have shown that prodigiosin inhibits cell proliferation and induces cell death in these lines. Various analogs of prodigiosin have also demonstrated in vitro anticancer activity. Additionally, prodigiosin's cytotoxic potential against pancreatic cancer, which claims 30,000 lives annually in the USA, raises hopes for a potential treatment for this highly fatal disease [136].

Violacein, a pigment derived from *Chromobacterium violaceum*, has been shown to induce apoptosis in human leukaemia cells by activating the TNF receptor 1, making it a promising candidate for a new category of cytotoxic drugs. An orange-yellow pigment (Beta-Carotene) extracted from *Rhodococcus maris*, has been associated with a lower risk of breast cancer. A red pigment from *Streptomyces venezuelae* and *Streptomyces peucetius* i.e. Doxorubicin is recognized as a potent anti-tumor agent [137].

Additionally, red bioactive compounds i.e. quinone-anthracycline and deinoxanthin are found in *Deinococcus radiodurans* and *Streptosporangium*, respectively, exhibit strong anti-tumor therapeutic potential [138].

#### 2.9.1.2 Anti-Leishmanial Ability of Bacterial Pigments of Soil

Leishmaniasis, a severe and disfiguring protozoan infection, affects over 12 million people globally. The treatments available are outdated, having been developed 50 years ago, and are known to be potentially toxic. Violacein, derived from *C. violaceum*, demonstrated only one-tenth of the effectiveness of pentamidine, a second-line drug used for treating leishmaniasis. While pentamidine is more effective than violacein, it poses significant toxicity risks, including alterations in electrocardiogram readings and renal and hepatic issues [139]. As a result, violacein may emerge as a preferable alternative for treating this life-threatening disease in the future. Meanwhile, other soil bacteria are being investigated for their potential to treat leishmaniasis and new drug discoveries are anticipated [133].

#### 2.9.1.3 Anti-viral Ability of Bacterial Pigments of Soil

Infectious diseases account for about 20% of global mortality, with viruses—one of the most formidable groups of microbes—responsible for one-third of these cases [140]. The speedy spread and outbreak of viruses have been exacerbated by modern advancements like air travel and the demographic shift toward urbanization. While disease outbreaks can be managed and diagnosed through fast diagnostics, the most effective prolonged public health method remains vaccine-based prevention [141].

To address this significant challenge, natural sources are being extensively screened for their antiviral properties, leading to the discovery of several bacterial pigments that successfully inhibit the viral replication cycle. These pigments either target viral components or alter host cell mechanisms crucial for viral replication. Violacein, extracted from *C. violaceum*, has demonstrated notable antiviral activity against FRhK-4, MA104 cells and HEp-2, showing a concentration-dependent greater cytotoxic impact [142]. Similarly, violacein from *Janthinobacterium lividum* XT1 has also exhibited antiviral potential [143].

#### 2.9.1.4 Anti-bacterial Ability of Bacterial Pigments of Soil

The rising prevalence of infectious diseases, driven by the increased occurrence of opportunistic pathogens and the growth of antibiotic resistance, serves as a serious alert for the medical community [144]. Currently, these drug-resistant infections result in an annual death toll of approximately 700,000 people worldwide, and if decisive measures are not implemented, this number could escalate to 10 million by 2050. The World Health Organization (WHO) has identified 12 bacteria that pose the greater risk to human life due to antibiotic resistance. Notably, *P. aeruginosa*, *Enterobacteriaceae*, and *Neisseria gonorrhoeae* are among the most critical. There is a pressing need for robust drug development to address these threats. Among natural sources, bacteria generate a range of pigments with antibacterial properties including violacein, carotenoids, melanins, flavins, quinones and monascins [145].

#### 2.9.1.5 Anti-fungal Ability of Bacterial Pigments of Soil

Over the last 2 decades, a wide range of toxic fungal and fungal-like diseases have been reported in both plants and animals, posing a significant threat to food security [146].

The conventional antifungal treatment, amphotericin B, is expensive and associated with toxic side effects, such as infusion-related reactions including chills, rigors, nephrotoxicity and fevers. To address these issues, researchers have been exploring natural sources, particularly plants and bacteria, for their antifungal ability. Within the pigmented bacteria studied for their antifungal properties, *S. marcescens* and *Neisseria* species have been extensively researched. Prodigiosin, a red tripyrrole pigment that forms distinctive pillar box colonies, was initially identified in *Serratia marcescens*. It has been shown to control cyclamen's grey mold effectively. When red pigment is extracted and purified from bacteria, it inhibits spore germination. Moreover, a combined application of prodigiosin and chitinolytic enzymes resulted in a synergistic inhibitory effect [147].

Similarly, from a soil bacterium i.e. *Neisseria* species, the red pigment extracted and purified shows great activity against many fungal species, including *Candida*, *Trichoderma* and *Aspergillus* [148].

#### 2.9.1.6 Immunosuppressive Ability of Bacterial Pigments of Soil

Immunosuppressant drugs are designed to weaken or inhibit the immune system of the body, often used to block rejection of transplanted organs like kidneys, heart or liver [149].

Several of these drugs have been obtained from nature-derived sources. In 1989, Nakamura and colleagues first documented the prodigiosin with immunosuppressive properties. Their research revealed that metacycloprodigiosin and prodigiosin were present in the culture broth of *Serratia*, and they found that these compounds selectively inhibited T-cells while demonstrating polyclonal inhibition in comparison to B-cells [136].

## 2.10 Role of Bacterial Pigments in Market Level

In recent years, trends like increasing globalization, restructuring, and internationalization have significantly impacted the pigment industry. According to, Global Industry Analysts, in 2017 the global demand for organic pigment reached 10 million tons compared to dyes. Originally, the global dye manufacturing industry was driven by suppliers from Germany, the United Kingdom, and Switzerland but it has shifted to Asia over the past 20 years. Many other industries like plastics, coatings, printing inks and paints are projected to rise more quickly but the largest consumer of natural dyes and pigments the textile industry will stay unchanged. There is also a rising focus on natural dyes due to restrictions on synthetic compounds, such as the European ban on azo dyes. The market value is anticipated to grow as consumer preferences increasingly favor environmentally friendly products (Figure 2.22) [136].

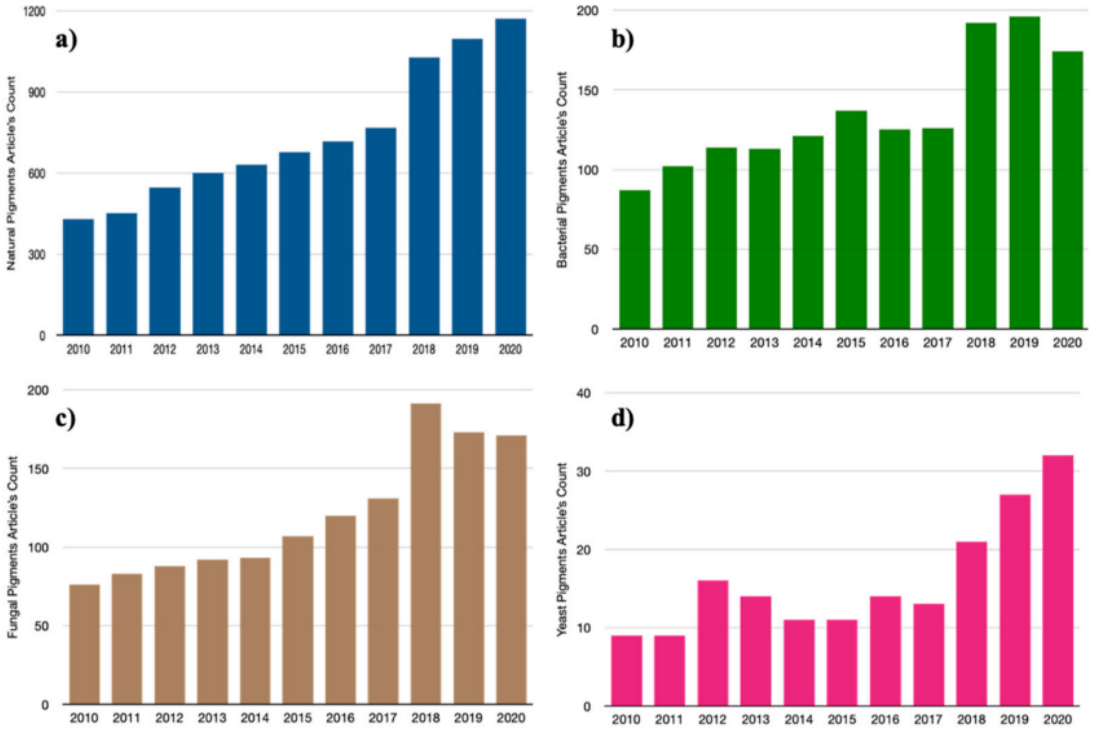



FIGURE 2.22: Over the last 10 years, the NCBI database has catalogued articles focusing on trends in (a) represents the Natural pigments, (b) represents the Natural Pigments and Bacteria, (c) represents the Natural Pigments and Fungi, and (d) represents the Natural Pigments and Yeast [150].

The bacterial strains are developed in such a way that they are inexpensive and their non-depleting substrates can make the cost of natural pigments more competitive alongside synthetic choices. By identifying cost-effective substrates for pigment production, it is expected that production costs will decrease. Thus, the production costs of bacterial pigments can be reduced by the following methods [136]:

- Utilizing agricultural waste products like molasses, pineapple waste, and sugarcane bagasse as growth mediums for bacterial cultivation.
- The necessity for costly genetic engineering is avoided by utilizing wild-type bacterial strains that are locally isolated.
- Implementing simple extraction methods.

Pigments extracted from bacteria offer promising prospects because of their improved compatibility with the environment and performance features, even though traditional grades are likely to remain prevalent in the organic market [136]. These opportunities help to establish a positive image and reputation for the brands and industries that adopt them, thereby fostering consumer trust in their products. Through putting efforts in marketing to promote the consumption of natural products by advertising campaigns that consistently increase the market, fueling innovation aimed at capturing a larger share. The latest data indicates that the global market for organic pigments is projected to approach approximately 4.89 billion dollars by 2024. Among microbial pigments, those sourced from microalgae and fungi are the most commonly used in the industry, whereas bacterial pigments are utilized less often [4].

The global market for microbial pigment has been evaluated across different categories, with carotenoids, for example, valued at approximately USD 1.7 billion in 2020. This market is expected to grow at a rate of 2.6%, potentially reaching nearly USD 2 billion soon. Based on 2021 AMR reports, Astaxanthin is a specific carotenoid that has a market value of USD 192.5 million in 2020, with projections indicating a significant increase to around USD 228.4 million by 2027 [151].

Currently, carotenoid production about 80-90% is achieved through the synthesis of chemicals due to its reduced price ($250–2000 per kg) with respect to carotenoids derived from plants, which range from $350 to $7500 per kg [152]. In contrast, carotenoid-rich biomass from various microorganisms, such as algae (e.g., *Haematococcus* sp., *Chlorella* sp.), is available on the market at $40–50 USD per kg [127]. This suggests that microbe-derived pigments have the potential to dominate the global market, potentially replacing synthetic and plant-derived pigments in the current landscape [7].

# Chapter 3

# Methodology

The flow chart of methodology can be noted in Figure 3.1.

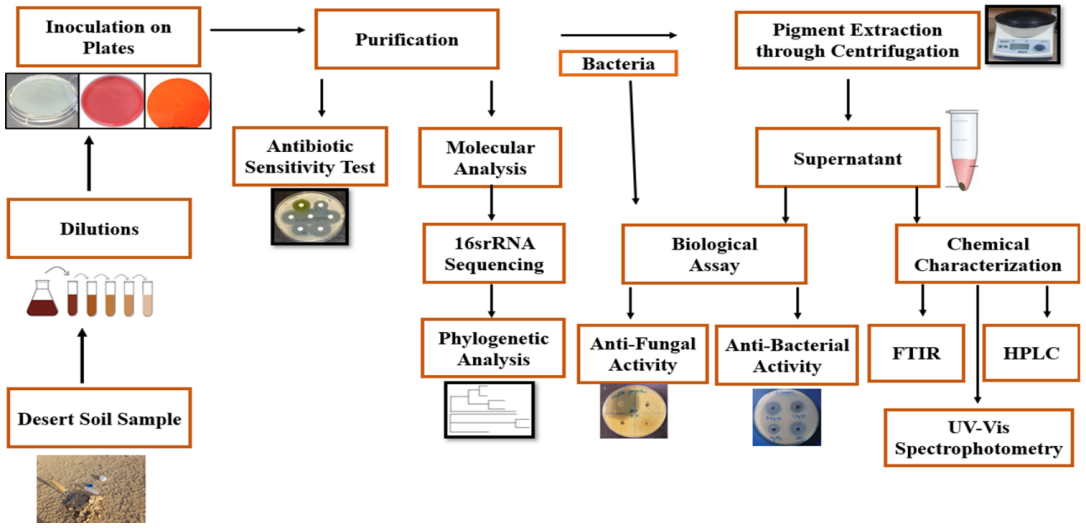



FIGURE 3.1: Flow chart of methodology.

## 3.1 Sample Collection

A sample of soil was collected from the desert of Bahawalpur where the latitude is 29.0700° N and the longitude is 71.4329° E (Figure 3.2). The trowel was used to clear the soil debris, after which the soil was gathered from a depth of 9 cm to obtain a surface sample. It was kept in an airtight plastic bag. After reaching the laboratory, it was put in the oven for drying at around 40°C [153].


FIGURE 3.2: : Location of the collection of Desert soil from Bahawalpur.

## 3.2 Preparation of Nutrient Agar

2.24g of Nutrient Agar was dissolved in 80 ml of distilled water. Once the media was prepared, it was autoclaved at 121°C for 1 hour. After the autoclave was done, it was poured onto 4 sterilized petri plates uniformly within the laminar flow and waited for 10-15 minutes for it to cool so that it could be solidified.

## 3.3 Preparation of Differential Media

Two differential media was used i.e. Congo Red Agar and Mannitol Salt Agar. To make CRA, 1.28g of agar was mixed with 40 ml of distilled water and for MSA, 4.44g of agar was dissolved in 40 ml of distilled water, both were autoclaved at 121°C for 1 hour. After the autoclaving, it was poured into 4 sterilized petri plates of each agar uniformly within the laminar flow and allowed to cool for 10-15 minutes to solidify.

## 3.4 Culturing on Media

The soil was weighed about 1.0g, dissolved in 10 mL of distilled water, and then serially diluted up to 10-8 dilutions [154]. In the laminar flow, 20$\mu$l of 10-6l sample was spread onto each Petri plate. The samples were then placed in an incubator. NA, CRA and MSA were incubated at 26°C for 2 days after wrapping it with parafilm.

## 3.5 Purification of Bacterial Strains

When the pigmented colonies were produced on NA, MSA and CRA then it was further cultured on these media to get the purified strains of bacterial colonies. As they produced pigmented colonies on NA orange and yellow colonies were formed, on CRA pink colonies were formed, yellow-colored colonies on MSA.

2.24g of NA was dissolved in 80 ml of distilled water, 1.28g of CRA was prepared in 40 ml of distilled water and 4.44g of MSA was dissolved in 40 ml of distilled water and then autoclaved at 121°C for 1 hour along with 10 petri plates. When the autoclave was done then these samples were taken to the laminar flow and poured the media in sterilized petri plates. After the petri plates were cooled down, the colonies of bacteria were picked from the red-hot loop and isolated in the specific media. NA, CRA and MSA were incubated at 26°C for 2 days after wrapping it with parafilm.

## 3.6 Preservation of Purified Strains

60% of glycerol stock was prepared to preserve the purified bacterial colonies. For preparation, 60 ml of glycerol and 40 ml of autoclaved distilled water were taken. Mixed it perfectly in the reagent bottle. 8 blue tips and 1.5ml of Eppendorf tubes were autoclaved at 121°C for 1 hour. 1ml of glycerol stock was poured into the Eppendorf tube and picked the bacterial colonies with the help of a sterilized loop suspended into the glycerol. After this eppendorf tubes were preserved at -20°C in a refrigerator [155, 156].

## 3.7 Antibiotic Sensitivity Test

The disc diffusion method determines bacterial growth inhibition under standard conditions. A large zone of inhibition will be produced by using an effective antibiotic [157, 158].

For the Disc Diffusion Antibiotic Susceptibility Test, 2.8g of Muller Hinton Agar was added to 100 ml of distilled water. Autoclaved the media at 121°C for 1 hour. After the autoclave was done, then poured into 4 sterilized petri plates. When the media was cool, then pure strain of bacteria was streaked on the Muller Hinton Agar plates. An antibiotic disc dispenser was used to dispense the disc (cephalexin, norfloxacin, cefotaxime and imipenem) into the agar. The sterilized loop was used to ensure the attachment of the disc to the petri plates. These plates were then incubated at 37°C for 24 hours after wrapping them with parafilm.

## 3.8 Molecular Analysis

### 3.8.1 16S rRNA

This analysis plays an important role in bacterial identification that are not yet characterized, poorly isolated, or exhibit unusual phenotypic traits. It is also a reliable tool for routine bacterial identification [159]. The pigmented plates were sent to Alphagenomics for DNA extraction. After DNA extraction, they were sent to Microgen Korea for 16S rRNA analysis.

### 3.8.2 NCBI Submission

The sequence was submitted to NCBI after the trimming of a low-quality sequence.

### 3.8.3 Phylogenetic Analysis

Phylogenetic analysis depicts the evolutionary history of different organisms, and the relationships between different organisms, and species by constructing a branching diagram [160]. 16S rRNA sequence was used for phylogenetic analysis.

1. At first, the sequence was retrieved in the form of a fasta file in Blastn.

2. In the next step, multiple sequence alignment was performed by using Clustal W.

3. The phylogenetic tree was constructed using the Neigbor-Joining Method that can be implemented through MEGA 11.

4. Finally, the resulting tree was visualized to interpret evolutionary relationships and the bootstrap analysis to assess the reliability of the tree.

## 3.9 Extraction of Pigments

95% Methanol was prepared and 1 ml methanol was placed into sterilized Eppendorf tubes. The pigmented colonies from NA, MSA, and CRA were picked with the help of a loop and put in the 1.5 ml sterilized Eppendorf tube. Vortex was done until the color of the pellet was absorbed in methanol.

After this centrifugation was done at 6000 rpm for 15 minutes then 3000 rpm for 10 minutes. The resulting supernatant absorbs the color and will be used for further analysis [161].

## 3.10 Chemical Characterization

UV-Vis Spectrophotometry, FTIR, and HPLC can help to characterize the pigment chemically.

### 3.10.1 Qualitative Analysis by UV-Vis Spectrophotometry

It is the method that measures the reduction of electromagnetic radiation caused by the substance that is absorbed in it [162]. The pigments were subjected to UV-vis analysis to check their maximum wavelength. Methanol was used as a standard. The 1 ml of supernatant was added to the cubit along with 1 ml of methanol and the UV was checked in wavelengths of 400nm-700nm.

### 3.10.2 Functional Group Analysis by Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR has been established as a method for the simultaneous analysis of organic components, encompassing chemical bonds and organic content such as protein, carbohydrates, and lipids [163]. FTIR was done to check the functional groups of pigmented colonies. The supernatant was used for this purpose. The spectral range for this purpose was 1300-4000$cm^{-1}$. Some functional groups that we needed to identify were:

- Carbonyl
- Hydroxyl
- Methyl
- Methyne
- Conjugated Double Bonds
- Alkanes
- Vinyl
- Disulfides
- Aliphatic compounds

### 3.10.3 Identification of Compounds by High-Performance Liquid Chromatography (HPLC)

It is a high-throughput liquid chromatography technique in which the separation, identification and quantification of individual components can be done within a mixture [164]. 1ml pigmented color and 1ml of methanol were added to the Eppendorf Tube. Column C-18 was used for reversed-phase HPLC. 10-20$\mu$L of concentrated sample was injected into the HPLC. The detection wavelength was

around 250 nm and the run time was 30-40 minutes. Moreover, the flow rate is 0.8-1.2mL/min.

## 3.11 Biological Assays

### 3.11.1 Antifungal Activity

The Agar well diffusion technique employed the anti-fungal properties of both pigment and its associated bacteria.

#### 3.11.1.1 Preparation of Potato Dextrose Agar (PDA)

1.56g of Potato Dextrose Agar was added to 40 ml of distilled water for fungal growth. The media was autoclaved for 1 hour at 121°C. After autoclaving, the media was poured into 2 sterilized petri plates. This was then allowed to cool down.

Once cooled down, the fungal strains *Aspergillus fumigatus* (ORO53856) and *Rhizopus delemar* (OQ9844419) were streaked onto 2 petri plates and were incubated at 27°C for 7 days.

#### 3.11.1.2 Preparation of PDA for wells

For fungal testing, 23.4g of PDA was added to 600 ml of distilled water. Autoclaved the media at 121°C for 1 hour along with 20 petri plates. Once the media was autoclaved, it was poured into 24 sterilized petri plates in the laminar flow hood.

When the media was cooled down, 3 wells were punched into the 16 agar plates with a sterilized borer and mentioned number according to the dilutions and on 4 plates 2 wells were punched into 4 plates for positive and negative control.

#### 3.11.1.3 Preparation of Concentrations

Three concentrations were prepared for each pigment color using methanol as a solvent of about 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml, and also 3 bacterial concentrations were prepared in distilled water of about 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml.

#### 3.11.1.4 Filling the wells

First, 1$\mu$l PDA was placed into the wells to create a consistent base. The prepared dilutions (pigment and bacteria) were added to the wells and labelled them.

#### 3.11.1.5 Control Groups

Amphotericin B was used as a positive control in both bacteria and pigment [165, 166]. The distilled water was poured into a bacterial control plate and methanol was added into a pigment control plate for the negative control. These plates were then incubated at 27°C for 3 days and then measured as the zone of inhibition.

#### 3.11.1.6 Incubation

The petri plates were incubated at 27°C for 3 days.

#### 3.11.1.7 Measurement

The zone of inhibition was measured with the help of a ruler in mm scale to identify the antifungal properties of the pigment and bacterial samples.

### 3.11.2 Antibacterial Activity

For Antimicrobial properties, the agar well diffusion method was used for both bacteria and their associated pigments.

#### 3.11.2.1 Preparation of Mannitol Salt Agar and Eosin Methylene-Blue Agar

4.44g of MSA was dissolved in 40 ml of distilled water for the growth of the gram-positive bacteria *Staphylococcus aureus* (SRR27533903) and autoclaved for 1 hour at 121°C. Then, it was poured onto 2 plates. Once the media was cooled, it was streaked with the *S. aureus* and placed in the incubator for 48 hours at 26°C. 1.46g of EMB was dissolved in 40 ml of distilled water and autoclaved for 1 hour at 121°C. Then pour it on 2 plates to grow gram-negative bacteria *Kerstersia gyiorum* (PQ666790). Once the media was cooled down, it was streaked with *K. gyiorum* and placed in the incubator for 24 hours at 37°C.

#### 3.11.2.2 Preparation of Muller-Hinton Agar and EMB for Wells

For microbial testing, 34.8g of Muller Hinton Agar and EMB was added to 600 ml of distilled water. Autoclaved the media at 121°C for 1 hour along with 24 petri plates. Once the media was autoclaved, it was poured into 20 sterilized petri plates in the laminar flow hood. When the media was cooled down, 3 wells were punched into the 16 agar plates with a sterilized borer and mentioned number according to the dilutions and on 4 plates 2 wells were punched into 4 plates for positive and negative control.

#### 3.11.2.3 Preparation of Concentration

Three conc. were prepared for each pigment color using methanol as a solvent of about 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml, and also 3 bacterial concentrations were prepared in distilled water of about 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml.

#### 3.11.2.4 Filling the wells

First, 1$\mu$l Muller-Hinton Agar was placed into the wells to create a consistent base, followed by addition of prepared dilutions (pigment and bacteria) and labelling.

#### 3.11.2.5 Control Groups

Ampicillin was used as a positive control in *S. aureus* both bacteria and pigment [167]. In contrast, for the negative control, the distilled water was poured into a bacterial control plate and methanol was added into a pigment control plate. Meanwhile, Ciprofloxacin was used as a positive control in *K. gyiorum* for bacteria and pigment [168]. For the negative control, the distilled water was poured into a bacterial control plate and methanol was added into a pigment control plate. These plates were then incubated at 37°C for 24 hours and then measured as the zone of inhibition.

#### 3.11.2.6 Incubation

The petri plates were incubated at 37°C for 24 hours.

#### 3.11.2.7 Measurement

After the incubation period, the zone of inhibition was measured with the help of a ruler in mm scale to identify the anti-microbial properties of the pigment and bacterial samples.

# Chapter 4

# Results and Discussion

## 4.1 Results

### 4.1.1 Results on Nutrient Agar

The desert soil was spread into the Nutrient Agar of about 20$\mu$l in 4 plates of NA, and orange and yellow colonies were formed on NA after the incubation at 26$^0$C for 2 days as shown in figure 4.1.

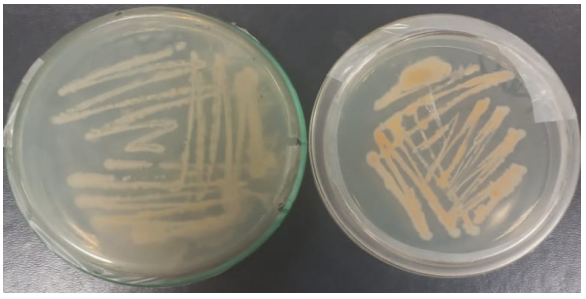


FIGURE 4.1: Yellow and Orange Colonies on Nutrient Agar.

### 4.1.2 Results on Differential Media

The desert soil was spread into Congo-Red Agar and Mannitol Salt Agar, about 20µl in every 4 plates for both agars. Pink-pigmented colonies formed on Congo-Red Agar after incubation at 26°C for 2 days. Meanwhile, yellow-pigmented

colonies were formed on Mannitol Salt Agar after incubation at 26°C for 2 days, as shown in Figure 4.2.

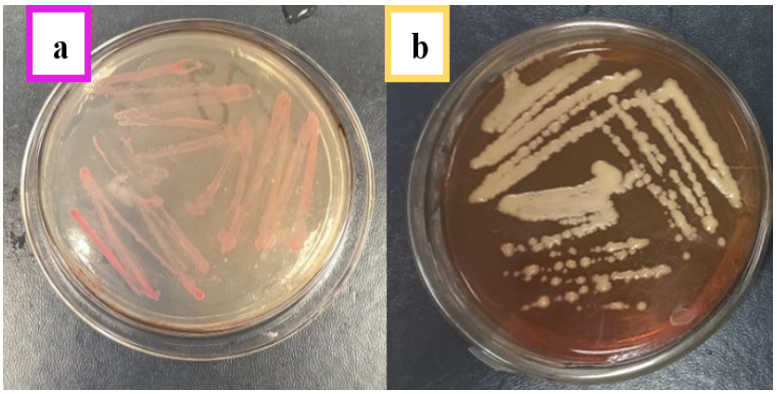



FIGURE 4.2: a) Pink colonies on Congo-Red Agar and b) Yellow colonies on Mannitol Salt Agar.

### 4.1.3 Determination of Antibiotic Resistance

The disc diffusion method was used to determine the antibiotic resistance of four bacterial strains as shown in Table 4.1.

TABLE 4.1: The results of Antibiotic resistance of bacteria.

| **Codes** | **Antibiotic Disk** | | | |
|---|---|---|---|---|
| | **Cephalexin** | **Norfloxacin** | **Cefotaxime** | **Imipenem** |
| NAO | - | - | - | 35mm |
| NAY | 4.5mm | 31mm | - | 4mm |
| MAY | - | 25.2mm | - | 43.5mm |
| COP | - | 22mm | - | - |

In this analysis, it has been seen that *Planomicrobium* sp. made a zone of inhibition only against imipenem (35mm), *Pseudarthrobacter* sp. made a zone of inhibition against cephalexin (4.5mm), norfloxacin (31mm) and imipenem (4mm), *Priestia aryabhattai* made a zone of inhibition against norfloxacin (25.2mm) and imipenem (43.5mm), *Priestia megaterium* only showed susceptibility against norfloxacin (22mm) and no zone of inhibition was formed against cefotaxime by any bacterial strains (Figure 4.3).

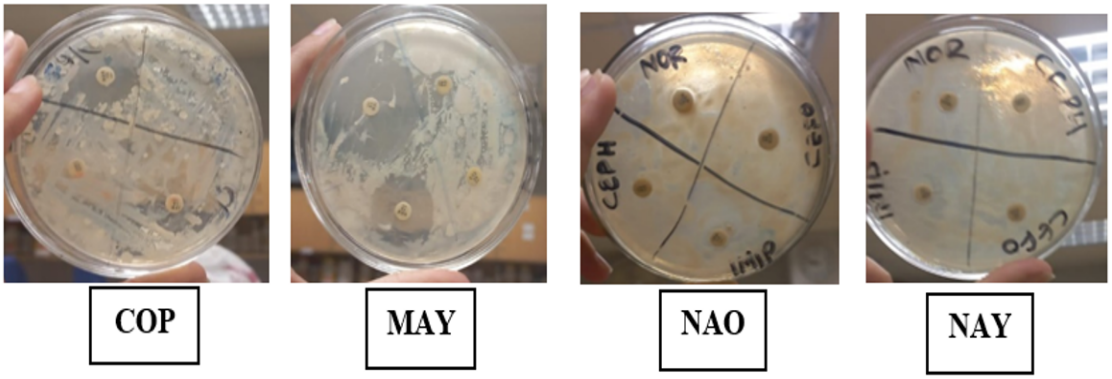



FIGURE 4.3: Results of Antibiotic resistance of Bacterial strains.

## 4.1.4 Results of Molecular Analysis

### 4.1.4.1 Molecular Analysis by 16S rRNA

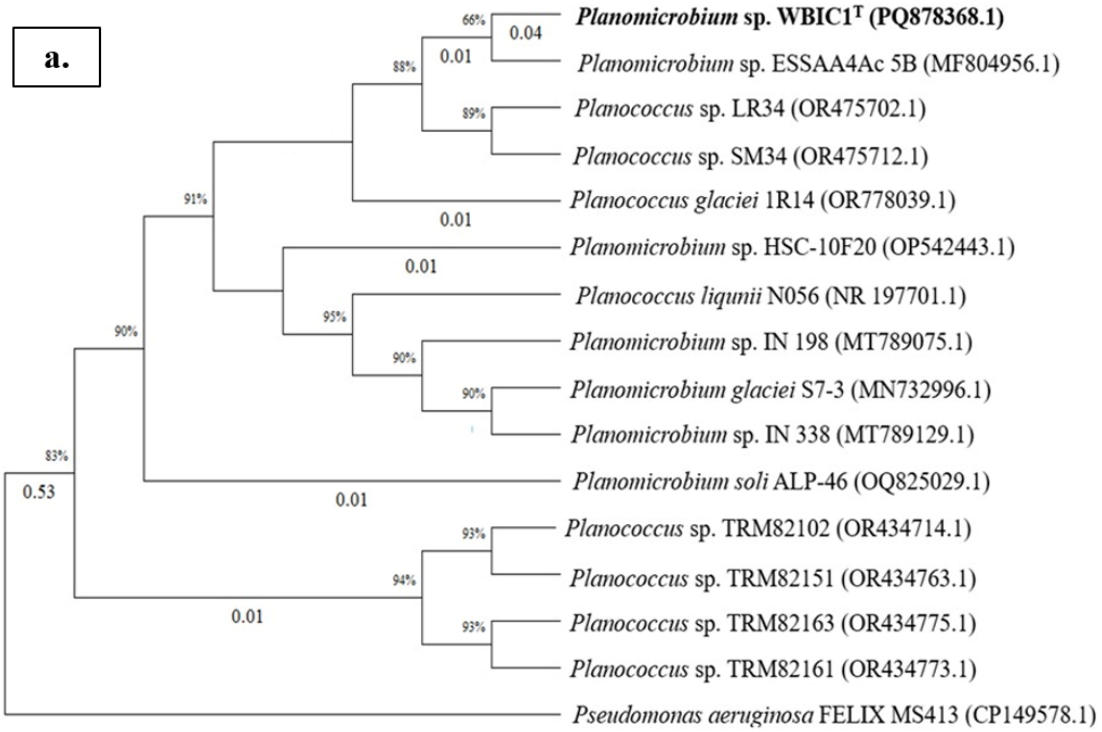
Four sequences were sent for 16S rRNA analysis following the extraction of microbial DNA from desert soil samples. The identified organisms were *Planomicrobium*, *Pseudarthrobacter*, *Priestia aryabhattai* and *Priestia megaterium* as presented in Table 4.2. The results of 16S rRNA sequencing were obtained through BLAST analysis, which compared the obtained sequences with those in the existing microbial databases, providing insights into the detected organism's taxonomic classification and phylogenetic relationships.

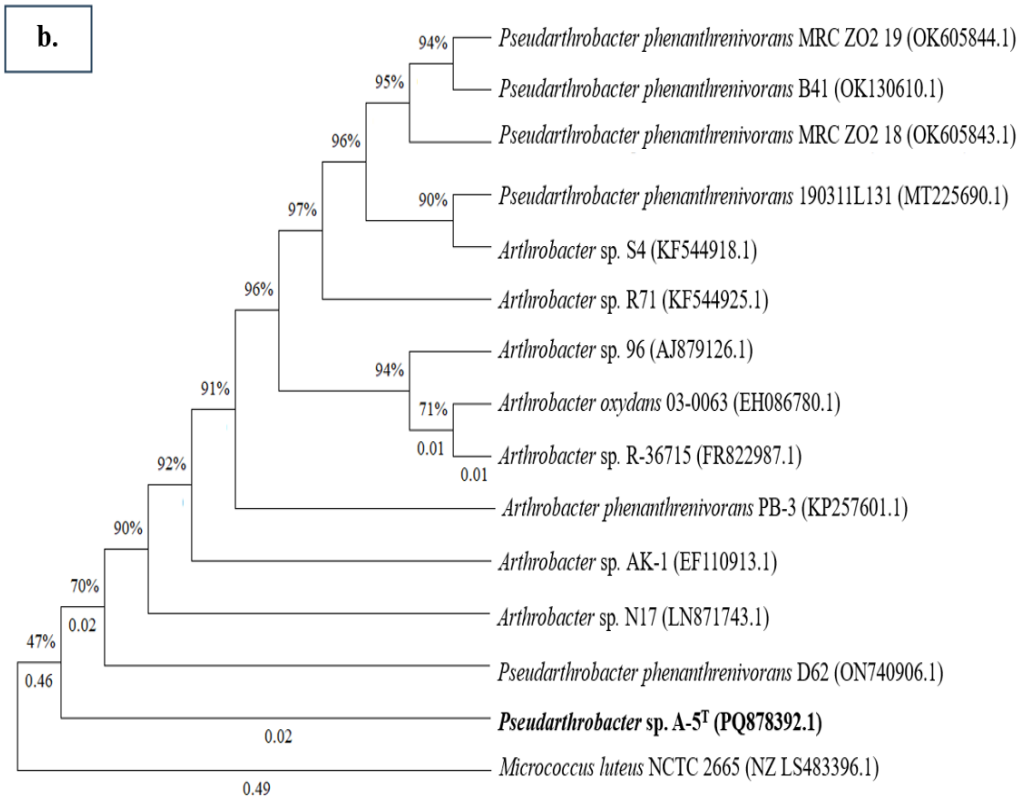
TABLE 4.2: The results of 16S rRNA sequenced through BLAST.

| S.No. | Sample ID | Bacterial Strain | Accession No. | Query Cover | %Identity |
|---|---|---|---|---|---|
| 1. | NAO | *Planomicrobium* sp. strain WBIC1 | PQ878368 | 95% | 96.15% |
| 2. | NAY | *Pseudarthrobacter* sp. strain A-5 | PQ878392 | 98% | 96.35% |
| 3. | MAY | *Priestia aryabhattai* strain G-12 | PQ661249 | 97% | 99.53% |
| 4. | COP | *Priestia megaterium* strain HG2 | PQ661253 | 97% | 98.97% |

#### 4.1.4.2 Molecular Analysis by Phylogenetic

To determine the phylogenetic analysis of four bacterial strains, their amplified sequence was obtained from NCBI using Blastn in fasta format. Table 4.2 shows a list of bacterial strains and their closely related species. Multiple sequence alignment was done using Clustal W, and the phylogenetic tree was constructed based on the Neighbouring Joining method in MEGA 11 software, as shown in Figure 4.4. The phylogenetic tree represents the evolutionary relationship among various *Planomicrobium* and *Planococcus* species with *P. aeruginosa* as an outgroup. The bootstrap values indicate the reliability of the branches, with values above 70% generally considered strong support. The branch length represents genetic distance, with shorter lengths indicating closer relationships. *Planomicrobium* sp. WBIC1$^{T}$ is placed separately, suggesting a distinct lineage. *Planococcus* species cluster together, showing a closer relationship among themselves. The scale bar values indicate evolutionary divergence, aiding in assessing genetic similarity as shown in Figure 4.4a. The figure 4.4b illustrates the evolutionary relationship among *Pseudarthrobacter*, *Arthrobacter* and *Micrococcus* species. Bootstrap value is mostly above 90% indicating strong support for most branches except a few with a lower value (47% - 70%). *Pseudarthrobacter phenanthrenivorans* strains form a well-supported clade, with closely related *Arthrobacter* species branching separately. *Pseudarthrobacter* sp. A-5$^{T}$ appears as a distinct lineage, suggesting potential novelty. *Micrococcus luteus* serves as an outgroup, providing a reference point for divergence. By branch lengths, genetic distances indicate varying degrees of relatedness among species. Figure 4.4c shows the evolutionary relationship among *Priestia*, *Bacillus* and *E. coli* species. The bootstrap values, mostly above 90% indicate strong support for most of the branches, though some nodes have lower support e.g. 69% and 70%. *P. aryabhattai* forms a well-defined clade with multiple strains clustering closely. *Bacillus* species are interspersed indicating their genetic similarity to *Priestia*. *E. coli* K-12 serves as an outgroup, providing a reference point for divergence. The branch length represents genetic distances, with shorter branches indicating closer relationships among certain species. *P. aryabhattai* G-12$^{T}$ is highlighted, suggesting its significance as a reference strain.


a.
***Planomicrobium* sp. WBIC1$^T$ (PQ878368.1)**
*Planomicrobium* sp. ESSAA4Ac 5B (MF804956.1)
*Planococcus* sp. LR34 (OR475702.1)
*Planococcus* sp. SM34 (OR475712.1)
*Planococcus glaciei* 1R14 (OR778039.1)
*Planomicrobium* sp. HSC-10F20 (OP542443.1)
*Planococcus liqunii* N056 (NR 197701.1)
*Planomicrobium* sp. IN 198 (MT789075.1)
*Planomicrobium glaciei* S7-3 (MN732996.1)
*Planomicrobium* sp. IN 338 (MT789129.1)
*Planomicrobium soli* ALP-46 (OQ825029.1)
*Planococcus* sp. TRM82102 (OR434714.1)
*Planococcus* sp. TRM82151 (OR434763.1)
*Planococcus* sp. TRM82163 (OR434775.1)
*Planococcus* sp. TRM82161 (OR434773.1)
*Pseudomonas aeruginosa* FELIX MS413 (CP149578.1)
66%
0.04
0.01
88%
89%
91%
0.01
0.01
95%
90%
90%
90%
83%
0.53
0.01
93%
94%
0.01
93%


b.
*Pseudarthrobacter phenanthrenivorans* MRC ZO2 19 (OK605844.1)
*Pseudarthrobacter phenanthrenivorans* B41 (OK130610.1)
*Pseudarthrobacter phenanthrenivorans* MRC ZO2 18 (OK605843.1)
*Pseudarthrobacter phenanthrenivorans* 190311L131 (MT225690.1)
*Arthrobacter* sp. S4 (KF544918.1)
*Arthrobacter* sp. R71 (KF544925.1)
*Arthrobacter* sp. 96 (AJ879126.1)
*Arthrobacter oxydans* 03-0063 (EH086780.1)
*Arthrobacter* sp. R-36715 (FR822987.1)
*Arthrobacter phenanthrenivorans* PB-3 (KP257601.1)
*Arthrobacter* sp. AK-1 (EF110913.1)
*Arthrobacter* sp. N17 (LN871743.1)
*Pseudarthrobacter phenanthrenivorans* D62 (ON740906.1)
***Pseudarthrobacter* sp. A-5$^T$ (PQ878392.1)**
*Micrococcus luteus* NCTC 2665 (NZ LS483396.1)
94%
95%
96%
90%
97%
96%
94%
71%
0.01
0.01
91%
92%
90%
70%
0.02
47%
0.46
0.02
0.49

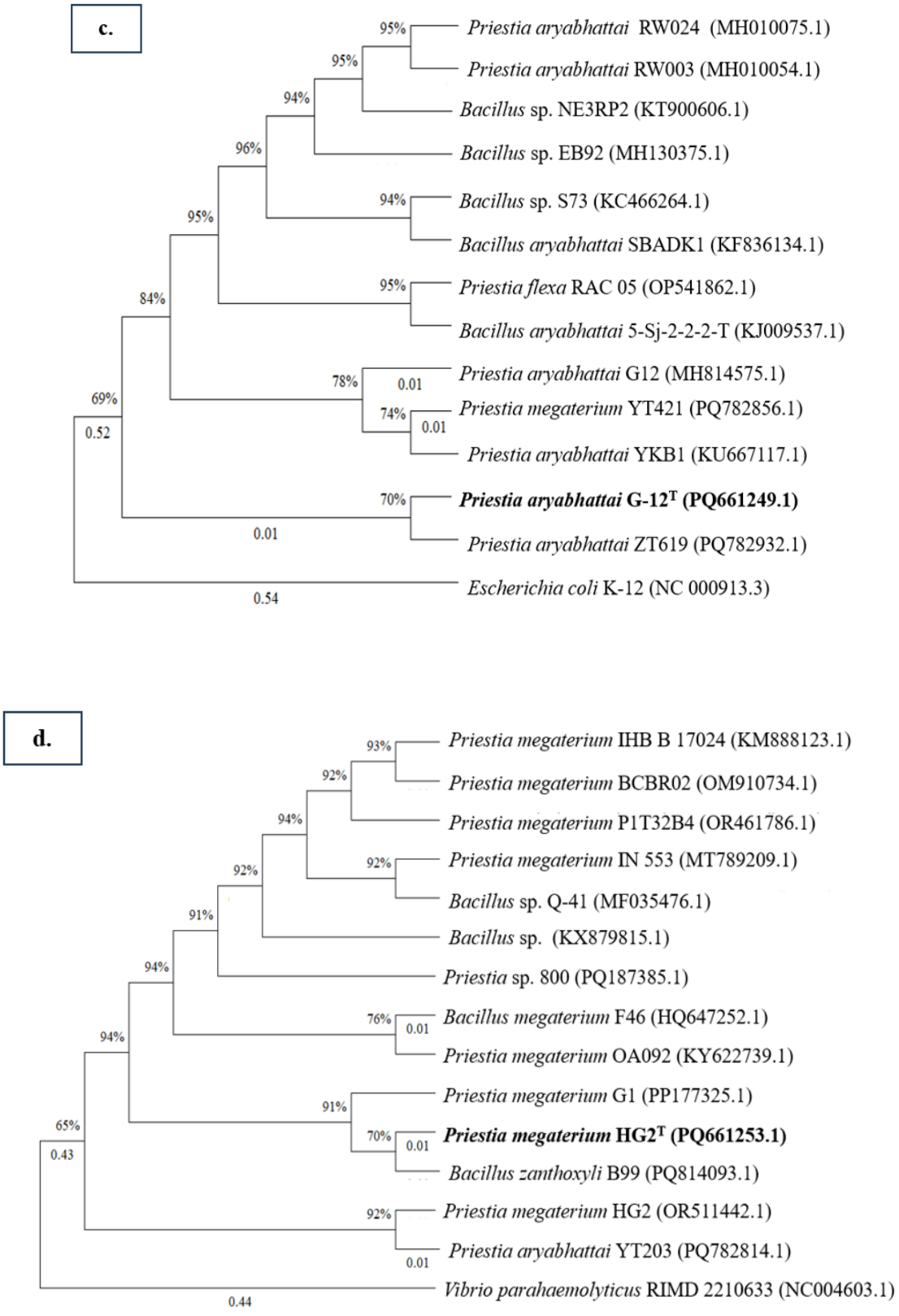



FIGURE 4.4: Phylogenetic tree of bacterial isolates based on 16S rRNA gene sequencing a) represents the phylogenetic analysis of *Planomicrobium* sp., b) represents the phylogenetic analysis of *Pseudarthrobacter* sp., c) represents the phylogenetic analysis of *P. aryabhattai*, and d) represents the phylogenetic analysis of *P. megaterium*.

The Figure 4.4d illustrates the evolutionary relationships among *P. megaterium*, *Bacillus* and *Vibrio* species. Most bootstrap values are above 90%, indicating strong support, though some nodes have lower values e.g. 65%, 70% and 76%. *P. megaterium* strains from a well-supported clade, with some closely related *Bacillus* species interspersed suggesting genetic similarities.

*P. megaterium* $HG2^T$ is highlighted indicating its significance as a reference strain. *Vibrio parahaemolyticus* serves as an outgroup, providing a divergence point for comparison.

### 4.1.5 Results of Pigment Extraction

After centrifugation, the pigment was extracted from Nutrient Agar, Congo-red Agar, and Mannitol Salt Agar. The following table 4.3 shows the result of pigments extracted from the differential media.

TABLE 4.3: Pigments extracted from selective and differential agar

| S.No | Media (Agar) | Bacterial Strains | Pigment | Picture |
|---|---|---|---|---|
| 1 | Nutrient Agar | *Planomicrobium* sp. strain WBIC1 | Orange |  |
| 2 | Nutrient Agar | *Pseudarthrobacter* sp. strain A-5 | Yellow |  |
| 3 | Mannitol Salt Agar | *Priestia aryabhattai* strain G-12 | Yellow |  |

Table 4.3: (Continued).

| S.No | Media (Agar) | Bacterial Strains | Pigment | Picture |
|---|---|---|---|---|
| 4 | Congo-Red Agar | *Priestia megaterium* strain HG2 | Pink |  |

### 4.1.6 Results of Chemical Characterization

#### 4.1.6.1 Qualitative Analysis by UV-Vis Spectrophotometry

The four extracted pigments underwent UV-vis spectrophotometry to detect the transparency and absorption of the pigments under 400-700nm. The following table 4.4 shows the result.

TABLE 4.4: Results of UV-vis spectrophotometry around 400-700nm of pigments.

| S.No. | Bacteria | Pigment | Transparency T% | Absorption |
|---|---|---|---|---|
| | | **400nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 41.3 | 0.384 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 56.8 | 0.247 |
| 3 | *P. aryabhattai* | Yellow | 52.1 | 0.283 |
| 4 | *P. megaterium* | Pink | 72.8 | 0.138 |
| | | **450nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 44.3 | 0.353 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 60.0 | 0.222 |
| 3 | *P. aryabhattai* | Yellow | 55.6 | 0.255 |
| 4 | *P. megaterium* | Pink | 70.6 | 0.151 |
| | | **500nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 48.1 | 0.318 |

Table 4.4: (Continued).

| S.No. | Bacteria | Pigment | Transparency T% | Absorption |
|---|---|---|---|---|
| 2 | *Pseudarthrobacter* sp. | Yellow | 64.0 | 0.194 |
| 3 | *P. aryabhattai* | Yellow | 61.2 | 0.213 |
| 4 | *P. megaterium* | Pink | 66.0 | 0.181 |
| | | **550nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 54.6 | 0.263 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 66.9 | 0.175 |
| 3 | *P. aryabhattai* | Yellow | 62.2 | 0.206 |
| 4 | *P. megaterium* | Pink | 77.1 | 0.113 |
| | | **600nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 56.5 | 0.248 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 69.0 | 0.161 |
| 3 | *P. aryabhattai* | Yellow | 63.7 | 0.196 |
| 4 | *P. megaterium* | Pink | 82.5 | 0.083 |
| | | **650nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 56.7 | 0.246 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 68.9 | 0.162 |
| 3 | *P. aryabhattai* | Yellow | 61.5 | 0.211 |
| 4 | *P. megaterium* | Pink | 83.0 | 0.081 |
| | | **700nm** | | |
| 1 | *Planomicrobium* sp. | Orange | 58.6 | 0.232 |
| 2 | *Pseudarthrobacter* sp. | Yellow | 69.7 | 0.157 |
| 3 | *P. aryabhattai* | Yellow | 62.2 | 0.206 |
| 4 | *P. megaterium* | Pink | 84.7 | 0.072 |

The absorption properties of bacterial pigments across different wavelengths demonstrate distinct trends as shown in Figure 4.5. At 400nm. *Planomicrobium* sp. (orange pigment) exhibited the highest absorption at 0.384 followed by *P. aryabhattai* yellow pigment at 0.283, *Pseudarthrobacter* sp. (yellow pigment) at 0.247 and *P. megaterium* (pink pigment) with the lowest absorption at 0.138. as the

wavelength increased to 450nm, absorption decreased for all the species, values ranging from 0.353 (*Planomicrobium* sp.) to 0.151 (*P. megaterium*).

At 500nm, absorption continued to decline, with *Planomicrobium* sp. recording the highest value (0.318) and *P. megaterium* the lowest (0.181). This trend persisted at 550nm where the absorption value ranged from 0.263 (*Planomicrobium* sp.) to 0.113 (*P. megaterium*). By 600nm, absorption further decreased, with the pink pigment of *P. megaterium* showing the lowest absorption (0.083). At 650nm and 700nm, absorption was minimal across all species, with *P. megaterium* recording the lowest values (0.081 and 0.072), respectively.

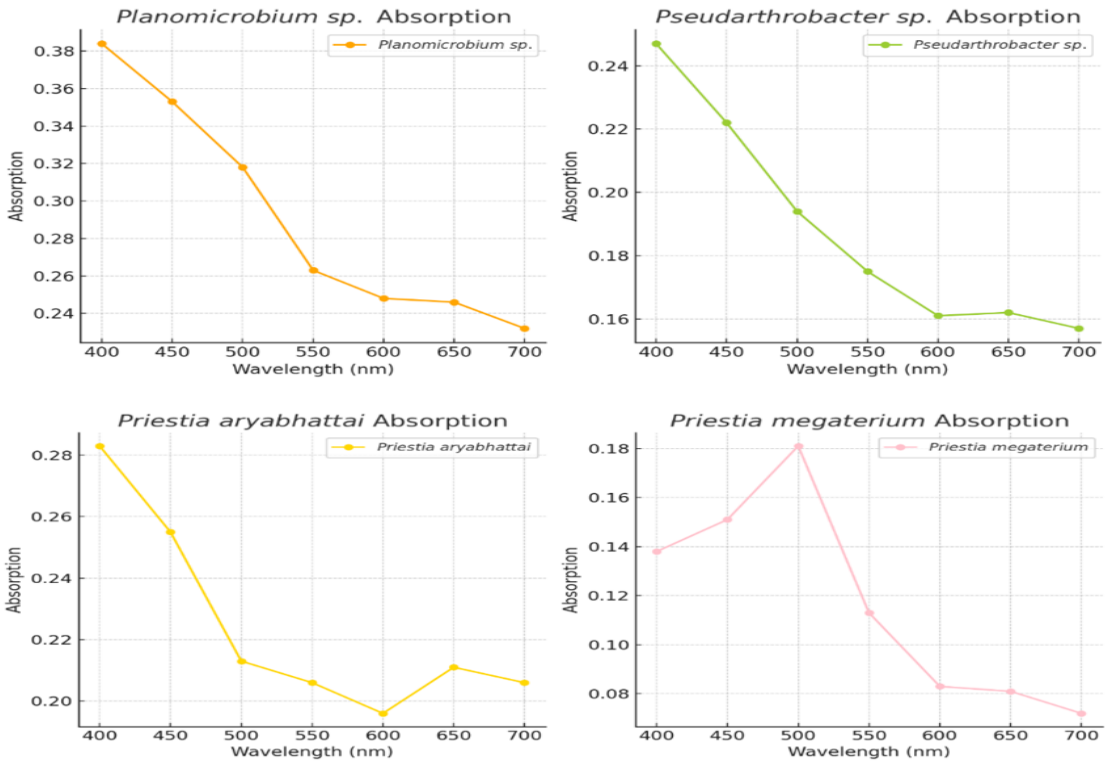



FIGURE 4.5: Graphical Representation of UV-Vis Spectrophotometry of pigments based on wavelength and absorbance.

### 4.1.6.2 Identification of the Pigmented Compounds by HPLC

The HPLC analysis of the combined bacterial pigments i.e. *Planomicrobium* sp., *Pseudarthrobacter* sp., *P. aryabhattai*, and *P. megaterium* revealed a comprehensive profile showing eight distinct peaks, each corresponding to various phytocompounds (Figure 4.6). The peaks appeared in vibrant shades of orange, yellow and

pink, showcasing the diversity and complexity of the phytochemical composition. The compounds include Beta cryptoxanthin, Zeaxanthin, Lutein, Violaxanthin, Prodigiosin, Cyclo Prodigiosin, Beta-carotene, and Lycopene as shown in Table .4.5

TABLE 4.5: Results of HPLC analysis of the bacterial pigments.

| **Peak No.** | **Retention Time (min)** | **Standard Retention Time Range (min)** | **Reference Standards** | **Identified Compounds** | **Refs** |
|---|---|---|---|---|---|
| 1 | 1.961 | 1–2.0 | Beta-Cryptoxanthin | Beta-Cryptoxanthin | [169] |
| 2 | 2.943 | 2.5–3.0 | Zeaxanthin | Zeaxanthin | [170] |
| 3 | 3.661 | 3.5–4.0 | Lutein | Lutein | [171] |
| 4 | 4.208 | 4.0–4.5 | Violaxanthin | Violaxanthin | [172] |
| 5 | 4.735 | 4.5–5.0 | Prodigiosin | Prodigiosin | [173] |
| 6 | 5.221 | 5.0–5.5 | Cyclo-Prodigiosin | Cyclo-Prodigiosin | [174] |
| 7 | 5.754 | 5.5–6.0 | Beta-carotene | Beta-carotene | [175] |
| 8 | 7.622 | 7.5–8.0 | Lycopene | Lycopene | [176] |

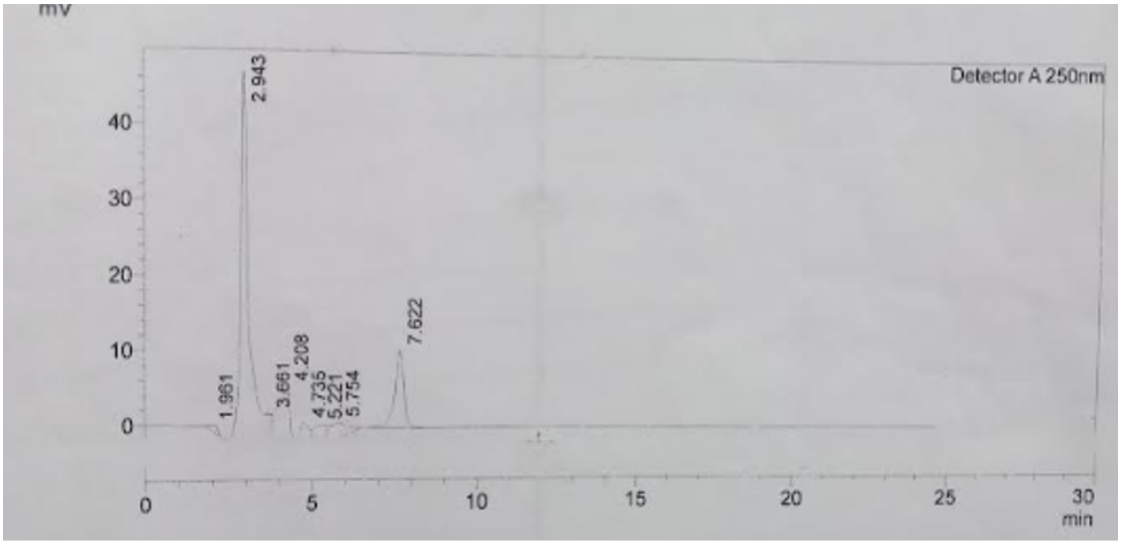



FIGURE 4.6: HPLC Chromatogram of bacterial pigments.

### 4.1.6.3 Functional Group Analysis by FTIR

The FTIR spectra of the bacterial pigments extracted in methanol were checked under 1300-4000cm-1, as shown in Table 4.6. Single bonds are represented around 2500-4000 wavenumber cm-1, triple bonds around 2000-2500 wavenumber cm-1, double bonds around 1500-2000 wavenumber cm-1, and fingerprinting below 1500 wavenumber cm-1. The peaks are shown in Figures 4.7 to 4.10.

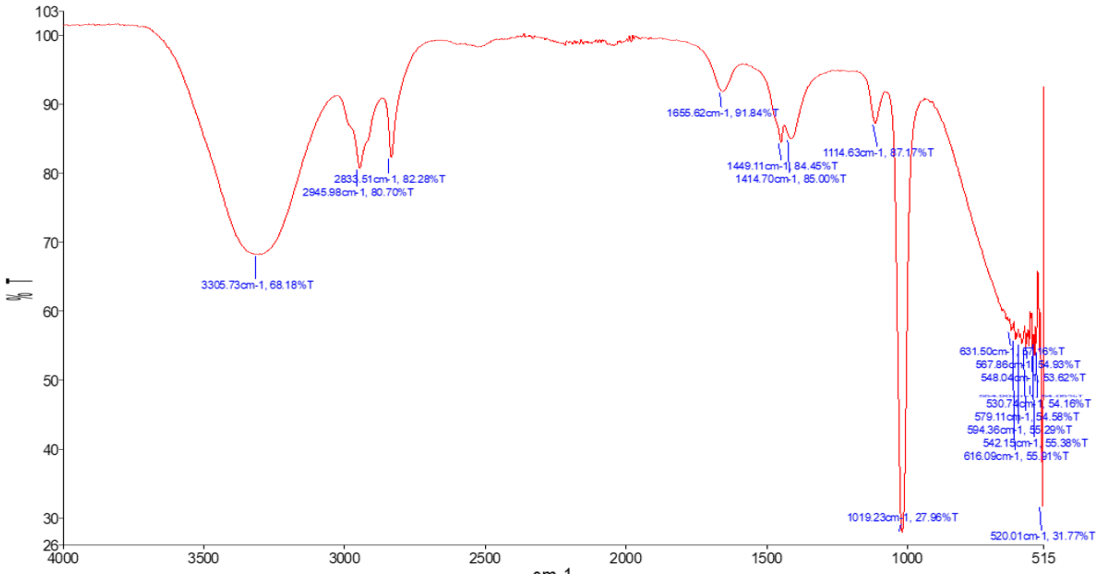



FIGURE 4.7: FTIR analysis of *Planomicrobium* sp.

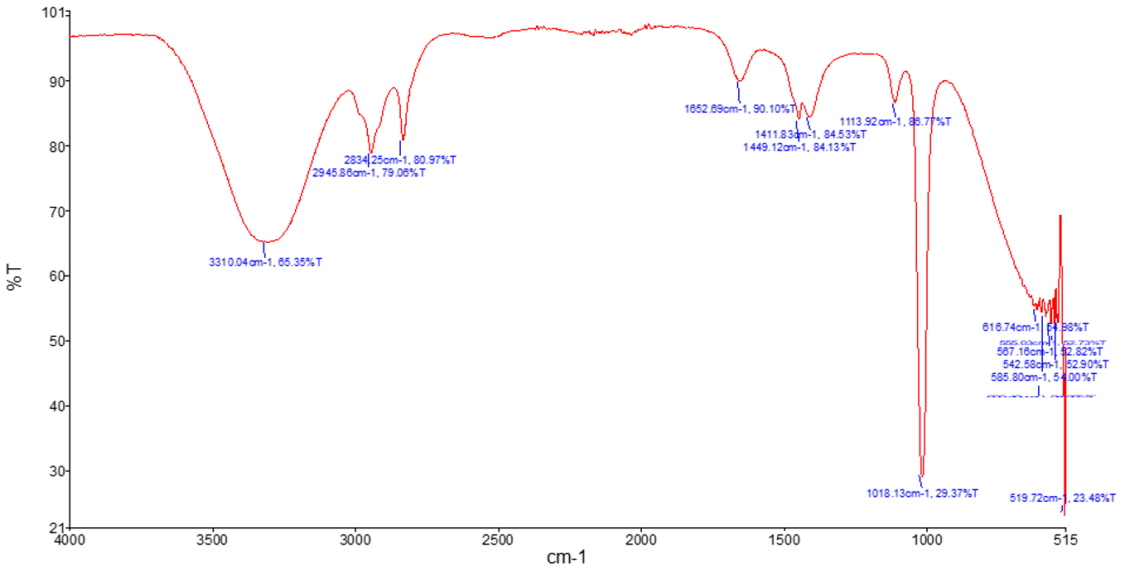



FIGURE 4.8: FTIR result of *Pseudarthrobacter* sp.

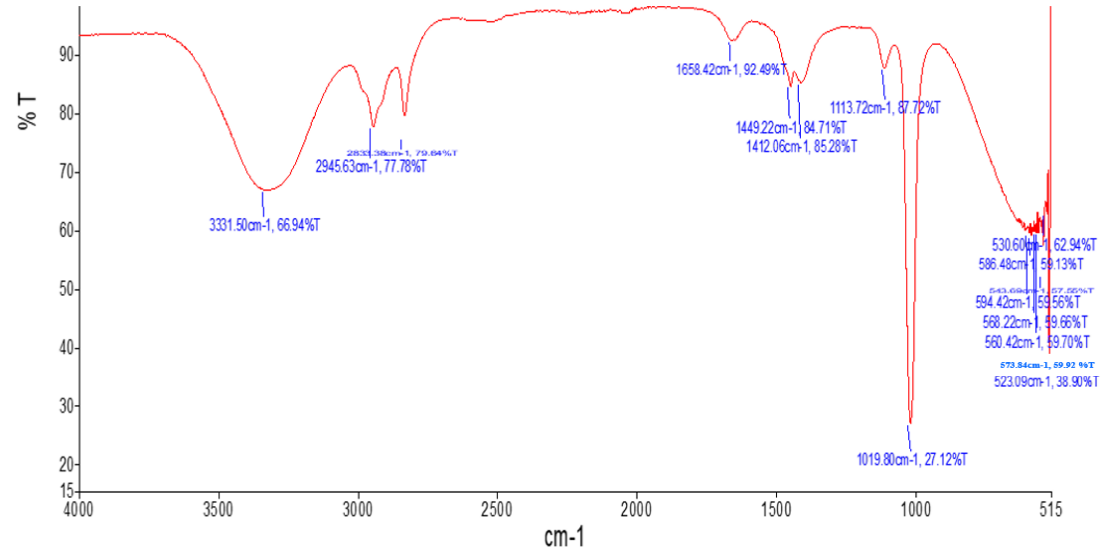



FIGURE 4.9: FTIR analysis of *P. aryabhattai*.

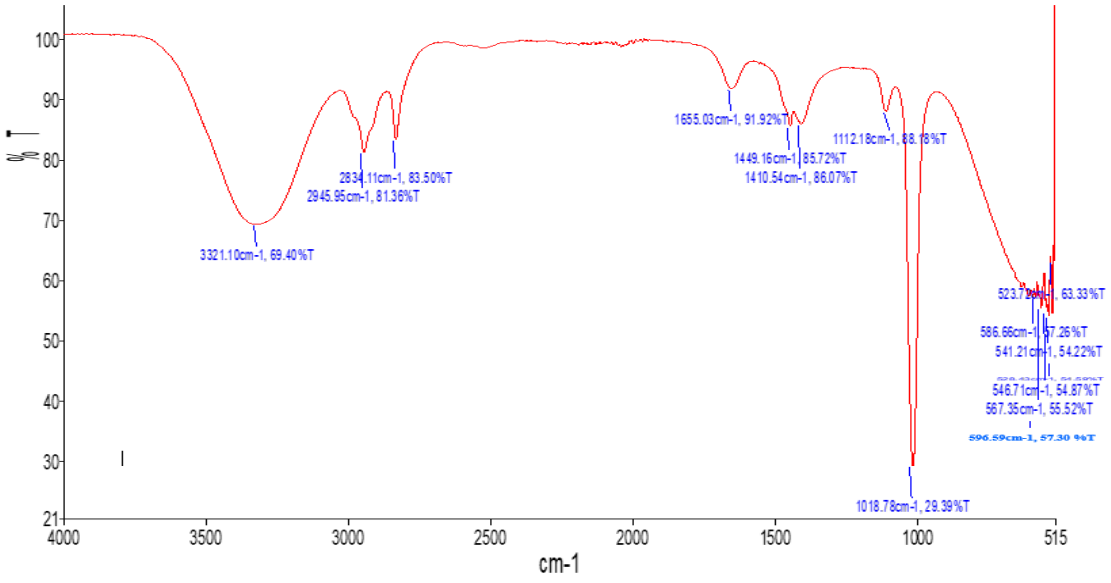



FIGURE 4.10: FTIR result of *P. megaterium*.

Table 4.6: Results of FTIR of the pigments.

| Bacterial Pigments | S.No. | Functional Group | Wavenumber ($cm^{-1}$) | Transparency (%T) | Types of Vibrations | Compound |
|---|---|---|---|---|---|---|
| ***Planomicrobium* sp.** | | | | | | |
| | 1 | OH | 3305.73 | 68.18 | Stretch | Hydroxy |
| | 2 | C-H | 2945.98 | 80.70 | Stretch | Alkanes |
| | 3 | O-$CH_3$ | 2835.51 | 82.28 | Stretch | Methyl-$CH_3$ |
| | 4 | $CONH_2$ | 1655.56 | 91.48 | Stretch | Carbonyl (Amide) |
| | 5 | C-H | 1449.11 | 84.45 | Bend | Methylene ¿$CH_2$ |
| | 6 | C-H | 1414.70 | 85.00 | Bend | Vinyl C-H |
| | 7 | C-C | 1114.63 | 87.17 | Stretch | Methyne ¿CH |
| | 8 | C-C | 1019.23 | 27.96 | | |
| | 9 | C-Br | 631.50 | 87.18 | Stretch | Aliphatic bromo compounds |
| | 10 | S-S | 616.09 | 55.91 | Stretch | Disulfide |
| | 11 | C-I | 542.15 | 55.38 | Stretch | Aliphatic iodo compounds |
| | 12 | C-I | 594.36 | 55.29 | | |

Table 4.6: (Continued).

| Bacterial Pigments | S.No. | Functional Group | Wavenumber ($cm^{-1}$) | Transparency (%T) | Types of Vibrations | Compound |
|---|---|---|---|---|---|---|
| | 13 | C-I | 567.88 | 54.93 | | |
| | 14 | C-I | 579.11 | 54.58 | | |
| | 15 | C-I | 530.74 | 54.16 | | |
| | 16 | C-I | 554.90 | 54.05 | | |
| | 17 | C-I | 548.04 | 53.82 | | |
| | 18 | C-I | 520.01 | 31.77 | | |
| ***Pseudarthrobacter* sp.** | | | | | | |
| | 1 | OH | 3310.04 | 65.45 | Stretch | Hydroxy |
| | 2 | C-H | 2945.86 | 79.06 | Stretch | Alkanes |
| | 3 | O-$CH_3$ | 2834.25 | 80.97 | Stretch | Methyl-$CH_3$ |
| | 4 | $CONH_2$ | 1652.69 | 90.10 | Stretch | Carbonyl (Amide) |
| | 5 | C-H | 1449.12 | 84.13 | Bend | Methylene ¿$CH_2$ |
| | 6 | C-H | 1411.83 | 84.53 | Bend | Vinyl C-H |
| | 7 | C-C | 1113.92 | 86.77 | Stretch | Methyne ¿CH- |

Table 4.6: (Continued).

| Bacterial Pigments | S.No. | Functional Group | Wavenumber ($cm^{-1}$) | Transparency (%T) | Types of Vibrations | Compound |
|---|---|---|---|---|---|---|
| | 8 | C-C | 1018.13 | 29.37 | | |
| | 9 | S-S | 616.74 | 54.98 | Stretch | Disulfide |
| | 10 | C-I | 555.03 | 52.73 | Stretch | Aliphatic Iodo Compounds |
| | 11 | C-I | 567.16 | 52.82 | | |
| | 12 | C-I | 542.58 | 52.90 | | |
| | 13 | C-I | 585.80 | 54.00 | | |
| | 14 | C-I | 519.72 | 23.48 | | |
| ***P. aryabhattai*** | | | | | | |
| | 1 | OH | 3331.50 | 66.94 | Stretch | Hydroxy |
| | 2. | C-H | 2945.63 | 77.78 | Stretch | Alkanes |
| | 3. | O-CH3 | 2833.38 | 79.64 | Stretch | Methyl -CH3 |
| | 4. | CONH2 | 1658.42 | 92.49 | Stretch | Carbonyl (Amide) |
| | 5. | C-H | 1449.22 | 84.71 | Bend | Methylene >CH2 |

Table 4.6: (Continued).

| Bacterial Pigments | S.No. | Functional Group | Wavenumber ($cm^{-1}$) | Transparency (%T) | Types of Vibrations | Compound |
|---|---|---|---|---|---|---|
| | 6. | C-H | 1412.06 | 85.28 | Bend | Vinyl C-H |
| | 7. | C-C | 1113.72 | 87.72 | Stretch | Methyne >CH- |
| | 8. | C-C | 1019.80 | 27.12 | | |
| | 9. | C-I | 530.60 | 62.94 | Stretch | Aliphatic iodo compounds |
| | 10. | C-I | 586.48 | 59.13 | | |
| | 11. | C-I | 543.69 | 57.55 | | |
| | 12. | C-I | 594.42 | 59.53 | | |
| | 13. | C-I | 568.22 | 59.66 | | |
| | 14. | C-I | 560.42 | 59.70 | | |
| | 15. | C-I | 573.84 | 59.92 | | |
| | 16. | C-I | 523.09 | 38.90 | | |
| ***P. megaterium*** | | | | | | |
| | 1 | OH | 3321.10 | 69.40 | Stretch | Hydroxy |

Table 4.6: (Continued).

| Bacterial Pigments | S.No. | Functional Group | Wavenumber ($cm^{-1}$) | Transparency (%T) | Types of Vibrations | Compound |
|---|---|---|---|---|---|---|
| | 2 | O-CH3 | 2834.11 | 83.50 | Stretch | Methyl-CH3 |
| | 3 | C-H | 2945.95 | 81.36 | Stretch | Methyl -CH3 |
| | 4 | CONH2 | 1655.03 | 91.92 | Stretch | Carbonyl (amide) |
| | 5 | C-H | 1449.16 | 85.72 | Bend | Methylene >CH2 |
| | 6 | C-H | 1410.53 | 86.07 | Bend | Vinyl C-H |
| | 7 | C-C | 1112.18 | 88.18 | Stretch | Methyne >CH- |
| | 8 | C-C | 1018.78 | 29.39 | | |
| | 9 | C-I | 523.72 | 63.33 | Stretch | Aliphatic iodo compounds |
| | 10 | C-I | 586.63 | 57.26 | | |
| | 11 | C-I | 541.21 | 54.22 | | |
| | 12 | C-I | 528.43 | 54.59 | | |
| | 13 | C-I | 546.71 | 54.87 | | |
| | 14 | C-I | 567.35 | 55.52 | | |
| | 15 | C-I | 596.59 | 57.30 | | |

The FTIR analysis of bacterial pigments from *Planomicrobium* sp., *Pseudarthrobacter* sp., *P. aryabhattai* and *P. megaterium* reveals common functional groups, indicating similarities in their biochemical composition. Hydroxyl (OH) groups ($\tilde{3}$300 $cm^{-1}$) and carbonyl ($CONH_2$) amide groups ($\tilde{1}$655 $cm^{-1}$) are consistently present, suggesting proteinaceous and peptide-like structures. Alkanes (C-H stretch $\tilde{2}$945 $cm^{-1}$) and methyl (-$OCH_3$ $\tilde{2}$835 $cm^{-1}$) groups imply lipid or carbohydrate associations. The presence of methyne (¿CH) ($\tilde{1}$114 $cm^{-1}$) and vinyl C-H bends ($\tilde{1}$410-1449 $cm^{-1}$) points to unsaturated hydrocarbon chains. Unique features include aliphatic iodo (C-I) and bromo (C-Br) compounds in *Planomicrobium* sp., while disulfide (S-S) bonds ($\tilde{6}$16 $cm^{-1}$) appear in *Pseudarthrobacter* sp., suggesting possible sulfur-containing compounds. The transparency (%T) values indicate varying pigment intensities, with *P. megaterium* exhibiting the highest transparency across most wavenumbers, implying potential structural variations in its pigment composition. These spectral similarities and differences highlight the biochemical diversity in bacterial pigment structures.

### 4.1.7 Results of Biological Assay

#### 4.1.7.1 Results of Antifungal Activity

The agar well diffusion method was used to assess the antifungal properties of both four bacterial strains and their pigments against pathogenic fungal strains i.e. *A. fumigatus* and *R. delemar* (Figure 4.11). For this test, three concentrations were prepared of bacteria in water i.e. 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml and pigments in methanol i.e. 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml. The antifungal activity of bacteria against *A. fumigatus* indicated that *Planomicrobium* sp. responds moderately (C1: 19.6mm, C2: 32.3mm, C3: 34.3mm) but doesn't surpass the positive control, indicating limited antifungal activity. *Pseudarthrobacter* sp. (bacteria) exhibits moderate activity in C1 (38.3mm), low activity in C2 (26mm) and no response in C3, showing a variable response. *P. aryabhattai* showed moderate activity across all concentrations (32mm, 24mm, 22.3mm). *P. megaterium* exhibited minimal activity (C1: 18mm, C3: 18.3mm) suggesting weak antifungal

properties. The antifungal activity of the pigment against *A. fumigatus* indicates that *Planomicrobium* sp. (orange pigment) showed minimal inhibition (C1: 10mm) with reduced activity. *Pseudarthrobacter* sp. (yellow pigment) showed high activity in C1 (46mm) and C2 (46.7mm), and moderate in C3 (10mm), suggesting significant potential as a pigment inhibitor against *A. fumigatus*, *P. aryabhattai* (yellow pigment) showed moderate inhibition in C1 and C2 but weaker in C3 showing limited potential while as *P. megaterium* (pink pigment) showed low inhibition in C1: 16.7mm. Moreover, the antifungal activity against *R. delemar* indicated that *Planomicrobium* sp. showed the highest activity in C1 (39mm), no activity in C2 and moderate activity in C3 (29mm), *Pseudarthrobacter* sp. showed variable results with moderate inhibition across all conditions, *P. aryabhattai* demonstrated relatively high inhibition in C3: 41.6mm, compared to other isolates, suggesting potential antifungal activity, *P. megaterium* showed inconsistent activity with weak inhibition across conditions. Moreover, the antifungal results of the pigment of these bacteria against *R. delemar* indicated that *Planomicrobium* sp. (orange color) showed low inhibition (C1: 30mm), *Pseudarthrobacter* sp. demonstrated significant inhibition in C3: 50mm indicating strong antifungal potential, *Priestia aryabhattai* showed moderate inhibition C1: 39.6mm and *P. megaterium* showed high inhibition C1: 51.6mm, suggesting strong antifungal activity. Thus, the bacteria *P. aryabhattai* showed the highest antifungal activity and in pigment, *P. megaterium* (pink) exhibited the highest antifungal activity against *R. delemar* (Table 4.7).

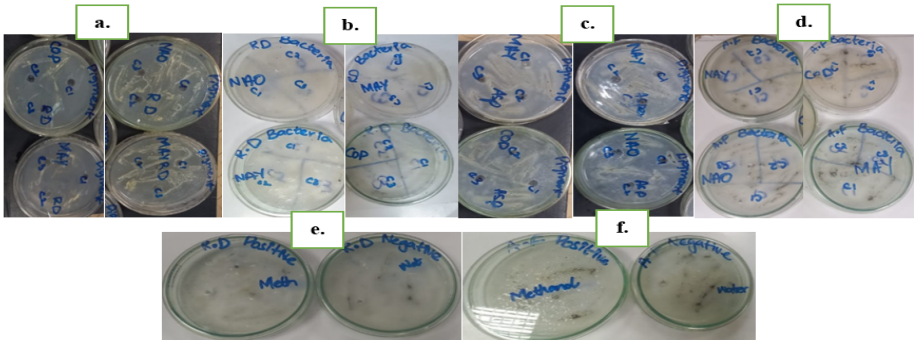



FIGURE 4.11: Results of anti-fungal a) shows the results of pigments against *R. delemar*, b) shows the result of bacteria *R. delemar*, c) shows the results of pigment against *A. fumigatus* d) shows the result of bacteria against *A. fumigatus*, e) shows the negative and positive control for *R. delemar*, f) shows the positive and negative control against *A. fumigatus*.

TABLE 4.7: Results of Anti-fungal activity of bacteria and its pigments.

| S.No. | Bacteria | C1 1000 $\mu$g/ml | C2 750 $\mu$g/ml | C3 500 $\mu$g/ml | Positive Control Amphotericin B | Negative Control Water or methanol |
|---|---|---|---|---|---|---|
| | | ***A. fumigatus* (Bacteria)** | | | | **Water** |
| 1. | *Planomicrobium* sp. | 19.6mm | 32.3mm | 34.3mm | 39mm | - |
| 2. | *Pseudarthrobacter* sp. | 38.3mm | 26mm | - | 39mm | - |
| 3. | *P. aryabhattai* | 32mm | 24mm | 22.3mm | 39mm | - |
| 4. | *P. megaterium* | 18mm | - | 18.3mm | 39mm | - |
| | | ***A. fumigatus* (Pigment)** | | | | **Methanol** |
| 1. | *Planomicrobium* sp. (Orange) | 10mm | - | - | 12mm | 5mm |
| 2. | *Pseudarthrobacter* sp. (Yellow) | 46mm | 46.7mm | 10mm | 12mm | 5mm |
| 3. | *P. aryabhattai* (Yellow) | 10mm | 20mm | 23mm | 12mm | 5mm |
| 4. | *P. megaterium* (Pink) | 16.7mm | 11.6mm | 5mm | 12mm | 5mm |

Table 4.7: (Continued).

| S.No. | Bacteria | C1 1000 $\mu$g/ml | C2 750 $\mu$g/ml | C3 500 $\mu$g/ml | Positive Control Amphotericin B | Negative Control Water or methanol |
|---|---|---|---|---|---|---|
| | | ***R. delemar* (Bacteria)** | | | | **Water** |
| 1. | *Planomicrobium* sp. | 39mm | - | 29mm | 15mm | - |
| 2. | *Pseudarthrobacter* sp. | 24mm | 36.3mm | 38mm | 15mm | - |
| 3. | *P. aryabhattai* | 27.3mm | 22mm | 41.6mm | 15mm | - |
| 4. | *P. megaterium* | - | 24.6mm | 29mm | 15mm | - |
| | | ***R. delemar* (Pigment)** | | | | **Methanol** |
| 1. | *Planomicrobium* sp. (Orange) | 3mm | 5mm | 6.7mm | 12mm | 5mm |
| 2. | *Pseudarthrobacter* sp. (Yellow) | - | - | 50mm | 12mm | 5mm |
| 3. | *P. aryabhattai* (Yellow) | 39.6mm | 13.3mm | 5mm | 12mm | 5mm |
| 4. | *P. megaterium* (Pink) | 51.6mm | 43mm | 35mm | 12mm | 5mm |

#### 4.1.7.2 Results of Anti-Bacterial Activity

The agar well diffusion method was used to determine the antibacterial properties of both four bacterial strains and their pigments against pathogenic one gram-positive bacteria (*S. aureus*) and one gram-negative bacteria (*K. gyiorum*) under three concentrations of bacteria in water i.e. 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml and pigments in methanol i.e. 1000$\mu$g/ml, 750$\mu$g/ml and 500$\mu$g/ml. The antibacterial activity of bacteria against *S. aureus* indicated that *Planomicrobium* sp. showed the most notable inhibition with a zone of inhibition ranging from 3 to 5mm across different conditions (C1, C2 and C3), indicating the antimicrobial activity, *Pseudarthrobacter* sp. demonstrated a consistent inhibition with zones ranging from 23mm to 31mm, which indicates the strong antibacterial activity against *S. aureus*, *P. aryabhattai* showed minimal inhibition C1: 5mm and no activity in C2 and C3 and *P. megaterium* showed no activity across all conditions. While the antimicrobial activity of the pigment of these bacteria indicated the orange pigment from *Planomicrobium* sp. and yellow pigment from *Pseudarthrobacter* sp. exhibited smaller zones of inhibition (3 to 5mm), indicating relatively weaker antimicrobial activity, the yellow color from *P. aryabhattai* showed a moderate inhibition zone of 25mm and pink color from *P. megaterium* did not show any inhibition against *S. aureus*.

Moreover, the results of bacteria and its pigment against *K. gyiorum* indicated that *Planomicrobium* sp. (C1: 5mm and C3: 5mm) and *Pseudarthrobacter* sp. (C1: 6mm) both show moderate inhibitions, *P. aryabhattai* showed stronger inhibition in C1: 30mm, and moderate in C2: 15mm, especially with *P. megaterium* showing no inhibition against *K. gyiorum*. Whereas the antibacterial activity of the pigments was also checked against *K. gyiorum*, it was shown that the orange color of *Planomicrobium* sp. showed moderate inhibition such as C1: 16mm, C2: 5mm and C3: 11mm, the yellow color of *Pseudarthrobacter* sp. showed consistent inhibition and making zone of inhibition in C1: 13mm, C2: 11mm and C3: 8mm, the yellow color of *P. aryabhattai* showed moderate inhibition such as in C1: 0.5 cm, C2: 15mm, and C3: 11mm, the pink color of *P. megaterium* showed minimal inhibition such as in C1 and C2: 5mm as shown in Table 4.8 and Figure 4.12.

TABLE 4.8: Results of Anti-bacterial activity of bacteria and its pigments.

| S.No. | Bacteria | C1 1000 $\mu$g/ml | C2 750 $\mu$g/ml | C3 500 $\mu$g/ml | Positive Control | Negative Control |
|---|---|---|---|---|---|---|
| | | ***S. aureus*(Bacteria)** | | | **Ampicillin** | **Water** |
| 1. | *Planomicrobium* sp. | 5mm | 3mm | - | 20mm | - |
| 2. | *Pseudarthrobacter* sp. | 23mm | - | 31mm | 20mm | - |
| 3. | *P. aryabhattai* | 5mm | - | - | 20mm | - |
| 4. | *P. megaterium* | - | - | - | 20mm | - |
| | | ***S. aureus*(Pigment)** | | | **Ampicillin** | **Methanol** |
| 1. | *Planomicrobium* sp. (Orange) | 3mm | - | - | 20mm | - |
| 2. | *Pseudarthrobacter* sp. (Yellow) | 5mm | - | - | 20mm | - |
| 3. | *P. aryabhattai (Yellow)* | - | - | 25mm | 20mm | - |
| 4. | *P. megaterium (Pink)* | - | - | - | 20mm | - |

Table 4.8: (Continued).

| S.No. | Bacteria | C1 1000 $\mu$g/ml | C2 750 $\mu$g/ml | C3 500 $\mu$g/ml | Positive Control | Negative Control |
|---|---|---|---|---|---|---|
| | ***K. gyiorum* (Bacteria)** | | | | **Ciprofloxacin** | **Water** |
| 1. | *Planomicrobium* sp. | 5mm | - | 5mm | 30mm | - |
| 2. | *Pseudarthrobacter* sp. | 6mm | - | - | 30mm | - |
| 3. | *P. aryabhattai* | 30mm | 15mm | - | 30mm | - |
| 4. | *P. megaterium* | - | - | - | 30mm | - |
| | ***K. gyiorum* (Pigment)** | | | | **Ciprofloxacin** | **Methanol** |
| 1. | *Planomicrobium* sp. (Orange) | 16mm | 5mm | 11mm | 30mm | - |
| 2. | *Pseudarthrobacter* sp. (Yellow) | 13mm | 11mm | 8mm | 30mm | - |
| 3. | *P. aryabhattai (Yellow)* | 5mm | 15mm | 11mm | 30mm | - |
| 4. | *P. megaterium (Pink)* | 5mm | 5mm | - | 30mm | - |

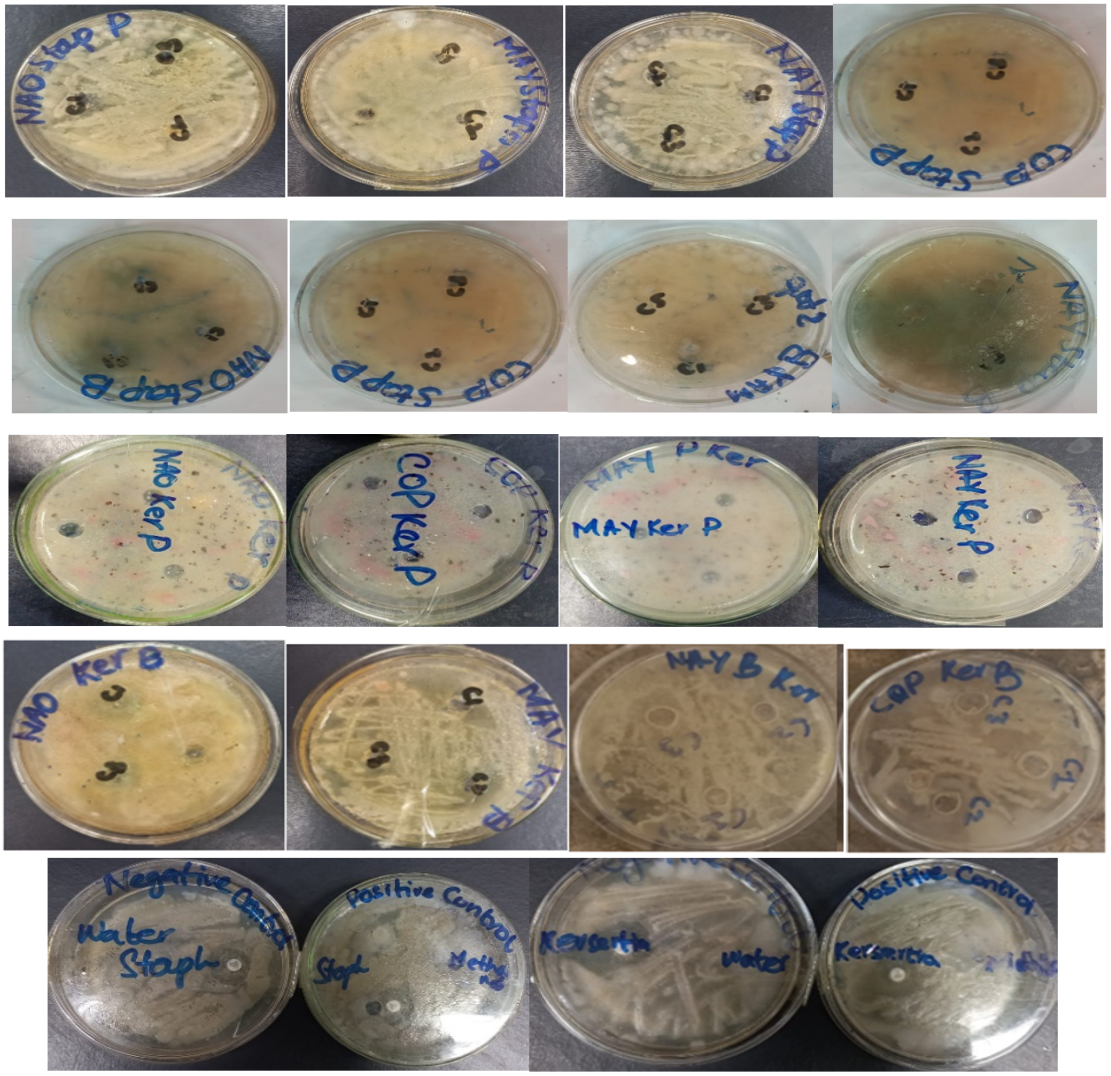



FIGURE 4.12: Results of Anti-bacterial activity of bacteria and its pigments.

## 4.2 Discussion

Soil harbors an immense and largely unexplored diversity of microorganisms. Innovative techniques particularly those utilizing rDNA and rRNA analyses, have revealed some aspects of this microbial richness [177]. One notable trait of many soil bacteria is the unique coloration of their colonies when they are grown in laboratory conditions. Studying the pigments derived from these bacteria is essential for understanding their significance in the survival and growth of the producing micro-organisms and for uncovering their potential uses in industries like food, cosmetics, pharmaceuticals, textiles and cosmetics [178, 179].

According to this study, 8 bacterial strains were selected on different media but 4 pigmented bacterial strains were taken from the desert sample from Bahawalpur. The isolation of 4 pigmented bacterial strains was identified according to the 16S rRNA analysis and chemical characterization as shown in Table 4.2. The two-colored strains belong to the genus *Priestia*, one from *Planomicrobium* and the last from *Pseudarthrobacter*. *Planomicrbium* sp, *Pseudarthrobacter* sp, *P. aryabhattai* and *P. megaterium* are gram-positive bacteria.

An antibiotic sensitivity test was done on bacterial strains to check their resistance against some antibiotics [157, 158]. According to the findings of antibiotic sensitivity of the bacteria in Table 4.1, it is plausible that *Planomicrobium* sp. exhibits resistance against norfloxacin, cephalexin and cefotaxime because of the production of beta-lactamases enzyme which breakdown the beta-lactam ring of cephalosporin making them ineffective [180].

Additionally, resistance to norfloxacin, a fluoroquinolone can occur through efflux pumps and porin mutations that reduce drug accumulation [181]. *Pseudarthrobacter* sp. exhibits susceptibility against cephalexin, norfloxacin and imipenem due to its lack of robust activity against beta-lactam antibiotics, fluoroquinolone and carbapenems [180, 181]. *P. aryabhattai* may exhibit susceptibility to both imipenem [182] and norfloxacin [183]. *P. megaterium* doesn't show susceptibility against cephalexin, cefotaxime and imipenem. In some previous research, it has been seen that *P. megaterium* also doesn't show any susceptibility against cefotaxime where the sample was taken from the blood culture of the patient [184]. It exhibits resistance to certain B-lactam antibiotics, such as imipenem and cephalexin, due to the production of the B-lactamase enzymes that degrade these antibiotics, and modifications in penicillin-binding proteins that reduce antibiotic binding [185]. It may exhibit susceptibility only to norfloxacin because this fluoroquinolone antibiotic effectively inhibits bacterial DNA gyrase, an enzyme essential for DNA replication in many bacteria [183].

Bacterial pigmentation plays a crucial role in various cellular functions and significantly contributes to the survival and protection of the organisms that produce it. However, obtaining bacterial pigments in a relatively pure and concentrated

form remains a significant industrial hurdle [16]. In this study, a pigment of different colors was successfully extracted from the specific bacterial strains: an orange pigment from *Planomicrobium* sp., a yellow pigment from *Pseudarthrobacter* sp., a yellow pigment from *P. aryabhattai*, and a pink pigment from *P. megaterium* as detailed in Table 4.3. The production of orange color from *Planomicrobium* sp. indicates the presence of carotenoid pigments. A study on psychrotrophic *Planomicrobium* sp., isolates identified and characterized these orange carotenoids, highlighting their biological activities [186].

The production of yellow color from *Pseudarthrobacter* sp. also indicates the presence of carotenoid pigments that serve as protective agents against environmental stresses, such as high solar irradiation prevalent in the desert ecosystem. Thus, the predominance of pigmented bacteria, including those producing carotenoids, in desert soils like the Mojave Desert underscores the importance of these pigments in protecting cells from harsh environmental conditions [187]. While the specific role of the genus Pseudarthrobacter has not been revealed, carotenoids in bacteria help the cells in oxidative stress due to their membrane-integrated antioxidant properties [188].

The production of pink color from *P. megaterium* indicates the presence of the tetrapyrrole group which contains cobalamin [189]. Similarly, the yellow pigment from *P. aryabhattai* is likely attributable to organic compounds like lactic acid that ferment mannitol [190]. Notably, many other organisms produce pigments that result in distinct colors with various biological properties. *Serratia marcescens* have prodigiosin containing a tetrapyrrole compound which produces a pink-to-red color and has antimicrobial and immunosuppressive properties [191], *Pseudomonas extorquens* produces pink pigment due to the presence of oxo-carotenoids [192] and *Micrococcus roseus* produces pink colored colonies due to the presence of canthaxanthin carotenoid derivatives [193].

Some other yellow-producing bacteria *Bacillus gibsonii* are isolated from lichen *Dirinaria aegialita* and have potential antifungal [194]. *Xanthomonas oryzae pv. oryzae* secretes xanthomodian, i.e. a membrane-bounded yellow-colored pigment which has antimicrobial properties [7]. *Streptomyces gresioaurianticus* JUACT 01

produces a yellow pigment that triggers apoptosis in HeLa and HepG2 cell lines while being non-toxic to human lymphocytes [195]. Lastly, *Deinococcus radiodurans* produce carotenoids, lipid-soluble pigments with characteristics of an orange color [7].

UV-vis spectroscopy is an analytical technique used to quantify an analyte by analyzing the amount of light absorbed by it. This method produces a spectrum by measuring light absorption as a function of its wavelength and frequency [196]. In this study as shown in Figure 4.5, the UV-absorbance of orange pigment from *Planomicrobium* sp. showed maximum absorption at 400 nm and decreased at 700 nm. This pattern suggests the presence of carotenoid compounds due to a broader band around 420 and 480 nm observed in the blue spectrum. It has conjugated double bonds that absorb light in the blue region [186].

The yellow pigment from *Pseudarthrobacter* sp shows a larger drop in absorption occurs between 400 nm and 450 nm indicating strong light absorption in the violet-blue region, absorption gradually tapers off from 500 nm to 700 nm showing minimal absorption. This pattern suggests the presence of carotenoids such as lutein or zeaxanthin which are known to absorb light in the violet-blue region around 400-500 nm and exhibit solvent-dependent absorption spectra [197]. The UV-absorbance of yellow pigment from *P. aryabhattai* showed fluctuations: a general decrease from 400 nm to 500 nm, a slight increase towards 650 nm and then a subsequent decline up to 700 nm. This pattern suggests the presence of carotenoid compounds, which are known to absorb the light in these regions. For instance, beta-carotene, a common yellow pigment, shows maximum absorption around 467 nm, characteristic of carotenoid derivatives [198].

The pink pigment from *P. megaterium* exhibited a strong wavelength-dependent behavior, with an increase in absorbance from 400 nm to 500 nm, followed by a steep decrease from 500 nm to 700 nm. According to this, a pink pigment exhibits absorption peaks around 535 nm as shown in the UV-vis spectrum of the purified pink pigment [199]. It shows the presence of prodigiosin a tetrapyrrole compound [200] and has garnered attention due to its biological activities including, antimicrobial, antimalarial and anticancer properties [201].

The HPLC was used to identify the compounds in the samples. In this study, the HPLC analysis of bacterial pigments revealed a comprehensive profile showing eight distinct peaks, each corresponding to various phytocompounds as shown in Figure 4.6. These peaks were observed at retention time ranging from 1.5 to 8 minutes, with notable peak areas suggesting the presence of bioactive phytochemicals as shown in Table 4.5. The identified compounds included beta-cryptoxanthin [169], zeaxanthin [170], lutein [171], violaxanthin [172], prodigiosin [173], cycloprodigiosin [174], beta-carotene [175] and lycopene [176]. The peaks appeared in vibrant shades of orange, yellow and pink, showcasing the diversity and complexity of the phytochemical composition.

The FTIR tool was used to identify the functional groups in the pigment extracted from the bacteria as shown in Table 4.6. FTIR spectroscopy has been employed to characterize the orange pigments produced by *Planomicrobium* sp., revealing key functional groups indicative of carotenoid compounds. In the study of *Planomicrobium* sp. GMMA, the FTIR spectrum displayed a broad peak of 3064 and 3562 $cm^{-1}$, corresponding to O-H and N-H stretching vibrations, which may explain the pigment's solubility in methanol and ethanol. Additionally, peaks at 2923 $cm^{-1}$ and 2854 $cm^{-1}$ were attributed to symmetric and asymmetric C-H stretching vibrations of aliphatic alkanes, while a peak at 1740 $cm^{-1}$ indicated the presence of ester carbonyl (C=O) groups. These findings suggest that the orange pigment comprises carotenoid derivatives with specific functional groups contributing to its chemical properties [186].

The FTIR analysis of *Pseudarthrobacter* sp. likely shows the characteristic peaks of carotenoids, including O-H, C-H, and C=C stretching. These functional suggest unsaturated, hydrocarbon-based pigment structures. Similar studies on yellow bacterial pigments confirm carotenoid-like spectral features [194].

The FTIR analysis of *P. aryabhattai* indicates the presence of Beta-carotene responsible for its yellow pigmentation. The key peaks at 2923 cm-1 and 2852 cm-1 (C-H stretching), 1745 cm-1 (C=O stretching), and 1465 cm-1 (C=C stretching) align with Beta-carotene while extracting the pigment from natural sources [202, 203]. While as, the FTIR results of *P. megaterium* reveal functional groups

such as hydroxyl (OH) at 3321cm$^{-1}$, methyl ($CH_3$) at 2834 cm$^{-1}$ and 2945 cm$^{-1}$, and carbonyl (amide) at 1655cm$^{-1}$ aligning with prodigiosin's characteristics peaks. These bands are consistent with those reported with prodigiosin, a red pigment primarily produced by *S. marcescens* including N-H/O-H stretching around 3300-3450 cm$^{-1}$, C-H stretching near 2900 cm$^{-1}$, and C=O stretching in amide/aromatic rings at 1600-1750 cm$^{-1}$ [204, 205].

All genus members have undergone antifungal activity against *A. fumigatus* and *R. delemar* of bacteria and their pigment as shown in Table 4.7. This study indicated that *Pseudartrobacter* sp. showed the highest antifungal activity against *A. fumigatus in both bacteria and pigment. Pseudarthrobacter* sp. produces secondary metabolites like non-ribosomal peptides and VOCs, which exhibit potent antifungal effects by disrupting fungal cell walls and metabolism [206, 207].

In *R. delemar*, *P. aryabhattai* showed antifungal activity due to its ability to produce various bioactive compounds, including secondary metabolites and hydrolytic enzymes. These substances can inhibit the growth of fungal pathogens by disrupting their cell wall and interfering with essential metabolic processes. For instance, a study demonstrated that the *P. aryabhattai* strain BPR-9 effectively suppressed the growth of several pathogenic fungi, like *Fusarium oxysporum* and *Alternaria solani*, highlighting its potential biocontrol [208].

While *P. megaterium* produces the bioactive compound including cobalamin, produces pink pigment i.e. essential for various metabolic processes in micro-organisms and may contribute to the bacteria's antagonism such as lipopeptides, which have demonstrated antifungal properties [209].

All genera of members also undergo antibacterial activity of both bacteria and their pigments against pathogenic strains of gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*K. gyiorum*) as shown in Table 4.8.

In this study, *Pseudarthrobacter* sp. bacteria have demonstrated notable antibacterial activity against *S. aureus*. Research on Arthrobacter strains, closely related to *Pseudarthrobacter*, revealed their ability to produce diffusible and volatile compounds that inhibit the growth of *S. aureus* [210].

These findings suggest that *Pseudarthrobacter* species can be effective in controlling *S. aureus* infection. Moreover, the yellow pigment produced by *P. aryabhattai* showed strong inhibition in *S. aureus* due to the presence of carotenoid compounds. These pigments possess strong antioxidants and antimicrobial properties enabling them to disrupt bacterial cell membranes and inhibit pigment production pathways. Additionally, they interfere with quorum sensing, a critical mechanism for biofilm formation and virulence factor regulation of *S. aureus* [209]. Meanwhile, the antibacterial properties of the bacteria and their pigments were also checked against *K. gyiorum*. It has been seen that the bacteria *P. aryabhattai* is known to produce metabolites with broad-spectrum anti-microbial activity, making it particularly effective against Gram-negative bacteria like *K. gyiorum* [208]. Moreover, the orange carotenoid pigment of *Planomicrobium* sp. has potent antimicrobial and anti-oxidant properties. In some research, it has been seen that the orange pigment bacterium *Planomicrobium* sp. could serve as an effective agent for antibacterial and antioxidant properties [186].

# Chapter 5

# Conclusion and Recommendations

## 5.1 Conclusion

This study highlights the diversity and significance of pigmented bacterial strains isolated from desert samples in Bahawalpur. Identifying and characterizing these bacteria, including *Planomicrobium* sp., *Pseudarthrobacter* sp., *P. aryabhattai*, and *P. megaterium*, revealed their unique pigment production, antibiotic resistance, and bioactive potential. The pigments extracted orange, yellow, and pink were linked to carotenoid and tetrapyrrole compounds, which play essential roles in cellular protection and environmental adaption. Antibiotic sensitivity tests showed *Planomicrobium* sp. resisted norfloxacin, cepha-lexin, and cefotaxime due to the beta-lactamase and efflux pumps. *Psedarthrobacter* sp. and *P. aryabhattai* were susceptible to imipenem and norfloxacin, indicating weaker resistance. *P. megaterium* resisted cephalexin, cefotaxime, and imipenem but remained susceptible to norfloxacin, likely due to beta-lactamase activity and penicillin-binding protein modifications. While, UV-Vis, HPLC, and FTIR confirmed the presence of bioactive compounds such such as Beta-Cryptoxanthin, Zeaxanthin, Lutein, Violaxanthin, Prodigiosin, Cyclo-Prodigiosin, Beta-carotene and Lycopene. Whereas, UV-Vis analysis detected carotenoid-specific absorption peaks (400–480 nm) in

*Planomicrobium* sp., *Pseudarthrobacter* sp. and *P. aryabhattai* while *P. megaterium* exhibited a peak around 535 nm, indicating tetrapyrrole structures such as prodigiosin. FTIR analysis identified functional groups, including hydroxy (OH), Carbonyl (C=O), aliphatic (CH) and aromatic compounds (CONH2), supporting the presence of these pigments. *Pseudarthrobacter* sp., exhibited the strongest anti-fungal and anti-bacterial activity, demonstrating its potential as a biocontrol agent.

## 5.2 Recommendations

Future research should focus on optimizing large-scale production methods, improving pigment stability for extended use, and conducting extensive toxicity and biodegradability studies to further enhance the application of microbial pigments. Collaboration with industrial sectors will be essential to integrate these pigments into commercial products, ensuring a sustainable and eco-friendly transition from conventional synthetic dyes.

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