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Full Length Research Paper

Kinetics of batch microbial degradation of phenols by indigenous binary mixed culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescence*

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The potential of various organisms to metabolize organic compounds has been observed to be a potentially effective means in disposing of hazardous and toxic wastes. Phenolic compounds have long been recognized as one of the most recalcitrant and persistent organic chemicals in the environment. The bioremediation potential of an indigenous binary mixed culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* was studied in batch culture 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 the different initial phenol concentrations. Increasing the initial phenol concentration from 100 to 500 mg/L increased the lag phase from 0 to 18 h and correspondingly prolonged the degradation process from 24 to 96 h. There was decrease in biodegradation rate as initial phenol concentration increased. Fitting data into three different kinetic models (Monod, Haldane, and Yano and Koga) showed that the difference in fit between the models was very small and thus statistically insignificant. Thus, the Yano and Koga model has been used to interpret the free cell data on phenol biodegradation. The kinetic parameters have been estimated up to initial phenol concentration of 500 mg/L. The r_{smax} decreased, while K_s and K_i increased with higher concentration of phenol. The r_{smax} has been found to be a strong function of initial phenol concentration.

Key words: Binary mixed culture, phenol, biodegradation, kinetic model, batch cultivation, bioreactor, primary culture, secondary culture, inoculum, 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; Banyopadhyay et al., 1998). This aromatic compound is water-soluble and highly mobile (Collins and Daugulis, 1997) and as such waste waters 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, representing a serious ecological problem due to their widespread use and occurrence throughout the environment (Fava et al., 1995).

Phenol is a listed priority pollutant by the U.S. Environmental Protection Agency (EPA, 1979) and is 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 docu-

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Nomenclature: K_s Half-saturation constant (mg/L); K_s, inhibition constant (g/L); K_s, specific phenol (substrate) consumption rate (mg/mg/h); and , maximum specific phenol (substrate) consumption rate (mg/mg/h).

mented (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 the greatest risk to humans (Prpich and Daugulis, 2005).

A variety of techniques have been used for the removal of phenol from industrial effluents and contaminated waters with bioremediation receiving the most attention due to its environmentally friendly, its ability to completely mineralize toxic organic compounds and of low-cost (Kobayashi and Rittman, 1982; Prpich and Daugulis, 2005). Microbial degradation of phenol has been actively studied and these studies have shown that phenol can be aerobically degraded by wide variety of fungi and bacteria 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), *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 various models, the Monod and Andrew (Haldane) equations have been extensively used to describe phenol biodegradation (Bandyopadhyay et al., 1998; Reardon et al., 2000; Oboirien et al., 2005). The Monod and Andrew (Haldane) equations are based on the specific growth rate (Bandyopadhyay et al., 1998; Reardon et al., 2000), but may also be related to the specific substrate consumption rate (Edwards, 1970; Solomon et al., 1994). Other kinetic models have been propagated. Sokol (1988) has reported a better fit for a modified Monod-Haldane equation while Schroeder 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, surprisingly, little work has been published on phenol microbial degradation kinetics based on specific substrate consumption rate using pure or mixed culture systems. The present study investigated the effect of initial phenol (substrate) concentration on the degradation potential of an indigenous binary mixed culture (local strains of *Pseudomonas aeruginosa* and *Pseudomonas fluorescence*) isolated from an oil-polluted swampy area of Warri in Niger-Delta region of Nigeria.

MATERIALS AND METHODS

Microorganisms

The microorganisms, binary mixed culture of *P. aeruginosa* and *P.fluorescence* being an indigenous 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 microorganism was maintained on nutrient agar slant and stored at $4 \pm 1^{o}C$ 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 0.5 g, $(NH_4)_2SO_4$ 0.5 g, deionized water 100 ml. Trace element solution B composition NaCl 0.5 g, $CaCl_2$ 0.02 g, $MnSO_4$ 0.02 g, $CuSO_4.5H_2O$ 0.02 g, H_3BO_3 0.01 g , deionized water 50 ml. Solution C composition $MgSO_4.7H_2O$ 0.5 g, deionized water 50 ml, solution D composition $FeSO_4$ 0.02 g, 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 D were autoclaved at $121^{o}C$ for 15 min. 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 loop 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 Brunwick gyratory shaker (G25-R model, N.J. U.S.A) for 48 h at a temperature of $30^{o}C$ and agitated with a speed of 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 the phenol as 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 litres working volume. 800 ml of the autoclaved mineral salt medium and 3 litres 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 litres of working volume. The bioreactor was operated for several hours at a temperature of $30^{o}C$, aeration rate of 3.0 vvm and agitation of 300 rpm. Culture broth was withdrawn at every 6 h for biomass and phenol determination.

Estimation of phenol concentration

The under graded phenol was estimated quantitatively by the spectrophotometric method using 4-amino antipyrene as colour indicator (Yang and Humphrey, 1975) at an absorbance of 510 nm.

Estimation of biomass concentration

The biomass concentration was estimated using the dry weight method. 50 ml sample of culture broth was withdrawn from the bioreactor and centrifuged (Gllenkamp centrifuge) at 4000 rpm for 20 min in plastic centrifuge tubes. The supernatant was decanted into small bottles and stored at $4^{o}C$ for subsequent phenol estimation. The pellets was re-suspended in de-ionized water and re-centrifuged. The supernatant was decanted and pellets rinsed off 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 $105^{o}C$ for between 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 second weight was used to estimate the dry weight of the biomass.

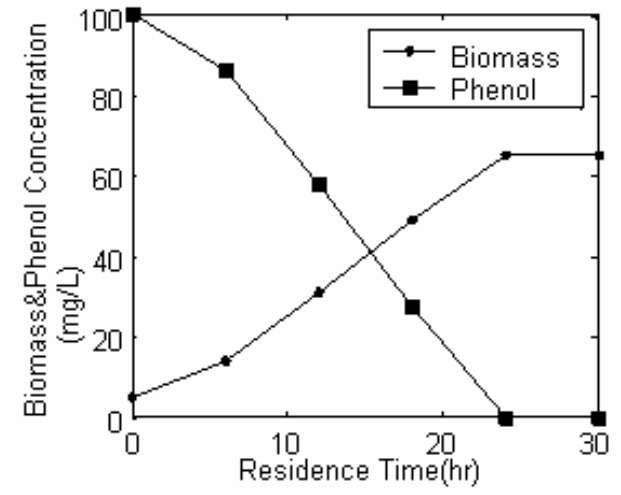

Figure 1a. A plot of 100 mgL phenol and biomass concentration as a function of time.

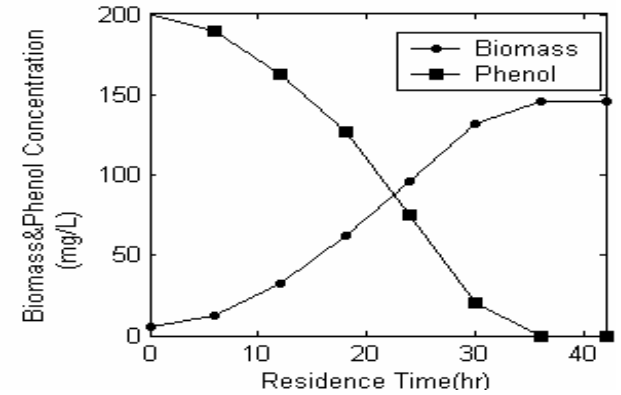

Figure 1b. A plot of 200 mgL phenol and biomass concentration as a function of time.

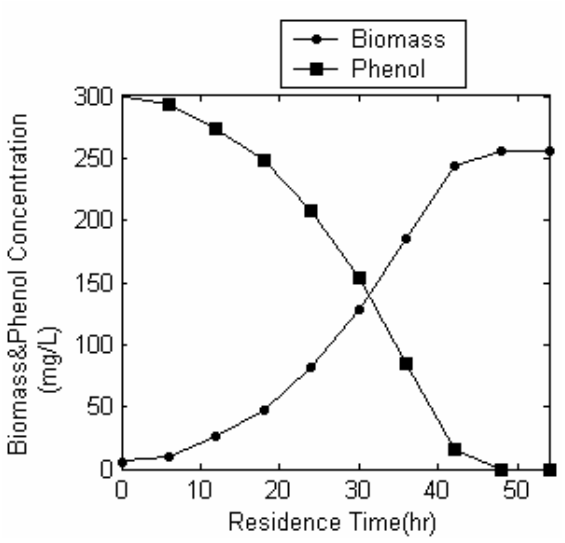

Figure 1c. A plot of 300 mgL phenol and biomass concentration as a function of time.

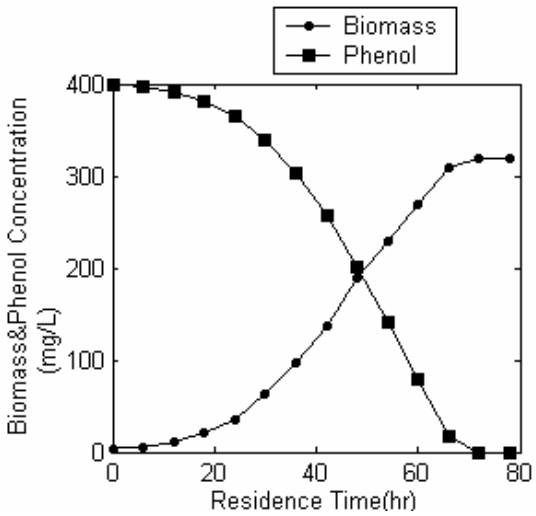

Figure 1d. A plot of 400 mgL phenol and biomass concentration as a function of time.

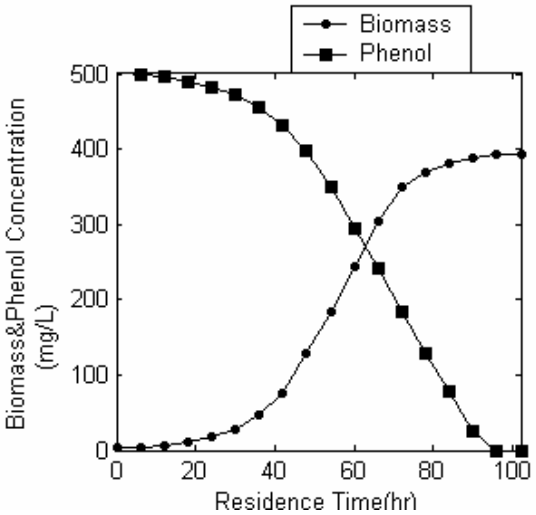

Figure 1e. A plot of 500 mg/L phenol and biomass concentration as a function of time.

RESULTS AND DISCUSSION

Five batch cultivation experiments were carried out using phenol as single limiting substrate for binary mixed culture of *P. aeruginosa* and *P. fluorescence.* Different initial phenol concentrations of 100 to 500 mg/L were used. The extent of phenol degradation using these different initial phenol concentrations was investigated for several batch residence times by intermittent sampling.

Figures 1a – e show the biodegradation potential of the indigenous binary mixed culture of *P. aeruginosa* and *P. fluorescence* 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 figure also depicts the typical cell growth curve. The cell

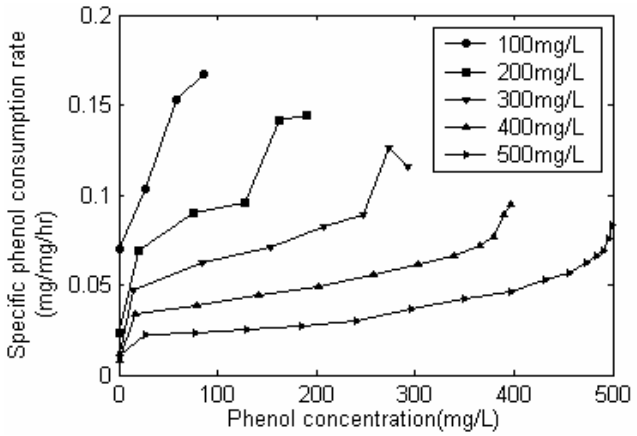

Figure 2. Specific phenol consumption rate as a function of phenol concentration.

growth curve has typical exponential and stationary phases with increasing lag phase. It could be seen from Figures 1a – e that the different initial phenol concentrations ranging from 100 – 500 mg/L were completely degraded (consumed) at different residence time of 24, 36, 48, 72, 96 h, and during these times the biomass correspondingly increased to a maximum of 65, 146, 256, 320, and 392 mg/L, respectively. No lag phase was observed for initial phenol concentration of 100, 200 and 300 mg/L as shown in Figures 1a – c. However, for initial phenol concentration of 400 and 50 mg/L corresponding lag phase of 6 and 18 h was observed respectively as shown in Figures 1d – e. Oboirien et al. (2005) reported a similar observation that no lag phase was observed for 100 and 250 mg/L of phenol but a lag phase was observed for 500 mg/L of phenol when a mixed culture of *P.aeruginosa* NCIB 950 and *P. fluorescence* NCIB 3756 was grown on phenol. Moreover, as shown in Figures 1a – e, which indicate the comparison of the time course for phenol substrate consumption of all the five batches, it is evident that the rate of degradation decreased with increase in the initial phenol concentration. Bandyopadhyay et al. (1998) and Ruiz-ordaz et al. (2001) reported a similar observation on *P. putida* and *Candida* sp. grown on phenol, respectively.

Evaluation of biokinetic parameters

Batch phenol degradation was carried out with free suspended cells of indigenous binary mixed culture of *P. aeruginosa* and *P. fluorescence* 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 binary mixed culture of the *P. aeruginosa and P. fluorescence* as shown in Figures 1a – e. 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) gave a similar report. Relatively very few data exist in the literature on this parameter. Most of the data available concern 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 2. As seen from this figure, the specific phenol consumption rate (r_s) decreases as the phenol concentration (s) decreases for each of the different initial phenol concentrations. 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) reported a similar observation.

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 that can be based on specific substrate consumption rate as proposed by Posten (Solomon et al., 1994) and which have been used by Zilli et al. (1993) and Schroder et al. (1997). In this work, phenol was completely degraded and kinetic models of Monod (1949), Haldane (Andrews) (1968), and Yano and Koga (1969) based on specific substrate consumption rate were fitted to the experimental batch data obtained for the indigenous binary mixed culture of *P. aeruginosa* and *P. fluorescence*. The classical method of obtaining kinetic parameters (constants) is to linearize kinetic models. Recently, non-linear least squares computer fitting of data to model equations has been used (Schroder et al., 1997; Reardon et al., 2000). The non-linear least square fitting routine of Matlab 6.5 software package (with Trust-Region Algorithm) was used to fit the kinetic models to the different batch experimental data. The parameters of Monod (K_s and r_{smax}), Haldane (K_s , r_{smax} , K_i), and Yano and Koga (K_s , r_{smax} , K_i) were fitted to the experimental calculated specific phenol consumption rate and the corresponding phenol concentration under the constraint that r_s never exceeds the maximum obtainable specific consumption rate (r_{smax}) and the results are presented in Table 1. As seen from the table, the difference in fit (based on the coefficient of correlation, R^2) between the three different models examined at the different initial phenol concentrations is very small and thus statistically insignificant as indicated by their SSE (sum of square errors) values. Yang and Humphrey (1975) made similar observations with Andrew's equation and two other models in describing phenol degradation by *P. putida* and *Trichosporon cutaneum*. However, it could be seen from the table that the inhibition constant (Ki) values seem very large. The reason for this may be due to the fact that the phenol (substrate) was not in close proximity to the inhibition constant. Hill and Robinson (1975) reported a similar observation. Since phenol has been used widely as a model inhibitory substrate, its biodegradation kinetics has been determined for many microorganisms and substrate inhibition was observed for all previously repor-

Table 1. Kinetic constants obtained from the fitting of batch experimental runs data from phenol degradation by binary mixed culture to some kinetic models.

Microorganism	Model	S_o (mg/l)	K_s (mg/l)	r_{smax} (h^{-1})	K_i (g/l)	SSE	R^2
Binary mixed culture (*P. aeruginosa* and *P. fluorescence*)	Monod	100	10.7	0.167	-	0.006	0.06
	Yano and Koga		10.7	0.167	5.2	0.006	0.06
	Haldane		9.6	0.167	26.8	0.006	0.05
	Monod	200	27.7	0.144	-	0.002	0.81
	Yano and koga		27.8	0.144	9.0	0.002	0.81
	Haldane		27.6	0.144	45.1	0.002	0.81
	Monod	300	66.3	0.126	-	0.002	0.79
	Yano and koga		66.2	0.126	11.0	0.002	0.79
	Haldane		66.3	0.126	50.4	0.002	0.79
	Monod	400	113.9	0.095	-	0.002	0.75
	Yano and koga		107.5	0.095	12.8	0.002	0.75
	Haldane		110.3	0.094	53.7	0.002	0.75
	Monod	500	211.6	0.083	-	-	0.70
	Yano and koga		200.3	0.083	14.1	0.002	0.70
	Haldane		207.0	0.083	50.0	0.002	0.70

Equations for kinetic models according to the table:

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

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

Yano and Koga (1969): $r_s = \frac{r_{s\max} S}{K_s + S + \frac{S^3}{K_i^2}}$

ted studies. Therefore, Yano and Koga model were chosen as the kinetic model to evaluate the degradation of phenol by the indigenous binary mixed culture of *P. aeruginosa* and *P.fluorescence.* As observed from the table, the Yano and Koga parameters (r_{smax} , K_s and K_i) show a definite trend of variation. The r_{smax} decreased while K_s and K_i increased as the initial phenol concentration increased. Considering the fact that K_s is inversely related to the affinity of the microbial system for the substrate (Pirt, 1975), this increase of K_s corresponds to a decrease in affinity of the bacteria (binary mixed culture of *P.aeruginosa* and *P.fluorescence*) for phenol. It is therefore evident that inhibition becomes prominent as initial phenol concentration increases. Thus, the r_{smax} is a strong function of initial phenol concentration (S_o). The variation of r_{smax} with S_o has been indicated in Figure 3 and also fitted by the fourth order polynomial fit from which r_{smax} at any value of S_o within the range of 500 mg/L of phenol concentration may be predicted.

Assessment of performance

Phenol has been used widely as a model inhibitory substrate and its biodegradation kinetics has been determined for many microorganisms. The performance of this indigenous binary mixed culture of *P. aeruginosa* and *P. fluorescence* 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_{smax}) varied from 0.001 to 2.6 h^{-1} (Folsom et al., 1990; Zilli et al., 1993; Schroder et al., 1997). Folsom et al. (1990) and Schroder et al. (1997) reported a $r_{smax\ value}$ of 2.6 $h^{-1}$and 0.4 h^{-1} for *P. cepacia G4,* respectively. While Zilli et al. (1993) reported a value of 0.0016 h^{-1} for *P. putida NCIMB 10015*. The r_{smax} value of 0.144 mg/mg/h for the binary mixed culture was lower than that of *P. cepacia* G4, however greater than that of *P. putida NCIMB 10015*. For equivalent initial phenol concentrations, the phenol degradation efficiencies obtained in this work were higher than those reported by Agarry (2006) for the monoculture of indigenous *P. aeruginosa and P. fluorescence,* respectively. This was in agreement with other workers (Ambujon, 2001; Guieysse et al., 2001; Prpich and Daugulis, 2005; Oboirien et al., 2005) that mixed culture offers an improved performance than mono or pure cul-

ture of microorganisms. However, it is in contrast with the observation of Zilli et al. (1993). They reported that pure culture of *P. putida* was efficient than the mixed culture of *Pseudomonas* species.

Conclusions

The present study shows the potential of the isolated indigenous binary mixed culture of *P. aeruginosa* and *P. fluorescence* for phenol wastewater treatment. The performance of the indigenous strain in biodegradation of phenol in the nutrient medium is excellent. The parameter K_s and K_i increased with the higher values of initial phenol concentration, while the other parameter r_{smax} decreased with the corresponding increase in the initial phenol concentration, indicating inhibition effect of phenol.

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