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Kinetics of growth and phenol degradation by *Pseudomonas* species isolated from petroleum refinery wastewater

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Abstract

Biodegradation of phenol by pure cultures of *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 was investigated in batch culture at maximum initial concentration of 1000 mg/l. The experiments aimed at determining the kinetics of growth and biodegradation by measuring biomass growth and phenol depletion as a function of time in shake-flask batch reactor. The progress of growth was adequately described by integrated Haldane model with kinetic constants μ_m 0.025 h^{-1}, K_s 12.718 mg/l, K_i 1632.086 mg/l for *Pseudomonas* sp. DAF1 and μ_m 0.024 h^{-1}, K_s 14.628 mg/l, K_i 2986.159 mg/l for *Pseudomonas* sp. RWW2 at 1000 mg/l initial phenol concentration. The average yield of biomass ($Y_{X/S}$) in cultures was 0.001 A_{600} units.l/mg. The cultures followed substrate inhibition kinetics. These Pseudomonads are potentially applicable in the treatment of phenol-containing wastewaters.

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Introduction

Phenol and other phenolic compounds are widely used in many processes in petro-chemical, industries, textile, plastic, pesticide, resin and paint manufacturing, etc. Therefore, phenols are major component of wastewater emanating from these industries. Depending on the chemical process and the volume of water used, phenol concentrations up to 10000 mg/l may be present in industrial wastewater (Fedorak and Hrudey, 1988). Being water-soluble, phenols are readily mobilized to contaminate aquatic and terrestrial environments. Phenol and its derivatives are considered priority pollutants due to their toxicity. At low concentration (5 µg/l), phenol is toxic to aquatic microorganisms (Santos *et al.*, 2009). Phenol is toxic to fish and can be lethal at concentration of 5 – 25 mg/l (Saha *et al.*, 1999). Phenol is toxic to humans; it irritates skin, damages kidneys, liver, muscle and eyes and is potentially carcinogenic (Michałowicz and Duda, 2007). In addition, phenol is well-known for its inhibitory effects on microorganisms. Thus, treatment of phenolic effluents is critical to maintaining our natural ecosystems.

Among the methods for removing phenols from polluted environments, biological treatment is generally preferable due to its effectiveness and non-toxic end products (Chang *et al.*, 1998; Aleksieva *et al.*, 2002). A wide range of bacteria, most commonly *Pseudomonas* species have been reported to degrade phenol (Kumar *et al.*, 2005; Nair *et al.*, 2008; Bajaj *et al.*, 2009). However, at high concentrations, phenol exhibit inhibitory effects on microbial cultures (Hill and Robinson, 1995; Yang and Humphery 1975). Substrate inhibition of microorganisms has been attributed to many mechanisms that affect the overall microbial growth process (Edward, 1970). Evaluation of substrate inhibition is thus an important consideration in the removal of phenol from environmental media via biodegradation processes. A number of mathematical models have been developed to quantify inhibitory effects of toxic substrates (e.g phenol) on microbial growth kinetics. Among the substrate inhibition models, the Haldane models is one of the most commonly used equation for describing the growth inhibition kinetics of microorganism (Goudar *et al.*, 2000).

Given wide distribution of *Pseudomonas* species and its diverse biodegradative capabilities, the applicability of the Haldane model for phenol degradation and its associated microbial growth by this bacterial species is of interest. To the best of our knowledge, phenol biodegradation potential of bacteria indigenous to Nigerian petroleum refinery effluent has not been widely investigated. Available literature on phenol degradation by bacterial isolates from Nigerian petroleum refinery wastewater did not investigate the kinetics of the process. Available studies on phenol degradation kinetics by Solomon and coworkers were on *Pseudomonas* species isolated from oil-polluted sites in Niger-Delta region of Nigeria (Ojumu *et al.*, 2005; Agarry and Solomon, 2008; Agarry *et al.*, 2008; Agarry *et al.*, 2010). In this study, we investigated phenol biodegradation and growth kinetics of *Pseudomonas* species isolated from Port Harcourt petroleum refinery wastewater.

Materials and methods

Bacterial strains and phenol degradation medium

Phenol-degrading *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 were isolated from Port Harcourt petroleum refinery wastewater as described by Nweke and Okpokwasili (2010).

The mineral salt medium used had the following composition per litre: 945 ml of deionized distilled water, 25 ml of buffer solution A, 25 ml of mineral salt solution B and 5 ml of trace element solution C. The composition of each solution was as follows:

Buffer solution A: $Na_2HPO_4.2H_2O$, 35 g; KH_2PO_4, 4 g; deionized distilled water, 1 litre.

Mineral salt solution B: $(NH_4)_2SO_4$, 10 g; $MgCl_2.6H_2O$, 2.0 g; $Ca(NO_3)_2$, 1.0 g; deionized distilled water, 1 litre.

Trace element solution C: Na_2-EDTA, 800 mg; $FeSO_4.7H_2O$, 300 mg; $MnCl_2.4H_2O$, 10 mg; $CoCl_2$, 2.0 mg; $CuSO_4$, 1.0 mg; H_3BO_3, 1.0 mg; LiCl, 0.5 mg; KBr, 2.0 mg, $BaCl_2.2H_2O$, 0.5 mg; $ZnSO_4$, 2.0 mg;

$Na_2MoO_4.2H_2O$, 3.0 mg, $SnCl_2.2H_2O$, 0.5 mg; deionized distilled water, 1 litre.

To prevent precipitations of salts, the water, buffer solution A, mineral salt solution B and trace element solution C were autoclaved separately at 121°C for 15 minutes. After cooling, all the solutions were mixed together and kept as stock solution from which known volumes were taken for biodegradation experiments.

Acclimation of bacterial strains for phenol biodegradation

Pure cultures of the bacterial strains were grown in half strength nutrient broth supplemented with increasing concentrations of phenol (20-100 mg/l) in fed batch mode. In each run, cultures were incubated for 48 h at room temperature in a rotary shaker operated at 120 rpm. After growing cells in 100 mg/l phenol, cells were harvested by centrifugation at 4000 rpm for 10 minutes. The harvested cells were washed twice in sterile mineral salt medium to avoid nutrient carryover. The washed cells were suspended in the mineral salt medium and the optical density adjusted to 0.8 at 600 nm. The cell suspensions were used as innocula in biodegradation studies.

Biodegradation of phenol and biomass production

Biodegradation of phenol was carried out by preparing 100 ml volumes of 10, 20, 50, 100, 200, 300, 500 and 1000 mg/l phenol in duplicate 250 ml Erlenmeyer flasks. Requisite amounts of phenol stock solutions prepared in the mineral salt medium were diluted to 99 ml using mineral salt medium. Thereafter, 1 ml of cell suspension was added. The resultant culture contained initial phenol concentration of 10, 20, 50, 100, 300, 500 and 1000 mg/l. The cultures were incubated at room temperature (28 ± 2°C) on a rotary shaker operated at 120 rpm. Samples were withdrawn periodically for biomass and phenol analysis. The optical densities (A_{600}) of the cultures were determined spectrophotometrically. The phenol remaining was determined quantitatively using 4-aminoantipyrene colorimetric method as modified from Folsom *et al.* (1990). The withdrawn culture was centrifuged at 4000 rpm for 15 minutes to remove the cells. Into 4 ml of the sample supernatant (or smaller aliquot of the supernatant diluted to 4 ml) contained in 15-ml screw-capped culture tubes were added 0.2 ml of 2N NH_4OH and 0.1 ml of 2 % 4-aminoantipyrene. The tubes were shaken to mix the contents. Thereafter, 0.1 ml of 8 % $K_3Fe(CN)_6$ was added and the contents were mixed. The absorbance (A_{500}) of the resultant red-coloured solution was determined. Phenol concentrations were calculated by making reference to the standard curve. The phenol degradation rates (Qs) were calculated from plots of $S_o - S$ (amount of phenol degraded) versus time of incubation ($t - t_o$). Similarly, the specific growth rates were taken from $\ln(X/X_o)$ versus t-t_o plots for each initial phenol concentration. In each case, values were taken as the maximum slope in the respective plots. The yield coefficients (Y) were determined from the mass balance by plotting X-Xo versus So-S.

$$Q_s = \frac{S_o - S}{t - t_o} \quad (1)$$

$$\ln \frac{X}{X_o} = \mu(t - t_o) \quad (2)$$

$$X - X_o = Y(S_o - S) \quad (3)$$

Where S_o = initial phenol concentration at time t_o (mg/l), S = phenol concentration at time t (mg/l), X = biomass concentration (A_{600}) at time t; X_o = biomass concentration (A_{600}) at time t_o

Progress curve analysis of biodegradation and biomass production

The microbial growth can be represented by a Haldane equation:

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_i}} \quad (4)$$

Where μ is the specific growth rate (h^{-1}), S is the substrate concentration (mg/l), μ_m is the maximum specific growth rate (h^{-1}), K_s is the half saturation coefficient (mg/l), K_i is the inhibition coefficient (mg/l).

Biomass production is given by the expressions:

$$\frac{dX}{dt} = \mu X \quad (5)$$

Substituting for μ (in equation 4) into equation 5:

$$\frac{dX}{dt} = \frac{\mu_m S X}{K_s + S + \frac{S^2}{K_i}} \quad (6)$$

According to mass balance:

$$S = S_o - \frac{(X - X_o)}{Y} \quad (7)$$

Where: X = biomass concentration, Y = Yield coefficient (x/s), S = substrate concentration, t = time, X_o = initial biomass concentration, S_o = Initial substrate concentration.

Substituting for S into equation 6

$$\frac{dX}{dt} = \frac{\mu_m \left(S_o - \frac{(X - X_o)}{Y}\right) X}{K_s + \left(S_o - \frac{(X - X_o)}{Y}\right) + \frac{\left(S_o - \frac{(X - X_o)}{Y}\right)^2}{K_i}} \quad (8)$$

Integrating equation 8 yields equation 9

$$\mu_m t = \left[\frac{K_s Y}{YS_o + X_o} + 1 + \frac{YS_o + X_o}{K_i Y}\right] \ln \frac{X}{X_o} - \frac{K_s Y}{YS_o + X_o} \ln \frac{YS_o + X_o - X}{YS_o} \quad (9)$$

The rate of biodegradation can be represented as:

$$\frac{dS}{dt} = -\mu \frac{X}{Y} \quad (10)$$

Substituting for μ (in equation 4) into equation 10:

$$\frac{dS}{dt} = -\frac{\mu_m S X}{\left(K_s + S + \frac{S^2}{K_i}\right) Y} \quad (11)$$

According to mass balance:

$$X = Y(S_o - S) + X_o \quad (12)$$

Substituting for X into equation 11

$$\frac{dS}{dt} = -\frac{\mu_m S[Y(S_o - S) + X_o]}{\left(K_s + S + \frac{S^2}{K_i}\right) Y} \quad (13)$$

Integrating equation 13 yields equation 14

$$\mu_m t = \left[\frac{K_s Y}{YS_o + X_o} + 1 + \frac{YS_o + X_o}{K_i Y}\right] \ln \frac{YS_o + X_o - YS}{X_o} - \frac{K_s Y}{YS_o + X_o} \ln \frac{S}{S_o} \quad (14)$$

The biomass production data were fitted into integrated Haldane model (equation 9) and the kinetic parameters were estimated by iterative process using Table Curve 2D v5.01. The yield coefficients obtained from the *X-Xo* versus *So-S* plots were used as initial estimates in the progress curve analysis. The kinetic parameters obtained from the biomass production data were used to simulate substrate depletion according to equation 14.

Results

Phenol degradation and biomass production

The bacteria utilized phenol as sole source of carbon and energy even at 1000 mg/l. The resident time vary according to the initial phenol concentration. *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 spent 288 h and 246 h respectively to completely degrade 1000 mg/l phenol. At low concentrations, phenol was rapidly degraded. *Pseudomonas* sp. DAF1 completely degraded 20 mg/l, 50 mg/l, 100 mg/l and 200 mg/l phenol within 3 h, 4.5 h, 6 h and 6.5 h respectively. Correspondingly, *Pseudomonas* sp. RWW2 completely degraded these lower concentrations of phenol within 6 h, 8 h, 10 h and 26 h respectively (plots not shown). Generally, degradation of phenol was faster with *Pseudomonas* sp. DAF1. The influences of initial concentration of phenol on the growth of the Pseudomonads during biodegradation of phenol in relation to the biomass yield are shown in Fig 1. The yield of biomass per unit mass of substrate consumed ($Y_{x/s}$) are shown in Table 1. Maximum yield of 0.0014 ODunits.l/mg was achieved by the bacterial strains.

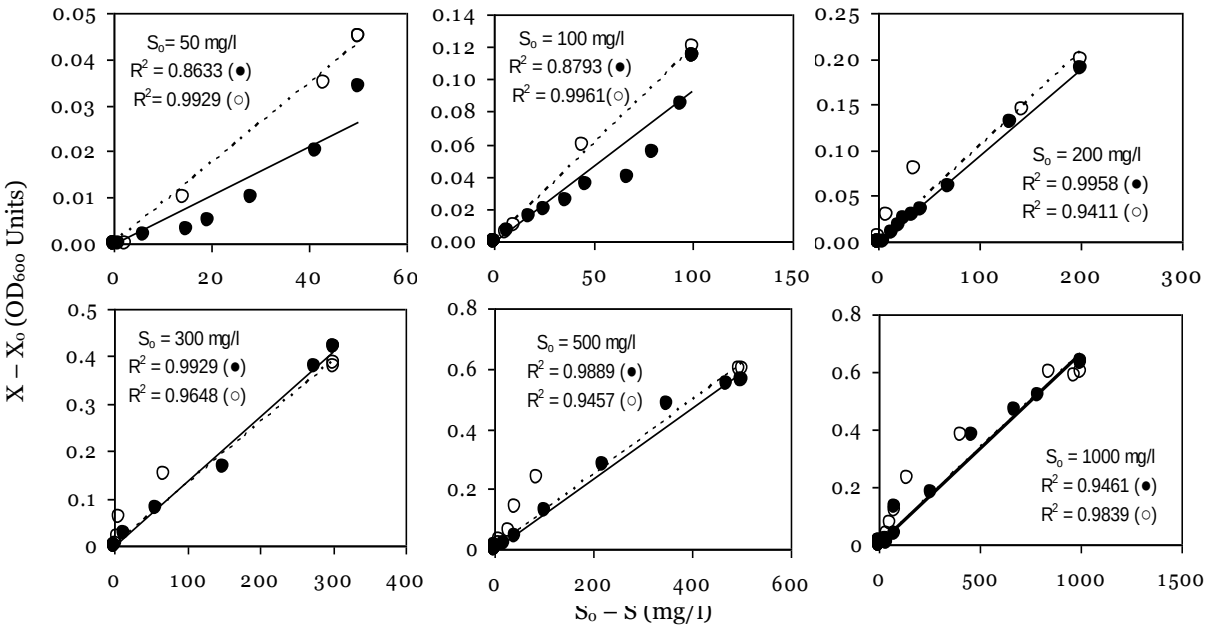

Fig. 1. Yield coefficients for *Pseudomonas* sp. DAF1 (●, solid regression line) and *Pseudomonas* sp. RWW2 (○, dotted regression line) growth on phenol.

The time course of bacterial growth and phenol depletion for 300 mg/l, 500 mg/l and 1000 mg/l phenol are shown in Fig. 2. The maximum optical density attained and the time taken by the organisms to reach stationary phase depends on the initial phenol concentration. Highest optical density (A_{600}) of 1.30 and 1.41 were attained by *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 respectively at 1000 mg/l phenol. The corresponding highest optical densities for the organisms at 500 mg/l were 0.58 and 0.62 respectively. The similar yield factor obtained for the organisms at different initial phenol concentration indicates that the highest optical density is dependent on the initial phenol concentration. The growth patterns has typical exponential and stationary phase with increasing lag phase. As the initial phenol concentration increased, the duration of lag phase increased, thus prolonging the time taken to achieve complete degradation of phenol. This observation corroborates reports of Oboirien *et al.* (2005). The stationary phases and further increase in the biomass after complete degradation of phenol could be attributed to intermediates of phenol metabolism. Similar observation has been reported for pure cultures of *Pseudomonas* and *Acinetobacter* species grown on phenol (Prpich and Daugulis, 2005; Hao *et al.*, 2002).

The dose-time biodegradation and growth pattern of the pure cultures indicated that the rate of growth and degradation decreased with increase in the initial phenol concentration. Similar observations have been reported for *Pseudomonas* species (Agarry and Solomon, 2008) and microbial consortium (Saravanan *et al.*, 2008).

Kinetics of phenol degradation and biomass production

The progress curve analysis indicated that the growth and the degradation data could be described with integrated Haldane's substrate inhibition model with R^2 values greater than 0.9. The kinetic parameters from the biomass production data are shown in Table 2. The growth of the bacterial strains and the biodegradation of phenol followed substrate inhibition pattern (Fig. 3). The rate of growth and degradation of phenol increased initially with increase in the concentration of phenol. However the rates subsequently decreased with increase in the concentration of phenol. The Haldane model described the overall growth of *Pseudomonas* sp. DAF1 (R^2 =0.959) better than *Pseudomonas* sp. RWW2 (R^2 = 0.639) at S_o range of 0 – 1000 mg/l. The kinetic parameters of the Haldane model are shown in Table 3.

Table 1. Yield coefficients for the bacterial growth on phenol estimated from linear plots

So (mg/l)	Yield coefficients (Yx/s) [A_{600} Units l/mg]	
	Pseudomonas sp. DAF1	*Pseudomonas* sp. RWW2
50	0.0005	0.0009
100	0.0009	0.0012
200	0.0010	0.0010
300	0.0014	0.0013
500	0.0012	0.0012
1000	0.0013	0.0014

Table 2. Progress curve Haldane's growth kinetics parameter values for phenol degradation

So (mg/l)	*Y* (A_{600} units.l/mg)	*Ks* (mg/l)	K_i (mg/l)	μ_m (h^{-1})	R^2
Pseudomonas sp DAF1					
300	0.0014	8.949	506.515	0.063	0.955
500	0.0011	113.92	1976.280	0.042	0.985
1000	0.0013	12.718	1632.086	0.025	0.977
Pseudomonas sp RWW2					
300	0.00128	4.005	506.475	0.039	0.949
500	0.0012	7.002	841.799	0.031	0.954
1000	0.0014	14.628	2986.159	0.024	0.948

However the Haldane model with R^2 values of 0.416 and 0.693 for *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 respectively did not describe the overall degradation rates well. The poor description of phenol degradation rate by *Pseudomonas* sp. DAF1 could be attributed to the interestingly higher degradation rate of phenol at S_o =200 mg/l. The estimated kinetic constants for the overall biodegradation of phenol were Q_s 74.075 mg/l/h, K_s 99.999 mg/l, K_i 161.606 mg/l (*Pseudomonas* sp. DAF1) and Q_s 32.607 mg/l/h, K_s 49.997 mg/l, K_i 192.255 mg/l (*Pseudomonas* sp. RWW2).

Discussion

Degradation of phenol occurs as a result of the activity of a large variety of microorganisms including bacteria, fungi and actinomycetes. Among the bacterial species that degrade phenol, *Pseudomonas* is widely applied. *Pseudomonas* species are widely distributed in nature and are known for their immense ability to grow on many organic compounds. The Pseudomonads used in this study were among the preponderant bacteria in a petroleum refinery wastewater (Nweke and Okpokwasili, 2010). The organisms are shown to possess innate ability to utilize phenol as source of carbon and energy. The pseudomonads could be said to be adapted to phenol, having been isolated from petroleum refinery wastewater containing 71.2 mg/l phenol (Nweke and Okpokwasili, 2010). Given that the organisms could grow and degrade phenol up to initial concentration of 1000 mg/l, they can be described as phenol resistant strains. They degraded low concentration of phenol relatively faster. Agarry *et al.* (2008) reported that at 72h, *Pseudomonas aeruginosa* NCIB 950 and *Pseudomonas fluorescence* NCIB 3756 growing under batch condition, degraded 30 mg/l phenol present in liquid effluent of a Nigerian petroleum refinery by 94.5% and 69.4% respectively. Similarly, Ojumu *et al.* (2005) reported a complete mineralization of 30 mg/l phenol within 60 h and 84 h by same species of *P. aeruginosa* and *P. fluorescence* respectively. Using the same *P. aeruginosa* NCIB 950 and *P. fluorescence* NCIB 3756, but acclimated in 5 mg/l phenol, 250 mg/l phenol was degraded by 100% and 41.5% at 72 h respectively (Oboirien *et al.*, 2005). The faster rate of degradation of phenol in this work could be attributed to acclimation of the Psudomonads in the phenolic petroleum refinery wastewater and further acclimation in culture medium containing up to 100 mg/l phenol.

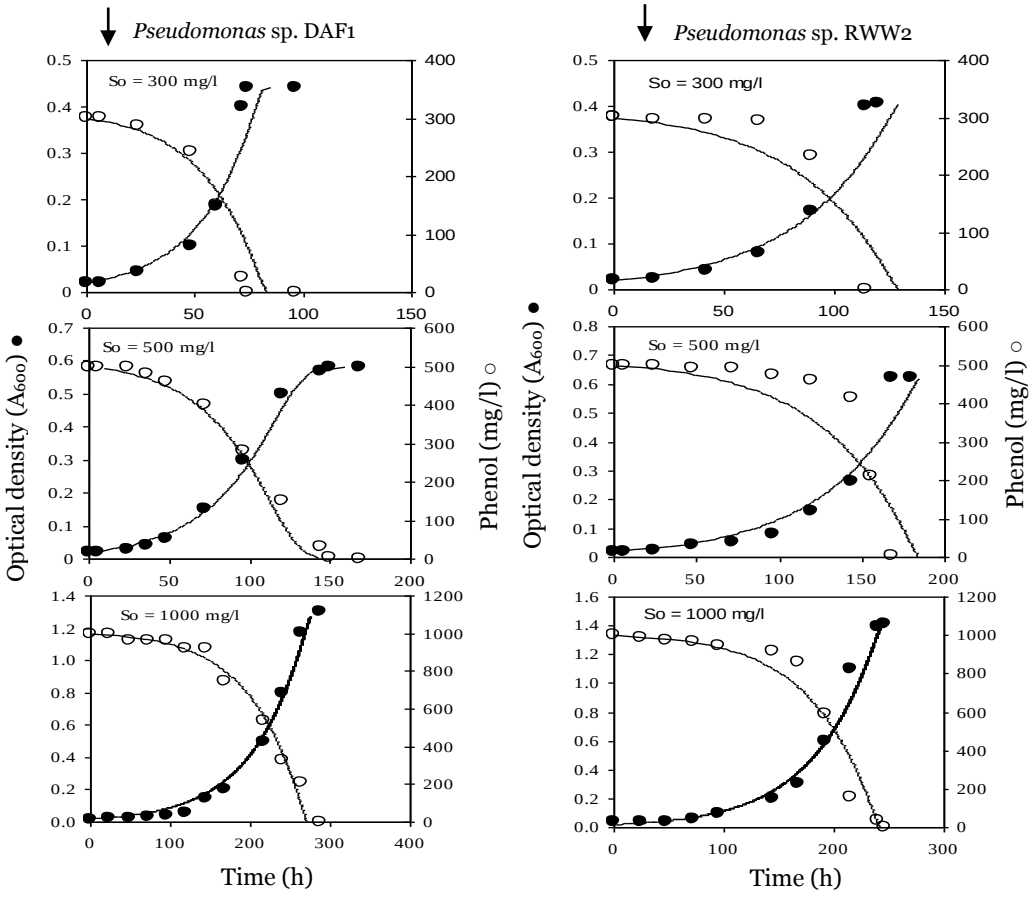

Fig. 2. Observed and predicted growth of *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 during biodegradation of phenol.

Relatively longer resident time was reported by Agarry and Solomon (2008). Using acclimated *Pseudomonas fluorescence* isolated from an oil-polluted area in Niger-Delta region of Nigeria, they reported resident time of 84 h, 120 h, 168 h, 252 h and 354 h for an initial phenol concentration of 100, 200, 300, 400 and 500 mg/l respectively. The *Pseudomonas* species used in this work was able to degrade 1000 mg/l. Degradation of phenol at high concentration by *Pseudomonas* species has been reported in literature. *Pseudoimonas aeruginosa* MTCC 4996 was capable of degrading 1300 mg/l phenol within 156 h (Kotresha and Vidyasagar, 2008). Jame *et al.* (2008), reported degradation of 600 mg/l phenol in 72 h by a *Pseudomonas* species. High concentration of 1000 mg/l phenol has also been degraded by *Pseudomonas* strains (Kumar *et al.*, 2005; Shourian *et al.*, 2009). At 1000 mg/l phenol, *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 with degradation rates of 5.6 mg/l/h and 7.31 mg/l/h respectively degraded phenol at lower rates than *Pseudomonas aeruginosa* and *Pseudomonas pseudomallei* reported by Afzal *et al.* (2007). The degradation rate of *Pseudomonas aeruginosa* ranged from 34.83 mg/l/h to 37.22 mg/l/h while that of *Pseudomonas pseudomallei* ranged from 23.95 mg/l/h to 29.04 mg/l/h in the presence of various salts.

In this study, integrated Haldane model with variables of maximum specific growth rate, biomass yield, half saturation and inhibition constants were used to evaluate the progress of cell growth during phenol degradation at different initial concentrations. In this regard, non-linear least squares computerized curve fitting procedure of Table Curve 2D was used to estimate kinetic parameters. The values of μ_m, K_s, K_i and $Y_{x/s}$ are shown in Table 2. Generally, the Haldane model gave good description of the experimental data with $R^2 > 0.9$.

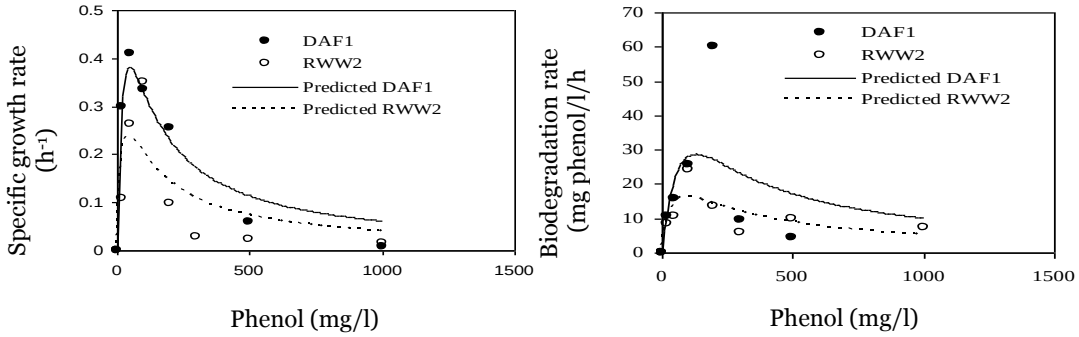

Fig. 3. Experimental and predicted specific growth and degradation rate of *Pseudomonas* sp. DAF1 and *Pseudomonas* sp. RWW2 during biodegradation of phenol

Table 3. Haldane kinetic parameters for biodegradation of phenol by batch cultures of *Pseudomonas* species

Bacteria	Phenol (mg/l)	μ_m (h^{-1})	K_s (mg/l)	K_i (mg/l)	Reference
P. putida ATCC 17484	0 – 700	0.534	0.015	470	Hill and Robinson, 1975.
P. putida ATCC 17484	< 200	0.388	1.06	903	Hutchinson and Robinson, 1990.
P. putida Q5	0 – 200	0.119	5.27	377	Kotturi *et al.*, 1991.
P. fluorescens 2218	0 – 600	0.618	71.4	241.0	Kumaran and Paruchuri, 1997
P. putida DSM 548	0 – 100	0.436	6.19	54.1	Monteiro *et al.*, 2000
P. putida MTCC 1194	0 – 500	0.305	36.33	129.79	Kumar *et al.*, 2005.
P. putida CCRC 14365	0 – 400	0.245	11.76	1185.79	Juang and Tsai, 2006
P. putida LY 1	0 – 800	0.217	24.4	121.7	Li *et al.*, 2010
Pseudomonas sp. DAF1	0 – 1000	0.799	25.276	84.591	This work
Pseudomonas sp. RWW2	0 – 1000	0.461	20.998	94.742	This work

The data indicated inhibitory effects of phenol at high phenol concentrations. The inhibition of microbial growth and biodegradation by phenol is a well-known phenomenon and has been reported in many organisms (Goudar *et al.*, 2000; Oboirien *et al.*, 2005; Okpokwasili and Nweke, 2006; Agarry and Solomon, 2008; Agarry *et al.*, 2008; Agarry *et al.*, 2010). Due to its potential to degrade phenol, pure cultures of the *Pseudomonas* species could be used as agents for treatment of petroleum refinery wastewater. Both cultures degraded phenol up to 1000 mg/l in mineral salts medium. The substrate inhibition due to phenol and the growth kinetics of the cultures were described using Haldane model. The biokinetics constants evaluated using the model showed good tolerance and growth of the culture during degradation of phenol. These indigenous bacteria from petroleum refinery wastewater have demonstrated capacity for adaptive growth in phenol and its complete degradation at relatively high concentration. They can be exploited for optimized removal of phenol from phenolic wastewater.

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