UJMR, Volume 2 Number 1 June, 2017 *ISSN: 2616 - 0668*
https://doi.org/10.47430/ujmr.1721.023
Received: 25th Feb, 2017 **Accepted:** 5th Apr, 2017

Growth Kinetics Modelling of Tributytin-Resistant *Klebsiella* SP. FIRD 2 In Cadmium Media

Abdussamad Abubakar[1*], Nazeef Idris Usman[1], Hadiza Ibrahim[2], Abdullahi Muhammad[3], Usman Sunusi[4], Ferdaus Mohamat-yusuff[5], and Salihu Ibrahim[3]
[1]Department of Microbiology, Faculty of Science, Bauchi State University, PMB 65, Itas Gadau Bauchi, Nigeria
[2]School of Dental Health Technology, Shehu Idris College of Health Science and Technology P.M.B 1050 Makarfi, Kaduna State, Nigeria
[3]Center For Biotechnology Research, Bayero University, PMB 3011 Kano, Nigeria
[4]Department of Biochemistry, Faculty of Basic Medical Science, Bayero University, PMB 3011 Kano, Nigeria
[5]Department of Environmental Sciences, Faculty of Environmental Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
*Corresponding author
Phone no.: +2348059535279; Email: abdussamadabubakar@yahoo.com

Abstract
Tributyltin (TBT) has been generally used as component of antifouling biocide in boat and ship paints to prevent the attachment of marine organism on the hull surface. TBT has been classified to be a very toxic compound, and poses significant danger to a broad diversity of organisms in the polluted environments due to the high concentrations. The growth kinetic of TBT-Resistant Bacterium containing cadmium was studied. In this study various cadmium concentrations ranging from 1 to 100 mg/L were used. Seven kinetic models (Haldane, Teissier, Monod, Yano, Luong, Aiba and Webb) were investigatedand the accuracy of the fitted model were evaluated using statistical analysis such as coefficient of determination, adjusted coefficient of determination (R^2) and root mean square (RMSE). Luong model were fitted to the experimental growth kinetics data and gave a very good fit. The calculated value for the Luong constants such as maximal growth rate, half saturation constant and half inhibition constant rate symbolized by u_{max}, k_s, and k_i, were 0.03405 hr^{-1}, 0.3 mg/L and 0 mg/L, respectively. Luong model also predicted the significant substrate concentration (S_m) value, at which specific substrate degradation rate falls to zero (98.93 mg/L). This is the first report of growth kinetics of TBT-Resistant bacterium by *Klebsiella* sp. FIRD 2 Containing Cadmium
Keyword: Cadmium, Growth, Kinetics models, *Klebsiella*sp. FIRD 2, Luong, TBT-resistant bacteria.

INTRODUCTION

Tributyltin (TBT) is an organotin compound mostly used as wood preservative, pesticide, bactericide, PVC stabilizer, fungicide, antifouling biocide in boat and ships paints to prevent attachments of the marine organism on the hull surface(Abubakar et al., 2015; Antizar-Ladislao, 2008; Andreia Cruzet al., 2007; Harino et al., 2008). The International Maritime Organization (IMO) in 1990s have banned the production, use, and export of TBT in developed countries, although some countries have continued to utilize this agent until its complete ban was effected in 2008 due to its toxicity (Rudel *et al*., 2003). More so, there are high concentrations in sediments from fresh and marine waters in many places across the world including north-west coast of Portugal, Strait of Johore, Malaysia, South Africa, Australia among others (Andreia Cruz et al., 2007; Du *et al*., 2014). TBT has deleterious effects in both prokaryotic and eukaryotic organisms (Antizar-Ladislao, 2008). 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 *et al*., 2004; Singh and Bragg, 1979). In eukaryotes, TBT effects imposex—superimposition of male characters onto gastropods females (Barroso *et al*., 2000; Gibbs and Bryan, 1996)and the immune system inhibition and endocrine disruption in humans (Dubey *et al*., 2006).

The environmental concentration levels, chemical and physical properties, distribution, toxicity, and human exposure of TBT in marine systems have been well studied and documented (Antizar-Ladislao, 2008; Bangkedphol *et al.,* 2009; Blackmore and Morton, 2001). However, most of published works regarding TBT compounds are mainly focusing on their toxicity levels, environmental fates, and properties. Efforts have been made to clean-up of the TBT-contaminated environments, since the discovery of the adverse effect of TBT antifouling agent on untargeted aquatic organisms.One of the major environmental concerns is the contamination of soil and marine generated by human activities due to the disposal of urban and industrial wastes. Metals are directly or indirectly involve in all aspects of growth, metabolism and differentiation of the biota. Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron manganese and zinc etc.) and are known as 'trace elements' (Bruins *et al.,*2000). Some heavy metals are harmful to microorganisms even at low concentration (zinc, cadmium, lead, copper, etc). Some have no biological role and are harmful to the microorganisms even at very low concentration (cadmium, copper, lead etc.). However, at high levels both of the essential and non-essential metals become toxic to the microorganisms(Nath *et al.*, 2012). Increasingly, heavy metals are found in microbial habitats due to several anthropogenic and natural processes. As such, in 1990,Gadd reported that microorganisms have developed mechanisms to tolerate the presence of heavy metals by either efflux or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration(Gadd, 1990). Contamination of marine by heavy metals has become a serious problem due to the increase in the addition of these metals to the marine, which cannot be degraded by microorganisms. These heavy metals not only influence the microbial population by affecting their biochemical activities, morphology and growth which result in the biomass and diversity decreased(Roane and Pepper, 2000), but also animals and plants, and the degree of toxicity varies from one microorganisms to another. Giller *et al.,*(1998) reported that the microbial metabolic activity and diversity might also decrease due to the presence of these metals, as well as affecting the quantitative and qualitative structure of microbial communities.

Microorganisms do not readily absorbed cadmium or captured them. These metals can damage the cell membranes, thereby changing the structure of the DNA,disrupt cellular functions, and alter enzymes specificity (Nath *et al.*, 2012). These heavy metals can exert their toxicity as a result of alterations in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Poole and Gadd, 1989). More so, they can also exert their toxicity through ligand interactions or displacement of essential metals from their native binding sites (Bruins *et al.,* 2000).

Previously, we have isolated a new TBT-resistant bacterium from contaminated surface sediment along Strait of Johor, Malaysia. The bacterium was identified as *Klebsiella* sp. FIRD2 (Abubakar *et al.,* 2015). Several works were reported on TBT-resistant, growth and degradation processes. Astoundingly, no work have so far been published on growth kinetics of TBT-resistant bacteriumcontaining cadmiumbased on specific substrate consumption. This works present the first report on growth ability of *Klebsiella* sp. FIRD2 containing various cadmium and the effect of initial substrate (TBT) concentration on its degradation.

MATERIALS AND METHODS

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, Fisher (Malaysia) and Merck (Darmstadt, Germany). TheMinimal Salt Mediaused contained the following (in g/L): 1.0 KH_2PO_4, 0.2 $MgSO_4.7H_2O$, 5 NH_4Cl, 0.01 $FeSO_4.7H_2O$, 0.01 $CaCl_2$, 5 NaCl, 5-yeast extract. The media contains1000 μg/L of TBT and various concentrations of cadmium in addition to the above compositions. Carbon sources if any added to the medium were sterilised separately and then mixed to the medium under aseptic conditions. For solid medium, (15 g/L) Bactor Agar was added to the minimal salt media. The isolates were maintained and sub-cultured in the Bactor Agar medium.

Flask Culture Experiments

A single colony of the strain from TBT agar plates was transferred to 5 mL sterile TBT medium. The tubes with cotton plugs were aerated on a rotary shaker at 150 rpm and incubated for 24 h at room temperature. Few percentage (%) of the culture was transferred to 50 mL of the TBT medium containing cadmium in 250 mL Erlenmeyer flasks and incubated on a rotary shaker at 150 rpm for 48 h at 30°C. Samples were collected after 6 h and TBT growth containing Cadmium were measured.

Growth Kinetics Modelling Experiment

Batch experiment was carried out using a shake flask studies at optimal conditions for TBT-resistant bacterium *Klebsiella* sp. FIRD 2. The flask was incubated for 48 h at room temperature and 150 rpm. The seed culture was transferred to 25 mL of TBT liquid media containing various initial cadmium concentrations ranging from 1 to 100 mg/L in 100 mL Erlenmeyer flasks and incubated on a rotary shaker at 150 rpm and at room temperature. 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.*, 2015a). In this study, the kinetic models as listed in Table 1 were used to represent the kinetics of cadmium. 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 1where S, S_m, μ, μ_{max}, K_s, K_i, and n are specific substrate concentration (mg/L), the above critical substrate concentration above which cell growth of TBT-resistant bacterium containing cadmium 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 cadmium, 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).

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

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

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 to 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).

RESULTS AND DISCUSSION

The TBT-resistant growth kinetics containing cadmium was determined by measuring the cell growth rate at different times for 48 h at different initial concentrations of the heavy metals.

Cadmium Growth Kinetics

For TBT-resistant growth kinetics containing Cadmium, Figure 1shows the resulting bacterial growth curve of *Klebsiella* sp. FIRD 2 at different Cadmium concentrations. The cell growth increased, reaching an optimal concentration at 1 mg/L, and then it started to decrease with an increase in cadmiumconcentration. The graphical results in Figure1,show that high cadmium concentration has an effect on the growth of *Klebsiella* sp. FIRD 2. The optimal growth of the bacteria was found to be at1 mg/L with an OD_{600} of 1.6437; then it started to decline with an increase in cadmium until the cadmium completely inhibited the growth of the TBT-resistant bacteria. It was found that a Cadmium concentration of 100 mg/L completely inhibits the growth of the bacteria, with an OD_{600} of 0.006917.

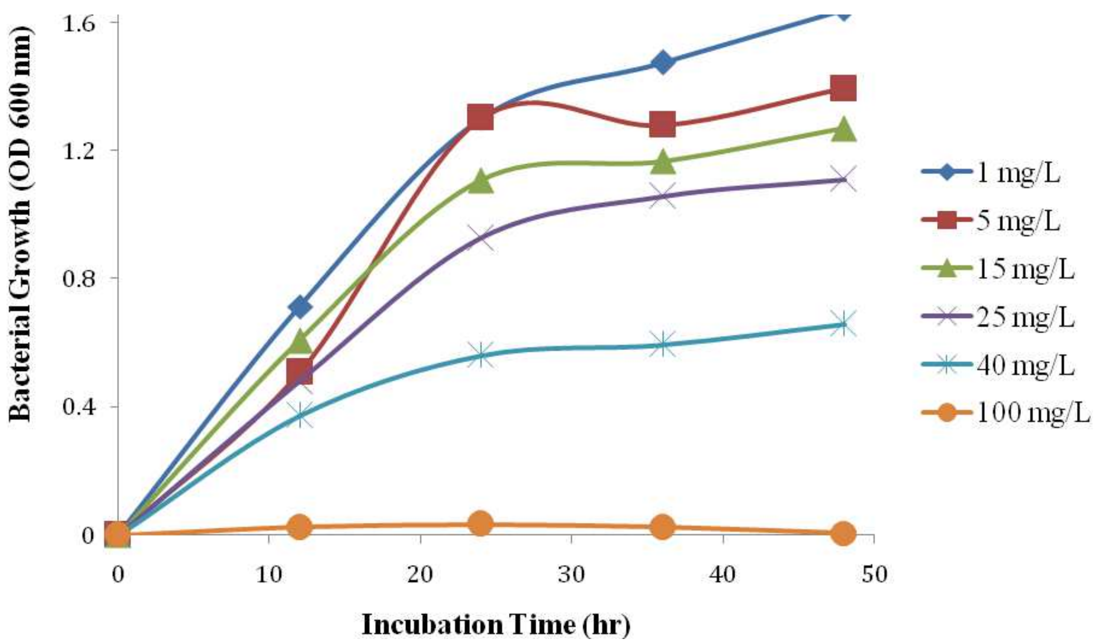

Figure 1. Effect of different cadmium concentration on *Klebsiella* sp. FIRD 2 growth containing TBT.Data represent mean ± STDEV, n=3.

The relationship between the specific growth rate (μ) of a population of microorganisms and the substrate (TBT) concentration (S) containing Cadmium is an important factor in the area of biotechnology. This association is characterized by a set of empirically derived rate laws called theoretical models. These models are nothing more than numerical expressions created to describe the behavior of a given system(Ibrahim *et al.,* 2015a). Based on the growth curves of *Klebsiella* sp., the specific growth rate (μ) for each initial cadmium concentration (S) was calculated. The gradient of line during the exponential phase provided the specific growth rate (Figure 2). The plot shows a definite increase in cell growth rate with increase in cadmium concentration until 1 mg/L, beyond which there was a decrease in cell growth rate as cadmium concentration increased, signifying cadmium inhibition kinetics.

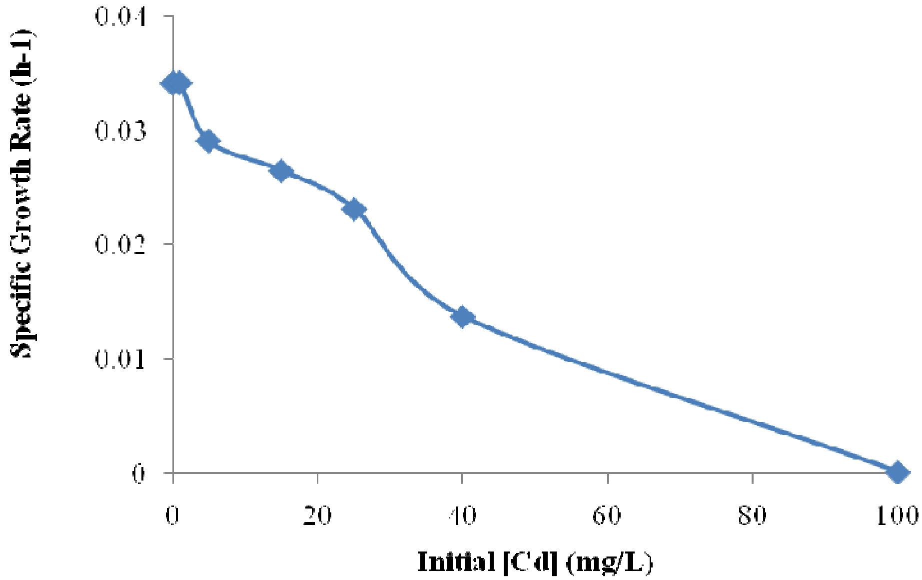

Figure 2. Replotted data of the growth rate against the substrate Cadmium Concentration on *Klebsiella* sp. FIRD 2 growth containing TBT

The results of the curve fitting are shown in Figure 3 as the data from the experimental values in batch studies were fitted to kinetic models. Models such as Teissier and Monod failed to fit the experimental data as their correlation coefficient R^2 was very low. All of the other models tested gave reasonably good fitting based on software output and by visual observation. The accuracy and statistical analysis of the seven kinetic models used shows that the best model was Luong with the lowest value for RMSE and the highest value for adjusted R^2. Table 2 presents the results of kinetic models with correlation coefficient R^2, RMSE and the adjusted R^2.

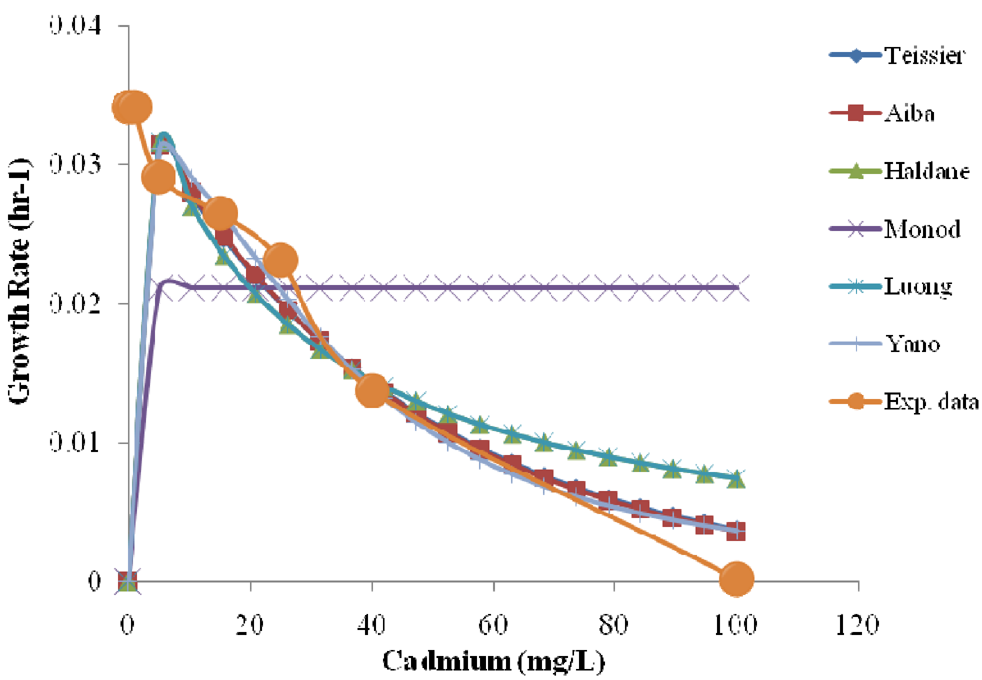

Figure 3. Cadmium growth kinetics resistance experimentalvalues with six different kinetic models.

The calculated value for the Luong constants in this work such as maximal growth rate, the Monodhalf saturation constant,the half inhibition constant, the maximum substrate inhibitory concentration rate and n symbolized by u_{max}, k_s, k_i, and $S_m$were 0.03405hr^{-1}, 0.3 mg/L, 98.93 mg/L, rand 0.7118 respectively. The value of μ_{max} estimated by Luong model (0.03405 hr^{-1}) was closer to the experimental value of 0.034153 hr^{-1} obtained at 1 mg/L. The value of S_m predicted by the Luong model (98.93mg/L) indicates that at and beyond this concentration, there will be no growth on TBT containing cadmium will be observed. The constant n was estimated to be 0.7118, indicating a non-linear correlation between specific growth and the initial substrate concentration. These estimations of parameters suggest that the Luong model best describes the inhibition kinetics of cell growth. This work is in compliance with the work of Othman *et al.,* (2013) where it was reported that Luong model is the best model that fitted the experimental data for the reduction of Molybdenum-to-Molybdenum blue by *Bacillus* sp. strain A.rzi with an R^2 of 0.99.

Table 2. Parameters estimation for different substrate-inhibition models

Model	μmax (hour-1)	Ks (mg/L)	Ki(mg/L)	K(mg/L)	Sm(mg/L)	R2	adjusted R2	RMSE	n
Haldane	0.03967	0.1306	22.93			0.9206	0.881	0.004669	
Teissier	1.014	11.91	12.84			0.595	0.3925	0.01055	
Monod	0.01784	0.4885				0.5341	0.4409	0.01012	
Yano	0.03582	0.6264	57.87	1.12		0.9365	0.8729	0.004825	
Luong	0.03405	0.3			98.93	0.9899	0.9745	0.00216	0.7118
Aiba	0.03575	0.3454	43.63			0.9717	0.9575	0.002791	
Webb	0.02779	0.179	69.22	23.77		0.8377	0.6754	0.007711	

Most of the studies concerning substrate inhibition on microbial growth are carried out using toxic substrate such as aromatic and halogenated hydrocarbons (Ahmad *et al.,* 2015; Chen *et al.,* 1991; Ibrahim *et al.,* 2015a; Sahinkaya and Dilek, 2007) and hence it can be deducted that at high concentration growth rate will be severely affected and the normal use of the monod model will fail. From a biological perspective, xenobiotic such as cadmium is toxic to biological system by virtue of its ability to inhibit enzymes and biological systems(Ibrahim *et al.,* 2015b). This indicates that the mathematical model developed based on enzyme inhibition such as Luong, Aiba, Haldane and others do indeed have biological basis or mechanistic in properties and hence the parameters may have true biological meaning and not just empirical in character(Halmi *et al.,* 2014).

Wayman and Tseng, (1976)described other models for the substrate inhibition kinetics developed during this period such as the discontinuous models.Halmi *et al.,* (2014) One of the reason for the development of the discontinuous model is the previous models developed such as Haldane, Andrews And Noack, and Webb can describe inhibitory effect on microbial growth but could not explain or adequately model for certain situations where the growth rate completely ceased or becoming zero at very high substrate concentration. Nevertheless, the discontinuous fitting profile of the Wayman and Tseng model is a major drawback(Mulchandani et al., 1989). Luong developed a continuous version of the above models and have found popular support due to its close agreement to experimental data in a number of cases(Hamitouche *et al.,* 2012; Nickzad *et al.,* 2012; Othman *et al.,* 2013) including this one. Luong model have a central attraction due to its ability to successful predicting the value of s_m, the maximum substrate concentration above which growth is completely inhibited.

Studies conducted by Othman *et al.,* (2013)on the reduction kinetics of heavy metals such as molybdenum reduction optimally reported Luong model as the best model.

In another report, Haldane has been reported to optimally fit the kinetics models mercury (Gluszcz *et al.*,2011), arsenate (Sukumar, 2010) and chromate (Halmi *et al.*, 2014; Soda *et al.*, 2006) reported a Haldane- type inhibition by the substrate metal ions thus indicating the applicability and ubiquity of this model in fitting growth or biotransformation rate of heavy metals.

CONCLUSION

The kinetics of bacterial growth and degradation can be modelled using different models available in the literatures. In this work, cadmium concentration ranging from 1 to 100 mg/L was used. The kinetic models were fitted to the experimental data and kinetic parameters were determined. Luong gave the most suitable kinetics model with an R^2 of 0.9899 for TBT-Resistant Bacterium By *Klebsiella* sp. FIRD 2, the values of μ_{max}, K_i, K_s and S_m were 0.03405 h^{-1}, 0 g/L, 0.3 mg/L, and 98.93 mg/L respectively. Amongst all the kinetic models, monod gave a poor R^2 of 0.5341.

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