

EFFECT OF MICROBIAL EXTRACT ON METABOLITE PRODUCTION OF INDUSTRIAL IMPORTANT BACTERIA

Project work

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

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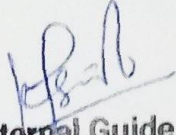
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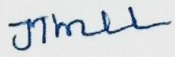
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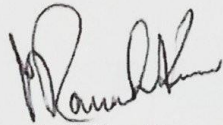
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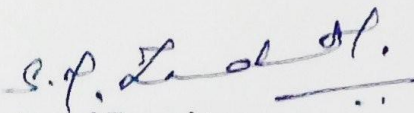
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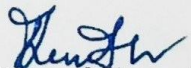

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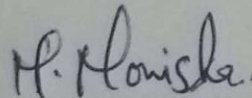

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DECLARATION

We, **M.MONISHA (41300023)** and **K.ASWATHI (41300006)** hereby declare that the Project Report entitled **"EFFECT OF MICROBIAL EXTRACT ON METABOLITE PRODUCTION OF INDUSTRIAL IMPORTANT BACTERIA"** is done by us under the guidance of **Dr.J.THEBORAL** (Internal guide) and **Dr.J.THEBORAL** (External guide) at **Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India**, is submitted in partial fulfillment of requirements for the award of **Bachelor of Science in Microbiology**.

DATE: 26.04.2024

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DECLARATION

We, K.ASWATHI(41300006) and M.MONISHA(41300023) hereby declare that the Project Report entitled **"EFFECT OF MICROBIAL EXTRACT ON METABOLITE PRODUCTION OF INDUSTRIAL IMPORTANT BACTERIA"** is done by us under the guidance of **Dr.PRABAKAR SINGH** (Internal guide) and **Dr.J.THEBORAL** (External guide) at **Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India**, is submitted in partial fulfillment of requirements for the award of **Bachelor of Science in Microbiology**.

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ABSTRACT

This research aims to explore natural pigments as eco-friendly alternatives to synthetic colorants, with potential applications across multiple industries. We have isolated pigment-producing microorganisms from various soil samples and upgrade their production by using microbial extract as biotic elicitor. Factors like temperature, pH, and salt concentration in the culture medium influence pigment production. Interestingly, when the bacterial isolate PB1 incubated at 37°C and with the concentration of 2.5 % NaCl and pH 7 resulted in highest level of pigment production, suggesting a factor-dependent aspect to pigment production. The application of microbial extract as biotic elicitor resulted in enhanced production of pigment which was confirmed through GC-MS analysis. This study provides a significant step towards finding sustainable alternatives to synthetic colorants. Natural pigments indeed offer promising solutions with their eco-friendly nature and diverse applications. Further refinement studies needed to employ the application of this bacterial pigment as bio colorant.

Keywords: Biotic elicitor, Microbial extract, Pigment, *Stenotrophomonas*

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LIST OF ABBREVIATIONS

FDA	Food and Drug Administration
UV	Ultra violet
TSB	Tryptone Soy Broth
RSM	Response Surface Method
MSA	Middle Stone Age
DSSC	Dye-sensitized solar cells
LHC	Light-harvesting complex
RC	Reaction Centre
MR	Methyl Red
VP	Voges-Proskauer
ITS	Internal Transcribed Spacer
PCR	Polymerase chain reaction

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CHAPTER 1

INTRODUCTION

1.1 PIGMENTS

Natural pigments have garnered global attention and are becoming increasingly significant in various industries. They can be derived from a range of sources such as ores, insects, plants, and microbes. Natural pigments offer several advantages, including anticancer properties, pro-vitamin A content, and desirable characteristics like stability to light, heat, and pH fluctuations. Moreover, natural colorants are considered safe due to their non-toxic, non-carcinogenic, and biodegradable nature, making them appealing alternatives to synthetic colorants (Sinha *et al.*, 2017).

1.2 ARTIFICIAL PIGMENTS

Numerous artificial synthetic colorants that are widely used in the production of food, dyes, cosmetics, and pharmaceuticals have several dangerous side effects. The development of processes for the production of pigments from natural sources is of great interest to the global community due to the negative effects of synthetic colorants. The initial appeal of synthetic dyes stemmed from several features, including ease of development, affordability, the absence of unwanted flavours, superior colouring capabilities, and the requirement for minimal usage. Nonetheless, the majority of the artificial dyes utilized had never undergone environmental or health risk assessments (Downham and Collins 2000). Synthetic dyes are typically composed of chemicals that are hazardous to human health, such as lead, mercury, copper, chromium, sodium chloride, benzene, and toluene. Several artificial colorants that were formerly approved for use in food, medicine, and cosmetics development by the Food and Drug Administration (FDA) were subsequently discovered to be carcinogenic (Rao *et al.*, 2017).

Due to apparent risks like benzidine, a bowel cancer cause, and carbon black, a pigment widely used in printing ink, which may be carcinogenic, the majority of synthetic dyes that were once used for a variety of applications have been discontinued (Rao *et al.*, 2017).

1.3 PIGMENT PRODUCTION FROM BIOLOGICAL SOURCES

In recent years, there has been an increase in the use of natural pigments in food, dye, cosmetic, and pharmaceutical manufacturing processes (Unagile *et al.*, 2005). Plants (Mizukami *et al.*, 1978) and microorganisms (Ryu *et al.*, 1989) are the two main sources of natural pigments. Ores, insects are a few examples of natural sources of pigments. The readily available, approved natural plant pigments have a lot of disadvantages, including low water solubility, instability to light, heat, or harsh pH, and seasonal availability. Because of the stability of the pigments produced (Raisanen *et al.*, 2002) and the accessibility of cultivation technology (Kim *et al.*, 1998), natural pigments derived from microorganisms are of great interest. Because microbial pigments are frequently more stable and soluble than those derived from plant or animal sources, they are of industrial interest (Gunasekaran and Poornima (2008). Microorganisms can grow quickly, which can result in high productivity and year-round product production (Jiang *et al.*, 2005).

1.4 MICROBES AS POTENTIAL SOURCE FOR PIGMENT PRODUCTION

Chromogenic bacteria are bacteria that produce pigment. Some bacteria's regular metabolism includes the production of pigment including fluorescent green, yellow, blue, golden, white, brown, and fluorescent green. A bacterium can be identified by its pigment; bacteria with pigment will produce cultures that have some colour. The process by which pigments are produced can vary between extracellular and intracellular microorganisms. Bacteria can make pigment, which is very helpful. The morphological traits, cellular functions, and pigment production of bacteria are linked to UV radiation, survival, pathogenesis, and protection. According to Rokade and Pethe (2016), extremophiles' vibrant pigments are necessary for both photosynthetic and respiratory processes. The bacteria produce certain pigments that have antimicrobial activity against infections. Pigments such as carotenoids, melanin, flavin, violacein, and prodigiosin demonstrated unique antimicrobial properties against a variety of pathogenic bacteria.

The solubility and stability of pigments as well as the ease of culturing microbes make natural pigments derived from microbes a more suitable option than plant-based pigments (Rao *et al.*, 2017). For a very long time, scientists believed that Earth's

cryosphere was too hostile to support life. Nonetheless, because of the harsh ecological circumstances, the glacial ecosystem is home to a variety of microbial communities that sustain active biochemical pathways (Anesio and Laybourn-Parry 2012; Anesio *et al.*, 2017; Hassan *et al.*, 2018; Rafiq *et al.*, 2019). Extremely low temperatures, oligotrophic conditions, freeze-thaw cycles, ultraviolet (UV) radiation, and increased salinity are just a few of the stressors that the microbial communities that live in the cryosphere are constantly exposed to. Microorganisms adapt multiple protective strategies, such as changing their morphology and producing different metabolites, to deal with these harsh conditions. The majority of pigment production takes place in the cytoplasm, where it serves a variety of ecological purposes and is a response to harsh environmental conditions (Pagano and Dhar 2015). Extreme physiochemical conditions, like those found in the cryosphere, offer microorganisms an ideal environment for producing pigments with special traits and uses. This review discusses pigments produced in the cryosphere by cold-adapted bacteria and fungi, as well as possible uses for them in a variety of industries. Additionally, the difficulties in using cryosphere microorganisms for pigment extraction are explored, along with condition optimization for maximum pigment yield.

1.5 PIGMENT-PRODUCING BACTERIAL SPECIES

Bacterial pigments indeed serve various functions and are produced by a wide range of bacteria for different purposes. Cyanobacteria, for example, use phycobilin pigments like phycocyanin and phycoerythrin to capture light energy during photosynthesis. *Serratia marcescens*, known for its red pigment prodigiosin, often appears in moist environments and can sometimes be found in bathrooms or on damp food. *Streptomyces coelicolor* produces prodigiosin and actinorhodin, with the former giving it a reddish color and the latter a blue colour. *Chromobacterium violaceum* synthesizes the purple pigment violacein, while *Thiobacillus versutus* produces natronochrome and chloronatronochrome (Browning *et al.*, 2003; Woodall *et al.*, 1997; Ahamed *et al.*, 2012). Isolating and culturing these pigment-producing bacteria involves using specific growth media tailored to their nutritional needs and environmental conditions. For example, nutrient agar or nutrient broth are common media used for general bacterial cultivation, while specialized media may be required to specifically encourage the growth of certain pigment-producing strains.

Environmental sources like water bodies, soil, plants, insects, and even human or animal bodies can be potential reservoirs for these bacteria, offering diverse opportunities for isolation and study.

1.6 REQUIREMENTS FOR BACTERIA-MEDIATED PIGMENT PRODUCTION

Differential Medias such as MacConkey agar, EMB agar, Macleod agar and TSB agar are very useful in indication and characterization of pigmented bacteria (Bhaswar and Cameotra 2011). Microorganisms produce different pigments such as quinines, carotenoids, favins, melanins, prodigiosins and mostly monascins, violacein (Mortensen 2006; Pantanella *et al.*, 2007; Bhawsar and Cameotra 2011; Venil *et al.*, 2013). The classification and identification of bacterial isolates are mostly through morphological and biochemical characteristics. Researchers are trying to use statistical analysis such as Response surface method (RSM) and Taguchi method (McClean *et al.*, 1997). To enhance the efficacy of bacterial pigments current technologies, have different potentials besides its challenges. Bacterial pigments that are produced by bioprocess market are difficult to estimate, due to lack of statistical regional method (Simova *et al.*, 2003). Global demand for dyes and pigments is hoped to reach almost 10 million tons by 2017 according to the analysts of Global industry. The largest consumers of organic and natural pigments are textiles industries (Joshi and Pandey 1999).

1.7 PROBLEM OF THE STUDY

Research on these pigment-producing bacteria is currently ongoing because the antimicrobial substance these bacteria produce has been effectively utilized in clinical treatment. Motivated by these facts, the study's objectives were to separate the microorganisms that produce pigment from their natural environments and assess the pigments' antibacterial properties against bacteria that cause harm to humans. The major objective of the work was to select the effective pigment-producing microbes from the natural source like soil. Then the selection of cheaper substrates that is economically productive and important and can be used commercially. And characterization of the isolate was made on the basis of morphology and biochemical tests.

CHAPTER 2

REVIEW OF LITERATURE

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2.1 DATA ON DAILY USAGE OF PIGMENTS

Our species' evolutionary threshold is marked by the emergence of symbolic material culture. Items having an informational function serve as stand-ins for language and, consequently, for the essential elements of modern human cognition. Symbolic artifacts were thought to have emerged relatively late in the Pleistocene and during the so-called Upper Palaeolithic revolution, which was the abrupt appearance of decorated bone tools, sculpted figures, and jewellery made of bone, ivory, shell, or stone in Europe some 40 thousand years ago. This evidence has been consistent for more than a century.

Research conducted over the last 20 years has seriously questioned the empirical underpinnings of this paradigm. Middle Stone Age (MSA) sites in southern and northern Africa have yielded perforated and ochred marine shell beads. These sites date to the Last Interglacial, which occurred over 70 year ago. The emergence of "modern behaviour," which is understood to be (i) a component of the speciation of anatomically modern humans and (ii) an explanation for the latter's eventual prevalence over coeval Eurasian humans, was suggested by these discoveries to be represented by such material symbols. Since these Eurasian populations, the Neandertals were anatomically primitive, it was assumed that they were also behaviourally primitive, meaning they lacked symbolism and possibly even language.

The energy consumption of the air conditioners can be effectively controlled, as well as the energy consumption between the inner and outer parts of the building, by regulating the amount of solar energy that passes through the glass. It aims to cut down on the amount of electricity used by using windows that control sunlight. Buildings with thermochromic coatings experience a decrease in heat island formation and the growth of a microclimate. The surface temperatures of thermochromic coatings typically range from 31.4 to 38.4°C in August to 26.1 to 31.6°C in September. These temperatures are 36.4 to 48.5°C in August and 29.8 to 42.3°C in September for general coatings. A different literature review states that a transition temperature of

about 35°C is appropriate for thermochromic glass coatings. Thermochromic pigments that are encapsulated are used to shield the thermochromic system from environmental factors, oxidation, aging, and chemicals. To minimize energy consumption during the winter, solar gains must be increased, and the pigments' molecular structure must be thermally reversible. In addition to being easy to process at low temperatures, sol-gel is essential for the formation of films with variable composition, microstructure, and thickness.

2.2 NEGATIVE IMPACTS OF CHEMICAL PIGMENTS

The effects of the sol-gel spray coating technique on the optical transmittance properties of the thermochromic coatings were examined after the coatings were applied to the glass surfaces. It was discovered that the phase transition performance of thermochromic coatings was extremely sensitive. The resulting coatings can serve as an efficient thermal sensor and indicator or be applied to glass in hotter climates. Microalgal biomass-derived fuels have drawn more attention during the past ten years. Because microalgae have high oil productivity, are compatible with a wide range of water sources, and can be grown on non-arable or unproductive land, microalgal biomass may offer significant advantages that complement traditional feedstock for these biomass-derived fuels.

The dry biomass of microalgae can contain up to 35–45% lipids, and the leftovers are disposed of as waste. This will be a problem in the future when mass production of microalgae biomass is planned for oil feedstock (especially in the tropical seas of Indonesia, which are spread out over 6 million km²). Investigating the value-adding of the waste microalgal biomass, including the photosynthetic pigments, is therefore crucial to applying zero waste concepts to the utilization of microalgal biomass. Since they are photosynthetic plants, microalgae generate large amounts of pigments like carotenoids and chlorophylls that are highly reflective of light. When photosynthesis occurs, microalgae use solar energy to synthesize glucose, lipids, proteins, and other highly valuable bioactive compounds. Photosynthesis is a process that requires both carbon dioxide and water. Microalgae are particularly efficient at harvesting and utilizing solar energy. A common microalgae model used to study photosynthesis is *Chlorella*, which is a simple species (Hoffmann, D- 2018).

A green alga with a diameter ranging from 2 to 10 μm , *Chlorella* is a member of the phylum Chlorophyta. Spread widely in fresh, brackish, and marine water, this microalga has a single chloroplast and is unicellular, coccoidal, and non-motile. In addition to being used in cosmetics, feed additives, and food supplements, *Chlorella* also yields photosynthetic pigments that may find use in the pharmaceutical and medical industries. In addition, dye-sensitized solar cells (DSSC) and photo-hybrid fuel cells could find use for photosynthetic pigments' capacity to absorb solar radiation and transform it into extremely excited electrons, or excitons. Two essential processes in the conversion of solar energy to high-exciton energy take place during photosynthesis (Nurachman – 2015).

The light-harvesting complex (LHC), a multiprotein complex that functions as the photosystem antenna, is initially responsible for gathering and transmitting solar energy. Entire chlorophyll (Chl) in chloroplasts is bound by the typical thylakoid membrane protein called LHC. The deep green hue of LHCs is a result of their elevated levels of chlorophylls and yellow/orange carotenoids, specifically β -Car, lutein, neoxanthin, violaxanthin, and zeaxanthin. Solar energy harvesting is significantly aided by the excited-state dynamics of carotenoids. Light-driven charge separation is the final method used to identify electron excitations in the photosystem's reaction centre (RC). It follows that the earlier photosynthetic activity is a crucial component of capturing and transferring solar energy, which can be used to develop DSSCs. On the other hand, the latter is relevant to the advancement of photo-hybrid fuel cells, where the exciton generated is utilized to reduce Arrhenius acid (H^+) and produce hydrogen. Photovoltaic processes in solar cells enable the direct conversion of solar radiation into electrical energy. A colloidal TiO_2 film electrode had dye absorbed on it in a DSSC created by O'Regan and Grätzel. By facilitating the absorption of solar energy, the dye in this device uses the principles of photosynthesis to convert solar energy into electrical energy (Civan 2020).

Ru-based complexes are effective sensitizers for Direct Solar Cells (DSSC) with high photovoltaic conversion efficiency, according to numerous reports and reviews of dyes. These sensitizers are non-renewable, extremely costly, and scarce. The heavy metal ions present in the Ru complexes are unfavourable to the environment and make them complex for synthetic routes. As a result, there is a lot of

research being done on the use of natural pigments found in fruits and vegetables as solar cell sensitizers. It is possible to produce and use the photosynthetic pigments of the tropical marine *Chlorella* sp. PP1 to sensitize solar cells. We tracked *Chlorella* sp. growth in the current study. PP1 was cultured in Walne medium to produce chlorophylls and carotenoids, both with and without light stress. We also investigated the characteristics of *Chlorella* sp. PP1's photosynthetic pigments are critical for absorbing sunlight. The development of solar cells using photosynthetic pigments as an antenna for light harvesting, as well as the growth profiles of *Chlorella* sp. PP1 under three distinct light exposures about photosynthetic pigment production, are all covered in this paper (Tkaczyk, 2020).

2.3 BIOREMEDIATION IN PIGMENT PRODUCTION

Because natural dyes are more biodegradable and therefore more environmentally friendly, there has been an increase in interest in their use. The earliest known human societies used natural dyes as colouring agents, demonstrating the long history of their use. But as soon as artificial colours were available, they began to lose their allure. Compared to their natural counterparts, these synthetic dyes were significantly more affordable, easily manipulated, available in a wide range of colours, and repeatable. The synthetic dye industry thrived due to recent advancements in the field, while the plant-based dye industries collapsed at the beginning of the twentieth century. Synthetic dye industries discharge hazardous chemical wastes into freshwater bodies like rivers and ponds, resulting in the growth of algae and the subsequent release of greenhouse gases. Additionally dangerous and carcinogenic to consumers were these industrial pollutants. The negative effects of synthetic dyes didn't become apparent until recently, forcing industries to change their approach. These drawbacks of synthetic dyes have led to their current classification as "toxic contaminants" and consequent decrease in usage. Though there are already plant-based natural dyes available, there are drawbacks to be aware of. Plants grown especially for dyeing purposes may be costly to grow. Moreover, when the pH is altered, plant-based pigments quickly denature and may not offer batch reproducibility. As a result, scientists adopted an alternative tactic utilizing microorganisms. Natural colors are usually produced by bacteria, yeasts, fungi, and algae, among other microbes. These microorganisms are known to synthesize several categories, such

as violacein's, carotenoids, flavones, phenazines, melanin's, and numerous others that are listed later in the paper (Dikshit, 2018).

2.4 BACTERIAL SPECIES FOR PIGMENT PRODUCTION

These pigments are necessary for the microorganisms' natural ability to adapt to challenging environmental conditions and carry out particular cellular processes, such as photosynthesis in photosynthetic microorganisms. Because of their unique qualities and beneficial traits that are good for the environment and human health, these naturally occurring pigments are now utilized in a variety of industries, including food, textiles, cosmetics, and pharmaceuticals. Several pigments have had their global markets for microbial pigments evaluated; for example, the carotenoids market was projected to reach USD 1.7 billion in 2020 and is predicted to grow at a rate of 2.6 percent to reach nearly USD 2 billion in the upcoming years. As per AMR (2021) reports, the market size of astaxanthin, a carotenoid type, was USD 192.5 million in 2020 and is expected to grow exponentially to approximately USD 228.4 million by 2027. Currently, chemical synthesis accounts for 80–90% of carotenoid synthesis because it is less expensive (\$250–2000 kg⁻¹) than carotenoids derived from plants (\$350–7500 kg⁻¹). However, the open market sells carotenoid-containing biomass from a wide variety of microorganisms, including algae (*Haematococcus* sp., *Chlorella* sp., etc.) for \$40–50 USD per kg. Therefore, in the current situation, pigments derived from microbes have the potential to completely replace synthetic and plant-based pigments on the global market (Mussagy, 2021).

CHAPTER 3

3.1 AIM

To study the effect of microbial extract as biotic elicitors on pigment production of industrial important bacteria.

3.2 OBJECTIVES

- To isolate potential bacterial and fungal from field-derived soil
- To characterize the bacterial and fungal isolates of the field-derived soil sample for their biochemical properties and identification
- To isolate and characterize industrial important bacterial species based on pigment producing capability
- To study the effect of microbial extract as biotic elicitor on pigment production of industrial important bacterial species

CHAPTER 4

MATERIALS AND METHODS

4.1 TEST CHEMICALS

Glass petri dishes, test tubes and measuring cylinders procured from was used for plating of cultures. Sterilized containers were used for collecting the samples. Chemicals such as NaCl, Ethanol, NaOH, HCl, and other analytical grade chemicals (SRL) were employed throughout the experiment. The studies were conducted using distilled water, and aseptic conditions were maintained throughout. Bacteriological media including nutritional agar, nutrient broth, lactose broth, blood agar, and MacConkey agar were utilized. Biochemical tests were performed using a Gram staining kit and reagents.

4.2 SAMPLE COLLECTION

The soil samples were collected from oil polluted area of Sathyabama Institute of Science and Technology in Chennai and used for isolation of pigment producing bacteria. The soil samples are also collected from garden area and Sathyabama Ocean Research Centre. The samples are kept in a test tube and labelled according to their collection place. Then they were processed with serial dilution method. The samples diluted for 10^5 and 10^7 were plated on *nutrient agar medium* by spread and pour plate method. The sample inoculated plates were cultured by incubating at the 37°C on the incubator (Figure 4.1).

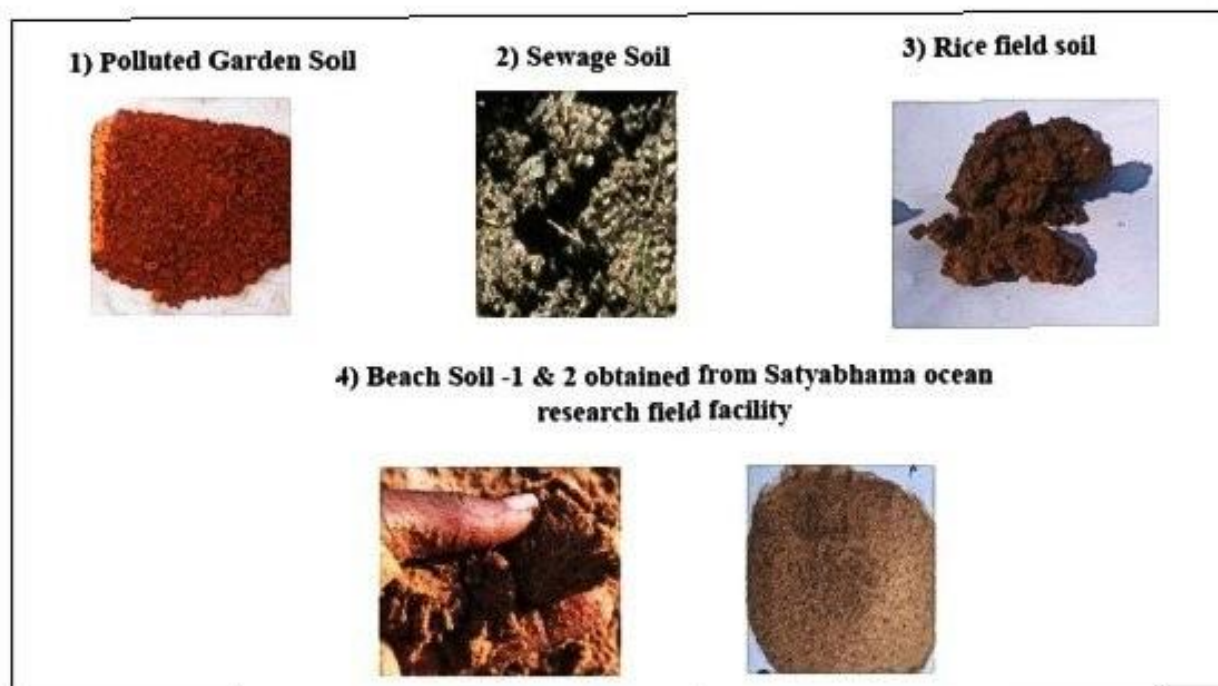


Fig 4.1 Different geological locations from where the soil samples were collected

4.3 CHARACTERIZATION OF ISOLATED PIGMENTED BACTERIA:

The bacterial isolates which showed pigment production were further studied for their characterization studies by Gram Staining and Biochemical tests. Morphological colony characters such as size, colour, texture, opacity, shape etc. were noted.

4.4 PURIFICATION AND MAINTENANCE OF PIGMENTED BACTERIA

Pigmented bacterial isolates were purified by streaking onto nutrient agar plate and it was incubated for 24 h at 30°C. Pigmented bacterial cultures were grown on different media and were maintained at 2–4°C temperature in refrigerator and sub cultured into the respective medium.

4.5 EFFECT OF FACTORS ON PIGMENT PRODUCTION

The effect of various physiological parameters such as temperature, pH and salt concentration were studied on the growth of the selected bacterial isolates to determine which gave maximum pigment production. Bacterial isolates were streaked on sterile nutrient agar slants (pH 7.4) and incubated at a varying range of

temperature, 10°C RT and 37°C for 24-48 hrs. Sterile Nutrient agar slants with different pH(5,6,9 and 12) were prepared. The isolated bacteria were streaked and kept for incubation at 37°C for 24-48 hrs.

4.6 IDENTIFICATION OF POTENTIAL PIGMENT-PRODUCING BACTERIA

Identification of the most promising bacterial isolate was done based on the morphological, culture and biochemical test.

4.6.1 Morphological and Biochemical Identification

The bacterial species undergone for Gram Staining, Spore staining and Capsule staining. Thin bacterial smears were prepared, and specific staining procedures were followed.

4.6.1 Biochemical tests

Biochemical tests are used to identify the bacterial species by differentiating them on the basis of the biochemical activities and metabolic capabilities. The bacterial isolate has been tested with Indole, MR-VP, citrate and catalase test.

4.7 MOLECULAR LEVEL IDENTIFICATION OF BACTERIAL SPECIES

4.7.1 Bacterial DNA extraction

50-100 mg bacterial cells that have been resuspended in 200 µl of water or isotonic buffer (e.g. PBS) or up to 200 mg of tissue were added to a ZR bashingbead lysis tube containing 750 µl Lysis Solution. It was secured in a bead fitted with 2ml tube holder assembly and processed at maximum speed for > 5 minutes. The ZR bashingbead lysis tube was centrifuged in a microcentrifuge at > 10,000 x g for 1 minute. 400 µl supernatant was transferred to a centrifuge tube and centrifuged at 7,000 x g for 1 minute. 1,200 µl of Bacterial DNA Binding Buffer was added to the filtrate in the centrifuge tube. 800 µl of the mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC column has a maximum capacity of 800 µl. The flow was discarded through the collection tube. 200 µl of DNA pre-wash buffer was added to the Zymo-Spin™ IIC column in new collection tube and centrifuged at 10,000 x g for 1 minute. 500 µl bacterial DNA wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC column was transferred

to a clean 1.5 ml microcentrifuge tube and 100 μ l (35 μ l) DNA elution buffer added directly to the column matrix. It was then centrifuged at 10,000 $\times$ g for 30 seconds to elute the DNA.

4.7.2 Quantity and quality check

The product was checked on a nanodrop of model 2000 from Thermo Scientific to quantify the concentration of the extracted DNA and also determine purity by measuring at 260/280 nm amount of proteins left in the DNA extract.

4.7.3 Polymerase chain reaction (PCR) analysis

The DNA was subjected to a cocktail mix and condition for the PCR. PCR reaction cocktail consisted of 10 μ l of 5 $\times$ Go Taq colourless reactions, 3 μ l of $MgCl_2$, 1 μ l of 10 mM of dNTPs mix, 1 μ l of 10 pmol each. The Internal Transcribed Spacer (ITS) gene for characterization of bacteria, ITS universal primer set which flank the 16S was used.

- 16SF: GTGCCAGCAGCCGCGCTAA
- 16SR: AGACCCGGAACGTATTCAC

Polymerase Chain Reaction (PCR) conditions include a cycle of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of each cycle comprised of 30 seconds denaturation at 94°C, 30 seconds annealing of primer at 55°C, 1.5 minutes extension at 72°C and a final extension for 7 minutes at 72°C.

4.7.4 Purification of amplified PCR product

The amplified fragments were purified with ethanol in order to remove the PCR reagents. Briefly, 7.6 μ l of sodium acetate 3M and 240 μ l of 95% ethanol were added to each about 40 μ l PCR amplified product in a new sterile 1.5 μ l eppendorf tube, mixed thoroughly by vortexing and kept at -20°C for atleast 30 minutes. Centrifugation was done for 10 minutes at 13000 Revolution per minute and 4°C followed by removal of supernatant (tube was inverted on tissue once) after which the pellet were washed by adding 150 μ l of 70 % ethanol and mixed, then centrifuged for 15 minutes at 7500g and 4°C. Again all supernatant was removed and tube inverted on paper tissue and allowed to dry in the fume hood at room temperature for 10-15 minutes. It was re-suspended with 20 μ l of sterile distilled water and kept in -20°C prior to sequencing.

The purified fragment was checked on a 2% Agarose gel ran on a voltage of 120 V for about 1 hour, to confirm the presence of the purified PCR product. The ladder used for bacteria is hyper ladder 1 from BioLone while 1 kb plus ladder from Invitrogen was used for bacteria. The expected base pair of the amplicon is around 850 bp. The amplified samples were then sent for sequencing.

4.7.5 Genome sequencing and annotation

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from applied biosystems using manufacturer' manual while the sequencing kit used was that of big dye terminator cycle sequencing kit. Bio-edit software and MEGA 6 were used for all genetic analysis. The classification/identification of organisms was performed by a local nucleotide BLAST search against the non-redundant version of the NCBI ref database

4.8 ELICITATION STUDIES

In order to enhance the pigment producing capability of the isolated bacteria, mixture of pathogenic fungal and bacterial extract have been added to the Nutrient broth inoculated with isolated bacteria. Then the culture incubated for 3-5 days at 37°C and then analysed for pigment production.

4.9 GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis is a powerful analytical technique used to identify and quantify compounds in a sample. GC-MS can be used to separate complex mixtures, quantify analytes, identify unknown peaks and determine trace level of contamination. The microbial extract treated and untreated control samples were analysed for GC-MS analysis.

4.9.1 Extract preparation

After the completion of the incubation process, the culture broth was divided into three separate funnels, with each funnel containing 500 mL of the broth and the solvent hexane added. Overnight, the solvent and the culture broth in the separating funnels formed distinct layers, indicating successful extraction of different compounds from the broth into each solvent. This solvent layer was carefully collected in separate beaker to ensure proper isolation of the extracted materials. To remove any remaining

traces of the solvents and to concentrate the extracted compounds, the beaker was placed in a water bath set at 60°C for drying. This step aimed to evaporate the solvent, leaving behind the concentrated extract from the culture broth. Once the extracts were fully dried and free of any residual solvents, they were transferred into test vials for further analysis and experimentation.

4.9.2 Gas chromatography–Mass spectrophotometry

The crude sample was sent for size- and polarity-based GC-MS analysis. The chemicals found in metabolites were identified using GC-MS. The GC-MS analysis was performed using a PerkinElmer GC Clarus 500 system and a gas chromatograph connected to a mass spectrometer. For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used in the electron impact mode. An injection volume of 2 µL was employed, and helium gas (99.999%) was used as a carrier gas at a continuous flow rate of 1 mL/min. The injector temperature was kept constant at 250°C, the ion-source temperature was kept constant at 200°C, and the oven temperature was programmed to begin at 110°C, then increased by 10 °C/min to 200°C, and then increased by 5 °C/min to 280°C, finishing with a 9-min isothermal at 280°C. Fragments ranging in size from 45 to 450 kDa were used in the mass spectra. The total GC–MS run time was 36 min, with a solvent delay of 0–2 min. The relative percentage quantity of each component was determined by comparing the average peak area of each component to the total areas.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Isolation of pigmented bacteria

The samples obtained were streaked on sterile nutrient agar plates and incubated at 37°C for 48 hours. A mixture of pigment producers and non-pigment producers were obtained on Nutrient agar plate (Figure 5.1) after incubation. 48 hours of incubation out of all colonies which morphologically different and pigmented colony was selected and named as PB1. Further the colony PB1 subcultured and also preserved using glycerol.(Fig 5.1)



Fig 5.1 Isolation of red pigment producing bacteria

5.2 EFFECT OF FACTORS INFLUENCING PIGMENT PRODUCTION

The pigment production is affected when the cells are exposed to stressful conditions which may include elevated temperatures, osmotic pressures, metabolic inhibition, etc. The yield of pigment from fermentation is increased as bacterial growth is restrained due to adaption to conditions of stress (Venil *et al.*, 2014). Effect of various physiological parameters such as temperature, pH, and salt concentration on the growth of the PB1 bacterial isolate was studied.

5.2.1 Effect of temperature

The bacterial isolate was streaked on sterile Nutrient agar slants and incubated at various temperatures, 100°C, RT and 37°C for 24-48 hours. Maximum growth and pigment production of the bacterial isolate was visually observed at RT and 37°C after 48 hours of incubation, whereas no growth was observed for all the isolates at 100°C (Fig.5.2). Although bacteria grow over a broad range of temperatures, reduction in pigment production at elevated temperatures is well documented (Shaikh, 2016).

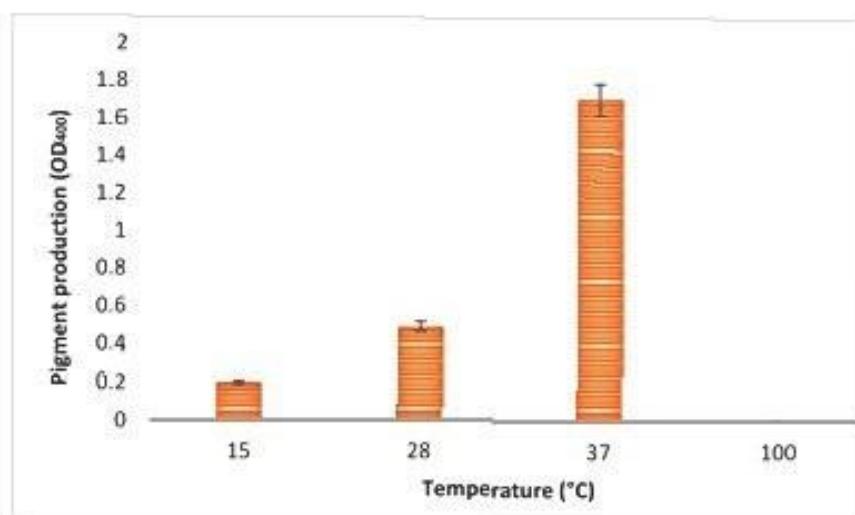


Fig 5.2 Effect of temperature on pigment production

5.2.2 Effect of pH

The bacterial isolate was streaked on sterile Nutrient agar slants of various pH (5.0, 7.0, 9.0 and 11.0) and incubated at 37°C for 24-48 hours. It was found that no growth was observed at pH 5 for the bacterial isolate. PB1 did not show pigment production at pH 12. Maximum growth and pigment production for bacterial isolate PB1 was seen at pH 7 after 48 hours of incubation (Fig 5.3). The bacterial isolate exhibited carotenoids production and growth in the range of pH 7 to 10, however, maximum carotenoids production observed to be 482.82 µg/g at pH 7 (Shatila, 2013).

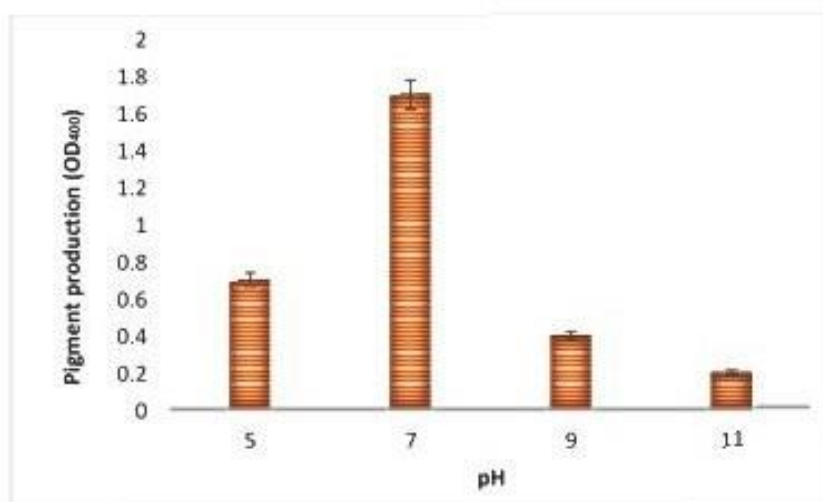


Fig 5.3 Effect of pH on pigment production

5.2.3 Effect of salt concentration

The bacterial isolate was streaked on sterile Nutrient agar slants with different salt (Sodium chloride) concentration 2.5%, 4.5%, 6.5% and 8.5% and incubated at 37°C for 24-48 hours. Maximum pigment production for the bacterial isolate PB1 was observed at 2.5%, after 48 hrs of incubation (Fig. 5.4). The bacterial isolate PB1 showed pigment production at the salt concentrations of 2.5, 4.5 and 6.5%. No pigment production was seen in 8.5 % of salt concentrations. Hence, with the increasing salt concentration (8.5%) there was no pigment production.

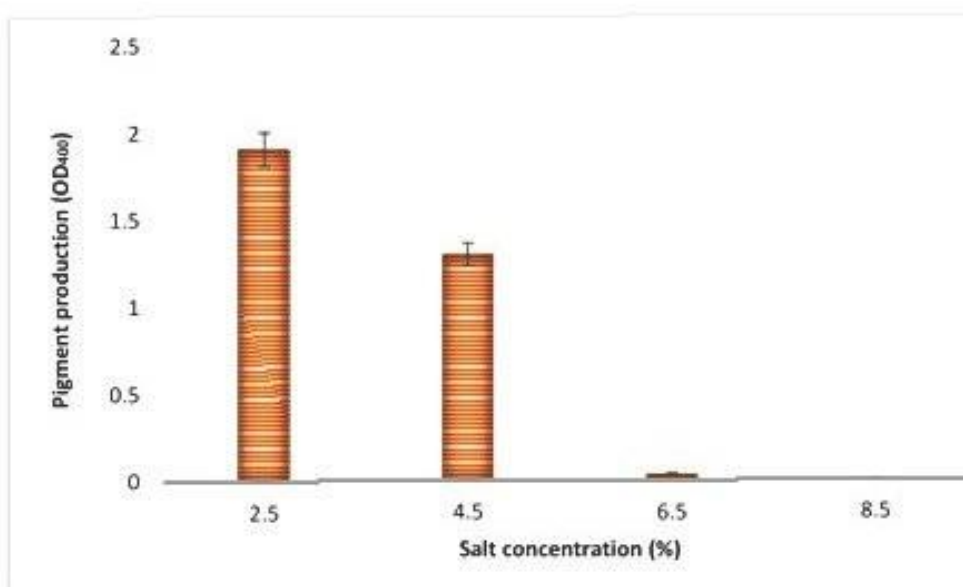


Fig 5.4 Effect of salt concentration on pigment production

5.3 IDENTIFICATION OF BACTERIAL ISOLATE PB1

5.3.1 Morphological and biochemical identification

The PB1 obtained was selected from the plate and its characteristics were noted down. Gram staining was performed to know the Gram nature of the isolates. Fig 5.5 and 5.6 illustrates the results of Gram staining and spore staining studies. For the bacterial isolate PB1, the colony shape, edge and surface was found to be round and smooth. However, the size of colonies varied.

Bacterial isolate PB1 belong to Gram negative, spore forming bacilli. Non-motile in nature. It can degrade tryptophan as it gave positive result for indole test. PB1 showed negative results for MR-VP and catalase test. It could utilize citrate as carbon source as evidenced from positive citrate utilization test.

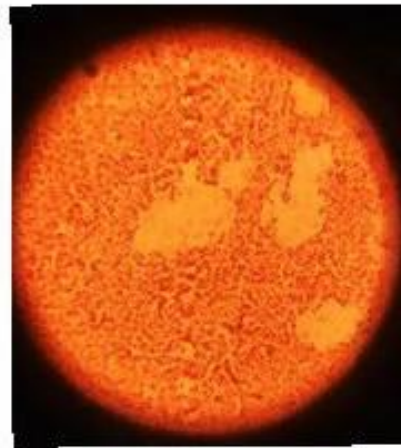


Fig 5.5 Gram staining (Gram negative bacilli shaped bacteria)

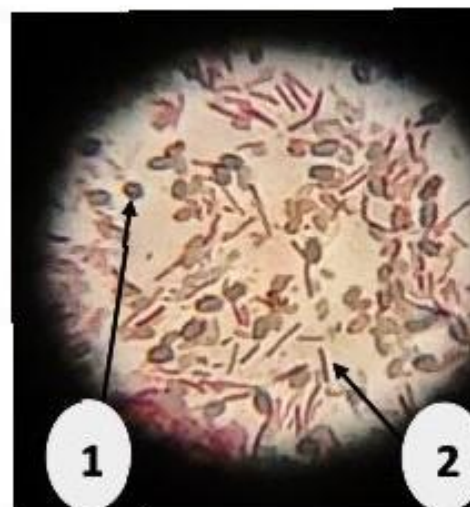


Fig.5.6 Spore staining (1. Free spores, 2 – Vegetative cells)

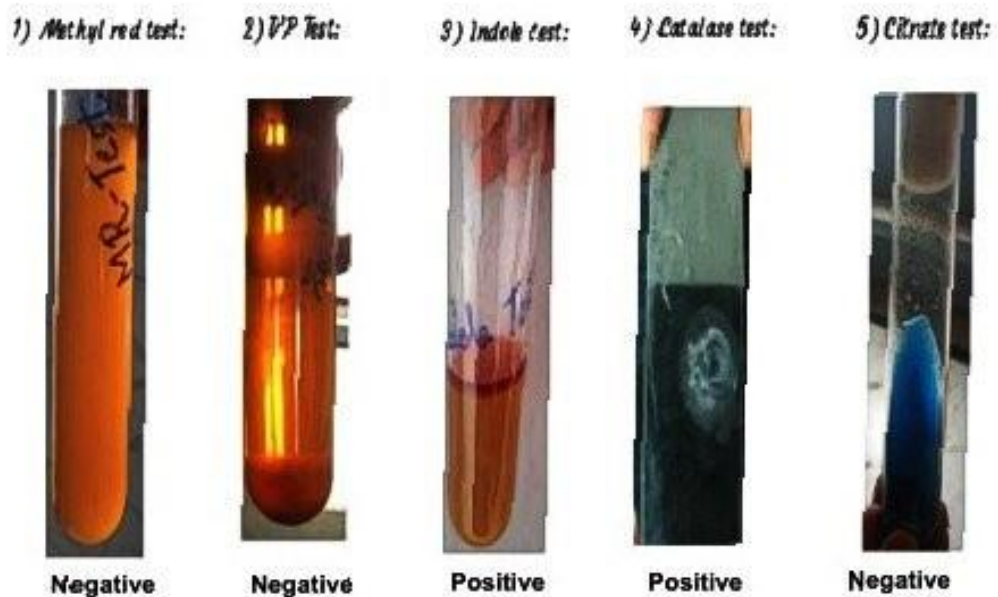


Fig 5.7 Responses of Bacterial isolate PB1 to biochemical tests

5.3.2 Molecular level identification of bacterial isolate PB1

The NCBI BLAST analysis of nucleotide sequences obtained from 16srRNA sequencing method revealed that PB1 is *Stenotrophomonas* species (Fig 5.8-5.10).

5.3.2.1 *Stenotrophomonas* spp

As the bacteria isolated from soil sample it could be *S. rhizophila*. *Stenotrophomonas* is a genus of Gram-negative bacteria, comprising at least ten species. The main reservoirs of *Stenotrophomonas* are soil and plants. *Stenotrophomonas* species range from common soil organisms (*S. nitritireducens*) to opportunistic human pathogens (*S. maltophilia*); the molecular taxonomy of the genus is still somewhat unclear. The most common species, *S. maltophilia* is very versatile and can be beneficial for plant growth and health, can be used in agriculture, biocontrol, bioremediation and phytoremediation strategies as well as the production of biomolecules of economic value. On the other hand, some of *S. maltophilia* strains are pathogenic to humans with multidrug resistant profile. *S. indulgences* can also cause or be part of polymicrobial infections in humans, especially small children. *Stenotrophomonas* can also be phytopathogenic unlike closely related genera *Xylella* and *Xanthomonas*. Members of the genus *Stenotrophomonas* have an important ecological role in the nitrogen and sulphur cycles.

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>0124_605_001_PCR_1A_27F_E09.ab1
GATGTCCCCACGAGTAGTTAATGAAAGCGAGGGCCTAACACATGCAAGTCGAACGGCAGCACAGATAAGAGCTTGCTC
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CTAATACCGCATACGACCTTCGGGTGAAAGCAGGGGACCTTCGGGCCCTTGC CGGATAGATGAGCCGATGTCGGATTAGC
TAGTTGGCGGGGTAAAGGCCACCAAGGCACGATCCGTAGCTGCTGAGAGGATGATCAGCCACACTGGAAC TGAGAC
ACGGTCCAGACTCTACGGGAGGCAGCAGTGGGGAATATTGGACAA TGGCGCAAGCCTGATCCAGCCATACCGCGTGGG
TGAAGAAGGCCTTCGGGTGTAAAGCCCTTTGTTGGGAAAAGAAAAGCAGTCGGCTAATACCCGGTTGTTCTGACGGTAC
CCAAAGAATAAGCACC GGCTAAC TTCGTGCCAGCAGCCGCGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGG
CGTAAAGCGTGCGTAGGTGGTTGTT?AAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGGACTGGGC
GACTAGAGTGTGGTAGAGGTAGTGGAAATCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGGAACATCCATGGCGA
AGGCAGCTACCTGGACCAACTGACACTGAGGCACGAAAGCGTGGGAGCAACAGGATTAGTACCCTGGTAGTCCAC
GCCCTAAACGATCGAACTGGATGTTGGGTGCAATTTGGCAGCAGTATCGAAGCTAACGCGTTAAGTTCGCCCGCTGGG
GAGTACGGTCGCAAGACTGAACTCAAGGAATTGACGGGGGCCGACAAAGCGGTGGAGTATGTGTTTATTTCATATGC
AACCGGAAGAACTTACCTGGTCTTGACGTGTGAGAACTT7CCAGAGATGGATTGGTGCCCTTCGGGAACCGAACCCAG
GTGCTGCATGGCTGTCTCACCTCCTTTCC

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Fig. 5.8 Nucleotide sequences of the bacterial isolate PB1

Descriptions

Graphic Summary

Alignments

Taxonomy

Sequences producing significant alignments

Download

Select columns

Show 100

select all

100 sequences selected

GenBank

Graphics

Distance tree of results

MSA View

Description	Scientific Name	Max score	Total Score	Query Cover	E value	Per ident	Acc. Len	Accession
☒ Shewanella sp. strain B-1049 16S ribosomal RNA gene, partial sequence	Shewanella sp.	1864	1864	90%	0.0	99.32%	1508	MN110222.1
☒ Shewanella sp. strain F814 16S ribosomal RNA gene, partial sequence	Shewanella sp.	1864	1864	90%	0.0	99.32%	1489	MN323165.1
☒ Shewanella macleodensis strain 16S rRNA, partial sequence, strain JCM 1369	Shewanella...	1864	1864	90%	0.0	99.32%	1489	LC379121.1
☒ Shewanella macleodensis strain F814 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1505	MN144280.1
☒ Shewanella macleodensis strain F814 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1503	MN144275.1
☒ Shewanella sp. LM91 chromosome, complete genome	Shewanella...	1864	5593	90%	0.0	99.32%	4317450	CP011483.1
☒ Shewanella sp. strain CPO 11.58 16S ribosomal RNA gene, partial sequence	Shewanella sp.	1864	1864	90%	0.0	99.32%	1219	GJ953849.1
☒ Shewanella macleodensis strain G1 P4 chromosome, complete genome	Shewanella...	1864	3728	90%	0.0	99.32%	4198852	CP014294.1
☒ Shewanella sp. JCM 20854 gene for 16S ribosomal RNA, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1507	LC133093.1
☒ Shewanella sp. 479c 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1129	KJ821158.1
☒ Shewanella sp. 479c 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1248	KJ821159.1
☒ Shewanella sp. 119c 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1406	KJ821156.1
☒ Shewanella sp. B2128 Strain chromosome	Shewanella...	1864	5593	90%	0.0	99.32%	4487489	CP024520.1
☒ Shewanella macleodensis strain F814 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1447	KM096602.1
☒ Shewanella macleodensis strain 42 16S ribosomal RNA gene, partial sequence	Shewanella...	1864	1864	90%	0.0	99.32%	1504	KJ054412.1

Fig 5.9 NCBI-BLAST result summary for the nucleotide sequences of PB1

Stenotrophomonas sp. strain BIS1040 16S ribosomal RNA gene, partial sequence

Sequence ID: [MN810222.1](#) Length: 1508 Number of Matches: 1

Range 1: 38 to 1067 [GenBank](#) [Graphics](#)

[Find Matches](#) [x](#) [123](#)

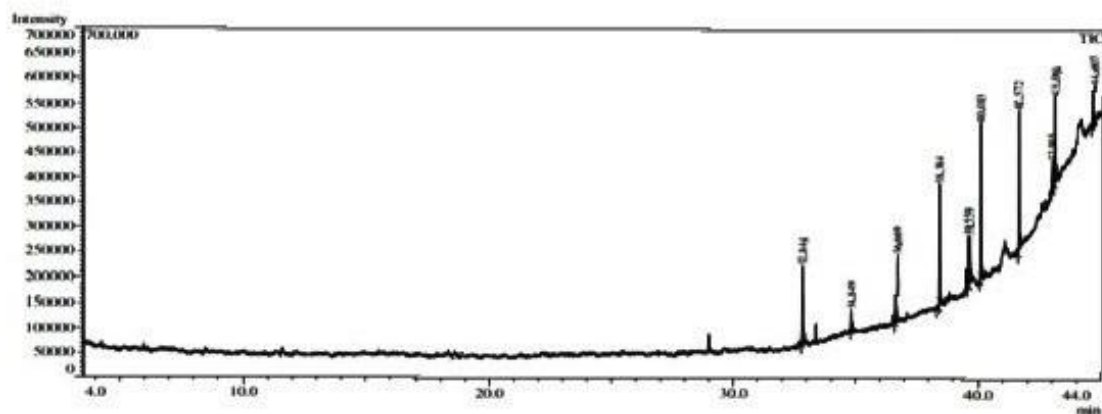
Score	Expect	Identities	Gaps	Strand
1864 bits(1009)	0.0	1024/1031(99%)	1/1031(0%)	Plus/Plus
Query 34	GGCCTAACACATGCAAGTCGAACGGCAGCACAGATAAGAGCTTGCCTTATGGGTGGCGA	93		
Sbjct 38	GGCCTAACACATGCAAGTCGAACGGCAGCACAG-TAAGAGCTTGCCTTATGGGTGGCGA	96		
Query 94	GTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAACGTAGGGAA	153		
Sbjct 97	GTGGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAACGTAGGGAA	156		
Query 154	ACTTACGCTAATACCGCATACGACCTTCGGGTGAAAGCAGGGGACCTTCGGGCCCTTGC	213		
Sbjct 157	ACTTACGCTAATACCGCATACGACCTTCGGGTGAAAGCAGGGGACCTTCGGGCCCTTGC	216		
Query 214	GGATAGATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAAGGCGACG	273		
Sbjct 217	GGATAGATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCACCAAGGCGACG	276		
Query 274	ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACCTGAGACACGGTCCAGACTC	333		
Sbjct 277	ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACCTGAGACACGGTCCAGACTC	336		
Query 334	CTACGGGAGGCAGCAGTGGGGAAATTGGACAATGGGCGCAAGCCTGATCCAGCCATACC	393		
Sbjct 337	CTACGGGAGGCAGCAGTGGGGAAATTGGACAATGGGCGCAAGCCTGATCCAGCCATACC	396		
Query 394	GCGTGGGTGAAGAAGGCCCTTCGGGTGTAAAGCCCTTTGTTGGGAAAGAAAAGCAGTCG	453		
Sbjct 397	GCGTGGGTGAAGAAGGCCCTTCGGGTGTAAAGCCCTTTGTTGGGAAAGAAAAGCAGTCG	456		
Query 454	GCTAATACCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCAGGCTAATTCGTGCCAG	513		
Sbjct 457	GCTAATACCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCAGGCTAATTCGTGCCAG	516		

Fig 5.10 Sequence alignment between the query sequence PB1 and *Stenotrophomonas sp*

Stenotrophomonas species, especially *S. maltophilia* and *S. rhizophila*, are often found in association with plants, such as cucumber, oilseed rape, potato, strawberry, alfalfa, sunflower, maize, rice, wheat, various weeds, willow and poplar. *Stenotrophomonas* can be isolated from the rhizosphere or from internal plant tissues, particularly from the vascular tissues of the root and stem (Stephen and Dirkhasse 2008).

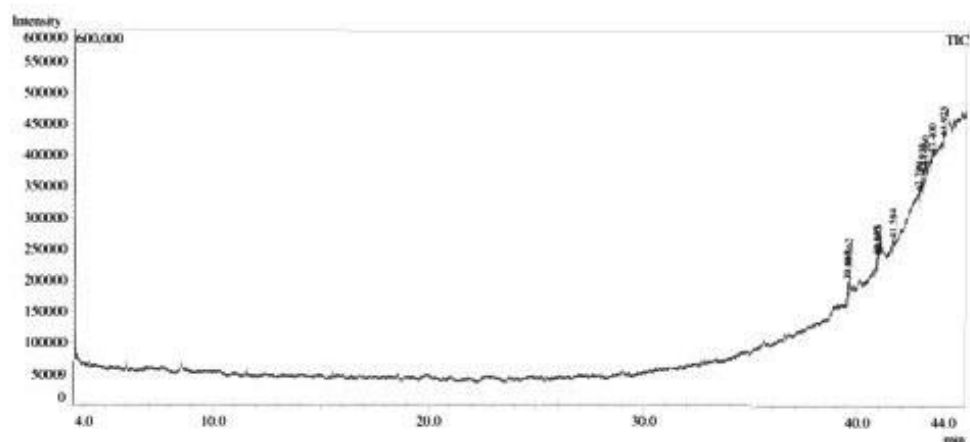
5.4 GC-MS-ANALYSIS

GC-MS analysis of untreated and treated bacterial culture revealed that there was a improved production of pigment as well as other metabolic componenets of bacterial extract obtained with solvent. This confirmed the positive influence of biotic elicitors on pigment production of bacterial isolate PB1(Fig 5.11 and Fig 5.12).



Peak#	R.Time	Area	Area%	Name
1	32.844	471870	10.62	9-OCTADECENOIC ACID (Z)-, METHYL
2	34.849	100538	2.26	Hexatriacontane
3	36.669	343504	7.73	HEXACOSANE
4	38.384	648454	14.60	HEXACOSANE
5	39.559	299156	6.73	9-Octadecenoic acid, 1,2,3-propanetriyl ester
6	40.013	836998	18.84	Tetracontane
7	41.572	770520	17.34	DOTRIACONTANE
8	42.945	210266	4.73	Oleoyl chloride
9	43.064	505176	11.37	DOTRIACONTANE
10	44.607	256136	5.77	Hexatriacontane
		4442618	100.00	

Fig.5.11 GC-MS report of bacterial extract (untreated control)



Peak#	R.Time	Area	Area%	Name
1	39.468	88670	13.14	6,11-HEXADECADIEN-1-OL
2	39.562	118284	17.53	9-Octadecenoic acid, 1,2,3-propanetriyl ester,
3	40.845	12714	1.88	1,4-METHANOAZULEN-7(1H)-ONE, OCT
4	40.875	34239	5.07	PHOSPHORIC ACID, 3-(DIMETHYLAMID
5	41.584	42234	6.26	Tetracosane
6	42.790	71689	10.62	Sebacic acid, di(2,2-dichloroethyl) ester
7	42.935	163998	24.30	OCTADECANOIC ACID, BUTYL ESTER
8	43.060	114177	16.92	2-(3-ACETOXY-4,4,10,13,14-PENTAMETI
9	43.400	13069	1.94	2-([2-(3,5-DIMETHYL-1H-PYRAZOL-1-Y
10	43.925	15826	2.34	(4-ALLYL-2-TERT-BUTYL-5-OXO-1,3-DI
		674980	100.00	

Fig 5.12 GC-MS report of bacterial extract treated with biotic elicitor

CHAPTER 6

SUMMARY AND CONCLUSION

The pigments derived from bacteria are gaining global significance due to their natural colour, ease of production, and non-toxic properties, making them a viable alternative to synthetic pigments. This research focuses on isolating pigment-producing bacteria from various soil collected from waste land, polluted soil and beach sand. One bacterial strain was isolated and labelled as PB1, and its morphological traits and Gram's nature were examined and found to be Gram negative, bacilli shaped spore-forming bacteria. The isolate PB1 produced positive result for indole, catalase and citrate utilization tests and showed negative result for MR-VP test. Further, the bacterial isolate PB1 was identified as *Stenotrophomonas* spp through DNA sequencing. The study also delves into understanding how different physical factors like temperature, pH as well as chemical factors like NaCl concentration affect pigment production and enumerated pH 7, 37°C temperature and 2.5 % NaCl concentration as optimal. Extraction of the pigments was carried out using hexane solvent. The biotic elicitation studies were carried out to enhance the pigment production which further validated with GC-MS analysis.

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