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Substrate Inhibition Kinetics of Phenol Degradation by *Pseudomonas aeruginosa* and *Pseudomonas fluorescence*

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Abstract: The effect of aeration and initial substrate concentration on the biodegradation of phenol by *Pseudomonas aeruginosa* NCIB 950, *Pseudomonas fluorescence* NCIB 3756 and their mixed was investigated in batch reactor. Increasing initial concentration from 100 ppm (100 mg L^{-1}) to 250 and 500 ppm increased the lag phase for pure culture *Pseudomonas aeruginosa* to 8 and 16 h, respectively. Mixed cultures had a lag phase only at concentration of 500 mg L^{-1}. There was increase in biodegradation rate when there was increase in aeration rate, but statically the effect of initial concentration was more significant at 95% confidence level. *Pseudomonas aeruginosa* had better degradation rate. Fitting data into the integrated Haldane Model, the kinetic parameters values derived from the model (μ_{max}, Ki and Ks), for pure culture in 250 and 500 ppm phenol are 0.46, 97, 450 and 0.38 h^{-1}, 195 and 450 mg L^{-1}, respectively while for mixed culture in 250 and 500 ppm phenol 0.85, 48, 250 and 0.58 h^{-1}, 127 and 450 mg L^{-1}, respectively were obtained.

Key words: Biodegradation, phenol, *Pseudomonas* sp., Haldane model, kinetic parameters

INTRODUCTION

The massive increase in the synthesis of organic chemicals by man has led to the production of wide variety of compounds, some of which are xenobiotic. Their xenobiotic character meant that their structures are not easily recognised by existing degradative enzymes and as a result they accumulate in the environment[1]. Phenol and its derivatives represent major organic pollutant in the effluent from many industrial activities such as oil refineries, chemical, petrochemical, pharmaceutical, metallurgical, pesticide products, paint and varnish industries, textile and also in the polymer industries like phenolic resins, bisphenol A, alkylphenols, caprolactums and adpic acid[2]. Phenol is a toxic and hazardous substance even at low concentrations[3,4]. The concentration of phenols in waste wasters varies from 10 to 300 mg L^{-1}[2].

Oil has established itself as the major source of revenue for Nigeria since 1970 and there has been a steady growth in the petrochemical industry of the nation. Refinery products and process constitute a very important source of pollution in the Nigerian aquatic ecosystem most especially due to the fact that some of the refineries are located near the coastal waters. Those that are located in the hinterland such as that of Kaduna discharge into fresh water ecosystems. Efficient treatment methods are necessary to reduce phenol concentration in wastewater to acceptable level, which is 5 ppm (US EPA). Several methods available for treatment of phenol, biological treatment is especially attractive because it has the potential to almost degrade completely without producing innocuous end products and minimum secondary waste generation[5].

Biological degradation of phenol has been extensively investigated and several studies have shown that phenol can be aerobically degraded by a wide variety of micro-organisms, including pure bacterial cultures such as *Aincetobacteria calcoaceticus*[6], *Alcaligenes eutrophus*[7,8], *Bacillius sterothermophilus*[9], *Burkholderia cepicia* G4[10,11], *Pseudomonas putida*[3,12-15]. A variety of kinetic substrate inhibition models have been

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used to describe the dynamics of microbial growth on phenol. Of the various inhibition models, the Andrews equation has been used extensively to describe phenol biodegradation[3,14-18]. The Andrews equation (Haldane) is based on the specific growth rate[19], but may also be related to the specific substrate consumption rate[20]. Other kinetic models have been propagated. Sokol[18] has shown a better fit for a modified Haldane equation but the kinetic parameter varies according to the history of the micro-organisms. Allsop *et al.*[19] reported a clear indication for intermediate production during phenol degradation in continuous culture after applying a step increase in the substrate feed concentration.

Proper design and operation of biological systems has potential of being the most cost effective way to dispose of toxic and hazardous chemicals (phenol) since almost complete oxidation is achieved[11]. Information about the kinetics of phenol degradation will improve process control and phenol removal efficiency in wastewater treatment plant. The present study investigated the effect of aeration and initial substrate concentration (phenol) the degradation process, also determined the phenol-inhibitory growth kinetics of two Pseudomonas species (*P. aeruginosa and P. fluorescens*) as well as the mixed cultures.

Mathematical formulation: According to Haldane[21] the growth of a micro-organism on an inhibitory substrate can be modeled in batch, well stirred tank reactor[22]. The specific rate of growth of the organism is given by:

$$\frac{\partial X}{\partial t} = \frac{\mu_{max} SX}{(S+Ks)\left(1+\frac{S}{Ki}\right)} \quad (1)$$

The initial conditions are (Xo, So).The cell concentration can be eliminated using overall mass balance and the definition of the yield coefficient $Y_{x/s}$

$$S = So - \frac{(X-Xo)}{Y_{X/S}} \quad (2)$$

We can define dimensionless variables and constants as follows:

$$Xo^* = \frac{Xo}{Y_{X/S}So} \qquad Ks^* = \frac{Ks}{So}$$
$$S^* = \frac{Xo}{So} \qquad Ki^* = \frac{Ki}{So} \quad (3)$$

These variables are inserted into equation (1.1) to eliminate X. The resulting equation is then integrated to give the Haldane Model:

$$\mu_{max} t = \frac{Ks^*}{1+X^*} \ln\left(\frac{1+X^*-S^*}{S^*X^*}\right) + \left(\frac{Ks^*+X^*+1}{Ki^*}+1\right) \ln\left(\frac{1+X^*-S^*}{X*}\right) + \frac{S^*-1}{Ki^*} \quad (4)$$

MATERIALS AND METHODS

Organisms: *Pseudomonas aeruginosa* NCIB 950 and *Pseudomonas fluorescence* NCIB 3756 used in this work was collected from the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The organism was maintained as direct stock culture from which inocula were prepared. It was grown on malt extract agar slant at 30°C for 5 days and stored at 40°C with regular sub-culturing.

Inoculum preparation: Eighty milliliter of 5 mg L^{-1} of phenol was measured into 250 mL conical flask and 20 mL of nutrient medium prepared according to Bondar *et al.*[23] and this was then inoculated with sub culture of the micro-organism. The innoculum was shaken continuously on an environment-controlled incubator shaker manufactured by New Brunswick Scientific Co. (USA) at 120 rpm for 72 h for growth of cells

Fermentation: Different fermentation runs were carried out on the batch fermentor unit of the double-unit Microform fermentor. Three liter of different concentrations of phenol (100, 250 and 500 g L^{-1}) was mixed with 800 mL of nutrient medium. 200 mL of inoculum was introduced aseptically and fermentation was carried out at different aeration rate of (1.5, 2.0 and 2.5 vvm) with agitation at rate of 400 rpm, temperature at 30°C, for 72 h, samples were every 8 h and the supernatant was analysed for phenol concentration and the filtrate for biomass analysis.

Analytical methods: Biomass concentration: The cell concentrations were determined by optical density and dry weight analysis. The optical density measurements were carried out at 600 nm wavelength using spectrophotometer (Bausch and Lomb). The dry weight was determined by filtration through a reweighed 1.2 μm filters (Whatman GF/C), this was then dried at 85°C to a constant weight. The filtrate were used for phenol concentration determination.

Phenol assay: The concentration of phenol was determined colorimetric method. The colorimetric method uses two reagents, 4-aminoantipyrine (AAP) (20.8 mM of AAP in 0.25 M $NaHCO_3$) and ferricyanide (83.4 mM of $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$), as colour generating substrates when combined with phenolic compounds. The colour generated at peak wavelength of 510 nm was directly proportional to the concentration.

Statistical analysis: In order to determine the biokinetics parameters of degradation of phenol using *pseudomonas* system, a non-linear regression Haldane Model was fitted using the original data, applying POLYMATH 5.1 for Windows© (2004-Control Data Corporation). SPSS package for windows operating system was used for the ANOVA analysis of the data.

RESULTS AND DISCUSSION

Biodegradation rate of microorganism: Figure 1a-c present the biodegradation rate of 250 mg L^{-1} phenol using *P. aeruginosa*, *P. fluorescence* and their mixed culture (i.e. *P. aeruginosa* and *P. fluorescence*). The biodegradation is faster with *P. aeruginosa* than with *P. fluorescence*, the biodegradation was completed with the former at 72 h fermentation with 2.5 vvm aeration while substantial amount of phenol (41.5%) remained with the latter at the same time under the same condition. Although Collins and Daugulis[24] reported that aeration rate greater than 0.5 vvm produce excessive foaming and lead to solvent and cell losses, present results showed that biodegradation rate was increased with increasing aeration rate for the various phenol concentrations and without foaming. Remarkable result was obtained with the mixed culture; there was complete mineralization within the 64 h fermentation regardless of the aeration rate. This may be as a result of co-metabolism, or transformation of the phenol compounds by the mixed cultures, where by each of the microbes gain a small amount of carbon and energy. Here the inhibitory problem associated with pure culture was solved by the synergy and co-metabolism in the mixed culture. According to Singleton[1], the problem of toxic intermediates produced by pure cultures produced cultures may be overcome by the use of mixed cultures or microbial consortia which have wider spectrum of metabolic properties.

Effect of initial concentration: Increase in the initial substrate concentration in a batch reactor prolonged the biodegradation process in all the experiment by increasing the duration of the lag phase as shown in Fig. 2 a-c for the pure culture (*Pseudomonas aeruginosa*) and Fig. 3 a-c for

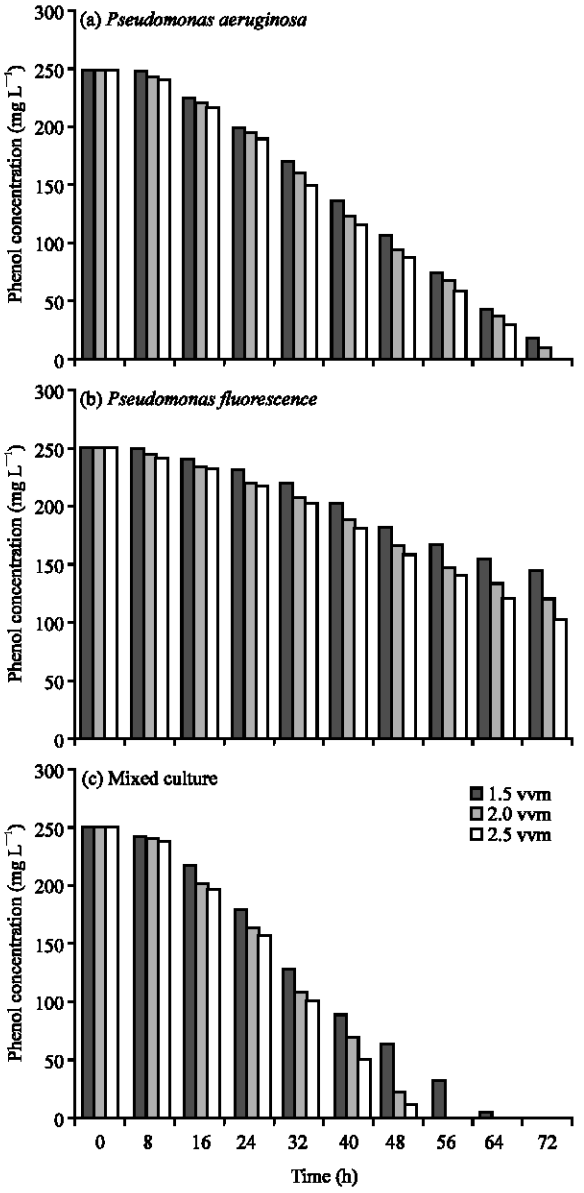

Fig. 1: Plot of phenol biodegradation with time using cultures of *Pseudomonas* species at various operating conditions

the mixed culture at different initial concentrations. In the pure culture, at 100 mg L^{-1} phenol no lag phase was observed but it became obvious as the initial concentration was increased to 250 and 500 mg L^{-1}, it extended to 8 and 16 h, respectively. This observation was supported by earlier works of Andrews[16], Colins and Daugulis[24], while for mixed culture no lag phase at 100 and 250 mg L^{-1} but at 500 mg L^{-1} the lag phase lasted for 8 h. The mixed culture would give significant advantage in phenol wastewater treatment

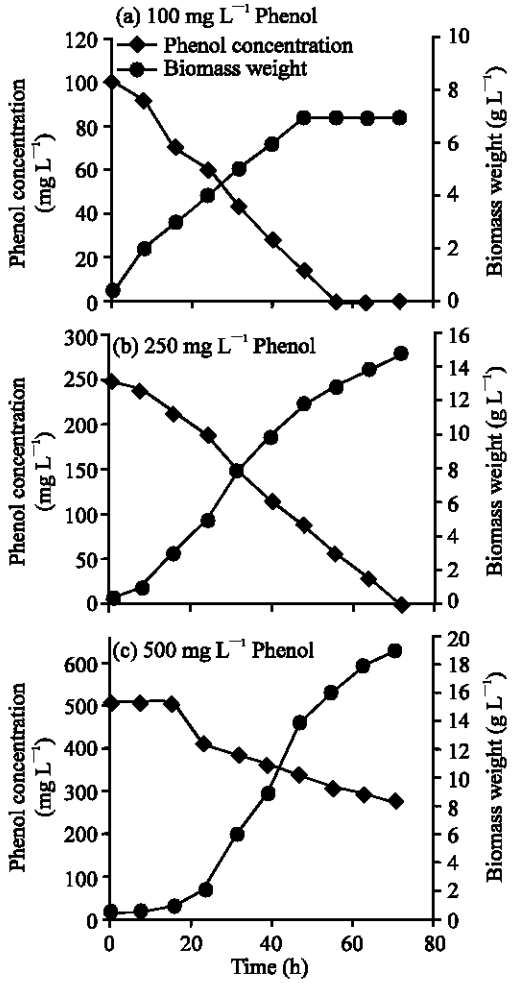

Fig. 2: Effect of initial phenol concentration and biomass changes with time in phenol biodegradation using cultures of *Pseudomonas aeruginosa* at 2.5 vvm

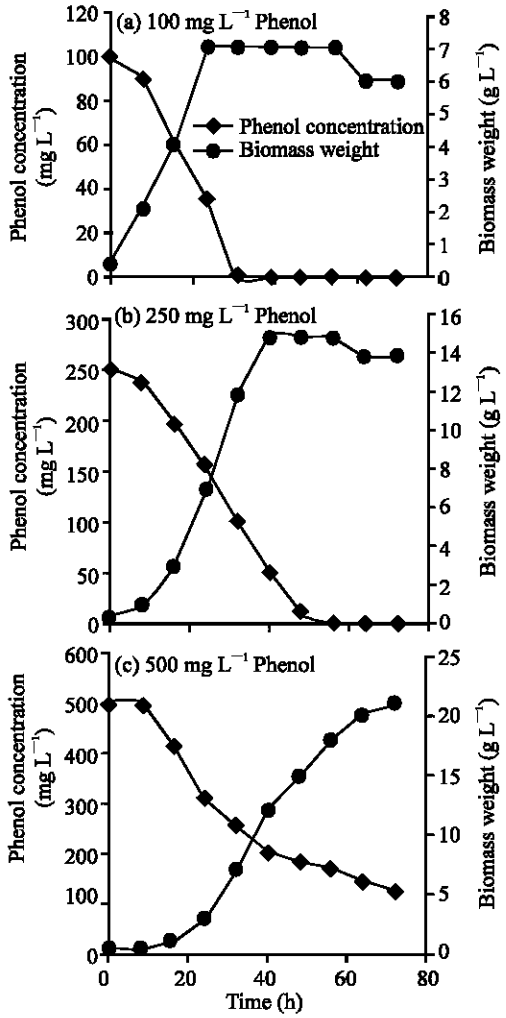

Fig. 3: Effect of phenol concentration and biomass changes with time in phenol biodegradation using mixed cultures of *Pseudomonas* species at 2.5 vvm

process by its rapid degradation rate. Although from these results there was increase in biodegradation rate when the aeration rate was increased the analysis of variance test showed that initial concentration was more important in the biodegradation of phenol.

Evaluation of model biokinetic parameters: Phenol has been used widely as a model inhibitory substrate, its biodegradation kinetics has been determined for many microorganisms. Reported values of maximum specific growth rates varied from 0.1 to 0.9[3,25,26]. Substrate inhibition was observed in all previously studies. The model derived values for Ki, Ks and U_{max}, for pure culture and the mixed culture cultivated in 250 and 500 mg L^{-1} phenol. Table 1 revealed an increase in the maximum specific growth rate and also a significant reduction in the inhibitory constants with the mixed culture. These

Table 1: Kinetic parameters of *Pseudomonas* species grown on phenol

	Pure culture (*P. aeruginosa*)		Mixed culture (*P. aeruginosa* and *P. fluorescence*)	
Kinetic parameters*	250 ppm	500 ppm	250 ppm	500 ppm
K_i	97.0 mg L^{-1}	195.0 mg L^{-1}	48.0 mg L^{-1}	127.0 mg L^{-1}
K_s	450.0 mg L^{-1}	450.0 mg L^{-1}	250.0 mg L^{-1}	450.0 mg L^{-1}
μ_{max}	0.46 h^{-1}	0.38 h^{-1}	0.85 h^{-1}	0.58 h^{-1}

* Parameters at 2.5 vvm aeration

accounted for easy and faster degradability of the substrate as aforementioned. The inhibitory problem associated with pure culture was solved by the synergy and co-metabolism in the mixed culture[1]. This confirms present speculation and suggestion of earlier work by Ojumu *et al.*[27] on the use of combination of these two microbes. It can be concluded therefore that the use of

mixed cultures or microbial consortia increases the degradation rate of phenol because of wider spectrum of metabolic properties as reported. Thus the possibility of up-scaling this bioremediation strategy to bulk dimension when a large volume of refinery and petrochemical effluent are available is therefore very promising.

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