

Full Length Research Paper

# Substrate inhibition kinetics of phenol degradation by binary mixed culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* from steady state and wash-out data

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Steady states of a continuous culture with an inhibitory substrate were used to estimate kinetic parameters under substrate limitation (chemo stat operation). Mixed cultures of an indigenous *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were grown in continuous culture on phenol as the sole source of carbon and energy at dilution rates of 0.01 – 0.20 h<sup>-1</sup>. 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 *P. fluorescens* and *P. 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 six inhibition models resulted in the best fit for Haldane, Yano and Koga, Aiba et al. and Teissier models, respectively. The  $r_{s,max}$  value of 0.322 mg/mg/h obtained from these model equations was comparable to the experimentally calculated  $r_{s,max}$  value of 0.342 mg/mg/h 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 date back to at least three decades. Most studies have been on the degradation of much higher concentrations ranging from 1000 – 2000 mg/L (Bettman and Rehm, 1984; Sokol, 1988; Schroeder et al., 1997). Only few workers have presented works on phenol degradation of concentrations lower than 250 mg/L (Hill and Robinson, 1975; Lakhoralal et al., 1992; Mordocco et al., 1990; 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-ordaz et al., 2001; Chang et al.,

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**Nomenclature:** CTR, Carbon dioxide transfer rate (mg/L/h); D, dilution rate (h<sup>-1</sup>);  $K_s$ , half-saturation constant (mg/L);  $K_i$ , inhibition constant (mg/L); OTR, oxygen transfer rate (mg/L/h);  $r_s$ , specific phenol (substrate) consumption rate (mg/mg/h);  $r_{s,max}$ , maximum specific phenol (substrate) consumption rate (mg/mg/h); S, C<sub>s</sub>, substrate concentration (mg/L);  $\mu_{max}$  maximum specific growth rate (h<sup>-1</sup>); X, biomass concentration (mg/L).

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).

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 Schroeder et al. (1997):

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

For steady state  $ds/dt = 0$ , hence

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

Where  $D$  is the dilution rate ( $h^{-1}$ ),  $X$  is biomass (cell) concentration (mg/L),  $r_s$  is substrate consumption rate (mg/mg/h),  $S$  is substrate concentration (mg/L) and  $S_0$  is the initial substrate concentration (mg/L).

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 a more effective and safer bioreactor operation.

A variety of kinetic substrate utilization and inhibition models have been used to describe the dynamics of microbial growth on phenol. Some of these models are as

shown in equations 3 to 9:

$$\text{Monod (1949): } r_s = \frac{r_{s,\max} S}{K_s + S} \quad (3)$$

$$\text{Haldane (Andrews, 1968): } r_s = \frac{r_{s,\max} S}{K_s + S + \frac{S^2}{K_i}} \quad (4)$$

$$\text{Aiba et al (1968): } r_s = r_{s,\max} \frac{C_{s,\text{exp}} \left( -\frac{C_s}{K_i} \right)}{K_s + C_s} \quad (5)$$

Teissier (Edwards, 1970):

$$r_s = r_{s,\max} \left[ \exp\left(-\frac{C_s}{K_i}\right) - \exp\left(-\frac{C_s}{K_s}\right) \right] \quad (6)$$

$$\text{Webb (Edwards, 1970): } r_s = r_{s,\max} \frac{C_s \left( 1 + \frac{C_s}{K_i} \right)}{K_s + C_s + \frac{C_s^2}{K_i}} \quad (7)$$

Yano and Koga (1969), (1):

$$r_s = r_{s,\max} \frac{C_s}{K_s + C_s + \frac{C_s^2}{K_1} + \frac{C_s^3}{K_2}} \quad (8)$$

Yano and Koga (1969), (2):

$$r_s = r_{s,\max} \frac{C_s}{K_s + C_s + \frac{C_s^3}{K_2}} \quad (9)$$

Of these various models, the Monod and Haldane (Andrew) 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 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 (Boyer and Humphrey, 1988; Li and Humphrey, 1989; Schroder et al., 1997) and that the Haldane model (Andrews, 1968) which is most fre-

quently used to describe substrate inhibition kinetics tends to over predict steady state concentration sometimes more than an order of magnitude (Allsop et al., 1993; Schroder et al., 1997). 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, a level that is lower than what has been investigated by previous studies.

## MATERIALS AND METHODS

### Microorganism

The microorganism, mixed culture of *P. fluorescence* and *P. aeruginosa* being indigenous bacteria 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  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$  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:  $\text{K}_2\text{HPO}_4$  1.0 g,  $\text{KH}_2\text{PO}_4$  0.5 g,  $(\text{NH}_4)_2\text{SO}_4$  0.5 g, deionized water 100 ml. Trace element solution B composition: NaCl 0.5 g,  $\text{CaCl}_2$  0.02 g,  $\text{MnSO}_4$  0.02 g,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.02 g,  $\text{H}_3\text{BO}_3$  0.01 g, deionized water 50 ml. Solution C composition:  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, deionized water 50 ml. Solution D composition:  $\text{FeSO}_4$  0.02 g, Molybdenum powder 0.02 g, deionized water 50 ml. To prevent the precipitation of  $\text{CaSO}_4$  and  $\text{MgSO}_4$  in storage, the water, buffer solution A, trace elements solution B, solution C and solution D were autoclaved at  $121^{\circ}\text{C}$  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 of *P. aeruginosa* and *P. fluorescence* was each 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 two different 250 ml Erlenmeyer conical flask. Each flask was then incubated in a New Brunswick gyratory shaker (G25-R model, N.J. U.S.A) for 48 h at a temperature of  $30^{\circ}\text{C}$  and agitated with a speed of 120 rpm. Thereafter, 10 ml of each primary culture was transferred into another 100 ml of feed medium in a two different 250 ml Erlenmeyer conical flask and the incubation process was repeated. This was the secondary culture that was each 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. Each of the secondary culture (*P. aeruginosa* and *P. fluorescence*) was then mixed in the ratio 1:1 (v:v) which was used as the binary mixed culture for the degradation studies.

### Experimental design to study the free suspended cell system

The continuous cultures were cultivated in a 7.5L New Brunswick Microferm Twin Bioreactor (PH – 22 model, N.J., U.S.A) described more fully elsewhere (Agarry, 2008). The reactor was equipped with a console for regulation of temperature, pH, aeration and agitation. The working volume of the bioreactor was 4 litres. All cultivations were carried out at  $30^{\circ}\text{C}$ . The pH was maintained at 7.0 by addition of  $1.0 \text{ molL}^{-1}$  sodium hydroxide solution and  $0.5 \text{ molL}^{-1}$  sulphuric acid solution. Aeration was done with compressed air at a flow of  $180 \text{ Lh}^{-1}$  (STP) and the stirrer speed was set at 300 rpm. A 501 U 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  $\text{O}_2$  and infra red (IR) absorption of  $\text{CO}_2$  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. 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. Different dilution rates ranging between 0.01 and  $0.20 \text{ h}^{-1}$  were investigated. For each dilution rate, three hydrodynamic residence times allowed before the first sample was taken. After that, at least three different samples were taken and the average values were used. 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

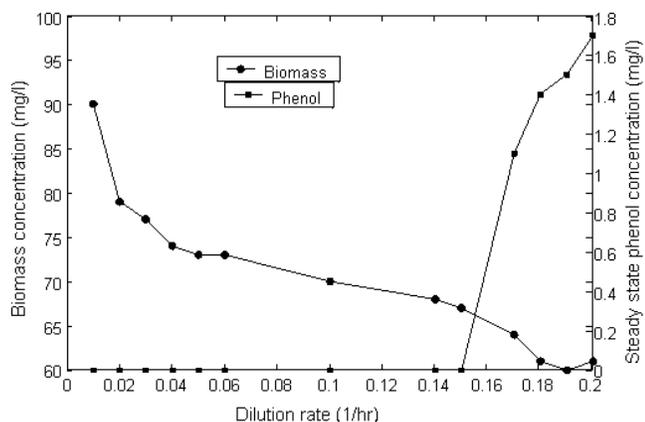
The undergraded phenol was estimated quantitatively by the spectrophotometric method using 4-amino antipyrene as colour indicator (Yang and Humphrey, 1975; Oboirien et al., 2005) at an absorbance of 510 nm.

### Estimation of biomass concentration

The biomass concentration was estimated using the dry weight method. A 50 ml sample of culture broth 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  $4^{\circ}\text{C}$  for subsequent phenol estimation. The pellets were re-suspended in de-ionized water and re-centrifuged. The supernatant was decanted and pellets rinsed off from the tube into a pre-weighed  $1.2 \mu\text{m}$  pore filter paper (Whatman GF/C). The filter paper was then dried in an oven at  $105^{\circ}\text{C}$  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 final constant weight was used to estimate the dry weight of the biomass.

## RESULTS AND DISCUSSION

Steady states were obtained for dilution rates of up to  $0.20 \text{ h}^{-1}$ . At dilution rates of  $0.21 \text{ h}^{-1}$ , 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

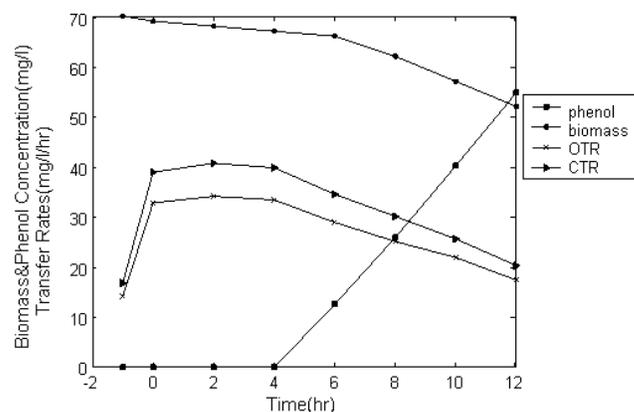


**Figure 1.** Experimental steady state data for the continuous degradation of phenol by binary mixed culture of *P. aeruginosa* and *P. fluorescence*.

seen from Figure 1 that biomass concentration decreased with increasing dilution rate while phenol was completely degraded (consumed) with an increase in the dilution rate up to  $0.15 \text{ h}^{-1}$  above which phenol removal progressively started to decrease. However, there was still a very high net conversion rate at the higher dilution rates. This behaviour may probably have been due to undetected localized wall growths, which have been predicted 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 and decreased phenol removal at higher dilution rate as observed in this study is in agreement with the observation of Agarry and Solomon, (2008) and Hill and Robinson (1975). Hill and Robinson (1975) 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 rates and a decrease in both biomass and phenol removal at high dilution rates. 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  $0.10 \text{ h}^{-1}$  with corresponding phenol feed concentration of  $100 \text{ mg/L}$ .

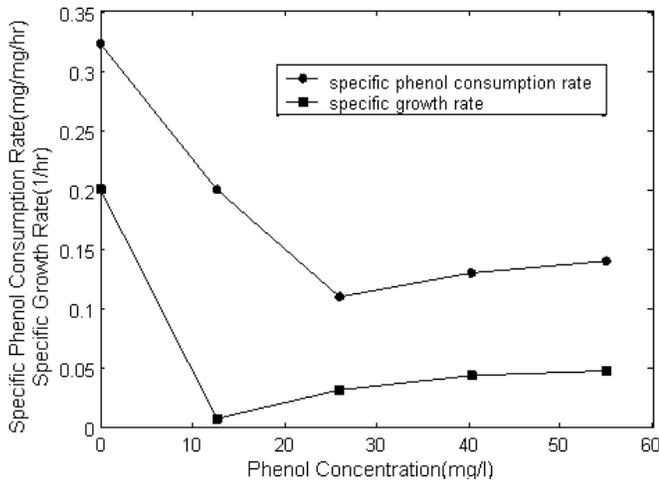
The dilution rate was increased to  $0.23 \text{ h}^{-1}$  and the corresponding phenol feed concentration was  $91 \text{ mg/L}$  after the dilution rate step. Figure 2 shows the biomass, phenol concentration, carbon dioxide transfer rate (CTR) and the oxygen transfer rate (OTR) as a function of time after the D-step ( $t=0$ ) for mixed culture of *P. fluorescence*



**Figure 2.** Experimental washout data of binary mixed culture of (*P. aeruginosa* and *P. fluorescence*) on phenol.

and *P. aeruginosa*. From Figure 2, it may 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 a 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 Figure 3. However, the specific phenol consumption rates and the specific growth rates start 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  $0.18 \text{ h}^{-1}$ , 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 take over and acts like a switch reducing metabolic activity considerably. The decrease in biomass concentration in combination with no phenol accumulation in the first 4 h reveals that the maximum flow through anabolism and catabolism is different and phenol begins to accumulate when the maximum specific substrate consumption rate was reached due to the decreasing biomass concentration.

However, the maximum specific growth rate ( $\mu_{max}$ ) was calculated from the wash out cultivation experimental data in which  $\ln(X/X_0)$  was plotted against time, (t) and a straight line obtained (not shown). The slope of this line



**Figure 3.** Specific phenol consumption and specific growth rates as function of phenol concentration for washout cultivation of binary mixed culture (*P. aeruginosa* and *P. fluorescense*).

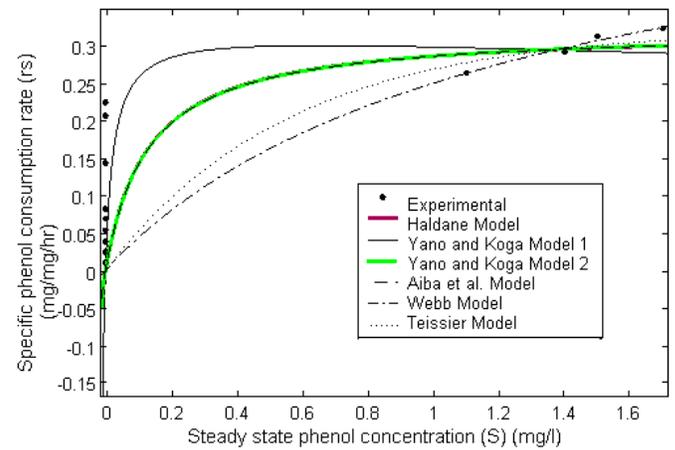
**Table 1.** Calculated specific phenol consumption rates for the continuous degradation of phenol by binary mixed culture of *P. aeruginosa* and *P. fluorescense*.

Dilution rate D (h <sup>-1</sup> )	Specific phenol consumption rate r <sub>s</sub> (mg/mg/h)
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

gives the maximum specific growth rates as obtained by

$$\ln(X/X_0) = (\mu_{\max} - D) t \quad (10)$$

The maximum specific growth rate was calculated to be 0.208 h<sup>-1</sup> and the corresponding maximum specific phenol consumption rate (r<sub>smax</sub>) to be 0.342 mg/mg/h. The  $\mu_{\max}$  value is considerably lower than those determined for *P. cepacia* G4 ( $\mu_{\max} = 0.30$  h<sup>-1</sup>) by Schroeder et al. (1998) from unstable steady state; *P. putida* ATCC17514 ( $\mu_{\max} = 0.567$  h<sup>-1</sup>) by Yang and Humphrey (1975) from continuous cultivation. On the other hand, the value is higher than the values obtained for *P. putida* F1 ( $\mu_{\max} = 0.11$  h<sup>-1</sup>) by Reardon et al. (2000), *P. putida* Q5 ( $\mu_{\max} =$



**Figure 4.** Specific phenol consumption rate as a function of steady state phenol concentration for binary mixed culture (*P. aeruginosa* and *P. fluorescense*).

0.119 h<sup>-1</sup>) by Kotturi et al. (1991) using batch cultivation, and *P. aeruginosa* ( $\mu_{\max} = 0.152$  h<sup>-1</sup>) by Agarry and Solomon (2008) under continuous 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<sub>s</sub>) was calculated (as shown in Table 1) from the steady state data obtained for mixed culture of *P. fluorescense* 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 Haldane (Andrew) (1968), Yano and Koga 1 (1969), Yano and Koga 2 (1969), Aiba et al. (1968) Teissier (Edwards, 1970) and Webb (Edwards, 1970) were fitted to experimental steady state data by plotting the specific phenol (substrate) consumption rate as a function of the steady state phenol concentration as shown in Figure 4 from which the kinetic parameters were estimated. The non-linear least squares fitting routine of MATLAB 6.5 software package was used and the results are presented in Table 2. The results in Table 2 revealed that all the tested models fitted the experimental data well. However, the best fit was obtained using the Haldane, Yano and Koga 2, Aiba et al. (1968) and Teissier models, respectively, as they all have lower RMSE values with statistically insignificant difference as shown by their Chi-square ( $\chi^2$ ) values being virtually the same. A similar observation has been reported by Pawlowsky and Howell (1973). They observed statistically insignificant difference between five inhibition models in their study of mixed culture bio-oxidation of phenol. Therefore, the models of Haldane, Yano and Koga 2, Aiba et al. (1968) and Teissier may respectively be proposed as the kinetic models to describe the continuous phenol degradation behaviour of binary mixed culture of *P. aeruginosa* and *P. fluorescense*.

**Table 2.** Fitting of the steady state data for binary mixed culture to some kinetic models.

Model	$r_{smax}$ mg/mg/h	$K_s$ (mg/L)	$K_1$ (mg/L)	$K_2$ (mg/L)	$K_i$ (mg/L)	RMSE	$\chi^2$
Yano and Koga 1	0.322	0.025	17.2	202	-	0.1150	0.0145
Yano and Koga 2	0.322	0.129	-	371	-	0.1145	0.0133
Teissier	0.322	0.559	-	-	1031	0.1089	0.0130
Aiba et al (1988)	0.322	0.129	-	-	1955	0.1092	0.0131
Haldane	0.322	0.128	-	-	858	0.1145	0.0131
Webb	0.231	0.478	2.49	-	0.968	0.1201	0.0206

## Conclusion

For mixed culture of *P. fluorescence* and *P. aeruginosa* grown on phenol as the limiting substrate, the experimentally calculated  $r_{smax}$  value from washout experiment was found to be 0.342 mg/mg/h and this was comparable to the  $r_{smax}$  value of 0.322 mg/mg/h obtained for the Haldane, Yano and Koga, Aiba et al. (1988) and Teissier model equations, respectively, from the steady state data. Mixed culture of *P. fluorescence* and *P. aeruginosa* may likely exhibit a considerable tendency for wall growth in continuous culture particularly at high dilution rate. The onset of localized wall growth with mixed culture of *P. fluorescence* and *P. aeruginosa* led to a decrease in the continuous flow phenol removal efficiency at high dilution rate concurrently with a decrease in biomass concentration.

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