REMOVAL OF TRIPHENYLMETHANE DYES USING *PSEUDOMONAS* SPECIES

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By

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ABSTRACT

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TAN YIN FENG

Triphenylmethane dyes are being used extensively in various industries and have become one of the main contaminants found in sewage waters. In order to minimize the toxic effects of triphenylmethane dyes on the environment and the organisms that live in it, scores of researches have been done to find the most efficient ways to remove the dyes in sewage waters or minimize their toxic effects before discharging them into the environment. In this study, the abilities of the bacteria *Pseudomonas* isolate OP2 in decolorizing two triphenylmethane dyes which are malachite green and crystal violet were investigated. The bacterium was firstly identified as *Pseudomonas aeruginosa* based on its 16S rRNA gene analysis. By inoculating the bacterium in LB broth supplemented with malachite green or crystal violet, it was discovered that malachite green which has lesser dimethyl groups compared to crystal violet exhibited lower toxic effects and could be decolorized at a concentration of as high as 1000 mg/L up to 90% while crystal violet could only be decolorized up to 70% at a low concentration of 10 mg/L. *P. aeruginosa* OP2 was able to decolorize both malachite green and crystal violet effectively at the temperatures of 25^{o}C, 30^{o}C and 37^{o}C and at pH values ranging from 6 to 8 with its highest growth rate manifested at 30^{o}C and pH 7. The growth of

bacteria to late log phase (OD_{600} =1) prior to the addition of dyes enabled a higher level of decolorization efficiency compared to the bacteria which were at lag phase when the dye was added. Besides, the growth rate of the bacteria was maximized under the shaking condition and hence exhibited a higher level of decolorization activity compared to the level of activity under the static condition. In addition, the toxicity levels of malachite green and its end products after the process of degradation were examined by using different types of bacteria with the results showing a marked decrease in the bacteriostatic effects of malachite green after it was biodegraded by *P. aeruginosa* OP2 . In short, *P. aeruginosa* OP2 had the ability to decolorize malachite green more efficiently compared to crystal violet under the optimum conditions which were at 30^{o}C, pH 7 and agitated at 200 rpm.

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Firstly, I would like to express my deepest gratitude and appreciation to my final year project supervisor, Assistant Professor Dr Kho Chiew Ling who had given me ample guidance throughout the project. It's only under her shrewd guidance that I am able to complete my project and thesis in the most presentable way. I would not be able to improve and step forward without her encouragement and advice. Next, I would like to express my gratitude to my family, especially my father, who had assisted me in my thesis writing and encouraged me whenever I felt stressful during the period of doing this project. I would have given up or lost my way if it wasn't for him. Last but not least, I would like to thank my FYP teammates, Hai Met, Calvinz, Yi Lun and Shi Ying who had assisted and encouraged me whenever I encountered problems in my project. Thank you.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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TAN YIN FENG

APPOVAL SHEET

This project report entitled "**REMOVAL OF TRIPHENYLMETHANE DYES USING *PSEUDOMONAS* SPECIES**" was prepared by TAN YIN FENG and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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DATE:_____

PERMISSION SHEET

It is hereby certified that **TAN YIN FENG** (ID No: 11ADB04630) has completed this final year project entitled "**REMOVAL OF TRIPHENYLMETHANE DYES USING *PSEUDOMONAS* SPECIES**" under the supervision of Dr. Kho Chiew Ling from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(TAN YIN FENG)

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LIST OF ABBREVATIONS

$BaCl_2$	Barium Chloride
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide-nucleotide BLAST
bp	Base pair
CI	Color Index
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
g	Acceleration of gravity
H_2SO_4	Sulphuric acid
kb	Kilo base pair
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
MH	Mueller-Hinton
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
tmr	Triphenylmethane reductase gene
U	Unit
UV-Vis	Ultra violet- Visible light

CHAPTER 1

INTRODUCTION

Dyes are substances that are used to add colors or change the colors of an object. Synthetic dyes are now more preferred compared to natural dyes as they cost less, have a wider range of colors and impart better on the dyed materials. Synthetic dyes are now being used extensively in many industries for the dyeing of plastics, food, wool, cotton, nylon and others (Hamid and Rehman, 2009). There are more than 0.7 million tons of synthetic dyes produced annually worldwide (Chawla and Saharan, 2014).

More than 10,000 different types of dyes and pigments are being used worldwide and it is estimated that 10% to 15% of the dyes enter the environment in the form of wastes every year and causes serious environmental problems (Ali and Akthar, 2014). Synthetic dyes are not readily biodegradable and when they are discharged into the environment, they are persistent due to their high stability against the effects of water, light, detergents, chemicals, temperature and microbial attacks (Michaels and Lewis, 1985; Couto, 2009). Synthetic dyes are difficult to decolorize due to their chemical structures and synthetic origins (Aksu, 2005). The presence of a small amount of dye such as 10 mg/l to 50 mg/l in the water is highly visible and it will affect the aesthetic value, the transparency of the water and the gas solubility of water bodies (Rajamohan and Karthikeyan, 2006). The discharge of these highly colored waste waters into rivers and lakes results in the reduction of dissolved oxygen

concentration in the water of these places and it also hinders the penetration of sunlight which in turn affects the photosynthetic activities of aquatic plants and the survival of various types of aquatics organisms (Sinoy, Mohan and Shaikh, 2011).

Some of the synthetic dyes such as triphenylmethane dyes can be toxic, carcinogenic or mutagenic and can be hazardous to health (Au, et al., 1978). Triphenylmethane dyes such as crystal violet and malachite green are the types of synthetic dyes that are extensively used in the textile industry (Wu, et al., 2008). Crystal violet was reported to be able to induce tumour growth in some species of fish while malachite green would induce hepatic tumour formation in rodents and cause reproductive abnormalities in rabbits and fish (Cho, et al., 2003; Cha, Doerge and Cerniglia, 2001).

It is thus crucial to treat waste waters before discharging them in the form of effluents. Several physiochemical methods such as chemical oxidation and reduction, physical precipitation and flocculation, photolysis, adsorption and etc have been used to remove synthetic dyes from waste waters (Azmi, Sani and Banerjee, 1998). However, these treatment methods are expensive, low in efficiency besides generating a large amount of sludge in the process (Wu, et al., 2008).

In view of this, the development of biological process by using microorganisms to treat waste waters provides an alternative way of treatment (Azmi, Sani and Banerjee, 1998). Microbial decolorization process has the advantage of being

environmentally friendly and low in costs compared to physiochemical treatment (Wu, et al., 2008). Decolorization of dye solutions by bacteria is known to take place in two ways, which is either the biosorption of the dyes on the microbial biomass or biodegradation of the dyes by bacterial cells. Biosorption involves entrapment of dyes in the matrix of the bacterial cells without degradation of the dyes, whereas in biodegradation, the dye structure is degraded into smaller compounds resulting in the decolorization of synthetic dyes (Shah, et al., 2013). Hence, bacteria that absorb dyes will be deeply colored, whereas those causing degradation will remain biocolorless.

The types of organisms being reported to have the ability to remove or degrade triphenylmethane dyes include fungi, yeast, actinomycetes and bacteria (Azmi, Sani and Banerjee, 1998). Fungi such as *Phanerochaete chrysosporium* are able to produce enzymes that are able to degrade dyes (Bumpus and Brock, 1988). Several types of bacteria species such as *Citrobacter sp., Kurthia sp., Bacillus sp.* and *Pseudomonas sp.* have been found to have the dye removing ability (Jang, et al., 2005; Sani and Banerjee, 1999; Azmi, Sani and Banerjee, 1998). It is also found that most strains of *Pseudomonas* sp. such as *Pseudomonas putida, Pseudomonas otitidis* WL-13 and *Pseudomonas aeruginosa* ETL-1 possess this ability (Chen, et al., 2007; Wu, et al., 2008; Shah, et al., 2013).

In this study, the triphenylmethane dyes removal activity under different culturing condition of *Pseudomonas* isolate OP2 which was previously isolated from the soil of oil palm plantation by Ng (2013) was observed. The species

level of OP2 was subsequently determined using 16S rRNA gene sequencing, followed by the BLASTn analysis.

The objectives of the study were as follows:

- To identify the species level of *Pseudomonas* isolate OP2
- To assess the malachite green and crystal violet removal activities of *Pseudomonas* isolate OP2
- To determine the mechanism of dye removal activity by *Pseudomonas* isolate OP2
- To examine the effect of different environmental parameters on decolorizaion of malachite green and crystal violet
- To determine the level of toxicity of malachite green before and after treatment with *Pseudomonas* isolate OP2

CHAPTER 2

LITERATURE REVIEW

2.1 Synthetic Dyes

Historically, natural dyes which are derived from plants, invertebrates or minerals without any synthetic treatment were used by human for coloring of clothes or textiles (Cardon, 2007). In 1856, the first synthetic dye, mauveine was discovered by William Henry Perkin. A few years after the discovery of mauveive, scientists especially those from Britain, Germany and France raced to formulate various dyes of new colors (Forster and Christie, 2013). The discovery of synthetic dyes had led to the replacement of natural dyes by the synthetic ones (Anderson, 2009). This being so as the synthetic dyes are cheaper to produce, have brighter and wider range of colors and are also able to impart desirable properties to the materials (Samantha, 2009). In the year 2013, it was estimated that approximately 34,500 types of synthetic dyes and pigments are listed under 11,570 Color Index Generic Names in the fourth edition of Color Index International of Society of Dyers and Colourists (SDC*)* and American Association of Textile Chemists and Colourists (AATCC) (Chromatic Notes, 2013).

2.1.1 Coloration of Dyes

Dyes are aromatic organic compounds that will selectively absorb the wavelengths of light in the range of 400 nm to 800 nm which are within the visible range of the electromagnetic spectrum and human eyes. When an object absorbs some of the wavelengths that are within the visible range, the wavelengths that are not absorbed will make the object appear to be visible and colored to human eyes (Christie, 2001).

Dyes appear in various colors due to the presence of chromophores in them. These chromophores alter the energy in the delocalized electron system of the aryl rings in the dyes, thereby enabling the dyes to absorb radiations within the visible range (StainsFile, 2005). Examples of chromophores are: ⁻C=C–, –C=N– and –C=O– (Mohan, 2004).

Besides chromophore, dyes also contain auxochromes with non-bonded electrons. These electrons attach themselves to chromophore, thereby modifying the ability of chromophore to absorb light (CellPath, 2006). Auxochromes which are attached to non-ionizing compounds would retain their ability to ionize, hence resulting in the intensification of the colors of the dyes by altering the wavelength and intensity of absorption (Rachita, 2014). Auxochromes will provide a site which allows the dyes to be chemically bonded to the fabric. Examples of auxochrome are: ⁻NH3, –COOH and –OH (CellPath, 2006).

2.1.2 Classification of Synthetic Dyes

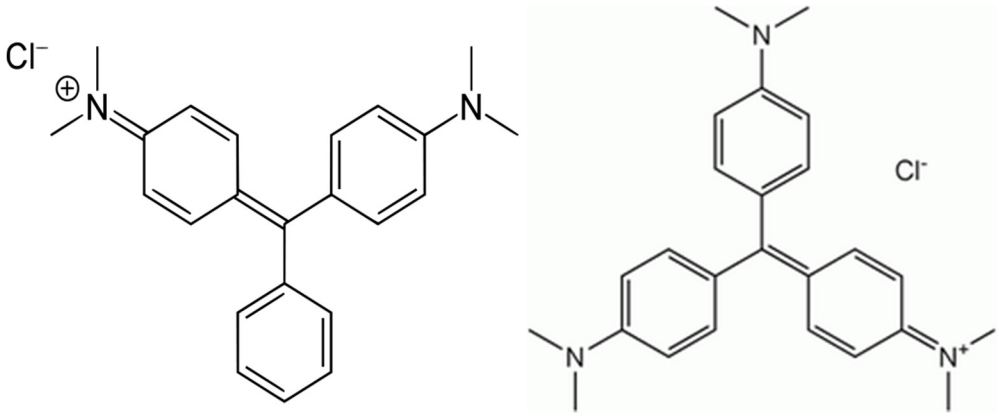
Synthetic dyes can be classified according to their chemical structures (Gregory, 1990). Based on the chemical classification system, synthetic dyes are classified according to the types of their chromophores such as nitro, azo, ethylenic and etc (Nassau, 2001). Dyes containing the same type of chromophore have similar characteristics. For example anthroquinone dyes are weaker and more expensive while azo dyes are stronger and more cost-effective (Gregory, 1990). The chemical classification system is widely used by synthetic dye chemists and dye technologists who are interested in the chemical constitution of the dyes (Hunger, 2003).

Classification of synthetic dyes according to their applications or usage is adopted by the Color Index (C.I.) (Hunger, 2003). It is a more preferable classification system to dye users who are concerned with the process of dyeing (Agarwal, 2006).

2.2 Triphenylmethane Dyes

Triphenylmethane dyes are brilliant and intense colored synthetic organic dyes with triphenylmethane $(C_6H_5)_3CH$ as their backbones. Triphenylemthane derivatives are one of the oldest synthetic dyes which provide vibrant bright colors and are highly colorfast (Wiley, 2002). They can be used to dye wool, silk, polyamide and etc. Due to their synthetic origins and complex aromatic structures, triphenylmethane dyes are more resistant to biodegradation (Godlewska, Przystas and Sota, 2009).

The range of colors for triphenylmethane dyes includes red, violet, blue and green (Rung International, 2008). Two of the well-known triphenylmethane dyes are malachite green and crystal violet as shown in Figure 2.1.



(a) (b)

Figure 2.1 : Chemical structures of (a) malachite green ; (b) crystal violet

2.2.1 Applications of Triphenylmethane Dyes

Triphenylmethane dyes are one of the groups of dyes that are most commonly used in the textile industry (Shah, et al., 2013). It is estimated that out of the total consumption of dyes for the purpose of dyeing cotton, wool, silk and nylon, 30% to 40% of it consists of triphenylmethane dyes (Carliell, et al., 1998). Besides that, triphenylmethane dyes can also be used for coloring food, fats, waxes, varnishes, cosmetics, paper, leather and plastics (Shah, et al., 2013). Moreover, malachite green is being used in the aquaculture industry as a fungicide, parasiticide and disinfectant to treat fungal and protozoa infection (Alderman, 1984; Parshetti, et al, 2006). Crystal violet which is commonly used in textile industry is also well known as a biological stain for staining specimens in bacteriological and histopathological processes (Shah, et al.,

2013). It is also being used as human and veterinary medicines for treating pinworm as well as a topical agent (Azmi, Sani and Banerjee, 1998).

2.2.2 Impact of Triphenylmethane Dyes

It is estimated that 280,000 tons of textile dyes are released into the environment as industrial effluents worldwide (Mass and Chaudhari, 2005). Triphenylmethane dyes present in industrial effluents post a serious environmental concern as they are very recalcitrant to microbial degradation (Pagga and Brown, 1986).

Moreover, even a small volume and a low concentration (10 mg/L to 50 mg/L) of these dyes which possess high tinctorial strength will create an obvious coloration (Ali and Akthar, 2014). The highly colored effluents will thus affect the gas solubility in water bodies and the amount of light penetration (Gupta and Rastogi, 2009). This will significantly decrease the photosynthetic activity in aquatic life and may post a threat to the sustainability of aquatic ecosystem (Gupta and Rastogi, 2009). Besides, the deposition of triphenylmethane dyes in the sediments of rivers has been found to cause tumour growths in some bottom feeding fish species (Diachenko, 1979). It has also been reported that the dye solutions can undergo anaerobic degradation to form potentially carcinogenic compounds which will eventually end up accumulating in food chains and possibly taken by humans (Banat, et al., 1996).

Furthermore, triphenylmethane dyes which also possess mutagenic and carcinogenic properties post a serious threat to human health. Malachite green for example, has adverse effects on human's immune system and reproductive system (Srivastava, Sinha and Roy, 2004). Besides that, cytogenetic toxicity of crystal violet and gentian violet resulted in high frequency of chromosomal breakage in Chinese hamster ovary cells as studied by Au et al. (1978). Moreover, according to another study, crystal violet was found to cause reduced RNA and protein synthesis and decreased oxygen consumption in the rabbit granulation tissues (Mobacken, Ahonen and Zederfeldt, 1974).

The increasing usage of dyes causes increasing concentration of dyes in the environment. Hence, government legislations regarding the removal of dyes from effluents before releasing them into the environment have become more and more stringent. Consequently, it becomes absolutely necessary to develop more efficient and cost-effective ways to remove dyes from effluents prior to releasing them into the environment in order to nullify or at least minimize their harmful effects.

2.3 Treatment of Triphenylmethane Dyes

2.3.1 Physical and Chemical Based Methods of Treatment

Many approaches such as physical and chemical processes have been used to remove dyes in effluents. However, most of these methods are costly and not environment friendly. Physicochemical methods such as photolysis, adsorption, floatation, precipitation, coagulation, reverse osmosis and chemical oxidation

are able to remove dyes effectively from effluents (Wang, et al., 2011). Nevertheless, there are also many limitations in these methods as they are very costly, energy intensive, limited in their usage and will produce toxic by-products and large amount of sludge (Godlewska, Przystas and Sota, 2014). The accumulation of sludge induces a problem concerning its disposal and the excessive use of chemicals to remove dyes will also cause secondary pollution (Pearce, Lloyd and Guthrie, 2003).

Activated charcoal is one of the commonly used methods that is able to remove dyes effectively but it is only able to treat small volume of effluents (Azmi, Sani and Banerjee, 1998). Besides, this method involves a slow process, incurs high costs and also exhibits regeneration and disposal drawbacks which have made it economically unfeasible (Azmi, Sani and Banerjee, 1998). Other techniques such as ozonation with the use of Fenton's reagent generate large amount of sludge which renders them undesirable (Pearce, Lloyd and Guthrie, 2003; Saratale, et al., 2009). Electrochemical oxidation is yet another very effective method that is able to transform dyes into non-hazardous products, but has also been abandoned due to its high requirement for electricity (Saratale, et al., 2009). The filtration technique which is suitable for all kinds of dyes also has its drawbacks and these include limited lifespan of the membrane used, the production of secondary waste and high investment cost (Saratale, et al., 2009).

2.3.2 Biological Methods of Treatment

The use of biological methods for the removal of synthetic dyes is gaining importance and popularity as they exhibit considerable advantages such as possessing eco-friendly nature, relatively inexpensive, less sludge production, non-toxic end products upon complete mineralization and much reduced water consumption compared to physicochemical methods (Saratale, et al., 2009; Forgacs, Cserhati and Oros, 2004).

Biological methods include biosorption, bioaccumulation and biodegradation of the synthetic dyes. In the biosorption method, dyes are bound to the biomass which consists of either living or dead cells without the use of metabolic energy (Swiatek and Krzywonos, 2014; Das and Charumathi, 2012). However, this method is not suitable for long term treatment as the dyes will accumulate in the biomass and need to be disposed of over time (Pearce, Lloyd and Guthrie, 2003).

Bioaccumulation is the accumulation of dyes by actively growing cells via their metabolic activities (Das and Charumathi, 2012). Bioaccumulation can only occur in living organisms through the transference of dyes or pollutants into the cells and accumulating in them (Swiatek and Krzywonos, 2014).

Biodegradation is an energy-dependent process involving the breakdown of complex dye structures into various byproducts by using various enzymes of microorganisms (Das and Charumathi, 2012). The effectiveness of biodegradation depends on the dye structure and concentration, adaptation of the microorganisms and their level of activities and biomass concentration

(Godleswska, Przystas and Sota, 2009). Biodegradation is a more effective mean for the treatment of dyes in waste waters compared to biosorption and bioaccumulation as it is able to reduce considerably the toxicity of dyes via the process of mineralization (Das and Charumathi, 2012).

2.4 Organisms involved in Dye Removal

Various types of organisms have been discovered to have the ability to remove dyes from wastewaters. Firstly, fungi are able to decolorize triphenylmethane dyes due to their production of non-specific-natured ligninolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase (Saratale, et al., 2009). These enzymes are able to decolorize dyes as they possess the ability to biodegrade highly recalcitrant environmental pollutants (Olukanni, et al., 2013; Das and Charumathi, 2012). Specifically, white rot fungi such as *Phanerochaete chrysosporium* is able to degrade azo dyes*, Trametes versicolor* is able to decompose anthraquinone, azo and indigo-based dyes while *Trametes hirsute* is capable of degrading triphenylmethane, indigoid and anthraquinone dyes (Forgacs, Cserhati and Oros, 2004). Besides white rot fungi, there are also other types of fungi such as *Hirschioporus larincinus, Inonotus hispidus, Phlebia tremallosa,* and *Coriolus versicolor* which exhibit such ability via their dye decolorization activities (Banat, et al., 1999; Saratale et al., 2009).

Secondly, yeast strains such as *Rhodotorulla, Candida tropicalis, Debaryomyces polymorphus* (Yang, et al., 2003) and *Issatchenkia occidentalis* (Ramalho, et al., 2004) are also useful in the removal of dyes from effluents

through the processes of biosorption, bioaccumulation or biodegradation. However, despite their ability to degrade dyes which are aromatic compounds, the utilization of aromatic compounds by yeasts are limited in scope and the degradation process is also rather slow (Das and Charumathi, 2012).

Besides, many different types of bacteria are also being tested in their ability to remove dyes from wastewaters. Bacteria such as *Bacillus thuringiensis* (Olukanni, et al., 2013), *Citrobacter* sp. strain KCTC 18061P (Jang, et al., 2005), *Kurthia* sp. (Sani and Banerjee, 1999), *Enterobacter asburiae* Strain XJUHX-4TM (Mukherjee, Bhandari, and Das, 2012), *Escherichia coli, Proteus* and *Pseudomonas* sp. are some common species of bacteria which are able to decolorize triphenylmethane dyes (Godleswska, Przystas, and Sota, 2014).

2.4.1 Bioremediation by *Pseudomonas* species

Michaels and Lewis (1986) reported that many bacteria are unable to degrade triphenylmethane dyes due to the cytotoxicity of these dyes. Nevertheless, the bacteria *Pseudomonas* species possesses the potential to remove such dyes as triphenylmethane dyes are less toxic to them (Chen, et al., 2007). *Pseudomonas* strain exhibits a remarkable color-removal capability when it is tested against high concentration of several types of triphenylmethane dyes (Wu, et al., 2008). For example, *Pseudomonas otitidis* WL-13 is able to decolorize malachite green and crystal violet through biosorption (Wu, et al., 2008). Whereas, triphenylmethane dyes such as brilliant blue and crystal violet can be degraded

by *Pseudomonas putida* and *Pseudomonas aeruginosa* ETL-1 (Chen, et al., 2007; Shah, et al., 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of Apparatus and Materials

All of the glasswares, consumables, media and non-heat labile chemicals were sterilized using the autoclave machine (Hirayama) at 121ºC for 15 minutes. Two types of triphenylmethane dyes, namely malachite green (QReC chemicals) and crystal violet (Bio Basic Inc.) were prepared by using deionized distilled water at a concentration of 10 g/L and 5 g/L respectively. Since triphenylmethane dyes are heat sensitive chemicals, a 0.22 µm filter membrane was used for filter sterilization. All the reagents that have been prepared based on the recipe are stated in Appendix A.

3.2 Bacteria species Used in this Study

The bacterium used in this study is *Pseudomonas* isolate OP2 which was recovered from the glycerol stock by streaking on LB agar (Conda Pronadisa). The bacteria cultured on the LB agar were maintained by storing them at 4ºC refrigeration and were subcultured once every two weeks for short term storage. As for long term storage, 20% (v/v) glycerol stocks of the bacteria were prepared using the overnight culture and stored in a -80ºC freezer (Thermo Scientific).

3.3 Spectroscopic Analysis of Dyes

The maximum absorbance wavelength of malachite green and crystal violet were determined using a nano spectrophotometer (Thermo Scientific NanoDrop 2000). The spectra of 20 mg/L of malachite green and 10 mg/L of crystal violet dye solutions were recorded within the range of 300-800 nm by using distilled water as a blank reference. The maximum absorbance wavelength would be used to calculate the percentage of decolorization throughout the dye removal assay.

3.4 General Operating Procedure for Optimization of Operating Parameters for Dye Removal Assay

The following is a general procedure for dye removal assay. Firstly, for malachite green, 1000 mg/L of the dye was added into a 100 ml Erlenmeyer flask containing 30 ml of sterile LB broth (Conda Pronadisa). As for crystal violet, 10 mg/L of the dye was added into the flask instead. An overnight culture was prepared by inoculating a single colony of *Pseudomonas* isolate OP2 into 5 ml of LB broth and incubated overnight in a shaking incubator (Labnet) at 30^{o}C and 200 rpm. The initial absorbance value of the LB broth which had been mixed with a particular dye was measured and then inoculated with 1% (v/v) of the overnight culture. The initial absorbance value of the mixture was measured using the predetermined maximum absorbance wavelengths which were 615 nm and 584 nm for malachite green and crystal violet respectively. The LB broth was used as the blank in the measuring process. The culture was then incubated at 30^{o}C in a shaking incubator (Labnet)

set at 200 rpm. At every 24 hours interval for 72 hours, 1.5 ml of the broth sample was collected from the test culture. By using a microcentrifuge (Stuart), the bacterial cells were pelleted down by centrifugation at 13000 rpm for 6 minutes. The final absorbance of the supernatant was measured. Then, by using the equation below, the initial and final absorbance of the mixture were used to determine the percentage of decolorization:

$$\text{Decolorization activity}(\%) = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \text{ x } 100\%$$

An optimum condition must be attained for the dye to be removed with high efficiency. Hence, in the dye removal assay by *Pseudomonas* isolate OP2, the following parameters which include dye concentration, temperature, pH value, bacterial growth phase and the incubation condition were studied.

3.4.1 Effect of Dye Concentration on the Decolorization Efficiency of Bacteria

The procedure of this study followed strictly the general operating procedure except for the dye concentration. The concentrations of malachite green added into the LB broth were at 100 mg/L, 200 mg/L, 500 mg/L, 1000 mg/L, 1500 mg/L and 2000 mg/L. Likewise, varying concentrations of crystal violet were added into the LB broth which were 10 mg/L, 50 mg/L, 100 mg/L, 200 mg/L and 500 mg/L. For each concentration of the mixture, duplicates of arrays were made.

3.4.2 Effect of Temperature on the Decolorization Efficiency of Bacteria

The bacterial cultures were prepared in accordance to the general operating procedure except for the incubation temperature. The cultures containing dyes were incubated in a shaking incubator at different temperatures which were 25ºC, 30ºC and 37ºC respectively. For every bacterial culture incubated under different temperatures, duplicates of arrays were made.

3.4.3 Effect of pH value on the Decolorization Efficiency of Bacteria

The general operating procedure was used to prepare the bacterial culture except for the pH of the broth. The pH of the LB broth was adjusted accordingly using 1 M sodium hydroxide or 1 M hydrochloric acid to pH 5, 6, 7, 8, 9 and 10. For each broth of differing pH, duplicates of arrays were made.

3.4.4 Effect of Bacterial Growth Phase on the Decolorization Efficiency of Bacteria

A 1% (v/v) overnight culture was added into a 30 ml LB broth and incubated at 30ºC and 200 rpm in a shaking incubator for the bacteria to grow to OD_{600} = 1, which is the late log phase of the bacteria. Then, the dye was added into the bacterial culture in accordance to the standard operating procedure. A control was made for this test for comparison purpose by inoculating 1% (v/v) overnight culture into the LB broth containing the dye. The test for dye removal efficiency was then conducted in its duplicate.

3.4.5 Effect of Aeration on the Decolorization Efficiency of Bacteria

The bacterial cultures were prepared in accordance to the general operating procedure. They were then incubated under two different incubation conditions which were the shaking condition at 200 rpm and the static condition. For each bacterial culture under different incubation conditions, duplicates of arrays were made.

3.5 Toxicity Test of Malachite Green before and after Treatment by *Pseudomonas* isolate OP2

The bacterial culture containing malachite green was prepared by following the general operating procedure. After 72 hours of incubation, the broth was collected and centrifuged at 13000 rpm for 6 minutes. The supernatant was sterilized using a 0.22 µm filter membrane to remove the bacterial residue. A LB broth containing 1000 mg/L of malachite green without bacteria inoculation was prepared as negative control.

In this toxicity test, six types of bacteria including *Staphylococcus epidermidis* 12228*, Micrococcus luteus, Staphylococcus aureus* ATCC 25923, *Salmonella thyphimunum, Escherichia coli* 25922, and *Klebsiella pneumonia* were used. First, a 0.5 McFarland was prepared by mixing 0.05 ml of 1.175% (w/v) $BaCl_2$ and 9.95 mL of 1% (v/v) H_2SO_4. Then, the bacteria colonies were added into a 0.85% (w/v) sterile saline solution. The mixture produced was subsequently compared with the 0.5 McFarland standard in terms of their turbidity. The mixture that was found comparable to the 0.5 McFarland standard was then

spread on the Mueller-Hinton agar plate using a sterile cotton swab. Two blank diffusion disks were placed on the agar surface. On each diffusion disk, 10 µL of either the control or filtered spent culture was added. The agar plate was incubated at 37ºC overnight, after which the diameter of the inhibition zone was measured to determine the degree of toxicity of the dye before and after treatment.

3.6 Species Identification of *Pseudomonas* Isolate OP2

3.6.1 Genomic DNA Extraction

An overnight bacterial culture was prepared by inoculating a single colony of *Pseudomonas* Isolate OP2 into a 5 ml LB broth and incubated overnight in a shaking incubator (Labnet) at 30ºC and 200 rpm. The Wizard Genomic DNA purification kit (Promega) was then used for genomic DNA extraction by following the protocol in the kit manual. The genomic DNA being extracted was run in 0.8% (w/v) agarose gel to verify the presence of genomic DNA. To estimate the size of the genomic DNA, a 1 kb DNA ladder (Thermo Scientific) was loaded beside the extracted genomic DNA. The DNA concentration was then measured using a nano spectrophotometer (Thermo Scientific NanoDrop 2000).

3.6.2 Amplification of 16S rRNA Gene for Species Identification

For species identification purpose, the DNA that had been extracted from isolate OP2 was used as a template for PCR to amplify the 16S rRNA gene region. The PCR reaction was carried out in a mixture containing sterile distilled water, 0.2 mM dNTP, 0.4 µM of forward and reverse primers each, 1x Taq buffer, 2 mM of $MgCl_2$, 1 U Taq polymerase (Fermentas), and 50 ng of DNA template with a total volume of 25 µL. The forward primer used was 16S-De-F with the sequence of 5'– AGAGTTTGATCCTGGCTCAG – 3' and the reverse primer was 16S-De-R with the sequence of 5' – GGTTACCTTGTTACGACTT – 3'. The PCR conditions were set with the initial denaturation at 95°C for 5 minutes, followed by 30 cycles at 95°C for 30 seconds, then at 55°C for 30 seconds and at 72°C for 1 minute and a final extension at 72°C for 10 minutes.

The PCR product was analyzed by running on 1.0% (w/v) agarose gel at 90 V for 45 minutes. A 1 kb DNA ladder (Thermo Scientific) was used to estimate the size of the PCR product.

3.6.3 Purification of PCR Product

PCR products of 100 µL were loaded into a well of a 1% (w/v) agarose gel. After electrophoresis at 90V for 45 minutes, the desired band within the agarose gel was cut out and purified using the PCR Clean-Up System Purification kit (Promega).

3.6.4 Determination of DNA Purity and Concentration

By using a nano spectrophotometer (Thermo Scientific NanoDrop 2000), the purity and concentration of purified PCR product was determined. This was done by placing 1 µL of the purified PCR product on the nano spectrophotometer. The purified PCR product was then run in a 1% (w/v) agarose gel to ensure the quality of the DNA before sending it for sequencing (First BASE).

3.6.5 Analysis of Sequencing Result

The Basic Local Alignment Search Tool (BLAST) was used to analyze the sequencing results. The sequencing result of 16S rRNA gene of OP2 was analyzed by searching for matches with the highest percentage of similarity using BLASTn.

CHAPTER 4

RESULTS

4.1 Species Identification of *Pseudomonas* Isolate

The species identification of the *Pseudomonas* isolate OP2 used in this study was determined by first carrying out the 16S rRNA gene amplification and purification followed by gene sequencing. Figure 4.1 shows the gel image of the purified PCR products of isolate OP2.

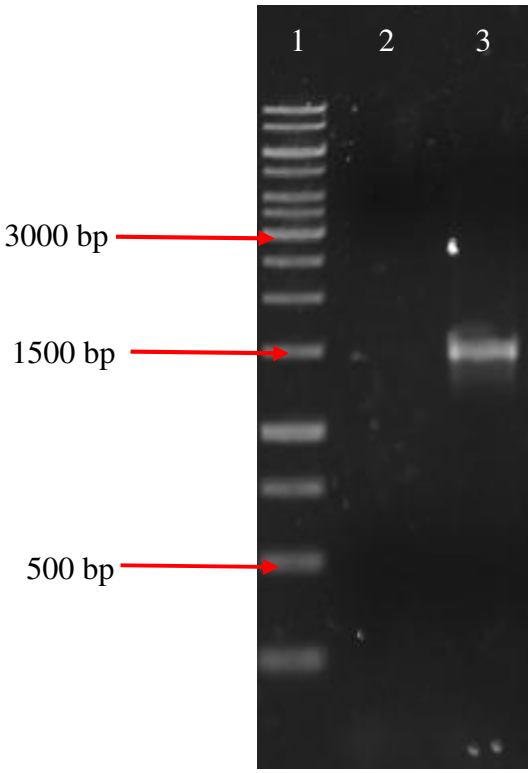


Figure 4.1: Gel image of purified PCR product of 16S rRNA gene of *Pseudomonas* isolate OP2. Lane 1: 1 kb DNA ladder; Lane 2: Negative control; Lane 3: The purified PCR product

The purified PCR products of 16S rRNA gene of *Pseudomonas* isolate OP2 were sequenced and analyzed using BLAST (Basic Local Alignment Search Tool). Based on the result shown in Table 4.1, OP2 isolate belongs to *Pseudomonas aeruginosa* species.

Table 4.1: BLASTn analysis of 16S rRNA gene amplified from *Pseudomonas* isolate OP2

Isolate	BLASTn Identify	Accession	Score	Query Cover (%)	E-value	Identity (%)
OP2	*Pseudomonas aeruginosa* strain fwzb12 16S ribosomal RNA gene, partial sequence	gi|523511865|gb|KF208493.1|	838	97	0.0	99
	Pseudomonas aeruginosa strain fwzb9 16S ribosomal RNA gene, partial sequence	gi|523511863|gb|KF208491.1|	852	97	0.0	99
	Pseudomonas aeruginosa strain ALK318 16S ribosomal RNA gene, partial sequence	gi|455509095|gb|KC456533.1|	852	97	0.0	99
	Pseudomonas aeruginosa strain ALK317 16S ribosomal RNA gene, partial sequence	gi|455509094|gb|KC456532.1|	852	97	0.0	99
	Pseudomonas aeruginosa strain HNYM41 16S ribosomal RNA gene, partial sequence	gi|379975145|gb|JN999891.1|	852	97	0.0	99

4.2 Spectroscopic Analysis of Dyes

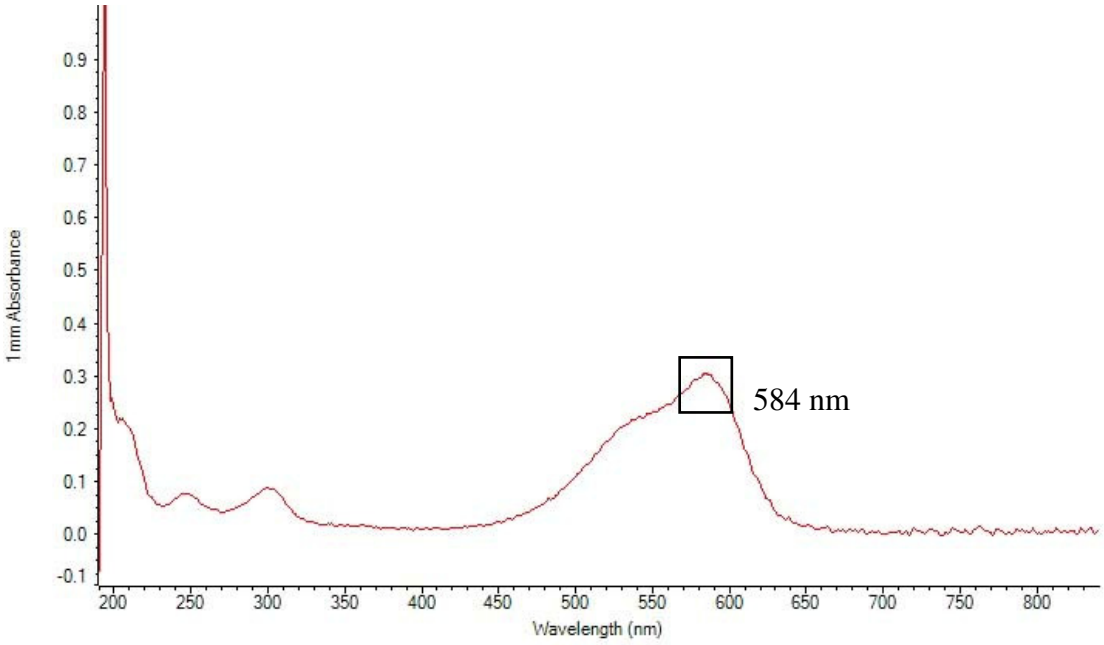
4.2.1 Pre-Treatment with *Pseudomonas* isolate OP2

The spectra of crystal violet and malachite green dyes before decolorization were determined (Figure 4.2). Based on the results, the maximum absorbance wavelengths for crystal violet and malachite green were at 584 nm and 618 nm respectively. These determined maximum absorbance wavelengths were used throughout the dye removal assays to determine the percentage of decolorization of the two dyes.

4.2.2 Post-Treatment with *Pseudomonas* isolate OP2

P. aeruginosa isolate OP2 was inoculated in the LB broth containing 100 mg/L of malachite green or 10 mg/L of crystal violet. After 72 hours, the maximum absorption peaks of crystal violet and malachite green which were 584 nm and 618 nm disappeared due to the decolorization of the dyes. The disappearance of the maximum absorption peaks was followed by an increase in the absorbance at a peak of 260 nm and 254 nm for crystal violet and malachite green respectively (Figure 4.3).

(a)



(b)

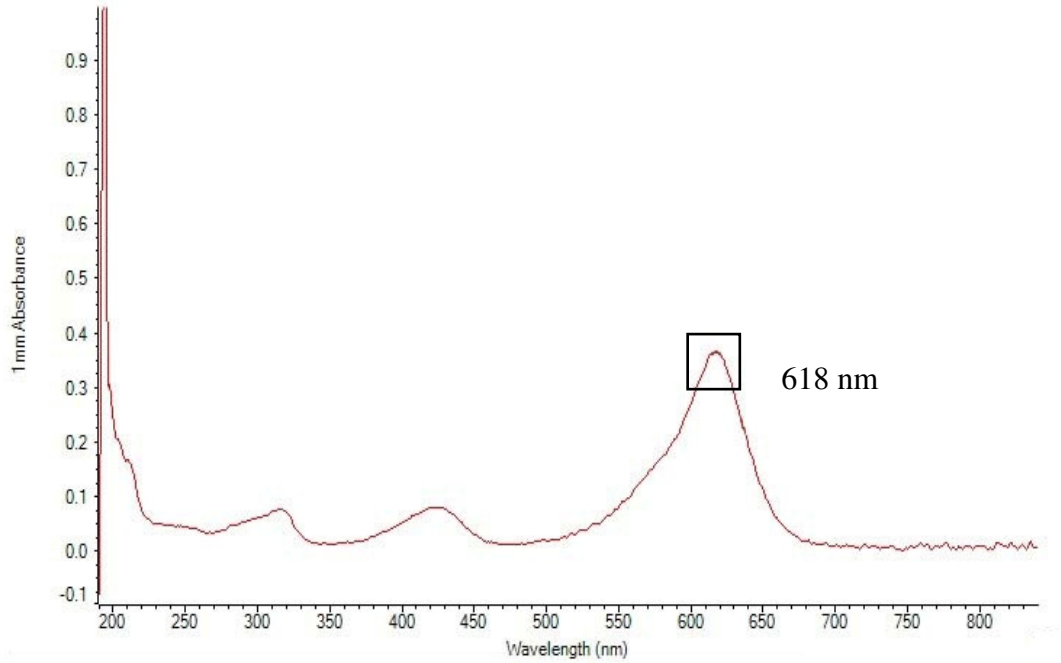
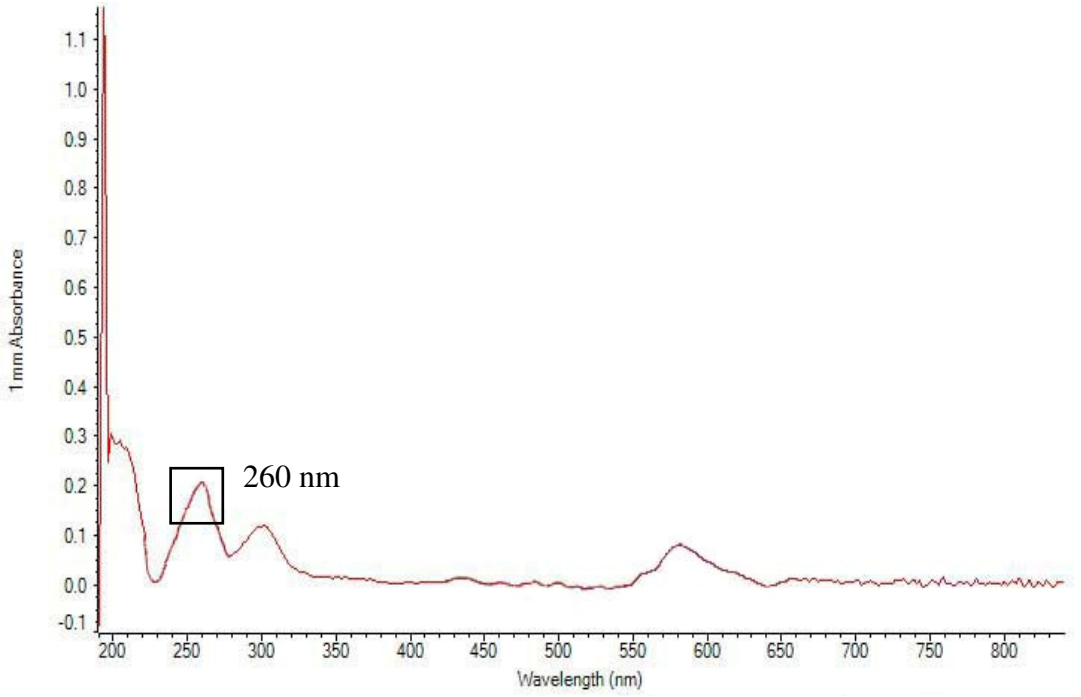


Figure 4.2: Spectroscopic analysis of dyes before decolorization. (a) Crystal violet; (b) Malachite green

a)



b)

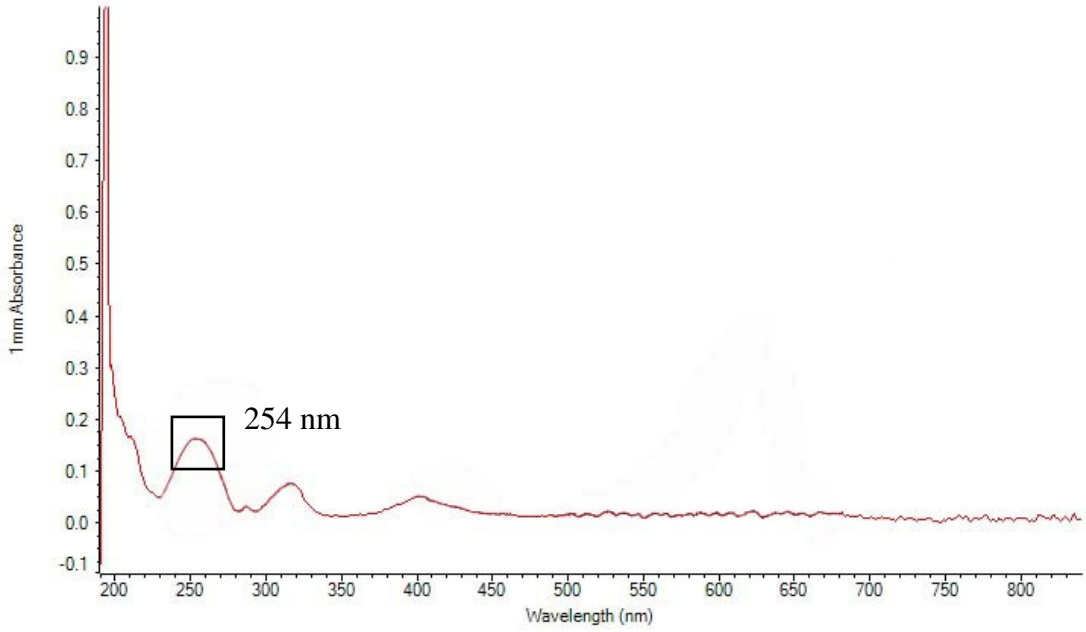
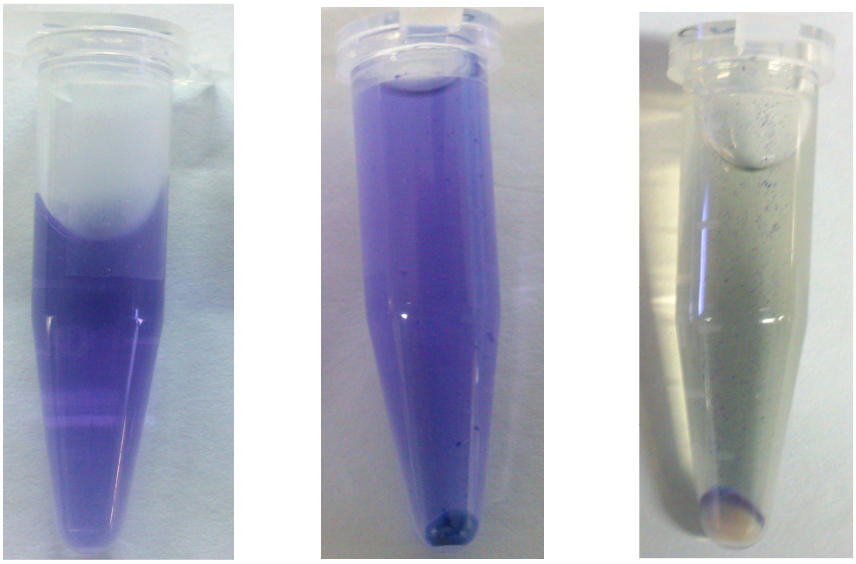


Figure 4.3 : Spectroscopic analysis of dyes after decolorization. (a) Crystal violet; (b) Malachite green

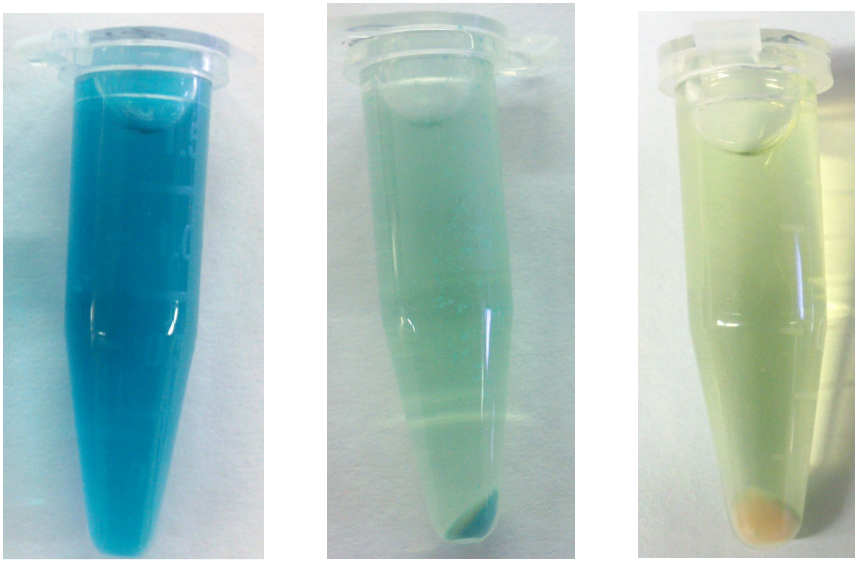
4.3 Mechanisms of Dye Removal

P. aeruginosa OP2 exhibited both bio-sorption and biodegradation properties in its dye removal mechanism. Based on the result obtained from crystal violet at a concentration of 10 mg/L and malachite green at a concentration of 100 mg/L after 24 hours of incubation, bio-sorption of dyes had taken place as the cell pellets were colored as shown in Figure 4.4(b) and Figure 4.5(b). And after 72 hours of incubation, most of the colors of crystal violet and malachite green had been removed as shown in Figure 4.4(c) and Figure 4.5(c) due to biodegradation.



(a) **(b)** **(c)**

Figure 4.4 : Crystal violet decolorization by *P. aeruginosa* OP2. (a) 10 mg/L of crystal violet before cell treatment; (b) after 24 hours of cell treatment; (c) after 72 hours of cell treatment



(a) (b) (c)

Figure 4.5: Malachite green decolorization by *P. aeruginosa* OP2. (a) 100 mg/L of malachite green before cell treatment; (b) after 24 hours of cell treatment; (c) after 72 hours of cell treatment

4.4 Effect of Dye Concentration on the Decolorization Efficiency of Bacteria

The effect of dye concentrations on the decolorization activities of *P. aeruginosa* OP2 were investigated with different concentrations of crystal violet and malachite green. Figure 4.6 shows the dye removal efficiency for crystal violet with the initial dye concentration ranging from 10 mg/L to 500 mg/L by *P. aeruginosa* OP2. After 72 hours of incubation, *P. aeruginosa* OP2 was able to decolorize crystal violet at a concentration of 10 mg/L up to 70%. Reduced dye decolorization activities were observed at the dye concentrations of 50 mg/L to 500 mg/L. Nevertheless, after prolonging the incubation period to 72 hours, the decolorization activities of crystal violet at the concentrations of 50 mg/L and 100 mg/L increased to 56% and 36% respectively. At the concentration of 500 mg/L, there was no decolorization of crystal violet in all

the three incubation periods due to the high level of toxicity of crystal violet that had completely inhibited the bacterial cell growth.

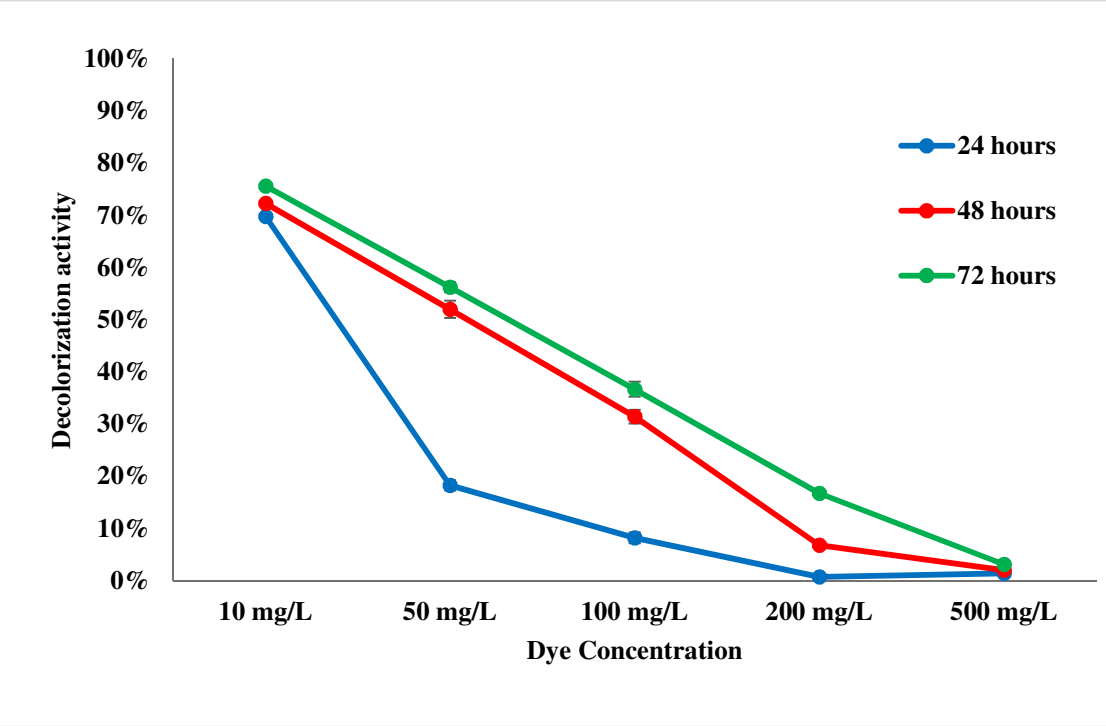


Figure 4.6: Effect of dye concentrations (10 mg/L to 500 mg/L) on decolorization of crystal violet at different incubation periods.

Figure 4.7 shows the malachite green removal efficiency with the initial concentration of malachite green ranging from 100 mg/L to 2000 mg/L. *P. aeruginosa* OP2 was able to decolorize malachite green up to 90% and above after 24 hours of incubation at the dye concentrations of 100 mg/L to 500 mg/L. At the dye concentration of 1000 mg/L, 62% of the dye was removed after 24 hours of incubation. The dye decolorization activity at this concentration then increased to 97% after 72 hours of incubation. At the dye concentration of 1500 mg/L, very low percentage of decolorization was observed even after 72 hours of incubation. There was no decolorization activity at the dye concentration of 2000 mg/L even after prolonging the incubation time to 72 hours as malachite green is toxic to the bacterial cells at this concentration.

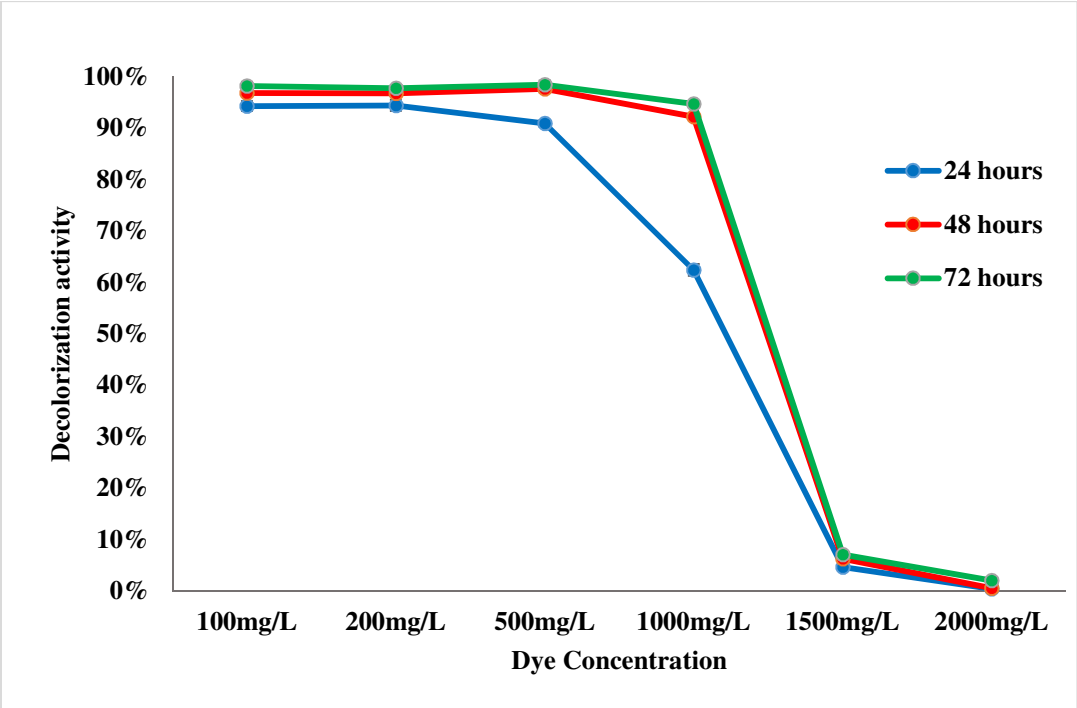


Figure 4.7: Effect of dye concentrations (100 mg/L to 2000 mg/L) on decolorization of malachite green at different incubation periods.

4.5 Effect of Temperature on the Decolorization Efficiency of Bacteria

The effects of temperatures on the decolorization activities of *P. aeruginosa* OP2 were investigated by using 10 mg/L of crystal violet and 1000 mg/L of malachite green incubated at 25°C, 30°C and 37°C. Figure 4.8 shows that *P. aeruginosa* OP2 decolorized crystal violet best at 30^0 C with 51% of decolorization activity compared to the other two temperature readings after 24 hours of incubation. The level of decolorization activity at 30^0 C then went up to 69% after 48 hours of incubation. The level of decolorization activities at all the three temperatures then increased to 70% and above after 72 hours.

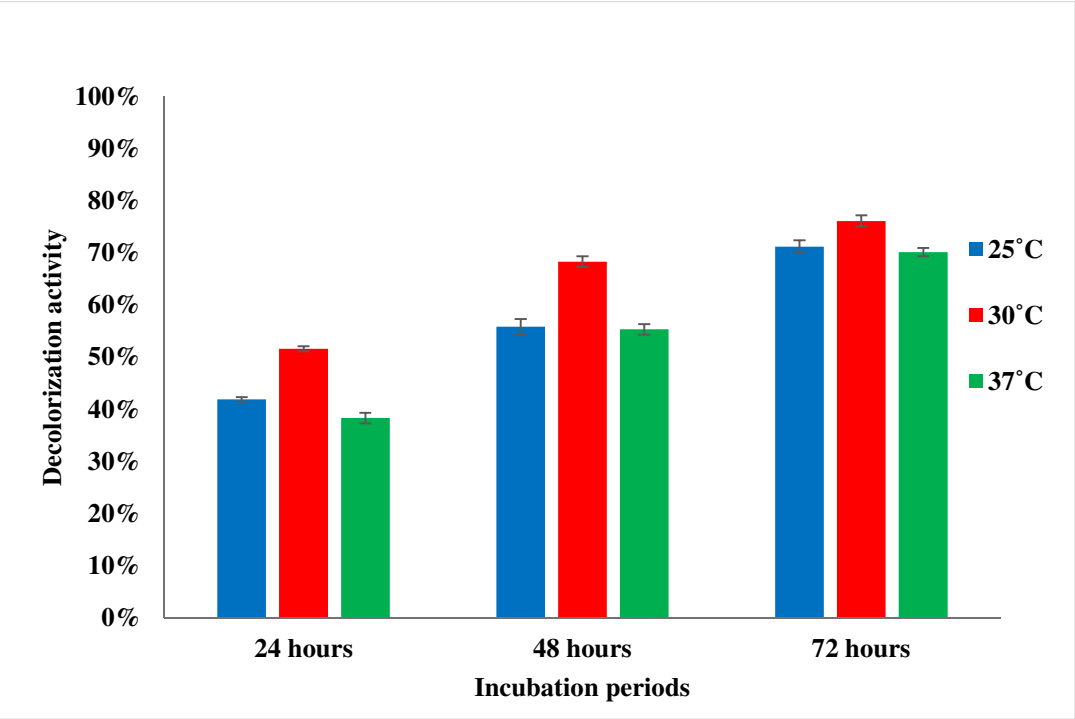


Figure 4.8: Effect of temperatures on decolorization of crystal violet at different incubation periods.

Based on Figure 4.9, *P. aeruginosa* OP2 incubated with malachite green at 30ºC exhibited the highest decolorization activity of 55% compared to only 35% and 26% of decolorization activities at 25ºC and 37ºC respectively after 24 hours of incubation. The decolorization activities of *P. aeruginosa* OP2 at all the three temperatures then went high up to 90% and above after 72 hours of incubation.

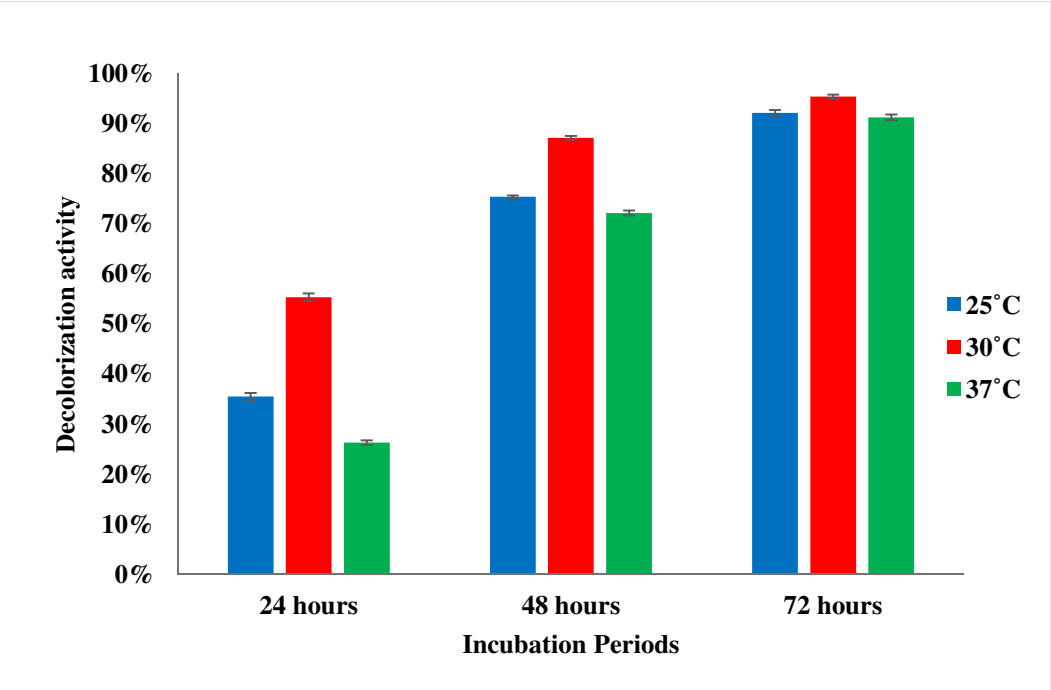


Figure 4.9: Effect of temperatures on decolorization of malachite green at different incubation periods.

4.6 Effect of pH value on the Decolorization Efficiency of Bacteria

The effects of pH values on the levels of decolorizing activities of *P. aeruginosa* OP2 on crystal violet and malachite green were investigated with pH values ranging from 5 to 10. Based on Figure 4.10, *P. aeruginosa* OP2 exhibited low levels of decolorization activities on crystal violet at pH 5, pH 9 and pH 10 in all the three different incubation periods. At the pH values ranging from 6 to 8, the levels of decolorization activities were within the range of 65% to 71% after 72 hours of incubation. *P. aeruginosa* OP2 exhibited the highest level of decolorization efficiency at pH 7 in all the three incubation periods.

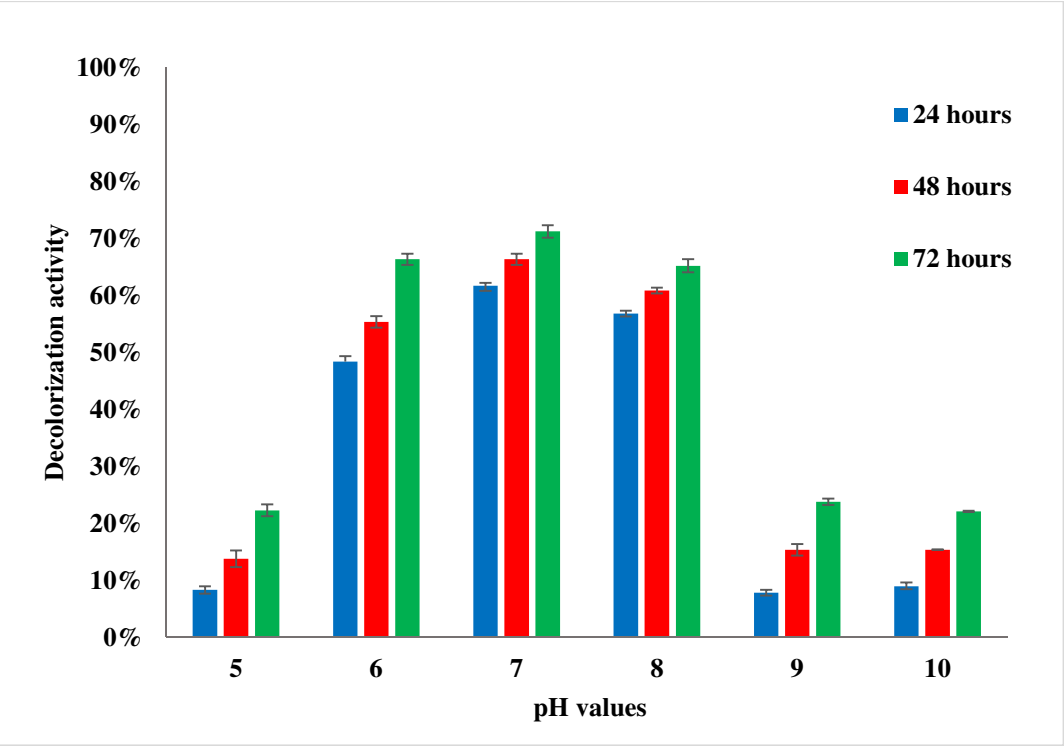


Figure 4.10: Effect of pH values on decolorization of crystal violet at different incubation periods.

Meanwhile, based on the results shown in Figure 4.11, *P. aeruginosa* OP2 exhibited extremely low levels of efficiency in decolorizing malachite green at pH 5 in all the three incubation periods. *P. aeruginosa* OP2 was able to decolorize malachite green at pH 6, pH 7 and pH 8 up to 90% and above after 72 hours of incubation with the highest level of decolorization activity of 97% exhibited at pH 7. The levels of decolorization activities at pH 9 and pH 10 in the same incubation period were lower at 78% and 76% respectively.

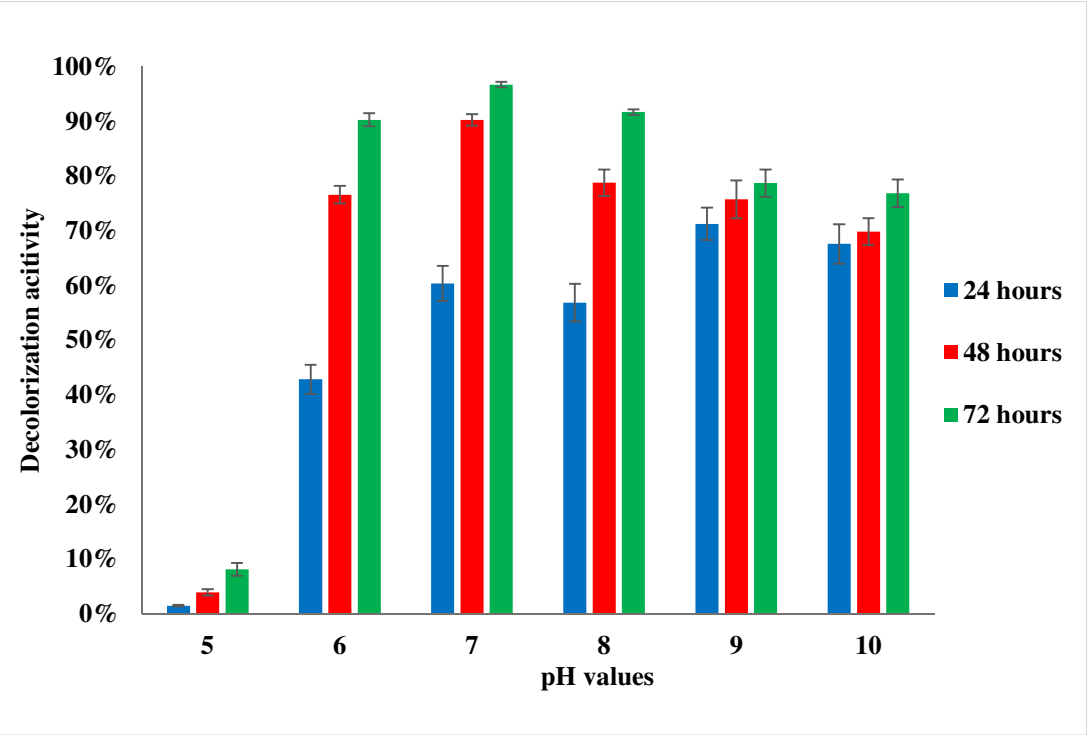


Figure 4.11: Effect of pH values on decolorization of malachite green at different incubation periods.

4.7 Effect of Bacterial Growth Phase on the Decolorization Efficiency of Bacteria

The study on the effects of bacterial growth phases on the dye decolorizing activities was done by growing a set of *P. aeruginosa* OP2 to OD_{600} = 1 before adding in 50 mg/L of crystal violet or 1000 mg/L of malachite green. Another set of *P. aeruginosa* OP2, also known as the control set, was prepared for comparison purpose by inoculating 1% (v/v) overnight culture into the LB broth containing the dye. Based on the result obtained as shown in Figure 4.12, the control culture added with crystal violet showed only 18% of dye decolorization activity while the set of *P. aeruginosa* OP2 which was grown to OD_{600} = 1 prior to the addition of crystal violet exhibited 42% of decolorization activity after 24 hours of incubation. The decolorization activities in both sets of cultures then improved after 48 hours of incubation and exhibited almost

identical levels of decolorization activities which were 67% and 64% respectively after 72 hours of incubation.

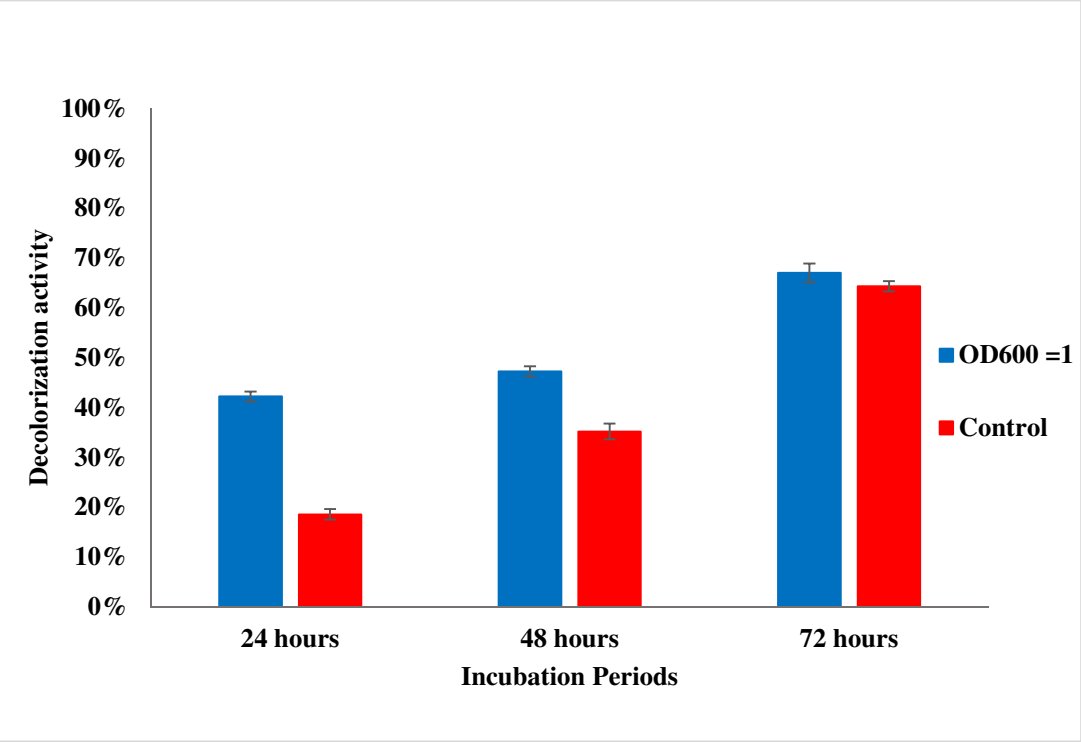


Figure 4.12: Effect of bacterial growth phases on decolorization of crystal violet at different incubation periods.

Figure 4.13 shows that *P. aeruginosa* OP2 decolorized malachite green up to 65% in the control culture compared to 86% in the culture which was grown to OD_{600} = 1 prior to the addition of malachite green after 24 hours of incubation. The levels of decolorization of the dyes in both sets of the cultures then improved after 48 hours of incubation and reached 95% and above after 72 hours of incubation.

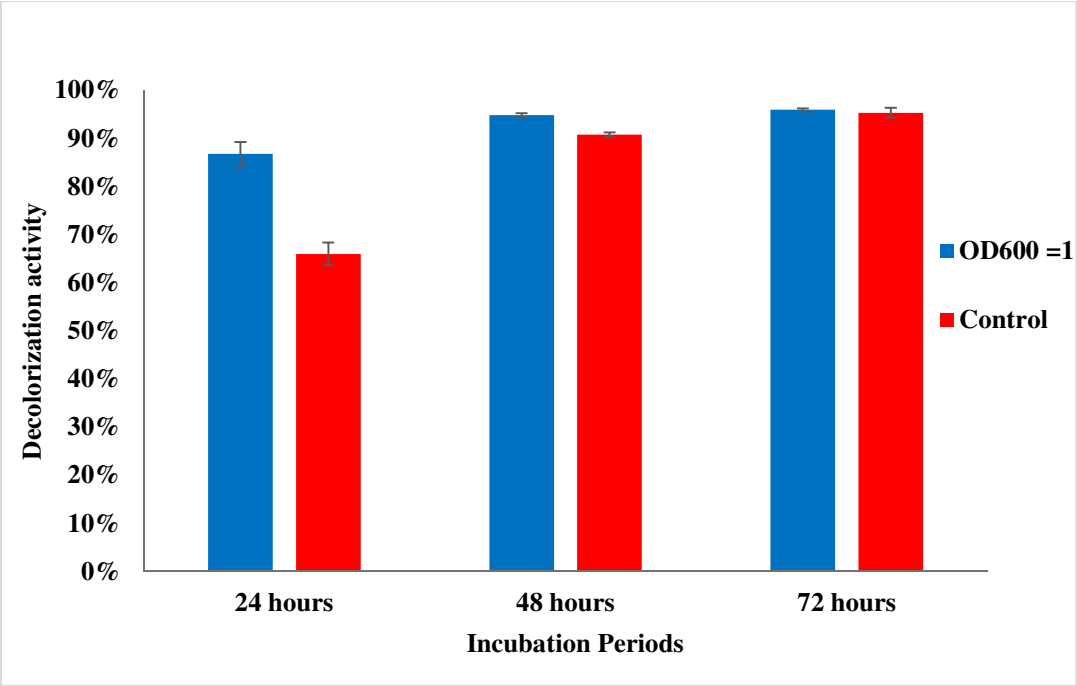


Figure 4.13 : Effect of bacterial growth phases on decolorization of malachite green at different incubation periods.

4.8 Effect of Aeration on the Decolorization Efficiency of Bacteria

The study was conducted to examine the effects of aeration on the level of efficiency of dyes decolorization. Two different conditions, which were the shaking and static conditions, were set. Based on the results shown in Figure 4.14, crystal violet was better decolorized under the shaking condition in all the three periods of incubation. The level of decolorization of crystal violet under the shaking condition was the highest at 58% while under the static condition, it was only 35% after 72 hours of incubation.

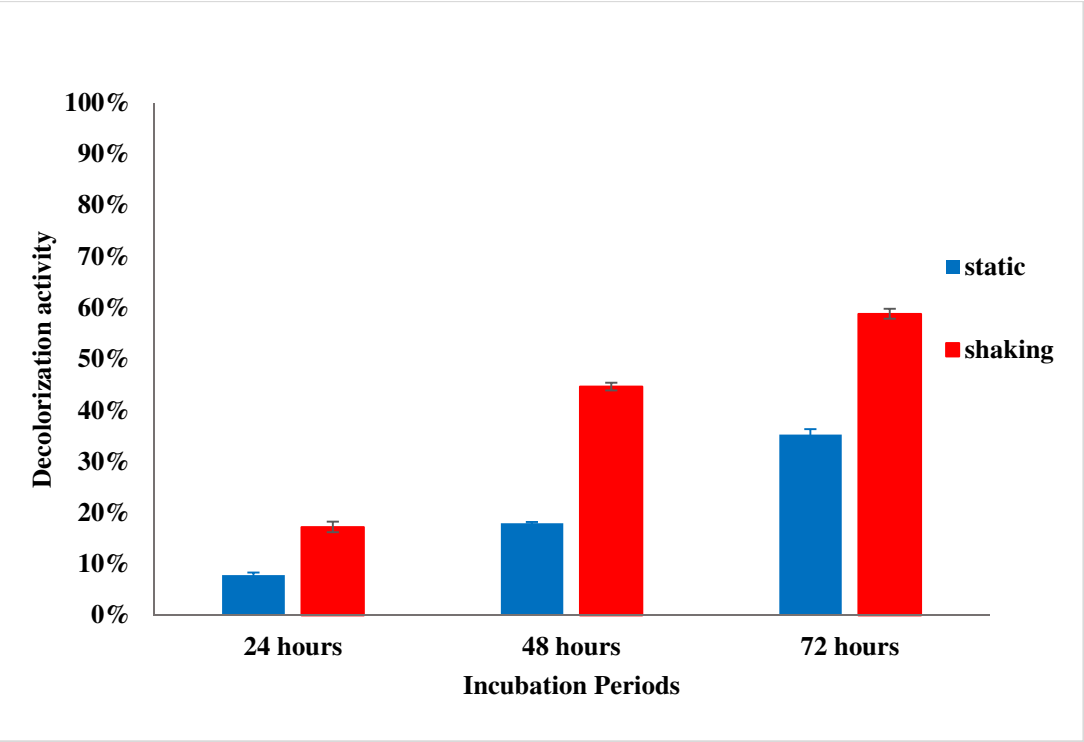


Figure 4.14: Effect of aeration on decolorization of crystal violet at different incubation periods.

Based on Figure 4.15, the decolorization pattern of malachite green was similar to that of crystal violet under the shaking and static conditions. The levels of decolorization of malachite green were much higher under the shaking condition compared to the static one in all the three periods of incubation. The level of decolorization of malachite green reached its peak at 95% after 72 hours of incubation under the shaking condition.

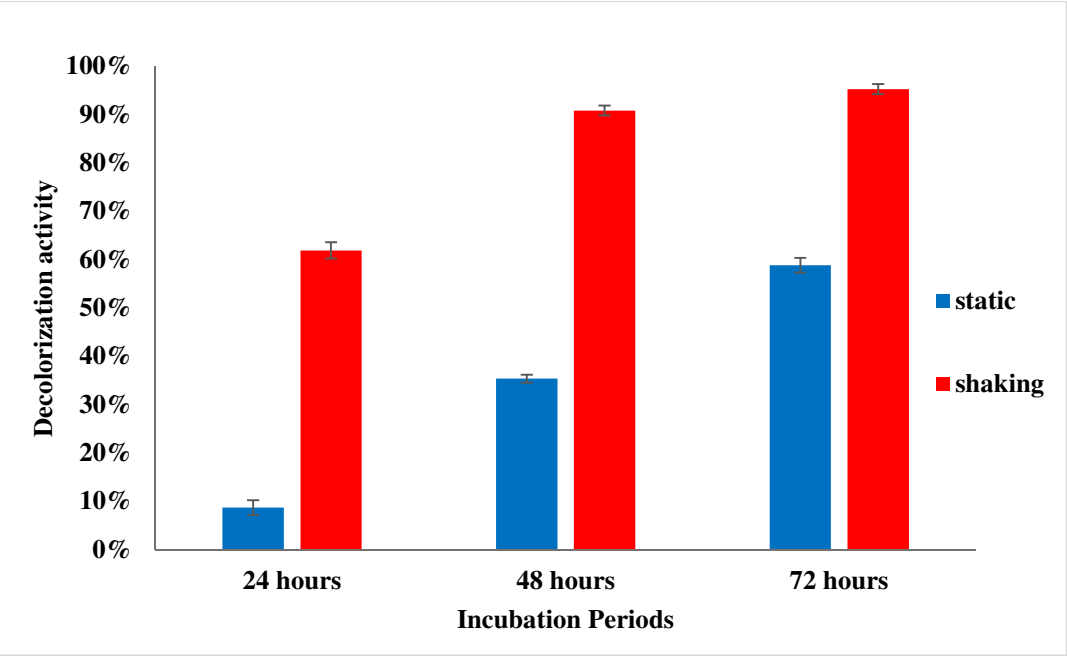


Figure 4.15: Effect of aeration on decolorization of malachite green at different incubation periods.

4.9 Toxicity Test of Malachite Green before and after Treatment by *P. aeruginosa* OP2

The toxicity of 1000 mg/L of malachite green before and after *P. aeruginosa* OP2 treatment was tested with six types of bacteria which consisted of three gram positive and three gram negative bacteria. Based on Table 4.2, all the gram positive bacteria used were susceptible to the toxic effects of malachite green as they showed inhibition zones of varying sizes before treatment. The toxicity of malachite green on these bacteria was then reduced as proven in the reduction of the inhibition zones after *P. aeruginosa* OP2 treatment. The test results in Table 4.2 also showed that all the gram negative bacteria were resistant to the toxicity of malachite green, hence no zone of inhibition was formed before or after treatment.

Table 4.2 Toxicity test of malachite green before and after treatment by *P. aeruginosa* OP2

	Size of Inhibition Zone (mm)	
	Before treatment	After treatment
Gram Positive Bacteria		
Staphylococcus aureus ATCC 25923	24	18
Staphylococcus epidermidis	31	22
Micrococcus luteus	18	11
Gram Negative Bacteria		
Salmonella trphimurium	0	0
Escherichia coli 25922	0	0
Klebsiella pneumonia	0	0

CHAPTER 5

DISCUSSION

5.1 Mechanism of Dye Removal

P. aeruginosa OP2 is deduced to be able to bio-adsorb and biodegrade crystal violet and malachite green based on the color changes of the cell pellets and the UV-vis spectral analysis of both dyes. Bio-sorption usually happens prior to the biodegradation process by the cells (Ogugbue and Sawidis, 2011). Based on Figure 4.4 (b) and Figure 4.5 (b), the cell pellets were deeply colored with crystal violet and malachite green which proved that the dyes were absorbed into the bacterial cells. After a period of 48 hours, the cell pellets were back to their original colors as shown in Figure 4.4 (c) and Figure 4.5 (c) which proved that the crystal violet and malachite green which had been adsorbed were biodegraded by *P. aeruginosa* OP2.

The biodegradation property of *P. aeruginosa* OP2 was further proven by the UV- vis spectral analysis. For those bacteria that exhibit only the property of biosorption, the absorption spectrum would show all peaks decreasing in proportion to each other with no appearance of new peaks (Shah, Patel, and Darji, 2013). While in the process of biodegradation, the major absorbance peak would completely disappear and new peaks will be produced (Jang, et al., 2005; Shah, Patel and Darji, 2013). The maximum absorbance peaks of crystal violet and malachite green were at 584 nm and 618 nm respectively as shown in Figure 4.2. After treatment by *P. aeruginosa* OP2, the maximum absorbance

peaks for both dyes decreased significantly. A new peak then appeared at 260 nm and 254 nm for crystal violet and malachite green respectively (Figure 4.3). These peaks most likely corresponded to the formation of leuco derivatives that are colorless upon degradation of crystal violet and malachite green as reported by Jang, et al. (2005) and Ye, et al. (2007).

Based on the research done by Jang et al. (2005), the enzyme triphenylmethane reductase detected in *Citrobacter* is able to reduce crystal violet and malachite green to their leuco forms. Besides, *Pseudomonas putida* is also found to contain the same enzyme (Chen, et al., 2007). Thus, it is believed that *P. aeruginosa* OP2 which comes from the same genus as *Pseudomonas putida* might also contain triphenylmethane reductase which will decolorize crystal violet and malachite green through the reduction process.

5.2 Effect of Dye Concentration on the Decolorization Efficiency of Bacteria

As shown in Figure 4.6 and Figure 4.7, when the concentrations of crystal violet and malachite green increased, the decolorization efficiency of *P. aeruginosa* OP2 decreased due to the toxicity of the triphenylmethane dyes. Triphenylmethane dyes of high concentration exhibited bacteriostatic effect which prolonged the lag phase by inhibiting cellular metabolic activities and cell growth of *P. aeruginosa* OP2 (Fischer, et al., 1944; Kerr and Gregory, 1969). The bacteriostatic effect of triphenylmethane dyes was due to the dye cations which combined with the acidic groups in the bacterial protein (Fry, 1957; Bourne, 2012). *P. aeruginosa* OP2 was able to decolorize crystal violet

at a concentration of lower than 500 mg/L and malachite green at a concentration of lower than 1500 mg/L as it is resistant to the toxic effects of both the dyes at these concentrations. Upon reaching the concentrations of 500 mg/L and 1500 mg/L respectively for crystal violet and malachite green, there was no decolorization of the dyes even after 72 hours of incubation as the bacterial growth had been completely inhibited by the toxicity of the dyes.

Malachite green is less toxic and is more easily decolorized compared to crystal violet due to their structural differences. The lipid solubility of dyes is affected by the number of alkyl groups present in the dyes (Fry, 1957). The higher the number of alkyl groups, the greater the lipid solubility of the dyes and hence the greater their antibacterial activity as bacterial plasma membrane is made up of lipoproteins (Fry, 1957). Malachite green which has only two dimethyl groups shows lower antibacterial activity compared to crystal violet which has three dimethyl groups (Sani and Banerjee, 1999). From the result obtained, *P. aeruginosa* OP2 was able to decolorize malachite green up to 90% at 1000 mg/L but was only able to decolorize crystal violet up to 70% at a concentration of as low as 10 mg/L.

The study conducted also shows that *P. aeruginosa* OP2 has a comparatively greater ability to decolorize malachite green than the other *Pseudomonas* species. For examples, the maximum malachite green decolorization activity of *Pseudomonas otitidis* WL-13 is achieved at a concentration of 180 mg/L (Wu, et al., 2008) while another species, *Pseudomonas putida* X5 is only able to decolorize malachite green at a low concentration of 50 mg/L though with 90%

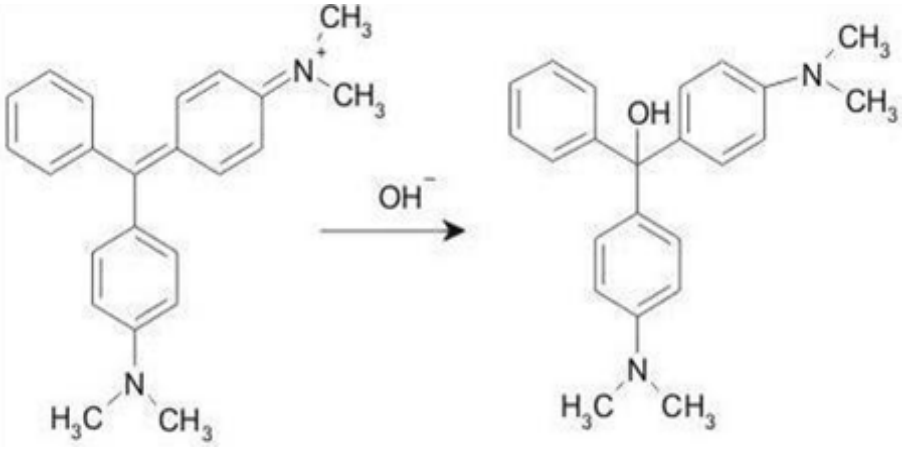
of decolorization efficiency (Nan, Zheng and Yao, 2012). Hence with its remarkably high level of dye decolorization ability, *P. aeruginosa* OP2 can be further exploited for treatment of dyes in waste waters.

5.3 Effect of Temperature on the Decolorization Efficiency of Bacteria

The study reveals that *P. aeruginosa* OP2 is able to grow and hence decolorize malachite green and crystal violet at the temperatures of 25^{o}C, 30^{o}C and 37^{o}C. Both crystal violet and malachite green are best decolorized at 30^{o}C and least at 37^{o}C after 24 hours of incubation. This indicates that 30^{o}C is likely the optimum growth temperature for *P. aeruginosa* OP2 as bacteria have the highest growth rate at its optimum temperature and hence the highest decolorization efficiency. Conversely, at a higher temperature of 37^{o}C, the oxygen solubility decreases and eventually causes a reduction in the metabolic activity of the aerobic natured *P. aeruginosa* OP2 (Bamforth and Singleton, 2005; Gan, et al., 2014). Since dye degradation is a metabolic process, shifting to higher temperature will lower the decolorization activity of *P. aeruginosa* OP2.

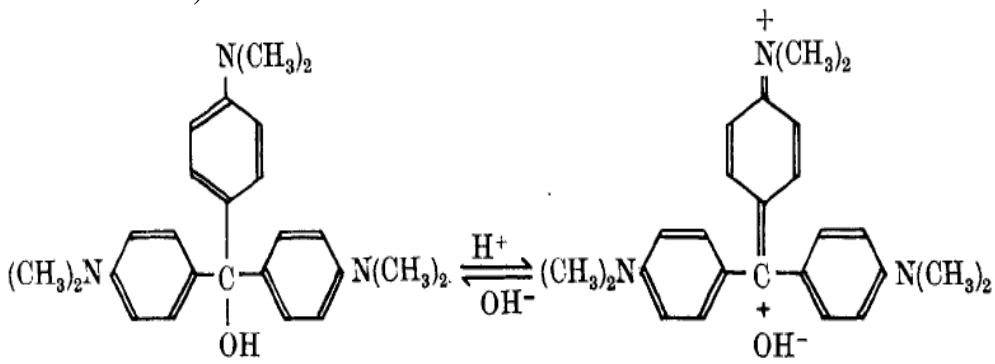
5.4 Effect of pH value on the Decolorization Efficiency of Bacteria

Based on the results shown in Figure 4.10 and Figure 4.11, both crystal violet and malachite green showed high levels of decolorization from pH 6 to pH 8 after 72 hours of incubation. This shows that *P. aeruginosa* OP2 is able to grow well and hence decolorize the dyes within this range of pH values. The optimum pH for *P. aeruginosa* OP2 to grow well and for its enzymes to function efficiently was at pH 7 as the decolorization activity was the highest at this pH value. The high levels of decolorization activities observed at pH 9 and pH 10 on malachite green might not be contributed by the decolorizing effects of *P. aeruginosa* OP2 on the dye. Instead, it could be due to the fading effects of the triphenylmethane dyes. This fading effect of the malachite green arises from an abundance of nucleophilic hydroxyl ions present in an alkaline solution which attack the electrophilic central carbon of malachite green and results in the formation of colorless carbinol as shown in Figure 5.1 (Fry, 1957). However, crystal violet did not show high decolorization activity at pH 9 and pH 10. This is because when a low concentration of crystal violet is used, an equilibrium between the dyes and carbinol can be established without precipitation of carbinol (Figure 5.2), and thus allows the color of crystal violet to remain the same. Conversely, a higher concentration of malachite green was used in the study, hence an equilibrium could not be achieved thus resulting in the formation of carbinol.



Malachite green Carbinol

Figure 5.1: Reaction of malachite green with hydroxyl ions (Raducan, et al., 2008)



Crystal violet

Carbinol

Figure 5.2: Equilibrium reaction of crystal violet and hydroxyl ions (Fry,1957)

It was also found that *P. aeruginosa* OP2 exhibited a very low efficiency in decolorizing crystal violet and malachite green at pH 5. This is because the growth of the bacterial cells are inhibited and the structures of the enzymes altered at such an acidic pH. Besides, according to Johnson (2009), triphenylmethane dyes are more toxic at a lower pH and this has made the bacterial cells more susceptible to their toxic effects (Johnson, 2009).

5.5 Effect of Bacterial Growth Phase on the Decolorization Efficiency of Bacteria

OD_{600} is the optical density that measures the light absorbance of a cell culture sample at 600 nm. The cell density and the growth phase of *P. aeruginosa* OP2 can be determined by measuring them at OD_{600}. When the OD_{600} = 1, it can be presumed that *P. aeruginosa* OP2 has reached its late log phase in which the cells have already adapted to the new environment and are in a rapidly growing and dividing state (Goldova, et al., 2011). At this phase, there are more bacteria cells available to bio-absorb and biodegrade malachite green and crystal violet. Hence they are exhibiting higher decolorization activities compared to the control culture. In the control culture, *P. aeruginosa* OP2 was not cultured to OD_{600} =1, hence it remained in its lag phase and took a longer time to adapt to the toxic environment created by the dye added into it. Therefore, the lag phase of *P. aeruginosa* OP2 was prolonged and this had resulted in a low level of decolorization activity after 24 hours of incubation as shown in Figure 4.12 and Figure 4.13. The control culture contained a sufficient number of *P. aeruginosa* OP2 bacterial cells after 72 hours of incubation, hence it also exhibited similar decolorization activity as the culture that had been cultured to OD_{600} = 1 prior to the addition of triphenylmethane dyes.

5.6 Effect of Aeration on the Decolorization Efficiency of Bacteria

P. aeruginosa OP2 was cultured with the dyes under both the shaking and the static conditions. And it was found that *P. aeruginosa* OP2 exhibited a higher level of efficiency in decolorizing crystal violet and malachite green under the shaking instead of the static condition (Figure 4.14 and Figure 4.15). This being so as. *P. aeruginosa* OP2 is an aerobic bacterium which depends on dissolved oxygen for its growth (MicrobeWiki, 2012; Bates, Phillips and Bryan, 2011). Under the shaking condition, the aeration in the flask was promoted, hence increasing the amount of dissolved oxygen in it. Besides, the nutrients were also distributed evenly in the mixture under the shaking condition which in turn maximized the nutrient uptake by *P. aeruginosa* OP2 (Expression Technologies Inc., 2003). Hence, the bacterial growth was increased which in turn contributed to a high level of decolorization of the dyes. Likewise, a low level of efficiency in the decolorization of dyes under the static condition was due to a low level of dissolved oxygen in the mixture. Moreover, the bacterial cells might settle to the bottom of the flasks under this condition and became oxygen depleted. This resulted in a slower rate of cell growth and in turn affected the dye decolorization process (Zuraida, Nurhashilna and Ku, 2013).

5.7 Toxicity Test of Malachite Green before and after Treatment by *P. aeruginosa* OP2

The toxicity of malachite green at 1000 mg/L was tested with six types of bacteria including gram positive and gram negative bacteria. From the result obtained (Table 4.2) no clear zone was observed when it was tested with gram negative bacteria. This is because gram negative bacteria are more resistant to malachite green due to the presence of their impermeable outer cell walls. Conversely, gram positive bacteria do not have outer cell walls. Instead their outer layers consist only of a thick layer of peptidoglycan. Hence, clear zones were observed before treatment by *P. aeruginosa* OP2 which shows that malachite green is toxic to gram positive bacteria. Then after treatment and biodegradation by *P. aeruginosa* OP2, leuco malachite green was predictably produced. It is less toxic compared to malachite green and hence the clear zones were reduced (Fessard, et al., 1999).

Another reason for gram negative bacteria to have higher resistance to tripheynlmethane dyes is due to their high isoelectric point compared to gram positive bacteria which have a lower one (Azmi, Sani and Banerjee, 1998). High isoelectric point indicates that there are lesser negative charges on the cell surfaces of the bacteria and vice versa. Under normal physiological environment, a portion of the cell surface anions such as the carboxylate and phosphate group of proteins and lipids will be neutralized by sodium ions or attached to calcium ions (Bourne, 2012). However, triphenylmethane dyes will displace the sodium and calcium ions and then combine with the anions to form unionized complexes which will cause toxicity to the cells (Danielli, 1944; Bourne, 2012). Hence, gram positive bacteria are more susceptible to the

toxicity of the dyes as there are more anions on the cell surfaces which attract the cationic dye molecules to form unionized complexes compared to gram negative bacteria (Silhavy, Kahne, and Walker, 2010). Conclusively, the more readily a dye forms unionized complex with anions, the greater is the toxicity of the dye.

5.8 Future perspectives

Since *P. aeruginosa* OP2 has the ability to decolorize malachite green at a high concentration of 1000 mg/L and decolorize crystal violet at a comparatively lower concentration, the ability of this bacteria to decolorize other triphenylmethane dyes should also be tested. Besides triphenylmethane dyes, the ability of *P. aeruginosa* OP2 to remove anthraquinone and azo dyes which also exist as pollutants in wastewaters should also be studied.

The level of toxicity of triphenylmethane dyes and their end products after treatment by *P. aeruginosa* OP2 should also be tested using mammalian cell line and plant tissues. The viability of these cells could be investigated to determine the toxic effects of the dyes and their end products to the environment and ecosystem. Triphenylmethane dyes may also exhibit differing levels of toxicity on different organisms such as bacteria, animals, plants and human beings and hence the effects of the dyes on these organisms should also be studied closely.

Moreover, biodegradation pathways of crystal violet and malachite green by *P. aeruginosa* OP2 can be studied further by using fourier transformed infrared radiation (FTIR), high pressure liquid chromatography (HPLC) and GC-MS. These steps are vital as identification of intermediates produced along the pathways allows a better understanding of the biodegradation process of triphenylmethane dyes and the possible enzymes involved in it (Gan, et al., 2014)

Triphenylmethane reductase is the enzyme that is predicted to be involved in the biodegradation of malachite green and crystal violet in this study. According to Jang et al. (2005), the *tmr* gene is responsible for the production of triphenylmethane reductase and hence the detection of this gene in *P. aeruginosa* OP2 should be carried out. However, resulting from a failed attempt to amplify the *tmr* gene in *P. aeruginosa* in previous research by Yeoh Loo (2014), it is suspected that the primers used which are specific to *tmr* gene in *Citrobacter* are not necessary specific to the DNA sequence in *P. aeruginosa*. A new set of primers can be designed for *tmr* gene amplification in *P. aeruginosa* OP2.

There is also a possibility that other types of enzymes such as lignin peroxidase and laccase instead of triphenylmethane reductase are responsible for the biodegradation process of dyes by *P. aeruginosa*. It had been reported by Azmi, Sani and Banerjee (1998) and Parshetti, et al. (2006) that lignin peroxidase is found in *Phanerochaete chrysosporium* fungi while laccase is present in *Agrobacterium radiobacter*. Both of these enzymes are also present in

Pseudomonas aeruginosa and they have the abilities to biodegrade triphenylmethane dyes such as crystal violet, malachite green and aniline (Bohlay, et al., 2012; Peter and Vandana, 2014; Zhang, et al., 2012). Hence, there is a high possibility that these enzymes are also present in *Pseudomonas* isolate OP2 and are involved in the biodegradation of the dyes.

CHAPTER 6

CONCLUSION

In this study, *Pseudomona*s isolate OP2 was identified as *P. aeruginosa* OP2 by the 16S rRNA gene analysis. *P. aeruginosa* OP2 is able to decolorize crystal violet and malachite green of differing concentrations through bio-sorption and biodegradation to different degrees. Malachite green at a concentration of 1000 mg/L was decolorized to 90% and above whereas crystal violet at a concentration of as low as 10 mg/L was only decolorized up to 70% after 72 hours of incubation. This being so as crystal violet is more toxic to *P. aeruginosa* OP2 than malachite green due to the structural differences of these two dyes. Besides, both crystal violet and malachite green were decolorized best by *P. aeruginosa* OP2 at 30^{o}C at pH 7 under agitation at 200 rpm. This is because the above conditions enable the aerobic *P. aeruginosa* OP2 to attain its maximum growth rate which in turn maximizes its decolorization activity. Besides, for the *P. aeruginosa* OP2 that was grown to late log phase (OD_{600} = 1), a high percentage of decolorization activity at 86% was observed after 24 hours of incubation. Finally, the toxicity of malachite green decreased after treatment by *P. aeruginosa* OP2. The end products exhibited lower levels of toxicity compared to malachite green.

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Appendix A

Reagents Preparation Recipe

Reagents	Ingredients per liter of solution
Luria-Bertani (LB) broth	10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl dissolved in dH_2O.
Luria-Bertani (LB) agar	10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar powder dissolved in dH_2O.
Mueller-Hinton (MH) agar	2 g of beef extract, 17.5 g of acid hydrolysate of casein, 1.5 g of starch, 17 g of agar powder dissolved in dH_2O.
Crystal violet stock solution (5 g/L)	0.15 g of crystal violet dissolved in 30 mL dH_2O.
Malachite green stock solution (10 g/L)	0.2 g of malachite green dissolved in 20 mL dH_2O.
10X TAE buffer	48.4 g of Tris base, 11.4 mL of glacial acetic acid, and 3.7 g EDTA dissolved in dH_2O.
1X TAE buffer	100 mL of 10X TAE buffer diluted in dH_2O.
EtBr solution	100 µL of stock EtBr dissolved in dH_2O.
0.85% (w/v) saline solution	0.85g NaCl dissolved in 100 mL dH_2O.
0.8 % (w/v) agarose gel	0.8 g of agarose powder dissolved in 100 mL 1X TAE buffer.
1.0 % (w/v) agarose gel	1.0 g of agarose powder dissolved in 100 mL 1X TAE buffer.