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Growth rate abolishment on phenol as a substrate by *Pseudomonas* sp. AQ5-04 best modelled using the Luong substrate inhibition kinetics

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ABSTRACT

Phenol shows inhibitory effects towards bacterial growth as reflected on the diminishing growth rates as the phenol concentration is increased. Although the Haldane model is often used to model this inhibitory profile, this is often based on popularity and not based on statistical evaluation in comparison with other growth inhibiting models such as Haldane, Aiba, Webb (Edward), Teissier, Yano and Koga, Monod, Han–Levenspiel and Luong. A phenol-degrading *Pseudomonas* sp. AQ5-04 showed a total absence of growth at the highest concentration of phenol tested, which was 2,000 mg L^{-1}. Statistical evaluation indicated that the best kinetic model for growth on phenol was Luong. The Luong's constants; maximal growth rate, half saturation constant for maximal growth, maximum substrate concentration that growth ceases, and curve parameter that defines the steepness of the growth rate decline from the maximum rate symbolized by μ_{max}, K_s, S_m, and n were 0.099 h^{-1}, 17.34 mg L^{-1}, 2,053.0 mg L^{-1}, and 0.801, respectively. The Luong model predicted that S_m value was close to the value of which no growth was observed experimentally suggesting the appropriateness of the model in adhering to observed values.

Keywords: Phenol-degrading; *Pseudomonas* sp.; Kinetics; Luong model; Statistics

1. Introduction

Phenols and phenolic compounds are injurious to organisms even at low concentrations with many of them categorized as hazardous pollutants due to their toxicity towards human health for various reasons [1–4]. Some of the phenolic compounds include chlorophenols, nitrophenols, methyl phenols, alkylphenols, aminophenols, butylhydroxytoluene, nonylphenol, and bisphenols A [5]. In Malaysia, the 2014 Environmental Quality Report showed that nearly all groundwater monitoring stations had phenol concentrations exceeding the National Guidelines for Drinking Water Quality Standard (0.002 mg L^{-1}) [6]. Phenol and phenolic compounds continue to be the top scheduled wastes generated in Malaysia as the demand for phenol by the industries are increasing annually [7]. There are various physicochemical methods for the removal of phenol pollution from the environment including chemical polymerization, advanced oxidation, and ion exchange [8] to name a few. However, a natural or biological method is still considered as the preferable way of controlling phenol pollution [9].

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Various mathematical models have been utilized to describe the effect of substrate to the growth of mixed or pure microbial cultures. Although the most widely used equation in describing substrate utilization linked to growth rate is the Monod equation [10], its limitation is that it cannot be used for biodegradation process that shows substrate inhibition towards the rate. Under this condition, the more appropriate models are the Haldane or other substrate-inhibiting models such as Aiba, Webb (Edward), Teissier, Yano and Koga, Monod, Han–Levenspiel, and Luong [11]. Hence, the utilization of considerable models available could replace the Haldane in some circumstances and discloses mechanistic process.

In this work, a phenol-degrading *Pseudomonas* sp. has been isolated from a waste site in the Langat River Basin where intensive studies for many years have shown the severity of the contamination of this area by industrial activities near and surrounding the basin [12]. The growth of the bacterium was observed to be inhibited as the phenol concentration was increased. Eventually, the growth rate was abolished at high concentration of phenol, a phenomenon which is not seen in many studies on phenol biodegradation in the *Pseudomonas* genus, where the Haldane and other kinetic models have been used instead of models that allow for the complete abolishment of growth rates such as Luong, Teissier, and Han–Levenspiel. This is probably due to the well perceived notion that the *Pseudomonas* genus is highly tolerant to toxicants including phenol [13,14]. To study this in a more accurate manner, the growth rates on phenol were obtained in a more accurate manner by employing the modified Gompertz model as a nonlinear curve fitting model [15]. In many other phenol biodegradation studies, the growth rates were obtained by taking the linear portion of the natural logarithm of cellular biomass, which is done manually. Using the growth rates data available in abundance, growth kinetic models were then evaluated. To date, a limited statistical test is used to accept the best model in modelling the kinetics of phenol biodegradation, and the most popular test is the coefficient of determination (R^2) [16–19] even though adjustment to the number of parameters used in the models must be made [10]. This adjustment can be made using an adjusted coefficient of determination (adjR^2), root mean square error (RMSE), corrected Akaike information criteria (AICc) to name a few [10]. The best model, after running through these exercises, was the Luong model. We suggest that future selection of the best kinetic model governing the growth rates on toxicants especially phenol should be done comprehensively.

2. Materials and methods

2.1. Isolation of phenol-degrading bacteria

The phenol-degrading bacterium AQ5-04 was isolated from a wastewater sample obtained from the Langat River Basin in 2014. This location receives water effluents from a pharmaceutical industry nearby. Four milliliters of wastewater sample was mixed with 40 mL of sterilized minimal salts medium (MSM) containing (g L^{-1}) of NaCl (0.1), $MgSO_4$ (0.1), K_2HPO_4 (0.4), KH_2PO_4 (0.2), Fe_2 (SO_4).H_2O (0.01), $MnSO_4$.H_2O (0.01), $(NH_4)_2SO_4$ (0.4), $NaMoO_4$.$2H_2O$ (0.01), and supplemented with 500 mg L^{-1} of phenol and incubated at 25°C on a shaking incubator at 150 rpm for 3 d [20]. The cultures were streaked onto MSM agar plates supplemented with 500 mg L^{-1} of phenol and incubated at 25°C for 3 d. Phenol-degrading activity was assayed using the 4-aminoantipyrine colorimetric assay at 510 nm [20]. Bacterial growth was measured using colony-forming-units (CFU mL^{-1}).

2.2. Molecular identification of the strain

AQ5-04 was partially identified through a molecular 16s rDNA gene sequence phylogenetic analysis. The genomic DNA was extracted using a commercial kit (GeneJet Genomic DNA purification kit, Thermo Scientific, Lithuania) and amplified using the following PCR universal primers; reverse: 5′-TAC GGT TAC CTT GTT ACG ACT T-3′ and forward: 5′-AGA GTT TGA TCC TGG CTC AG-3′ [21]. PCR was carried out under the following conditions: 1st cycle at 96°C for 4 min as an initial denaturation; 30 cycles at 94°C for 1 min for denaturing followed by an annealing stage at 58°C for 1 min, an extension stage carried out at 72°C for 1 min and a final extension at 72°C for 7 min. The sequence was further deposited at the NCBI Gen bank and assigned an accession number of KT693288. For the phylogenetic tree analysis, twenty 16s rDNA sequences were obtained from Genbank showing the closest identity to *Pseudomonas* species. The evolutionary analysis was carried out using MEGA6 [22]. A neighbour-joining method involving closest nucleotide sequences sourced from the BLASTn exercise was utilized to infer evolutionary history. The Maximum Composite Likelihood method was utilized to calculate the evolutionary distances [22]. In the analysis, codon positions included were the 1st + 2nd + 3rd. Also, missing data and gaps were removed from all positions resulting in a final 1,377 positions presented in the final dataset.

2.3. Growth kinetics on phenol

Growth kinetics was studied using a batch culture of the bacterium grown in MSM with phenol supplemented at concentrations ranging from 0 to 2,000 mg L^{-1}. The initial inoculum of the bacterium was standardized at an OD_{600} nm of 0.1. The maximum specific growth rate of the bacterium, μ_m, to be utilized in the secondary inhibition kinetics modelling was calculated using the modified Gompertz model [2,10,23] as follows:

$$y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}\left(\lambda - t\right)+1\right]\right\} \quad (1)$$

2.4. Fitting of the data

A Marquardt algorithm was used to fit the modified Gompertz and several inhibition kinetic models (Table 1) by nonlinear regression. This algorithm reduces the sums of squares of the residuals. Nonlinear regression was carried out using the CurveExpert Professional software (Version 1.6).

2.5. Statistics of the growth kinetics

Statistical analysis of the residuals was carried to select the best model using approaches such as the corrected

Table 1
Kinetic models for growth of *Pseudomonas* sp. AQ5-04 on phenol

Author	Degradation rate
Monod	$\mu_{max}\frac{S}{K_S+S}$
Haldane	$\mu_{max}\frac{S}{S+K_S+\frac{S^2}{K_i}}$
Teissier	$\mu_{max}\left(1-\exp\left(-\frac{S}{K_i}\right)-\exp\left(\frac{S}{K_S}\right)\right)$
Aiba	$\mu_{max}\frac{S}{K_s+S}\exp\left(-KP\right)$
Yano and Koga	$\frac{\mu_{max}S}{S+K_S+\left(\frac{S^2}{K_1}\right)\left(1+\frac{S}{K}\right)}$
Luong	$\mu_{max}\frac{S}{S+K_S}\left[1-\left(\frac{S}{S_m}\right)^n\right]$

μ_{max}, maximal growth rate (h^{-1}); K_s, half saturation constant for maximal degradation (mg L^{-1}); S_m, maximal concentration of substrate tolerated (mg L^{-1}); and *m*, *n*, *K*, curve parameters; *S*, substrate concentration (mg L^{-1}); *P*, product concentration (mg L^{-1}).

Akaike Information Criterion or AICc, adjusted coefficient of determination (R^2), root mean square error (RMSE) accuracy factor (AF), and bias factor (BF) [10].

The RMSE was calculated according to Eq. (2) as follows:

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}\left(Pd_i - Ob_i\right)^2}{n-p}} \tag{2}$$

where *n* number of experimental data; Pd_i predicted values by the model; Ob_i experimental data; *p* parameters number of the model.

In general, the model having a smaller number of parameter results in a smaller RMSE value [10].

The coefficient of determination or R^2 has been very popular in describing the goodness of fit of models. However, in nonlinear regression, the method does not consider the number of parameters of models and hence does not freely provide comparative analysis. To overcome this issue, an adjusted R^2 that takes into account the number of parameter of models is used to compute the quality of nonlinear models according to the formula below

$$\text{Adjusted}\left(R^2\right) = 1 - \frac{\text{RMS}}{s_y^2} \tag{3}$$

$$\text{Adjusted}\left(R^2\right) = 1 - \frac{\left(1-R^2\right)\left(n-1\right)}{\left(n-p-1\right)} \tag{4}$$

where S_y^2 is the total variance of the *y*-variable and RMS is the residual mean square.

The Akaike information criterion (AIC) handles the trade-off associated with the goodness of fit between the models well as the complexity of the model. This is established upon information theory. The technique offers a relative approximation from the information lost for every single period a given model will be employed to represent the procedure that creates the info or data. To have a result of a set associated with the expected model, the majority of the desired design would become the model showing the particular minimal value for AIC. This unique value is frequently an adverse value, with for instance; an AICc value of −10 is preferred over a value of −1. The formula incorporates some variables penalty where the more the variables, the higher the AIC value indicating a less parsimonious model. AIC discourages the use of more complicated models (overfitting) in fitting experimental data. When the data in a study are small concerning the parameters' number, a corrected version of AIC; the Akaike information requirements (AIC) with correction or AICc is utilized instead [24]. AICc is calculated using the following equation:

$$\text{AICc} = 2p + n\ln\left(\frac{\text{RSS}}{n}\right) + 2\left(p+1\right) + \frac{2\left(p+1\right)\left(p+2\right)}{n-p-2} \tag{5}$$

where *n* number of data points; *p* parameter numbers of the model.

AICc considers the particular change in the model goodness-of-fit. Also, the model also takes into account the number of parameters of the models. The model showing the smallest AICc value is likely to be the correct model [25].

Another goodness-of-fit of models are the AF and BF adapted from common use in predictive microbiology for bacterial growth in food science [26]. The statistics calculates the perfect match between experimental and predicted values. As a rule, a BF value > 1.0 indicates a model which is fail-safe a value < 1.0 indicates a model that is fail-dangerous. On the other hand, the AF is always ≥ 1.0, with precise models giving values nearing to 1.0.

$$\text{Bias factor} = 10^{\left(\sum_{i=1}^{n}\log\frac{\left(Pd_i/Ob_i\right)}{n}\right)} \tag{6}$$

$$\text{Accuracy factor} = 10^{\left(\sum_{i=1}^{n}\log\frac{\left|\left(Pd_i/Ob_i\right)\right|}{n}\right)} \tag{7}$$

Assessment of normality for the residuals was carried out using the GraphPad Prism® 6 (Version 6.0, GraphPad Software Inc., USA). Residual for the *i*th observation in regression model can be mathematically represented as follows:

$$e_i = y_i - f\left(x_i;\hat{\beta}\right) \tag{8}$$

where the *i*th response from a given dataset is denoted by y_i while at each set of the *i*th observation, the vector for the explanatory variables is x_i [27].

3. Result and discussion

3.1. Molecular identification of the bacterium and characterization of growth and degradation on phenol

Isolate AQ5-04 was identified using a molecular phylogenetic analysis of the 16S rDNA sequence. Molecular identification begins with a BLASTn exercise on the NCBI Gen Bank database. The result shows a 99% similarity to *Pseudomonas* spp. The percentage of replicate trees (1,000 replicates) is shown next to the branches (Fig. 1) based on a bootstrap exercise [28]. The bacterium is linked to several *Pseudomonas* species clades such as *Pseudomonas azotoformans* and *Pseudomonas cedrina* but with low bootstrap values. At this stage, the bacterium was tentatively identified as *Pseudomonas* sp. AQ5-04. The optimization results demonstrate that the optimal temperatures of between 25°C and 35°C for both phenol degradation and bacterial growth, pHs of between 7 and 7.5 for phenol degradation and between 6 and 7.5 for growth, ammonium sulphate as the best nitrogen source for both degradation and growth on phenol and sodium chloride concentrations of between 0.1 and 0.15 g L^{-1} for both degradation and growth on phenol, respectively. The best ammonium sulphate concentrations supporting phenol degradation was between 0.4 and 0.5 g L^{-1} and between 0.3 and 0.6 g L^{-1} for growth on phenol (data not shown).

3.2. Growth kinetics

The specific growth rate was obtained from a primary modelling exercise using the modified Gompertz model (Fig. 2) as this model has often been used to model growth curve on phenol as a substrate [2,23].

The specific growth rates were then plotted against initial phenol concentrations and a secondary modelling exercise this time utilising various kinetics models (Fig. 3).

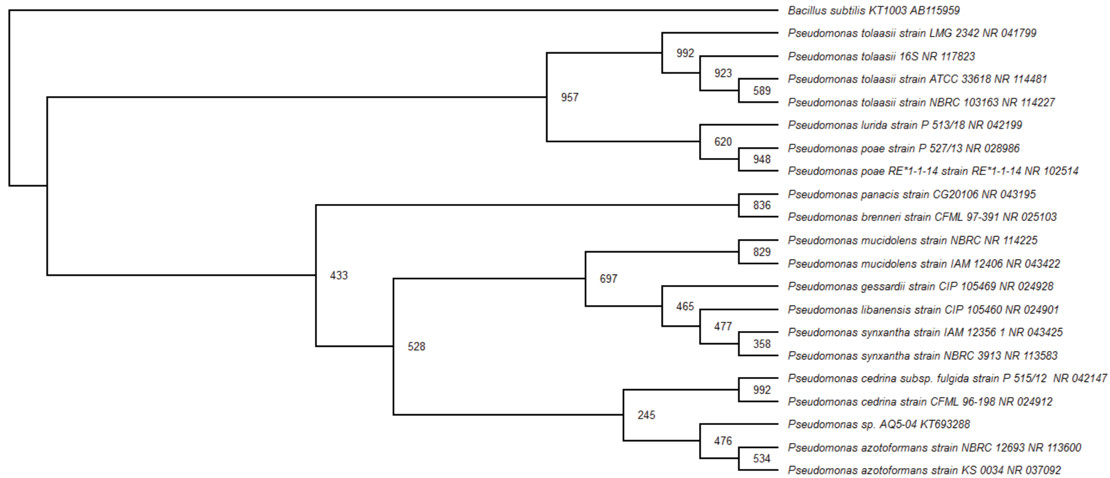

Fig. 1. Neighbour-joining tree based on partial 16s rRNA sequence demonstrating the phylogenetic relatedness of strain AQ5-04 to other bacterial species. *Bacillus subtilis* strain KT1003 AB115959 was the outgroup.

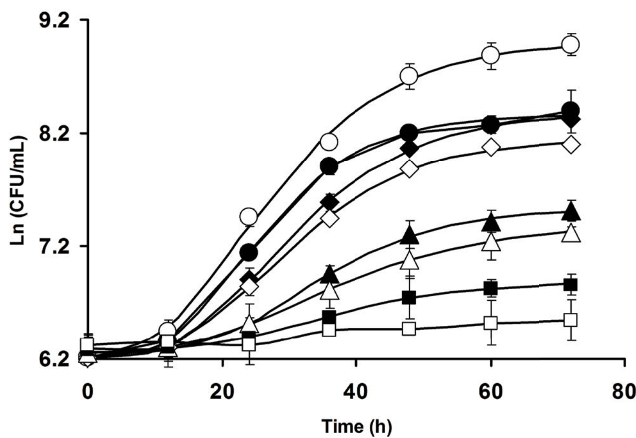

Fig. 2. Growth on various phenol concentrations (100 (●); 200 (○); 500 (◆); 800 (◇); 1,100 (▲); 1,400 (△); and 1,700 (■) mg L^{-1}) modelled according to the modified Gompertz model (line). There was no growth at 2,000 mg L^{-1} (□) phenol. Error bars indicate the mean ± standard deviation (n = 3).

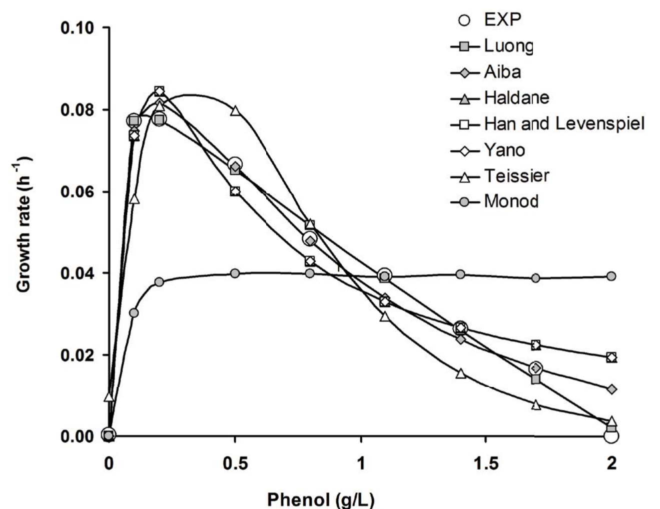

Fig. 3. Curve fitting of the growth rate of *Pseudomonas* sp. using various models.

Table 2
Statistical analysis of kinetic models

Model	p	RMSE	R^2	adjR^2	AF	BF	AICc
Luong	4	0.00	1.00	0.99	–76.60	0.98	1.04
Aiba	3	0.01	0.97	0.96	–72.74	0.97	0.98
Haldane	3	0.01	0.97	0.96	–73.89	0.99	1.10
Han and Levenspiel	4	0.01	0.91	0.81	–48.95	0.99	1.10
Yano	4	0.01	0.91	0.81	–48.95	0.99	1.10
Teissier	4	0.01	0.89	0.79	–45.90	0.88	1.27
Monod	2	0.03	–4.00	–5.66	–49.95	0.88	1.49

SSE, sums of squared errors; RMSE, root mean squared error; R^2, coefficient of determination; adjR^2, adjusted coefficient of determination; AICc, corrected Akaike information criterion; BF, bias factor; AF, accuracy factor.

The statistical analysis and accuracy of all the six kinetic models used indicated that Luong was the best model with small values for RMSE and AICc, uppermost adjusted R^2 values, F-test and with BF and AF nearest to unity (1.0; Table 2). The Luong's constants; maximal growth rate, half saturation constant for maximal growth, maximal concentration of substrate tolerance and curve parameter that defines the steepness of the growth rate decline from the maximum rate symbolized by μ_{max}, K_s, S_m, and n (± standard error) were 0.099 ± 0.017 h^{-1}, 17.34 ± 5.0 mg L^{-1}, 2,053.0 ± 56.0 mg L^{-1}, and 0.801 ± 0.202, respectively. Models such as Luong, Teissier, and Han–Levenspiel were developed due to the limitations of previous models such as Haldane, Andrews and Noack, Webb, and Yano in that these models failed to explain some situations where growth rate became zero at very high substrate concentration [18]. To date, the majority of phenol-degrading microbial works reported or utilized the Haldane's model (Table 3) in isolation or after statistical assessment of other available substrate inhibition models and to the best of our knowledge, the Luong model has

Table 3
Various phenol-degrading microorganisms growth curves models on phenol and their kinetic constants

Microorganism	Best model	Temperature °C	Max phenol concentration (mg L^{-1})	μ_{max} (h^{-1})	K_s (mg L^{-1})	K_i (mg L^{-1})	S_m, K_1 or K_2 (mg L^{-1})	Reference
Pseudomonas putida	Haldane	26 ± 0.5	500	0.436	6.19	54.1	–	[29]
Rhodococcus AQ5NOL1	Haldane	35	1,110	0.11	99.03	354	–	[30]
Pseudomonas putida	Haldane	30	–	0.569	18.539	99.374	–	[31]
Mixed consortium	Han–Levenspiel	27	800	0.4029	110.93		790	[18]
Pseudomonas sp.	Haldane	29 ± 2	400	0.0324	0.0324	0.0324	–	[32]
Pseudomonas sp.	Webb (Edward)	29 ± 2	400	0.0238	0.0238	–	400	[32]
Mixed bacterial culture	Luong	30	350	1.04	153.2	–	540	[17]
Bacillus cereus MTCC 9817	Luong	30	–	0.755	925.8	–	1,859.3	[10]
Pseudomonas IES-Ps-1	Monod	35	2,000	0.38	111	–	2,000	[4]
Pseudomonas IES-S	Monod	35	2,000	0.63	77	–	2,174	[4]
Bacillus IES-B	Luong	35	2,000	1.2	102	–	2,190	[4]
Pseudomonas fluorescence	Haldane	30	–	0.229	0.374		729	[33]
Pseudomonas fluorescence	Yano and Koga	30	–	0.229	0.377	–	411	[33]
Pseudomonas fluorescence	Aiba	30	–	0.229	0.376		2,008	[33]
Sulfolobus solfataricus 98/2	Haldane	80	–	0.094	77.7	319.4	93	[2]
Candida tropicalis PHB5	Haldane	30	2,400	0.3407	15.81	169.0	–	[23]
Mixed consortium of bacteria	Haldane	30	800	0.1301	99.84	220.9	–	[34]
Alcaligenes faecalis B6-2	Haldane	30	1,410	0.48	188.16	469.23	297.1	[35]
Alcaligenes faecalis B8-1	Haldane	30	1,410	0.14	32.85	447.44	121.2	[35]
Alcaligenes faecalis D3-1	Haldane	30	1,410	0.38	267.3	1,847.82	702.8	[35]
Acinetobacter johnsonii D1	Haldane	30	1,410	0.55	483.83	2,582.63	1,117.8	[35]
Pseudomonas putida MTCC 1194	Haldane	25	1,500	0.041	640.05	216.59		[14]
Mixed microbial culture	Haldane	25	700	0.3057	257.5	162.6		[36]
Pseudomonas AQ5-04	Luong	30	–	0.10	–	1,000.02	2,050	Present study

not been used as the best model after statistical evaluation for governing growth rates on phenol by the *Pseudomonas* genus. In addition, this is probably the first time a complete abolishment of growth rates on phenol was observed for the *Pseudomonas* genus.

The Luong equation can then be replaced with the calculated model parameters as follows:

$$\mu = 0.099\frac{S}{S+17.34}\left[1-\left(\frac{S}{2,053.0}\right)^{0.801}\right] \quad (9)$$

4. Conclusion

In this study, a complete cessation of growth at a very high phenol concentration by *Pseudomonas* sp. strain AQ5-04 was observed and the use of various kinetic models in conjunction with a comprehensive statistical treatise of the model suggest that the Luong model was the best in fitting the growth rate at various phenol concentrations as opposed to the popular Haldane model. The Luong model allows for the modelling of the maximum concentration of substrate that results in the complete cessation of growth rate.

Symbols

A — Cell number lower asymptote (modified Gompertz model fitting parameter)
y_{max} — Cell number upper asymptote (modified Gompertz model fitting parameter)
e — Exponent (2.718281828)
t — Sampling time (modified Gompertz model constant)
K_s, K_i — Half saturation and inhibition coefficients for secondary growth rate models (Haldane, Luong, etc.), mg L^{-1}
q — Specific degradation rate, h
q_{max} — Maximum specific degradation rate, h
S — Phenol concentration, mg L^{-1}
S_m — Phenol concentration at which $\mu = \mu_{max}$
y_i — ith response from a given data set
x_i — Vector for the explanatory variables

Greek

μ — Specific growth rate, h^{-1}
μ_{max} — Maximum specific growth rate in secondary growth model (Haldane, Luong, etc.), h^{-1}
μ_m — Maximum specific growth rate (modified Gompertz model fitting parameter)
λ — Lag time (modified Gompertz model fitting parameter)

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Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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