Bisphenol A: Degradation by *Pseudomonas aeruginosa* From Local Water Source

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***Abstract* – A *Pseudomonas* sp. was isolated from lakes (Section 2 and Section 7) and sewer (SIRIM BERHAD), Shah Alam, Selangor by using *Pseudomonas* isolated agar for about 24 hours of incubation. There about 4 species of Pseudomonas sp. based on observation after 24 hours of incubation and it was found that one of the species which is species A was identified as *Pseudomonas aeruginosa*. This is because only *Pseudomonas aeruginosa* (species A) show green pigmentation on it and species B, C and D not showing any color of pigmentation. Thus, *Pseudomonas aeruginosa* was chosen based on the average reading of absorbance (540nm) as sole species for the growth kinetics studies. Growth kinetics studies on the *Pseudomonas aeruginosa* show the bacterial able to adapt in the nutrient broth containing BPA. The rough idea on how *Pseudomonas aeruginosa* degrades BPA is the bacterial cell will used carbon in BPA as one of carbon source. The morphology *Pseudomonas aeruginosa* was identified by gram staining method which the cells were in rod-shape and Gram negative.**

***Keywords* – Bisphenol A degradation, *Pseudomonas aeruginosa*, *Pseudomonas* isolated agar, nutrient broth**

1. INTRODUCTION

Bisphenol A (BPA) also known as 2,2-bis(4-hydophenyl)propane is a chemical compound that colourless and can dissolve inorganic solvent. It is a chemical compound that usually used in product that based on plastics and as a epoxy resins. Example in plastics based product is in manufacturing plastics bottle and for epoxy resins usually used to coat inside metal product such as cans and bottle caps.

BPA was able to synthesis in 1981 by A.P Dianin and he also the person who to discover BPA as the potential commercial chemical in 1930s. In late 1940s to 1971 another synthetic compound which known as diethylstilbestrol (DES) is more potent than BPA, thus BPA was abandoned as the synthetic estrogen.

In chemical reaction, BPA is produce by the condensation of phenol and acetone with the presence of catalyst (hydrochloric acid or styrene DVB) in order to complete the condensation process (figure 1.1). The molar ratio of phenol: acetone is 3:1 or 10:1 which at 60-80ºC (acid catalysis).

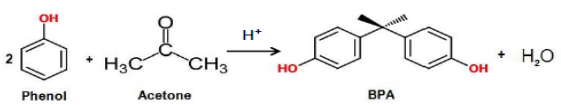


Figure 1.1: Chemical synthesis of BPA [1]

In the industry, BPA can easily discharged into terrestrial aquatic and marine environments from its manufacturing or treatment facilities which will disturb the endocrine system of humans and aquatic life. There are 4 ways how BPA can be harmful to human and aquatic organisms and release into environment which are through water source, air, soil and plastics product. Studies by McKay show that the distribution of BPA contamination in different environments was found that 25% BPA can be found in soil while 25% in sediment and about 50% can be found in water source [2], thus the studies was focus on the local water source because of the contamination of BPA mostly occurred in water. The solubility of BPA in water in the range between 120-300µg/mL [3].

In removal of BPA from polluting environment, there are several method and it's was found that the most effective to remove BPA without it polluting environment is by using microbial degradation. Microbe plays important role in the BPA removal from environments [4]. There are other several organisms capable to degrade BPA other than bacteria such as fungi and planktons.

The best method to remove or degrade BPA from environment is through the microbial degradation and *Pseudomonas* sp. was the most capable in degrading BPA with 90% rate of BPA removal compared to other species such *Streptomyces* sp. (<90%). Most of research on bacterial capable in degrading BPA was done in western countries which *Pseudomonas* sp. maybe not identical to *Pseudomonas* sp. in Malaysia. Thus, most important purpose of this research is to find the *Pseudomonas* sp. which isolated from local water source in Shah Alam, Malaysia and capable in degrading BPA. Other objective of this research is to study whether *Pseudomonas* sp. able to grow in different concentration of BPA and also to study growth kinetic of *Pseudomonas* sp. in different concentration of BPA. Lastly, this research is to study the morphological of *Pseudomonas* sp. which able to degrade BPA.

2. MATERIALS AND METHODS

2.1 Isolation of *Pseudomonas* species

The medium for *Pseudomonas* agar plate was prepared using 24.2g of *Pseudomonas* isolation agar base in 500ml of

distilled water and 5ml of glycerol. At boiling rate, it was dissolve completely. Then, the medium was autoclaved at 121°C for 15 minutes and allow the medium to cool (50°C). The *Pseudomonas* isolation agar base was poured into the petri dish about half of petri dish (34 ml), then agar base then was allowed to solidify and wrapped with Para film. For the collection of water sample, the water sample was poured into sample bottles and labelled with A, B and C. The pH of water sample was measured and recorded (pH 5-6) and the sample bottle was placed in ice box at 4ºC. For the isolation of *Pseudomonas* sp., about 0.1mL of water sample A was transferred into *Pseudomonas* isolation agar. The petri dish was wrapped with Para film and was done thrice by using the same base agar. The entire step was repeated by using water sample B and C. After all of it was done, all petri dish was incubated at 37ºC for 24 hours. After 24 hours, observation of growth of *Pseudomonas* sp. was done.

2.2 Maintaining the *Pseudomonas* strain

The isolation *Pseudomonas* isolation agar with addition of glycerol was prepared and boiled. The medium must be well mix or stirred in order to make sure the nutrient was well distributed. About 5mL of *Pseudomonas* isolation agar was transferred into each of test tube and the cap was placed loosely on the tubes and the test tube was sterilized. The caps of test tube contain nutrient agar was tighten and allowed to cool. Then, an inoculating loop was heated first then the allowed inoculating loop cool for a few second and the colony of *Pseudomonas* sp. was transferred to the surface of the *Pseudomonas* isolation agar. Lastly, the caps of test tube were tightened and incubate at 37ºC for 24 hours. After there was a growth *Pseudomonas* sp., the test tubes was refrigerate at -20ºC.

2.3 Study of Growth kinetics of *Pseudomonas* sp. in Shake Flask resistant to BPA.

The isolated colonies is inoculated into the nutrient broth that containing BPA at different concentration of BPA (10ppm, 20ppm, 30ppm, 40ppm and 50 ppm). *Pseudomonas* sp. strain were cultivated in a Erlenmeyer flask (with a capacity of 250 ml) with 100 ml of working volume which 90mL of nutrient broth and 10mL of BPA at 37ºC with agitation speed of 150 rpm in incubator shaker. Growth of the bacterial species was monitored by measuring optical density at 540 nm (OD_{540}) using a Shimadzu UV-Visible double-beam Spectrophotometer at every 3 hours gaps for about 3 days. The blank used for absorbance reading of cultivate *Pseudomonas* sp. is nutrient broth.

In this case, the growth kinetics study will be specified on Pseudomonas sp. in the different concentration of BPA. In growth kinetics, the maximum specified growth rate of *Pseudomonas* sp. can be calculated is based on the increase in the cell mass over a period of time. The equation that used to calculate maximum specific growth rate, μ_{max} of the *Pseudomonas* sp. growth as follows [5];

$$\mu_{max} = K = In\frac{m_{t2}}{m_{t1}} \div (t2 - t1)$$

where;

μ_{max} = maximum specific growth rate
K = growth rate constant
m_t = biomass at different time

2.4 Estimation of Bacterial Cell Mass

The Optical Density (OD) of the culture broth at 540 nm was measured periodically using Spectrophotometer and about 2.0mL sample of the culture was withdrawn from the flask and centrifuged at 10000 rpm for 10 minutes for every 3 hours of sampling. The supernatant was poured out and the remaining cell is suspended in distilled water and the cell suspension was centrifuged again at same parameter. The supernatant was again poured out and the remaining cell was washing slowly using of distilled water on the suspension of cells. Then, transfer the falcon tube with small opening into a desiccator at room temperature overnight. After 24 hours, the falcon tube containing dry cell was weight. The difference between the final weight of falcon tube and pre-weight falcon tube were used to estimate Cell Dry Weight (CDW).

2.5 Study of Morphological of *Pseudomonas* sp. using Gram staining

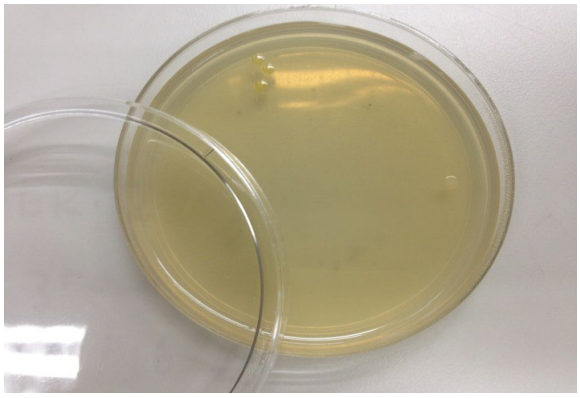
In Gram staining the first consideration that need to make sure was the correct preparation of the smear on the glass slide. Then, sterile the loop first and smear the cell on the glass slide. Next, the smear was dropped with crystal violet staining reagent and heat-fixed for about 1 minute. The glass slide then was washed gently and indirectly with tap water for 2 second. The glass slide then was flooded with Gram iodine for 1 minute and wash gently and indirectly with tap water for 2 second. Next, the glass slide was added drop by drop until the decolourizing agent make the glass slide become clear. Then, safranin was added to counterstain the cell on the glass slide and wait for 1 minute. The glass slide then was washed gently and indirectly with tap water until no colour appears and was dried with absorbent paper. Lastly, the glass slide was observed under the microscope where the Gram negative bacteria will stain pink/red and Gram positive will stain with blue/ purple colour. For this case the bacteria known was *Pseudomonas aeruginosa* which supposedly stain with pink/red because it belongs to Gram negative bacteria.

3. RESULTS AND DISCUSSION

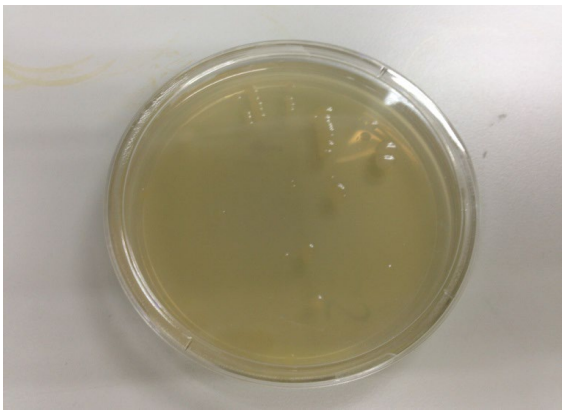
3.1 Isolation of *Pseudomonas* sp.

For the isolation of *Pseudomonas* species from the local water source, the water sample that had been collected from 3 different places which are lakes (Seksyen 2 and Seksyen 7) and sewer (SIRIM, Seksyen 2). The water samples then were spike on *Pseudomonas* isolation agar and incubate for 24 hours and the result are as follow:

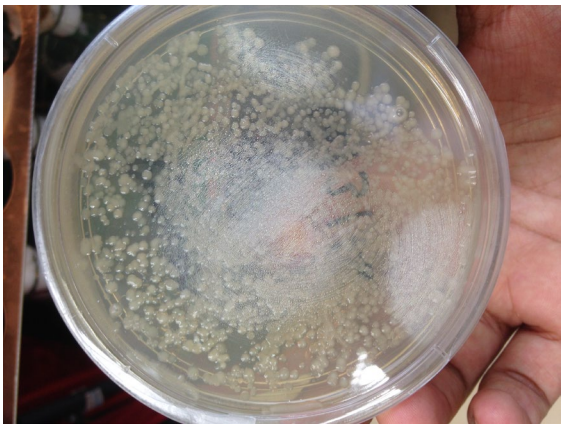
A. Seksyen 2 (Lake)



B. Seksyen 7 (Lake)

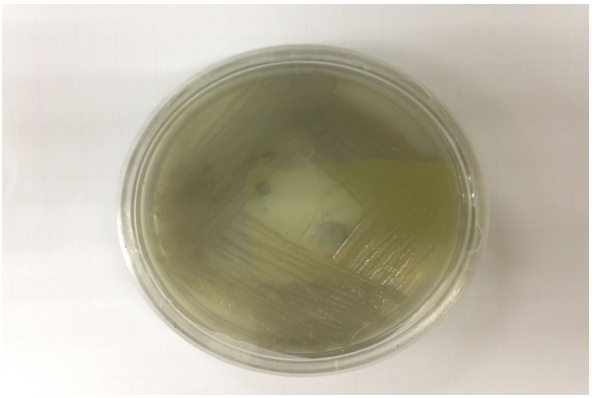


C. SIRIM, Seksyen 2 (Sewer)

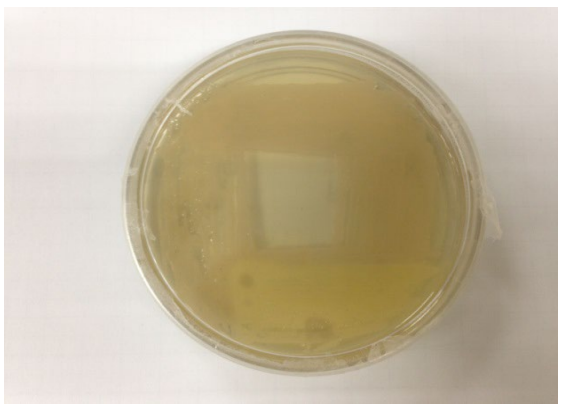


All 3 water samples show a positive growth of bacterial cell but water sample for sewer near SIRIM, Seksyen 2 show most growth compared two other water samples and based on observation it show there are brown colour and white colour of *Pseudomonas* species that appear in the *Pseudomonas* isolation agar. Then, a few isolated colonies was choose from the brown and white colour *Pseudomonas* species and subculture it and triplicate each of the type of *Pseudomonas* species. After 24 hours of incubation brown and white colour *Pseudomonas* species, it was observed that have 4 types of *Pseudomonas* species and labelled as Species A, B, C and D based on the appearance of the growth and only species A show blue-green pigmentation in it growth. The results are as follow:

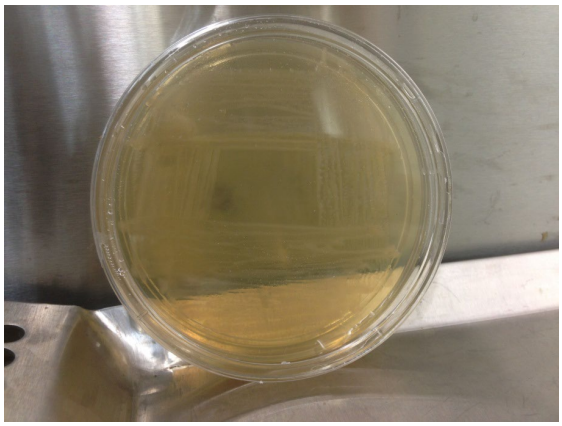
I. Species A



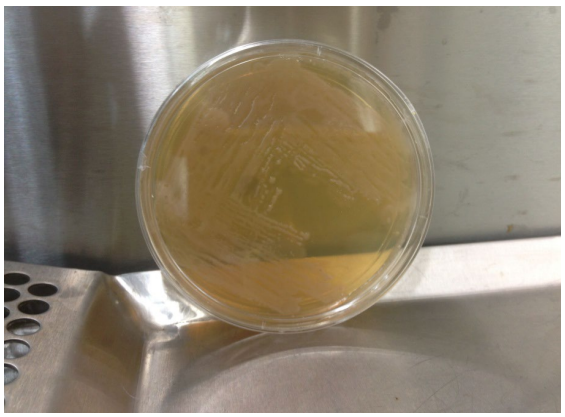
II. Species B



III. Species C



IV. Species D



3.2 Growth of *Pseudomonas* sp. in BPA environments

After 4 types of species were obtained, the experiments continue to look whether *Pseudomonas* sp. can survive in BPA. The experiment was conduct in shake flask by using nutrient broth as the growth medium and 10ppm concentration of BPA which is the working volume 100mL (90mL of nutrient broth + 10mL of 10ppm concentration of BPA). All 4 types of *Pseudomonas* sp. (species A, B, C and D) isolated from sewer (Seksyen 2, SIRIM, Shah Alam) were inoculated in the shake flask and each of it was triplicate and incubates for 24 hours. After the incubation, 3mL of medium was pipette to test the absorbance of each shake flask to confirm there is growth of *Pseudomonas* sp. The table 3.2.1 shows the result after 24 hours incubation.

Table 3.2.1: Absorbance reading of different type of species of *Pseudomonas* sp.

Type of Species	**Absorbance Reading (540 nm)**			**Average absorbance reading**
A1	2.293	2.308	2.391	**2.3307**
A2	2.28	2.309	2.273	**2.2873**
A3	2.292	2.262	2.25	**2.2680**
B1	2.01	2.023	2.043	2.0253
B2	2.065	2.013	2.021	2.0330
B3	2.092	2.022	2.036	2.0500
C1	2.218	2.172	2.146	2.1787
C2	2.18	2.153	2.177	2.1700
C3	2.164	2.157	2.145	2.1553
D1	1.36	2.006	2.032	1.7993
D2	2.037	1.967	1.948	1.9840
D3	1.997	2.016	1.995	2.0027

Species A the highest average absorbance reading compared to species B, C and D of *Pseudomonas* sp. thus, species A (figure 3.1.2) was selected as the *Pseudomonas* sp. for the studies growth kinetic in Erlenmeyer flask.

3.2.1 Confirmation of *Pseudomonas aeruginosa.*

After a week, species A was subculture into a new media of *Pseudomonas* isolation agar and the growth of species A in the isolation agar show green pigmentation which usually produce by *Pseudomonas aeruginosa*. This due to the pyocyanin produce by *Pseudomonas aeruginosa* which give green pigmentation to the species A. Pyocyanin is a redox-active phenazine which is to kill mammalian and bacterial cell through the generation of reactive oxygen intermediates [6]. Thus, green pigmentation of species A give confirmation it is *Pseudomonas aeruginosa*.

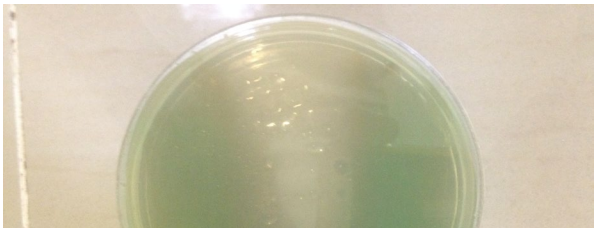


Figure 3.2.1: Green pigmentation produce by of *Pseudomonas aeruginosa* after 24 hours incubation.

3.3 Growth Kinetics of *Pseudomonas aeruginosa* in Different Concentrations of BPA

The highest peak of average absorbance reading is 2.0617 which is the growth of *Pseudomonas aeruginosa* at 20ppm of BPA during the exponential phase. After the exponential phase, the growth become at stationary phase when the absorbance reading for next hours become constant which at 12 and 15 hours the absorbance reading is 1.9970 and 2.0267. Then, the growth pattern of *Pseudomonas aeruginosa* enter death phase when after 15 hours the absorbance keep fluctuate which is at 18, 21 and 24 hours the absorbance reading is 0.9077, 0.8673 and 0.7907. For maximum specific growth rate, $\mu_{max,}$ of *Pseudomonas aeruginosa* at 20ppm, increase of cell mass per time happen at 12 until 18 hours which the value is 0.4079, 0.0191 and 0.9378and at 21 and 24 hours the value for μ_{max} is , -1.1337 and -0.1189 which indicate there are no increase of cell. This show that after 18 hours *Pseudomonas aeruginosa* already enter stationary phase or death phase.

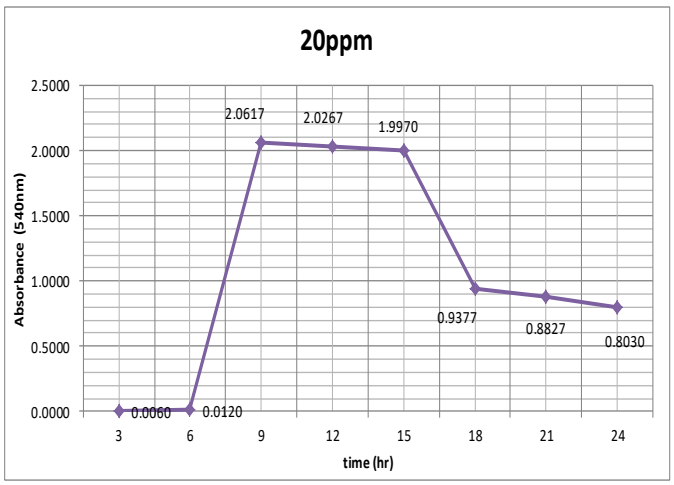


Figure 3.3.1: Growth curve of *Pseudomonas aeruginosa* at 20ppm of BPA.

The lowest peak of average absorbance reading during exponential phase is 1.9473 which is at 50ppm of BPA. After exponential phase, the growth of *Pseudomonas aeruginosa* enter stationary phase which is at 12 and 15 hours, average reading of absorbance slightly increase and decrease which almost show constant and the value is 1.9660 and 1.9337. Then, the average absorbance reading fluctuates at 18, 21 and 24 hours which the value is 0.9190, 0.8157 and 0.7630. At this phase the growth of

Pseudomonas aeruginosa was already enter death phase from stationary phase. For the maximum specific growth rate, the number of cell per hours start to appeared at 9 hours until 15 hours which the value is 0.0906, 0.0959 and 0.0339 and after 15 hours, it show that the cell at stationary or death because the value μ_{max} show negative value which is -0.1812, -0.5474 and -0.0959.

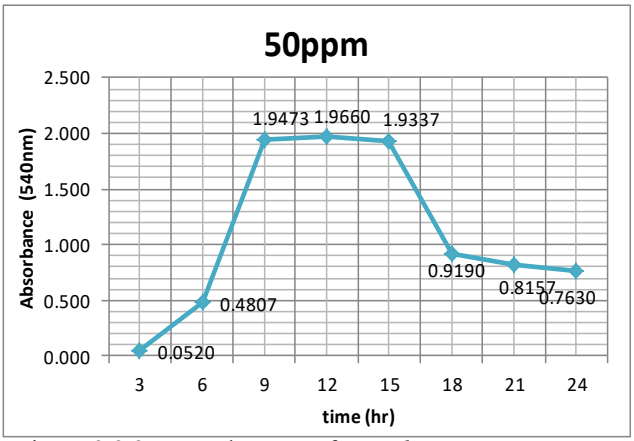


Figure 3.3.2: Growth curve of *Pseudomonas aeruginosa* at 50ppm of BPA.

At 20ppm and 50ppm of BPA, *Pseudomonas aeruginosa* show it has no problem to adapt at medium contain BPA thus it show that BPA still not toxic to the *Pseudomonas aeruginosa*. The rough idea how *Pseudomonas aeruginosa* degrades BPA is by using carbon that BPA as the source of carbon in order the cell to grow and divide. Other than that, the observation that can be made through the average absorbance reading at different concentration of BPA was the *Pseudomonas aeruginosa* became less effective with increase concentration of BPA. This happen at the exponential phase growth of *Pseudomonas aeruginosa* which at 10ppm, 20ppm and 30ppm the value of average absorbance reading still at value above two then when the concentration of BPA enter 40ppm and 50ppm the average absorbance reading is below two. If the experiment is continuing for example until 100ppm and above maybe the average absorbance reading will continue to drop (at exponential peak of growth). Figure 3.3.3 show the as the concentration BPA increase the average absorbance become decrease at 9 hours (exponential peak) of *Pseudomonas aeruginosa* growth.

The correlation between μ_{max} and concentration of BPA that can be made in this experiment was the number of cell per hour, μ_{max} in different concentration show inconsistent value of cell produce per 3 hours of sampling. Supposedly, μ_{max} must be increase at exponential growth and slowly decrease at stationary phase. Both of 20ppm and 50ppm show inconsistent of μ_{max} values which for 20ppm the µmax only start at 12 hours of sampling while 50ppm at 9 hours of sampling. The value of μ_{max} supposedly to decrease as the concentration of BPA increase to show that the as the concentration BPA increase, the BPA itself become toxic to *Pseudomonas aeruginosa* which effect the μ_{max} value.

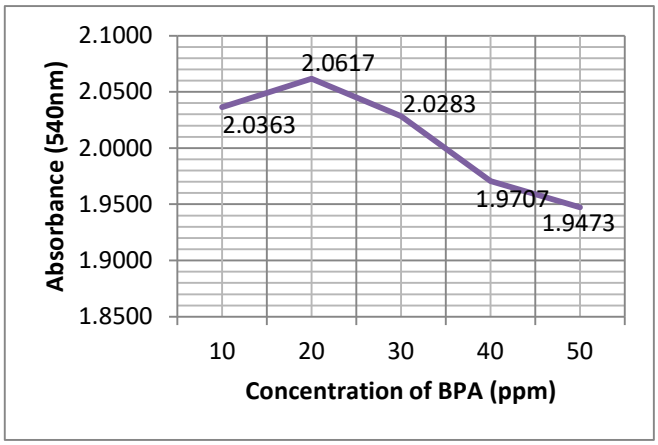


Figure 3.3.3: Growth of *Pseudomonas aeruginosa* (exponential peak) at different concentration of BPA.

3.4 Morphological study of *Pseudomonas aeruginosa* by Gram staining

Gram staining is technique that often been used to compared two large group of bacteria which is Gram positive and Gram negative group of bacteria by based on their different cell wall constituent. Gram positive bacteria usually will stain with purple/violet color and gram negative will stain with red stain. In the case of *Pseudomonas aeruginosa*, the bacterium is known as Gram negative bacteria. The bacteria cannot retained the violet color during the decolorizing process because it peptidoglycan is thinner compared to Gram positive bacteria. During this study also the shape of Pseudomonas sp. can be observed under the microscope. It was found that the shape of *Pseudomonas aeruginosa* is in rod shape.

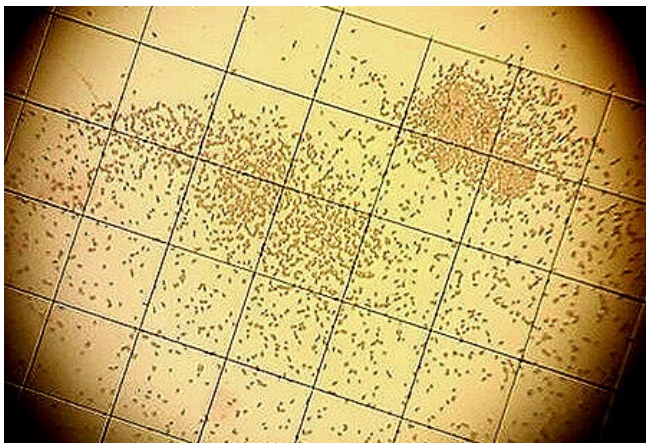


Figure 3.4.1: Observation of *Pseudomonas aeruginosa* under microscope.

4. CONCLUSION AND RECOMENDATION

In conclusion, it was found that *Pseudomonas aeruginosa* can be found in the local water source in Malaysia. It also can be observe that there are several species of *Pseudomonas sp.* exist and it was able to be isolated. It was found species A was *Pseudomonas aeruginosa* based on the green pigmentation which produce by pyocyanin of the bacteria. It also shows a positive growth in medium containing Bisphenol A (BPA). This species able to grow in

3 days because of BPA can be substitute to carbon source from the medium of bacteria (it means as it consume BPA as carbon source, it also degrade the BPA).

One of the recommendation is that can be made for the further improvement of this research is continue to increase the BPA concentration until the maximum concentration of BPA where the *Pseudomonas aeruginosa* able to grow or survive. Other than that, after 3 days of growth *Pseudomonas aeruginosa* the sample of broth is test through the High Performance Liquid Chromatography in order to make sure there is little or no presence of BPA in the broth. The morphology of *Pseudomonas aeruginosa* is further studies about it mortality and endospore and also the biochemical studies of *Pseudomonas aeruginosa* need to add on this research. Lastly, this research must continue to calculate the efficiency of *Pseudomonas aeruginosa* for the degradation of BPA in order to be specific in it capability in degradation of BPA.

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