

# **SCREENING AND ISOLATION OF DYE DEGRADING BACTERIA FROM SOIL SAMPLE**

Submitted in partial fulfillment of the requirements for the award of Bachelor of  
Science degree in B. Sc. Microbiology by

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**INSTITUTE OF SCIENCE AND TECHNOLOGY**  
**(DEEMED TO BE UNIVERSITY)**

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**BONAFIDE CERTIFICATE**

This is to certify that this Project Report is the bonafide work of E.VANISRI  
(Reg. No. 40300072) who carried out the project entitled **SCREENING AND  
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## DECLARATION

I **E. VANISRI** hereby declare that the Project Report entitled **SCREENING AND ISOLATION OF DYE DEGRADING BACTERIA FROM SOIL SAMPLE** done by me under the guidance of **Dr. R.P. OVIYA** (Internal) and **Dr. SARAVANAN** (External) at **CENTRE FOR ANIMAL LABORATORY AND RESEARCH, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **MICROBIOLOGY**.

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## **ABSTRACT**

Textile dye industry waste is one of the most serious problems in the environment. The presence of dyes in the environment causes harmful effects on plants, animals and humans. Industrial dye degradation is any of the numbers of processed by which dyes are broken down, ideally into harmless products. So, biological degradation, being eco-friendly and cheap methodology is taken into account as an efficient methodology for removal of dyes. The bacteria are isolated from different sources were subjected to acclimatization with the following dyes such as Safranin, crystal violet, malachite green in basal media studies. The most promising bacterial isolate was used for the further dye degradation studies. This decolorization and degradation potential increased the applicability of these microorganisms for the dye removal. The enzyme analysis and GCMS and UV methods are done. The present study isolated E1 strain has ability to degrade all the three dyes. The probable isolates obtained must be additionally investigated relating to various factors and further steps of dye degrading Activities.

Keywords: Analytical dye, isolation, Degradation, Decolourization.

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## LIST OF ABBREVIATIONS AND SYMBOLS

|                                               |
|-----------------------------------------------|
| ➤ NaCl- sodium chloride                       |
| ➤ $\text{FeCl}_3$ - Iron(III) chloride        |
| ➤ $\text{Na}_2\text{SO}_4$ -Sodium sulphate   |
| ➤ RB- reactive Blue                           |
| ➤ DR- Direct red                              |
| ➤ FTIR- Fourier transform infrared            |
| ➤ UV- vis- Ultraviolet–visible spectroscopy   |
| ➤ GCMS- Gas chromatography mass spectrometry  |
| ➤ nm- nanometer                               |
| ➤ ES- Electron shuttles                       |
| ➤ ELISA-Enzyme-linked immunosorbent assay     |
| ➤ OD- optical density                         |
| ➤ $\text{H}_2\text{O}_2$ - Hydrogen peroxide. |
| ➤ AQS- anthraquinone sulfonate                |
| ➤ AQDS- anthraquinone disulfonate             |



## **CHAPTER-1**

### **INTRODUCTION**

In modern life, rapid industrialization and urbanization resulted in the discharge of large amount of waste into the environment, which in turn creates pollution. Water is essential for survival and existence of life on planet Earth (Sakpal *et al.*, (2021). The waste waters were released into the environment. It causes severe effect on the environment. The most environmental problem faced due to the textile dyeing industry is that the industry produces large volumes of high strength of aqueous waste effluents. The discharge of dye effluents containing recalcitrant residue into the rivers and lakes (Thoker Farook Ahmed *et al.*, (2012). It is responsible for about 20% of global clean water pollution from dyeing and finishing products. Washing synthetics releases an essential 0.5M tons of microfibers into the ocean and river in a year. The discharge from different sources such as textile industries, cosmetics, paper mills, pulp industries, dyeing and dye intermediates and pigments are produced in these industries. Especially in textile industries produced more than 70% of the total quantity of waste in India (Rajeswari *et al.*, (1992)).

The textile industry accounts for largest consumption of dyestuffs at nearly 80%. It is estimated that over 280,000 tons of the textile dyes are discharged in industrial effluent every year worldwide. Therefore, pollution from these discharge contaminated with dye stuff is becoming alarming (Pandey *et al.*, (2007). Textile industries waste water is highly coloured which mainly block the penetration of sunlight thereby retarding the growth of aquatic animals and plants, it is also contains the dissolved toxic substances and carcinogens (Chung *et al.*, (2016). Dyes also affect the internal organs like kidney, liver and gastro intestinal tract. In addition, the formation of tumors, cancers and various allergies have been associated with the presence of some of these dyes in waste waters (Sponza *et al.*, (2002). Most of the methods used in textile effluent treatment are channeled towards color removal. These methods are either ineffective or expensive and more over generate more toxic products that are recalcitrant to biodegradation process (Maier, J.*et al.*, (2004).

## **1.1 Classification of dyes**

Dyes are classified into two groups namely,

1.1.1 Natural dye

1.1.2. Synthetic dye

### **1.1.1 Natural Dye**

Before 1856, the natural dyes derived from aniline (One of the simplest compounds of coal tar) were obtained as a black precipitate and discovered as 2 purple color which is used for dyeing the silk. The major disadvantage of natural dye is that it is not stable in sunlight. To overcome this, synthetic dyes are synthesized(Sakpal and Tarfeet *al.*,(2021).

### **1.1.2 Synthetic Dye**

Different chemical compounds are used to make synthetic dyes. There are three categories of synthetic dyes. Cellulose fiber dyes, synthetic fiber dyes, and protein fiber dyes are the three types.

#### **1.1.1.1 Cellulose Fibre dye**

Plants such as cotton, linen, ramie, rayon, and lyocell are sources of cellulose fiber.

a). Receptive Color

b). Dry direct

c). Indigo Colors

d). Dyeing Sulfur

#### **a). REACTIVE DYE**

Reactive dyes are the most important dye compared to other dyes. These dyes can produce high pigmentation, can manipulate under wide temperature ranges,

permanent attachment of dye to fibers and more reactive groups can form a covalent bond to multiple fibers (Parimal Pal *et al.*, (2017).

#### **b). Direct dye**

Since the uptake of direct dyes is not sufficient, the uptake of direct dyes can be improved by combining them with inorganic electrolytes to create sodium 3 sulphate or sodium chloride. When the solubility of the direct dye can be decreased in the dye bath phase, NaCl or Na<sub>2</sub>SO<sub>4</sub> is added for aggregation (Burkinshaw and George Salihu *et al.*, (2017).

#### **c). Indigo dyes**

The most common application for indigo dyes is the dyeing of blue denim, for which there is massive global manufacturing. Insoluble in water, indigo dyes became soluble through alkaline reduction. Indigo is used in the textile dyeing process in its water-soluble form, which oxidises when exposed to air and transforms back into its insoluble form for excellent fabric binding (Alicia Paz *et al.*, (2017).

#### **d). Sulphur Dyes**

The best colouring qualities and lowest cost are found in sulphur dyes. These are converted to water-soluble compounds by using sodium sulphide in the colouring procedure. Due to the existence of disulfide bonds, the structures of sulphur dyes are complicated (Khattab *et al.*, (2019).

##### **1.1.1.1 PROTEIN FIBER DYES**

Protein fiber dyes are obtained from animal sources like wool, silk, mohair, and cashmere. They are suitable at high pH levels since cloths are dyed with water-soluble acid dyestuff to obtain insoluble dye on the fiber.

a). AZO DYES

b). ANTHRAQUINONE DYES

c). TRIARYL METHANE DYES

d). PHTHALOCYANINE DYES

#### **a) Azo dyes**

Azo dyes have been utilized in the industrial sector to a degree of 60–70% due to their low cost, high stability, and intense colour. Azo dyes are made up of auxochrome, chromophores, and decolorized electron systems containing conjugated double bonds. Azo dye loses color when the bonds break down and has  $-N=N$  accounts for colouring. These colours are joined to unsaturated carboxyl, sulphonyl, and aliphatic groups on one side and aromatic compounds on the other (Sriram *et al.*, (2013).

#### **b) Anthraquinone dye**

The second important family of protein fibre dyes is anthraquinone dyes, but because they are so expensive, they are less commonly utilized. Excellent qualities of these dyes are their fastness, vibrant hues, and water solubility (Cenek Novotny *et al.*, (2005).

#### **c) Triarylmethane dye**

Triarylmethane is a hydrocarbon that is used to make dyes. The major fibers that these dyes are used for are wool and silk protein fibers. When their chemical structure only contains one sulfonic acid, they can be employed as indicator (Gizem Basaran Dindaas *et al.*, (2019).

#### **d). Phthalocyanine dyes**

Blue and green hues are created using phthalocyanine dyes. These pigments have great colorfastness to light, oxidation resistance, chemical stability, and water solubility. When they come into touch with the metal Cu, they turn poisonous. (Maria Cristina Silva *et al.*, (2011).

### **1.1.2.3 SYNTHETIC FIBER DYES**

Synthetic fibers are made up of polyester, acrylic, polyamide, and polypropylene. The synthetic fibers are dyed using two dyes namely, Disperse dyes, and Basic dyes

#### **a). DISPERSE DYES**

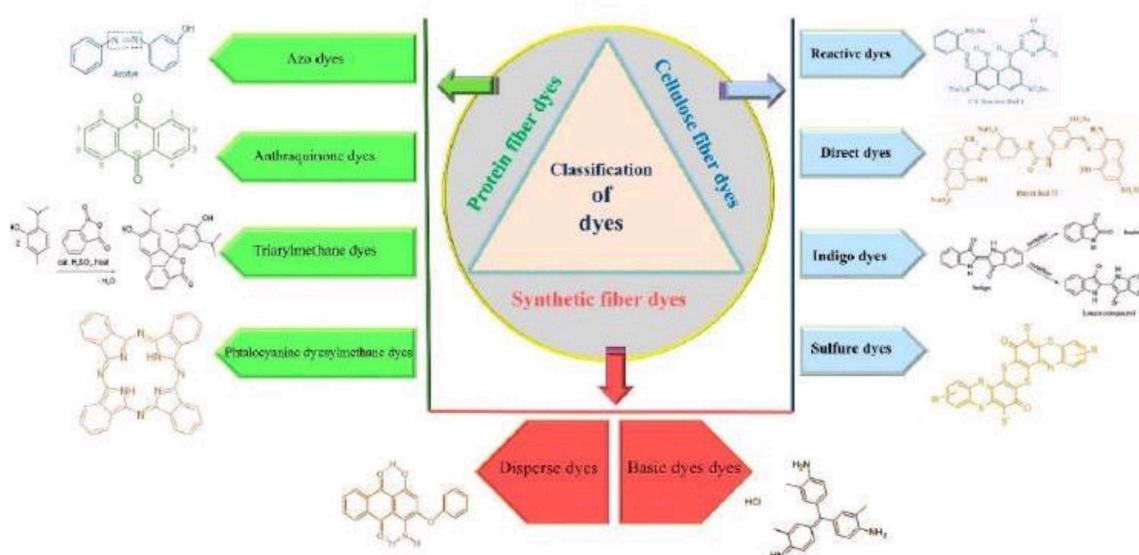
#### **b). BASIC DYES**

### a) Disperse dyes

The tiniest molecule above all other dyes is found in disperse dyes. These colors can withstand extreme heat. A high temperature dyeing solution is made using the dye and dispersion agent combination (Bayramoglu *et al.*, (2020).

### b) Basic dyes

Basic dyes are converted into vibrant cationic salts that are used to dye fabrics with anionic fibres. As a result, it is known as a cationic dye (Thakur *et al.*, (2018).



**Figure 1.1 Schematic chart of synthetic dye classification.**

As the dye wastewater is not handled, synthetic dyes can pose major risks. Environmental harm is done by the industry's effluent discharge, which is damaging. Due to the 6 oxidative stress and low protein content caused by soil pollution, plant growth is decreased (Vikrant *et al.*, (2018) Wastewater discharge byproducts may have an impact on human organs. It can directly aggravate skin conditions including allergies and asthma. By resulting in cancer, gene mutation, and tuberculosis, it indirectly influences the food chain. (Khan *et al.*, (2018).

By considering this, there are three different methods for the degradation and decolorization of textile dye effluent, Physical treatment, Chemical treatment, and Biological treatment.

### **Physical treatment :**

As the dye wastewater is not handled, synthetic dyes can pose major risks. Environmental harm is done by the industry's effluent discharge, which is damaging. Due to the oxidative stress and low protein content caused by soil pollution, plant growth is decreased. Wastewater discharge byproducts may have an impact on human organs. It can directly aggravate skin conditions including allergies and asthma. By resulting in cancer, gene mutation, and tuberculosis, it indirectly influences the food chain (Ghasemi *et al.*, (2016).

### **Chemical treatment:**

Coagulation/Flocculation and Ozonation are chemical techniques. For textile dye effluent, coagulation can be employed as a pretreatment. This method entails the use of chemicals like FeCl<sub>3</sub>, FeSO<sub>4</sub>, alum, lime, etc. where insoluble materials are eliminated (Zhang *et al.*, (2015). Utilizing ozone as an oxidant, ozonation removes the hazardous textile effluent component. This method has several restrictions because it needs the right pH, salts, temperature, etc. (Adnan *et al.*, 2016). The aforementioned procedure is more costly and has some restrictions

### **Microbial treatment**

Biodegradation is a very important characteristic for toxic chemicals in comparison to physical and chemical degradation because if the rate is high, the concentration and subsequently the toxic effect will be reduced quickly, whereas extremely persistent chemicals can retain their toxic effect for many years to come.

However, microbial detoxification and degradation offer a practical and environmentally friendly alternative (F.J. Cervantes *et al.*, (2001) Promising findings have been obtained from extensive study in the field of biological dye effluent

decolourization and degradation, however the majority of this work was conducted with single model compounds .

However, industrial textile wastewater presents the additional complexity of dealing with unknown quantities and varieties of many dyes and presents the high amount of auxiliary chemicals and heavy metals (Lokendrasingh *et al.*). *Proteus sp*, *Enterococcus sp*, *Streptococcus sp.*, *Bacillissubtilis* and *Staphylocococcus sp.* are some of the common bacterias that are present on the soil. The analytical dyes were used for the dye degradation of bacterias (Wuhrmannk *et al.*, (1980). Malachite green, Saffranin, Crystal violet are the analytical dyes used in the degradation process.

The probable isolates obtained must be additionally investigated relating to varied factors like dye degrading capability, media composition affecting dye degradation and mechanism of dye degrading activity. Dyes are natural or synthetic colored organic compounds having the property imparting their color the other substances such as textile fibres (Sriram *et al.*, (2013).

Bioremediation has become a key microbial tool to deal with different pollutants, is a key of research area in the field of environmental science (Sailesh *et al.*, (2015).A number of bacteria, fungi, yeasts, algae and actinomycetes have been capable of decolorizing a range of analytical dyes. A number of biological and physio-chemical methods have been developed of the efficient removal of industrial analytical dyes. Particularly, bacteria are the most frequently applied organisms for degradation of analytical dyes, as they are generally fast to multiply rapidly under aerobic, anaerobic, facultative condition as well as in extreme environmental conditions as well as salinity and wide range variations in both PH and temperature (Balapure *et al.* (2014)

Attempts to isolate the pure bacterial cultures capable of degrading dyes started way back in 1970s with isolation of *Bacillus subtilis*, *Aeromonas hydrophobia*. Bacteria capable of dye decolourization / biodegradation either in pure cultures. However, comprehensive solutions for analytical dyes removal are far from reality, which calls for continued search for new organisms and technologies..

This study aimed to isolate and characterize an efficient bacterial strain, which exhibited the remarkable ability to degrade Malachite green, Safranin and Crystal violet.



## **CHAPTER 2**

### **REVIEW OF LITERATURE**

#### **2.1 DEGRADATION OF AZO DYES BY AZO REDUCTASE**

Saranraj, *et al.*, (2011) characterized that under aerobic circumstances, the enzyme azoreductase totally breaks down azo dyes. There is no prior treatment performed before collecting and storing the dye effluent. The pour plate technique is used to extract the bacterium that decolorizes dyes. Colonies are then selected and streaked for purity. The strains underwent the biochemical test while being stored while being preserved in the nutrient agar medium. putting an inoculum of bacteria in a flask and shaking it with a machine for four days. The dye solution is collected every 24 hours, and a decolorization and biodegradation assay is conducted. The deterioration of the dye is evident in a shift in colour from black to brown.

#### **2.2 DEGRADATION OF ACID ORANGE 10 BY *PSEUDOMONAS PUTIDA***

Tripathi and Srivastava *et al.*, (2011) defined that they employed five distinct strains in this publication to decolorize acid orange 10 in a batch reactor. To find the ideal conditions, they have experimented with several parameters like media condition, pH, temperature, and initial dye concentration. They gathered several germs and injected them into a medium rich in nutrients. These inoculated bacteria are transferred and kept in top shape in a flask filled with acid orange 10. Samples are collected at predetermined intervals, centrifuged, and their optical densities for supernatants are calculated, along with the percentage of decolorization. The author's research for this journal includes Conical Flask Assay, Parameter Optimization, Concentration 11 Studies, Bio-Decolorization and Biodegradation Analysis. This suggests that *Pseudomonas putida* MTCC102 can deteriorate and discolors.

#### **2.3 DEGRADATION OF REACTIVE BLUE 160 BY BDN CULTURE**

KshamaBalapure, *et al.*, (2014) analyzed that eight strains that can decolorize and breakdown reactive blue 160 (RB 160) have been identified from the polluted soil.

These strains are together referred to as mixed culture BDN. BDN can be employed in bioremediation to decolorize 27 structurally distinct colours. Genomic DNA in mixed culture BDN contains RB160. Primers were used to amplify the 16rRNA gene, which was then subjected to ARDRA (Amplified Ribosomal DNA Restriction examination) examination and sequenced by an automated DNA analyzer. Enzymatic experiments have shown that submerging an ultrasonic probe into a suspended cell mass causes a release of a crude enzyme. The activities of laccase, tyrosinase, lignin peroxidase, and NADH-DCIP reductase have been determined.

## **2.4 DEGRADATION OF DIRECT RED 81 BY NBNJ6 CULTURE**

NishantJunnarkar, *et al.*, (2005) defined that the new bacterial consortium uses the model dye Direct Red 81 (DR 81) for quick decolorization. Starch and casein are added to the medium to speed up the decolorization process, and high-performance thin-layer chromatography is used to further analyse the results. The bacterial consortium discolours nine azo dyes with various structural compositions. On the basis of the decolorization on liquid media, the 10 distinct bacteria are identified from the soil sample. To identify the type of bacteria, gramme staining is done on these isolated bacteria in Bushnell and Hass medium, which contains the only source of carbon. These ten isolated bacteria are combined to create NBNJ6, which completely removes direct red 81's hue. This journal examines how culture 12 circumstances, the impact of the co-substance on the dye.

## **2.5 DEGRADATION OF MALACHITE GREEN, SAFFRANIN, CRYSTAL VIOLET BY *PSEUDOMONAS FLUORESCENS***

Manivannan, *et al.*, (2011) characterized that utilizing microorganisms recovered from polluted soil, textile dyes such as Malachite Green, Saffranin and Crystal Violet have been broken down. By measuring the spectral density of the supernatant where there is a drop in absorbance, the decolorization may be seen. Comparative studies have been conducted to identify the best decolorizer and degrader bacteria by contrasting isolated bacteria with various colours. *Pseudomonas fluorescens*, *Bacillus*, and

*Escherichia coli* are the three isolated bacteria. *Pseudomonas fluorescens*, one of these microorganisms, has been found to be an efficient colour remover.

## **2.6 DECOLOURIZATION OF TEXTILE DYE BY *TRAMETES HIRSUTA***

Mohd Zaini Nawah, *et al.*, (2013) analyzed that the 16s rRNA DNA is amplified and extracted. The basic local alignment search tool is used to further sequence and analyze the amplified 16s rRNA. Due to the presence of additional intermediates such as aromatic amines, which can impair cell survival, the decolorization of pigments by bacteria in the late phase of growth is more successful than in a different phase. When the pH is neutral, where there is a quick reduction when it is very acidic or alkaline, the process of degradation is more effective. The pace of enzyme activity is accelerated by temperature; however a high temperature reduces cell viability. The supernatant is examined via HPLC to ascertain dye degradation. Colour removal is affected by factors like pH, temperature, and oxygen content. One co-metabolite is glucose.

## **2.7 IDENTIFICATION OF POTENT STAIN FOR DECOLORIZATION OF INDIGO**

(Ajibola *et al.*, (2005) ) discovered that the pH of the effluent affects both degradation and decolorization. At various pH levels, *Clostridium perfringens* degrades colour. It has been determined that the degradation of dye is significantly influenced by the effluent concentration. Degradation is more efficient at low concentrations than highly concentrated effluent. One of the isolated bacteria that breaks down the dye in aerobic circumstances is *Bacillus cereus*. Under unfavorable circumstances, *B.cereus* produces spores that are less effective at degrading the target material. Under aerobic conditions, *E. coli* is the most effective decolorizer and may break down the dye by up to 97% when the effluent is dilute.

## **2.8 DEGRADATION OF REACTIVE RED 195 BY *PAENIBACILLUS* SPP. R2**

(Mohd Zaini Nawah, *et al.*, (2013) discovered that the 16s rRNA DNA is amplified and extracted. The basic local alignment search tool is used to further sequence and analyze the amplified 16s rRNA. Due to the presence of additional intermediates such

aromatic amines, which can impair cell survival, the decolorization of dyes by bacteria in the late log phase is more successful than in a different phase. When the pH is neutral, where there is a quick reduction when it is very acidic or alkaline, the degradation is more effective. The rate of enzyme activity is accelerated by temperature, however a high temperature reduces cell viability. The supernatant is examined via HPLC to ascertain dye degradation. Colour removal is affected by factors like PH, temperature, and oxygen content. One co-metabolite is glucose.

## **2.9 DEGRADATION OF BROWN 706 BY *PSEUDOMONAS AERUGINOSA***

(AsadUllah Khan, *et al.*, (2021) stated that 15 distinct bacterium stains have been isolated. The most potent decolorizer across isolated bacterial strains is *Pseudomonas aeruginosa*. By homogenizing the bacteria, which releases the intracellular product, and subjecting the resulting mixture to filtering and extraction, the metabolites produced following decolorization are identified. One of the recognized metabolites is P-Xylene, which is produced when an azo-substituted benzene ring is deaminated, methylated, and the azo-linkage is broken by the azo reductase enzyme. Heavy metals, organic, and inorganic chemicals in waste dye effluent have an adverse effect on organisms. Bacterial metabolites can be toxic at large amounts and hinder the development of microorganisms. As a result, it is discovered that the microbe can effectively destroy colour at low concentrations. Microbes use glucose as a source of carbon for growth.

## **2.10 IMPORTANCE OF ELECTRON SHUTTLES**

JianboGuo, *et al.*, (2010) characterized that the redox mediators, such as electron shuttles (ES), enable microbial energy metabolism by transferring electrons from electron donor substances to microbes or from microbes to electron acceptor substances. It is possible to artificially induce ES, which results in electron flow and microbial energy metabolism. As a result, ES can be induced to breakdown azo dye. There are various reduction processes, including chemical, indirect/mediated, and direct enzymatic reduction. By including redox-mediating substances like anthraquinone sulfonate (AQS) and anthraquinone disulfonate (AQDS), the azo

degradation can be started. Aerobic bacteria with azoreductase, which may breakdown azo dyes, have been found, including K22 and KF46. After purification, these bacteria no longer contain flavin.

## **CHAPTER 3**

### **AIM AND OBJECTIVES**

#### **3.1 AIM**

To isolate and to determine the potent bacteria for textile dye degradation from a soil sample.

#### **3.2 OBJECTIVES**

1. Collection of soil sample.
2. Isolation of morphologically different isolates.
3. Screening of dye degrading bacteria.
4. Selection of potent bacteria.
5. Production, and Optimization of dye degradation by potent isolate.
6. Characterization of dye degradation samples by FTIR , GCMS.

## **CHAPTER 4**

### **MATERIALS AND METHODS**

#### **4.1 DESCRIPTION OF SAMPLING SITE**

Soil samples were collected in sterile polythene bags from Tada falls and hills. Soils from the area of Tada falls is have various properties;

1. Sufficient but not excessive nutrient supply
2. Correct and Good Texture
3. Perfect density and Temperature
4. Good relative humidity so that the presence of bacterium is very high
5. No chemical or any other toxic contents are found
6. Plant pathogens and insect pests are not found as it is a reserved forest
7. Plenty of organic matters
8. Healthy pH

#### **4.2 COLLECTION AND ISOLATION OF SOIL SAMPLE**

We took the soil samples from Tada falls and around the places of Tada falls. Humus and other debris will be present in the higher layer because five centimeters of the top layer were removed. A sterile container was used to collect a few grams of soil samples, which were then brought to the lab.

With each 9 ml of sterile distilled water, soil samples were serially diluted. From the obtained soil samples, cultivable bacterial populations were isolated and counted using the usual spread plate method with nutrient agar medium. For bacterial growth, all of the plates are incubated at room temperature.

#### **4.3 ISOLATION OF MORPHOLOGICALLY DIFFERENT BACTERIA FROM SOIL SAMPLE**

One gram of the collected soil sample was inoculated into 9 ml of nutrient broth and incubated for 1 day to enrich the microbial growth. One ml of incubated soil sample was transferred to 10<sup>-1</sup>, test tube and mixed thoroughly. From 10<sup>-1</sup> tube one ml was transferred to 10<sup>-2</sup> tube. Same steps were done and serially diluted up to 10<sup>-6</sup> tubes. One ml from the last tube was discarded. The nutrient agar medium is prepared. 0.1ml of diluted sample from each tube was transferred to the prepared nutrient agar plate. A sterile L-rod spread plate was made and all the plates were incubated at 28°C for 1 week. (Saravanan *et al.*, (2012); Gayathri *et al.*, (2010).

#### **4.4 SCREENING AND SELECTION OF POTENT ISOLATE FOR ANALYTICAL DYE DEGRADING BACTERIA FROM SPOT INOCULATION METHOD**

By using the spot inoculation method, all the bacterial isolates were tested for three different analytical dye degradation. Three different textile dyes are used to produce the nutritional agar before it is sterilized and a plate is made. Each isolate was spot-inoculated onto the agar surface, and it was then left to sit for three days at 28°C. We looked for zones of clearance on the plates.(Kannan *et al.*, (2019).

#### **4.5 DETERMINATION OF POTENT BACTERIA BY THE LIQUID METHOD**

Liquid media were used to validate all of the positive isolates using the spot inoculation procedure. Three different analytical dyes were used to make nutritional broth, which is then poured into the test tube and given time to sterilize. The positive isolates from the spot inoculation were sterilized, then injected in the liquid medium and incubated at 28°C and decolourized.



## **4.6 PRODUCTION AND OPTIMIZATION OF DYE DEGRADING ENZYME FROM POTENT STRAIN ISOLATES.**

### **4.6.1 INOCULUM PREPARATION**

For the preparation of inoculum, 10 ml of nutrient broth was prepared and inoculated with potent bacterial culture and incubated at 28°C for 48 hours.

### **4.6.2 Collection of Supernatant**

After incubation the culture broth was centrifuged at 7,000 rpm for 10 min at 4°C. The supernatant was collected and analyzed for further studies.

### **4.6.3 Decolourization Assay by uv-vis spectrum**

Five ml aliquots of the powerful bacterial culture were taken at different culture times (2, 4, 6, 8 and 24 hours), After incubation, the culture broth was centrifuged at 7,000 rpm for 10 min at 4°C to remove the bacteria. The supernatant was then tested for dye decolorization using a spectrophotometer (Shimadzu UV-2401 PC model, Yoto, Japan). The percentage of decolorization was determined using the formula below.

$$\text{Decolorization (\%)} = \frac{[(\text{Absorbance at } t_0) - (\text{Absorbance at } t_1)] \times 100}{(\text{Absorbance } t_0)}$$

Where  $t_0$  refers to the initial absorbance, OD  $t_1$  refers to the absorbance after incubation. Dye decolorization was investigated with potent bacterial strain (Manjinder *et al.*, (2005).

### **4.6.4 Optimization of degraded potent bacteria**

The prospective bacteria culture was inoculated onto nutritional broth to create the bacterial inoculum, which was then maintained in a rotary shaker for 18 hours at 28 °C. The inoculum was each 5 ml of nutrient broth culture.

#### **4.6.5 Effect of Carbon**

To investigate the impact of the carbon source, 100 ml of screening media (replacing the original) was replaced with 1% of each of the following carbon sources: glucose, fructose, sucrose, mannitol, and starch in a series of flasks. The screening medium was created using 0.1% of each colour (crystal violet, saffranin, malachite green). All of the tubes underwent a 120-hour incubation period at 28 °C in a rotating shaker.

#### **4.6.6 Effect of Nitrogen source**

About 5 ml of an 18-hour prospective bacterial strain was inoculated into a series of tubes containing 0.1% of dyes (Crystal violet, saffranin, malachite green) on screening media to evaluate the impact of nitrogen supply. (replacement with nitrogen source) using 1% of various nitrogen sources (peptone, urea, beef extract, malt extract). All of the tubes underwent a 120-hour incubation period at 28 °C in a rotating shaker.

#### **4.6.7 Effect of PH**

About 5 ml of an 18-hour inoculum was added to a series of tubes containing 0.1% dye (crystal violet, saffranin, malachite green) in a screening medium with various pH values (5, 6, 7, 8, and 9), in order to evaluate the impact of pH. All of the tubes underwent a 120-hour incubation period at 28 °C in a rotating shaker.

#### **4.6.8 Effect of incubation**

A 5 ml 18-hour prospective bacterial strain inoculum was placed into a series of flasks containing 0.1% of Crystal violet, saffranin and malachite geendyes in a screening medium with incubation durations of 24, 48, 72, 96, and 120 hours to evaluate the influence of incubation length. For 120 hours, all of the tubes were incubated at 28°C on a rotating shaker with 120 rpm. (Muhammad Ikram *et al.*, (2022).

#### **4.6.9 Effect of temperature**

A prospective bacterial strain was inoculated into a series of tubes containing 0.1% of dyes (crystal violet, safranin and malachite green) in screening media at different temperatures (25°C, 30, 35, 40, and 45°C) in order to evaluate the influence of temperature for roughly 5 ml 18 hours. All of the tubes underwent a 120-hour incubation period at 28 °C in a rotating shaker.

#### **4.7 BIODEGRADATION ANALYSIS OF DYES BY UV- VIS, FTIR AND GCMS**

Decolorized materials were examined by UV vis, FTIR, and GC-MS analysis in order to study the products generated following dye degradation. The isolate was injected into sterile nutritional broth that included 1000 g of malachite green per 100 ml. Potent strain was used to inoculate the broth, which was then cultured for 24 hours at room temperature in separate flasks. The decolored broth was then centrifuged in a cooling centrifuge for 15 minutes at 10,000 rpm. After lyophilizing the supernatant, the samples were examined for UV and FTIR. (Tripathi, and Srivastava *et al.*, (2011).

#### **4.9 MORPHOLOGICAL CHARACTERIZATION OF H1 STRAIN**

##### **4.9.1 GRAM STAINING**

On the surface of the sterilized glass slide, the loop of culture is dispersed after being removed from the slant. Let the heat help the smear to dry. Crystal violet stain should be applied over the culture; after 20 seconds, remove with water. After one minute of iodine solution application, thoroughly rinse the slide with water once more. Wash with water after adding decolorizer till the colourless solution appears. Safranin, a secondary stain, was applied to the smear and left for 30 seconds. The smear was then rinsed with water and given time to air dry. With the use of an immersion oil bright field microscope, the prepared slide was examined. (Tripathi and Amit Sapra *et al.*, (2022).

#### **4.9.2 MOTILITY**

The specific colony is selected, and it is then injected into the sterile nutritional broth. A cover slip with petroleum jelly on the corner edges holds the loop of culture that has been removed from the broth. Inverted towards the cavity slide, the clean cavity slide was preserved. A drop of broth was left hanging in the slide's hollow when the slide was taken. A 40x objective lens is used to observe the slide to look for bacterial movement in a drop of broth.

#### **4.9.3 SPORE STAINING**

The method developed by Schaeffer and Fulton is used for spore staining. The culture is spread out on the glass slide and heated to fix it. Schaeffer and Fulton's spore stain A, the primary stain, was washed on the smear for 30 seconds before being followed by Schaeffer and Fulton's spore stain B. After being cleaned and air dried, the slide was examined with a 100x oil immersion objective lens.

#### **4.9.4 CAPSULE STAINING**

A clean glass slide was stored with a drop of nigrosin stain on it. On the nigrosin stain, culture was applied in a loop. After the slide had dried by air, it was examined with a 100x oil immersion objective lens.

### **4.10 BIOCHEMICAL CHARACTERIZATION OF H1 STRAIN**

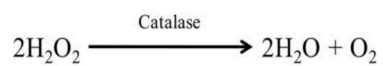
#### **4.10.1 OXIDASE**

In a clean petri dish, the culture loop is taken and applied to an Oxidase disc. In less than 60 seconds, a purple disc was seen to form. The N, N-dimethyl-p-phenylenediamine, oxalate, ascorbic acid, and a-naphthol-containing oxidase disc will react with the oxidase enzyme and turn purple.

#### **4.10.2 CATALASE**

Hydrogen peroxide in the amount of 2 ml was added to a clean test tube. The hydrogen peroxide tube was filled with a loopful of culture that had been placed within a sterile glass rod. Air bubbles that would indicate the presence of catalase were

looked for in the tube. Hydrogen oxide is broken into water and oxygen by the catalase enzyme. A bubble of air is created when oxygen is released.



## **CHAPTER 5**

### **RESULTS AND DISCUSSIONS**

#### **5.1 ISOLATION OF MORPHOLOGICALLY DIFFERENT BACTERIA**

The Tada falls and hills yielded a total of 32 morphologically unique bacterial colonies. In the soil samples that were obtained, there were  $1.3 \times 10^5$  to  $1.6 \times 10^6$  cfu of bacteria per gramme of soil. On nutritional agar, the majority of the bacterial isolates generated non-pigmented colonies. Fourteen of the 32 isolates were pigmented and eighteen were not. Yellow, red, white, and orange are all colours.

From soil samples collected in Saudi Arabia, Dhaban and Bakhali (2016) identified bacterial colonies with various morphologies. Additionally, 27 morphologically distinct bacterial colonies were identified in this investigation (Figure 5.1)



**Figure 5.1 Isolation of morphologically different bacteria**

#### **5.2. SCREENING AND SELECTION OF POTENT DYE DEGRADING BACTERIA FROM ISOLATES**

All 32 isolates' biodegradation activity was tested using the spot method in minimum agar that also included analytical dyes like crystal violet, saffranin and malachite green. A maximum of five isolates degraded saffranin dye, compared to eleven and seven isolates each that degraded malachite green and crystal violet. (Table 5.1)

The liquid approach was used to screen all the positive isolates from the spot method, and all five showed deterioration in three colours. For all three strains, the E1 isolate

out of 5 demonstrated greater degrading activity. Consequently, the strain E1 was chosen as a powerful isolate for further research (Table 5.2)

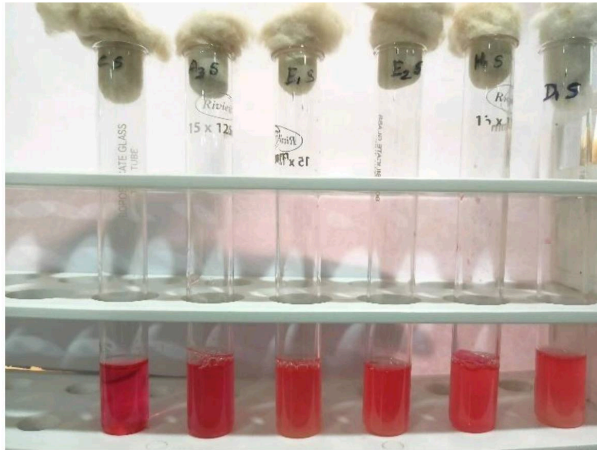
**Table 5.2 List of positive and negative results of screening test of all isolates.**

| <b>S.NO</b> | <b>Strain number</b> | <b>Malachite green</b> | <b>Crystal violet</b> | <b>Saffranin</b> |
|-------------|----------------------|------------------------|-----------------------|------------------|
| A1          | TP1                  | +                      | –                     | +                |
| A2          | TP2                  | –                      | –                     | –                |
| A3          | TP3                  | ++                     | +                     | –                |
| A4          | TP4                  | –                      | –                     | –                |
| B1          | TP5                  | +                      | –                     | +                |
| B2          | TP6                  | –                      | –                     | –                |
| B3          | TP7                  | +                      | –                     | –                |
| B4          | TP8                  | –                      | –                     | –                |
| C1          | GA1                  | ++                     | –                     | –                |
| C2          | GA2                  | –                      | –                     | –                |
| C3          | GA3                  | +                      | +                     | +                |
| C4          | GA4                  | +                      | +                     | –                |
| D1          | GA5                  | +                      | +                     | –                |
| D2          | GA6                  | +                      | –                     | –                |
| D3          | GA7                  | +                      | –                     | –                |
| D4          | GA8                  | –                      | –                     | –                |
| E1          | GA9                  | ++                     | –                     | +                |
| E2          | GA10                 | +                      | +                     | +                |

|    |      |   |   |   |
|----|------|---|---|---|
| E3 | TF1  | + | – | – |
| E4 | TF2  | – | – | – |
| F1 | TF3  | – | – | – |
| F2 | TF4  | – | – | – |
| F3 | TF5  | + | – | – |
| F4 | TF6  | + | – | – |
| G1 | TF7  | – | – | – |
| G2 | TF8  | – | – | – |
| G3 | TF9  | – | + | – |
| G4 | TF10 | + | – | – |
| H1 | TF11 | + | + | – |

+ Positive for Degradation; - Negative for degradation





**Saffranin**



**Malachite Green**



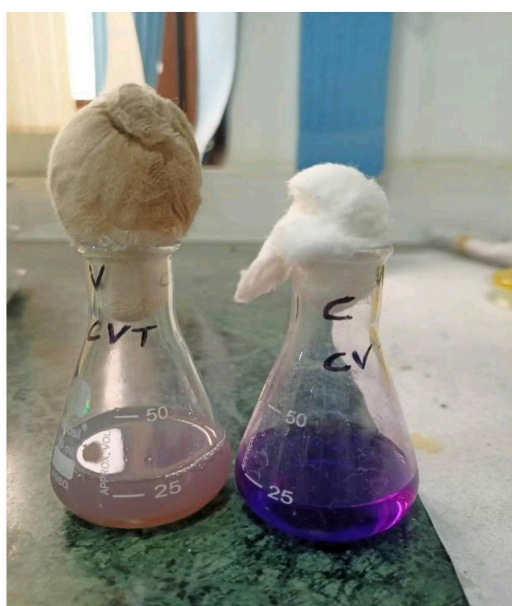
**Crystal violet**

**Figure. 5.2 Determination of degradation of dyes by potent isolate for different concentrations.**

### 5.3. DECOLORIZATION OF ANALYTICAL DYES OF POTENT ISOLATE

The dyes such as Crystal violet, saffranin and malachite green have been decolorized by different strains that are isolated stains from Parangipettai estuarine area. The decolorization is monitored with respect to variations in PH (Srinivasan *et al.*, (2014).

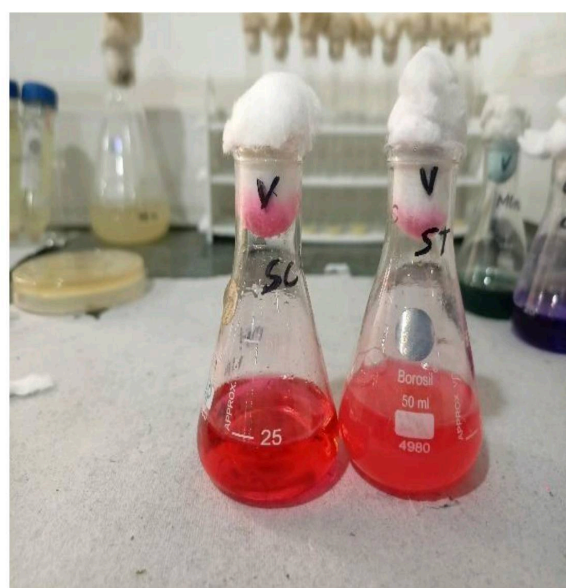
In this present study, the potent isolate E1 showed good decolourisation for various concentrations from 1mg to 10mg/ per 100 ml in 120 hours incubation. After 120 hours of incubation the H1 isolate showed 70% degradation in crystal violet dye, 67 % degradation in saffranin dye and 57% degradation in malachite green dye (Table 5.3) figure 5.3



Test

Control

**a) Crystal violet**



Test

Control

**b) Saffranin**



Test

Control

**c) Malachite green**



Test

Control

**d) Mixed dyes**

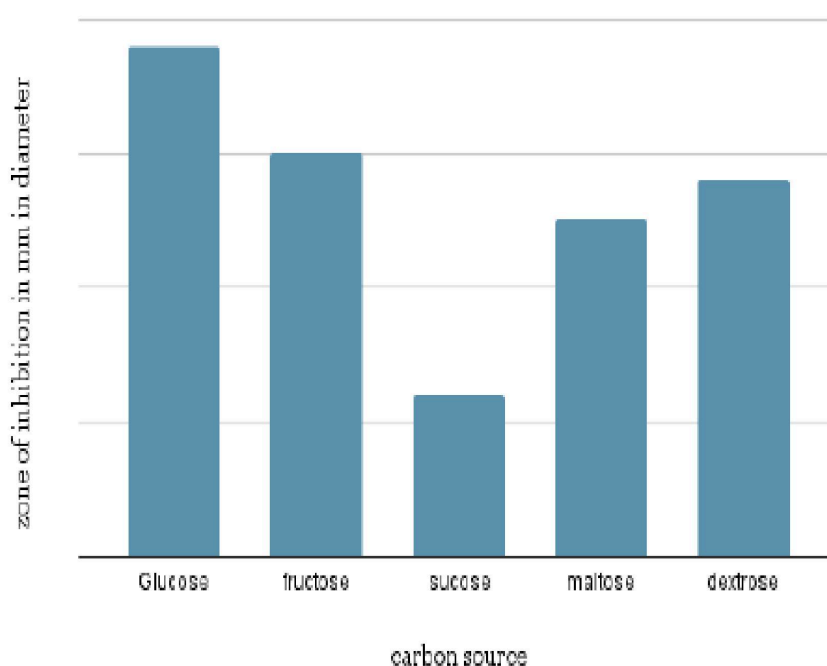
**Figure 5.3 Decolourization of analytical dyes**

**Table 5.3. Decolourisation percentage of textile dyes by E1 isolate**

| Dyes            | Initial OD value | Final OD value | % of decolourization |
|-----------------|------------------|----------------|----------------------|
| Crystal violet  | 1.935            | 0.522          | 70                   |
| Safranin        | 1.022            | 0.475          | 55                   |
| Malachite green | 1.045            | 0.458          | 83                   |
| Mixed dyes      | 1.533            | 0.766          | 50                   |

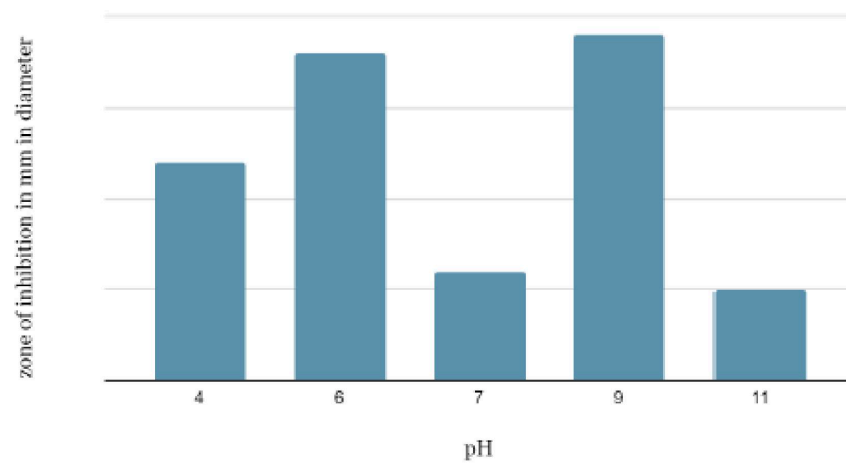
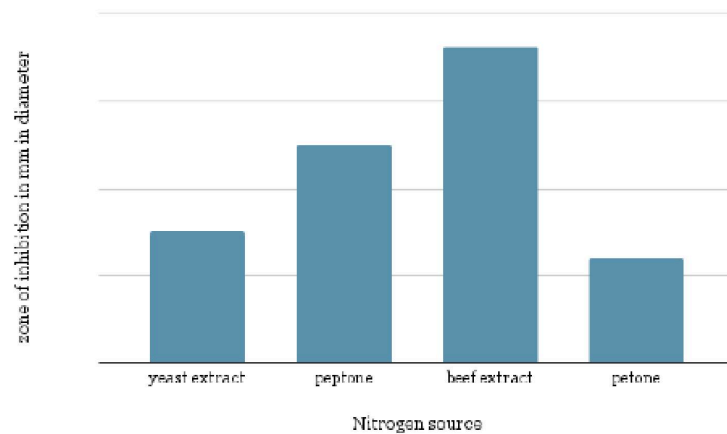
## 5.4 OPTIMIZATION STUDIES OF POTENT ISOLATE FOR DYE DEGRADATION

The efficiency of biological decolorization of synthetic dye solutions including malachite green was explored by Daneshver [7], who also noted that decolorization was dependent on dye concentration, pH, and temperature, but that pH had the greatest impact on dye removal. In the current work, the degradation of the dye was well-optimized using glucose (a carbon source), beef extract (a nitrogen source), pH 7, temperature 28, and 10% inoculum.



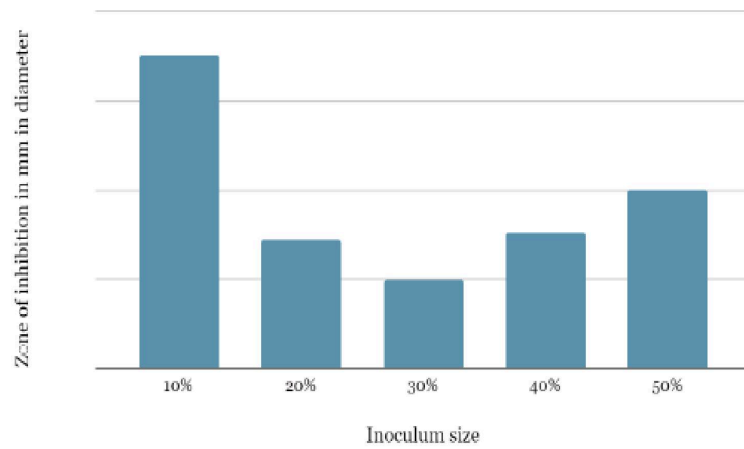
**Graph 5.4 (a) Optimization of carbon source**

**Graph 5.4 (b) optimization of nitrogen source**



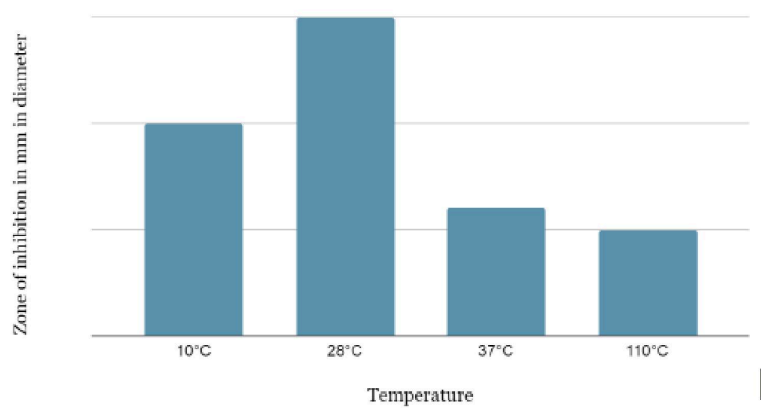
**Graph 5.4 (c) Optimization of pH.**

**Graph 5.4 (c) pH**



**Graph 5.4 (d) Optimization of Inoculum size.**

**Graph 5.4 (d) Inoculum size**



**Graph 5.4. (e) Optimization of temperature**

**(e) temperature**

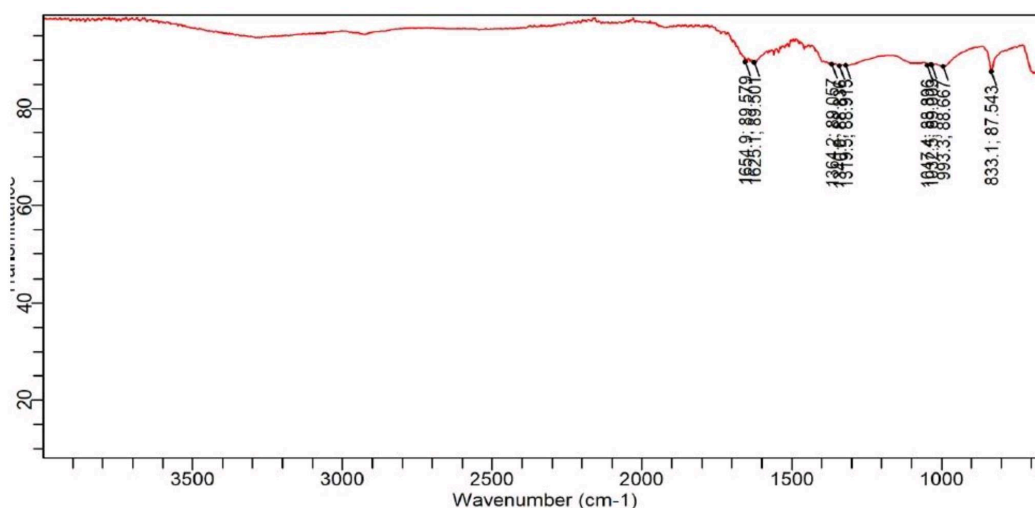
**Graph 5.5 (a-e) Optimization of dye degradation by potent isolate**

## 5.5 CHARACTERIZATION OF DEGRADED DYES BY E1 ISOLATES

FTIR stands for “Fourier transform infrared” and it is the most common form of infrared spectroscopy. All infrared spectroscopies act on the principle that when infrared (IR) radiation passes through a sample, some of the radiation is absorbed. The Absorbance range is from 600-10000  $\text{cm}^{-1}$ . The radiation that passes through the sample is recorded. The analytical dyes such as crystal violet, safranin, Malachite green were the samples given for analyse. The peak value of test and control of the samples were noted. The functional groups were analysed. (figure 5.5)(Table 5.5).

### MALACHITE GREEN

#### Before degradation (control)



**Figure 5.5(a) ftir analyses of malachite green**

| Wave number $\text{cm}^{-1}$ | Functional groups |
|------------------------------|-------------------|
| 833.06001                    | ZTP, MAA          |
| 1032.47258                   | Alkyl amine       |
| 1047.38193                   | Polysaccharide    |
| 1319.47759                   | C-O               |

**Table 5.5(a) Functional groups**



After degradation(Test)

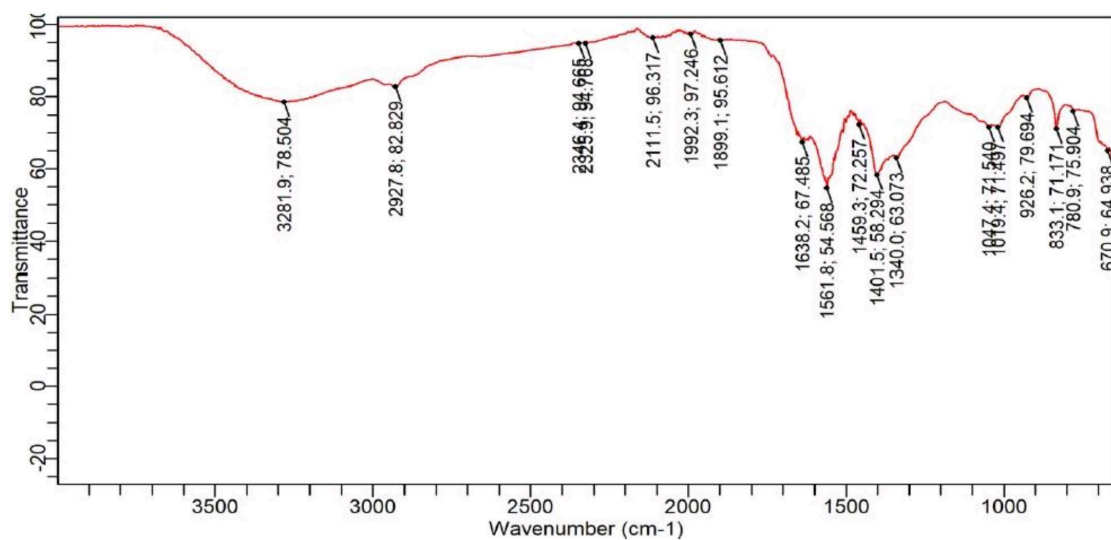


Figure 5.5 (b) ftir analyses of Malachite green

Table 5.5 (b)Functional groups

| Peak number | Wave number cm-1 | Functional groups                 |
|-------------|------------------|-----------------------------------|
| 1           | 670              | Halogen compounds                 |
| 2           | 1401.47903       | Streching –C=O inorganic compound |
| 3           | 2927.82387       | Carboxylic acid                   |
| 4           | 1459.25276       | CH aliphatic bending group        |



## CRYSTAL VIOLET

Before degradation (control)

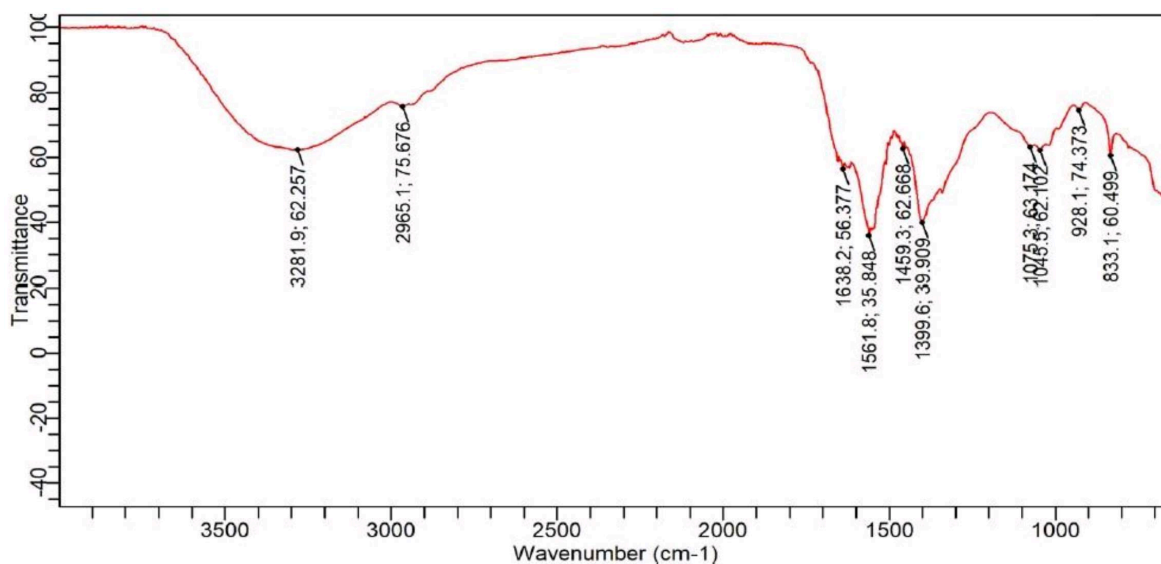
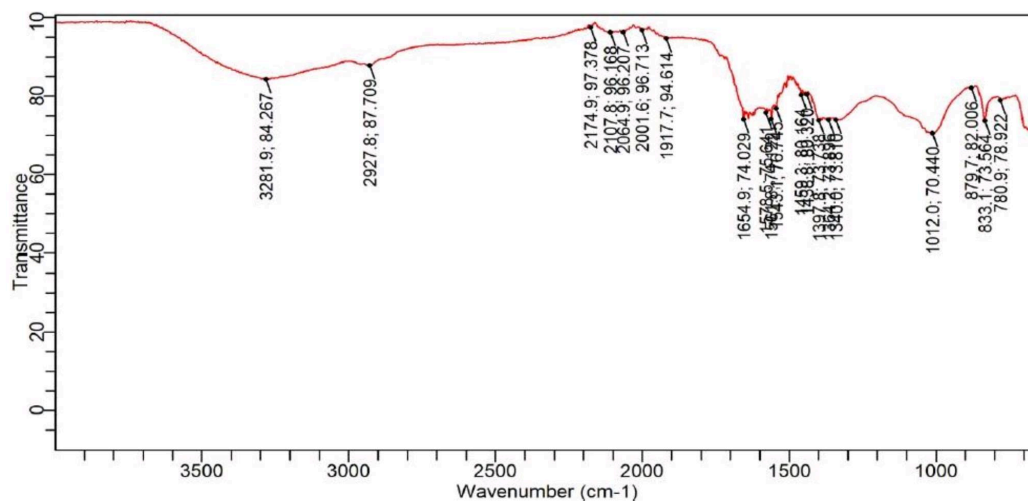


Figure 5.5(c) ftir analyses of Crystal Violet

Table 5.5(c) Functional group

| Peak number | Wave number cm-1 | Functional groups          |
|-------------|------------------|----------------------------|
| 1           | 833.06001        | ZTP, MAA                   |
| 2           | 1075.33697       | TEOS                       |
| 3           | 2965.09725       | carboxylic acid            |
| 4           | 1459.25276       | CH aliphatic bending group |

### After degradation(Test)



**Figure 5.5(d) ftir analyses of crystal violet**

**Table 5.5(d) Functional group**

| Peak number | Wavenumber cm-1 | Functional groups                    |
|-------------|-----------------|--------------------------------------|
| 1           | 1364.20565      | C-N amide III band                   |
| 2           | 1438.75241      | Stretching –C=O inorganic compound   |
| 3           | 2107.80955      | C=C conjugated                       |
| 4           | 2927.82387      | CH and CH2 streching aliphatic group |

## SAFFRANIN

Before degradation (control)

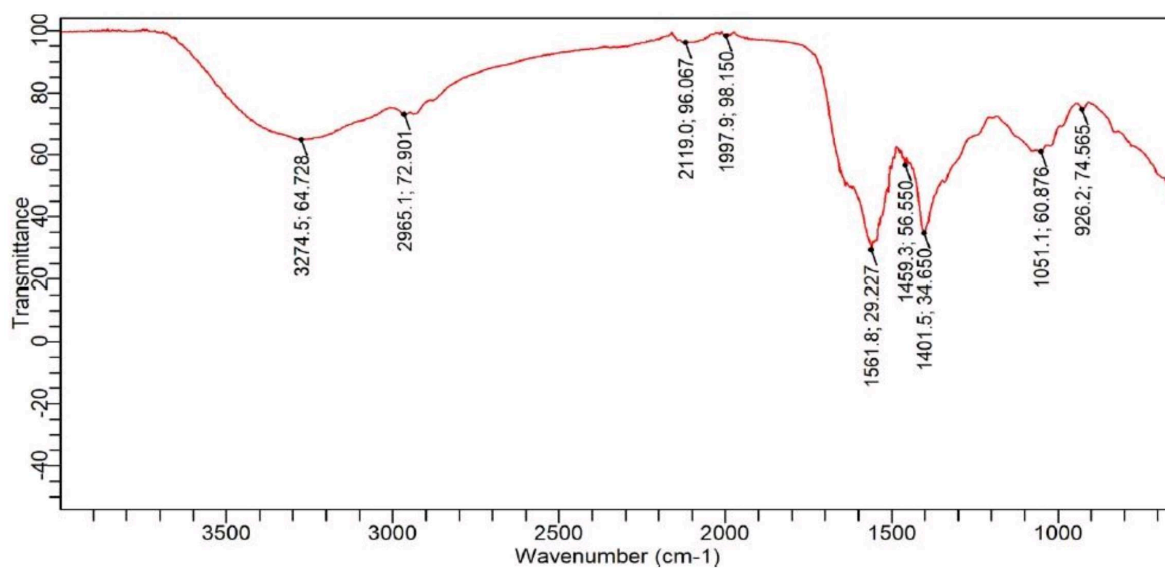


Figure 5.5(e)ftir analyses of Saffranin

Table 5.5(e) Functional groups

| Peak number | Wave number cm-1 | Functional groups                 |
|-------------|------------------|-----------------------------------|
| 1           | 1459.25276       | Streching-C=O inorganic carbonate |
| 2           | 1051.10927       | Polysaccharide                    |
| 3           | 2965.09725       | Carboxylic acid                   |
| 4           | 3274.46629       | N-H stretching of amideIII        |

After degradation(Test)

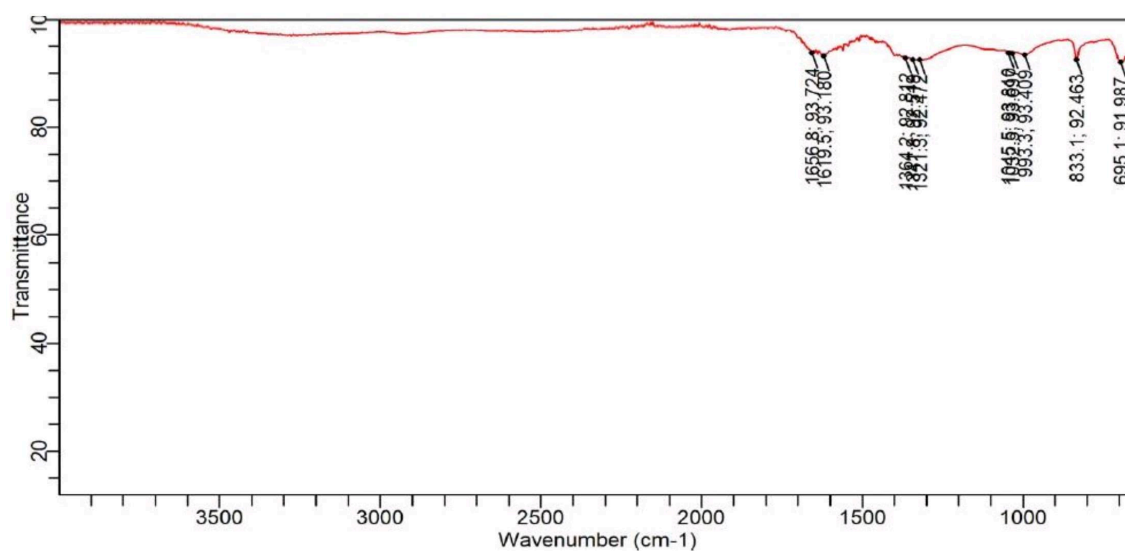


Figure 5.5(f) ftir analysis of saffranin

Table 5.5(f) Functional groups

| Peak number | Wave number cm-1 | Functional groups |
|-------------|------------------|-------------------|
| 1           | 695.14851        | Halogen compounds |
| 2           | 833.06001        | ZTP, MAA          |

|   |            |              |
|---|------------|--------------|
| 3 | 1045.51826 | Alkyl amine  |
| 4 | 1321.34126 | Alkyl ketone |

### MIXED DYES

Before degradation(control)

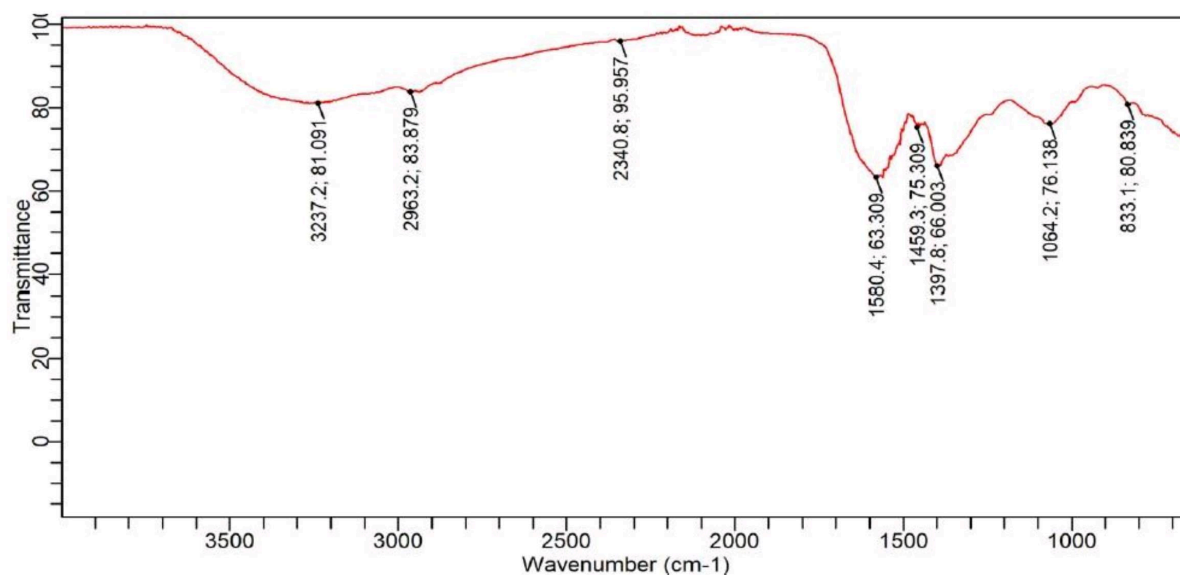


Figure 5.5(g) ftir analyses of mixed dyes

Table 5.5 Functional groups

| Peak number | Wave number cm-1 | Functional groups |
|-------------|------------------|-------------------|
| 1           | 1064.15495       | Alkyl amine       |
| 2           | 2340.76816       | C=Conjugated      |

|   |            |                 |
|---|------------|-----------------|
| 3 | 2963.23358 | Carboxylic acid |
| 4 | 1580.39124 | Diketones       |

After degradation(test)

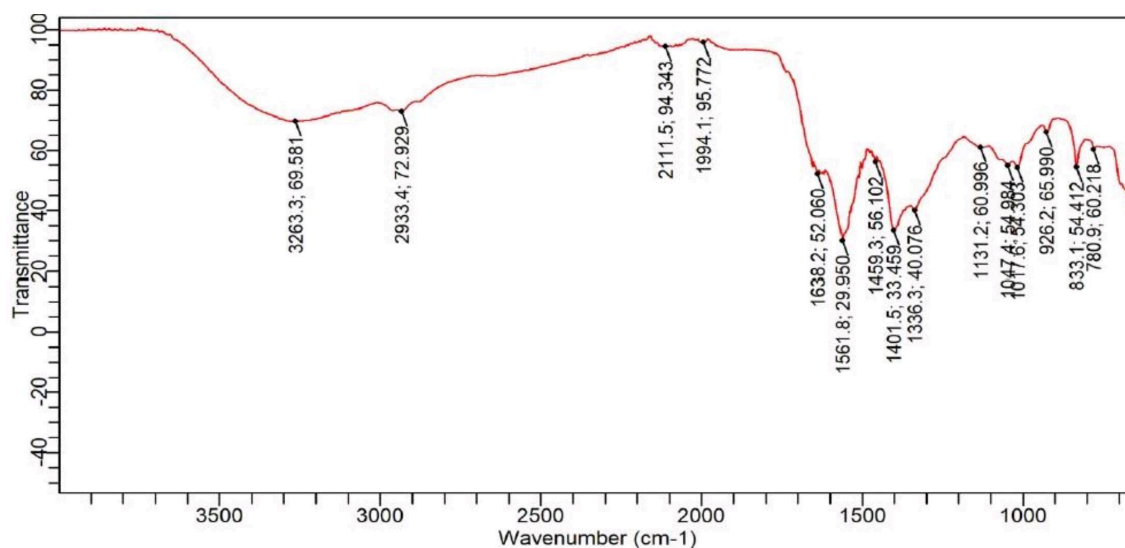


Figure 5.5 (h) ftir analyses of mixed dyes

Table 5.5(h) Functional groups

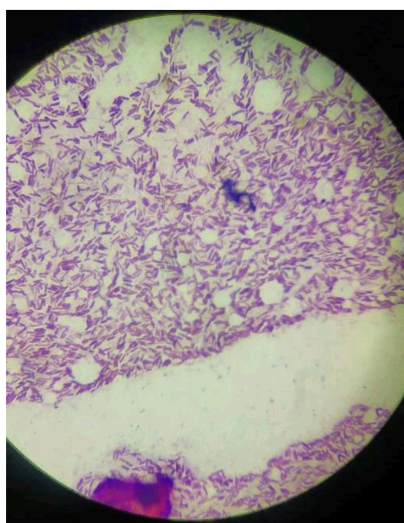
| Peak number | Wave number cm-1 | Functional groups |
|-------------|------------------|-------------------|
| 1           | 1047.38193       | Ethanol           |

|   |            |                                   |
|---|------------|-----------------------------------|
| 2 | 1131.24703 | Polysaccharide                    |
| 3 | 1336.25061 | Aromatic primary amine CN scratch |
| 4 | 1561.75455 | Diketone                          |

## **5.6 MICROSCOPIC OBSERVATION**

### **5.6.1 GRAM STAINING**

The H1 potent isolate showed violet color rod shaped bacteria in gram staining. Hence the E1 isolate is found to be Gram positive rod shaped bacteria.(Figure 5.7.1)



**Figure 5.6.1 Microscopic observation of Gram staining**

### **5.6.2 MOTILITY TEST:**

The potent isolate E1 showed motile form in hanging drop motility method.

### **5.6.3 SPORE STAINING**

In spore staining the potent isolate E1 showed red color spores whereas vegetative cell green color. It is confirmed that H1 isolate is spore producers

#### **5.6.4 CAPSULE STAINING**

Colorless rod shaped bacteria seen around black background. This confirms the presence of a capsule in E1 isolate.



**Figure 5.6.4 Capsule stainin**

## **CHAPTER 6**

### **SUMMARY AND CONCLUSION**

#### **SUMMARY**

Textile dye industry waste is one of the most serious problems in the environment. The presence of dyes in the environment causes harmful effects on plants, animals and humans. The textile dyes significantly compromises the aesthetic quality of the water bodies. Some of the dyes are carcinogenic and can cause skin and eye irritation. Microbial degradation is most valuable, environment free compare to other degradation process. With this view, the present study initiated to isolate potential bacteria having ability to degrade dye degradation. Soil samples was collected and transport to laboratory in sterile container. Totally 27 morphologically different bacteria were isolated from three different soil samples. Out of 27 isolates, -7 bacteria showed positive for crystal violet, - 16 bacteria showed positive for Malachite green, -4 for Saffranin. Out of 27 positive bacteria against dyes, the strain **E1** showed positive results for all the three dyes. 10 mg concentration of all three dyes were prepared for control and test and E1 strain was inoculated into the test at the medium. The



degradation time was noted. The tests and control of degraded dye was then centrifuged to collect the supernatant for 7000 rpm in 10 minutes at 4°C. Then, FTIR, GCMS were carried out. The present study isolated E1 has ability to degrade all three dyes.

## CONCLUSION

Microorganisms from Tada falls and hills are the promising source for microbial metabolites. Bacterial isolate E1 showed promising source for analytical dye degradation. Finding of the present work evidenced that the Tada falls area investigated in this study for potential bacterial resources for environmental applications. The present study concluded that the potent isolate E1 showed good degradation activity in all aspects. Further field study, other characterization such as structure elucidation proves is potential.

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