UNIVERSITI
MALAYSIA
KELANTAN

Identification and Characterization of Batik Wastewater Bacteria and Determination of its Reactive Orange 16 Decolorization Ability

Nurul Najatul Alia binti Hussin

J20A0600

A reported submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bioindustry Technology) with Honours

FACULTY OF BIOENGINEERING AND TECHNOLOGY

UMK

2024

DECLARATION

I declare that this thesis entitled **Identification and Characterization of Batik Wastewater Bacteria and Determination of its Reactive Orange 16 Decolorization Ability** is the results of my own research except as cited in the references.

Signature : 

Student's Name : NURUL NAJATUL ALIA BINTI HUSSIN

Date : 18/1/2024

Verified by:

Signature : _____

Supervisor's Name : _____

Stamp : _____

Date : _____

ACKNOWLEDGEMENT

I would want to express my gratitude to Allah SWT, the Almighty, the Most Gracious, and the Most Merciful, for His blessings throughout the time I spent studying and finishing this thesis. The final prophet Muhammad (peace be upon him), his family, and his companion all get blessings from Allah SWT.

First of all, I want to express my biggest gratitude to my family and friends for their support and affection. They have consistently inspired me to follow my ambitions and shown faith in me. My parents, Hussin Bin Zainuddin (father) and Zakiah Binti Jaafar (mother), have always supported and encouraged me, and for that I am very thankful. Without them, I could not have completed this.

I would like to thank Dr. Wan Hasnidah binti Wan Osman, my thesis adviser, from the bottom of my heart for all of her help and support during my graduate studies, especially with Final Year Project 1. I would like to express my gratitude to Dr. Wee Seng Kew, my co-supervisor, for his thoughtful criticism and recommendations, particularly during Final Year Project 2. In every sense of the term, Dr. Wee Seng Kew has been a mentor to me, and I am very appreciative of his willingness to share his knowledge and skills with me.

I am also grateful to my fellow graduate students for their companionship and support. They provide a motivating and supportive atmosphere for learning and development. My special gratitude goes out to my friends (Syazwin Atika, Nurul Izzah, and Aina Natasha) for their support and friendship during my graduate studies, as well as for their readiness to assist me with my study.

With deep humility and a grateful heart, I acknowledge the blessings of Allah throughout this endeavour. My strength and resolve were fuelled by my faith, and I am eternally thankful for the guidance and support He provided me on this path. May this work serve as a testament to the power of faith and self-belief. Alhamdulillah, for the strength to persevere and the grace to see this endeavour through to completion.

Lastly, I would like to share one of Nelson Mandela's most inspirational quotes: "It always seems impossible until it's done." This quote helped me stay motivated during the writing of my thesis. It acted as a continual reminder that, with perseverance and determination, even the most overwhelming obstacles can be surmounted.

Identification and Characterization of Batik Wastewater Bacteria and Determination of its Reactive Orange 16 Decolorization Ability

ABSTRACT

Unlocking sustainable solutions for batik wastewater treatment remains a pressing challenge due to the prevalent use of harsh chemicals. In response, this study delves into a bioremediation approach, focusing on the decolorization of Reactive Orange 16, a ubiquitous azo dye found in batik wastewater. The objectives of this study are (1) Identification and characterization of bacteria isolated from batik industry wastewater using biochemical and molecular methods, and (2) Evaluation of the isolated bacteria's ability to decolorize Reactive Orange 16 dye. This study was isolated bacteria from batik wastewater that could decolorize Reactive Orange 16 dye. Molecular and biochemical techniques, including polymerase chain reaction (PCR) and 16S rRNA gene sequencing, were employed for the identification and characterization of the isolated bacteria. Subsequent BLAST analysis confirmed and classified the obtained sequences, enhancing the precision and reliability of the findings. The key findings reveal the successful isolation of three bacteria (Y1, P1, and W1) from batik wastewater, achieving a significant reduction to 2 mg/L within just six days. Through BLAST analysis, these bacteria were identified as Klebsiella sp., Citrobacter sp., and Gamma proteobacteria sp., respectively, underscoring their potential for bioremediation in batik wastewater treatment. The significance of strain Y1, identified as Klebsiella sp., for its rapid and efficient decolorization of Reactive Orange 16, highlights its potential for long-term bioremediation in the batik industry. This discovery not only demonstrates the efficacy of strain Y1, but also the larger potential of bacteria isolated from batik wastewater for environmentally friendly treatment methods.

Keywords: azo dye, Reactive Orange 16, isolated bacteria, decolorize, BLAST

Pengenalpastian dan Pencirian Bakteria Air Sisa Batik serta Penentuan Keupayaan Penyingkiran Warna Oren Reaktif 16.

ABSTRAK

Penyelesaian lestari untuk rawatan air sisa batik masih menjadi cabaran yang mendesak disebabkan penggunaan bahan kimia yang keras secara meluas. Sebagai tindak balas, kajian ini meneroka pendekatan bioremediasi, memberi tumpuan kepada penyingkiran warna Reactive Orange 16, sebatian pewarna azo yang biasa ditemui dalam air sisa batik. Objektif kajian ini adalah (1) Pengenalan dan pencirian bakteria yang dipisahkan daripada air sisa industri batik menggunakan kaedah biokimia dan molekul, dan (2) Penilaian keupayaan bakteria yang dipisahkan untuk menyingkirkan pewarna Reactive Orange 16. Kajian ini memisahkan bakteria dari air sisa batik yang dapat menyingkirkan pewarna Reactive Orange 16. Teknik-teknik molekul dan biokimia, termasuk reaksi rantai polimerase (PCR) dan pengurutan gen 16S rRNA, telah digunakan untuk pengenalan dan pencirian bakteria yang dipisahkan. Analisis BLAST seterusnya mengesahkan dan mengkelaskan jujukan yang diperoleh, meningkatkan ketepatan dan kebolehpercayaan dapatan. Dapatan utama mendedahkan pemisahan yang berjaya tiga bakteria (Y1, P1, dan W1) dari air sisa batik, mencapai pengurangan yang signifikan kepada 2 mg/L dalam tempoh hanya enam hari. Melalui analisis BLAST, bakteria-bakteria ini dikenal pasti sebagai Klebsiella sp., Citrobacter sp., dan Gamma proteobacteria sp., masing-masing, menekankan potensi mereka untuk bioremediasi dalam rawatan air sisa batik. Kepentingan strain Y1, dikenal pasti sebagai Klebsiella sp., untuk penyingkiran warna Reactive Orange 16 yang cepat dan cekap, menonjolkan potensinya untuk bioremediasi jangka panjang dalam industri batik. Penemuan ini tidak hanya menunjukkan keberkesanan strain Y1, tetapi juga potensi yang lebih besar bakteria yang dipisahkan dari air sisa batik untuk kaedah rawatan yang mesra alam.

Kata Kunci: pewarna azo, Oren Reaktif 16, bakteria yang diasingkan, pelunturan, BLAST

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BOD	Biochemical oxygen demand	3
COD	Chemical oxygen demand	3
DO	Dissolved oxygen	3
RO16	Reactive orange 16	3
PCR	Polymerase Chain Reaction	4
BLAST	Basic Local Alignment Search Tool	4
O2	Oxygen	14
OD	Optical density	23
UV	Ultraviolet	24

LIST OF SYMBOLS

L	Litre	1
^{o}C	Celsius	15
Ml	Millilitre	23
µL	Microlitre	23
Rpm	Revolution per minute	23
V	Volt	24
mA	Milliampere	24
Min	Minute	26
G	Gram	26
mg/L	Milligram per litre	27
Nm	Nanometre	27
Bp	Base pair	31
%	Percentage	32

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The widespread adoption of synthetic dyes in batik, fuelled by their economic appeal, raises concerns about the potential consequences for the preservation of traditional techniques, the environment, and the cultural identity embodied in this art form. In addition, it also has significant amounts of contaminants, such as high colour intensity, carcinogenic dyes, and harmful heavy metals. The effluents must be treated before release to ensure that they are safe, clean, and have no negative impact on the environment or human health . Boiling processes contribute significantly to organic pollutant loading, due to the frequent practice of releasing their effluents without proper treatment or with insufficient treatment (Zakaria et al., 2023).

Throughout the boiling phase in batik production, approximately 40 to 65 litres of wastewater are generated for every kilogram of fabric processed (Ghaly et al., 2014). This wastewater carries a substantial amount of dye remnants, the concentration of which fluctuates based on the specific dye molecules used (Yaseen et al., 2019). Additionally, industrial effluent comprises persistent compounds that pose challenges for natural degradation processes (Velusamy et al., 2021).

Moreover, in a specific batik industry, the discharge of effluents containing maroon-colored dyes and soda silicate, along with waste batik wax, has resulted in water pollution and soil contamination. Environmental concerns within the batik sector contribute to the degradation of plant life and wildlife habitats, as well as skin irritations such as allergies, sensitivities, and itching due to the presence of coloring agents and batik waxes. Additionally, individuals may suffer from burns caused by hot materials used in the process. Another issue in the batik business is air pollution. During the chanting process, wax was burnt, and the cloth was bathed in boiling water to remove the molten

wax. Air pollution was caused by the use of fire, whether it was made of wood or something else (Yaacob et al., 2015).

The most widely used artificial chemical in the paper and textile sectors is azo dye, which frequently reacts poorly to conventional wastewater treatment techniques. Large volumes of sludge are produced by physio-chemical treatment techniques, which are not commercially viable. As a result, more research is being conducted on their biodegradation (Yang et al., 2003). Azo reductase is the first enzyme involved in the bacterial decolorization of azo dyes because it breaks or reduces azo bonds in an anaerobic environment (Zimmermann et al., 1982). RO16 (reactive orange 16) is an anionic dye. Its limited biodegradability endangers the ecology since it affects the environmental system (Abdulhameed et al., 2019).

1.2 Problem Statement

There are significant amounts of pollutants in the wastewater from batik that are hazardous to human health and the environment. These contaminants include pigmentation, different compounds, dissolved and suspended materials, chemical and biological oxygen demand (BOD), and disagreeable odours (Zakaria et al., 2023). In addition to polluting the aquatic environment, wastewater from the batik industry, in particular wastewater in the form of liquid, can generate odour. The odour is caused by the suspension of solids from the batik colouring ingredient. High temperatures cause a decrease in dissolved oxygen (DO) level in water, which kills many organisms and disrupts the aquatic ecosystem's equilibrium. One potential respond to this problem is to use microorganisms in the batik industry to remove dyes (Muchtasjar et al., 2019).

Nevertheless, employing microorganisms for dye removal in this context presents notable limitations. A primary obstacle stems from insufficient understanding regarding the optimal conditions necessary for bacterial growth and efficacy within batik wastewater. The efficiency of bacterial dye removal may be affected by the wastewater's composition and the presence of additional pollutants.

Moreover, the selection of appropriate bacterial strains for dye elimination is paramount, yet it presents challenges given the diverse range of dye compositions and characteristics. Furthermore, the presence of other microorganisms in the wastewater may

disrupt bacterial growth and functionality through resource competition or inhibition of bacterial activity. Some dyes used in batik industry might be harmful or inhibitive to microbial organisms. Toxic dyes or their breakdown byproducts can inhibit microbial growth and activity, limiting their efficacy in dye removal. This toxicity problem is exacerbated if the effluent contains various colours or other chemical pollutants (Bal et al., 2022).

1.3 Objectives

The objectives of this study:

1. To identify the isolated bacteria collected from batik industry wastewater by biochemical and molecular methods.
2. To evaluate the ability of the isolated bacteria from batik industry wastewater to decolorize the Reactive Orange 16 dye.

1.4 Scope of Study

In this study, three unique bacterial species have been isolated from the wastewater of the Batik Industry, serving as the primary material for the current investigation. These bacteria were selected for their potential to decolorize the RO16 dye, aligning with one of the central objectives of this project.

In this research, gel electrophoresis was utilized to examine and segregate DNA fragments derived from bacteria. Subsequently, polymerase chain reaction (PCR) was employed for the detection and identification of bacteria. The sequencing method was instrumental in accurately identifying the bacteria being studied at a molecular level, entailing the precise determination of the arrangement of nucleotide bases (A, T, C, and G) within the bacterial DNA or RNA. The selected tool for this analysis was the Basic Local Alignment Search Tool (BLAST), which matches a given sequence against a database of known sequences to detect similarities or homologous sequences. BLAST aids in identifying and characterizing unknown sequences by comparing them with sequences that have established functions or annotations.

1.5 Significance of Study

The results of this research will contribute to mitigating water pollution, particularly stemming from dye contamination. Additionally, employing microorganisms for dye removal in the batik sector could prove economically advantageous in the long run. Despite potential initial expenses, cultivating bacteria can be done at a low cost and sustained through conventional bioreactor systems. Once established, operational costs may be lower compared to the continuous procurement and disposal of chemicals typically needed for traditional dye removal methods.

The use of microbes for dye removal in batik industry wastewater has the potential to provide several environmental benefits, as traditional dye removal methods frequently require the use of chemicals, such as oxidising agents or coagulants, which can introduce additional pollutants into the environment. The use of bacteria can minimise or eliminate the need for such chemicals, resulting in a more ecologically friendly method to colour removal.

Additionally, microbes can be used in a variety of wastewater treatment systems, ranging from small-scale batik businesses to major industrial plants. Their capacity to adjust to changing conditions makes them an adaptable option for a wide range of requirements. This work will provide further research, especially for firms hoping to employ microbes to reduce dye-related water pollution. Lastly, this study might advance science, especially in the area of wastewater dye removal.

CHAPTER 2

LITERATURE REVIEW

2.1 Dye

A wide range of industries employ dyes, including paper, paint, leather, food, and cosmetics. There are around twenty-five different kinds of dye groups based on the chemical structure of the chromophore (Benkhaya et al., 2020). Thousands of dyes are combined to create textile dyes, which are used to colour a variety of clothing items. Precursors to dyes are called dye intermediates. Many chemical techniques may be used to synthesise them from basic materials like benzene and naphthalene. (Abe et al., 2019).

2.2 Natural Dye

Natural dyes are colourants made from organic materials, mostly plants but sometimes minerals and animals. They are taken out using different techniques such as soaking, boiling, or grinding, and they are then used to colour wood, yarns, textiles, and other things. Natural dyes provide a multitude of advantages over synthetic dyes. Their biodegradability reduces their influence on the environment. They frequently have hypoallergenic qualities, which makes them kind to skin and appropriate for those with sensitive skin. Additionally, their distinct colours, which have minute variances and flaws, give fabrics and artwork a sense of rustic appeal. The majority of natural fibres, including cotton, linen, wool, and silk fibre, as well as certain synthetic fibres like nylon and polyester, are coloured using natural dyes. The primary obstacles faced by natural coloured fabrics, however, are shade reproducibility, the absence of a well-defined standard application procedure, and the shade's inadequate long-term performance under light and water exposure (Gupta, 2019).

2.3 Synthetic Dye

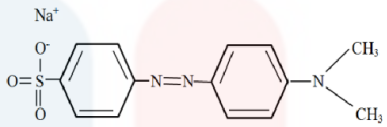
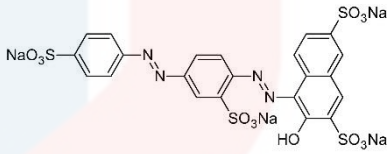
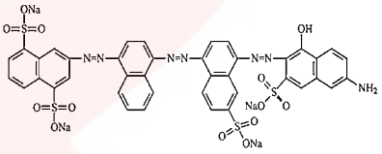
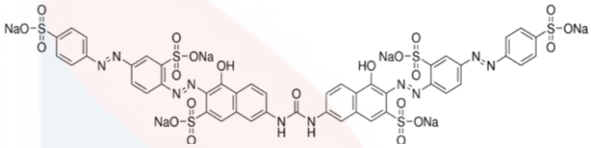
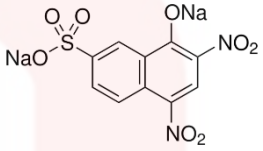
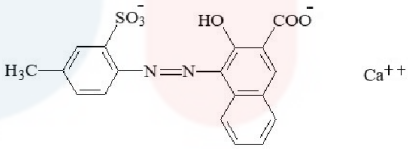
Synthetic dyes are colourants that are manufactured artificially through chemical reactions and procedures. To create their brilliant colours, they are generated from petroleum or coal tar and involve complicated organic compounds. Synthetic dyes are extremely difficult to remove. This is due to their complex aromatic structure, which gives physicochemical, thermal, and optical stability (Aljeboree et al., 2017). Due to their composition of compounds that were previously only found in coal tar, synthetic dyes are sometimes referred to as "coal tar dyes". They are classed depending on their chemical composition and how they are used in the dyeing process. Acid Dyes, Azo Dyes, Basic Dyes, and Mordant Dyes are a few examples.

2.4 Azo Dye

Azo dyes are the most common form of synthetic dye, responsible for a wide range of colours found on our clothing, food, and countless other items. Unlike their natural equivalents, these bright pigments are created in labs through complicated chemical reactions and procedures. Azo dye is commonly used as a dying procedure in the batik industry, and it has a variety of consequences on the water environment, including loss of dissolved oxygen, obstruction of aquatic photosynthetic activity, and toxicity to plants, animals, and people (Didier de Vasconcelos et al., 2021).

Azo dyes are chemical compounds that have an azo group present (-N=N-). This unique chemical connection is responsible for their brilliant colours. Consider small nitrogen atoms shaking hands and sharing a double bond that resonates with light, resulting in a kaleidoscope of colours depending on the molecules around them. Azo dye includes sulphide (SO_3^{2-}) or other electron donor groups, resulting in electron deficit; this process promotes azo dye breakdown by microbes (Barragán et al., 2007).

Table 2.1 Classification of azo dyes based on number of azo linkages.

Chromophore	Azo dye	Chemical structure
Monoazo	Methyl Orange	
Diazo	Red Ponceau S	
Triazo	Direct Blue 71	
Poliazo	Direct Red 80	
Naphthol	Naphthol Yellow S	
Azo lakes	Lithol Rubine BK	

2.4.1 Impact of Azo Dye

The primary consequences of breaking the main azo links, aromatic amines, are primarily responsible for the environmental concerns associated with the disposal of untreated azo dyes. These are considered to be significant carcinogens and represent a serious risk to human health. As a result, research efforts have been directed towards creating methods for handling industrial synthetic dye effluents, especially those that contain azo dyes. The suggested treatments included chemical, physical, and biological procedures, with a focus on bioremediation because of its excellent decolorization of azo dyes in contaminated environments and cheap cost. (A. L. Singh et al., 2015).

Table 2.2 Effect of Azo dye

Name of the dye	Effects	References
Reactive brilliant red	Inhibits function of human serum albumin	(Li et al., 2010)
Acid Violet 7	Induce chromosomal aberration, lipid peroxidation, acetyl cholinesterase in mice	(Ben Mansour et al., 2010)
Disperse Red -1 & Disperse Orange -1	Increase the frequency of micronuclei in human lymphocytes	(Chequer et al., 2009)
Reactive Black 5	Decrease urease activity, arginine ammonification rate in terrestrial ecosystem	(Topaç et al., 2009)
Disperse Blue 291	Mutagenic, Cytotoxic, genotypic effects, formation of micronuclei, DNA fragmentation in human hepatoma cells	(Tsuboy et al., 2007)
Direct Blue 1	Mutagenic	(Reid et al., 1984)

2.5 Reactive Orange 16 Dye

A RO16 (reactive orange 16) is classed as an azo reactive dye. This dye is one of the most widely used and widely produced reactive azo dyes. RO16 has excellent coloration qualities, is highly water soluble, and has excellent covalent bonding with both natural and synthetic textile fibres (Fungaro et al., 2013). This dye can pollute the environment since it is very poisonous to the environment and destructive owing to its mutagenesis qualities. Because it is water soluble, it may be easily absorbed by the body. As a result, it must be removed before the wastewater may be released into the environment (Vandevivere et al., 1998). RO16 and other synthetic colours are notoriously difficult to remove. Because of their complex aromatic structure, they exhibit physicochemical, thermal, and optical stability (Aljeboree et al., 2017).

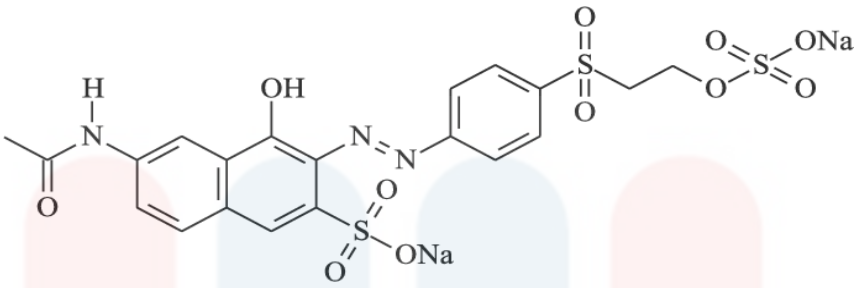


Figure 2.1: The chemical structure of RO16

(Source: (Gomes et al., 2011)

2.6 Biological Method

Utilizing biological approaches, such as employing biological processes like bioremediation, offers an environmentally friendly means of eliminating dye from textile wastewater, requiring minimal costs and shorter operational durations. Suggestions have been made to utilize biological agents such as algae, bacteria, fungi, and yeasts, which possess the ability to degrade and absorb different synthetic dyes (Ali et al., 2013). In this research, a biological method utilizing isolated bacteria is employed for removing dye from batik industry wastewater.

Table 2.3 : Method for dye removal

Method	Rationale of method	Advantages	Disadvantages	References
Biological method	Uses different microbes like bacteria, fungi, algae, yeast, and enzymes to degrade and decolourise dyes.	- Simple, economically attractive, and environmentally friendly process - Large number of species can be used in consortiums or pure cultures e.g., bacteria, fungi.	- Requires optimally favourable environment. - Requires management and maintenance of the microorganisms and/or physiochemical pre-treatment.	(Samsani et al., 2020)

- Good dye removal efficiency - High removal of biochemical oxygen demand and suspended solids (BAS) - Anaerobic bacteria are suitable for large scale application.	- Slow process - Generation of biological sludge and uncontrolled degradation products

2.7 Microbial

A microorganism, sometimes known as a microbe, is a living thing that is too tiny to view with the naked eye yet observable under a microscope. The study of microorganisms is known as microbiology. Microbes include bacteria, fungus, archaea or protists, viruses, and algae. Microbes can be found in the air, on our bodies, and in water. Microbes help to keep the environment clean by eliminating toxins from water and soil and decomposing organic debris from dead plants and animals. Although certain microbes are hazardous, the vast majority are really beneficial (Moellering Jr, 2011).

2.7.1 Pure Bacterial Culture

A pure bacterial culture is a colony of bacteria that has been cultivated under controlled conditions from a single cell. This indicates that there is genetic homology among all the bacteria in the culture. As if they were descended from a single ancestor, the strains are studied one at a time by researchers, preventing interference from other bacterial species. The genome sequencing of these strains and proteome analyses to determine specific proteins and evaluate their immunoproteomic antigenicity are made

possible by obtaining a pure culture of bacteria. In the end, this makes it easier for these proteins to be synthesised and used as antigens in serologic tests (Lagier et al., 2015).

2.7.2 Mixed Bacterial Culture

A mixed bacterial culture refers to a community of bacteria comprising two or more distinct strains or species coexisting within the same habitat. These mixed cultures exhibit greater microbial diversity compared to pure bacterial cultures, which consist of a single strain or species. Mixed bacterial populations can naturally occur in diverse environments, including soil, water bodies, and the human digestive system. They can also be intentionally cultivated or utilized for various purposes such as treating wastewater, remedying environmental contamination, and facilitating fermentation processes. Microorganisms serve as inherent agents for environmental purification. Research has shown that mixed bacterial or bacteria-fungus combinations exhibit enhanced capabilities for decolorizing dyes compared to pure cultures (Tang et al., 2022).

Table 2.4 : Biodegradation of dyes in coloured wastewater using bacterial cultures.

Dye/coloured wastewater studied	Organisms used	Comments	Reference number and year
Azo dyes	Bacillus cereus, Sphaerotilus natans, Arthrobacter sp., activated sludge.	Dye reduction under anoxic conditions involving non-enzymatic intracellular reduced flavin nucleotides	(Wuhrmann et al., 1980)
C.I. Acid Orange 6			
C.I. Basic Violet 1	Field-collected and laboratory cultures	Basic dye transformed under aerobic conditions	(Michaels et al., 1986)
C.I. Basic Violet 3			

87 different acid, basic, direct, mordant, and reactive dyes	Activated sludge	Short term aerobic biodegradation tests. Around 13% biodegraded and 23% absorbed	(Pagga et al., 1986)
C.I. Mordant Yellow 3 **C.I. Acid Red 27** **C.I. Acid Yellow 23 and 21**	Mixed bacterial consortium	Reduction of dye under anaerobic conditions followed by oxidation of amine metabolites after re-aeration. Aerobic desulphonation of sulphonated naphthalene	(Haug et al., 1991)
Reactive dyes	Anaerobic sludge	Reactive dye decolorised via reduction	(Senio et al., 1994)

2.8 Dye Removal Using Microorganism

Utilizing microorganisms or their enzymes, either alone or in combination with physicochemical methods, offers enhanced outcomes with greater cost-effectiveness. Microbes not only provide a non-toxic solution but also possess the capability to remove highly complex synthetic colors. The effectiveness of dyestuff treatment is influenced by the activity and adaptability of microorganisms. Microorganisms accomplish the decolorization of textile dyes through two mechanisms: adsorption onto microbial biomass or biodegradation of dyes by cells or enzymes (Jamee et al., 2019).

2.9 Bacterial Methods for Dye Decomposition

Oxidase enzymes in bacteria aid in the breakdown of synthetic colours. In the case of azo dyes, azo reductase is the most important enzyme in decolorization since it breaks down azo bonds. Some bacteria have been examined for their ability to breakdown

colours under aerobic conditions. Aerobic circumstances make it easier for mono- and dioxygenase enzymes to catalyse the incorporation of oxygen from O2 into organic molecules' aromatic rings (Sarayu et al., 2010) . Some aerobic bacteria use oxygen catalysed azo reductases to degrade azo compounds (Lin et al., 2010).

2.10 Factors Influencing the Performance by Bacteria in Decolorization.

2.10.1 pH

The most effective pH range for dye removal is often neutral or slightly alkaline; dye removal effectiveness is significantly reduced at very acidic or strongly alkaline pH values. As a result, coloured wastewater is frequently buffered to improve the cell culture's ability to remove colour. (Buthelezi et al., 2012). Since most industrial textile processes occur in alkaline environments, it is very crucial to be tolerant of high pH. Usually, the ideal range is between 6.0 and 10.0.

2.10.2 Temperature

High temperatures within a certain range accelerate colour fading in many systems; the exact range varies from system to system. The temperature needed to reach the maximum rate of colour removal corresponds with the cell culture's optimal growth temperature, which is normally between 35 and 45°C. Enzyme denaturation or a reduction in cell viability are the likely causes of the colour removal efficiency drop at higher temperatures. (Chang et al., 2001). Additionally, bacteria exhibit temperature ranges conducive to efficient growth and function, emphasizing the importance of maintaining suitable temperatures for successful color removal.

2.10.3 Dye Concentration

The efficiency of dye removal can be influenced by the concentration of the dye substrate, influenced by various factors. These include the dye's (and co-contaminants')

increased toxicity at increasing concentrations and the enzyme's capacity to effectively identify the substrate at extremely low quantities, which may exist in some wastewater samples. Elevated dye concentrations may surpass the bacteria's capacity to effectively degrade them. Moreover, complex dye structures or the presence of multiple colors may pose challenges for bacterial breakdown. When selecting suitable bacterial strains and optimizing process parameters, it's crucial to consider the unique properties of the colors involved.(Shah et al., 2013).

2.10.4 Dye Structure

The basic component of a dye that gives it colour is called a chromophore; it is a conjugated system of atoms that absorbs particular light wavelengths. The electronic transitions that occur within the chromophore, wherein the energy of absorbed photons stimulates electrons to higher energy levels, are correlated with this absorption. The exact wavelengths absorbed that result in the sense of colour are determined by the energy differential between these levels (Hunger, 2007).

Higher rates of colour loss are observed in dyes with simpler structures and lower molecular weights. It has been demonstrated that oxidation is influenced by the kind of substituents on the aromatic ring. Research has indicated that azo phenols are more easily broken down by enzymes when methyl and methoxy substituents provide electrons, but chloro, fluoro, and nitro substituents withhold electrons prevent oxidation (L. Singh et al., 2014).

2.11 Qualitative Study in RO16 dye

2.11.1 Spectrophotometer

The colour and strength of the dyestuffs itself, whether in solution or on the fibre, are the most important variables to measure, and the spectrophotometer is a physical instrument that is ideally suited for this task. In practical colour measurement applications, some of the methods for obtaining and interpreting spectrophotometric data

into usable information are discussed. Examples of these include measuring production dye-mixture solutions and print pastes to control colour before using on fabric, standardising both water soluble and some insoluble dyes, estimating dyes on fibre both qualitatively and quantitatively, and researching the mechanism of the dyeing process itself (Simon, 1949).

2.12 Qualitative Study in Identification and Characterization of Bacteria

2.12.1 Gel Electrophoresis

Based on their size and charge, DNA fragments (or other macromolecules like RNA and proteins) may be separated using the gel electrophoresis technique. A current is run through a gel containing the target molecules during the electrophoresis process. Depending on their size and charge, these molecules flow through the gel at different rates or in different directions, which helps to separate them from one another. As a result, DNA fragments are carefully separated according to size using gel electrophoresis. Nucleic acids can also be separated using agarose gel electrophoresis. Nucleic acid separation has been shown to be an effective and dependable process using gel electrophoresis. Large DNA fragments can be separated from low % gels thanks to agarose's high gel strength (Devor EJ, 2010).

2.12.2 Polymerase Chain Reaction (PCR)

The PCR method is based on the amplification of DNA sequences specific to a certain bacterium. This is accomplished by repeated heating and cooling cycles, which allow primers (short DNA sequences) to bind to target areas and DNA polymerase enzymes to synthesise complementary DNA strands. Each cycle exponentially raises the target DNA copy number, allowing detection of even small amounts of bacterial DNA. The design of primers is what gives PCR its specificity. These primers are designed to match specific sections of the bacterial genome, resulting in amplification of just the appropriate target sequences. This enables differentiation between closely related bacteria, which is a major improvement over older approaches.

There are various advantages of using PCR to identify microorganisms. Compared to culture-based approaches, PCR is quicker, frequently offering. For example, specific PCR methods are designed to identify the existence of antibiotic resistance genes in bacterial isolates or the presence of pathogenic microorganisms. It is a sensitive and fast technology that has revolutionised study and diagnosis of microorganisms. In PCR experiments, primers specific to certain sequences have been utilised to verify the existence or non-existence of target microorganisms or particular characteristics linked to them, such virulence and resistance to antibiotics. (Anderson et al., 2004).

2.13 Quantitative Study

2.13.1 Basic Local Alignment Search Tool (BLAST)

A bacterial DNA sequence is compared to a large database of known sequences using the Basic Local Alignment Search Tool, or BLAST. Consider it a massive repository of microbial blueprints. BLAST quickly searches through this library of DNA patterns, identifying the closest matches and finally providing the name of the bacteria. When studying bacteria and their enzymatic capabilities, the BLAST (Basic Local Alignment Search Tool) approach is often employed to compare and evaluate DNA or protein sequences (Rognes et al., 2016).

BLAST is used for much more than just naming bacteria. It helps quickly diagnose infections in clinical settings, which improves patient outcomes and allows for more tailored therapy. BLAST guarantees food safety in the food business by promptly detecting any infections that may be present in ingredients. Because BLAST can track individual bacteria in soil and water, it is useful for environmental monitoring and helps safeguard ecosystems.

Two approaches have been used in the past to find new enzymes. One method, known as "genome hunting," is locating open reading frames in a microorganism's genome. On the other hand, homology alignment of sequences stored in databases is necessary for data mining. The BLAST method is used to find conserved sections

between sequences, and homologous protein sequences are found to be good candidates for further investigation and characterisation (Luo et al., 2012).

Table 2.5: The possibility types of bacteria from BLAST analysis.

NO	SCIENTIFIC NAME	PERCENTAGE IDENTITY
1	*Pseudomonas sp. strain* MBL0288	99.90%
2	*Pseudomonas pseudoalcaligenes strain* ASW2	99.90%
3	*Pseudomonas khazarica strain* TBZ2	99.90%
4	*Pseudomonas sp. strain* PrPy123	99.90%
5	*Pseudomonas mendocina strain* CE13	99.90%
6	*Pseudomonas oleovorans strain* NIOSSD020#290	99.90%
7	*Stutzerimonas stutzeri strain* NIOSSD020#429	99.79%
8	*Pseudomonas sp. strain* B-BETUL-7	99.79%
9	*Pseudomonas sp. strain* V5R.22	99.69%
10	*Pseudomonas sp. strain* Bu15_40	99.69%

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Several raw materials were used in this project, including Peptone, Crystal Violet Solution, Iodine Solution, Acetone/Ethanol, Nutrient Agar, Reactive Orange 16 Dye, Hydrochloric Acid (HCL), and Sodium Hydroxide (NaOH), as well as three isolated microorganisms from wastewater from the batik industry (Y1, P1, W1).

Chemical nutrients include Nutrient Broth, Hydrogen Peroxide, Oxidase Reagent and TAE Buffer. Additionally, some manufacturer-provided kits were employed for this experiment, including the PCR Purification Kit and the PrimeWay Genomic DNA Extraction Kit.

3.2 Methods

Figure 3.1 shows the study flow chart for identifying isolated bacteria from batik wastewater and assessing R016 decolorization abilities.

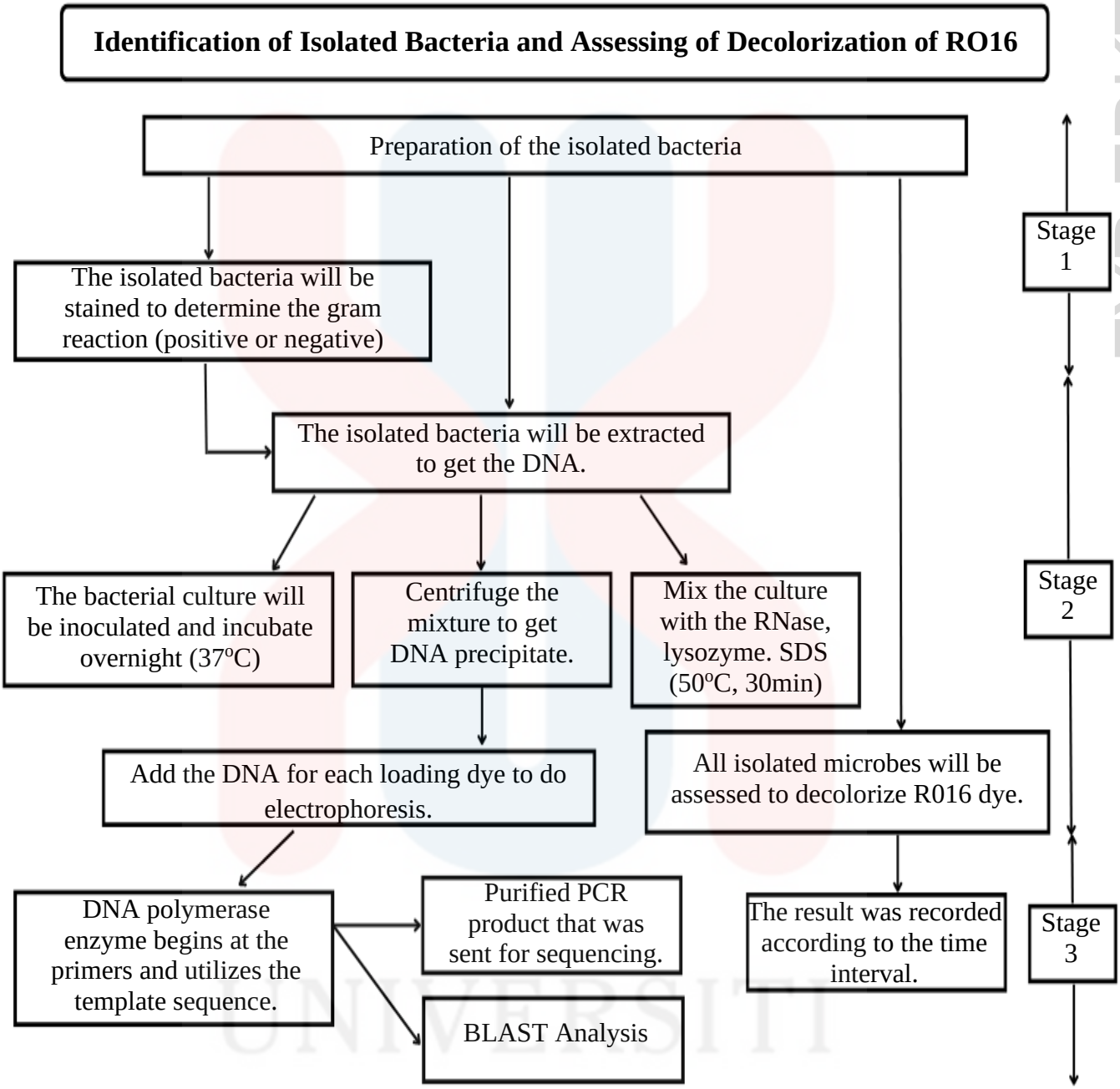


Figure 3.1: Research flow for identifying isolated bacteria from batik wastewater and assessing R016 decolorization abilities.

3.2.1 Gram staining

A single loop of saline solution was placed onto a dry, clean glass slide. Using a sterile loop, a single bacterial colony was collected from agar plates and spread into the saline solution on the slide. The smear was allowed to air dry before carefully passing the slide through a flame. Subsequently, the smear was stained with crystal violet for 1 minute, followed by a water rinse. Next, the smear was stained with iodine for 1 minute before another water rinse. Then, the smear underwent a 30-second wash with acetone followed by a water rinse. Finally, the smear was counter-stained with safranine for 1 minute, rinsed with water, and left to air dry. The slide was examined under the microscope with various lenses. As a result of the tests, bacteria with a blue or purple appearance were classified as Gram-positive, whereas bacteria with a pink appearance were classified as Gram-negative (Oztas Gulmus et al., 2020).

3.2.2 Biochemical test

The biochemical parameters of the isolates (oxidase and catalase) were determined using the procedures outlined by Prescott and Harley.

Catalase test

A little amount of bacteria was smeared over the dry slide with a sterilised inoculating loop. The bacteria's smear was then covered with one drop of hydrogen peroxide. The appearance of air bubbles as a result of the observation indicates that the test was positive, whilst the absence of bubbles indicates that the test was negative.

Oxidase test

The filter paper was soaked in the oxidase agent, N-tetramethyl-p-phenylenediamine dihydrochloride, and allowed to dry before picking the colony from the plate and rubbing it onto the filter paper. The result may be determined by examining the colour changes on the filter paper. If the colour changes, the outcome is positive; otherwise, the result is negative (Pollack et al., 2018).

3.2.3 DNA extraction using PrimeWay Genomic Kit (KIT-9020-50)

A 500 ml volume of nutritional broth has been prepared and the broth was poured into seven conical flasks, each with 50ml, and six test tubes, each with 5ml. The bacterial culture was then inoculated into six test tubes and incubated overnight at 37°C in an orbital shaker at 200 rpm. The next day, the inoculated bacteria was transferred from the test tubes to the conical flask. It was then incubated for four hours at 200 rpm in orbital shaker 37°C. Next, the spectrophotometer was used to measure the bacterial culture's OD size.

Genomic DNA extraction was conducted using the PrimeWay Genomic DNA Extraction Kit (no: KIT-9020-50) according to the manufacturer's instructions. A 1 ml aliquot of bacterial culture was centrifuged at 8000 rpm for 5 minutes, and the supernatant was discarded. The bacterial pellet was then suspended in 180µl TLB1 Buffer along with 25µl proteinase K solution and vortexed. Subsequently, the sample was incubated at 56°C for 1 hour before another vortexing step. Following this, 200µl TLB2 Buffer was added, and the mixture was incubated at 70°C for 10 minutes. The sample was then mixed with 210µl ethanol using vortexing. A PrimeWay Genomic Column was placed in a collection tube, and the lysate was loaded onto the column and centrifuged for 1 minute.

The PrimeWay Genomic Column was loaded with 500µl of Wash Buffer T1 and centrifuged for 1 minute. The liquid flow-through was discarded, and the column was repositioned into the tube. Following this, the column was filled with 600µl of Wash Buffer T2, centrifuged for 1 minute, and the flow-through was discarded. The column was then placed back into the tube, and the tube was centrifuged for an additional minute to remove any remaining ethanol residue. Subsequently, the column was transferred to a new centrifuge tube, and 100µl of Elution Buffer was added to the centre of the column membrane. After standing for 1 minute, the DNA was eluted by centrifugation for 1 minute.

3.2.4 Agarose gel electrophoresis

1ml of 6X loading dye was placed onto the parafilm. Using a micropipette, 5ml of DNA was added to each loading dye and the mixture was mixed by pipetting up and down. For the marker, 1:1:4 ml of Hindill; loading dye; deionized water was pipetted up

and down onto the parafilm and mixed. The solution was then transferred into the gel well. The marker filled the first lane. The gel tank was closed, the power supply was turned on, and the gel was run for 45 minutes at 80V and 200mA. After finishing, the power was turned off, the leads were disconnected, and the gel was removed from the casting tray. Finally, the gel was analysed using a UV transilluminator.

3.2.5 Polymerase Chain Reaction (PCR)

The genome of the unknown bacterial strains will serve as the template DNA. Universal primers (forward primer 68F and reverse primer 1392F) were used to amplify the DNA's 16S rRNA gene region. The 50µl total volume used for the amplification reaction had the following components: deionized water (40.5µl), dNTPs (1µl), 10X Taq Buffer contain $MgCl_2$ (5µl), forward primer 68F (1µl), reverse primer 1392 (1µl), Taq DNA Polymerase (0.5µl), and DNA template (1µl). The PCR process was run as follows: 1 cycle (pre-denaturation) at 94 C for 4 minutes, 30 cycles (denaturation) at 94 C for 1 minute, 30 cycles (annealing) at 53.8 C for 1 minute, 30 cycles (extension) at 72 C for 1 minute, and 1 cycle (final extension) at 72 C for 7 minutes.

The PCR-amplified samples underwent examination through agarose gel electrophoresis. Following this, the PCR products were purified and sequenced using methods outlined in previous studies. Utilizing an appropriate marker, the size of the band produced by the product's gel run was determined. Subsequently, the band was carefully excised from the gel under UV light, and purification was conducted in accordance with the PCR purification technique provided by the manufacturer (PCR Purification Kit no: KIT-9050-50). The PCR products were then sent for sequencing analysis through service procurement, and the sequencing data (results) were analysed using GenBank.

Table 3.1: PCR reaction component and their required volume

For a 50 µL reaction volume:	
Components	**Volume**
Deionized water/Nuclease water	40.5 µl
dNTPs	1 µl
10X Taq Buffer contain $MgCl_2$	5 µl
Forward primer 68F	1 µl
Reverse primer 1392	1 µl
Taq DNA Polymerase	0.5 µl
(PCR product) DNA template	1 µl

Table 3.2 : PCR Reaction Condition

Steps	Temperature (°C)	Time (min)	Cycle
Pre-Denaturation	94	4	1
Denaturation	94	1	30
Annealing	53.8	1	30
Extension	72	1	30
Final Extension	72	7	1

3.2.6 Blast Analysis Of 16s rRna Gene Sequence

BLAST alignment compares each location of the search sequence to each position of the sequences in the database. If the nucleotides match, BLAST provides a positive

score to that site. During alignment, BLAST may introduce gaps. Each gap insertion lowers the alignment score; however, if enough nucleotides align as a result of the gap, the negative impact is balanced, allowing the gap in the alignment. The most similar sequence appears at the top of the BLAST results list.

3.2.7 Preparation of RO16 Dye

In order to prepare RO16, 1 g of dye was mixed and dissolved in 800 ml of distilled water. The volume was then adjusted once again by adding distilled water. In order to get the mixture's pH down to 8.0, 0.1 M hydrochloric acid (HCL) or NaOH were added. The solution will then be stored in a bottle of dark reagent.

3.2.8 Preparation of Dye Calibration Curve

In order to plot the calibration curve, RO16 dye solution was prepared from dye standard stock solution by utilizing the dilution technique. A spectrophotometer was used to get readings of 2, 4, 6, 8, and 10 mg/L. Then plotted the absorbance curve after that.

The following formula was used to calculate the volume of stock solution needed for each dilution:

Volume of stock solution (mL) = (Desired concentration (mg/L) * Final volume (mL)) / Stock concentration (mg/L)

3.2.9 Decolorization of RO16 Dye Using Bacterial Culture

For this experiment, the dye solution was prepared with a concentration up of 6 mg/L. For an interval of 24 hours, three separate sterile conical flasks containing different bacterial cultures were inoculated aerobically at 37°C in a 200-rpm rotary shaker. Within 24 hours, the exact same quantity of already made dye solution was added to each flask. One control flask was also prepared, but it contained no bacteria and only sterile dye and nutritional broth. Using a micropipette, 1 ml aliquots of the culture medium were taken out of each flask at various intervals. After being transferred to the centrifuge tube, each

aliquot was centrifuged for 20 minutes at 10,000 g. From each tube, the clear supernatant was removed for further observation.

3.2.10 Analysis of Dye Decolorization Using Spectrophotometer

Each cuvette has been filled with the prepared samples. At a wavelength of 493 nm, the absorbance value of the cuvettes was measured in the spectrophotometer. The data for each value was measured and recorded to plot the graph.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The biochemical and molecular methods for identifying bacteria from batik industry wastewater will be covered in this chapter. Understanding the microbial composition in such wastewater is crucial due to its environmental impact and implications for wastewater treatment processes. The identification of specific bacterial strains holds significant importance, particularly in the context of developing effective remediation strategies and mitigating environmental pollution. Each bacterial strain's DNA was extracted, and subsequent analysis utilizing polymerase chain reaction (PCR) techniques facilitated their identification. The utilization of NCBI BLAST and MEGA 11 software enabled precise identification of each bacterium, while sequencing provided further insights into their genetic composition. Additionally, quantifying the decolorization of Reactive Orange 16 (RO16) through spectrophotometric analysis provides valuable insights into the efficacy of bacterial strains in pollutant removal.

4.2 Isolated Bacterial Culture, Gram Staining. Biochemical Test

In this study, three microorganisms were identified in nutrient agar using the streaking technique, as indicated in table 4.1. The streaking technique was employed for bacterial isolation due to its effectiveness in obtaining pure bacterial cultures from mixed samples. This method involves spreading a diluted sample of the wastewater onto the agar surface in a systematic manner, allowing individual bacterial colonies to form and be

isolated for further analysis. These particular strains, Y1, W1, and P1, were selected based on their distinct morphological characteristics observed during initial observation.

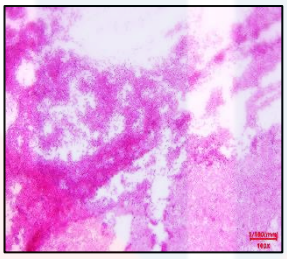
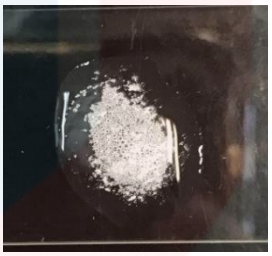
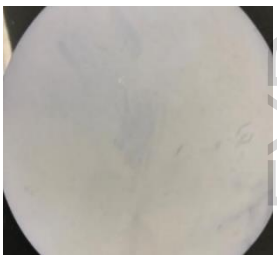
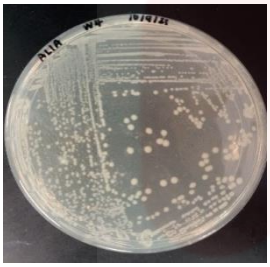
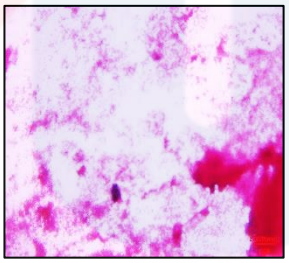
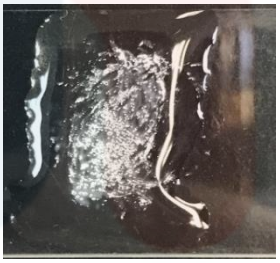
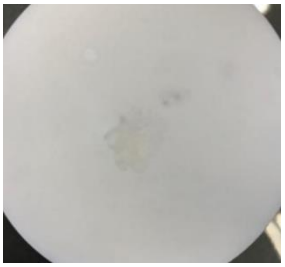
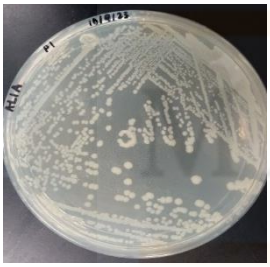
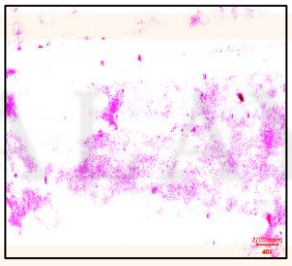
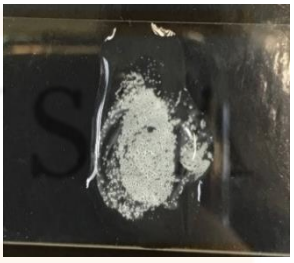
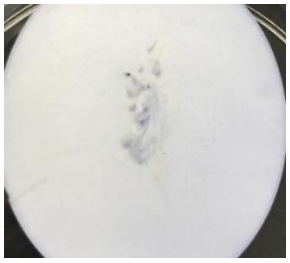
The initially observed bacteria in the observation were identified as Y1, the second microbes were identified as W1, and the last microbes were identified as P1. These bacteria were then examined by gram staining and biochemical tests (oxidase and catalase) to further characterize their properties. Additionally, their DNA was extracted and subjected to molecular identification through polymerase chain reaction (PCR) analysis.

The gram staining test revealed that the Y1 strain, which was classified as Gram-positive because of its thick peptidoglycan cell wall, kept the crystal violet stain. The existence of the enzyme that degrades hydrogen peroxide is shown by its catalase-positive character, which is demonstrated by the creation of bubbles. But the negative oxidase test indicates the absence of cytochrome c oxidase, which points to a lack of ability to use aerobic respiration. Put more simply, the Y1 strain cannot rely on oxygen for energy but instead possesses a thick cell wall and the ability to degrade hydrogen peroxide.

W1 had a gram-negative characteristic and a thin peptidoglycan cell wall that was encased in an outer membrane. Unlike their Gram-positive counterparts, Gram-negative bacteria are able to easily decolorize during the Gram staining process, indicating their identification. The positive catalase test result indicated the existence of the enzyme, which was further examined. This enzyme is essential for the detoxification of hydrogen peroxide, which poses a possible risk to cells. On the other hand, the oxidase-negative finding suggested that cytochrome c oxidase was absence, which is a critical part of the electron transport chain that is essential for aerobic respiration.

The P1 strain sustained the crystal violet stain because of its strong cell wall, which is mostly made of peptidoglycan, and displayed typical Gram-positive characteristics. This characteristic is consistent with a positive catalase test, which shows that the catalase enzyme is present and effectively breaks down hydrogen peroxide into components that are safe for consumption. Moreover, the positive oxidase test indicates that P1 has cytochrome c oxidase, which is an essential part of the electron transport chain that facilitates aerobic respiration. In addition, these data depict a gram-positive bacterium that possesses the metabolic machinery necessary for effective oxygen consumption in addition to defence mechanisms against oxidative stress.

Table 4.1 The characterization of isolated bacteria

Sample Name	Isolated Bacteria	Gram Staining	Catalase Test	Oxidase Test
Y1				
	Colony colour: Yellowish Colony form: Circular	Colour: Pink Outcome: Gram negative	Outcome: Positive	Outcome: Negative
W1				
	Colony colour: White Colony form: Circular	Colour: Pink Outcome: Gram negative	Outcome: Positive	Outcome: Negative
P1				
	Colony colour: Pink Colony form: Circular	Colour: Pink Outcome: Gram negative	Outcome: Positive	Outcome: Positive

4.3 Gel Electrophoresis For PCR

Gel electrophoresis serves as a molecular detective by revealing a molecule's size, purity, and amplification success. The process involves subjecting DNA fragments to an electric field within a gel matrix, causing them to migrate based on size. The gel composition typically consists of agarose, providing a porous medium through which DNA molecules can travel. In this procedure, gel electrophoresis was conducted at 80V and 200mA for 45 minutes, ensuring optimal separation of DNA fragments. The results, displayed graphically on the gel, allow for the visualization of amplified PCR products. To aid in determining the sizes of DNA fragments, the HindIII marker, a pre-digested DNA combination with known fragment sizes, was utilized. By running the HindIII marker alongside the PCR product, the sizes of the unknown fragments can be compared to the known sizes of the marker bands.

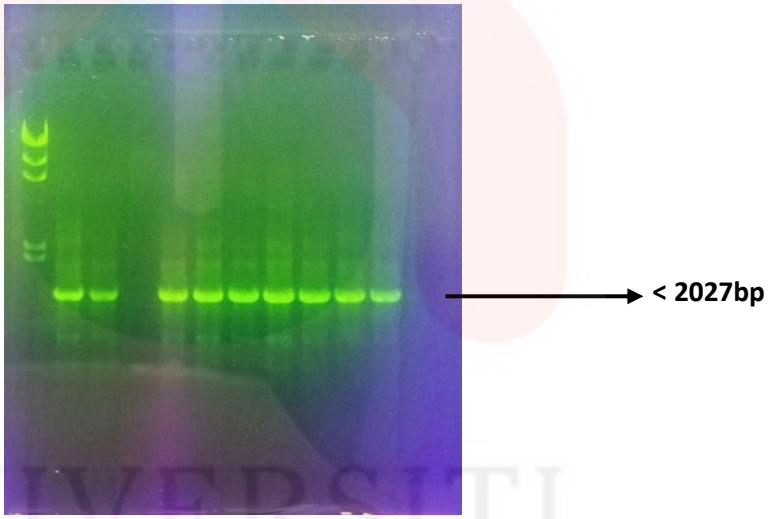


Figure 4.1 Gel electrophoresis band for PCR product.

4.3.1 16S Rrna Gene Sequence Analyses

In this study, the 16S rRNA gene sequences were amplified using universal primers and subsequently subjected to gel electrophoresis for visualization and confirmation of amplification. Following this step, BLAST searches were conducted to compare the obtained sequences with those deposited in the NCBI database. The presence of varying numbers of base pair nucleotides among the isolates further underscores the genetic diversity within the bacterial community.

The accession numbers documented in Table 4.4 provide a reference to the specific sequences in the NCBI database, facilitating further analysis and comparison with existing data. Additionally, each isolated microbe is associated with a unique percentage identification value, reflecting the degree of similarity between its sequence and those in the database.

The 16S rRNA sequencing of the first strain Y1 revealed a potential genetic resemblance to other known bacterial strains, specifically Klebsiella pneumoniae and Klebsiella quasipneumoniae, with a highest percentage identity of 97% in the BLAST search. These close matches on both forward and reverse strands suggest a high level of sequence conservation, implying functional relevance of the targeted genes shared by these closely related bacteria. In the context of industrial wastewater treatment, the presence of Klebsiella species like K. pneumoniae is significant as they are known for their ability to degrade various organic pollutants and may play a role in bioremediation processes.

Strain W1 exhibited promising matches with Citrobacter sp. and Citrobacter amalonaticus, both members of the Citrobacter genus, with a high percentage identity of 93% in nucleotide databases. The conservation of sequences in both forward and reverse strands suggests potential functional similarities among the discovered genes. Citrobacter species are known for their metabolic versatility and ability to degrade a wide range of organic compounds. In the context of industrial wastewater treatment, strains like W1 may offer bioremediation potential for pollutants commonly found in wastewater effluents.

The final strain, P1, demonstrated considerable matches with uncultured gamma proteobacteria, particularly with a strain named "Uncultured gamma proteobacterium isolate DGGE gel band 2 16S ribosomal RNA gene, partial sequence," with a high degree of sequence conservation reaching up to 100% identity. While there is no cultured reference sequence, the shared genetic composition suggests potential evolutionary and ecological traits shared between P1 and these uncultured bacteria. In the context of industrial wastewater treatment, the presence of gamma proteobacteria may indicate their involvement in various biogeochemical processes, including nutrient cycling and pollutant degradation.

Table 4.4 The potential of strain hits by Y1 forward and reverse strand in BLAST

No	Description	Max Score	Total Score	Query Cover	E-Value	Percentage Identity
1	Klebsiella quasipneumoniae strain L22 chromosome, complete genome	453	3584	36%	3e-122	97%
2	Klebsiella pneumoniae strain KPLSN chromosome, complete genome	453	3584	36%	3e-122	97%
3	Klebsiella pneumoniae strain KPLSX chromosome, complete genome	453	3584	36%	3e-122	97%
4	Klebsiella pneumoniae strain S-P-N-054.01 chromosome	451	3526	36%	1e-121	97%
5	Klebsiella quasipneumoniae strain SH1GKU 16S ribosomal RNA gene, partial sequence	449	449	36%	4e-121	97%
6	Klebsiella pneumoniae gene for 16S rRNA, partial sequence, strain: NBRC 14441	449	449	36%	4e-121	97%

Table 4.5 The potential of strain hits by W1 forward and reverse strand in BLAST.

No	Description	Max Score	Total Score	Query Cover	E-Value	Percentage Identity
1	Citrobacter amalonaticus strain JVSP20 16S ribosomal RNA gene, partial sequence	259	259	87%	2e-64	93%
2	Citrobacter sp. 387F 16S ribosomal RNA gene, partial sequence	255	255	87%	2e-63	93%
3	Citrobacter amalonaticus strain ThlvkrLrS1 16S ribosomal RNA gene, partial sequence	254	254	87%	8e-63	93%
4	Citrobacter amalonaticus strain JVSP20 16S ribosomal RNA gene, partial sequence	259	259	87%	2e-64	93%
5	Citrobacter sp. strain UYEF32 16S ribosomal RNA gene, partial sequence	254	254	87%	8e-63	93%
6	Citrobacter sp. strain strain shz1 16S ribosomal RNA gene, partial sequence	254	254	87%	8e-63	93%

Table 4.6 The potential of strain hits by P1 forward and reverse strand in BLAST.

No	Description	Max Score	Total Score	Query Cover	E-Value	Percentage Identity
1	Uncultured gamma proteobacterium isolate DGGE gel band 2 16S ribosomal RNA gene, partial sequence	69.4	69.4	7%	8e-07	100%
2	Chromohalobacter israelensis partial 16S rRNA gene, isolate israelensis 2	62.1	62.1	7%	1e-04	97%
3	Chromohalobacter israelensis partial 16S rRNA gene, isolate israelensis 2	69.4	69.4	9%	8e-07	95%
4	Uncultured gamma proteobacterium isolate DGGE gel band 2 16S ribosomal RNA gene, partial sequence	80.5	80.5	12%	4e-10	91%

4.4 Standard Curve Preparation of Reactive Orange 16

A standard curve for RO16 was established within the concentration range of 2 to 10 mg/L by diluting a 1000 mg/L stock solution of RO16 with distilled water. The absorbance reading of RO16 was measured using a spectrophotometer at a wavelength of 493 nm. Subsequently, the collected data was used to plot the graph.

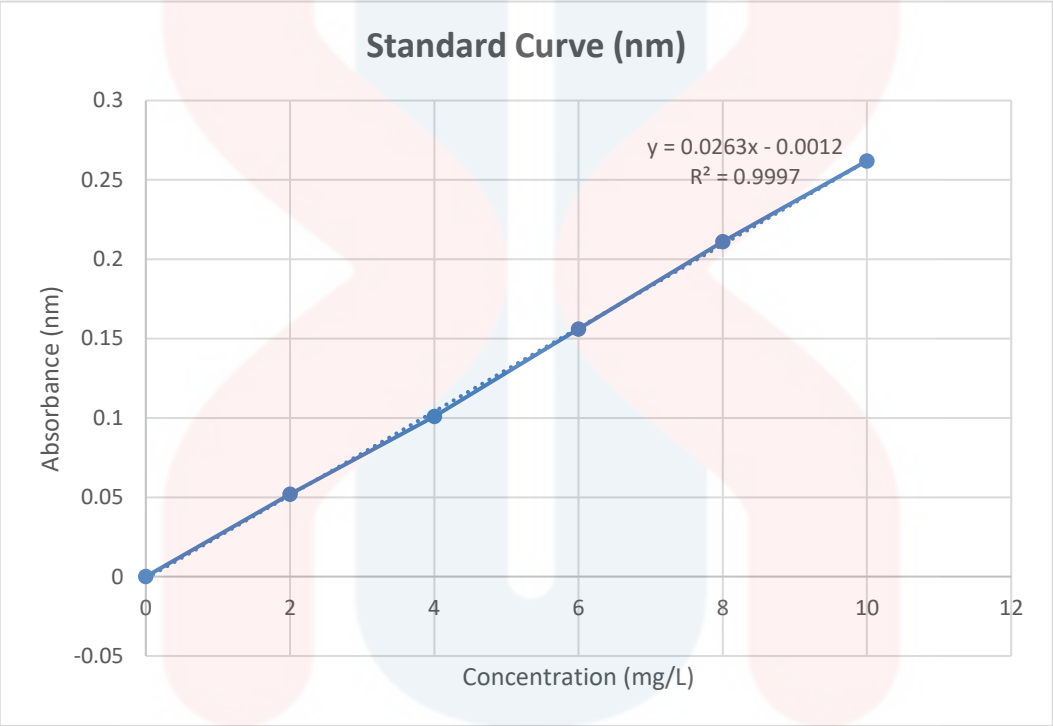


Figure 4.2 Preparation of Dye Calibration curve of RO16

4.5 Decolorization assay of Reactive Orange 16

The isolated bacteria Y1, W1, and P1 have demonstrated their capability to decolorize Reactive Orange 16 (RO16), as depicted in Figure 4.3. The choice of Reactive Orange 16 as the target for decolorization was motivated by several factors. Firstly, RO16 is a commonly used azo dye in the textile industry, including in batik production, and its discharge into wastewater streams poses environmental concerns due to its persistence and potential toxicity. Secondly, azo dyes like RO16 are known to be challenging to degrade using conventional treatment methods, often requiring harsh chemicals or advanced oxidation processes, which can be costly and environmentally damaging. By demonstrating the decolorization ability of the isolated bacteria towards RO16, this study contributes to the development of eco-friendly strategies for treating wastewater

contaminated with azo dyes, thereby addressing both environmental and industrial concerns.

4.5.1 By using decolorize concentration

RO16 concentration was calculated using their absorbance value, as illustrated in figure 4.3. Figure 4.3 shows the data as a line graph with concentration (mg/L) on the y-axis and time (in days) on the x-axis. The graph is constructed from up of four lines: one for the control (no strain) and three for the three strains, Y1, P1, and W1.

As the concentration of RO16 decreased over time for each strain, it became obvious that all three strains were successful to some extent in decolorizing RO16 overall. The most efficient strain was strain Y1, which was followed by strains P1 and W1. On the sixth day, the RO16 concentration had been decreased to around 2 mg/L by Y1, P1, and W1. None of the strains completely decolorized RO16 by day 6, the end of the experiment.

Strain Y1 exhibited the quickest rate of decolorization, as seen by its steeper slope on the line graph, which represents the rate of decolorization. Throughout the experiment, it rapidly decolorized RO16 and decreased its concentration by approximately 30 mg/L on the first day. Although strain P1 decoloured more slowly than strain Y1, it did so yet more quickly than strain W1. In the first day, there was a 20 mg/L drop in its RO16 concentration, which subsequently slowed down. The pace at which strain W1 decolorized was the slowest. After the first day, the concentration of RO16 was only lowered by around 10 mg/L, and it subsequently remains rather constant.

In terms of decolorization rate and efficiency, Y1 emerged as the obvious effective. In the shortest amount of time, it reached the lowest concentration of RO16. Although P1 was slower and did not reach as low of a concentration as Y1, it was still somewhat effective. Of the three strains, W1 was the least productive. It reduced the RO16 concentration to a modest degree and had the slowest rate of decolorization.

The differences in decolorization capabilities across strains might be explained by a variety of factors. These could include the possibility that various strains have varying metabolic capacities, with some having a higher capacity to break down RO16 than others. Subsequently, the strains' capacity to decolorize RO16 may be impacted by

differences in the enzymes they produce or the degrees to which they are active. Furthermore, certain strains could be able to decolorize RO16 more successfully than others because to their increased tolerance to it.

In the context of the batik industry and wastewater treatment, these findings have significant implications. The demonstrated effectiveness of strains Y1, P1, and W1 in decolorizing RO16 highlights the potential for employing microbial agents in the treatment of batik industry wastewater contaminated with azo dyes. By harnessing the decolorization capabilities of these bacterial strains, it may be possible to develop sustainable and cost-effective treatment strategies for mitigating the environmental impact of dye discharge.

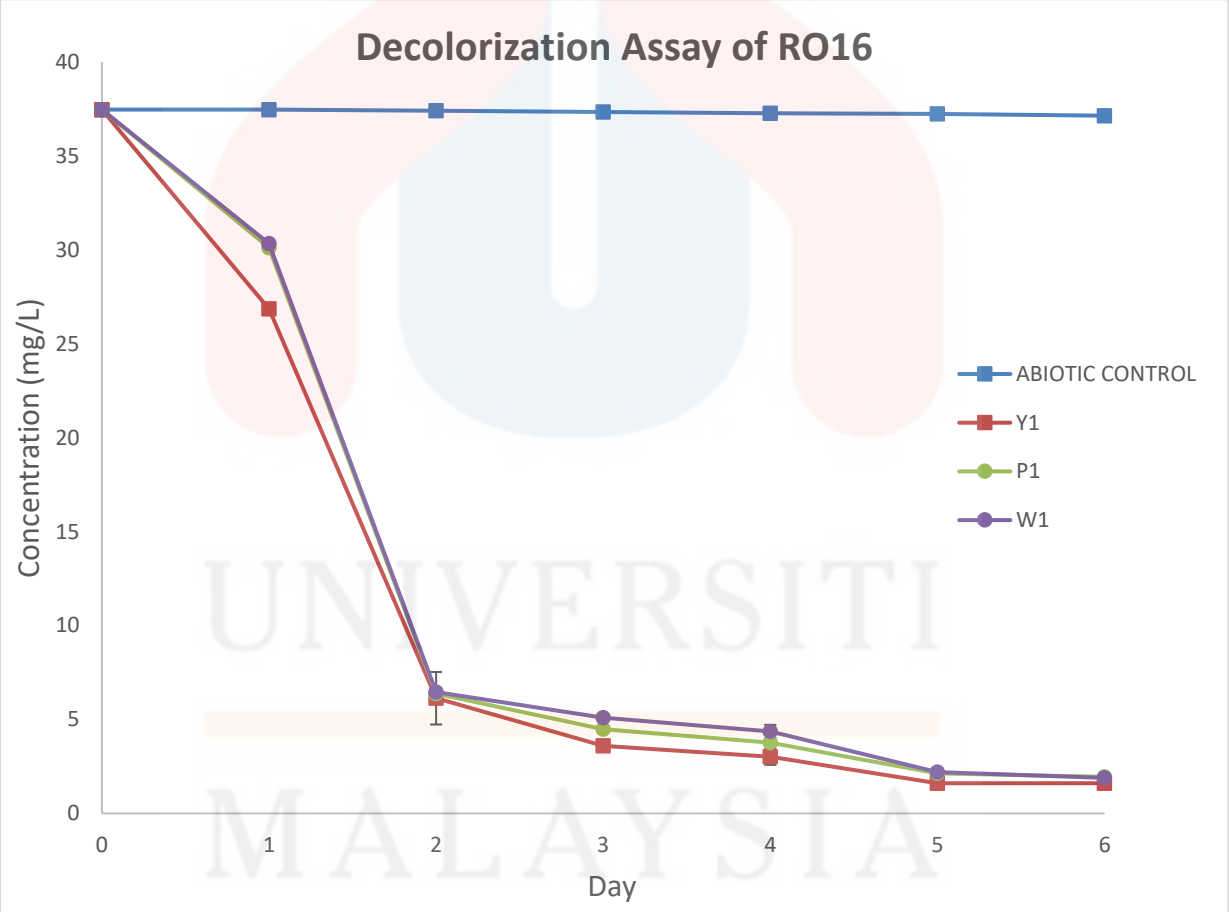


Figure 4.3 Decolorization Assay of RO16

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In summary, this study investigated the potential of bacteria isolated from batik industry wastewater for bioremediation of Reactive Orange 16 (RO16), a commonly used azo dye known for its persistence and toxicity in the environment. Key findings reveal three promising bacterial isolates, Y1, P1, and W1, demonstrating significant decolorization capabilities against RO16. Notably, isolate Y1 emerged as the most efficient decolorizer, achieving a remarkable decolorization rate exceeding 96% within a six-day period.

Further characterization through BLAST analysis identified these isolates as Klebsiella sp., Citrobacter sp., and Gamma proteobacteria sp., respectively. These taxonomic affiliations are significant as these bacterial genera are well-documented for their ability to degrade various azo dyes, including RO16.

These findings provide compelling evidence for the potential utilization of bacteria isolated from batik wastewater as a sustainable and environmentally friendly approach for RO16 bioremediation. Compared to traditional wastewater treatment methods relying on harsh chemicals, bioremediation offers a more environmentally sound alternative with minimal impact.

However, further research is necessary to optimize the decolorization process for enhanced efficacy and scalability. This includes investigating factors such as optimizing growth conditions, exploring different dye concentrations, and potentially implementing co-culture strategies with other decolorizing bacteria. Additionally, larger-scale studies are crucial to assess the feasibility and economic viability of utilizing these bacteria in practical wastewater treatment applications within the batik industry.

In conclusion, this study lays the groundwork for the development of sustainable bioremediation strategies, offering a promising solution to mitigate the environmental impact of azo dyes in the batik industry and beyond.

5.2 Recommendations

Based on the success of this study in identifying decolorizing bacteria from batik wastewater, further exploration is warranted to optimize the bioremediation of Reactive Orange 16 (RO16) and potentially other azo dyes. To enhance efficacy and pave the way for larger-scale applications, several specific recommendations are proposed.

Firstly, it is recommended to investigate the optimal temperature, pH, and nutrient composition for each bacterial isolate to maximize their decolorization activity. This could involve conducting experiments to determine the temperature range (e.g., 25°C to 40°C) and pH levels (e.g., pH 6 to pH 9) at which the bacterial isolates exhibit the highest decolorization rates. Additionally, assessing the impact of different nutrient compositions, such as carbon and nitrogen sources, on decolorization efficiency could further optimize the process.

Next, it is suggested to evaluate the potential for synergistic effects by co-culturing different decolorizing isolates or combining them with other dye-degrading microorganisms. By investigating the interactions between bacterial strains and their collective ability to degrade RO16, synergistic effects may enhance overall decolorization efficiency. Prioritizing the identification of compatible bacterial combinations and optimizing co-culture conditions could amplify decolorization rates and expand the applicability of bioremediation strategies.

Furthermore, analyzing the cost-effectiveness of using these bacteria compared to traditional treatment methods is essential. This assessment should consider factors such as initial setup costs, operational expenses, and environmental benefits associated with bioremediation. By quantifying the economic feasibility and environmental advantages of bacterial bioremediation, decision-makers can make informed choices regarding the adoption of sustainable treatment technologies.

Implementing these recommendations can refine and optimize the bioremediation process, paving the way for a sustainable and environmentally friendly solution for treating azo dye-contaminated wastewater in the batik industry and beyond. By addressing specific parameters, exploring synergistic effects, and assessing cost-effectiveness, the practical implications of these recommendations include improved treatment efficiency, reduced environmental impact, and enhanced feasibility for industrial-scale implementation.

REFERENCES

Abdulhameed, A. S., Mohammad, A., & Jawad, A. H. (2019). Modeling and mechanism of reactive orange 16 dye adsorption by chitosan-glyoxal/TiO2 nanocomposite: application of response surface methodology. *Desalination and Water Treatment, 164*, 346-360.

Abe, F. R., Machado, A. L., Soares, A. M., de Oliveira, D. P., & Pestana, J. L. (2019). Life history and behavior effects of synthetic and natural dyes on Daphnia magna. *Chemosphere, 236*, 124390.

Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals—concepts and applications. *Chemosphere, 91*(7), 869-881.

Aljeboree, A. M., Alshirifi, A. N., & Alkaim, A. F. (2017). Kinetics and equilibrium study for the adsorption of textile dyes on coconut shell activated carbon. *Arabian journal of chemistry, 10*, S3381-S3393.

Anderson, M., Bollinger, D., Hagler, A., Hartwell, H., Rivers, B., Ward, K., & Steck, T. R. (2004). Viable but nonculturable bacteria are present in mouse and human urine specimens. *Journal of clinical microbiology, 42*(2), 753-758.

Bal, G., & Thakur, A. (2022). Distinct approaches of removal of dyes from wastewater: A review. *Materials Today: Proceedings, 50*, 1575-1579.

Barragán, B. E., Costa, C., & Marquez, M. C. (2007). Biodegradation of azo dyes by bacteria inoculated on solid media. *Dyes and pigments, 75*(1), 73-81.

Ben Mansour, H., Ayed-Ajmi, Y., Mosrati, R., Corroler, D., Ghedira, K., Barillier, D., & Chekir-Ghedira, L. (2010). Acid violet 7 and its biodegradation products induce chromosome aberrations, lipid peroxidation, and cholinesterase inhibition in mouse bone marrow. *Environmental Science and Pollution Research, 17*, 1371-1378.

Benkhaya, S., M'rabet, S., & El Harfi, A. (2020). Classifications, properties, recent synthesis and applications of azo dyes. *Heliyon, 6*(1).

Buthelezi, S. P., Olaniran, A. O., & Pillay, B. (2012). Textile dye removal from wastewater effluents using bioflocculants produced by indigenous bacterial isolates. *Molecules, 17*(12), 14260-14274.

Chang, J.-S., Chou, C., Lin, Y.-C., Lin, P.-J., Ho, J.-Y., & Hu, T. L. (2001). Kinetic characteristics of bacterial azo-dye decolorization by Pseudomonas luteola. *Water research, 35*(12), 2841-2850.

Chequer, F. M. D., Angeli, J. P. F., Ferraz, E. R. A., Tsuboy, M. S., Marcarini, J. C., Mantovani, M. S., & de Oliveira, D. P. (2009). The azo dyes Disperse Red 1 and Disperse Orange 1 increase the micronuclei frequencies in human lymphocytes and in HepG2 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 676*(1-2), 83-86.

Didier de Vasconcelos, G. M., Mulinari, J., de Arruda Guelli Ulson de Souza, S. M., Ulson de Souza, A. A., de Oliveira, D., & de Andrade, C. J. (2021). Biodegradation of azo dye-containing wastewater by activated sludge: a critical review. *World Journal of Microbiology and Biotechnology, 37*(6), 101.

Fungaro, D. A., Borrely, S. I., & Carvalho, T. E. (2013). Surfactant modified zeolite from cyclone ash as adsorbent for removal of reactive orange 16 from aqueous solution. *American Journal of Environmental Protection, 1*(1), 1-9.

Ghaly, A., Ananthashankar, R., Alhattab, M., & Ramakrishnan, V. V. (2014). Production, characterization and treatment of textile effluents: a critical review. *J Chem Eng Process Technol, 5*(1), 1-19.

Gomes, L., Miwa, D. W., Malpass, G. R., & Motheo, A. J. (2011). Electrochemical degradation of the dye reactive orange 16 using electrochemical flow-cell. *Journal of the Brazilian Chemical Society, 22*, 1299-1306.

Gupta, V. K. (2019). Fundamentals of natural dyes and its application on textile substrates. *Chemistry and technology of natural and synthetic dyes and pigments, 2019*.

Haug, W., Schmidt, A., Nörtemann, B., Hempel, D., Stolz, A., & Knackmuss, H. (1991). Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Applied and environmental microbiology, 57*(11), 3144-3149.

Hunger, K. (2007). *Industrial dyes: chemistry, properties, applications*: John Wiley & Sons.

Jamee, R., & Siddique, R. (2019). Biodegradation of synthetic dyes of textile effluent by microorganisms: an environmentally and economically sustainable approach. *European journal of microbiology and immunology, 9*(4), 114-118.

Lagier, J.-C., Edouard, S., Pagnier, I., Mediannikov, O., Drancourt, M., & Raoult, D. (2015). Current and past strategies for bacterial culture in clinical microbiology. *Clinical microbiology reviews, 28*(1), 208-236.

Li, W.-Y., Chen, F.-F., & Wang, S.-L. (2010). Binding of reactive brilliant red to human serum albumin: insights into the molecular toxicity of sulfonic azo dyes. *Protein and peptide letters, 17*(5), 621-629.

Lin, J., Zhang, X., Li, Z., & Lei, L. (2010). Biodegradation of Reactive blue 13 in a two-stage anaerobic/aerobic fluidized beds system with a Pseudomonas sp. isolate. *Bioresource technology, 101*(1), 34-40.

Luo, X., Yu, H., & Xu, J. (2012). Genomic data mining: An efficient way to find new and better enzymes. *Enzyme Eng, 1*, 104-108.

Michaels, G. B., & Lewis, D. L. (1986). Microbial transformation rates of azo and triphenylmethane dyes. *Environmental Toxicology and Chemistry: An International Journal, 5*(2), 161-166.

Moellering Jr, R. C. (2011). Discovering new antimicrobial agents. *International journal of antimicrobial agents, 37*(1), 2-9.

Muchtasjar, B., Hadiyanto, H., & Izzati, M. (2019). *Microbial degradation of batik waste water treatment in Indonesia.* Paper presented at the IOP Conference Series: Earth and Environmental Science.

Oztas Gulmus, E., & Gormez, A. (2020). Identification and characterization of novel thermophilic bacteria from hot springs, Erzurum, Turkey. *Current Microbiology, 77*(6), 979-987.

Pagga, U., & Brown, D. (1986). The degradation of dyestuffs: Part II Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere, 15*(4), 479-491.

Pollack, R. A., Findlay, L., Mondschein, W., & Modesto, R. R. (2018). *Laboratory exercises in microbiology*: John Wiley & Sons.

Reid, T. M., Morton, K. C., Wang, C. Y., & King, C. M. (1984). Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environmental mutagenesis, 6*(5), 705-717.

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ, 4*, e2584.

Sarayu, K., & Sandhya, S. (2010). Aerobic biodegradation pathway for Remazol Orange by Pseudomonas aeruginosa. *Applied biochemistry and biotechnology, 160*, 1241-1253.

Senio, E., Carliel, C., Barclay, S., Naidoo, N., Buckley, C., & Mulholland, D. (1994). Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes. *Water Sa, 20*(4), 341-344.

Shah, M. P., Patel, K. A., Nair, S. S., & Darji, A. (2013). Optimization of environmental parameters on microbial degradation of Reactive Black dye. *Journal of Bioremediation and Biodegradation, 4*(3).

Simon, F. T. (1949). The Spectrophotometer as an Aid to Dyeing. *Textile Research Journal, 19*(9), 567-574.

Singh, A. L., Chaudhary, S., Kayastha, A. M., & Yadav, A. (2015). Decolorization and degradation of textile effluent with the help of Enterobacter asburiae.

Singh, L., & Singh, V. P. (2014). Textile dyes degradation: a microbial approach for biodegradation of pollutants. In *Microbial degradation of synthetic dyes in wastewaters* (pp. 187-204): Springer.

Tang, K. H. D., Darwish, N. M., Alkahtani, A. M., AbdelGawwad, M. R., & Karácsony, P. (2022). Biological removal of dyes from wastewater: a review of its efficiency and advances. *Tropical Aquatic and Soil Pollution, 2*(1), 59-75.

Topaç, F. O., Dindar, E., Uçaroğlu, S., & Başkaya, H. S. (2009). Effect of a sulfonated azo dye and sulfanilic acid on nitrogen transformation processes in soil. *Journal of Hazardous Materials, 170*(2-3), 1006-1013.

Tsuboy, M., Angeli, J., Mantovani, M., Knasmüller, S., Umbuzeiro, G., & Ribeiro, L. (2007). Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI Disperse Blue 291 in the human hepatic cell line HepG2. *Toxicology in vitro, 21*(8), 1650-1655.

Vandevivere, P. C., Bianchi, R., & Verstraete, W. (1998). Treatment and reuse of wastewater from the textile wet-processing industry: Review of emerging technologies. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology, 72*(4), 289-302.

Velusamy, S., Roy, A., Sundaram, S., & Kumar Mallick, T. (2021). A review on heavy metal ions and containing dyes removal through graphene oxide-based adsorption strategies for textile wastewater treatment. *The Chemical Record, 21*(7), 1570-1610.

Wuhrmann, K., Mechsner, K., & Kappeler, T. (1980). Investigation on rate—Determining factors in the microbial reduction of azo dyes. *European journal of applied microbiology and biotechnology, 9*, 325-338.

Yaacob, M. R., Ismail, M., Zakaria, M. N., Zainol, F. A., & Zain, N. F. M. (2015). Environmental Awareness of Batik Entrepreneurs in Kelantan, Malaysia—An Early Insight. *International Journal of Academic Research in Business and Social Sciences, 5*(4), 338-347.

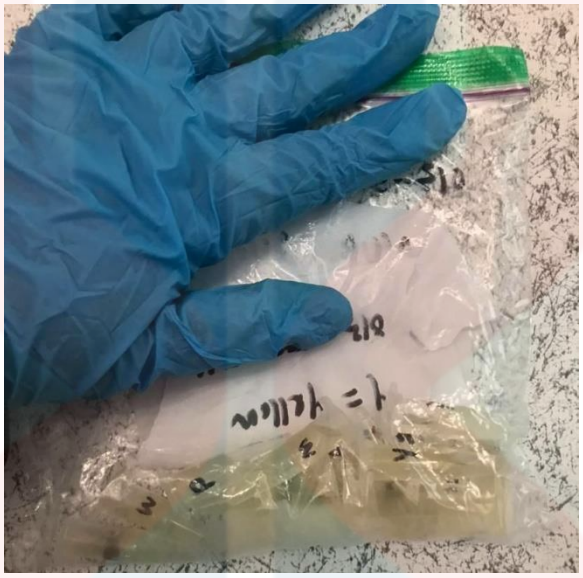
Yang, Q., Yang, M., Pritsch, K., Yediler, A., Hagn, A., Schloter, M., & Kettrup, A. (2003). Decolorization of synthetic dyes and production of manganese-dependent peroxidase by new fungal isolates. *Biotechnology Letters, 25*, 709-713.

Yaseen, D., & Scholz, M. (2019). Textile dye wastewater characteristics and constituents of synthetic effluents: a critical review. *International journal of environmental science and technology, 16*, 1193-1226.

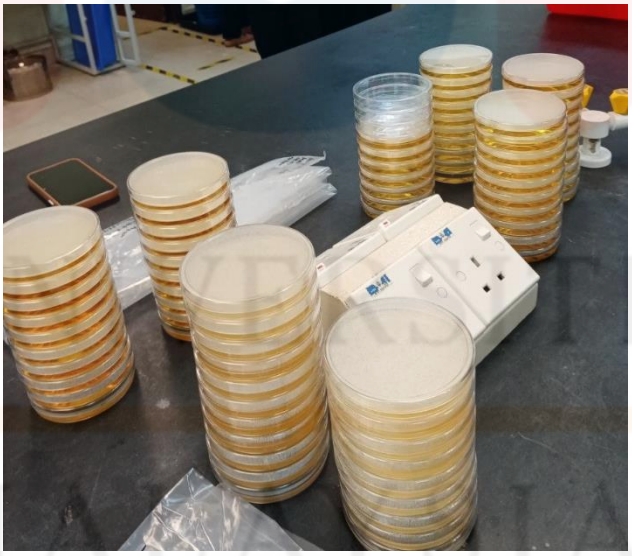
Zakaria, N., Rohani, R., Wan Mohtar, W. H. M., Purwadi, R., Sumampouw, G. A., & Indarto, A. (2023). Batik Effluent Treatment and Decolorization—A Review. *Water, 15*(7), 1339.

Zimmermann, T., Kulla, H. G., & Leisinger, T. (1982). Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by Pseudomonas KF46. *European Journal of Biochemistry, 129*(1), 197-203.

APPENDIX

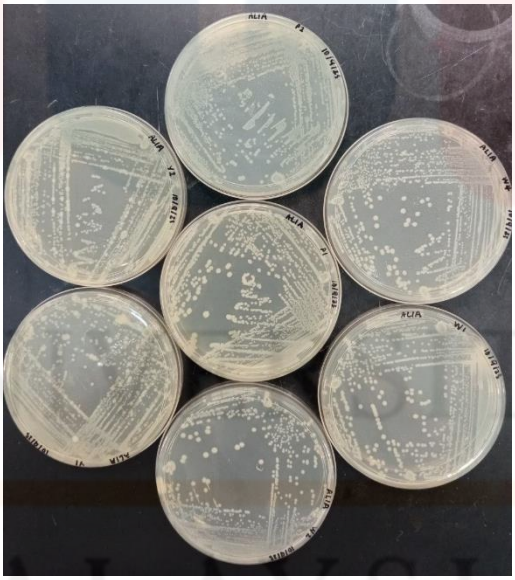


Appendix 1: The freezer stock of the three strains bacteria that were kept in -18ºC.



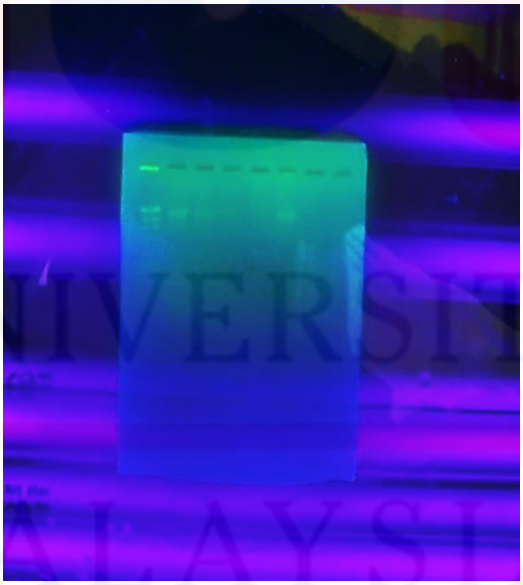
Appendix 2: The nutrient agar after undergoing the sterilization using autoclave.

Appendix 3: Three bacterial strains (Y1, P1, W1) were stored in a freezer, with multiple copies available for each strain.



Appendix 4: The growth of isolated bacteria after they was streaked in the nutrient agar.

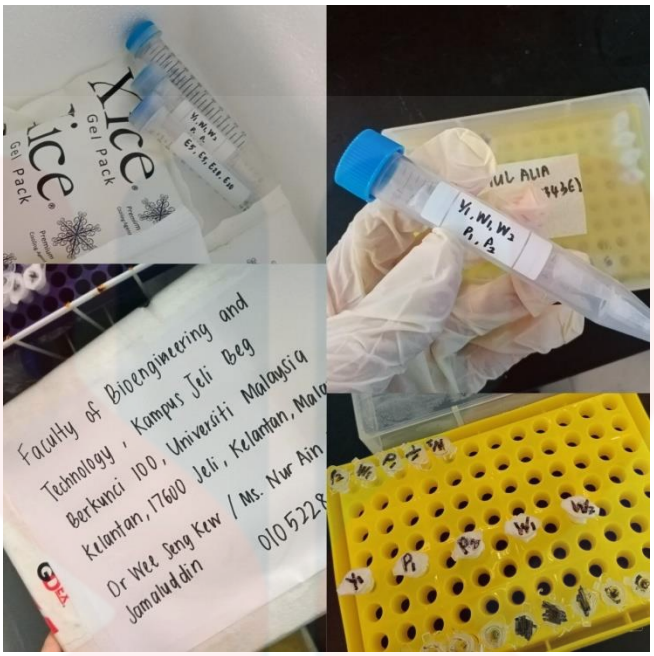
Appendix 5: The preparation of gram staining procedure and observing their gram negative or positive.



Appendix 6: The result of gel electrophoresis after DNA extraction.

Appendix 7: The result of gel electrophoresis after PCR purification.

Appendix 8: The PCR product (Y1, P1,P2,W1,W2) before they been sent for sequencing.



Appendix 9: Preparation before sent the PCR product for sequencing.

Appendix 10: The preparation of nutrient broths before undergoing the sterilization using autoclave. These broths will be used for growing the bacteria.

Appendix 11: The colour of nutrient broth after adding the R016 dye turn to orange for each of the conical flasks. The centrifuge had been used to get the clear supernatant.

Appendix 12: Left cuvette shows the colour of broth that did not contain the bacteria which is the control and right cuvette shows the colour of broth that contain the bacteria.