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Modelling Growth Kinetics of *Klebsiella* sp. FIRD 2 on TBT-Resistant Containing Lead

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ABSTRACT: Tributyltin (TBT) is one of the most toxic substances ever deliberately introduced into the marine environment. The high toxicity of TBT has resulted in a wide range of adverse effects on biological systems ranging from bacteria to mammals and from the molecular to the community level. One of the most deleterious effects of TBT is imposex. The growth kinetics of TBT-Resistant Bacterium containing lead was studied. In this study various lead concentrations ranging from 1 to 100 mg/dm^3 were used. Seven kinetic models (Teissier, Monod, Yano, Luong, Aiba, Webb, and Haldane,) were investigated and the accuracy of the fitted models were evaluated using statistical analysis such as coefficient of determination, adjusted coefficient of determination (R^2) and root mean square (RMSE). Aiba model was fitted to the experimental growth kinetics data and gave a very good fit with an R^2 of 0.98 and RMSE of 0.0042 respectively. The calculated value for the Aiba constants such as maximal growth rate, half saturation constant and half inhibition constant rate symbolized by μ_{max}, k_s, and k_i, were 0.038 hr^{-1}, 0.38s mg/dm^3 and 34.38 mg/dm^3 respectively. This is the first report of growth kinetics of TBT-Resistant bacterium by *Klebsiella* sp. FIRD 2 Containing lead. © JASEM

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

Tributyltin (TBT) is one of the most toxic anthropogenic compounds deliberately introduced into the environment. It has been extensively used as additives in antifouling paints, plastics, biocides and wood preservatives (Hoch, 2001). It has been reported that TBT has some harmful effects in both prokaryotic and eukaryotic organisms (Antizar-Ladislao, 2008). Inhibition of immune system, endocrine disruption in humans (Dubey *et al.,* 2006) and imposex - superimposition of male characters onto gastropods females are some examples of the toxic effect of TBT in eukaryotes (Barroso *et al.*, 2000; Hoch, 2001). Inhibition of amino acids uptake and growth and interference of TBT with the biological membrane is an example in prokaryotes (Cruz *et al.,* 2012; Jude *et al.,* 2004).

Due of its toxicity to marine organisms, the International Maritime Organization (IMO) in 2008 totally contraband the use of TBT as a constituent of antifouling paints. Yet, there are continuing high concentrations in sediments from fresh and marine waters in many places across the globe including the Strait of Johore, Malaysia (Abubakar *et al.,* 2015; Harino *et al.,* 2008; Zulkifli *et al.,* 2010).

A fraction of heavy metals are essential as trace elements or trace metals to all living organisms, but excessive concentrations can cause severe toxic effects to microorganisms such as bacteria, fungi, algae etc (Poli *et al.,* 2009). The existence of heavy metals at the contamination site is a major restrictive factor for bioremediation, as many organisms cannot withstand high concentration of heavy metals thus losing their capacity to degrade the contaminants (Ibrahim *et al.,* 2015a). Although relatively high levels of these elements occur in natural environment, their presence as a contaminant in ecosystems results mainly from anthropogenic activities (Lima e Silva *et al.,* 2012; Trevors *et al.,*1985).

Some heavy metals such as zinc, copper, nickel, and iron are essential to metabolic reactions and are required as trace elements by the living organisms. Others like lead, cadmium, mercury, and silver have no apparent biological role and are harmful to the organisms, even at very low concentrations (Hughes & Poole, 1989). The toxic effects of heavy metals on microorganisms are persuaded by a multitude of factors such as organic matter, speciation, concentration of chelating agents, and pH (Duxbury,

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1986; Nwuche and Ugoji, 2008). The existence of those elements in the environment can result in impacts on ecosystems, with changes in the biomass, diversity of microbial communities and cycling of elements (Lima e Silva *et al.,* 2012; Sobolev and Begonia, 2008). There are a lot of published papers describing the action of heavy metals on microorganisms. As such, there are studies on the effects of toxic metals in the physiology of metal tolerant bacteria, in comparison to those about their inhibitory or deleterious effects on susceptible organisms (Gupta *et al.,*1992). This paper describes the effect of various lead concentrations on the growth kinetics of TBT-resistant bacterium; *Klebsiella* sp. FIRD 2.

MATERIALS AND METHOD

Bacterial strain: Previously, isolated TBT-resistant bacterium 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 25 gL^{-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***:** Tributyltin chloride (TBTCl) 96%, was purchased from Sigma, Aldrich USA. Other chemicals used are analytical grade that were obtained from recognized chemicals suppliers, Merck (Darmstadt, Germany) and Fisher (Malaysia). The Minimal Salt Media used contained the following (in g/L): 5 NH_4Cl, 0.01 $CaCl_2$, 1.0 KH_2PO_4, 0.01 $FeSO_4.7H_2O$, 0.2 $MgSO_4.7H_2O$, 5 NaCl, 5-yeast extract. In addition to the above compositions, the media contains 1000 µg/L of TBT and various concentrations of lead. Carbon sources if any added to the medium were sterilised separately and then mixed to the medium under aseptic conditions.

Growth Kinetics Experiments: Batch experiment was carried out using a shake flask in a 250 mL Erlenmeyer flask containing 50 mL of the TBT medium was incubated on a rotary shaker at 150 rpm for 48 hr at room temperature. Samples were drawn after every 6 hr and TBT growth containing lead 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 25mL of TBT liquid media containing various initial lead concentrations ranging from 1 to 100 mg/dm^3 in the 250 mL Erlenmeyer flask. Samples were collected at different time intervals and measured for cell growth (Agarwal *et al.,* 2009; Ahmad *et al.,* 2015; Gokulakrishnan and Gummadi, 2006; Ibrahim *et al.,* 2015b).

Statistical Analysis: To decide whether there is a statistically substantial difference between models with different number of parameters, in terms of the quality of fit, the same experimental data was statistically assessed through various methods such as the root-mean-square error (RMSE), coefficient of determination (R^2) and the adjusted coefficient of determination (R^2) (Halmi *et al.*, 2014).

Mathematical Model**:** In this study, the kinetic models as listed in Table 1 were used to represent the kinetics of lead. 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).

The rate of bacterial growth and degradation can be represented as cell production rate. The formula for various kinetics models is as shown in Table 1 where S, S_m, μ, μ_{max}, K_s, K_i, and n are the specific substrate concentration (mg/dm^3), the above critical substrate concentration above which cell growth of TBT-resistant bacterium containing lead completely stops (mg/dm^3), cell growth rate (hr^{-1}), maximum cell growth rate (hr^{-1}), saturation constant or half velocity constant (mg/dm^3), inhibition constant (mg/dm^3), and the exponent representing the impact of the substrate to μ_{max}, respectively. For each initial concentration of lead, 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 the values were expressed as mean standard deviation (Gokulakrishnan and Gummadi, 2006).

Table 1: Various kinetic models for effect of substrate on TBT cell growth containing various lead concentrations

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

RESULT AND DISCUSSION

Effect of Initial Concentration on Growth Curve: Figure 1 shows the time course profile of the TBT-resistant growth curve of *Klebsiella* sp. FIRD 2 by the shake flask culture. The culture is able to resist upto 40 mg/dm^3 lead in almost 48 hr. It was observed that the time taken for the culture to grow in the presence of lead is depended on its initial concentration of the lead. It was found that the bacterial growth decreased with an increase in lead concentration. At a concentration of 1 mg/dm^3 the bacterial isolate grows optimally with an OD_{600} of 1.388 while at a concentration of 100 mg/dm^3, it completely inhibits the growth of *Klebsiella* sp. FIRD 2 as shown in Figure 1

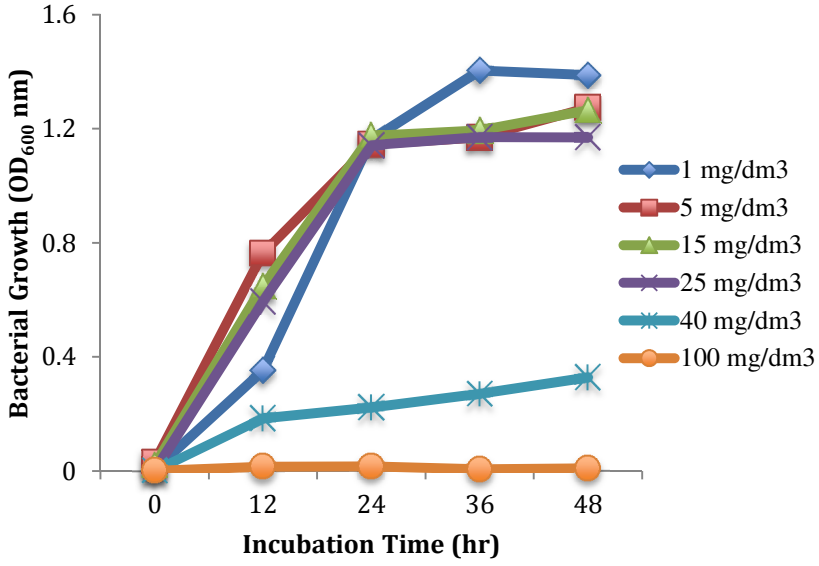

Fig 1: Effect of various lead concentrations on *Klebsiella* sp. FIRD 2 growth containing TBT. Data represent mean ± STDEV, n=3.

Microbial Growth Kinetics: Based on the growth curves of *Klebsiella* sp. FIRD 2 (Figure 1), the specific growth rate (μ) for each initial lead concentration (S) was calculated. One of the valuable tool in biotechnology is the relationship between the specific growth rate (μ) of a population of microorganisms and the substrate concentration (S) which are 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.,* 2015b).

The results of the curve fitting are shown in Figure 2.

Models such as Luong and Monod failed to fit the experimental data and were omitted. All of the other models tested gave reasonably good fitting based on software output. The accuracy and statistical analysis of the six kinetic models used showed that the best model was Aiba with the highest value for adjusted R^2 and lowest value for RMSE. The R^2 and adjusted R^2 values were also excellent for Aiba with their values being close to 1.0 as shown in Table 2, which 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).

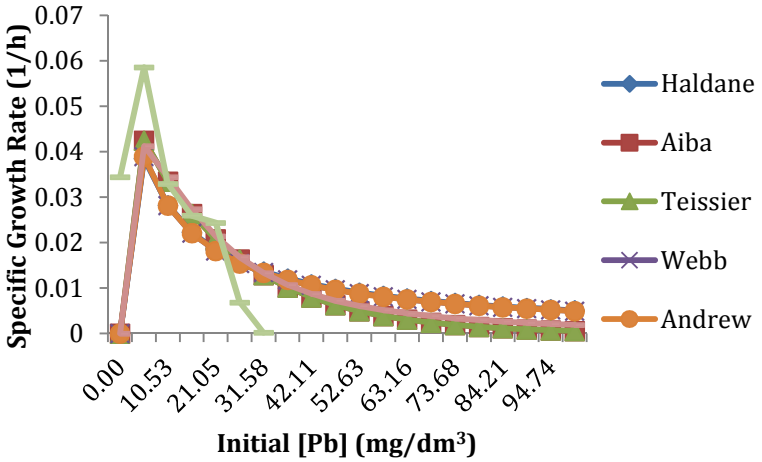

Fig 2: Fitting experimental data with the different kinetics model.

The growth kinetic constant of the batch culture as shown in Table 2 reported the value μ_{max} and *Ks* by nonlinear regression method as per Monod. The calculated value for the Aiba constants in this work such as maximal growth rate, half saturation constant and half inhibition constant rate symbolized by μ_{max}, k_S, and k_i, were 0.03759 hr^{-1}, 0.3748 mg/dm^3 and 34.38 mg/dm^3, respectively.

The μ_{max} value obtained needs to be cautioned, as the 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 (Halmi *et al.,* 2014) and in this case, Aiba value was approximately 0.0424 hr^{-1} at 5.2632 mg/dm^3 lead (Figure 2). The constant n was found to be 1.234, indicating a non-linear correlation between specific growth and the initial substrate concentration.

Table 2: Kinetic Model Parameter

Model	μ_{max} (hr^{-1})	K_s (mg/dm^3)	K_i(mg/dm^3)	K(mg/dm^3)	S_m(mg/dm^3)	R^2	Adjusted R^2	RMSE	n
Aiba	0.038	0.38	34.38			0.98	0.96	0.0042	
Webb	0.079	0.47	1.017	6.03		0.83	0.65	0.025	
Monod	0.018	0.71				0.75	0.69	0.012	
Yano	0.035	0.43	22.3	1.89		0.95	0.89	0.007	
Luong	0.048	0.38			94.23	0.97	0.94	0.005	1.23
Teissier	1.022	8.40	9.18			0.42	0.13	0.019	
Haldane	0.043	0.32	16.64			0.96	0.93	0.006	

In most of the studies concerning substrate inhibition on microbial growth are carried out using toxic substrate such as aromatic hydrocarbons and hence it can be concluded that at high concentration growth rate will be severely affected and the normal use of the monod model will fail (Halmi *et al.,* 2014). Lead currently have no known biological function in bacteria and may disturb the normal functioning of an organism if bioaccumulate (Hynninen, 2010). Pb^{2+} cause toxicity by interacting with nucleic acids, by binding to essential respiratory proteins (Vallee and Ulmer, 1972), and through oxidative damage by production of reactive oxygen species (Stohs and Bagchi, 1995). Pb^{2+} enter bacterial cells via transport systems for essential divalent cations, such as Mn^{2+} (Hynninen, 2010; Tynecka *et al.,* 1981) and Zn^{2+} (Grass *et al.,* 2002; Laddaga and Silver, 1985).

As described by Wayman and Tseng, (1976), there were other models for describing substrate kinetics inhibition established during this period, such as the discontinuous models. Discontinuous model was developed due to the fact that in the previous models developed (Webb, Andrews And Noack, and Haldane), only inhibitory effect on microbial growth were described but could not explain or adequately model for certain situations where the growth rate completely ceased or becoming zero at very high substrate concentration. While Luong in 1987 developed a continuous type of the model above that has popular support due to its close agreement to experimental data in some cases (Halmi *et al.,* 2014; Hamitouche *et al.,* 2012; Nickzad *et al.,* 2012; Othman *et al.,* 2013). A central attraction of the Luong model is its ability to successfully predict the value of S_m, the maximum substrate concentration above which growth is completely inhibited.

Conclusion: The kinetics of *Klebsiella* sp. FIRD 2 on TBT-Resistant containing lead was studied under aerobic condition. The kinetic models were fitted to the experimental data and kinetic parameters were determined. It was observed that the best model that fit the present study is Aiba model having the highest R^2 value of 0.98 and lowest RMSE value of 0.0042 and predicting reasonable kinetic coefficient values. Amongst all the kinetic models, Teissier gave a poor R^2 of 0.42.

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