# Decolourisation and Degradation of Reactive Orange 16 (RO16) Dye by Co-Immobilize Dye Degrading Microbe and Coconut Fronds Biochar

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## DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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## ABSTRACT

Industrial wastewater is one of the main sources of environmental pollution. Textile industry was one of the industries that contributes towards environmental pollution. This is because the textile industry utilized large quantities of dye and discharge the dye as wastewater. The effluent from the textile industry can affect human health and also the environment. This is due to the dye compound in textile wastewater was proved to be carcinogenic. The dye compound that usually used is azo dye. There are several methods to treat wastewater such as physical, chemical and biological method. The biological method is used as this method is low cost and can reduce the sludge production after the decolourisation treatment. In this project, dye degrading microbes and coconut fronds biochar were immobilized using sodium alginate. This is to identify the efficiency of immobilized dye degrading microbes with coconut fronds to decolourize and degrade the azo dye which is Reactive Orange 16. Decolourisation activity was compared with the three controls which are alginate beads, coconut fronds biochar beads and immobilized dye degrading microbes. The result showed the percentage of decolourisation using immobilized dye degrading microbe with coconut fronds biochar was 79.29% which is the highest percentage of decolourisation. The immobilized dye degrading microbes with coconut fronds biochar and three controls were subjected for FTIR analysis. The sample was analyzed every 24 hours for 72 hours after decolourisation assay. The changes on the functional groups in Reactive Orange 16 that can be observed from FTIR spectra suggesting possible degradation of azo RO16.

Keywords: Immobilized dye degrading microbes, decolourisation, Reactive Orange 16,

biochar

**Penyahwarnaan dan Penguraian Pewarna Reaktif Oren 16 (RO16) oleh Pembantu Bacteria Pengurai Warna Tergerak-sekat dan Arang Bio Pelepah Kelapa**

## ABSTRAK

Air buangan industri adalah salah satu sumber utama pencemaran alam sekitar. Industri tekstil merupakan salah satu industri yang menyumbang kepada pencemaran alam sekitar. Ini kerana industri tekstil menggunakan sejumlah besar pewarna dan mengeluarkan pewarna sebagai air kumbahan. Efluen dari industri tekstil boleh menjejaskan kesihatan manusia dan juga alam sekitar. Ini disebabkan oleh sebatian pewarna dalam sisa buangan tekstil terbukti karsinogenik. Kompaun pewarna yang biasanya digunakan ialah pewarna azo. Terdapat beberapa kaedah untuk merawat air sisa seperti kaedah fizikal, kimia dan biologi. Kaedah biologi digunakan kerana kaedah ini adalah kosnya yang rendah dan boleh mengurangkan pengeluaran enapcemar selepas rawatan penyahwarnaan. Dalam projek ini, bakteria pengurai warna dan arang bio pelepah kelapa akan digerak-sekatkan menggunakan natrium alginat. Ini adalah untuk mengenal pasti kecekapan bakteria pengurai warna dengan bantuan arang bio pelepah kelapa untuk penyahwarnaan dan penguraian pewarna azo iaitu Reaktif Oren 16. Pengujian penyahwarnaan dibandingkan dengan ketiga-tiga control iaitu manik alginat, manik arang bio pelepah kelapa dan bacteria pengurai warna yang tergerak sekat. Hasilnya memperlihatkan peratusan penyahwarnaan dengan menggunakan bakteria pengurai warna dan arang bio pelepah kelapa adalah 79.29% yang merupakan peratusan tertinggi dalam penyahwarnaan. Bakteria pengurai warna dan arang bio pelepah kelapa dan tiga kawalan telah dianalisis untuk analisis FTIR. Sampel dianalisa setiap 24 jam selama 72 jam selepas ujian penyahwarnaan. Perubahan pada kumpulan berfungsi di RO16 yang boleh diperhatikan dari spektrum FTIR mencadangkan kemungkinan penguraian azo RO16.

Kata Kunci : gerak-sekat bacteria penguraian pewarna, penjerapan, Reaktif Oren 16, arang bio

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| NA | Nutrient agar |
| LB | Luria Bertani broth |
| NaOH | Sodium hydroxide |
| HCL | Hydrochloric acid |
| CaCl2 | Calcium chloride |
| KBr | Potassium bromide |
| M | Molar mass |
| mm | Millimetres |
| g | Grams |
| mL | Millilitre |
| w | Weight |
| v | Volume |
| nm | Nanometres |
| µm | Micrometre |
| rpm | Rotation per minutes |
| $cm^{-1}$ | Reciprocal centimetre |
| $A_0$ | Initial absorbance |
| $A_t$ | Observed absorbance |
| ℃ | Degree Celsius |
| % | Percent |
| C | Carbon |
| H | Hydrogen |

| | |
|---|---|
| N | Nitrogen |
| O | Oxygen |
| FTIR | Fourier Transform Infrared Spectroscopy |
| CF | Coconut fronds |
| RO16 | Reactive Orange 16 |
| IR | Infrared |
| GC-MS | Gas chromatography-mass spectrometry |
| AL | Sodium alginate beads |
| AL+BC | Coconut fronds beads |
| AL+MC | Immobilized dye degrading microbes |
| AL+BC+MC | Immobilized dye degrading microbes with coconut fronds biochar |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background Study

Waste management is very important in industrial sectors in order to have a sustainable ecosystem. Industrial wastewater is one of the main sources of environmental pollution. The industry that had a great influence on wastewater is the textile industry (Carmen & Daniel, 2012). Textile industry utilized large quantity of dye and discharged the dye as wastewater effluents (Kandisa & Saibaba, 2016). A report from Guadie et al., (2017) stated that dyeing process releases roughly around 10-15% of dye into the wastewater stream.

Many studies had reported that effluent from the textile industry can harm the environment especially to the aquatic ecosystem and human health. The examples of harm such as inhibit the light penetration system into deep layer of water which can decrease the rate of photosynthetic activities and leads to the depletion of dissolve oxygen (DO). The wastewater effluents from textile industry can cause harmful impacts towards human health as it contains lots of chemical such as ammonia, sulphide, lead and heavy metal (Hossain et al, 2018).

Azo dye is widely used as a textile dye. It has one or more of the azo group (-N=N-) that attach with one or more of aromatic structures (Gregory, 1990). The dye can be classified into a few types based on their application such as direct dyes, acid dyes, disperse dyes, mordant dyes, vat dyes and basic and cationic dyes (Samanta & Konar, 2011)

In order to reduce the wastewater from textile industry, many methods have been used mainly physical and chemical methods. The example of physical and chemical methods is ion exchange, adsorption and membrane separation. Lade et al., (2015) had reported this method had a few disadvantages which are the production of sludge after the decolourisation of dye, high-cost production and high energy requirement. Therefore, an alternative method is needed which is the biological method. A study from Lade et al., (2015) had proven that the biological method can decolourise and degrade the dye. This method also can reduce the production of sludge, the cost and energy production.

In this study, the combination of the physical and biological method was used to decolourise and degrade Reactive Orange Dye 16 (RO16). The Reactive Orange Dye 16 will be degraded by dye degrading microbe with the aid of agriculture waste adsorbent which is coconut fronds biochar. Biochar is a biological adsorbent that can be produced from various sources for instance agriculture waste. The examples of agriculture waste such as rice husk, corn cob, coconut fronds and coffee waste (Li et al., 2017). Biochar is widely used as an adsorbent. By using the biochar, the cost of production can be cut off and the capability of biochar to remove the heavy metal from wastewater is higher (Niazi et al., 2016).

In of this study, a locally isolated dye degrading bacteria *Bacillus sp*. (UMKDG 1) was used as dye degrading microbe to decolourise and degrade the RO16. The bacteria

were isolated at batik factory located at Kota Bharu. Then, the UMKDG 1 was immobilized with the coconut fronds biochar by mixing the solution into the sodium alginate to produce the immobilized beads. The decolourisation and degradation of RO16 by immobilized beads was observed and analysed at time range 0 hours until 72 hours. Then, the decolourisation of dye was analysed using UV-Vis spectroscopic analysis. The degradation of dye was analyse using Fourier Transform Infrared Spectroscopy (FTIR) analysis.

Based on the result obtained, the decolourisation assay of immobilized dye degrading microbes with coconut fronds biochar shows a higher decolourisation percentage compared to other controls. Meanwhile, the changes on the functional groups in Reactive Orange 16 that can be observed from FTIR spectra suggesting possible degradation of azo RO16.

## 1.2 Objective

The objectives of this study are:

1. To immobilize dye degrading microbe on coconut fronds biochar by using sodium alginate.
2. To investigate the decolourisation of Reactive Orange 16 dye by the immobilized dye degrading microbe with coconut fronds biochar.
3. To investigate the degradation of Reactive Orange 16 dye by immobilized dye degrading microbe coconut fronds biochar.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Textile effluent

Textile industry is one of the large industries that produce severe pollution in the environments (Chen, 2003). Textile industry consumes large amounts of dye and water for wet processing of textiles. The effluent of textile is the wastewater contains the dye and their metabolites in ecosystems. The textile effluent produces a high amount of agents such as chemical oxygen dissolved (COD) biological oxygen dissolved (BOD) and chemical trace metal like Zn, As and Cu (Antoni et al, 2017). Therefore it can causes harm to the environment especially the aquatic system. The example is the reduction in sunlight penetration, which in turn decreases photosynthetic activity, dissolved oxygen concentration, and water quality. Moreover, a report from Saratale et al., (2011), indicate that textile dyes and effluents have toxic effects on the germination rates and biomass of several plant species which have important ecological functions, such as providing a habitat for wildlife, protecting soil from erosion and providing the organic matter that contributed significantly to soil fertility. Dye is resistant towards the exposures such as water or light due to their chemical structure (Robinson et al., 2001). Azo dye is the common dye used in the textiles industry as azo dye has stability and availability of the variety of colours (Gomaa, 2016).

## 2.2 Dye

Dye is organic compounds that have molecules which responsible for dyeing ability which are chromophoric groups and basic or acid auxochromic groups. In dye molecules, there is two key component which are the auxochromes and chromophores. The auxochromes responsible to supplement the chromophore makes the molecule soluble in water and enhanced the affinity the molecule to attach towards the fibres. Meanwhile, the chromophore is responsible in producing the colours (Gupta & Suhas, 2009). Dye is included as colouring substances and dye can stay long (Hoffmann & Puszynski, 2010). The dyeing process can lead to the production of zinc, chromium, lead and copper. The copper produce is toxic towards aquatic ecosystems (Parvathi et al, 2011).

Due to their chemical structures, dye is difficult to decolourize as the dye is not easily faded upon the exposure to water, sweat, light, chemicals and microbial attack. Dye can be classified by their application to the fiber and their chemical structure. Dye is classified as acidic, disperse, azo, basic, anthroquinone based, metal complex and diazo. Basically, the azo dye compound is the most used synthetic dye and widely used in the textile industry (Kolodkin & Murin, 2006). A report from Gupta & Suhas (2009), stated almost 65 – 70% of azo dye was produced. Although, the dye is classified based on basis structure was a suitable system and has many advantages such as it can readily identify dyes as belonging to a group and having characteristic properties. The example is an azo dye which is cost-effective, strong and anthraquinone dyes which are weak and expensive. Dye is considered as toxic due to it carcinogenicity. The carcinogenicity

causes the skin and eye irritation, oral ingestion and inhalation and skin sensitisation. (Gupta & Suhas, 2009)

### 2.2.1 Classification of Dye

Dyes are classified according to their method of application to the substrate. The classification based on application is advantageous before considering chemical structures. The classification of dye and description of each classes of dye are summarized in Table 2.1. It is also worth to point out that classification by application is the principal system adopted by the Colour Index (C.I.) (Gupta & Suhas, 2009).

Table 2.1: The classification of dye and description of each classes of dye.

| Class | Description |
|---|---|
| **Azo dye** | • Has a linkage called azo bond linkage (-N=N-)<br>• Used in food, printing, leather and widely in the textile industry |
| **Reactive dyes** | • Reactive dyes have pendant side chains that can combine covalently with the substrate such as in textiles as a colourant as it formed the covalent bond with the substrate during the colouring process.<br>• Widely used with cellulose as the covalent bond link to the hydroxyl groups of cellulose and produce the high resistance to washing. |
| **Vat dye** | • Wat dye is used in notably cotton and cellulosic fibre due to their water insolubility. |

| | |
|---|---|
| | • Glucoside or indicant is the indigo that can found in natural vat dyes. |
| **Sulphur dye** | • The range of sulphur dye is dull compared to another dye<br>• Sulphur dye is used widely in blends of cellulosic fibers with synthetic fibers and dyeing textile cellulosic materials. |
| **Acid dyes** | • Acid dye has azo chromophoric systems such as antraquinone or triphenylmethane which is soluble in the water.<br>• Acid dyes usually used in nylon, silk and wool which is applied in low pH ( 3.0 – 7.0) |
| **Disperse dye** | • Disperse dyes are widely used in textile colouration and disperse dye is one of the synthetic colourants for hydrophobic substrates.<br>• Disperse dye produce a lot of wastewater because these dye is insoluble or sparingly soluble in water and need to use high quantities and amount of dyes and water and the proportion of dye remain in water is high. |
| **Basic dye** | • Basic dye has low migration properties which is due to the high substantivity of the dye for the substrate.<br>• Basic dye sre water soluble where it produces the coloured cations in solution which can attract electrostatically to substrate with negative charge, |
| **Direct dye** | • Direct dye is reacted by forming van der Waals and hydrogen bond.<br>• Direct dye is classified based on a few characteristics such as chromophore. Example of chromophore is dioazine, stilbene and azo. |

Adapted from (Benkhaya *et al.*, 2017)

### 2.2.2 Azo Dye

Azo dye is widely used in textile industry due to the variety and availability of colours (Shobana & Hangam, 2012). According to a report from Chung, (2016), it states that 50% of dyes are produced worldwide and more than 2000 azo dyes are used. Azo dye can be detected by the presence of one or more of azo group (-N=N-) that are chromophores. The aromatic and other groups such as chloro (-Cl), nitro (-NO2), hydrolysis (-OH), carboxyl (-COOH), sulfonic groups (-SO3H) and amino (-NH3) are associated with the azo group to produce a different type of azo groups (Kolodkin & Murin, 2006).

Azo group is formed by coupling component which is when phenol or amine is reacted with diazonium ion or known as a diazo component. The reaction of nitrous acid from sodium nitrite ($NaNO_2$) and hydrochloric acid (HCl ) on aromatic primary amines at 0°C will generate diazonium ions (Kiernan, 2001).

The azo dye components can affect human health. The example of azo dye component is benzidine which can cause bladder cancers. There is a higher probability for dye workers to have bladder cancers. Therefore, the azo dyes are carcinogenicity and mutagenicity to human and also animals (Chung, 2016).

### 2.2.3 Reactive Orange 16

Reactive orange 16 has two sulfonate groups, which have negative charges in aqueous solution. The molecular structure of reactive orange 16 is shown in Figure 2.1 and general characteristics of RO16 are summarized in Table 2.2.

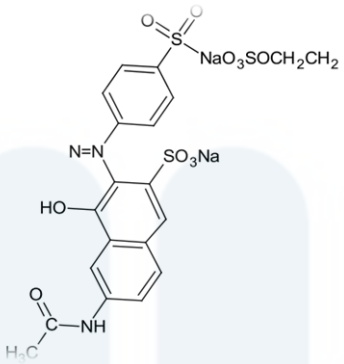


Figure 2.1: The molecular structure of Reactive Orange 16
Source: Shobana & Hangam,(2012).

Table 2.2: Properties of Reactive Orange 16

| Properties | Description |
|---|---|
| **Name of IUPAC** | Disodium (3Z)-6-acetamido-6-acetamido-4-oxo-3- [[4-(2- sulfonatooxyethylsulfonyl) phenyl]hydrazinylidene]naphthalene-2-sulfonate |
| **General Name** | Reactive Orange 16<br>Reactive Orange 7<br>Remazol Brilliant Orange 3R<br>Brilliant Orange 3R |
| **Molar mass, g/mol** | 617.54 |
| **Chemical formula** | $C_{20}H_{17}Na_20_{11}S_3$ |
| **Maximum wavelength** | 494 |
| **H-Bond Accepter** | 13 |
| **H-Bond Donor** | 2 |

Adapted from (Won et al., 2006)

## 2.3 Methods for Removal of Textiles Dyes.

There are several types of technique that can be used to remove the dye from textile industry such as chemical, physical and biological methods.

### 2.3.1 Chemical methods

There are a number of methods are used to remove dye by chemical techniques such as ozonation, oxidation, electrolysis, photochemical and neutralization (Robinson et al., 2001). Oxidation is one of chemical methods that are broadly used to decolourise dye because the process is quite simple compared to other methods. In oxidation process, the molecules of dye will be oxidized and broken down into small colourless molecules depends on the strength of oxidant employed and also the dye structure. The example of colourless molecules are aldehydes, water, nitrogen, sulphates and carbon dioxide (Singh & Arora, 2011).

The other method of oxidation is direct chemical oxidation. Direct chemical oxidation is being used for decolourisation of water soluble dye in textile wastewater such as reactive dyes, direct metal and acid. This method use chlorine in the solid form of sodium hypochlorite to decolourise the water soluble dye. However, the decolourisation of reactive dye need a long time to treatment, on the other hand, vat dyes are resistant towards chlorine decolourization (Singh & Arora, 2011).

Electrocoagulation also one of the chemical method that widely used on the removal of organic matter in textile wastewater. This method is an economically feasible

method whereby electrocoagulation method is investigated on a laboratory scale on the textile dye. This method contains real or synthetic wastewater sample for the removal of organic matter. This method also prized to produce less quantity of sludge as the uses of excess chemical can be avoided. In addition, current density, dyestuff, initial pH and anode material will be effect the electrocoagulation process to decolourise the dye (Singh & Arora, 2011).

### 2.3.2 Physical methods

The physical methods that commonly used to treat textile effluent such as flotation, adsorption, sedimentation, ultra-filtration, flocculation and coagulation ( Singh & Arora, 2011). Based on a report from Saratale et al., (2011), the effective ways to remove the sulphur and disperse dyes are coagulation-flocculation but this method has very low capacity towards direct, vat, reactive and acid dyes. This method has some limitation because of the huge amounts of sludge production and low colours removal efficiency.

In the textile industry, filtration method is generally used such as membrane filtration as this methods can provide the possibilities of the hydrolysed dyestuff and dyeing auxiliaries to be separated simultaneously. Reduce the colour is to indicate the separation of dye is occur. This method also useful for bleaching the dye (dos Santos et al., 2007). Membrane filtration also has some disadvantages such as the potential of the membrane are fouling and need further treatment for secondary waste production streams and high investment cost (Saratale et al., 2011).

Adsorption is also one of the methods that frequently used as a tertiary treatment in the textile industry. In adsorption, activated carbon generally used in decolourisation as it is an effective adsorbent. Activated carbon is expensive. Therefore, some researchers have found and used the low-cost adsorbent materials such as coconut fronds, spent coffee as a replacement of expensive activated carbon (Antoni et al, 2017).

### 2.3.3 Biological Methods

Biological methods are an alternative method that uses in decolourisation and degradation of dye. These methods are widely used in many studies as this method is cost-effective compared to physical and chemical method. In biological method, the microorganisms are used to decolourise and degrade the dyes from wastewater (Shah, 2011). Biological methods are divided into two types which are anaerobic and aerobic treatment. Anaerobic treatment occurs when the organic compounds are converted to methane and carbon dioxide with the absence of free oxygen while aerobic treatment is a treatment that uses of microorganism to degrade the organic component with the presence of free oxygen that has dissolved in wastewater (Singh & Arora, 2011).

A study from Shah, (2011) isolate a few strains of bacteria from the textile effluent such as *Bacillus, Pseudomonas, Acinetobacter* and *Legionella.*. Ajibola et al., (2005), had found *Bacillus* and *Legionella* were used in effluent treatment. From a study of Shah, (2011), *Bacillus subtilis* was able to decolourise 91% of Red RR and 61% of Yellow RR. The bacteria inoculums were inoculated into the flasks containing azo dyes with trace amounts of glucose, sucrose and yeast extract and incubated for 4 days.

## 2.4 Mechanism of Dye Decolourization by Microorganisms

From the previous study, the degradation of azo dye using the dye degrading microbe were associated with the symmetric cleavage of azo dye (-N=N-) in either aerobic or anaerobic conditions whereby this symmetric cleavage of azo dye can be mediated through many types of mechanism such as low molecular weight, enzyme chemical reduction by biogenic reductant such as sulphide. The reaction takes place either in extracellular or intracellular. However, recent study reports about the involvement of tyrosinase, peroxide, laccase, MG reductase and NADH-DCIP reductase in decolourization of azo dye (Singh, 2015).

A report from Singh, (2016), stated many types of microorganisms grows in biological treatment systems such as bacteria, actinomycetes and fungi. These microorganisms are used in biodegradation as biological agents. Microbial degradation is usually used for complete mineralization of organic molecules and degrade the dye. Other than degrade the dye, the microbes also able to absorb the heavy metal, concentrate and also accumulate inside cell or cell walls. The example of the microorganisms that previously isolated to degrade azo compounds are *Enterococcus sp, Bacillus subtilis, yeast Proteus sp* and *Streptococcus sp.*

### 2.4.1 Degradation of Dye by Bacteria

Generally, the degradation of azo dye has two stages. The first stages are the formation of colourless aromatic amines. Then, the second stages are the degradation of

aromatic amines which can only be obtained and occur at anaerobic condition. The microbial degradation of azo dyes was about the reduction of –N=N-. The reduction can occurred either intracellular or extracellular with different mechanisms such as enzymes or low molecular redox mediators. The enzyme that reduced the azo dyes either specialized enzymes or non-specialized enzymes. The specialized enzymes were only catalysing the reduction of azo dyes. While the non-specialized enzymes, it catalyses the reduction of a wide range of compounds including the azo dyes. The specialized enzymes of reducing azo dye called as azoreductase.

The other biological degradation of azo dyes is indirectly reduced enzymatically reduced electron carriers. Mohammed et al., (2018) reported that anaerobic azoreductases were flavin reductases. It also stated that Flavin dependent reductases generate the Flavin such as FADH2, FMNH2 and riboflavin. The Flavin was able to stimulate the azo dye reductions. The reduced Flavin nucleotides and redox mediators used azo dyes as electron acceptors to enhance the reduction. The reduction of azo dyes using Flavin reductases also resulted in colourless aromatic amine.

### 2.4.2 Biodegradation of Dye by Fungal

Bacteria and fungi both are degraders of organic matters but due to the advantages of fungi in enzyme production, fungi are known better as degrader of organic matters. Regardless of their taxonomic position, fungi can be classified as brown-rot fungi, white-rot fungi and soft-rot fungi based on the basis of descriptions and technical decay. This is because of the metabolic pathway and enzymes system that involved in the breakdown of lignins and carbohydrates which are actually distinct in these fungi. Therefore, decay

types are more taxonomic importance rather than just modified in one or a few specific enzymes activities. The production of extracellular enzymes such as peroxides and phenoloxides are physiological characteristic importance of decay fungi (Singh, 2017)

The use of fungi in microbial degradation of dye is widely used. According from Lavanya, (2014), it reported a study as an example from a study that used *Gliocladium virens* to degrade Congo red. Fungi are able to degrade the dye due to their excretion of extracellular enzymes. It also because of the non-stereo selective and non-specific enzyme system that includes laccase, lignin peroxidase (LiP) and manganese peroxide (MnP). Lavanya, (2014) reported the ability of immobilized white-rot fungus *Phanerochaete chrysporium* using entrapment with ca-alginate to decolourize different of recalcitrant azo dyes.

The study biodegradation on dyes degradation using fungi *Phlebia radiata*, *Phenerocheate chrysporium*, *Pleurotus sp*. and *Trametes versicolor* to degrade the dye. It resulted in the production of enzyme laccase which is related to dye degradation and also lignin. *Phenerocheate chrysporium* is one of the well-characterized white rot fungi that has been implicated in degradation of dyes. This fungus is mainly due to it expression of some non-specific extracellular enzymes such as ligninolytic peroxides that involve in dye degradation.

White fungi rot can produce a few types of enzymes such as cellulose and lignin which not only able to degrade natural polymers but also can degrade the synthetic chemicals (Singh, 2016). Therefore, according to Singh (2017), it states that these fungi are effective to degrade many types of groups of dyes. Then, by using fungi in dye degradation, the formation of hazardous anilines can be avoided due to the fungi oxidative

mechanisms. The formation of anilines is formed from the reductive cleavage of azo dyes by bacteria.

The fungi can remove the dye either in living or dead through a few methods such as bioaccumulation, bio-sorption, biodegradation and also enzymatic mineralization using manganese peroxide and lignin peroxide (Singh, 2017). Fungal bio-sorption has been widely studied because of large disposal amount of waste from fermentation industry and the amenability of bacteria to morphological and genetic manipulations (Singh, 2016).

**i) Algae**

The uses of *Chlorella vulgaris, Oscillsteria tenuis* and *Chlorella pyreniodosa* in decolourise and degrade more than thirty azo compounds had been reported from (Yan & Pan, 2004). It reported that they were able to degrade the azo dye into the simpler structure which is a simple aromatic amine. Algae have a significant role in removal of aromatic and azo dyes in stabilization ponds (Lavanya et al., 2014).

**ii) Yeast**

Lavanya (2014), stated that yeast species can decolourise the azo dyes. It also states that yeast species are promising dye adsorbent as it capable to uptake the higher concentration of dye. The example of yeast species are *Saccharomyces cerevisiae Galactomyces geotrichum* and *Trichosporon beigelii*. There is some benefits in using

yeast which is the yeast are fast growth and they also have the ability to resist in unfavourable environments. Besides, yeast also founds to be efficient in treating high strength organic wastewater (Jafari et al, 2014).

### 2.4.3 Enzymatic Dye Decolourisation

Enzymatic dye decolourisation are divided into four groups such as azo reductases, NADH-DCIP reductases and malachite green reductase, lignin peroxide (LiP) and laccase (Singh, 2015).

#### i. Azo Reductases

Azo reductases is called as oxygen- tolerant which it can reduce the azo group (Barragán et al., 2007). Azo compound is commonly used as the sole carbon and energy to the aerobic bacteria to reductive the cleavage of azo linkage (Guasch et al., 2010). Azo reductases are flavoproteins (NAD(P) H: Flavin oxidoreductase) which are located in extracellular or intracellular at the site of the cell membrane that required electron donors such as NADH, NADPH and FADH for reduction of azo bonds. Then, $FADH_2$, $FMNH_2$, NADPH and NADH will act as co-factor that will provide 'H' in reduction process at cytoplasm. The co-factor that release from the cell lysis is one of the possible reason for the azo dye reduction rate is higher using cell extract compare to the resting cell (S. Singh, 2015). The reaction catalysed by azo reductase has been shown in Figure 2.2 The NAD(P)H-dependent reduction of azo compound corresponding to the

amines when being catalysed by azo reductase in azo dye degradation (Leelakriangsak & Borisut, 2012).

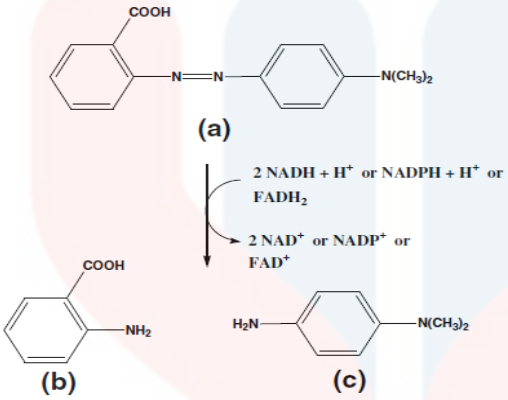


Figure 2.2: Reaction catalyzed by azo reductases. a Methyl red; b 2-Amino benzoic acid; c p-dimethyl amino aniline
Source: Singh, (2015).

### ii. NADP-DCIP Reductase and Malachite Green Reductase

NADP-DCIP reductase and Malachite Green (MG) reductase are known as non-specific reductase which also uses in decolourization of dye. NACP-DCIP reductases belong to the bacterial mixed function group system whereby NACP-DCIP reductases play a part in the detoxification of xenobiotic compounds. The mechanism of reduction process using this reductase is occur when DCIP substrate which is in blue colour is reduced by NADH-DCIP enzyme by using NADH as an electron donor. The DCIP will become colourless after reduction. Figure 2.3 shown the reaction catalysed by NADH-DCIP reductase. Next, the biodegradation of malachite green is reduced by MG-reductase and convert malachite green into leucomalachite green by using NADH as an electron

donor, the reaction catalyzed by MG reductase has been shown in Figure 2.4 (Singh, 2015).

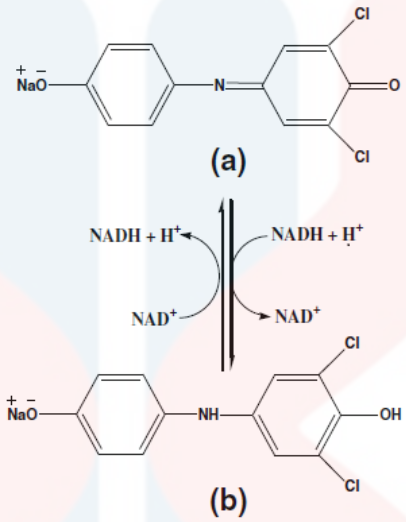


Figure 2.3: Reaction catalysed by NADH-DCIP reductase, **a**. Oxidised form of DCIP; **b**. Reduced form of DCIP
Sources: Singh, (2015).

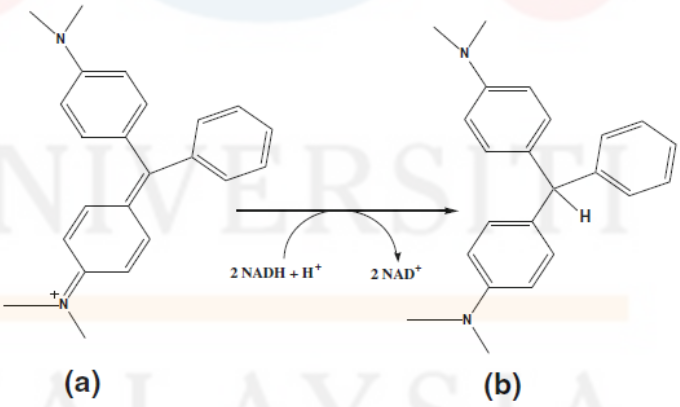


Figure 2.4: Reaction catalysed by MG reductase. **a** Malachite green; **b**. Leucomalachite green
Sources: Singh, (2015).

### ii. Lignin Peroxidase (LiP)

LiP is an enzyme that belongs to oxidoreductases family. The specific name of LiP is 1, 2-bis (3, 4-dimenthoxyphenyl) propane-1,3diol: hydrogen-peroxide oxidoreductase. LiP is N-glycosylated protein with the molecular weight range 23 and 47 kDa. LiP contains heme that located at the active site. The heme is involved in oxidation reaction which is catalysed by the side chain of lignin and related compound to form reactive radical.

A study from (Singh, 2015), suggested the role of lignin peroxidase in dye decolourisation. It stated that there is a significant induction in lignin peroxidase activity in decolourisation of azo dye. Therefore, it is proved by in vitro dye decolourisation assay with purified protein, the reaction catalysed by lignin peroxide has been shown in Figure 2.5, (Singh, 2015).

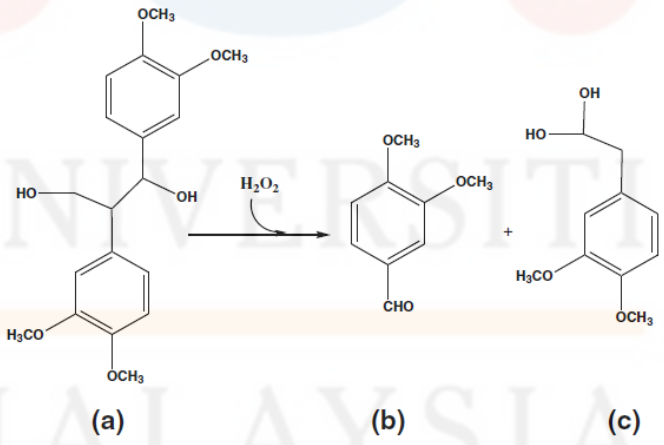


Figure 2.5: Reaction catalyzed by lignin peroxide. **a** 1,2-bis (3,4-dimenthoxyphenyl) propane-1 3-diol; **b** 3,4-dimenthoxybenzaldehyde; **c** 1-(3,4-dimenthoxyphenyl) ethane-1,2-diol

Sources : Singh,(2015).

**iv. Laccase**

Laccase is one of multi-copper oxidase protein family and with the presence of oxygen that acts as an electron acceptor in the catalyses the oxidation of substituted phenolic and non-phenolic compounds. Laccase is divided into two groups such as blue laccase and yellow-brown laccase. Blue laccase has blue colour spectra while yellow-brown laccase had no typical blue oxidase spectra. This types can be detected in EPR spectra. The previous study from (Singh, 2015) had found laccase activity in *pseudomonas syringae.* Then, the decolourisation of azo dye was done by a non-specific free radical mechanism to avoid the formation of toxic aromatic amines. A report from Singh, (2015) reported the ability of laccase to decolourise a few of textile dye by using the purified laccase that isolated from *pseudomonas desmolyticum* and *Bacillus* sp.

## 2.5. Factors affecting bacterial decolourisation

In bacterial decolourisation, there are several physico-chemical parameters which are temperature, level of agitation, oxygen, dye structure, supplement of different carbon and nitrogen sources and pH that will directly affect the performance in bacterial decolourisation. To increase and make the process more efficient, it essential to prior determination of the effect of each factor on the bacterial decolourisation of azo dyes (Lavanya et al., 2014).

### i. Effects of Oxygen and Agitation

Decolourisation of azo dyes can occur under different conditions. It can occur under aerobic conditions, facultative anaerobic and under strictly anaerobic conditions by different trophic groups of bacteria. Under anaerobic conditions, the feeding of a carbon source such as simple and complex substrates could affect the decolourisation process. This because most of azo dye reduction to amines occurs during active bacterial growth. The example of simple substrates is starch, ethanol and glucose while the complex substrates are tapioca and whey (Saratale et al., 2011).

Therefore, under anaerobic conditions of reductive enzymes, it is assumed that the activities of decolourisation are higher but a small amount of oxygen was needed for the oxidative enzymes that involve in azo dye biodegradation. Saratale et al., (2011), reported that oxidative and reductive enzymes play a part during bacterial degradation of azo dyes. Therefore, it recommends to decrease the concentration of oxygen in the solution to get a better effective of colour removal aeration and agitation.

### ii. Effects of carbon and nitrogen sources supplements

Azo dyes are lack in carbon sources. The biodegradation process of azo dyes is very difficult without the addition of carbon and nitrogen sources. During reduction of azo bond in decolourisation of azo dyes, it was reported that reducing equals from several of carbon sources are transferred to the dye. Then, the capability of acidogenic bacteria was observed in anaerobic consortia that able to convert the soluble substrates such as acetic acid, volatile organic acids and methanol that act as competitive substrates for

methanogenic and acetogenic bacteria. Thus, the addition of carbon sources in stimulating the decolourisation is less effective. This is might be due to the preference of the cells in assimilating the extra carbon over using the carbon source from the dye compound. Then, the addition of nitrogen sources which are beef extract, peptone, yeast extract and urea can regenerate the NADH. In the reduction of azo dyes using microorganisms, NADH act as an electron donor thus the decolourisation is more effective (Chen et al., 2003).

### iii. Effects of Temperature

A report from Saratale et al.,( 2011), stated that the environmental temperature in microorganisms is directly established organismal temperature. This is because of the respond of the microbial cell to the temperature changes by adaptation enzymatic and biochemical mechanisms. Temperature is an important factor in the process associated with microbial vitality. From the report, it also states that the decolourisation rate is increased when the temperature is up to the optimal temperature and then there is a minimal reduction in decolourisation activity. The decolourisation rate is decreased at higher temperature probably cause from the denaturation of azo reductase enzyme and loss of cell viability. However, there also some azoreductase in certain whole bacterial cell is thermostable and able to remain active up to 60°C over short periods of time (Pearce et al., 2003).

### iv. Effects of pH

The medium pH is an important factor in decolourization as it has the major effect in efficiency of dye decolourization. The optimal pH in removal colour is between 6.0 and 10.0. At the optimal pH, the decolourisation rate is high as it tends to rapidly decrease at strongly acid or strongly alkaline pH. Next, the transport of dye molecules across the cell membrane also effected by pH. This is pH is considered as rate-limiting step in decolourization of azo dyes. The increase in pH during biological reduction of azo bond is due to the formation of aromatic amine metabolites. The altering of pH between 7.0 – 9.5 will cause a little effect on the dye reduction process. A study from Chang et al. (2001) found that when the pH is increased from 5.0 to 7.0, the dye reduction rate is slightly increased, then the rate become insensitive to pH within range 7.0 to 9.5.

### v. Effects of Dye Concentration

The decolourisation rate is gradually decreased when the dye concentration is increase. This is due to the toxic effect of dyes with regard to the inadequate biomass concentration or improper cell to dye ratio as well as the blockage the active sites of azoreductases by molecules with a different structure. A report from Chen et al.,(2003), stated the result observed from bacteria decolourisation using various reactive azo dyes as example reactive group with sulfonic acid ($SO_3H$) groups. The report specified that at high concentration of dyes, the reactive azo dye with sulfonic acid ($SO_3H$) groups on their aromatic rings inhibit the growth of microorganisms. It found that the higher concentration of dye can be reduced when bacterial co-culture is used instead of pure

culture. This is probably due to the synergistic effect of microorganisms (Saratale et al., 2011); (Van Der Zee & Villaverde, 2005).

## 2.6 Immobilization

Immobilization technique is used to preserve the catalytic and viability functions of the enzymes or cells by localised the enzymes or cells in solid support material. The advantages of this technique over dispersed cell are high resistant towards toxicity, the density of cell is higher, the operation cost and residual sludge are lower (Sneha et al., 2015). There are some techniques that can be used for immobilization such as covalent binding, adsorption, encapsulation and affinity immobilization.

Adsorption is the simplest method where it uses physical interaction between carrier surface and bacteria. Next, encapsulation technique used synthetic polymers and natural polymers to encapsulate the bacteria. The example of synthetic polymers is polyacrylamide and example of natural polymers are agarose, collagen and alginate (Das & Adholeya, 2015). Then, the factors that will influences immobilized enzymes are physical nature as carrier, physical structure of the carries (e.g.; pore size) and the presence of substrates or inhibitors (Datta et al., 2013).

Das & Adholeya, ( 2015), reported there were few factors in the selection of carriers. The carriers should light weight, non-biodegradable in test conditions and have good mechanical strength. Generally, there are two types of material that used in immobilization of microorganisms which are inorganic and organic. Inorganic carrier usually used electrostatic attachment between the carrier material and the cell to

immobilize microorganisms. This carrier can resist to microbial degradation and also cost effective. The example of inorganic carrier are ceramics, clay and porous glass.

Meanwhile, organic carriers have larger varieties of reaction groups such as hydroxyl, amino and carboxyl. This reaction group are responsible for adsorption capacity. With the presence of reaction groups, the organic carrier material has higher absorptivity compared to the inorganic carrier material. The organic material is grouped into two types which are a natural and synthetic polymer. The example of organic carrier agar, chitosan and alginate (Das & Adholeya, 2015).

## 2.7 Bioadsorbent

Bioadsorbent is a biological material that used to decolourise the dye from the solution. Bioadsorbent is a modified adsorbent from chemical adsorbent that used agriculture waste as material such as corn cob and spent coffee fronds to adsorb the dye (Feng et al., 2012). A study from Farooq et al., (2010) report the benefits of bioadsorbent such as the materials can be recycled, the availability of material, low-cost of production as the material is made from the agriculture waste and the process to make the bioadsorbent is quite simple.

### 2.7.1 Biochar

Biochar is a carbon rich solid bioadsorbent. Biochar is made from slow biomass pyrolysis. Several applications of biochar such as to remove the dye, reduce the

greenhouse and improve the soil quality (Gwenzi et al., 2017). Biochar has a large surface area that can process aromatic area and oxygen functional groups. It a fine grained and highly porous (Venkateswarlu, 2018).

The physical point of view of biochar is biochar has a low bulk density. This is due to biochar structure which is a porous structure that leading to high water capacity and high specific surface area. In the chemical point of view, biochar has poly-condensed aromatic structure. This structure will lead the changes of the biomass to the biochar. The biomass changes from brown to the black colour which are caused by the degradation process during the thermos chemical (Venkateswarlu, 2018).

Biochar can be produced by using various agriculture wastes such as pinewood, rice husk and wood bark at the different pyrolysis conditions. The example of pyrolysis conditions are heating transfer rate, temperature and time to adsorb metals from water (Li *et al.*, 2017). Report from Lehmann (2007), stated there are two aspects that increase the value of biochar. The first aspect is biochar has high stability from decay and second aspects it ability to retain the nutrients compared to another soil organic matter. Then, there are three environmental benefits using biochar which are the changes of climate, to reduce environmental pollution and also to improve the soils (Lehmann, 2007).

The particulate of biochar form is can clearly distinguish it from others forms of organic matters. They are commonly perceived as macromolecular associations that entrapped in fine pores and macromolecules. The particulate form is used in protection against decay by compartmentalization (Lehmann, 2007).

### 2.7.2 Production of Biochar

Biochar has different chemical and physical properties (Nartey & Zhao, 2014) and is produced from pyrolysis of biomass. The difference of properties of biochar was determined by temperature applied during the pyrolysis process.

The production of biochar at high temperature will produce biochar that has a large pore size, high surface area and will be more effective in non-selective sorption of contaminant. The biochar that contains high surface area as efficient sorbents to reduce environmental pollutions. While using low pyrolysis temperature, the biochar produced will have more polar functional groups effective in the sorption of polar compounds (Yavari et al., 2014).

The process of low temperature pyrolysis is using moderated heat between 400°C until 500°C to heat the biomass which is under complete or partial exclusion of oxygen. In addition to heat the biomass in low temperature pyrolysis, the exothermic process will take place on biomass and release a multitude of gaseous components. Then, the gases and heat release can be collected and can be used to produce energy carriers such as bio-oil and electricity (Lehmann, 2007).

The uses of pyrolysis technology will cut off the production cost. This technology will produce a high energy recovery from waste, produce less contamination and high energy recovery compared to the activated carbon. Biochar is the precursor for producing activated carbon. Activated carbon has higher surface area and porosity compare to the biochar but the production cost of activated carbon is quite high. Activated carbon also will form toxic after sorption and need for regeneration and the loss of sorption efficiency after regeneration make limitations (Yavari et al., 2014).

## 2.6 UV-Visible Spectrophotometer

UV-Visible spectrophotometer is an analytical instrument that used for UV-Vis spectroscopic analysis which is used to measure the absorbance of UV or visible radiation through an analyte. The configuration of spectrophotometer has been categorized into a few types such as into single beam, split beam or double beam types (Fereja et al., 2015).

A range of techniques has been developed to analysed different types of samples. For example, when to determine the analyte molecule contains a chromophore, the direct spectrophotometric determinations are developed and used. In the other hand, the indirect determinations are used when to determine analyte molecule that does not contain a suitable chromophore. On these instances, the analyte is made to quantitatively react with a molecule containing a chromophore and correlating the diminution of absorbance with the concentration of the analyte or by reacting with a reagent, which produces a chromophoric group (Fereja et al., 2015).

The UV-Vis spectrophotometer is deviations from the Beer-Lambert law. According to this law, it stated that the absorbance is linearly proportional to the concentration of chromophores. Different spectrophotometer has different amount of stray light, which the light is received at the detector but not anticipated in a spectral band isolated by the monochromator. The higher absorbance value, the higher the amount of light being absorbed by the molecule (Wilson, 2010).

## 2.7 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is infrared spectroscopy whereby the infrared radiation is passed through a sample (Ganzoury et al., 2015). The changes in vibrational and rotational status of molecules result from the adsorption in the infrared region. This is because of the absorption frequency is influence with the vibrational frequency of the molecules while the adsorption intensity depends on the effectively the infrared photon energy can be transferred to the molecule and it also depends on the changes in the dipole moment that occur as a result of molecular vibration. As a consequence, a molecule will absorb infrared light only if the absorption causes a change in the dipole moment (Amand & Tullin, 1999). FTIR spectroscopy also widely used in investigating materials in gaseous, solid and liquid phase. There are two types of molecular vibrations: one that changes the bond length (stretching) and others that changes the bond angle (bending) (Moraes et al., 2008).

FTIR is also used for determination of quantitative of complex mixtures, identification of organic and inorganic materials, determination of molecular orientation on solution and polymers and the differentiation of geometric and structural isomers. The application of FTIR is compound identification, quantitative analysis of one or more known species, structural elucidation and lastly measurement of the fundamental properties of molecules (Berthomieu & Hienerwadel, 2013).

# CHAPTER 3

# METHODOLOGY

## 3.1 Material

### 3.1.1 Chemical and Reagent

Luria Bertani broth and distilled water were used for media preparation. For the preparation of solid media, the nutrient agar, Reactive Orange 16 dye (RO16) were used. Then, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were added to control the pH (Features, 1993). The immobilization of the dye degrading bacteria, 2% (w/v) sodium alginate solution and 0.2M calcium chloride were used.



### 3.1.2 Apparatus and Equipment



The equipment and apparatus that were used in media preparation were media bottle, pH meter, autoclave machine, centrifuge, spatula, magnetic stirrer, hotplate, electronic balances and petri dishes while sieve and auto sieve shaker, oven, zipper bag and furnace are required in preparing the biochar. The next step is to immobilise the dye degradation microbe. The apparatus and material that required to immobilise the dye

degrading microbe are filter paper, flask, and syringe without needle, needle, and filter tunnel and lastly beaker. Next, for decolourisation assay, centrifuge machines, incubator, cuvettes and spectrophotometer was needed. Then, FTIR analysis was used in dye degradation analysis.

### 3.1.3 Bacteria Strain

Dye degrading bacteria used in this study is *Bacillus sp*. (UMKDG1) that was isolated from batik factory at Kota Bharu. Nutrient agar with 0.1% w/v RO16 was used to maintain the dye degrading bacteria (Pou, 2018).

## 3.2 Method

### 3.2.1 Preparation of Biochar

Agriculture waste that used as biochar in this experiment is coconut fronds. Coconut fronds were collected at Jeli district. The coconut fronds cushion was used in the preparation of biochar. The coconut fronds were washed and sundried. After sundried, the coconut fronds were further dried by using oven in 100°C at 24 hours in order to remove all the moisture inside the coconut fronds. Then, the coconut fronds were blended into the powder. The powdered coconut fronds were sieved into the particle size between 0.125 mm to 0.075 mm by using sieve and auto sieve shaker after the coconut fronds was completely dry. After the powdered coconut frond was sieve, the powdered coconut fronds was burned in a furnace at 600°C for 2 hours and the samples will left to cool down and store in sterile conditioner for further studies (Pohs, 2017).

### 3.3.2 Media Preparation

To prepare 1 litre of Luria Bertani broth (LB) with 0.1% (w/v) of RO16, 20g of LB powder were dissolved with 500ml of distilled water and 100ml of 1% w/v of Reactive Orange Dye stock solution. The pH of the mixture was adjusted to pH 8.0 with an applicable amount of 0.1M hydrochloric acid (HCl) or 0.1M sodium hydroxide (NaOH). Then, the mixture was topped up with 1 litre of distilled water. Next, the media broth was sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

For the preparation of agar media, 28g of nutrient agar (NA) powder was topped up with distilled water 1 litre. Then, the agar media was autoclaved at 121°C, 15 psi for 15 minutes. Then, the nutrient agar was poured into the petri dish and left for solidified.

### 3.2.3 Inoculum Preparation

UMKDG 1 was inoculated into 100ml of LB broth in 250ml Erlenmeyer flask. Then, the culture was incubated overnight until the culture reach the optical density (OD) 0.6 at 600nm under a static condition at 30°C (Joshi et al, 2015).

### 3.2.4 Beads Preparation

The coconut fronds biochar was mixed with the cell suspensions of UMKDG 1 in ratio biochar and cell suspension is 5:100 (w/v) for 2 hours. Next, the 2% (w/v) of sodium alginate solution was added to the mixture with the same amount as the mixture. Then the

mixture was dropwise by using sterilized syringe into the 2% (w/v) of $CaCl_2$ solution. $CaCl_2$ act as cross-linking agent to form the immobilized beads. Then, the bead was leave for 1 hour in $CaCl_2$ to allow it to harden (Chen et al, 2012).

As the control, three other types of beads were also prepared for positive and negative controls. Sodium alginate bead was prepared by mixing 2g of sodium alginate with 200 ml of distilled water for 2 hours. Then, the mixture was autoclaved at 121°C, 15 psi for 15 minutes. The mixture then releases into droplets into the 2% (w/v) of $CaCl_2$ solution and left for 1 hour for hardening.

Immobilized dye degrading microbes were prepared by mixing 100 ml of overnight culture with 200 ml of 2% of sodium alginate solution for 2 hours. The mixture then releases into droplets into the 2% (w/v) of $CaCl_2$ solution and left for 1 hour for hardening.

The biochar bead was prepared by mixing 5 g of coconut fronds biochar with 200 ml of % of sodium alginate and mixed for 2 hours. Next, the solution was autoclaved at 121°C, 15 psi for 15 minutes. The mixture then releases into droplets into the 2% (w/v) of $CaCl_2$ solution and left for 1 hour for hardening (Chen et al, 2012).

### 3.2.5 Decolourization Assay by Immobilized Beads

The immobilized beads and three controls were added into the LB broth that contains 0.1% (w/v) of Reactive Orange 16 separately. The mixture was incubated at 30°C for 72 hours. After the incubation process is completed, the mixture was further centrifuged for 10 minutes at 10000 rpm. To measure the decolourization efficiency, the supernatant was taken and measured by observed the decrease of intensity dye in UV-Vis

spectrophotometer (Sneha *et al*., 2015) at range 492 nm (Gomaa, 2016). LB broth was served as blank. The decolourization assay was conducted in duplicate. The percentage of decolourization of dye was calculated using the formula:

$$\text{Decolourization efficiency (\%)} = \frac{A_0 - A_t}{A_0} \times 100\%$$

$A_0$ = initial absorbance, $A_t$ = observed absorbance (Guadie et al., 2017)

### 3.2.6 Dye Degradation Analysis

#### i FTIR Analysis

After the decolourization assay, the immobilized dye degrading microbes with coconut fronts and three control was taken out from incubator and air dried by using laminar flow then sent for FTIR analysis. Other than that, Reactive Orange 16 powder had also sent for FTIR analysis. Reactive Orange 16 powder was used as the standard. The samples were collected at scanning range 400-2000 cm at several times points (Shobana & Hangam, 2012).

# CHAPTER 4

# RESULT AND DISCUSSION

## 4.1 Decolourisation Assay by Immobilized Beads

Decolourisation assay has been carried out to test the ability of dye degrading microbes (UMKDG1) to decolourise and degrade the Reactive Orange 16 (RO16). The decolourisation assay was carried out at 30°C in static conditions for continuously 72 hours (h) and the result was taken at several time points. A report from Shinkafi et al., (2015) stated that the microbes able to metabolise the azo dyes under aerobic and anaerobic. The microbes can efficiently degrade and mineralize the azo dye under anaerobic conditions compared to the aerobic conditions. Under anaerobic conditions, the microbes reduce the azo dye by using azo reductases. Azo reductases will produce the colourless amine which are mutagenic, toxic and possibly carcinogenic to animals. Under aerobic conditions, the microbes are not completely ready to metabolise the azo dyes. Therefore, while undergoing degradation process, the microbes formed the intermediate product. This intermediate product will disrupt the metabolic pathways and the dyes cannot actually mineralise (Shinkafi et al., 2015).

The decolourisation assay was conducted using co-immobilized dye degrading microbe with biochar and 3 controls. The dye degrading microbes was immobilized with coconut fronds biochar labelled as (AL+BC+MC) to increase the decolourisation activity. The controls are alginate beads (AL), biochar beads (AL + BC) and immobilized dye degrading microbe (AL + MC). The concentration of Reactive Orange 16 was 0.1% (v/v).

The entrapment method using sodium alginate as immobilizing support was very convenient as the sodium alginate was easy to prepare, low cost and non-toxic. Alginate is a natural anionic poly-saccharide that composed of repeated units of α-L-guluronic acid and β-D-mannuronic acid residues. Alginate supports are usually prepared by cross linking of mannuronic acid with guluronic acid residues in the presence of divalent cations like $Co^{2+,}$ $Ba^{2+}$ and $Ca^{2+}$. By using the immobilization, the concentration of microbes can be maintained and the decolourization can occur in a long period. Since the catalytic stability is often improved by immobilization, microbes may degrade a higher concentration of toxic compounds than their free counterpart (Bilal & Asgher, 2015)

In summary, the decolourisation efficiency of 0.1% RO16 can be observed within 24h. Decolourisation of RO16 by AL+BC+MC was the highest compared to the three controls which were 38.74%. Then, followed by with AL+ MC, AL + BC and lastly AL which are 8.07%, 0.10% and 0.04% respectively. The result of the decolourisation of immobilized dye degrading microbes and the controls are shown in Table 4.1.

Then, decolourisation activity for AL+BC+MC was carried out for 72 hours. Based on the result in Table 4.1, decolourisation activity of AL+BC+MC is still the highest compared to the 3 control which is 79.29%. Then, followed by AL+ BC, AL + MC and lastly AL which were 67.58%, 66.35% and 66.35%.

Table 4.1 Percentage of decolourisation of 0.1% RO16 for 24 hours and 72 hours.

| Type of samples | Percentage of decolourisation assay of 0.1% RO16 for 24 hours | Percentage of decolourisation assay of 0.1% RO16 for 72 hours |
|---|---|---|
| AL+BC+MC | 38.74 | 79.29 |
| AL + BC | 0.10 | 67.58 |
| AL + MC | 8.07 | 66.39 |
| AL | 0.04 | 66.35 |

Biochar is one of solid by-product of pyrolysis. Theoretically, biochar was used as an adsorbent for pollutant due to its large specific area, high charge density, abundance of pores and high surface area (Yang et al., 2016). Many studies had used biochar to decolourize the dye as an example a study from Saba et al., (2014) that used rice husk biochar to decolourize Reactive Black. With the aid of microbes, the decolourization of azo dye can be increased and also the azo dye also can be degraded. Therefore, by immobilizing the dye degrading microbe with coconut fronds biochar, the percentage decolourisation was higher compared to controls.

In Figure 4.1, is shown that the percentage of decolourisation by co-immobilized dye degrading microbe with coconut fronds biochar (AL+MC+BC) was the highest and increase every 24h. AL+BC+MC was able to decolourise and degrade the azo dye due to the presence of microbes. According to Abu Talha et al., (2018), the immobilized cell was an efficient method of degradation. This is because of the large surface of biochar, the contact between microbes and dye significantly increases and resulted the decolourization rate higher at higher concentration. With the aid of active site on biochar, the microbes that present of the surface of biochar can easily and further degrade the dye as the active site of biochar will collect and adsorbed the bulk solution. Thus, by

degrading the bulk, the microbes also renewing the pores of biochar that will use in subsequent adsorption (Abu Talha et al., 2018).

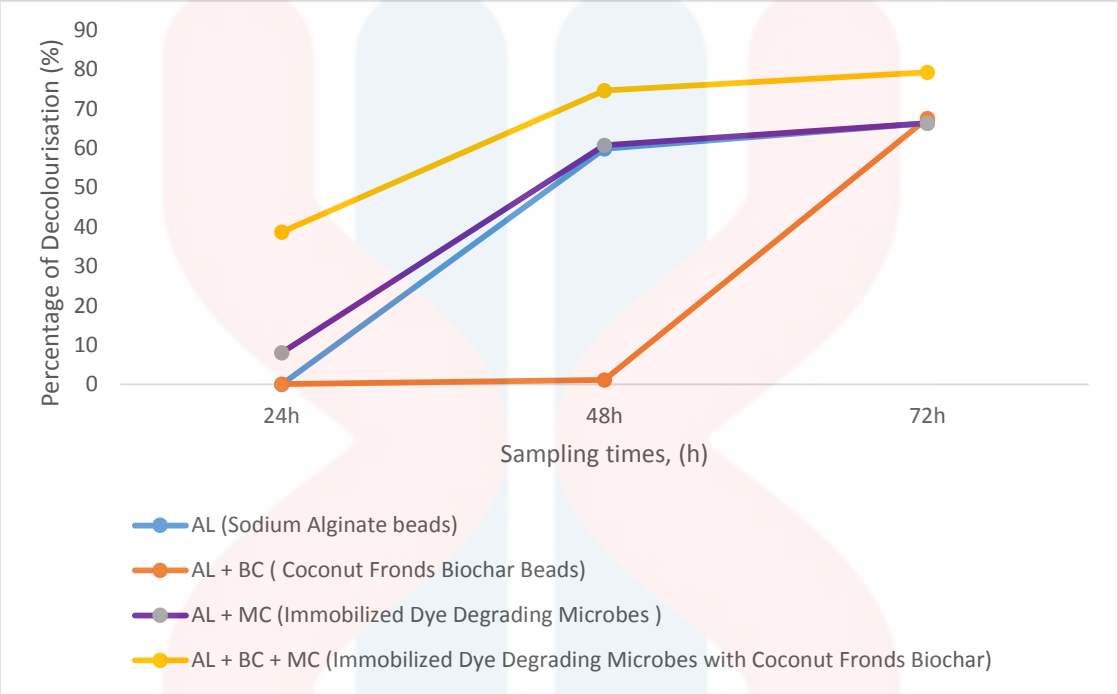


Figure 4.1: Decolourization assay of co-immobilized dye degrading microbe with biochar and 3 controls of 0.1% RO16 at 24, 48 and 72 hours.

Decolourisation assay of 0.1% RO16 was carried out by using the immobilized dye degrading microbe with biochar (AL+MC+BC) and three controls. The controls were carried out in the same condition with AL+MC+BC which were 30°C in static conditions. In the summary after 72 hours, the percentage decolourisation of these three controls, the biochar beads (AL+BC) was the highest result, 67.58%. Then, followed by with immobilized dye degrading microbe (AL+MC) which was 66.39% and lastly alginate beads (AL) 66.35%.

AL+BC was able to decolourise the azo dye because of the presence of biochar. Biochar was used as an adsorbent for pollutant due to the large specific surface area and abundance of pores. The biochar was derived from pyrolysis method at the higher

temperature. A study from Jindo et al. (2014) stated that the higher temperature of pyrolysis of biochar led the biochar with high adsorption characteristics, large surface area and high carbon content. Based on Figure 4.1, the percentage decolourisation of AL+BC was slightly slower compared to AL+BC+MC and other control at 24h and 48h. The AL+BC needed a longer time to decolourise azo dye as there was no aids from others to increase the decolourisation rate and to degrade the azo dye.

The decolourisation of RO16 by AL+MC was slightly higher compared to the AL. Bilal & Asgher, (2015) reported the alginate beads turns coloured after contact with dyes however the sodium alginate only able to remove 7-15% colours of dyes. AL+MC was slightly higher than AL because of the presence of microbes. The microbes not only decolourise the azo dyes but also degrade the dye. Sudha & Saranya (2014), reported that the azo dye was reduced by microbe. The microbe secreted enzymes as examples azo reductase, laccase and peroxide then reduced the dyes. After that, it further degrades into the simpler compounds and utilized it as their energy source.

## 4.2 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectroscopy is infrared spectroscopy whereby the infrared radiation is passed through a sample (Ganzoury et al., 2015). The changes in vibrational and rotational status of molecules result from the adsorption in the infrared region. This is because the absorption frequency depends on the vibrational frequency of the molecules while the adsorption intensity is depends on the effectively the infrared photon energy can be transferred to the molecule. It also depends on the changes in the dipole moment that occur as a result of molecular vibration. As a consequence, a molecule will absorb

infrared light only if the absorption causes a change in the dipole moment (Amand & Tullin, 1999). FTIR spectroscopy also widely used in investigating materials in gaseous, solid and liquid phase. There are two types of molecular vibrations: one that changes the bond length (stretching) and others that changes the bond angle (bending) (Moraes et al., 2008).

The degradation of azo dye was identified through the changes in the FTIR spectrum for the immobilized beads and controls. The information such as position and sizes of adsorption of the immobilized dye degrading bacteria has been determined through the peaks in the infrared region. By comparing the result obtained with the standard (RO16 powder), there were changes in spectrum from range 500$cm^{-1}$ to 1700$cm^{1.}$ This can be observed in Figure 4.2, 4.3 and 4.4. The changes indicate that AL+BC+MC and three controls changed the structure of RO16 after decolourisation assay.

The specific peaks at 1650$cm^{-1}$ to 1500$cm^{-1}$ on the FTIR spectra of RO16 indicated the presence of -N=N- double bond stretching which represent of the azo group in the dyes (Lade *et al*, 2015). Therefore, the disappearing of this peak after decolourisation of RO16 using AL+BC+MC indicated the possible was a reductive breakdown of the azo dye group.

The result of FTIR in 24h (Figure 4.2), 48h (Figure 4.3) and 72h (Figure 4.4) was analysed. It showed that all the samples had a same change absorbance peak at had a changes of peak at 1635$cm^{-1}$, 1614$cm^{-1}$ and 1594$cm^{-1}$. According to a study from Lade et al.,(2015), the changes of absorbance at peak 700-900$cm^{-1}$ was indicated there were changes in the benzene ring structure of azo dye. Therefore, based on the result obtained, the absorbance peak of AL+BC+MC and three controls indicated some changes from 24h

until 72h. Based on the result, it can conclude that the AL+BC+MC and three controls were undergoing degradation process to break down the azo group.

As shown in Figure 4.2, after 24 hours of decolourisation assay, only AL+BC+MC and AL+BC had changes of absorbance peaks at 1419cm$^{-1}$, 1409cm$^{-1}$, and 1026cm$^{-1}$ were indicates the carbonyl (-C=C) and sulfonate group (Dos Santos et al., 2007). Meanwhile, in Figure 4.3 and 4.4 show a few changes after 48 and 72 hours of decolourisation assay. Only AL and AL+BC had detected the carbonyl (-C=C) and sulfonate group at peak 1416cm$^{-1}$, 1409cm$^{-1}$ and 1025cm$^{-1}$ (Singh et al., 2015). In 48 hours of decolourisation assay, it was shown that the AL and AL+BC were not fully broken down the azo group as the spectra were shifted from 1660cm$^{-1}$ to 1600cm$^{-1}$. The intensity of spectra was medium and different compared to the AL+BC+MC and AL+MC. This can be observed in Figure 4.3.

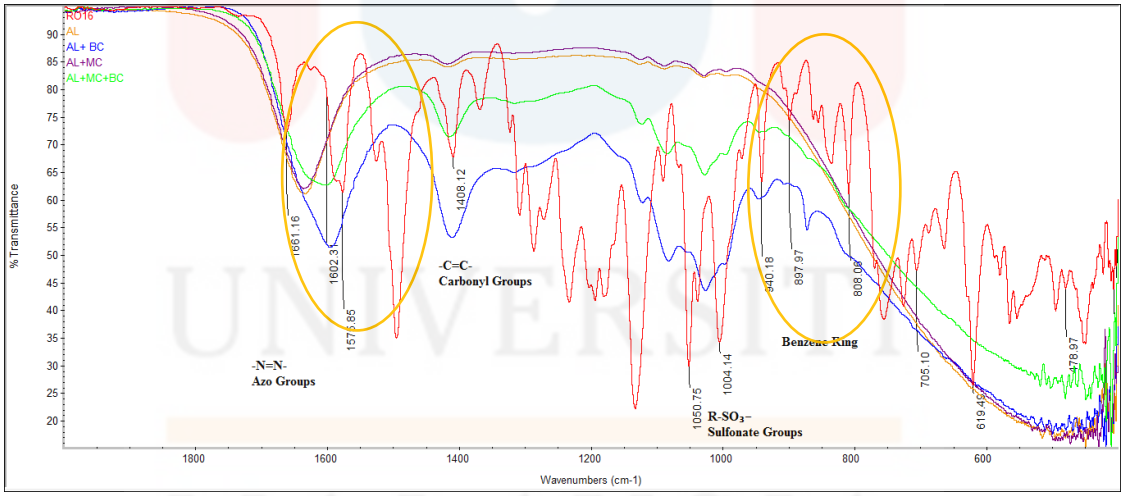


Figure 4.2: FTIR result for decolourisation after 24 hours , ——— : Reactive Orange 16 (RO16) ( Standard), ——— : Alginate beads , ——— : Coconut fronds biochar Beads, ——— : Immobilized dye degrading microbes, ——— : Immobilized dye degrading microbes with biochar.

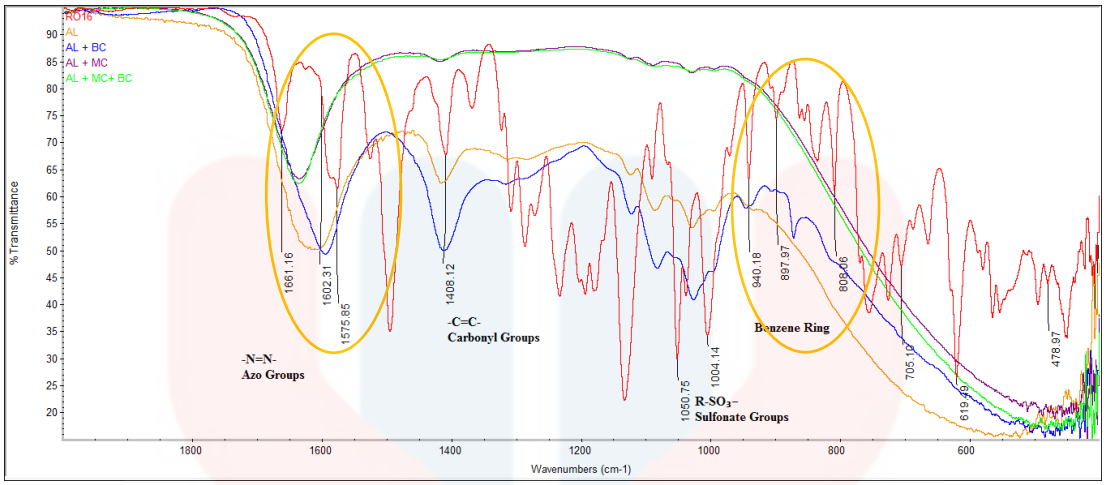


Figure 4.3: FTIR result for decolourisation after 48 hours, ——— :Reactive Orange 16 (RO16) ( Standard), ——— : Alginate beads , ——— : Coconut fronds biochar Beads, ——— : Immobilized dye degrading microbes, ——— : Immobilized dye degrading microbes with biochar.

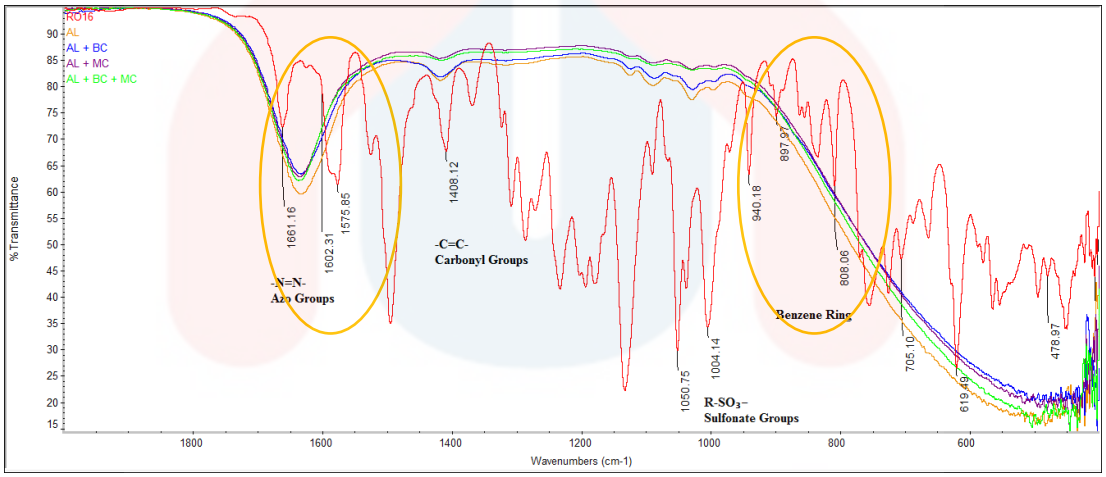


Figure 4.4: FTIR result for decolourisation after 72 hours, ——— :Reactive Orange 16 (RO16) ( Standard), ——— : Alginate beads , ——— : Coconut fronds biochar Beads, ——— : Immobilized dye degrading microbes, ——— : Immobilized dye degrading microbes with biochar.

The microbial degradation of azo dyes such as RO16 can be carried out under aerobic and anaerobic condition. According to the report from Sudha & Saranya, (2014), it stated that the degradation of azo dyes was able to degrade under aerobic condition but the intermediate pathway cannot be completely mineralized. However, in the anaerobic condition, the azo linkages can easily reduce with digester sludge. In anaerobic

conditions, the reduction of azo dyes is the reductive cleavage of azo linkages which resulting in the formation of colourless aromatic amines.

Generally, the degradation of azo dye has two stages. The first stages are the formation of the colourless aromatic amine. Then, the second stages are the degradation of aromatic amines which can only be obtained and occurred at the anaerobic condition. The microbial degradation of azo dyes was about the reduction of –N=N-. The reduction can occur either intracellular or extracellular with different mechanisms such as enzymes or low molecular redox mediators. The enzyme that reduced the azo dyes either specialized enzymes or non-specialized enzymes. The specialized enzymes were only catalysing the reduction of azo dyes. While the non-specialized enzymes, it catalyses the reduction of a wide range of compounds including the azo dyes. The specialized enzymes of reducing azo dye called as azoreductases.

The other biological degradation of azo dyes is indirectly reduced enzymatically reduced electron carriers. A report from Mohammed et al., (2018) stated that anaerobic azoreductase was Flavin reductase. It also reported that Flavin dependent reductases generate the Flavin such as FADH2, FMNH2 and riboflavin. The Flavin was able to stimulate the azo dye reductions. The reduced Flavin nucleotides and redox mediators used azo dyes as electron acceptors to enhance the reduction. The reduction of azo dyes using Flavin reductases also resulted in the colourless aromatic amine.

The breakdown of RO16 might produce by products which are 2-(4-aminophenyl sulfonyl)ethyl hydrogen sulphate and 6-acetamindo-4-hydroynaphtalene-2-sulfonic acid (Dea & All, n.d.). The reaction was shown in Figure 4.5

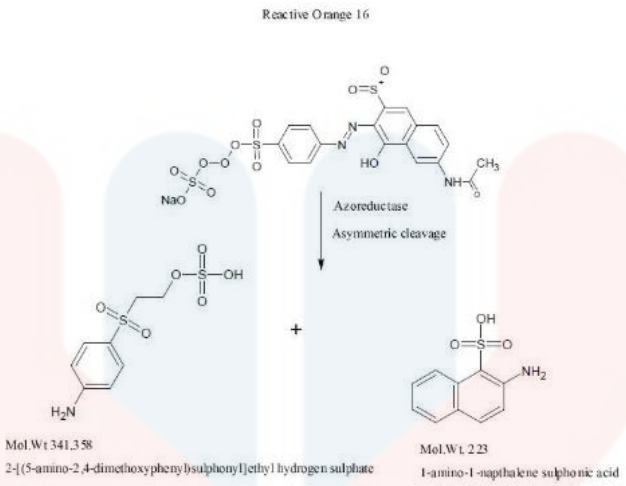


Figure 4.5: The mechanism of Reactive Orange 16
Sources : (Dea & All, n.d.)

The result obtained in Figure 4.2, 4.3 and 44 can be improved if the samples are mix with KBr (potassium bromide). The immobilized beads (AL+BC+MC) and three controls mix with the ratio is 1:20 where 0.02g of sample and top up with KBr until the final weight is 0.4g. Then, the sample is ground and desorbed for 60°C for 24 hours and pressed to obtain the IR-transparent pellets. By using FTIR spectrum, the absorbance of sample can be recorded and collected at scanning range 400-4000 cm. The FTIR spectrum is first calibrated with pure KBr before measuring the absorbance the sample (Shobana & Hangam, 2012). Overall, based on Figure 4.1, it can be concluded that the AL+BC+MC not only able to speed up the decolourisation assay but it also able to degrade the RO16.

# CHAPTER 5

## CONCLUSION AND RECOMMENDATION

In this project, the dye degrading microbe was successfully immobilized with coconut fronds biochar using sodium alginate. As for control, three controls which are alginate beads, Coconut fronds Biochar beads and immobilized dye degrading microbe were prepared. The decolourisation and degradation of 0.1% Reactive Orange 16 were conducted under static condition, pH 8 at 30℃ for 72 hours. At 24 hours, the decolourisation assay of immobilized dye degrading microbes with coconut fronds biochar showned a significant result as the sample are able to decolourise the dye in short of time. The percentage decolourisation of Reactive Orange 16 (RO16) by using immobilized dye degrading microbes with coconut fronds biochar is the highest percentage which is 38.74% in 24 hours and 79.29% in 72 hours compared to other controls. This immobilized dye degrading microbes with coconut fronds biochar indicated that by using the dye degrading microbes with aid of biochar can increase and improve the decolourisation and also degradation of RO16. FTIR analysis was to support that the immobilized dye degrading microbes with coconut fronds biochar is able to decolourise and degrade the RO16. From the FTIR result, it can be concluded that immobilized dye degrading microbes with coconut fronds biochar is able to degrade the RO16.

As the recommendation, scanning electron microscopy (SEM) can be used to investigate the morphology of immobilized dye degrading microbes with coconut fronds biochar, biochar beads, immobilized beads and alginate from before and after decolourisation. Additionally, immobilized dye degrading microbes with coconut fronds biochar also can be analysed by using gas chromatography spectrometry (GC-MS) to identify the compound after the decolourisation and degradation of RO16.

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# APPENDICES

Table A.1: Decolourisation Assay of 0.1% RO16

| Time of sampling (hour) | Decolourisation Assay (%) | | | |
|---|---|---|---|---|
| | AL | AL + BC | AL + MC | AL + BC + MC |
| **0th** | 0 | 0 | 0 | 0 |
| **24th** | 0.04 | 0.10 | 8.07 | 38.74 |
| **48th** | 59.86 | 1.19 | 60.72 | 74.66 |
| **72th** | 66.35 | 67.58 | 66.39 | 79.29 |

Table A.2: Decolourisation Assay of Alginate Beads

| **Time of sampling (hour)** | **Optical Density at 492nm** | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | Average |
| **0th** | 2.506 | 2.516 | 2.515 | 2.506 |
| **24th** | 2.506 | 2.504 | 2.506 | 2.505 |
| **48th** | 1.004 | 1.005 | 1.009 | 0.770 |
| **72th** | 0.853 | 0.852 | 0.857 | 0.854 |

Table A.3: Decolourisation Assay of Coconut Fronds Biochar Beads

| **Time of sampling (hour)** | **Optical Density at 492nm** | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | Average |
| **0th** | 2.511 | 2.511 | 2.512 | 2.511 |
| **24th** | 2.489 | 2.488 | 2.481 | 2.486 |
| **48th** | 2.484 | 2.478 | 2.481 | 2.481 |
| **72th** | 0.842 | 0.770 | 0.829 | 0.814 |

Table A.4: Decolourisation Assay of Immobilized Dye Degrading Microbes

| **Time of sampling (hour)** | **Optical Density at 492nm** | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | Average |
| **0th** | 2.485 | 2.492 | 2.92 | 2.490 |
| **24th** | 2.288 | 2.290 | 2.290 | 2.289 |
| **48th** | 0.975 | 0.977 | 0.981 | 0.978 |
| **72th** | 0.845 | 0.815 | 0.829 | 0.837 |

Table A.5: Decolourisation Assay of Immobilized Dye Degrading Microbes With Coconut Fronds Biochar

| **Time of sampling (hour)** | **Optical Density at 492nm** | | | |
|---|---|---|---|---|
| | 1st | 2nd | 3rd | Average |
| **0th** | 2.512 | 2.504 | 2.503 | 2.506 |
| **24th** | 1.533 | 1.536 | 1.537 | 1.535 |
| **48th** | 0.635 | 0.636 | 0.638 | 0.635 |
| **72th** | 0.507 | 0.528 | 0.522 | 0.519 |

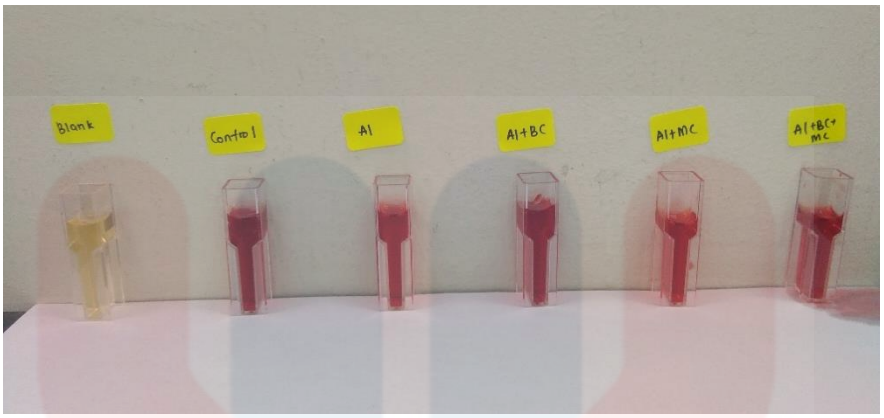


Figure A.1: Decolourisation of 0.1% Reactive Orange 16 at 24h

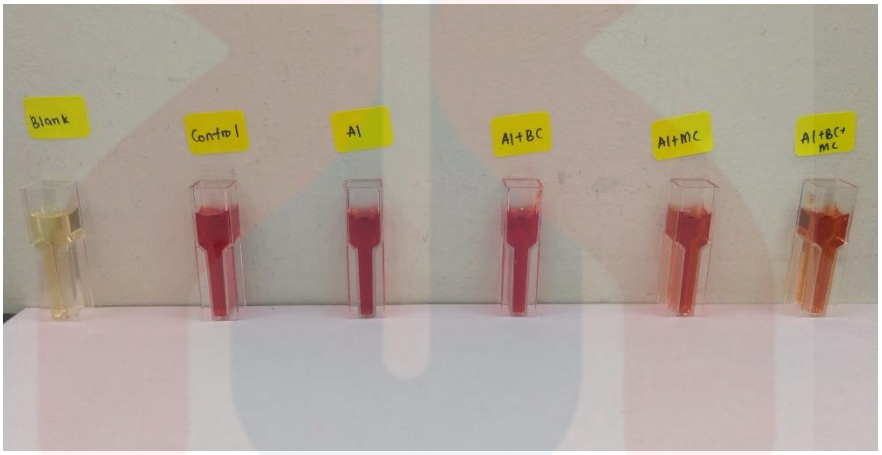


Figure A.2: Decolourisation of 0.1% Reactive Orange 16 at 48h.

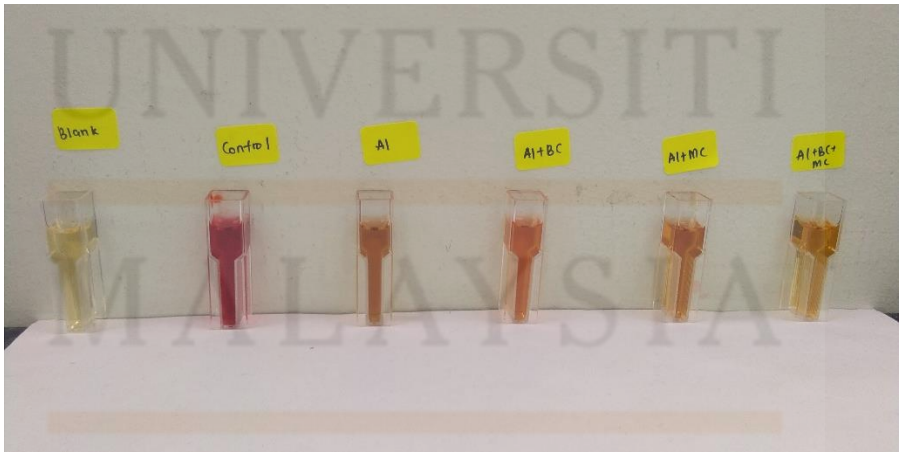


Figure A.3: Decolourisation of 0.1% Reactive Orange 16 at 72h.