Chapter

Isolation and Identification of Carbazole Degrading Bacteria from Lake Water

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Abstract

Heterocyclic hydrocarbon compounds have been identified as one of the major components of water pollution that occurs as a result of urbanization. It has been known that the presence of these compounds is hazardous and remain in the environment for a long period of time. This study was conducted to isolate and identify heterocyclic hydrocarbon degrading bacteria from lake water by genomic DNA extraction and sequencing as well as measure the degradation rate of the bacteria using Gas Chromatography Flame-Ionization Detector (GC-FID). The water sample was collected from west campus lake of Universiti Malaysia Sarawak where six strains of bacteria that has degrading ability was isolated using sub-culturing technique on MSM double layer agar plates. The genomic DNA of bacteria designated as strain IM1, IM2, IM3, IM4, IM5 and IM6 were extracted and amplified using Polymerase Chain Reaction (PCR). The isolates were then sequenced and were identified *as Bradyrhizobium sp.*, *Ochrobactrum sp.*, *Pseudomonas aeruginosa sp. and Burkholderia sp.* All six isolates possessed the ability to utilize carbazole as sole carbon and energy source as the degradation rate of carbazole was measured using GC-FID analysis. After 12 days of incubation, IM2 showed 96.37% degradation while the other five isolates were able to degrade 100% of the carbazole. Thus, bacteria isolated from this study may provide great benefit for bioremediation.

Keywords: heterocyclic hydrocarbon, carbazole, degrading bacteria, bioremediation

1. Introduction

Heterocyclic hydrocarbon has been known to manifest various cytotoxic effect such as severe toxicity, cytotoxicity, mutagenicity, carcinogenicity and photo-induced toxicity [1–3]. Gas, petroleum and coal are the three main natural resource of hydrocarbons. One of the examples of hydrocarbon causing toxic affect to the environment is the accidental crude oil spill in seawater [4]. It was reported that a black bag containing 15 to 20 liters of oil was dumped in the seawater of Redang island in Terengganu [5]. The marine life along the shore was affected, especially turtles landing on the island to lay eggs. Moreover, the toxic chemicals found in the crude oil could also affect human health especially kidney system, liver and sensory system. Some of the hydrocarbons that have their degradation product detected in

industrial waste water, contaminated sites are carbazole, biphenyl, dibenzofuran, dibenzothiophene and fluorine.

Carbazole is one of the heterocyclic hydrocarbons that is highly mutagenic and toxic especially to aquatic organism as documented in a recent study [1, 2, 6]. However, this compound has also been widely used in the raw industry for various purpose such as dye productions, medicines and plastics. Carbazole compound is a derivation of shale oil, crude oil and creosote and it has been proven as non-human carcinogen although however its derivatives such as N-methylcarbazole, which are detected in automobile emission and cigarette smokes are highly genotoxic and are now classified as IARC Group 2B carcinogens [1, 7].

Biodegradation has been known to be a technology that provides various benefits for organic pollutants. As it has been understood for so long, microorganism was able to degrade pollutant, bioremediation sets a goal to transform organic pollutant to various type of nontoxic or environmental friendly metabolites or mineralize it into water and carbon dioxide [8]. Many researches have been done using carbazole as sole carbon source where various bacteria with degradable properties has been isolated from different type of environment by enrichment culture method. Most carbazole degrading bacteria are gram negative and aerobic such as *Microbacterium esteraromaticum* strain SL6 [9].

2. Objectives

1. To isolate and identify carbazole degrading bacteria strains from lake water environment that are able to contribute in bioremediation.

2. To assess degradation ability and degradation rate of isolated degrading bacteria strains using gas chromatography.

3. Literature review

3.1 Aromatic hydrocarbon

Aromatic compounds are organic molecules that contains one or more aromatic rings. The three major categories of aromatic compounds are polycyclic aromatic hydrocarbons (PAHs), heterocyclic, and substituted aromatics. Polycyclic aromatic hydrocarbon (PAH) has carcinogenic and mutagenic properties that are connected to some chemical properties such as dipole moment, electrophilic potency, intramolecular and subcellular binding, electronegativity or L- and K- region reactivity, hydrophobicity and others.

Whereas heterocyclic hydrocarbon which is also known as heterocycles are compound that contains at least one atom other than carbon and some or all the atoms are joined in rings. Heterocyclic hydrocarbon has high boiling and melting point, low water solubility and low vapor pressure. These characteristics proves that heterocyclic hydrocarbon can stay in the environment for a long period of time [10]. **Figure 1** shows the molecular structure of aromatic heterocyclic organic compound known as carbazole.

Heterocyclic hydrocarbon is by products of incomplete combustion of organic materials such as coal, petroleum, tar and gas [11]. According to Bamforth and Singleton [12], heterocyclic hydrocarbon can be found easily in groundwater, soil and sediments and also the atmosphere. Some areas such San Diego Bay, California,

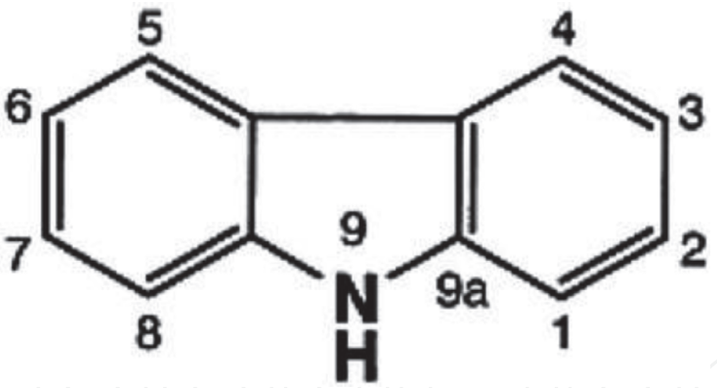


Figure 1.
Molecular structure of carbazole.

Central Pacific Ocean, intertidal sediments, gas works site soils, sewage sludge-contaminated soils, aquifers and groundwater and atmospheric air pollution have shown presence of high concentration of heterocyclic hydrocarbon. Heterocyclic hydrocarbon also originates from two major sources which are natural and anthropogenic sources. Heterocyclic hydrocarbon does not only occur during partial combustion of organic material but also occur with fossil fuels such as petroleum and coal. Carbazole and dibenzothiophene which are heterocyclic compounds often exist together with PAH and some other aromatic compounds as they are components of crude oil, shale oil and creosote [13].

3.2 Health and environmental concerns

Heterocyclic hydrocarbon is a major concern in urban and industrial areas as some of them have been classified as carcinogenic, mutagenic and teratogenic [14, 15]. The properties of heterocyclic hydrocarbon are that they are relatively insoluble in water and they are also highly lipophilic. Another found property is that this compound ban be degraded by some bacteria in soil [14, 15]. Whereas in the atmosphere, heterocyclic hydrocarbon can react with different kind of pollutants such as ozone, nitrogen oxides and sulfur dioxide.

Studies have shown that heterocyclic hydrocarbon are strongly bio concentrated or metabolized [16]. Human are exposed to most PAH through their eating diet that consist of marine lives. For heterocyclic hydrocarbon such as carbazole, tobacco smoking and breathing from polluted air are the routes of exposure [17, 18]. Carbazole has also been classified as "benign tumorigen" by Nojiri and Omori [19]. Moreover, heterocyclic hydrocarbons are potential carcinogens that can produce tumors. Benzo(a)pyrene, a common heterocyclic hydrocarbon, is shown to cause lung and skin cancer in laboratory animals. When ingested, heterocyclic hydrocarbons are absorbed very fast into the gastrointestinal tract. This is because of their high lipid solubility [12, 20]. In general, the more number of benzene rings present, the more harmful the heterocyclic hydrocarbon would be.

3.3 Bioremediation

Bioremediation is defined as a process where microorganisms or their enzymes are used to degrade contaminants to its original condition. Microbial degradation is natural mechanism to demolish hydrocarbon pollutants (and crude oil) from the environment [21, 22]. Research has shown that, in order to induce the ability of certain microbes to degrade or transform toxic or pollutants, bioremediation and biotransformation have been the most successful method chosen [23, 24]. The main

objective or aim in bioremediation is to destroy or remove contaminants by using microbes or degrading bacteria and stimulate them with nutrients and other chemicals to aid the degradation process. The presence of suitable microbes and the ideal environmental conditions such as the right amount of nutrient, oxygen and suitable pH and temperature for microbes to grow are some of the factors that also lead to bioremediation [25].

In this process of bioremediation, different microbes will act upon in parallel or sequence in order to degrade the compound. Two approaches are widely used which is in-situ where contaminants are treated at the site and ex-situ approach where contaminants are removed to be treated elsewhere. The ability of variety of microbes to degrade different kinds of pollutants proves that bioremediation is a crucial and important technology that can be used in different conditions [26]. Previous study by Alvarez et al. [27] stated that bioremediation has been proven to be an environmental-friendly technique as the degrading agents are microbes that can be easily decomposed. Therefore, this method appears to fulfill the characteristics of the demanding, growing industry as it is sustainable, easy to implement and cost effective. **Table 1** shows some carbazole degrading bacteria that has been reported in previous literature that are able to contribute to bioremediation.

3.4 Degradation of heterocyclic hydrocarbon

According to Surajit Das from National Institute of Technology, Rourkela Odisha, India, the toxicity, mutagenic properties as well as high carcinogen of PAH in nature has cause a great environmental concern to scientist. Therefore, researchers have collected many marine bacteria that has potential in bioremediation. A research done by Latha and Laithakumari [28] has also shown on how the efficiency of degradation can be increased when a catabolic plasmid from *Pseudomonas putida* that has genotype of hydrocarbon degradation is inserted in a marine bacterium. Some examples of marine bacteria that has been taken and used for bioremediation are *Neptunomonas naphthovorans, Lutibacterium anuloederans*, and *Cycloclasticus spirillensus* [23, 29].

According to research by Nojiri and Omori [19], the structural analog of dioxin and some carbazole-degrading enzymes that plays the same role as dioxin degrading enzymes are the factors that made carbazole known as model compound for bioremediation and led to more study on bacterial degradation of carbazole. There are three main degradation pathways of carbazole that has been identified which are the angular deoxygenation, lateral deoxygenation and hydroxylation pathway of carbazole that can be catalyzed with different enzymes. **Figure 2** shows lateral deoxygenation carbazole at C3 and C4 [9, 30–32].

The common reaction by carbazole degrading bacteria would be hydroxylation as identified by Lobastova et al. [30], who studied on hydroxylation of Aspergillus *flavus* VKM F-1024 by carbazole, where hydroxycarbazole were produced as major product. Nojiri et al. [33] explained on the angular deoxygenation where oxidation

Bacteria Strain	Medium	Product
Achromobacter sp. SL1	Carbon	Anthranilic acid, catechol
Pseudomonas sp. SL4	Carbon	Anthranilic acid, catechol
Microbacterium esteraromaticum SL6	Carbon	Anthranilic acid, catechol
Sphingomonas sp. GTIN11	Nitrogen	Anthranilic acid

Table 1.
Carbazole degrading bacteria, adapted from Salam et al. [9].

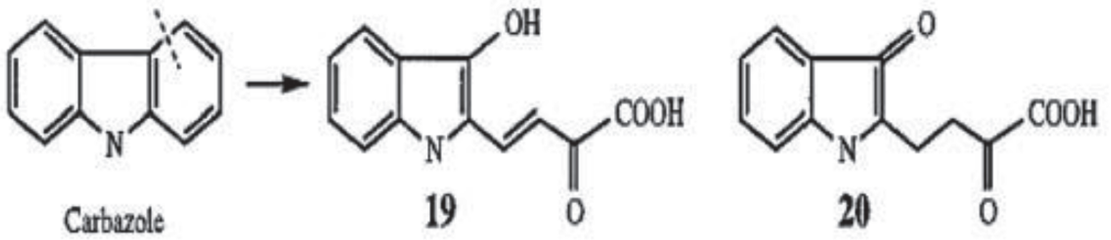


Figure 2.
Lateral deoxygenation of carbazole at C3 and C4.

occur at the ring-fused position of carbazole which is induced by a multicomponent enzyme known as carbazole 1,9a- dioxygenase (CARDO) in an angular position. Based on literature, carbazole degradation pathway has been well reported and many types of carbazole degrading bacteria has been identified successfully.

4. Materials and methods

4.1 Sample collection

Five hundred ml of water sample was collected from lake water from west campus at University Malaysia Sarawak (UNIMAS). The location was at latitude 1.468616 and longitude 110.430536. The samples obtained were stored in falcon tube and transported to the laboratory at room temperature (**Figure 3**).

4.2 Preparation of minimal salt media (MSM) broth and agar

A standard formulation was used to prepare the Minimal Salt Media (MSM). All the chemical compounds were placed in 1 L conical flask after weighing using precision digital balance. Than the pH was adjusted to 7.2 0.2 at 25 °C. The chemical compounds used to prepare the media are as in the **Table 2**: Minimal Salt Media mixture;

The MSM double layer agar was prepared by adding 3 g of Bacto agar (Difco) into 200 ml prepared MSM broth in two conical flasks. Next, the MSM agar solution was autoclaved at 121 °C/15 atm for 1 hour and 30 minutes. Then, one of the conical flasks containing MSM agar solution was supplemented with 2 ml of 10X carbazole

Figure 3.
Location of sampling from a lake located at UNIMAS west campus, Sarawak.

Chemical Compound	Weight
KH2PO4,	1.36 g
(NH4)2SO4,	0.5 g
MgSO4.7H2O	0.2 g
CaCI.2H2O	0.01 g
FeSO4.7H2O	0.005 g
MnSO4. 7H2O	0.0025 g
NaMoO4.2H2O	0.0025 g
Na2HPO4	0.00213 g

Table 2.
Minimal salt media mixture [34].

stock and stirred to mix it well. Finally, the agar solution was poured into agar plates forming double layer and stored in fridge at 4 °C for further use.

4.3 Enrichment culture and isolation of degrading bacteria

4.3.1 First enrichment culture

Enrichment cultures were undertaken where 2 ml of sample were added into test tube with 8 ml of Minimal Salt Media(MSM) and 0.05%(w/v) substrate(carbazole). The test tubes were then incubated at 30 °C and shaken at 200 rpm until color change was observed indicating heterocyclic hydrocarbon degradation.

4.3.2 Second enrichment culture

For the second enrichment culture, 2 m1 of bacteria samples was transferred from the first enrichment culture into another 8 ml of MS media and 0.1% (w/v) substrate (Carbazole) as the sole carbon source. Next, the samples were incubated at 30 °C and shaken at 200 rpm for another two to four weeks until color change observed.

4.3.3 Isolation of pure colony

After second enrichment, the bacteria were inoculated on MS double layer agar plate. Heterocyclic hydrocarbon which is carbazole was added as the sole carbon source before incubating them for several days. Then the bacteria were sub cultured until pure colony is observed. Colonies that shows clear zone were picked by inoculating needles and purified by streaking several times on a fresh new plate. The growing bacteria colonies were observed as the pure cultures are obtained to be used in the next procedure.

4.4 Glycerol stock preparation and test

The pure colony of carbazole degrading bacteria isolated were inoculated into the MSM broth and incubated for 24 hours. Next, the bacteria culture was transferred into sterile 2 ml.

Eppendorf tube and sterile glycerol were added to make the final concentration 20% and 40%. Finally, the glycerol stocks were stored in −80 °C. After 3 weeks

stored, the bacteria from glycerol stock was streaked on MSM agar where growth were observed.

4.5 Gram staining

A single colony from culture plate of isolated degrading bacteria was picked using sterile inoculation loop and mixed with a few drops of distilled water to form a smear on microscopic slide. Then, the smear was air dried and heat fixed by passing through the flame on bunsen burner for a few seconds. Next the primary stain, crystal violet was applied on the smear for 1 minute and then rinsed with water. Then the smear was covered with iodine for one minute before rinsing with water and ethyl alcohol was used to decolorize the slide for.

30 seconds. After decolorizing, safranin was added for 30 seconds before rinsing with water. Then the slides were observed under light microscope to identify and distinguish between gram positive and gram-negative bacteria.

4.6 Molecular characterization

4.6.1 Genomic DNA extraction of isolated bacteria

For genomic DNA extraction, the bacteria isolated, that has pure colony on a plate was washed with 2 ml of TE buffer. Then, 1 ml of the mixture was transferred into new Eppendorf tube and centrifuged at 10000 rpm for 2 minutes in order to obtain pellet. Next, the pellet will be resuspended with 500 μl TE buffer, vortex until pellet dissolved and divided into two tubes with 750 μl in each tube. Then, 65 μl of 10% sodium dodecyl sulphate (SDS) and 10 μl proteinase K were added before incubating for 1 hour in incubator and flipped every 10 minutes. Next, 825 μl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and placed on shaker for 45 minutes. After that, the tubes were centrifuged for 15 minutes at 10000 rpm until double layer is formed. Then the upper layer of the mixture was extracted into new sterile 1.5 ml tubes. Next, 200 μl of isopropanol will be added before spinning for 15 minutes at 10000 rpm and the tubes were flipped a few times. Once the pellet of DNA was obtained, the supernatant was removed, and the pellet was air dried before adding 50 μl TE buffer to dissolve the pellet and stored at −20 °C for further analysis. Then, the extracted genomic DNA was also analyzed by using agarose gel electrophoresis to confirm presence of DNA.

4.6.2 Polymerase chain reaction

Polymerase Chain Reaction (PCR) was carried out by using universal primers which is forward primer 27F and reverse primer 1492R to amplify 16S rRNA gene. **Table 3** shows the sequence for PCR.

All PCR reagents were pipetted into 0.5 μl Eppendorf tube and mixed gently to make sure all mixtures were collected at the bottom of the tube. **Table 4** shows the reaction mixture used for polymerase chain reaction. Next, the tubes containing

Primer	Sequences
27F	5’ AGAGTTTGATCCTGGCTCAG 3’
1492R	5’ TACGGCTACCTTGTTACGACTT 3’

Table 3.
Polymerase chain reaction universal primer [35].

PCR Reagents	Volume (μl)
Sterile distilled water (dH2O)	9.5
Master Mix	12.5
Forward primer(27F)	1.5
Reverse primer(1492R)	1.5
DNA template	1.5
Silicon oil	10

Table 4.
Reaction mixture for polymerase chain reaction.

Phase	Temperature and Duration
Initial denaturation	95 °C for 5 minutes
Denaturation	95 °C for 30 seconds
Annealing	50 °C for 30 seconds
Extension	72 °C for 1 min 30 seconds

Table 5.
PCR profiles.

PCR mixture were placed in DNA thermal cycler machine and performed according to parameters shown in **Table 5**.

4.6.3 Agarose gel electrophoresis (AGE), DNA purification and sequencing

Agarose gel 1%was prepared for gel electrophoresis where 0.75 g of agarose powder was added to 50 ml of distilled water in a conical flask which is then heated for about 40 seconds in microwave until agarose powder is fully dissolved. Next, 1 ml of 50X TAE buffer was added to the mixture and the mixture was poured into agarose gel electrophoresis tray with comb to let it solidify. After about 30 minutes when the gel is solidified, the PCR products and was loaded into respective wells and the gel was run at 100 V,400 mA for 30 minutes. After 30 minutes, the gel was stained with ethidium bromide for 10 minutes and then rinsed with distilled water for 5 minutes. Then, the gel is viewed under the UV light to observe the DNA band.

Once bands were observed, the PCR products were purified using DNA purification kit. Firstly, 20 μl of PCR mixture was mixed with 35 μl of binding buffer and the mixture was then transferred into a high pure filter tube that had been inserted into a collection tube. Next, the tube was centrifuged for 15 seconds at 5000 rpm and then the flowthrough in the collection tube was discarded. Then, 800 μl of washing buffer was added into the high pure filter tube which was then centrifuged at 5000 rpm for 15 seconds. The flowthrough in the collection tube was then discarded. Washing using buffer was done twice to enhance the result. Next the collection tube was replaced and the high pure filter tube was inserted into another sterile collection tube. Then, 30 μl of elution buffer was inserted into high pure filter tube and centrifuged for 15 seconds at 5000 rpm. The flowthrough, which was the purified PCR product in the collection buffer was collected and transferred into new 1 mL tube. Then, the purified DNA was sent to First BASE Laboratories Sdn. Bhd. Company for sequencing and the sequence data obtained were analyzed using MEGA software and BLAST from NCBI [36].

4.7 Gas chromatography-flame ionization detector (GC-FID) analysis

The gas chromatography-flame ionization detector (GC-FID) was conducted to measure the degradation rate of the bacteria strains. The bacteria strains were grown back using enrichment culture method in different tubes and each tube was harvested on day 0, day 3, day 6, day 9 and day 12. Next, 1 ml of ethyl acetate was added to 1 ml of each sample and the mixture is vortex for around 10 seconds until a double layer could be observed from the mixture. Then the upper layer was pipetted into a vial tube [35]. The samples were then analyzed using gas chromatography-flame ionization detector machine and the data was collected. The degradation percentage were calculated using the formula given below. The percentage of residual substrate are calculated first using the Eq. 1. Once the percentage is obtained, the degradation percentage are calculated using the formula in Eq. 2.

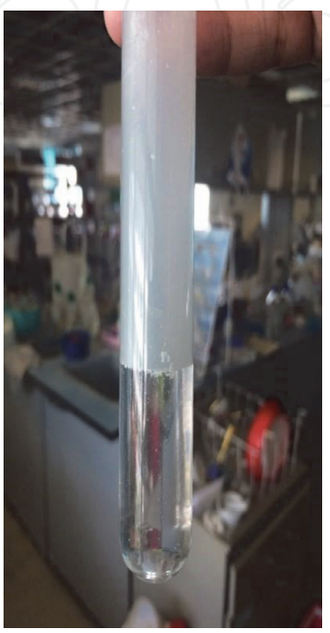
$$\text{Percentage of residual substrate} : (\text{average peak area average control}) \times 100 = \% \quad (1)$$

$$\text{Degradation percentage} : 100 - \text{Percentage of residual substrate} = X\% \quad (2)$$

5. Result

5.1 Enrichment cultures and streaking on MSM agar plates

After preparing media, enrichment cultures were done where 2 ml of lake water was added into a test tube containing 8 ml of MSM prepared before and 0.1% of carbazole was also added as sole carbon source. The cultures are then sub cultured two times and color change was observed and compared with the control. Another test tube was also prepared but without any water samples as a control. **Table 6** shows change in color observed on first, second and third enrichment medium for the lake water sample collected from Unimas lake. In tube C, it could be observed major color change from cloudy to greenish yellow after three weeks. On the second and third enrichment the substrate residue become smooth and the color change to pale yellow compared to the control which is clear after two weeks.

Control media **Tube A**	

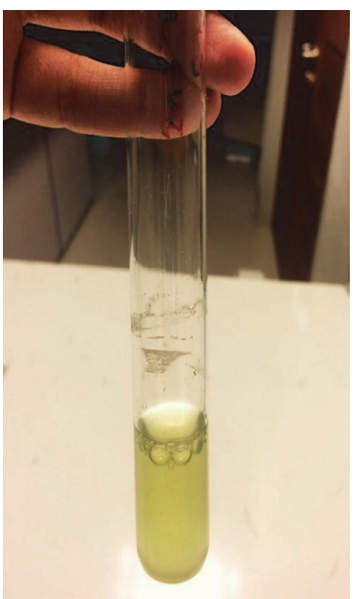
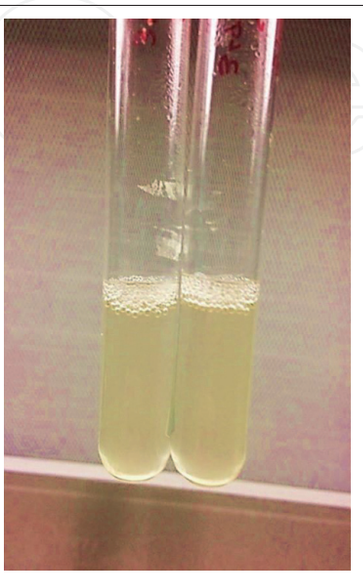
First enrichment **Tube B (before) Tube C (after 3 weeks)**	
Second enrichment Tube C (after 2 weeks)	
Third enrichments **Tube D and Tube E** **(after 2 weeks)**	

Table 6.
Observation on first, second and third enrichment.

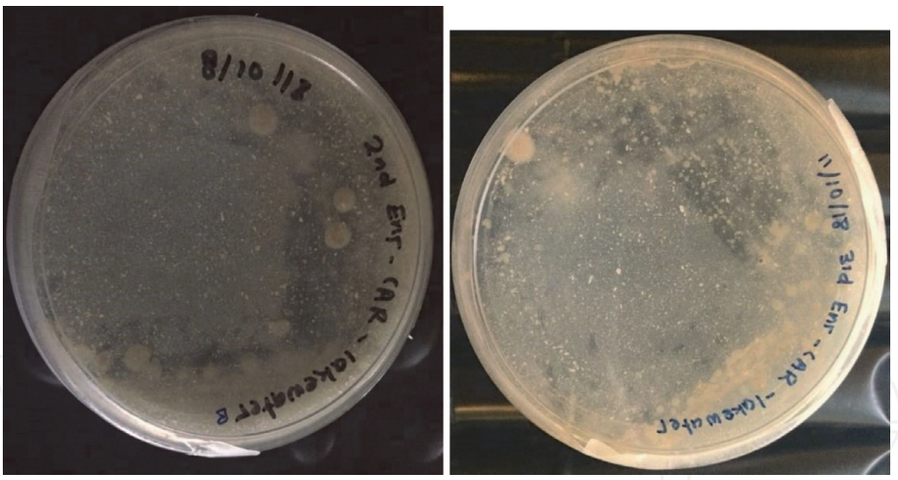


Figure 4.
Streaking results of second enrichment and third enrichment of lake water samples.

The **Figure 4** below shows bacteria growth observed on MSM double layer agar after streaking with second and third enrichment cultures. Both plates are able to form clear or hollow zone proving degradation of substrate carbazole.

5.2 Isolation of carbazole degrading bacteria and gram staining

The **Figure 5** below shows the growth of bacteria isolated from second and third enrichment plates which was cultured on MSM broth supplemented with 0.1% carbazole as sole carbon source. It could be observed that IM2 and IM3 turned to dark yellow while IM6 is cloudier but still turned yellow in color. IM1, IM3 and IM5 became light yellow in color compared to control from **Table 6**.

After isolating six different bacteria by streaking on MSM double layer agar plates supplemented with 0.1% carbazole, it could be observed that not all of them produce clear or hollow zones. Despite that, all the isolates showed growth on agar plate proving that carbazole was utilized efficiently by the bacteria. Next, gram staining was done for all isolates to distinguish between Gram positive and Gram-negative groups of bacteria before proceeding with DNA extraction and sequencing (**Table 7**).

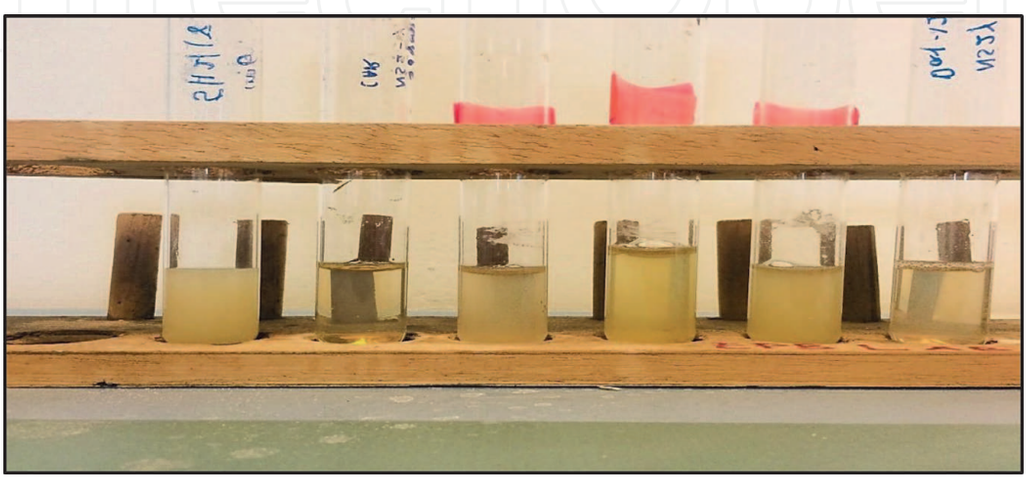
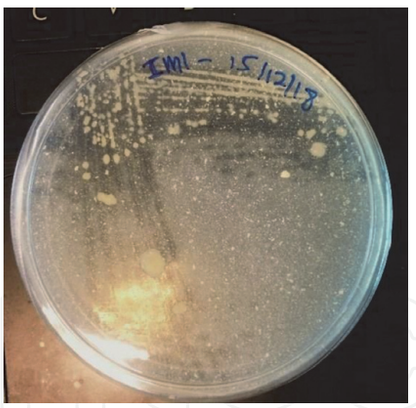
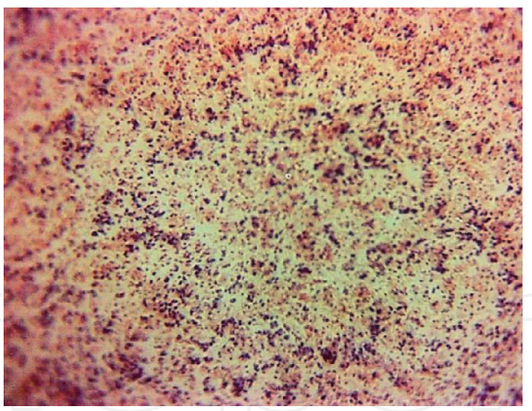
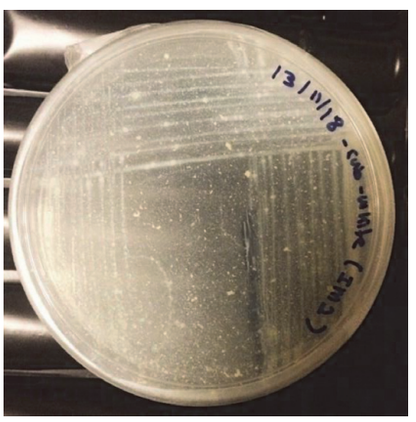
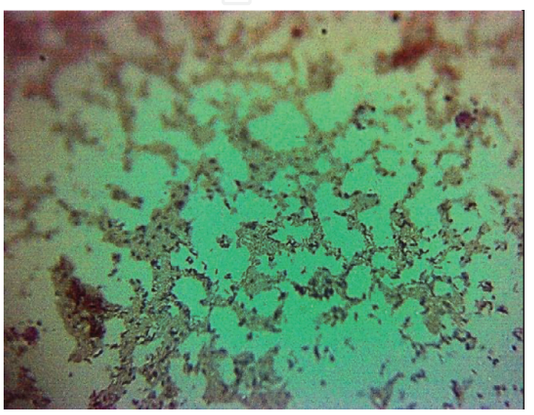
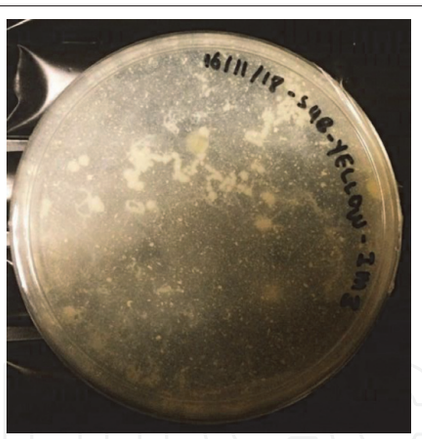
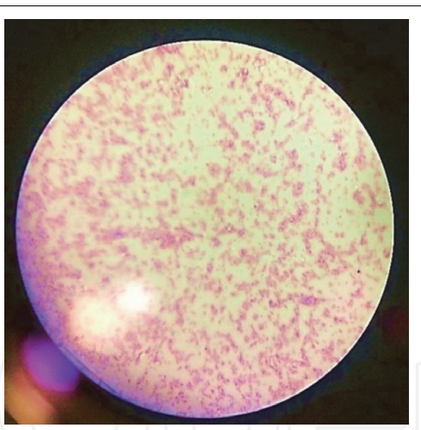
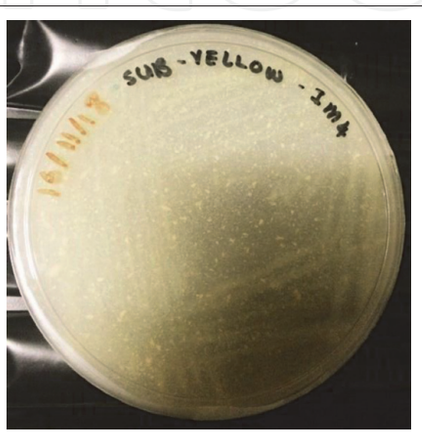
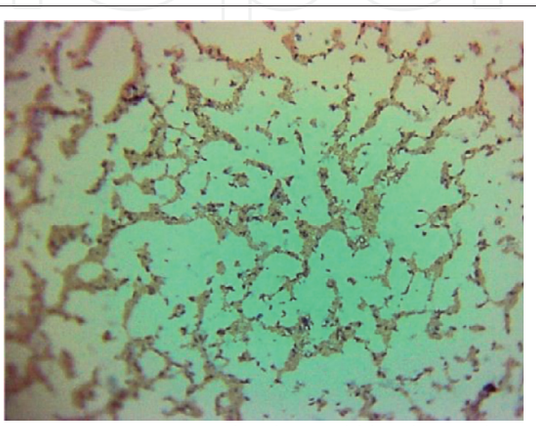


Figure 5.
Six bacteria isolated, from right, IM1, IM2, IM3, IM4, IM5 and IM6.

Pure colony of isolated bacteria	Gram staining results
 **IM1**	 Gram negative: Pink color could be observed
 **IM2**	 Gram negative: Pink color could be observed
 **IM3**	 Gram negative: Pink color could be observed
 IM4	 Gram negative: Pink color could be observed

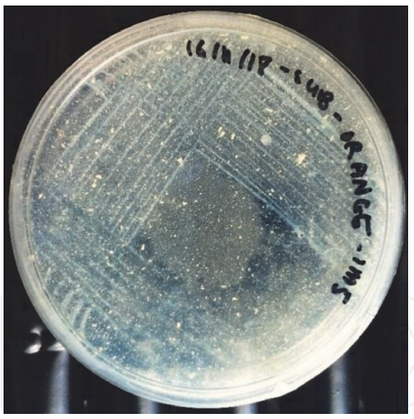
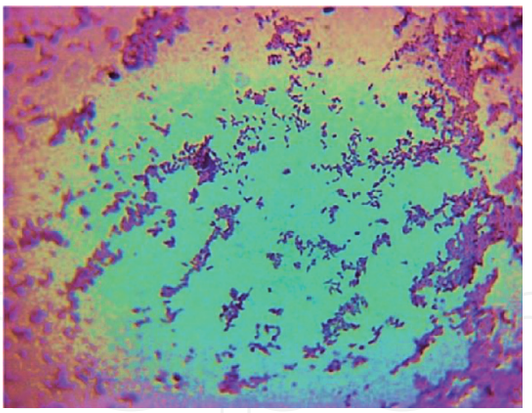
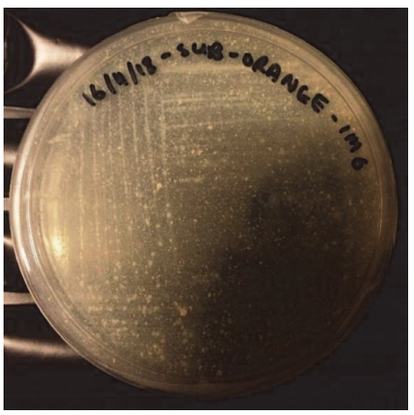
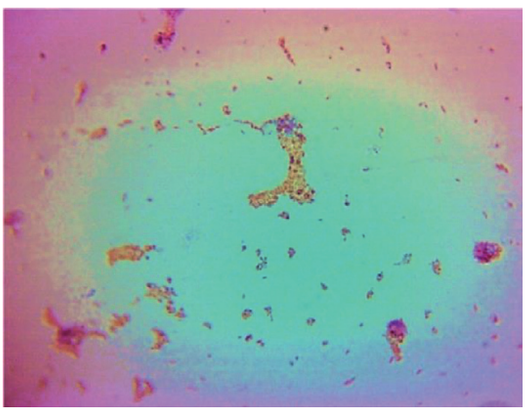
Pure colony of isolated bacteria	Gram staining results
 IM5	 Gram negative: Pink color could be observed
 IM6	 Gram negative: Pink color could be observed

Table 7.
Table of isolated bacteria and gram staining results.

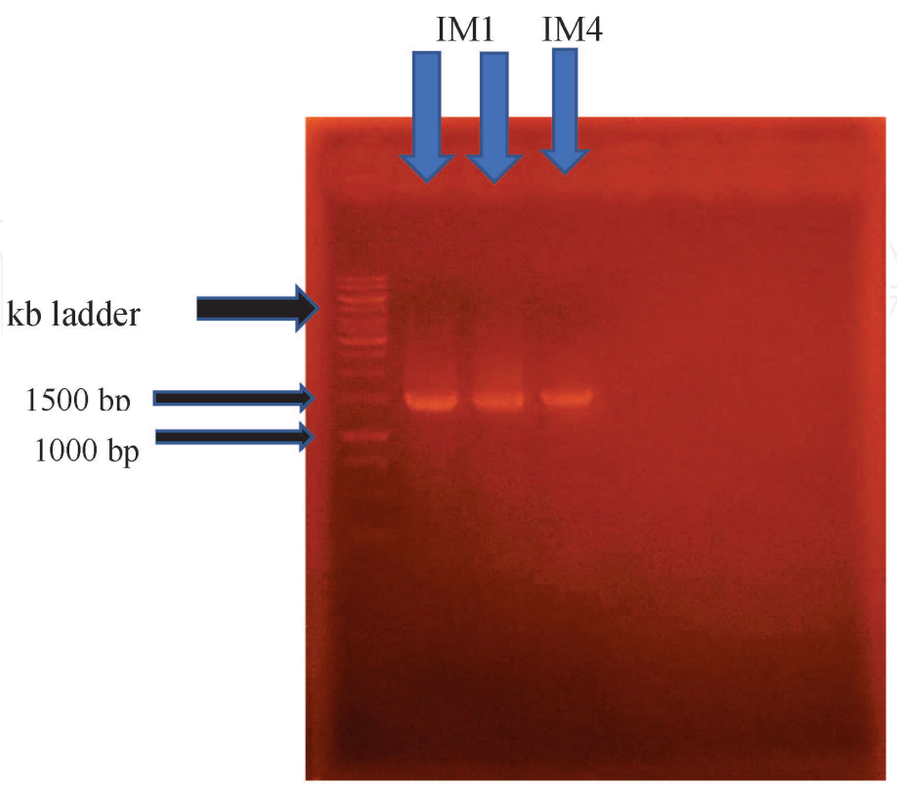


Figure 6.
Clear band on IM1 and IM4 genomic DNA fragment at 1500 bp.

5.3 Molecular identification

5.3.1 Agarose gel electrophoresis (AGE)

After the carbazole degrading bacteria were isolated from lake water sample, DNA Extraction was done continued with PCR and Agarose Gel Electrophoresis

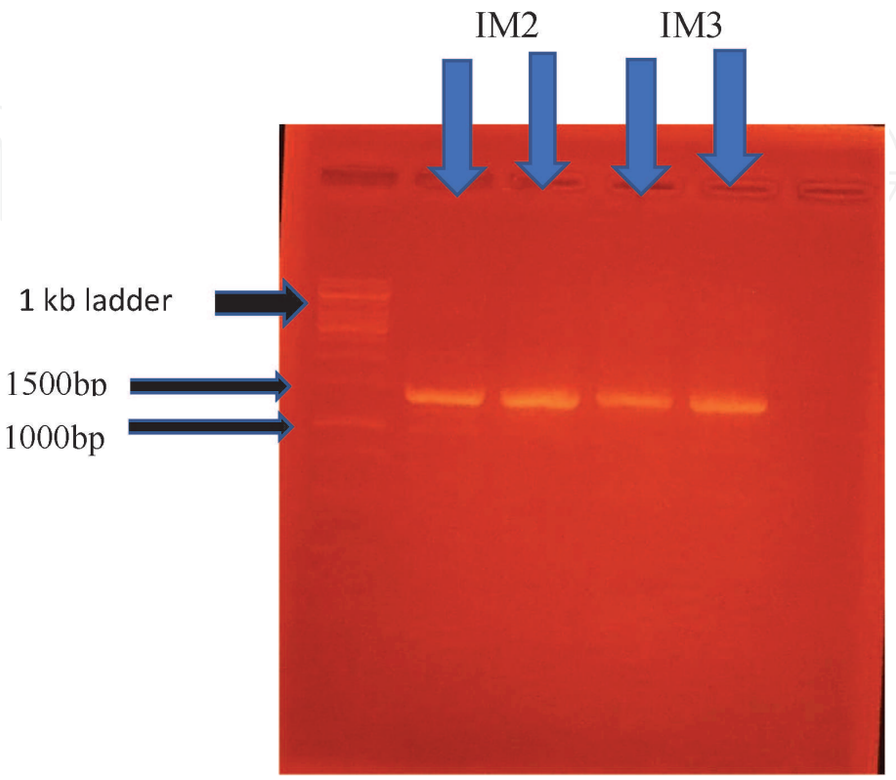


Figure 7.
Clear band on IM2 and IM3 genomic DNA fragment at 1500 bp.

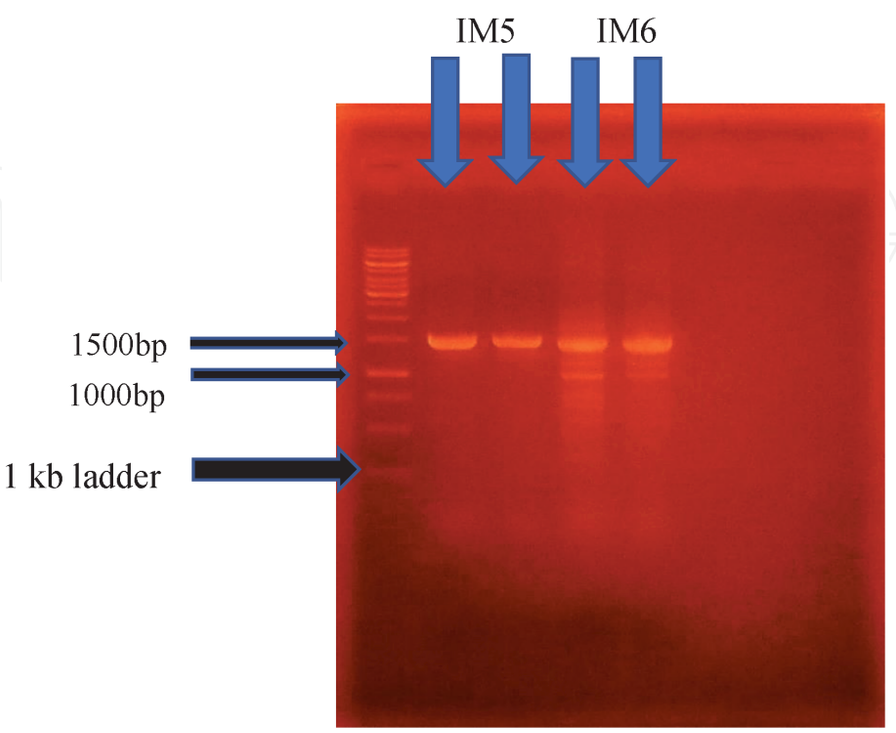


Figure 8.
Clear band on IM5 and IM6 genomic DNA fragment at 1500 bp.

(AGE) to identify the bacteria. All lanes produced intact, clear bands. The band for each isolated bacterium from IM1 to IM6 are shown at 1500 bp as in **Figures 6–8**.

5.3.2 Sequencing analysis

The nucleotide sequence retrieved from the company was processed using MEGA 6 software. The nucleotide sequence with weak signals was cut off leaving only the nucleotide sequence with strong signal which was then analyzed using BLAST tool. From the analysis, one of the six isolated bacteria showed weak,

Isolated strain	Identification	Percentage of Similarity
IM1	*Bradyrhizobium sp.*	90%
IM2	*Ochrobactrum sp.*	99%
IM3	*Pseudomonas aeruginosa sp.*	96%.
IM4	*Burkholderia sp.*	100%
IM5	*Burkholderia sp.*	100%
IM6	*Ochrobactrum anthropic sp.*	100%

Table 8.
Sequencing analysis results.

Days	Control	3	6	9	12
Average peak area	4432.86	481.64	215.34	101.7	0
Percentage of residual substrate (%)	100	10.865	4.858	2.294	0
Degradation percentage (%)	0	89.13	95.14	97.71	100

Table 9.
Summary of GC-FID analysis of IM1 samples.

Days	Control	3	6	9	12
Average peak area	6688.91	6311.12	1078.71	849.48	309.38
Percentage of residual substrate (%)	100	94.35	16.13	12.7	4.63
Degradation percentage (%)	0	5.65	83.87	87.3	95.37

Table 10.
Summary of GC-FID analysis of IM2 samples.

Days	Control	3	6	9	12
Average peak area	4590.37	4432.86	2777.48	2647.27	0
Percentage of residual substrate (%)	100	96.57	60.51	57.67	0
Degradation percentage (%)	0	3.43	39.49	42.33	100

Table 11.
Summary of GC-FID analysis of IM3 samples.

Days	Control	3	6	9	12
Average peak area	4432.86	623.3	0	0	0
Percentage of residual substrate (%)	100	14.06	0	0	0
Degradation percentage (%)	0	85.94	100	100	100

Table 12.
Summary of GC-FID analysis of IM4 samples.

Days	Control	3	6	9	12
Average peak area	4432.86	1704.71	1635.69	308.38	0
Percentage of residual substrate (%)	100	38.46	36.9	6.96	0
Degradation percentage (%)	0	61.54	63.1	93.04	100

Table 13.
Summary of GC-FID analysis of IM5 samples.

Days	Control	3	6	9	12
Average peak area	4432.86	389.16	0	0	0
Percentage of residual substrate (%)	100	8.78	0	0	0
Degradation percentage (%)	0	91.22	100	100	100

Table 14.
Summary of GC-FID analysis of IM6 samples.

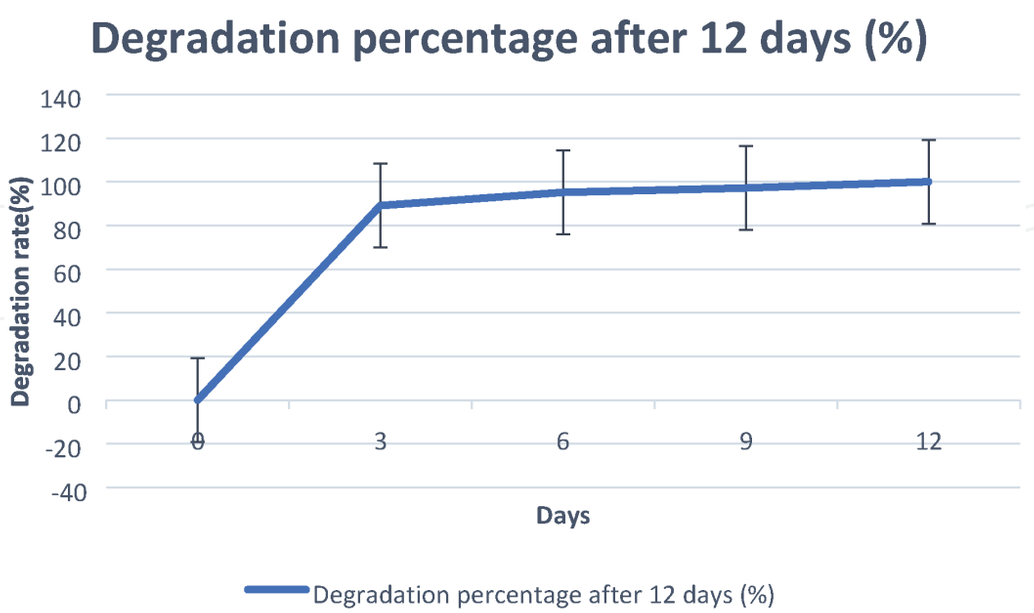


Figure 9.
The degradation percentage of IM1 strain on CAR after 12 days incubation.

overlapping signals giving result with lower percentage of identification. **Table 8** shows the sequencing analysis results of six isolated bacteria strains.

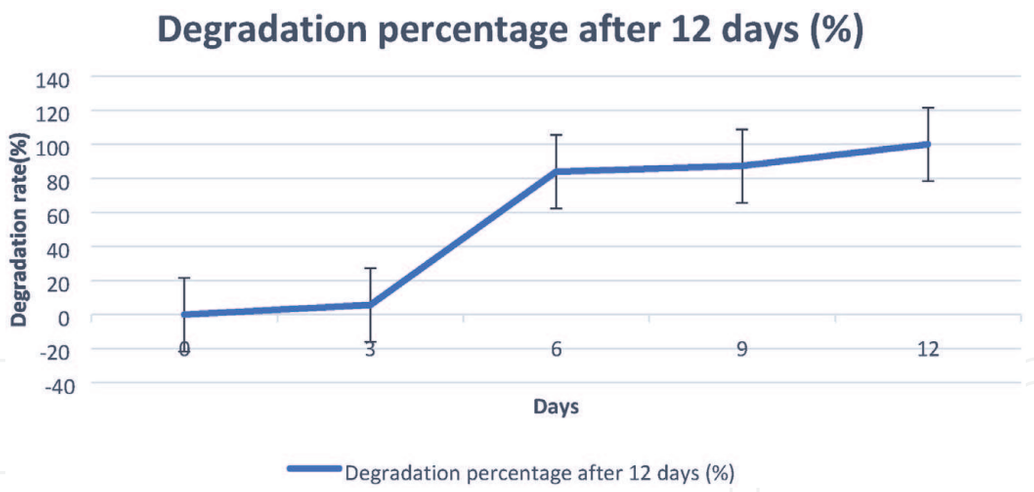


Figure 10.
The degradation percentage of IM2 strain on CAR after 12 days incubation.

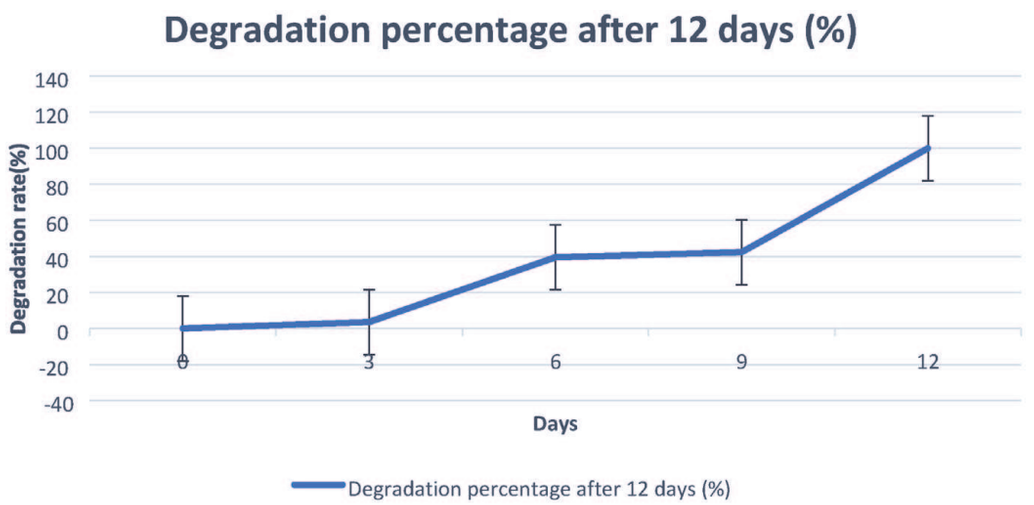


Figure 11.
The degradation percentage of IM3 strain on CAR after 12 days incubation.

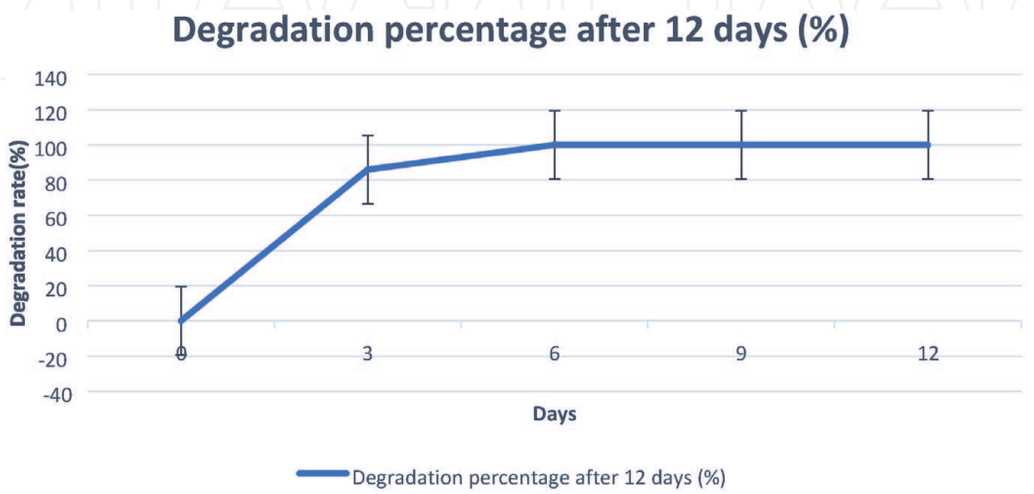


Figure 12.
The degradation percentage of IM4 strain on CAR after 12 days incubation.

5.4 Carbazole degradation analysis

5.4.1 Gas chromatography-flame ionization detector (GC-FID) analysis

The isolated bacteria strains degradation rate on CAR was analyzed from day 0, day 3, day 6, day 9 and day 12 using GC-FID analysis. From the chromatogram obtained, the degradation rate of six isolated strains could be summarized as shown in **Tables 9–14**. **Figures 9–14** shows the percentage of degradation after 12 days for six isolated strains.

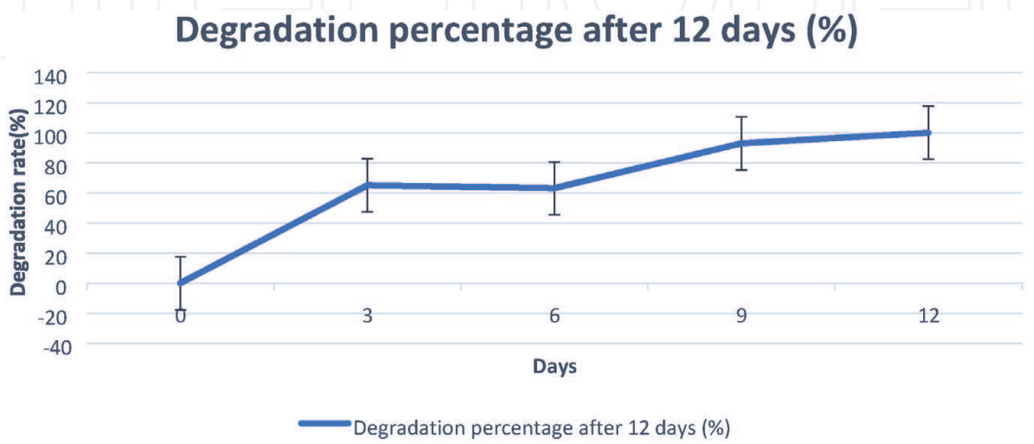


Figure 13.
The degradation percentage of IM5 strain on CAR after 12 days incubation.

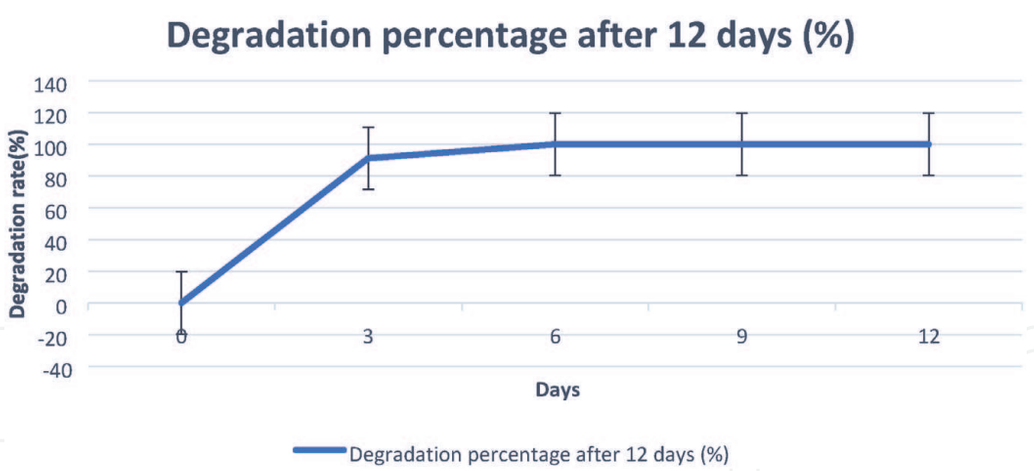


Figure 14.
The degradation percentage of IM6 strain on CAR after 12 days incubation.

6. Discussion

6.1 Enrichment and isolation of carbazole degrading bacteria

Many different types of bacteria exist in our natural environment according to Zulkharnain and Taka [37]. Although most research focus on isolating heterocyclic hydrocarbon degrading bacteria from sewage or site with high probability of contamination, these degrading bacteria could be found anywhere in the environment and not just in certain area. Therefore, lake environment is chosen to discover

different type of novel carbazole degrading bacteria. The project started by taking lake water from Unimas west campus lake, Sarawak to isolate heterocyclic hydrocarbon mainly carbazole degrading bacteria. Sampling was done at the side of the lake closer to land as the number of bacteria are higher at coastal, shallow area [38]. Once, sample was taken, enrichment was done using minimal salt media (MSM) with carbazole as sole carbon source where the degradation of carbazole could be observed in **Table 6**. After around three to four weeks of the first enrichment, color change could be observed where the enrichment turned to clear yellow from cloudy white before. Here, based on study by Zulkharnain and Taka [37], it proves that the bacteria present in the enrichment had utilize the carbazole as sole carbon source and degrade it causing color change. It also explains that the accumulation and production of different metabolites during degradation caused the color change [37]. Another research by Stope et al. [39] and Maeda et al. [40] explained that the change in color of the enrichment was due to the meta cleavage product formation which are the intermediates. When second and third enrichment was done, color change could be observed in a faster rate within two weeks as degradation rate increases. According to the concept where 3-ring structure is more stable and recalcitrant than 2d-structure, bacteria takes about 10 days to degrade fully [37].

From the enrichment, MSM agar plates supplemented with carbazole were prepared and used in this experiment to provide a selective condition and avoid growth of any other bacteria [41]. Double layer agar was used in this experiment to observe the clear zones easily. Streaking was done to observe the growth and degradation on MSM agar plates. After two weeks, clear or hollow zones could be observed on the plates in **Figure 4**. According to Takahashi et al., [42], clear zones that appear on the bacterial colony is because of the mineralization of insoluble CAR by bacteria. As clear zone could be observed, this proves the ability or capability of bacteria to degrade the substrate and utilize it as sole energy source.

Once clear zone was observed, sub-culturing was done to isolate pure colony of bacteria that shows degradation where six bacteria were isolated and designated as IM1, IM2, IM3, IM4, IM5 and IM6 strains. Based on the result shown, all the bacteria were able to grow on MSM agar supplemented with carbazole plate. Although, not all the six isolated showed clear zones or halo formation, the isolates were able to grow on the plates proving the capability of using the substrate. To observe the production of intermediate product and confirm the degradation, the isolated bacteria were inoculated on MSM liquid media as in liquid media it is easier to degrade the substrate because the bacteria becomes free moving cell and it mimics the original or natural condition of the bacteria in lake water. The MSM culture with bacteria was placed on orbital shaker to make sure the bacteria get enough nutrient as the shaker moves in circular motion.

6.2 Gram staining

The isolated bacteria were then characterized morphologically by gram staining. Based on results in **Table 7**, all isolated bacteria (IM1, IM2, IM3, IM4, IM5, IM6) were identified as Gram negative, rod shaped bacteria after viewing under light microscope with 100X magnification with immersion oil. To differentiate between Gram-positive and Gram-negative bacteria, it has been known that gram staining is the best and easiest method so far. Bacteria that can retain the primary strain which is crystal violet (purple color) are Gram-positive bacteria while Gram-negative bacteria classifications are the ones that could not retain the primary strain, where it will stain pink with safranin instead [43]. Based on a few studies, some Gram-negative bacterium such as *Pseudomonas sp.* LD2 [44], *Achromobacter sp.* Strain CAR1389 [45] and *Sphingomonas sp.* GTIN1 1 [46] has been reportd to utilize and

degrade carbazole. This shows that most or many of the heterocyclic hydrocarbon degrading bacteria are Gram-negative.

6.3 Molecular characterization

The molecular characterization was done firstly by DNA extraction procedure based on method in 3.6.1. Detergent which is SDS and proteinase K was used to break the cells and digest contaminated protein in the cell. The digestion of protein process was done under optimal condition of 37°C and incubated where the tubes were flipped every 10 minutes to increase digestion. Once the mixture became viscous, it shows that the digestion process has ended. To take up the DNA in aqueous phase, phenol: chloroform: isoamyl (25:24:1) was added and placed on rotary shaker to maximize the bacterial DNA take up. Centrifugation was done to form the double layer of organic and aqueous phase and to separate the upper layer containing DNA into new tubes. Next, isopropanol was added before centrifuging leaving the precipitate of DNA at the side and bottom of the tube. For further amplification using PCR machine, the DNA precipitate was redissolved in TE buffer.

According to method in 3.6.2. amplification was done using thermal cycler before 16S rDNA sequencing to identify the bacteria. In the PCR amplification, three main process were involved which are denaturation, annealing and extension phase which is all repeated for 30 cycles, therefore producing a million copies in around 2 hours. As shown in AGE analysis in the results, all six isolates (IM1, IM2, IM3, IM4, IM5, IM6) was successfully amplified using 27F as forward and 1492R (reverse) primers showing clear, intact band which is located at 1500 bp after being compared with 1Kb DNA ladder in lane 1. According to Angeline [47], the total of two primers and the sequence of the target DNA is the size of the PCR products.

One of the most common technique to separate DNA was used in this experiment which is gel electrophoresis. In this experiment, according to method in 3.6.3., 1% of gel was used with 1 ml of 50X TAE and 50 ml of distilled water. As the resolution of DNA band depends on the concentration of gel, most concentration of the gels are made in range 0.7% to 2% [48]. The four major factors that influence the migration rate of DNA, according to Cheng and Zhang [48] are, the concentration of agarose where lower concentration will produce better results for large DNA fragments. Then, the size of DNA is also a major factor where smaller fragments tend to migrate in a faster rate compared to larger fragments. Furthermore, the DNA conformation where DNA in supercoiled form is faster than in linear form. Lastly, during electrophoresis, the voltage supplied plays important role as well as the lower the voltage the slower the migration rate. After the bands of PCR were viewed under UV light, the remaining products were purified using the right protocol before sending for sequencing to identify the isolated carbazole degrading bacteria.

6.4 The identification of isolated bacteria

The sequence data for strain IM1, IM2, IM3, IM4, IM5 and IM6 was obtained from the First BASE Laboratories Sdn Bhd which are shown in **Table 8**. All the sequence data were analyzed using MEGA software before the species were determined molecularly using BLAST programming from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

From the analysis, it is shown that the signals for IM1 and IM3 signs were not strong enough resulting in lower identification percentage. For IM1strain, the BLAST result showed that the bacteria belong to the genus of *Bradyrhizobium sp.* with 90% identity compared to the database. *Bradyrhizobium sp.* is a gram negative, nitrogen fixing bacteria that is rod in shape. It is most commonly found in soil and it

is slow growing compared to *Rhizobium sp.* A study by Qu and Spain [49], showed the first clear evidence for the initial steps in biodegradation of nitroanilines by *Bradyrhizobium JS329.* Although not many studies could be found on degradation of carbazole by *Bradyrhizobium sp.,* a study on isolation and characterization of species that degrades polycyclic and heterocyclic aromatic compounds under extremely low oxygen conditions, found that Shinorhizobium sp. C9 (AF227756), Mesorhizobium amorphae (AJ271899), Mesorhizobium sp. SH2851 (AY141983) and Rhizobium ciceri (U07934) has the ability to degrade heterocyclic hydrocarbons [35].

For IM2 strain, it was discovered that the bacteria belong to the genus *Ochrobactrum sp.* with 99% identity to the database while IM6 was also discovered to be another type of *Ochrobactrum* species with a specified name which is *Ochrobactrum anthropi sp*. with 100% identity. This species is known to be gram negative, rod shaped, non-pigmented, aerobic bacteria. This explains how the two isolates could be from the same species as it is hard to differentiate when the bacteria are not pigmented. Novel strain of *Ochrobactrum anthropi* HM-1 has been isolated from oil-contaminated soil where the degradation potential has been reported that contributes to bioremediation of used engine oil polluted sites [50].

With similarity of 97% to the database, IM3 strain was found to be *Pseudomonas aeruginosa sp. this* species is a pathogenic bacterium that could cause disease to plant, animal and even human. This bacterium is also a gram negative, rod shaped bacteria that could be found widely in environment, especially in soil, water and plants. In an experiment where potent biodegradation of crude oil was assessed, *Pseudomonas aeruginosa sp. have* been reported to degrade at the percentage of 58% of crude oil with direct or indirect assistance of glycerol or rhamnolipid. As *Pseudomonas aeruginosa sp. has* the ability to utilize vegetable oil or glycerol as sole carbon source [51]. Crude oil is mainly involved in biodegradation as the major component of crude oil are volatile hydrocarbon that needs to undergo biodegradation.

IM4 and IM5 strain has been confirmed to belong to the genus of *Burkholderia sp.*

with 100% similarity to the database after the 16S rDNA sequencing was done. *Burkholderia sp.* are obligate aerobic, gram-negative bacteria that is well known for their antibiotic resistance. Some family of *Burkholderia sp.* such as *Burkholderia mallei sp.* are known to be pathogenic to mostly horses and other related animals. Based on a study by Inoue [52], 27 carbazole utilizing bacteria was isolated from environment where three of them were *Burkholderia sp. strain* NE-7, *Burkholderia sp.a and Burkholderia sp. strain* NW-1. When hybridization was done, CAR gene was found in these isolates proving the ability to utilize carbazole as sole carbon source.

6.5 Gas chromatography-flame ionization detector (GC-FID) analysis

As the rate of degradation cannot be determined through direct observation, GC-FID analysis was used in order to obtain the result of the degradation rate of carbazole by the six (IM1, IM2, IM3, IM4, IM5, IM6) isolated bacteria. The GC-FID machine was used by analyzing the residual substrate after 12 days incubation period of the bacteria supplemented with carbazole where pattern of degradation was observed and compared. In previous study by Okoh et al. [53], the degradation of crude oil by Pseudomonas aeruginosa was determined by measuring the reduction rate of crude oil. Hedlund et al. [54] had also done analysis using GC-FID where the degradation rate of substrate was calculated by looking at the disappearance of substrate.

In this experiment, the sample were analyzed every two days until day 12. Based on the chromatogram observed from the analysis result in 4.4, the peak of substrate, CAR was detected at the retention time from 14.0 to 15.0 minutes and the pattern of CAR utilization are revealed to vary among all isolates. This could be due

to the sundry nature or environment of hydrocarbon present at the location from which the isolates were taken [55]. For IM1 strain, the percentage of residual carbazole was seen to be decreasing from 10.87% on day 3 to 0% on day12 while the degradation percentage showed gradual increase from 89.13% on day 3, 95.14% on day 6, 97.71% on day 9 and 100% on day 12. This shows that *Bradyrhizobium sp.* possess the ability to degrade carbazole gradually with highest percentage on day 12 resulting on zero amount of carbazole left.

For IM2 strain, the percentage of residual of CAR was high on day 3 with 94.35% but reduced greatly to 16.13% on day 6 and subsequently to 12.7% on day 9 and 4.63% on day 12. For the degradation rate, on the third day, the degradation rate was low at 5.65% but increase sharply on day 6 up to 83.87 and 87.3% on day 9 followed by 95.37 on day 12. Therefore, the degradation rate was slower on the first 3 days and increased a lot from the sixth day. However, it could be observed that this *Ochrobactrum sp.* does not totally degrade CAR after 12 days as there was still some residual substrate left. Thus, indication could be made by observing the plotted degradation graph that the strain might need more time to completely degrade the substrate compared to other bacteria.

The percentage of residual substrate for IM3 isolate, decreased from 96.57% on day 3 followed by 60.51% on day 6 and 57.67% on day 9. Whereas the degradation percentage on day 3 was low at 3.43% and increased to 39.49% on day 6, 42.33% on day 9 and finally to 100% on day 12. The *Pseudomonas aeruginosa sp. which* had been widely analyzed as a degrader showed that the strain took 12 days to completely utilize and degrade carbazole as sole carbon source based on the analysis result.

For IM4 strain, it could be observed from the result that the degradation rate was one of the highest compared to other isolates. The percentage of residual carbazole reduced to 14.06% on day 3 and was fully utilize leaving 0% from day 6 onwards. The degradation rate went down to 85.94% on the third day and increase fully to 100% on day 6. For IM5 strain which was identified as the same species with IM4, the rate of degradation however was different from IM4 strain with residual substrate percentage of .38.46% on day 3 and gradually falling to 36.9% on day 6, 6.96% on day 9 and was totally utilized by day 12. The degradation rate on day 3 was 61.545 which was higher compared to IM4 strain and gradually decreased to 93.04% on day 9 and 100% on day 12. Thus, both IM4 and IM5 strain which was identified as *Burkholderia sp.* could have been different type of stains showing different level of degrading ability.

The IM6 strain which was identified as *Ochrobactrum anthropi sp.* has the highest degradation rate based on the GC-FID analysis result where it was shown that the degradation rate on day 3 was up to 91.22% and the substrate, CAR was totally degraded by day 6 onwards. The residual substrate left on day 3 was 8.78% from 100% on the first day and by day 6 there was no substrate left. Based on study by Abulgasem Alenabi [56], the degradability of bacteria on CAR or other substrate was believed to be influenced by the adaptability of isolates to utilize the substrate as sole carbon source. The optimum growth factors such as temperature, pH and presence of nutrients also plays an important role on the capability of bacteria to degrade substrate [57].

7. Conclusion and recommendations

As a conclusion, all the objectives were successfully achieved. The appearance of clear zone on MSM agar plates and color change in MSM broth of all six isolates proved to be carbazole degrading bacteria as they were able to utilize the substrate carbazole as sole carbon source. All six species of bacteria isolated from Unimas lake

water was successfully identified using 16S rDNA sequencing which was found to be *Bradhyrhizobium sp., Ochrobactrum sp., Burkholderia sp*. and *Pseudomonas aeruginosa sp. all* these bacteria were found to be gram negative bacteria through gram staining analysis. As for the second objective of measuring the degradation rate, all the six isolates were able to degrade carbazole after 12 days of incubation when tested using GC-FID analysis where *Burkholderia sp*. and *Ochrobactrum sp.* showed high degradation rate. Thus, this proves that all the six species are a good candidate for bioremediation.

For the recommendations, the bacterial growth parameter such as pH, agitation and temperature can be manipulated to increase the degradation rate on the substrate used. The degrading ability of isolated bacteria strains could also be tested with other heterocyclic compound such as dibenzofuran and dibenzothiophene. Next, when preparing and handling samples for GC-FID analysis, precautions should be applied in order to gain more accurate results. Moreover, in order to fully understand on the degradation pathway of isolates, experiment on detection of metabolites produced can be done in the future.

A. APPENDICES

A.1 DNA Sequence for six isolates

IM1:

GGCAGTGGTGCTGCTTACCATGCAAGTCGAGCGCCCCCTGGGGTAGCGG
CTTACTGGTGAGTAACGCGTGGGATCTCCCTTTTGCTACAGAATAACATACG
GAAACTTGTGCTAATACAGTATGTGCCCTTTGGGGGAAAGACTTATGGGCA
AAGGATGAGCCCGCGTTGGATTACCTAGTTGGTGGGGTAAATGCCTACCAA
GGCGACTATCCATATCTGGGCTGAGAGGATGATCAACCCCGTTGTGACTGA
AACACAGCCCAAACTCCTACGGGAGGCAGCAACGGGGAATATTGGACAATG
GGTCCAAGCCTGAGCCATTCGTGCCCTGAGAGAGATGAAGGTCTTACGGTT
GAAAAGCTCTTTCACCGGCGAAGATAATGACGGTAACCGGAGAAGAAACC
CCGGCTAACTGCGTGCCAACCTCCGCG GAAAGACGAAGGGGGCTAACGTTG
CTCGGATTTACTGGGTGTAAACCGCACGGACGACGATCGATTAGTCAGGGA
TGAAATCCCACGGCTCACCCCTGGAGCTGCCTTTGAGACTGTATATCTGGAG
TATGTATCAGGTGAGTG GAGTTCCGATTGCAAAGTTGACATTCTCATATAT
CTGTCGGAACACTA TAGGTGAAAGCTGATAGGACATGACCTAGTGAGACAG
AGGTGCA CAAGCGTGGGGAGCTATCTGGAGCACGTACGTTGGAAGTTG
ACTGCATT GACCATCATGGCTGCGCGCACGTCCGTATACTGCCTATGTG
TCTCT GATGCCGGACGAACTCAACTCTCTGGTGAGGACGTTCTCGCGAGAC
TAGCCTCTTTTCTACTCGAACCGTTCACTCACGCGCGATAGCAG GATG
TTTCTTCATTGCTAAGTGACCCTCTAGACGCTGCTCCACACGTGACC TATTG
ACTGTCGCATGAAGCAGATGCTATCTCATCTACCGCTCTTAGCAGTT CACG
TAGCCCTTAAGCTCGATCAAAAATCTGAGATCTTTTGTCTAGTTTGA
CATTATTAGCTTATCCAATGCTTGGCACGTGAATCGTCTGTCTAGTTCGC
CATCAAAAGCGACTTTCGGCTATCATACATCTTAGGAGAGATGCTGGCG G
ACTGAACGCCCTTTATGCATCGATCAGCCAGTTCCACT GAAATGCCTTGTG
CGCAGCCTTGACTGCTAGTATTCCAGAACTTTCACGC GAGACCTCAGATTCG
ATTAGTGAACTGTGTAATCAGGCATCGATGACGCCG.

IM2:

CNNNNNNNGNGGNNGCTNANNNATGCAAGTCGAGCGCCCCGCAAGGG
GAGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGCTACGGAA
TAACTCAGGGAAACTTGTGCTAATACCGTATGTGCCCTTCGGGGGAAA
GATTTATCGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTGGTGAGG

TAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGAT
GAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTGAAGATAATGACGG
TAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACG
TAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCG
GAACTGCCTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTG
GAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAA
CACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAATGTTAGCCGTTGGGGAGTTTACTCTTCGGTGGCGCAGC
TAACGCATTATACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAA
CTCATAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATACCGGTCGCG
GANNCANAGATGTGTCTTTCAGTTCGGCTGGACCGGATACAGGTGCTG
CATGGCTGTCGTCAGCTCTNGTCGGGAGATGTTGGGTTAAGTCCG
CAACGAGGGCAACCCTNGCCCTTAGTTGCCAGCATTTAATTGGGNNNTC
TAAGGGACTGCCGGGGATAACCCACAGAAAGGGGGGGATGACGG
CAAGNCCCNAGGGCCCTTACGGGCTGGGTNACACNGGGTTANAATGGGGG-
GAANGGGGGGCACNAACCCCNGAGGGGGAGCTATTTCCCAN NACNCTNC
CAATTCGGAATGCNCTTGGAACCCGGGGCCTAAAATG GAAACCNTT.

IM3:

CCTGCATTGGGGGCAGCTACCATGCAAGTCGAGCGGATGCAACGG
GAGTTTGCTCCGGGGTTCAGCGGCGGACGGGTGAGTAATGCCTAG
GAACCTGCCCGGTAGCGGGGGATAACTTCCGGAAACGGGCGCTAATACCG
CATACGCCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTTTGGAT
GAGCCTATGTCGGATTAGCTAGTTGGTGGGGAAAGGCCTACCAAGGCGAC
GATCCATAACTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
CAATGGGCGAAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGT
CTTCGGATTGTAAAGCACTTTTGGTTGGGAGGAAGGGCAGCGAGTTAAT
ACCTTGCTGATTTGACGGTACCTGCAGAATAAGCACCGGCTAACTTCG
TGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCGCGCAGGTGGTTCAGAAAGATGGATGTGA
AATCCCCGAGGCTCAACCTGGGAACTGCATTTTAACTACTGAGCTAGAG
TGCGGTAGAGGGGAGGTGGGAATTCCGCTGTGTAGCAGTGAAATGCGT
AGATATGCGGAGGAACACCGATGGCGAAGCAGACTCCTGGGATAACAC
TGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGACTTCTGA
GCTTTGAAGCGCAGCTAACACGTGAAATTGACCGCCTGGGGAGTACAG
TCGCGAGATTATAACTCTCAAGGAGTTGACACGGGACCCCACAGACGC
TGGGATGATGTGTATTATATCGATGAGACGCGCAAAACTTACCTACCG
CTTTACATGTCTGAATGCTTACAGAATTTGATTGGTTCTACGAGACTC
GAACACAGTGCTGCATGCTGTCGTCAGCTCGGTCTGGATGTGGGTAG
TTCCGTACGAGCGCACTGGCATAGTGCTACCACTGGACTCGTACTGT
ACTGGCAGTGACAGTCGAGCTGTGGAGTGAGTAAGGCTCTAGTACCTA
GGCTAAGGCTAGCTCATACGTCAGTCAGTACCTGTCGCATGAAGCG
GATCTCTATCCGATGACG ATCG

IM4:

NNNNNNNNNGCATGCTTACNNTGCAGTCGAACGGCAGCACGGGT
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ATACGATCTACGGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGG

TTGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAG
GCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGA
CAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGC
CTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTTGGCTCTAATA
CAGTCGGGGGATGA CGGTACCGGAAGAATAAGCACCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGTGCGCAGGCGGTTTGCTAAGACCGATGTGAAATC
CCCGGGCTCAACCTGGGAACTGCATTGGTGACTGGCAGGCTAGAGTA
TGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGAT
GTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGCCAATACTGACGC
TCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCTTTCCTTAGTAAC
GTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTA
AANNCNAAGGACT.

IM5:

NNNNNNNNGNNNGGCATGCTTACNNTGCAAGTCGAACGGCAGCA
CGGGTGCTTGCACCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATC
GGAACATGTCCTGTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAA
TACCGCATACGATCTACGGATGAAAGCGGGGGACCTTCGGGCCTCGC
GCTATAGGGTTGGCCGATGGCTGATTAGCTAGTTGGTGGGGTAAAGG
CCTACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATT
TTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAA
GAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCTTGGC
TCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGG
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ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCTACGCAAGACACTCTA
AGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAATCC
TCNTGGCCCTTATGGGTAGGGCTTCCACGTCNNACATGGTCCGAACC
AAGGTTTCCANCCCCCGAGGGGAACTATCCCAAAAACNANTCTAAT
CCGAATGCCCTGCAACCCAGGGCTAAACTGGATCCTTAANCCCGANNNNT
GCCCGGGGAAACTTCCCGGTTTTTACCCCCCCNNCCCCCGGGAGGGGTTT
TCCAAAGGGGGTNNAC.

IM6:

NNNNNNNNNNNGNNGCTTACCATGCATGTCGAGCGCCCCGCAAGGGGA
GCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGCTACGGAA
TAACTCAGGGAAACTTGTGCTAATACCGTATGTGCCCTTCGGGGGAAAGA
TTTATCGGCAAAGGATCGGCCCGCGTTGGATTAGCTAGTTGGTGAGG
TAAAGGCTCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGA
TCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCC
GCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTCACCGGTG

AAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAG
CAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGC
GTAAAGCGCACGTAGGCGGACTTTTAAGTCAGGGGTGAAATCCCGGGG
CTCAACCCCGGAACTGCCTTTGATACTGGAAGTCTTGAGTATGGTAGA
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AGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
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CAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCATAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCTAAGCAACGCGCAGAACCTTACCAGCCCTTGACATACCGGTCG
CGGACACAGAGATGTGTCTTTCAGTTCGGCTGGACCGGATACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGGGCAACCCTCNCCCTTAGTTGCCAGCATTTAGTTGGGGA
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NCAAGTCTCCTGGGCCTTACGGCTGGGGTACCACCGGGCTACAATGGG
GGGGACNGGGGGCACCGAGACCCCNAGGGGGNCTNNTTNCCAAANCN
NNNCAGTCGGATTGCNNNNCNACNCCGGGGCTGAAGTGGAATCCGNG
GAATCCGGANNCCATGCCCGGGGAANNCTTCCGGGCTTNTAACCCCCC
CGTCNCCCTGGGGATTGGTTTTCCCAAAGGNTTGTTTACCCCAGGGG
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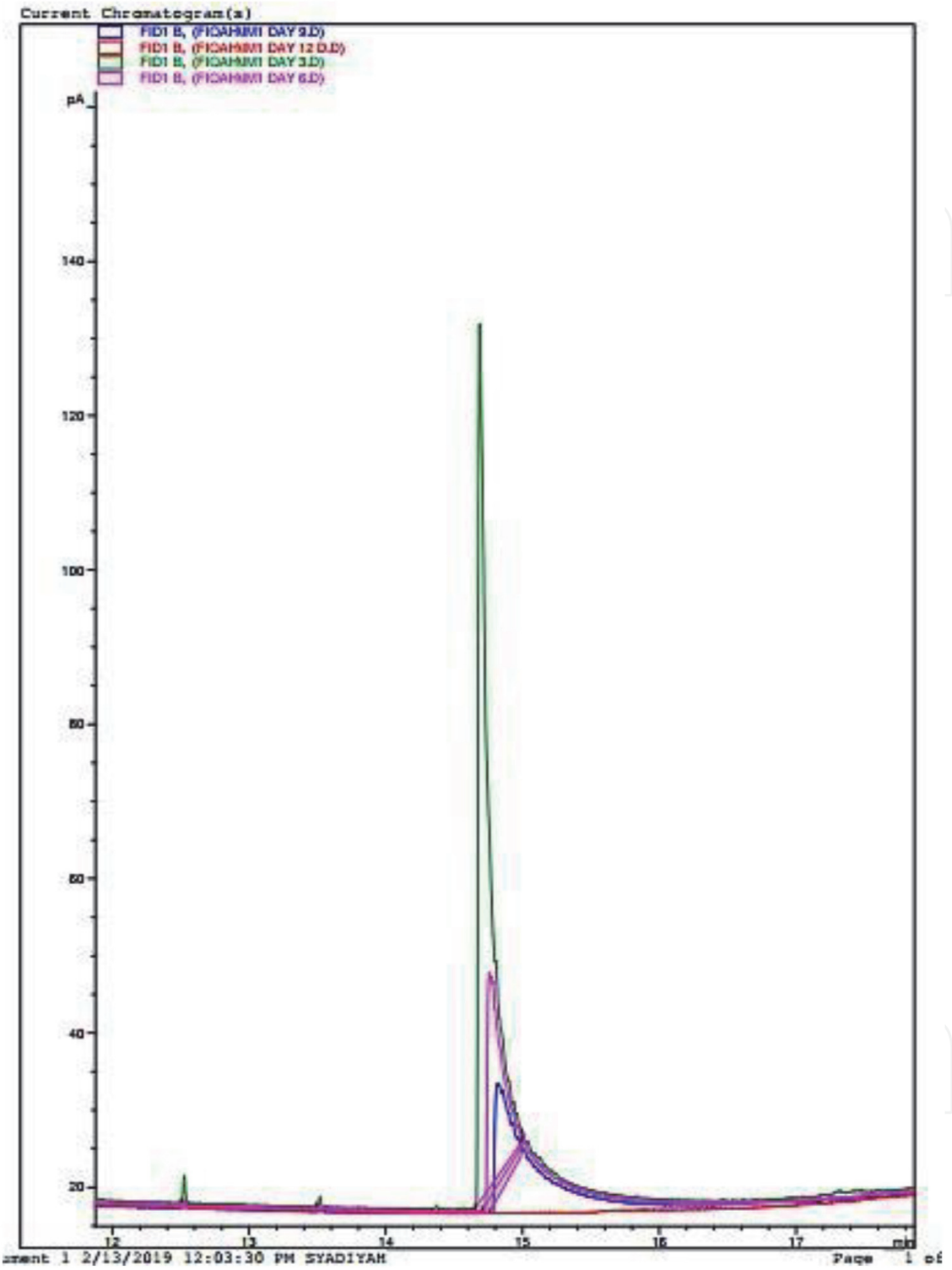
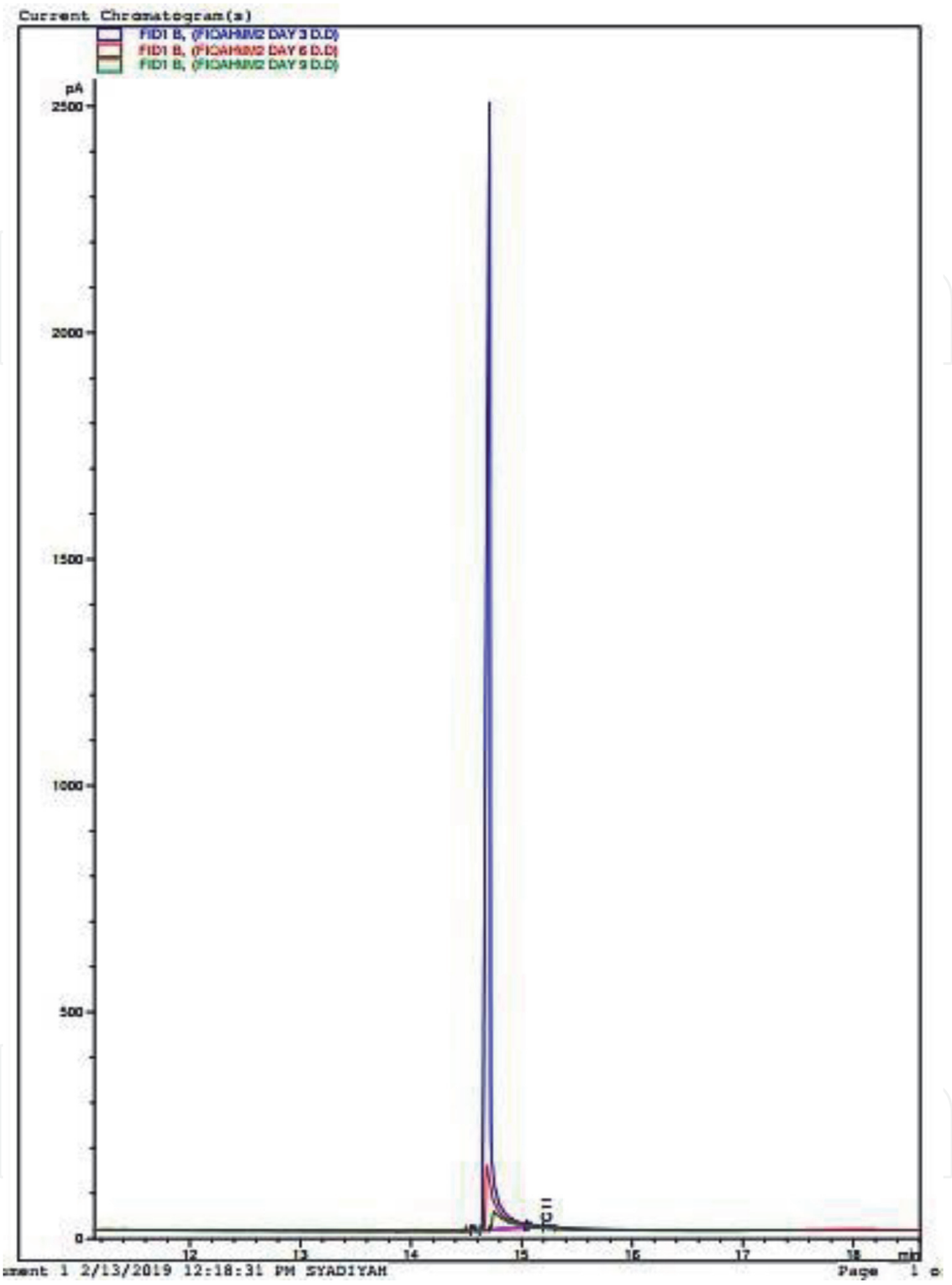
A.2 Overlay of GC-FID results

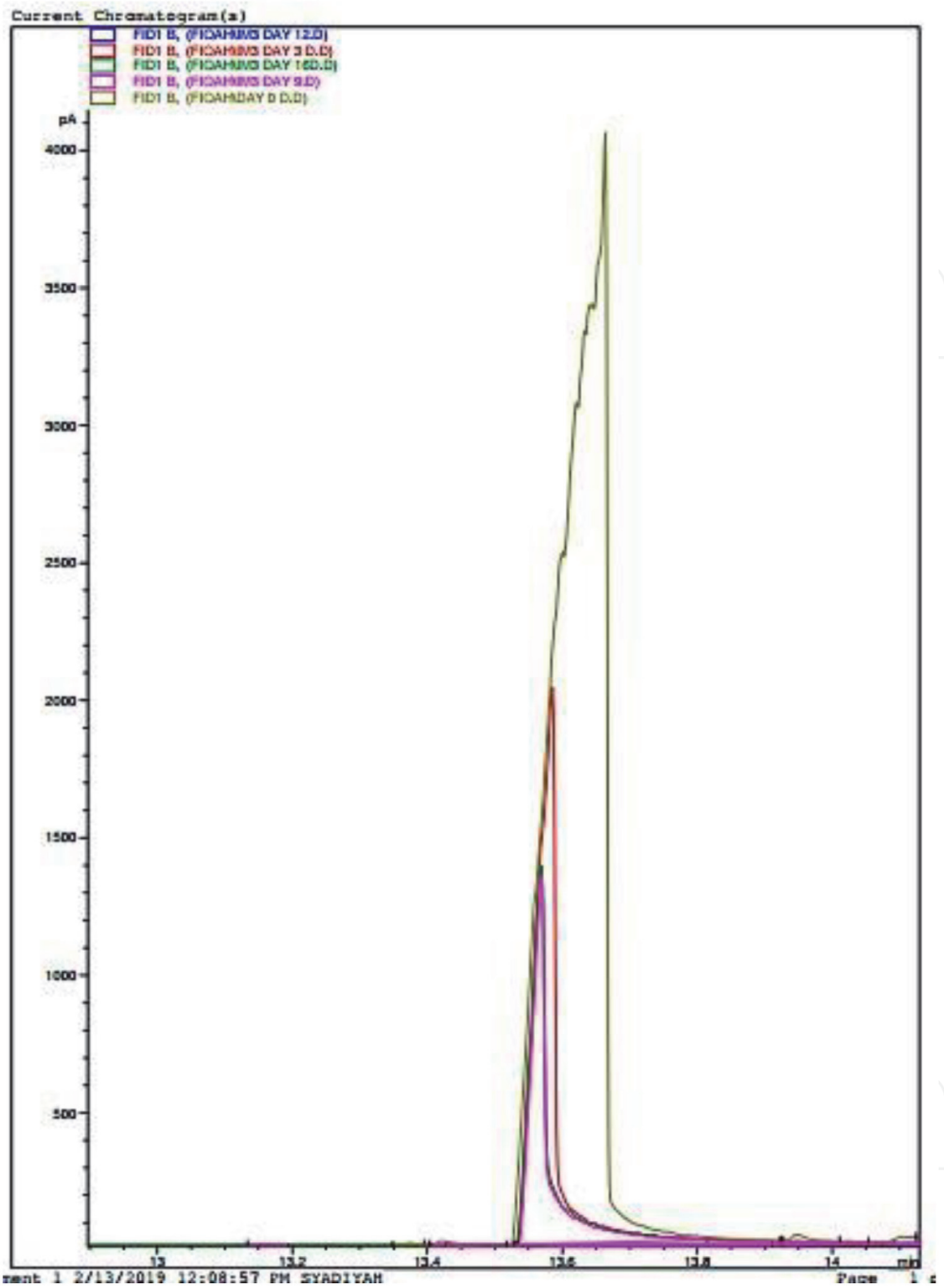
IM1:



IM2:

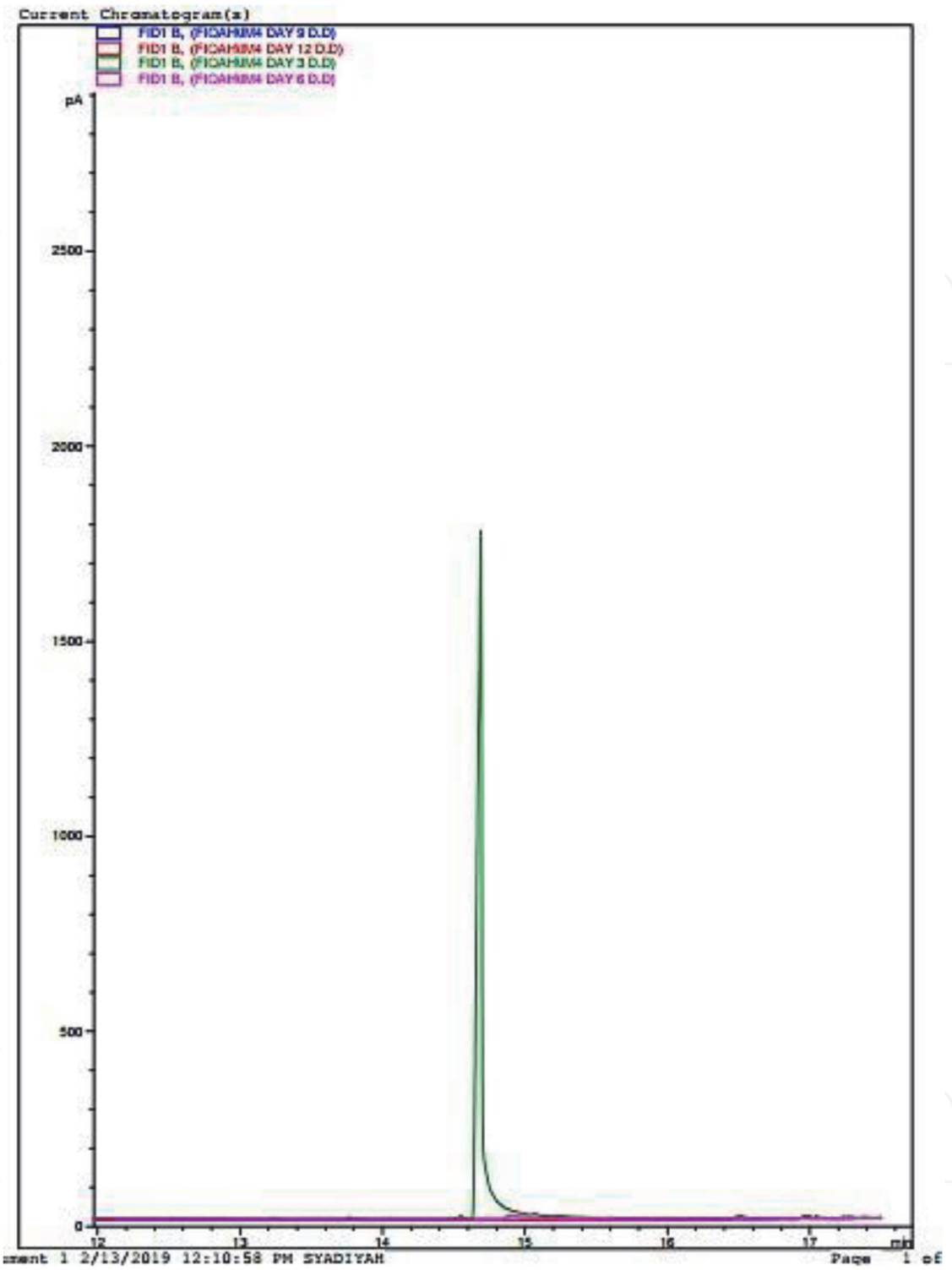

Current Chromatogram(s)
FID1 B, (FIDAHIM2 DAY 3 D.D)
FID1 B, (FIDAHIM2 DAY 6 D.D)
FID1 B, (FIDAHIM2 DAY 9 D.D)
pA
2500
2000
1500
1000
500
0
12
13
14
15
16
17
18
min
2/13/2019 12:18:31 PM SYADIYAH
Page 1

IM3:


Current Chromatogram(a)
FID1 B, (FIOAHIM3 DAY 12.D)
FID1 B, (FIOAHIM3 DAY 3 D.D)
FID1 B, (FIOAHIM3 DAY 16D.D)
FID1 B, (FIOAHIM3 DAY 9.D)
FID1 B, (FIOAHDAY 0 D.D)
pA
4000
3500
3000
2500
2000
1500
1000
500
13
13.2
13.4
13.6
13.8
14
min
ment 1 2/13/2019 12:08:57 PM SYADIYAH
Page 1

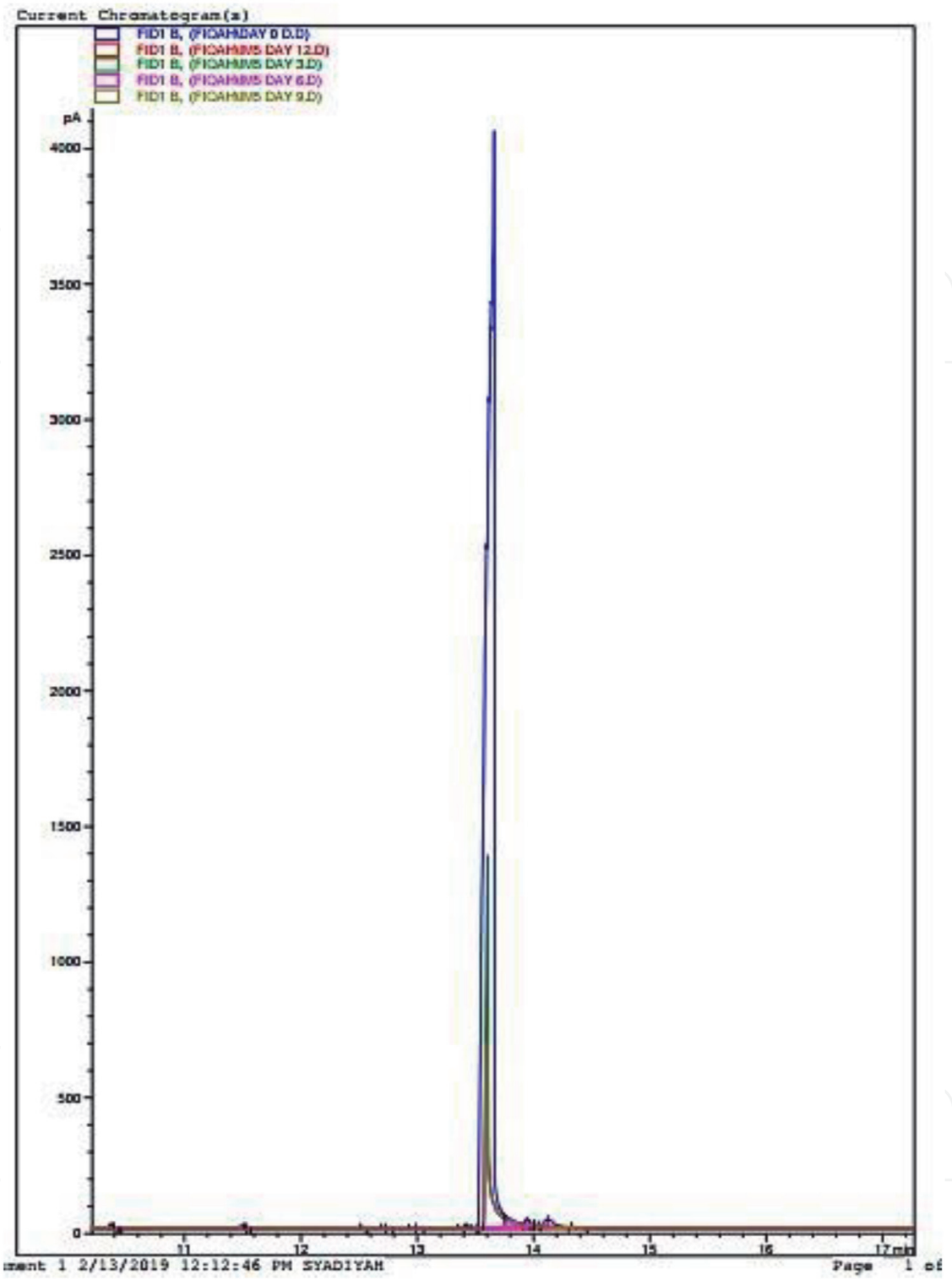
IM4:

IM4:

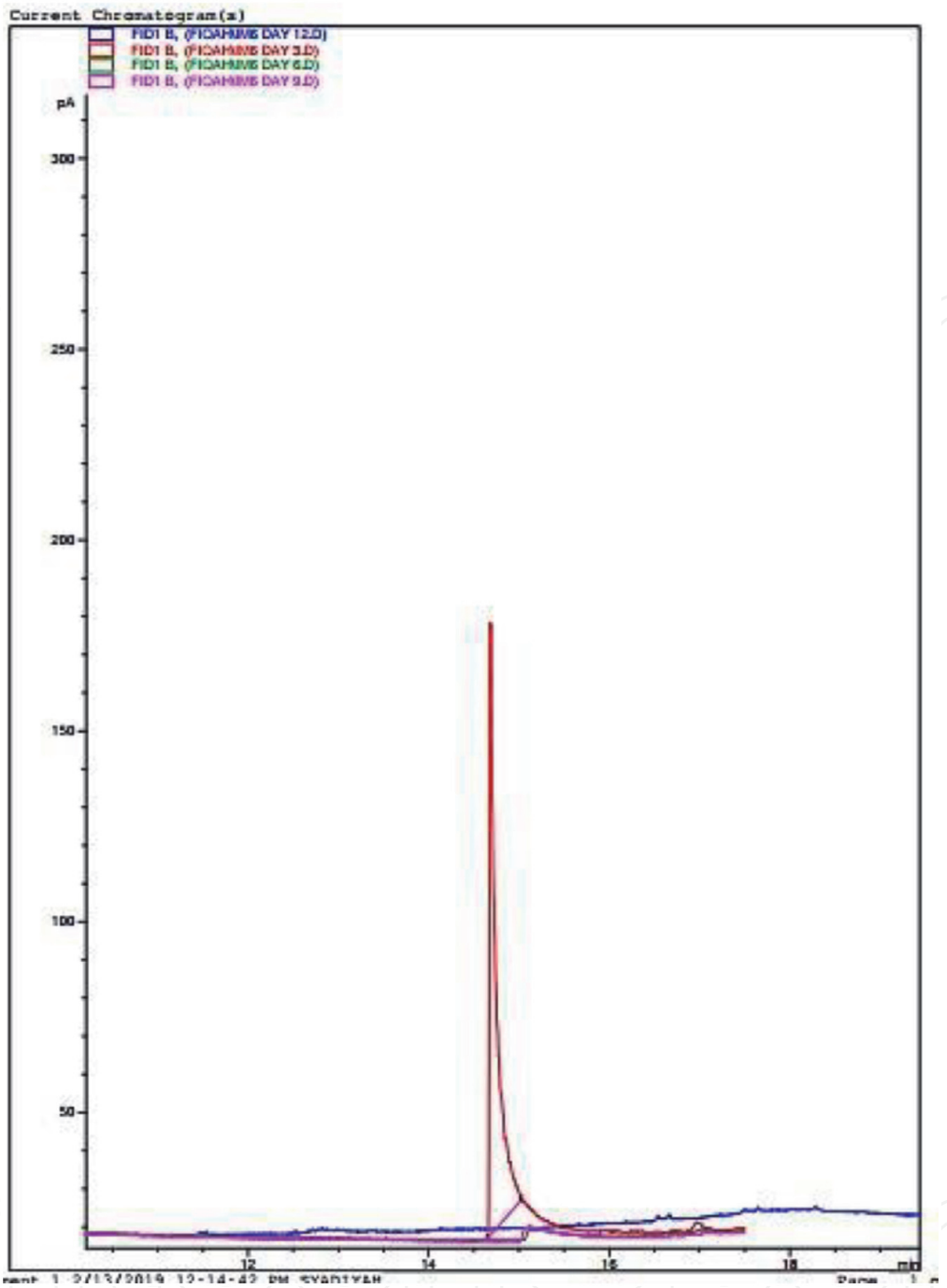

Current Chromatogram(s)
FID1 B, (FIDAHIM4 DAY 9 D.D)
FID1 B, (FIDAHIM4 DAY 12 D.D)
FID1 B, (FIDAHIM4 DAY 3 D.D)
FID1 B, (FIDAHIM4 DAY 6 D.D)
pA
2500
2000
1500
1000
500
0
12
13
14
15
16
17
min
2/13/2019 12:10:58 PM SYADIYAH
Page 1 of

IM5:

IM5:


Current Chromatogram(s)
FID1 B, (FIOAH\DAY 0 D.D)
FID1 B, (FIOAH\IM5 DAY 12.D)
FID1 B, (FIOAH\IM5 DAY 3.D)
FID1 B, (FIOAH\IM5 DAY 6.D)
FID1 B, (FIOAH\IM5 DAY 9.D)
pA
4000
3500
3000
2500
2000
1500
1000
500
0
11
12
13
14
15
16
17min
ment 1 2/13/2019 12:12:46 PM SYADIYAH
Page 1 of

IM6:



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