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# KINETICS AND MASS BALANCE RELATIONSHIP BETWEEN LACCASE *PSEUDOMONAS AERUGINOSA* NR.22 WITH SUBSTRATE BISPHENOL A

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***Abstract***

*The strain Pseudomonas aeruginosa NR.22 (Ps.NR.22); an extracellular laccase producer isolated from a polluted lake in Malaysia has been proved to grow very well in 1000mg/L Bisphenol A (BPA). Therefore, the kinetics of BPA utilization and bacterial growth is investigated to determine the possibility of using Ps.NR.22 as an alternative method in the bisphenol A (BPA) removal from aqueous solution and contaminated open water bodies. This study has been modelled using a shake flask system with the ability of the strain to remove about 90% of BPA; recorded after 72 hours of incubation. The kinetic parameters are verified to improve the biological removal of BPA from the environment. Effect of various parameters such as contact time, pH, temperatures, initial BPA concentrations and cell dosages has been carried out in this study. The optimum conditions for the removal of BPA within the experiment range of variables studies were 250mg/L of initial BPA concentration, 6x106 g/L of cells, pH value of 6.0 and 92 min of contact time. Under these conditions the maximum removal efficiency was 90.5% using 1000mg/L and 100% using 250mg/L BPA.*

***Keywords****: Kinetic model; Mass balance, Substrate utilization; Pseudomonas aeruginosa NR.22; Wastewater; Laccase*

## Introduction

The environment is continuously affected by different types of organic pollutants. These organic pollutants may exert adverse effect to the living organisms. One type of the organic pollutants is referred as Endocrine Disruptor Chemicals (EDC) which has the ability to cause harmful effect to the reproductive system, cardiovascular disease as well as cancer. Bisphenol A is a chemical that falls under EDC which are widely used around the world mainly as a monomer in the production of polycarbonate plastic and epoxy resins. According to *L. Mita et al.* [1], BPA is produced approximately three million tons and has very wide applications and consequently become one of the pollutant source that directly affect the environment and living organisms when it is not properly treated. The main factor that causes environmental contamination is from the production of polymer by using BPA as the monomer which results in the constant release of this substance into the water bodies and soil at a very high concentration. Researchers have focused on the counter measure for BPA exposure problem for many years and have found several solutions such as chemical degradation as well as biodegradation. However, the chemical treatment of BPA is not favored as it causes other environmental issues. Therefore, biodegradation is much more preferred and one of the methods

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is through the use of Laccase enzyme that is produced by microorganism. *A.E. Abd El Monssef et al.* [2] stated that Laccases are enzymes that belong to the oxidase group and it has been extensively studied due to its ability to oxidize phenolic compound. Laccase enzyme usually made up of 15 to 30% of carbohydrate and has the molecular mass of 60 to 90kDa. This enzyme can be found and widely distributed in higher plants as well as microorganism. One of the microorganisms that are able to produce this enzyme is the Pseudomonas sp. This species is a versatile species that have very wide metabolic pathways and able to withstand extreme conditions. Therefore, BPA degradation has been proposed using Laccase enzyme produced by this species as an alternative to counter the BPA pollution problem. In order to utilize this species as a mean to degrade BPA in the environment, it is important to study on the kinetic of the species with respect to the BPA utilization for the microbial growth. *A. Talaiekhozani et al* [3] stated that the kinetic models are important in order to determine the optimal conditions for the organic matter degradation with microorganism activity in the environment. In this study, the kinetics of BPA utilization and the bacterial growth is investigated to search for the possibility of using the Pseudomonas aeruginosa NR.22 as an alternative for BPA removal from contaminated open water bodies and aqueous solution. The aim of the study was: (1) investigation of Pseudomonas aeruginosa to remove the BPA from aqueous solution, (2) fitting the kinetic model to analyze the experimental data, (3) determination of degradation behavior of BPA, (4) determination of cell dosage on the BPA degradation.

## Materials and Methods

### *Reagent, chemicals and pH*

All material and chemicals were collected from various sources and quality as listed. Bacteriological peptone (Ultrapure, protein = Nx6.38 ≥ 76.5%), yeast extract (Y1625, Sigma-Aldrich), Pseudomonas agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate ($KH_2PO_4$, V90004, Vetec™ reagent grade, 99%, Vetec), ammonium chloride (09718, NH4Cl, BioUltra, ≥99.5%, Sigma), polyvinylalcohol (Aldrich), calcium chloride (Sigma-Aldrich), acetone (Sigma), ethanol ($CH_3CH_2OH$ analytical grade, Sigma-Aldrich), sodium hydroxide (NaOH, Fluka) and hexane ($CH_3(CH_2)_4CH_3$, 95%, Sigma-Aldrich).

### *Pseudomonas strain and estimation of bacterial cell mass*

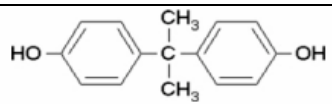
*Pseudomonas aeruginosa* NR.22 (Ps.NR.22) was isolated from a lake in Shah Alam, Selangor, Malaysia [4]. It is a Gram negative-rod shaped bacterium. The strain was maintained at -20ºC in *Pseudomonas* glycerol agar. The enrichment medium was a modification of medium by Nik Raikhan (150rpm, 37°C, 24h) [4]. The inoculum was prepared using nutrient broth (NB) at pH = 6.0, 150 rpm at 37°C [5, 6]. The amount of 8% (v/v) cells was used. The Optical Density (OD) of the culture broth at 540nm 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 h of sampling. This method was adapted and modified using *N.H. Nik Raikhan and M.Y.M. De Valda* [7]. Figure 1 depicted the strain.

### *Preparation of BPA solution and laccase assay.*

The BPA (Merck) was used without further purification to the concentration of 1000mg/L (Table 1). The experimental solutions were obtained by series of dilution. Laccase activity was determined spectrophotometrically at 436nm as described by *N.H. Nik Raikhan and M.Y.M. De Valda* [7] with 5ppm BPA as a substrate in a reaction mixture containing 50 mM phosphate buffer (pH = 6.0) ($\varepsilon 465 = 48{,}000 M^{-1}cm^{-1}$), 37°C, 30min. One unit of enzyme activity was defined as the amount of laccase that increased the absorbance by 0.001 units per min at 37°C; the activities were expressed in U/mL. All assays are repeated thrice to gain the mean ±sd of three applications. All spectrophotometric measurements were carried out using a UV–Vis spectrophotometer (SPEKOL 2000, Germany).

**Fig. 1.** *Pseudomonas aeruginosa* Nr.22; left is 24 hour growth on slant agar (NA) and pyocianin pigment production on Cetrimide agar on the right)

**Table 1.** Characteristic of the BPA

| | |
|---|---|
| **The molecular structure** |  |
| **Molecular formula** | $C_6H_5C_{lO}$ |
| **Molecular weight** | 228.1g/mol |
| **$\lambda_{max}$** | 278nm |
| **Basic solubility in water** | 120mg/L |

***Resistance to various BPA concentrations***

BPA concentrations of 250 to 1000 ppm were used to study the resistance of the Ps.NR.22. The cultivated medium was incubated at 37°C for 92 hours in thrice application with one control.

***Analysis of Degradation Products Using HPLC and GC-MS***

After each indicated interval (6, 12, 18 to 72 h), the residual of non-degraded BPA was periodically extracted with 40 ml of hexane and ethyl acetate followed by filtration with a 0.45µm microfilter. The residual BPA in the culture was quantitatively determined using HPLC according to table 2.

**Table 2.** HPLC characteristic used to run the test on the residual BPA

| | |
|---|---|
| **HPLC** | Alliance HPLC system (Waters 2695, Milford, MA) attached to a photodiode array detector (PDA, Waters 2998) |
| **Column** | Symmetry® C18 (4.6×250mm, 5µm) |
| **Retention time** | 5 min |
| **Mobile phase** | 50% acetonitrile and 50% water |
| **Signal monitoring at** | 250 nm |
| **Flow rate** | 0.6mL/min |

***Kinetic models***

All experiments were carried out with Ps.Nr.22 strain at 37°C in 250mL Erlenmeyer flasks operating at 150 rpm to elucidate the optimum conditions (pH, contact time and initial BPA concentration). The effect of pH on biodegradation was studied by adjusting BPA solutions (25 mg/L) to different pH values (4.5–8.0) with the addition of 0.1M HCl or NaOH. BPA degradation experiments were accomplished by using 10–100mg/L BPA concentrations. The amount of BPA degraded by the strain was calculated using modification of *D. Swapna Sundari et al.* [8] using the below mass balance equation:

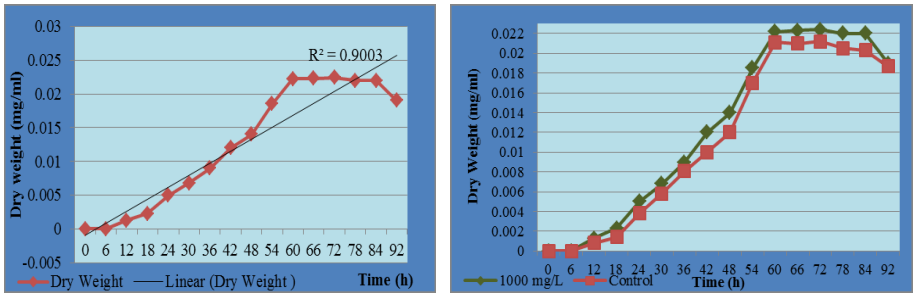
$$q_e = (C_0 - C_e)V/W, \quad (1)$$

where: $C_0$ is the initial of BPA concentration, $C_e$ is equilibrium of BPA concentrations in the solution (mg/g), V is the volume of the solution in liter (L) and W is the mass (g) of bacterial cells mass.

## Results and Discussion

### *Stability of Ps.Nr.22 in high BPA concentration*

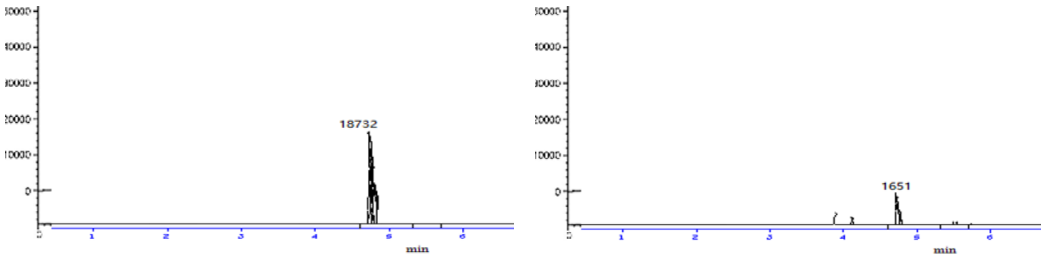
Potential of Ps.Nr.22 as bio-indicator as well as a natural 'bio-absorbant' for the BPA to be eradicated from water bodies has been studied and our results happens to support the hypothesis. Most researchers have demonstrated that several processes including the usage of hollow fibre microfiltration membrane, single walled carbon nanotubes–ultrafiltration, coagulation and adsorption, ultrafiltration, RO, NF and photo-catalysis are capable of removing BPA from aqueous environments with different removal efficiencies and strategies. Suggesting whole-cell usage for BPA removal would need strong background study thus could only be done with great cell stability in such an extreme environment. Figure 2a shows the growth profile of the Ps.Nr.22 with the first 6 hours of laq phase and steady growth from 54-92 hours of incubation. Figue 2b illustrates the growth of Ps.Nr.22 in control medium (without BPA) and in 1000mg/L BPA. The results have revealed that Ps.Nr.22 is showing the same significant growth curve with better growth stability in the high BPA concentration in 92 hours.




a. b.

**Fig. 2.** Growth profile of Ps.Nr.22:
a. the 92 hours incubation in 1000mg/L BPA;
b. Dry weight in 1000mg/L versus control in 92 hours of incubation.

Figure 3 shows the HPLC chromatogram of BPA metabolites at the early incubation and after 3 days of incubation. The recorded retention was 4.919min. Laccase has bio-degraded mostly 90% of the BPA in short 3 days using the nutrient broth support medium containing the peptone, beef extract and sodium chloride to support early growth and the very short laq phase at concentration varies from 250 to 1000mg/L.




a. b.

**Fig. 3.** HPLC Chromatogram of BPA metabolites:
a. at the early incubation (0 hour) with BPA 1000ppm was incubated with Ps.Nr.22 in nutrient broth (NB);
b. After 3 days of incubation. The retention time of BPA in the chromatogram was 4.919min.
pH and cell dosage effect on the BPA degradation

***Kinetic and mass balance interaction of the laccase enzyme using contact time and initial BPA concentration***

We have managed to point out the kinetic and mass balance interaction of the laccase enzyme expressed by the Ps.Nr.22 in the BPA degradation. The plot of BPA removal efficiency at various contact times is depicted in figure 4. It reveals a "one-stage kinetic" reaction through a total 92h of a rapid initial degradation without a significant of slower BPA uptake. The maximum removal efficiency was observed at 92h, which was then chosen as the experimental contact time for the whole study. Theoretically, kinetic reaction on enzymatic scales must go through a two-stage reaction with the rapid adsorption during the first few hours follow by much slower interaction on the next hours. Nevertheless, the interaction of laccase with BPA in this study has performed high activity was probably due to the abundant availability of active sites on the enzyme related to the surface of the BPA itself. The effect of initial BPA concentration ($C_0$) at different contact times (6-92h) on the bio-degradation of BPA is presented in figure 4. The percentage of removal (%R) decreased from 100 to 90.5±0.01% with the increment of $C_0$ in the range of 250-1000mg/L. This could have been contributed by the electrostatic repulsions between the negative surface charge of the laccase enzyme and the BPA anions in the solution.

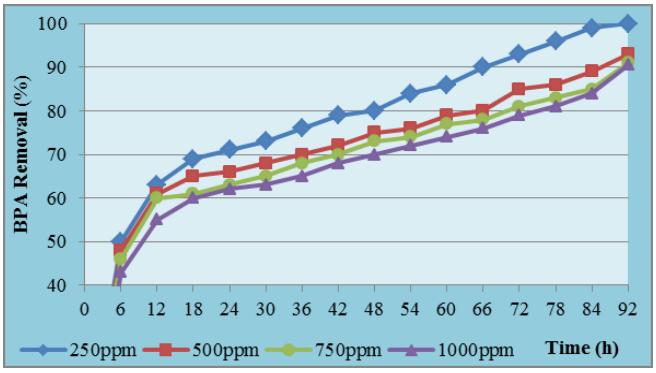



**Fig. 4.** Effect of contact time and initial BPA concentration to the BPA removal (%)

The BPA removal has followed the 1st-order kinetic enzyme; therefore by taking figure 5 as a one-stage kinetic reaction of a rapid BPA initial degradation, we will earn an equation namely Eq. 2, where A is the BPA and A* is the product of biodegradation as a whole single substrate-product interaction.

$$A \rightarrow A^* \text{ (ex: conformational change of a molecule)} \quad (2)$$

or

$$A \rightarrow B + C \text{ (dissociation of a molecule into two molecules)} \quad (3)$$

$$v = kAB \quad (4)$$

$$v = kA \quad (5)$$

$$dA/dt = -kA \quad (6)$$

$$A(t) = A_0 e^{-kt} \quad (7)$$

where: A is BPA

We might as well strongly believe that BPA has been degraded into two species of products following Eq. 3. By using the definition of the rate law given by the mass action law as $v = kAB$ (Eq. 4), we will get the rate of reactions as $v = kA$ (Eq. 5). When we continue with the time evolution of the concentration of the substrate A (BPA), the equation will be written as $dA/dt = -kA$ (Eq. 6) which will be then finalized as $A(t) = A_0 e^{-kt}$ (Eq. 7) after the integration. This is when $A_0$ is the initial concentration of substrate A [$A_0 = A(0)$].

Finally, we have observed an exponential decrease of the BPA concentration over time which is illustrated in figure 5.

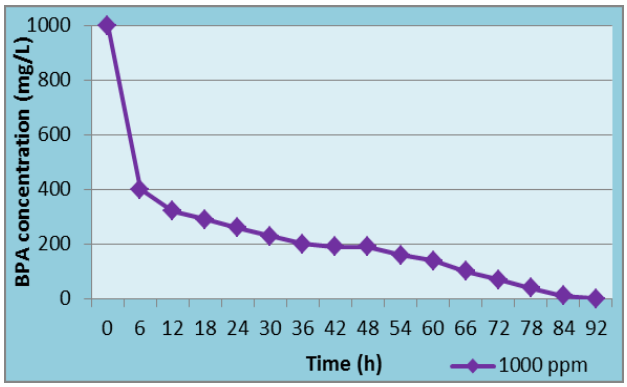



**Fig. 5.** 1st-order kinetics: exponential decrease of the BPA concentration

On the long run, BPA will be converged to 0, meaning it has been fully degraded. The half-life $\tau_{1/2}$ of the BPA is defined by the time it takes for its concentration to decrease from its initial value $A_0$ to half of this value, $A_0/2$ which is

$$A_0/2 = A_0 e^{-k\tau_{1/2}} \text{ therefore } \tau_{1/2} = \ln 2/k \qquad (8)$$

Note that $\tau_{1/2}$ does not depend on the initial value $A_0$. So half of BPA is around 6 min or lesser. One of the many things to understand about enzymes reaction is that they do not follow the law of mass action directly. As the concentration of substrate is increased, the rate of the reaction increases only to a certain extent, reaching a maximal reaction velocity at high substrate concentration. This is in contrast with the mass action law, which, when it is applied directly to the reaction containing the enzyme, it will predict the reaction velocity to increase linearly as the substrate increases. The original level of laccase produced by most species is relatively low in the absence of any inducer which in this study is referring to the BPA concentrations. Laccase (EC 1.10.3.2) belongs to a group of copper-containing polyphenol oxidases that can catalyze the four-electron reduction of $O_2$ to $H_2O$ coupled with the oxidation of phenolic compounds. As a ligninolytic enzyme produced by this species, laccase exhibits a broad substrate specificity and unique ability of biodegradation [4]. Laccase has been widely applied in many fields, such as delignification of lignocellulosic biomass, detoxification of recalcitrant pollutants, decolorization of industrial dyes and textile dye effluents, biological bleaching in pulp and paper industries, juice and wine clarification, and biosensors [5-7].

## Conclusion

In conclusion, the study of the kinetic and mass balance relationship of the BPA degradation by Pseudomonas aeruginosa Nr.22 (Ps.Nr.22) was well recorded. We have confirmed the species as a suitable alternative method of Bisphenol A (BPA) removal from aqueous solution and contaminated open water bodies

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