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Full Length Research Paper

Substrate utilization and inhibition kinetics: Batch degradation of phenol by indigenous monoculture of *Pseudomonas aeruginosa*

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The biodegradation potential of an indigenous monoculture of *Pseudomonas aeruginosa* was studied in batch fermentation using synthetic phenol in water in the concentration range of (100 - 500) mg/L as a model limiting substrate. The effect of initial phenol concentration on the degradation process was investigated. Phenol was completely degraded at different cultivation times for various initial phenol concentrations. Increasing the initial phenol concentration from 100 to 500 mg/L, increased the lag phase from 0 to 24 h and correspondingly prolonged the degradation process from 54 to 168 h. This implies that there was decrease in biodegradation rate as initial phenol concentration increased. Four substrate utilization models were examined, and out of these, the adapted Miura model was found to be the best fit for description of kinetics. The $r_{s\max}$ decreased and K_s increased with higher concentration of phenol. The $r_{s\max}$ has been found to be a strong function of initial phenol concentration. The bacterial culture followed substrate inhibition kinetics and the specific phenol consumption rates were fitted to five inhibition models. The Haldane and Yano and Koga inhibition models were found to give the best fit. Therefore, the biokinetic constants estimated using these models show good potential of the monoculture of *Pseudomonas aeruginosa* and the possibility of using it in bioremediation of phenolic waste effluents.

Key words: *Pseudomonas aeruginosa*, phenol, biodegradation, kinetic model, batch cultivation, bioreactor, primary culture, secondary culture, bioremediation.

INTRODUCTION

Phenol and its derivatives are the basic structural unit in a wide variety of synthetic organic compounds (Annadurai et al., 2000). It is an organic, aromatic compound that occurs naturally in the environment (Prpich and Daugulis, 2005), but is more commonly produced artificially from industrial activities such as petroleum processing, plastic manufacturing, resin production, pesticide production, steel manufacturing and the production of paints and varnish (Mahadevaswamy et al., 1997; Bandyopadhyay et al., 1998). This aromatic compound is water-soluble and highly mobile (Collins and Daugulis, 1997). Wastewaters generated from these industrial activities contain high concentrations of phenolic compounds (Chang et al., 1998) which eventually may reach down to streams, rivers, lakes and soil, and represent a serious ecological problem (Fava et al., 1995). Phenol is a listed priority pollutant by the U.S. Environmental Protection Agency (EPA, 1979) and is

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considered to be a toxic compound by the Agency for Toxic substances and Disease Registry (ATSDR, 2003). The adverse effects of phenol on health are well documented (Calabrese and Kenyon, 1991) and death among adults has been reported with ingestion of phenol ranging from 1 to 32 g (Prpich and Daugulis, 2005). The low volatility of phenol and its affinity for water make oral consumption of contaminated water a greatest risk to humans (Prpich and Daugulis, 2005).

A variety of techniques involving physical, chemical and biological methods have been used for the removal of phenol from industrial effluents and contaminated waters. Bioremediation have received the most attention due to its environmental friendliness, its, ability to completely mineralize toxic organic compounds and its low-cost (Kobayashi and Rittman, 1982; Prpich and Daugulis, 2005). Microbial degradation of phenol with different initial concentrations ranging from 50 – 2000 mg/L have been actively studied using shake flask, fluidized-bed reactor, continuous stirred tank bioreactor, multistage bubble column reactor, air-lift fermenter and two phase partitioning bioreactor methods (Bettmann and Rehm, 1984; Sokol, 1988; Annadurai et al., 2000; Reardon et al., 2000; Ruiz-ordaz et al., 2001; Oborien et al., 2005; Prpich and Daugulis, 2005; Saravanan et al., 2008). These studies have shown that phenol can be aerobically degraded by wide variety of fungi and bacterial cultures such as *Candida tropicalis* (Ruiz-ordaz et al., 2001; Chang et al., 1998; Ruiz-ordaz et al., 1998), *Acinetobacter calcoaceticus* (Paller et al., 1995; Hao et al., 2002), *Alcaligenes eutrophus* (Hughes et al., 1984; Leonard and Lindley, 1998), *Pseudomonas putida* (Hill and Robinson, 1975; Kotturi et al., 1991; Nikakhtari and Hill, 2006) and *Burkholderia cepacia* G4 (Folsom et al., 1990; Solomon et al.,1994).

A variety of kinetic substrate utilization and inhibition models have been used to describe the dynamics of microbial growth on phenol. Of these, the Monod and Andrew (Haldane) equations have been extensively used to describe phenol biodegradation and are based on the specific growth rate (Bandyopadhyay et al., 1998; Reardon et al., 2000; Oboirien et al., 2005), but may also be related to the specific substrate consumption rate (Edwards, 1970; Solomon et al., 1994). Other kinetic models have also been propagated. Sokol (1988) has reported a better fit for a modified Monod-Haldane equation, while Schroder et al. (1997) have shown a better fit for Yano and Koga equation amongst the tested inhibition models. In spite of the rather extensive use of phenol biodegradation processes, little work has been published on phenol microbial degradation kinetics based on specific substrate consumption rate ($r_{s\max}$) using mono or mixed culture systems. The present study investigates the effect of initial phenol (substrate) concentration on the degradation potential of an indigenous monoculture of *Pseudomonas aeruginosa* isolated from an oil-polluted swampy area of Warri in Niger-Delta region of Nigeria and to determine its kinetics at these different phenol concentrations.

MATERIALS AND METHODS

Microorganism

The microorganism, monoculture of *P. aeruginosa* being an indigenous bacteria strain isolated from an oil-polluted area in Niger-Delta region of Nigeria was procured from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The bacterial culture was maintained on nutrient agar slant and stored at 4 ± 1°C for further use.

Culture medium and inoculum preparation

The mineral salt medium used was modified from the one suggested by Bettman and Rehm (1984). The medium had the following composition per litre: 700 ml deionized water, 100 ml buffer solution A, 100 ml trace elements solution B, 50 ml solution C and 50 ml solution D. Compositions of each solution were as follows: Buffer solution A composition: K_2HPO_4 1.0 g, KH_2PO_4 0.5g, $(NH_4)_2SO_4$ 0.5g, deionized water 100ml. Trace element solution B composition: NaCl 0.5g, $CaCl_2$ 0.02g, $MnSO_4$ 0.02g, $CuSO_4.5H_2O$ 0.02g, H_3BO_3 0.01g, deionized water 50ml. Solution C composition: $MgSO_4.7H_2O$ 0.5g, deionized water 50 ml, Solution D composition $FeSO_4$ 0.02g, Molybdenum powder 0.02 g, deionized water 50 ml. To prevent the precipitation of $CaSO_4$ and $MgSO_4$ in storage, the water, buffer solution A, trace elements solution B, solution C and solution D were autoclaved at 121°C for 15 m. After cooling, all the solutions were then mixed together and kept as stock solution from which known quantities were taken for the cultivation of the microorganisms

A primary culture was prepared by transferring two loops full of microorganisms from an agar slant culture into 100 ml of feed medium containing 20 ml of mineral salt medium and 80 ml of 50 mg phenol solution in a 250 ml Erlenmeyer conical flask. This was then incubated in a New Brunswick gyratory shaker (G25-R model, N.J. U.S.A) for 48 h at 30°C and agitated at 120 rpm. Thereafter, 10 ml of the primary culture was transferred into another 100 ml of feed medium in a 250 ml Erlenmeyer conical flask and the incubation process was repeated. This was the secondary culture that was used as the inoculum for the degradation studies as this ensures that the organisms had fully adapted to growth on phenol as the sole source of carbon and energy.

Experimental design to study the free suspended cell system

The experimental studies were carried out in a New Brunswick Microferm Twin Bioreactor (pH - 22 model, N.J., U.S.A) with 4 l working volume. Autoclaved mineral salt medium (800 ml) and 3 l of phenol solution (100 mg/L) were measured into the bioreactor vessel and 200 ml of the inoculum was introduced aseptically to make up 4 l of working volume. The bioreactor was operated for several hours at 30°C, aeration rate of 3.0 vvm and agitation of 300 rpm. Culture broth was withdrawn every 6 h for biomass and phenol determination.

Estimation of phenol concentration

The undergraded phenol was estimated quantitatively by measuring its absorbance at 510 nm wavelength using UV - visible

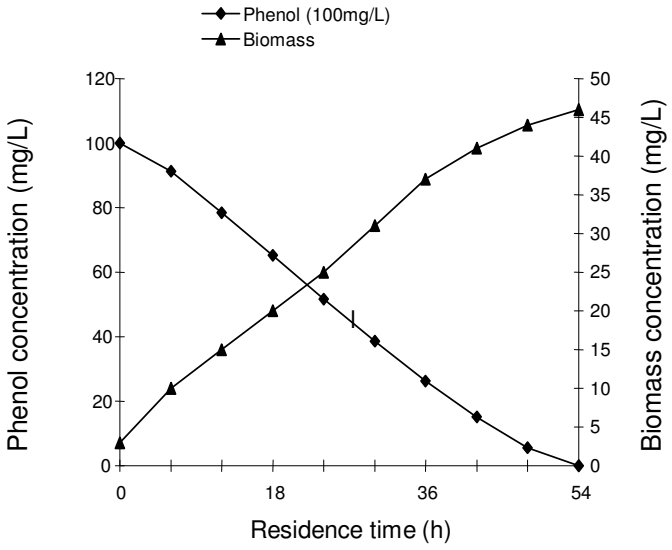

Figure 1. Experimental data obtained from batch degradation of phenol (100 mg/L) by monoculture of *P. aeruginosa.*

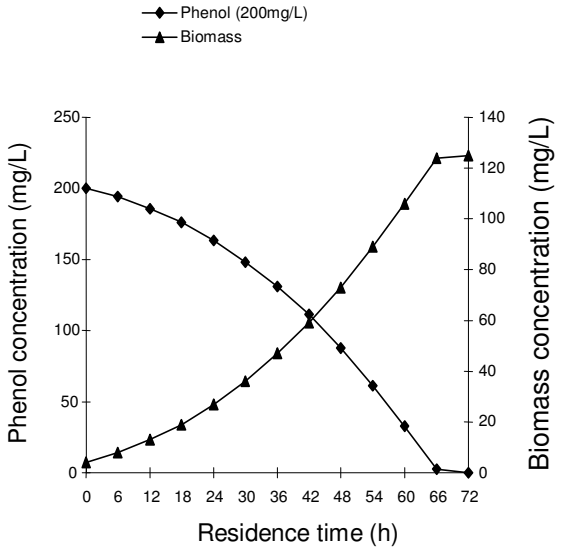

Figure 2. Experimental data obtained from batch degradation of phenol (200 mg/L) by *P. aeruginosa.*

Spectrophotometer (Lambda 35, Perkin-Elmer, USA) and 4 - amino antipyrene as colour indicator (Yang and Humphrey, 1975; Oboirien et al., 2005).

Estimation of biomass concentration

The biomass concentration was estimated using the dry weight method. A sample of culture broth (50 ml) was withdrawn from the bioreactor and centrifuged (Gallenkamp centrifuge) at 4000 rpm for 20 min in plastic centrifuge tubes. The supernatant was decanted into small bottles and stored at 4℃ for subsequent phenol estimation. The pellets was re-suspended in de-ionized water and re-centrifuged. The supernatant was decanted and pellets rinsed off

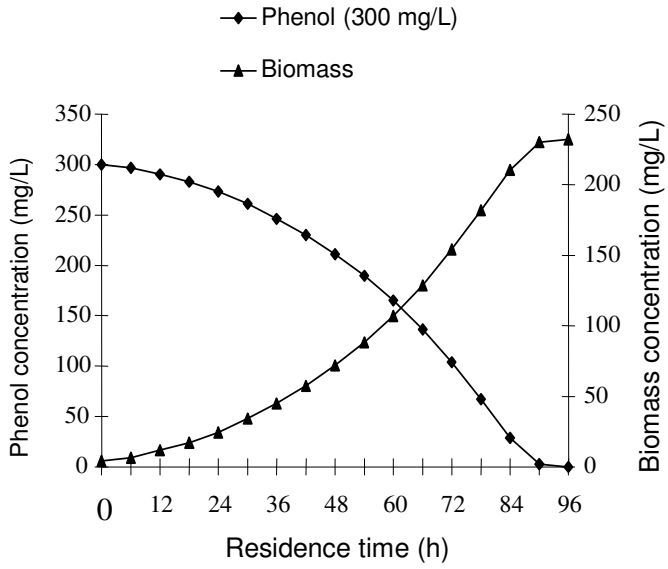

Figure 3. Experimental data obtained from batch degradation of phenol (300 mg/L) by of *P. aeruginosa.*

from the tube into a pre-weighed 1.2 µm pore filter paper (Whatman GF/C). The filter paper was then dried in an oven at 105℃ for 12 - 24 h, cooled in a dessicator at room temperature and re-weighed until a constant dry weight was obtained. The difference between the pre-weighed filter paper and the final constant weight was used to estimate the dry weight of the biomass.

RESULTS AND DISCUSSION

Five batch cultivation experiments were carried out using phenol as single limiting substrate for monoculture of *P. aeruginosa.* The extent of phenol degradation using different initial phenol concentrations (100 – 500 mg/L) was investigated for several batch residence times by intermittent sampling.

Figures 1 – 5 show the biodegradation potential of the indigenous monoculture of *P. aeruginosa* in degrading synthetic phenol waste in the concentration range of 100 to 500 mg/L. Since the degradation proceeds with biomass (cell mass) growth, the Figures also depict a typical cell growth curve with increasing lag phase. Various initial phenol concentrations ranging from 100 – 500 mg/L were completely degraded (consumed) at different residence times of 54, 72, 96, 120 and 168 h, respectively. At these times the biomass correspondingly increased to a maximum of 46, 125, 232, 280 and 385 mg/L, respectively. No lag phase was observed for initial phenol concentrations of 100 and 200 mg/L as shown in (Figures 1 and 2). Similar observations have been reported by Oboirien et al., 2005; Saravanan et al., 2008; Agarry and Solomon, 2008. However, for initial phenol concentrations of 300, 400 and 500 mg/L respectively, corresponding lag phase of 6, 12 and 18 h was observed

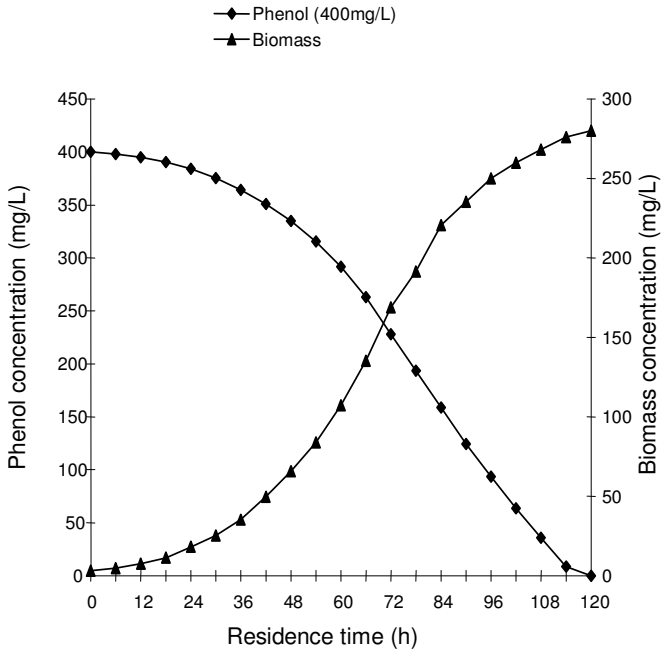

Figure 4. Experimental data obtained from batch degradation of phenol (400 mg/L) by *P. aeruginosa.*

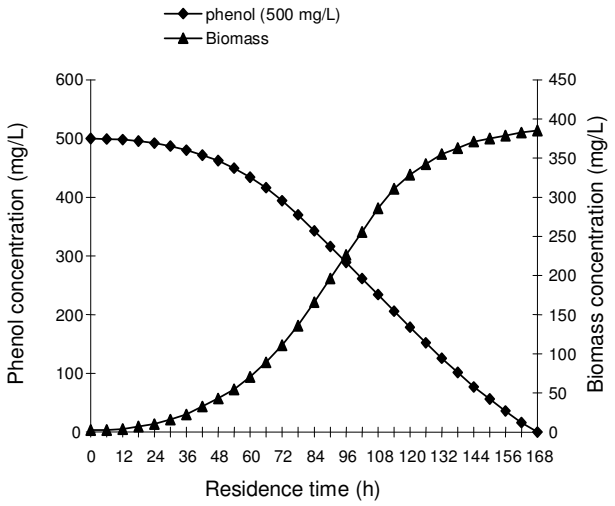

Figure 5. Experimental data obtained from batch degradation of phenol (500 mg/L) by *P. aeruginosa.*

Figures 3 - 5). It is evident from Figure 6, which compares the time course for phenol substrate consumption of all the five batches that the rate of degradation decreases with increase in the initial phenol concentration. Bandyopadhyay et al. (1998); Ruiz-ordaz et al. (2001); Agarry and Solomon (2008) reported similar observations for *P. putida, C. tropicalis* and *Pseudomonas fluorescence* grown on phenol, respectively.

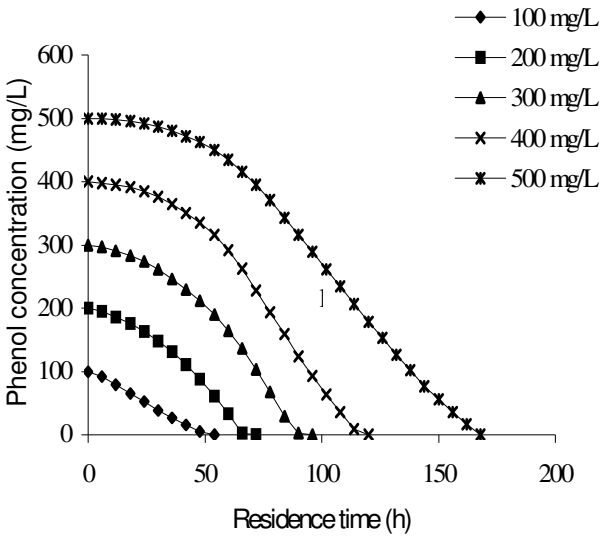

Figure 6. Effect of initial phenol concentration on phenol degradation by *P. aeruginosa.*

Evaluation of biokinetic parameters

Batch phenol degradation was carried out with free suspended cells of indigenous monoculture of *P. aeruginosa* under different initial phenol concentrations as stated above. In this work, phenol well known as an inhibitory substrate under different concentrations (100 – 500 mg/L) was completely degraded by the monoculture of *P. aeruginosa* as shown in Figure 6. According to Prpich and Daugulis (2005), the rate of substrate consumption was suggested to be the most important measure of microbe performance. Zilli et al. (1993) have similar results but relatively less data exists in the literature on this parameter. Most of the data available concerns specific growth rate. It was on this basis that the specific phenol (substrate) consumption rate was calculated and plotted against phenol concentration as shown in Figure 7. As seen from this Figure, the specific phenol consumption rate (r_s) decreases as the phenol concentration (S) decreased. Therefore, it seems that there is also an influence of the initial phenol concentration on the specific phenol consumption rate. Hinteregger et al. (1992) and Abd-El Hameidshalaby (2003) have reported similar observations.

According to Layokun et al. (1987), the growth of microorganisms corresponds to the degradation (consumption) of the substrate. Hence, the growth of microorganisms on phenol can be described by the most commonly used kinetic models. Posten has proposed that these models can be based on specific substrate consumption rates (Solomon et al., 1994), which have been also used by Zilli et al. (1993) and Schroder et al. (1997). The classic method of obtaining kinetic parameters (constants) is to linearize kinetic models.

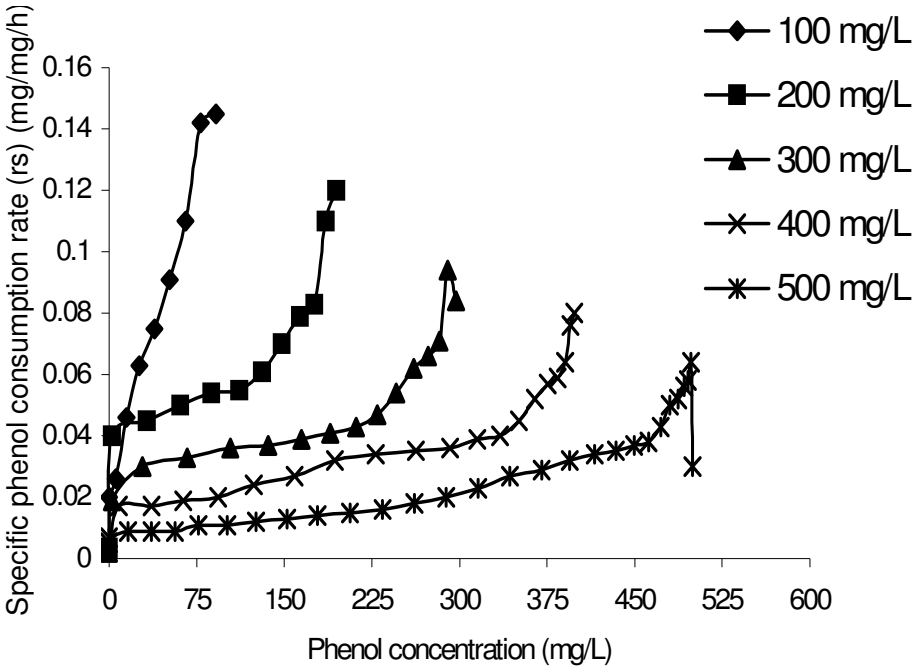

Figure 7. Specific phenol consumption rate vs phenol concentration for monoculture of *P. aeruginosa.*

However, non-linear least squares computer fitting of data to model equations are being used (Reardon et al., 2000; Schroder et al., 1997; Saravanan et al., 2008). The non-linear least square fitting routine of MATLAB 6.5 software package was used to fit the Eckenfelder, Monod, Moser and the adapted Miura models to the different batch experimental data. The parameters of these models (K_s and $r_{s\max}$) were fitted to the experimental calculated specific phenol consumption rate and the corresponding phenol concentration under the constraint that r_s never exceed the maximum obtainable specific consumption rate ($r_{s\max}$). The results from Table 1 have revealed that for initial phenol concentration (S_o) of 100 mg/L, the adapted Miura (R^2 = 0.9311, RMSE = 0.0130) and Eckenfelder (R^2 = 0.9036, RMSE = 0.0144) models show a better fit as compared to other models. However, amongst the two models, the adapted Miura model showed the best fit. For initial phenol concentration (S_o) of 200 mg/L, once again, the adapted Miura (R^2 = 0.6290, RMSE = 0.0187) and Eckenfelder (R2 = 0.6290, RMSE = 0.0192) models, show a better fit. This indicates that the mode of 200 mg/L phenol utilization is well represented by the two models. Nonetheless, based on the lower RMSE value, the adapted Miura model showed the best fit. For initial phenol concentration (S_o) of 300 mg/L, the adapted Miura (R^2 = 0.7592, RMSE = 0.0120) and Eckenfelder (R^2 = 0.7504, RMSE = 0.0118) models show a better fit but the adapted Miura model showed the best fit. For initial phenol concentration of 400 mg/L, the adapted Miura (R^2 = 0.8220, RMSE = 0.0014) and Eckenfelder (R^2 = 0.8007, RMSE = 0.0091) models indicated a better fit. Yet again the adapted Miura model showed the best fit (having a higher R^2 value). The adapted Miura (R^2 = 0.8593, RMSE = 0.0011) and a strong function of initial phenol concentration (S_o). The variation of $r_{s\max}$ with S_o has been indicated in Figure 8. It is also fitted by the fourth order polynomial trendline from which $r_{s\max}$ at any value of S_o within the range of 100 - 500 mg/L of phenol concentration can be predicted. However, the observation of substrate inhibition due to phenol can be modeled using substrate inhibition models described in literature (Schroder et al., 1997). The experimental results of specific phenol consumption rate variation with initial phenol concentration were fitted to five inhibition models namely Haldane (1968); Yano and Koga (1969); Aiba et al. (1968); Teissier (Edward, 1970); Webb (Edward, 1970) shown in Figure 9. The model with the best fit was selected on the basis of highest correlation coefficient (R^2) and the least root mean square error (RMSE). The kinetic parameters of the models ($r_{s\max}$, K_s and K_i) were estimated using the non-linear regression routine of MATLAB 6.5. The results shown in Table 2 revealed that between the five models, Haldane and Yano and Koga models have a correlation coefficient (R^2) greater than 0.90 and a root mean square error (RMSE) less than 1%. This indicates a very good fit to the batch

Table 1. Kinetic constants obtained from the fitting of batch experimental runs data of phenol degradation by *P. aeruginosa* to substrate utilization kinetic models.

Model	S_o (mg/L)	K_s (mg/L)	$r_{s\max}$ (mg/mg/h)	RMSE	R^2
Monod	100	23.8	0.145	0.0188	0.8347
Moser		853.4	0.143	0.0178	0.8701
Adapted Miura		0.91	0.145	0.0130	0.9311
Eckenfelder		0.0017	-	0.0144	0.9036
Monod	200	79.8	0.120	0.0203	0.5845
Moser		905.3	0.073	0.0242	0.4623
Adapted Miura		1.63	0.110	0.0187	0.6290
Eckenfelder		0.0005	-	0.0192	0.6290
Monod	300	139.8	0.094	0.0134	0.6808
Moser		1015.9	0.053	0.0191	0.3928
Adapted Miura		4.41	0.079	0.0120	0.7592
Eckenfelder		0.0003	-	0.0118	0.7504
Monod	400	208	0.080	0.0113	0.6973
Moser		11460	0.055	0.0131	0.6086
Adapted Miura		13.8	0.065	0.0014	0.8220
Eckenfelder		0.0002	-	0.0091	0.8007
Monod	500	331.2	0.064	0.0098	0.6682
Moser		13590	0.038	0.0119	0.5277
Adapted Miura		40.6	0.051	0.0011	0.8593
Eckenfelder		0.0001	-	0.0073	0.8177

experimental data. The difference in the R^2 and RMSE values between the two models is statistically insignificant. Thus, both the Haldane and Yano and Koga inhibition models may be proposed as the best models to describe the phenol degradation behaviour of monoculture of *P. aeruginosa*. Yang and Humphrey (1975); Agarry and Solomon (2008) have reported similar observations with Haldane's equation and two other models in describing phenol degradation by *P. putida*, *T. cutaneum* and *Pseudomonas fluorescence*, respectively.

Assessment of performance

Phenol has been used widely as a model inhibitory substrate and its biodegradation kinetics have been determined for many microorganisms (Bandyopadhyay et al., 1998; Oboirien et al., 2005; Saravanan et al., 2008; Agarry and Solomon, 2008) The performance of this indigenous monoculture of *P. aeruginosa* is being compared with well known effective degraders of phenol with emphasis on maximum specific substrate consumption rate. Reported values of the maximum specific substrate consumption rate ($r_{s\max}$) varied from 0.001 to 2.6 h^{-1} (Zilli et al., 1993; Folsom et al., 1990; Schroder et al., 1997). Folsom et al. (1990) and Schroder et al. (1997) reported a $r_{s\max}$ value of 2.6 and 0.4 h^{-1} for *Burkholderia cepacia* G4, respectively. Whereas, Zilli et al. (1993) reported a value of 0.0016 h^{-1} for *P. putida* NCIMB 10015. The $r_{s\max}$ value of 0.294 mg/mg/h (using the value obtained for Yano and Koga model) for the monoculture of *P. aeruginosa* was comparatively lower than the value obtained by Schroder et al. (1997) for *B. cepacia* G4, however, higher than the value for *P. putida* NCIMB 10015. Using the Haldane model, for equivalent initial phenol concentrations, the phenol degradation efficiencies obtained in this work ($r_{s\max}$ = 0.457) were higher than those reported by Agarry and Solomon (2008) for the monoculture of indigenous *P. fluorescence* ($r_{s\max}$ = 0.357). More also, the performance of the indigenous monoculture of Pseudomonas aeruginosa in the degradation of pure phenol and phenol present in the refinery waste water effluents are compared as shown in Table 3. The results revealed that local isolates of P. aeruginosa was more effective in the degradation of pure phenol than phenol present in the refinery waste water effluents. This showed that the biodegradation of phenol present in refinery waste water effluents is being inhibited by other compounds in the waste effluents. This corroborates the report of Reardon et al. (2000) that degradation or the removal of one compound can be inhibited by another compound in the mixture.

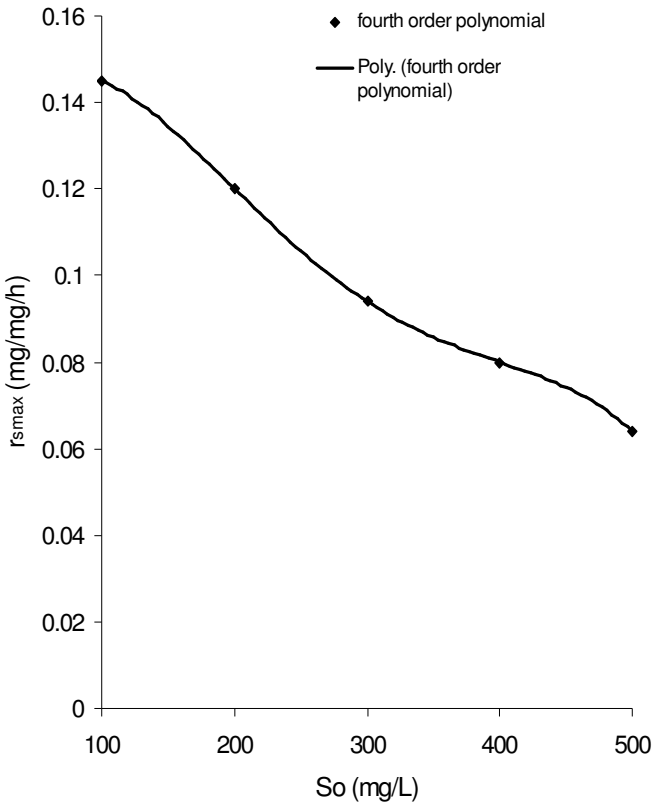

Figure 8. Variation of $r_{s\max}$ with So for phenol degradation by monoculture of *P. aeruginosa.*

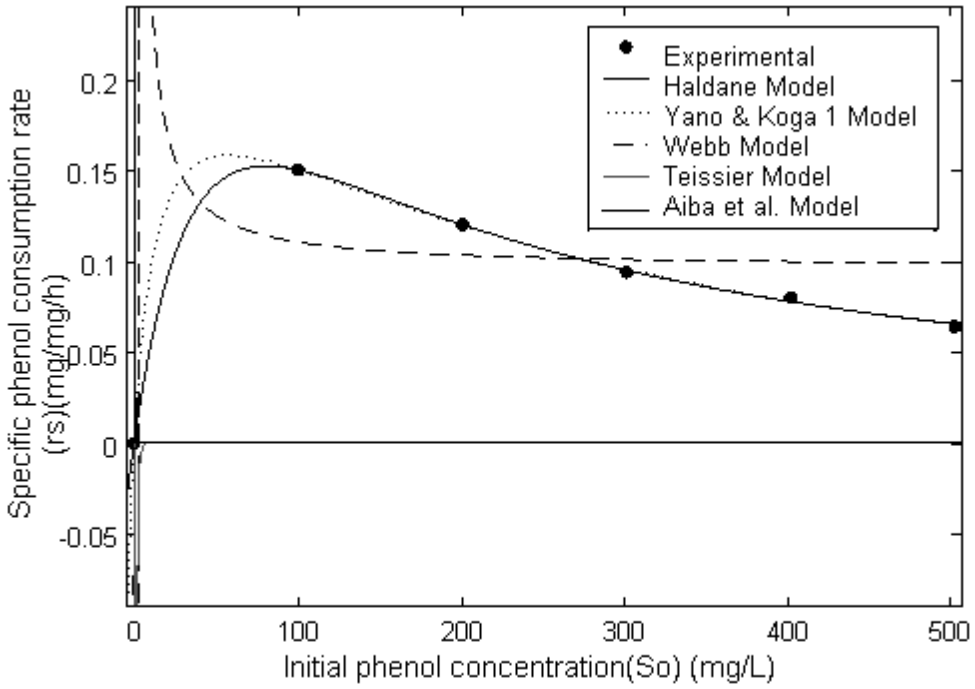

Figure 9. Experimental and predicted specific phenol consumption rate of *P. aeruginosa* due to some kinetic models.

Table 2. Kinetic parameters value obtained from five inhibition models fitted to the biodegradation data of *P. aeruginosa.*

Model	$r_{s\max}$ (mg/mg/h)	K_s (mg/L)	K_1 (mg/L)	K_2 (mg/L)	K_i (mg/L)	R^2	RMSE
Yano and Koga 1	0.294	42.7	172	678	-	0.9994	0.0019
Teissier	0.565	0.441	-	-	0.605	-3.349	0.1351
Aiba et al	0.565	0.441	-	-	0.605	-3.35	0.1351
Webb	4.821	-1.631	0.543	-	29	0.8073	0.0348
Haldane	0.458	99.9	-	-	86.1	0.9993	0.0017

Table 3. Comparison of the biodegradation of pure phenol and phenol present in refinery waste water by indigenous monoculture of *P. aeruginosa.*

Organism	Type of waste	Residence time (h)	% Phenol degradation	References
P. aeruginosa	Phenol in refinery waste water (28 mg/L)	48	82.6	Aremu (2003)
P. aeruginosa	Phenol in refinery waste water (30 mg/L)	48	90	Ojumu et al. (2005)
		60	100	,,
P. aeruginosa	Pure phenol (100 mg/L)	48	94.4	This study
		54	100	,,

Conclusion

The present study shows the potential of the isolated indigenous monoculture of *P. aeruginosa* for phenol wastewater treatment. The performance of the indigenous strain in biodegradation of phenol in the nutrient medium is excellent. The parameter K_s increased, while $r_{s\max}$ decreased with the higher values of initial phenol concentration, indicating an inhibition effect of phenol.

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NOMENCLATURE

K_s, Half-saturation constant (mg/L); K_i, Inhibition constant (g/l); r_s, Specific phenol (substrate) consumption rate (mg/mg/hr); $r_{s\max}$, Maximum specific phenol (substrate) consumption rate (mg/mg/hr); S, C_s, Substrate (phenol) concentration (mg/L).

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Appendix

Equations for kinetic models according to Tables 1 and 2

Monod (1949): $r_s = \frac{r_{s,\max} S}{K_s + S}$

Adapted Miura (Layokun et al., 1987): $r_s = \frac{r_{s,\max} S/X}{K_s + S/X}$

Moser (Layokun et al., 1987): $r_s = \frac{r_{s,\max} S^2}{K_s + S^2}$

Eckenfelder (Layokun et al., 1987): $r_s = KS$

Haldane (Andrews, 1968): $r_s = \frac{r_{s\max} S}{K_s + S + \frac{S^2}{K_i}}$

Aiba et al. (1968): $r_s = r_{s_{\max}} \frac{C_{s_{\exp}} \left(- C_s / K_i \right)}{K_s + C_s}$

Teissier (Edwards, 1970): $r_s = r_{s_{\max}} \left[\exp\left(- C_s / K_i \right) - \exp\left(- C_s / K_s \right) \right]$

Webb (Edwards, 1970): $r_s = r_{s_{\max}} \frac{C_s \left(1 + C_s / K_i \right)}{K_s + C_s + C_s^2 / K_1}$

Yano and Koga (1969): $r_s = r_{s_{\max}} \frac{C_s}{K_s + C_s + C_s^2 / K_1 + C_s^3 / K_2^2}$