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Implication of Copper II Ion in the Modelling of Growth Kinetics of TBT-Resistant *Klebsiella* sp. FIRD 2

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Abstract

Tributyltin (TBT) is one of the most toxic substances ever deliberately introduced into the marine environment. It is an organotin compound mostly used as wood preservative, pesticide, bactericide, PVC stabilizer, fungicide, antifouling biocide in boat and ships paints to prevents attachments of the marine organism on the hull surface.We studied the TBT-Resistant Bacterium by *Klebsiella* sp. FIRD 2 containing copper II ion and modelled it using several kinetic models such as Monod, Haldane, Luong, Aiba, Teissier, Yano, and Webb and estimated the accuracy of the fitted model using statistical analysis such as coefficient of determination (R^2), adjusted coefficient of determination (adj R^2), and root mean square (RMSE). Aiba model was the best model to the experimental growth kinetics data determined and gave a very good fit. The estimated value for the Aiba constants in this work such as maximal growth rate, half inhibition constant, and half saturation constant rate designated as u_{max}, k_i and k_s were 0.1265 hr^{-1}, 8.061 mg/Land 0.8300 mg/L respectively. The true u_{max} where the gradient for the slope is zero for the Aiba model was approximately 0.061093 hr^{-1} at 1 mg/Lcopper.

Keywords: Growth Kinetic models, *Klebsiella* sp. FIRD 2, Copper, TBT-resistant bacteria.

INTRODUCTION

One of the most toxic substances often deliberately introduced into the aquatic environment is Tributyltin (TBT) (Andreia Cruz *et al.*, 2014). It has been used as additives in the preservation of wood, paper, and textile materials (Hoch, 2001). Furthermore, TBT has been widely used as the active component in antifouling paints used in boats and ship hulls to prevent attachments of the marine organism on the hull surface (Antizar-Ladislao, 2008; Abubakar *et al.*, 2015). As such, TBT escaping out from the paint soon became a global concern as this affects non-target organisms and those biomagnified in the food chain. Thus, constituting an important contaminant in aquatic environments (Hu *et al.*, 2006; Mohamat-yusuff *et al.*, 2014).The International Maritime Organization (IMO) in 2003, worked for the banning of TBT through Anti-Fouling System (AFS) Convention (Mohamat-yusuff *et al.*, 2014). In 2008, it has succeeded in banning the application of TBT-based paints which came into law. More than 60 countries including Malaysia signed the convention into law. Even though TBT has been banned worldwide, a relatively high concentration of TBT can still be found around coastal areas of Peninsular Malaysia (Harino *et al.*, 2008). The levels reported from the sediments at the Straits of Johor, Malaysia are among the highest in the ASEAN region (Harino *et al.*, 2008; Abubakar *et al.*, 2015).

Antizar-Ladislao, (2008) reported that TBT has adverse effects in both eukaryotic and prokaryotic organisms.

In eukaryotes for example, TBT has effect in imposex - superimposition of male characters onto gastropods females (Barroso*et al.,* 2000; Hoch, 2001)—and the disruption of endocrine in humans and inhibition of immune system (Dubey*et al.,* 2006). In prokaryotes for instance, it can be referred as the interference with biological membranes (Cooney and Wuertz, 1989; Cruz *et al.,* 2012), and the uptake of amino acids inhibition and growth (Jude e*t al.,* 2004). Normally, dibutyltin (DBT) and monobutyltin (MBT) compounds are less toxic than TBT, and seem to exert toxicity through their interaction with membrane lipids. It has been reported that organotins compounds are toxic to microorganisms such as Gram-negative and Gram-positive bacteria collected from sediment; however, the latter showed increased sensitivity to TBT (Mendo *et al.,* 2003).

Heavy metals are persistent and stable environmental contaminants since they cannot be destroyed or degraded. The mechanism whereby these heavy metals affects the environment, in the soil and plant relation has not been fully understood (Wyszkowska *et al.,* 2008). Thus, they tend to accumulate in soils and sediments (Montuelle *et al.,* 1994). Some heavy metals (zinc, cadmium, manganese, nickel, copper) for example are required in trace amounts as nutrients, they become strongly inhibitory for microorganisms at relatively low concentrations (Nies, 1999; Sevgİ *et al.,* 2010). Some such as copper, lead, cadmium have no biological role and are harmful to the microorganisms even at very low concentration. The toxicity exerts its effect through ligand interactions or through essential metals displacement from their active binding sites, which result in the interference with oxidative phosphorylation, alterations in the conformational structure of proteins and nucleic acids(Said and Lewis, 1991; Bruins*et al.,* 2000). This study has therefore aimed at describing the effect of various copper concentrations on the growth kinetics of TBT-resistant bacterium; *Klebsiella* sp. FIRD 2.

Materials and Methods

Bacterial Strain

TBT-resistant bacterium was previously isolated from contaminated surface sediment at Kong Kong Laut along Strait of Johor, Malaysia. The bacterium was identified as *Klebsiella* sp. FIRD2 (Abubakar *et al.*, 2015). The isolate was maintained on slants/plates agar containing Bactor Agar 25gL^{-1}added to the minimal salt media. The isolate was maintained and sub-cultured every ten days in the Bactor Agar medium.

Chemicals and Media

All chemicals were used without additional purification. Purchased from Sigma-Aldrich Co. USA. Tributyltin chloride (TBTCl) 96%, Methanol HPLC grade. All kinetic experiments were conducted with the Minimal Salt Media containing (gL^{-1}): 5 NaCl, 5 NH_4Cl, 0.01 $CaCl_2$, 0.2 $MgSO_4.7H_2O$, 0.01 $FeSO_4.7H_2O$, 1.0 KH_2PO_4, 5-yeast extract. The media contains1000 μg/L of TBT and various concentrations of copper in addition to the above compositions. TBT stock was prepared from 96% TBTCl, 21,000 μg/L (1μL in 20 cm^3 methanol) and kept in the dark at 4°C (Wuertz *et al.,*1991). All gradient concentrations were prepared using the dilution formulae $M_1V_1=M_2V_2$ from the stock.

Kinetic experiments

Shake flask studies were carried out in 250 cm^3 Erlenmeyer flask containing 50 cm^3 of the TBT medium was incubated on a rotary shaker at 150 rpmfor 48 hr at room temperature (27ºC).Samples were drawn after every 6 h and TBT growth-containing copper was measured. The initial temperature and pH of the medium was adjusted to room temperature and neutral pH, which were the environmental temperature and pH. The seed culture was transferred to 25cm^3 of TBT liquid media containing various initial copper concentrations ranging from 1 to 100 mg/L in the 250 cm3 Erlenmeyer flask. Samples were collected at different time intervals and measured for cell growth (Gokulakrishnan and Gummadi, 2006;Agarwal *et al.,* 2009; Ahmad *et al.,* 2015; Ibrahim *et al.,* 2015).

Mathematical Model

Agarwal *et al.,*(2009) reported that Inhibition of substrate occurs at high substrate concentrations. Primarily, it is caused by more than one substrate molecule binding to the active site, and/or often by different parts of the substrate molecules binding to different sub-sites within the substrate-binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction (Agarwal *et al.,* 2009). At various initial copper concentrations, the specific growth rates (μ) have been calculated and data were tested in different deterministic models as per dms. The dms shows different substrate inhibition models that have been used to explain cell growth kinetics of copper. All the kinetic models were fitted to the experimental data by using a curve fitting toolbox available from MATLAB R2012a based on Windows vista (Singh *et al.,* 2008).

dms: Growth models considered for the data evaluation

Author	μ (Growth rate)	References
Monod	$\mu_{max}\dfrac{S}{K_s + S}$	(Monod, 1949)
Haldane	$\mu_{max}\dfrac{S}{S + K_s + \left(\frac{S^2}{K_i}\right)}$	(Haldane, 1930)
Luong	$\mu_{max}\dfrac{S}{K_s + S}[(1 - (\frac{S}{S_m})^n]$	(Luong, 1987)
Aiba	$\mu_{max}\dfrac{S}{K_s + S}exp^{(\ S/K_i)}$	(Aiba et al., 1968)
Teissier	$\mu_{max}\left(1 - \exp\left(-\frac{S}{K_s}\right)\right)$	(Teissier, 1942)
Yano	$\dfrac{\mu_{max\,S}}{S + K_s + \left(\frac{S^2}{K_I}\right)(1 + \frac{S}{K})}$	(Yano*et al.*, 1966)
Webb	$\dfrac{\mu_{max}S(1 + (\frac{S}{K}))}{K_s + S + (\frac{S^2}{K_I})}$	(Webb, 1963)

Substrate inhibition occurs generally at high substrate concentrations. It is primarily caused by more than one substrate molecule binding to an active site, and/or often by different parts of the substrate molecules binding to different sub-sites within the substrate-binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction cell growth kinetics.

The formula for various kinetics models as indicated by dms where S, S_m, K_s, K_i, μ, μ_{max}, and n are specific substrate concentration (mg/L), the above critical substrate concentration above which cell growth of TBT-resistant bacterium containing copper completely stops (mg/L), cell growth rate (hr^{-1}), maximum cell growth rate (hr^{-1}), saturation constant or half velocity constant (mg/L), inhibition constant (mg/L), and the exponent representing the impact of the substrate to μ_{max}, respectively. For each initial concentration of copper, specific growth rate was calculated based on the linear portion of the growth against time in an exponential phase. The specific growth rate (μ) in exponential phase was calculated by the following equation:

$$\mu = \frac{X_2 - X_1}{t_2 - t_1} \qquad (1)$$

where X_1 and X_2 are the cell dry weight obtained at time t_1 and t_2, respectively. All experiments were conducted in triplicates under identical conditions and all results had a mean standard deviation (Gokulakrishnan and Gummadi, 2006).

RESULTS AND DISCUSSION

In sites co-contaminated with organic compounds and heavy metals, heavy metal toxicity inhibits the activity of organic degrading microorganisms, impacting both their physiology and ecology, thus reducing the growth and biodegradation rate of the organic compounds(Roane *et al.*, 2001; Okpokwasili and Nweke, 2005). Heavy metals toxicity to microorganisms is dependent on its bioavailability.

Quantification of bioavailable metal concentration is an important step in the process of standardizing experiments to determine the impact of metals on organic pollutant growth and biodegradation (Okpokwasili and Nweke, 2005).The effect of various copper concentrations on TBT-resistant growth kinetics was determined by measuring the cell growth rate at different times interval for 48 hr with different initial concentrations of the heavy metals.

Effect of Various Copper Concentrations on the Growth Rate of Cultures

Figure 1 shows the result for TBT-resistant bacterial growth curve of *Klebsiella* sp. FIRD 2 at various copper concentrations. The bacterial growth decreased with an increase in copper concentration, thus, reaching an optimum concentration at 1 mg/L. Therefore, it can be concluded that copper has a significant effect on the growth of the bacterial isolate. At a concentration of 100 mg/L, it completely inhibits the growth of *Klebsiella* sp. FIRD 2 while at a concentration of 1 mg/L the bacterial isolate grows optimally with an OD_{600} of 1.489 as shown in Figure 1.Copper concentration has been shown to have an inhibitory effect at higher concentration (Sevgİ *et al.*, 2010).

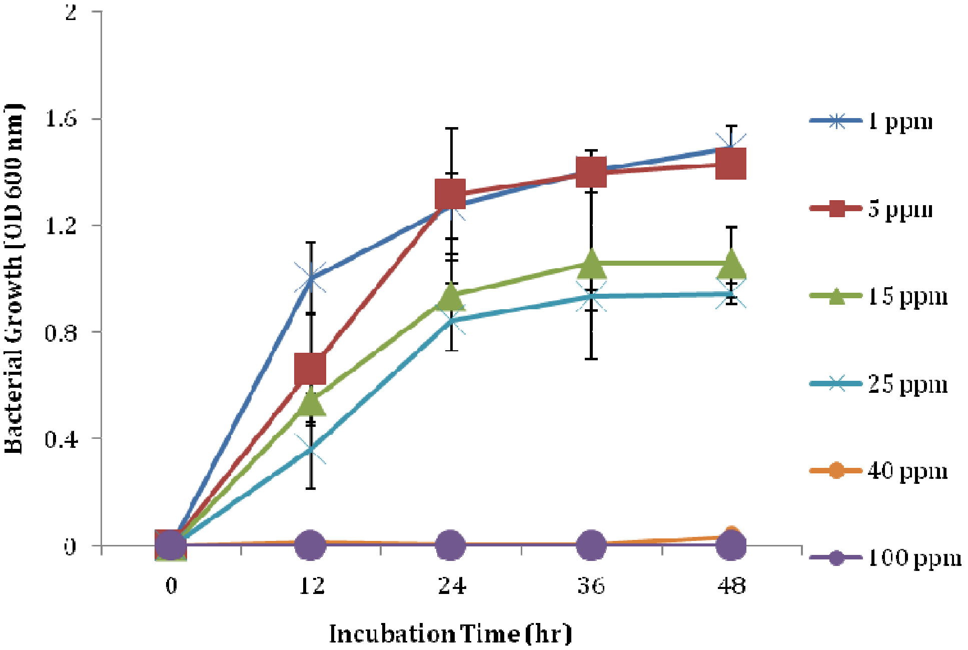

Figure 1. Effect of various copper concentrations on *Klebsiella* sp. FIRD 2 growth containing TBT. Data represent mean ± STDEV, n=3.

Effect of Initial Substrate Concentration on Specific Growth Rate

Based on the growth curves of *Klebsiella* sp., the specific growth rate (μ) for each initial copper concentration (S) was calculated. The plot shows a definite increase in cell growth rate with increase in copper concentration until 1 mg/L, beyond which there was a decrease in cell growth rate as copper concentration increased, signifying copper inhibition kinetics. A plot of specific growth rate vs. specific substrate consumption rate containing copper (Figure 2) according to Equation (1) was used to evaluate the growth of the isolate, which is a valuable tool in biotechnology. This relationship is represented **by** a set of empirically derived rate laws referred to as theoretical models. These models are nothing but mathematical expressions generated to describe the behavior of a given system (Okpokwasili and Nweke, 2005; Ibrahim *et al.*, 2015). The classical models, which have been applied to microbial population growth, include the Gompertz and Verhulst function (Gompertz, 1825; Okpokwasili and Nweke, 2005). The Gompertz function was originally formulated for actuarial science for fitting human mortality data but it has also been applied deterministically to organ growth (Causton, 1977). The Gompertz function is based on an exponential relationship between specific growth rate and population density (Okpokwasili and Nweke, 2005).

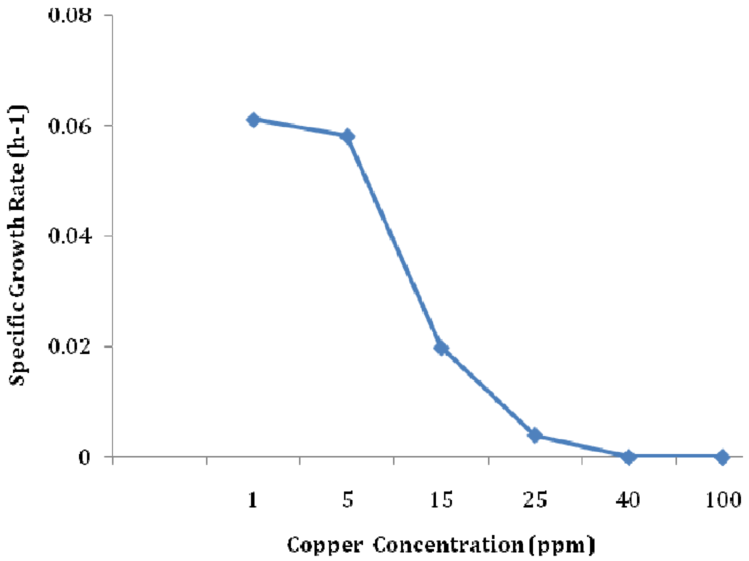

Figure 2. Specific growth rate as a function of initial substrate (TBT) concentration containing copper

Exploration of best-kinetic model for *Klebsiella* sp. FirD2 containing copper

Figure 3 showed the comparative plots of the model predicted growth rates and the experimental one solved by MATLAB R2012a as given in Table 1. Among various models used to fit the present experimental data for different initial copper concentration verses specific growth rates. All of the other models tested apart from Luong and Monod gave reasonably good fitting based on software output and by visual observation. Aiba model showed fit well reasonably as determined by correlation coefficient R^2, Adjusted R^2, and root mean square error (RMSE), it have highest R^2 and Adjusted R^2 value and lowest RMSE value as shown in Table 2. This could be attributed based on the models themselves, which are considered more refined from the standpoint of development of these models (Dey and Mukherjee, 2010).

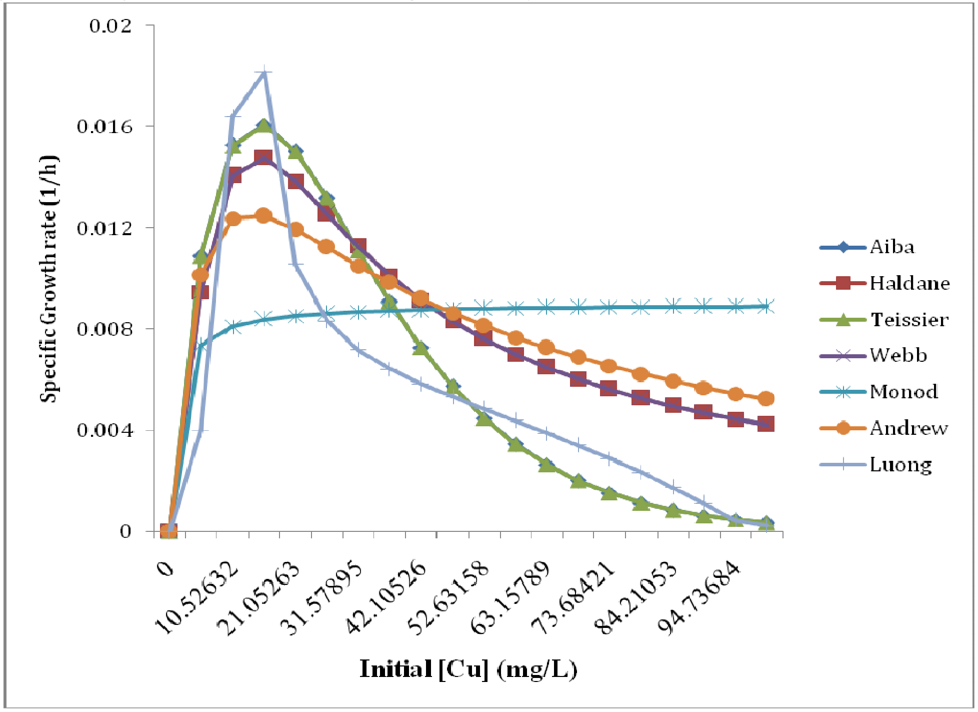

Figure 3. Fitting experimental data with the different kinetics model.

The growth kinetic constant of the batch culture as shown in Table 2 reports the value μ_{max} and *Ks* by nonlinear regression method as per Monod. Halmi *et al.*, (2014) reported the needs to be cautioned that the μ_{max} value obtained based on curve fitting interpolation is not the true value as the true μ_{max} should be where the gradient for the slope is zero and in this case, the calculated value for the Aiba constants in this work such as the half inhibition constant, maximal growth rate, and the Monod half saturation constant, symbolized by k_i, μ_{max}, and k_s, were 8.0610 mg/L,0.1265 hr^{-1}, and 0.8300 mg/L, respectively. In addition, Luong model predicted maximum substrate inhibitory concentration rate (*Sm*) of 99.89 mg/L that was closer to the experimental value of 100 mg/L but gave a poor R^2 value of 0.5837, and the exponent representing the impact of the substrate to μ_{max} (n). The constant n was found to be 1.727, indicating a non-linear correlation between specific growth and the initial substrate concentration.

Table 2. Parameters estimation for different substrate-inhibition models

Model	μ_{max}(h-1)	*Ks* (mg/L)	*Ki*(mg/L)	K(mg/L)	S_m(mg/L)	R^2	Adjusted R^2	RMSE	n
Haldane	1.12	13.5	0.2854			0.9616	0.9424	0.006644	
Teissier	0.999	2.342	2.915			0.9386	0.9078	0.008401	
Monod	0.016	0.7384				0.5134	0.416	0.02115	
Yano	0.3323	4.364	26.32	0.2782		0.9779	0.9558	0.005816	
Luong	0.01974	0.6812			99.89	0.5837	0.1675	0.02525	1.727
Aiba	0.1265	0.83	8.061			0.999	0.9985	0.0020445	
Webb	0.7755	9.125	0.4216	5.366		0.9597	0.9193	0.007861	

Initially, Aiba model was proposed for product inhibition in alcohol fermentation, where specific growth rate decreases as the product concentration increases (Aiba *et al.*, 1968).Exponential term to take care of the product inhibition could be well replaced with substrate concentration. Aiba's exponential model, though has been widely used to analyze product inhibition, fails to give the critical value of inhibitory substrate/product concentration(Agarwal *et al.*, 2009).

Conclusion

The growth kinetics of *Klebsiella* sp. FIRD 2 containing copperdoes not follow a simple Monod's kinetics. Substrate inhibition is exhibited in batch experiments carried out in a shake flask and it was found that Aiba model was able to describe the growth kinetics with an R^2 of 0.999. The maximum growth rate of this bioprocess in batch was found to be 0.1265hr^{-1}, while the half inhibition constant, and the Monod half saturation constant, symbolized by k_i, and k_s, were 8.0610 mg/L, and 0.8300 mg/L, respectively. Thus, *Klebsiella* sp. FIRD 2 is resistant to copper concentration within the range of 1 - 40 mg/dl.

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