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Bio-Colorants: Characterizing Pigment Producing Microbes from Peels, Soil and Textile Effluent

by

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I dedicate this work to my family, friends, and teachers. With heartfelt gratitude, I especially dedicate this thesis to my beloved mother(late), whose love and prayers continue to guide me every step of the way, and to my father, whose unwavering support and encouragement have been my greatest strength. I am also deeply grateful to my sister and brothers for always standing by my side, supporting me throughout my life, and especially during my journey toward earning my Master's degree. Their valuable advice, unconditional love, and endless prayers have been a source of strength and motivation for me.

CERTIFICATE OF APPROVAL

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Abstract

Bacterial pigments are bioactive compounds, offering applications in pharmaceuticals, textiles, and food industries. Due to environmental concerns associated with synthetic dyes, this study aims to isolate, characterize, and analyze pigment-producing bacterial strains from natural sources as sustainable alternatives. The objectives include pigment extraction, molecular analysis, and evaluation of antimicrobial, cytotoxic, and larvicidal activities. For bacterial isolation, samples were collected from fruits and vegetable Peels (FVP), compost Soil (CS), and textile effluents (TE). Culturing and isolation were performed on selective and differential media followed by Gram staining and biochemical characterization. Molecular identification of bacterial species was conducted using 16S rRNA sequencing, followed by NCBI sequence submission and Phylogenetic analysis. An antibiotic sensitivity test was performed using Doxycycline, Ceftriaxone, Streptomycin, and Imipenem to evaluate bacterial resistance profiles. Pigments were extracted in methanol and analyzed using UV-vis spectrophotometry, FTIR, and HPLC. A biological evaluation of pigments was done using antibacterial, antifungal, cytotoxicity (brine shrimp lethality assay), and larvicidal assays. *Pseudomonas nitroreducens* strain ES-18, *Klebsiella* sp. KL-1 strain, *Kerstersia gyiorum*, *Chryseobacterium* sp. S5 strain, *Serratia marcescens* strain XC 19, *Psychrobacter pulmonis* PIGB 167 and *Acinetobacter* sp. NII-56 were isolated bacterial strains, producing pigments like green, pinkish-red, blue, yellow, pinkish orange, brown, and purple respectively. Phylogenetic analysis established evolutionary relationships, confirming the distinct clustering of all the species with a significant bootstrap value. Antibiotic sensitivity testing revealed that Imipenem was more effective ($P<0.05$) compared to other antibiotics. UV-vis and FTIR confirmed the presence of carotenoids, prodigiosin, flexirubin, and other bioactive compounds. HPLC analysis identified key bioactive compounds, including riboflavin, pyocyanin, carotenoids, and benzoic acid, suggesting potential therapeutic applications. Antimicrobial assays showed significant activity, with *P. pulmonis* and *Chryseobacterium* sp. exhibiting the highest inhibition against *Staphylococcus aureus* (15mm) and *Aspergillus fumigatus* (31mm). Statistical analysis

(ANOVA) confirmed the significant inhibitory effect ($P<0.05$). Cytotoxicity assays showed moderate toxicity (LC 50 10-30μg/mL), while larvicidal activity was low confirming their safety for potential applications. The study identifies bacterial pigments as promising natural alternatives to synthetic dyes, offering potential applications in pharmaceutical and environmental sustainability.

Keywords : Bacterial pigments, antimicrobial activity, cytotoxicity, FTIR, UV-vis, HPLC, antibiotic resistance, phylogenetic analysis

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Abbreviations

CS	Compost Soil
EMB	Eosin Methyl Blue
FTIR	Fourier Transform Infrared Spectroscopy
FVP	Fruits and Vegetable Peels
HPLC	High-Performance Liquid Chromatography
MHA	Muller Hinton Agar
MRVP	Methyl Red/Voges Proskauer
PDA	Potato Dextrose Agar
TE	Textile Effluents
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron

Chapter 1

Introduction

A dye is a colored substance soluble in its application medium, such as water or solvents. It imparts color to materials by penetrating their fibers or surfaces and forming a chemical bond. In contrast, a pigment is an insoluble solid that provides color by reflecting light and requires a binder to adhere to surfaces [1].

Color is one of the first features that the human eye notices and evaluates when observing any object, such as food, flowers, plants, clothing, animals, or even other people. Humanity has always been fascinated by color, both aesthetically and socially. Historically, dyes and pigments have played a crucial role in trade, and today, virtually all commercial products incorporate color at some stage of their production. Pigments and dyes are chemical compounds that absorb and reflect specific wavelengths of light, typically between 400 and 800 nm, making certain colors apparent to the human eye [2]. These colors play a crucial role in initial human perception and are part of the primary system responsible for processing visual information. Colors are also linked to emotions, used to organize and categorize objects, and play a significant role in forming personal styles and identities [3, 4].

Pigments in living organisms serve various functions, ranging from increasing visual beauty as attractants for pollination to providing internal protection, such as defense against oxidative stress induced by ultraviolet (UV) radiation. Due to

their bioactive properties, pigments have attracted significant interest from industries, as they are natural compounds with numerous health benefits. These include anti-inflammatory, antioxidant, and antimicrobial effects, and in some cases, they have been linked to the prevention and management of chronic diseases like certain cancers and cardiovascular conditions [5, 6].

Although dyes and pigments are both used to provide color to objects, there are key differences between them. Dyes are water-soluble and disperse at the molecular level, which gives them brighter and more vibrant colors but makes them less stable when exposed to light. In contrast, pigments are naturally insoluble in water and are dispersed as particles. Their color properties remain unaffected by the medium in which they are incorporated, allowing them to maintain consistent light absorption and reflection [7, 8].

Nearly 800,000 tons of dyes are produced annually worldwide. Textile industries use a lot of the dyes that are produced. Globally speaking, China is the top producer and exporter of dyes. After China, India is the world's second-largest producer and exporter of dyes. In addition to China and India, the US, Brazil, the UK, and Germany are significant producers of dyes [9].

The regional geography of the worldwide textile dyes market is dynamic, with the Asia Pacific region leading the way with over 35% of the market. The main reason for its importance is the vast base of textile manufacturing in nations like Bangladesh, India, and China. Manufacturers of core dyes worldwide include Huntsman, Archroma, and others. About 35% of the market is held by the top 3 companies. With a market share of over 43%, China is the largest market, followed by North America and Europe, with respective shares of roughly 20% and 19% [10]. As of 2023, the dyes market is primarily led by several key regions worldwide. North America encompasses nations such as the U.S., Canada, and Mexico. In Europe, major contributors include Germany, the U.K., France, Italy, Russia, and Turkey. The Asia-Pacific region is driven by countries like China, Japan, South Korea, India, Australia, Indonesia, Thailand, the Philippines, Malaysia, and Vietnam. South America includes leading markets such as Brazil, Argentina, and Colombia. Lastly, the Africa and Middle East region features prominent players

like Saudi Arabia, the UAE, Egypt, Nigeria, and South Africa [11]. Located in the vibrant business district of Jodia Bazar, Karachi, Khaju Sons is a prominent importer of chemicals and dyes in Pakistan. Ninety percent of Pakistan's imports originate from this area [10].

Pigments can be natural or synthetic. Natural pigments are colorants derived from natural sources such as plants, animals, and microbes. In laboratories, chemical substances like toluene, benzene, sodium chloride, chromium, copper, lead, and mercury are combined to create synthetic pigments [12]. Synthetic pigments are artificially created and come in a variety of colors. They are widely used in sectors such as coatings, paints, polymers, and ceramics. Examples include bismuth vanadate, black, iron oxide yellow, and cadmium red. Certain synthetic artificial pigments such as chromium oxide and titanium dioxide are mineral-based and are well-regarded for their durability and long-lasting properties [13, 14]. Synthetic pigments are preferred for their stable synthesis processes, cost-effectiveness, and economic benefits. For instance, synthetic pigments such as Tartrazine, which provides orange/yellow hues, are priced at approximately 700–800 USD per 100 grams, whereas microbial carotenoids can cost around 1000 USD per 100 grams. The price variations are influenced by the type of pigment, the extraction methods used, and the various conditions of the bioprocess, including both upstream and downstream factors [15, 16]. The pigments are classified as small-scale pollutants due to their low concentrations, ranging from ng/L to μg/L [17].

Synthetic pigments often lack biodegradability, contributing to unsustainable practices. Additionally, there are documented instances of synthetic pigments being linked to carcinogenic effects and allergic reactions. In the food industry, certain synthetic pigments used for coloring can be toxic. Issues such as allergenicity, hyperactivity in children, and potential cancer-causing activity have led to the discontinuation of many synthetic food colorants [18]. Synthetic pigments have been a significant element of the pigment industry for ages, despite their adverse effects on both human health and the environment. As a result, many industries are investing in research and development to find new colors and sources, driving market trends and fostering innovation. One significant issue with these synthetic

additives is their potential to cause cell damage through oxidation, which can lead to immunosuppression and, in severe cases, carcinogenesis [19].

In the past, natural products and their bioactive natural metabolites have been used in treatments and medicine for hundreds of years, as well as the ongoing discovery of new drugs, remains a vibrant and active field [1]. Natural materials such as fruits, flowers, leaves, roots, and bark can be utilized to extract natural colors or pigments. Plant-based pigments are vulnerable to rapid denaturation when there is a change in pH, which impacts their reproducibility [20]. Moreover, the cultivation of plant pigments requires extensive plantation regions, pest management, and dependence on rainfall, among other factors that influence crop quality and lead to potential losses [21–23]. Although these pigments are natural, organic, and biodegradable, they still present environmental challenges due to the significant irrigation water required and, in some cases, the necessity of pesticides for pest control [13].

Additionally, pigments derived from animals, particularly mollusks and insects, were commonly used in the classical era but have fallen out of favor due to production difficulties and concerns about animal exploitation [24]. Insects produce a variety of pigments, including aphids, pterins, melanin, anthraquinones, tetrapyrroles, ommochromes, and papiliochromes [25]. However, extracting these pigments is challenging due to the high costs associated with insect farming and purification processes [26]. Additionally, there have been reports of allergic reactions linked to these pigments [27].

Microorganisms offer a rich source of novel bioactive compounds because they are easily renewable and can produce higher yields compared to higher organisms [28]. Among these microbial bioactive compounds, natural pigments have garnered significant industry interest. This is driven by a growing demand for new, safe, quickly degradable, and environmentally friendly products that have minimal adverse effects. Microbial pigments which are created as either primary or secondary metabolites, provide an ecofriendly and non-toxic alternative [29].

Natural pigments are vital for microorganisms, helping them adapt to extreme environments, protect against solar radiation, and engage in photosynthesis. In this context, microbial pigments are noted for their significant therapeutic and nutritional benefits, and the industry is increasingly acknowledging these valuable properties [30].

Microorganisms, influenced by environmental factors, produce a diverse array of pigments with distinct characteristics that reflect their interaction with their ecosystems. Many different types of microbial pigments such as carotenoids, phenazines, flavins, quinones, violaceins, monascines, melanin, and indigodines have been identified across different environments [31]. Research shows that natural pigments offer benefits like anticancer properties, pro-vitamin A activity, strong photostability, thermal stability, and pH resilience. They also contribute to biodiversity preservation by reducing the environmental impact associated with synthetic dye production. Among microorganisms that produce natural pigments, fungi, bacteria, and yeast are particularly significant. In contrast, bacteria provide several unique advantages, such as a short life cycle, minimal sensitivity to seasonal and climatic variations, ease of scaling up, and the ability to produce pigments in a range of colors and shades [32].

Microbial pigments are produced by microorganisms in various environments like water, soil, compost, organic matter, forest and industrial waste. For example, Monascus species from soil and compost produce red and orange pigments like monascorubrin, while Chromocbacterium violaceum in aquatic habitats produces a violet pigment called violacein. *Bacillus* sp. commonly found in forests and organic matter produces yellow pigments such as carotenoids. Industrial wastes especially the food industry produce microbes like *Aspergillus* sp., produce yellow and red pigments like Aspergillic acid. Additionally, fruit and vegetable peels are home to *Penicillium* sp., which produces blue and green pigments [33].

The peels of fruits and vegetables are rich in organic compounds that can serve as substrates for pigment-producing bacteria. Research has shown that peels from fruits such as oranges, pomegranates, bananas, and papayas, as well as vegetable scraps like carrots, tomatoes, potatoes, and beetroot, provide an excellent medium

for bacterial growth and pigment production. For example, *Serratia marcescens* isolated from these peels can produce the red pigment prodigiosin, which is known for its antimicrobial properties [34].

Compost soil, enriched with decomposing organic matter, harbors a variety of bacteria capable of synthesizing pigments. Bacteria isolated from compost, such as *Bacillus* spp. and *Pseudomonas* spp., have been found to produce pigments like carotenoids and violacein. These pigments not only impart color but also play a role in nutrient cycling and stress resistance in soil environments [35].

The effluents from textile dyeing processes are rich in organic compounds that can support the growth of pigment-producing bacteria. Studies have identified bacterial strains such as *Micrococcus* and *Rhodococcus* that utilize these effluents to produce dyes like indigo and various quinones. These pigments can degrade pollutants and help in bioremediation, thus offering an eco-friendly solution to wastewater management [36].

In this context, bacterial pigments hold significant potential for emerging biotechnological applications. They offer the possibility of rapid mass production and can be applied across various industrial sectors, including functional food production, drug development, and biomedical therapies. This study aims to highlight the beneficial properties of several important bacterial pigments and explore their potential applications in biomedicine and human health.

1.1 Problem Statement

The widespread use of synthetic dyes is depleting non-renewable resources and causing serious environmental and health concerns. Although natural dyes derived from plants and animals provide a more sustainable option, their limited durability and high production costs pose significant challenges. Therefore, it is essential to rapidly identify eco-friendly microbial dyes to address this issue.

1.2 Research Gap

A significant portion of research on bacterial pigments has concentrated on well-known bacterial strains from limited sources. There is potential to investigate novel pigment-producing bacteria from previously unexplored sources.

1.3 Research Questions

1. Which pigment-producing bacterial species can be isolated from different sources?

2. Are the bacterial pigments extractable or not?

3. What are the possible applications of the extracted bacterial pigments?

1.4 Scope of Study

The production of bacterial pigments provides significant economic opportunities in biotechnology and sustainable industries. Unlike synthetic pigments, bacterial pigments are biodegradable with minimal environmental pollution and associated health risks. Their application holds immense potential for developing safer, non-toxic industrial products in different sections like Pharmaceuticals, Food, Textiles, and Cosmetics aligning with global demands for sustainability and environment friendly.

1.5 Aim

To identify (screen out) pigment-producing bacterial species from natural sources with the potential to use as organic dyes, in different industries.

1.6 Objectives

- To isolate and characterize the pigment-producing bacterial strains from natural sources.
- To extract pigment from isolated bacterial strains.
- To perform the molecular analysis of extracted pigments.
- To assess the antimicrobial activity of isolated bacterial strains and extracted pigments against selected bacterial and fungal strains.
- To evaluate the cytotoxic activity of isolated bacterial strains and extracted pigments.
- To evaluate the larvicidal activity of isolated bacterial strains and extracted pigments.

1.7 Impact on Society

Bacterial pigments offer eco-friendly and sustainable alternatives across various domains with therapeutic properties and diverse industrial applications.

Chapter 2

Literature Review

2.1 Pigments

Pigments are widely utilized across various industries and come in a broad spectrum of colors, with some being water-soluble. The non-toxic nature of pigments produced by microorganisms makes them environmentally friendly options for use in dyes, food products, pharmaceuticals, cosmetics, and other industrial applications. In the quest for sustainable and eco-friendly bioproducts, microorganisms offer a promising source of valuable bioproducts for diverse industrial sectors. Microbial pigments, in particular, present several advantages. They can be cultivated in bioreactors under controlled physical and chemical conditions, utilizing industrial residues and by-products as sources of carbon and nitrogen, which helps reduce production costs [37]. This method also minimizes industrial waste by transforming it through microbial metabolism [38, 39]. Additionally, the production of microbial pigments does not require cultivable land and is minimally impacted by climate change [40]. Advances in synthetic biology allow for genetic modifications to optimize production, including the expression of genes in model organisms like *E. coli* [41–45]. Microbial pigments provide a crucial alternative to traditional synthetic pigments, offering environmental benefits. Additionally,

natural pigments are valued for their medicinal properties, including antimicrobial, antioxidant, additive, color-enhancing, and anti-cancer effects. They are also cost-effective [37].

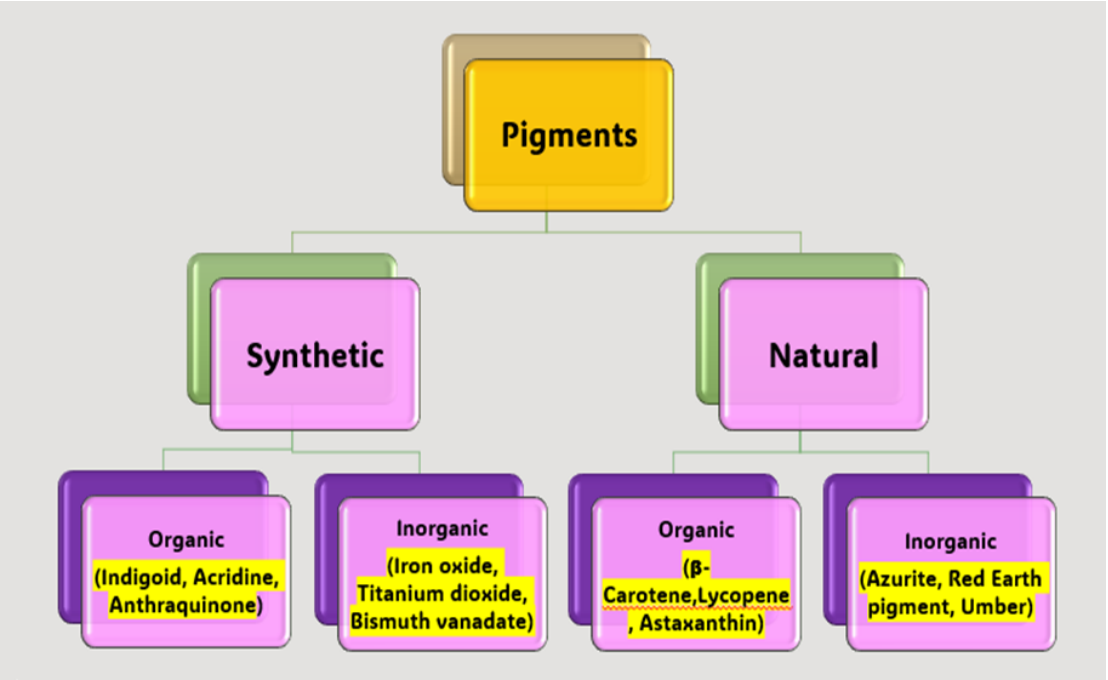

FIGURE 2.1: Classification of Pigments: Synthetic and Natural, Further Divided into Organic and Inorganic Categories with Examples [9]

Synthetic pigments fall into two main categories: artificial organic pigments and synthetic inorganic pigments. Artificial organic pigments are often ignored as environmental pollutants despite their varied chemical and physical properties. Synthetic inorganic pigments can be mineral-derived or chemically made; for example, iron oxide is natural when extracted from rocks but is considered synthetic when manufactured [46]. Organic pigments sourced from minerals may carry heavy metal pollution, which poses potential issues for industrial applications. Consequently, there are numerous global regulations designed to limit the presence of arsenic, lead, and other heavy metals in products such as cosmetics [13, 47]. Mineral pigment usage also presents environmental risks. For example, irrigation water contaminated with these pigments can affect agriculture and food safety. If not properly managed, waste from this production process can enter the food chain, leading to bioaccumulation in fish and other aquatic organisms [48].

2.2 Sources of Natural Pigments

Natural dyes, obtained from plants, minerals, and animals, are coloring agents that can impart a range of hues to textiles, leather, food, and medicinal products. This traditional dyeing practice has been utilized by ancient civilizations for millennia [49]. Categorized based on their origins as plant, animal, and mineral dyes, they contribute to a wide array of applications [50].

Natural dyes provide excellent biodegradability and typically exhibit greater environmental compatibility. Derived from animal or plant sources without synthetic additives, they are known for being non-toxic, non-allergenic, and non-carcinogenic [51]. Minerals, vegetables, and animals serve as the primary sources of natural dyes [52].

2.2.1 Plant Source (Fruit, Vegetables, and other Plants)

In ancient times, plant species were the primary source of natural colorants, with approximately 500 plant species verified as capable of producing dyes. These plant-based dyes are not only renewable and biodegradable but also offer potential health and medicinal benefits.

Various plants, including roots, stems, leaves, bark, berries, and seeds, can be utilized to extract dyes. The concentration and composition of these coloring agents can vary significantly depending on factors such as the plant's age and the time of year when it is harvested. [53].

2.2.2 Animal Source

Natural dyes also have remarkable origins in the animal kingdom, notably in ancient cultures where red dyes were highly valued for their vibrant colors.

Several animal-derived sources have been historically important for producing these dyes [54, 55].

TABLE 2.1: Showing the animal dyes sources and description [56, 57]

Dye	Source	Description	References
Lac	Resin from lac insect (*Laccifer lacca*)	Early red dye made from the insect's resin, used for centuries.	[56]
Kermes	Kermes insect (*Kermes ilicis*)	Ancient scarlet dye valued for its vibrant red color, popular in textiles of historical eras.	[57]
Cochineal	Cochineal insect (*Dactylopius cacti*)	Brought to Europe in the 1500s; creates a range of red and purple hues.	[56]
Tyrian Purple	Mollusks (*Murex brandaris, M. trunculus*)	Prestigious purple dye from Mediterranean mollusks, celebrated for its durability and richness.	[56]

2.2.3 Mineral Sources

Mineral-based sources constitute another category of natural dyes, stemming from earthy pigments that derive their coloring attributes from oxides or hydrated oxides of manganese. Examples include chrome yellow, nankin yellow, prussian blue, iron buff, and manganese brown. These dyes exhibit minimal solubility in water and other solvents, requiring binders to adhere to fiber surfaces. Iron oxides and titanium dioxide are used in paints, coatings, cosmetics, and sunscreens for their color and UV-blocking properties. Despite their durability-enhancing properties, many mineral colorants are recognized as toxic, restricting their applications. Hence, the term "natural dye" encompasses both dyes sourced from vegetable origins and minerals. Additionally, certain minerals like seru, cow urine, cow dung, and egg albumin are directly utilized as coloring agents [50].

TABLE 2.2: Benefits and drawbacks of plants, animals, minerals, and microbial pigments [57–60]

Parameters	Plants	Animal	Minerals	Microbial
Production Cost	×	×	✓	×
Biological breakdown	✓	✓	×	✓
Uncontaminated	×	×	×	✓
Sustainable/Renewable	✓	×	×	✓
Use of substrate	✓	×	×	✓

Table 2.2 continued from previous page

Parameters	Plants	Animal	Minerals	Microbial
Climate-resilient	×	×	✓	✓
Genetic modification	✓	×	×	✓

2.3 Microbial Sources

2.3.1 Microalgae

Microalgae genera, including *Dunaliella*, *Chlorella*, *Nostoc*, *Nanochloropsis*, *Scenedesmus*, *Haematococcus*, *Phaeodactylum*, *Muriellopsis*, *Spirulina*, *Polysiphonia*, and *Arthrospira*, produce various types of pigments such as chlorophylls, carotenoids, and phycobiliproteins (PBSs). These phycobiliproteins are non-toxic, water-soluble proteins. These pigments are mainly found in Cyanobacteria, red algae (*Rhodophyta*), and *Cryptophyta* [61]. Microalgae, including species like *Spirulina* and *Haematococcus pluvialis*, are valuable sources of pigments such as phycobiliproteins and astaxanthin, respectively. These pigments offer vibrant colors and antioxidant properties, making them ideal for food, cosmetics, and nutraceuticals. Microalgae-based pigments are sustainable since they can grow in controlled environments without seasonal limitations, unlike plant or animal pigments. Additionally, *Spirulina* produces phycocyanin, a blue pigment widely used in natural food colorants and dietary supplements [62]. Phycobiliproteins are widely utilized in pharmaceutical, food, cosmetics, and biomedical sectors due to their strong absorbance and fluorescence qualities along with their antioxidant and free radical scavenging abilities [63].

2.3.2 Fungi

Fungi from families such as *Chlorociboriaceae*, *Monascaceae*, *Sordariaceae*, *Trichocomaceae*, *Xylariaceae*, *Nectriaceae*, *Hypocreaceae*, *Pleosporaceae*, *Cordycipitaceae*, and *Chaetomiaceae*, are recognized as strong pigment producers [64]. Fungi

produce a diverse range of pigments, including melanins, carotenoids, and polyketides like flavins, phenazines, monascins, violacein, quinones, and indigo, which provide a wide array of colors. These fungal pigments are also associated with significant bioactive properties, such as anticancer, antioxidant, antimicrobial, anti-inflammatory, and immunosuppressive activities [65]. In laboratory trials, studies have shown that bead milling is an effective method for extracting carotenoids from *Sporobolomyces ruberrimus* H110, with pigment levels assessed using spectrophotometric analysis [66].

One such pigment is Arpinkred^{TM} pigment (also known as Natural red^{TM}), which is the first red color produced commercially by a fungus. It is derived from the soil-isolated strain of *Penicillium oxalicum* var. *armeniaca* CCM 8242. A large number of these pigments are derived from polyketides, which are widely generated by most *Ascomycetous* fungi. Ascomycetes fungi such as *Neurospora* spp. and *Monascus* spp. illustrate an important field that needs more research [67]. Fungal pigments have long served as valuable taxonomic tools and are commercially utilized for applications such as protein detection, cell staining, and as alternatives to synthetic dyes in the textile industry [68–70].

2.3.3 Yeast

Yeasts like *Rhodotorula* and *Phaffia rhodozyma* are known for producing valuable pigments such as carotenoids, including torulene, torularhodin, and astaxanthin. These pigments serve diverse roles, including antioxidant activity, vitamin A precursors, and natural colorants in food and pharmaceuticals. Notably, astaxanthin is widely used in aquaculture to enhance the pigmentation of fish and crustaceans, while β-carotene finds applications in cosmetics and health supplements due to its provitamin A properties and immune-boosting effects [71]. Yeasts are known for producing pigments in orange, yellow, and red, used in wine, aquaculture, and medicine [72].

2.3.4 Bacteria

Microbial pigments sourced from bacteria, fungi, and microalgae have diverse biotechnological applications across the cosmetic, food, textile, and pharmaceutical industries. These pigments contribute to the survival and adaptation of microorganisms by protecting them from ultraviolet radiation and ROS, as well as offering antibacterial and fungicidal properties. Such functions help microorganisms maintain their ecological niches and compete effectively for resources. Furthermore, some pigments contribute to photosynthetic processes within cells, assisting in energy production [73]. The primary pigments produced by bacteria include carotenoids, phenazines, quinones, prodigiosines, violacein, and aryl polyenes, some of which may be esterified through a dialkyl resorcinol system. These bacterial pigments are increasingly recognized for their potential medicinal applications due to their properties such as antibiotic, antimalarial, and anticancer activities. Among these, melanins are typically black, brown, or olive; carotenoids appear in shades ranging from red, yellow, and pink to violet; and blue pigments are also commonly observed in bacteria [74]. As *Spirulina* becomes increasingly popular among health-conscious consumers, there is active research in the food, cosmetics, and pharmaceutical industries focused on enhancing production and optimizing the extraction of phycocyanin. This blue photosynthetic pigment-protein complex possesses antioxidative and anti-inflammatory properties. Promising results have been demonstrated with bead milling for the extraction of phycocyanin and phenolic compounds from *Arthrospira spirulina* [75].

2.4 Bacterial Pigments

Bacterial pigment production is vastly varied, with Actinobacteria being a common group where it is observed. Numerous genera, including *Streptomyces*, *Nocardia*, *Thermomonospora*, *Microbispora*, *Streptosporangium*, *Rhodococcus*, and *Kitasatospora*, are known for producing a variety of pigments. These pigments are primarily utilized in industries such as textiles, food, and cosmetics [76]. Moreover, pigments like quinones, melanin, violaceins, and indigoidines have demonstrated

strong antimicrobial properties [77].

In addition to their antimicrobial capabilities, these pigments also serve as antioxidants, bioindicators, and even anticancer agents, making them increasingly significant in biomedical research [78].

Among the numerous bacterial pigments, the following possess the properties needed to serve as promising therapeutic agents in the biomedical field.

TABLE 2.3: Showing bacterial pigments, their biological activities, and associated pigment-producing genera.

Pigment	**Pigment producing genera**	**Color / Shade**	**Biological Function**	**Ref.**
Violacein	*Iodobacter* *Collimonas* *Pseudoalteromons* *Chromobacterium* *Alteromonas* *Streptomyces*	Purple	Antiviral Anticancer Antitumoral Antiparasitic Antibiotic	[77, 78]
Indigoidine	*Dickeya* *Clavibacter* *Arthrobacter* *Corynebacterium* *Bacillus*	Indigo	Antibiotic Antioxidant Signaling	[79, 80]
Melanin	*Rhizobium* *Klebsiella* *Pseudomonas* *Streptomyces* *Brevibacterium*	Dark brown	Anticancer UV protection Antioxidant	[81–83]
Carotenoids	*Paracoccus* *Pantibacter* *Erwinia* *Flavobacterium*	Red/Orange	Cytotoxicity Antioxidant Antibiotic	[84–87]
Prodigiosin	*Serratia* *Vibrio* *Streptomyces* *Hahella* *Zooshikella*	Deep red	Biocontrol Algacidal Antibiotic Anti-inflammatory Antidiabetic Anticancer	[88–91]

Table 2.3 continued from previous page

Pigment	Pigment producing genera	Color / Shade	Biological Function	Ref.
Rhodopsins	*Hallobacterium (Archea)* *Halorubrum (Archea)* *Anabaena (Eubacteria)* *Gloeobacter (Eubacteria)* *Natromonas (Archae)*	Light pink	Cell behavior modulator Active transport signaling	[92, 93]
Pyoverdine	*Pseudomonas*	Yellowish green	Bioluminescence Iron uptake Virulence factor Antibacterial activity	[94, 95]
Pyocyanin	*Pseudomonas*	Greenish blue	Cytotoxicity Virulence factor Iron uptake	[96, 97]

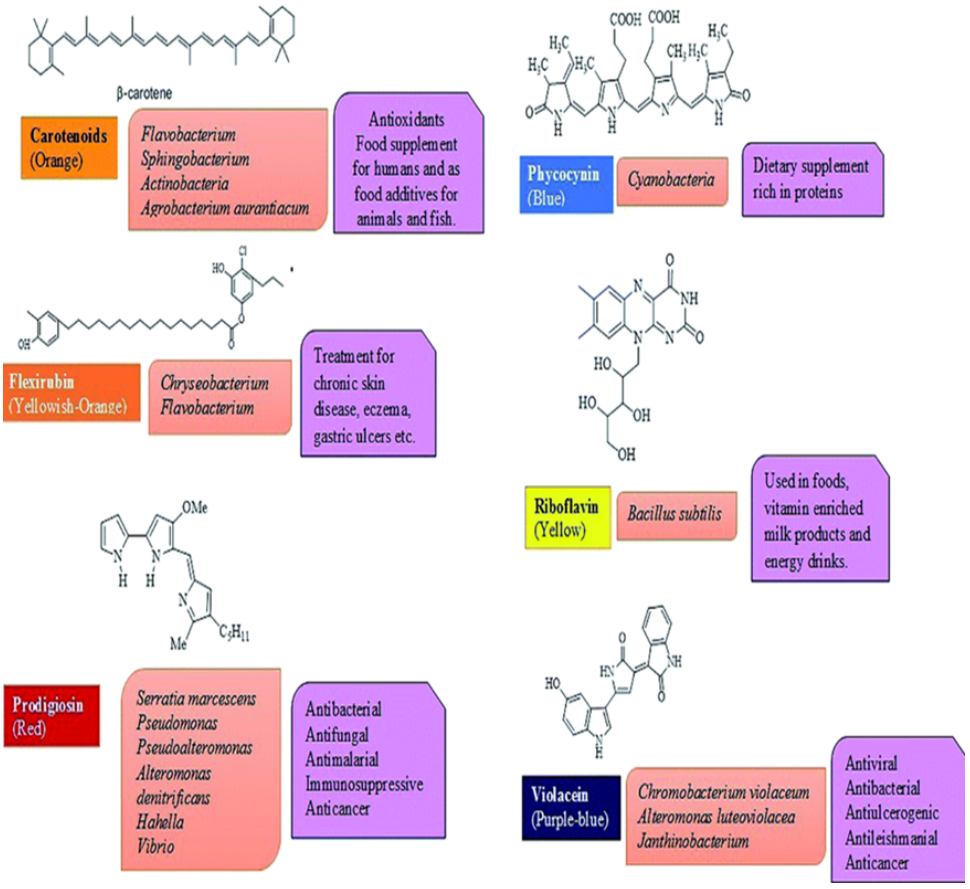

FIGURE 2.2: Microbial Pigments: Structures, Sources, and Applications in Health and Industry [78].

2.4.1 Indigodine

This powerful natural blue pigment is formed by the condensation of two L-glutamine molecules, showcasing its impressive potential in a variety of applications. This pigment is produced by bacteria, though its biosynthesis genes are often repressed, leading to reduced or absent production. Originally discovered in the 1960s in *Pseudomonas indigofera*, indigoidine has since been identified in various bacterial genera like *Arthrobacter*, *Erwinia*, *Corynebacterium*, *Photorhabdus*, *Streptomyces*, and more [81].

Despite its structural and biosynthetic characterization, the biological roles of indigoidine remain largely unexplored. Indigoidine is believed to help bacteria resist oxidative stress by acting as an antioxidant and protecting them from reactive oxygen species. Furthermore, it has shown antibacterial activity, especially against *Vibrio fischeri*. Recent studies also suggest that its production may give bacteria a competitive edge in their environment, potentially by suppressing other organisms or through intracellular signaling roles that influence cellular processes like motility. However, further research is needed to better understand the full range of its antibiotic properties [82].

2.4.2 Melanin

Melanin is a complex, polymeric pigment produced through fermentative oxidation by eukaryotes and microorganisms. In microbes, melanin is noted for its high molecular weight and stability, making it difficult to analyze due to its insolubility in most organic solvents, acids, and water [83]. Melanin has two main types: eumelanin, which is dark brown and sulfur-free, and pheomelanin, which is yellow or reddish and contains sulfur [84].

Bacteria such as *Bacillus*, *thuringiensis*, *Streptomyces*, *Rhizobium*, and *Pseudomonas aeruginosa* are known to produce melanin [85]. In particular, melanin plays a key role in protecting organisms from UV radiation by absorbing ultraviolet rays and

neutralizing reactive oxygen species, minimizing radiation damage. Its physicochemical properties also allow it to function as a drug carrier, semiconductor, cation exchanger, and absorber of X-rays and gamma rays [86]. Melanin's versatility extends to biomedical applications, where it is recognized for its photoprotective, antioxidant, anticancer, and heat-stable properties. Recently, melanin nanoparticles have been explored for therapeutic uses, such as in chemotherapy and photothermal treatments, positioning melanin as a promising candidate for future biotechnological and medical advancements [87].

Melanin biosynthesis in many fungi involves hydroxynaphthalene units from the acetate/malonate pathway, regulated by polyketide synthases (PKSs) and controlled by specific gene clusters [98].

Rao and colleagues [78] documented melanin production in a diverse range of microorganisms, including *Cryptococcus neoformans*, *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Paracoccidioides brasiliensis*, *Aspergillus fumigatus*, *Sporothrix schenckii*, as well as *Alteromonas nigrifaciens*, *Vibrio cholerae*, *Shewanella colwelliana*, and several Streptomyces species. Additionally, recent research highlighted the notable melanin production in bacteria isolated from marine sponges, indicating these microorganisms as promising candidates for industrial melanin production [99].

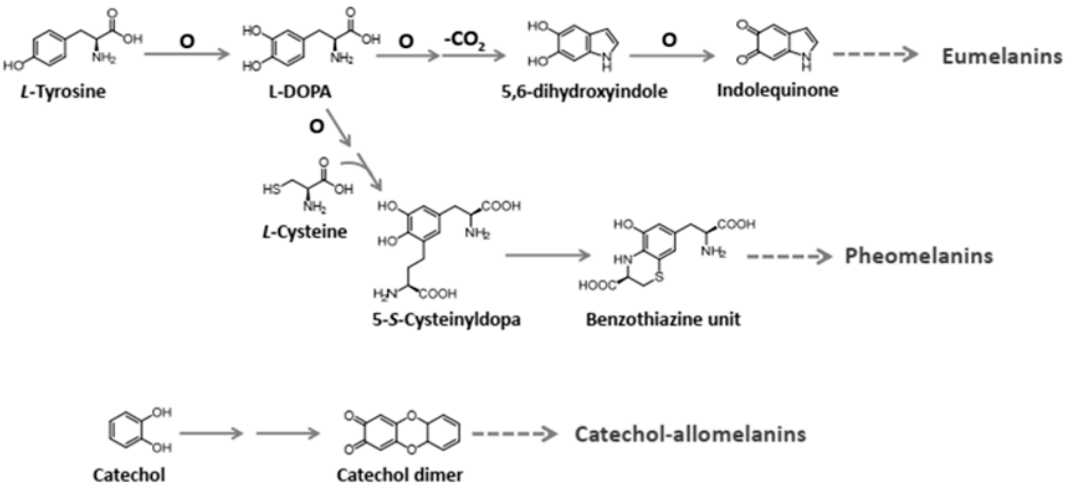

FIGURE 2.3: Biosynthetic Pathways of Melanins: Formation of Eumelanins, Pheomelanins, and Catechol-allomelanins from Precursors L-Tyrosine, L-Cysteine, and Catechol [99]

2.4.3 Carotenoids

Carotenoids are a diverse group of pigments known for their bright red, yellow, and orange colors. Produced by various organisms, including non-photosynthetic bacteria and higher plants, these pigments play vital roles in photosynthesis and environmental protection. These hydrophobic molecules are generally associated with photosynthetic membranes, where they are thought to be bound to pigment-protein complexes rather than moving freely within the membrane. Carotenoids are categorized into two groups: xanthophylls like canthaxanthin, zeaxanthin, and astaxanthin; and carotenes like -carotenes and β-carotenes [79]. Carotenes are particularly valued in the industry for their health benefits, such as enhancing the immune system, preventing cancer, and offering antioxidant properties. Carotenoids are used in nutritional supplements and cosmetics due to their antioxidant potential [80].

Over 700 carotenoid types have been identified, with β-carotene, canthaxanthin, astaxanthin, lycopene, zeaxanthin, and lutein being the most commercially significant. In bacteria, carotenoids help organisms adapt to extreme environments, such as areas with high radiation or low temperatures [77]. Given their wide distribution and numerous health benefits, carotenoids are an emerging field in biotechnology, with astaxanthins, canthaxanthins, and zeaxanthins standing out for their biomedical applications. For example, astaxanthin from Pontibacter korlensis AG6 has demonstrated antioxidant, antibacterial, and anticancer properties against breast cancer cells. New genetic engineering methods are being explored to enhance the production of these carotenoids using bacterial strains. Carotenoid biosynthesis starts with the enzyme phytoene synthase catalyzing the condensation of two geranyl pyrophosphate (GGPP) molecules to form phytoene, which is then converted to lycopene through desaturation and isomerization. Lycopene is cyclized into various carotenes and further modified by hydroxylases and ketolases to produce xanthophylls. GGPP is synthesized from C5 precursors via either the mevalonic acid (MVA) pathway in archaea, fungi, and many bacteria, or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in photosynthetic organisms [100, 101].

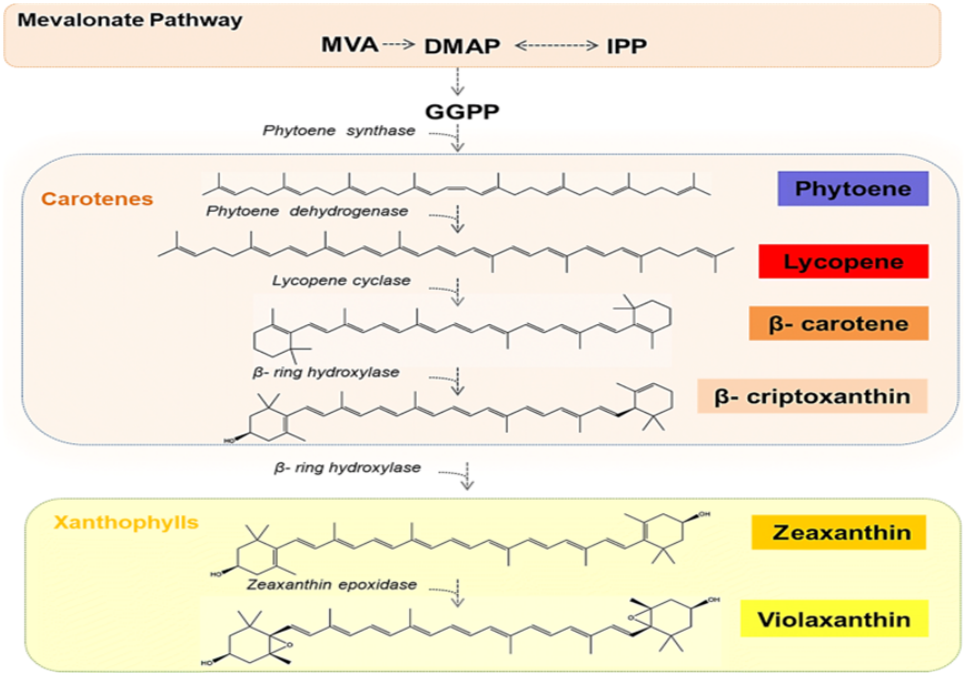

FIGURE 2.4: Schematic representation of biosynthesis of carotenoids using the Mevalonate pathway [100, 101]

Many microorganisms synthesize carotenoids, often in response to stressful environmental conditions, though not all are of industrial significance. Bacteria are particularly advantageous for carotenoid production due to their short life cycles, metabolic flexibility, and ease of cultivation.

They can also be genetically engineered to enhance production [102]. Microbial carotenoids such as torularhodin and torulene are produced in large quantities by several genera of microorganisms [103, 104].

TABLE 2.4: Microorganisms known for carotenoid production and their specific species [105, 106]

Microorganism Type	**Specie**	**Carotenoid Production**
Bacteria	*Nostoc commune*	Cyanobacterium producing diverse carotenoids
	Rhodococcus maris	Known for pigment synthesis
	Micrococcus roseus	Produces pink to red carotenoids
	Mycobacterium sp. *LEMMJ01*	Carotenoid-producing bacterial strain
	Bradyrhizobium sp.	Contributes to carotenoid biosynthesis

Table 2.4 continued from previous page

Microorganism Type	Specie	Carotenoid Production
	Anabaena variabilis	Cyanobacterium with photosynthetic pigments
	Halobacterium salinarum	Produces bacterioruberin and other pigments
	Paracoccus sp.	Known for carotenoid diversity
	Mycobacterium kansasii	Associated with carotenoid synthesis
	Pseudomonas putida	Produces various carotenoids
Microalgae	*Haematococcus pluvialis*	Rich in astaxanthin production
	Dunaliella salina	High β-carotene content
Yeast	*Phaffia rhodozyma*	Produces astaxanthin
	Rhodotorula glutinis	Known for its red pigments
Filamentous Fungi	*Mucor circinelloides*	Produces β-carotene and other pigments

2.4.4 Alkaloid Pigments

Some microbial pigments are categorized as alkaloids, which are low molecular weight compounds containing nitrogen derived from amino acids [107]. Pigment groups such as prodigiosines, tambjamines, and betalains fall into this alkaloid category. In microorganisms, these pigments, often secondary metabolites, are usually released into the environment and can function as reserve substances. Research on polyketide-derived alkaloids, such as citrinin, shows that these compounds are produced and degraded during the growth of the fungus *P. citrinum* [108].

2.4.4.1 Prodigiosins and Tamjamines

Prodigiosines are hydrophobic red pigments synthesized by *actinomycetes*, *serratia* species, and certain marine bacteria, with prodigiosin being the most prominent,

particularly from *Serratia marcescens*. These pigments occur in linear forms like prodigiosin and undecylprodigiosin, and in cyclic structures such as cyclononylprodigiosin and butyl-meta-cycloheptylprodiginine [109, 110].

Tambjamines are yellow pigments similar to prodiginines, but they have a bipyrrole core linked to a primary amine. Tambjamine A and C are examples found in bacteria like *Pseudoalteromonas* [111–113].

Prodigiosin, a vibrant red pigment from the prodiginine family, first identified in *Serratia marcescens*, a Gram-negative bacterium. It is produced during the stationary phase through the condensation of 2-octenal and proline into a linear tripyrrole structure and is also present in smaller amounts in genera like *Janthinobacterium*, *Streptomyces*, and *Vibrio* [103].

From a biomedical perspective, prodigiosin shows significant potential due to its broad range of pharmaceutical properties. It exhibits antimicrobial, algicidal, anti-inflammatory, anticancer, antimalarial, and antidiabetic activities, and can also modulate the immune system. Notably, it induces apoptosis in cancer cells by disrupting pH regulation, oxidative phosphorylation, and mitochondrial function [114].

Prodigiosin is also effective against various bacteria, including Gram-positive species like *S. aureus* and *B. subtilis*, as well as Gram-negative bacteria such as *Salmonella enterica* and *E. coli* [79].

Despite its promising properties, the precise antibacterial mechanisms of prodigiosin remain unknown, although it has been demonstrate to act as a bacteriostatic agent in *Escherichia coli* by influencing multiple physiological processes. Efforts are being made to optimize production methodologies for prodigiosin and to develop drug delivery systems through encapsulation techniques. Its potential in developing new treatments and pharmaceuticals continues to attract interest from microbiologists and clinicians [115].

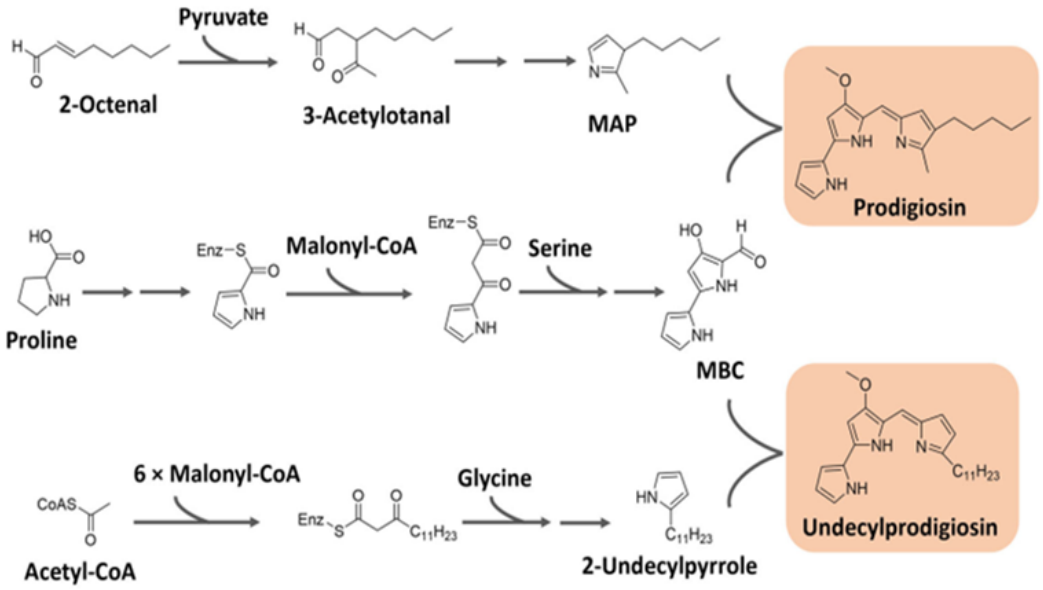

FIGURE 2.5: Biosynthetic pathways for the production of prodigiosin and undecylprodigiosin from primary precursors [109]

Prodiginines are synthesized through a bifurcated pathway involving the condensation of 4-methoxy-2, 2'-bipyrrole-5-carbaldehyde (MBC) with either 2-methyl-3-pentylpyrrole (MAP) or 2-undecylpyrrole. MBC is formed from proline, serine, and malonyl-CoA, while MAP and 2-undecylpyrrole are derived from 2-octenal and pyruvate, or acetyl-CoA and glycine, respectively [109]. Gene clusters such as the Pig cluster in Serratia and the Red cluster in Streptomyces regulate this pigment production [116].

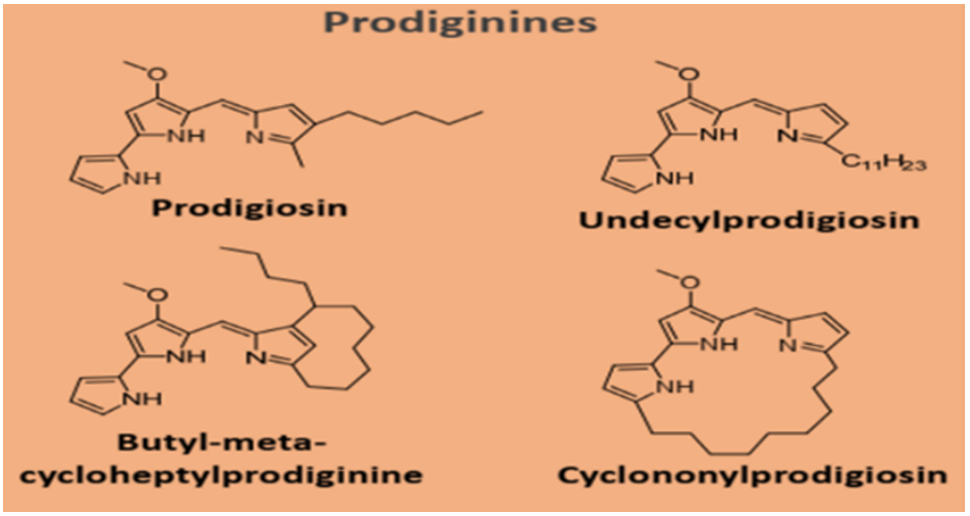

FIGURE 2.6: Different structures of prodigiosin [109]

Kurbanoglu et al. [117] noted that while Serratia marcescens is the main producer of prodigiosin, this red pigment with a linear tripyrrole structure is also synthesized by various other bacteria, such as Hahella chejuensi, Streptomyces sp. and Vibrio species. Prodigiosin exhibits a wide range of bioactive properties, including antibacterial, antifungal, and anticancer activities [118].

2.4.4.2 Betalains

These water-soluble pigments contain betalamic acid as their chromophore and are classified as yellow betaxanthins or red violet betacyanins based on light absorption. Betacyanins, which absorb at 535–540 nm, are formed from betalamic acid and cyclo-DOPA, while betaxanthins, absorbing at 460–480 nm, arise from reactions between betalamic acid and various amines [119, 120]. These pigments are rare, mainly found in Caryophyllales plants and certain fungi like Amanita muscaria, which produces pigments such as Muscapurpurin, Muscaurin I, and Vulgaxanthine I. [121].

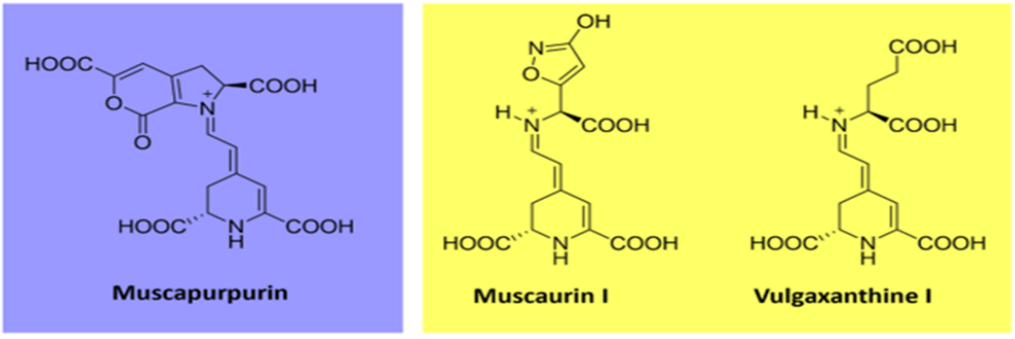

FIGURE 2.7: Molecular structures of Musca purpurin, Muscaurin I, and vulgaxanthine I pigments derived from *Amanita muscaria* [121]

Betalains are derived from the aromatic amino acid tyrosine, which is produced via the shikimate pathway. Tyrosine is first converted to L-DOPA through hydroxylation. The enzyme 4,5-dioxygenase then transforms L-DOPA into betalamic acid. This betalamic acid can interact with amino acids or other amine-containing compounds, resulting in the formation of betaxanthins. On the other hand, betacyanin are created when betalamic acid reacts with cyclo-DOPA, a substance

formed from L-DOPA through oxidation and cyclization. As a result, betanidin forms and undergoes glycosylation to form a variety of betacyanin [122].

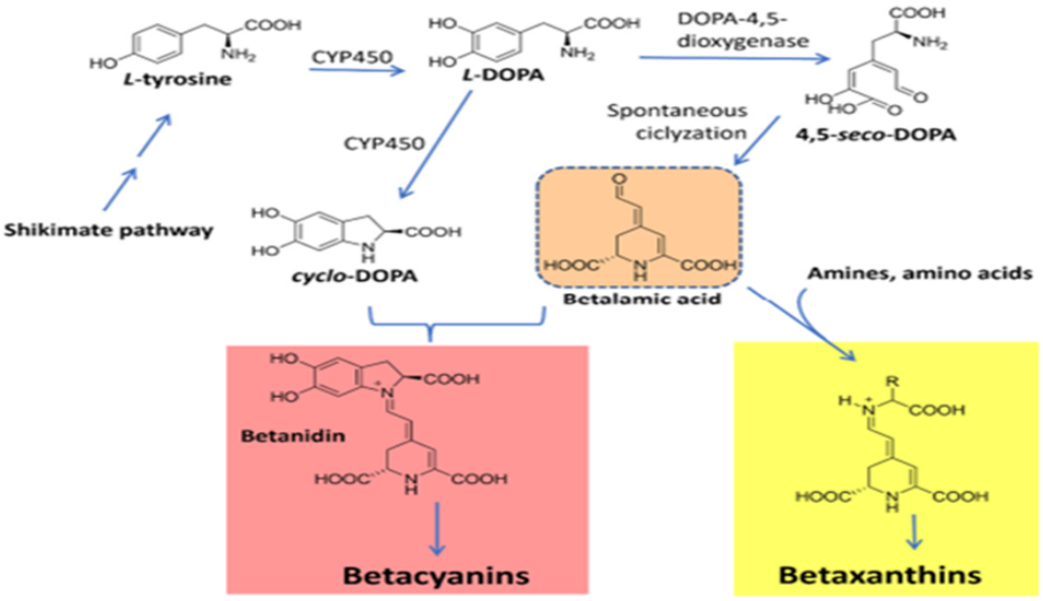

FIGURE 2.8: Biosynthetic pathway for the production of betalains, including betacyanins and betaxanthins, from L-tyrosine via betalamic acid [122]

2.4.4.3 Violacein

Violacein is a purple indolocarbazole dye with a molecular mass of 343.341 g/mol, formed from two tryptophan molecules. It is insoluble in water but soluble in alcohols, DMSO, ethyl acetate, and partially in dioxane. Found mainly in aquatic and cold environments, it is primarily produced by heterotrophic species, particularly *Chromobacterium violaceum*. Other genera like *Janthinobacterium*, *Alteromonas*, and *Pseudoalteromonas* also produce this pigment [77].

First identified in 1942, violacein was found to inhibit soil amoebas from ingesting bacteria, suggesting its role in bacterial defense. Its intracellular production may serve as a defense mechanism, with the pigment's vesicles potentially damaging nearby membranes. Recent studies have linked violacein to a lethal effect on the amphibian fungus *Batrachochytrium dendrobatidis*. In addition to its antibacterial, antiparasitic, antiviral, and antitumor properties, violacein has been evaluated for its genotoxic effects in cell lines. In the past five years, new strains from

genera such as *Pseudoalteromonas*, *Iodobacter*, *Janthinobacterium*, and the newly identified *Massilia* have been discovered as violacein producers. The pigment has shown promise in cancer treatment, inducing apoptosis in MCF-7 breast cancer cells and being proposed as a therapeutic agent for leukemia [78].

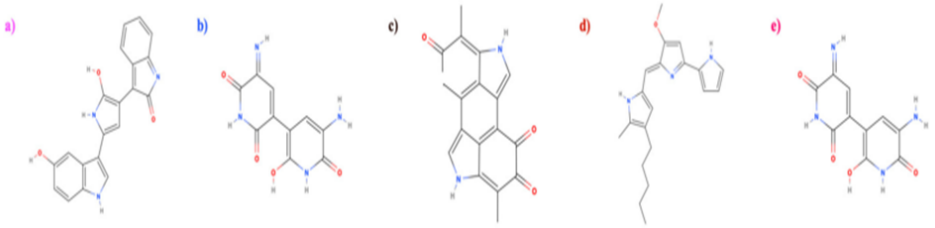

FIGURE 2.9: Bacterial pigments molecular structures Violacein, Indigoidine, Melanin, Prodigiosin and Rhodopsin [123]

2.4.4.4 Other Alkaloid Pigments

Phenazines, are a group of aromatic N-heterocyclic pigments produced by several bacterial species, such as *Burkholderia*, *Nocardia*, *Pseudomonas*, *Streptomyces*, and *Vibrio* [124]. These pigments can display a variety of colors, such as blue, yellow, green, purple, brown and red. They are synthesized through the shikimate pathway. A well-known example is pyocyanin, a blue pigment produced by *Pseudomonas aeruginosa* [125].

2.4.5 Rhodopsin

Rhodopsin is an essential visual pigment in the photoreceptor cells of retinal rods, allowing vertebrates to see in low light. Initially thought to exist only in higher organisms, research in the 1980s found a similar pigment called halorhodopsin in Archaea, specifically *Halobacterium halobium*, which acts as a light-driven sodium pump. Subsequently, scientists identified three more types of microbial rhodopsins in *H. halobium*, known as bacteriorhodopsins. These rhodopsins work in conjunction with halorhodopsin to facilitate the movement of ions across membranes using light as the energy source [92]. Recent research has revealed a variety of rhodopsins in different microorganisms, which perform diverse functions ranging

from ion flux mediation to photo mobility and phototaxis. These rhodopsins are valuable models for studying active membrane transport and signaling mechanisms. Bacteriorhodopsins, in particular, can influence cellular behavior through their luminescence-induced proton exchange capabilities, offering potential applications in manipulating animal cell growth, metabolism, and differentiation. Additionally, this technology is being explored for reprogramming human fibroblasts into neural cells, to enhance neural regeneration. These developments represent some of the early biomedical applications of bacteriorhodopsins [93].

2.4.6 Pyocyanin and Pyoverdines

The genus *Pseudomonas* is known for producing a variety of pigments that are crucial for its pathogenicity. Notable among these are pyocyanins and pyoverdines, both produced by *Pseudomonas aeruginosa*. Pyocyanin, a greenish-blue phenazine derivative, is involved in iron metabolism by reducing iron and releasing it from transferrins. It also exhibits pro-inflammatory effects and generates free radicals, contributing to cellular damage and pathogenicity [126, 127]. Conversely, pyoverdine is a bioluminescent greenish-yellow siderophore with a strong affinity for Fe(III). It extracts iron from the environment and the host's iron transport proteins, which is essential for bacterial iron acquisition and pathogenesis. High levels of pyoverdine are found in the sputum of cystic fibrosis patients [128–130]. Research has primarily focused on inhibiting these pigments to manage *Pseudomonas* infections, particularly in immunocompromised patients or those with cystic fibrosis, where treatment is challenging. While studies have mainly addressed their roles as virulence factors, recent research has identified additional potential biological activities for pyocyanin, including antibiotic properties and cytotoxicity against pancreatic cancer cells, though its toxicity poses concerns [131].

2.4.7 Flavin Pigments

Flavins are yellow pigments found in plants and most microorganisms. They have a pteridine structure and an isoalloxazine ring. The main water-soluble flavin is

riboflavin, or vitamin B2, which converts into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both vital for bodily functions. Roseoflavin is an antimicrobial pigment produced by certain Streptomyces species. Another type, 5-deazaflavins, found in Archaea, has carbon instead of nitrogen at position five. [132, 133].

FIGURE 2.10: Structures of different types of flavins [132]

Riboflavin is produced from guanosine triphosphate (GTP) and ribulose - 5 - phosphate (Ru5P), where GTP provides the pyrimidine component and nitrogen atoms, while Ru5P supplies the carbon for the isoalloxazine ring. Riboflavin synthase then catalyzes the final step, converting the intermediate 6, 7 - dimethyl - 8 - ribityllumazine (DrL) through a dismutation reaction [134]. In contrast, deazaflavins are synthesized from diaminouracil and tyrosine, using 50-deoxyadenosyl radicals to form their unique heterocyclic structure [135, 136].

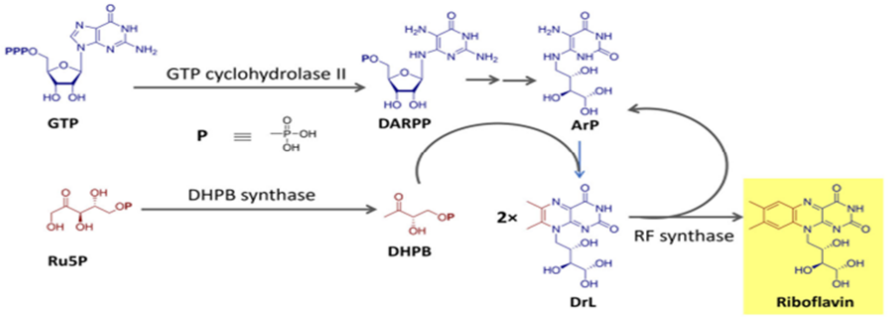

FIGURE 2.11: Biosynthetic pathway for riboflavin production from GTP and Ru5P through intermediates DARPP, ArP, and DHPB [137]

2.4.8 Tetrapyrrole-Containing Pigments

Tetrapyrroles are essential in microorganisms for light absorption, electron transfer, and protecting cells from oxidative stress by detoxifying reactive oxygen species

(ROS) [138]. They are crucial in photosynthetic organisms for managing oxidative stress and are structurally defined by four interconnected pyrrole rings. These pigments form the heme group found in hemoglobin and cytochromes, and are also key components of chlorophyll and bilins. [139, 140].

Tetrapyrrole biosynthesis starts with δ-aminolevulinic acid (ALA), which is synthesized from glycine and succinyl CoA in some bacteria, fungi, and animals, or from -ketoglutarate in algae, archaea, most bacteria and plants. Two ALA molecules combine to form porphobilinogen (PBG), a pyrrole-containing compound, which is then further condensed by porphobilinogen deaminase to produce the tetrapyrrole ring known as uroporphyrinogen III [139]. This ring undergoes further modifications and metal ion complexation to produce various tetrapyrrole-containing compounds, including heme, chlorophyll, and coenzyme B12 as depicted in Figure 2.12.

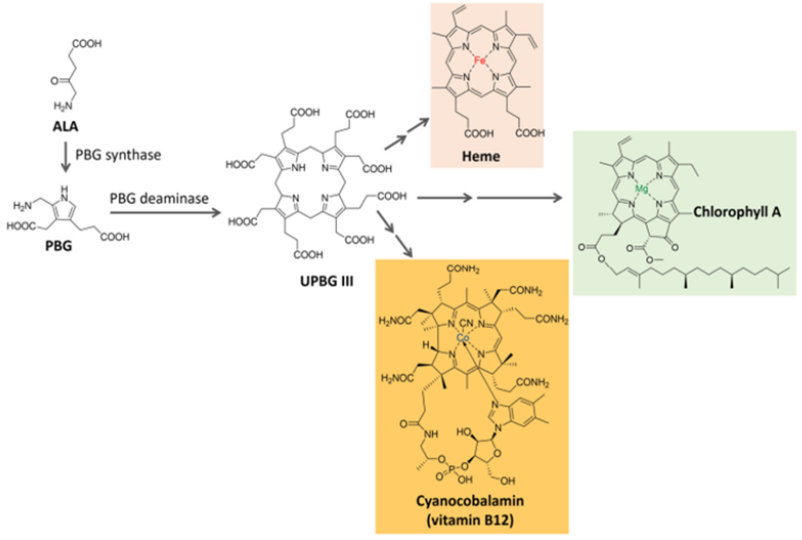

FIGURE 2.12: Tetrapyrrole macrocyclic compounds biosynthetic pathways representation [139]

Chlorophylls, essential for photosynthesis, are the most widespread pigments on Earth, capturing light to convert it into chemical energy. Tetrapyrrolic macrocycles, commonly incorporating magnesium and typically esterified at the C-17 position, predominantly absorb light in the violet-blue and yellow-orange/red regions of the spectrum. Present in plants, algae, and cyanobacteria, chlorophylls (a-d) collaborate with carotenoids and proteins within light-harvesting complexes.

The variations in their pyrrolic macrocycle structure significantly impact their light absorption characteristics [141, 142].

Bacteriochlorophylls, which are related to chlorophylls, are present in anoxygenic photosynthetic bacteria. These pigments often exhibit absorption bands shifted compared to chlorophylls, extending their light absorption range beyond 700 nm and below 400 nm. Bacteriochlorophyll a, for instance, is a widely distributed pigment in photosynthetic bacteria and is known for its purple coloration [143, 144].

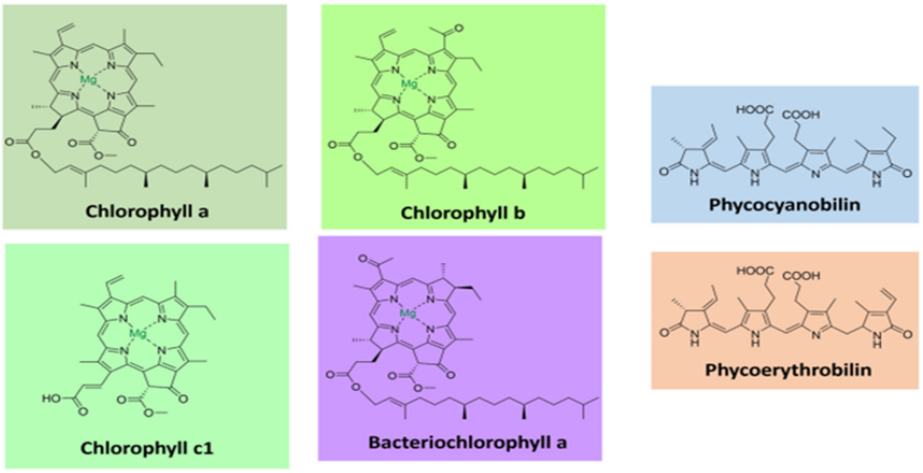

FIGURE 2.13: Molecular structures of different tetrapyrrole containing pigments [142]

PBPs are categorized into three main types based on their color: phycocyanin (blue), allophycocyanin (Bluish-green) and phycoerythrin (red). Due to their high absorption capacity, PBPs are commonly used as fluorescent markers in techniques such as microscopy and flow cytometry [145].

2.4.9 Polyketide Pigments

Polyketides are diverse compounds created by the sequential condensation of malonyl-CoA derivatives via the acetate/malonate pathway, facilitated by polyketide synthases. Several microbial pigments in this category are discussed below [146].

2.4.9.1 Quinones

Certain natural pigments are derived from a quinone structure, which is a fully conjugated cyclic dione, and they vary in color from yellow to red. Examples include Nigrodiquinone A from *Nigrosproa* sp. (yellow), Aspergiolide A from *Aspergillus* sp. (red), arpink red from *Penicillium oxalicum* (red), and bikaverin from *Fusarium* sp. (red) [147].

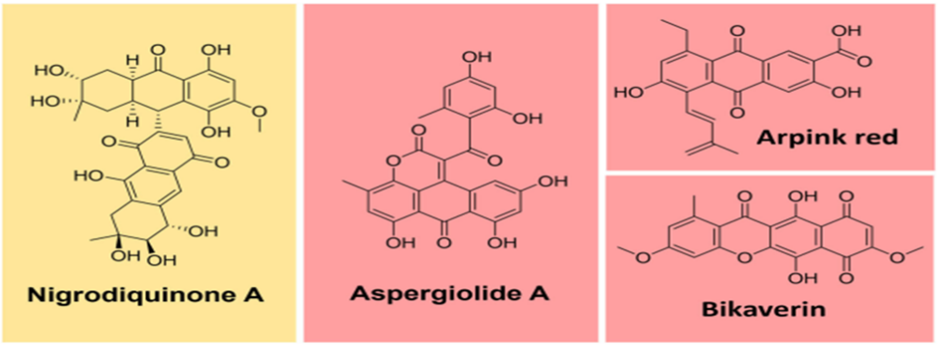

FIGURE 2.14: Molecular structures of quinone pigments [147]

Quinones are produced by oxidizing suitable phenolic compounds.They can be synthesized through either the acetate/mevalonate pathway or the shikimate pathway using phenolic precursors. The acetate/mevalonate pathway involves the condensation of acetyl-CoA and malonyl-CoA to form a polyketide chain, which then undergoes specific folding and cyclization, as illustrated in Figure 2.15 [148].

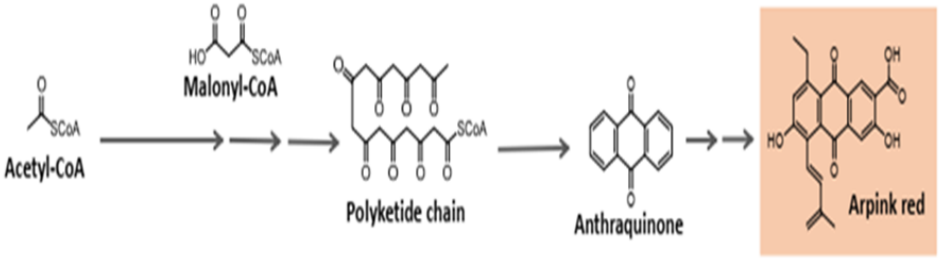

FIGURE 2.15: Illustration of the biosynthetic pathway leading to the production of Arpink red, starting from acetyl-CoA and malonyl-CoA to form a polyketide chain, followed by conversion into anthraquinone and its subsequent transformation into the final pigment [148]

2.4.9.2 Azaphilones

Azaphilone pigments, produced by various fungal genera such as *Aspergillus* and *Monascus* feature a distinctive bicyclic chromophore structure. Six key pigments are recognized within this group: Monascin and Ankaflavin -These yellow pigments have λmax values ranging from 330 to 450 nm. Rubropunctatin and Monascorubrin -These are orange pigments with λmax values between 460 and 480 nm. Monascorubramine and Rubropunctamine- These red pigments display λmax values from 490 to 530 nm [149].

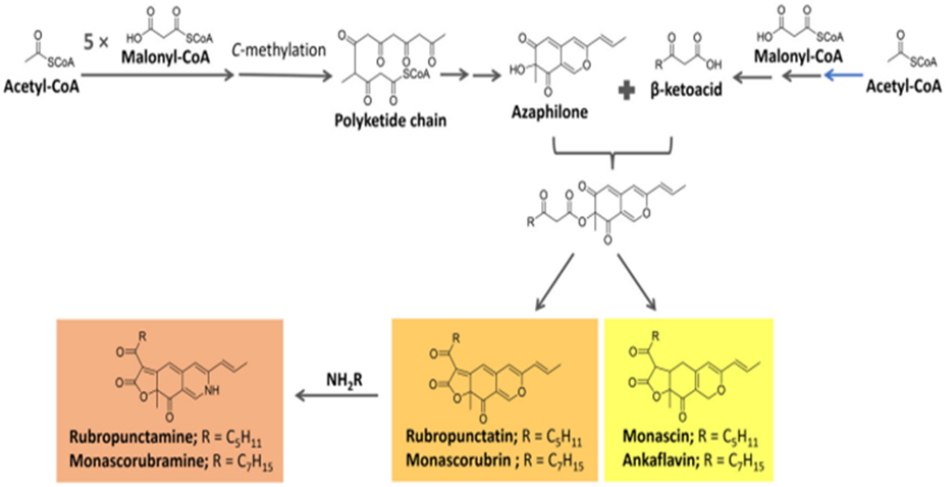

FIGURE 2.16: Biosynthetic pathway of azaphilone derivatives, leading to pigments like rubropunctamine, monascorubramine, monascin, and ankaflavin [150]

Azaphilone pigments are synthesized from acetate/malonate pathway precursors, with polyketide synthase (PKS) and fatty acid synthase (FAS) enzymes playing key roles. Their formation involves esterification of a polyketide-derived chromophore with a -ketoacid. An aminophilic reaction between orange pigments and primary amines produces red compounds by replacing oxygen in the heterocyclic ring with nitrogen. Five main biosynthetic pathways, regulated by specific genes and enzymes, govern azaphilone production. In Monascus species, seven gene clusters related to this process have been identified [151].

2.4.10 Phenol Pigments

Styrylpyrones are yellow polyphenolic pigments primarily found in fungi of the *Hymenochaetaceae* family, such as *Phellinus* and *Inonotus*, as well as in some plants like *Lauraceae* and *Ranunculaceae* [152, 153]. The first styrylpyrone, hispidin (compound), discovered in 1889, is a precursor to fungal luciferin in bioluminescent fungi and is found in various fungal fruiting bodies [154].

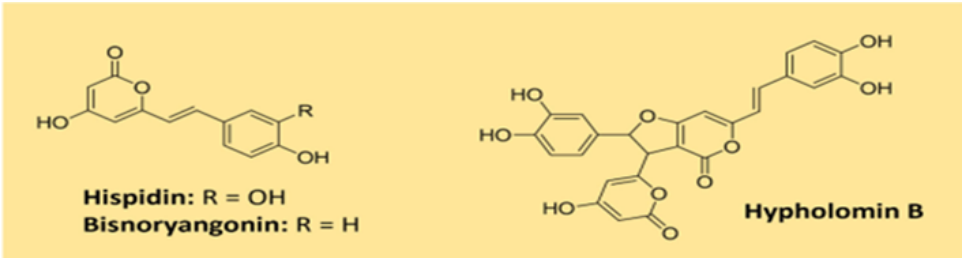

FIGURE 2.17: Structure of fungal Styryl pyrone pigments [152, 153]

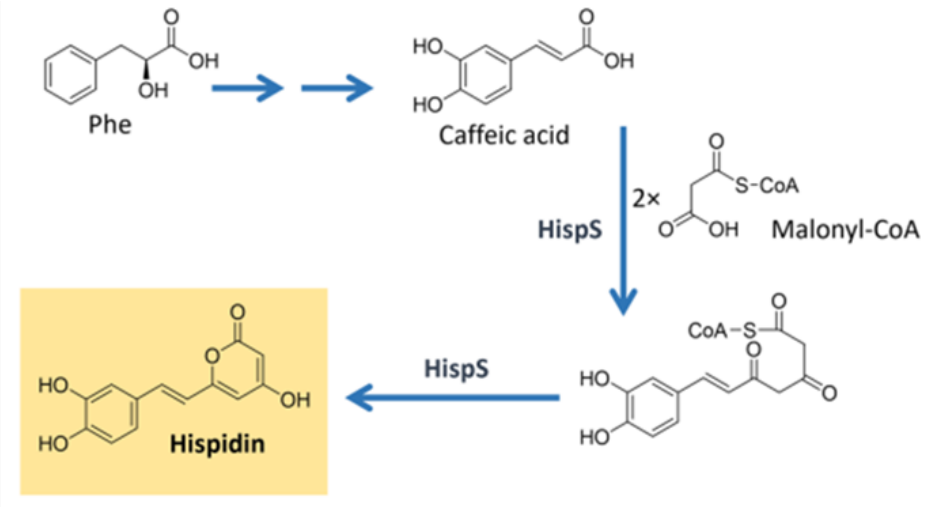

FIGURE 2.18: Biosynthesis of hispidin from phenylalanine via caffeic acid and malonyl-CoA intermediates [154]

The Styrylpyrone structure is created from phenylpropanoid units through the shikimate pathway and malonate units via the acetate/malonate pathway, with the aid of type I polyketide synthases. More complex styrylpyrones, such as dimeric and polymeric forms, are probably produced by the oxidative coupling of these monomeric units [154].

Polyphenolic pigments extracted from genetically modified microorganisms demonstrate significant antioxidant properties. Flavonoids, anthocyanin, nagirenim are well known examples produced in *Saccharomyces cerevisiae* through metabolic engineering [155], as well as curcuminoids.

2.5 Industrial Applications Microbial Pigments

Microbial pigments are widely used in different industries. Their applications are as follows.

2.5.1 Pharmaceutical Industry

These industries utilize pigments from various sources, such as microorganisms, algae, and plants, due to their bioactive properties. Many pigments, with their antibacterial and antifungal effects, are valuable for use in pharmaceutical and cosmetic products, helping to increase shelf life and improve product safety. Some examples of pigments with antimicrobial properties include prodiginines, which target viruses; pyocyanin, which is effective against bacteria; and violacein, which combats fungi. Microbial pigments like melanin, prodigiosin, and violacein have shown potential in providing anticancer, anti-inflammatory, and antioxidant benefits [155–157].

2.5.1.1 Anti-cancer Effects

Prodigiosin, a compound derived from Serratia marcescens, possesses notable bioactive properties and shows promise as a potential drug candidate for treating cancer and neurodegenerative diseases. It has been tested against various tumor cell lines, including those associated with pancreatic cancer, epithelial carcinoma, breast cancer, and lung cancer, yielding encouraging results. Conversely, violacein, extracted from *Chromobacterium violaceum*, has also been recognized as an antitumor agent. It is capable of inducing cell death in osteosarcoma and

rhabdomyosarcoma cells. In addition to its antitumor effects, violacein provides anti-inflammatory benefits by promoting the release of interleukins IL-4 and IL-10 [158]. Pyocyanin, produced by *Pseudomonas aeruginosa* inhibits the growth of cancer cell lines by producing reactive oxygen species [159].

2.5.1.2 Antiviral Activity

A class of prodigiosin produced by *Serratia marcescens*, shows antiviral activity against herpes simplex virus. In vitro studies have shown that violacein inhibits HIV-1 reverse transcriptase by 94.3% at a concentration of 1 mM and reduces the binding between the SARS-CoV-2 spike protein and ACE2 receptor by 53% at 2 mM. It also shows antiviral activity against polio virus, and simian rotavirus SA11 by minimizing viral replication efficiency [160].

2.5.1.3 Antifungal Activity

Violacein has antifungal activity comparable to fungicides like bavistin and drugs such as amphotericin B, used to treat conditions like candidiasis and mucormycosis. *Bacillus gibsonii* produced a yellow-colored pigment posing antifungal activity and can be used as antifungal ointment. Pyocyanin produced by *Pseudomonas aeruginosa* shows antifungal activity against different fungal species such as *Candida albicans*, *Candida glabarata*, *Cryptococccus neoformans*, and *Fusarium oxysporum* [161].

2.5.1.4 Antimicrobial Activity

Recent studies have also identified pigments such as rubropunctamine, sclerotiorin, and bostrycoidin from *Talaromyces verruculosus*, *Penicillium multicolor*, and *Fusarium solani*, respectively. These pigments possess antimicrobial and antioxidant activities against both Gram-negative and Gram-positive bacteria. Additionally, pigments like those from *Penicillium mallochi* have shown antibiofilm properties, expanding the therapeutic potential of natural pigments. *Bacillus*

cereus strain called cerein produced a green pigment that shows bactericidal properties against other *Bacillus cereus* strains. Pyocyanin also possesses antibacterial properties against gram negative and positive bacteria [162]. A new species of *Micrococcus*, isolated from the Persian Gulf, produced a yellow pigment with antibacterial properties, although the pigment's identity was not determined in the study. This pigment was effective against *S. aureus*, *P. aeruginosa*, and *E. coli*. Additionally, in the marine environment, a melanin-producing strain of *Streptomyces* showed the ability to inhibit the growth of other bacterial species, including *Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* [163].

2.5.1.5 Antiparasitic Activity

Research on psychrotolerant bacteria from Antarctica has uncovered bioactive pigments from *Pseudomonas* sp. with antiparasitic properties, highlighting the potential of psychrophiles [164]. Pigments like violacein and prodigiosin are gaining attention for their antimicrobial and antiparasitic activities, with ongoing efforts to scale up production for medical and agricultural applications. Many prodigiosin and their synthetic derivatives are also potent anti-protozoan compounds [165].

2.5.2 Cosmetic Industry

Microalgae and cyanobacteria are valuable sources of pigments like carotenoids, phycocyanins, and chlorophylls, which produce a range of colors, such as yellow, orange, red, green, and blue. These pigments are widely used in product development, particularly in cosmetics [166]. Various bacterial and fungal species are also known for producing melanin, a pigment with strong UV protection properties. This makes melanin an ideal ingredient in skin care products, especially sunscreens. When microbial melanin is extracted and incorporated into cosmetic formulations, it offers significant photoprotective benefits for safe human use. Studies have revealed that pigments like rubropunctamine and monascin, derived from the fungus *Monascus purpureus*, can enhance sunscreen efficacy, increasing photoprotection

by 36.5% and 13%, respectively [167]. Melanin extracted from fungi has been tested as a sunscreen additive, yielding positive results in boosting sun protection, highlighting the potential of microbial pigments in enhancing Sun protection factor performance [168].

Microbial pigments acquire a wide range of bioactive properties, making them promising candidates for use in the cosmetics industry. Potential applications include sunscreens, makeup, anti-aging products, skin lightening treatments, as well as permanent dyes for tattoos. Despite their potential, these applications are not yet fully explored by the industry [169]. Zoz et al. [170] highlighted that some microbial pigments, such as the lycopene carotenoid pigment, have pro-vitamin A activity and could serve as natural color additives for both food and cosmetic products due to their red hue.

Fungal melanin, in particular, shows great potential for sunscreen formulations because of its sustainability and scalability. A study by Jeong-Joo Oh [171] and colleagues investigated melanin derived from *Amorphotheca resinae* and found that it exhibited superior antioxidant activity compared to conventional antioxidants. The study also showed that the pigment did not exhibit cytotoxic effects on human keratinocytes, suggesting that fungal melanin could be used as a safe, broad-spectrum sunscreen ingredient. This research indicates the potential for fungal melanin to replace or reduce the need for synthetic sunscreens in cosmetic products [172].

2.5.3 Food Production Industry

Microbial pigments have numerous applications in the food industry. Natural pigments, such as β-carotene and astaxanthin produced by microbes (Table 2.5) are utilized in various food industrial applications. Several microbial pigments, such as riboflavin from *Bacillus subtilis*, β-carotene and lycopene from *Blakeslea trispora*, and astaxanthin and canthaxanthin from *Haematococcus lacustris*, are commercially produced worldwide. Astaxanthin, a red pigment from Haematococcus pluvialis, is commonly used to enhance the coloration of salmon, shrimp,

and egg yolks, and is included in animal feeds like "Red Meat." Carotenoids, including astaxanthin, are vital in fish diets, improving pigmentation in skin, flesh, and eggs, as shown in studies on koi carp. Astaxanthin also enhances color and oxidative stability in pork and supports faster growth in animals like trout. Additionally, microbial canthaxanthin, produced by bacteria such as *Bradyrhizobium* and *Lactobacillus pluvalis*, is used as a feed additive for poultry and salmon [173].

Chlorophylls from microalgae like *Tetraselmis*, *Chlo*rella, and *Arthospira* are commonly used as green colorants in the food industry [174], while *Blakeslea trispora*, a fungus producing β-carotene, is approved for food use in the European Union [175]. Microencapsulation and nanoencapsulation technologies are used to improve the stability of microbial pigments. These methods protect pigments from environmental factors like heat, light, and oxygen, extend their shelf life, and improve solubility, especially for hydrophobic pigments like carotenoids. Encapsulated pigments are already used in products like yogurts, cakes and soft drinks [176, 177].

2.5.4 Textile Industry

The use of microbial pigments for fabric dyeing is still uncommon and underexplored, but recent studies have shown promising results. The process typically involves preparing a dye solution with color-fixing additives, followed by hot dyeing (60–80^{o}C), washing, and drying [178]. One study successfully used the violacein pigment from *Chromobacterium violaceum* to dye cotton and silk, achieving stable violet/purple coloration. Additionally, dark green pigment from *Sclerotinia fungus* was used to dye cotton fabric, showing color durability after washing and sun exposure [179, 180].

Bisht et al. [181] examined antimicrobial pigments for their application in bioactive textiles. They successfully applied a red pigment extract from *Rhodonellum psychrophilum* to cotton, silk, and rayon fabrics, although the color did fade over time. Additionally, a red pigment sourced from *Talaromyces albobiverticillius*, derived from the fermentation of agro-industrial waste, was applied to cotton and

demonstrated both antimicrobial and antioxidant properties, highlighting its potential for textile applications.

Fungi like *Scytalidium cuboideum* and *S. ganodermophthorum* produce red and yellow pigments suitable for dyeing cotton, nylon, and polyester fabrics, while *Cuvularia lunata* offers melanin for silk and wool, and various fungi provide red pigments for leather. *Talaromyces verruculosus* produces a red pigment that was successfully used to dye cotton, showing good color stability and safety for human use. *Nigrospora aurantiaca* produces bostrycin, a red pigment that dyes various fabrics with varying shades and shows some color loss after washing.

Additionally, microalgae-derived chlorophylls from *Caulerpa lentillifera* are being explored for silk dyeing, though challenges such as cost and process improvements remain. Despite these challenges, microbial pigments present a promising and innovative opportunity for more sustainable textile dyeing solutions [182].

TABLE 2.5: Pigment producing bacteria with colors that can be used in industrial applications [183]

Compound	**Color**	**Pigment producing bacteria**	**Industrial Applications**
Astaxanthin	Pink-red	*Agrobacterium aurantiacum, Paracoccus carotinifaciens, Xanthophyllomyces dendrorhous*	Antioxidants in dietary supplements, aquaculture feed, and cosmetics
Canthaxanthin	Dark-red	*Bradyrhizobium species, Haloferax alexandrines*	Food and feed coloring, cosmetics
Zeaxanthin	Yellow	*Flavobacterium sp. Paracoccus zeaxanthinifaciens*	Nutraceuticals, vision health supplements
	Creamy	*Achromobacter*	Food additives and pharmaceuticals
	Brown	*Bacillus*	Food coloring, textiles
	Orange-Yellow	*Brevibacterium species*	Functional food additives, poultry feed coloring

Table 2.5 continued from previous page

Compound	**Color**	**Pigment producing bacteria**	**Industrial Applications**
	Greyish to Creamish	*Corynebacterium michiganense*	Food and industrial dyes
Indigoiodine	Blue	*Corynebacterium insidiosum*	Textile and fabric dyeing, biological stains
Prodigiosin	Red	*Serratia marcescens, Serratia rubidaea*	Pharmaceuticals (anticancer, immunosuppressive agents), textiles
	Bluish-Red	*Rugamonas rubra, Streptoverticillum rubrireticuli, Vibrio gaogenes, Alteromonas rubra, Rhodococcus maris*	Cosmetics, industrial dyes
Staphyloxanthin, Zeaxanthin	Golden Yellow	*Staphylococcus aureus*	Antioxidant additives, food colorants
Violacein	Purple	*Chromobacterium violaceum, Janthinobacterium lividum*	Antibacterial and anticancer agents, cosmetics
Pyocyanin	Blue-Green	*Pseudomonas aureginosa*	Biomedical research, biosensors
Xanthomonoadin	Yellow	*Xanthomonas oryzae*	Food dyes and Industrial coatings

Chapter 3

Methodology

3.1 Methodology Chart

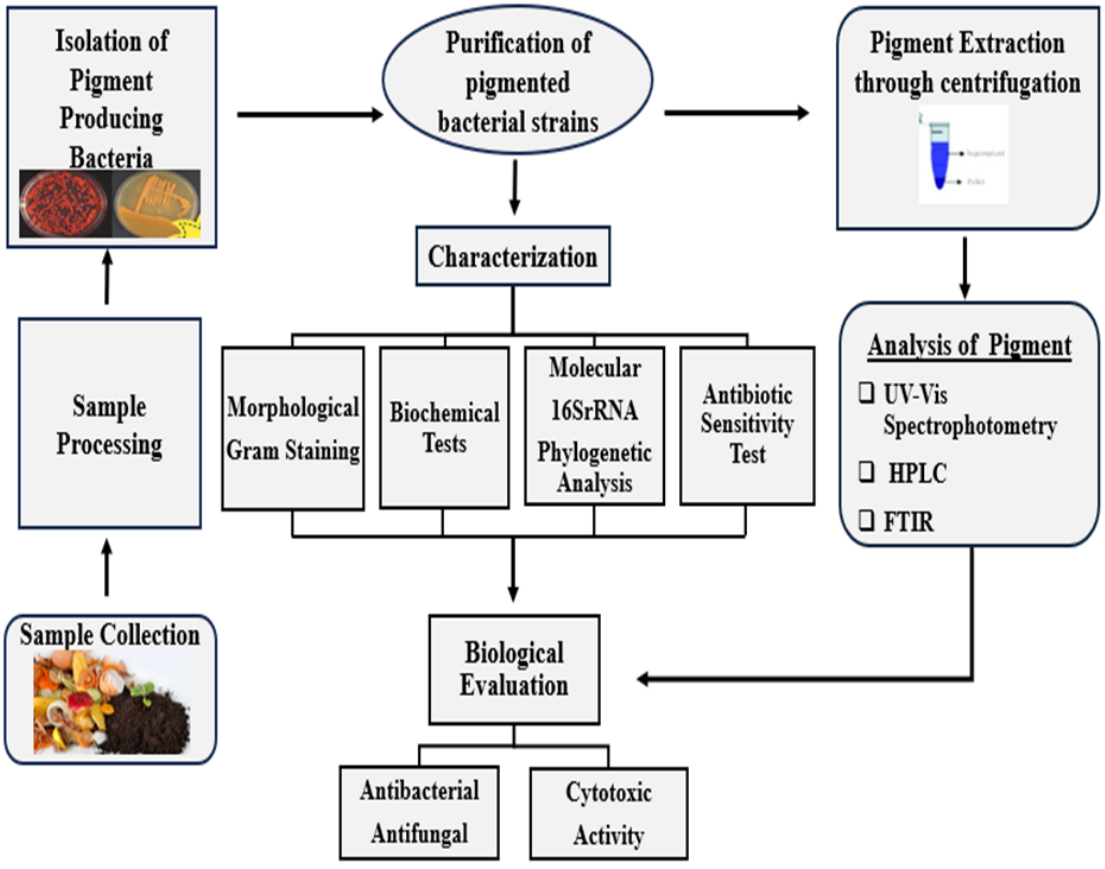

FIGURE 3.1: Research methodology flow diagram

3.2 Materials

3.2.1 Equipment

Ice Box (5 liter), Autoclave, Incubator, Vortex, Microscope, Shaker, pH Meter, Centrifuge, Measuring Balance, Hot Air Oven, Laminar Flow Hood, Spectrophotometer, FTIR equipment and, HPLC Equipment and Magnetic stirrer.

3.2.2 Apparatus

Falcon Tubes, Sterile Polythene Bags, Sterile Plastic Bottles, Beakers (100ml to 1000 ml), Petri Dishes (90mm), Glass test tubes, Spirit Lamp, Conical Flask.

Eppendorf Tubes (1.5ml), Micropipette, Dropper, Gloves, Whatman No. 1 Filter Paper and Inoculation Loop, Spirit lamp, Glass slides, and Cover slips.

3.2.3 Chemicals

Nutrient Agar, Nutrient Broth, Eosin Methyl Blue (EMB) Agar, Blood Agar, MacConkey Agar, Tryptic Soy Agar, Minimal Salt Agar Media, Ethanol, Crystal violet, Gram iodine, Safranin, Distaining solution, Dimethyl Sulfoxide (DMSO)

Distilled Water, Oxidase, Catalase, Methyl Red / Voges-Proskauer (MRVP), Urease Agar, Simmons-Citrate Agar, Indole, Triple Sugar Iron, Muller Hinton Agar, Potato Dextrose Agar, and Sea salt.

3.3 Method: Sample Collection and Processing

Three types of samples were collected. The details are given below.

3.3.1 Sample 1: Fruits and Vegetable Peels (FVP)

Peels of freshly chopped fruits and vegetable samples (Mango, Banana, Plum, Peach, Cucumber, and tomatoes) were collected from kitchen waste. Collected peels were rinsed under running tap water followed by washing with sterile distilled water to remove dust and debris.

They were cut into small pieces (1-2 cm), mixed, and stored in an airtight plastic jar at room temperature 37^{o}C for one week. After one week, peel samples were homogenized using a sterile mortar and pestle, slurry was created by mixing the distilled water with crushed peel samples in a ratio of 1:10 (w:v).

All samples were blended in a ratio of 1g of sample to 10 ml of distilled water [184].

3.3.2 Sample 2: Compost Soil (CS)

A bag of 250g compost was purchased from Gulzar-e-Quaid Nursery Farm in Rawalpindi. It was then mixed with the garden soil and was left there for one month. Then, a 20g compost soil sample was collected from a depth of 10cm using a sterile corer and spatula. The sample was placed in a sterile polyethylene bag (6 $\times$ 10 inches) and stored in the refrigerator at -4^{o}C for further analysis.

A mixture of CS samples was passed through a sterile sieve to remove large debris and obtain a homogenous fine sample. 1-10 grams of compost was added in 90 mL of sterile distilled water to make a slurry. The mixture was vortexed for 10-15 minutes to maximize microbial release from the soil.

Serial dilutions of the compost slurry were created by adding 1 mL of slurry to 9 mL of sterile water, resulting in a 10^{-1} dilution, with further dilutions up to 10^{-6} [185].

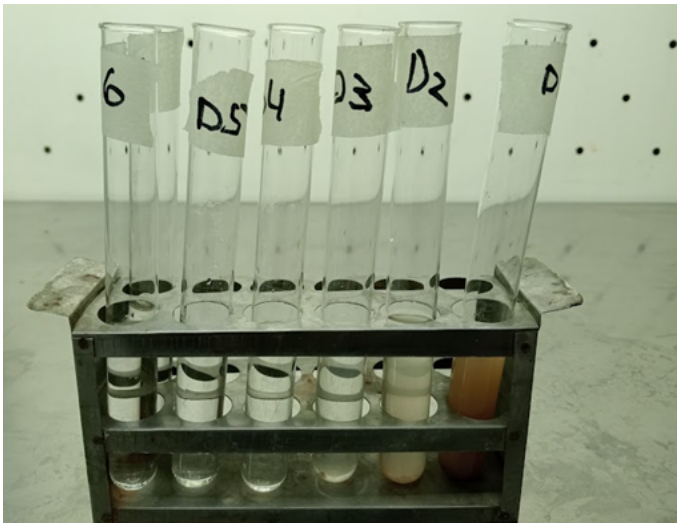

FIGURE 3.2: Dilutions of compost soil

3.3.3 Sample 3: Textile Effluent (TF)

Four samples of Textile dyes effluent were collected from discharge outlet of textile industry (Mian Mehmood Textiles) located in Faisalabad. The collected samples were labeled as follows (Table 3.1).

TABLE 3.1: Abbreviations of textile effluent samples

Sample	**Abbreviation**
Black Effluent	S1
Burgandy Effluent	S2
Pink Effluent	S3
Mixture of Black, Burgandy and Pink	SM

There were 4 samples of different colors Black S1, Burgandy S2, Pink S3, and a mixture of samples 1,2 and 3 named SM. 20 ml of each sample were collected in sterile plastic bottles. They were labeled as S1, S2, S3, and SM. Further, these bottles were stored in ice boxes until they were transported to the laboratory. In the laboratory, they were kept at -4^{o}C until they were used. Before culturing, the effluent was kept at room temperature for a few hours to remove to allow to settle the large particulates. Samples were filtered through a Whatman filter to remove debris. 1 mL of the filtered effluent was added to 9 mL of sterile distilled water

to create a 10^{-1} dilution. Serial dilutions were prepared up to 10^{-5} by taking 1 mL from each dilution and adding it to 9 mL of sterile water, creating a range of dilutions (10^{-2}, 10^{-3} etc.).

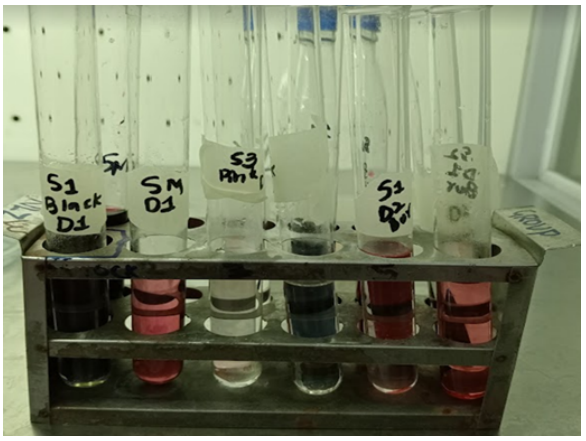

FIGURE 3.3: Dilutions of Textile effluents

3.4 Culturing and Isolation of Pigment Producing Bacteria

3.4.1 From FVP and CS Samples

3.4.1.1 Isolation on Nutrient Agar

To determine the presence of bacteria in fruit and vegetable peels and compost soil, samples were cultured on nutrient agar. Nutrient agar was prepared by weighing 1.3 g of the powder and mixing it with the 100ml distilled water. This mixture was then autoclaved at 121^{o}C for two hours to ensure sterilization. Once the autoclaving was complete, the nutrient agar was poured into autoclaved Petri plates within a laminar flow hood and allowed to cool for about 10 minutes for solidification. Then, 100 μL of each dilution from the fruit and vegetable peels (FVP) and compost soil (CS) samples were placed onto the nutrient agar plates. A sterile glass rod was used to evenly spread the samples across the agar surface. The plates were then incubated at 37^{o}C for 24 to 72 hours. After this period, the plates were observed for pigmented bacterial growth such as red, yellow, orange,

or blue colors. Pigment-producing colonies were carefully picked and transferred onto fresh nutrient agar plates for isolation. These new plates were incubated again under the same conditions to confirm the production of pigments.

3.4.1.2 Isolation on Differential Media

3.4.1.2.1 MaCconkey Agar

200ml of distilled water was added to 10g of dry MacConkey agar powder. The mixture was autoclaved at 121 ^{o}C for 15-20 minutes, then poured into Petri dishes and allowed to solidify under a laminar flow hood. 100 μL of each dilution of FVP and CS were applied to the agar plates and spread evenly with a sterile glass rod. The plates were incubated at 37 ^{o}C for 24-72 hours and checked for bacterial growth, focusing on pigment-producing colonies (red, yellow, orange, or blue). These colonies were sub cultured onto fresh media plates to obtain pure isolates and confirm pigment production.

3.4.1.2.2 Eosin Methylene Blue Agar

200 ml of distilled water was mixed with 7.4 g of dry EMB powder, and autoclaved for 15 to 20 minutes at 121^{o}C. After autoclaved, the media was poured onto Petri dishes under a laminar flow hood and allowed to solidify at room temperature.

100 μL of each dilution of FVP and CS were applied to the agar plates and spread evenly with a sterile glass rod. The plates were incubated at 37 ^{o}C for 24-72 hours and checked for bacterial growth, focusing on pigment-producing colonies (red, yellow, orange, or blue). These colonies were sub cultured onto fresh media plates to obtain pure isolates and confirm pigment production.

3.4.1.2.3 Blood Agar

4.9 g of Blood Agar was mixed with 100 mL of distilled water and sterilized by autoclaving at 121^{o}C for 2 hours. The media was then poured into four autoclaved

Petri plates and allowed to solidify at room temperature. Next, 100 μL of each dilution of FVP and CS was spread on the Blood Agar plates using a sterile glass rod. The plates were incubated at 37^{o}C for 24 to 72 hours and examined for bacterial growth, particularly colonies with pigment production (red, yellow, orange, or blue pigments).

3.4.1.3 Purification of Bacterial Strains

To purify bacterial strains from fruit and vegetable peels, as well as compost soil, differential media such as EMB Agar and MacConkey Agar were prepared following the above protocols. The media was poured into the autoclaved Petri plates. The inoculum from previously cultured on differential media was further streaked on these media plates for purification. And kept in an incubator for 24 hours at 37^{o}C.

3.4.2 Isolation of Pigment-Producing Bacteria from TE

For the isolation of pigment-producing bacteria from textile dye effluent, the following media were used.

3.4.2.1 Nutrient Agar

Nutrient agar media was prepared as per 3.4.1.1. A total of 100 μL from each dilution (10^{-3} and 10^{-5}) of 4 TE samples was spread on nutrient agar plates using a sterile glass rod. The plates were incubated at 37^{o}C for 24 to 72 hours and then checked for bacterial growth, focusing on any colonies displaying pigments such as red, yellow, orange, or blue.

3.4.2.2 Luria Bertani Agar

To prepare Luria-Bertani (LB) media, 10 g of dry powder was mixed with 400 ml of distilled water, stirred continuously, and autoclaved at 121^{o}C for 15 to 20

minutes. The sterilized media was poured into 16 Petri dishes and allowed to solidify at room temperature. Using a sterile glass rod, 100 μL of each dilution was spread on the LB agar plates, which were then incubated at 37^{o}C for 24 to 72 hours. After incubation, the plates were examined for bacterial colonies with red, yellow, orange, or blue pigments.

3.5 Gram Staining

Gram staining is a technique developed by Hans Christian Gram in 1884 to classify bacteria based on their cell wall structure using crystal violet and safranin dyes. Solutions of Crystal violet, iodine, Safranin, and destaining were prepared as per the protocol defined [186]. The procedure involves cleaning glass slides with 75% ethyl alcohol and placing a drop of bacterial suspension from a purified culture. After air-drying, the slides were heated over a spirit lamp for 60 seconds to fix the bacteria. A drop of crystal violet was applied for 30 seconds, followed by rinsing with distilled water. Next, 3-4 drops of gram iodine were added for one minute, then rinsed. The slide was washed with a 95% ethanol decolorizer and rinsed again. Finally, 3-4 drops of safranin were applied for one minute before placing a cover slip on top. The process was repeated for 7 bacterial strains. Under a microscope at 40x magnification, Gram-negative bacteria appeared pink, while Gram-positive bacteria appeared purple.

3.6 Biochemical Characterization

For biochemical characterization, different biochemical tests were performed to identify and classify microorganisms by pure bacterial strains obtained [187].

3.6.1 Catalase Test

The catalase test differentiates between catalase-positive and catalase-negative bacteria, particularly gram-positive cocci like *Staphylococcus* and *Streptococcus*. It

also indicates whether microorganisms are aerobic or anaerobic. Colonies from 24-hour incubated samples were placed in hydrogen peroxide using a sterile loop. This process was repeated for each sample.

3.6.2 Mannitol Salt Agar

Mannitol Salt Agar is a selective and differential medium used to test salt utilization in bacteria. Media was prepared for 7 plates as per the manufacturer defined on the label (HIMEDIA) and incubated at 37^{o}C for 48 hours.

3.6.3 Indole Test

The indole test involves inoculating a pure bacterial culture into a sterile test tube containing tryptone broth and incubating it at 37^{o}C for 24-48 hours. This allowed the bacteria to metabolize tryptophan and produce indole if the enzyme tryptophanase was present. After incubation, 0.5 mL of Kovac's reagent was added, and the tubes were gently shaken.

3.6.4 Simmon Citrate Test

The citrate utilization test is used to determine whether certain bacterial strains can utilize citrate. Media was prepared for 7 plates as per the manufacturer's defined on the label (HIMEDIA) and incubated at 37^{o}C for 48 to 72 hours.

3.6.5 Urease Test

The urea utilization test involves using bacterial samples to observe their ability to metabolize urea. Media was prepared for 7 plates as per the manufacturer's defined on the label (HIMEDIA) and incubated at 37^{o}C for 48 to 72 hours.

3.6.6 Oxidase Test

A piece of filter paper was soaked with a small quantity of a recently prepared 1% reagent solution. A sterilized loop was used to pick a well-isolated colony from a bacterial plate, and this colony was subsequently transferred onto the treated filter paper. A positive reaction was identified by a vibrant deep-purple color appearing within 5-10 seconds. A delayed positive reaction was characterized by coloration occurring between 10-60 seconds, whereas a negative reaction indicated either the absence of coloration or coloration happening after 60 seconds. This test was performed for 7 bacterial strains.

3.6.7 Methyl Red Voges Proskauer Test

Media was prepared for 7 test tubes as per the manufacturer's defined on the label (HIMEDIA). The broth mixture was left to incubate at 35^oC for a minimum of 48 hours following inoculation. Five teaspoons of the methyl red detection solution were applied to each test tube after the incubation period. Next, each tube's subsequent color shift was observed.

3.6.8 Triple Sugar Iron Test

The test that based on fermentation, with or without gas production, of glucose, lactose, and sucrose. Media was prepared for 7 plates as per the manufacturer defined in the label (HIMEDIA). The plates after wrapping with parafilm were stored in an incubator at 37^oC for 48-72 hours.

3.7 Molecular Characterization Using 16S rRNA

The 16S rRNA sequencing procedure was used for identifying and classifying isolated strains at the molecular level. It involved the extraction of DNA, amplification, and sequencing of the 16S rRNA gene, which is a conserved region found in

the prokaryotic ribosome [188]. To determine the 16S rRNA of isolated bacterial strains, the samples were sent to Microgen Korea.

3.8 NCBI Submission

The bacterial strain sequences were submitted to the NCBI portal.

3.9 Phylogenetic Analysis

Phylogenetic analysis is the study of evolutionary relationships among organisms or genes based on genetic, molecular, or morphological data. It depicts the evolutionary history, common ancestry, and divergence of lineages often represented through phylogenetic trees [189]. The phylogenetic analysis was done to find the evolutionary relationships and taxonomic classifications of isolated pigment-producing bacteria. The amplified 16S rRNA sequences of 4 bacterial isolates were obtained from NCBI using Blast n in FASTA format for phylogenetic analysis. Then, Multiple sequence alignment was done by Clustal W, and a phylogenetic tree was constructed based on the maximum likelihood method in MEGA 11 software.

3.10 Antibiotic Sensitivity Test

In the antibiotic sensitivity test, different types of antibiotics such as doxycycline, ceftriaxone, streptomycin, and imipenem were used, and each type is effective against different bacteria. In this study, the Disk diffusion method was used. It is extensively used antibiotic susceptibility test in determining which type of antibiotic should be used in infection treatment. It depends upon bacterial growth inhibition measured under standard conditions [190]. Muller Hinton Agar media was used. It was prepared as per the instructions given by the manufacturer (HIMEDIA). Pure strains of bacteria were taken by a sterile loop from each plate

and streaked on the Muller Hinton agar plates. Plates were allowed to dry for approximately 5 minutes. An antibiotic disc dispenser was used to dispense the disk containing the antibiotic onto the plate. Each disc was gently pressed using sterile loop to agar to ensure that each disc is attached to agar media. These plates were then preserved in an incubator at 37^oC for 24 hours after wrapping with parafilm. After 24 hours of incubation, measure the zone sizes in millimeters using a ruler. Enter the zone sizes into the disk diffusion log.

3.11 Extraction of Pigments

To extract bacterial pigments, the following methodology was used. Bacterial strains were cultured under optimal conditions. For pigment extraction, organic solvents like acetone, methanol, ethanol, or chloroform are commonly used. Here, 95% methanol was used for extraction. The bacterial pellet was resuspended in 1 ml solvent of 95% methanol, and the mixture was allowed to vortex for several minutes to ensure cell lysis and pigment release.

After sufficient vortex, the mixture was centrifuged at 6000rpm for 15-20 minutes to separate the solvent containing the extracted pigment from the cell debris. The process was repeated 2-3 times [191]. The supernatant was carefully collected in another Eppendorf tube and stored for analysis by UV-spectrophotometer, HPLC, and FTIR.

3.12 Analysis of Pigments

3.12.1 Spectrophotometric Analysis

Using a UV spectrophotometer, the maximum absorption spectra of the acquired bacterial pigments were measured [192]. To determine the maximum absorption spectra, the produced bacterial pigment was suspended in 1 ml 95% methanol

solution in the wavelength range of 400 to 700 nm, with 1 ml 95% methanol serving as a blank. The pigments were measured against 400 to 700nm wavelengths.

3.12.2 FTIR

FT-IR technique indicates the bonds existed in the compound and consequently be used to determine functional groups of the molecule [193]. It gives both qualitative and quantitative analysis of samples. For FTIR analysis, seven pigments pellet - yellow, pink, brown and blue from compost soil and green, pink and blue from FVP samples were added in 1.5 ml Eppendorf tubes with 1 ml of 95% methanol and vortex for 10 minutes. The samples were sent to QAU for FTIR. The following functional groups were checked with peaks 1300 - 4000 cm^{-1} against extracted pigments.

- Methyl
- Conjugated double bonds
- Keto-groups
- Hydroxy group
- Disulphides
- Aliphatic iodo compounds
- Methyl
- Carbonyl (Amide)
- Methylene
- Vinyl
- >CH-Methyne

3.12.3 HPLC

HPLC stands for High performance Liquid Chromatography, is a powerful technique used to separate, identity, and quantify the components of a mixture [194]. For HPLC analysis, 1ml of 95% methanol was added to 1ml of bacterial pigment in the Eppendorf tube. C-18 column was used for reversed phase HPLC. The

concentration of the sample injected for HPLC was 10-20 μL. The wavelength for detection was 200-600 nm with 30-40 minutes run time. The sample flow rate was 0.8-1.2ml/min.

3.13 Biological Evaluation

3.13.1 Antibacterial Activity

It is a laboratory test that is used to evaluate the effectiveness of a substance or compound against bacteria [195]. For the anti-bacterial test, the agar well diffusion method was employed to determine the properties of bacterial pigments and bacteria as well. It includes the following protocol.

3.13.1.1 Isolation of Bacterial Strains

The bacterial strains *Staphylococcus aureus* (SRR27533903) and *Kerstersia gyiorum* (PQ666790) were used as pathogenic strains in the anti-bacterial test. This strain of *S. aureus* can cause skin and soft tissue infections, bloodstream infections, pneumonia, and endocarditis, particularly in immunocompromised individuals [196]. *K. gyiorum* is associated with chronic wounds, bloodstream infections, and respiratory tract infections, often in hospitalized patients or those with underlying health conditions [197]. Media was prepared according to the instructions provided by the manufacturer and autoclaved for 1 hour at 121^{o}C.

3.13.1.2 Concentration Preparation

For each of the 7 pigments, 3 concentrations of each pigment were prepared using methanol as a solvent about 1000 μg/mL, 750 μg/mL, and 500 μg/mL. The same concentrations of 7 bacteria were prepared by using distilled water.

3.13.1.3 Media Preparation for Anti-Bacterial Test

16.2g of MHA was added to 640ml distilled water and autoclaved at 121^{o}C for 1 hour with 32 petri plates. Once the media was autoclaved, it was poured onto the sterile petri plates and allowed to solidify in the laminar flow hood. After solidification, sterile wells, approximately 6-8 mm in diameter, were created in the agar plates using a sterile cork borer. Each plate contains 3 wells, while 1 well was formed in the control plates.

3.13.1.4 Control Groups

Ciprofloxacin was used as a positive control for both pigment and bacteria against *K. gyiorum* due to its broad-spectrum activity against gram-negative bacteria [198]. In contrast, Ampicillin was used as a positive control against *S. aureus* because it is a beta-lactam antibiotic effective against non-resistant strains of *S. aureus* [199]. In negative control, methanol was added for pigments while water was used for bacteria.

3.13.1.5 Inoculation and Wells Filling

The bacterial strains were inoculated onto the Muller Hinton Agar petri plates. Each well was filled with 1μ MHA to create a consistent base. The prepared dilutions i.e. pigment and bacteria were added to the well. After that, both bacterial strains were streaked on each MHA plate and labeled well. The plates were then incubated at 37^{o}C for 24 hours.

3.13.1.6 Measurement of Zone of Inhibition

The zone of inhibition was measured after the incubation period, with the help of a ruler to identify the anti-bacterial properties of pigments and bacteria.

3.13.2 Antifungal Activity

It is an assay that determines the ability of a test substance to inhibit the growth of fungi [200]. For the anti-fungal test, agar well diffusion was employed to check the properties of bacterial pigments and bacteria as well. It includes the following protocol.

3.13.2.1 Culturing of Fungal Strains

To perform the antifungal test, the pathogenic fungal strains were inoculated on Potato Dextrose Agar (PDA) from stock culture. PDA was prepared as per manufacturer (HIMDIA). The media was autoclaved for 1 hour at 121^{o}C. After autoclave, the media was poured into 2 petri plates and allowed to solidify in the laminar flow hood. Then, the fungal strains *Aspergillus fumigatus* (ORO53856) and *Rhizopus delemar* (OQ9844419) were inoculated onto the petri plates with a sterile loop and incubated at 27^{o}C for 7 days. A. fumigatus can cause invasive aspergillosis, particularly in immunocompromised patients, leading to severe respiratory infections and systemic fungal disease [201]. *R. delemar* is associated with mucormycosis, a severe fungal infection that can affect various parts of the body, including the sinuses, lungs, and brain, particularly in immunocompromised individuals [202].

3.13.2.2 Dilution Preparation

For each of the 7 pigments, 3 concentrations of each pigment were prepared using methanol as a solvent about 1000 μg/mL, 750 μg/mL, and 500 μg/mL. The same concentrations of 7 bacteria were prepared by using distilled water.

3.13.2.3 Media Preparation for Anti-Fungal Test

PDA was prepared as per the manufacturer's instructions (HIMEDIA). Once the media was autoclaved, it was poured onto the sterile petri plates and solidified in

the laminar flow hood. After solidification, sterile wells, approximately 6-8 mm in diameter, were created using a sterile cork borer. Each plate contains 3 wells, while 1 well was formed in the control plates.

3.13.2.4 Control Groups

Amphotericin B was used as a positive control for both pigment and bacteria because it is used as a broad-spectrum antibiotic against these fungal strains [203]. In negative control, methanol was added for pigments while water was used for bacteria.

3.13.2.5 Inoculation and Wells Filling

The fungal strains were inoculated onto the PDA petri plates. Each well was filled with 1μ PDA to create a consistent base. The prepared dilutions i.e. pigment and bacteria were added to the well. After that, both fungal strains were streaked on each PDA plate and labeled well. The plates were then incubated at 27^{o}C for 3 days.

3.13.2.6 Measurement of Zone of Inhibition

The zone of inhibition was measured with (mm) scale.

3.13.3 Brine Shrimp Lethality Bioassay

Using brine shrimp (*Artemia salina*), the extracted pigments' cytotoxic activity was assessed. For this test, 1g of (*Artemia salina*) cysts were immersed in 500ml distilled water with 5% saline with a pH kept between 7.5 and 8.5. Throughout the procedure, a steady supply of oxygen was kept available with a light source as shown in figure 3.4 below.

After hatching the eggs, add 20 shrimps to each sample with concentrations 10, 20, and 30 μL. Repeat the process with each concentration for 7 bacterial pigment

samples with 1 control as methanol. The process was further repeated with each concentration for bacterial samples with 1 control as water. Keep the plates in the incubator at 37°C for 24 hours. Observed the death count of the larvae, by using the following %mortality formula and calculated the LC50 [204].

$$\% \, Mortality = \frac{No. \, of \, dead \, Shrimp}{Total \, No. \, of \, Shrimps} \times 100$$

FIGURE 3.4: Growth of brine shrimp (*Artemia salina*)

3.13.4 Larvicidal Activity

The cytotoxicity of pigments was evaluated by using worms at the larvae stage (*Caenorhabditis elegans*). For this test, 210 worms at the larvae stage were used. The test was performed against seven bacterial pigments and strains from FVP and CS samples. Add 5 worms to each sample at concentrations 20, 50, and 70 μL. Repeat the process with each concentration for 7 bacterial pigment samples with 1 control as methanol.

The process was further repeated with each concentration for bacterial samples with 1 control as water. Keep the plates in the incubator at 37°C for 3 days.

Observe the death count of the larvae, by using the following %mortality formula [205].

$$\%\ Mortality = \frac{No.\ of\ dead\ Shrimp}{Total\ No.\ of\ Shrimps} \times 100$$

After mortality, LC50 was calculated.

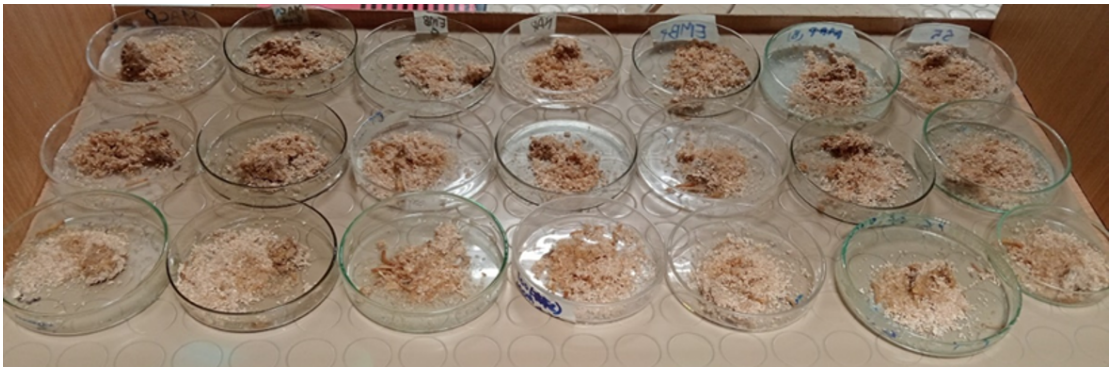

FIGURE 3.5: Test for Larvicidal activity

Chapter 4

Results

4.1 FVP and CS

4.1.1 Culturing and Isolation on Nutrient Agar

Culturing of FVP and CS revealed that all 4 plates of FVP samples on Nutrient agar showed green pigmented colonies after incubation at 37^{o}C for 48 hours as shown in figure 4.1 (A). While CS sample cultured and isolated plates showed yellow-pigmented colonies on Nutrient agar after incubation at 37^{o}C for 48 hours as shown in Figure 4.1 (B).

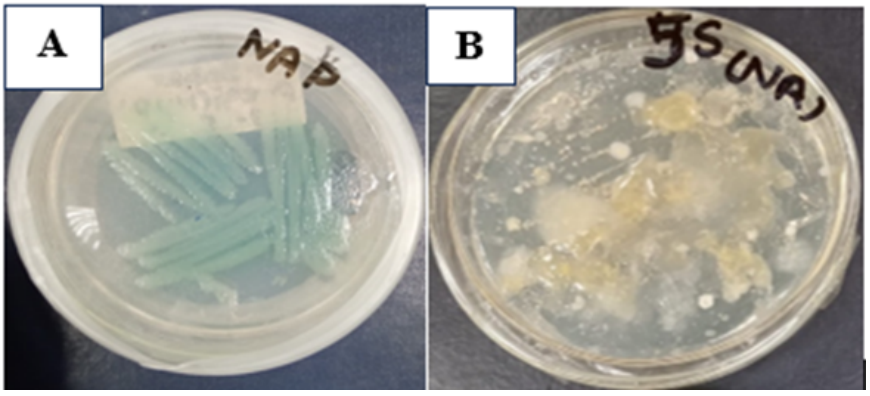

FIGURE 4.1: Results on Nutrient Agar (A) Green pigmented colonies on Nutrient Agar (Fruits/vegetable peels); (B) Yellow colonies on Nutrient Agar (Compost soil)

4.1.2 Culturing and Isolation on Differential Media

The FVP samples were streaked on differential media such as MacConkey agar, Eosin Methyl Blue agar and Blood agar. Dark Pink pigmented colonies were formed on MacConkey agar (A), Reddish pink colonies were formed on EMB agar (B), while colorless colonies were formed on Blood agar (C) after incubation at 37^{o}C for 48 hours as shown in Figure 4.2. However, in compost soil samples MacConkey agar showed light pink pigmented colonies (a) and brown pigmented colonies (b), EMB agar showed purple pigmented colonies (c), and colorless colonies were formed on blood agar (d) after incubation at 37^{o}C for 48 hours as shown in Figure 4.3.

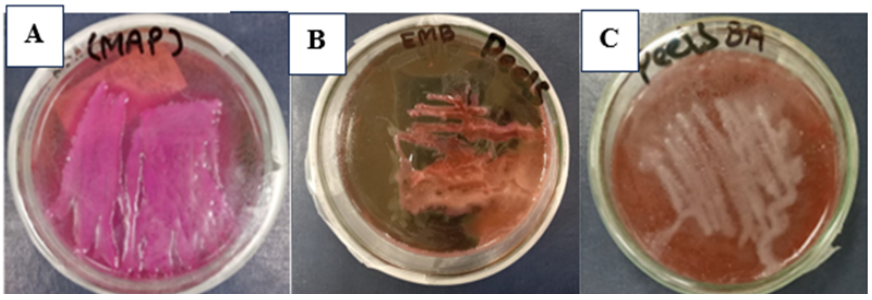

FIGURE 4.2: (A) Dark pink colonies on MacConkey agar, (B) Reddish Pink Colonies on EMB agar and, (C) Colorless colonies on blood agar

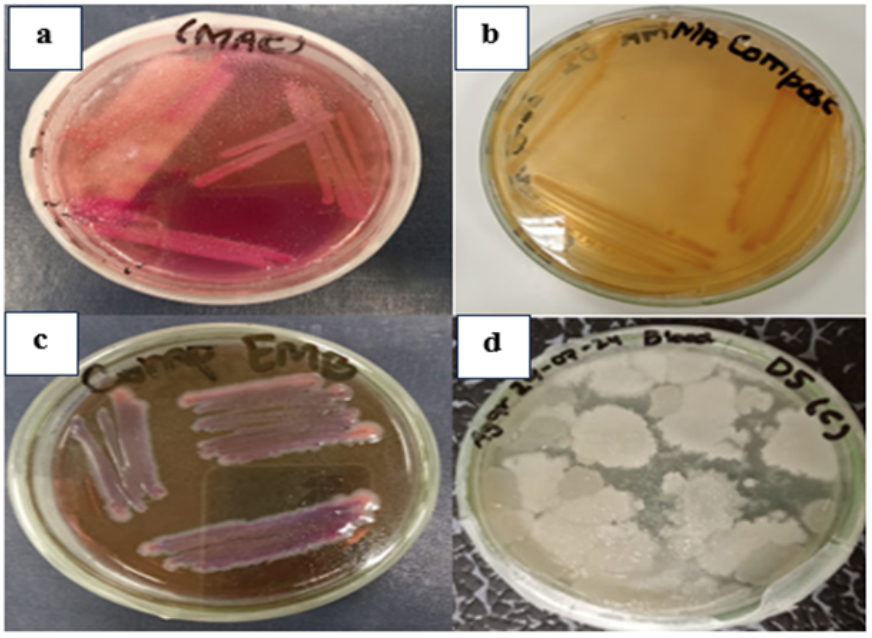

FIGURE 4.3: (a) Light pink colonies on MacConkey agar, (b) Brown colonies on MacConkey agar (c) Purple Colonies on EMB agar and, (d) Colorless colonies on blood agar

4.2 TE

4.2.1 Culturing and Isolation on Nutrient Agar and Selective Media

The textile effluent samples from dilutions 10-3 and 10-5 were streaked on nutrient agar plates and did not show pigmented colonies after incubation at 37^{o}C for 48 hours as shown in Figure 4.4.

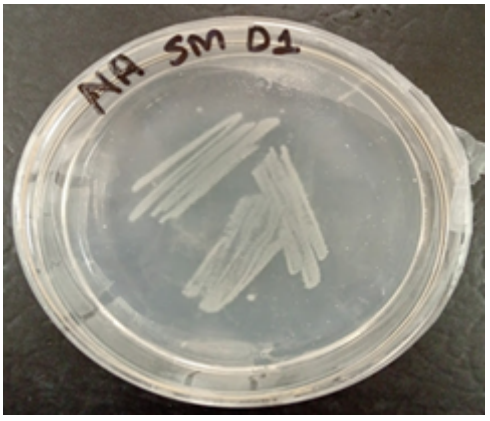

FIGURE 4.4: Colorless colonies on Nutrient Agar

The textile effluent samples were streaked on Tryptic soy agar in each of the 16 plates. Colorless colonies were formed on TSA after incubation at 37^{o}C for 48 as shown in Figure 4.5. These colonies were not further continued for study.

FIGURE 4.5: Colorless colonies on TSA

4.3 Gram Staining

Gram staining was performed for 7 isolated strains and out of 7 strains, 4 strains were gram-positive while the other 3 strains were gram-negative. Gram negative strains were retrieved from FVP samples, while Positive strains were taken from FVP sample as shown in Table 4.1 and Figure 4.6.

TABLE 4.1: Showing gram staining of isolated strains and their abbreviations

Sample	**Sample ID and Abbreviations**	**Gram Stain**
FVP	NAP = Nutrient Agar Peels	-ive
	MAP = MacConkey Agar Peels	-ive
	EMBP = EMB Peels	-ive
CS	NACY= Nutrient Agar Yellow	+ive
	MACP =MacConkey Agar Compost Pink	+ive
	MACB = MacConkey Agar Compost Brown	+ive
	EMBC = EMB Compost	+ive

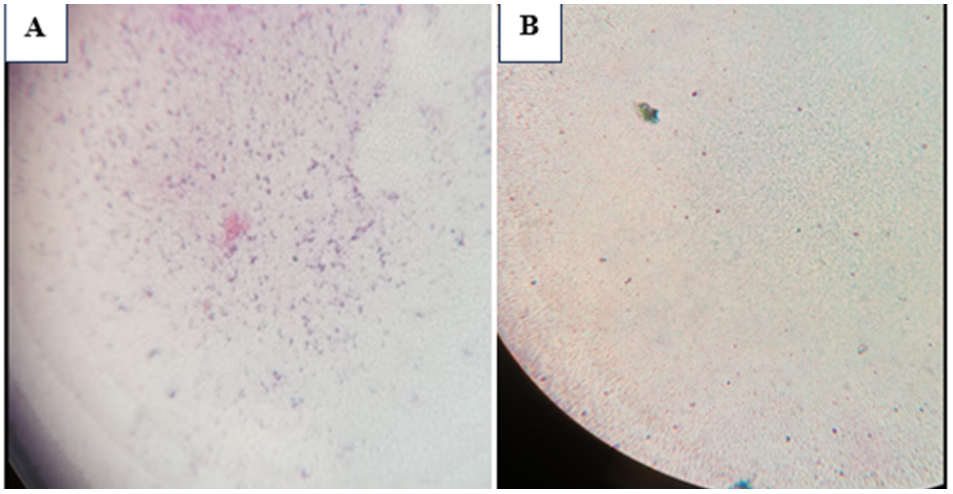

FIGURE 4.6: (A) Gram-positive strain from EMBC; (B) gram-negative strain from NAP

4.4 BioChemical Characterization

The results of the bi^{o}Chemical characterization of 7 bacterial strains are shown in Table 4.2 below.

TABLE 4.2: Results of bioChemical characterization of isolated bacterial strains

Code	**Catalase Test**	**Oxidase Test**	**Urease Test**	**Indole Test**	**Simmon Citrate Test**	**Mannitol Salt Test**	**Triple Sugar Iron Test**	**Methyl Red Test**
NAP	+ive	-ive	+ive	-ive	+ive	+ive	+ive	+ive
MAP	+ive	-ive	-ive	-ive	-ive	-ive	-ive	+ive
EMBP	+ive	-ive	+ive	-ive	-ive	-ive	+ive	+ive
NACY	+ive	-ive	+ive	-ive	+ive	+ive	+ive	+ive
MACB	+ive	-ive	+ive	-ive	+ive	+ive	+ive	+ive
MACP	+ive	-ive	+ive	-ive	+ive	+ive	-ive	+ive
EMBC	+ive	-ive	+ive	-ive	+ive	+ive	-ive	+ive

4.4.1 Catalase Test

Some bacteria produce the enzyme catalase to protect themselves from hydrogen peroxide (H_2O_2), a toxic byproduct of aerobic metabolism that can damage their DNA, proteins, and lipids. Catalase breaks down H_2O_2 into water (H_2O) and oxygen gas (O_2), preventing cellular harm. Bacteria that have the catalase genes produce catalase-positive results while bacteria that do not have catalase genes produce catalase-negative results. The rapid formation of bubbles indicates the presence of catalase enzyme [206].

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

All of the 7 strains were catalase positive as shown in Figure 4.7 and Table 4.2.

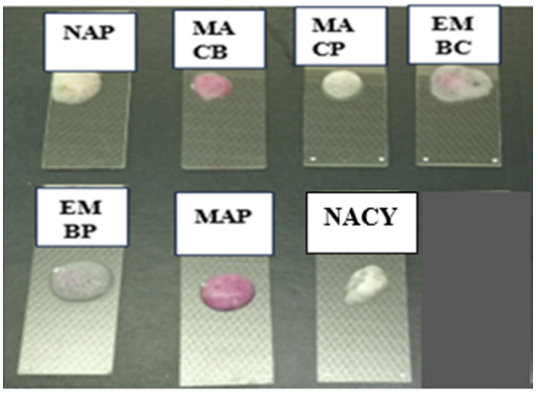

FIGURE 4.7: All strains-catalase positive

4.4.2 Oxidase Test

Aerobic respiration uses oxygen to generate ATP by transferring electrons through a chain of proteins, creating an energy gradient. In some bacteria, the enzyme cytoChrome c oxidase plays a key role in passing the final electrons to oxygen. The oxidase test is an important test to differentiate between oxidase-positive *Pseudomonadaceae* from oxidase-negative *Enterobacteriaceae* and is also useful for identifying various bacterial species. This test uses a colorless oxidase reagent, which reacts with cytoChrome c oxidase if present, donating electrons and producing a bluish or purplish color within 30 seconds. The blue or purplish colors are regarded as oxidase positive while colorless are oxidase negative [207]. All of the strains were oxidase-negative as shown in figure 4.8 and Table 4.2.

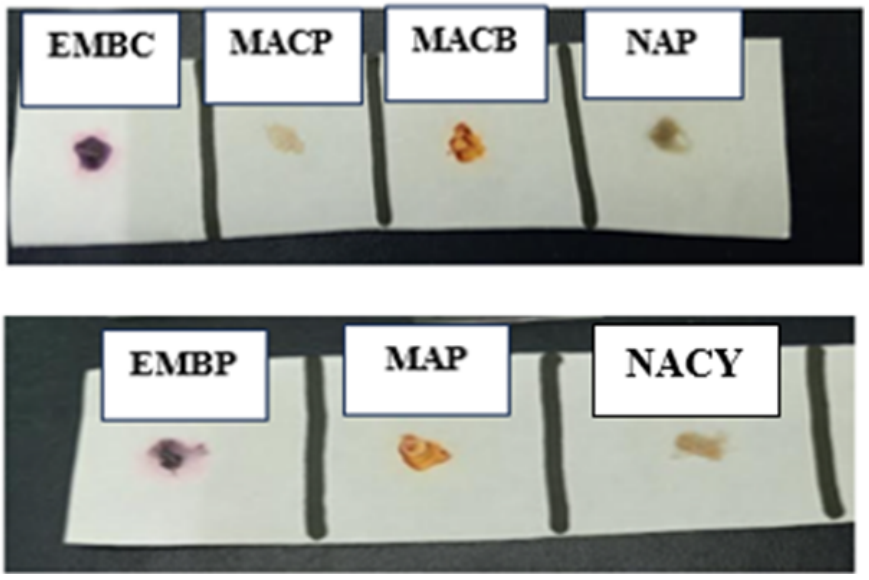

FIGURE 4.8: All oxidase-negative strains

4.4.3 Urease Test

Urease is an enzyme that continuously breaks down urea into carbon dioxide and ammonia. In the urease test, a special medium containing 2% urea and the pH indicator phenol red is used to detect this activity. When urease breaks down urea, ammonia is released, raising the pH and causing the color to shift from yellow (pH 6.8) to bright pink (pH 8.2). A positive result turns the medium

bright pink due to alkaline conditions, as seen with *Proteus mirabilis*. A negative result shows no color change (yellow or orange), typical of *Escherichia coli*. This test is commonly used to differentiate urease-positive bacteria, especially within the *Enterobacteriaceae* family [208]. Out of 7 strains, 1 was urease negative while 6 were urease positive as shown in figure 4.9 and Table 4.2.

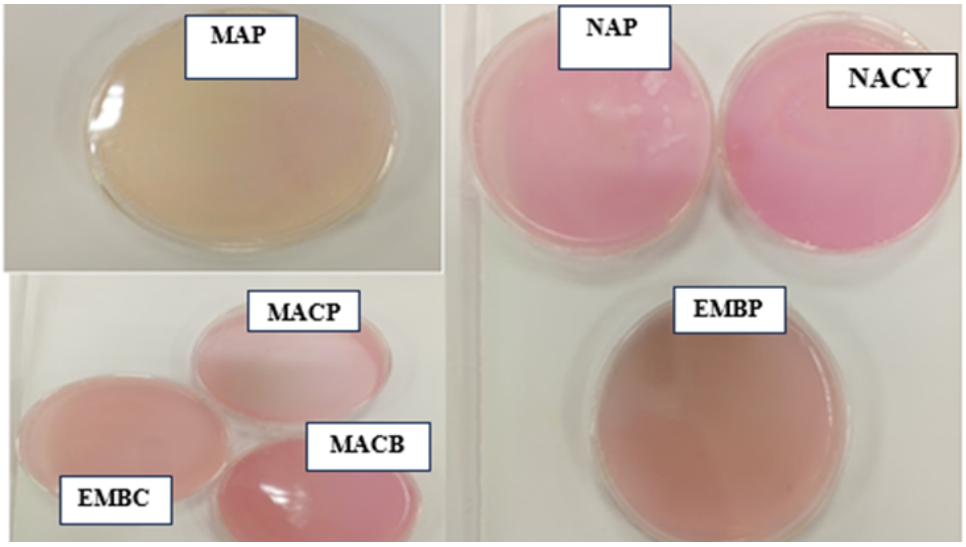

FIGURE 4.9: All urease-positive bacteria, except MAP - urease-negative

4.4.4 Indole Test

Some bacteria can break down the amino acid tryptophan using the enzyme tryptophanase, producing indole, pyruvic acid, and ammonia. To perform the indole test, the culture medium must contain enough tryptophan to support this reaction. If a bacterium can degrade tryptophan, it will release indole as a byproduct. The test detects indole by adding p-dimethylaminobenzaldehyde (DMAB) under acidic conditions, which reacts with indole to form a red-colored compound called rosindole, confirming the presence of tryptophanase activity. A red or pink layer on top, as seen with *Escherichia coli*. A negative result remains colorless or slightly yellow, typical of *Enterobacter cloacae*. This test helps differentiate indole-producing bacteria, especially within the *Enterobacteriaceae* family [206]. All of the 7 strains were indole negative as shown in figure 4.10 and Table 4.2.

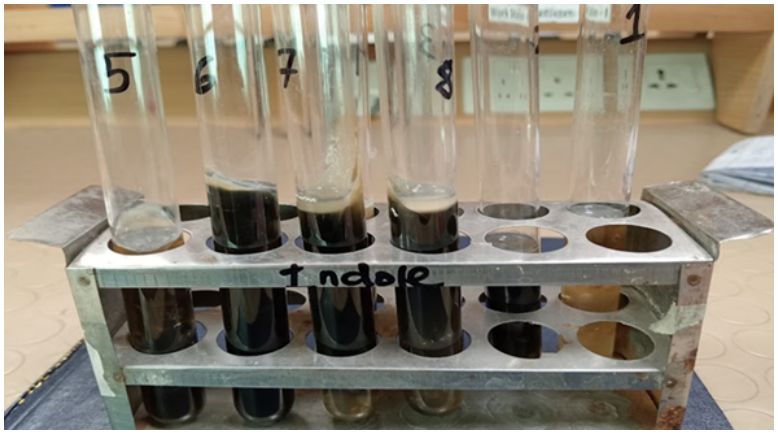

FIGURE 4.10: Indole test negative results for all strains

4.4.5 Simmon Citrate Test

The citrate test, part of the IMViC series, helps differentiate *Enterobacteriaceae* based on their ability to use citrate as their sole carbon source. In Simmons citrate medium, bacteria that can import and metabolize citrate produce alkaline byproducts, raising the pH and changing the bromothymol blue indicator from green to blue. This reaction occurs as citrate is broken down into oxaloacetate and further metabolized, releasing CO_2, which reacts with sodium to form sodium carbonate. A positive result turns the medium blue due to alkaline by-products, as seen with *Enterobacter aerogenes*. A negative result keeps the medium green, indicating no citrate utilization, typical of *Escherichia coli* [206]. Out of 7 strains, 5 were citrate positive while 2 were citrate negative as shown in figure 4.11 and Table 4.2.

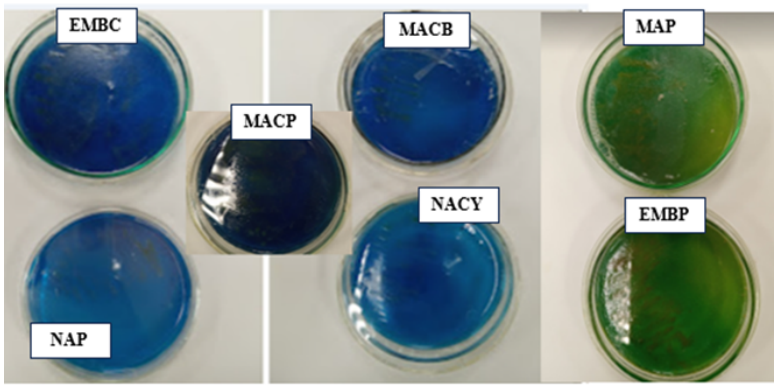

FIGURE 4.11: All strains are citrate positive; MAP and EMBP are citrate negative.

4.4.6 Mannitol Salt Agar Test

Mannitol Salt Agar (MSA) is a selective and differential medium used to isolate salt-tolerant bacteria, particularly *Staphylococcus* species. If the organism ferments mannitol, the medium turns yellow (positive), indicating acid production, as seen with *Staphylococcus aureus*. If mannitol is not fermented, the medium remains red (negative), typical of *Staphylococcus epidermidis*. Non-halophilic bacteria like *Escherichia coli* do not grow due to the high salt concentration [206]. Out of 7 strains, 5 were mannitol positive while, the remaining 2 strains were mannitol negative as shown in Figure 4.12 and Table 4.2.

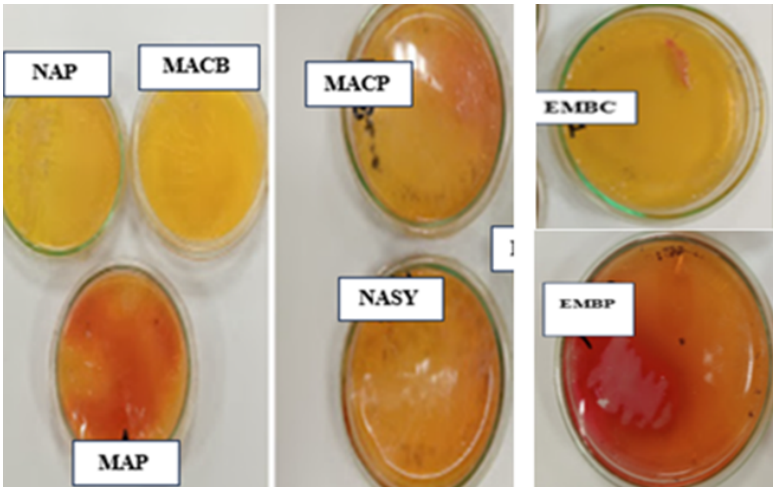

FIGURE 4.12: All Mannitol positive except EMBP and MAP

4.4.7 Triple Sugar Iron Test

The Triple Sugar Iron (TSI) agar test differentiates bacteria based on their ability to ferment glucose, lactose, or sucrose, and produce hydrogen sulfide (H_2S) or gas. A yellow slant and butt indicate glucose and lactose/sucrose fermentation (positive), while a red slant with a yellow butt shows glucose-only fermentation. Black precipitate signifies H_2S production, and gas bubbles indicate gas formation. *Escherichia coli* shows full sugar fermentation, *Salmonella produces* H_2S, while *Pseudomonas* gives a completely red result (no fermentation) [206]. Out of 7 strains, 4 showed yellow slant and butt, 1 showed red slant yellow butt while the

remaining 2 strains showed black precipitates with yellow butt as shown in Figure 4.13 and Table 4.2.

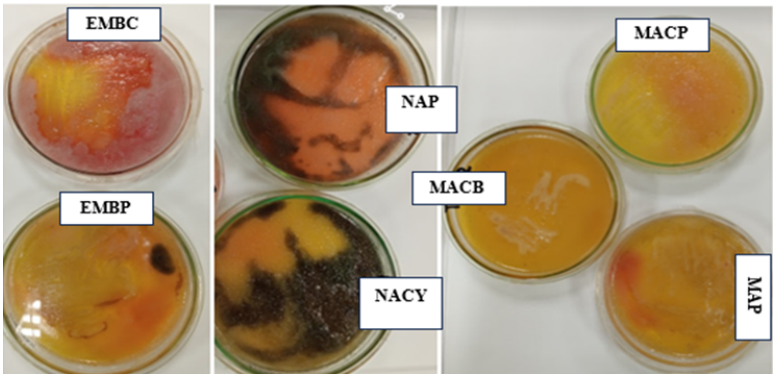

FIGURE 4.13: All TSI positive, except MAP, MACP and EMBC

4.4.8 Methyl Red Test

The Methyl Red (MR) test identifies bacteria that perform mixed acid fermentation, producing stable acids and lowering the pH. A positive result shows a red color after adding methyl red, indicating strong acid production, as seen in *Escherichia coli*. A negative result appears yellow or orange, indicating less acid production, typical of *Enterobacter aerogenes*. This test helps differentiate members of the *Enterobacteriaceae* family based on their fermentation pathways [206]. All of the 7 strains were methyl-red positive as shown in Figure 4.14 and Table 4.2.

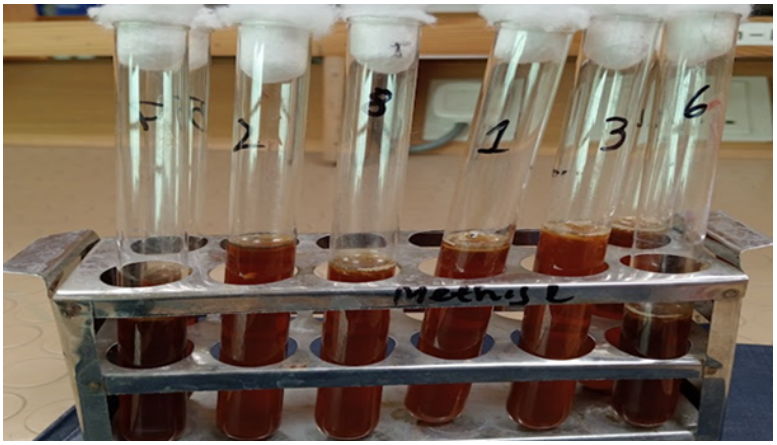

FIGURE 4.14: Methyl red all strains positive results.

4.5 16sRNA and NCBI Submission

The results of 16sRNA of bacterial species are presented in Table 4.3.

TABLE 4.3: Results of 16sRNA

Sample Source	**Sample ID**	**Bacterial Specie Name**	**Pigment**	**Query Coverage**	**Percentage Identity**	**Accession ID**
FVP	NAP	*Pseudomonas nitroreducens* strain ES-18	Green	76%	93.90%	SUB 15201586
	MAP	*Klebsiella* sp. Strain KL-1	Pinkish red	95%	87.92%	PQ661259
	EMBP	*Kerstersia gyoirum*	Dark blue	96%	97.75%	PQ661279
CS	NACY	*Chryseobacterium* sp. S5	Yellow	94%	89.60%	PQ892031
	MACP	*Serratia marcescens* strain XC 19	Pinkish Orange	94%	97.64%	PV382956
	MACB	*Psychrobacter pulmonis* strain PIGB167	Brown	94%	93.62%	PQ661263
	EMBC	*Acinetobacter* sp. NII-56	Purple	98%	84.17%	PV382871

The above sequences were submitted to the NCBI submission portal. The results for strains from FVP cultured on NA, MAC, and EMB agar identified *Pseudomonas nitroreducens* strain ES-18, *Klebsiella* sp. KL-1 and *Kerstersia gyoirum* species with query coverage and percentage identities, sequence similarity, respectively, with NCBI accession IDs as mentioned in Table 4.3. The results for strains from CS cultured on Nutrient agar, MACP, MACB, and EMBC agar identified *Chryseobacterium* sp. THG-SQA1, *Serratia marcescens* strain XC 19, *Psychrobacter pulmonis* strain PIGB167, and *Acinetobacter* sp. NII-56 species with query coverage, percentage identity, and NCBI accession IDs as mentioned in Table 4.3.

4.6 Phylogenetic Analysis

The phylogenetic tree was constructed using the maximum likelihood method in MEGA 11 software.

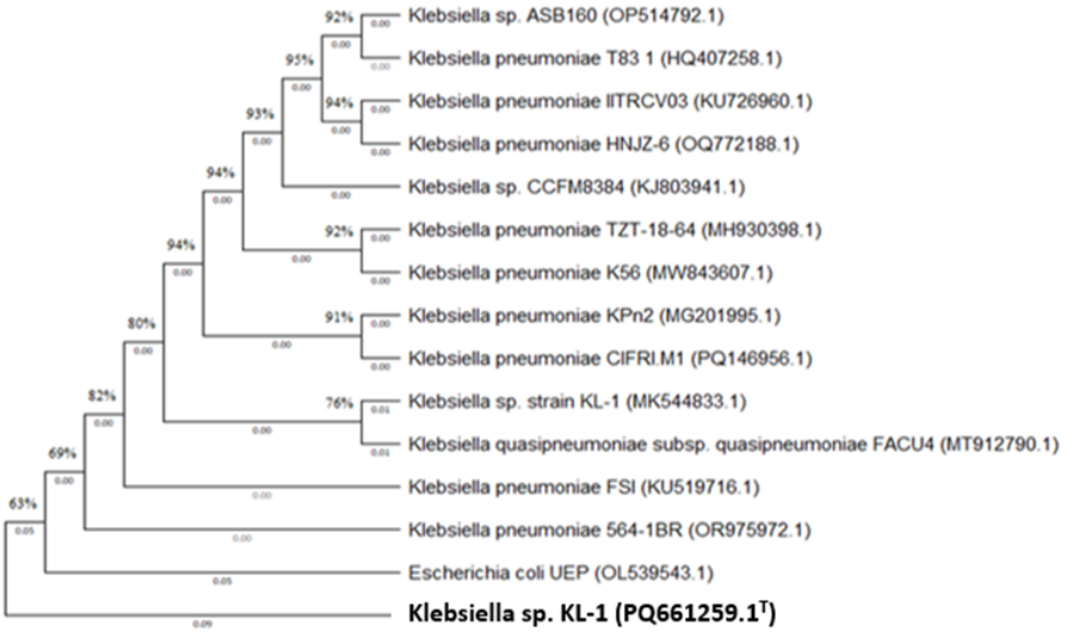

FIGURE 4.15: Phylogenetic tree of *Klebsiella* sp. KL-1 (PQ661259) from FVP sample

The phylogenetic tree analysis highlights Klebsiella sp. KL-1 (PQ661259.1^T) is the specie of interest, positioned within the Klebsiella pneumoniae complex. The closest relatives include *Klebsiella quasipneumoniae subsp. quasipneumoniae* FACU4 (MT912790.1) and *Klebsiella pneumoniae* FSI (KU519716.1), indicating a strong evolutionary relationship. The bootstrap values, ranging from 63% to 95%, provide confidence in the clustering, with higher values suggesting greater reliability in the branching structure. The outgroup, *Escherichia coli* UEP (OL539543.1), serves as a distinct lineage, reinforcing the divergence between *Klebsiella* and *Escherichia* species. The positioning of *Klebsiella* sp. KL-1 suggests that it shares a significant genetic similarity with other *Klebsiella pneumoniae* strains while maintaining its unique phylogenetic identity.

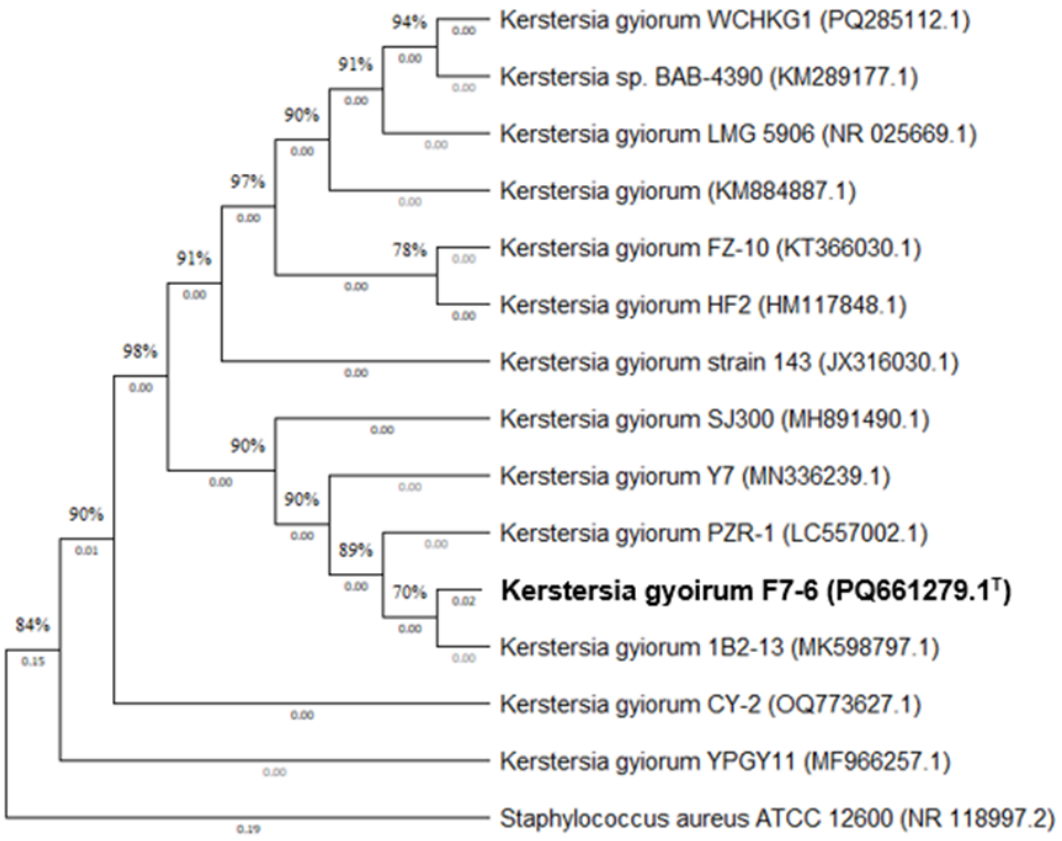

FIGURE 4.16: Phylogenetic analysis of *K. gyiorum* F7-6 (PQ661279.1^T) from FVP samples

The phylogenetic tree based on bootstrap values shows that *Kerstersia gyiorum* F7-6 (PQ661279.1T) clusters closely with *Kerstersia gyiorum* 1B2-13 (MK598797.1)) with a weak bootstrap support of 70%, indicating some genetic divergence. This cluster is moderately supported (89%) as its groups with *Kerstersia gyiorum* PZR-1 (LC557002.1) and *Kerstersia gyiorum* Y7 (MN336239.1) while a broader clade with 90% support includes *Kerstersia gyiorum* SJ300 (MH891490.1) *Kerstersia gyiorum* strain 143 (JX316030.1) and *Kerstersia gyiorum* HF2 (HM117848.1). The highest support (98%) is observed in a cluster of multiple *Kerstersia gyiorum* strains, reinforcing their close evolutionary relationships. The tree also includes *Staphylococcus aureus* ATCC 12600 (NR 118997.2) as an outgroup, highlighting its phylogenetic divergence from *Kerstersia gyiorum* with a distinct branch. The varying bootstrap values suggest strong to moderate confidence in most relationships, confirming the genetic relatedness within *Kerstersia gyiorum* while showing divergence among certain strains.

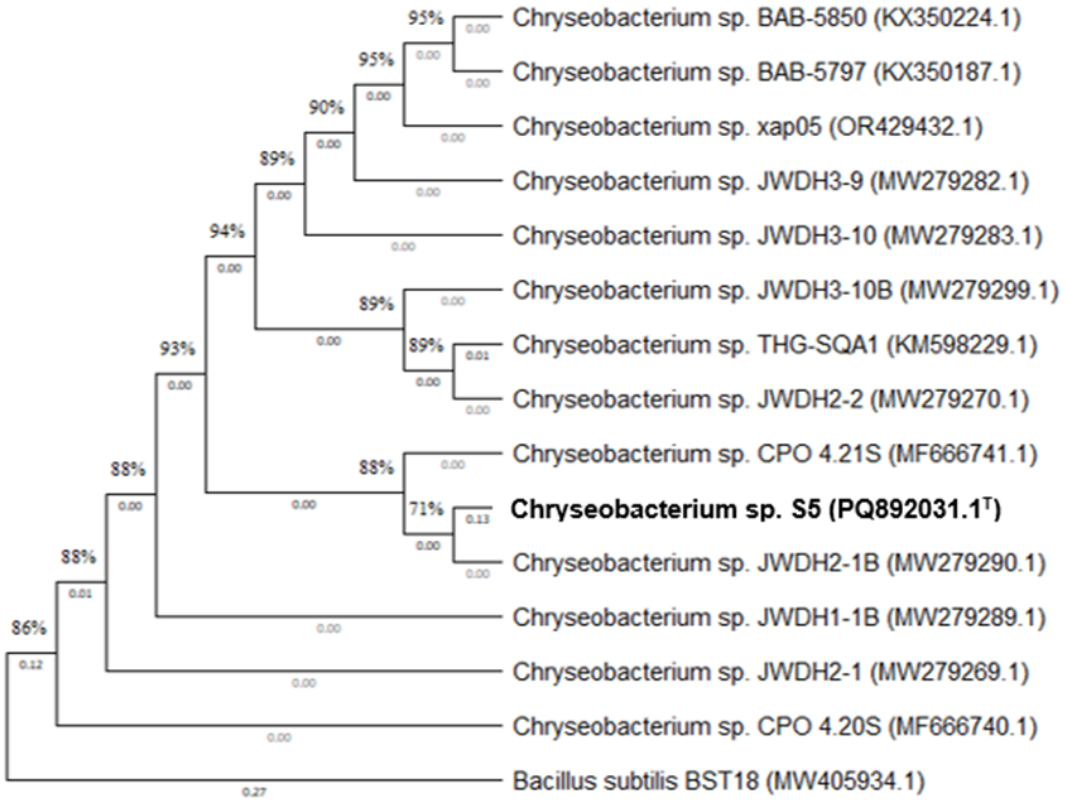

FIGURE 4.17: Phylogenetic analysis of *Chryseobacterium* sp. S5 (PQ892031.1^T) from CS sample

The phylogenetic tree shows that *Chryseobacterium* sp. S5 (PQ892031.1) clusters with *Chryseobacterium* sp. JWDH2-1B (MW279290.1) and *Chryseobacterium* sp. JWDH1-1B (MW279289.1) with a weak bootstrap support of 71%, indicating some divergence within this subgroup. This cluster is part of a larger clade with *Chryseobacterium* sp. JWDH2-1 (MW279269.1) and *Chryseobacterium* sp. CPO 4.20S (MF666740.1), supported by a moderate bootstrap value of 88%. Higher up, *Chryseobacterium* sp. S5 shares ancestry with *Chryseobacterium* sp. JWDH2-2 (MW279270.1) and *Chryseobacterium* sp. CPO 4.21S (MF666741.1) within a clade supported by 89%. The strongest bootstrap values (above 90%) support the grouping of *Chryseobacterium* sp. strains in the upper branches, particularly *Chryseobacterium* sp. BAB-5850 (KX350224.1) and *Chryseobacterium* sp. BAB-5797 (KX350187.1) at 95%. The tree includes *Bacillus subtilis* BST18 (MW405934.1) as an outgroup, showing significant divergence from *Chryseobacterium* sp., supported by a long branch. Overall, the bootstrap values indicate strong confidence in most

clades, while *Chryseobacterium* sp. S5 is moderately supported in its subgroup, suggesting evolutionary distinctiveness within *Chryseobacterium* species.

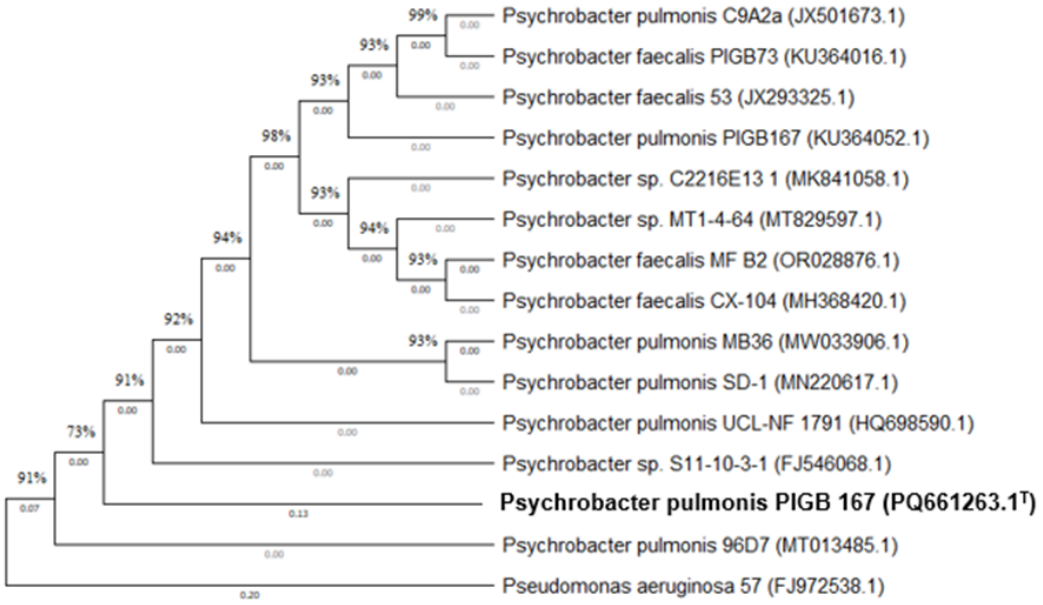

FIGURE 4.18: Phylogenetic analysis of *Psychrobacter pulmonis* PIGB 167 (PQ661263.1^T) from CS sample

The phylogenetic tree reveals that *Psychrobacter pulmonis* PIGB 167 (PQ661263.1) clusters with *Psychrobacter* sp. S11-10-3-1 (FJ546068.1) with strong bootstrap support of 91%, indicating a well-supported evolutionary relationship. This cluster is part of a larger clade that includes *Psychrobacter pulmonis* UCL-NF 1791 (HQ698590.1) and *Psychrobacter pulmonis* SD-1 (MN220617.1), supported by a moderate bootstrap value of 73%, suggesting some genetic divergence within the group. Further, *Psychrobacter pulmonis* MB36 (MW033906.1) and *Psychrobacter faecalis* CX-104 (MH368420.1) form a highly supported clade with values above 93%. The highest bootstrap values (98-99%) support the close relationships among *Psychrobacter pulmonis* and *Psychrobacter faecalis* strains, particularly *Psychrobacter pulmonis* C9A2a (JX501673.1) and *Psychrobacter faecalis* PIGB73 (KU364016.1). The tree includes *Pseudomonas aeruginosa* 57 (FJ972538.1) as an outgroup, which diverges significantly from *Psychrobacter* species, confirmed by a long branch. The bootstrap values indicate strong confidence in most evolutionary relationships, with *Psychrobacter pulmonis* PIGB 167 showing close ties to

other *Psychrobacter* species but also some divergence, suggesting distinct genetic variation within this lineage.

4.7 Antibiotic Sensitivity

Different antibiotics were used to test the sensitivity of bacterial species. These include Doxycycline, Ceftriaxone, Streptomycin, and Imipenem. The results are shown in Figure 4.19.

TABLE 4.4: Results of Antibiotic Sensitivity Test

Antibiotic	**NAP**	**MAP**	**EMBP**	**NACY**	**MACP**	**MACB**	**EMBC**
Doxycycline	0	0	0	0	0	0	5mm
Ceftriaxone	0	0	0	0	0	2mm	0
Streptomycin	0	0	0	0	0	4mm	0
Imipenem	0	5mm	10mm	15mm	5mm	8mm	6mm

TABLE 4.5: Variance analysis showing significant showing significant effect of treatment on response variable F-value and p-value.

Source	**Sum of Squares (SS)**	**Df**	**F-value**	**p-value**
Antibiotic	2.2086	3	10.36	0.00015
Residual	1.7057	24	-	-

Table 4.4 and 4.5 show antibiotic sensitivity results which depicts that Imipenem is significantly more effective than other antibiotics, with the highest zone of inhibition ranging from 5mm to 15mm in all samples except NAP. However, Doxycline, Ceftriaxone, and Streptomycin showed little to no zone of inhibition. The statistical analysis supports this with an F-value of 10.36 and a highly significant p-value 0.00015 confirming that Imipenem differs significantly in its inhibitory effect. The sum of squares (SS) values indicate greater variance between antibiotic groups than within-group variance, reinforcing that Imipenem is the most potent antibiotic, while the others show minimal or no inhibition against the tested bacterial strains.

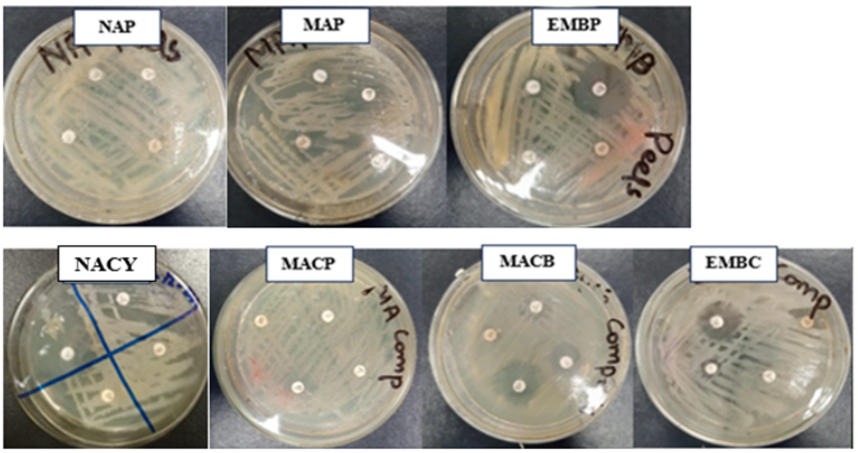

FIGURE 4.19: Results of bacterial pigments showing maximum ZOI against Imipenem

4.8 Pigment Extraction

Microbial pigments from FVP and CS were extracted by adding methanol. The Table 4.6 and Figure 4.20 shows the extracted pigments of different colors from the differential media.

TABLE 4.6: Results of extracted pigments from selective and differential media

S. No.	Source	Sample ID	Agar Media	Pigment
1	FVP	NAP	Nutrient Agar	Green
2		MAP	MacConkey Agar	Pinkish red
3		EMBP	Eosin Methyl Blue	Dark blue
4	CS	NACY	Nutrient Agar	Yellow
5		MACP	MacConkey Agar	Pinkish Orange
6		MACB	MacConkey Agar	Brown
7		EMBC	Eosin Methyl Blue	Purple

The pigments extracted from FVP samples showed green pigment from NAP, dark pink pigment from MAP (*Klebsiella* sp. KL-1) and blue pigment from EMBP (*Kerstersia gyiorum*). Whereas, pigments extracted from CS samples showed yellow pigment from NACY (*Chryseobacterium* sp. S5), pinkish orange pigment from MACP, brown pigment from MACB (*Psychrobacter pulmonis* strain PIGB167), and purple pigment from EMBC.

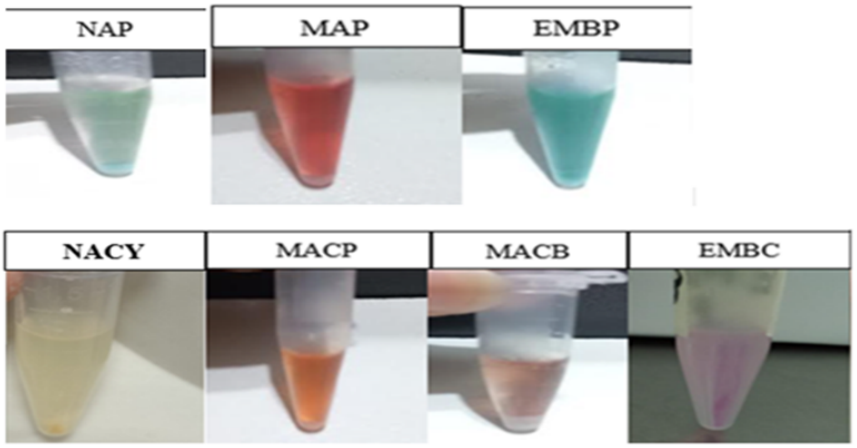

FIGURE 4.20: Bacterial pigments isolated from FVP and CS

4.9 Characterization of Pigments

4.9.1 UV-Vis Spectrophotometry

The extracted pigments were subjected to UV-vis spectrophotometry to detect the transmission and absorption of pigments within the 400nm to 700nm wavelength range. The results are shown in appendix A and Figures 4.21 and 4.22.

The pigments extracted from FVP samples exhibit distinct absorption patterns. NAP graph (A) represented by green curve showed maximum absorption 0.91 and gradually decreases towards 700nm with significant dip at 600nm. MAP (*Klebsiella* strain KL-1) graph (B) indicated with pink curve shows absorption fluctuations from 400-600nm, with maximum absorption of 0.24 at 450nm and 0.23 at 600nm respectively, before decreasing from 600-700nm. EMBP (*Kerstersia gyiorum*) graph (C) indicated by blue curve exhibit high fluctuation absorption pattern with multiple peaks. It showed maximum absorption values of 0.4 at 400nm, 0.44 at 500nm, and 0.3 at 650nm followed by a decline at 700nm.

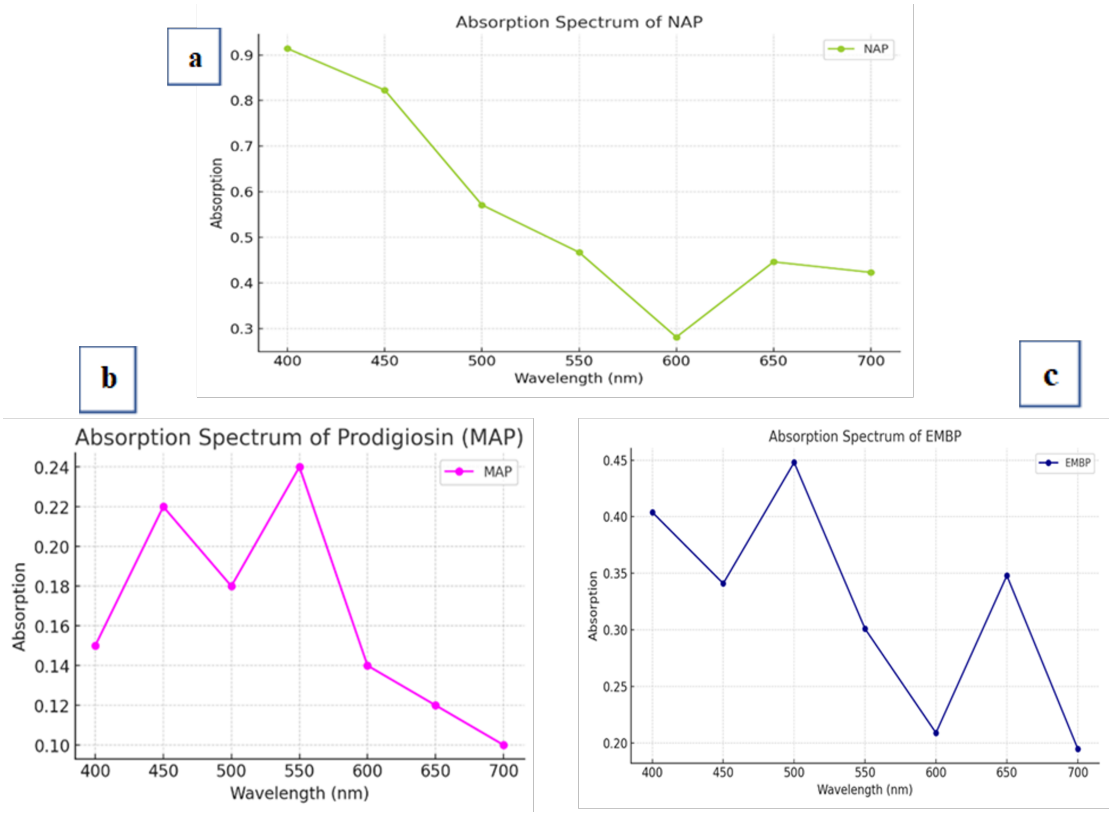

FIGURE 4.21: Graph showing Absorption Spectrum of (a) NAP, (b) MAP (*Klebsiella* strain KL-1), and (c) EMBP (*Kerstersia gyiorum*) across wavelengths 400-700nm.

The pigment extracted from CS samples exhibit different absorption patterns. NACY (*Chryseobacterium* sp. S5) graph (a) represented by yellow curve shows maximum absorption of 0.8 at 400nm, gradually decreasing towards 700nm. MACP graph (b) represented by orange curve followed a similar decreasing pattern with a peak absorption of 0.5 at 400nm, declining steadily. MACB (*Psychrobacter pulmonis* strain PIGB167) graph (c) represented by brown curve exhibits a highly variable pattern maintain low absorption from 400-550nm, followed by a sharp peak of 0.6 at 570nm, then decreasing. EMBC graph (d) represented by purple curve displays multiple peaks with the highest absorption of 0.5 at 520nm, along with other fluctuations.

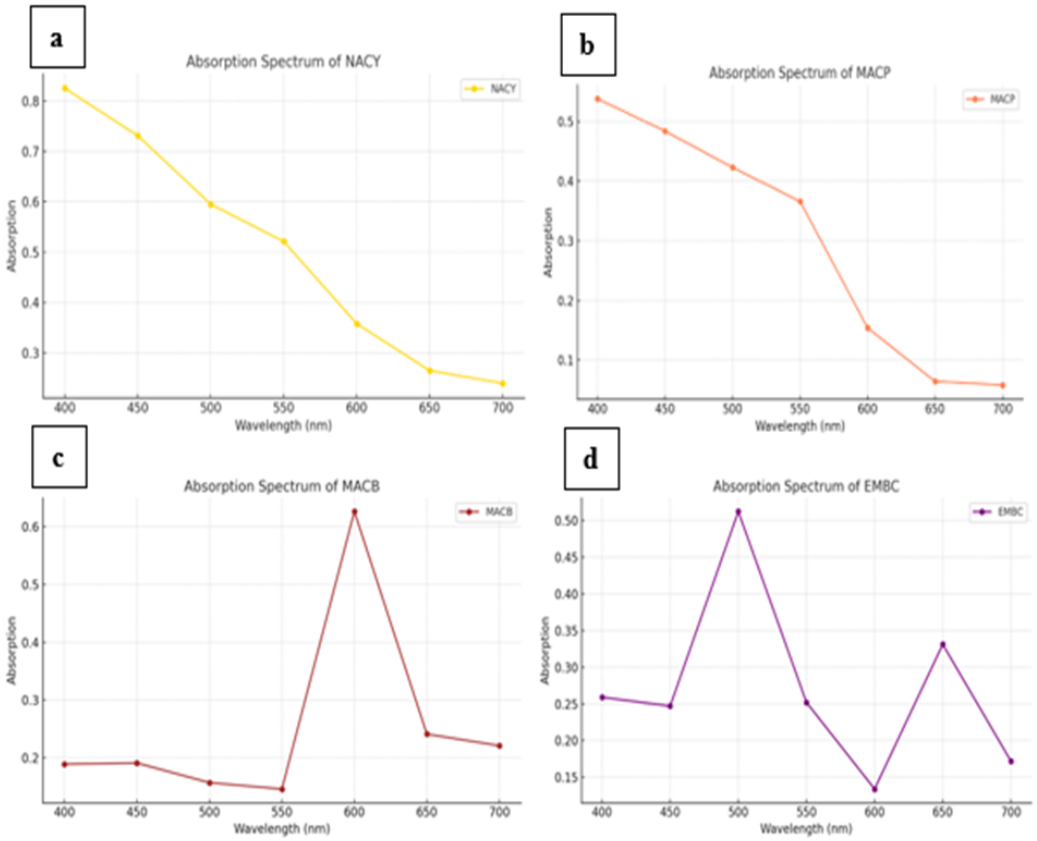

FIGURE 4.22: Graph showing Absorption Spectrum of (a) NACY (*Chryseobacterium* sp. S5) (b) MACP (c) MACB (*Psychrobacter pulmonis* strain PIGB167) and (d) EMBC across wavelengths 400-700nm.

4.9.2 FTIR Analysis

The molecular analysis of bacterial pigments was analyzed through FTIR technique. The results of FTIR are as shown in Table 4.7 and Figures 4.23, 4.24, 4.25, 4.26, 4.28, and 4.29 [209].

TABLE 4.7: Results of FTIR analysis

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond		Functional Group
1	*Pseudomonas nitroreducens*	3328.74	70.88	H-bonded Stretch	OH	Hydroxy group
		2945.19	80.19	C-H Stretch		Methyl

Table 4.7 continued from previous page

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond	Functional Group
		2833.04	81.88	C-H Stretch	Methyl
		1654.92	94.80	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1449.03	85.46	C-H bend	Methylene
		1410.55	86.15	C-H in-plane Bend	Vinyl
		1115.06	88.44	C-C Stretch	>CH-Methyne
		1020.36	25.95	C-C Stretch	>CH-Methyne
		622.77	63.05	C-H bend	Alkyne
		595.34	62.66	C-S Stretch	Disulphides
		578.75	63.36	C-S Stretch	Disulphides
		567.32	63.30	C-I Stretch	Aliphatic iodo Compounds
		560.91	60.85	C-I Stretch	Aliphatic iodo Compounds
		546.54	58.91	C-I Stretch	Aliphatic iodo Compounds
		535.15	61.38	C-I Stretch	Aliphatic iodo Compounds
		528.73	57.25	C-I Stretch	Aliphatic iodo Compounds
		521.41	65.88	C-I Stretch	Aliphatic iodo Compounds
2	*Klebsiella* sp.	3331.91	68.78	H-bonded OH Stretch	Hydroxy group
		2946.76	81.84	C-H Stretch	Methyl
		2834.46	84.23	C-H Stretch	Methyl
		1652.14	91.10	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1449.14	86.03	C-H bend	Methylene
		1410.30	86.14	C-H in-plane Bend	Vinyl
		1114.64	88.13	C-C Stretch	CH-Methyne
		1018.21	30.72	C-C Stretch	CH-Methyne
		617.84	54.28	C-H bend	Alkyne
		607.07	55.83	S-S Stretch	Disulphides
		584.20	54.64	C-S Stretch	Disulphides
		574.75	54.69	C-S Stretch	Disulphides

Table 4.7 continued from previous page

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond	Functional Group
		567.58	53.97	C-I Stretch	Aliphatic iodo Compounds
		555.87	52.92	C-I Stretch	Aliphatic iodo Compounds
		550.62	50.76	C-I Stretch	Aliphatic iodo Compounds
		538.16	51.33	C-I Stretch	Aliphatic iodo Compounds
		523.23	31.85	C-I Stretch	Aliphatic iodo Compounds
		517.10	124.71	C-I Stretch	Aliphatic iodo Compounds
3	*Kerstersia gyoirum*	3321.02	71.55	H-bonded OH Stretch	Hydroxy group
		2944.85	80.62	C-H Stretch	Methyl
		2832.89	82.06	C-H Stretch	Methyl
		1652.02	95.34	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1449.25	85.53	C-H bend	Methylene
		1414.96	86.34	C-H in-plane Bend	Vinyl
		1115.64	88.67	C-C Stretch	>CH-Methyne
		1020.50	25.34	C-C Stretch	>CH-Methyne
		616.75	62.85	C-H bend	Alkyne
		595.16	61.95	C-S Stretch	Disulphides
		567.05	61.26	C-I Stretch	Aliphatic iodo Compounds
		559.74	61.49	C-I Stretch	Aliphatic iodo Compounds
		553.59	64.02	C-I Stretch	Aliphatic iodo Compounds
		541.19	64.08	C-I Stretch	Aliphatic iodo Compounds
		529.09	61.13	C-I Stretch	Aliphatic iodo Compounds
		522.62	56.97	C-I Stretch	Aliphatic iodo

Table 4.7 continued from previous page

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond	Functional Group
4	*Chryseobacterium*	3305.70	67.38	C-H Stretch	Alkyne
		2946.12	81.33	C-H Stretch	Methyl
		2834.28	83.19	C-H Stretch	Methyl
		1654.79	90.34	R-CO-NH2 Stretch	Carbonyl (Amide)
		1449.16	84.55	C-H bend	Methylene
		1411.67	84.91	C-H in-plane Bend	Vinyl
		1113.84	86.96	C-C Stretch	>CH-Methyne
		1017.49	29.89	C-C Stretch	>CH-Methyne
		630.87	54.95	C-H bend	Alkyne
		593.05	53.68	C-S Stretch	Disulphides
		579.11	52.73	C-S Stretch	Disulphides
		571.75	51.88	C-S Stretch	Disulphides
		545.10	54.19	C-I Stretch	Aliphatic iodo Compounds
		536.25	56.35	C-I Stretch	Aliphatic iodo Compounds
		526.53	50.43	C-I Stretch	Aliphatic iodo Compounds
		519.08	9.83	C-I Stretch	Aliphatic iodo Compounds
5	*Serratia marcescens*	3319.99	71.12	H-bonded OH Stretch	Hydroxy group
		2944.36	79.78	C-H Stretch	Methyl
		2832.74	81.44	C-H Stretch	Methyl
		1654.52	95.70	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1448.99	85.54	C-H Bend	Methylene
		1414.41	86.39	C-H in-plane Bend	Vinyl
		1114.10	88.92	C-C Stretch	>CH-Methyne
		1020.55	25.07	C-C Stretch	>CH-Methyne
		547.21	63.52	C-I Stretch	Aliphatic iodo Compounds
		584.78	63.09	C-I Stretch	Aliphatic iodo Compounds

Table 4.7 continued from previous page

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond	Functional Group
		531.44	63.26	C-I Stretch	Aliphatic iodo Compounds
		616.00	63.57	C-H bend	Alkyne
		567.98	64.69	C-I Stretch	Aliphatic iodo Compounds
		539.29	66.48	C-I Stretch	Aliphatic iodo Compounds
		522.87	42.21	C-I Stretch	Aliphatic iodo Compounds
6	*Psychrobacter pulmonis*	3321.52	68.82	H-bonded OH Stretch	Hydroxy group
		2947.38	81.27	C-H Stretch	Methyl
		2834.35	84.15	C-H Stretch	Methyl
		1651.55	90.87	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1449.36	85.88	C-H bend	Methylene
		1408.42	85.88	OH bend	(Hydroxy) Phenol
		1113.57	87.68	C-C Stretch	>CH-Methyne
		1017.97	30.58	C-C Stretch	>CH-Methyne
		614.13	56.36	C-H bend	Alkyne
		594.80	55.00	C-S Stretch	Disulphides
		578.11	55.04	C-S Stretch	Disulphides
		548.09	50.94	C-I Stretch	Aliphatic iodo Compounds
		535.71	53.97	C-I Stretch	Aliphatic iodo Compounds
		530.33	54.59	C-I Stretch	Aliphatic iodo Compounds
		522.70	49.90	C-I Stretch	Aliphatic iodo Compounds
		519.22	53.30	C-I Stretch	Aliphatic iodo Compounds
7	*Acinetobacter* sp.	3323.09	71.21	H-bonded OH Stretch	Hydroxy group
		2945.21	80.21	C-H Stretch	Methyl

Table 4.7 continued from previous page

#	Bacterial Pigment	Wave Number (cm^{-1})	Transparency (%)	Bond	Functional Group
		2832.99	82.23	C-H Stretch	Methyl
		1655.13	94.60	R-CO-NH_2 Stretch	Carbonyl (Amide)
		1449.03	85.43	C-H bend	Methylene
		1409.19	86.09	OH bend	(Hydroxy) Phenol
		1114.49	88.25	C-C Stretch	>CH-Methyne
		1020.13	26.01	C-C Stretch	>CH-Methyne
		611.22	62.14	C-H bend	Alkyne
		599.58	61.74	C-S Stretch	Disulphides
		585.91	61.32	C-S Stretch	Disulphides
		560.23	59.34	C-I Stretch	Aliphatic iodo Compounds
		546.92	59.85	C-I Stretch	Aliphatic iodo Compounds
		529.00	65.36	C-I Stretch	Aliphatic iodo Compounds
		519.73	10.69	C-I Stretch	Aliphatic iodo Compounds

The FTIR analysis of bacterial pigments isolated from FVP and CS samples revealed a diverse range of functional groups. NAP from FVP sample exhibited functional groups with wave numbers such as hydroxyl (OH,3328.74 cm^{-1}), methyl/methylene (-CH3, -CH2, 2945.19, 2833.04, 1449.03 cm^{-1}), amide (-C=O, 1654.92 cm^{-1}), and vinyl (-CH=CH2, 1410.55 cm^{-1}), along with C-H bending (622.77 cm^{-1}), C-C stretches (115.06, 1020.36 cm^{-1}), and C-S (595.34, 578.75 cm^{-1}), and C-I (560.91-521.41 cm^{-1}) bonds, revealed alcohols, alkanes , proteins, unsaturated carbons and halogenated compounds.

Similarly, *Klebsiella* sp. KL-1 from FVP identified H-bonded OH (3331.91 cm^{-1}), methyl (2946.76, 2834.46 cm^{-1}), carbonyl amide (1652.41 cm^{-1}), methylene (1449. 14 cm^{-1}), vinyl (1410.30 cm^{-1}), and alkyne (617.84 cm^{-1}) structures with disulfides (607.07, 584.20, 574.75 cm^{-1}) and strong C-I absorption (567.58-517.10 cm^{-1}).

Kerstersia gyiorum from FVP sample showed a similar composition, including H-bonded OH (3321.02 cm^{-1}), methyl (2944.85, 2832.89 cm^{-1}), carbonyl amide (1652.02 cm^{-1}), vinyl (1414.96 cm^{-1}), along with methyne (1115.64, 1020.50 cm^{-1}), alkyne (616.75 cm^{-1}), disulfide (595.16 cm^{-1}) and aliphatic iodo compounds (567.05-522.62 cm^{-1}).

However, MACP from CS sample contained similar functional groups including H-bonded OH (3319.99 cm^{-1}), carbonyl amide (1654.52 cm^{-1}), and aliphatic iodo compounds (547.21-522.87 cm^{-1}).

Psychrobacter pulmonis PIGB 167 from CS sample exhibited H-bonded OH (3321.52 cm^{-1}), methyl (2947.38, 2834.35 cm^{-1}), carbonyl amide (1651.55 cm^{-1}), methylene (1449.36 cm^{-1}), phenol (1408.42 cm^{-1}), alkyne (614.13 cm^{-1}), disulfide (594.80, 578.11 cm^{-1}) and aliphatic iodo compounds (548.09-519.22 cm^{-1}), while EMBC from CS sample exhibited phenol (1409.19 cm^{-1}), alkyne (611.22 cm^{-1}), disulfides (599.58, 585.91 cm^{-1}), and aliphatic iodo compounds (560.23-519.73 cm^{-1}).

Overall, these pigment analysis highlights functional groups such as hydroxyl, carbonyl, sulfur-rich and halogenated compounds.

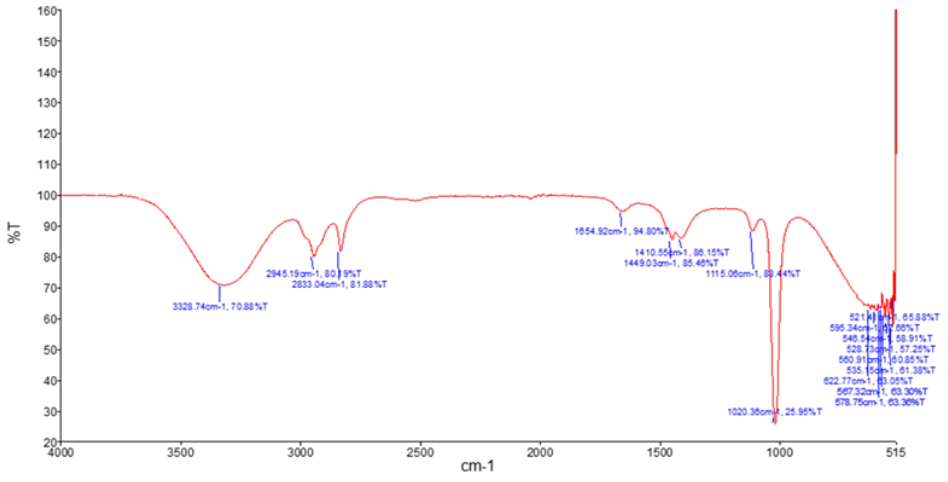

FIGURE 4.23: FTIR results of NAP (*Pseudomonas nitroreducens*)

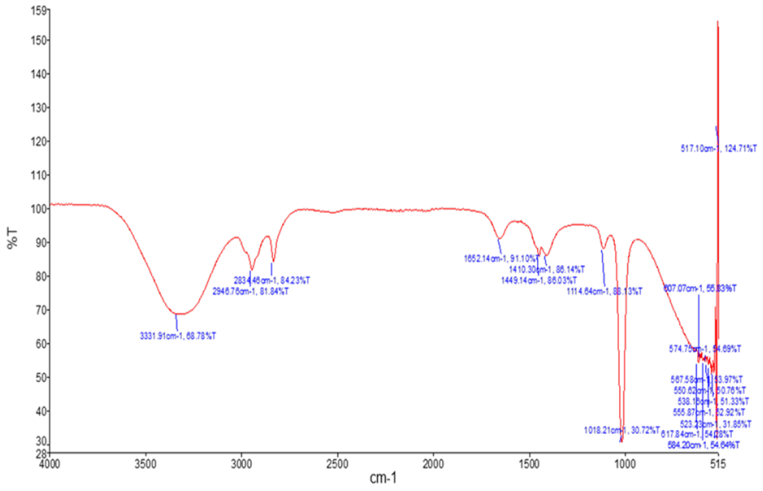

FIGURE 4.24: FTIR results of MAP (*Klebsiella* sp. Strain KL-1)

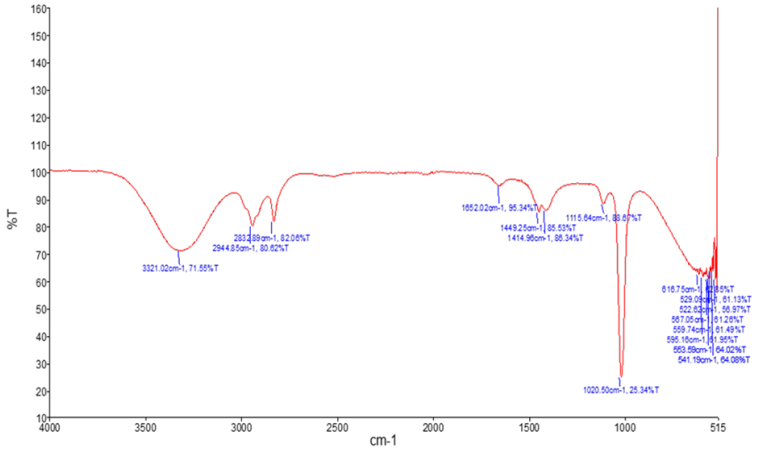

FIGURE 4.25: FTIR results of EMBP (*Kerstersia gyiorum*)

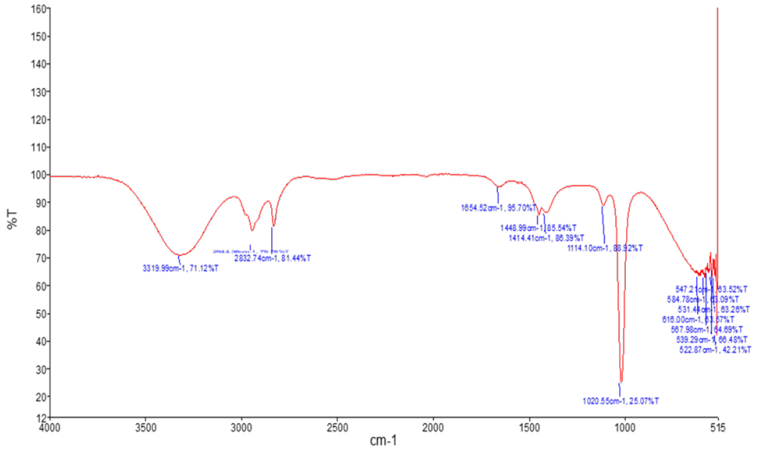

FIGURE 4.26: FTIR results of NACY (*Chryseobacterium* sp. strain S5)

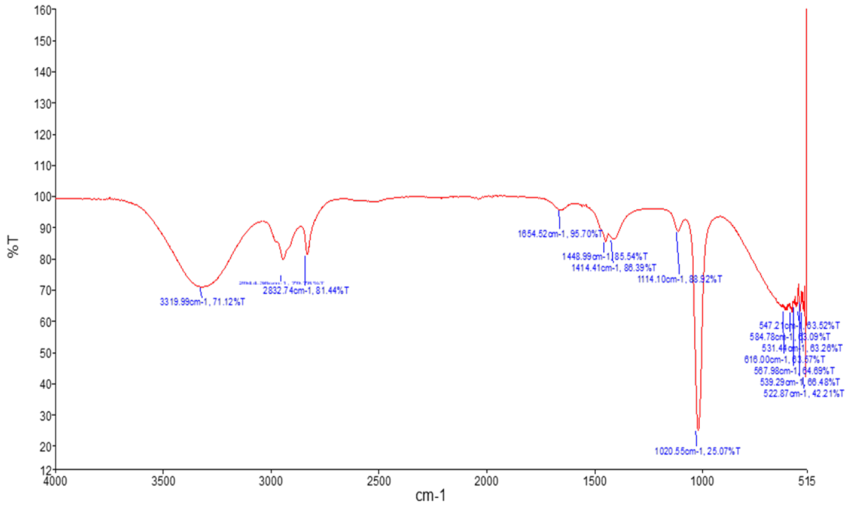

FIGURE 4.27: FTIR results of MACP(Serratia marcescens)

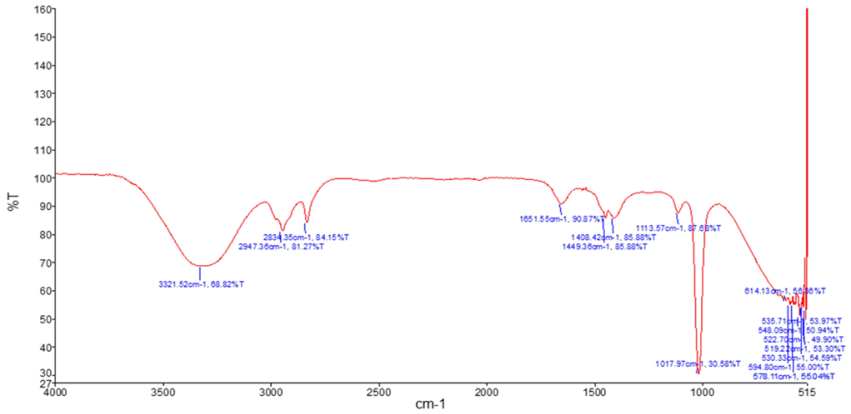

FIGURE 4.28: FTIR results of MACB (*Psychrobacter pulmonis* strain PIGB167)

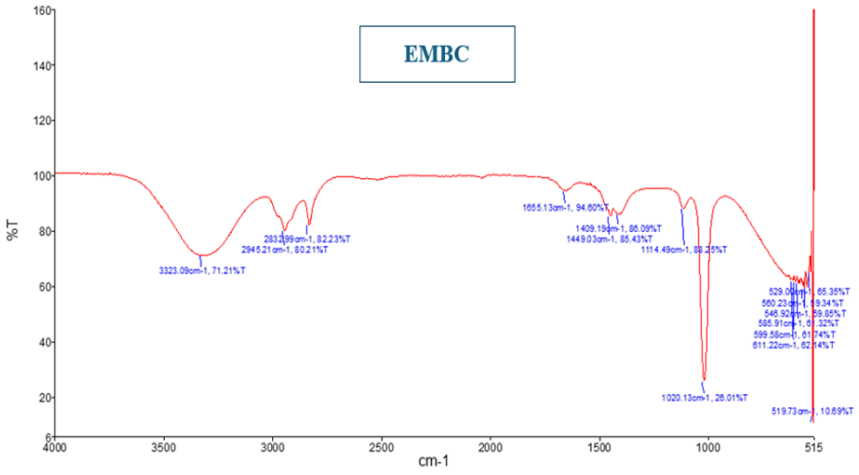

FIGURE 4.29: FTIR results of EMBC (*Acinetobacter* sp.)

4.9.3 HPLC Analysis

The HPLC results of bacterial pigments from FVP and CS samples are shown in Tables 4.8 and 4.9 below and Figures 4.30 and 4.31.

TABLE 4.8: HPLC results of FVP samples.

Peak No.	**Retention Time (min)**	**Standard Retention Time Range (min)**	**Reference Standard**	**Identified Compound**	**Ref**
1	2.219	2.2 - 2.4	Acetic Acid	Acetic Acid	[210]
2	2.709	2.7 - 2.8	Lactic Acid	Lactic Acid	[210]
3	3.196	3.1 - 3.3	Succinic Acid	Succinic Acid	[210]
4	3.740	3.6 - 3.8	Malic Acid	Malic Acid	[211]
5	4.702	4.6 - 4.8	Fumaric Acid	Fumaric Acid	[212]
6	5.551	5.5 - 5.6	Adipic Acid	Adipic Acid	[213]
7	7.617	7.6 - 7.7	Benzoic Acid	Benzoic Acid	[214]
8	10.308	10.3 - 10.6	Propanoic acid	Propanoic Acid	[215]

The absorption spectrum of pigments extracted from *Kelbsiella* sp., and *Kersteria gyiorum* (FVP) samples was obtained at 250 nm. The HPLC profile revealed 8 peaks with retention time 2.219 (Acetic acid), 2.709 (Lactic acid), 3.196 (Succinic acid), 3.740 (Malic acid), 4.702 (Fumaric acid), 5.551 (Adipic acid), 7.617 (Benzoic acid), and 10.308 (Propanoic acid) as given in Table 4.8 and Figure 4.30.

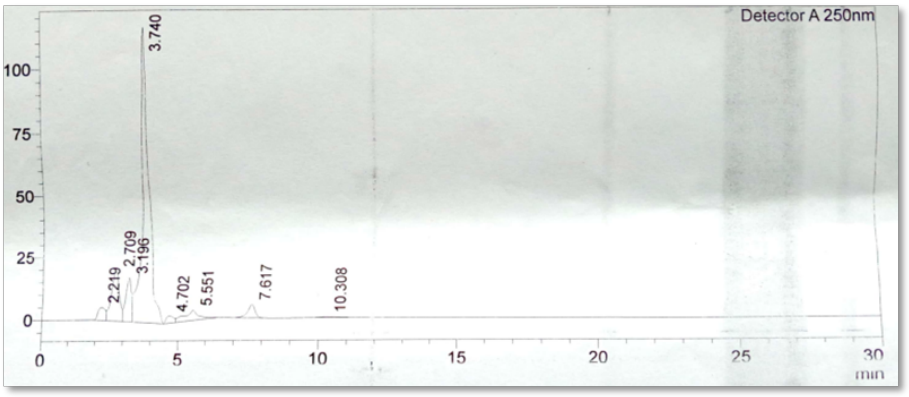

FIGURE 4.30: HPLC graph of FVP samples

TABLE 4.9: HPLC results of bacterial pigments from soil samples

Peak No.	**Retention Time (min)**	**Standard Retention Time Range (min)**	**Reference Standard**	**Identified Compound**	**Ref**
1	2.815	2.7- 2.9	Zeaxanthin	Zeaxanthin	[216]
2	3.158	3.1 - 3.3	Tyrosol	Tyrosol	[217]
3	3.585	3.5 - 3.7	Pyocyanin	Pyocyanin	[218]
4	4.008	4.0 - 4.2	Catechol	Catechol	[219]
5	4.198	4.1 - 4.3	Eumelanin	Eumelanin	[220]
6	4.753	4.7 - 4.8	Lutien	Lutein	[221]
7	5.678	5.6 - 5.8	Carotenoids	Carotenoids	[222]
8	7.619	7.6 - 7.7	β-Carotene	β-Carotene	[221]
9	16.754	16.7 - 16.9	Rutin	Rutin	[223]

The absorption spectrum of pigment extracted from *Chryseobacterium* sp. S5 and *Psychrobacter pulmonis* PIGB 167 (CS) samples was obtained at 250 nm.

The HPLC profile revealed 9 peaks with retention time 2.815 (Zeaxanthin), 3.158 (Tyrosol), 3.585 (Pyocyanin), 4.008 (Catechol), 4.198 (Eumelanin), 4.753 (Lutien), 5.678 (carotenoids), 7.619 (β-Carotene), and 16.754 (Rutin) as given in Table 4.9 and Figure 4.31.

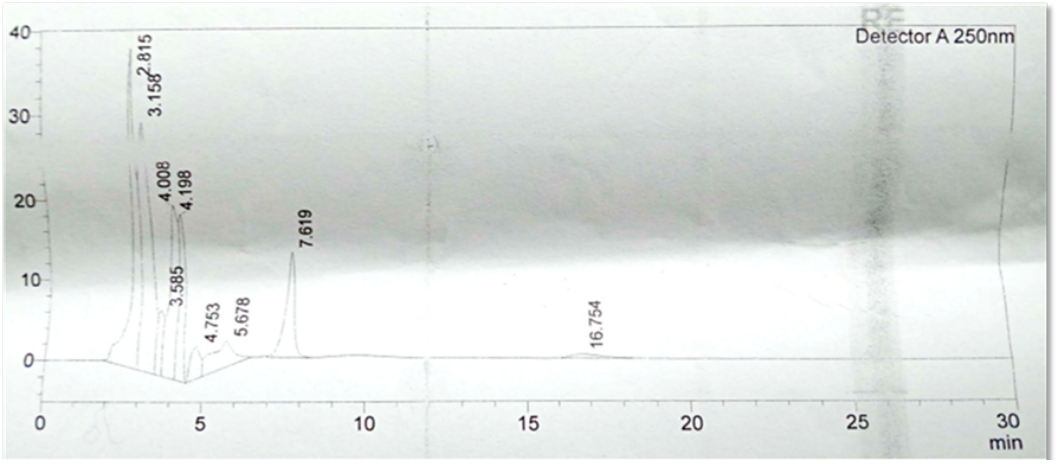

FIGURE 4.31: HPLC graph of CS samples

4.10 Biological Evaluation

4.10.1 Antibacterial Activity

The antibacterial assay was employed for both bacteria and bacterial pigments. Table 4.10 and Figure 4.32 below show the results of the antibacterial activity of pigments.

TABLE 4.10: Results of antibacterial activity of pigments against *S. aureus* and *K. gyiorum*

		Pigment								
Src	**Bacteria Pigment**	***S. aureus***			***K. gyiorum***			**Positive Control**		**Negative Control (Methanol) (mm)**
		C[1]	**C[2] (mm)**	**C[3]**	**C[1]**	**C[2] (mm)**	**C[3]**	**(Ampicillin) (mm)**	**(Ciprofloxacin) (mm)**	
FVP	(Pyocyanin) *Pseudomonas nitroreducens*	56	0	0	0	0	0	20	30	0
	(Prodigisin) *Klebsiella* sp.	0	0	0	0	0	0	20	30	0
	(Pyocyanin) *Kerstersia gyoirum*	0	0	0	0	0	0	20	30	0
CS	(Flexirubin) *Chryseobacterium* sp.	5	0	0	0	0	0	20	30	0
	(Carotenoids) *Serratia marcescens*	0	0	0	0	0	0	20	30	0
	(Carotenoids) *Psychrobacter pulmonis*	15	10	0	5	0	20	20	30	0

Table 4.10 continued from previous page

Src	Bacteria Pigment	Pigment								
		S. aureus			***K. gyiorum***			**Positive Control**		**Negative Control**
		C^1	**C^2**	**C^3**	**C^1**	**C^2**	**C^3**	**(Ampicillin) (mm)**	**(Ciprofloxacin) (mm)**	**(Methanol) (mm)**
			(mm)			**(mm)**				
	(Violacein) *Acinetobacter*	0	0	0	0	0	10	20	30	0

C^1 (1000μg/ml), C^2 (750μg/ml), C^3 (500μg/ml).

TABLE 4.11: Statistical analysis (ANOVA) of pigments for antibacterial

Group	Degrees of Freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	F-Statistic	P-Value
Between Groups	7	25.5512	3.6502	5.7645	0.0001
Within Groups	48	30.3943	0.6332		
Total	55	55.9455			

The results of the antibacterial activity of pigments indicate that NAP (56mm), *Psychrbacter pulmonis* (15mm) at C^1, and (10mm) at C^2 showed the highest inhibition against *S. aureus*. In comparison, other pigments exhibit minimal such as *Chryseobacterium* sp. (5mm) or no inhibition activity. *K. gyiorum* was largely unaffected by all test pigments except *P. pulmonis* exhibited a zone of inhibition (5mm) at C^1 and (20mm) at C^3, and EMBC (10mm) at C^3.

The ANOVA (F = 5.7645, P = 0.0001) results confirm statistically significant differences among the pigment groups.

The positive control Ampicillin (20mm) for *S. aureus* and Ciprofloxacin (3mm) for *K. gyiorum* consistently showed inhibition, validating the experimental setup.

The negative control methanol had no effect further confirming the accuracy of the results.

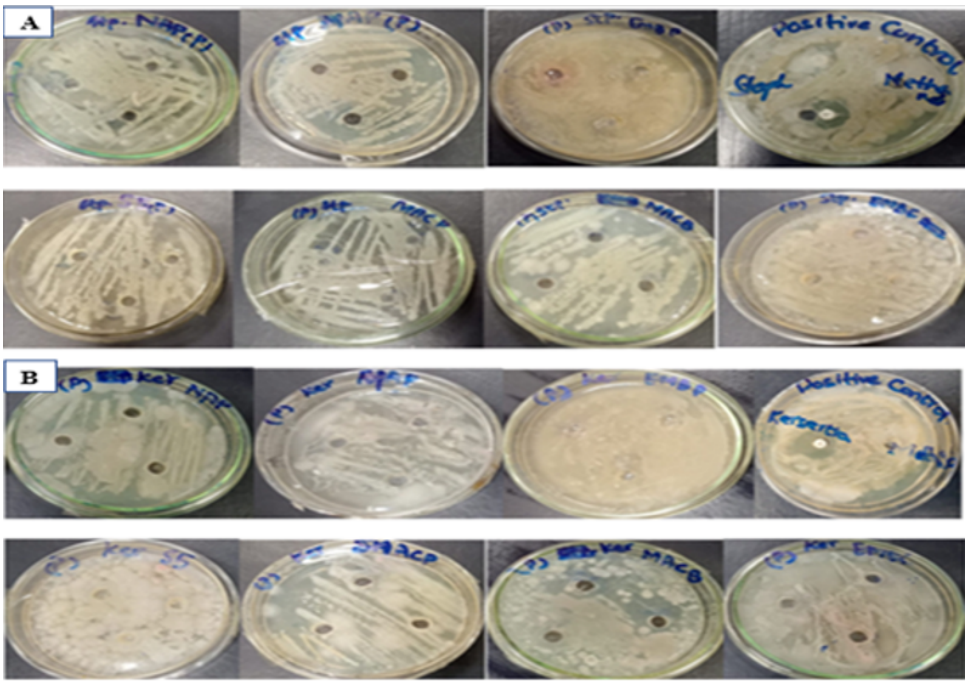

FIGURE 4.32: Antibacterial results of pigments against (A) *S. aureus* and (B) *K. gyiorum*

Table 4.12 and Figure 4.33 below show the results of the antibacterial activity of pigmented bacteria.

TABLE 4.12: Results of antibacterial activity of pigmented bacteria against *S. aureus* and *K. gyiorum*

		Pigmented Bacteria								
Src.	**Bacteria**	**S. aureus**			**K. gyiorum**			**Positive Control**		**Negative Control**
		C^1	**C^2**	**C^3**	**C^1**	**C^2**	**C^3**	**(Ampicillin) (mm)**	**(Ciprofloxacin) (mm)**	**(Methanol) (mm)**
			(mm)			**(mm)**				
FVP	*Pseudomonas nitroreducens*	0	0	0	0	0	0	20	30	0
	Klebsiella sp.	0	0	0	0	0	14.6	20	30	0
	Kerstersia gyoirum	0	0	0	0	0	0	20	30	0
CS	*Chryseobacterium* sp.	0	0	0	5	0	0	20	30	0
	Serratia marcescens	0	0	0	0	0	0	20	30	0

Table 4.12 continued from previous page

Src.	Bacteria	Pigmented Bacteria								
		S. aureus			K. gyiorum			Positive Control		Negative Control
		C^1	C^2 (mm)	C^3	C^1	C^2 (mm)	C^3	(Ampicillin) (mm)	(Ciprofloxacin) (mm)	(Methanol) (mm)
	Psychrobacter pulmonis	0	35	0	0	0	0	20	30	0
	Acinetobacter	0	0	0	0	0	6	20	30	0

C^1 (1000μg/ml), C^2 (750μg/ml), C^3 (500μg/ml).

TABLE 4.13: Statistical analysis of pigment bacteria for anti-bacterial.

Group	Degrees of Freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	F-Statistic	P-Value
Between Groups	7	23.2062	3.3152	12.6295	4.6938e-9
Within Groups	48	12.5997	0.2625		
Total	55	35.8059			

The anti-bacterial results of pigmented bacteria at different concentrations varied significantly, as indicated by ANOVA results (F=12.6295, P= 4.6938e-9). The p-value corresponding to the F-statistic of one-way ANOVA is lower than 0.05, suggesting that one or more treatments are significantly different. At C^1 only *Chryseobacterium* sp. showed inhibition (5mm) against *K. gyiorum*, while at C^2 *P. pulmonis* showed inhibition (35mm) against *S. aureus*.

However, at C^3 *Klebsiella* sp. showed (14.6mm) and EMBC showed (6mm) inhibition against *K. gyiorum*. The positive control Ampicillin (20mm) for *S. aureus* and Ciprofloxacin (30mm) for *K. gyiorum* consistently showed inhibition, validating the experimental setup. The negative control methanol had no anti-bacterial effect further confirming the accuracy of the results.

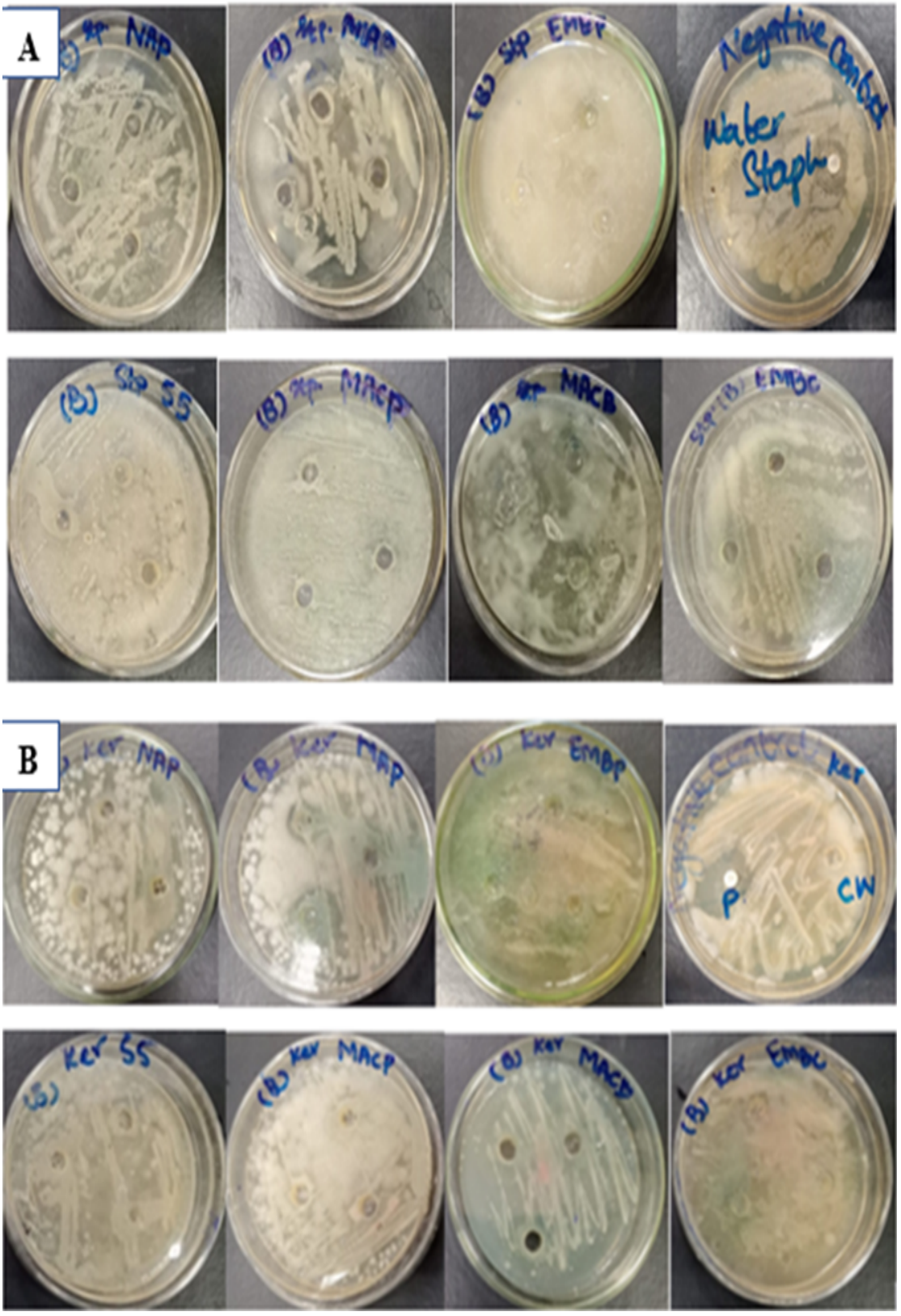

FIGURE 4.33: Antibacterial results of pigmented bacteria against (A) *S. aureus* and (B) *K. gyiorum*

4.10.2 Antifungal Activity

The anti-fungal activity of both bacterial pigments and bacteria was employed against *Aspergillus fumigatus* and *Rhizopus delemar*.

Table 4.14 and Figure 4.34 show the antifungal results of bacterial pigments.

TABLE 4.14: Anti-fungal results of bacterial pigments

		Pigments							
Src	**Bacteria Pigment**	***A. fumigatus***			***R. delemar***			**+ive Control**	**-ive Control**
		C^1	**C^2**	**C^3**	**C^1**	**C^2**	**C^3**	**(Amphotericin B) (mm)**	**(Methanol) (mm)**
		(mm)			**(mm)**				
FVP	(Pyocyanin) *Pseudomonas nitroreducens*	30	25	25	6	14	15	8	4
	(Prodigisin) *Klebsiella* sp.	15	10	15	23	23	22	8	4
	(Pyocyanin) *Kerstersia gyoirum*	19	25	25	11.6	11.6	28	8	4
CS	(Flexirubin) *Chryseobacterium* sp.	24	25	31	13	15	10	8	4
	(Carotenoids) *Serratia marcescens*	20	20	15	26	20	11.6	8	4
	(Carotenoids) *Psychrobacter pulmonis*	25	15	10	13	15	18	8	4
	(Violacein) *Acinetobacter*	10	5	5	16	11.6	15	8	4

C^1 (1000μg/ml), C^2 (750μg/ml), C^3 (500μg/ml).

TABLE 4.15: Statistical analysis (ANOVA) of bacterial pigments for Anti-fungal.

Group	**Degrees of Freedom (DF)**	**Sum of Squares (SS)**	**Mean Square (MS)**	**F-Statistic**	**P-Value**
Between Groups	7	15.4265	2.2038	5.8649	0.0001
Within Groups	48	18.0365	0.3758		
Total	55	33.463			

The anti-fungal activity of bacterial pigments at different concentrations C^1, C^2, and C^3 varies with ANOVA results (F=5.8649, P=0.0001) confirming a statistically significant difference among groups.

For *A. fumigatus*, NAP (30mm) at C^1, *Chryseobacterium* sp. (31mm) at C^3, and *P. pulmonis* (25mm) at C^1, showed the highest inhibition, and *Klebsiella* sp. (15mm) at C^1, and C^3.

K. gyiorum (25mm) at C^2, and C^3 showed moderate inhibition, while EMBC had the lowest inhibition (10mm) at C^1 and (5mm) at C^2 and C^3.

For *R. delemar*, *Klebsiella* sp. (23mm) at C^1 and C^2, MACP (26mm) at C^2, *K. gyiorum* (28mm) at C^3, exhibit strong activity whereas, NAP (15mm) at C^3 *Chryseobacterium* sp. (15mm) at C^2, *P. pulmonis* (18mm) at C^3, and EMBC (16mm) at C^1 had moderate effect. The positive control *Amphotericin B* (8mm) consistently inhibiting both fungi, validating the test, while negative control Methanol (4mm) had not significant effect.

The positive control *Amphotericin B* (8mm) consistently inhibiting both fungi, validating the test, while negative control Methanol (4mm) had not significant effect.

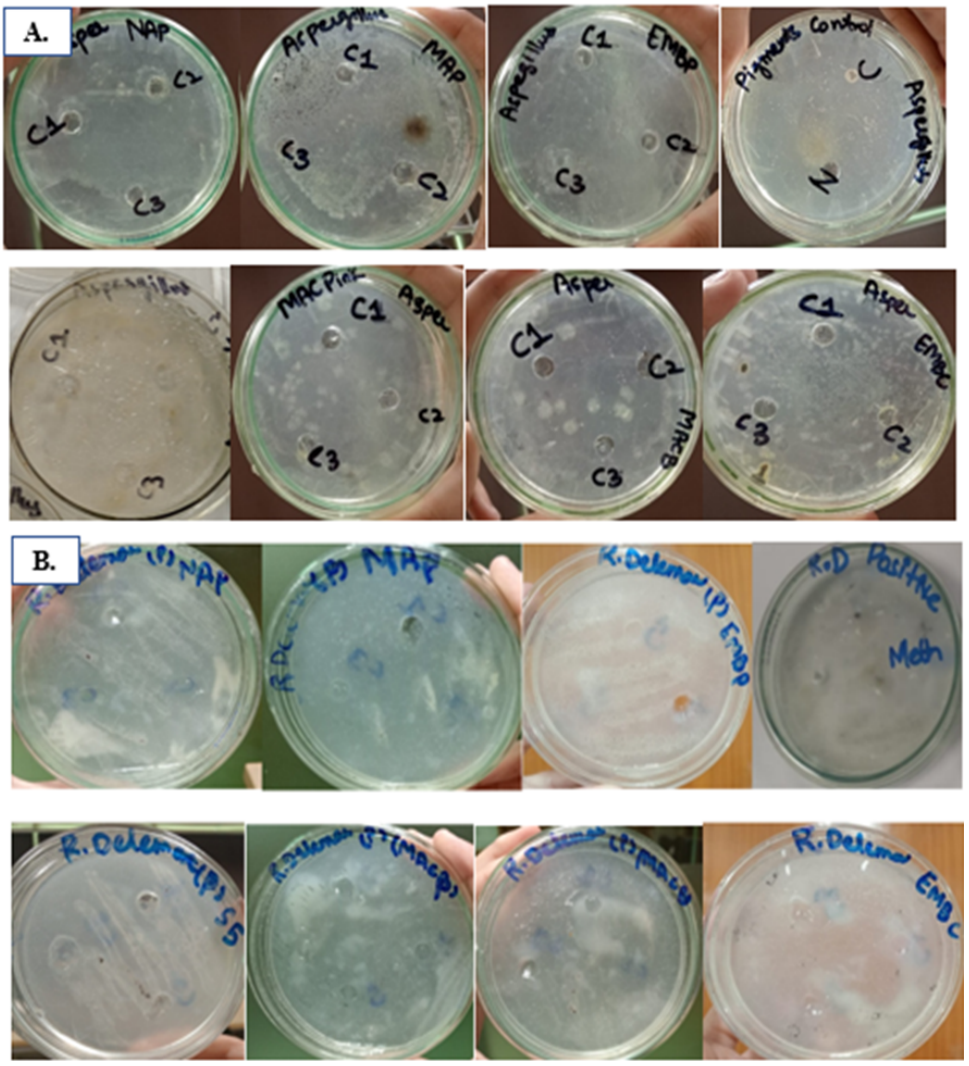

FIGURE 4.34: Anti-fungal results of bacterial pigments against (A) *A. fumigatus* and (B) *R. delemar*

Table 4.16 and Figure 4.35 below show the results of the anti-fungal activity of pigmented bacteria.

TABLE 4.16: Anti-fungal results of pigmented bacteria

		Pigmented Bacteria							
Src	**Bacteria**	***A. fumigatus***			***R. delemar***			**Positive Control**	**Negative Control**
		C^1	**C^2**	**C^3**	**C^1**	**C^2**	**C^3**	**(Amphotericin B) (mm)**	**(Methanol) (mm)**
		(mm)			**(mm)**				
FVP	*Pseudomonas nitroreducens*	24	24	0	5	8	8	1	8
	Klebsiella sp.	20	25	26	10	0	5	1	8

Table 4.16 continued from previous page

Src	Bacteria	Pigmented Bacteria							
		A. fumigatus			***R. delemar***			**Positive Control (Amphotericin B) (mm)**	**Negative Control (Methanol) (mm)**
		C^1	**C^2**	**C^3**	**C^1**	**C^2**	**C^3**		
		(mm)			**(mm)**				
	Kerstersia gyoirum	30	30	30	5	0	0	1	8
CS	*Chryseobacterium* sp.	5	28	26.3	10	18	15	1	8
	Serratia marcescens	5	5	06	15	35	35	1	8
	Psychrobacter pulmonis	5	33	05	25	30	30	1	8
	Acinetobacter	0	12	16	30	35	38	1	8

C^1 (1000μg/ml), C^2 (750μg/ml), C^3 (500μg/ml).

TABLE 4.17: Statistical analysis (ANOVA) of pigmented bacteria for Anti-fungal.

Group	**Degrees of Freedom (DF)**	**Sum of Squares (SS)**	**Mean Square (MS)**	**F-Statistic**	**P-Value**
Between Groups	7	22.2889	3.1841	2.6388	0.0216
Within Groups	48	57.9205	1.2067		
Total	55	80.2094			

The anti-fungal activity of bacterial pigments at different concentrations C^1, C^2, and C^3 varies with ANOVA results (F=2.6388, P=0.0216) confirming a statistically significant variations among pigmented bacteria. NAP exhibit strong inhibition against *A. fumigatus* (24mm) at C^1 and C^2 but minimal impact on *R. delemar* (5-8mm). *Klebsiella* sp. showed moderate inhibition against *A. fumigatus* (20-26mm), and weak (0-10mm) against *R. delemar*. *K. gyiorum* exhibit strong inhibition (30mm) at all concentrations against *A. fumigatus* but largely ineffective against *R. delemar* (0-5mm). *Chryseobacterium* sp. showed minimal inhibition of *A. fumigatus* at C^1 (5 mm) but strong activity at C^2 and C^3 (28-26.3

mm), with moderate inhibition of *R. delemar* (10-18 mm). MACP exhibited weak inhibition of *A. fumigatus* (5-6 mm) but strong activity against *R. delemar* (15-35 mm). *P. pulmonis* showed variable inhibition against *A. fumigatus* (5-33 mm) but strong antifungal effects on *R. delemar* (25-30 mm). EMBC had no effect on *A. fumigatus* at C^1 but moderate inhibition at C^2 and C^3 (12-16 mm), while it was highly effective against *R. delemar* (30-38 mm).

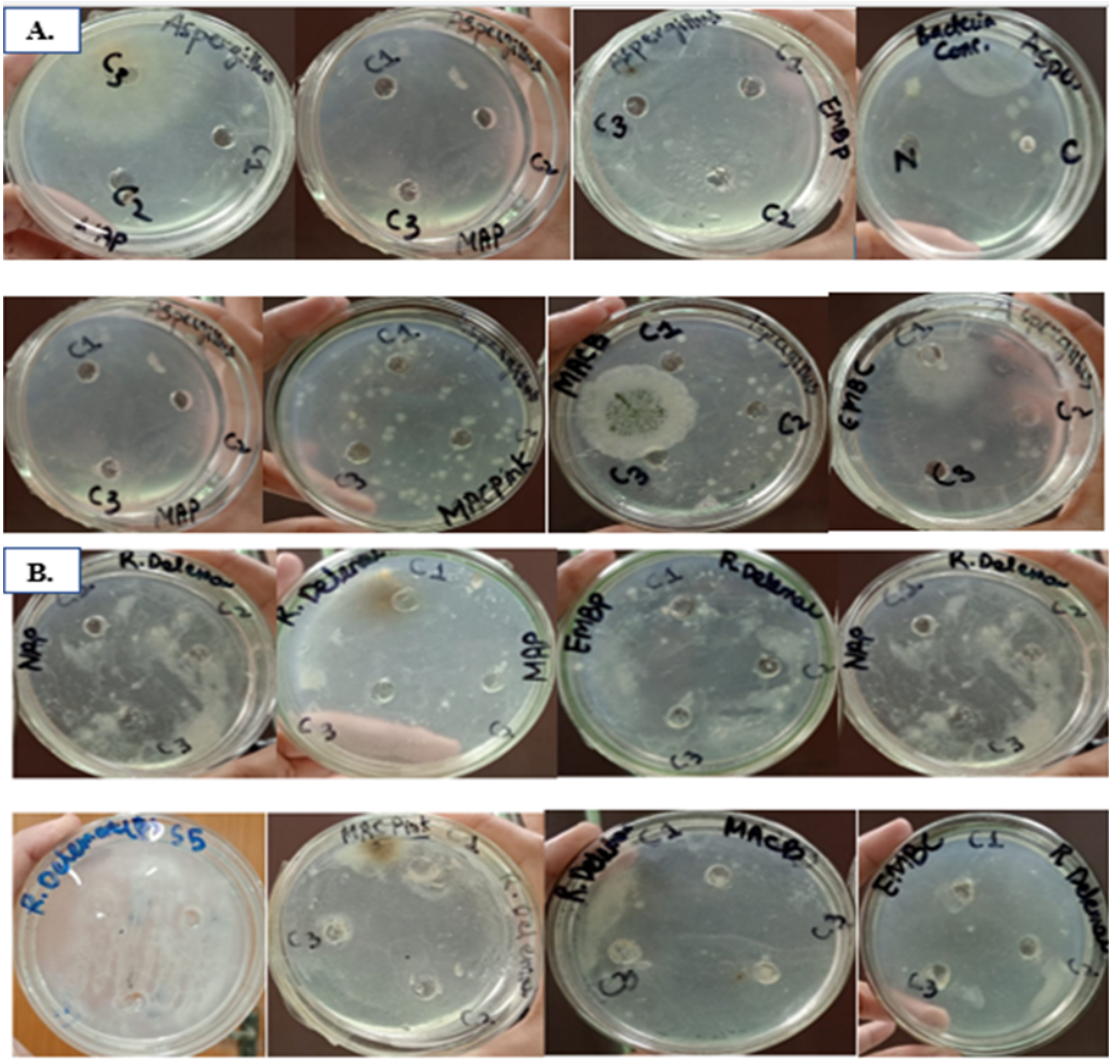

FIGURE 4.35: Anti-fungal results of pigmented bacteria against (A) *A. fumigatus* and (B) *R. delemar*

4.10.3 Cytotoxicity Assay- Brine Shrimps

For cytotoxic analysis, brine shrimps were used against 7 bacterial pigments and bacteria at different concentrations. Methanol was used as a control for pigments,

while water was used as a control for bacteria. Table 4.18 and Figure 4.36 show the mortality % and LC-50 of pigments and bacteria.

TABLE 4.18: Mortality % and LC-50 of pigments and bacteria against brine shrimps

S. No.	**Sample**	**Type**	**10 μL Mortality (%)**	**20 μL Mortality (%)**	**30 μL Mortality (%)**	**LC50 (%)**
1	(Pyocyanin) *Pseudomonas nitroreducens*	Pigment	45	55	60	15
2	(Prodigisin) *Klebsiella* sp.		60	65	80	10
3	(Pyocyanin) *Kerstersia gyoirum*		60	75	85	10
4	(Flexirubin) *Chryseobacterium* sp.		55	60	80	10
5	(Carotenoids) *Serratia marcescens*		50	65	75	30
6	(Carotenoids) *Psychrobacter pulmonis*		20	30	50	30
7	(Violacein) *Acinetobacter*		65	70	85	10
8	Control (Methanol)		45	50	55	30
9	*Pseudomonas nitroreducens*	Bacteria	5	15	20	0
10	*Klebsiella* sp.		10	15	20	0
11	*Kerstersia gyoirum*		15	20	30	0
12	*Chryseobacterium* sp.		5	10	20	0
13	*Serratia marcescens*		10	30	40	0
14	*Psychrobacter pulmonis*		10	30	40	0
15	*Acinetobacter*		5	20	35	0
16	Control (Water)		10	20	30	0

Table 4.18 reveals the results of mortality % and LC-50 of pigments and bacteria. Among the pigment samples, *Klebsiella*, *K. gyiorum*, *Chryseobacterium* sp. and EMBC exhibited the highest toxicity with mortality reaching upto 85% at 30 μL and an LC 50 of 10% indicating their high toxicity. Conversely, NAP, MACP and *P. pulmonis* showed lower toxicity, with *P. pulmonis* having the lowest mortality

(50% at 30 μL) and an LC50 of 30%, similar to methanol control. In contrast, bacterial samples exhibit lower mortality, with LC 50 values at 0%, reflecting minimal toxicity within the test range. *K. gyiorum* and MACP showed slightly higher bacterial toxicity, reaching 30–40% mortality at 30 μL, while other samples remained below 35%. The control (water) shows the slight mortality of 30% at 30 μL. LC 50 value $\leq$10% indicate high toxicity, 11-30% show moderate toxicity, and >30 or no LC 50 indicate low or negligible toxicity.

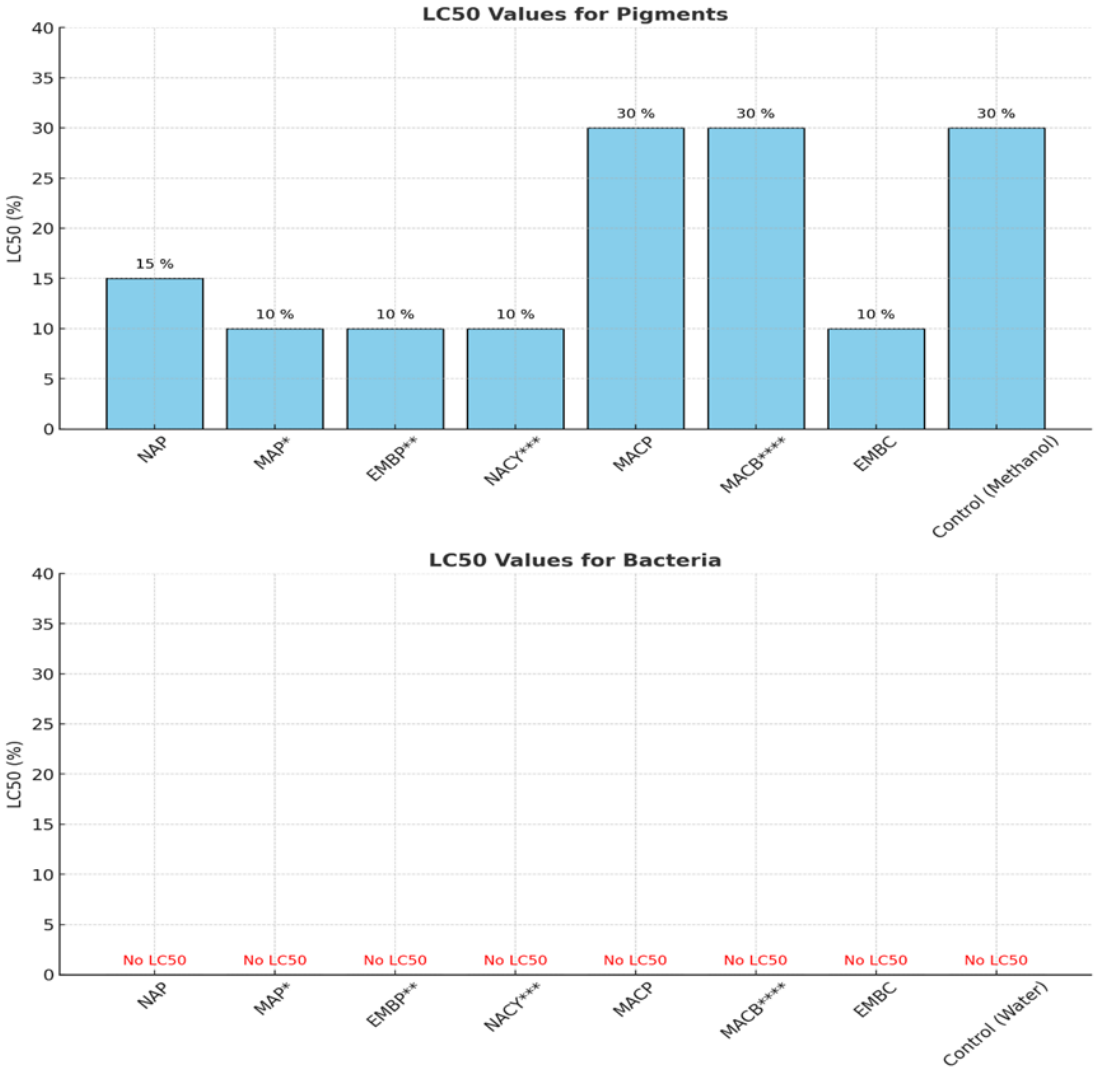

FIGURE 4.36: LC 50 of pigments and bacteria against brine shrimps

4.10.4 Cytotoxicity Assay-Larvicidal Activity

The toxicity of pigments and bacteria was analyzed by larvicidal test. Worm larvae were exposed to different concentrations of pigments and bacteria respectively. Methanol was used as a control for pigments, while water was used as a control

for bacteria. Table 4.19 and Figures 4.37 and 4.38 show the results of larvicidal activity for both pigment and bacteria.

TABLE 4.19: Results of Larvicidal activity of pigments and bacteria with mortality % and LC-50

#	**Sample**	**Type**	**20 μL Mortality (%)**	**50 μL Mortality (%)**	**70 μL Mortality (%)**	**LC50 (%)**
1	(Pyocyanin) *Pseudomonas nitroreducens*	Pigment	40	40	60	60
2	(Prodigisin) *Klebsiella* sp.		60	60	80	0
3	(Pyocyanin) *Kerstersia gyoirum*		20	40	60	60
4	(Flexirubin) *Chryseobacterium* sp.		20	60	60	42.5
5	(Carotenoids) *Serratia marcescens*		60	60	80	0
6	(Carotenoids) *Psychrobacter pulmonis*		20	30	40	0
7	(Violacein) *Acinetobacter*		20	40	60	60
8	Control (Methanol)		20	40	40	0
9	*Pseudomonas nitroreducens*	Bacteria	20	40	40	0
10	*Klebsiella* sp.		40	40	60	60
11	*Kerstersia gyoirum*		60	80	80	0
12	*Chryseobacterium* sp.		0	20	20	0
13	*Serratia marcescens*		0	0	20	0
14	*Psychrobacter pulmonis*		0	20	20	0
15	*Acinetobacter*		20	20	40	0
16	Control (Water)		0	0	0	0

Table 4.19 shows the moderate toxicity levels of pigments with LC 50 values ranging from 42.5% to 60%, except for *Klebsiella* sp., MACP, and *P. pulmonis*, which donot show LC 50. Chryseobacterium sp. showed the highest toxicity with lowest LC 50 (42.5%), NAP and *K. gyiorum* showed LC 50 (60%). In contrast bacterial

samples displayed generally lower toxicity, with none achieving LC 50 value except *Klebsiella* sp. (60%). While controls (Methanol and water) indicated lower to no toxicity. LC 50 value ≤10% indicate high toxicity, 11-30% show moderate toxicity, and >30 or no LC 50 indicate low or negligible toxicity.

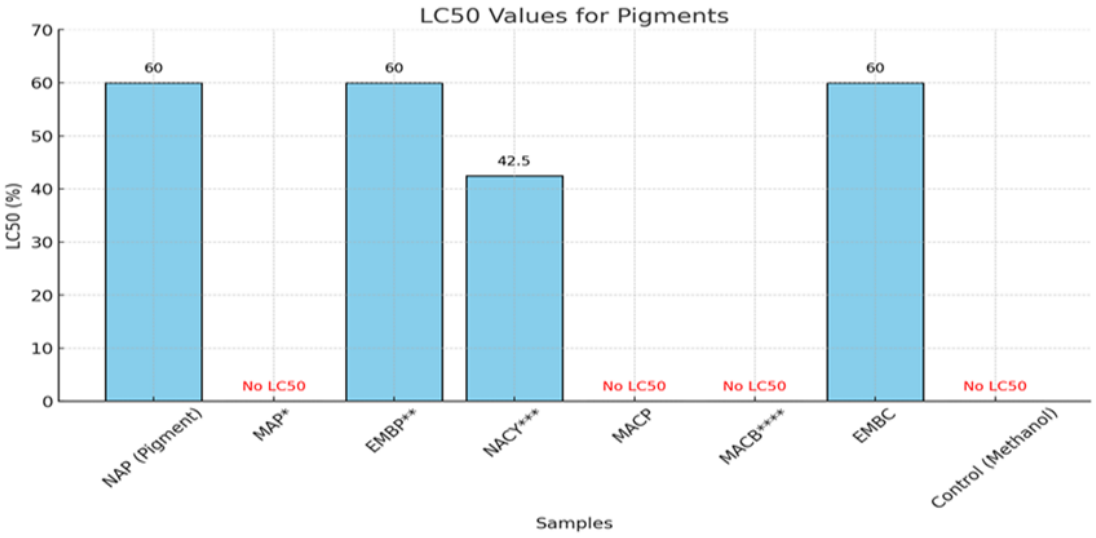

FIGURE 4.37: LC 50 of pigments against larvicidal activity

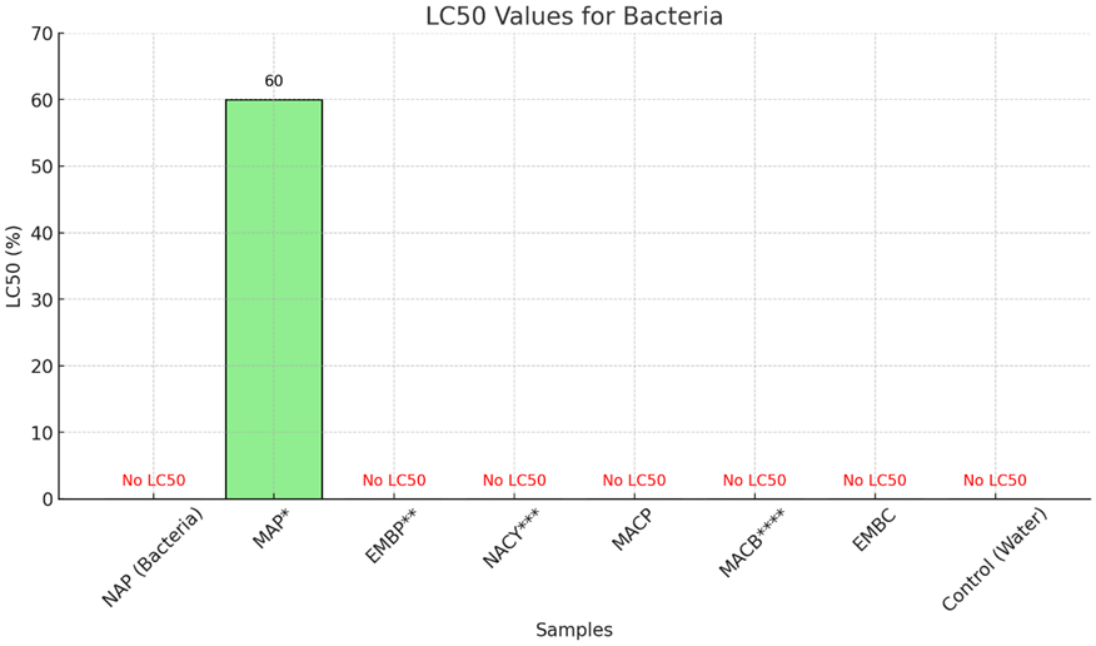

FIGURE 4.38: LC-50 of pigments and bacteria against larvicidal activity

Chapter 5

Discussions

Bacterial pigments are bioactive compounds produced by various microorganisms, with a varied range of colors such as red, yellow, orange, green, violet, and blue. These pigments incude prodigoisin, violacein, flexirubin, carotenoids, and pyocycnin have acquired attention for their potential in different industries including food, pharmaceutical, and cosmetic. Recent studies have successfully isolated pigment-producing bacteria from diverse sources including soil samples and fruit and vegetable wastes [224]. In this study, 7 bacterial pigments have been isolated. The species identified in 16SrRNA from FVP samples are *Pseudomonas nitroreducens* strain ES-18 which exhibited green color and belong to genus *Pseudomonas*, *Klebsiella* sp. which exhibited pink color and belongs to the genus *Klebsiella*, and *K. gyiorum* displayed a blue color and belongs to the genus *Kerstersia*. In CS samples, *Chryseobacterium* sp. showed a yellow color and belongs to the genus *Chryseobacterium*, *Serratia marcescens* showed pinkish orange color and belong to genus Serratia, *P. pulmonis* exhibited a brown color and belongs to the genus Psychrobacter and *Acinetobacter* sp. showed purple color and belong to genus *Acinetobacter* shown in Table 4.3.

Antibiotic sensitivity test was performed against pigmented bacterial species as shown in Table 4.4. In this study, it has been seen that *Klebsiella* sp., *K. gyiorum*, and *Chryseobacterium* sp. are generally susceptible to imipenem, a carbapenem antibiotic effective against many gram-negative bacteria, due to the absence of

carbapenemase enzyme, allowing imipenem to inhibit cell wall synthesis. However, resistance to doxycycline, ceftriaxone, and streptomycin is common in all species due to mechanisms like beta-lactamase production and efflux pumps [225–227]. Studies have reported a resistance rate of 32% to ceftriaxone in *Klebsiella* isolates [228]. A study on *Chryseobacterium* sp. reported that this species is resistant to aminoglycosides, tetracyclines, chloramphenicol, erythromycin, and clindamycin highlighting the multi-drug resistance of this species [229]. *P. pulmonis* showed susceptibility against imipenem and ceftriaxone due to the absence of a beta-lactamase enzyme that would otherwise hydrolyze these antibiotics. The susceptibility to streptomycin is due to the absence of an aminoglycoside - modifying enzyme, and resistance to doxycycline is due to the presence of efflux pumps that expel the antibiotic from the bacterial cell wall reducing its intracellular concentration and effectiveness [230].

Bacterial pigments are essential for various cellular functions, playing a key role in the survival and production of organisms that produce them. However, obtaining bacterial pigment in a relatively pure and concentrated form remains a significant industrial hurdle [224]. In this study, different pigments were extracted from FVP bacterial strains, such as Pinkish brown pigment from *Klebsiella* sp. and blue pigment from *K. gyiorum*. However, pigments extracted from CS samples include yellow pigment from *Chryseobacterium* sp. and purple pigment from *P. pulmonis* as shown in Table 4.6. Some strains of *Klebsiella* sp. like *Klebsiella pneumoniae* produces pink color indicates the presence of prodigiosin [231]. A study has reported pink pigment production by *Enterobacter* sp. strain PWN1 from fruit and vegetable waste samples [232]. Additionally, *Trichothecium roseum* also produces pink pigment from rotten fruit and vegetable peels [233]. *K. gyiorum* produces a blue pigment, pyocyanin due to interactions between bacterial metabolites and the dyes present in the EMB media [234]. *P. aeruginosa* also produces a blue-green pigment called pyocyanin from soil and, fruits and vegetable peels [235]. *Chryseobacterium* sp. is known to produce yellow to yellow-orange pigment, indicating the presence of flexirubin-type pigment [236]. Additionally, other species like *Xanthomonas*, *Micrococcus luteus*, and *Exiguobacterium aestuarii* produce yellow pigment, when isolated from soil samples [237, 238]. *P. pulmonis* showed brown

pigment due to the presence of eumelanin. A study indicates the presence of black to brown pigment produced by *Psychrobacter*-like bacteria, strains KC 40(T) and KC 65 while, the bacteria were isolated from the marine crustacean Sea of Japan [239].

UV-Vis spectrophotometry is commonly used for identifying compounds and quantifying substances based on their light absorption properties [192]. In this study, the UV-vis spectrum of *Klebsiella* sp. at 535nm as shown in Figure 4.21 (b) suggests the presence of prodigiosin. It is also indicated in the study of *Serratia marcescens*, which produces prodigiosin at 535nm [240]. *Chryseobacterium* sp. exhibit peaks around 400nm with subsequent decline up to 700nm, suggesting the presence of flexirubin-type pigments. They are characterized by their yellow-orange color and absorb light primarily in the blue region of the spectrum, around 400 nm. The presence of these pigments protects bacteria from oxidative stress [241]. Research on *Chryseobacterium artocarpi* CECT 8497 demonstrated antioxidant properties of flexirubin [242]. The absorption peaks of *P. pulmonis* at 520nm and 570nm are characteristics of carotenoids pigments. These pigments play a crucial role in light harvesting and photoreception. A study on *Rhodoblastus acidophilus* examined carotenoid to bacteriochlorophyll energy transfer. The research highlighted that carotenoids like rhodopsin and rhodopinal exhibit absorption features in the 525 - 570nm range which aligns with the peak of my study [243].

FTIR analysis was done to determine the presence of functional groups in bacterial pigments [193]. In this study, as shown in the Table 4.7, *Klebsiella* sp. showed bands between 3331 to 517 cm^{-1}, a study on *K. pneumonia* SNA2 the FTIR spectrum within this range showing absorption band at 1,652.8 cm^{-1}, attributed to the ester carbonyl (C=O) stretch. Other significant peaks were observed at 1,236.54 cm^{-1}, corresponding to C–O and C–N stretches, and at 1,719.74 cm^{-1}, also indicating the ester carbonyl group. These functional groups are characteristic of prodigiosin, a red pigment produced by various bacterial species [244]. *K. gyiorum* showed bands between 3321 to 522 cm^{-1}, a similar study on marine Bacillus circulans reported FTIR absorption bands corresponding to functional groups such as hydroxyl (OH), methyl (CH_3), carbonyl (C=O), and amide groups,

showing indicative lipoprotein surfactants which align with the bands observed in *K. gyiorum*. Similar bands in *Pseudomonas aeruginosa* have been associated with pyocyanin, a phenazine pigment with antimicrobial properties. This suggests the potential presence of pyocyanin or a related compound in K. gyiorum, as also observed in *Pseudomonas fluorescens* and *Pseudomonas putida* [245].

The FTIR analysis of the flexirubin-type pigment from Chryseobacterium sp. reveals functional groups such as alkynes (3305.70 cm^{-1}), methyl groups (2946.12 and 2834.28 cm^{-1}), and carbonyl amides (1654.79 cm^{-1}). These findings match previous studies on Chryseobacterium sp. flexirubin pigments, confirming their structure and antioxidant properties [246]. Research on flexirubin-type pigments from Flavobacterium johnsoniae has demonstrated comparable FTIR spectra, further supporting the structural similarities across different bacterial sources [246]. *P. pulmonis* PIGB - 167 showed bands 3321 to 519 cm^{-1}. In a related study, the FTIR analysis of *P. pulmonis* strain T-15 exhibited absorption peaks at 3392.79 cm^{-1} (O–H stretch), 2924.09 cm^{-1} (C–H stretch), and 1653.00 cm^{-1} (C=O stretch), among others. Comparing these studies, both *P. pulmonis* strains show FTIR absorption bands corresponding to hydroxyl, methyl, and carbonyl groups, suggesting similarities in their biochemical compositions, particularly in the production of polysaccharides. The presence of specific absorption bands corresponding to functional groups such as hydroxyl, methyl, and carbonyl in *P. pulmonis* strains may suggest the presence of carotenoid compounds [247].

HPLC analysis of FVP and CS was done at an absorption spectrum of 250nm as shown in Tables 4.8 and 4.9. The HPLC profile of *Klebsiella* sp. and *K. gyiorum* from FVP samples revealed 8 peaks with retention times 2.219 (Acetic acid), 2.709 (Lactic acid), 3.196 (Succinic acid) [210], 3.740 (Malic acid) [211], 4.702 (Fumaric acid) [212], 5.551 (Adipic acid) [213], 7.617 (Benzoic acid) [214], and 10.308 (Propanoic acid) [215]. The absorption spectrum of pigment extracted from *Chryseobacterium* sp. S5 and *Psychrobacter pulmonis* PIGB 167 from (CS) samples were obtained at 250nm. The HPLC profile revealed 9 peaks with retention times 2.815 (Zeaxanthin) [216], 3.158 (Tyrosol) [217], 3.585 (Pyocyanin)

[218], 4.008 (Catechol) [219], 4.198 (Eumelanin) [220], 4.753 (Lutien) [221], 5.678 (carotenoids) [222], 7.619 (β-Carotene) [221], and 16.754 (Rutin) [223].

The antibacterial activity as shown in the Tables 4.10 and 4.12 of the yellow pigment of *Chryseobacterium* sp. against *S. aureus* is due to presence of flexirubin-like pigments known for their antimicrobial properties. However, the bacterial strain of *Chryseobacterium* sp. also shows less anti-bacterial properties against *K. gyiorum* due to the presence of flexirubin-like pigments [247]. The brown pigment of *P. pulmonis* shows antibacterial activity against *S. aureus* and *K. gyiorum* at high concentrations due to phenolic or other bioactive compounds in the pigment. The bacterial strain of *P. pulmonis* also showed antibacterial activity against S. aureus due to the presence of bioactive compounds. Similar studies have reported the antimicrobial potential of pigments from *P. pulmonis* due to their ability to interfere with gram-positive bacterial structures like the thick peptidoglycan layer, suggesting their promising role in developing novel therapeutics [248]. *Klebsiella* sp. KL-1 strain showed antibacterial activity against *K. gyiorum* due to the production of bacteriocins or other small antimicrobial peptides that target closely related bacterial strains. Studies indicate that *Klebsiella* strains can produce such bioactive compounds, which are effective at low concentrations [249].

The anti-fungal activities of pigments and bacteria are mentioned in the Tables 4.13 and 4.15. Pinkish brown pigment from *Klebsiella* sp. exhibited maximum activity against *R. delemar* due to the presence of phenolic or quinoid compounds that target fungal activity by targeting the fungal membrane sterols or oxidative stress pathways. Additionally, the strain showed the highest anti-fungal activity against *A. fumigatus* due to the presence of microbial peptides that inhibit fungal cell walls [250]. Blue pigment from *K. gyiorum* and the strain both showed highest susceptibility against *A. fumigatus* due to presence of pyocyanin derivatives which disrupt fungal respiration and cell wall integrity [251]. The yellow pigment of *Chryseobacterium* sp. and the strain showed maximum anti-fungal susceptibility against *A. fumigatus* is due to presence of flexirubin-like pigments known for their antimicrobial properties [247]. The brown pigment and the strain *P. pulmonis*

both showed the highest antifungal susceptibility against *R. delemar* due to the presence of antimicrobial peptides that inhibit the fungal cell wall [249].

The LC-50 values of bacterial pigments against brine shrimps as mentioned in Table 4.18 shows LC-50 range from 10–30 μg/mL for *Klebsiella* sp. (Pinkish red), *K. gyiorum* (Blue), *Chryseobacterium* sp. (Yellow) and *P. pulmonis* (Brown) which determines the cytotoxicity of these pigments against brine shrimps. While specific studies directly correlating these pigments' LC50 values with cytotoxic effects are limited, existing literature provides insights into potential applications and cytotoxic nature of bacterial pigments. For instance, prodigiosin produced by *S. marcescens* shows anticancer, antibacterial and anti-inflammatory properties. Similarly, pyocyanin produced by *P. aeruginosa* possesses antimicrobial and biofilm-disrupting capabilities, despite its cytotoxic nature. Additionally, certain carotenoids inhibit anti-oxidant and anticancer properties despite being cytotoxic [252]. The bacterial strains did not possess LC 50 value, regarding them as non-cytotoxic. The bacterial strains did not possess LC 50 value, regarding them as non-cytotoxic. For instance, flexirubin isolated from *Chryseobacterium artocarpi* CECT 8497 was classified as non-toxic in toxicity studies [242].

The LC50 values of bacterial pigments and their respective strains against worm larvae are presented in Table 4.19, with LC50 values ranging from 40 to 60 μg/mL, which are considered to be non-cytotoxic. The pinkish-red pigment from *Klebsiella* sp. did not show LC -50 and specific strain showed less cytotoxicity at 60 μg/mL, which is considered safe. In contrast, *S. marcescens* known for producing prodigiosin (red pigment) has been associated with cytotoxic effects ranging from 51 -79% cytotoxic effect [253]. The blue pigment from *K. gyiorum* shows LC 50 of 60 μg/mL, and the specific strain did not show LC 50 make the pigment and bacteria not cytotoxic. This aligns with findings [252], that this pigment might possess anti-microbial properties like *Klebsiella* sp. *Chryseobacterium* sp. showed LC 50 of 42.5 μg/mL, while the relevant strain did not show LC-50 considered it to be non-toxic. It shows that they can be used as bio-pesticides due to their anti-microbial properties. For instance, *S. marcescens* which produces prodigiosin have been investigated for its insecticidal properties [254].

Chapter 6

Conclusion and Future Recommendations

6.1 Conclusion

The growing need for sustainable and eco-friendly alternatives in various industries has led to increased interest in microbial pigments. This research successfully isolated and characterized several pigment-producing bacterial strains from different natural sources including fruits, vegetable peels (FVP), and compost soil (CS). Bacterial strains were screened, and their pigments were analyzed using spectrophotometric, FTIR, and HPLC techniques. Furthermore, the biological activities of these pigments including antimicrobial, cytotoxicity, and larvicidal effects were assessed, highlighting their potential for industrial and therapeutic applications.

The first objective of this research was to isolate and characterize pigment-producing bacterial strains from natural sources. The results demonstrated that seven bacterial strains *Pseudomonas nitroreducens* strain ES-18, *Klebsiella* sp., *K. gyiorum*, from FVP samples, while *Chryseobacterium* sp. S5 strain, *Serratia marcescens* strain XC 19, *Psychrobacter pulmonis* PIGB 167 and *Acinetobacter* sp. NII-56 from CS sample were successfully isolated and identified, each producing distinct

pigments in green, pinkish-red, blue, yellow, pinkish orange, brown, and purple respectively.

Gram staining and biochemical tests revealed diverse metabolic characteristics. 16Sr RNA confirmed bacterial identification and phylogenetic relationships. The second objective was to extract pigment from isolated bacterial strains. The pigments were successfully extracted using methanol.

The extracted pigments include pinkish-red, blue, yellow, and brown respectively. The third objective aimed to perform molecular analysis of extracted pigments. UV-Vis spectroscopy confirmed distinct absorption peaks suggesting potential pigment classification (e.g. carotenoids, prodigiosin, and flexirubin). FTIR analysis indicated functional groups such as hydroxyl, carbonyl, amide, and alkyne suggesting the presence of bioactive compounds. HPLC analysis identified key bioactive compounds such as carotenoids, pyocyanin, and lactic acid.

The fourth objective was to assess the antimicrobial activity of isolated bacterial strains and their extracted pigments against selected bacterial and fungal strains. The findings showed *P. pulmonis* and *Chryseobacterium* sp. exhibited significant antibacterial activity against *S. aureus* and *K. gyiorum* with statistical validation ($P<0.05$). *Klebsiella* sp. pigment showed notable antifungal activity against *R. delemar* while *K. gyiorum* pigment was highly effective against *A. fumigatus* with statistical validation ($P<0.05$). The fifth objective was to evaluate the cytotoxic and larvicidal activities of bacterial strains and extracted pigments.

Cytotoxicity results of brine shrimps assay showed that *Klebsiella* sp. *K. gyiorum*, and *Chryseobacterium* sp. pigments had moderate toxicity (LC50 10-30 μg/mL). However, bacterial strains themselves showed moderate toxicity supporting their safe applications in biological studies. In larvicidal results, pigments demonstrated moderated toxicity with *Chryseobacterium* sp. showing the highest larvicidal effect. Bacterial strains had relatively lower toxicity, suggesting potential eco-friendly applications.

6.2 Future Recommendations

6.2.1 Optimization of Pigment Production

Investigate alternative culture conditions, including temperature, pH, and nutrient availability, to enhance pigment yield. Explore genetic engineering and metabolic pathway modifications to increase pigment biosynthesis efficiency.

6.2.2 Expanding Applications

Explore how these pigments can be used in new fields, such as creating biodegradable plastics, developing advanced wound-healing materials, or even contributing to eco-friendly building materials. This can open up a variety of practical and impactful uses.

6.2.3 Toxicological and Biocompatibility Studies

Perform in vivo toxicity assessments in animal models to ensure the safety of bacterial pigments for human applications.

Evaluate possible allergenic and inflammatory responses to bacterial pigments in cosmetic and pharmaceutical formulations.

6.2.4 Industrial Applications

Explore the application of bacterial pigments as eco-friendly textile dyes. Investigate their use in food coloring and packaging to replace synthetic colorants. This step is essential for translating research into real-world applications.

6.2.5 Sustainable and Large-Scale Production

Develop cost-effective bioreactors and fermentation processes for large-scale production. Assess the environmental impact and sustainability of bacterial pigment extraction compared to synthetic dyes.

6.2.6 Bio Pesticidal Potential

Conduct field trials to validate the effectiveness of bacterial pigments as larvicides. Explore synergistic formulations with existing biopesticides for enhanced efficacy.

6.2.7 Interdisciplinary Partnership

Partner with experts in fields like materials science and medicine to discover innovative uses for these pigments. For example, they could be applied in cutting-edge technologies like nanotechnology or in specialized medical treatments such as targeted drug delivery systems.

This study contributes to the growing body of research on microbial pigments and their potential industrial applications. By implementing suggested future directions, bacterial pigments can be harnessed for various sustainable applications, ultimately benefiting both industry and the environment.

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An Appendix

TABLE 1: UV-Vis absorption spectrum of bacterial pigments from 400-700nm.

S. No.	Source	Sample ID	Pigment	Wavelength 400nm	
				Transmission	**Absorption**
1	FVP	NAP*	Green	12.2	0.914
2		MAP**	Dark pink	72.8	0.138
3		EMBP***	Dark blue	39.5	0.404
4	CS	NACY****	Yellow	15.0	0.825
5		MACP*****	Pinkish orange	29.0	0.538
6		MACB******	Brown	64.7	0.189
7		EMBC*******	Purple	55.1	0.259
				Wavelength 450nm	
1	FVP	NAP*	Green	15.0	0.823
2		MAP**	Dark pink	70.6	0.151
3		EMBP***	Dark blue	45.6	0.341
4	CS	NACY****	Yellow	18.6	0.731
5		MACP*****	Pinkish orange	32.8	0.484
6		MACB******	Brown	64.5	0.191
7		EMBC*******	Purple	56.6	0.247
				Wavelength 500nm	
1	FVP	NAP*	Green	26.9	0.571
2		MAP**	Dark pink	66.0	0.181
3		EMBP***	Dark blue	35.6	0.448
4	CS	NACY****	Yellow	25.4	0.595
5		MACP*****	Pinkish orange	37.7	0.423
6		MACB******	Brown	69.6	0.157
7		EMBC*******	Purple	30.7	0.512
				Wavelength 550nm	
1	FVP	NAP*	Green	52.4	0.281

Table 1 continued from previous page

S. No.	Source	Sample ID	Pigment		
				Transmission	**Absorption**
2		MAP**	Dark pink	82.5	0.083
3		EMBP***	Dark blue	61.9	0.209
4	CS	NACY****	Yellow	43.8	0.358
5		MACP*****	Pinkish Orange	70.1	0.154
6		MACB******	Brown	23.7	0.626
7		EMBC*******	Purple	73.4	0.134
				Wavelength 650nm	
1	FVP	NAP*	Green	35.8	0.446
2		MAP**	Dark pink	83.0	0.081
3		EMBP***	Dark blue	44.9	0.348
4	CS	NACY****	Yellow	54.3	0.265
5		MACP*****	Pinkish Orange	86.2	0.064
6		MACB******	Brown	57.5	0.241
7		EMBC*******	Purple	46.6	0.331
				Wavelength 700nm	
1	FVP	NAP*	Green	37.7	0.423
2		MAP**	Dark pink	84.7	0.072
3		EMBP***	Dark blue	63.8	0.195
4	CS	NACY****	Yellow	57.6	0.240
5		MACP*****	Pinkish orange	87.4	0.058
6		MACB******	Brown	60.2	0.221
7		EMBC*******	Purple	67.4	0.172

*(*Pseudomonas nitroreducens*) **(*Klebsiella* sp. Strain KL-1), ***(*Kerstersia gyiorum*), ****(*Chryseobacterium* sp. 5S), *****(*Serratia marcescens*), ******(*Psychrobacter pulmonis* strain PIGB167) *******(*Acinetobacter* sp.)