# Biodegradation and detoxification of Azo dyes using halophiles/halotolerant bacteria

A Dissertation for

Course code and Course Title: GBT 651 Dissertation

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc in Biotechnology

by

**SUMANA SOMEN BISWAS**

22PO470020

ABC ID : 248-684-949-730

PRN : 201902525

Under the Supervision of

**DR. SANIKA SAMANT**

School of Biological Sciences and Biotechnology
Discipline of Biotechnology

**GOA UNIVERSITY**

**APRIL 2024**

Examined by:

Seal of the School

## DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled,

**"Biodegradation and detoxification of Azo dyes using halophiles/halotolerant bacteria"**

is based on the results of investigations carried out by me in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr Sanika Samant and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities / College will be not be responsible for the correctness of observations / experimental or other findings

given the dissertation.

I hereby authorise the University/college authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

Sumana Somen Biswas

Seat no: 22PO470020

Date: 08/04/2024

Place: Goa University

# COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Biodegradation and detoxification of Azo dyes using halophiles/halotolerant bacteria**" is a bonafide work carried out by Ms Sumana Somen Biswas under my supervision in partial fulfilment of the requirements for the award of the degree of Master's in Science in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

Dr Sanika Samant

Date: 8/4/2024

SBSB: Biotechnology * Taleigao Plateau, Goa 403206 * GOA UNIVERSITY

Signature of Dean of the School/HoD of Dept

School/Dept Stamp

Dean of School of Biological Sciences & Biotechnology Goa University, Goa-403206

Date: 8/4/24

Place: Goa University

# PREFACE

In an era where the consequences of water pollution are increasingly evident, the investigation into dye degradation has become important. This dissertation delves into the intricate realm of isolating halophiles and degrading commercial dyes and lab made wastewater from riverine source. Through meticulous experimentation and analysis, this thesis aims to shed light on the isolation of halophiles present in the water sample and their potential for degrading commercial dyes which can cause an effect on the environment. By presenting findings and insights collected from rigorous research, this work strives to contribute to the growing body of knowledge on dye degradation.

The degradation of dyes is a complex phenomenon that involves several variables, including chemical interactions, the environment, and the dye's own properties. The practical implications of the discoveries serve as a motivating factor in addition to the intellectual challenge. Knowing the mechanisms underlying dye deterioration allows us to create solutions to extend the colour's useful life in a variety of materials, such as textiles and artwork. Consequently, the two goals in mind during the research: first, to understand the basic laws governing dye stability; second, to apply this understanding to real-world problems that benefit society and business.

# ACKNOWLEDGEMENT

The success and outcome of this dissertation required a lot of guidance and assistance from many individuals and I am extremely privileged to have gotten this all along with the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them .

Firstly I would like to express my sincere gratitude to our Dean Prof Bernard F. Rodrigues and Ex Dean Prof Savita Kerkar for providing us with the facilities and opportunity to do the dissertation work at Goa University.

Additionally, I would like to express my gratitude to my professors, Dr. Savita Kerkar, Dr Meghanath Prabhu, Dr Samantha Fernandes D'mello, Mrs. Dviti Volvoikar, Miss Snesha Bhomkar, and Miss Snigdha Mayekar, for their encouragement and assistance.

Foremost, I would like to express my sincere gratitude to my guide, Dr. Sanika Samant (School of Biological Sciences and Biotechnology) for her guidance and immense knowledge. I would also like to thank research scholar Miss Fazila Shaikh.

Also, I would like to extend my sincere esteems to all our non-teaching staff Mr. Serrao, Mr. Ashish Kurtiker, Mr. Samir Malik, Ms. Sandhya, and Ms. Jaya for providing us with all the necessary requirements.

I would especially like to thank my friends and classmates for their inspiration and support. Finally, I want to express my gratitude to Mr. Somen Biswas and Mrs. Susmita Biswas, my parents, for their unwavering support and affection.

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF GRAPHS

# LIST OF ABBREVIATIONS

| SERIAL NO. | ABBREVIATIONS | FULL FORM |
|---|---|---|
| 1 | CR | Congo red |
| 2 | CFU | Colony forming unit |
| 3 | °C | Degree Celsius |
| 4 | D/w | Distilled water |
| 5 | g | Gram |
| 6 | g/L | Gram per litre |
| 7 | mL | Mililitre |
| 8 | mg/L | Kilograms per litre |
| 9 | MG | Malachite Green |
| 10 | % | Percentage |
| 11 | psi | Pound per square inch |
| 12 | rpm | Rotations per minute |

# ABSTRACT

Untreated industrial effluent contaminates water and interferes with microorganisms' natural cleaning cycle, posing serious environmental risks. The present study focuses on the isolation of halophiles/halotolerant from the riverine source and to analyse the physicochemical parameters of water along with morphological and biochemical characterization. Additionally, lab-made wastewater and commercially available colours were degraded by the bacterial isolates, and the percentage of degradation was calculated. Studies on optimization were conducted in relation to the deterioration of commercial dyes.

Thus the research work carried out showed that the bacteria present in the water sample showed good dye degradation potential against the commercial dye and the lab made watseater. Thus the bacteria isolated from industrial water can be utilised to carry out degradation of dyes in the environment

**Key words**: Wastewater, Biochemical characterization, Dye Degradation, Optimization studies

# TABLE OF CONTENTS

# CHAPTER 01

## INTRODUCTION

## 1.1 Background

One of the essentials for maintaining and extending life is water. Hence it's critical to provide high-quality water for a variety of uses. However, given the widespread pollution brought on by industrial, agricultural, and residential activities, this is getting harder by the passing days. Phenols, dyes, detergents, insecticides, pesticides, and heavy metals are a few of the often occurring contaminants.

Water pollution has many sources. Human activities are usually the main cause of water pollution. These include industrial dumping, chemical wastes, use of herbicides, etc. The wastewater produced can be divided into sewage wastewater, domestic wastewater, wastewater from institutions, industrial wastewater and agricultural wastewater (Henze, M., & Comeau, Y.,2008).

Industrial wastewater and wastewater from institutions are one of the leading causes of irreversible damage to the ecosystem. Major industries and institutions usually set up their own treatment plants but small industries and institutions cannot afford to do so. Depending on the industries the effluents often contain harmful components such as organic and inorganic compounds, paints, dyes, suspended solids, etc. These not only affect humans but also nature.

Due to the rapid development of numerous industries, including textiles, leather goods, paper manufacturing, and food technology, dyes have emerged as one of the primary causes of water pollution. The use of dyes has increased, leading to an unregulated release of dyes into water bodies (Etezad & Kiakhani, 2021).

Dyes are a vital resource in paper, paint, culinary, cosmetic, textile, etc. Dyes are also used in educational institutions and laboratories to perform several tests (Qiu H et al., 2022). There are almost three thousand azo dyes available, and the leather, textile, paper, food colouring, and cosmetic manufacturing industries heavily utilise Sandolan Yellow, Maxilon Blue GRL, and Astrazon Red GTLN (Sudha et al., 2014). Textile dyes, which come in thousands of varieties, are used to colour a wide range of fabrics. The majority of clothing today is coloured with synthetic or artificial dyes. Dyes are able to absorb light in the visible spectrum (400–700 nm) and have at least one chromophore (Varjani et al., 2020).

Dyes vary in structure, molecular mass, polarity and solubility due to presence of different chromophore or auxochrome groups present in them. Based on their structures, azo dyes, nitro dyes, phthalein dyes, triphenylmethane dyes, indigoid dyes, and anthraquinone dyes are categorised. On the other hand, colours such as acid, basic, vat, ingrain, disperse, mild, and reactive are categorised according to how they are used (Varjani et al., 2020). The intensity of dye colour may increase due to the presence of auxochromes (Piaskowski et al., 2018).

Natural dyes are now being replaced by synthetic dyes as they retain their colours even after several washing. The colour associated with dyes destroy artwork (Setiadi et al., 2006) and prevent light from diffusing into the water, which lowers the amount of dissolved oxygen and slows down the pace at which aquatic life photosynthesizes (Ajaz et al., 2020).

Azo dyes are the most significant category of commercial dyes and adaptable colourants. These colourants have been used extensively in industries around the world due to their large range of colours, ease of use, and inexpensive cost of production especially when compared to natural dyes (Piaskowski et al., 2018). Azo dyes are synthetic dyes used widely and make around 70% of the dyes (Selvaraj et al., 2021).

Dyes act as mutagenic, carcinogenic and toxic agents which act as environmental pollutants and affect the food chain by causing biomagnification (Sandhya, 2010). Hence it is necessary to reduce the dye concentration and decolourize the dye using physical, chemical or biological methods (Setiadi et al., 2006). Thus using a single technique for removal does little to reduce the dye concentration. Several different techniques are used as a combination for treatment of dyes. Approximately 10–15% of dyes are discharged which can cause environmental pollution (Singh et al., 2015).

Various methods are used to treat wastewater, which include physical, chemical and biological methods (Varjani et al., 2020). Compared to physical and chemical methods , biological methods are more viable economically and environmentally (Varjani et al., 2020).

Chemical processes and physical processes include membrane processes, filtration with coagulation, ozonation with coagulation and adsorption, sonication, irradiation etc (Piaskowski et al., 2018). As a result of their somewhat complex requirements, the overall cost of micropollutant removal rises.

Biological methods of dye degradation by use of bacterias, fungus and other microorganisms serve as the better source since they are easy to set up, have low cost, are eco friendly, etc. (Iqbal et al .,2011).

Bacteria have the highest potential because they have a short life cycle and can break down substances fast into metabolites that are significantly less hazardous and gentle on the environment. Bacteria can grow concurrently on the surface of several substrates without being constrained by their surroundings ( Kolekar et al., 2012). Some bacteria have evolved to degrade specific classes of dyes. For example, strains of *Pseudomonas*, *Bacillus*, and

*Escherichia coli* are known to degrade azo dyes, while other bacteria like *Shewanella* and *Geobacter* can degrade azo and anthraquinone dyes under anaerobic conditions.

Fungi are extensively used for dye degradation due to their ability to produce a wide range of enzymes capable of breaking down complex dye molecules. One of the most studied fungi for this purpose is *Phanerochaete chrysosporium*, a white-rot fungus known for its lignin-degrading enzymes. These enzymes can also break down aromatic dye molecules by oxidising them, leading to their degradation.Fungi degrade dyes through a process called decolorization, where the colour of the dye fades as it undergoes degradation. This process can result in the mineralization of dye molecules into simpler, non-toxic compounds like carbon dioxide, water, and biomass. They also exhibit broad substrate specificity, enabling them to degrade various types of dyes, including azo, anthraquinone, triphenylmethane, and heterocyclic dyes. This versatility makes fungi suitable for treating complex dye mixtures found in industrial wastewater. (Hassan et al., 2013; Rani et al., 2014).

Due to their strong metabolic capacity, azo dyes can be broken down by a variety of microbes. Unfortunately, because of the extreme conditions in dye-polluted environments, many of them cannot be employed as degrading agents (Amoozegar et al., 2010).

The halotolerant and halophilic microorganisms have the natural ability to withstand high osmotic pressure during the treatment of azo dye wastewater. Halotolerant and halophilic microorganisms are unique groups of organisms that can live in high salinity water (Giovanella et al., 2020; Guo et al., 2020), during the process of treating and degrading dye wastewater, it would be ideal to obtain microbial strains that could tolerate such high-salt concentrations. Azo dyes can be decolored by halophilic microorganisms, including fungi, bacteria, and algae, in situations with high salinity.

For example, at salinities between 2 and 10%, *Halomonas sp.* can decolorize reactive black 5, remazol bright violet 5R, and reactive orange 16 (Montanez-Barragan et al., 2020). *Shewanella putrefaciens* could completely remove 100 mg each of Reactive Black-5, Direct Red-81, Acid Red-88, and Disperse Orange-3 in about eight hours (Amoozegar et al., 2010). Because halotolerant/halophilic microorganisms can survive in an excessively high-salt environment, they would be the best option to decolorize and degrade azo dyes with a high-salt content in wastewater. Some strains with the ability to tolerate extreme conditions, such as high salt, include *Pichia pastoris, Streptomyces halophilus, and Scheffersomyces spartinae,* etc (Song et al., 2017)

This study aims to isolate and morphologically characterise halophilic bacterial strains and evaluate the dye degradation potential using the comercial dyes Malachite Green and Congo Red.

## 1.1.1 Malachite Green

### 1.1.1.1. Introduction

Malachite green is an organic chloride salt used for its anti-fungal qualities in aquaculture, used as a counter-stain in histology, as a green dye,. as an antibacterial agent. It also functions as an environmental contaminant, a carcinogen, a teratogenic agent, and a fluorochrome.

### 1.1.1.2 Chemical and physical properties

1. Chemical name : [4-[[4-(dimethylamino)phenyl]-phenylmethylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium;chloride
2. Formula : C23H25ClN2
3. Mass : 364.9 g/mol

4. Colour : Green crystals with metallic lustre
5. Max absorbance : 616.9 nm

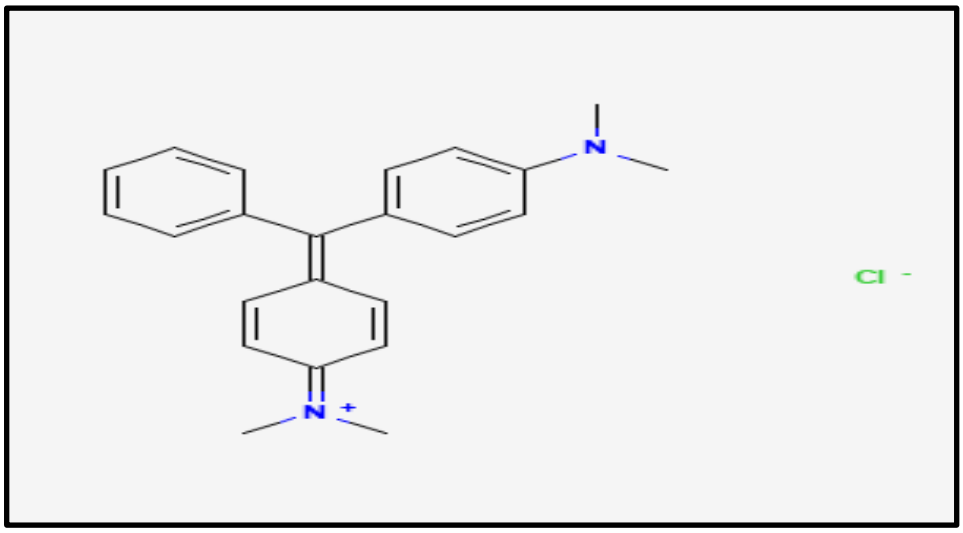



**Fig 1.1 :** chemical structure of MG dye. (Pubchem, national centre of Biotechnology, 2023)

### 1.1.1.3. Uses of malachite green

1. Malachite green has been utilised as a direct dye in the textile industry to colour fabrics, jute, leather, particularly silk and wool.
2. It has been employed as a biological stain in microscopy to visualise tissues and cells, particularly in histology and bacteriology.
3. It has been used in aquaculture to treat fungal and protozoan infections in fish eggs and fish (Hasimoto et al., 2011).
4. In the past, it was used as an antiseptic in the medical and veterinary fields to treat fungal infections on skin and wounds.

### 1.1.1.4. Side effects of malachite green

The toxicity of the dye is increased by concentration, temperature, and length of exposure. There have been reports of chromosomal breaks, carcinogenesis, teratogenicity, and pulmonary toxicity. One of the histological consequences of MG is multi-organ tissue damage. Fish

exposed to MG experience notable alterations in blood biochemical markers. Leucomalachite green, the reduced form of MG, has been discovered to be present in eggs, fry, muscles, serum, liver, and other tissues. Toxicological signs in certain mammals include developmental problems, organ damage, mutagenicity, and carcinogenicity.

## 1.1.2. Congo Red

### 1.1.2.1. Introduction

Congo Red is an indicator dye that is blue-violet at pH 3.0 and red at pH 5.0. It is an acid dye used especially in testing for hydrochloric acid in gastric contents. It is also used in histology to test for amyloidosis.

### 1.1.2.2. Chemical and physical properties

1. chemical name : disodium;4-amino-3-[[4-[4-[(1-amino-4-sulfonatonaphthalen-2-yl)diazenyl]phenyl]phenyl]diazenyl]naphthalene-1-sulfonate
2. Formula : C32H22N6Na2O6S2
3. Mass : 696.083 g/mol
4. Colour : brownish red powder
5. Max absorbance : 495 NM

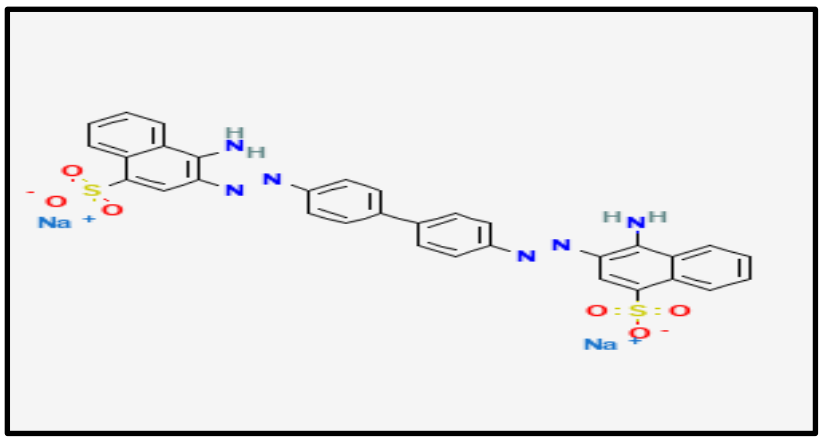


**Fig 1.2**: chemical structure of CR dye. (Pubchem, National Centre of Biotechnology, 2023)

### 1.1.2.3. Uses of Congo red

1. Congo red dye is primarily used as a histological dye for staining amyloid protein deposits in tissue samples, aiding in the diagnosis of amyloidosis.
2. It's also utilized in various scientific research applications, including studying protein folding and aggregation, as well as in textile dyeing and as a pH indicator.
3. In industries, Congo red dye is primarily used in textile dyeing processes to impart vibrant red colors to fabrics.
4. Additionally, it's employed in paper and leather industries for dyeing and finishing products. Congo red can also be used in quality control processes to detect the presence of certain substances and in research laboratories for various analytical applications, including as a pH indicator and in the study of protein aggregation.

### 1.1.2.4. Side effects of Congo red

CR has a harmful effect on the human body and can cause a number of diseases that, in high concentrations, can be deadly. These diseases include cytotoxic (genotoxic, hemotoxic, and neurotoxic), carcinogenic, and mutagenic substances (Siddique et.al., 2023). Furthermore, there have been accounts of Congo red exposure leading to negative outcomes like allergic responses and skin irritation. In rats, teratogenicity was detected at higher doses. Additionally, it was observed that more frequent doses prevented and delayed coagulation (Rosoff, 1974).

## 1.2. Aims and Objectives

### 1.2.1. Aim

Assessment of biodegradation and detoxification ability of azo dyes by marine halophiles.

### 1.2.2. Objectives

1. Isolation of azo dye degrading bacteria by enrichment culture method
2. An Evaluation of morphological and biochemical characteristics of the isolated microorganisms
3. Determination of the dye degradation ability of the potential isolate(s) in the lab-made dye wastewater.

## 1.3. Hypothesis

The degradation of dyes can be influenced by various factors such as exposure to light, temperature, pH, and chemical reactions with other substances present in the environment. One hypothesis is that during degradation, the dye molecules undergo structural changes, breaking down into smaller compounds, which may be less colorful or completely colorless.

This study attempts to evaluate the ability of halophiles/halotolerant bacteria to degrade commercial dyes and lab made wastewater.

## 1.4. Scope

Dye degradation using halophiles is promising due to their unique enzymatic capabilities and tolerance to extreme conditions. Halophiles, microorganisms that thrive in high salt concentrations, possess enzymes that can efficiently degrade various dyes, including synthetic ones used in industries like textiles and printing. Their ability to function in saline environments widens the potential applications for treating wastewater from such industries, offering an eco-friendly solution to pollution problems. Additionally, the use of halophiles for dye degradation can potentially reduce the need for costly chemical treatments and minimize environmental impact. However, further research is needed to optimize processes and understand the full potential of halophiles in this context.

# CHAPTER 02

## LITERATURE REVIEW

One of the most prevalent byproducts of commercial or industrial operations is industrial wastewater, which is the water utilized in almost every industry's commercial product manufacturing process. This processed water must be treated before it is disposed off. For the reason that potential breakdown products from anaerobic microbial action can be toxic, mutagenic, and carcinogenic, synthetic azo dyes, which are commonly found in textile industry effluents, pose a threat to the environment (Conneely et al ., 1999).

While the industrial sector's recent rapid development has brought comfort to the population, it has also produced enormous amounts of wastewater that contain dangerous substances including hydrocarbons, nitrogen, phosphorus, and heavy metals that have an adverse effect on both the environment and human health. Industrial wastewater treatment has seen a number of technological advances, with a focus on hybrid systems that recover resources from effluent in a feasible, cost-effective, and time-saving manner. the secondary treatment of wastewater, which is often carried out by trickling filtering, anaerobic digestion, and aerated lagoons before being dispensed into water bodies (Sathya et al., 2022)

The capacity of halophilic and halotolerant bacteria to decolorize azo dyes has been the subject of numerous investigations. For instance, it was found that *Shewanella putrefaciens* could completely eliminate 100 mg/l of Reactive Black-5, Direct Red-81, Acid Red-88, and Disperse Orange-3 in about 8 hours when 40 g/l NaCl was present (Amoozegar et al., 2010).
According to a study, facultative anaerobes were separated via enrichment on media containing yeast extract and textile dye discharge effluents, resulting in the isolation of a mixed and pure culture. As temperatures increase above 60 °C, both cultures develop and decolonize. At 50 °C, the percentage of decolorization ranged from 67 to 84 % for mixed cultures and from 70 to

99 % for pure cultures. The cultures grew most effectively at 55 °C in aerobic circumstances (Banat et al., 1997).

A different investigation included a variety of soil and sludge samples that were gathered from the areas surrounding waste disposal facilities and textile dyeing businesses. These were employed to enrich microbial communities while Acid Violet-17, a triphenylmethane dye, was present. The ability of 25 isolates to decolorize AV-17 dye applied at a rate of 10 mgl-1 in mineral salts medium agar plates was tested. To create a bacterial consortium, five bacterial isolates from the species *Bacillus*, *Alcaligenes*, and *Aeromonas* were chosen based on their greater capacity for decolorization. The consortium successfully decolored Acid Violet-17 (86%), Acid Blue-15 (85%), Crystal Violet (82%), Malachite Green (82%), and Brilliant Green (85%), among other TPM colors. The consortium is envisaged to be utilized in the construction of economical and effective treatment methods for wastewater from the textile processing industry (Meehan et al., 2001).

Investigations were conducted on the decolorization and detoxification of a textile industry effluent using *Trametes trogii* laccase in the presence and absence of laccase mediators. At the greatest enzyme concentration tested, laccase alone was unable to effectively decolorize the effluent; with 9 U/mL reaction mixture, less than 10% decolorization was achieved. Laccase mediators were evaluated at concentrations ranging from 0 to 1 mM to improve effluent decolorization. The majority of possible mediators improved the effluent's decolorization, with 1-hydroxybenzotriazol (HBT) demonstrating the greatest efficacy. At 50 °C, pH 5, 20% effluent, and 1 mM HBT were the conditions that led to optimal decolorization (Khlifi et al., 2010).

The subsequent investigation sought by Roy *et al.*, 2019 to separate and characterize microorganisms that degrade Crystal Violet from industrial effluents in order to potentially apply them in bioremediation. A photoelectric colorimeter was used to test the bacteria's decolorizing activity following aerobic incubation at various intervals for the isolates. Using a mineral salt medium with various concentrations of Crystal Violet dye, environmental parameters like pH, temperature, initial dye concentration, and inoculum size were optimized. In a mineral salt medium containing up to 150 mg/l of Crystal Violet dye, 10% (v/v) *Enterobacter sp*. inoculums showed complete decolorizing effectiveness.

Lately, a study performed on extreme halophilic/halotolerant bacteria screened from the salt fields of Tibet, including *Enterococcus*, unclassified *Enterobacteriaceae*, *Staphylococcus*, *Bacillus*, and *Kosakonia*, revealed to decolorize azo dyes in high-salt industrial wastewater under high-salt and low oxygen conditions. 600 mg/l of Congo red, Direct Black G (DBG), amaranth, methyl red, and methyl orange could totally decolorize in 24, 8, 8, 12, and 12 hours, respectively, under ideal circumstances. The microflora demonstrated high degradative ability in addition to its capacity to withstand variations in salt concentrations ranging from 0 to 80 g/l. Based on a phytotoxicity study, it was demonstrated that the halophilic/halophilic bacteria could convert the hazardous DBG dye into metabolites of low toxicity. Based on intermediates found using liquid chromatography-mass spectrometry (LC–MS), a novel mechanism for the microflora's degradation of DBG was postulated (Qiu H et al., 2022).

Further, Kolekar et al., 2012 studied the formation of aerobic granules from textile wastewater sludge and evaluated their capacity to degrade dyes by subjecting them to varying concentrations of reactive blue 59 (RB59). The granules maintained a greater dye loading of up to 5.0 g/l and effectively broke down reactive blue 59. The notable upregulation of

azoreductase and cytochrome P-450 enzymes suggested their important function in the dye degradation process, but genotoxicity investigations proved the dye's biotransformed product to be non-toxic.

Another study on dye decolourisation was carried out using a halophilic bacterium *Salinivibrio kushneri* isolated from salterns. Water soluble dyes like Coomassie brilliant blue, Safranin, and Congo red were used. Within 48 hours more than 80% of decolorization was observed in CBB and Congo red dyes. The highest rate of decolourisation was observed in CR, followed by CBB and then by safranin. Using UV spectroscopy and FTIR, the decolorization peaks were observed where the peaks indicated the breakdown of dyes upon decolorization. Therefore the study has shown that the potential of *S. kushneri* to decolorize dyes with higher concentrations is at a faster rate (John J et al., 2020).

A study done by Song et al., 2017 on *Pichia occidentalis*, a salt-tolerant yeast with the ability to decolorize a variety of azo dyes, was recently discovered. Comprehensive studies were conducted on this yeast's characterization, degradation route, detoxifying effects, and enzyme analysis. The findings indicated that the following values for strain G1's ideal development and metabolism: 2.0 g/l glucose, 0.6 g/l ammonium sulfate, 0.08 g/l yeast extract, 30 g/l NaCl, 160 rpm, 30°C, in pH 5.0. In ideal circumstances, almost 98% of 50 mg/l Acid Red B (ARB) may be decolorized in less than 16 hours. Furthermore, strain G1 degraded and clearly detoxified ARB via a potential pathway that included the TCA cycle, decolorization, and deamination/desulfonation activities in order of likely order. Furthermore, it was determined that NADH-DCIP reductase was the primary reductase for decolorization and that laccase, manganese peroxidase, and lignin peroxidase were significant oxidoreductases for the additional breakdown of decolorization intermediates.

Recently, Tian F et al., 2021 studied a halophilic bacterial consortium that was enriched at 5% salinity. The decolorization of the dye metal yellow G was observed from 1% to 15% salinity and with dye concentrations of 100 - 400 mg/l under normal conditions. The study showed that maximum decolorization was observed at 200 - 400 mg/l, and the decolorization was 98.3% after 6 hours. This indicated that the halophilic consortium had the capability to endure high concentration; thus, it could be used for a wide range of azo dye concentrations.

This review aims to isolate and characterize halophiles from salt water and investigate their potential dye-degrading properties. It was conducted to see trends and advancements in the field of industrial wastewater effluent treatment. The review emphasizes that industrial wastewater treatment is a global issue and is actively researched.

# CHAPTER 03

## METHODOLOGY

## 3.1. Collection of Sample

Sample collection was carried out on 1st December, 2023 from Betim Verem Saligao road, Bardez, Penne de Franca, Goa ferry. The sample was collected from five different spots from the start to the end of the ferry. The sample was collected in plastic bottles (500ml) which were previously washed thoroughly with distilled water twice and rinsed with the sample water on the spot. The water samples were brought to the laboratory and 200ml of water sample from each bottle was mixed in a beaker.

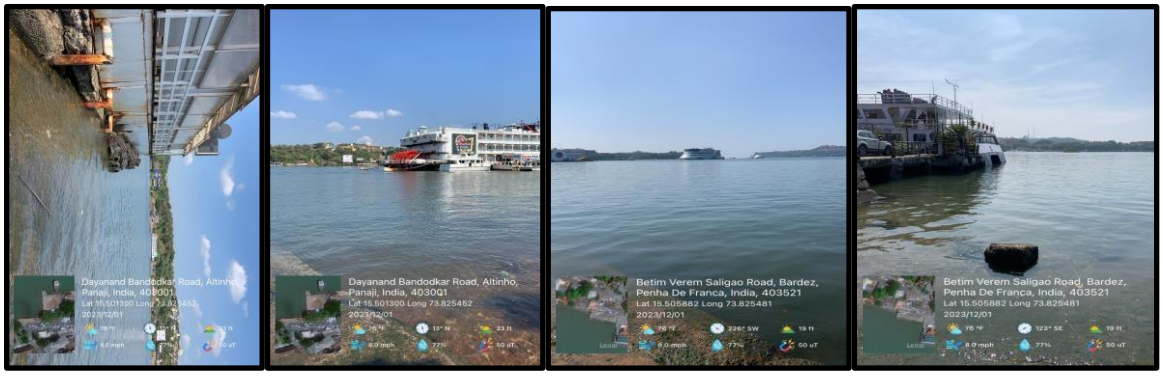



**Fig 3.1** : Betim Verem Saligao road, Bardez, Penne de Franca, Goa.

## 3.2. Analysis of physico chemical parameters of the sample

### 3.2.1. Measurement of temperature of the water sample

A glass thermometer was used to analyse the temperature of the water samples. The temperature was recorded immediately after the collection of samples to avoid errors in the result.

### 3.2.2. Determining the pH of the water sample

A digital pH metre (Eutech instruments) was used to record the sample's pH. Using pH buffer standards in the acidic (pH 4), neutral (pH 7), and alkaline (pH 9) pH ranges, the pH metre was standardised. The pH probe was inserted into a 100 ml glass beaker containing the sample, the

reading was recorded as displayed on the screen. The readings were recorded in triplicates and the average was calculated. Following each measurement, the probe was thoroughly cleaned with distilled water and wiped carefully (Kulasekaran et al., 2015).

### 3.2.3. Determination of salinity of the water sample

A refractometer was used to record the salinity of the water sample. The sample stage was wiped thoroughly. A drop of the sample was added to the stage and the salinity was observed.

### 3.2.4. Analysis of colour and odour of the sample

The color and odour of the sample were observed using olfactory and visual sensory methods.

## 3.3. Isolation of halophiles/halotolerant bacteria from water sample

### 3.3.1. Selection of media

Nutrient broth and Minimal broth without dextrose was chosen for the enrichment and growth of bacteria from the water sample.

### 3.3.2. Enrichment of media

Nutrient broth was used to obtain isolates for bacteria growing in the water sample. Components of nutrient broth were weighed and a flask containing 100 ml of nutrient broth was prepared and their pH was adjusted to 7+0.2. The flask was then autoclaved at 121°C for 20 mins at 15 psi and allowed to cool. The flask was then inoculated with 5 ml of sample and placed on a rotary shaker incubator at 110 rpm for three days at 30+2°C. The nutrient broth flasks were kept on a rotary shaker incubator for three days during which enrichment of the culture took place. Turbidity was observed visually and the OD of the broth was recorded at

0.8 which indicated growth of microorganism. (R.S Shertate & P.R Thorat, 2014), ( Qiu H et al., 2022)

Similarly, Minimal broth without dextrose were prepared which contained 2% and 5% NaCl concentration along with malachite green (MG) and Congo red (CR) dye. Components of minimal broth without dextrose were weighed. To these flasks 2g of NaCl was added to make the salt concentration to 2% and 5g of NaCl was added to make the salt concentration to 5%. Two flasks of 100 ml minimal media without dextrose for each concentration were prepared which contained 100 mg/l CR dye and two flasks of 100 ml minimal media without dextrose for each concentration were prepared which contained 15 mg/l MG dye and each of their pH was adjusted to 7+0.2. The flasks were then autoclaved at 121°C for 20 mins at 15 psi and allowed to cool. To the cooled flasks under sterile conditions approximately 2.5ml of sterile dextrose was added. From the enriched nutrient medium 5 ml of the grown culture was added to each of the flasks containing 2% and 5% salt concentration to enable growth of only those organisms that can tolerate the provided salt concentration. The flasks were then placed on a rotary shaker incubator at 110 rpm for three days at 30$\pm$2°C. Decolourisation and turbidity was observed visually which indicated growth of microorganism.

### 3.3.3. Serial dilution and Spread plating of sample

0.85% of sterile saline was prepared. The workstation was surface sterilised using 70% ethanol. Four sets of six eppendorf tubes were placed on the stand and were labelled as $10^{o}$, $10^{-1}$, $10^{-2}$, $10^{-3}$ , $10^{-4}$ and $10^{-5}$ respectively. In 10° tubes 1 mL incubated minimal broth of CR in 2% NaCl was added under sterile conditions and in the remaining five tubes 0.9 mL of saline was added. From 10° tubes 0.1 mL of culture was transferred in $10^{-1}$ tubes and mixed and so on. The procedure was repeated with MG in 5% NaCl, MG in 2% NaCl and MG in 5% NaCl flasks.

Minimal medium agar plates amended with 2% and 5% NaCl concentrations, along with 15 mg/l malachite green (MG) dye and 100 mg/l Congo red (CR) dye, respectively, were prepared separately under sterile conditions. 0.1ml of the serially diluted sample from each set was pipetted out and spread plated using an L-shaped glass spreader that was dipped in 70% ethanol and flamed. All the dilutions were spread plated on minimal agar CR and MG dye plates. The plates were then incubated at 30°C till there was visible growth observed.

### 3.3.4. Colony counting

To determine the viable count of the bacteria on the agar plates, colony counting was done. By counting each colony dot once, one may determine the size of the colonies. Usually, the plates containing more than 150–250 colonies were counted by splitting them into four equal quadrants. To calculate the CFU, one quadrant's count is multiplied by the total number of quadrants. A colony counter, a tool frequently used to quantify the concentration of bacterial colonies, can also be used to measure the colonies.(Brugger et al., 2012)

### 3.3.5. Isolation of distinct colonies

From the plates that were serially diluted, distinct colonies were separated. The colonies were selected and streaked on minimum CR and MG agar plates in an aseptic manner using a sterile nichrome loop. For twenty-four hours, the plates were incubated at 30°C. (Stanier et al., 1987)

### 3.3.6. Maintenance of cultures

The isolated bacterial cultures were maintained at 4°C in the refrigerator. The bacterial cultures were sub-cultured under sterile conditions on minimal agar slants. Sub-cultured tubes were checked regularly for contamination.

## 3.4. Morphological tests of grown colonies

### 3.4.1. To examine the growth characteristics of the bacterial colonies

The colonies obtained after incubation were observed for their growth characteristics.

### 3.4.2. Gram staining of isolated microorganisms

The visually distinct isolates were Gram stained and observed under a compound microscope (Lawrence and Mayo XSZ-N107T). The isolated species was thinly smeared onto a sterile glass slide. Heat was used to repair the smeared slides. After applying crystal violet, each smear was held for one minute. After that, distilled water was used to wash the crystal violet until the extra color was removed. After applying Gram's iodine solution and letting the smears sit for a minute, the iodine residue was carefully removed by giving them a gentle wash with distilled water. After a minute, the decolourizer was added and rinsed. After using safranin as a counterstain for thirty seconds, the area was cleaned. After air drying, the stained slides were examined using an oil immersion objective at a magnification of 100X.

## 3.5. Biochemical Characterization Studies

The iMVIC test was performed using the KB001 kit from Himedia.

### 3.5.1. Indole test :

Indole test was carried out to determine the ability of microorganisms to produce indole.

50 µl of fresh culture of GUCR2 and GUCR5 were separately inoculated to the indole well and incubated for 2 days at room temperature. 2 drops of Kovacs reagent were added to the well after incubation and observed for colour change.

### 3.5.2. Methyl red test :

Methyl Red test was performed to determine the ability of microorganisms to produce high concentrations of acid end products 50 µl of fresh culture of GUCR2 and GUCR5 were separately inoculated to the MR well and incubated for 2 days at room temperature. 2 drops of methyl red reagent were added to the well after incubation and observed for colour change. Development of red colour was taken as MR positive whereas yellow indicates that the organism is MR negative.

### 3.5.3. Voges Proskauer test :

Voges Proskauer test was performed to determine the ability of microorganisms to produce non acidic or neutral end products 50 µl of fresh culture of GUCR2 and GUCR5 were separately inoculated to the VP well and incubated for 2 days at room temperature. 2 drops of Baritts A and 2 drops of Baritts B reagent were added to the well after incubation and observed for colour change. Development of red colour represented a positive result whereas the absence of red colour was interpreted as negative result.

### 3.5.4. Citrate utilisation test :

Citrate utilisation test was performed to determine the Ability of microorganisms to ferment Citrate as sole source of carbon. 50 µl of fresh culture of GUCR2 and GUCR5 were separately inoculated to the citrate well and incubated for 2 days at room temperature. Positive result was indicated by a change of colour from Green to blue and retention of green colour is a negative result.

### 3.5.5. Determination of the ability of microorganisms to utilise carbohydrates

The sugars present were glucose, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose. Positive result was indicated by a change of colour from pinkish red/red to yellow and pinkish red/red to red as a negative result.

## 3.6. Assessment of dye degradation ability of bacteria

### 3.6.1. Using commercial dyes

#### 3.6.1.1. Selection of dyes.

In the present study, commercial dyes Congo red (CR) and Malachite green (MG) were used for analysing the dye degradation potential of the isolated colonies.

### 3.6.2. Optimisation studies

#### 3.6.2.1. Effect of temperature on degradation

The isolates GUCR2 and GUCR5 were separately inoculated in Minimal Davis (without dextrose) media containing CR (100mg/L) with 2% and 5% NaCl respectively. Control flasks for each were kept under sterile conditions. Similar procedure was followed for Malachite green dye (15 mg/l). These flasks were kept at different temperatures ( 22°C, 32°C and 40°C) to check the effect of temperature on degradation. The flasks were incubated for 10 days. Using calibrated micropipettes, 5 ml of the inoculation broth was transferred to centrifuge tubes, and the tubes were spun for 5 minutes at 10,000 rpm. Four ml of the supernatant were poured into glass cuvettes. From Day 0 to Day 10, a spectrophotometric reading of the supernatant was obtained at each λmax wavelength after every alternate day, using D/W and control broth as blanks. Percentage degradation of the colony degrading the dyes was also calculated. Percentage Degradation = [initial absorbance - final absorbance ] ÷ final absorbance × 10

### 3.6.2.2. Effect of pH on degradation

The isolates GUCR2 and GUCR5 were separately inoculated in Minimal Davis (without dectrose) media containing CR (100mg/L) with 2% and 5% NaCl respectively. Control flasks for each were kept under sterile conditions. Similar procedure was followed for Malachite green dye (15 mg/l). These flasks were kept at different pH (5, 7, 8, 9.2) to check the effect of pH on degradation. The flasks were incubated for 10 days. Using calibrated micropipettes, 5 ml of the inoculation broth was transferred to centrifuge tubes, and the tubes were spun for 5 minutes at 10,000 rpm. Four ml of the supernatant were poured into glass cuvettes. From Day 0 to Day 10, a spectrophotometric reading using UV vis spectrometry (Toshvin Analytical Pvt ltd) of the supernatant was obtained at each λ max wavelength after every alternate day, using D/W and control broth as blanks. Percentage degradation of the colony degrading the dyes was also calculated.

### 3.6.2.3. Effect of incubation (aeration) conditions

The isolates GUCR2 and GUCR5 were separately inoculated in Minimal Davis (without dectrose) media containing CR (100mg/L) with 2% and 5% NaCl respectively. Control flasks for each were kept under sterile conditions. Similar procedure was followed for Malachite green dye (15 mg/l). These flasks were kept at room temperature in static and shaker condition to check the effect of incubation (aeration) on degradation. The flasks were incubated for 10 days. Using calibrated micropipettes, 5 ml of the inoculation broth was transferred to centrifuge tubes, and the tubes were spun for 5 minutes at 10,000 rpm. Four ml of the supernatant were poured into glass cuvettes. From Day 0 to Day 10, a spectrophotometric reading using UV vis spectrometry (Toshvin Analytical Pvt ltd) of the supernatant was obtained at each λmax wavelength after every alternate day, using D/W and control broth as blanks. Percentage degradation of the colony degrading the dyes was also calculated.

## 3.6.2. Lab made wastewater degradation

Industrial wastewater samples were placed in flasks with 2% and 5% saline to test for deterioration. Two flasks containing the specific sample and saline were constructed in order to measure the rate of deterioration of each sample. A control flask was also maintained in a similar manner, but without inoculation. In a 4:1 ratio, the saline and wastewater samples were introduced. Following the inoculation of the flasks with the corresponding isolates, the flasks were incubated at 110 rpm on the rotary shaker. Following this, the absorbance of the flask contents was measured for alternate days at each respective λ maxima of the dye, and the graphs were plotted. The percent degradation ability of each isolates of the lab water sample was also determined (Sriram N and Reetha D, 2015).

# CHAPTER 04

## ANALYSIS AND CONCLUSIONS

## 4.2. Analysis of physico chemical parameters of sample

### 4.2.1. Measurement of temperature water sample

The respective water sample temperature was measured using a glass thermometer. The analysis of the sample was done expeditiously to minimize errors in the results. The temperature range of the sample was found to be around 30 to 31°C (**Table 4.1**). The temperature provides information on the conditions required for the survival and proliferation of microbial species; a higher temperature provides a more suitable environment and amplifies the growth rate of microbes as an increase in temperature increases enzyme activity that causes cells to grow faster.

**Table 4.1 :** Measurement of temperature of water sample

| Sites | Temperature |
|---|---|
| Site 1:15.501300 $^{o}$N / 73.825452 $^{o}$E | 30°C |
| Site 2 :15.501300 $^{o}$N / 73.825452 $^{o}$E | 31°C |
| Site 3: 15.501300 $^{o}$N / 73.825452 $^{o}$E | 31°C |
| Site 4: 15.501300 $^{o}$N / 73.825452 $^{o}$E | 30°C |
| Site 5: 15.501300 $^{o}$N / 73.825452 $^{o}$E | 31°C |

### 4.2.2. Determination of pH of water sample

The sample's average pH was discovered to be 8.02 (**Fig 4.1**). The pH scale runs from 7 to 14, where a number less than 7 denotes acidic properties and a value greater than 7 suggests alkaline properties. The highest level of alkalinity is found at pH 14, while the highest degree of acidity is found at pH 0. A pH metre measures the sample's pH and temperature. (Ariswati et,al., 2020).

According to guidelines from the World Health Organization and the National Drinking Water Quality Standard, the appropriate pH range for drinking water is between 6.5 and 8.5

(Rehmanian et al., 2015). The pH values of the provided samples were found to be fairly near to the neutral range, with a minor tendency toward the basic range. It can be concluded that the sample's hydrogen ion activity is within the typical range for drinking water.

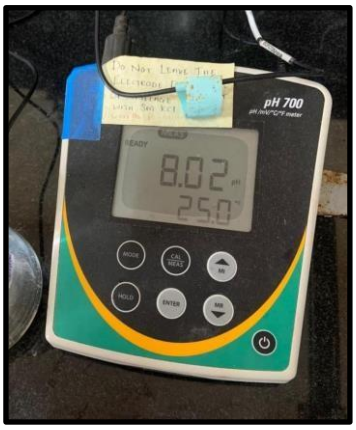



**Fig 4.1** : pH of water sample.

## 4.2.3. Determination of salinity of water sample

Facultative halophiles grow best at salinities greater than 2% but can also thrive with less salt, whereas obligatory halophiles are defined as those that require 2% or more salt (Schneegurt, 2012). The sample's salinity used in this experiment was found to be 28 PSU (2.8% )(**Fig 4.2**) suggestive of facultative halophile in the sample tested.

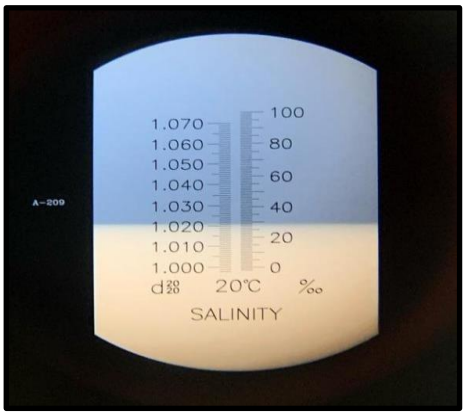



**Fig 4.2 :** Salinity of the water sample

### 4.2.4. Analysis of odour and colour of sample

No colouration was observed in the water sample. No traceable odour was noted.

## 4.3. Isolation of halophiles/halotolerant bacteria from water sample

The water sample was added to flasks containing Minimal Davis Broth that was incorporated with CR and MG dye amended with 2% and 5% NaCl, respectively, and left on a rotary shaker for four days, during which the culture was enriched. Indicators such as turbidity and decolorization were used to track the expansion of the bacterial growth.

### 4.3.1. Serial dilution and Spread plating of sample

Individual colonies were observed on agar plates labelled 2% CR, 5% CR, 2% MG and 5% MG, at concentration $10^{-4}$ and $10^{-5}$ (**Fig 4.3, Fig 4.4, Fig 4.5, Fig 4.6**).Agar plates at concentration $10^{0}$ , $10^{-1}$ , $10^{-2}$ and $10^{-3}$ showed matte growth.

### 4.3.4. Colony counting

Volume of inoculum = 0.1ml

**Fig 4.2 :** Viable count of bacteria isolated from water sample.

| | Average viable count (CFU/mL) |
|---|---|
| CR in 2% NaCl | $22.84 \times 10^6$ |
| CR in 5% NaCl | $31 \times 10^6$ |
| MG in 2% NaCl | $13.18 \times 10^6$ |
| MG in 5% NaCl | $15.13 \times 10^6$ |

The average viable count of bacteria on CR in 2% NaCl was found to be $22.84 \times 10^6$ CFU/mL.

The average viable count of the bacteria on CR in 5% NaCl was found to be $31 \times 10^6$ CFU/mL.

The average viable count of the bacteria on MG in 2% NaCl was found to be 13.18 × $10^6$ CFU/mL. The average viable count of the bacteria on MG in 5% NaCl was found to be 15.13 × $10^6$ CFU/mL.

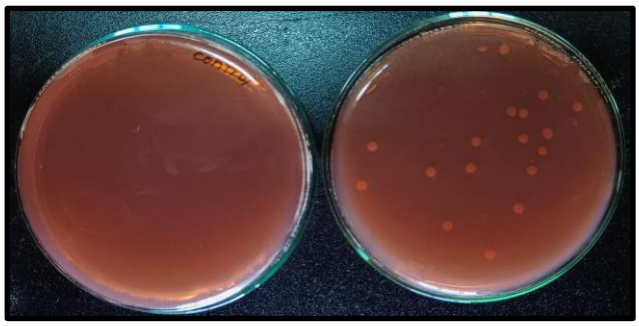


**Fig 4.3** : Viable count on 2% NaCl concentration CR plates

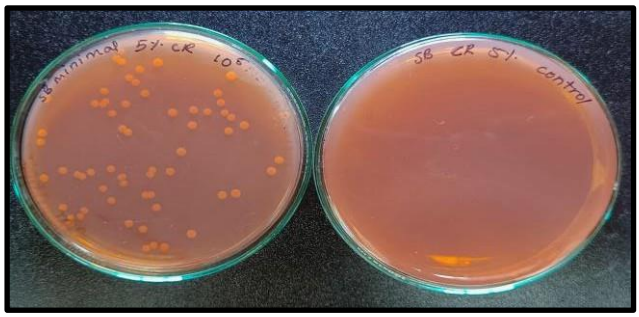



**Fig 4.4 :** Viable count on 5% NaCl concentration CR plates

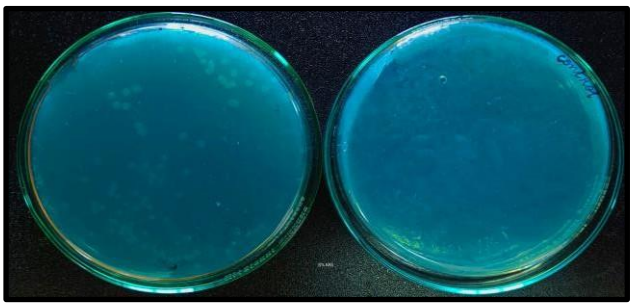


**Fig 4.5 :** Viable count on 2% NaCl concentration MG plates

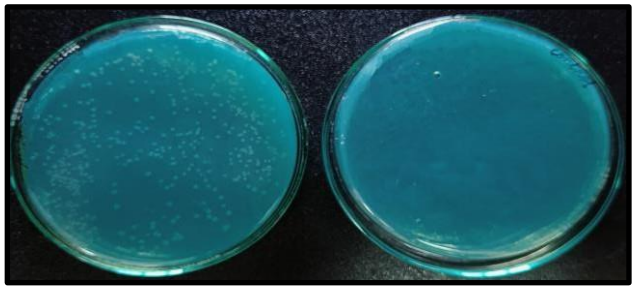


**Fig 4.6 :** Viable count on 5% NaCl concentration MG plates

### 4.3.5. Isolation of distinct colonies

Plates containing CR in 2% NaCl, CR in 5% NaCl, MG in 2% NaCl, and MG in 5% NaCl were serially diluted to isolate morphologically different colonies. Using a sterile nichrome loop, the colony was removed, and under aseptic circumstances, it was streaked on Minimal Davis agar plates incorporated with CR and MG dye respectively with the appropriate NaCl concentration. After the plates were incubated at 37°C, growth was evident.

### 4.3.6. Maintenance of cultures

The refrigerator was kept at 4°C for the isolated bacterial cultures. On minimum agar slants, the bacterial cultures were subcultured in sterile conditions. The sub-cultured tubes were checked regularly for any contamination.

## 4.4. Morphological tests of grown colonies

### 4.4.1. Colony characteristics

A collection of bacteria that originate from the same mother cell and grow and reproduce to create a colony is known as a bacterial colony. The physical features of a bacterial colony on an agar plate are known as colony morphology were noted (**table 4.3**). The bacterial colonies' edge, form, elevation, pigmentation, and texture are crucial characteristics that act as crucial identifying criteria (Aryal S., 2022).

**Table 4.3:** Colony characteristics of the bacteria isolates on the respective media plates amended with dye.

| | **GUCR2** | **GUCR5** | **GUMG2** | **GUMG5** |
|---|---|---|---|---|
| **Edge** | Smooth | Smooth | Smooth | Smooth |
| **Shape** | Circular | Circular | Circular | Circular |
| **Elevation** | Raised | Raised | Raised | Raised |
| **Pigmentation** | Light pink | White | Light Blue | Light Blue |
| **Texture** | Mucoid/sticky | Mucoid/sticky | Mucoid/sticky | Mucoid/sticky |

## 4.4.2. Gram staining of isolated microorganisms

The Gram-positive bacteria stained blue/purple and the Gram-negative stained red in colour. GUCR2 was found to be Gram positive rod (**Fig 4.7 (a))** which means they have a thick peptidoglycan layer in their cell walls. GUCR5 was found to be Gram negative rod (**Fig 4.7 (b**)).

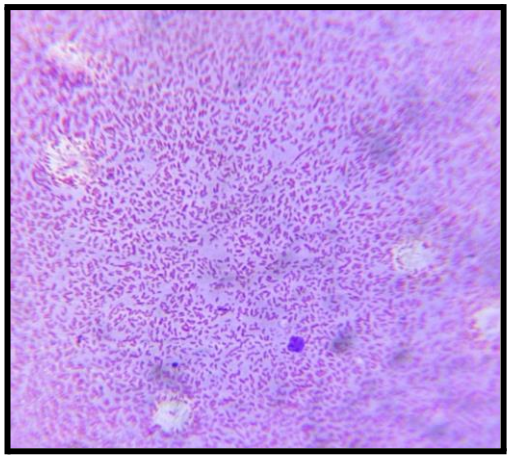
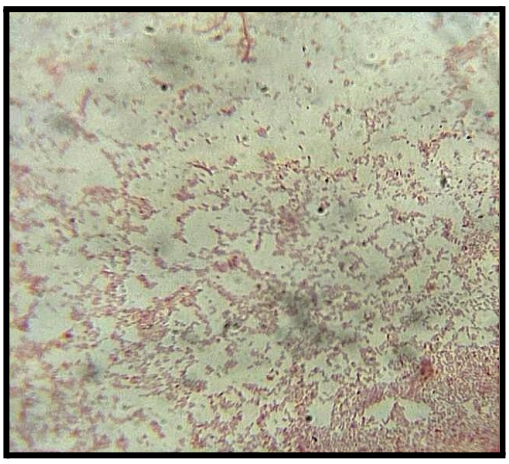
(a)  (b) 

**Fig 4.7 :** Gram staining **(a)** GUCR 2 : Gram +ve rods **(b)** GUCR5 : Gram -ve rods

## 4.5. Biochemical tests of bacterial isolates from water sample.

**Table 4.4** : Biochemical tests

| | GUCR2 | GUCR5 | GUMG2 | GUMG5 |
|---|---|---|---|---|
| **Indole** | - | - | - | - |
| **Methyl Red** | + | + | + | + |
| **Voges Proskauer** | - | - | - | - |
| **Citrate utilisation** | - | + | - | + |
| **Glucose** | + | - | + | - |
| **Adonitol** | - | - | - | - |
| **Arabinose** | + | - | + | - |
| **Lactose** | + | - | + | - |
| **Sorbitol** | + | - | + | - |
| **Mannitol** | + | - | + | - |
| **Rhamnose** | + | - | + | - |
| **Sucrose** | - | - | - | - |

In the present study, none of the samples showed positive results for the Indole test. All the samples tested showed positive results for the methyl Red test. The cultures tested positive may belong to the Genus *Salmonella* (Sriram and Reetha, 2015). GUCR5 and GUMG5 showed positive results for the citrate utilisation test as there was colour change from green to Prussian blue. This may belong to Genus *Bacillus, Salmonella, Pseudomonas, Klebseilla* (Khan and Joshi, 2019).

GUCR2 and GUMG2 showed positive results for the utilisation of the sugars Sucrose, Mannitol, Sorbitol, Lactose, Adonitol and Glucose.

According to the manual provided with the kit, GUCR2 and GUMG2 matched with the *Enterobacteriaceae* species *Escherichia vulneris* whereas GUCR5 and GUMG5 did not match with any of the species present in the provided manual.


**Fig 4.8** : Results for Biochemical tests

## 4.6. Assessment of dye degradation ability of bacteria

### 4.6.1. Using commercial dyes

#### 4.6.1.1. Selection of dyes

Environmental contamination is caused by the pervasive presence of organic dyes in wastewaters from the paper, textile, and clothing industries. These wastewaters contaminated with dyes contain extremely dangerous, carcinogenic, non-biodegradable pigments that can harm both people and the environment. Dyes are observably visible in water at very low concentrations (below 1 ppm) and cause significant harm to watery habitats. As a result, it is crucial and necessary to remove wastewater containing coloured organic dyes (Chiu Y et al., 2019).

Cotton was once dyed with Congo red (CR). To dye tissues for microscopic inspection, it is still widely employed. It is employed in histology and biochemistry to detect amyloids and stain microscopic sections, particularly those containing erythrocytes and cytoplasm (Gurr E, 1977). It is a common anionic dye in the textile industry, possessing a complicated chemical structure with several diazo aromatic groups (Gharbani P et al., 2008).

The triphenylmethane group's malachite green dye is widely utilised in the textile and apparel sectors. In the textile business, malachite green has been used as a direct dye to colour textiles, jute, leather, especially silk and wool.In specifically, in histology and bacteriology, it has been used as a biological stain in microscopy to visualise tissues and cells (Roy et al., 2020 and Hasimoto et al., 2011)

## 4.6.2. Optimization studies

### 4.6.2.1. Spectrophotometric Analysis of the effect of temperature on dye degradation

Bacterial metabolism is significantly impacted by temperature. According to Chen et al. (2018), high temperatures can impede enzyme activity and damage the enzyme's active core, whereas low temperatures can result in insufficient enzyme activity.

Temperature was one of the primary factors influencing the dyes' decolorization effectiveness in this investigation. The optimum temperature recorded by GUCR2 and GUCR5 to breakdown CR was 30-32°C **(Graph 4.1a, 4.1b)**. At 32°C, the decolorization rate increased to 71.63% by GUCR2 and 82.23% by GUCR5. Nevertheless, the microflora's capacity to decolorize the dyes rapidly diminished as the temperature rose, but it was still able to do so at 40°C for concentrations of GUCR2 and GUCR5, respectively, with degradation rates of 52.95% and 63.28% after 12 days of incubation (**Graph 4.1e, 4.1f)**).

GUCR2 and GUCR5 showed 38.05% and 35.57% degradation of Malachite Green respectively at the end of 12 days of incubation at 32°C (**Graph 4.1e, 4.1f**). After 12 days of incubation, GUCR2 and GUCR5 were still able to decolorize the dyes at 40° **(Graph 4.1c, 4.1d)** with degradation rates of 29.37% and 23.13% respectively. However, this ability to do so quickly decreased as the temperature rose. The optimal temperature was found out to be 32°C.

This could be because some thermotolerant halophilic bacteria lost their ability to degrade due to the high temperature inhibiting their activity (Jadhav et al., 2008) or because some enzymes' active centers inactivated at high temperatures, reducing their capacity to degrade. At 40°C, the flora retained some degrading activity because certain bacteria, like *Bacillus* and *Enterococcus*, were present and have a certain amount of heat tolerance. Consequently, the high temperature employed in this investigation may cause the halophilic bacteria to adapt.

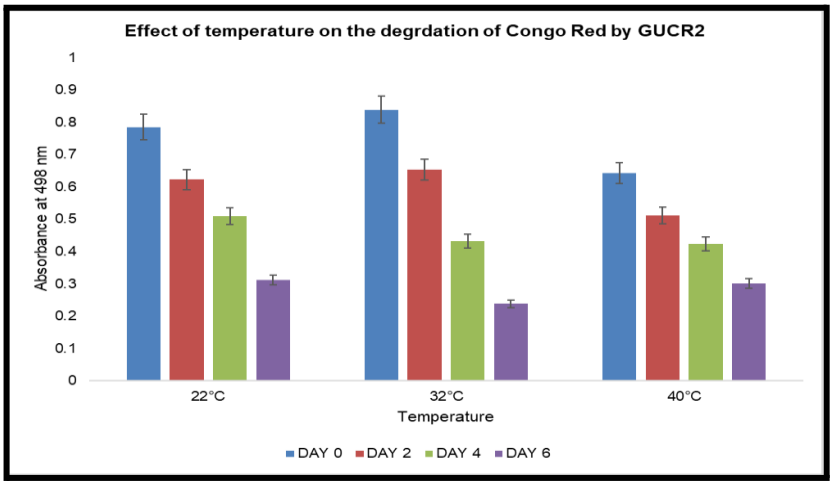



**Fig 4.1 (a)** : Effect of temperature on the degradation of Congo Red by GUCR2

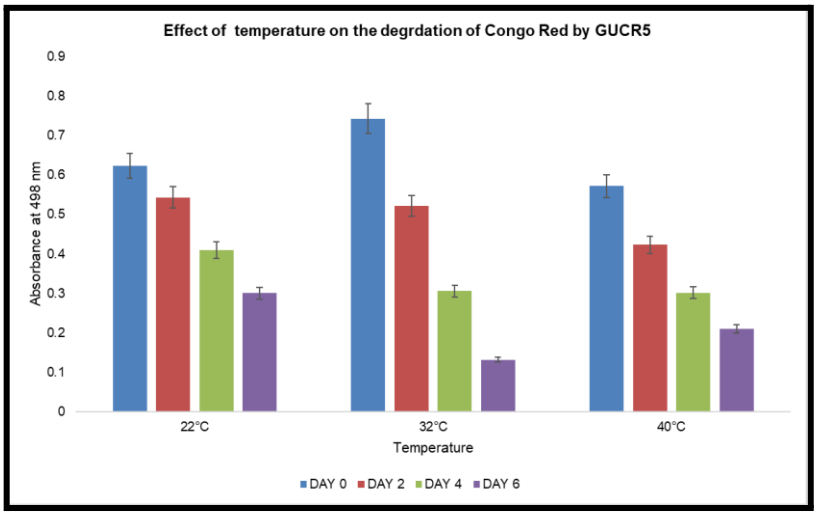



Fig 4.1 (b) : Effect of temperature on the degradation of Congo Red by GUCR5

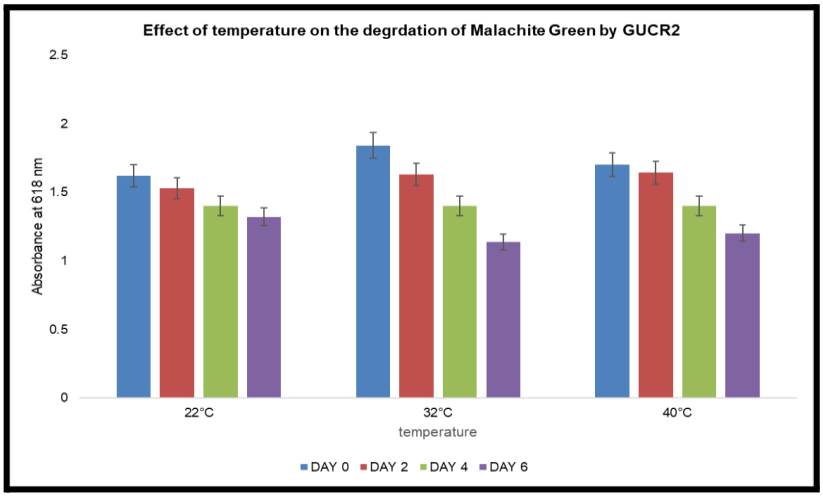



**Fig 4.1 (c)** : Effect of temperature on the degradation of Malachite Green by GUCR2

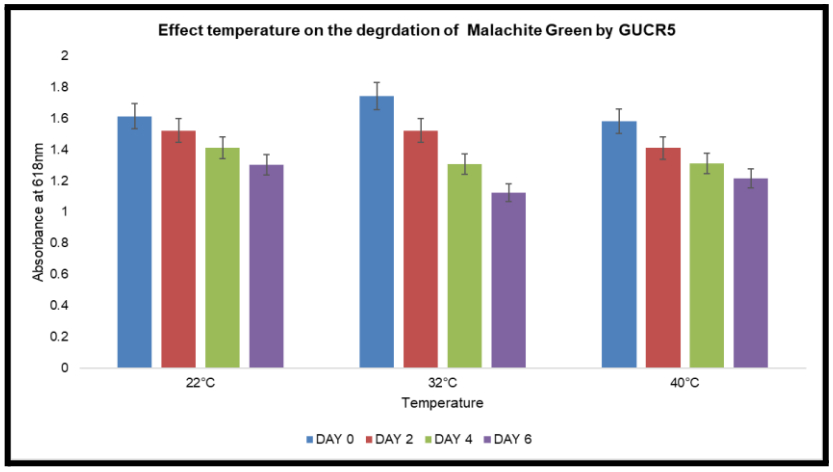



**Fig 4.1 (d)** : Effect of temperature on the degradation of Malachite Green by GUCR5

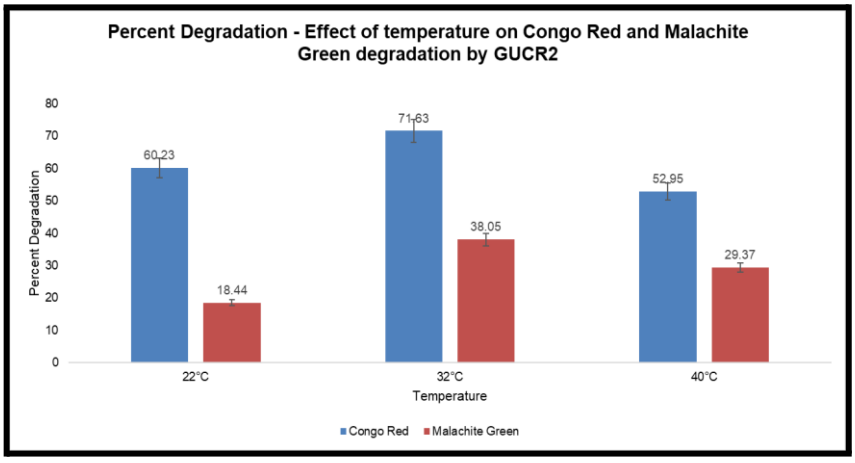



**Fig 4.1 (e)** : Percentage Degradation - Effect of temperature on the degradation of Congo Red and Malachite Green by GUCR2

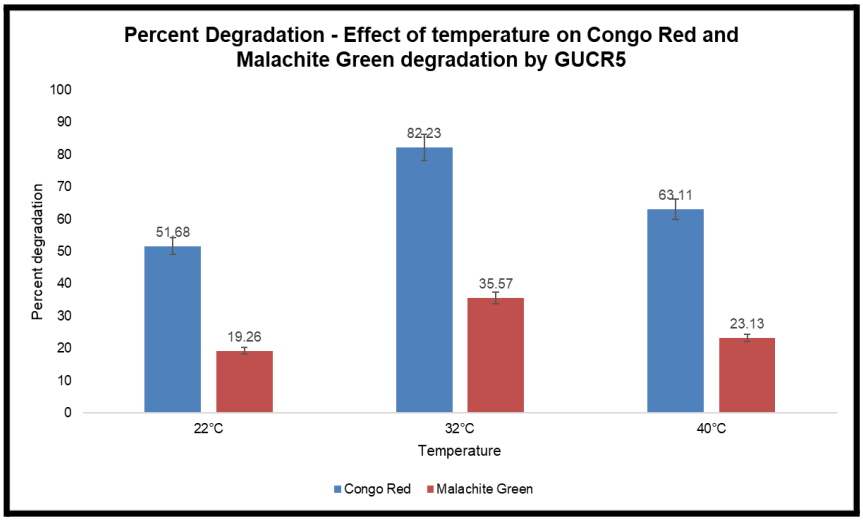



**Fig 4.1 (f)** : Percentage Degradation - Effect of temperature on the degradation of Congo Red and Malachite Green by GUCR5

### 4.6.2.2. Spectrophotometric Analysis of the effect of pH on dye degradation

pH is typically regarded as one of the primary elements impacting the degradation of dyes since it is essential to the movement of cell components across the membrane (Chem et al., 2018). It was observed that GUCR2 was able to degrade Congo Red over a wide pH range of 5–9. GUCR2 exhibited a comparatively stable degradation of CR with an effective degradation rate of 52.48% at pH 8 on day 6 with a decrease in the absorbance value to 0.201 **(Graph 4.2a)**. On the other hand, the Congo Red degradation rate by the microflora started to modestly rise at pH 8 when the reaction substrate turned alkaline. As the pH increased above 8 (alkaline conditions), it was observed that the percent degradation on CR decreased. Since the growth of GUCR2 was impacted by increase in pH thus, the pace of breakdown of CR was noticeably slower.

GUCR5 showed a relatively persistent effect on Congo Red degradation, with an effective degradation rate of 54.76% at pH 8 with a decrease in the absorbance value to 0.204 (**Graph 4.2b)**. The growth of GUCR5 was affected at pH of 9 and hence rate of degradation was notably slower than at pH 8. At pH 8, the degradation was at its maximum, while at pH 5, it was at its lowest, at 26.72% (**Graph 4.2e).**

It was perceived that at pH 7 the pace at which GUCR2 broke down Malachite Green was highest. The rate of MG degradation peaked under neutral conditions at 96.33% at pH 7 by day 6 (**Graph 4.2c)**. On the other hand, a pH of 9 had an effect on the proliferation of the microflora, and the rate of breakdown was notably slower than at pH 7. The lowest degradation was observed at pH 9 with a degradation percentage of 12.72 % at the end of 12 days of incubation (**Graph 4.2f**).

The rate at which GUCR5 degraded Malachite Green started to rise around pH 7. Under neutral conditions, the rate of degradation of MG peaked on day six, with a breakdown percentage of

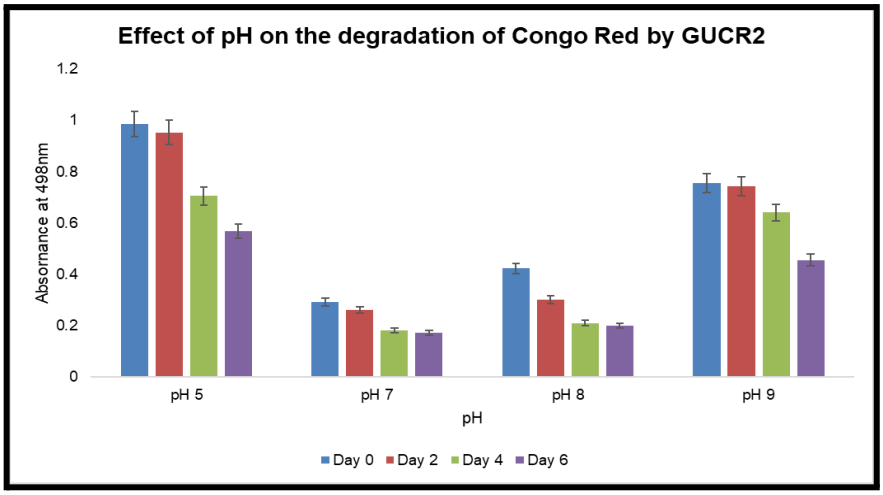



**Fig 4.2 (a)** : Effect of pH on the degradation of Congo Red by GUCR2

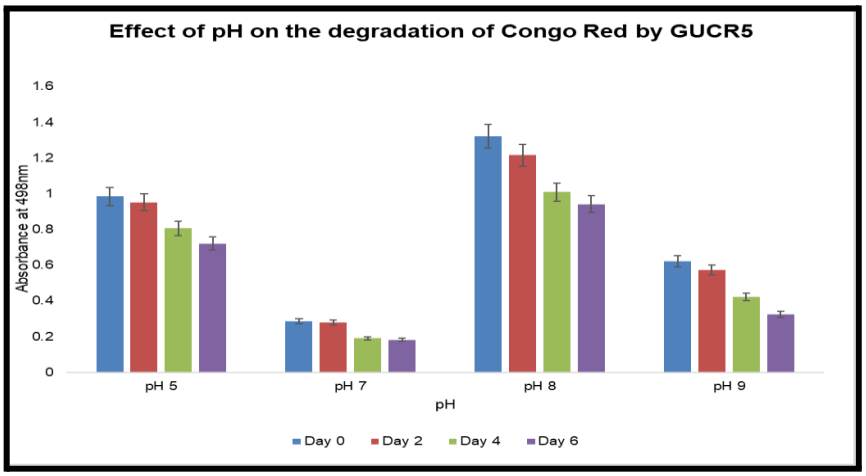



**Fig 4.2 (b)** : Effect of pH on the degradation of Congo Red by GUCR5

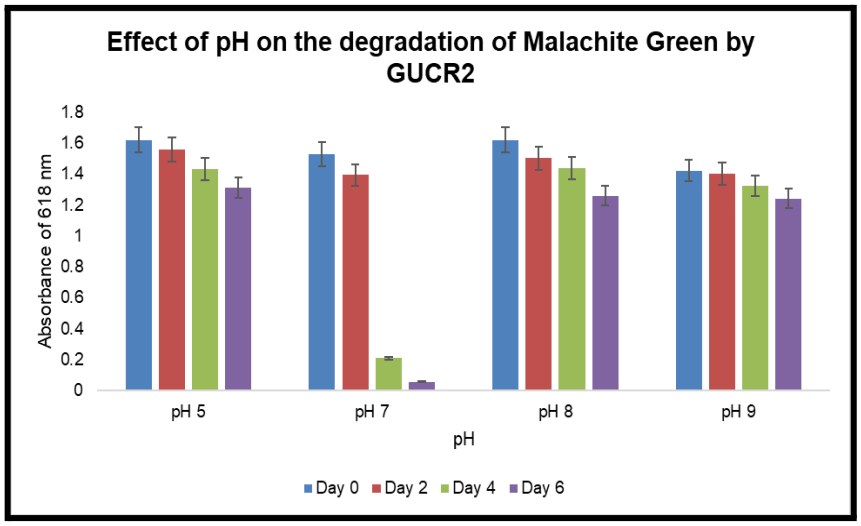



**Fig 4.2 (c)** : Effect of pH on the degradation of Malachite Green by GUCR2

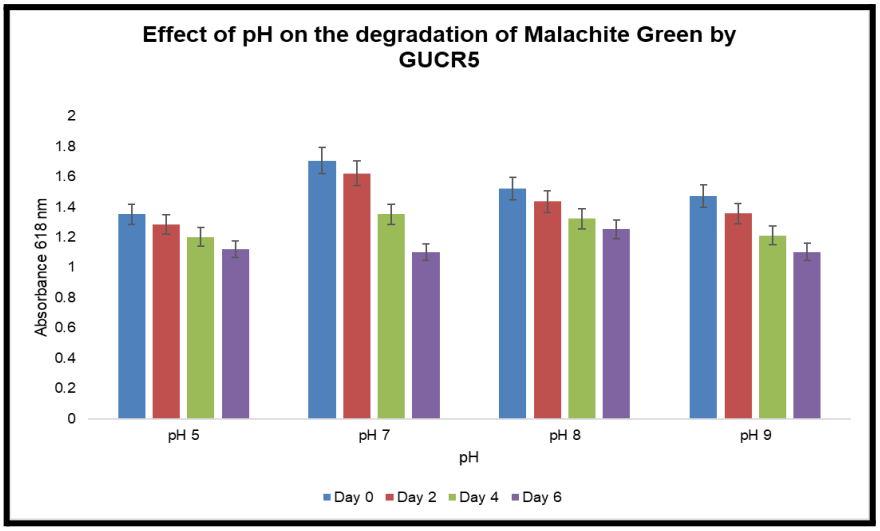



**Fig 4.2 (d)** : Effect of pH on the degradation of Malachite Green by GUCR5

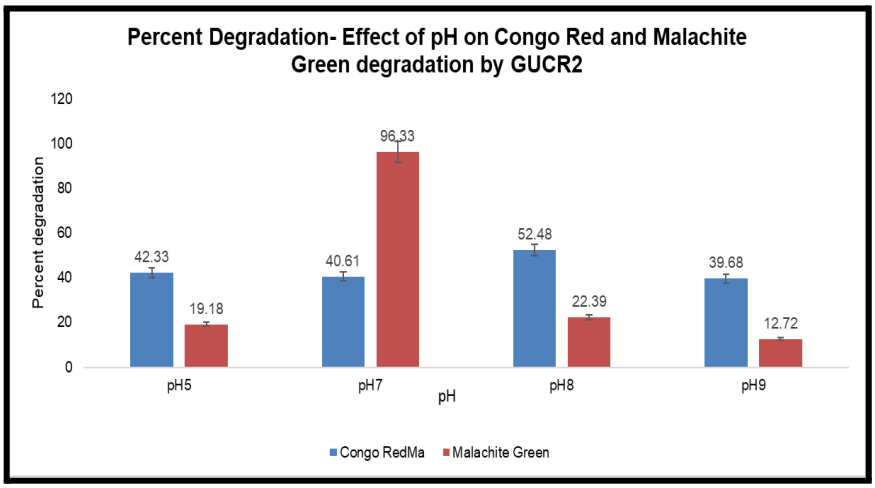



**Fig 4.2 (e)** : Percentage Degradation - Effect of pH on the degradation of Congo Red and Malachite Green by GUCR2

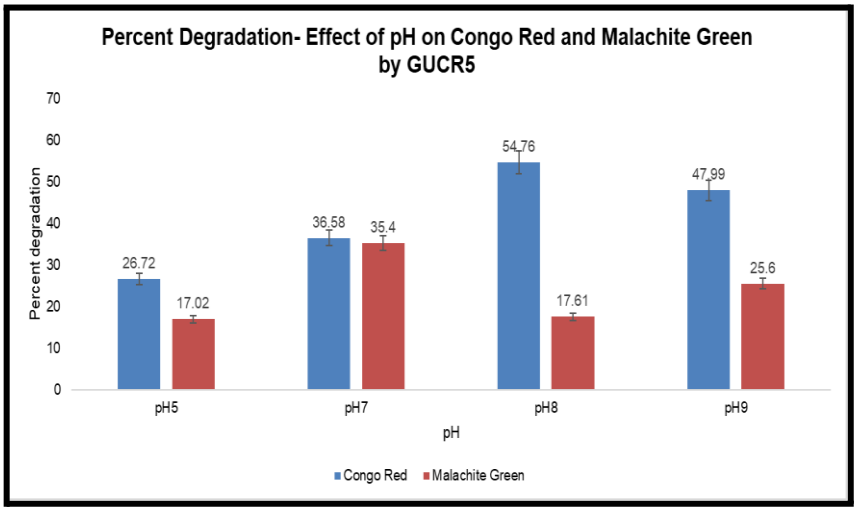



**Fig 4.2 (f)** : Percentage Degradation - Effect of pH on the degradation of Congo Red and Malachite Green by GUCR5

35.40% (**Graph 4.2d)**. However, the growth of the microflora was affected by a pH of 8, and the rate of breakdown was noticeably slower than at pH 7. After 12 days of incubation, pH 8 showed the least amount of degradation with a percent degradation of 17.61% (**Graph 4.2f).** Since the water sample is from riverine source, it could be contaminated by discharges from different sources from nearby surroundings which could contain metal cations. Conclusively. the optimal pH could be 7 to 8. The pH level of effluent from printing and dyeing industries was previously reported to be between 7 and 8 (Wang et al., 2013). Thus, halophilic/halotolerant microorganisms in this study can degrade the azo dyes used by printing and dyeing industries across a wide variety of pH value. Thus, the isolated microflora in the present study could have a strong potential for industrial application in the future.

#### 4.6.2.3. Spectrophotometric Analysis of the effect of incubation condition on dye degradation

A major factor influencing the rate at which halophilic and halotolerant bacteria decolorize is their oxygen level. After 12 days of incubation at 110 rpm, the degradation rate of GUCR2 under shaker conditions was 96.33% for Malachite Green and 55.33% for Congo Red. Nevertheless, the decolorization rate dropped to 24.62% for MG and 47.78% for CR after 12 days of incubation when the flasks were maintained in a static condition (**Graph 4.3a**).
The degradation rate of GUCR5 under shaker conditions was 41.32% for MG and 97.05% for CR after 12 days of incubation at 110 rpm. Nevertheless, after 12 days of incubation, when the flasks were kept in a static state, the decolorization rate decreased to 38.27% for MG and 55.81% for CR (**Graph 4.3b**).

The decolorization rate falls with increasing revolutions because oxygen might compete with the enzymes involved in degradation for reaction centres. These outcomes agree with those of

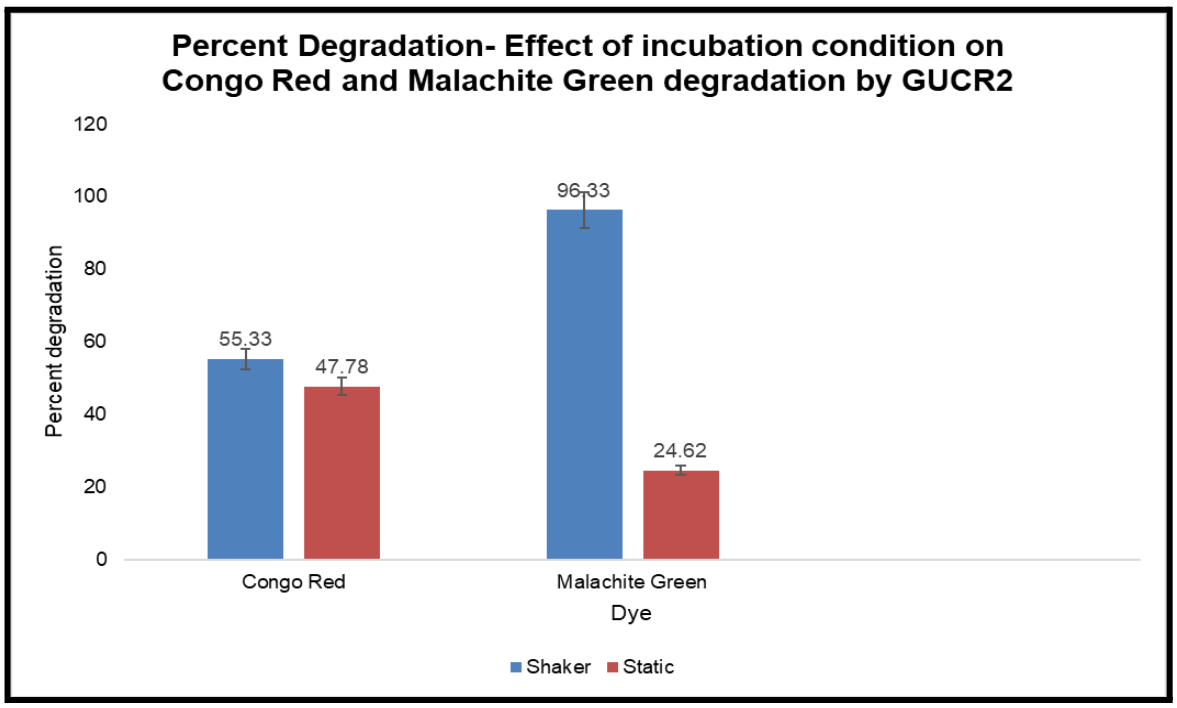



**Fig 4.3 (a)** Effect of incubation condition on Congo Red and Malachite Green by GUCR2

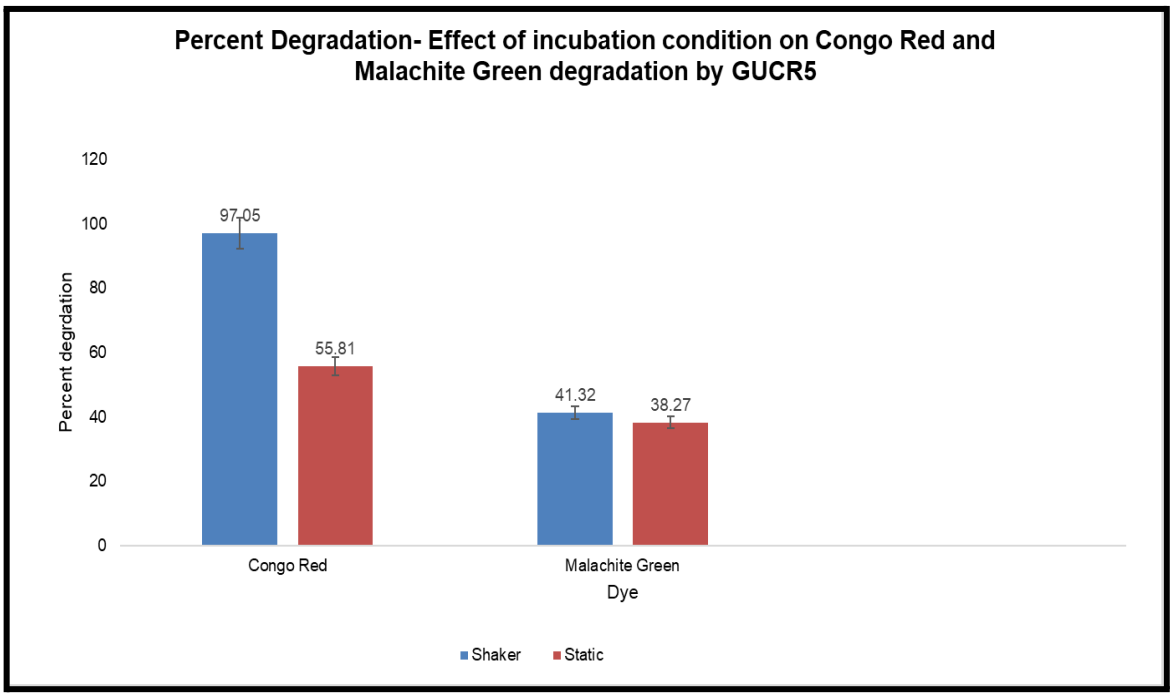



**Fig 4.3 (b)** Effect of incubation condition on Congo Red and Malachite Green by GUCR5

Rania Al-Tohamy et al. (2020), who discovered that the yeast strain SSA-1575 totally decolored Reactive Black 5 (RB5) in less than 24 hours when the number of revolutions was 200. However, only 13% of RB5 was damaged when the shaker was rotated at 110 rpm.

## 4.6.3. Optimised media condition for effective dye degradation

### 4.6.3.1. Spectrophotometric Analysis of Congo red dye degradation

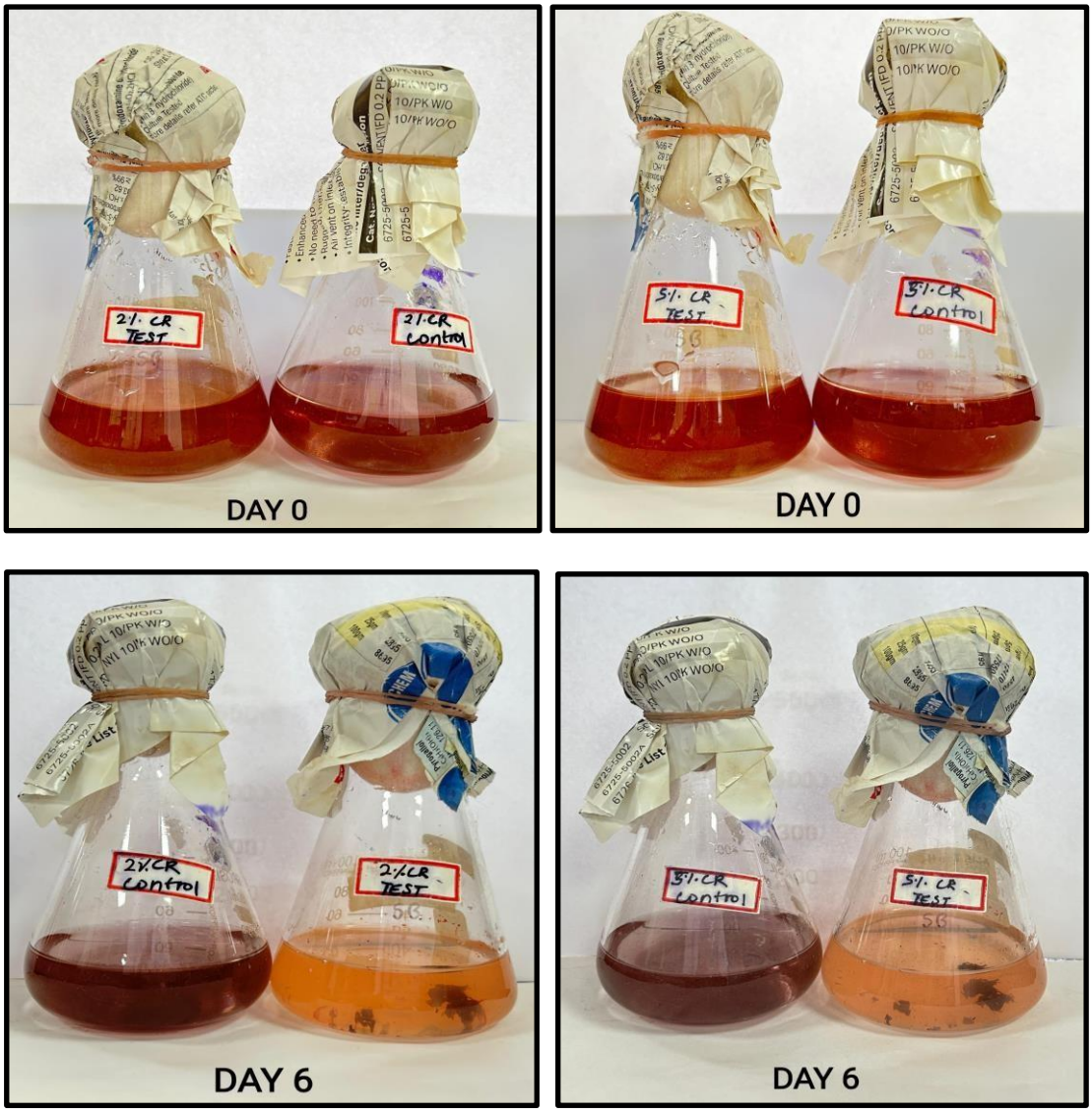



**Fig 4.9 :** Decolourisation of the commercial dye CR

In the present study, (**Fig 4.9**) GUCR2 and GUCR5 showed positive results for dye degradation. The highest percentage degradation was obtained to be 97.05% by GUCR5 followed by 93.19% by GUCR2 after 6 days of incubation. The study on dye degradation was done using graphical analysis. From the graph (**Graph 4.4a**) it is seen that the maximum degradation was observed on day 6 by GUCR5 with a decrease in absorbance value to 0.020 and a percentage degradation of 97.05%. There was a difference in the absorbance values between day 0 and day 6. Similarly, with GUCR2 there was a difference observed in the percentage degradation between day 4 to be 50.63% and day 6 to be 93.19% **(Graph 4.6)**.

### 4.6.3.2. Spectrophotometric Analysis of Malachite green dye degradation

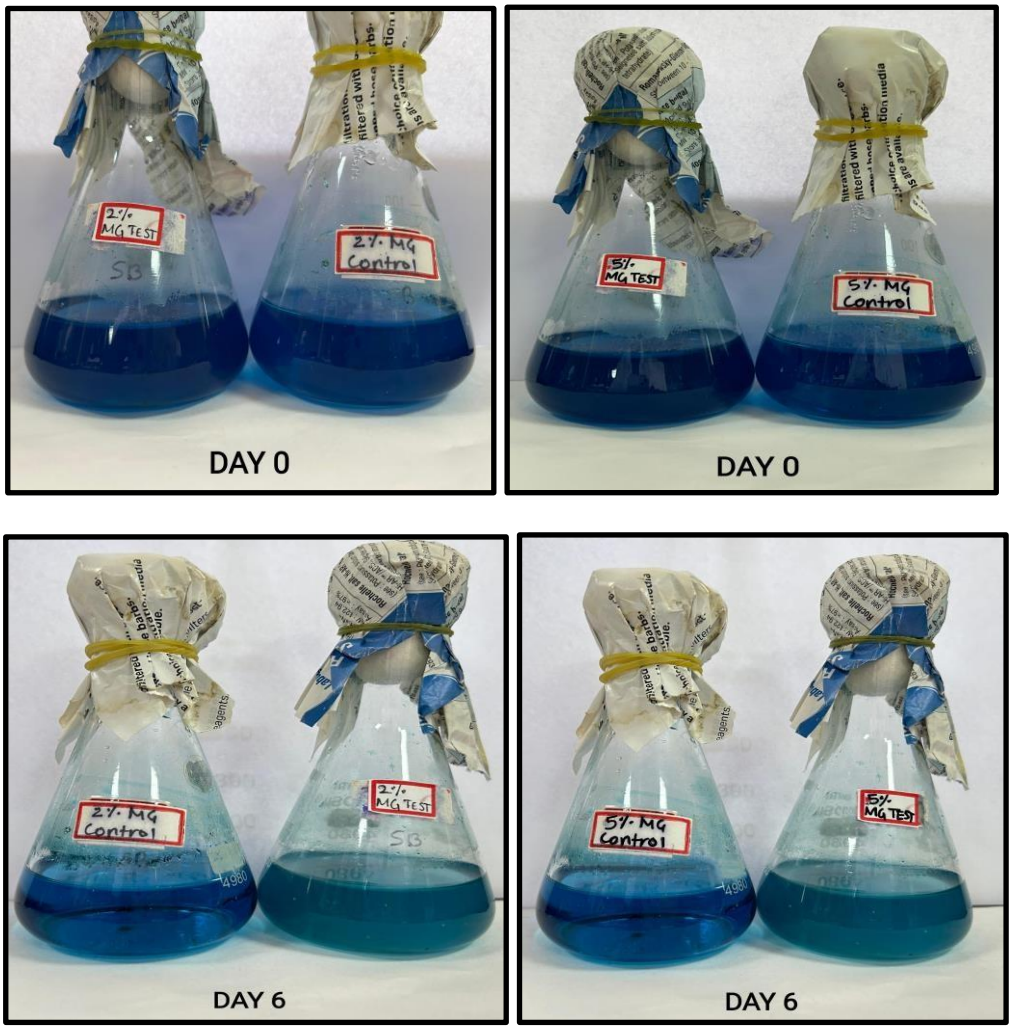



**Fig 4.10** : Decolourisation of commercial dye MG

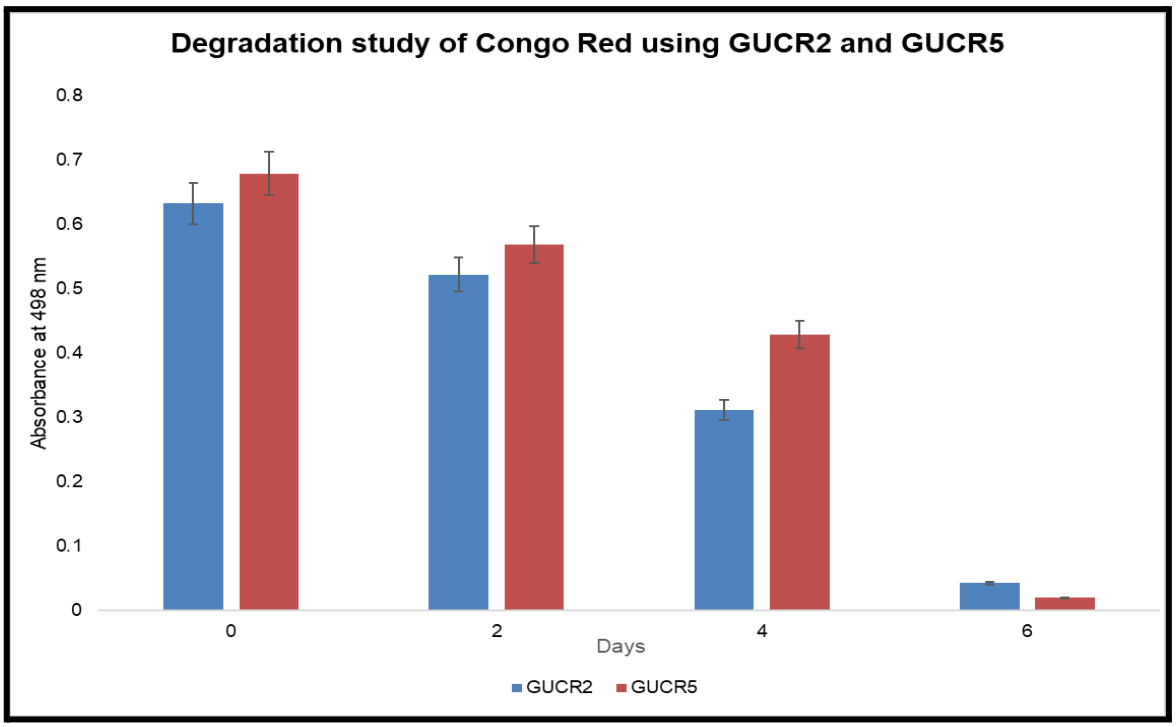



**Fig 4.4 (a)** : Percentage Degradation - Degradation of Congo Red

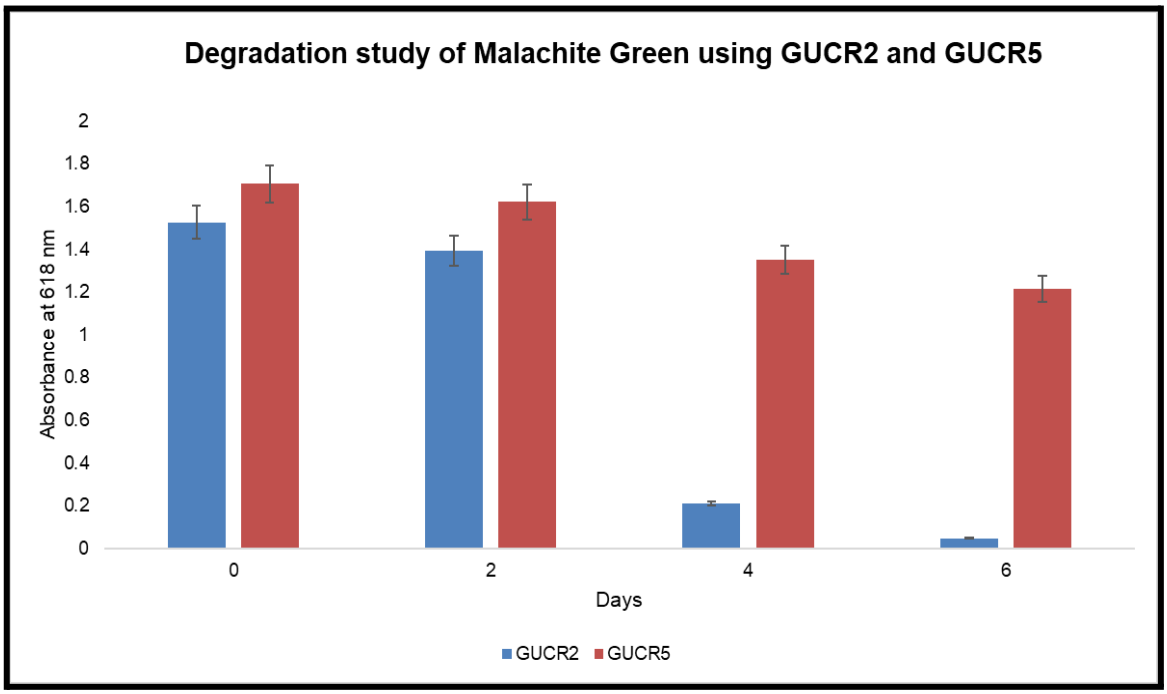



**Fig 4.4 (b)** : Percentage Degradation - Degradation of Malachite Green

GUCR2 showed the highest percentage of deterioration (96.92%) of MG after 6 days of incubation, whereas GUCR5 showed the lowest percentage of degradation (28.72%). Based on the graph (**Graph 4.4b**), it can be observed that GUCR2 showed the maximum degradation of MG on day 6, with a decrease in the absorbance level to 0.047 and a percentage degradation of 96.92%. Between day 0 and day 6, there was a change in absorbance values. The percentage deterioration in the flask with GUCR2 was found to fluctuate between days 4 and 6, coming in at 86.21% and 96.92% respectively (**Fig 4.10**) **(Graph 4.6)**.

## 4.6.3. Spectrophotometric Analysis of lab made wastewater degradation

The mixture of pollutants found in industrial effluent is highly variable and includes organic products, polymers, and inorganic chemicals and metals. The full degradation of dyes requires the employment of appropriate technologies in order to guarantee the safety of the effluents. Industrial wastewater is treated using both chemical and physical techniques. Since biodegradation poses few or no risks, it is an environmentally benign process. The deterioration of wastewater produced in a lab was done in this study (Palamthodi S. et al, 2011).

In the present work (**Fig 4.11**), the study on dye degradation potential of GUCR2 and GUCR5 was studied (**Graph 4.5**). From graph (**Graph 4.6)**, GUCR2 showed a maximum degradation percentage of 97.01% with an absorbance value of 0.056 at the end of 12 days of incubation, whereas GUCR5 showed 77.56% degradation with an absorbance value of 0.702 after 12 days of incubation. After 48 hours of incubation, GUCR2 showed a percentage degradation of 12.33% whereas GUCR5 showed only 8.92% degradation after 48 hours of incubation. Thus, it can be said that

GUCR2 was able to degrade the lab-made wastewater more efficiently.


**Fig 4.11 :** Decolourisation of lab made wastewater

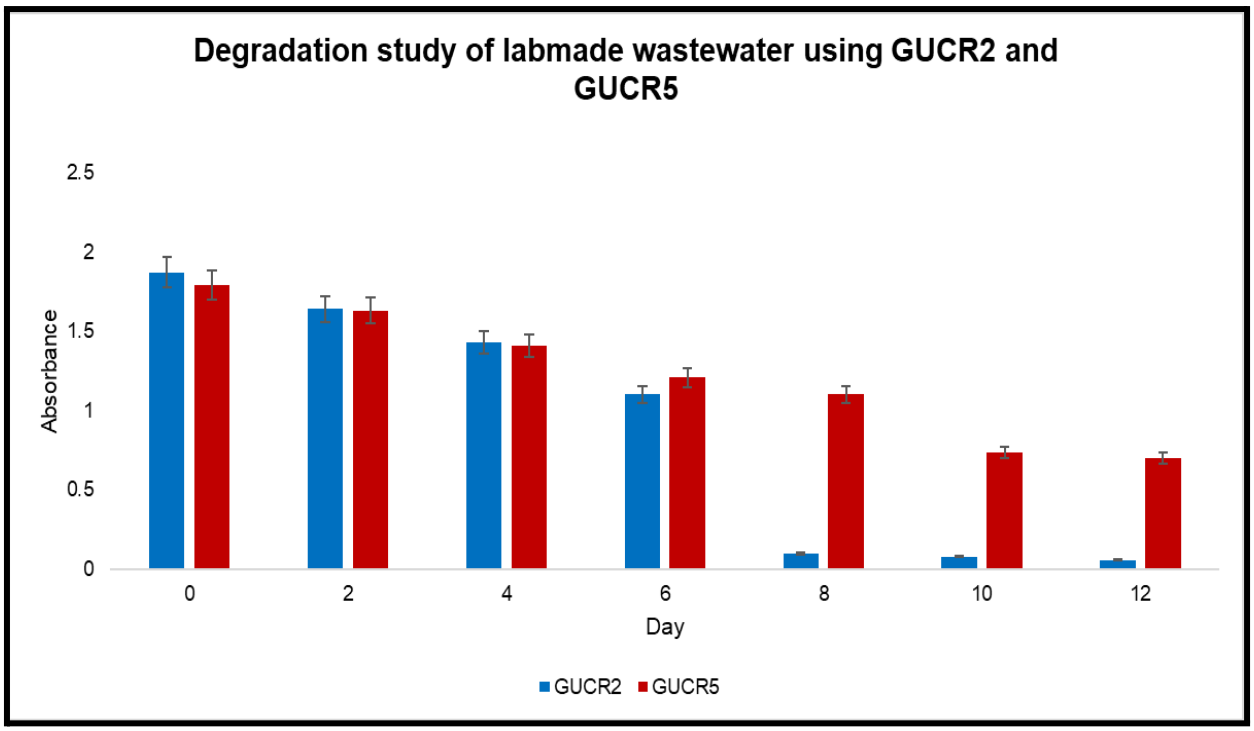



**Fig 4.5** : Percentage Degradation - Degradation of Lab Made wastewater

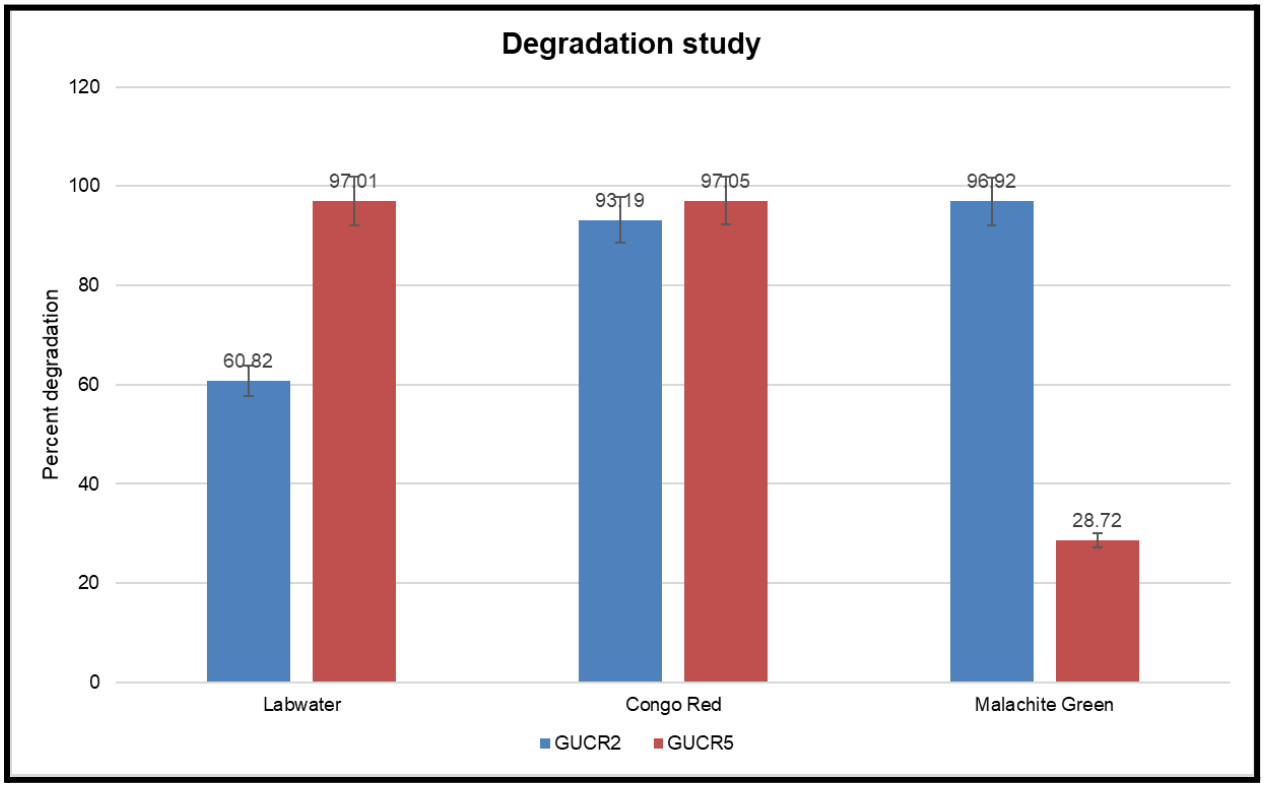



**Fig 4.6** : Percentage Degradation - Degradation of commercial dyes and lab made wastewater

From the study it could be concluded that the cultures isolated from the water sample using 2% and 5% NaCl concentration showed potential to degrade the azo dye contaminated saline water. It can also be explored for future study pertaining to enzyme study, toxicity study and germination assay.

The riverine sample in the current study had a pH that was almost neutral, a normal temperature, and a salinity that was not consumable, according to the physicochemical examination of the water. After serial dilution and spread plating, colonies were isolated based on their unique morphological characteristics and subsequently examined for their ability to degrade the dyes Malachite Green and Congo Red. The conclusion drawn from the data was that the bacterial isolates from the riverine sample had a decent chance of breaking down both the lab-made wastewater and commercial dyes. GUCR2 showed the greatest capacity to degrade the wastewater produced in the lab. Malachite Green was most effectively degraded by GUCR2, but GUCR5 degraded Congo Red to the greatest extent (97.05%), followed by GUCR2 (93.19%).

# SUMMARY

The rapid progression of industrialization and urbanisation around the world has escalated the detrimental effects of environmental pollution on public health. Although pollution generated by human activities are the most challenging, the effects of water pollution are constantly rising with the amount of wastewater generated. The textile industry accounts for the largest consumption of dyes, at nearly 80%, this study attempts to isolate bacterial strains from riverine source and exploit their potential to degrade such dyes. The water sample was collected from a riverine source from which the bacterial colonies were isolated using minimal broth with 2% and 5% NaCl concentration incorporated with the commercial dyes. Physicochemical testing was carried out of the water sample. These were followed by media enrichment, serial dilution, spread plating and isolation of morphologically distinct colonies. The samples were subjected to multiple biochemical tests such as indole, methyl red, Voges-Proskauer, citrate utilisation and sugars. Two commercially used dyes, Congo Red and Malachite Green were selected for the study on which dye degradation was tested. These dyes are widely used in the textile industries and laboratories which have numerous detrimental effects to human life and the environment as well. GUCR2 showed maximum degradation in Malachite Green and Congo red. The isolated colonies GUCR2 and GUCR5 were then used to test the degradation of lab-made wastewater. It was concluded that the GUCR2 showed maximum degradation of the lab-made wastewater.

# REFERENCES


1. Asad, S., Amoozegar, M. A., Pourbabaee, A. A., Sarbolouki, M. N., & Dastgheib, S. M. M. (2007). Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. Bioresource Technology, 98(11), 2082–2088. https://doi.org/10.1016/j.biortech.2006.08.020

2. Amoozegar, M. A., Hajighasemi, M., Hamedi, J., Asad, S., & Ventosa, A. (2010). Azo dye decolorization by halophilic and halotolerant microorganisms. Annals of Microbiology, 61(2), 217–230. https://doi.org/10.1007/s13213-010-0144-y

3. Abdeltawab, N. F., & Farag, M. A. (2022). Biodegradation, decolorization, and detoxification of Di-Azo Dye Direct Red 81 by halotolerant, Alkali-Thermo-Tolerant bacterial mixed cultures. Microorganisms, 10(5), 994. https://doi.org/10.3390/microorganisms10050994

4. Ayed, L., Cheriaa, J., Laadhari, N., Cheref, A., & Bakhrouf, A. (2009). Biodegradation of crystal violet by an isolatedBacillus sp. Annals of Microbiology, 59(2), 267–272. https://doi.org/10.1007/bf03178327.

5. Ariswati, H. G., & Titisari, D. (2020). Effect of temperature on ph meter based on arduino uno with internal calibration. Journal of Electronics, Electromedical Engineering, and Medical Informatics, 2(1), 23-27.

6. Aryal, S. (2016). Spread plate technique-principle, procedure and uses. Journal of Microbiology Information, 2(3), 121-124.

7. Al-Tohamy, R., Kenawy, E. R., Sun, J., & Ali, S. S. (2020). Performance of a newly isolated salt-tolerant yeast strain Sterigmatomyces halophilus SSA-1575 for azo dye decolorization and detoxification. Frontiers in Microbiology, 11, 1163.

8. Benkhaya, S., M'rabet, S., & Harfi, A. E. (2020). Classifications, properties, recent synthesis and applications of azo dyes. Heliyon, 6(1), e03271. https://doi.org/10.1016/j.heliyon.2020.e03271

9. Brugger, S. D., Baumberger, C., Jost, M., Jenni, W., Brugger, U., & Mühlemann, K. (2012). Automated counting of bacterial colony forming units on agar plates. PloS one, 7(3), e33695.

10. Chen, C., Chang, C., Ho, C., Tsai, T., & Liu, S. (2008b). Biodegradation of crystal violet by a Shewanella sp. NTOU1. Chemosphere, 72(11), 1712–1720. https://doi.org/10.1016/j.chemosphere.2008.04.069

11. Conneely, A., Smyth, F., & McMullan, G. (1999). A study of the microbial degradation of metal phthalocyanine textile dyes by high-performance liquid chromatography and atomic absorption. Journal of Porphyrins and Phthalocyanines, 03(07), 552–559. https://doi.org/10.1002/(sici)1099-1409(199908/10)3:6/7

12. Chiu, Y. H., Chang, T. F. M., Chen, C. Y., Sone, M., & Hsu, Y. J. (2019). Mechanistic insights into photodegradation of organic dyes using heterostructure photocatalysts. Catalysts, 9(5), 430.

13. Chen, Y., Feng, L., Li, H., Wang, Y., Chen, G., & Zhang, Q. (2018). Biodegradation and detoxification of Direct Black G textile dye by a newly isolated thermophilic microflora. Bioresource technology, 250, 650-657.

14. Guo, G., Li, X., Tian, F., Liu, T., Yang, F., Ding, K., et al. (2020). Azo dye decolorization by a halotolerant consortium under microaerophilic conditions. Chemosphere 244:125510. doi: 10.1016/j.chemosphere.2019.125510

15. Giovanella, P., Vieira, G., Ramos Otero, I. V., Pais Pellizzer, E., de Jesus Fontes, B., and Sette, L. D. (2020). Metal and organic pollutants bioremediation by extremophile microorganisms. J. Hazard. Mater. 382:121024. doi: 10.1016/j.jhazmat.2019.121024

16. Gurr, E. (2012). Synthetic dyes in biology, medicine and chemistry. Elsevier.

17. Gharbani, P., Tabatabaii, S. M., & Mehrizad, A. (2008). Removal of Congo red from textile wastewater by ozonation. International Journal of Environmental Science & Technology, 5, 495-500.

18. Henze, M., & Comeau, Y. (2008). Wastewater characterization. Biological wastewater treatment: Principles modelling and design, 33-52.

19. Hashimoto, J. C., Paschoal, J. a. R., De Queiroz, J. F., & Reyes, F. G. R. (2011). Considerations on the use of malachite green in aquaculture and analytical aspects of determining the residues in fish: a review. Journal of Aquatic Food Product Technology, 20(3), 273–294. https://doi.org/10.1080/10498850.2011.569643

20. Imran, M., Crowley, D. E., Khalid, A., Hussain, S., Mumtaz, M. W., & Arshad, M. (2015). Microbial biotechnology for decolorization of textile wastewaters. Reviews in Environmental Science and Bio/Technology, 14, 73-92.

21. Iqbal, H., Asgher, M., and Bhatti, H. N. (2011). Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of trametes versicolor. Bioresources 65, 103–107. doi: 10.1515/HF.2010.121

22. John, J., Dineshram, R., Hemalatha, K. R., Dhassiah, M. P., Dharani, G., & Kumar, A. (2020). Bio-Decolorization of Synthetic Dyes by a Halophilic Bacterium Salinivibrio sp. Frontiers in Microbiology, 11. https://doi.org/10.3389/fmicb.2020.594011

23. JCDR - Congo-red formaldehyde, Fungal morphology, Fungal stain, Iodine-glycerol, Lactophenol cotton blue. (n.d.). https://www.jcdr.net/article_fulltext.asp?issn=0973-709x&year=2014&month=July&volume=8&issue=7&page=DL01&id=4535#:~:text=Congo%20red%20is%20a%20diazo,of%20fungal%20elements%20in%20tissue.

24. Jadhav, U. U., Dawkar, V. V., Ghodake, G. S., & Govindwar, S. P. (2008). Biodegradation of Direct Red 5B, a textile dye by newly isolated Comamonas sp. UVS. Journal of Hazardous Materials, 158(2-3), 507-516.

25. Kolekar, Y. M., Nemade, H., Markad, V. L., Adav, S. S., Patole, M. S., & Kodam, K. M. (2012). Decolorization and biodegradation of azo dye, reactive blue 59 by aerobic granules. Bioresource Technology, 104, 818–822. https://doi.org/10.1016/j.biortech.2011.11.046

26. Kulasekaran, A., Gopal, A., Lakshimipathy, R., & Alexander, J. (2015). Modification in pH measurements for getting accurate pH values with different pH meters irrespective of aging and drifts in the meters. International Journal of ChemTech Research, 8(5), 16-24.

27. Khlifi, R., Belbahri, L., Woodward, S., Ellouz, M., Dhouib, A., Sayadi, S., & Mechichi, T. (2010). Decolourization and detoxification of textile industry wastewater by the laccase-mediator system. Journal of Hazardous Materials, 175(1-3), 802-808.

28. Mishra, A., Takkar, S., Joshi, N. C., Shukla, S., Shukla, K., Singh, A., Manikonda, A., & Varma, A. (2022). An Integrative Approach to Study Bacterial Enzymatic Degradation of Toxic Dyes. Frontiers in microbiology, 12, 802544. https://doi.org/10.3389/fmicb.2021.802544

29. Methylene blue staining. (n.d.). https://microbiologie-clinique.com/Methylene-Blue-Stain.html

30. Meehan, C., Bjourson, A. J., & McMullan, G. (2001). Paenibacillus azoreducens sp. nov., a synthetic azo dye decolorizing bacterium from industrial wastewater. International journal of systematic and evolutionary microbiology, 51(5), 1681-1685

31. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 11294, Malachite Green. Retrieved August 30, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Malachite-Green.

32. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 11313, Congo red. Retrieved December 14, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Congo-red.

33. Nayak, S., Gonsalves, V., & Nazareth, S. (2012). Isolation and salt tolerance of halophilic fungi from mangroves and solar salterns in Goa - India. ResearchGate. https://www.researchgate.net/publication/268300760_Isolation_and_salt_tolerance_of_halophilic_fungi_from_mangroves_and_solar_salterns_in_Goa_-_India

34. Oladoye, P. O., Bamigboye, O. M., Ogunbiyi, O. D., & Akano, M. T. (2022). Toxicity and decontamination strategies of Congo red dye. Groundwater for Sustainable Development, 19, 100844. https://doi.org/10.1016/j.gsd.2022.100844

35. Parshetti, G. K., Parshetti, S., Telke, A. A., Kalyani, D., Doong, R., & Govindwar, S. P. (2011). Biodegradation of Crystal Violet by Agrobacterium radiobacter. Journal of Environmental Sciences, 23(8), 1384–1393. https://doi.org/10.1016/s1001-0742(10)60547-5

36. Piaskowski, K., Świderska-Dąbrowska, R., & Zarzycki, P. K. (2018). Dye Removal from Water and Wastewater Using Various Physical, Chemical, and Biological Processes. Journal of AOAC INTERNATIONAL, 101(5), 1371–1384. https://doi.org/10.5740/jaoacint.18-0051

37. Qiu, H., Shen, F., Yin, A., Liu, J., Bi-Yu, W., Liu, Y., Xiao, Y., Jiang, H., & Xu, B. (2022). Biodegradation and detoxification of Azo dyes by Halophilic/Halotolerant microflora isolated from the salt fields of Tibet Autonomous Region China. Frontiers in Microbiology, 13. https://doi.org/10.3389/fmicb.2022.877151

38. Rahmanian, N., Ali, S. H. B., Homayoonfard, M., Ali, N. J., Rehan, M., Sadef, Y., & Nizami, A. S. (2015). Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia. Journal of chemistry, 2015, 1-10.

39. Roy, D. C., Biswas, S. K., Sheam, M. M., Hasan, R., Saha, A. K., Roy, A. K., Haque, M. E., Rahman, M., & Tang, S. (2020). Bioremediation of malachite green dye by two bacterial strains isolated from textile effluents. Current Research in Microbial Sciences, 1, 37–43. https://doi.org/10.1016/j.crmicr.2020.06.001

40. Roy, D. C., Biswas, S. K., Saha, A. K., Sikdar, B., Rahman, M., Roy, A. K., ... & Tang, S. S. (2018). Biodegradation of Crystal Violet dye by bacteria isolated from textile industry effluents. PeerJ, 6, e5015.

41. Shertate, R. S., & Thorat, P. R. (2015). Biodegradation and Detoxification of a Textile Azo Dye Reactive Blue 171 by Marinobacter sp. NB-8. Indian Journal of Geo-Marine Sciences, 44(10), 1604–1607. http://nopr.niscair.res.in/bitstream/123456789/34987/1/IJMS%2044%2810%29%2016 04-1607.pdf

42. Setiadi, T., Andriani, Y., & Erlania, M. (2006). Treatment of textile wastewater by a combination of anaerobic and aerobic processes: A denim processing plant case. Proceedings of the Southeast Asian Water Environment, 1.

43. Sandhya, S. (2010). Biodegradation of azo dyes under anaerobic condition: role of azoreductase. Biodegradation of azo dyes, 39-57.

44. Song, L., Shao, Y., Ning, S., and Tan, L. (2017). Performance of a newly isolated salt-tolerant yeast strain Pichia occidentalis G1 for degrading and detoxifying azo dyes. Bioresour. Technol. 233, 21–29. doi: 10.1016/j.biortech.2017.02.065

45. Sarkar, S., Echeverría-Vega, A., Banerjee, A., & Bandopadhyay, R. (2021). Decolourisation and Biodegradation of Textile Di-azo Dye Congo Red by Chryseobacterium geocarposphaerae DD3. Sustainability, 13(19), 10850. https://doi.org/10.3390/su131910850

46. Sathya, K., Nagarajan, K., Carlin Geor Malar, G., Rajalakshmi, S., & Raja Lakshmi, P. (2022). A comprehensive review on comparison among effluent treatment methods and modern methods of treatment of industrial wastewater effluent from different sources. Applied Water Science, 12(4), 70.

47. Sriram, N., & Reetha, D. (2015). Isolation and characterization of dye degrading bacteria from textile dye effluents. Central European Journal of Experimental Biology, 4(2), 5-10.

48. Siddiqui, S. I., Allehyani, E. S., Al-Harbi, S. A., Hasan, Z., Abomuti, M. A., Rajor, H. K., & Oh, S. (2023). Investigation of Congo red toxicity towards different living organisms: a review. Processes, 11(3), 807.

49. Tian, F., Yong-Bo, W., Guo, G. C., Ding, K., Yang, F., Wang, H., Cao, Y., & Liu, C. (2021). Enhanced azo dye biodegradation at high salinity by a halophilic bacterial consortium. Bioresource Technology, 326, 124749. https://doi.org/10.1016/j.biortech.2021.124749

50. Wang, Z. W., Liang, J. S., & Liang, Y. (2013). Decolorization of Reactive Black 5 by a newly isolated bacterium Bacillus sp. YZU1. International Biodeterioration & Biodegradation, 76, 41-48.

# APPENDIX I

## Preparation of Media

- **Nutrient agar**

  5g of peptone, 1.5g Beef extract. 1.5 Yeast extract, 5gNaCl

  15 g of Agar was dissolved in 900 ml of Distilled Water with constant stirring. The pH was adjusted to 7.4 ± 0.2 using Dilute HCl or NaOH. (Hi media)

- **Nutrient broth**

  Yeast extract 2g/L, Beef extract 1g/L, Peptone 5g/L, Sodium chloride 5g/L (Himedia)

- **Minimal Broth Davis without Dextrose**

  ammonium sulfate 1.0 g/L , dipotassium phosphate 7.0 g/L , magnesium sulfate 0.1 g/L , monopotassium phosphate 2.0 g/L , sodium citrate 0.5 g/L (Himedia)

- **Minimal Broth Davis without Dextrose Agar plates**

  ammonium sulfate 1.0 g/L , dipotassium phosphate 7.0 g/L , magnesium sulfate 0.1 g/L , monopotassium phosphate 2.0 g/L , sodium citrate 0.5 g/L, Agar 15g/L (Himedia)

Note: All the media to be sterilised by autoclaving At 121°C at 15 psi for 20 mins.

# APPENDIX II

## A) Reagents preparation

- **Saline**

  0.85g NaCl was dissolved in 100mL of Distilled Water.

- **Dextrose**

  10ml sterile dextrose in 100 ml distilled water

## B) Reagents used

- **Gram's staining**

  Gram's crystal violet (primary stain), Gram's iodine, Gram's decolorizer, Safranin.