Isolation and identification of dye degrading bacteria from textile sludge to eradicate environmental hazards and maintain a sustainable environment

Submitted by

Afia Kamal

13336012

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the Bachelor of Science in Biotechnology

Department of Mathematics and Natural Sciences

BRAC University

October 2022

Declaration

I hereby declare that,

1. The thesis submitted by me is my original work on account of completing my Undergraduate degree.
2. The thesis does not contain any information or work published or written by any third party, except where it is appropriately cited through accurate reference.
3. The thesis does not contain material that has been accepted or published for any other degree or diploma at any other university or institute.
4. I have acknowledged all the main sources of help

Student's Full Name and Signature:

Afia Kamal

13336012

Approval

The thesis **"Isolation and identification of dye degrading bacteria from textile sludge to eradicate environmental hazards and maintain a sustainable environment**" submitted by Afia Kamal (13336012) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of B.Sc. In Biotechnology in November 2021.

Examining Committee-

Supervisor

Senior Lecturer, Ms. Romana Siddique
Department of Mathematics and Natural Sciences,
BRAC University

Departmental Head (Chair)

Professor and Chairperson, F M Yusuf Haider
Department of Mathematics and Natural Sciences,
BRAC University

Ethic Statement

1) This material is the author’s own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

Abstract

Bangladesh's textile industries contribute to the country's enormous wealth and prosperity, but they are also silently destroying the country's water bodies and surrounding areas. Textile dye wastewater is brightly colored and contains a wide range of chemicals, toxins, and heavy metals. Under the Bangladesh Environment Conservation Act, 1995, and the Environment Conservation Rules, 1997, the government designated textile dyeing industries as "red industries" (the most polluting) and made Effluent Treatment Plants (ETPs) mandatory for the factories. However, the majority of them lack ETP because chemical and physical methods require constant monitoring and are extremely costly to operate. Industry is budding, and so is pollution. Microbial bioremediation offers an easy and affordable solution to this problem and helps to maintain a sustainable environment. Keeping the above situation in mind, research on isolating a dye-degrading bacterium is preferred. In this study, one decolorizing bacterial isolate has been isolated from industrial textile sludge with the ability to decolorize 1% of commercially available Reactive Violet 5R (RV5R) dye. About 91.16% of RV5R dye was decolorized after 48 hours of incubation at 37^0C and 80 rpm. The absorbance was measured at 510 nm for the supernatant with the help of a spectrophotometer. The whole cell culture and the supernatant of the culture as an inoculate showed the same result. The study was also conducted under aerobic conditions without using any co-substrate and using the Reactive Violet 5R (RV5R) dye as the only carbon and energy source for the bacterial isolate. The cultural, morphological, and physiological characteristics of that bacteria isolate have been detected. Through analyzation with ABIS Microbiology software, the isolate is identified as either *Actinobacillus rossi* (85.9% similarity) or *Acromonas salmonicida subsp. smithia* (82.2% similarity). For further molecular identification, the DNA was extracted, and PCR amplification of the 16S rRNA gene was conducted. The gel electrophoresis was performed to check whether the band was present or not. The future goal is to identify the bacterium isolate at the genomic level and identify the gene or genes that synthesize enzymes capable of decolorizing textile dyes. If everything goes as planned, this bacterium will undoubtedly contribute to the creation of a sustainable environment for our country.

Dedicated to

Everyone who helped me complete my thesis

Acknowledgement

I am grateful to Allah for my good health and well-being, which enabled me to finish this thesis. My bachelor's thesis would not have been completed without the continual blessings of Almighty in every stage of my life.

I would like to thank my father for everything and absolutely everything, as well as my siblings for their unconditional support and words of encouragement during the most challenging times.

First and for most my deepest regards, gratitude and appreciation goes to my research supervisor, Ms. Romana Siddique, Senior lecturer, Biotechnology Program, Department of Mathematics and Natural Sciences, BRAC University. Without her supervision, collaboration, and excellent ideas, the work would not have been possible to complete. I appreciate how she went beyond to make this day happen. I thank her from the bottom of my heart for being patient with me. For everything she has done and will continue to do for me, I will be eternally grateful.

I would like to thank and express my heartiest gratitude to Professor A F M Yusuf Haider, Ph.D., Professor and Chairperson of Department of Mathematics and Natural Sciences, BRAC University and Iftekhar Bin Naser, Ph.D., Assistant Professor of Department of Mathematics and Natural Sciences, BRAC University for his active cooperation and encouragement.

I would like to be grateful to those helping hands around me throughout the research. I take this opportunity to express gratitude to all of the Department faculty members for their help and support.

Afia Kamal

October 2022

Table of contents

Table of Contents

List of figures

List of tables

List of abbreviations

ETPs	Effluent Treatment Plants
RV5R	Reactive Violet 5R
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
RMG	Readymade Garments
DoE	Department of Environment
SM	Salt Media
NB	Nutrient Broth
NA	Nutrient Agar
LB	Luria Broth
T_1N_1	Tryptone Salt Agar
gm	Gram
ml	Milliliter
rpm	Rotation Per Minute
TSI	Triple Sugar–Iron
MIU	Motility Indole Urea
MR	Methyl Red
VP	Voges-Proskauer
OD	Optical Density
nm	Nanometer
PCR	Polymerase Chain Reaction

Chapter One: Introduction and Background Knowledge

1.1 Introduction:

Bangladesh's textile industries contribute significantly to the country's wealth and prosperity, but they are also silently destroying the country's water bodies and environment. Textile dye wastewater is a complex mixture of chemicals, toxic compounds, and heavy metals. The government classifies textile dyeing industries as "Red industries" (the most polluting) under the Bangladesh Environment Conservation Act, 1995, and the Environment Conservation Rules, 1997, and has made Effluent Treatment Plants (ETPs) mandatory for the factories. Yet, the majority of them lack an ETP because chemical and physical methods require constant monitoring and are extremely costly to operate. Industry is budding, so is pollution. Microbial bioremediation provides an easy and affordable solution to this problem, as well as aids in the preservation of a sustainable environment. In light of the foregoing, research into isolating a dye-degrading bacterium has been opted.

Textile manufacturing uses over 10,000 different dyes. Azo dyes, which make up 60 to 70% of all dyes in the industry, have a complex structure and are synthetic in nature. They created high-intensity hues. However, when broken down and metabolized, they have been found to exhibit carcinogenic evidence upon reductive cleavage. Even if the color of our clothes has nothing to do with cancer, azo and other chemicals do not dissolve, but rather evaporate into the air we breathe or are absorbed through our skin. These dyes have the potential to alter the physical and chemical properties of soil, degrade water bodies, and harm the environment's flora and fauna. It was also discovered that the toxic nature of dyes kills soil microorganisms, lowering agricultural productivity (Mohamed A. Hassaan, 2017).

The textile industry's environmental impact calls for the development of novel and effective technologies to reduce the presence of dyes in wastewaters before they are discharged into the environment. To treat textile effluent, a variety of biological, chemical, and physical methods have been used. The existing physical, chemical, and photochemical approaches for treating such dyeing effluents have drawbacks such as excessive costs, operational and technical difficulties, and the production of large amounts of sludge as well as toxic substances. To overcome these drawbacks, biological approaches such as biodegradation and biosorption by both live and dead microbial biomass in aerobic, anaerobic, or combined treatment processes with a variety of bacteria, fungi,

yeasts, and algae have been investigated over the last few decades (Robinson T, 2001). Azo dyes are typically regarded as xenobiotic chemicals that are resistant to biodegradation. Nonetheless, some bacteria have been shown in recent years to be capable of converting azo dyes to non-colored compounds or even fully mineralizing them under environmental circumstances. Moreover, it has received increasing interest due to its high effectiveness, low sludge production, and environmental friendliness (K. Chen, 2003).

1.2 Azo Dye:

Dyes are natural and synthetic compounds that color products and make the world more beautiful, but they are also considered pollutants in some water resources. Dyes are used to give substances, particularly fabrics, their colors. These dyes are colored by chromophores, which are light-absorbing functional groups. Azole, nitro, and carbonyl groups are the most common chromophores. Auxochromes, which are functional groups that increase the color intensity, are also important components of dyes. Hydroxyl, amino, sulfonate, and carboxylate groups are the most common chromophores. The chromophore of azo dyes is a nitrogen-to-nitrogen double bond. These dyes are created by taking a diazonium salt and adding it to a strongly activated aromatic system (Aljamali, 2015).

The removal of dyes is one of the most difficult aspects of textile wastewater treatment. The majority of the more than 10,000 dyes used in textile processing are Azo compounds, which are molecules with one or more Azo (N=N) bridges connecting substituted aromatic structures. Discharge of Azo dyes is undesirable not only because many Azo dyes and their breakdown products are toxic to aquatic life and mutagenic to humans, but also because many Azo dyes and their breakdown products are toxic to aquatic life and mutagenic to humans. The first reaction in the biological metabolism of Azo dyes is reductive cleavage of the Azo bond, which results in the formation of aromatic amines. Several mixed and pure bacterial cultures have been reported to decolorize Azo dye in anaerobic/anoxic conditions. This reaction is nonspecific in terms of organisms and dyes under these conditions. Direct enzymatic, indirect (mediated), and chemical Azo dye reduction are among the mechanisms used. Only a few aerobic bacterial strains capable of growing on Azo dyes have been isolated. These organisms have a limited range of substrates. Aromatic amine degradation is influenced by their chemical structure and environmental factors.

Simple aromatic amines can now be mineralized under methanogenic conditions, according to new research. Sulfonated aromatic amines, on the other hand, are resistant to mineralization and require specialized aerobic microbial consortiums. (Waleed Mohammed Sheet Alabdraba, 2014). Azo dyes are classified into two types based on their hydrophobicity: (i) hydrophobic azo dyes that are taken up by the bacterial cell and reduced within the cell, and (ii) hydrophilic dyes that are reduced outside the bacterial cell. Various azo dyes are also widely available for commercial use. Because azo dyes are non-fluorescent, fluorescent probes are used to track the pathway attached to the azo dye by the alkyl bond. Textile azo dyes contain synthetic dye, reactive dye, acid dye, sulfur dye, basic dye, oxidation dye, anthraquinone dye, acridine dye, and a variety of other colorants. The primary reasons for the use of different types of azo dyes in the dying process are their various usage purposes, namely cellulosic fiber, protein fiber, and synthetic fiber. Because not all dyes are fixed to the fiber during the dying process, some of the unfixed dyes are released into the environment via effluent, causing pollution. (Shrabana Sarkar, 2017).

1.3 Reactive Violate 5R:

One of the azo dyes is the Reactive Violet 5R with the chemical name trisodium; (3Z)-5-acetamido-3-[[2- hydroxy-4-(2-sulfonatooxyethylsulfonyl) phenyl] hydrazinylidene]-4-oxo-4a,8a- dihydronaphthalene-2,7-disulfonate; copper with the molecular formula, C20H16N3Na3O15S4. Reactive Violate 5R is a polar, water-soluble azo dye, with N11 being the most active site involved in the degradation process. It has hydrophobic (phenyl rings) and hydrophilic (hydroxyl groups) parts in its chemical structure. (Hayat Anouar, 2014)

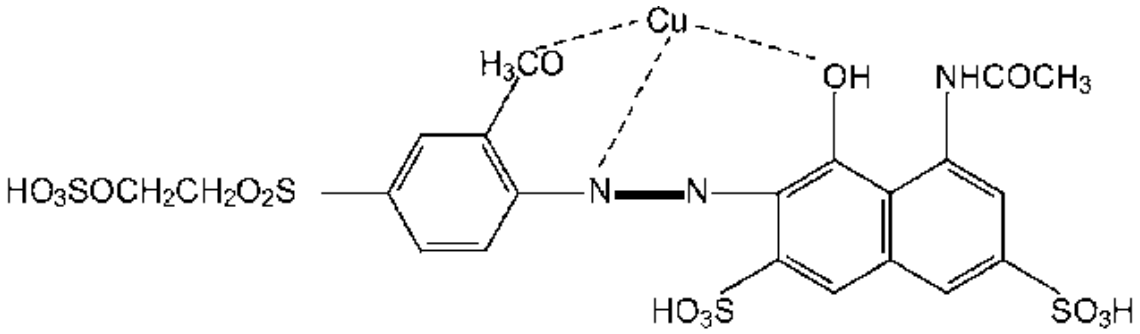

Figure 1: structure of azo dye Reactive Violet 5R (RV5R)

1.4 Effluent treatment plant in textile industry:

Due to its high consumption of water resources and treatment of individual or combined effluents for no environmental pollution generation, the textile sector will continue to be vitally important in water conservation (i.e., polluting colorants). Critical analyses of industrial effluents (total wastewater and raw reusable stream characterization) and removal of all pollutants from final effluents are required to meet both discharge criteria for sewerage systems, watercourses, and textile reuse standards within economically viable limits. Textile organic dyes are a special category of organic pollutants that must adhere to strict limits in final effluents discharged or not in natural water resources. This fact necessitates the removal of color and/or dye from final effluents (especially industrial effluents). Dye removal from textile effluents in controlled conditions and with strict reproducibility is a problem that can be solved by using appropriate mechano-physico-chemical and biological treatment procedures. (Zaharia Carmen, 2012).

In the below figure, difference methods that use in textile industry effluent treatment are showed. This figure is collected from (Shrabana Sarkar, 2017).

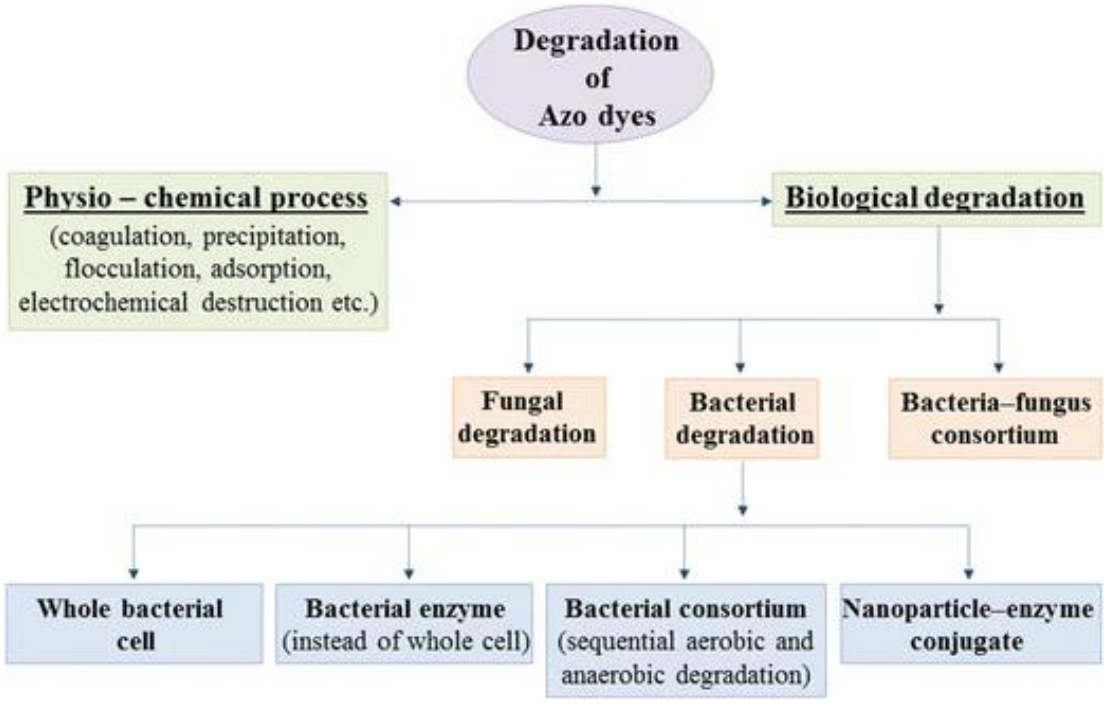

Figure 2: Different possible methods of synthetic azo dye degradation

All physical and chemical methods are expensive and produce enormous amounts of sludge, which pollutes the environment. As a result, the cost-effective and safe removal of polluting dyes remains a priority. The use of microorganisms in bioremediation has been identified as a cost-effective and environmentally friendly method of disposing of textile effluent. A number of studies have recently focused on microorganisms that are capable of degrading and absorbing dyes from wastewater. Decolonization of dyes is said to be possible with a wide range of microorganisms.

1.5 Biological treatment:

Biological treatments are defined as the artificial or natural reproduction of self-purification phenomena found in nature. Biological treatments can be conducted in aerobic, anaerobic, or combined anaerobic/aerobic conditions. In large-scale textile effluent treatment, biological treatment with "activated sludge" was the most common. Most azo dye metabolites are rapidly degraded in aerobic conditions due to oxidation of the substituents or side branches. The main microorganisms contributing to biodegradation of organic compounds are bacteria (e.g. Bacillus subtilis, Aeromonas Hydrophilia, Bacillus cetreus, Klebsiella pneunomoniae, Acetobacter liquefaciens, Pseudomonas species, Pagmentiphaga kullae, Sphingomonas, etc.), fungi (e.g., white-rot fungi: Phanerochaete chrysosporium, Hirschioporus larincinus, Inonotus hispidus, Phlebia tremellosa, Coriolus versicolor, etc.), algae (e.g. Chlorella and oscillotoria species) etc (Zaharia Carmen, 2012).

There are several claims in the literature that bacteria that can aerobically reduce azo dyes in a cometabolic fashion can also use these dyes as their sole source of carbon and energy. Aerobic reductive metabolism of azo dyes necessitates the use of specific enzymes known as "aerobic azoreductases," which catalyze these reactions in the presence of molecular oxygen. (Waleed Mohammed Sheet Alabdraba, 2014) The reduction of the azo dye is usually non-specific and decolorization is faster during the bacterial degradation process. A wide range of aerobic and anaerobic bacteria such as Pseudomonas sp., Bacilus subtilis, Geobacillus sp., Escherichia coli, Rhabdobacter sp., Enterococcus sp., Staphylococcus sp., Corneybaterium sp., Lactobacillus sp., Xenophilus sp., Clostridium sp., Acinetobacter sp., Micrococcus sp., Dermacoccus sp., Rhizobium sp., Proteus sp., Morganella sp., Aeromonas sp., Alcaligenes sp., Klebsiellla sp., Shewanella sp., and Alishewanella sp. have been extensively reported for resulting good biodegradation of azo dyes (Shrabana Sarkar, 2017)

1.6 Significance to the field:

Textiles that emit colored effluents can contain toxic materials such as reactive dyes, synthetic azo dyes, and a variety of other hazardous chemicals. By changing the pH, this causes water pollution, a loss of environmental balance, and an increase in the chemical oxygen demand (COD) and biological oxygen demand (BOD) of water. It also changes the organic-inorganic chemical composition of the water. Because of the low light penetration and oxygen consumption, this colored waste has a severe toxic effect on the aquatic ecosystem. As a result, because every ecosystem is interconnected, the biosphere's overall integrity is jeopardized, with unpredictable consequences for human health. (Shrabana Sarkar, 2017)

Although, according to recent research, nanoparticle-microbial enzyme conjugates are also highly effective at removing azo dye from textile waste in a matter of minutes. However, due to some gaps between academia and industry, these methods are currently limited to the laboratory, and their industrialization remains a challenge (Shrabana Sarkar, 2017)

Thus, the development of any eco-friendly and cost-effective method that may address the drawbacks of physical or chemical methods of dye removal is a recent global priority. Microbial techniques for textile dye degradation will be environmentally sustainable and more lucrative than physio-chemical processes.

1.7 Objective:

- The present study was focused on the isolation and identification of potent bacterial isolates from textile dying effluents.
- The study was conducted to provide substantial evidence of the efficiency of the bacteria isolate to decolorize the dye azo dye reactive violet 5R.
- This will eventually lead to the treatment of waste effluents along with the elimination of a dye’s carcinogenic properties and the utilization of the isolate to mitigate environmental hazards and maintain a sustainable environment.

Chapter Two: Literature Review

2.1 Studies on Bacteria strains capable of azo dye decolorization

Many microorganisms capable of dye decolorization at lab scale have been discovered in the past few decades, but there are few reports on their applicability in treatment processes. Attempts to establish a bacterial culture capable of degrading azo dyes began in the 1970s with claims of a Bacillus subtilis strain capable of doing so (Hiroyuki Horitsu, 1977). *Bacillus sp., Alcaligenes sp.,* and *Aeromonas sp.* bacterial isolates from soil and sludge samples have been shown to have high decolorization capacity (Deepak Kumar Sharma, 2004). Kodam et al. reported that an unidentified bacterium, KMK 48, completely decolorized the sulfonated azo dyes Reactive Red 2, Reactive Red 141, Reactive Orange 4, Reactive Orange 7, and Reactive Violet 5. Under strict aerobic conditions, the best decolorization occurred within 36 hours of incubation at room temperature and neutral pH (K.M. Kodam, 2005). A bacterial consortium (consortium GR) comprised of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168 was discovered to rapidly decolorize and degrade the widely used sulfonated reactive dye Green HE4BD as well as many other reactive dyes. Consortium GR has significantly stronger decolorization activity than the individual strains. Consortium GR also demonstrated substantial biodegradation and decolorization capabilities for a combination of reactive dyes as well as wastewater from the dye production sector. This opened up the prospect of using consortium GR for the treatment of dye-polluting industrial wastes (Govindwar, 2008).

Klebsiella oxytoca and *Bacillus subtillis* showed an excellent potential to decolorize Reactive Black in 6 hours. (Maulin P Shah, 2013). A bacterial consortium BMP1/SDSC/01 consisted of six bacterial isolates (*Bacillus cereus, Bacillus mycoides, Bacillus subtilis, Bacillus sp. Micrococcus sp. Pseudomonus sp.)* exhibited good decolorization, degradation and detoxification of red, green, black, and yellow dyes and mixture of all dyes within 24 hours (Rashid Mahmood, 2014). In another study, a bacterial consortium consisting of *Providencia rettgeri* strain HSL1 and *Pseudomonas sp.* SUK1 has been investigated, and it showed 98-99 % decolorization and detoxification of azo dyes viz. Reactive Black 5 (RB 5), Reactive Orange 16 (RO 16), Disperse Red 78 (DR 78) and Direct Red 81 (DR 81) within 12 to 30 hours (Harshad Lade, 2015). Another report showed that *Staphylococcus spp*. Has potential to decolorize malachite green within 9 hours (Saravanan, 2015). Also, 11 consortia developed using combination of three to five of *Bacillus*

cereus, Bacillus subtilis, Pseudomonus fluorescence, Pseudomonus aeruginosa, E. coli showed capability to decolorize and degrade textile dye effluent (N. Sriram, 2015).

According to M.E. Karim et al. (2018), the developed bacterial consortium was much more effective than monocultures in decolorizing single dyes as well as mixtures of dyes, indicating the potential of mixed microbial consortiums as a potent bioremediation agent for the cost-effective removal of diverse dyes from dying. They also stated that the dyes were not used as the only source of energy by the bacteria; instead, they required adequate co-substrates, such as glucose and yeast extract, to induce dye decolorization and related metabolism. Novacron Orange FN-R, Novacron Brilliant Blue FN-R, Novacron Super Black G, Bezema Yellow S8-G, and Bezema Red S2-B were all commercially available reactive dyes utilized in this research. The isolated bacteria were identified as *Neisseria sp., Vibrio sp., Bacillus sp., Bacillus sp., and Aeromonas sp.* The results indicated that decolorization percentages in monoculture vary from no apparent decolorization (Bezema Red S2-B by Ek-5) to 90% decolorization (Novacron Brilliant Blue FN-R by Ek-13), whereas decolorization percentages in bacterial consortium vary from 65% (Bezema Yellow S8-G) to 90% (Novacron Brilliant Blue FN-R by Ek-13) (Novacron Brilliant Blue FN-R and Novacron Super Black G). The paper describes the cosubstrates-mediated decolorization process, in which a bacterial consortium outperformed monocultures as an effective dye decolorizer (Md. Ekramul Karim, 2018).

2.2 Textile industry and their effluent treatment in Bangladesh

In recent years, the textile industry, driven by the readymade garments (RMG) sector, has emerged as Bangladesh's most important economic sector. RMG exports totaled USD24.6 billion in 2014, accounting for 80.9 percent of total export revenues and 14.2 percent of GDP, with an annual growth rate of 12 percent from 2010 to 2014.The increasing trend is expected to continue, with an ambitious export target of $50 billion in 2021 and $66.25 billion by 2030 (Restiani, 2017). In 2019, there were around 4.62 thousand garment industries in Bangladesh. Despite an increase over the previous year, there was a decrease from 2013, when Bangladesh had over 5.88 thousand garment manufacturers. (Stipp, 2021).

From an article published in 2017 by A S M Tareq Amin, setting up ETP is a need for receiving DoE authorization for factory setting. The Department of Environment (DoE) is striving to impose

Effluent Treatment Plants (ETPs) in the country's dye houses. According to the Director General of the DoE, 52 percent of Bangladesh's current industries have created ETP, while the remaining 48 percent have not [Bangladesh Textile Today, July-August 2011]. Smaller industries, notably those focused on the local market, cannot afford to install a competent ETP. Many of these industries discharge straight into water bodies. However, the factories that have ETPs are not all running them 24 hours a day, and many of them are not working effectively. Many of those ETPs are being retained to show them to prospective buyers and to keep them secure from DoE inspection. There is, without a doubt, another side to the coin. Many industries (mostly medium-to-large size) have state-of-the-art ETPs that are always in use. Top management at such factories is concerned about environmental protection and complies with DoE standards. Frequently, they discharge products of higher quality than the law demands. In many of these situations, the efficiency of their ETP may simply be enhanced to save money (Amin, 2017).

2.3 Hazardous Effect of Textile Dye Effluents on the Environment

Bangladesh's textile and apparel industries are extremely significant, providing enormous prospects for the country's economy. However, uncontrolled and haphazard clustering industrial expansion has grave environmental consequences. A material balancing technique was established in the research to describe the trajectory of environmental consequences connected with Bangladesh's textile dying industry (2011–2021). Textile companies in Bangladesh produced around 1.80 million metric tons of fabric in 2016, resulting in approximately 217 million m3 of effluent including a variety of contaminants. If the textile industry continues to use traditional dyeing processes, wastewater output is expected to exceed 349 million m3 by 2021 (Laila Hossain, 2018).

The usage of water, its treatment, and the disposal of effluent are all major environmental concerns involved with textile manufacturing. At all phases of production, dyes contribute to total toxicity. Dye baths may also include heavy metals and have significant levels of BOD, COD, color, toxicity, surfactants, fibers, and turbidity. They account for a small proportion of total liquid effluent, but they can contribute a significant number of total pollutants. It is important to remember that textile effluents are highly colored and salty, include non-biodegradable chemicals, and have a high biochemical and chemical oxygen demand (BOD and COD). It has been observed that the presence

of metals and other coloring chemicals inhibits microbial activity and, in certain situations, causes the biological treatment system to fail. Suspended particles, BOD, COD, nitrogen, phosphate, temperature, hazardous compounds (phenol), chromium and other heavy metals, pH-value, alkalinity, acidity, oils and grease, sulfide, and coliform bacteria are all pollution criteria in textile effluents, according to the USEPA. Bangladesh's Department of Environment has backed these efforts and demanded that they be closely monitored in the country's textile effluents. Textile mill wastewater effluents include significant levels of harmful contaminants, with heavy metals being particularly prevalent. The majority of textile industry effluents are discharged into rivers untreated; as a result, a significant amount of accessible water is polluted by textile effluents, and water-borne diseases cause nearly two-thirds of infections in Bangladesh. The textile mill, in fact, represents a diverse range of industries, with operations and processes as varied as its products. Industrial pollution is one of the problems that Bangladesh is currently facing, and several efforts are being made to control it in various industries so that people can live in a disease-free environment. Textile industry effluent is one of the major sources of pollution, and increasingly strict effluent discharge permit limitations have been implemented (Shuchismita Dey, 2015).

Chapter Three: Materials and Methods

3.1 Place of Research:

The research of isolation, characterization of dye-degrading bacterial isolates, decolorization potential of bacterial isolates, and identification of that bacteria was carried out at the Microbiology Research Laboratory under the Department of Mathematics and Natural Sciences, BRAC University.

3.2 Sample Collection:

The sludge sample was collected in a sterile plastic bag from the soil contaminated by untreated textile wastewater from a small-scale textile industry in the Turag riverbed near Tongi. After collection, this dye-contaminated sludge was preserved at 40°C in a refrigerator, and the sample was used within 24 hours of collection to isolate the dye-degrading bacteria.

Figure 3: Untreated textile wastewater outlet in Turag riverbed near Tongi; from where the sludge sample was collected to carry out the research.

3.3 Dyes:

At first, six different industrial Azo dyes were used. The dyes were commercially available as Asudel Blue BR, Asudel Red GN2, Asudel Yellow G2, Reactive Violet 5R, Reactive Red 3BX

and Reactive Yellow 4GL. Stock solutions (1%) of these dyes were prepared by dissolving 0.1g of each dye into 10ml or 1g of each dye into 100ml of distilled water, then filled and stored in bottles at room temperature.

3.4 Media:

SM (salt media) Broth, Nutrient Broth (NB), Nutrient Agar (NA), Luria Broth (LB) media were used throughout the whole research. Also, T_1N_1 (Tryptone Salt Agar) media was used for stock culture.

3.4.1 SM (salt media) Broth:

The whole decolorization study was carried out using the SM broth. The medium consists of -

- KH_2PO_4 -1.9gm/L,
- K_2HPO_4 -0.6gm/L,
- NH_4Cl -1gm/L,
- Peptone -10gm/L,
- and Yeast Extract - 1gm/L.

The pH of the medium was adjusted to 6.8–6.4.

3.4.2 T_1N_1 Media:

T_1N_1 (Tryptone Salt Agar) media composition for 100 ml –

- Tryptone – 1 gm
- NaCl – 1 gm
- Agar – 0.6 gm - 0.75 gm

Three ml of T_1N_1 media were prepared into sterile vials. The media was inoculated with the final dye-degrading bacterial isolate, which showed the best result and was kept at room temperature for preservation.

3.5 Enrichment and Isolation of dye degrading bacteria:

To isolate the dye degrading bacteria from sample collection, at first 1g of sludge sample was inoculated into 200ml conical flask containing 100ml sterilized SM broth with 1ml of each stock solution (1%) of six different Azo dyes. The flasks were incubated at shaker incubator with 80 rpm at 37^0C.

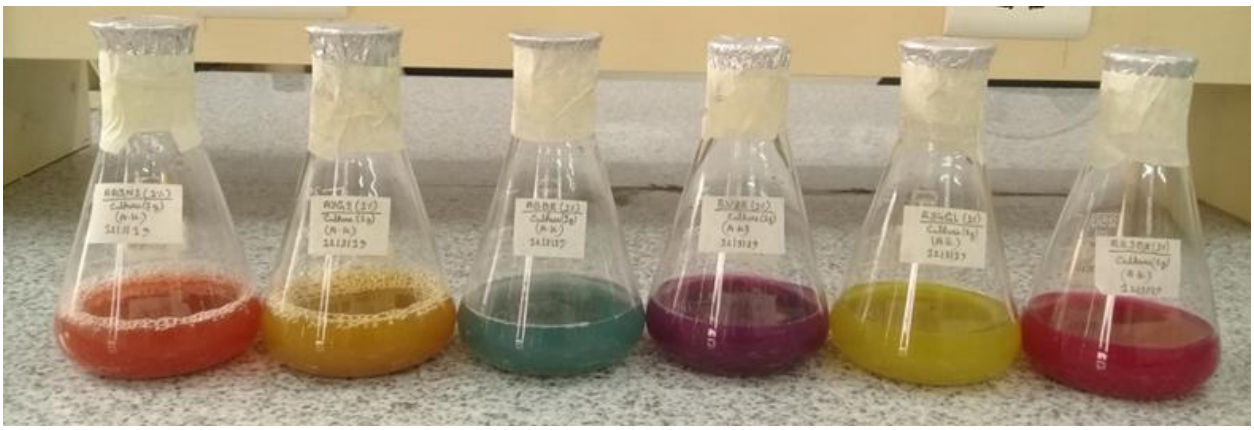

Figure 4: Six different Azo dyes were inoculated with 1gm of sludge sample (inoculation day)

All flasks were observed for around two weeks. After 24 hours of incubation, the flask containing Reactive Violet 5R was almost decolorized, and within 48 hours the dye was visibly completely decolorized. The rest of the five flasks were not properly decolorized, and some of them took 10 to 12 days to decolorize. Thus, further study is intended to focus on using the Reactive Violet 5R as the only dye to isolate the dye-degrading bacteria.

Therefore, after 24 hours of incubation (37^0C, 80 rpm), 1ml of enriched broth culture from that 1% RV5R SM broth (1st flask, A) was transferred to another fresh 1% Reactive Violet 5R dye supplemented SM broth (2nd flask, B) and incubated at the same condition. Again, 1 mL of broth from that flask (2nd flask, B) was transferred into another one (3rd flask, C) after 24 hours. This repetaed cycle was done to observe and ensure the decolorization potantial of the isolates and enriched the culture. Within 36 hours of period the 1st flask A containing Reactive Violet 5R dye was perceptibly complete decolorized, so 1ml of that broth was transferred into another 1% RV5R 100ml SM broth (4th flask, A1). The 2nd flask B, 3rd flask C were decolorized in 3rd and 5th days respectively. On the contrary, the 4th flask A1 was decolorized within 48 hours. Further for the

enrichment and isolation of the putative dye decolorizing bacteria, another two cycles of above serial transfer were performed. These series also yielded identical results.

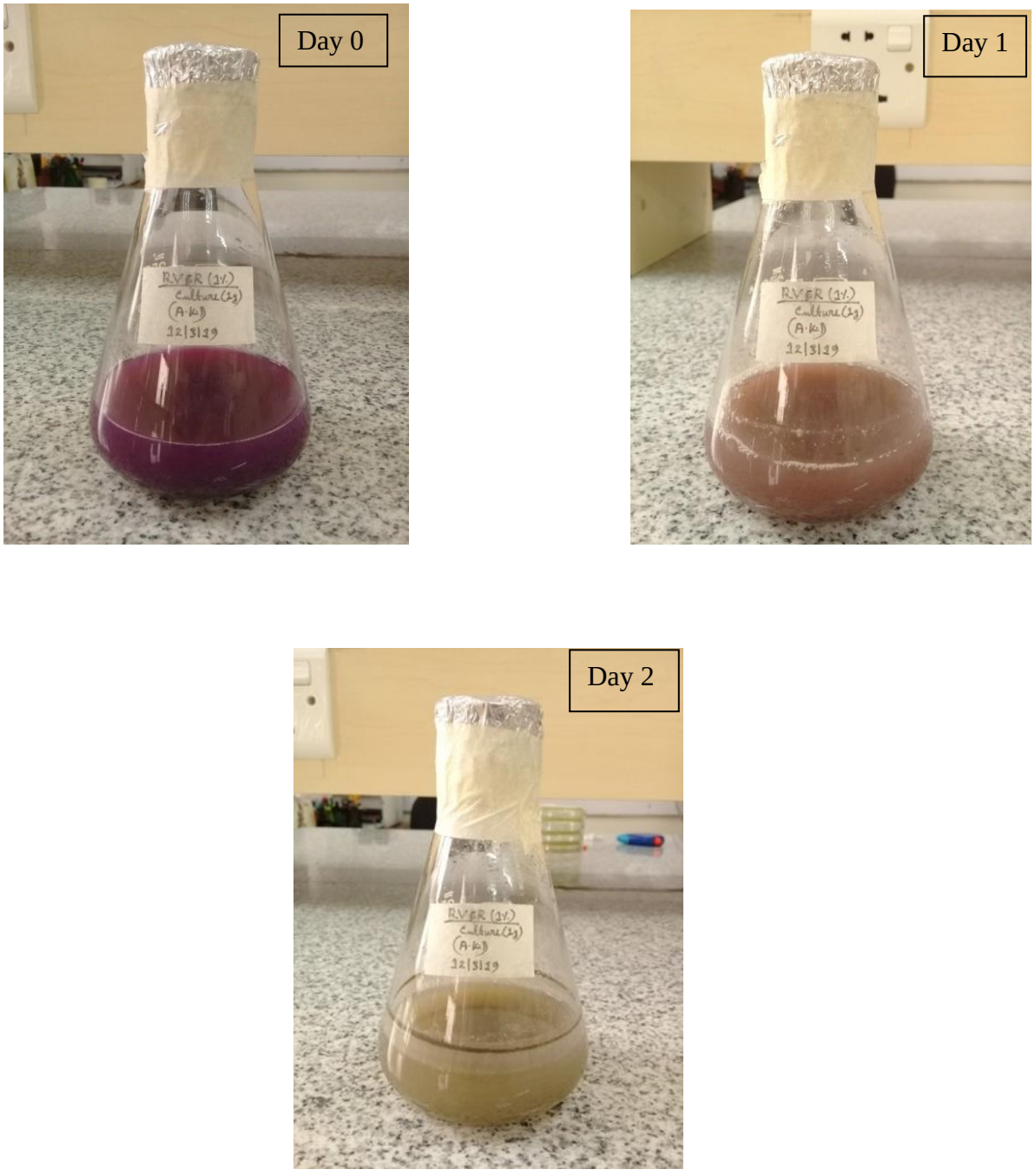

Figure 5: 1% RV5R SM broth inoculated with 1gm sludge sample (1st flask A).

From left to right - inoculation day (day 0), after 24h (day 1), after 48h (day 2).

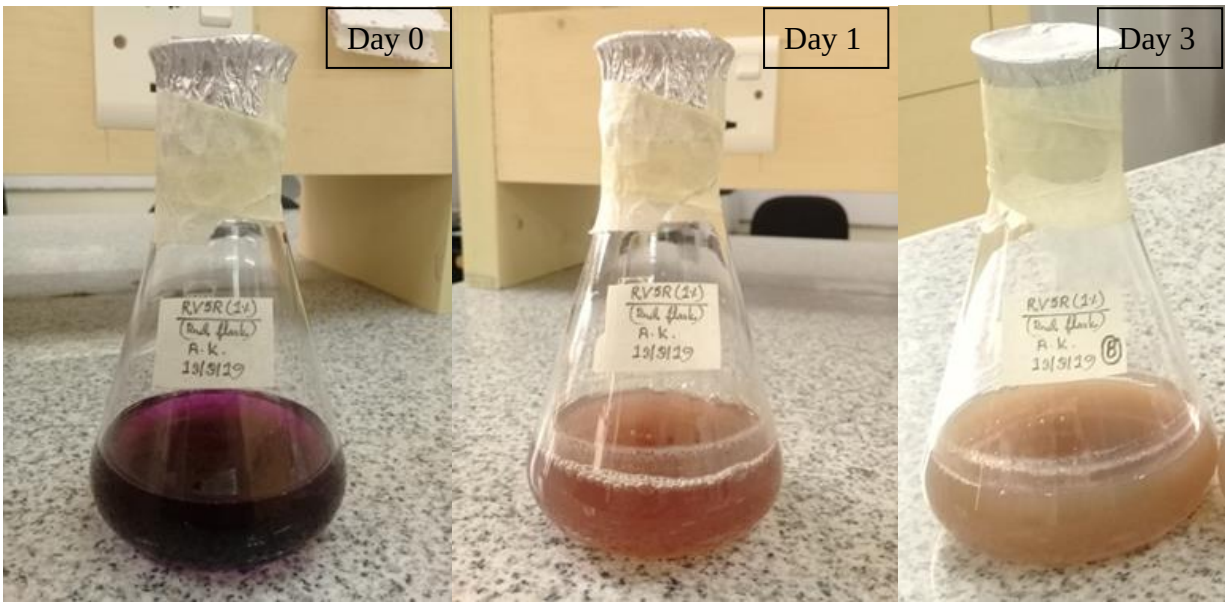

Figure 6: 2nd flask B. From left to right - inoculation day (day 0), after 24h (day 1), after 72h (day 3).

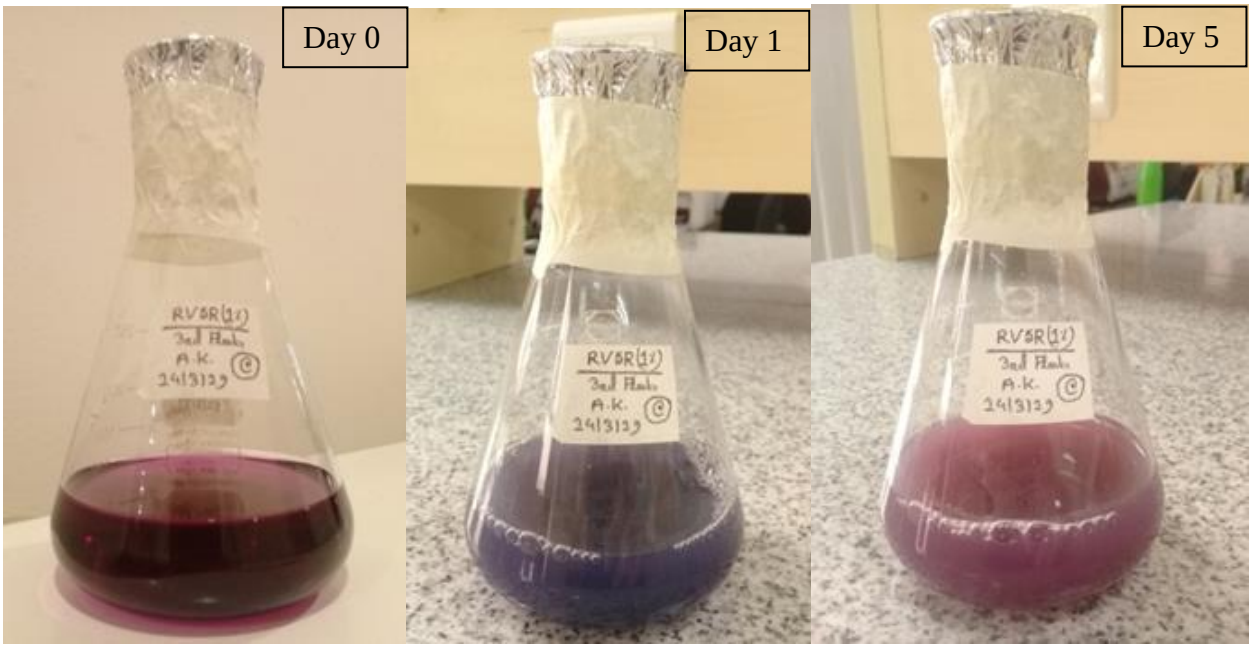

Figure 7: 3rd flask C. From right to left - inoculation day (day 0), after 24h (day 1), after 5days.

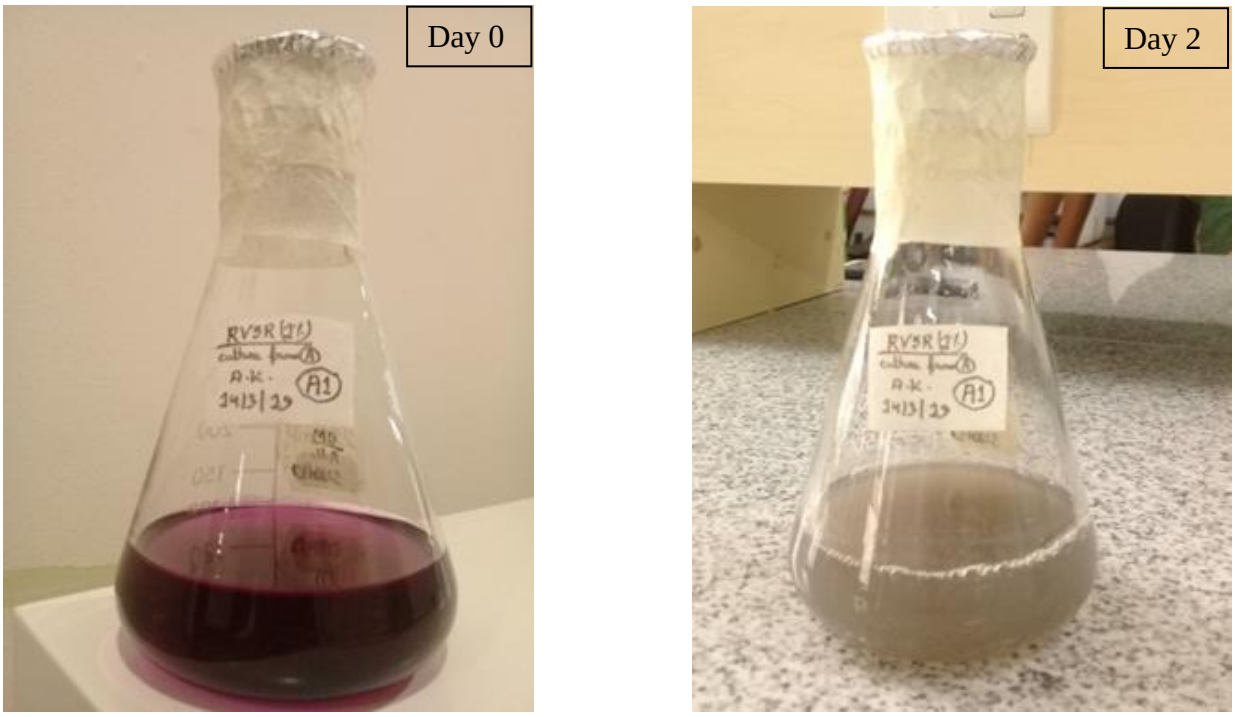

Figure 8: 4th flask A1(directly inoculated from flask A after complete decolorization within 2 days). From right to left - inoculation day (day1), after 48h (day 2).

3.6 Screening of the dye degrading bacterial isolate:

Serial dilution of the enriched broth (1st flask A series) was made up to 10^{-6} dilution and aliquots of 10^{-5} and 10^{-6} were spread on nutrient agar (NA) plates and incubated at 37^0C for 24 hours. After incubation, from the spread plates, morphologically distinct and prominent 19 different colonies were identified based on their cultural characteristics (e.g., colony color, form, margin, surface, and elevation). Each of the colonies was named AR1, AR2, AR3 …….AR18, AR19 correspondingly. All 19 different colonies were purified via streaking on the different NA plates. The purified bacterial isolates were inoculated in Luria-Bertani (LB) broth to cultivate.

Further, all the 19 morphologically distinct bacterial isolates were tested for their ability to decolorize 1% RV5R dye. Thus, 1ml overnight grown culture from LB broth of these 19 isolates were used to inoculated into 100ml of SM broth containing 1ml of 1% RV5R dye.

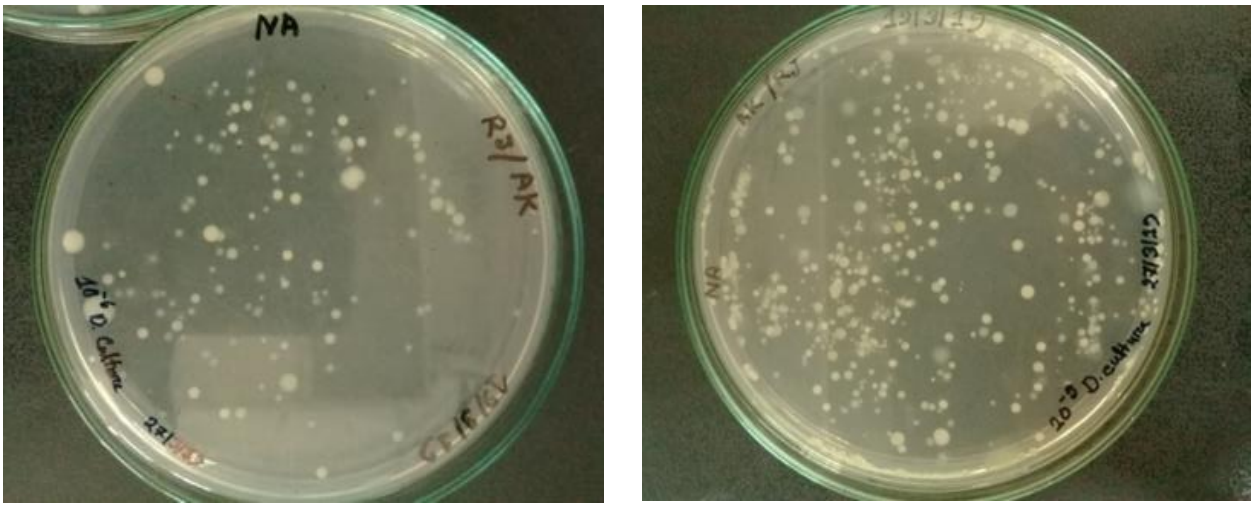

Figure 9: on left (10^{-6} dilution spread plate), on right (10^{-5} dilution spread plate).

Among all the 19 colonies, AR7 containing flask showed 90 % decolorization within 2 days.

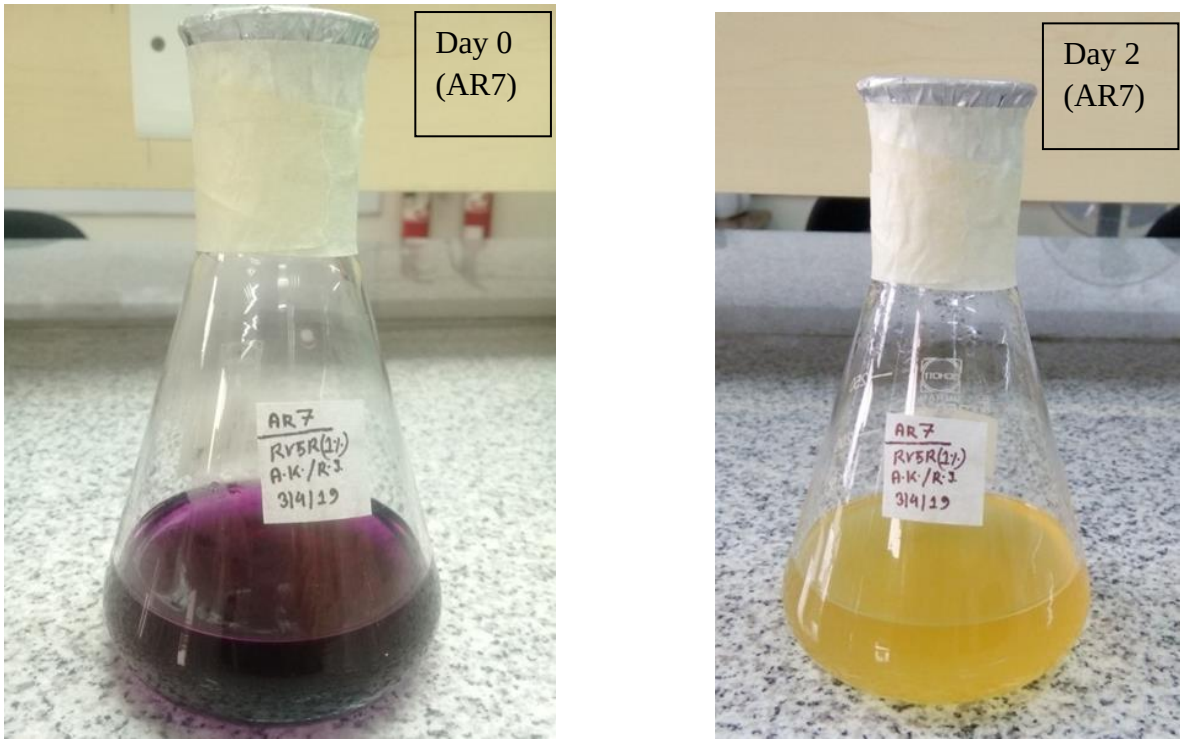

Figure 10: dye degradation of AR7 isolate. From left to right - inoculation day (day0), after 48h (day 2).

AR7 was then inoculated into another 3 flasks following the same procedure, and all the flasks were 50% decolorized within 24 h, and almost above 90% decolorization happened within 48 hours. A control was used, only containing 1% RV5R to differentiate the decolorization rate.

Figure 11: dye degradation of AR7 isolate after 24 hours (day1).

Figure 12: dye decolorization of AR7 isolate after 48 hours (day2).

Afterward, AR7 was reinoculated using 1 ml of LB cultured AR7 isolate into 100 ml of 1% RV5R SM broth, including 1 flask containing the supernatant only. The supernatant was collected after centrifuging the 5ml overnight culture of AR7 isolate using LB broth at 4000 rpm for 15 minutes. Within 48 hours, all flasks, including those containing the only supernatant, were also 90% decolorized.

Figure 13: Dye decolorization of AR7 isolate after 24 hours (day1). The 1st flask from the right contains the supernatant as inoculate.

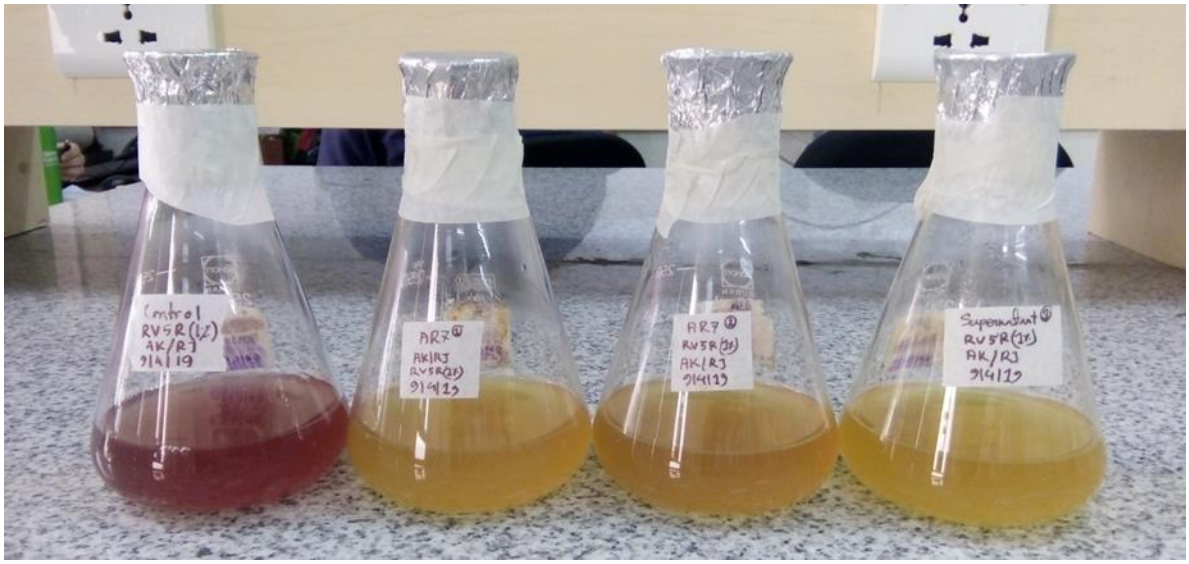

Figure 14: dye decolorization of AR7 isolate after 48 hours. The 1st flask from the right contains the supernatant as the inoculate.

As this AR7 bacterial strain showed more than 90% degradation of the added dye, this strain was selected for further study. The isolated AR7 bacterial strain was repeatedly screened out by streaking it on 100ml of NA medium plates incubated at 37^0C for 24 hours. After the incubation, plates were observed for single colony. Then, the screened culture was transferred to LB media and stored at 4^0C for further study.

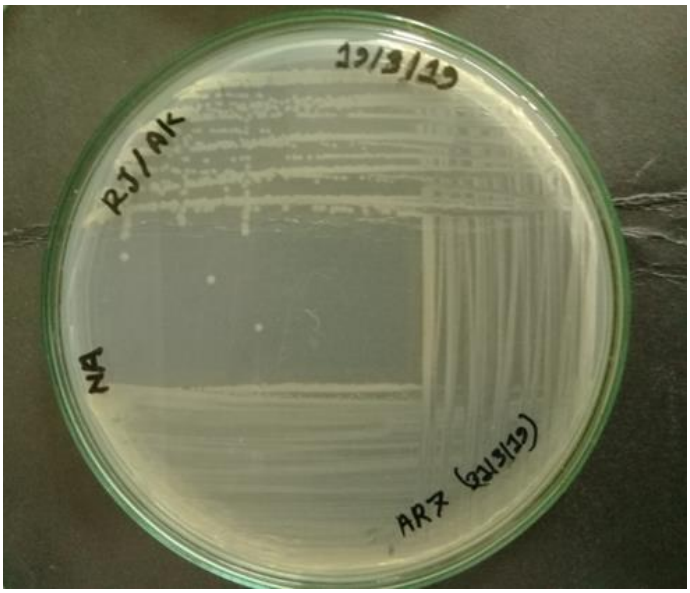

Figure 15: streak plate of pure culture of AR7 isolate.

3.7 Identification of the selected isolate:

The screened bacterial isolates AR7 used in this study were identified based on cultural, morphological, physiological, and biochemical characteristics using the protocol given in the Microbiology Laboratory Manual (James G. Cappuccino, 2014). Further, presumptive identification was performed using ABIS Online software.

3.7.1 Cultural characteristics of selected bacterial isolate:

cultural characteristics of selected bacterial isolates AR7 was carried out by streaking of single colony on NA plate and incubate at 37˚C for 24 hrs. After 24 hours of incubation, the cell size, form, colony color, margin, surface and elevation of the isolated bacteria AR7 was examined and tabulated.

3.7.2 Morphological characteristic of selected bacterial isolate:

Gram's reaction was used to validate the isolates' morphological characterizations. Gram staining of the AR7 isolate was performed to study the cell shape and arrangement and sporulation. A loopful of signal colonies from an overnight cultured AR7 isolate was smeared onto a glass slide with a drop of distilled water. After the spreading was done, the glass slide was heat fixed. Then the slide was flooded with crystal violet and left to stand for 30 seconds before being washed. After that, grams iodin were given for 1 minute and washed away. Then, it was flooded with 100% ethanol and washed away. After washing with ethanol immediately, the slide was counterstained with safranin, which was poured for 45 seconds and washed away. After all these steps, the glass slide was kept for drying. When drying was done, it was observed under the microscope. The gram-positive cells would appear violet, while the gram-negative cells would appear red.

3.7.3 Physiological characteristic of selected bacterial isolate:

To note the physiological characteristic of the AR7 isolate, growth on MacConKey Agar Plate and Growth response at different NaCl concentration was carried out.

3.7.3.1 Growth on MacConKey Agar Plate:

MacConkey agar is a selective and differentiating agar that only grows gram-negative bacteria and can further distinguish them based on their lactose metabolism. Lactose fermentation produces organic acids, particularly lactic acid, which lowers the agar's pH. In acidic conditions, MAC contains a pH indicator that turns pink. Lactose-fermenting gram-negatives (lactose fermenters) will produce pink colonies, whereas non-lactose fermenters will produce off-white opaque colonies. (Jung & Hoilat., October 5, 2021.) Loopful of AR7 isolate was streaked on MacConkey agar plate and incubated for 24 hours at 37^0C. After 24 hours of incubation, the growth and color were observed.

3.7.3.2 Growth response at different NaCl concentration (%):

This test was used to observe the saline tolerance of isolated AR7. Here, different concentrations of salt (6.5%, 7%, 8% and 10%) was mixed with the 10g/l Brain heart infusion broth, 10g/l peptone, 1g/l dextrose along with 0.016g/l bromocresol purple. Single AR7 bacterial colony from fresh plate was transferred with sterile inoculating loop to 6.5%, 7%, 8% and 10% NaCl containing

broths. The broths were incubated at 37^0C. Observation was made at 24 hours and every day for a period of 3 days if no change occurred. High salt tolerant bacteria would grow after incubation, giving a positive result. Bacteria would be unable to grow and make the negative test result.

3.7.4 Biochemical characteristics of the selected bacterial isolate:

The biochemical transformations that occur both outside and inside the cell are governed by biological catalysts called enzymes. All of the necessary media for biochemical tests were prepared in test tubes, flasks, and Petri dishes. All biochemical tests were carried out in accordance with the requirements outlined in the Microbiology Laboratory Manual. (James G. Cappuccino, 2014). To investigate the biochemical activities of AR7 isolate following test were done:

- Starch Hydrolysis
- Lipid Hydrolysis
- Casein Hydrolysis
- Gelatin Hydrolysis
- The triple sugar–iron (TSI) agar test
- MIU test
- Nitrate Reductase test
- Catalase test
- Oxidase test
- The IMViC tests -
    - Indole production test,
    - Methyl red test,
    - Voges-Proskauer test,
    - Citrate utilization test

3.7.4.1 Starch Hydrolysis:

Starch hydrolysis is used to test the utilization of starch by Bacteria by producing the enzyme Amylase. The hydrolytic activities of this exoenzyme are demonstrated using starch agar. Starch agar plate was prepared and using an aseptic technique, a single line streak inoculation of AR7

isolate was made on the agar surface. The plate was then incubated for 24 hours at 37^0C. After the 24h incubation period, the starch agar plate was flooded with the Gram's iodine solution, allowing the iodine to remain with the medium for 30 seconds. Then the excess was poured off. Finally, the culture was examined for the presence or absence of a blue-black color surrounding the growth of AR7 isolate.

3.7.4.2 Lipid Hydrolysis:

The hydrolytic activities of the exoenzyme lipase are demonstrated using tributyrin agar. Tributyrin agar plate was prepared and using aseptic technique, a single line streak inoculation of AR7 isolate was made on the agar surface. The plate was then incubated for 24 hours at 37^0C C. after incubation, the tributyrin agar plate culture was examined for the presence or absence of a clear area, or zone of lipolysis, surrounding the growth of AR7 isolate.

3.7.4.3 Casein Hydrolysis

The hydrolytic activity of proteases exoenzymes is demonstrated using milk agar. Milk agar was prepared and using aseptic technique, a single line streak inoculation of AR7 isolate was made on the agar surface. The plate was then incubated for 24 hours at 37^0C. The milk agar plate cultures were examined after 24h incubation period for the presence or absence of a clear area, or zone of proteolysis, surrounding the growth of each of the bacterial test organisms.

3.7.4.4 Gelatin Hydrolysis:

Gelatin liquefaction is accomplished by microorganisms that produce gelatinase, a proteolytic extracellular enzyme that hydrolyzes the protein into amino acids. To conduct the test, nutrient gelatin deep tubes were used to demonstrate the hydrolytic activity of gelatinase. The medium consists of nutrient broth that has been supplemented with 12% gelatin. Following stab inoculation and incubation for 48 hours, the cultures were placed in a refrigerator at 4°C for 30 minutes. If the cultures remained liquefied, that means they produced gelatinase and demonstrated rapid gelatin hydrolysis. All solidified cultures should be re-incubated for an additional 5 days. Then it was refrigerated for 30 minutes and observed for liquefaction.

3.7.4.5 The triple sugar–iron (TSI) agar test:

The TSI agar slant was inoculated by means of a stab-and-streak procedure. This required the insertion of a sterile, straight needle from the base of the slant into the butt. Upon withdrawal of the needle, the slanted surface of the medium was streaked. After that the TSI agar tube was incubated for 24 hours at 37^0C. After the incubation, the color of both the butt and slant of all agar slant cultures was examined. Based on observations, the type of reaction that had taken place (acid, alkaline, or none) and the carbohydrate that had been fermented (dextrose, lactose, and/or sucrose, all, or none) in each culture was determined. The culture was also examined for the presence or absence of blackening within the medium and also determined whether or not each organism was capable of H_2S production.

3.7.4.6 MIU test:

MIU is an abbreviation for Motility Indole Urea. This single-tube test helps distinguish the organisms based on motility, urease, and indole production. (MIU Test: Uses, Principle, Composition, Procedure, Result Interpretation and keynotes, 2021, july 27) A well-isolated single colony of AR7 was taken with an inoculating needle and stabbed into the medium leaving 1/3 part from the bottom of the tube. A loose-fitting cotton plugin was used as a test tube. After inoculation the tube was Incubated at 37°C for 24 hours. The tube was observed for growth, motility, and color change of the medium.

3.7.4.7 Nitrate Reductase test:

The reduction of nitrates can be measured by growing organisms in a nitrate broth medium. To carry out the test a single colony of AR7 bacteria isolate was inoculated into nitrate broth containing Durham tube, with the aid of sterile loop. Following overnight incubation of the cultures, the isolate's ability to reduce nitrates to nitrites was determined by the addition 5 drops of two reagents: Solution A, which is sulfanilic acid, followed by Solution B, which is α-naphthylamine. After reduction, the addition of Solutions A and B results in an immediate cherry red color. Cultures that do not produce a color change point to one of two possibilities: (1) The organism did not reduce nitrates, or (2) the organism possessed such powerful nitrate reductase enzymes that nitrates were rapidly reduced beyond nitrites to ammonia or even molecular nitrogen.

A small amount of zinc powder is added to determine whether nitrates were reduced past the nitrite stage. Nitrates are converted to nitrites by zinc.

3.7.4.8 Catalase test:

Catalase-producing organisms degrade hydrogen peroxide rapidly. By adding the substrate H_2O_2 to an appropriately incubated culture, catalase production can be determined. To determine the catalase production of the AR7 isolate, a slide method was carried out. A sterile loop was used to collect a small sample of AR7 Isolate and transferred to the slide. The slide was placed in the Petri dish and then one drop of 3% hydrogen peroxide was placed on the sample. Without mixing it, the Petri dish was covered to contain any aerosols. Observation was taken place for the immediate presence of bubble formation.

3.7.4.9 Oxidase test:

The ability of bacteria to produce cytochrome oxidase can be determined by adding the test reagent p-aminodimethylaniline oxalate to plate medium colonies. The cotton swabs method may also be used to determine the oxidase presence. Sterile cotton swab was used as a substrate for the oxidase test. With a sterile cotton swab, a heavy loopful of the AR7 isolate was obtained. One or two drops of p-aminodimethylaniline oxalate reagent was dropped on isolate. Then isolate was observed for the appearance of a purple color within 30 seconds of contact with the oxidase reagent, indicating a positive test.

3.7.4.10 Indole production test:

Kovac's reagent, which produces a cherry red reagent layer, can be used to detect the presence of indole. In this test, SIM agar, which contains the substrate tryptophan, was used. Using aseptic technique, AR7 isolate was inoculated into deep tube by means of a stab inoculation. Two tubes were prepared as a negative control (*Pseudomonasaeruginosa*) and positive control *(E. coli*). The tubes were Incubated for 24 to 48 hours at 37°C. After that the color of the reagent layer in each culture was observed. Based on your observations, determine and record whether or not each organism was capable of hydrolyzing the tryptophan.

3.7.4.11 Methyl red test:

The Methyl-Red is a test for mixed-acid fermentation capability. The AR7 isolate was inoculated into MR-VP tube by means of a loop inoculation. Two other tubes were prepared as positive control (*E. coli*) and negative control (*Klebsiella pneumoniae*). All tubes were incubated for 24 to 48 hours at 37°C. After inoculation five drops of the methyl red indicator was added to the remaining aliquot of each culture. The color of all cultures was examined and observed to determine whether or not each organism was capable of fermenting glucose with the production and maintenance of a high concentration of acid.

3.7.4.12 Voges-Proskauer test:

The Voges-Proskauer test determines whether some organisms can produce nonacidic or neutral end products from organic acids produced during glucose metabolism, such as acetylmethylcarbinol. The AR7 isolate was inoculated into MR-VP tube by means of a loop inoculation. Two other tubes were prepared as positive control (*Klebsiella*) and negative control (*E.coli).* 10 drops of Barritt's reagent A were added into the tubes and then the cultures were Shaked. Immediately 10 drops of Barritt's reagent B were also added and Shaked. The cultures were reshaked every 3 to 4 minutes. The color of the cultures was examined 15 minutes after the addition of Barritt's reagent. The result was observed to determine whether or not each organism was capable of fermenting glucose with ultimate production of acetylmethylcarbinol.

3.7.4.13 Citrate utilization test:

Some microorganisms can use citrate as a carbon source for energy in the absence of fermentable glucose or lactose. Simmons citrate agar slants was used to inoculate AR7 isolate into tube by means of streak inoculation. Two tubes were prepared to serve as positive *(Klebsiella)* and negative (*E. coli*) control. All cultures were incubated for 24 to 48 hours at 37°C. Following incubation, the result was observed to determine whether or not each organism was capable of using citrate as its sole source of carbon.

3.8 dye decolorization assay:

The dye decolorization experiments were carried out in 200ml conical flasks containing 100ml of SM broth along with 10ml of 1% Reactive Violet 5R dye solution. 1 ml of overnight cultured AR7 isolate was transferred into the flasks. One of the flasks was inoculated only with the supernatant of the cell, which was obtained via centrifuge. A control only containing the dye was also used. All the flasks were incubated in the shaker incubator for 4 days at 37^0C, 80 rpm. For four days, samples were drawn at 24-hour intervals for optical density (OD) analysis. About 5 ml of culture suspension was centrifuged at 4000 rpm for 15 minutes for removal of the biomass. The degree of the decolorization of the 1% RV5R dye was assessed by the measured absorbance at 510 nm of the supernatant with the help of a spectrophotometer at the maxima (m) of the respective dye.

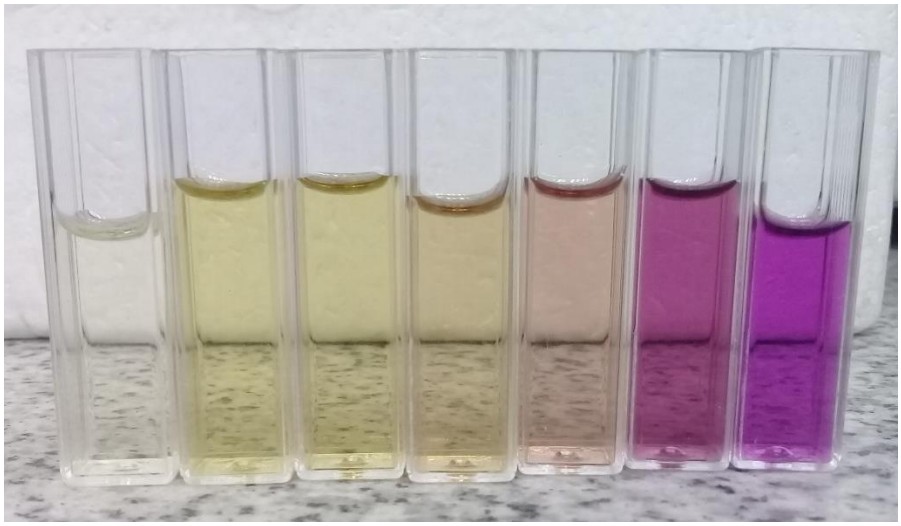

Figure 16: supernatant of the dye decolorized cultural broth in cuvette to record OD

The decolorization assay was measured in terms of percentage decolorization using spectrophotometer. The percentage decolorization was calculated from the following formula -

$$\text{Percentage (\%) of decolorization} = \frac{(A-B)}{A} \times 100$$

Where, A = Initial OD

B = Final OD

3.9 Identification of isolate using ABIS Microbiology Software:

A presumptive identification was performed by using ABIS Online software. This software is used to determine the identification of bacterial strains based on morphology, biochemical characteristics, cultural characteristics, ecology, and pathogenicity data. This was done by individually inputting the morphology, biochemical characteristics, and cultural characteristics results obtained for the AR7 isolate. The software also gives users a brief description of the biochemical tests that must be performed in order to identify each organism. ABIS's results also show very good taxa identification with a high percentage of matches made by ABIS software.

3.10 DNA extraction PCR amplification of 16S rRNA gene

For the further molecular identification of the obtained isolate, the DNA was extracted, and PCR amplification of the 16S rRNA gene was carried out. The total genomic DNA was extracted from the pure AR7 isolate culture using the Wizard® Genomic DNA Purification Kit, which is designed.

The procedure for the isolation of DNA from gram-negative bacteria was followed. After DNA extraction, PCR amplification of the 16S rRNA gene from bacterial isolate AR7 was conducted using the universal primers: F:fD1 = (5'-AGAGTTTGATCCTGGCTCAG-3'') and R:rP2 = (5'-ACGGCTACCTTGTTACGACTT-3''). Thermal cycling comprises the following steps: 94^0C for 5 minutes, followed by 35 cycles of 94^0C for 45 seconds, 55^0C for 1 minute, 72^0C for 1 minute, and a final step at 72^0C for 10 minutes. The PCR product was then cleaned by using the Wizard® SV Gel and PCR Clean-Up System kit in accordance with the directions of the manufacturer.

3.11 Agarose Gel Electrophoresis:

After that PCR product was cleaned and the amplified DNA was separated by 1% agarose gel electrophoresis at 70 voltage and visualized by UV transilluminators. The gel electrophoresis was performed to check whether the band one present or not.

3.12 Preservation of bacterial isolate:

The AR7 bacterium isolate was inoculated into 3 mL of T1N1 agar by stabbing it from the nutrient agar plate. To allow the bacteria to reach a log phase, the vial was incubated for 5 hours. Following that, 200 L of sterile glycerol was added to the vial, which was then sealed with parafilm and stored

at room temperature. For long-term preservation, the isolate was also stored in 30% glycerol at -80°C.After the PCR amplification, the PCR product was purified and stored for a long time at -20^0C.

Chapter Four: Results and Observation

4.1 Potent dye degrading bacterial isolate isolation:

In this study, after a series of decolorization experiments out of 19 morphologically distinctive bacterial isolates, the AR7 isolate showed the best decolorization rate of the 1% Reactive Violet 5R Azo dye degradation within 48 hours. The decolorization experiment was done using both the whole cell culture and only the supernatant of the cell as an inoculate; both cases yielded identical results.

4.2 Cultural characteristics:

The cultural characteristic of the AR7 isolate is -

- From: Circular
- Elevation: Convex
- Margin: Entire
- Surface: Smooth
- Color: Cream / Whitish yellow

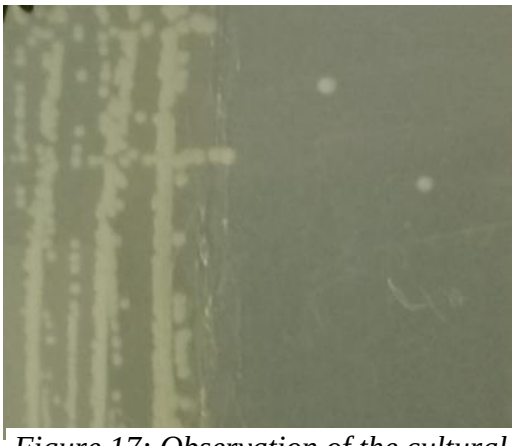

Figure 17: Observation of the cultural characteristic of AR7 in NA streak plate

4.3 Morphological characteristic (Gram's Reaction):

Cell morphology of the AR7 isolate is –

- Gram staining - Gram Negative Rod
- Shape - short rod,
- Arrangement - single, pair.

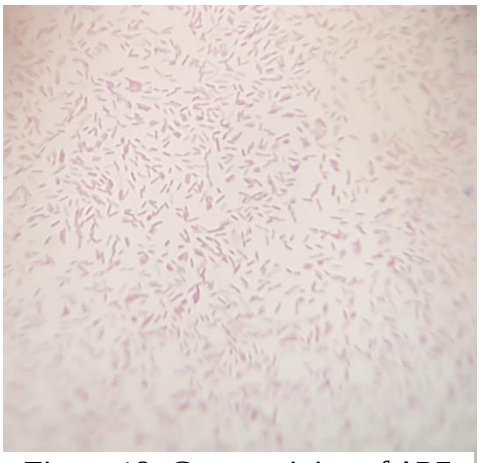

Figure 18: Gram staining of AR7 (Gram Negative Rod)

4.4 Physiological characteristics:

4.4.1 Growth response at different NaCl concentration:

AR7 isolate's growth rate increased as the concentration of the salt increased.

- 6.5% NaCl = No growth
- 7% NaCl = No growth
- 8% NaCl = Scanty
- 10% NaCl = Moderate

Figure 20: No growth at 6.5% NaCl conc.

Figure 19: No growth at 7% NaCl conc.

Figure 22: Scanty growth at 8% NaCl conc.

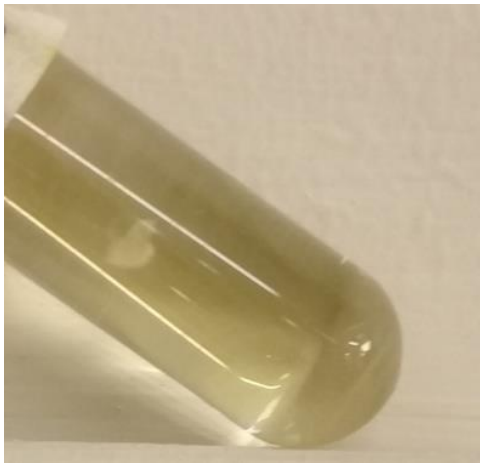

Figure 21: Moderate growth at 10% NaCl conc.

4.4.2 Growth response at MacConKey Agar Plate:

The AR7 isolate showed growth on the MacConkey Agar plate. The culture of the plate turns off-white opaque colonies, identifying it as a non-lactose fermenter.

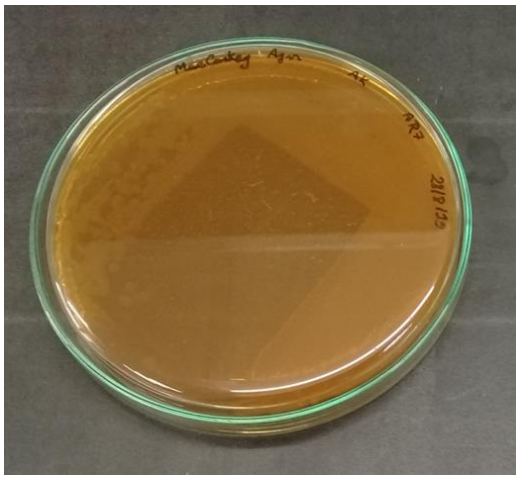

Figure 23: Growth on MacConKey Agar plate, non-lactose fermenter.

4.5 Biochemical characteristics:

4.5.1 Starch Hydrolysis:

There was not a clear zone of hydrolysis surrounding the growth of the isolate and also medium turned blue-black, indicating the absence of starch-splitting enzymes and representing a negative result.

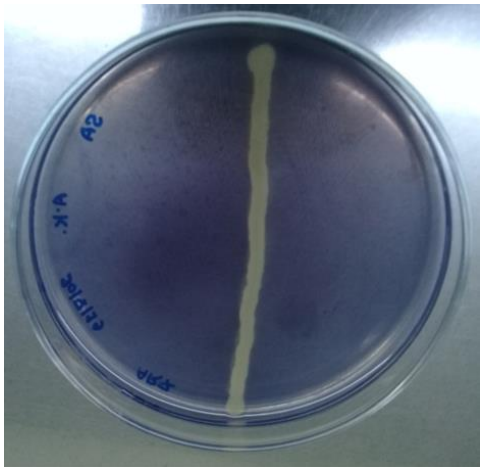

Figure 24: Starch hydrolysis (Negative test result)

4.5.2 Lipid Hydrolysis:

There was an absence of a clear area surrounding the growth of AR7 isolate indicating a negative result.

Figure 25: Lipid hydrolysis (Negative test result)

4.5.3 Casein Hydrolysis:

Here, the medium surrounding the AR7 isolate's growth remains opaque in the absence of protease activity, which is a negative reaction.

Figure 26: Casein hydrolysis (Negative test result)

4.5.4 Gelatin Hydrolysis:

The AR7 cultures remained liquefied, which means they produced gelatinase and demonstrated rapid gelatin hydrolysis, showing positive results.

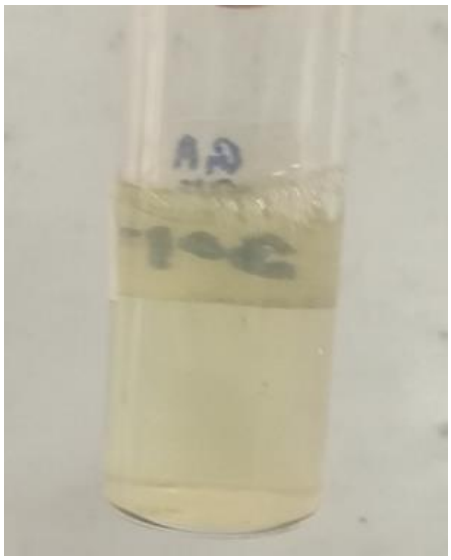

Figure 27: Gelatin liquefaction (Positive test result)

4.5.5 The triple sugar-iron (TSI) agar test:

- An alkaline/Acidic (red slant, yellow butt) reaction: only glucose fermentation occurred.
- Blackening of the medium: Occurs in the presence of H2
- No Gas production: Absence of Bubbles or cracks in the agar indicate the no production of gas.

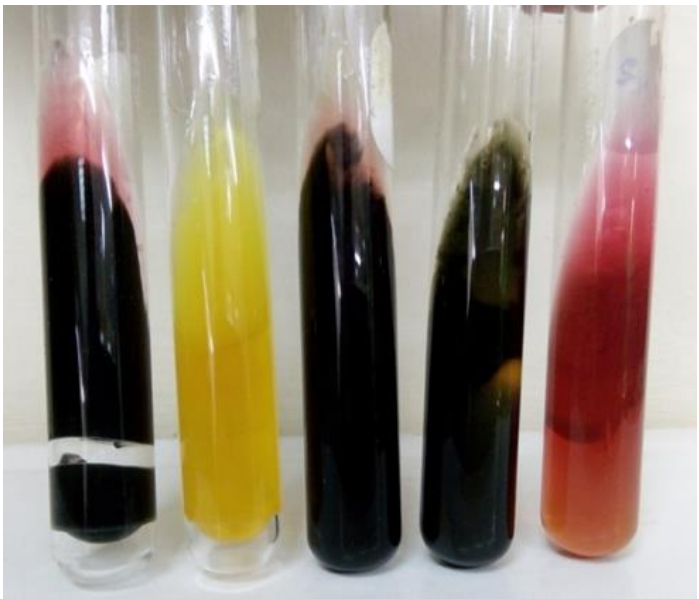

Figure 28: Left to right: Salmonella paratyphi, E.coli, sample1 AR7(red slant yellow butt), sample2 AR7(red slant, yellow butt), Pseudomonas.

4.5.6 MIU test:

Non motile, and Urease test positive.

Figure 29: MIU test (non motile, urease test positive)

4.5.7 Nitrate Reductase test:

AR7 showed positive nitrate reductase test.

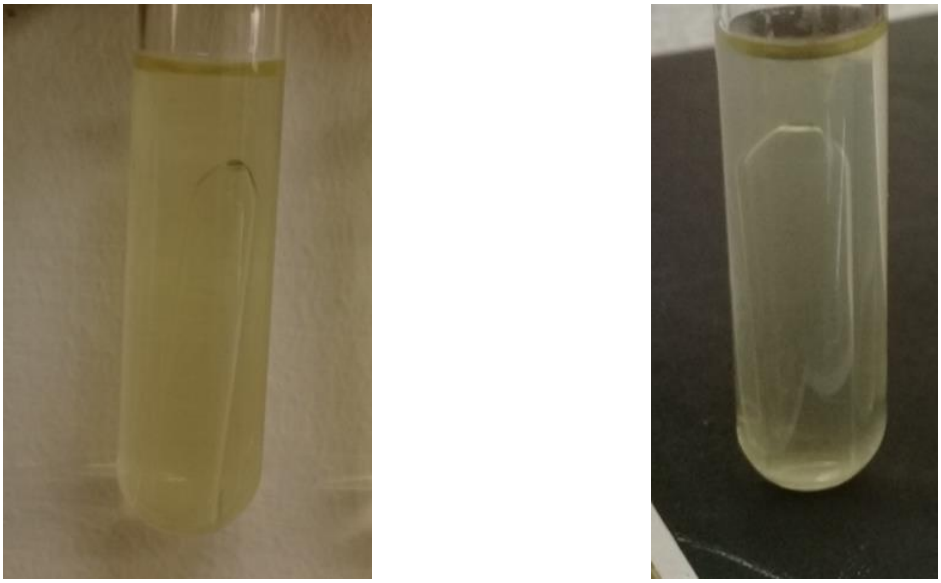

Figure 30: no color changes after adding solution A and B (left), no color changes after addition of zinc powder (right), indicating positive nitrate reductase test.

4.5.8 Catalase test:

Here, the presence of bubbles of oxygen indicated a positive catalase test.

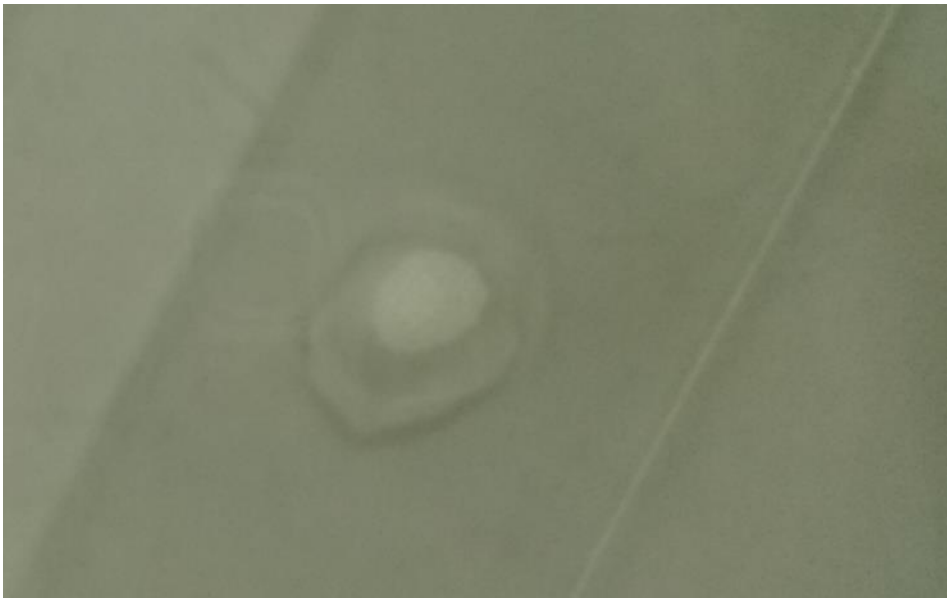

Figure 31: Catalase test (Positive)

4.5.9 Oxidase Test:

The presence of pink color on the culture resulted in a positive oxidase test.

Figure 32: Oxidase test (positive)

4.5.10 Indole production test:

No purple ring formation indicated a negative indole test.

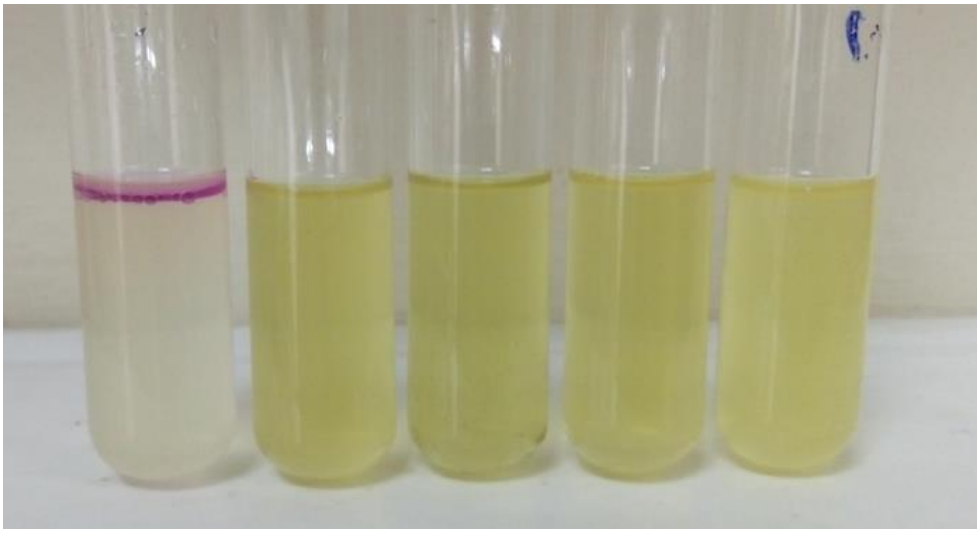

Figure 33: Left to right: Positive control (E. coli), sample tube 2,3,4, Negative control (Pseudomonasaeruginosa)

4.5.11 Methyl red test:

AR7 showed a negative Methyl red test.

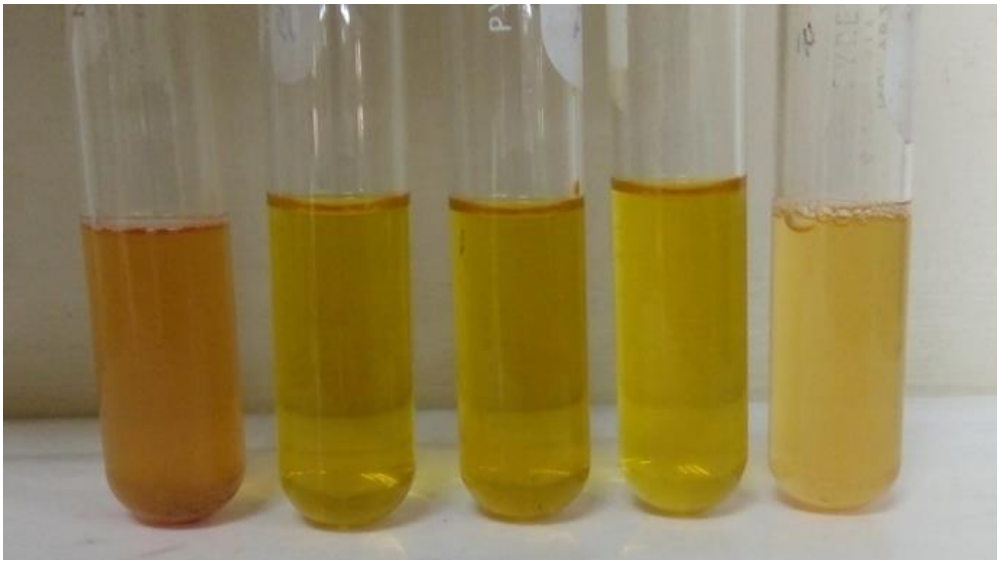

Figure 34:Left to right: Positive control (E.coli), sample tube1,2,3, Negative control (Klebsiella pnuemoniae)

4.5.12 Voges-Proskauer test:

No color changes indicated a negative Voges-Proskauer test.

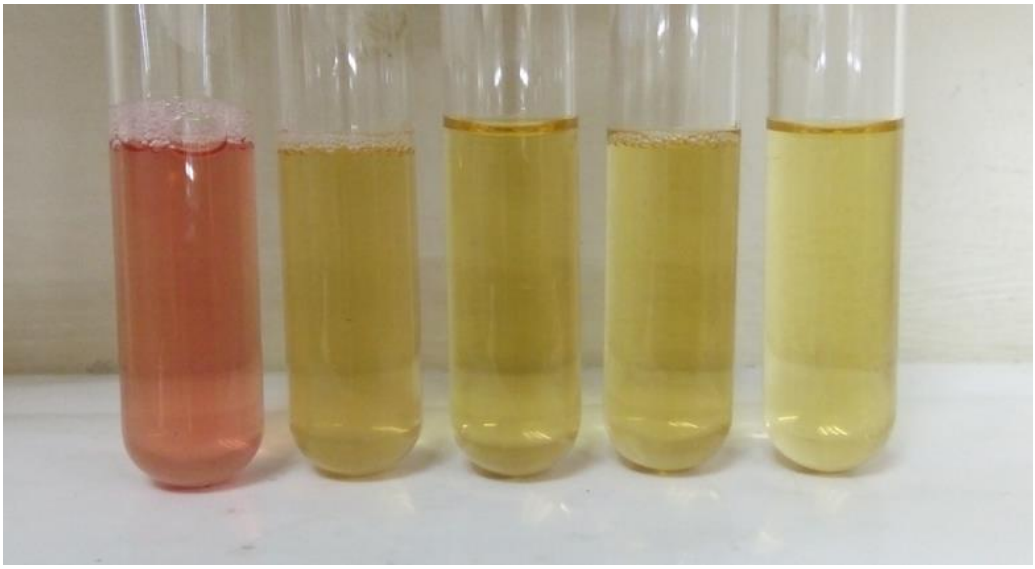

Figure 35: Left to right: positive control (Klebsiella), sample tube1,2,3, negative control (E.coli)

4.5.13 Citrate utilization test:

The culture remaining green indicated the negative Citrate utilization test.

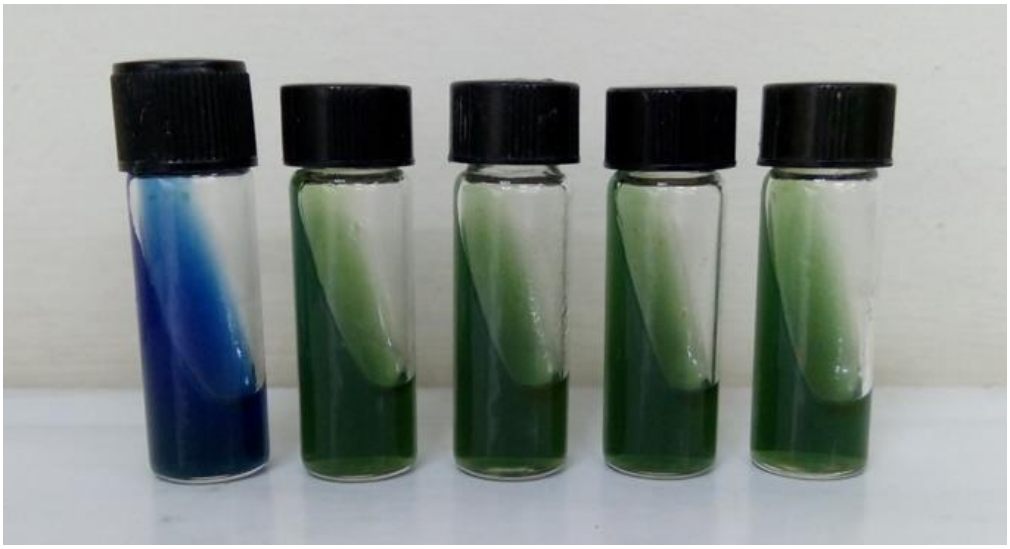

Figure 36: Left to right: positive control (Klebsiella), sample tube 1,2,3, negative control (E.coli)

Test Parameters	Observation
Colony on nutrient agar/ Cultural characteristic	Form: Circular, Elevation: Convex, Margin: Entire, Surface: Smooth, Color: Cream / Whitish yellow.
Gram Staining	Gram negative rod
Cell Morphology	Short rod, single, pair
Motility	Non motile
Catalase test	Positive
Oxidase test	Positive
Urease test	positive
H_2S production	Positive
Nitrate Reduction test	Positive
Citrate utilization	Negative
Voges Proskauer test (VP)	Negative
Methyl Red test (MR)	Negative
Indole test	Negative
Starch hydrolysis	Negative
Casein hydrolysis	Negative
Lipid Hydrolysis	Negative
Gelatin hydrolysis	Positive
Triple Sugar Iron test	Acidic but alkaline slunt, acidic but and slunt, no gas production, H_2S production. Slightly Glucose fermenter.
MacConKey agar plate	Growth on MacConKey agar plate. Gram negative non lactose fermenter
Growth response at different NaCl concentration -	
6.5% NaCl	No growth
7% NaCl	No growth
8% NaCl	Scanty growth
10% NaCl	Moderate growth

Table 1: The cultural, morphological, physiological and biochemical characteristics of the selected bacterial isolate AR7.

4.6 Absorbance reading:

Control

Time	nm	Abs
day1	510	0.869
day 2 (24h)	510	0.818
day 3 (48h)	**510**	**0.817**
day 4 (72h)	510	0.806

Table 2: 4 days Absorbance reading at 510nm of control

AR7 (Flask 1)

Time	nm	Abs
day1	510	1.131
day 2 (24h)	510	0.668
day 3 (48h)	**510**	**0.1**
day 4 (72h)	510	0.135

Table 3:4 days absorbance reading at 510nm of AR7 (flask1)

AR7 (Flask 2)

Time	nm	Abs
day1	510	1.037
day 2 (24h)	510	0.478
day 3 (48h)	**510**	**0.13**
day 4 (72h)	510	0.157

Table 4: 4 days absorbance reading at 510nm of AR7 (flask 2)

AR7 (Flask 3)

Time	nm	Abs
day1	510	0.882
day 2 (24h)	510	0.148
day 3 (48h)	**510**	**0.19**
day 4 (72h)	510	0.122

Table 5: 4 days absorbance reading at 510nm of AR7 (flask3)

AR7 (Flask 4)

Time	nm	Abs
day1	510	1.082
day 2 (24h)	510	0.278
day 3 (48h)	**510**	**0.145**
day 4 (72h)	510	0.188

Table 6: 4 days absorbance reading at 510nm of AR7 (flask 4)

AR7 (Flask 5)

Time	nm	Abs
day1	510	1.27
day 2 (24h)	510	0.478
day 3 (48h)	**510**	**0.178**
day 4 (72h)	510	0.121

Table 7: 4 days absorbance reading at 510nm of AR7(supernatant) (flask 5)

4.7 Decolorization of dye Reactive Violate 5R (RV5R) by bacterial isolate AR7:

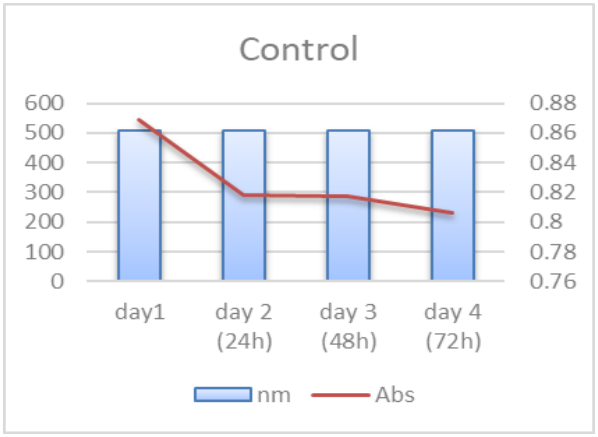

Figure 37: decolorization of RV5R by control

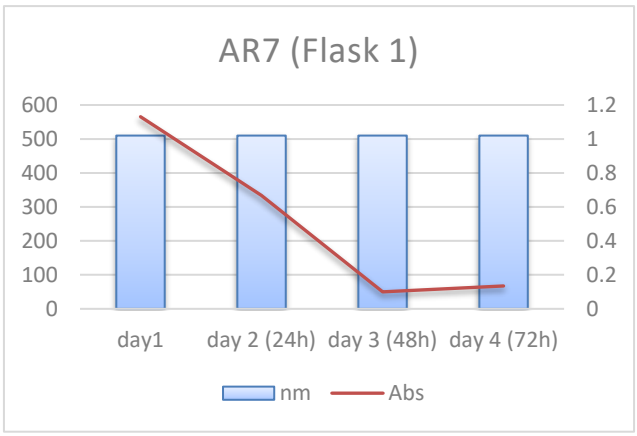

Figure 38: decolorization of RV5R dye by AR7 isolate

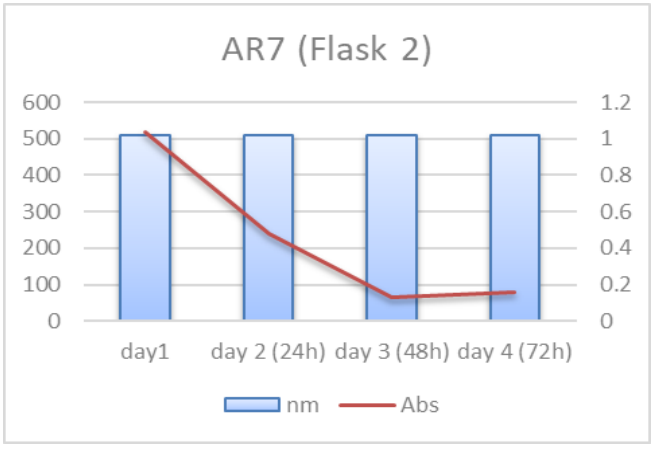

Figure 39: decolorization of RV5R dye by AR7 isolate

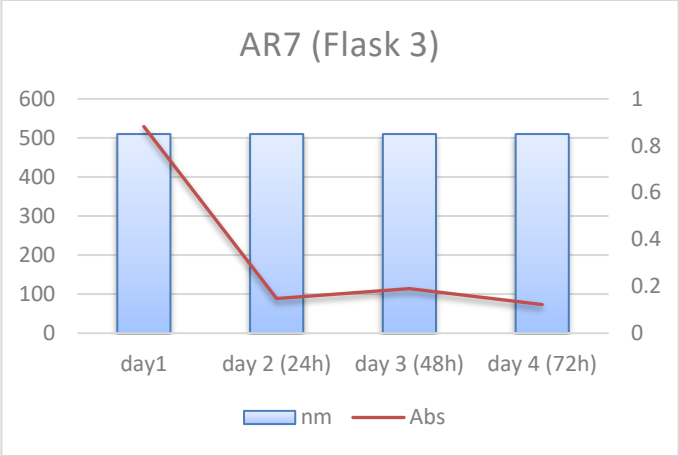

Figure 40: decolorization of RV5R dye by AR7 isolate

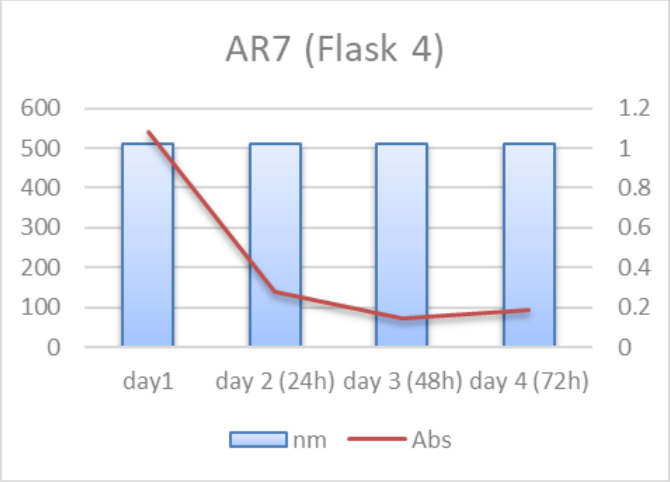

Figure 41: decolorization of RV5R dye by AR7 isolate

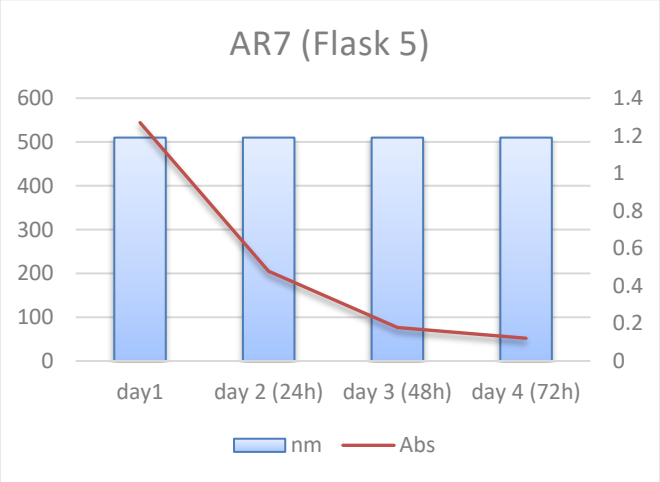

Figure 42: decolorization of RV5R dye by AR7 isolate (supernatant)

4.8 decolorization assay:

- **AR7 (Flask 1):** Decolorization rate **91.16%**
- **AR7 (Flask 2):** Decolorization rate **87.46%**
- **AR7 (Flask 3):** Decolorization rate **86.17%**
- **AR7 (Flask 4):** Decolorization rate **86.60%**
- **AR7 (Flask 5):** Decolorization rate **90.47%**

4.9 Agarose Gel Electrophoresis:

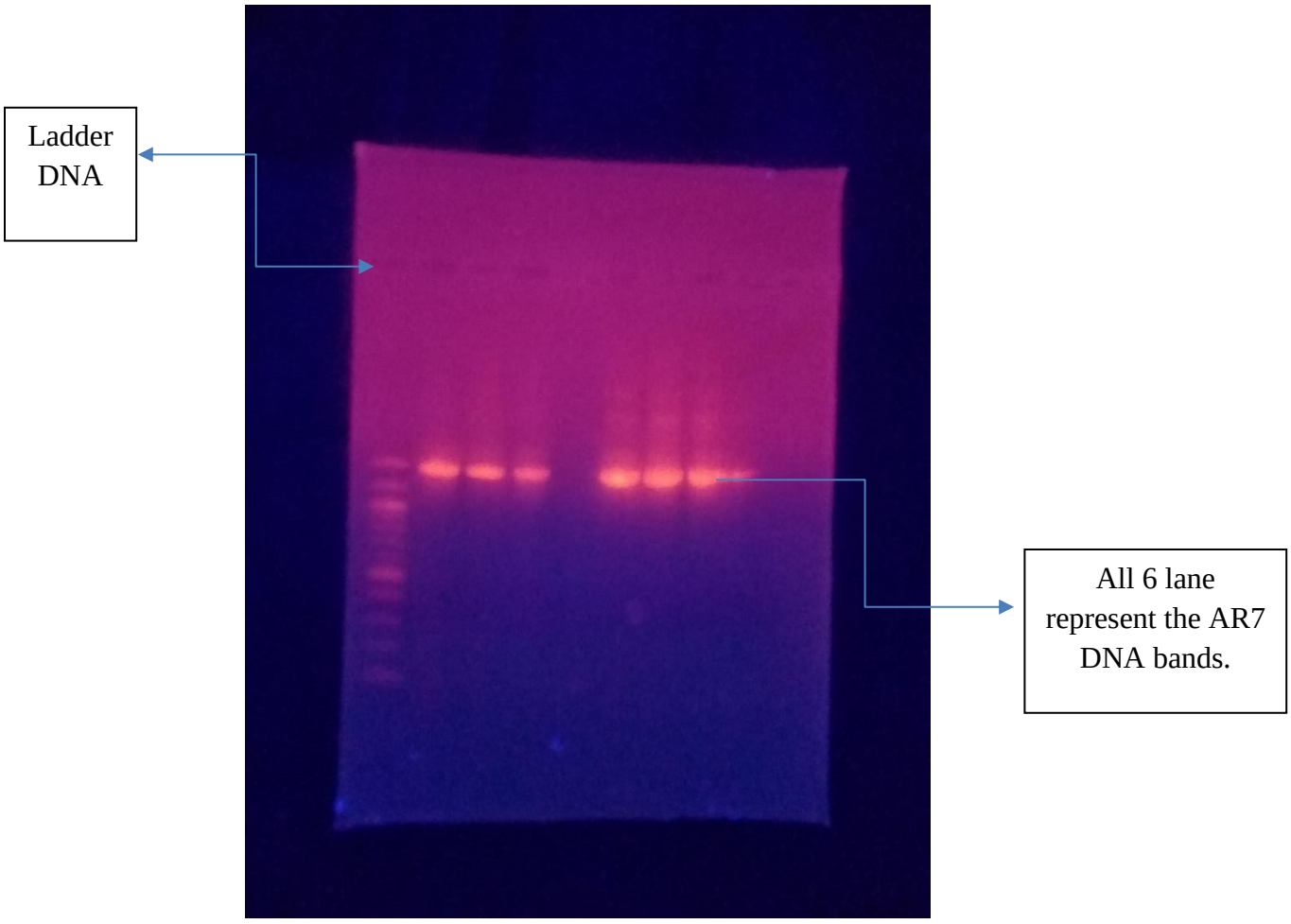

Figure 43: Presence of DNA band on agarose gel electrophoresis

4.10 Identification of isolate using ABIS Online software:

Through ABIS Online software, presumptive identification of the selected RV5R dye degrading bacterial isolate AR7 was obtained. Two plausible bacteria were identified as the feasible result of AR7.

Figure 44: ABIS result of AR7(1)

Figure 45: ABIS result of AR7 (2)

AR7 could be identified as either of these two bacteria that were obtained from this project are *Actinobacillus gracillus (85.9% similarity)* and Aeromonas salmonicida subsp. Smithia (82.2% similarity). These two bacteria were identified on the basis of cultural, morphological, physiological, and biochemical tests, microscopic observation, and ABIS software.

Chapter Five: Discussions and Conclusion

5.1 Discussion:

In this study, one isolate was obtained for the ability to decolorize **91.16%** of the 1% Reactive Violet 5R (RV5R) dye within 48 hours. The whole cell culture and the supernatant of the culture displayed the same result. The optimum decolorizing parameters of the study were: concentration of dye 1%, inoculum size (0.5% v/v), temperature (37^0 C), pH (6.5-6.4), with a rotation of 80 rpm. The absorbance was measured at 510 nm for the supernatant with the help of a spectrophotometer. The study was also conducted under aerobic conditions without using any co-substrate and using the Reactive Violet 5R (RV5R) dye as the only carbon and energy source for the bacteria isolate. This isolate was later identified on the basis of gram reaction, colony characteristics, cell morphology, and biochemical tests. After identification, the results are analyzed with software called ABIS Microbiology. There is a high probability that this isolate could be *Actinobacillus rossi* (85.9% similarity) or *Acromonas salmonicida subsp. smithia* (82.2% similarity).

The cultural, morphological, and physiological characteristics of the isolated bacteria have been detected. This particular isolate is circular in form, convex elevation, entire margin, smooth surface, and whitish yellow color. It is a gram-negative rod. The rod shape is short, and they are always arranged in single and pair configurations. This isolate showed growth on the MacConkey Agar plate. The culture of the plate turns off-white opaque colonies, identifying it as a non-lactose fermenter. It's also shown scanty growth at 8% NaCl concentration and moderate growth at 10% NaCl concentration, proving that this bacteria's growth increases along with the NaCl concentration increase. This specific culture produced gelatinase and demonstrated rapid gelatin hydrolysis. It also slightly ferments glucose, producing H_2S. It is a nonmotile bacteria. This bacterium is also capable of hydrolyzing the substrate urea and is able to reduce nitrates to nitrites. This isolate is capable of catalase activity and producing cytochrome oxidase.

The bacterium isolate was inoculated into 3 mL of T_1N_1 agar sealed with parafilm and stored at room temperature. For long-term preservation, the isolate was also stored in 30% glycerol at -80°C. For the further molecular identification of the obtained isolate, the DNA was extracted, and PCR amplification of the 16S rRNA gene was carried out. The gel electrophoresis was performed to check whether the band one present or not. After the PCR amplification, the PCR product was purified and stored for a long time at – 20^0C. Further studies on molecular characterization of the

isolated bacteria, DNA sequencing, and phylogenic analysis are required to validate the isolate as a promising bioremediation agent. Furthermore, field research and other characterizations, such as structure elucidation, will demonstrate that there is potential. The future goal is to identify the bacterium isolate at the genomic level and also determine which stretches of DNA contain the genes that cause the decolorization and which stretches carry regulatory instructions, turning genes on or off. If everything goes as planned, this bacterium will undoubtedly contribute to the creation of a sustainable environment for our country.

5.2 Conclusion:

One of the industries that heavily uses synthetic chemicals as dyes is the textile industry. Textile dyes are the most significant polluters of the environment. Textile industry wastewaters pose a threat to the environment due to the large amount of chemically different dyes used. A large portion of these dyes end up in the environment through wastewater. Researchers are particularly interested in azo dyes due to their potential toxicity. Biological methods are a better way to remove this azo dye from textiles. Several microorganisms capable of dye decolorization at lab scale have been discovered in the past few decades, but there are few reports on their applicability in treatment processes. However, despite more than decades of concerted efforts, the microbial dye decolorization research still lacks direction in environmental context. Therefore, this research needs a framework to carry out environmentally relevant studies which take into account microbe-dye-environment interactions. In this study, the newly isolated bacteria *Actinobacillus rossi* (85.9% similarity) or *Acromonas salmonicida subsp. smithia* (82.2% similarity) has demonstrated potentiality for its 1% Reactive Violet 5R dye degradation within 48%. From the overall findings, it can be concluded that this isolated bacterium could effectively be used as an alternative to the physical and chemical processes of textile effluents as they have a high potential for being able to decolorize or degrade Reactive Violet 5R.

References

References

Aljamali, N. M. (2015). Review in Azo Compounds and its Biological Activity. *Biochem Anal Biochem, 4*(2), 169. doi:10.4172/2161-1009.1000169

Amin, A. S. (2017, May 31). Textile effluents, their treatment and discharge in Bangladesh. *Textile Today*.

Deepak Kumar Sharma, H. S. (2004). Isolation and characterization of microorganisms capable of decolorizing various triphenylmethane dyes. *Journal of Basic Microbiology, 44*, 59-65. Retrieved from https://doi.org/10.1002/jobm.200310334

Govindwar, R. G.-S.-S. (2008). Decolorization and biodegradation of reactive dyes and dye wastewater by a developed bacterial consortium. *Elsevier Ltd*, 999-1015. doi:10.1007/s10532-010-9360-1

Harshad Lade, A. K. (2015). BIODEGRADATION AND DETOXIFICATION OF TEXTILEAZO DYES BY BACTERIAL CONSORTIUM UNDER SEQUENTIALMICROAEROPHILIC/AEROBIC PROCESSES. *EXCLI Journal, 14*, 158-174.

Hayat Anouar, E. A. (2014). Density functional theory study of Reactive Violet 5R azo dye. *ISSR Journals, 2028-9324*(9), 1362-1367. Retrieved from http://www.ijias.issr-journals.org/

Hiroyuki Horitsu, M. T. (1977). Degradation of p-Aminoazobenzene byBacillus subtilis. *European J. Appl Microbiol., 4*, 217–224. Retrieved from https://doi.org/10.1007/BF01390482

James G. Cappuccino, N. S. (2014). Microbiology: A Laboratory Manual (Subscription), 10th Edition. In N. S. James G. Cappuccino, *Microbiology: A Laboratory Manual (Subscription), 10th Edition* (p. 560 pages). Pearson; 10th edition (January 24, 2013).

Jung, B., & Hoilat., G. J. (October 5, 2021.). MacConkey Medium. *StatPearls Publishing LLC*.

K. Chen, J. W. (2003). Decolorization of the textile dyes by newly isolated strains. *J. Biotechnology., 101*, 57-68.

K.M. Kodam, I. S. (2005). Microbial decolorization of reactive azo dyes under aerobic conditions. *World Journal of Microbiology & Biotechnology, 21*, 367–370. doi:10.1007/s11274-004-5957-z

Laila Hossain, S. K. (2018). Evaluation of present and future wastewater impacts of textile dyeing industries in Bangladesh. *Environmental Development, 26*, 23-33. Retrieved from https://doi.org/10.1016/j.envdev.2018.03.005

Maulin P Shah, K. A. (2013). Optimization of Environmental Parameters on Microbial Degradation of. *Journal of Bioremediation & Biodegradation*, 3-4. doi:10.4172/2155-6199.1000183

Md. Ekramul Karim, K. D. (2018). Decolorization of Textile Reactive Dyes by Bacterial Monoculture andConsortium Screened from Textile Dyeing Effluent. *Journal of Genetic Engineering and Biotechnology*. Retrieved from https://doi.org/10.1016/j.jgeb.2018.02.005

MIU Test: Uses, Principle, Composition, Procedure, Result Interpretation and keynotes. (2021, july 27). *Universe84a.com*.

Mohamed A. Hassaan, A. E. (2017). Health and Environmental Impacts of Dyes: Mini Review. *American Journal of Environmental Science and Engineering*, *1*(3), 64-67. doi:10.11648/j.ajese.20170103.11

N. Sriram, D. R. (2015). Isolation and Characterization of dye degrading bacteria from dye effluents. *Central European Journal of Experimental Biology*, 5-10.

Rashid Mahmood, F. S. (2014). Enhancing the Decolorizing and Degradation Ability of Bacterial Consortium Isolated from Textile Effluent Affected Area and Its Application on Seed Germination . *The Scientific World Journal* , 9.

Restiani, P. (2017). *WATER GOVERNANCE MAPPING REPORT:TEXTILE INDUSTRY WATER USE IN BANGLADESH*. SWEDEN TEXTILE WATER INITIATIVE.

Robinson T, M. G. (2001). *Bioresour Technol*, *77*, 247–55.

Saravanan, N. M. (2015). Isolation of dye degrading bacteria from textile effluent. *Journal of Chemical and Pharmaceutical Research*, 2214-2218.

Shrabana Sarkar, A. B. (2017). Degradation of Synthetic Azo Dyes of Textile Industry: a Sustainable Approach Using Microbial Enzymes. *Water Conserv Sci Eng*, 2, pages 121–131 .

Shuchismita Dey, A. I. (2015). A Review on Textile Wastewater Characterization in Bangladesh. *Resources and Environment*, 15-44.

Stipp, D. H. (2021, March 29). Number of garment factories in Bangladesh from 2010 to 2019. *Statista Research Department*. Retrieved from https://www.statista.com/statistics/987697/bangladesh-number-garment-factories/

Waleed Mohammed Sheet Alabdraba, M. B. (2014). Biodegradation of Azo Dyes—A Review. *International Journal of*, *1*(4), 179-189.

Zaharia Carmen, S. D. (2012). Textile Organic Dyes – Characteristics, Polluting Effects and Separation/Elimination Procedures from Industrial Effluents – A Critical Overview. *IntechOpen*.