DECOLORISATION AND DEGRADATION OF REACTIVE BLACK 5 (RB5) DYE BY CO-IMMOBILIZED DYE DEGRADING MICROBE WITH SPENT COFFEE GROUND BIOCHAR

NORSHAFIQAH BINTI SHAHBUDIN

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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.

_____

Student Name: NORSHAFIQAH BINTI SHAHBUDIN

Matric No :

Date :

Approved by:

_____

Supervisor Name: DR AINIHAYATI BINTI ABDUL RAHIM

Stamp :

Date :

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TABLE OF CONTENT

DECLARATION **ii**

ACKNOWLEDGEMENT **iii**

TABLE OF CONTENT **iv**

LISTS OF TABLE **vii**

LIST OF FIGURES **viii**

LIST OF ABBREVIATIONS AND SYMBOLS **x**

ABSTRACT **xi**

ABSTRAK **xii**

CHAPTER 1 INTRODUCTION **1**

1.1 Research Background 1

1.2 Research Objectives 5

CHAPTER 2 LITERATURE REVIEW **6**

2.1 Textile Effluents 6

2.2 Treatment methods for industrial effluents 7

2.3 Dyes 7

2.3.1 Azo dye 8

2.3.2 Reactive dyes 9

2.4 Biosorption and bioaccumulation 11

2.4.1 Biochar 12

2.5 Biodegradation of azo dye 14

2.5.1 Enzyme involve in degradation of dye 14

2.5.2 Mechanism of azo dye degradation 15

2.6 Immobilization of microbial cell 15

CHAPTER 3 METHODOLOGY 17

3.1 List of material and apparatus 17

3.1.1 Reagents and chemicals 17

3.1.2 Apparatus and equipment 17

3.1.3 Bacterial strain 18

3.2 Method 18

3.2.1 Preparation of media 18

3.2.2 Inoculum Preparation 19

3.2.3 Biochar Preparation 19

3.2.4 Immobilisation of dye degrading bacteria 19

3.2.5 Decolorisation assay by immobilized beads 20

3.2.6 Fourier Transform Infrared Spectroscopy (FTIR) analysis 21

CHAPTER 4 RESULTS AND DISCUSSION 22

4.1 Decolorisation Assay by Dye Degrading Bacteria 22

4.1.1 Decolorisation of Reactive Black 5 on Different Dye Concentration 22

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR) Analysis 29

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS 40

REFERENCES 43

LISTS OF TABLE

NO		PAGE
4.1	Comparison of the percentage of decolorisation for 0.1% (w/v) Reactive Black 5 and 0.01% (w/v) Reactive Black 5 by UMKDG 1 after 3 days of decolorisation assay at 597 nm wavelength.	30

LIST OF FIGURES

NO		PAGE
2.1	Structure of Reactive Black 5	10
2.2	Structure of Reactive Orange 16	11
4.1	Result of decolorisation assay for 0.1% (w/v) Reactive Black 5	23
4.2	Result of decolorisation assay for 0.01% Reactive Black 5	24
4.3	FTIR spectra for Reactive Black 5 powder and the co-immobilized dye degrading bacteria with biochar beads for decolorisation assay of 0.1% (w/v) Reactive Black 5.	30
4.4	FTIR spectra for beads before decolorisation	31
4.5	FTIR spectra for Reactive Black 5 powder and immobilized bacteria beads for decolorisation assay of 0.01% (w/v) Reactive Black 5.	32
4.6	FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 1st day decolorisation of 0.1% RB5.	34
4.7	FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 4th day decolorisation of 0.1% RB5.	34

4.8 FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 8th day decolorisation of 0.1% RB5. 35

4.9 FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 1st day decolorisation of 0.01% RB5. 36

4.10 FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 2nd day decolorisation of 0.01% RB5. 37

4.11 FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 3rd day decolorisation of 0.01% RB5. 37

4.12 Proposed degradation pathway of Reactive Black 5 by *Aeromonas hydrophilla* (Bouraie & Din, 2016) 39

LIST OF ABBREVIATIONS AND SYMBOLS

NaOH	Sodium hydroxide
HCl	Hydrochloric acid
LB	Luria broth
℃	Degree Celcius
psi	Pounds per square inch
rpm	Revolution per minute
FTIR	Fourier Transform Infrared Spectroscopy
(w/v)	Weight per volume
OD	Optical density
RB 5	Reactive Black 5
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide

Decolorisation and Degradation of Reactive Black 5 (RB5) Dye by Co-Immobilized Dye Degrading Microbe with Spent Coffee Ground Biochar

ABSTRACT

Water pollution is the most threatening pollution to biodiversity especially the aquatic life. One of the source of water pollution comes from the disposal of effluents from dye-based industries such as textile industry. The colorant from dyestuff interfere the aquatic ecosystem by lowering light penetration, gas solubility and interference in phytoplankton's photosynthesis. Azo dye is highly toxic as it possesses electron withdrawing nature, which later develops en electron deficiency and become reduced to carcinogenic amino compound. The main purpose of this study is to immobilize dye degrading microbe, UMKDG-1, on spent coffee ground biochar and to analyze the decolorisation and degradation of Reactive Black 5 dye by the immobilized dye degrading microbe. All the decolorisation assay were carried out in batch using 0.1% and 0.01% (w/v) Reactive Black 5, pH8 and incubated at 30℃ under static condition. The control for this experiment are Reactive Black 5 dye powder, alginate beads, biochar beads and microbial beads. Decolorisation efficiency on different initial dye concentration (0.1% and 0.01%, w/v) was determined. It was found that higher decolorisation rate was recorded for decolorisation assay of 0.01% (w/v) Reactive Black 5 (30.32%). However, unsuitable FTIR analysis method was used which resulted in the same spectra formation when compared to control, lead to inaccurate determination of the degraded metabolites.

Keywords: Reactive Black 5 dye, decolorisation, dye degrading microbe, microbial immobilization.

Penyahwarnaan dan Penguraian Pewarna Hitam Reaktif 5 oleh Bakteria Pengurai Warna Tergerak-Sekat Bersama Bio-Arang Sisa Kopi

ABSTRAK

. Pencemaran air merupakan pencemaran yang paling mengancam biodiversiti terutama sekali hidupan akuatik. Salah satu sumber pencemaran air berasal daripada pelepasan efluen dari industri berasaskan pewarna seperti industri tekstil. Warna daripada bahan pewarna mengganggu ekosistem akuatik melalui pengurangan penembusan cahaya, keterlarutan gas serta mengganggu proses fotosintesis fitoplankton. Pewarna azo sangat bertoksik kerana ia mempunyai sifat mengeluarkan elektron, yang kemudiannya akan menyebabkan kekurangan elektron lalu tindak balas penurunan berlaku menghasilkan sebatian amino karsinogenik. Matlamat utama kajian ini adalah untuk menggerak-sekat bakteria pengurai warna, UMKDG-1 pada bio-arang sisa kopi serta untuk menganalisis penguraian pewarna Hitam Reaktif 5 oleh bakteria pengurai warna. Kajian ini telah dijalankan secara berkelompok dengan menggunakan pewarna Hitam Reaktif 5 dengan peratus kepekatan 0.1% dan 0.01% (berat/isipadu), pH8, serta suhu inkubasi 30℃ dalam keadaan statik. Serbuk pewarna Hitam Reaktif 5, manik alginat, manik bio-arang serta manik mikrob telah digunakan sebagai kawalan di dalam kajian ini. Keberkesanan penguraian warna pada pewarna yang mempunyai peratus kepekatan awal berbeza (0.1% dan 0.01%, berat/isipadu) telah ditentukan. Kajian mendapati bahawa keberkesanan penguraian warna yang tinggi ditunjukkan pada penguraian 0.01% pewarna Hitam Reaktif 5 (30.32%). Namun begitu, kaedah analisis FTIR yang kurang sesuai menyebabkan penghasilan spektrum yang sama seperti spektrum kawalan. Ini telah menyebabkan penentuan metabolit terurai yang kurang tepat.

Kata kunci: Pewarna Hitam Reaktif 5, penguraian warna, bakteria pengurai warna, bakteria tergerak-sekat

CHAPTER 1

INTRODUCTION

1.1 Research Background

Human activities had created pollution along the way to achieve their goals. Water pollution is the most threatening pollution to biodiversity especially the aquatic life. The major cause of the water pollution comes from the disposal of effluents from dye-based industries. One of the common dye-based industry is textile industry. Textile industry had consumed a massive amount of water and develop huge amount of waste water. The waste water then been disposed to the water body which later went into waste water treatment facility. Effluents containing dyes are complex, and most of them are non-biodegradable and toxic to aquatic and non-aquatic biota (Shah, 2014). About 1.5 million liters average discharge of dye-bearing effluents were discarded per day in India, which become the main cause of chronic and acute toxicity to the living organisms (Chakraborty et al, 2003). In addition, colorant from the dyestuff interfere the aquatic ecosystem by lowering light penetration, gas solubility and interference in phytoplankton's photosynthesis (Hassan et al., 2013). Dyes are classified into acid dyes, reactive dyes, direct dyes, basic dyes, disperse dyes,

azoic dyes, mordant dyes, oxidation-based dyes, vat dyes, naphtol dyes and sulphur dyes (Mustroph, 2014).

Azo dye widely used in textile industry is an aromatic component bind together by azo (-N=N-) chromophores (Shah, 2014). Azo dye is highly toxic where the main cause of its chronic toxicity is the electron-withdrawing nature of the azo group, which then develops an electron deficiency and become reduced to carcinogenic amino compounds (Gupta et al., 2006). Azo dyes have one or more azo bonds (-N=N-) and can be classified into monoazo or diazo according to their number of azo bonds. Azo dyes are used in textile industries because of its nature where they have varieties of color, good stability and solubility, and low cost (Panja et al., 2016). Although azo dyes are beneficial and important in textile industry, it cause pollution to the environment because of its nature of complex composition, high solubility, toxicity and low degradability (Panja et al., 2016). Azo dye are xenobiotic compounds, thus they are recalcitrant in conventional water treatment plants (Steffan et al., 2004). Reactive Black 5 (RB5) is one of the azo dye.

Various method can be used to decolourize azo dye namely chemical method, physical method and biological method. However, some method might end up producing toxic byproduct and large volume of sludge as a result of incomplete degradation. Conventional processes such as physico-chemical treatment tends to produce huge volume of toxic sludge and the degradation only effective for some azo dye as different dye contain different structure that need to be degraded completely. The examples of dangerous aromatic amines formed by degradation of azo dyes are some alkylated derivatives of aniline, *o*-toluidine, naphtylamine derivatives and benzidine derivatives (Tauber et al., 2005).

On contrary, biological method may propose a better solution for complete degradation of azo dye. By using microbial degradation method, a single step of degradation process can be carried out where the decolorisation of azo dye and mineralization of metabolites formed occur at the same time (Steffan et al., 2004). This could reduce cost, low energy requirement and simple structural set up required compared to conventional method. From the research done by Steffan et al (2004), faster degradation rate of ethyl orange was achieved for the microbial consortium entrapped in alginate beads coated with Eudragit and stored in $CaCl_2$. In addition, immobilization of the bacterial culture who act as biocatalyst in the mineralization process purpose is to protect microbial cell from possible toxic effects release by the metabolites produce or changes in environment condition. This creates a demand for the development of treatment concepts which provide irreversible decolorisation and complete mineralization of azo dye in single step.

Disposal of huge volume of azo dyes from dye-based industries to water body is a major cause of water pollution. The structures of the azo dye consists of coupling of diazotized amine with either a phenol or an amine and also the present of azo linkage(s) (Hassan et al.,2013). Hassan et al., (2013) also state that reactive azo dye are completely recalcitrant to conventional wastewater treatment processes that up to 90% of reactive dyes could remain unaffected after activated sludge treatment. This situation had raised health concern as most azo dyes are known for its carcinogenic and mutagenic effects towards human being and aquatic biota.

Conventional physico-chemical treatment method are not always suitable for each dye that it do not provide complete degradation and conversion to CO_2 as textile azo dyes are difficult to degrade completely. This method also has disadvantages which include intensive

energy requirements, huge chemical and power consumption, complex structural set- up, high expenditure and formation of hazardous byproducts. Bioremediation of dyeing industry effluents by using microorganisms have shown capability to overcome this problem as it require low costs, simple structural set-up, easy to operate, wider application and most importantly it is environmental-friendly (Shah, 2014). Bacterial species including *Bacillus sp.* and *Pseudomonas sp.* have been reported to have capability in decolorizing and detoxifying a wide range of azo dyes (Hassan et al., 2013). The microbial degradation involves reductive cleavage of azo bonds of the dyes which results in simpler compound formation and colorless aromatic amine.

This study aims to investigate the decolorisation and degradation of Reactive Black 5 in Luria broth (LB) by immobilized dye degrading microbe. The dye degrading microbe used was UMKDG-1, which previously isolated from the effluents of a batik factory. The dye degrading bacteria was immobilized with spent coffee ground biochar by using sodium alginate and calcium chloride. For the decolorisation assay, the physicochemical parameters used were pH 8, temperature 30℃, static condition and dye concentration of 0.1% and 0.01%. The duration for each decolorisation assay was 72 hours. The analysis of the degradation were carried out by Fourier Transform Infrared (FTIR) analysis at wavelength range from 400 – 2000 cm^{-1}. The disappearance of a few peaks suggests that there might be changes in the functional groups in the dye, which possibly caused by the microbial degradation activity and adsorption by the biochar.

1.2 Research Objectives

The objectives of this study are:

1. To immobilize dye degrading microbe (UMKDG-1) on spent coffee ground biochar by using sodium alginate and calcium chloride.
2. To analyze the decolorisation of Reactive Black 5 dye by co-immobilized dye degrading microbe with biochar on different dye concentrations through decolorisation assay.
3. To study the degradation of Reactive Black 5 dye by co-immobilized dye degrading microbe with biochar on different dye concentration through FTIR analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Textile Effluents

Production of textile fiber products in textile industries consume a huge amounts of water and the effluents generated contains variety of dissolved dyes and contaminants. The release of textile effluents into the environment during textile fiber dyeing and finishing processes is a major cause of water pollution (Pang & Abdullah, 2013). Wastewater treatment through physical, biological or chemical method usually costly and resulting in the production of large amount of sludge. Pollutant in textile effluents were vary and depends on the chemical and treatment process applied (Tufekci et al., 2007).The commonly present pollutants include suspended solids, biodegradable organic matter and toxic organic compounds such as phenol, heavy metal, and colour (Tufekci et al., 2007). The existance of dyes even in a very low concentration in effluent is highly visible and degradation product from those textile dyes are mostly carcinogenic (Al-Prol et al., 2017). In addition, coloured textile effluents discharged into water body would causes reduction in dissolved oxygen concentration and create toxic conditions to aquatic life (Bouraie & Din, 2016). According to Goncalves et al.,(2000), colour normally detectable at a dye concentration exceeding 1 mgL^{-1} and an average concentration of 300 mgL^{-1} has been reported from textile manufacturing processes. Around 10% of approximately 10 000 different dyes and pigments

produced annually worlwide may be found in wastewater (Deveci et al., 2004). The color need to be removed from water body because the color interferes in sunlight penetration into waters, decelerate photosynthesis, inhibits the growth of aquatic life and interferes with gas solubility (Banat et al., 1996).

2.2 Treatment methods for industrial effluents

The methods used for treatment of industrial effluents come under two classes, abiotic and biotic methods. According to Vijayaraghavan & Yun, (2008), abiotic method namely precipitation, adsorption, ion exchange, membrane technology and electrochemical technology. A lot of discussion on the negative aspects of this type of treatment method, which lead to a conclusion that these treatment method are costly, non-environment friendly, and most of the time depends on the waste concentration. Different combination of chemical, physical and biological method are used to decolorise, degrade and adsorb residual dyes to enhance the removal efficiency (Mahmood et al., 2015). In accordance, another initiatives for a more efficient, eco-friendly and cost effective techniques for wastewater treatment were initiated. Biological method for treating dyes are attractive for it requires low costs and promotes complete degradation to avoid the formation of toxic aromatic amines that are produced by the reductive cleavage of azo bonds (Mahmood et al., 2015).

2.3 Dyes

Dye is a colored substance that has an affinity to the substrate to which it is being applied. Natural and synthetic dyes are used widely in textile industries. Dye may be

classified according to their chemical structure. For example, dyes are classified as azo, anthraquinone, cyanine, carbonyl, nitro, styryl, sulfur or phthalocyanine dyes (Mustroph, 2014). Apart from that, dyes are classified by their application in textile dyeing, such as acid, basic, cationic, direct, disperse, reactive, solvent or vat dyes. The color of a molecule become apparent from the ability of the molecule to absorb electromagnetic radiation in the visible range of 380nm to 780nm (Mustroph, 2014). The characteristics of the resulting absorption band are the maximum of the absorption band, the intensity of the absorption band, and the shape of the absorption band, which determine the observed color (Mustroph, 2014). Arnold (2009) mentioned that, according to Wilts color theory, a dye is made up two parts, auxochromes and chromophores. According to Hunger (2003), auxochromes refers to the functional group in a dye molecule which are saturated, and the presence of these functional group compound increase the color yielding power of a compound. This is done by altering the overall energy in the electron system and contribute to solubility and adherence of the dye to the fiber. (Rocca, 2012) gives example of auxochromes are such as $–NR_2$, $-NH_2$, -NHR, -COOH, -OH, $-SO_3H$ and$–OCH_3$ groups. Chromophores are the functional groups which are unsaturated and it determines the color of the dyes (Hunger, 2003). Examples of chromophores are –N=N, -C=C-, -C=N, $-NO_2$, -NO- and –C=O.

2.3.1 Azo dye

Azo dye is a synthetic dye commonly used in textile industry, cosmetics, foods colorant, printing and pharmaceuticals industries. Azo dye has a variety of colors and

become favorable choice in textile industry for its stability, non-biodegraded ability and high solubility. Azo dyes are expressed by one or more azo bond (-N=N-). It is considered as electron-deficient xenobiotic compounds where they are bearing the functional group R-N=N-R' in which R and R' could be either alkyl or aryl group (Sharma & Foster, 2014). Azo dye is a mutagenic and toxic compound. Therefore, the removal of azo dye from effluent in water body is a huge challenge to prevent the formation of toxic byproduct and toxicity release to environment especially to aquatic ecosystem. The toxicity of azo dye is caused by the electron-withdrawing characteristic of the azo group that create electron deficiency and then reduced to carcinogenic amino compounds (Gupta et al., 2006).

2.3.2 Reactive dyes

Reactive dye is a dye that is chemically active radical groups reacts chemically with fibers. Most of reactive dyes dissolve in water. The molecular structure of reactive dye include a parent dyes, a bridging group and reactive groups. The parent dyes include azo, anthraquinone and phthalocyanine. The reactive groups includes triazine, acrylamide and vinysulfone (Lewis, 2011). Chemical reaction that occur between dyes and fibers during dyeing create new covalent bonds. The covalent bonds enhance the color fastness. Reactive dyes are easy to use and possesses bright color. The dyeing process is accompanied by the hydrolysis of the reactive dyes. Hence, there is a post procesing stage to fully wash the hydrolyzed dye (Lewis, 2011).

2.3.2.1 Reactive Black 5

Reactive Black 5 (RB5) is a diazo dye generally used in dye-based industry. The bonding that occurs during dyeing give the RB5 fair fastness properties. RB5 are most commonly used in dyeing and coloration of cellulosic fibers, and can also be applied on wool and nylon. RB5 is also known as 2,7-naphtalenedisulfonicacid as it is an azo compound with two aryldiazenyl moieties places at positions 2 and 7 of a multisubtituted naphthalene. Figure 2.1 shows the structure of RB5 where two azo bonds could observed at the N=N bond which indicate that it is a diazo dye.

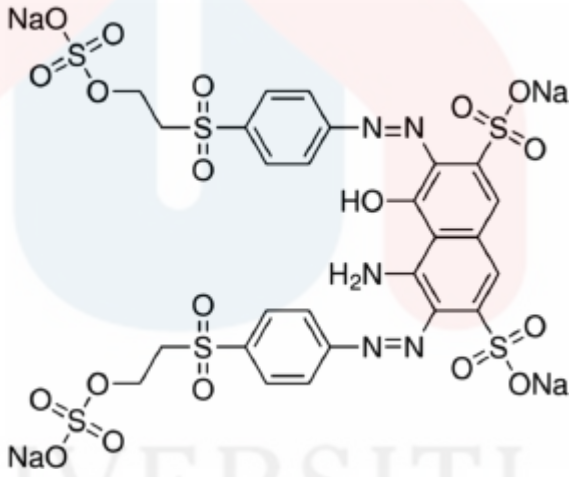

Figure 2.1: Reactive Black 5 structure
Sources: Bouraie & Din, 2016

2.3.2.2 Reactive Orange 16

Reactive Orange 16 is a monoazo dye with two sulfonate groups, which have negative charges in aqueous solution. It is commonly used for dyeing process of cellulose fibers. The general names of Reactive Orange 16 are Brilliant Orange 3R, Reactive Orange 7 and Remazol Brilliant Orange 3R. Figure 2.2 shows the structure of Reactive Orange 16 where the azo bond could be observed at the N=N bond.

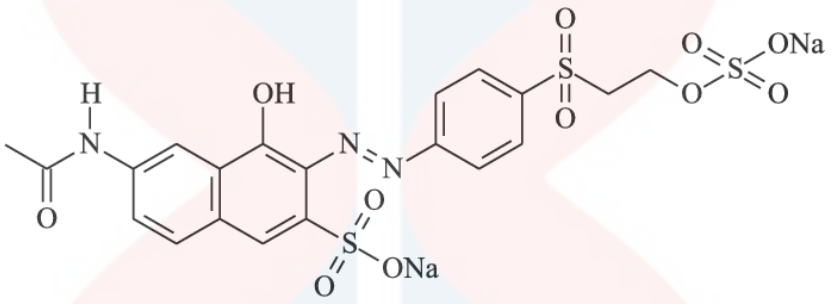

Figure 2.2: Reactive Orange 16 structure
Source: (Gomes, Miwa, & Malpass, 2011)

2.4 Biosorption and bioaccumulation

Biosorption and bioaccumulation are the biological method used in wastewater treatment. Biosorption could be defined as a process where the passive uptake of toxicants by dead or inactive biological materials or by materials sources derived from biological sources. This method is due to a number of metabolism-independent processes which take place in cell wall, where the mechanism used for the pollutant uptake depends on the type of biomass used. Bioaccumulation is the use of living cells in taking up toxicants where the toxicant could be transported into the cell and accumulate intracellularly across the cell membrane and through the cell metabolic cycle. (Vijayaraghavan & Yun, 2008). Biosorbent is the material used for removal of metals/dyes which classified under several categories namely bacteria, fungi, algae, industrial wastes, agricultural wastes and other

polysaccharides materials (Vijayaraghavan & Yun, 2008). Generally, all types of biomaterials possesses good adsorption capacities towards all types of metal ions.

2.4.1 Biochar

Biochar is a carbon-rich product obtained from biomass thermochemical conversion in a closed container with a little or no available air (Nartey & Zhao, 2014). Examples of biomass that can be used to produce biochar are woods, leaves, agricultural waste, organic waste, kitchen waste and forest residues. Carbonization process of biomass removes impurities inside it and only carbon left after the process which make biochar rich in carbon. Biochar can be used as adsorbent. The reasons why biochar fit to be biosorbent are they have high surface area, large pore volume, plenty of functional groups, stable molecular structure and good adsorption performance (Deng, Zhang, & Wang, 2017). The porosity of biochar depends on the lignin content in the biomass used to make biochar (Nartey & Zhao, 2014). Biochar can be prepared by several methods such as pyrolysis, hydrothermal carbonization, gasification, torrefaction and other method (Nartey & Zhao, 2014).

2.4.1.1 Application of biochar

Conversion of waste product into biochar can be considered as a proper way of disposal and recycling. The potential biochar applications include carbon sequestration, improvement of soil fertility, pollution remediation, and also agricultural byproduct / waste recycling (Ahmad et al., 2014), the properties of biochar is determine by several parameters which are pyrolysis temperature, residence time, heat transfer rate and feedstock type. High pyrolysis temperature would produce biochar that is suitable and effective for the sorption of

organic contaminants. This is because high temperature in pyrolysis make the biochar have high surface area, micro porosity and hydrophobicity (Ahmad et al., 2014). Meanwhile, biochar obtained from low temperature process are effective for removal of inorganic or polar organic contaminants by oxygen-containing functional groups, electrostatic attraction and precipitation (Ahmad et al., 2014). Biochar natures have shown potential as a very useful environment sorbent for organic and inorganic contaminants in soil and water. Thus, the selection of suitable biochar is crucial in determining the efficiency of its function.

2.4.1.2 Spent coffee ground biochar

Coffee is one of the most abundance agricultural products all over the world and the second most traded commodities (Anastopolous et al., 2017). Therefore, huge amounts of coffee waste, especially spent coffee ground (SGC) were produced. SCG refers to powdered organic remnants obtained after coffee is been extracted from the beans by steam under high pressure (Kim, 2014). SCG are small particles of arganic material appoximately 20 μm, made up of fiber and complex lignin structures with a high surface areas (Kondamundi et al., 2008). Anastopoulos et al (2017) stated that SGC is a residue with fine particle size, high humidity (80-85%), organic load, and acidity which obtained from treatment of raw coffee powder with hot water or steam for instant coffee preparations. Statistics show that 1 ton of green coffee would generate about 650 kg of SCG, and approximately 2 kg of SCG are obtained from 1 kg of soluble coffee powder.

Spent coffee ground biochar (SCGB) have been widely used in adsorption or amendment of heavy metals that contaminate water or soil (Kim et al., 2014). In addition, SCGB had shown potential in adsorption of dye and colorant from wastewater. Anastopoulos

et al (2017) had suggested that –OH, C=O and amino groups might participate in adsorption process in dye removal based on the FTIR band studies before (pure) and after (loaded material) adsorption in order to find out changes in adsorption of different bands to determine which functional group are involve.

2.5 Biodegradation of azo dye

Degradation of azo dye could be done by three method; chemical method, physical method and biological method. The combination of these methods could also be done to achieve complete degradation. Azo dye is hard to break down due to its azo linkage. The degradation of azo dye would produce aromatic amines, which is more toxic than the parent compounds. Biodegradation propose the use of microbial cells such as aerobic bacteria, lignin-degrading fungi and yeast in order to break down the azo linkage and mineralize the byproduct from degradation process. The decolorisation process occur by the reductive cleavage of the azo bonds (Steffan et al., 2005).

2.5.1 Enzyme involve in degradation of dye

Enzymatic degradation of azo dye involve the cleavage of azo bonds (N=N) at a chromophore group through reduction, either in aerobic or anaerobic conditions. Singh et al (2015) mention that the reduction process may involve various mechanism involving the roles of enzymes, low molecular weight redox mediators, chemical reduction by biogenic reductants such as sulphide or the combination of the mechanisms. The methods involves in degradation of azo dyes includes direct enzymatic method, direct chemical method and biological method (mediated). Enzymes involve in decolorisation of dyes are laccase and azoreductase which already show promising potential and ability. Apart from that, there are several other enzymes such as manganese peroxidase, lignin peroxidase and polyphenol

oxidase which also have promising future in decolorization and degradation of azo dye (Singh, Singh, & Singh, 2015).

2.5.2 Mechanism of azo dye degradation

Biodegradation of azo dye may take place in aerobic and anaerobic condition. The enzymes are the main factor in the mechanism of biodegradation of recalcitrant compounds. In general, the mechanism of microbial degradation of azo dyes involve reductive cleavage of azo bonds (N=N) with the assistance of azo-reductase under aerobic conditions involves the transfer of four electrons (reducing equivalent). Then it proceed through two stages at the azo linkage and at each stage, two electrons are transferred to the azo dye. Azo dye act as final electron accepter which results into dye decolorisation and the formation of colorless solution. The produced intermediate metabolite such as aromatic amines are further degraded aerobically (Kulandaivel et al., 2014). Hence the presence of oxygen tends to inhibit the azo bond reduction activity since aerobic respiration may dominate utilization of NADH to azo bonds, causing the electron used in respiration rather than decolorisation. Most of the research done involving the anaerobic decolorization of azo dye was conducted using mono cultures from various selection of potential bacteria species such as *Bacillus, Pseudomonas, Aeromonas* and *Micrococcus* were found to be effective in anaerobic degradation for several types of dyes (Saratale et al., 2009)

2.6 Immobilization of microbial cell

Immobilization is the method to attach or entrap the microbial cell in a suitable matrix. Microbial cells are immobilized on biochar to protect them from possible toxic effects release from the metabolism produce or changes in environment condition (Steffan et al., 2004). The uses of free suspension in wastewater treatment results in a lot of disadvantages such as

substrate inhibition, low stability, low degradability of cell for high concentration of pollutants and high possibility of the microbial cell to emerge from the wastewater treatment system. However, immobilization of the microbial cell would overcome this problem as it have several advantages such as high mechanical strength, high metabolic activity and resistance to toxic chemical. The microbial cell are resistance to toxic chemical due to high concentration of biochar and diffusion barriers that protects the biofilm from toxic compounds (Elakkiya et al., 2016)

Immobilization process requires support material to bind microbial cell on the biochar. Support material can be divided to two types namely organic and inorganic. Organic support material includes acrylamide, alginate, agar and polyurethane, while the inorganic support material include zeolite, activated charcoal and glass. There are several criteria in choosing a suitable support material which are non-biodegradable, non-toxic, non-pollute, contain high mechanical and chemical stability, simple immobilization procedure and low expenses. The methods that can be used to prepare the immobilized cells are encapsulation, gel entrapment, covalent bonding, cross linking and adsorption (Elakkiya et al., 2016). Entrapment of microbial cell in alginate beads was considered as the mildest immobilization method to preserve cells viability and to achieve all the advantages of heterogenous catalysis (Steffan et al., 2004).

CHAPTER 3

METHODOLOGY

3.1 List of material and apparatus

3.1.1 Reagents and chemicals

For media preparation, the reagents and chemical used were Luria broth (LB), Reactive Black 5 powder, sodium hydroxide (NaOH), hydrochloric acid (HCl), and nutrient agar (NA) powder. For immobilization of dye degrading bacteria, the chemicals and reagents used were 1% (w/v) sodium alginate solution and 0.2 M calcium chloride.

3.1.2 Apparatus and equipment

For media preparation, the apparatus and equipment needed were autoclave machine, media bottle, spatula, pH meter, magnetic stirrer, hotplate, petri dishes and electronic balance. In immobilization of dye degrading bacteria, syringe without needle, filter funnel, filter paper, flasks and beakers were needed. For decolorisation assay, incubator, centrifuge machine, centrifuge tubes, cuvettes, and spectrophotometer were used.

3.1.3 Bacterial strain

The dye degrading bacteria that used in this study is UMKDG-1. The bacterial strain was isolated from the effluent of batik factories in Kota Bharu (Pou, 2018). The strain was maintained on nutrient agar (NA) media.

3.2 Method

3.2.1 Preparation of media

Luria broth (LB) was used for inoculum preparation. 25 g of LB powder was dissolve with 500ml distilled water solution. The mixture then adjusted to pH 8.0 with appropriate amount of 0.1M sodium hydroxide (NaOH) solution or 0.1M hydrochloric acid (HCl) before it been top up with distilled water to 1 liter. For decolorisation assay, Luria broth with 0.1% and 0.01% dye was used. 25g of LB powder was dissolved with 500ml distilled water and 10ml of 1% (w/v) of Reactive Black 5 stock solution was added into the solution. The mixture then adjusted to pH 8.0 with appropriate amount of 0.1M sodium hydroxide (NaOH) solution or 0.1M hydrochloric acid (HCl) before it been top up to 1 liter. Thus, it will produce 1liter of LB broth with 0.01% Reactive Black 5. For LB broth with 0.1% RB 5, the volume of 1% RB 5 stock solution added was 100 ml. Then, the media broth was autoclaved at 121 ℃, 15 psi for 15 minutes to sterilize the broth. Agar media was prepared by mixing 28g of nutrient agar (NA) with distilled water and top up to 1 liter. The solution then autoclaved at 121 ℃, 15 psi for 15 minutes. After that, the solution were poured into petri dishes and leave to solidified. 1% dye stock solution was prepared by mixing 1.0g of RB5 powder with distilled water and top up to 100 ml. The solution then autoclaved at 121 ℃, 15 psi for 15 minutes.

3.2.2 Inoculum Preparation

For inoculum preparation, single colony of UMKDG 1 was inoculated to 100ml of Luria broth (LB) in 250ml Erlenmeyer flask. The culture was incubated overnight in incubator shaker at 30℃ until the culture reach optical density (OD) of 0.6 at 600 nm by using spectrophotometer.

3.2.3 Biochar Preparation

Spent coffee ground was collected from Ruby Coffee stall in University Malaysia Kelantan. The spent coffee ground was washed and sun dried. Then, it was oven dried at 100 ℃ for 24 hours to remove completely the moisture content inside the material. After that, the material was grinded into powder and sieved into particle size at the range of 0.125 mm to 0.075 mm using sieve and auto sieved shaker. Then, the spent coffee ground was placed inside crucible and sent for furnace at 500℃ for one hour. The material was leave to cool down before being stored inside a sterile container for further studies.

3.2.4 Immobilisation of dye degrading bacteria

The spent coffee ground biochar was mixed with cell culture of bacteria UMKDG 1 for 2 hours. The ratio of biochar and cell culture is 5:100 (w:v). Then the mixture were mixed with 2% (w/v) sterilized sodium alginate solution with the same volume as the mixture. For the formation of immobilized beads, the mixture were released into droplets into excess of 2% $CaCl_2$ solution using sterilized syringe aseptically. The beads were stirred in $CaCl_2$ solution for an hour for hardening. After that, the beads were filtered and rinsed with distilled water to remove excess $CaCl_2$. Another three types of beads were also prepared for positive

and negative controls. Sodium alginate beads were prepared by mixing 2 g of sodium alginate powder in 200 ml distilled water and was sent for autoclave at 121 ℃, 15 psi for 15 minutes. The solution then release into droplets into excess 2% $CaCl_2$ and left for an hour for hardening. Biochar beads were prepared by mixing 5 g of spent coffee ground biochar with 200 ml of 1% sodium alginate solution and was sent for autoclave at 121 ℃, 15 psi for 15 minutes. The solution then release into droplets into excess 2% of $CaCl_2$ and left for an hour for hardening. Immobilized microbe beads were prepared by mixing 100 ml of overnight culture with 100 ml of 2% sodium alginate solution which had been autoclaved at 121 ℃, 15 psi for 15 minutes. The solution then release into droplets into excess 2% of $CaCl_2$ and left for an hour for hardening. All of the beads were then filtered and rinsed with distilled water to remove excess $CaCl_2$.

3.2.5 Decolorisation assay by immobilized beads

The immobilized beads and the other three controls were added to the sterile LB broths which contain 0.1% and 0.01% (w/v) of RB5 respectively and incubated at 30 ℃ under static condition for 72 hours. 2 ml of the mixture were pipetted out and centrifuged at 10 000 rpm for 10 minutes. The supernatant were withdraw and the absorbance were measured at 597 nm by using spectrophotometer. The decolorisation assay were carried out in triplicates. The percentage of decolorisation by the immobilized beads were calculated using the following formula:

Decolorisation (%) = $\frac{Initial\ absorbance - observed\ absorbance}{Initial\ absorbance} \times 100\%$

(Tripathi and Srivastava, 2011)

3.2.6 Fourier Transform Infrared Spectroscopy (FTIR) analysis

Small amounts of immobilized beads and control dye samples were air dried. They were sent for FTIR analysis to analyze the changes of functional group. FTIR analysis were done in the mid-IR region of 400 cm^{-1} to 2 000 cm^{-1}. Reactive Black 5 was used as the standard for the FTIR analysis with another three controls which were the alginate beads, alginate with biochar beads and also immobilized dye degrading microbe beads.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Decolorisation Assay by Dye Degrading Bacteria

4.1.1 Decolorisation of Reactive Black 5 on Different Dye Concentration

The decolorisation assay of 0.1% (w/v) and 0.01% (w/v) of Reactive Black 5 by isolate UMKDG 1 have been carried out in 30 ℃, pH 8, static condition. The decolorisation assay were done by using alginate beads (AL), alginate with biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC). The decolorisation assay for 0.1% (w/v) of Reactive Black 5 were carried out for 4 days and been prolonged to 8 days. Meanwhile, for 0.01% (w/v) of Reactive Black 5, the decolorisation assay were carried out continuously in 3 days. Based on Figure 4.1, the color for 0.1% (w/v) of Reactive Black 5 changes slightly for the 4th day of decolorisation assay for all the sample and controls. However, on the 8th day of decolorisation assay, a significant changes could be observed and shown through the calculation by formula mentioned earlier.

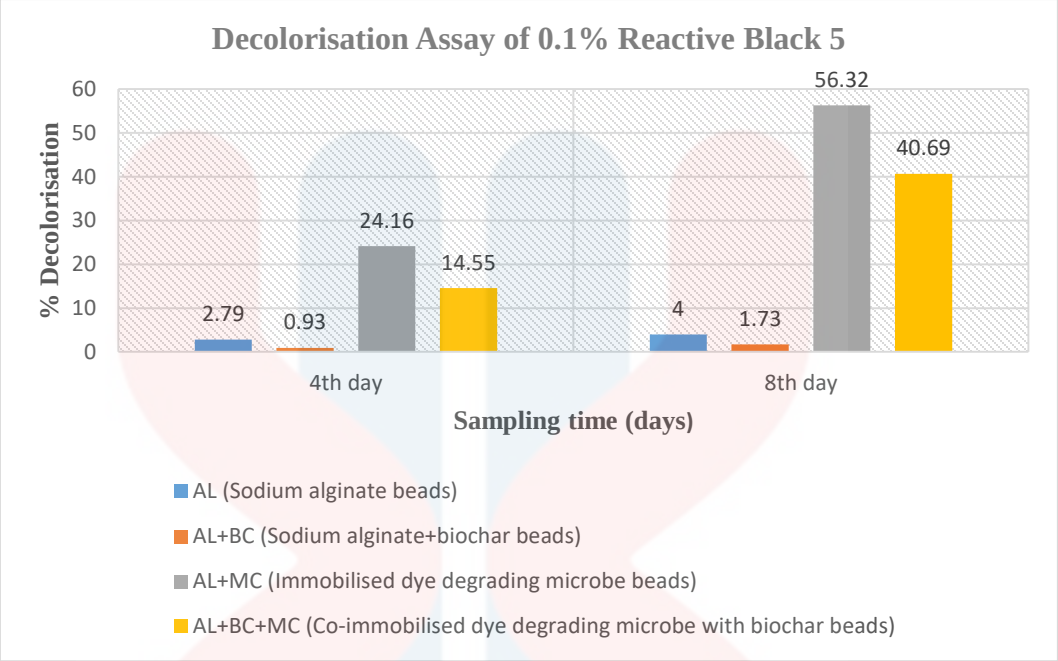

Figure 4.1: Result for decolorisation assay of 0.1% (w/v) Reactive Black 5

The decolorisation percentage of co-immobilized dye degrading microbe with biochar beads for 4th day was 14.55%, and the percentage increase to 40.69% on 8th day. The dye degrading bacteria UMKDG 1 shown a significant decolorisation activity towards 0.1% (w/v) Reactive Black 5 but a longer time is needed for complete decolorisation. For the first 4 days, the color changes were intangible as the percentage decolorisation for all beads including controls did not exceed 25%. The decolorisation assay was prolonged to 8th days to observe a significant color changes in the dye. As predicted, the decolorisation percentage increased to 40.69%. This result is in accordance with the findings from Shah (2014), where the percentage removal of dye would increase when the incubation period increase.

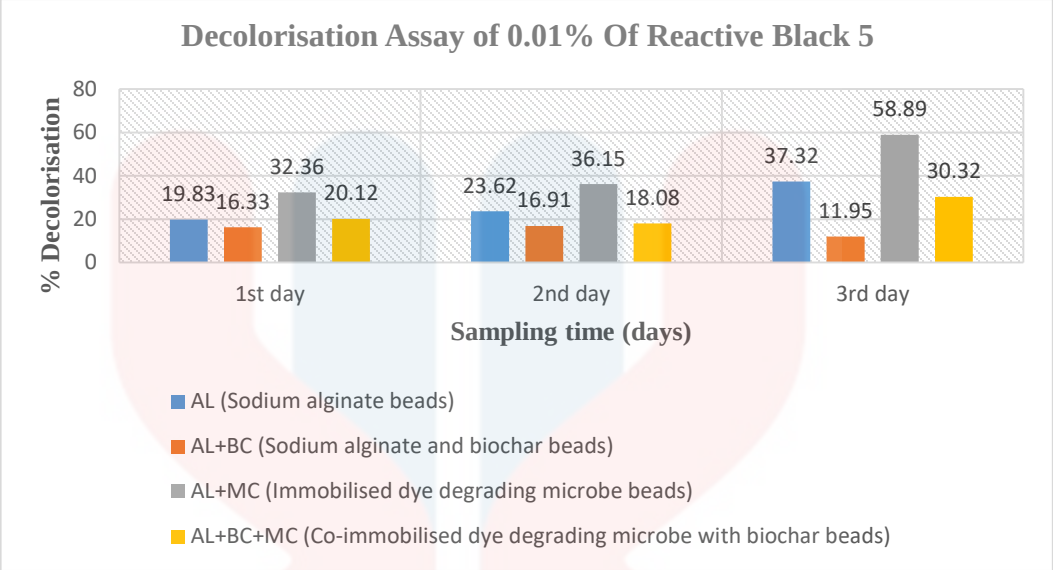

Figure 4.2: Results for decolorisation assay of 0.01% (w/v) Reactive Black 5

According to the results in Figure 4.2, the color of 0.01% (w/v) Reactive Black 5 changed little by little throughout the sampling hours for decolorisation using co-immobilized dye degrading microbe with biochar beads. The decolorisation percentage for co-immobilized dye degrading microbe with biochar beads was 20.12% on Day 1, reduced to 18.08% on Day 2 and gradually increase to 30.32% on Day 3. The result for decolorisation percentage on Day 2 might possesses some error because the absorbance reading increase slightly. This may due to the configuration of the cuvette or the position of the cuvette holder (Mukherjee, 2013) . The other possible reason is the biochar particle might diffusing out from the immobilized beads into the dyed LB broth, resulting in interference in the sample although the sample was already centrifuged. According to Steffan et al.,(2005), the immersion of immobilized bacterial beads in Eudragit solution resulted in increasing mechanical properties of the beads, as it were coated with an external polyacrylamide resin layer which give strength to their structure and avoid biomass leakage. Hence, biomass

leakage might happen which cause the spent coffee ground biochar particle diffuse out from the alginate beads. This problem shall be avoided by increasing the concentration of calcium chloride used in the hardening of the beads to more than 2% concentration to get a harder bead with stronger mechanical properties. Other than that, increasing the concentration of sodium alginate solution used in immobilisation may also increase the hardness of the beads.Another way to lower the biomass leakage is to use another polymer matrice for immobilization. Usha et al.(2010) stated that various matrix could be used for immobilization studies such as carrageenan, alginate, agar, polyurethane foam and polyacrylamide-hydrazide. Usha et al. (2010) studies found out that polyurethane foam matrix has lower(2×10^{1} CFU/ml) biomass leakage compared to Ca-alginate matrix (1×10^{13} CFU/ml) after undergo repeated washing during recycling of immobilized microbial systems for 19 cycles and 25 cycles respectively.

By referring to Table 4.1, it proposed that efficiency in color removal decrease when the color concentration increase. Hence, a longer time required to decolorize the dye at a higher concentration. Similar result obtained by Shah (2014) where the increasing dye concentration from 50-200 mg/l caused continuous decrease in decolorization and growth observed. This probably anticipated by the toxic effect of dyes and also the low reduction capability of the bacterial cells to degrade and transferred dye through the cell membrane (Sani & Banerjee, 1999). This is supported by the findings from (Bouraie & Din, 2016) where the decrease in decolorization ability at higher adsorbate concentration might be due to the toxicity of the dye. Azo dyes normally contain one or more sulphonic-acid groups on aromatic rings, hence might inhibit the growth of microorganisms. Apart from that, the toxicity at higher dye concentration may be because of the presence of heavy metals (metal-

complex dye) and/or the presence of non-hydrolyzed reactive groups which may decelerate the bacterial growth (reactive dyes). Identically, reduction in decolorization at low concentration of the adsorbate may due to the decrease in enzyme activity to recognize the adsorbate efficiently (Bouraie & Din, 2016).

Table 4.1: Comparison of the percentage of decolorisation for 0.1% (w/v) Reactive Black 5 and 0.01% (w/v) Reactive Black 5 by all samples after 3 days of decolorisation assay at 597nm wavelength.

Sample	Percentage of decolorisation (%) for 0.1% RB5	Percentage of decolorisation (%) for 0.01% RB5
AL	2.79	37.32
AL+BC	0.93	11.95
AL+MC	24.16	58.89
AL+BC+MC	14.55	30.32

Furthermore, another possible reason for slow decolorization activity is the uses of non-optimal process parameters during the decolorization assay. Shah, Patel, & Darji (2013) and Shah (2014) found that the optimum parameters on decolorization of dye by *Bacillus spp.* ETL-1982 and *Bacillus spp.* ETL-1949 were pH 9 and incubation temperature 37 ℃ under static condition. Meanwhile, in this study, pH 8 and incubation temperature 30 ℃ were used in decolorisation assay by UMKDG 1, which also a *Bacillus spp.* Although the proposed range of suitable pH for bacterial dye degradation is from pH 6-11, the optimum pH always be either 8 or 9, depends on the bacterial type. However, higher temperature caused a decreased in all parameters, apparently due to the production of large amount of metabolic heat, thus inhibit the microbial growth and enzyme formation (Al-Prol et al., 2017). Besides, the incubation time of decolorisation assay also give effect towards decolorisation efficiency. In this study, continuous 3 days incubation time was used for decolorisation assay of 0.01% Reactive Black 5 and continuous 8 days for 0.1% Reactive Black 5. Result obtained from the study done by Al-Prol et al (2017), found out the decolorisation percentage was increased by the increasing incubation period until reach the optimum decolorization at the 7^{th} day of incubation period with the percentage decolorization 93.8% for Reactive Black 5 and 79% for Reactive Blue 19. However, longer incubation period which is 10^{th} day showed no additional decolorisation percentage where the percentage removal remain the same as 7^{th} day. This is due to nutrient depletion from the medium and also accumulation of some toxic secondary metabolite which inhibit fungal growth and show negative effect on overall degradation activity (Al-Prol et al., 2017).

Based on Figure 4.1 and Figure 4.2, the dye degrading bacteria possess potential ability to degrade Reactive Black 5 dye. This is shown by the decolorisation percentage of microbial beads which are 56.32% on 8th day for 0.1% (w/v) Reactive Black 5 and 58.89% on 3rd day for 0.01% Reactive Black 5. However, this study suggest that the co-immobilized dye degrading bacteria with biochar beads show lower decolorisation percentage compared to immobilized dye degrading microbe beads, which propose that the biochar used might be less efficient for the decolorisation assay. This is supported by the results which show that the decolorisation percentage for biochar beads are the lowest compared to other beads. These results are on contrary with the findings from Kulandaivel, Kaleeswari, & Mohanapriya (2014), where bacteria immobilized with saw dust by gel entrapment method using sodium alginate as polymer matrix, has the ability to degrade dyes at higher concentration than microbes alone. This might due to the less efficient biochar resulted from low pyrolysis temperature (500 ℃) used in biochar preparation. By referring to the previous study by Nakamura et al (2003) where different pyrolysis temperature were used (800 ℃, 1000 ℃, 1200 ℃) on coffee ground to produce charcoal for removal of Acid Orange 7, the result showed that charcoal produced at higher temperature had the highest removal ratio. Charcoal produced at 1200 ℃ also had higher degree of reduction in dye concentration, higher specific surface area, bigger pore volume and lower pore radius compared to charcoal at lower pyrolysis temperature. An activation process for charcoal is necessary to imcrease the adsorption ability (Nakamura et al., 2003).

4.2 Decolorisation Analysis

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Reactive Black 5 consist of two aryldiazenyl placed at position 2 and 7 of a multi-subtituted naphthalene. It is a bis (azo) compound, a sulfone and an organic sodium salt (Gunawardena, 2014). The spectra was observed at the wavelength 400cm^{-1} to 2000cm^{-1} showed peaks at 1214.72cm^{-1} (C-N stretch, amine), 1120.23cm^{-1} (S=O stretch, sulfone), 1042.43cm^{-1} (S=O stretch, sulfoxide), 889.88cm^{-1} (C=C bending, alkene), 663.85-733.66cm^{-1} (C-H bending, monosubtituted benzene derivatives) and 413.94-614.32cm^{-1} (secondary amines). A quite similar result of FTIR spectra for Reactive Black 5 was obtained from previous study done by Bouraie & Din, (2016) where the spectra showed peaks at 3000 - 3718 cm^{-1}(N-H stretch), 2968-28888cm^{-1} (CH_2), 2161cm^{-1} (C-S and S-O stretch), 1626-1748cm^{-1} (C=C stretch), 1387-1596cm^{-1} (N-H), 1078-1256cm^{-1} (O-C), 1011-1078cm^{-1}(alkenes) and 386-644cm^{-1} (secondary amines).

After Reactive Black 5 degradation, significant difference could be observed from the FTIR spectrum for both RB5 concentration. By referring to Figure 4.3, for 0.1% (w/v) RB5, a continuous changes could be observed from Day 1 to Day 8. For Day 1, changes could be seen at 663.85-733.66cm^{-1} where the peak was completely disappeared, which is the C-H bending of the monosubtituted benzene derivates. The peaks at 889.88cm^{-1} - 1214.72cm^{-1} were reduced in intensity, which was observed because of C=C, S=O, C-N and

C-O. The vibration range from 1300-1500cm^{-1} was completely disappeared. New peak formed at 1597.25cm^{-1} (N-H bending, aliphatic primary amines), 1081.82cm^{-1} (C-O stretch) and 1026.09cm^{-1} (C-N stretch, amine). The vibration range from 400-455.88cm^{-1} for mono-subtituted benzene derivatives.

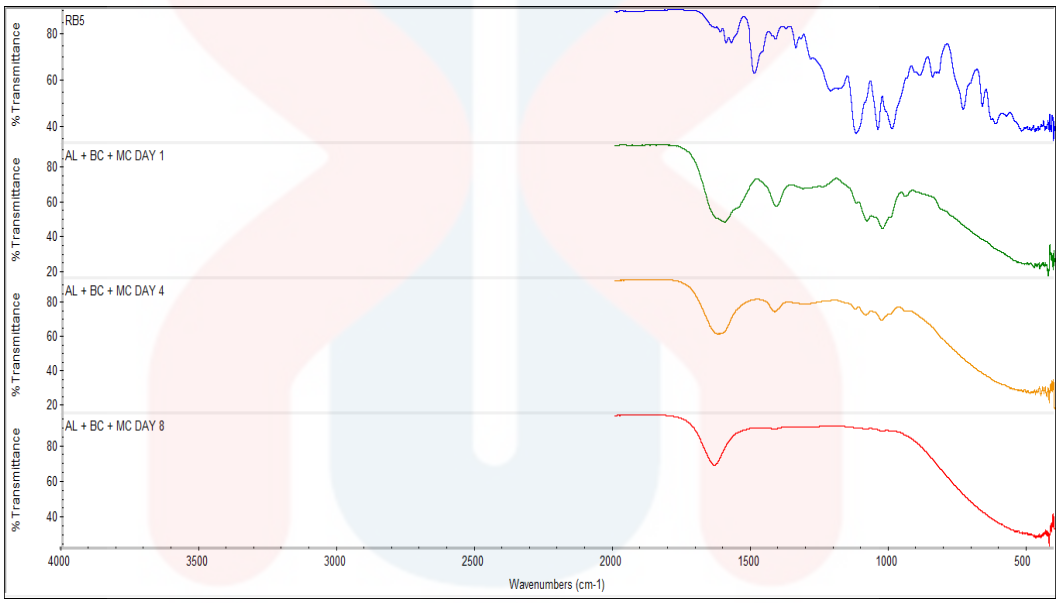

Figure 4.3: FTIR spectra for Reactive Black 5 powder and the co-immobilized dye degrading bacteria with biochar beads for decolorisation assay of 0.1% (w/v) Reactive Black 5.

For Day 4, there are significant changes in the FTIR spectra as several peaks show decreasing intensity such as at 1026.09cm^{-1} (C-N stretch) and 1081.82cm^{-1} (C-O stretch), which indicates the bond stretching is getting weaker and it is going to deform. The vibration range at 800-900cm^{-1} was disappeared while the vibration range 400-480 cm^{-1} remains. The peaks at range 1000-1060cm^{-1} indicates detection of aliphatic sulfoxide (sulfonic acid, sodium salt) which come from the $-SO_3$ stretch. The peaks at range 1550-1650cm^{-1} show the

detection of secondary amine from NH deformation. The presence of aromatic amine at peak range 400-800 cm^{-1} in the degraded sample suggest that degradation had taken place.

For Day 8, most of the peaks have completely disappeared and the peak at 1650cm^{-1} (secondary amines) become more intense. By referring to Figure 4.4, FTIR spectra of Day 8 is similar to the beads before decolorisation. This suggest that the Reactive Black 5 dye was completely degraded. This might due to the late FTIR analysis on the sample, thus the dye had enough time to be degraded inside the beads and the results cannot be taken into account. The peaks observed after decolorisation were for O-H, C-H, C=O, C-C, and C-N clearly indicates the removal of amine from the degradation product (Bouraie & Din, 2016).

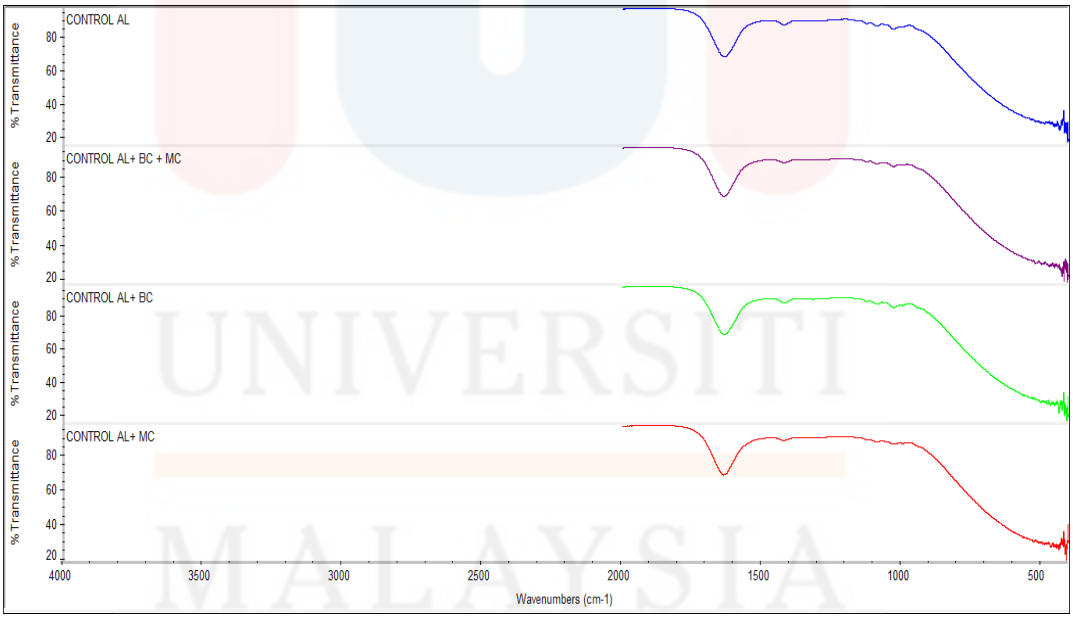

Figure 4.4: FTIR spectra for beads before decolorisation

According to Figure 4.5 which shows the FTIR analysis on degradation of 0.01% RB5 for co-immobilized dye degrading microbe beads with biochar, all the spectra show the similar peak with empty control beads in Figure 4.4. This was due to the error in FTIR sample preparation where the beads sample were kept for a few days before it were sent for FTIR analysis. This proposed that the dye might be continuously degraded inside the beads throughout the time hence the results were not valid.

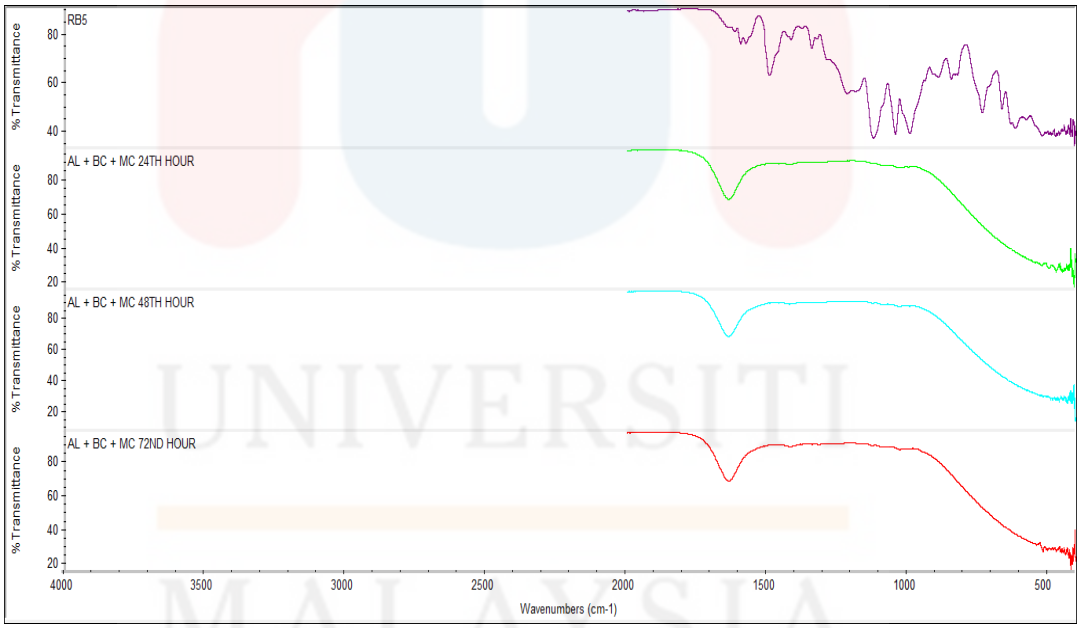

Figure 4.5: FTIR spectra for Reactive Black 5 powder and co-immobilized dye degrading microbe with biochar beads for decolorisation assay of 0.01% (w/v) Reactive Black 5.

According to Zubbair et al. (2018), the peak characteristic of azo bond at (630, 683.5, 119.2, 1655, 2250) cm^{-1} are correspond to –C=C-H:C-H, =C-H, C-H, and S-H. Meanwhile, the peaks (1018.45-111.03), (1411.94-1450.52), (2522.98-2839.31) and 3356.25 which corresponding to C-O, C-C, O-H, and both primary and secondary amine found to be dominant in the degraded reactive dyes. Reactive Black 5 is quite hard to be degraded because it contain sulfo group. Zubbair et al. (2018) stated that azo compound with hydroxyl or amino groups were more likely to be degraded compare to azo compound with methyl, methoxy, sulfo or nitro groups.

Figure 4.6, 4.7 and 4.8 shows the FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for decolorisation of 0.1% RB5 on 1^{st}, 4^{th} and 8^{th} day respectively. From Figure 4.6, the degradation process had started to occur as the beads sample peaks of AL+MC and AL+BC+MC intensity are different from the RB5 powder peak. However, for AL and AL+BC beads, the peaks intensity were quite similar as both beads did not contain microbe whom carried out the degradation process. Changes could be seen at 663.85-733.66cm^{-1} where the peak was completely disappeared, which is the C-H bending of the monosubtituted benzene derivates. The peaks at 889.88cm^{-1} - 1214.72cm^{-1} were reduced in intensity, which indicates the presence of of C=C, S=O, C-N and C-O that show the azo characteristics.

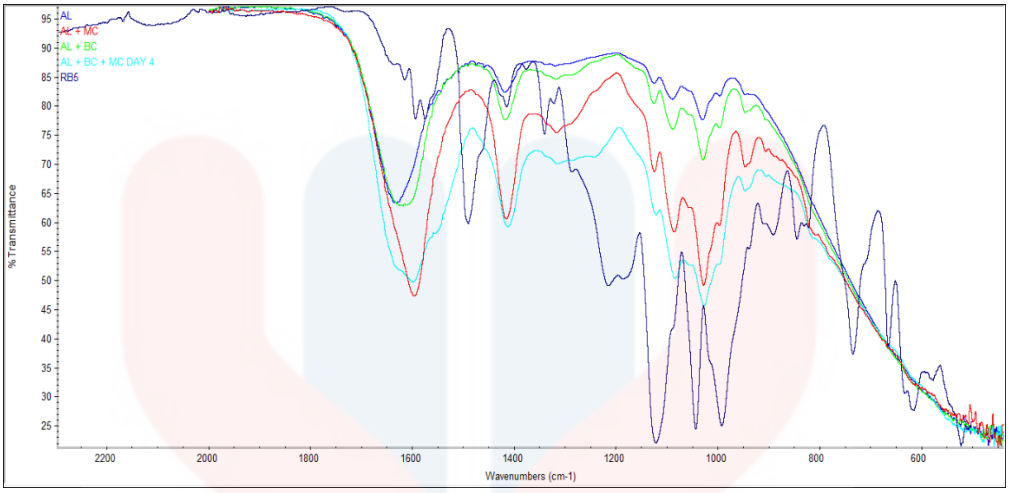

Figure 4.6: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 1st day decolorisation of 0.1% RB5.

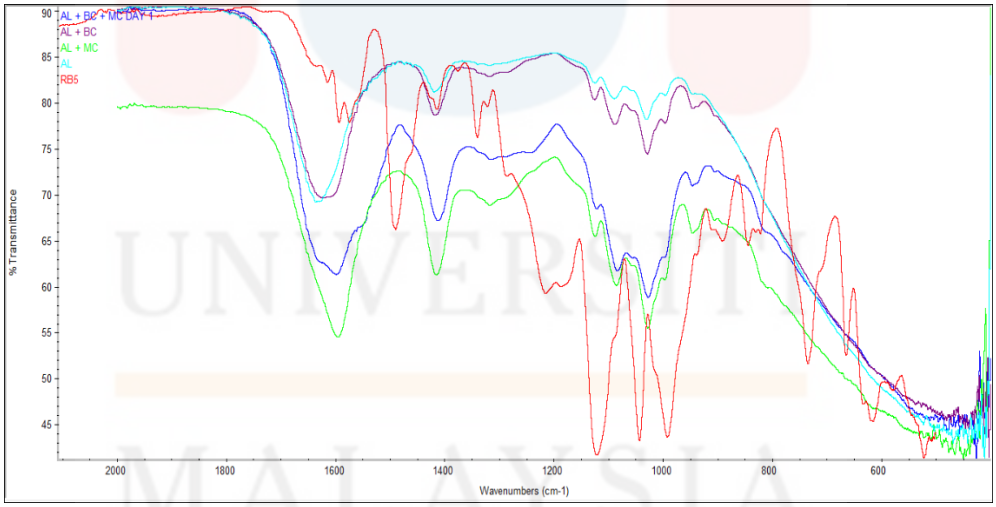

Figure 4.7: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 4th day decolorisation of 0.1% RB5.

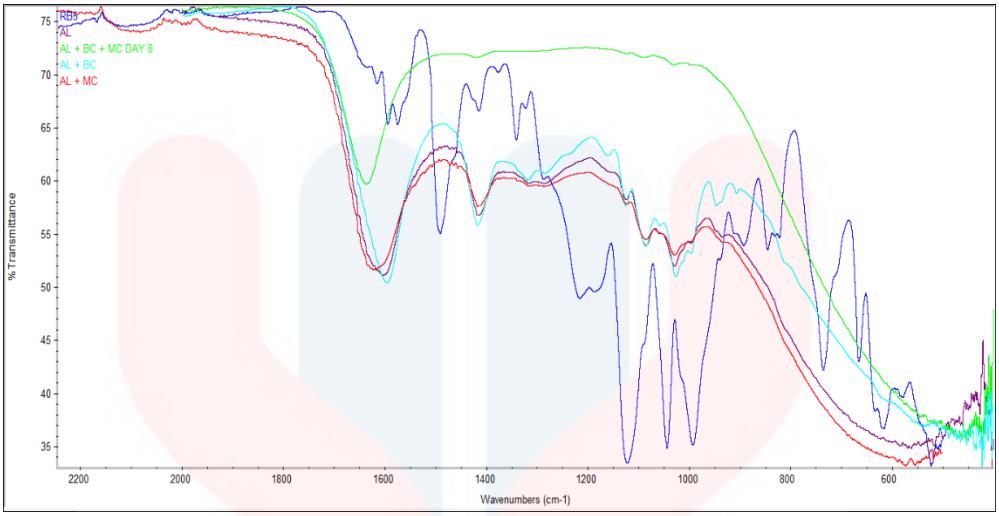

Figure 4.8: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 8th day decolorisation of 0.1% RB5.

For Figure 4.7, there are significant changes in the FTIR spectra as several peaks show decreasing intensity for AL+MC and AL+BC+MC such as at 1026.09cm^{-1} (C-N stretch) and 1081.82cm^{-1} (C-O stretch), which indicates the bond stretching is getting weaker and it is going to deform. The vibration range at 800-900cm^{-1} was disappeared while the vibration range 400-480 cm^{-1} remains. The peaks at range 1000-1060cm^{-1} indicates detection of aliphatic sulfoxide (sulfonic acid, sodium salt) which come from the $–SO_3$ stretch. The peaks at range 1550-1650cm^{-1} show the detection of secondary amine from NH deformation. The presence of aromatic amine at peak range 400-800 cm^{-1} in the degraded sample proposed that degradation had taken place. Meanwhile, for AL and AL+BC peaks, it remains the same as in 1st day which suggest no degradation had taken place. For Figure 4.8, most of the peaks have completely disappeared and the peak at 1650cm^{-1} (secondary amines) become more intense. The AL+BC+MC peaks was similar to the peaks for bead before decolorisation in Figure 4.4 which suggest complete degradation had taken place. Meanwhile, peaks for AL,

AL+BC and AL+MC had become slightly similar which showed that the dye were not completely degraded inside the beads.

Figure 4.9, 4.10, and 4.11 showed the FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for decolorisation of 0.01% Reactive Black 5. The results were inaccurate as the FTIR analysis was conducted a few days after the samples were taken out from the dyed LB broth. The spectra showed that the dye were degraded completely inside the beads throughout the period, thus leaving the same spectra for the three days of decolorisation assay.

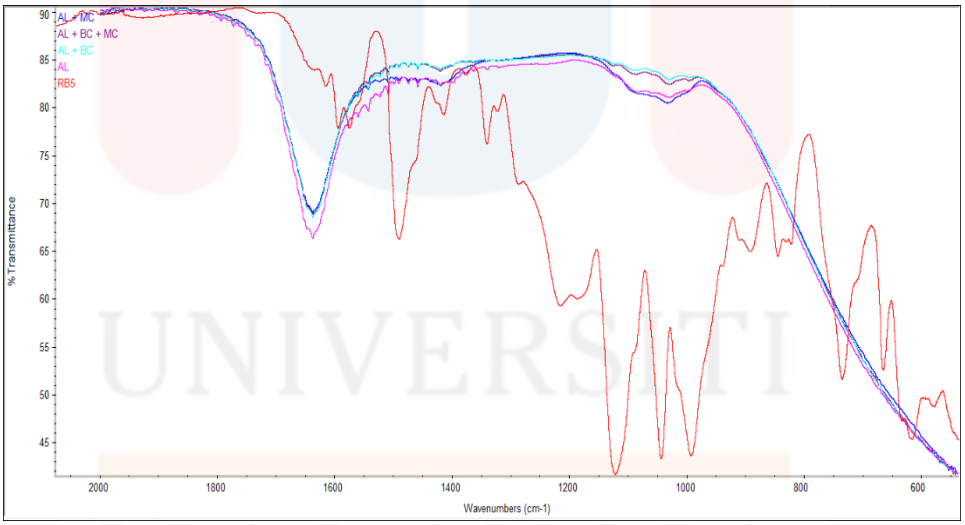

Figure 4.9: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 1st day decolorisation of 0.01% RB5.

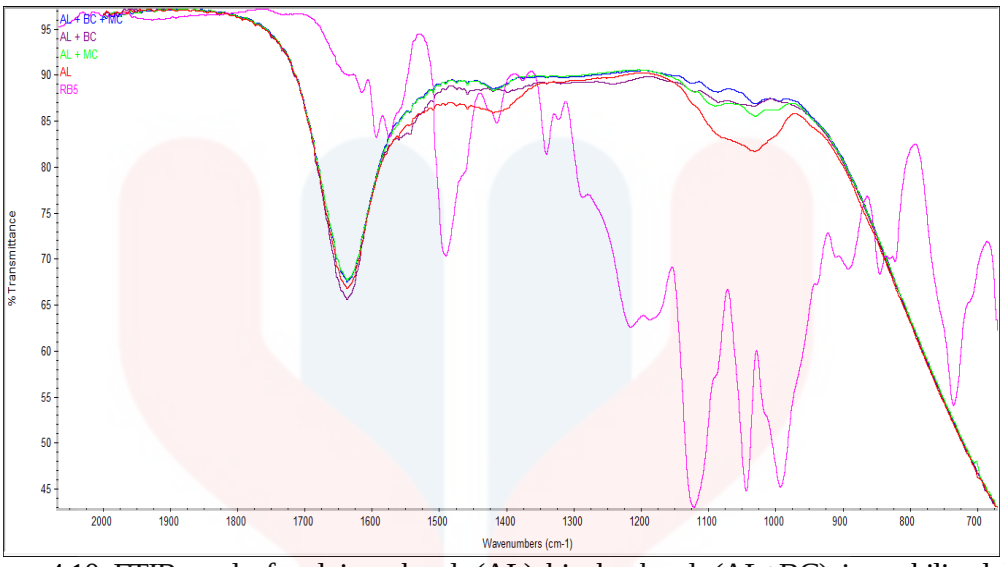

Figure 4.10: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 2nd day decolorisation of 0.01% RB5.

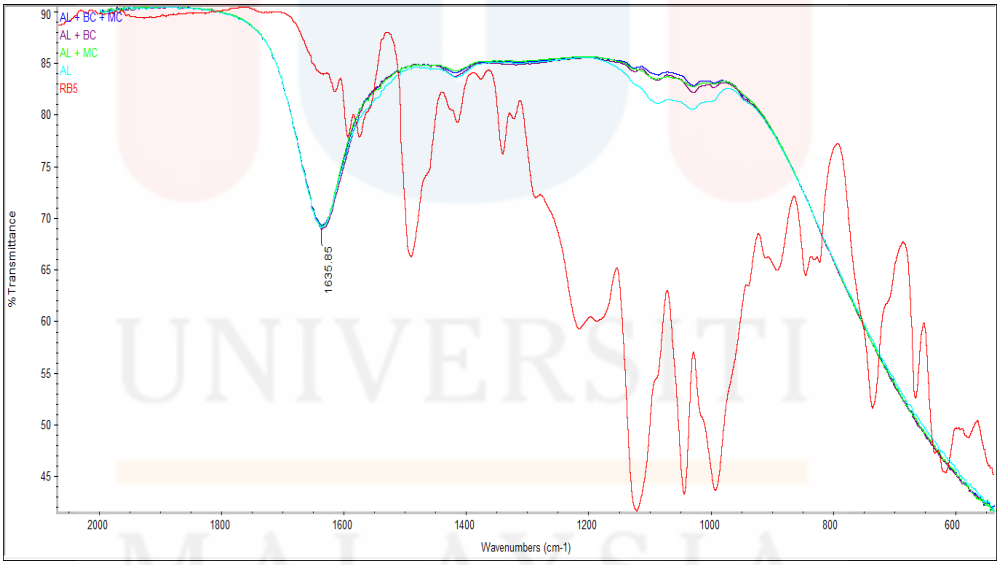

Figure 4.11: FTIR results for alginate beads (AL), biochar beads (AL+BC), immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC) for 3rd day decolorisation of 0.01% RB5.

Figure 4.12 shows the proposed degradation pathway obtained from the previous study done by Bouraie & Din,(2016) which may be compared to this study as both study used bacteria for dye degradation. During the dye degradation, there is asymetric cleavage of azo bonds in Reactive Black 5 which result in formation of 4-aminobenzenesulphonyl and the naphthalene part of the dye was degraded and form smaller compounds (Bouraie & Din, 2016). Further degradation of naphthalene part happen with opening of ring, cause rising in formation of carboxylic group, is confirmed from the FTIR data obtained. Hence, this show that from the analytical method used, Reactive Black 5 is degraded to intermediate compounds as a result of cleavage of azo bond (N=N). The intermediate formed are identified as 4-aminobenzenesulphonyl, napththalene derivatives and carboxylic, which are devoid of any chromophores like azo group (N=N) and thus are colorless (Bouraie & Din, 2016). From Bouraie & Din,(2016) study, the results suggest that the decolorization of dye proceed via the cleavage of azo bond resulting in formation of aromatic amines.

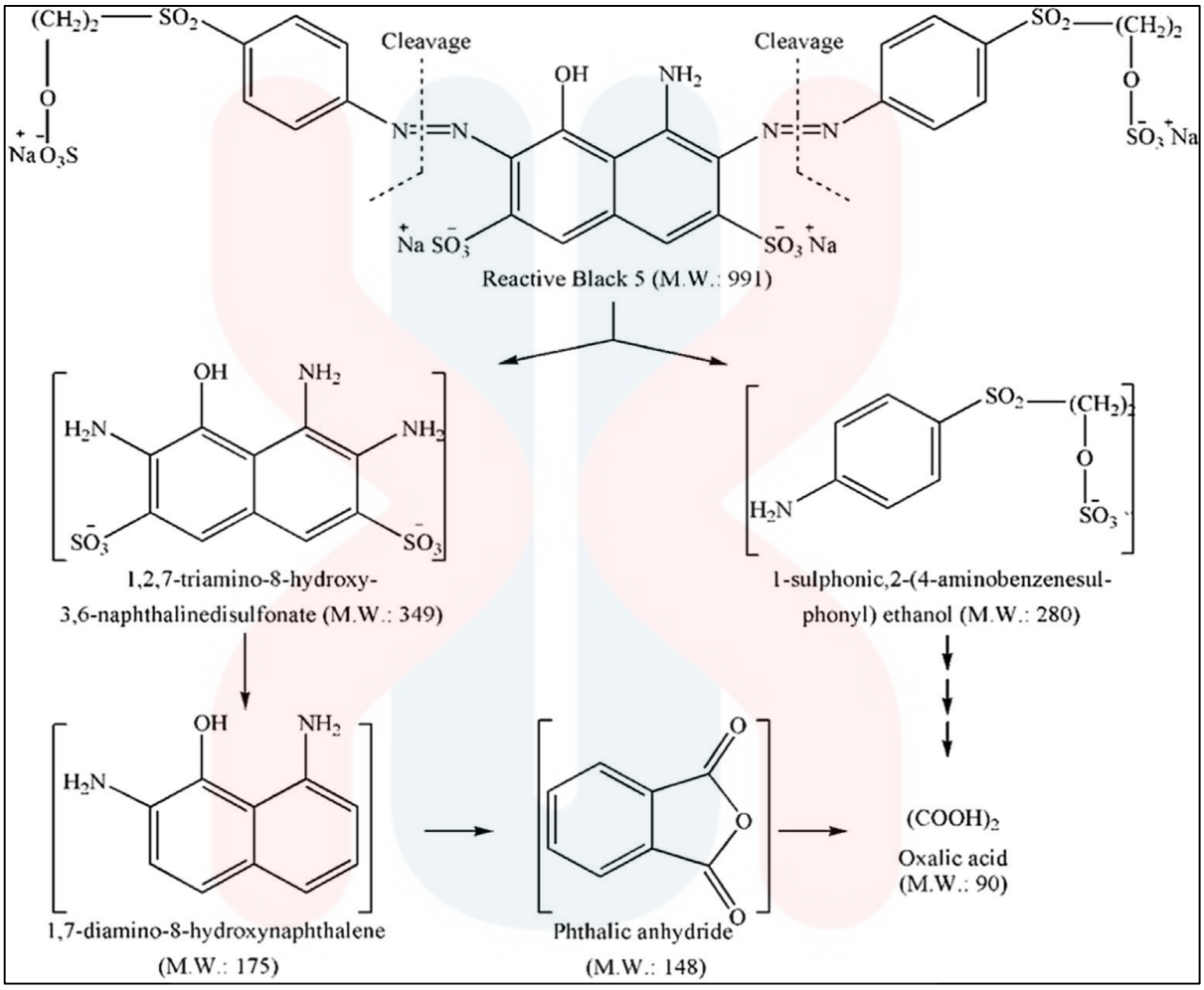

Figure 4.12: Proposed degradation pathway of Reactive Black 5 by *Aeromonas hydrophilla* (Bouraie & Din, 2016)

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The present study demonstrated that the bacterial strain UMKDG 1 possesses a good potential for textile industrial effluent treatment. Based on the presented data, dye concentration has significant effects on the rate of decolorisation activity by the dye degrading bacteria. A good decolorisation performance was shown on dye concentration 0.01% (w/v) within 72 hours. The FTIR analysis suggested that there were changes in functional group during the decolorisation activities. The peak changes observed in the range of 200cm^{-1} to 2 000cm^{-1} had shown that the degradation process had taken place in immobilized dye degrading microbe beads (AL+MC) and co-immobilized dye degrading microbe with biochar beads (AL+BC+MC). However, the determination of functional group for 0.01% (w/v) of Reactive Black 5 was unsuccessful due to further degraded metabolite because of late FTIR analysis conducted. Therefore, it is important to determine the optimum concentration of dye to enhance the decolorisation rate of UMKDG 1 on diazo dye such as Reactive Black 5.

For recommendation, more studies can be carried out to find out the optimum pH medium, temperature and concentration for decolorisation of different type of azo dye such as monoazo, diazo and triazo dye. In present work, the optimum pH for diazo dye decolorisation by UMKDG 1 was not yet be determine due to time constraint. A higher range of pH level may be tested in decolorisation assay as the wastewater polluted by dye mostly high in alkalinity. Therefore, the effect of number of azo bonds on pH could be further studied.

The effect of carbon source and nitrogen source supplemented on the media in decolorisation assay could also be studied to know which carbon and nitrogen source enhance the performance of dye degrading activity by dye degrading bacteria. Various carbon source namely glucose, sucrose, lactose and maltose, and nitrogen source such as NH_4SO_3 and NH_4NO_3 could be added to the media used.

Other than that, study on different pyrolysis temperature in biochar preparation could also be carried out to find out the most effective temperature that form the desired pores suited for the immobilization of bacteria. As for the present study, the temperature 550 ℃ used was quite ineffective for the decolorisation process, hence temperature higher than it could be investigated. A higher temperature would bring out the biochar's ability and efficiency to the fullest in providing more pores for bacteria to form biofilm and immobilized.

In addition, viability test could be done to determine the viability of the bacterial cell after being immobilized inside the beads and the viability period inside the bead. This is important to make sure the decolorisation and degradation of dye was due to the enzyme release by the bacteria, not by the adsorption on the biochar or the polymer binding matrix.

On the other hand, Scanning Electron Microscopy (SEM) could be carried out for characterization of the biochar as a bacterial support matrix. The formation of biofilm on the biochar's surface could be observed and the bacterial cell densities inside the micro pores can be found out. From this, the suitable pyrolysis temperature for biochar could be determine as different temperature would produce different pore size.

Furthermore, Gas Chromatography-Mass Spectrometry (GC-MS) analysis could be conducted to propose the possible degradation pathway of the dye and to determine the intermediate metabolites formed. High Performance Liquid Chromatography (HPLC) analysis is another method that can be used to find out the intermediate metabolites formed during the decolorisation process of Reactive Black 5 dye.

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