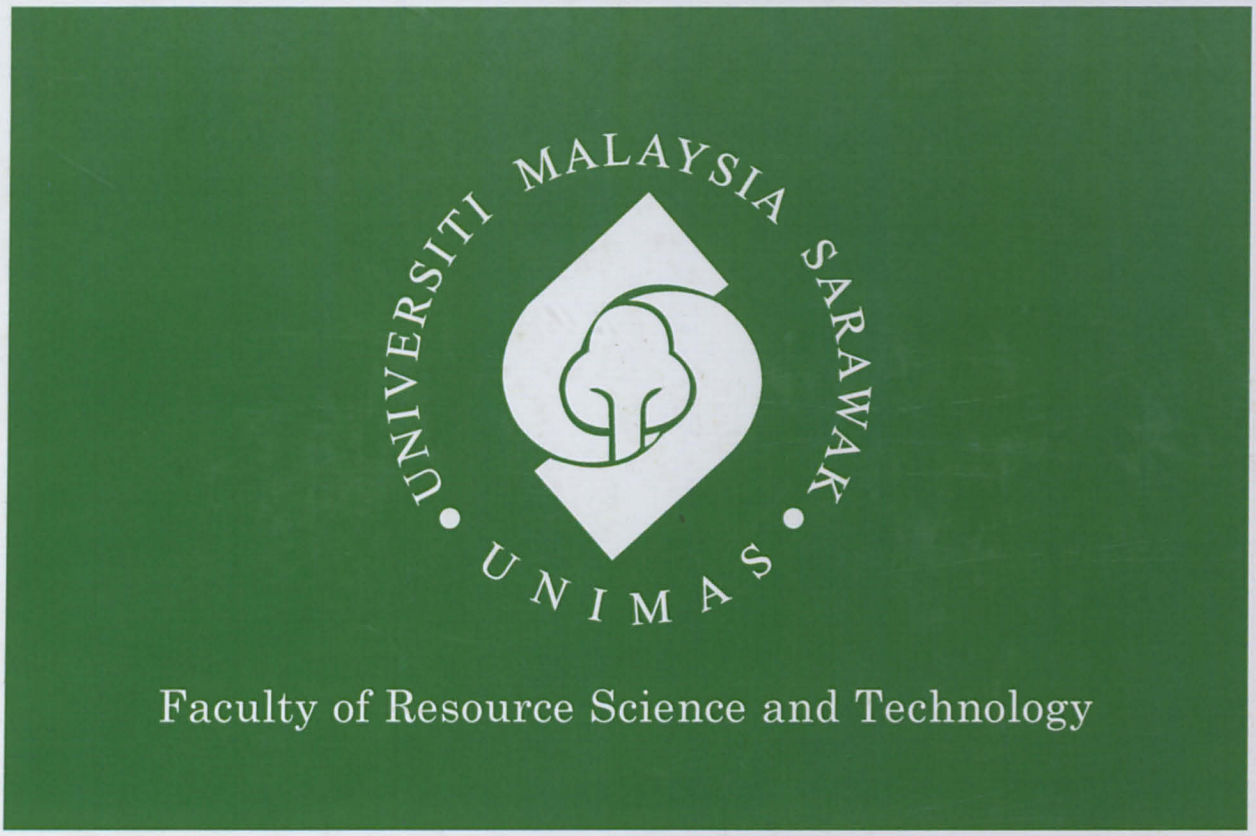

EVALUATION OF HETEROCYCLIC HYDROCARBON DEGRADATION BY USING IMMOBILIZED BACTERIA

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Bachelor of Science with Honours
(Resource Biotechnology)
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Evaluation of Heterocyclic Hydrocarbon Degradation by Using Immobilized Bacteria

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A final year project submitted in partial fulfillment of the requirement for the degree of
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I also place on record, my sense of gratitude to one and all, who directly or indirectly, have lent their hand in this venture.

DECLARATION

I hereby declare that this Final Year Project (FYP) entitled "Evaluation of heterocyclic hydrocarbon by using immobilized bacteria" is a *bona fide* and genuine research carried out by me under the guidance and supervision of Dr. Azham Zulkharnain, Department of Molecular Biology, Faculty of Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS). I also declare that this Final Year Project has not been submitted in any form of another degree or diploma at any university. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given. FRST shall have the rights to preserve, use and disseminate this FYP report in print or electronic format for academic or research purpose.

Date: 13 MAY 2015

Signature:

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LIST OF ABBREVIATIONS

A: Absorbance

BPH: Biphenyl

DBF: Dibenzofuran

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

GC/MS: Gas Chromatography-Mass Spectrometry

HOPDA: 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid

MB: Marine Broth

MSM: Marine Salt Medium

OD: Optical Density

PAH: Polycyclic Aromatic Hydrocarbon

PBS: Phosphate Buffered Saline

PCB: Polychlorinated Biphenyl

PCDF: Polychlorinated Dibenzofuran

POP: Persistent Organic Pollutants

PVA: Polyvinyl Alcohol

Rpm: Revolutions per Minute

2'-OH-HOPDA: 2-hydroxy-6-(2hydroxyphenyl)-6-oxo-2, 4-hexadienoic acid

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Evaluation of Heterocyclic Hydrocarbon Degradation by using Immobilized Bacteria

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ABSTRACT

There are some bacteria known to have the ability to break down heterocyclic hydrocarbon compounds. However, they are not efficient enough to degrade these compounds as the bacteria require a long time to grow, fully degrade hydrocarbon compounds, and the environment for growing bacteria is easily contaminated. Thus, in this study, the bacteria were immobilized and grew inside the gellan gum. The bacteria involved are *Thalassospira* sp. strain M01 and M155 bacteria which have the ability to degrade Biphenyl (BPH) and Dibenzofuran (DBF) respectively. By immobilizing these bacteria, the rate of degradation is proven to be faster than free bacteria and they can be reused. Furthermore, they are safe from any possible contamination. Immobilization also improves the degrading enzyme induction and reusability of bacteria.

Keywords: Biphenyl, Dibenzofuran, Gellan gum, Immobilized bacteria.

ABSTRAK

Terdapat beberapa bakteria yang diketahui memiliki kemampuan untuk mengdegradasi sebatian heterosiklik hidrokarbon. Walaubagaimanapun, bakteria ini tidak cukup cekap untuk mengdegradasi sebatian ini kerana mereka memerlukan masa yang lama untuk membiak, untuk mengdegradasi sebatian sepenuhnya dan persekitaran di mana bakteria ini membiak mudah tercemar. Justeru, melalui kajian ini, bakteria tersebut telah diperangkap dan dibiak di dalam gam gellan. Antara bakteria yang terlibat ialah Thalassospira sp. M01 dan M155 yang berkemampuan untuk mengdegradasi Biphenil (BPH) dan Dibenzofuran (DBF). Melalui pemerangkapan bakteria, kadar degradasi terbukti lebih cepat berbanding bakteria bebas dan mereka boleh digunakan semula. Selain itu, mereka juga bebas daripada sebarang kemungkinan untuk tercemar. Teknik pemerangkapan juga telah meningkatkan kadar pengaktifan enzim pengdegradasi dan penggunaan semula bakteria.

Kata kunci: Biphenil, Dibenzofuran, Gam gellan, Pemerangkapan bakteria.

1.0 INTRODUCTION

Every year, large varieties of commercially synthesized chemicals are irresponsibly discharged into containment such as land, water bodies and atmospheric space. Within these chemicals, lies heterocyclic hydrocarbon compounds which is known for their carcinogenic and neurotoxic behavior (Singh, 2011). Hydrocarbons such as Biphenyls (BPHs) and Dibenzofurans (DBFs) are widely spread in environment as the by-products from incomplete incineration of organic matter, emission sources from stationary matter such as coal-fired, automobile exhausts, and area source matter such as forest fires. If these compounds managed to enter the living food chain, they will accumulated inside the consumer and at a certain point causing detrimental effect, for example, serious and chronic health problems and genetic defects in human. In animals, they will disrupt in bodily functions such as developmental deformities, reproductive failure, sex changes and leading to death (Samantha, Singh & Jain, 2002).

Among the variety of hydrocarbon degradation methods available, bioremediation technology is believed to be non-invasive and economically effective (Kota et al., 2014). In addition, they can degrade the toxic by-products faster than the conventional treatment system since specialized organisms are used in high densities. Nowadays, biodegradation of BPH and DBF has been the subject of a large body of research for over three decades (Leigh et al., 2007). Physical and chemical conditions such as the organic substances which consist of nitrogen, carbon and phosphorus, oxygen and pH are the keys for a successful bioremediation (Maliji, Olama & Holail, 2013).

Despite discovering various types of microorganisms which are able to degrade highly stable and toxigenic heterocyclic hydrocarbons, the problem of environmental pollution caused by these hydrocarbons remains unsolved (Koukkou & Drainas, 2008). One of the factors is because of the abundance of these compounds which become toxic to cells and limiting the degrading ability of microorganisms involved in degradation. The bioremediation kinetics also very limited and few months are required for hydrocarbons degradation (Wannoussa, 2015). Thus, modifications are needed to increase the effectiveness of these microorganisms in degrading hydrocarbons which were thought as recalcitrant. One of the known methods to increase the efficiency of these microorganisms is through immobilization technique. The commonly used substances as immobilization medium for bacteria in heterocyclic hydrocarbon degradation are Calcium Alginate and Polyvinyl Alcohol (PVA) because the methodologies are simple, relatively mild and not toxic to the cells. In turns, they are susceptible to degradation and have a low mechanical stability (Ahmad et al., 2011).

Thus, gellan gum is chosen as the gelling agent in this study as the gel is water soluble, stable over a wide pH ranges from 2 to 10, non-toxic and suggested to be used in fermentation technology due to its thermal and mechanical stability. Gellan gum also provides protection for BPH and DBF degrading bacteria, namely *Thalassospira* sp. strain M01 and M155 against the high concentration of BPH and DBF compounds. This study aimed to formulate immobilized bacteria by using different matrices and to measure the degradation performance by immobilized bacteria in comparison to free bacteria. By understanding the degradation performance in both immobilized and free bacteria, different parameters to figure out the optimum conditions needed for degradation activity can be carried out by manipulating several factors.

2.0 LITERATURE REVIEW

2.1 Bioremediation

Bioremediation refers to the complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds and cell protein or transformation of complex organic compounds to other simpler organic compounds by biological agents such as microorganisms. Biodegradability of heterocyclic compounds is determined its complexity which depends on number, type and position of substituent on heterocyclic ring. The use of microorganisms is to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including Biphenyls and Dibenzofurans. However, bioremediation is only restricted to biodegradable compounds and unfortunately, not all these compounds are susceptible to fast and whole degradation. Moreover, biological processes are usually very specific as it requires important site factors such as the presence of metabolically proficient microbial colonies, optimal growth conditions and suitable supply of nutrients (Vidali, 2001).

However, bioremediation offers a better environment clean up strategy for certain types of pollution such as oil spillage which are caused by accident during transportation by ship or oil pipelines (Patil et al., 2012), and discharges of chemical effluents into receiving water bodies. Microbial bioremediation is the primary technique in natural decontamination. Depending on the site of pollution and pollutants, bioremediation is considered safer and cost effective compared to other solutions such as landfilling or combustion of contaminated materials which will lead to far more serious problems in the future including accumulation of toxic substances in soil and causing the soil to be infertile for plants growth (Vinãs et al., 2005).

2.2 Heterocyclic Hydrocarbon

Heterocyclic hydrocarbons are toxic compound composed of four or more fused rings (Singh & Ward, 2004). It contains the hydrocarbon components that have been known belong to the family of carcinogens and neurotoxic organic pollutants. They can be found in emission of hydrocarbons such as coal and petroleum products and also formed naturally at high-temperatures or incomplete combustion of organic materials. One of the main environmental crises today which rise from hydrocarbon contamination is the result of activity involving petrochemical industry. Furthermore, discharges of petroleum by-products are the main concern to ecosystem as it will cause predicament towards biotic and abiotic components in future if left untreated (Qi et al., 2012).

2.3 Biphenyl

Biphenyl is an aromatic hydrocarbon which forms colorless crystals at room temperature with molecular formula of $(C_6H_5)_2$ and acts as intermediate for the formation of a host of other organic compounds, for examples the emulsifiers and optical brighteners (Wu, Feng & Boyd, 2003). Apart from the effluents discharged by chemical industry, BPH can be formed anywhere and anytime, intentionally or unintentionally. For example, in a chemistry laboratory and even occurs naturally in crude oil, coal tar, and natural gases. BPH possess the ability able to stop the growth of fungi and molds and thus, is used as preservatives (E_{230}) especially in preservation of citrus fruits and woods during their transportation. Even though BPH application has been reduced in recent years, it still remains in environment (Ang, 2009). Nevertheless, BPH is toxic and brings harm to human health but it can be degraded biologically by conversion into non-toxic compounds.

2.3.1 Polychlorinated Biphenyls

BPH is notable as an initial material for the formation of Polychlorinated Biphenyls (PCBs), which were utilized widely as dielectric fluids and heat transfer agents in the past years due to their non-flammability, high boiling point, chemical stability, and electrical insulating properties (Joutey, 2013). PCBs are synthetic, heterocyclic compounds that are produced in large quantities during the past century and following their low biodegradability and high stability in environment, PCBs are characterized into one of the 12 groups of compounds known as Persistent Organic Pollutants (POP) (Prządò, Kafarski & Steininger, 2007).

2.3.2 Effects of PCBs to human health

PCBs are capable to cause grave danger diseases by affecting humans immune system and suppressing immune system and thyroid function, causing heart disease by increasing risk of cardiovascular disease, diabetes and hypertension, bring hormonal impacts through altering of sex hormone system by lowering the age of girl reaches puberty and decrease levels of testosterone hormone in male, causing asthma and infectious respiratory disease and also worsen the ability to learn in which the PCBs linked with cognitive problems and diminish the ability to learn and memorize (Fiedler, 2015).

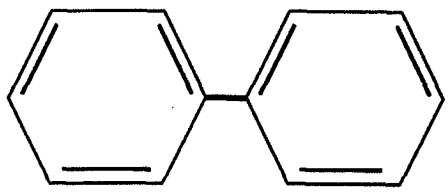

Figure 1: Structure of BPH

2.4 Dibenzofuran

Dibenzofuran (DBF) is a white, crystalline or sand like powder that is derived from the production of coal tar and often used as insecticides and to produce other chemicals. DBF is a polycyclic aromatic hydrocarbon (PAH) with molecular formula of $C_{12}H_8O$, molecular weight of 168.19, melting point at 86.5°C, boiling point at 287°C and slightly soluble in water, soluble in ethanol and very soluble in ether, benzene and acetic acid. DBF is produced from phenol and benzene in the incineration gas exhaust streams. Prior to the particle collection equipment and a subsequent chlorination at lower temperatures on particle surfaces is identified to be a potential source of Polychlorinated Dibenzofuran (PCDF) (Ryu, Mulholland & Chu, 2003).

2.4.1 Effects of DBF to human health

Generally, inhaling DBF contaminated air or come in contact with DBF compounds can cause irritation to the eyes, nose, throat or skin and under severe conditions, rashes may appear on skin and exposure to sunlight can worsen the conditions. As stated in the report by Agency for Toxic Substances and Disease Registry (n.d.), DBF can enter human body systems by inhalation of contaminated air, consumption of contaminated food or water and can also be absorbed into body systems once it comes in contact with the skin. A short-term exposure to DBF can cause skin, eye, nose, and throat irritation, meanwhile a long-term exposure can lead to rashes and growths to appear on the skin and the skin may also change color.

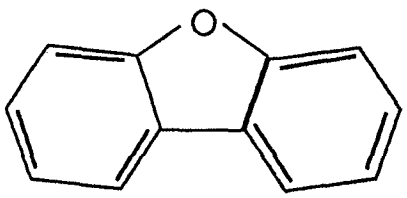

Figure 2: Structure of DBF

2.4.2 Environmental occurrence of DBF

DBF is a typical component of environmental pollutant and has been detected in air, ground, water, fuel, various gases, fly ash from the municipal incinerators and diesel exhaust gas particulates, and also cigarette smoke. DBF is one of the components of coal tar and prior to the growth of a nationwide gas pipeline systems, gas was manufactured locally through the process of coal distillation. Meanwhile, the coal tar residue that is not sold for roofing and road surfacing materials will be disposed at the sites nearby the gasification plants (Prijambada et al., 2012).

2.5 Gellan gum

Gellan gum is a high molecular weight polysaccharide gum which produced as a result of fermentation product by pure culture of the bacteria *Sphingomonas elodea*. Physically, gellan gum is water soluble and in form of white powder. Structurally, it composes a tetrasaccharide repeating unit of two β-D-glucoses, one β-D-glucuronate, and one α-L-rhamnose (Kirchmajer et al., 2014). The gelling property of gellan gum makes it valuable because only a few kinds of polysaccharides able to form gel (Niita & Nishinari, 2005). Gellan gum becomes gels when positively charged ions such as potassium, calcium, magnesium, or sodium salts are added. Thus, the texture and thickness of gellan gum can be controlled by using these salts.

3.0 MATERIALS AND METHODS

3.1 Preparation Method

3.1.1 Preparation of ONR7a suspension

ONR7a is a mineral salt medium (MSM) that is used to grow marine bacteria. In this experiment, all the reagents needed for ONR7a preparation was used according to standard formulation by DSMZ (German Collection of Microorganisms and Cell Cultures GmbH) and weighed by using digital balance before transferred into a 1 L Schott media storage bottle. Distilled water of 800 ml was added into the media bottle and the pH was adjusted to 7 by using a pH meter. Remaining 200 ml of distilled water was added to a volume of 1 L and autoclaved at 121°C/15 atm for 90 min. The ONR7a was kept at room temperature for future use.

Table 1: Composition of 1 L ONR7a

Chemical compounds	Weight, g
NaCl	22.79
Na_2SO_4	3.98
KCl	0.72
NaBr	0.083
$NaHCO_3$	0.0318
H_3BO_3	0.027
NaF	0.0026
NH_4Cl	0.27
$Na_2HPO_4.7H_2O$	0.047
$MgCl_2.6H_2O$	11.18
$CaCl_2.2H_2O$	1.102
$SrCl_2.6H_2O$	0.0024
$FeSO_4.7H_2O$	0.002

3.1.2 Preparation of 500 ml Marine Broth

Marine broth (MB) is necessary for the growth of marine bacteria. Difco Marine broth 2216 powder of 18.7 g was weighed and transferred in a 500 ml media bottle. Next, distilled water was added until the volume reached 500 ml before autoclaved it at 121°C/15 atm for 90 minutes and kept in a fridge at 4°C.

3.1.3 Preparation of 1 L Phosphate Buffered Saline

Phosphate Buffered Saline (PBS) is required to wash the BPH and DBF degrading bacterial pellet and prepared according to Cold Spring Harbor Protocols. Distilled water of 800 ml was measured with a graduated cylinder and transferred into a 1 L media bottle. The solutes were dissolved within 3 to 5 min to ensure that there are no remaining salt particles in the solution before pH was adjusted to 7.4 by using a pH meter. The final volume was adjusted to 1 L of distilled water. The solution was autoclaved and kept at room temperature for further use.

Table 2: Composition of 1 L PBS

Chemical compounds	Weight, g
NaCl	8.00
KCl	0.20
Na_2HPO_4	1.44
KH_2PO_4	0.25

3.1.4 Preparation of 250 ml Sodium Hydroxide Solution

The formula for preparing Sodium Hydroxide solution (NaOH) is provided by Monash Scientific in their website. Distilled water of 50 ml was measured by using a graduated cylinder and poured into a 250 ml media bottle. NaOH of 40 g (1 mole) was weighed and dissolved in the bottle. Distilled water was poured until the volume reached 250 ml. The NaOH solution was autoclaved and kept at room temperature for further use.

3.2 Enrichment preparation of heterocyclic hydrocarbon degrading bacteria

3.2.1 Preparation of stock substrate

Biphenyl and Dibenzofuran stock substrate was prepared at concentration of 0.1%. BPH and DBF substrate of 0.5 g were weighed and transferred into 15 ml falcon tubes. Next, 5 ml of Dimethylformamide (DMF) was added into both of the tubes and the mixture of stock substrates were kept at room temperature for future use.

3.2.2 Preparation of enrichment culture

For each substrate, two tubes of enrichment cultures were prepared. Next, 5 ml of ONR7a and 0.5 ml of MB were added into 15 ml glass tubes. Then, 50 µl, 0.1% of both BPH and DBF stock substrates were pipetted into the tubes. *Thalassospira* sp. strain M01 and M155 were inoculated by using inoculating loop and inserted into their respective glass tubes. The mixtures were incubated in a rotary shaker at 150 rpm, room temperature until there is a sign of color change.

3.3 BPH and DBF degrading bacteria growth conditions

3.3.1 Growth of *Thalassospira* sp. strain M01 and M155.

All procedures were conducted under sterile conditions and sterilization techniques were practiced. The growth of these bacteria required consecutively 3 days.

On the first day of bacteria growth, 100 µl of *Thalassospira* sp. and M155 from the enrichment culture and 100 µl of 0.1% of BPH and DBF stock substrates were pipetted into two units of 100 ml Erlenmeyer flask according to their respective bacteria. Next, 5 ml of MB was added into each of the flasks and incubated in a rotary shaker at 150 rpm, 27°C for 24 hours.

On the next day, the cultures from the first day were sub-cultured. New medium was prepared by adding 50 ml of MB and 0.1%, 100 µl of each stock substrate into new Erlenmeyer flasks. The cultures from the first day were transferred into their respective flasks and incubated in rotary shaker at 150 rpm, 27°C for 24 hours.

On the third day, the cultures were ready for harvesting. Bacterial cultures from the second day were transferred into 50 ml falcon tubes. The falcon tubes were centrifuged for 10 min, 7000 rpm at room temperature. After centrifugation was completed, the supernatant was discarded and the pellet obtained was washed and resuspended with 5 ml PBS. Next, 45 ml of PBS was added into each of the falcon tubes before the cultures were centrifuged the again at 7000 rpm for 10 min at temperature of 27°C. The washed and resuspended pellet was repeated for two times and at the third time of centrifugation, the supernatant was removed and pellets obtained were kept in fridge at 4°C and ready for immobilization procedures.

3.4 Immobilization of BPH and DBF degrading bacteria

Gellan gum of 0.375% or 0.375 g was mixed with 50 ml sterile distilled water in a 100 ml beaker and heated on a hot plate until the mixture turned clear, which means that all the gum has completely dissolved. The temperature was monitored frequently so that it does not went above 75°C as the mixture required longer time to cool down. 0.06 g of $CaCl_2$ was added into the mixture to cross-linked with gellan gum and produce linear gel that is necessary for solidifying of gum mixtures. After the temperature of the mixture reduced to approximately 50°C, the pH was justified to 7 by using NaOH solution and a pH meter. The temperature was always checked and maintained at approximately 42°C and stirred continuously to prevent the gum mixture from solidifying. At approximately 45°C, bacterial pellet obtained from centrifugation process was added into the mixture and dispersed by continuously stirred it.

Beads were formed by using a 2.5 ml syringe and dropped the gum mixture into Naturel Blend of Canola and Sunflower oil. The gum mixture was dropped at interval of one to two seconds so that the beads can gained a perfect sphere shape and prevented them from clumping together. After all the gum mixture has been dropped into canola oil, the beads were filtered from the oil and washed twice with Tween 80 solution to remove the oil from the beads. The Naturel Blend Canola and Sunflower oil can be reused for another immobilization process, so it is kept at room temperature. Lastly, the beads were washed with tap water and rinsed thoroughly before stored in sterile distilled water and kept in fridge at 4°C.

3.5 BPH and DBF degradation by free and immobilized bacteria

The medium needed for both BPH and DBF degradation composed of 100 ml ONR7a and 0.1% of their respective substrate. Free bacteria, in form of bacterial pellet obtained from centrifugation process also acted as the control. Both free and immobilized bacteria were added into each separate medium and incubated in rotary shaker at 150 rpm, room temperature. The changes of color indicated that the degradation activity has occurred. For BPH, the medium color changed from cloudy to yellowish and cloudy to orange for DBF.

3.6 Measuring the rate of degradation by free and immobilized bacteria

BPH and DBF degradation rate were measured by determining their optical density (OD) using a UV-visible light spectrophotometer at A_{434}. About 1.5 ml of the matrix of degradation for both free and immobilized bacteria were pipetted into eppendorf tubes. Then, the tubes were centrifuged at 7000 rpm for 2 min. After that, the supernatant was pipetted and transferred into cuvette for spectrophotometry. Firstly, the spectrophotometer was zeroed by passing light of interested wavelength, 434 nm through a blank solution which in ONR7a. Next, the same wavelength of light was passed through the mediums of degradation which were obtained hourly for 7 consecutive hours.