# Study of pH and Shaking Rates on Fast Biodegradation of Toxic BPA by *Pseudomonas aeruginosa* NR.22 Isolated from Malaysian Pond

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***Abstract*— Biodegradation is the decomposition of materials by the bacteria, fungi, or other biological substances. Material that can be composed by the living creature is known as biodegradable material. This terms is widely used through the ecology, waste management, biomedicine, and bioremediation. Biodegradable matter serve as a carbon sources for microorganism and it is generally organic material. 100 ppm of Bisphenol A (BPA) will be the carbon source for the Pseudomonas aeruginosa (*P. aeruginosa*) and will be degraded. In this study pH and shaking will be tested. Firstly, the study on the pH effect will be conducted in order to obtain the optimum pH for the fast biodegradation of BPA. After that, by using the optimum pH, the study of shaking rate will be conducted. The shaking rate will be investigated to obtain optimum shaking. Laccase assay is done to relate to the biodegradation. Biodegradation process that use those parameters will be estimated by using the High Performance Liquid Chromatography (HPLC) and the percentage of the BPA degradation could be determined. The correlation between the optimum pH and shaking rate could be done at the end of the experiment. And it can be conclude that the most optimum pH is at 6.3 while the most optimum shaking rate is at 130 ppm. This finding will be discussed in this writing.**

***Keywords*— Bisphenol A (BPA), biodegradation, *Pseudomonas aeruginosa (P.aeruginosa)***

## I. INTRODUCTION

*P. aeruginose* is a Gram-negative bacillus that is commonly 1-3µm in length. It is typically an organism with the strict probe that grows on many solid media and can grow at both 37 and 42°C. It has a grape like odor. It does not ferment carbohydrates. This organisms commonly found in moist environments and can be found in water or soils. Sometimes, it is also can be found on fruits, vegetables and flowers. [1]

Due to that, people with high risk of serious infections with *P. aeruginosa* are advised not to consume fruits and undercooked vegetables. [2] There are many moist environment that can be colonized by the *P. aeruginosa* such as swimming pools, hot tubs, contact-lens solution, illicit injectable drugs, and other inner soles of sneakers. [1] *P. aeruginosa* also can be found in the aerators and trap sinks also on inadequately cleaned bronchoscopes. [3]

*P. aeruginosa* has been studied for its ability of BPA removal from aqueous system. It is found to be the best strain to be employed in the process of BPA removal. The extracellular laccase is produced when the *P. aeruginosa* grown in the nutrient broth. This enzyme catalyse the oxidation of various substituted phenolic. And in this case, it also can catalyse the oxidation of BPA by using molecular oxygen as an electron acceptor.

Laccases act on wide range of substrates and this makes them highly useful biocatalyst for various biotechnological applications. [4] It also have broad substrate specificities of this enzyme to catalyse the oxidation of the environmental pollutants such as BPA. Beside *P. aeruginosa*, there are many organisms that have the ability to degrade BPA. Those organisms will be discussed later in the next subtopic of this chapter. There are many method to biodegrade the BPA and also the way to analyses the amount of BPA being biodegraded. All of these also will be discussed in the next subtopic.

## II. METHODOLOGY

### *A. Materials*

Cetrimide agar is used during the isolation of the *P. aeruginosa*. The component of this agar is Cetyltrimethylammonium bromide, a quaternary ammonium and cationic detergent. This component allows for the selective isolation of *P. aeruginosa*. The Cetyltrimethylammonium bromide will cause the release of nitrogen and phosphorus from the bacterial cell when have contact with bacteria. In addition, organisms other than *P. aeruginosa*. Are unable to withstand this germicidal activity. Nutrient broth (NB) is used during the growth curve experiment. All this materials is sterilized by autoclaving at 121°C for 3 hours together with the apparatus such as shake flasks, pipette tip, beaker. This broth also being used during the experiment on pH and shaking rates. Besides that, spectrophotometer is being used to measure the Optical Density (OD) of the growth of *P. aeruginosa* also for the laccase assay. Besides that, BPA is being used. *P. aeruginosa* is sub-cultured from the Dr.Nik Raikhan stock cultured. All the media is prepared with the distilled water. Media of pH is 6.3-7.1 and sterilized by autoclaving at 121°C for 20 minutes. Acetonitrile also being used for the HPLC analysis. Potassium dihydrogen phosphate ($KH_2PO_4$) will be used during the laccase assay.

### *B. Growth Curve of P. aeruginosa.*

The seed culture of the *P. aeruginosa* is prepare 24 hours before the experiment. Then the *P. aeruginosa* is placed in the 250ml of the nutrient broth of three 500 ml shake flasks. The shaking flask then is place in the shaker at the temperature of 35°C.

Figure 1: Seed culture of *P. aeruginosa*

The sample of the *P. aeruginosa* is taken every 2 hours and is placed into the eppeendorfs tube in order tu determine the dry cell weight of the cells. The eppendorfs tube without the sample is weigh first. The eppies is place into centrifuge at 4000 rpm for 20 minutes. After 20 minutes, the supernatant is drawn out and the eppies containing the cell is placed into the oven for 24 hours. After that, the dried residue is weigh and then is calculated by using equation above.

$$Dry\ Mass\ \frac{mg}{L} = \frac{[Weigh\ of\ Eppies + Dried\ Residue(mg) - Weight\ of\ Eppies\ (mg)]}{Volume\ Used\ (L)} X\ 1000$$

Figure 1: Dry mass equation

### *C. Effect of pH on the Biodegradation of BPA*

The inoculum of *P. aeruginosa* is prepared 24 hours before the experiment is conducted. 100ml of NB is prepared in the 250ml shake flasks. The pH of the NB is adjusted from 6.3-6.7. After the Nutrient Broth is prepared, 10 ml of 100ppm of BPA is placed into the each NB. The flasks will be shake at 150 rpm at 38°C for 48 hours. The experiment will be repeated with the different pH of 6.3-6.7. After 48 hour, the sample is analyse by the HPLC for the determination of the percentage of BPA left in the sample and the optimum pH for the fast biodegradation of BPA.

### *D. Effect of Shaking Rates on the Biodegradation of BPA*

The seed culture of *P. aeruginosa* is prepared 24 hours before the experiment is conducted. 100ml of NB is prepared in the 250ml shake flasks with the optimum pH obtained from the experiment of effect of pH. 10ml of 100ppm of BPA is placed into the NB. The shaking rate will be tested: 130, 140 and 150 rpm. The flasks will be shake at different shaking rate for 48 hour at 38°C. After 48 hour, the sample is analyses by the HPLC for the determination of the percentage of BPA left in the sample and the optimum shaking rates for the fast biodegradation of BPA.

### *E. Laccasu Enzyme Assay*

100 ml of nutrient broth was used for fermentation of *P. aeruginosa* for 24 hours. The broth then is centrifuged for 10000 rpm for 20 minutes after the fermentation process so that the biomass is separated and we could obtain the crude laccase enzyme. Then the activity of crude laccase enzyme is analyze by using UV-spectrophotometer at 465 nm and mixture of 1:1 of enzyme and 50 mM of phosphate buffer as the blank. To find the standard curve, 1 ml of crude enzyme is mixed with 1 ml of phosphate 50 mM buffer and 1 ml of BPA at concentration of 1 ppm. The step is repeated by using the different concentration of BPA which is 2,3,4,5 ppm BPA [16]

### *F. HPLC Analysis*

HPLC analysis is being done according to the parameter in the table below:

Table 1: Parameter for HPLC analysis

| **Parameters** | **Value** |
|---|---|
| BPA concentration | 100 ppm |
| Mobile phase | Water: Acetonitrile = 60:40 |
| Flowrate | 1 ml/min |
| Temperature | 35°C |
| Pressure | 225 bar |
| Detector | UV at 230 nm |
| Injection | 10 µL |

All these parameter is being used in order to analyze the degradation of BPA in this study.

## III. RESULTS AND DISCUSSION

### *A. Growth Curve of P. aeruginosa.*

Based on the conducted experiment, there is a result obtained. The result is shown in the table above. From the result obtained, a graph is plot in order to study the relationship between the time and the microbial activity.

Table 2: Table of Growth curve of *P. aeruginosa*

| Time (Hours) | Dry Weight (mg/ml) |
|---|---|
| 0 | 0 |
| 2 | 0 |
| 4 | 0.0011 |
| 6 | 0.0024 |
| 8 | 0.0034 |
| 10 | 0.0068 |
| 12 | 0.0091 |
| 14 | 0.0121 |
| 16 | 0.019 |
| 18 | 0.02351 |
| 20 | 0.0231 |
| 22 | 0.023 |
| 24 | 0.0223 |
| 26 | 0.022 |
| 28 | 0.0211 |
| 30 | 0.0191 |

Based on the table above, a graph of cell dry weight against time is plotted and it is shown in the figure 2.

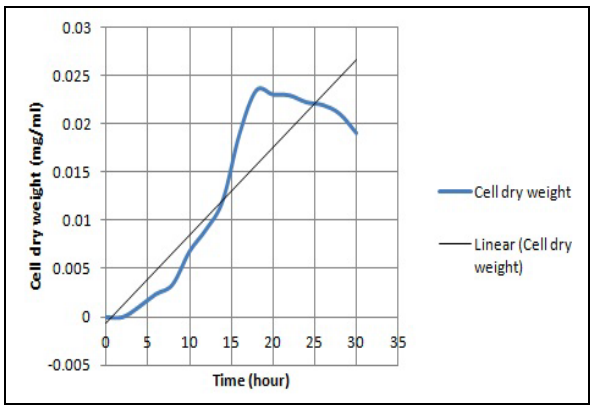


Figure 2: Graph of growth curve of *P. aeruginosa*

Based on the graph it is shown that the lag phase occur at the 0th

until the 2th hour. This phase occur when the microorganism is introduced into the medium. It takes some time to adjust the bacteria with a new environment. In this phase, cellular metabolism is accelerated and cell is increasing in size. The bacteria are unable to replicate at this phase, thus, there is no increase in cell mass. Besides, it is shown on the graph that the exponential phase is started from 4th hour until 18th hour. During this phase, the bacteria is vigorously growing and in a diving state condition. At this phase, the metabolic activity began to increase and the replication by binary fission is occur at a constant state. At this phase, the medium has exploited at the maximal rate, thus, the culture reach the maximum growth rate. The number of bacteria also increase exponentially that will result in balanced growth. The stationary phase occur in a short time which is from 18th hour until 22th hour. As the nutrient is being used for the growth of the bacteria, the reproduction rate will become slow and finally stop to replicate. Finally, the death phase is start to occur at 24th hour and decline until it stop at 30th hour. The depletion of nutrients and the accumulation of waste product and other toxic has facilitated the death phase of the bacteria. At this stage, the bacteria is completely lose it ability to replicate. Individual bacteria begin to die cause by the unfavorable condition. This result is expected based on the theory explained by [14] and the graph is following the theory stated by them.

### *B. Retention Time of BPA*

Before run the HPLC from the sample, the retention time of the BPA need to be determined in order to locate the region of the retention time of BPA.

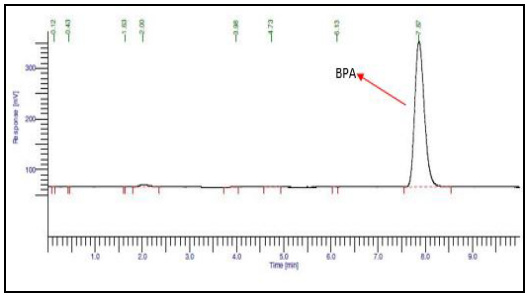


Figure 3: Retention time of BPA

Based on the figure above, it is shown that the retention time of BPA lies on the 8th minute. The retention time of BPA may vary due to the parameter that has been used. As mention before, the parameter of BPA used are:

Table 2: Parameter of HPLC has being used

| Parameter | Value |
|---|---|
| BPA concentration | 100 ppm |
| Mobile phase | Water: Acetonitrile = 60:40 |
| Flowrate | 1 ml/min |
| Temperature | 35ºC |
| Pressure | 225 bar |
| Detector | UV at 230 nm |
| Injection | 10 µL |

The retention time we obtained is different from [5] is shown below:

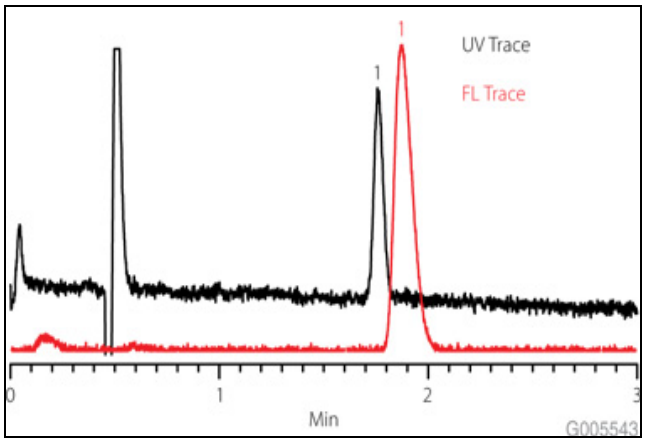


Figure 4: Retention of BPA time from [5]

Table below show the parameter being used:

Table 3: Parameter of HPLC has being used to find the retention time of BPA [5]

| Parameter | Value |
|---|---|
| BPA concentration | 100 ppm |
| Mobile phase | Water: Acetonitrile = 60:40 |
| Flowrate | 0.4 ml/min |
| Temperature | 35ºC |
| Pressure | 225 bar |
| Detector | UV at 230 nm |
| Injection | 1 µL |

Based on the [5], the retention time of BPA obtained is different because of the parameter that being used. It is shown that the flowrate used by [5] is 0.4 ml/min why the flowrate used in this experiment is 1 ml/min. Besides, the amount of injection also different which is in this experiment, the amount of injection used are 10 µL compare to [5] they use 1 µL. Due to this modification, the retention time obtained become different. But the reading from the experiment still can be accepted as the retention time may vary because of the parameter used.

### *C. Effect of pH on the Biodegradation of BPA*

In order to study the degradation of BPA, an initial reading is being taken. The initial reading is shown in figure 5. Based on figure 5, the retention time of BPA is between 8th and 9th minute. The Reading is still accepted since the region of retention time of BPA still can be determined.

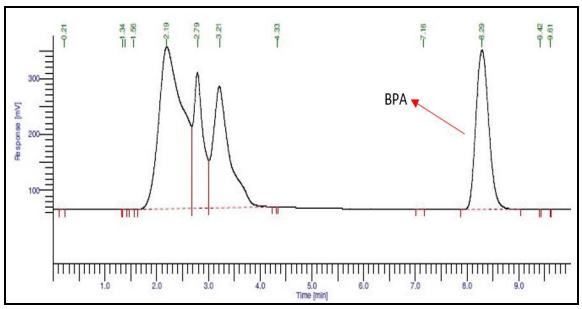


Figure 5: Initial Reading (100ppm)

The result of the study with the different pH is shown below.

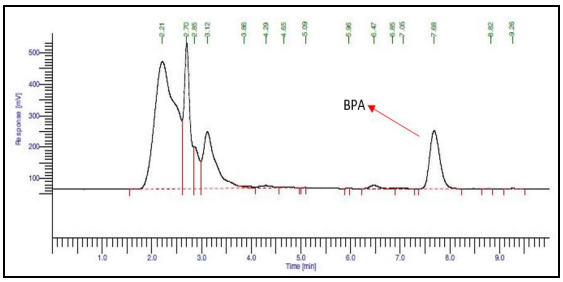


Figure 6: pH of 6.3

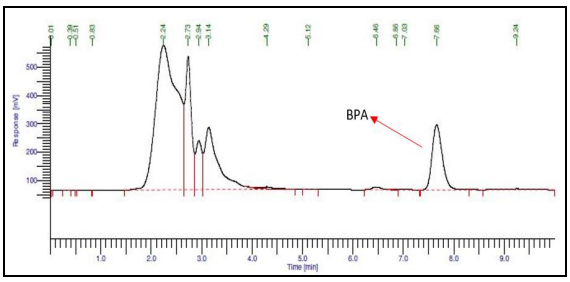


Figure 7: pH of 6.5

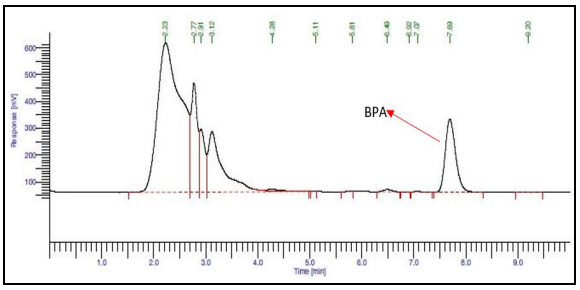


Figure 8: pH of 6.7

Table below show that the data collection on the degradation of BPA by the different pH. This analysis is made by using HPLC. The results is shown in table below. The initial reading of BPA before the experiment is $4.9x10^6$ uV*sec

Table 4: Summary table of Effect of pH on the Biodegradation of BPA

| pH | Value of BPA left from the HPLC graph uV*sec($10^6$) | Percentage of BPA Degraded (%) | Concentration of BPA left (ppm) |
|---|---|---|---|
| 6.3 | 2.6 | 47 | 53 |
| 6.5 | 3.2 | 35 | 65 |
| 6.7 | 3.7 | 24 | 76 |

The parameter of pH were selected to optimize the degradation efficiency of BPA. 100 ppm and 2 days incubation in incubation shaker (150 rpm at 38ºC) were fixed variable. Media pH were varied from 6.3-6.7 and based on the table 4, the highest degradation value of 47% were observed at pH 6.3. A slight reduction were observed at pH 6.5 which is 35%. The lowest degradation percentage was obtained at pH 6.7. Even though there was variation in degradation efficiency, the strain were able to survive at both low and high pH levels

### *D. Effect of Shaking Rates on the Fast Biodegradation of BPA*

In order to study the degradation of BPA, an initial reading is being taken. The initial reading is shown in figure 9. Based on figure 9, the retention time of BPA is between 8th and 9th minute. The Reading is still accepted since the region of retention time of BPA still can be determined.

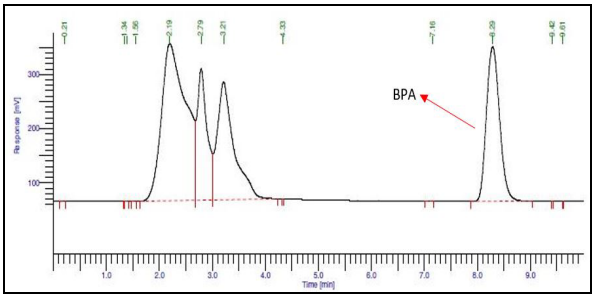


Figure 9: Initial Reading (100ppm)

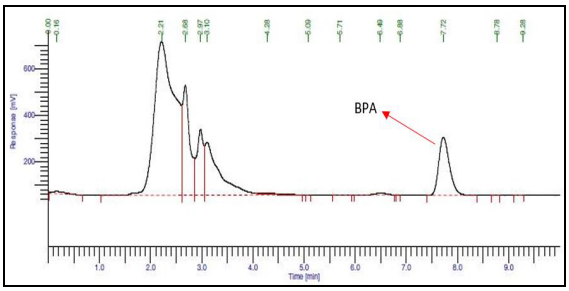


Figure 10: Shaking rate of 130rpm

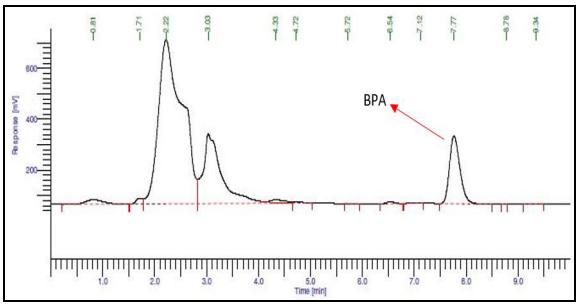


Figure 11: Shaking rate of 140rpm

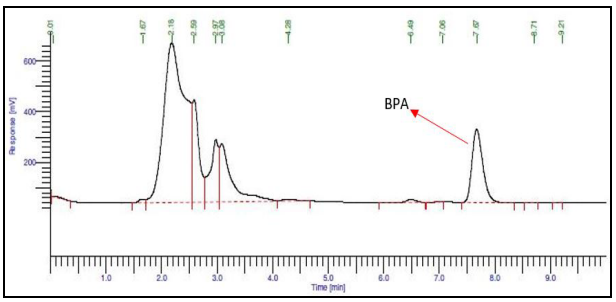


Figure 12: Shaking rate of 150rpm

Table below show that the data collection on the degradation of BPA by the different shaking rate. This analysis is made by using HPLC. The results is shown in table below. The initial reading of BPA before the experiment is $4.9x10^6$ uV*sec

Table 5: Summary table of Effect of Shaking Rates on the Fast Biodegradation of BPA

| rpm | Value of BPA left from the HPLC graph uV*sec($10^6$) | Percentage of BPA Degraded (%) | Concentration of BPA left (ppm) |
|---|---|---|---|
| 130 | 3.6 | 26 | 74 |
| 140 | 3.7 | 24 | 76 |
| 150 | 3.9 | 20 | 80 |

The parameter of shaking were selected to optimize the degradation efficiency of BPA. 100 ppm and 2 days incubation in incubation shaker (150 rpm at 38ºC) were fixed variable. The shaking rate were varied from 130-150 rpm and based on the table 5, the highest degradation value of 26% were observed at 130 rpm.

A slight reduction were observed at shaking rate of 140 rpm which is 24%. The lowest degradation percentage was obtained at a shaking rate of 150 rpm which is 20%. Even though there was variation in degradation efficiency, the strain were able to survive at both low and high shaking rate levels.

### *E. Laccase Enzyme Assay*

Enzyme assay for laccase is done in order to observe and determine the BPA degradation activity. One unit of enzyme activity is defined as the amount of enzyme that increase the absorbance by 0.001 units per min at 37°C and is expressed as U/ml [16]. A sample is collected 24 hours after the fermentation in order to determine the BPA degradation ability of the P. aeruginosa with the initial BPA concentration of 5 ppm. The sample is being centrifuged at 10000 rpm and 4°C for 20 minutes so that the biomass is removed. After that, 1 ml of the sample was mixed with 1 ml of crude laccase enzyme and 1 ml of 50 mM phosphate buffer. The mixer is let to be reacted for 30 minutes before the absorbance reading was determine by using UV-spectrophotometer. Graph below show the standard curve for the laccase assay.

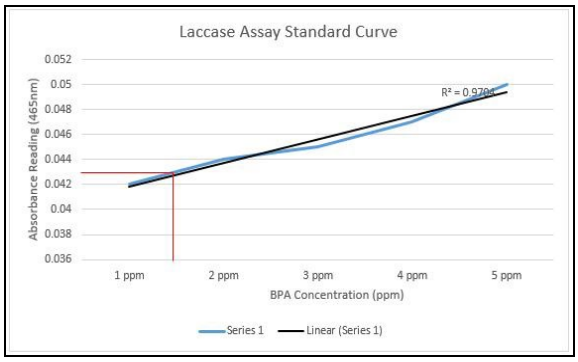


Figure 13: Standard Curve of Laccase Assay

From the reading obtain, it is shown that the absorbance value is 0.043 from the sample which is corresponds to the BPA concentration of 1.5 ppm based on the interpolation that has been done on the standard curve. Based on the results obtained, it is shown that there is a BPA degradation activity occurred during the 24 hours of fermentation process as the concentration of the BPA is decreased from 5 to 1.5 ppm after the fermentation process.

### *F. Discussions*

The study on the characterisation of *Pseudomonas aeruginosa* isolated from fresh water culture system [13] have been done before. *Pseudomonas* are important phytopathogens and agents of human infections while other strains and species exhibit bioremediation and bio control activities. A specific study of *Pseudomonas* species in the environment may lead to more complete understanding of the ecological significance of these microorganisms. A study on the growth curve of *P. aeruginosa* has been done in order to study the bacteria activity. Based on the results, it is shown that the *P. aeruginosa* is actively replicate during 4$^{th}$ hour until 18$^{th}$ hour. At this stage, the degradation of BPA is at its best condition, thus the efficiency of degradation rise up during 4$^{th}$ hour until 18$^{th}$ hour.

Besides, several study also have been done on the toxic effect of BPA upon long-term exposure and on the possible mechanisms of BPA's on various life forms [11]. This is the first report regarding simultaneously isolation of three different bacterial strains from coastal regions of Chennai, Tamil Nadu, India, having the ability to survive and degrade 1000 ppm BPA in seawater in an effective manner. Although there are abundant bacteria that have the ability to degrading BPA, those with high BPA biodegradability are limited in number [8]. In this work, three different kind of bacteria that is highly BPA tolerant is being used.

[10] Has let the isolated bacteria to grow in the medium supplemented with different concentration of BPA. In the present study, 70 colonies were inoculated to grow in M medium supplemented with a variety of different concentration (250 ppm, 500 ppm, and 1000 ppm) of BPA. The colonies number was reduced gradually according to the BPA concentration and their resistance. Even there are many bacteria capable of degrading BPA at 250 ppm, their biodegradability at high BPA (1000 ppm) was not significant. [7] reported that under the environmental conditions and with strain has been cultivated in mineral medium, BPA was not easily being degraded. In this study, BPA in nutrient broth was degraded by *P. aeruginosa* at 100 ppm for 2 days.

There are many factor has been suggested as the factors that affecting the growth and composition of bacterial communities such as oxygen, pH, salinity, temperature, co-substrate and organic carbon [12]. In our study, two parameters pH and shaking rates were selected and the effect on the microorganism was tested. Generally bacteria need physiological pH inside their cells as other living organism do [6]. A narrow range of pH value (from 6.3-6.7) have been tested on BPA degradation by *P. aeruginosa* NR.22 and maximum degradation and cell growth were observed at pH 7.0 [15]. But, in this study, the highest degradation level was found at a pH of 6.3 and it subsequently decrease when the pH of medium being altered.

When bacteria in liquid media are shaken, the cultures respond with the increased growth rate and final cell number. This response is attributed to aeration of the media by shaking. But there are several papers comparing shaken and stationary cultures, let alone studying the effect of shaking without the presence of ambient air for oxygenation. The study on shaking rate impersonate the effect of agitation for the fermentation. The suitable shaking rates will give a good result. [9] Show that the shaking rate increase the growth rate and reduce the size of *Brevundionas diminuta*. In this study, the shaking rate also increase the growth rate of *P. aeruginosa* resulting its effectiveness in degraded the BPA because the optimum the shaking rate, the increase the growth of *P. aeruginosa* thus, the higher degradability of BPA. In this study also, the high degradation of BPA occur at the shaking rate of 130 RPM. The degradation is subsequently decrease when the shaking rate increase

Besides that, the crude laccase enzyme assay also an important approach that has to done. This assay will prove that the laccase is an enzyme that able to degrade the BPA. *P. aeruginosa* is one of the microbe that able to secrete the laccase when there is a presence of BPA in their life medium. The ability to secrete the laccase makes the *P. aeruginosa* as a special microbe as it also a versatile bacteria. With this finding, now it will be easier to extract the laccase as it can be secreted from the *P. aeruginosa*. This also will help the environment as the *P. aeruginosa* can be found everywhere. With the presence of *P. aeruginosa*, the BPA can be degraded and the industry that involve with BPA can use the *P. aeruginosa* as their material to handle the abundance of BPA that contain in their waste water.

## IV. Conclusion

There is a need to eliminate or minimize the presence of endocrine disruptor chemicals in the environment. The current level of PA production is big and risks associated with BPA are complicated. There are many challenge in order to remove the BPA from the environment. Microbe-mediated removal has been suggested in order to achieve a better degradation on BPA since the BPA itself cannot be degraded by water. This deal with the isolation of *P. aeruginosa* and its ability to degrade BPA efficiently under the difference pH and also shaking rate. This study suggest that the best pH for the biodegradation of BPA is at pH 6.3 while the ideal shaking rate is 130 rpm. For the future approach, it is recommended that to try the experiment with the others parameters so that a wide range of finding can be obtained.

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