BIODEGRADATION OF HETEROCYCLIC HYDROCARBON USING BIOLOGICAL ACTIVATED CARBON

Chia Choon Hiung

Bachelor of Science with Honours (Resource Biotechnology) 2013
Biodegradation of Heterocyclic Hydrocarbons Using Biological Activated Carbon

Chia Choon Hiung (26082)

A final project report submitted in partial fulfilment of the Final Year Project II (STF 3015) Course

Supervisor: Dr. Azham Bin Zulkarnain
Co-Supervisor: AP Dr. Zainab Ngaini

Resource Biotechnology
Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak
2013
Acknowledgement

Firstly, I would like to thank my parents for guiding and giving me spiritual and financial support throughout the years of study in University Malaysia Sarawak (UNIMAS) and for the moment of accomplishing this Final Year Project. Secondly, I would like to express my appreciation to my supervisor, Dr. Azham Bin Zulkhamain for his assistance, supervision, blessings, as well as encouragements in guiding me at the every stages of the project. Thank you very much for all the useful advices that aided me in completing this project. I would also like to thanks to my co-supervisor, AP Dr. Zainab Ngaini for supplying the materials and reagents that I needed in this project and provided me the knowledge regards the chemistry field. Moreover, I am grateful to the postgraduate students of Molecular Genetics Laboratory (MGL), especially to Jane Sebastian, Simon Ngieng, Christy, and Farith for their knowledge sharing, advices given, willingness to help, and commitment in assisting me for my research project. To the lab assistance, Ms Sheila Unggau, thank you for constantly providing the materials and apparatus in the laboratory so that I am able to run my experiments smoothly throughout the project. Apart from that, I would like to express my deepest grateful to Mr. Benedict from Chemistry Department for the help with the Gas Chromatography-Mass Spectrometry.

Finally, I would like to thank to all my friends, especially Ng Li Ying, Kenneth Lim Chin Chiaw, Yeap Kim Heng and Wong Ha Chung for most of the moment working together and sharing materials and reagents in the laboratory. Thank you very much for all the experiences and knowledge that is very useful for this project.
I declared that this project entitled “Biodegradation of Heterocyclic Hydrocarbons Using Biological Activated Carbon” is the result of my own research except as cited in the references. This project has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Chia Choon Hiung  
Resource Biotechnology Programme  
Department of Molecular Biology  
Faculty of Resource Science and Technology  
University Malaysia Sarawak
Table of Contents

Acknowledgement I
Declaration II
Table of Contents III
List of Abbreviations V
List of Tables and Figures VI
Abstract VII

1.0 Introduction 1

2.0 Literature Review 3
  2.1 Biodegradation 3
  2.2 Biological Activated Carbon 4
  2.3 Heterocyclic Hydrocarbon 5
  2.4 Heterocyclic Hydrocarbon Degrading Bacteria 6
  2.5 Gas Chromatography Mass Spectrometry 7

3.0 Materials and Methods 9
  3.1 Media Preparation 9
    3.1.1 Preparation of ONR7a 9
    3.1.2 Preparation of Substrate Concentration 10
    3.1.3 Preparation of ONR7a (Liquid Media) with Substrate 10
    3.1.4 Preparation on Marine Agar (Solid Media) 10
  3.2 Enrichment of *Thalassospira Profundimarisis* 11
  3.3 Preparation of Sample and Control Unit 11
  3.4 Enumeration of Marine Bacteria 12
  3.5 Measurement of Organic Compound 13

4.0 Result 15
  4.1 Growth Confirmation of Isolated 15
    4.1.1 Growth on ONR7a (Liquid Media) 15
    4.1.2 Culture Enrichment on Marine Agar 16
  4.2 Enumeration of Marine Bacteria 17
  4.3 Measurement of Organic Compound 20

5.0 Discussion 23
  5.1 Growth Confirmation of Isolated 23
  5.2 Enumeration of Marine Bacteria 24
  5.3 Measurement of Organic Compound 25

6.0 Conclusion 28
7.0 References
8.0 Appendices

Appendix A
Appendix B
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAC</td>
<td>Biological Activated Carbon</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl Formamide</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agencies</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas Chromatography Mass Spectrometer</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Sodium Sulphate</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbon</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
</tbody>
</table>
Lists of Tables and Figures

Table 1  ONR7a composition.  
Table 2  The average of Colony Forming Units (CFU/ml).  
Table 3  Carbazole degradation of control and sample unit in terms of peak area.  
Table 4  Carbazole degradation of control and sample unit in terms of percentage.  
Figure 1 (a)  100ml of ONR7a supplemented with 0.2% of substrate concentration on Day 0.  
Figure 1 (b)  100ml of ONR7a supplemented with 0.2% of substrate concentration on Day 14.  
Figure 2  Culture enrichment on Marine Agar.  
Figure 3 (a)  Control unit on Day 0.  
Figure 3 (b)  Control unit on Day 15.  
Figure 3 (c)  Sample unit on Day 0.  
Figure 3 (d)  Sample unit on Day 15.  
Figure 4  Growth curve of the *Thalassospira profundimaris* in control and sample unit.  
Figure 5  Carbazole degradation of control and sample unit in 14 days.  
Figure 6  The efficiency of carbazole degradation rate of the sample unit based on the control unit.  
Figure 7  The CFU/ml from Day 0 to Day 15 of the experiment.  
Figure 8  The carbazole concentration analysis from Day 0 to Day 12 of the experiment.  

Pages

9  
17  
20  
21  
15  
15  
16  
18  
18  
18  
19  
21  
22  
32  
34
Biodegradation of heterocyclic hydrocarbons using biological activated carbon

Chia Choon Hiung (26082)

Resource Biotechnology Programme
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

The disturbance on ecological system caused by environmental pollution has raised public concerns. Recently, heterocyclic hydrocarbon has been claimed as one of the major pollutants. Therefore, it is essential to develop an efficient system that is capable of degrading heterocyclic hydrocarbon. In this study, the positive Thalassospira Sp. Strain M02 that is capable of degrading specific substrate was selected and grew in ONR7a liquid medium supplemented with heterocyclic hydrocarbon. Activated carbon was introduced into the ONR7a medium together with the growth of bacteria to develop biological activated carbon. The detection of bacteria growth on activated carbon was carried out by serial dilution on every three days from day 0 to day 15. In the meanwhile, the degradation of carbazole was quantified by using Gas Chromatography-Mass Spectrometry (GC-MS) method on every two days from day 0 to day 14. The colony forming units (CFU) in the log phase was higher and the carbazole was degraded to a lower concentration in the biological activated carbon unit as compared to control unit. As for the conclusion, the biodegradation of carbazole using biological activated carbon has shown to be more effective than the control unit by a difference of 94% degradation efficiency on day 12.

Keywords: Biodegradation, heterocyclic hydrocarbon, biological activated carbon

ABSTRAK


Kata kunci: biodegradasi, hidrokarbon heterocyclic, karbon aktif
1.0 INTRODUCTION

In recent days, the public has raised their concern towards the risk of environmental pollution. Environmental pollution is the accumulation of highly concentrated harmful compounds at a certain site (Diaz, 2004). This phenomenon usually appears as a consequence from human activities. Therefore, pollutants are used to be the compounds that presence in the use of industrial origin (Diaz, 2004). However, there is also a minority of pollutants that are produced through the metabolism of natural compounds into bioavailable form that is harmful to the environment (Diaz, 2004). Environmental pollution usually occurred at sites of chemical and pharmaceutical industries, pulp and paper bleaching factories, mining area, agriculture site, and etc (Dua et al., 2002; Rieger et al., 2002).

According to Briggs (2003), environmental pollutions are frequently more chronic in developing countries. This is because developing countries lack the finance to treat environmental pollution and the environmental legislation is weak. Hence, the management of environmental pollution is often inefficient or ignored. However, constant exposure to environmental pollution can raise health risk towards the society (Briggs, 2003). Certain pollutants such as PAH and heterocyclic hydrocarbon are well-known for its mutagenic and carcinogenic properties (Eisentraeger et al., 2008; Monna, et al., 1993). If these pollutants are transferred to human through seafood consumption, human populations are at a high risk of getting cancer. In order to solve the problem of environmental pollution, bacteria are introduced for pollutant degradation.
Bacteria have developed strategies over three billion years to metabolize available compound into energy to fulfill their needs (Diaz, 2004). On the other hand, activated carbon is well known for its high adsorptive capacity. Therefore, this study is being carried out to combine the ability of bacteria to degrade chemical pollutants and the high adsorptive capacity of activated carbon in order to produce a more effective bioremediation method.

The objectives of this study are to:

1. Identify the bacteria species that are capable of degrading carbazole.
2. Develop BAC using the identified bacteria.
3. Measure the biodegradation rate of heterocyclic hydrocarbon by using biological activated carbon.
2.0 LITERATURE REVIEW

2.1 Biodegradation

Biodegradation is a process of metabolizing organic compound into energy and metabolic end products by bacteria (Zekri & Chaalal, 2005). In other words, it is a biological oxidation process carried out by bacteria to utilize the environmental organic compound as food source (Zekri & Chaalal, 2005). According to Zekri and Chaalal (2005), the study of biodegradation arises in the middle of the 1960's. Until the 1990's, there are 20 genera of bacteria, 14 genera of fungi and one algae genus were found to have the ability of degrading hydrocarbon (Bartha & Atlas, 1997). The habitats of these microorganisms are found to be from salt water, fresh water and soil (Zekri & Chaalal, 2005). Bartha and Atlas (1997) also added that different species of bacteria and fungi are unique for degrading specific organic compound.

In the recent industrial field, biodegradation of organic compound is improved by combining with the ozonation process (Woo et al., 1997). This is because biodegradation by ozonation increase the amount of biodegradable organic compound (Woo et al., 1997). The effect of preoxidation with ozone before BAC treatment helps to increase the amount of biodegradable organic compound (Takeuchi et al., 1997). Certain parties proclaim that biodegradable organic compound might be removed by the BAC system. However, Woo et al. (1997) has proved and concluded that ozonation was responsible for the higher removal of organic compound. The addition of ozonation in BAC treatment is usually applies in the raw water treatment and is more complicated because ozonation, adsorption and biodegradation are occurring simultaneously (Takeuchi et al., 1997).
2.2 Biological activated carbon

Activated carbon is one of the materials which are widely used for the water treatment. This is because activated carbon is well known for its high adsorptive capacity and more convenient to be use (Ghosh, et al., 1999; Imai, et al., 1995). However, the limited adsorptive capacity of activated carbon requires frequent renewal and is uneconomic. Hence, Rice and Robin comes out with an idea to introduce suitable microorganism, which aid in biodegradation, onto the activated carbon for wastewater treatment (Walker & Weatherley, 1999). According to de Jonge et al. (1996), under appropriate terms and conditions, introduction of suitable microorganism onto activated carbon can leads to the combination of sorption and biodegradation.

Now, the combination of activated carbon with biodegradation is called “biological activated carbon (BAC) treatment” (Takeuchi et al., 1997). The adsorptive properties serve to create a suitable environment for the growth of microorganism by enriching the substrate and oxygen concentration on the activated carbon (Ghosh et al., 1999). Therefore, the environment created by the activated carbon tends to induce the growth of biomass and creates a biofilm around the activated carbon (Ghosh et al., 1999).

The biological activity of microorganism on activated carbon involves in the degradation of biodegradable organic compound (Ghosh et al., 1999). These compound are being degraded when the absorbed biodegradable organic compound diffuse out of the BAC and being metabolized in the biofilm (Olmstead & Weber, 1991). On the other hand, the non-biodegradable organic compound is left to be absorbed by activated carbon (Ghosh et al., 1999). With the degradation of biodegradable compound in the biofilm, the absorptive capacity is being regenerated for more non-biodegradable organic compound adsorption (Ghosh et al., 1999). The BAC treatment is more preferable by the regulatory agencies and
industry in wastewater treatment because it requires lower cost and gives higher performance as compared to the conventional activated carbon adsorption treatment process (Takeuchi et al., 1997).

2.3 Heterocyclic hydrocarbon

Heterocyclic hydrocarbon is a compound of hydrocarbon and nitrogen, sulfur, or oxygen (Eisentraeger et al., 2008). This compound is usually found as pollutant in air, soil, sewage sludge, marine environments, and freshwater sediments (Eisentraeger et al., 2008). The present study on heterocyclic hydrocarbon is more onto its ecotoxicity and mutagenicity because the available data on heterocyclic hydrocarbon’s toxicity is still limited (Eisentraeger et al., 2008).

Polycyclic aromatic hydrocarbon (PAH), two or more fused aromatic ring, is a compound related with heterocyclic hydrocarbon. The only difference between PAH and heterocyclic hydrocarbon is the basic element of PAH only composed of hydrogen and carbon (Boldrin, et al., 1993). PAH is usually produced from human activities and natural events, such as industrial processes, forest fire and petroleum seeps (Geiselbrecht et al., 1998). If these pollutants are not treated with care and exposed to the marine organisms, it may be transferred to humans through seafood consumption (Geiselbrecht et al., 1998). So, bioremediation is essential as the prevention step.

A variety of PAHs are found in the marine environment (Geiselbrecht et al., 1998). Fluorene is known to be one of the PAH which gives a great environmental concern to the public due to its toxicity, mutagenic and carcinogenic properties (Monna, et al., 1993). Other examples of hazardous PAHs are the polychlorinated derivatives of dibenzo-p-dioxin (DD) and dibenzofuran (DBF) (Monna et al., 1993). These compounds are usually
produced from herbicides, combustion of dust or bleaching of pulp at paper mills (Monna et al., 1993). According to Monna et al. (1993), biodegradation by ozonation of PAH is possible but the information is still so rare and the catabolic pathways of these compounds still remain unknown.

2.4 Heterocyclic Hydrocarbon Degrading Bacteria

Heterocyclic hydrocarbon degrading bacteria are bacteria that have the potential to degrade heterocyclic hydrocarbon in nature. One of the examples for heterocyclic hydrocarbon degrading bacteria is *Pseudomonas resinovorans*, which is a gram negative bacteria that is capable of degrading carbazole to 2'-aminobiphenyl-2,3 diol via angular dioxygenation catalyzed by 1,9a-dioxygenase (CARDO) and also to transform dioxin compound and Polycyclic Aromatic Hydrocarbons (PAHs) (Habe et al., 2001; Inoue et al., 2006; Nojiri et al., 1999; Urata et al., 2006).

Another example of heterocyclic hydrocarbon degrading bacteria is *Thalassospira profundimaris*. *T. profundimaris* is a class of alphaproteobacteria that uses hydrocarbons as the sole source and mostly detected in petrol-oil-degrading consortia (Liu et al., 2007). *T. profundimaris* is a gram-negative bacteria with curved rod shape, about 0.8 to 2.3 µm long and 0.3 to 0.8 µm wide (Liu et al., 2007). This bacteria cell is non motile, non-flagellated and non-spore forming that usually grows between 10 to 37 °C in the presence of sodium chloride (Liu et al., 2007).
2.5 Gas Chromatography Mass Spectrometer

Gas chromatography mass spectrometer (GC-MS) is actually a combination technique of the gas chromatography and mass spectrometer. Gas chromatography has the function to separate sample molecules based on their chemical properties without identifying its structural information (Harrison & Gases, 2011). On the other hand, mass spectrometer plays the role of breaking the components into ionized species according to their mass to charge ratio (Harrison & Gases, 2011). In order for qualitative identification and quantitative measurement, Roland Gohlke and Fred McLafferty come out with an idea to combine the two techniques in the mid of 1950s (Harrison & Gases, 2011). Nowadays, GC-MS is widely being used for the purpose of structural determination of unknown organic compound in complex mixtures, measurement of molecular weights and elemental composition of unknown organic compound, and identification and quantification of volatile and semi-volatile organic compounds in complex mixtures (Hites, 1997).

In GC-MS, carrier gas plays an important role in transporting the sample from the gas chromatography column towards the mass spectrometer (Hites, 1997). The carrier gas must be inert to prevent any reaction among itself or with the samples (Hites, 1997). Usually there are two typical choice of gas can be use in GC-MS, which are hydrogen and helium (Hites, 1997). Hydrogen is capable of providing shortest analysis time due to its lower viscosity provide a higher phase of velocity (Hites, 1997). On the other hand, helium gives a better overall performance and peak resolution (Hites, 1997). However, separation efficiency and velocity is still the factor for the choice of carrier gas (Hites, 1997).
GC-MS is widely used by the Environmental Protection Agencies (EPA) in the quantitation of pollutants in drinking and wastewater (Hites, 1997). This is because GC-MS is available for the quantitative measurement of volatile and semi-volatile pollutants that are found in the wastewater (Hites, 1997). Besides, GC-MS can identify the specific pollutants which the EPA is interested with (Hites, 1997). Hence, GC-MS is known to be the best technique to identify volatile pollutants in wastewater.
3.0 MATERIAL AND METHOD

3.1 Media Preparation

3.1.1 Preparation of ONR7a

According to Dyksterhouse et al. (1995), the ingredients as showed in Table 1 were dissolved with 1 liter of distilled water in 1 liter of Schott Duran bottle. Magnetic stirrer was used for the stirring until all the substances were completely dissolved. 0.01g of the yeast extract was added to the ONR7a at pH 7.8.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>22.79</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>11.18</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3.98</td>
</tr>
<tr>
<td>TAPSO</td>
<td>1.3</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.102</td>
</tr>
<tr>
<td>KCl</td>
<td>0.72</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0.27</td>
</tr>
<tr>
<td>NaBr</td>
<td>0.083</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.04715</td>
</tr>
<tr>
<td>NaHPO₃</td>
<td>0.031</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.027</td>
</tr>
<tr>
<td>SrCl₂·6H₂O</td>
<td>0.0024</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.002</td>
</tr>
</tbody>
</table>
3.1.2 Preparation of Substrate Concentration

0.1% of substrate concentration was prepared by the following calculation.

\[
0.2\% = \frac{X}{100 \text{ ml of media}}
\]

\[
X = \frac{(0.2 \times 100 \text{ ml})}{100} = 0.2 \text{ g of substrate}
\]

0.2 g of heterocyclic hydrocarbon was weighed and transferred into a test tube. Then, 2 ml of dimethyl formamide (DMF) was added into the test tube to dissolve the substance completely.

3.1.3 Preparation of ONR7a (Liquid media) with Substrate

0.2% w/v of substrate was mixed with 100 ml of autoclaved ONR7a in the conical flask.

3.1.4 Preparation of Marine Agar (Solid media)

The following calculation was used to determine the amounts of Marine Agar needed.

\[
1000 \text{ ml of distilled water} = 37.4 \text{ g of Marine broth powder}
\]

\[
\text{If } 300 \text{ ml} = X \text{ g of Marine broth powder}
\]

\[
X = \frac{(300 \text{ ml} \times 37.4)}{1000 \text{ ml}} = 11.22 \text{ g}
\]
11.22 g of Marine broth was dissolved in 300 ml of distilled water and 1.5% w/v of Bacto Agar was added to aid in the solidifying of Marine agar. In this condition, 4.5 g of Bacto Agar was added into the 300 ml of prepared marine broth and the magnetic stirrer was used to stir the broth. Then, the broth was heated in the oven for 10 minutes to completely dissolve all the powder in the broth. The media was autoclaved for 1 hour and 30 minutes before poured in plates in the laminar flow hood. The plates were allowed to cool for 15 minutes to solidify.

3.2 Enrichment of \textit{T. profundimaris} strain M02

\textit{T. profundimaris} strain M02 was first being enriched in ONR7a broth supplemented with carbazole. The enriched process was started with the adding of 100 ml of ONR7a, 2 ml of dissolved carbazole, and one loopful of colony into a 250 ml conical flask. The broth was incubated at room temperature on a rotary shaker of 150 rpm for growth (Maeda, 2009). It takes between 4 to 7 days for the complete grow of bacteria. The growth of carbazole degrading bacteria was identified by colour change. Then, 100 µl of the broth was streaked on the prepared marine agar. The streaked marine agar was allowed to grow at room temperature on bench for overnight.

3.3 Preparation of Sample and Control Unit

Two 250 ml conical flasks were prepared with each one labelled as control and sample. For the conical flask labelled with control, 100 ml of ONR7a, 2 ml of dissolved carbazole, and one loopful of colony from the overnight marine agar plate was added into the flask. In the mean while, the conical flask labelled with sample was added with 100 ml of ONR7a, 2 ml
of dissolved carbazole, one loopful of colony from the overnight marine agar plate, and 1 g of activated carbon. Both flasks were incubated at room temperature on a rotary shaker of 150 rpm (Maeda et al., 2009).

3.4 Enumeration of Marine Bacteria

Enumeration of bacteria was carried out from day 3 until day 15 by serial dilution. For the control unit, 1 ml of the broth was pipette and used in 10 times dilution ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, and $10^{-10}$). Each dilution factor was spreaded in duplicate on marine agar plate by using a bent glass spreader. The agar plates were left overnight on bench at room temperature. The colony forming units was calculated by using the following formula (Lanza & Klimanskaya, 2006):

\[
\text{Colony Forming Units (CFU)} = \frac{\text{Number of colonies} \times \text{volume added (ml) \times dilution factor}}{\text{dilution factor from original broth}}
\]

As for the sample unit, 0.1 g of powdered BAC was extracted from the flask and used in 10 times dilution ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, and $10^{-10}$). Each dilution factor was spreaded in duplicate on marine agar plate by using a bent glass spreader. The agar plates were left overnight on bench at room temperature. The colony forming units was calculated by using the same formula as used for the control unit.

This method was repeated for day 3, 6, 9, 12, and 15 with the 10 times dilution as mentioned above ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, and $10^{-10}$). The mean of
the number of colony on the duplicate plate was used as the number of colony for the CFU counts. All the CFU from day 3 to day 15 were calculated and recorded.

3.5 Measurement of Organic Compound

Concentration of carbazole was measured every 2 days from day 2 to day 14. 3 ml of broth was pipette from the control or sample unit. The extracted broth was first being transferred into a 50 ml clean beaker and reduced the sample pH to less than 2 with 3.0 M concentrated Hydrochloric acid (HCl). A pH meter was used as the indicator for sample pH measuring. Then, 3 ml of acidic sample was being pipette into another clean beaker. The sample was transferred to the fume hood, followed by addition of 3 ml of ethyl acetate by using glass pipette. The solution was shook vigorously to make sure it mixes well. The forming layer is observed and the desired clear layer at the top of the solution was transferred into a new beaker. The sample is extracted for three times with ethyl acetate to get pure sample.

After the extraction of carbazole with ethyl acetate, the sample was added with anhydrous sodium sulphate (Na$_2$SO$_4$) to absorb the water content. Next, the sample was filtered with Millex Syringe-Driven Filter unit and dried in the fume hood. This process took about 20-30 minutes. The sample was then dissolved in 2.5 ml of dichloromethane and stored in 4 ml of amber screw capped vial for degradation rate analysis.

The detection of heterocyclic hydrocarbon degradation was performed by using Gas Chromatography-Mass Spectrometry method QP2010 PLUS (SHIMADZU). Gas Chromatography was equipped with SE-54 capillary column (30 m x 0.32 mm x 0.25 μm). The flow rate of the carrier gas (helium) was 2 ml/min. The column temperature was
programmed from 50 °C (held 1 min) to 220 °C with an increasing rate of 10°C/min. The injector temperature was 200 °C and the detector temperature was 200 °C.
4.0 RESULTS

4.1 Growth Confirmation of isolated

4.1.1 Growth on ONR7a (Liquid media)

The degradation of carbazole was indicated by the changing colour of broth from chalky colour to yellow colour. The growth confirmation was positive and the yellow colour was started to appear on the sixth day of incubation.

Figure 1: (a) 100ml of ONR7a supplemented with 0.2% w/v of substrate concentration on day 0.
(b) 100ml of ONR7a supplemented with 0.2% w/v of substrate concentration on day 14.