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Inhibition Kinetics of Phenol Degradation by Binary Mixed Culture from Continuous Culture and Wash-Out Data

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Abstract: Steady states of a continuous culture with an inhibitory substrate was used to estimate kinetic parameters under substrate limitation (chemo stat operation) Mixed cultures of an indigenous *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* were grown in continuous culture on phenol as the sole source of carbon and energy at dilution rates of 0.010-0.20 h^{-1}. Using different dilution rates several steady states were investigated and the specific phenol consumption rates were calculated. In addition, phenol degradation was investigated by increasing the dilution rate above the critical dilution rate (washout cultivation). The results showed that the phenol degradation by mixed culture of *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* can be described by simple substrate inhibition kinetics under substrate limitation but cannot be described by simple substrate inhibition kinetics under washout cultivation. The phenol consumption rate (degradation rate) increased with increase in dilution rate. Fitting of the steady state data from continuous cultivation to various inhibition models resulted in the best fit for the Teissier kinetic inhibition model. The r_{smax} value of 0.322 mg/mg/hr obtained from the Teissier equation was comparable to the experimentally calculated r_{smax} value of 0.342 mg/mg/hr obtained under washout cultivation.

Key words: Continuous cultivation, washout cultivation, steady state, substrate inhibition, bioreactor, primary culture, secondary culture, kinetic parameters, mixed culture

INTRODUCTION

Phenol is a reasonably common wastewater contaminant (Li and Humphrey, 1989), which has been found to be either toxic or lethal to fish and most types of microorganisms at relatively low concentrations (Hill and Robinson, 1975). Studies on microbial means of treating or removing phenols dates back to at least three decades. Most studies have been ocn the degradation of much higher concentrations. Only few workers have presented works on phenol degradation of concentrations lower than 250 mg L^{-1} (Hill and Robinson, 1975; Lakhwala *et al*., 1992; Mordocco *et al*., 1999; Oboirien *et al*., 2005). Microbial degradation of phenol have 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 *et al*., 1998, 2001; Chang *et al*., 1998) *Acinetobacter calcoaceticus* (Palter *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).

Phenol degradation can be achieved in batch, fed batch or continuous mode. In continuous culture, the influent phenol concentration and dilution rate controls cell number (Mordocco *et al*., 1999). Thus, to maintain a high cell number continuous cultures need to operate at high dilution rates when the influent concentration is low and at low dilution rates when the influent concentration is high. Nonetheless, in a free cell system the maximum dilution rate that can be achieved is limited by the need to prevent cell washout (Mordocco *et al*., 1999). Furthermore, a bioreactor used in a waste treatment should be operated in such a way that practically all the substrates introduced are oxidized (Sokol, 1988) or degraded. Any change in the operation parameters may cause either a transient increase in the substrate concentration in the bioreactor or washout of the cell from the bioreactor. In the latter, the cell concentration in the bioreactor will fall to zero, while the substrate concentration will increase until it reaches the concentration in the feed (Sokol, 1988).

For a continuous culture (chemostat operation) and a single inhibitory substrate, the material balance on substrate is given by the equation:

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$$ds/dt = D(S_0-S) - r_s X \quad (1)$$

For steady state ds/dt = 0, hence

$$D(S_0-S) = r_s X \quad (2)$$

It has been reported that for a given dilution rate three steady states can be obtained when an inhibitory substrate is utilized in a continuous culture reactor (Yano and Koga, 1969; Schroder *et al*., 1997). The first one is a high conversion stable steady state, the second is an unstable steady state and the third is a trivial washout state (Pawlowsky *et al*., 1973). At low dilution rates, the bioreactor is relatively stable to fluctuating loads while at high dilution rates close to the maximum, stable and unstable steady states are close together and small perturbations of the substrate feed concentrations can lead to washout of the cells (Schroder *et al*., 1997). Therefore, knowledge of the microbial inhibition kinetics is important and can lead to more effective and safer bioreactor operations.

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 (1949) and Andrews (1968) equations has been extensively used to describe phenol biodegradation (Bandyopadhay *et al*., 1998; Reardon *et al*., 2000). The Monod and Andrew (Haldane) equations are based on the specific growth rate (Solomon *et al*., 1994; Edwards, 1970) but may also be related to the specific substrate consumption rate (Solomon *et al*., 1994; Edwards, 1970). Other kinetic models have 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 in their study of inhibition kinetics of phenol degradation from unstable steady state data amongst the tested inhibition models. However, it is reported that kinetic parameters estimated from non-steady state like washout experiments are often widely different from that for steady state conditions (Li and Humphrey, 1989; Schroder *et al*., 1997; Boyer and Humphrey, 1988) and that the Haldane model (Andrews, 1968) which is most frequently used to described substrate inhibition kinetics tends to over predicts steady state concentration sometimes more than an order of magnitude (Schroder *et al*., 1997; Allsop *et al*., 1993). 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 phenol utilization kinetics of a mixed culture of an indigenous *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* under steady state and non-steady state (washout) conditions using an influent phenol concentration of 100 mg L^{-1} a level lower than what has been earlier investigated.

MATERIALS AND METHODS

Microorganism: The microorganism, mixed culture of *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* being indigenous strains 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°C±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_2 Hpo_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 solution D were autoclaved at 121°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 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 NewBrunwick gyratory shaker (G25-R model, N.J. U.S.A) for 48 h at a temperature of 30°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 continuous cultures were cultivated in a 7.5 L NewBrunswick Microferm Twin Bioreactor (PH-22 model, N.J., U.S.A) described more fully elsewhere (Agarry, 2007). The reactor was equipped with a console for regulation of temperature, pH, aeration and agitation. The working volume of the bioreactor was 4 L. All cultivations were carried out at 30°C. The pH was maintained at 7.0 by addition of 1.0 mol L^{-1} sodium hydroxide solution and 0.5 mol L^{-1} sulphuric acid solution. Aeration was done with compressed air at a flow of 180 L h^{-1} (STP) and the stirrer speed was set at 300 rpm. A 501U peristaltic pump (Watson Marlow, Falmouth, United Kingdom) was used to supply the phenol feed medium to the reactor while a second peristaltic pump of the same kind operated at a higher flow was used to withdraw culture broth by a constant level overflow to maintain a constant culture volume. Mass flow of the phenol feed medium was controlled by balances. The exhaust gas from the bioreactor was analyzed for oxygen and carbon dioxide content using the paramagnetic properties of oxygen for O_2 and Infra Red (IR) absorption of CO measurement. To start the continuous runs a batch culture was initiated by addition of 200 mL of the inoculum to the bioreactor containing 3.8 L of medium with a phenol concentration of 100 mg L^{-1}. After the exponential growth had ended, continuous pumping of feed medium was started and smoothly increased for several hours until the required dilution rate was reached. Measurements were started when the steady state had been established. For the washout experiment, the dilution rate was increased above the critical dilution rate.

Estimation of phenol concentration: The undegraded 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. Fifty mililiter 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°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 saw then dried in an oven at 105°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.

RESULTS AND DISCUSSION

To characterize the model system under substrate limiting conditions, phenol degradation by mixed culture of *pseudomonas fluorescence* and *Pseudomonas aeruginosa* was studied in continuous culture (chemo stat operation). Different dilution rates ranging between 0.010 h^{-1} and 0.200 h^{-1} were investigated. For each dilution rate, three hydrodynamic residence times were waited before the first sample was taken. After that, at least three different samples were taken and the average values were used.

Steady states were obtained for dilution rates of up to 0.20 h^{-1},. At dilution rates of 0.21 h^{-1} and above wash out of the cells was observed. Figure 1 shows the relationship between biomass concentration and effluent phenol concentration with dilution rate at steady state for mixed culture of *P.fluorescence* and *P. aeruginosa.* It could be seen from the figure that biomass concentration decreased with increasing dilution rate while phenol was completely degraded (consumed) except at high dilution rate. This behaviour may probably have been due to undetected localized wall growths, which have been predicted by Howell as reported by Hill and Robinson (1975) that wall growth tends to increase the net conversion rate of phenol in a biological reactor. The decrease in biomass concentration as observed is in agreement with the observation of Hill and Robinson

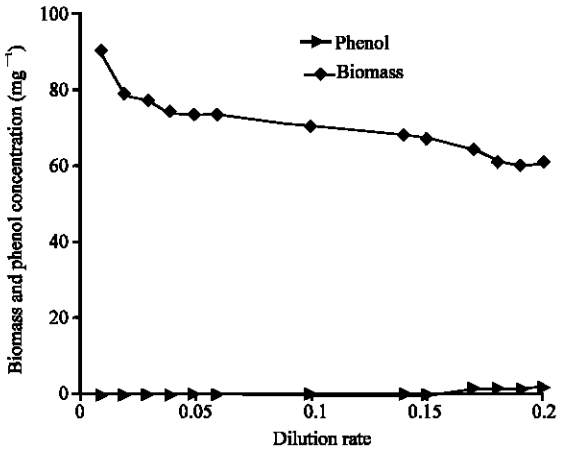

Fig. 1: Experimental steady state data for the growth of binay mixed culture in phenol limited continuous culture

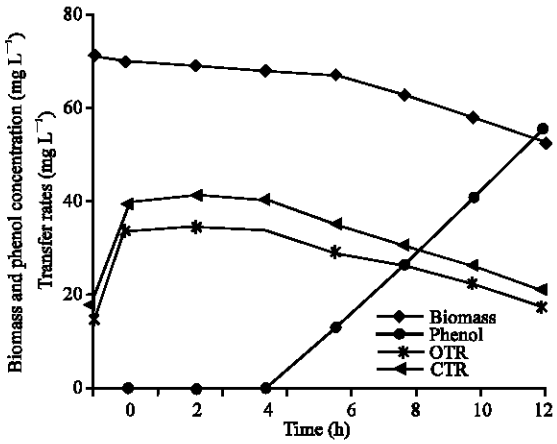

Fig. 2: Wash-out experimental data of phenol degradation by binary mixed culture

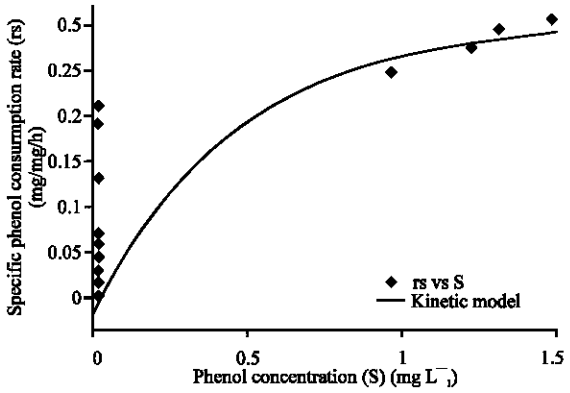

Fig. 3: Specific phenol concentration rate vs phenol concentration for binary mixed calture

Table 1: Calculated specific phenol consumption rates for the continuous degradation of phenol by binary mixed culture

Dilution rate D (hr^{-1})	Specific phenol consumption rate ,r_s (mg/mg/hr)
0.01	0.011
0.02	0.025
0.03	0.039
0.04	0.054
0.05	0.069
0.06	0.082
0.10	0.143
0.14	0.206
0.15	0.224
0.17	0.263
0.18	0.291
0.19	0.312
0.20	0.322

(1975) who reported the observation of wall growth that resulted in the decrease of biomass concentration and phenol removal at steady state as dilution rate increased. Anselmo and Novais (1992) and Schroder *et al.* (1997) both observed an increase in biomass concentration and a complete degradation of phenol at low dilution rate and a decrease in both biomass and phenol removal at high dilution rates. It could also be observed that the biomass concentration was relatively stable (no significant decline) at the higher dilution rate. It has been shown by mass balances that phenol degradation by pure culture of *Pseudomonas cepacia* G4 is complete at dilution rates of up to the critical one (Schroder *et al.*, 1997)

To estimate the maximum specific growth rate and the maximum specific phenol (substrate) consumption rate for phenol a wash out experiment was carried out. The wash out experiment was started out of a steady state with a dilution rate of 0.10 h^{-1}. with corresponding phenol feed concentration of 100 mg L^{1}.

The dilution rate was increased to 0.23 h^{-1} and the corresponding phenol feed concentration was 91 mg L^{-1} after the dilution rate step. Figure 2 shows the biomass, phenol concentration, CTR and the OTR as a function of time after the D-step (t = 0) for mixed culture of *P.fluorescence* and *P. aeruginosa*. From the figure, it could be seen that immediately after the D-step (step change) the OTR and CTR increased to higher values and remained nearly constant for about 4 h. This indicates an increased and constant metabolic activity for this time span. During this period there was no phenol accumulation but the biomass concentration was decreasing. After about 6 h there was a sharp decrease of OTR and CTR with corresponding accumulation of phenol and a more rapid decrease of biomass concentration. This demonstrates a decrease in the metabolic activity. During this period of increasing phenol accumulation and decreasing biomass concentration, the specific rates that were obtained also decreased as a result of an increasing inhibitory effect as shown in Fig. 3. However, the specific phenol consumption rates and the specific growth rates starts to increase again that cannot be explained by simple substrate inhibition kinetics. This behaviour indicates an adaptation of the organisms to higher phenol concentrations whose effect is contradictory to the increasing substrate inhibition. It is most likely that the organisms increase their enzyme level or concentration. In general, it probably seems that the mixed culture of *P. aeruginosa* and *P. fluorescence* can grow at a dilution rate of 0.18 h^{-1}, for some time. During this time, the biomass and the transfer rates decrease slightly while phenol does not accumulate. Eventually, inhibition or other metabolic changes takes over and acts like a switch reducing metabolic activity considerably. The decrease of biomass concentration in combination with no phenol accumulation in the first 4 h reveals that the change in anabolic metabolism is slower than in the catabolic metabolism, which adds a time constant to the process. Or probably, the maximum flow through anabolism and catabolism is different and phenol

Table 2: Fitting of the steady state data obtained for binary mixed culture to various kinetic models

Model	r_{smax} (Mg/mg/hr)	K_s (Mg L^{-1})	K_1 (Mg L^{-1})	K_2 (Mg L^{-1})	K_i (Mg L^{-1})	R^2
Yano and Koga 1	0.078	0.385	1793	36.4		0.196
Yano and Koga 2	0.011	2901	-	352.7		-31.7
Teissier	0.322	0.558	803.7	-		0.206
Aiba *et al*	0.322	0.129	1955	-		0.201
Haldane	0.011	2924	3209	-		-31.75
Webb	0.011	624.9	3189	-	95.4	-31.75
Monod	0.011	326.7	-	-		-3172

begins to accumulate when the maximum specific substrate consumption rate was reached due to the decreasing biomass concentration.

However, the maximum specific growth rate (μ_{max}) was calculated from the wash out cultivation experimental data in which ln x /x_o was plotted against time, (t) and a straight line obtained (not shown). The slope of this line gives the maximum specific growth rates as obtained by

$$\ln(x/x_o) = (\mu_{max}\text{-}D)\, t \quad (3)$$

The maximum specific growth rate was calculated to be 0.208 h^{-1}.and the corresponding maximum specific phenol consumption rate (r_{smax}) to be 0.342mg/mg/h. The μ_{max} value is considerably lower than those determined for *Pseudomonas cepacia* G4 (μ_{max} = 0.30 h^{-1}) by Schroder *et al.* (1997) from unstable steady state; *Pseudomonas putida* ATCC17514 (μ_{max} = 0.567 h^{-1}) by Yang and Humphrey (1975) from continuous cultivation. On the other hand, the value is comparable with the values obtained for *Pseudomonas putida* F1 (μ_{max} = 0.11hr^{-1}) by Reardon *et al.* (2000) and *Pseudomonas putida* Q5 (μ_{max} = 0.119 h^{-1}) by Kotturi *et al.* (1991) using batch cultivation. According to Layokun *et al.* (1987) and Solomon *et al.* (1994) growth of organism is a consequence of substrate consumption; hence the specific substrate (phenol) consumption rate (r_s) was calculated (as shown in Table 1) from the steady state data obtained for mixed culture of *P. fluorescence and P. aeruginosa*. From the table, it could be observed that the specific phenol consumption rate increased with increased dilution rate at steady state. Six inhibition kinetic models of Andrew (Haldane, 1968; Yano and Koga, 1969). Aiba *et al.* (1968) Teissier (Edwards, 1970) and Webb (Edwards) and a non inhibitory model of Monod (1949) were fitted to experimental steady state data by plotting the specific phenol (substrate) consumption rate as a function of steady state phenol concentration as shown in Fig. 4 from which the kinetic parameters were estimated. The non-linear least squares fitting routine of Matlab 6.5 software package was used the results are presented in Table 2. From the table, it could be seen that the best data approximation (or best fit) based on the correlation

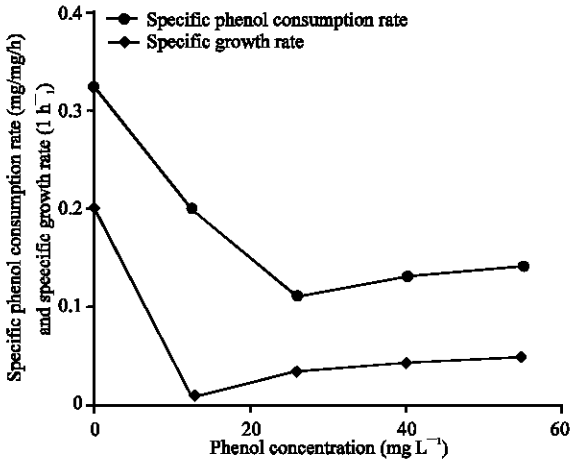

Fig. 4: Specific phenol consumtration rate and specific growth rate as function of phenol concentration in wash-out experiment

coefficient (R) was achieved using Teissier model (Edwards, 1970). It should be mentioned that one of the poorest fit of all the tested model equations was achieved for the Monod (1970) model which is most commonly used to describe non-inhibitory growth kinetics. This indicates that phenol should be considered as an inhibitory substrate and to be modeled by an appropriate inhibition kinetic equation.

CONCLUSION

For mixed culture of *Pseudomonas fluorescence and Pseudomonas aeruginosa* grown on phenol as the limiting substrate, kinetic parameters applicable to the Teissier substrate inhibition model were estimated or evaluated from continuous culture experiment. The results are $r_{s\,max}$= 0.322 mg/mg/hr, K_i = 803.7 mg/L, K_s = 0.558 mg L^{-1}.

The experimentally calculated r_{smax}value from washout experiment was found to be 0.342 mg/mg/hr and this was comparable to the r_{smax} value obtained for the Teissier equation. Mixed culture of *Pseudomonas fluorescence and Pseudomonas aeruginosa* exhibits a considerable tendency for wall growth in continuous culture particularly at high dilution rate. Similar to the

observation of Howell as reported by Hill and Robinson (1975) the onset of localized wall growth with mixed culture of *P. fluorescence and P. aeruginosa* led to an increase in the continuous flow phenol removal efficiency concurrently with a decrease in biomass concentration.

NOMENCLATURE

K_s Half-saturation constant (mg L^{-1})
K_i Inhibition constant (g L^{-1})
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 concentration (mg L^{-1})

Appendix: Equations for kinetic models according to Table 2:

Monod (1949) :

$$r_s = \frac{r_{s,max} S}{K_s + S}$$

Haldane (Andrews, 1968) :

$$r_s = \frac{r_{s\,max} S}{K_s + S + \frac{S^2}{K_i}}$$

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):

$$r_s = r_{s_{max}} \frac{C_s}{K_s + C_s + C_s^2 / K_1 + C_s^3 / K_2^2}$$

Yano and Koga (1969):

$$r_s = r_{s_{max}} \frac{C_s}{K_s + C_s + C_s^3 / K_2^2}$$

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