CHAPTER 4

BIODEGRADATION STUDIES

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4.1 INTRODUCTION

Bacteria, yeasts and fungi capable of growing on phenolic compounds are found in soil and water environments. List of these microorganisms is given in Table 2.3. of Chapter 2. It is clear from Table 2.4. of Chapter 2 that *Pseudomonas sp* is the most widely used organism for phenols removal. However, most of the work reported in the literature is with *Pseudomonas putida.* The information pertaining to the kinetics of degradation of phenol and its derivatives by *P. aeruginosa* is not available. Thus degradation studies on phenol, catechol and 3-aminophenol would provide a good database. These compounds have been selected as representative substituted groups from our target phenolic compounds.

It is known that to metabolize inhibitory compounds like phenol, bacteria carry genes, which are capable of detoxifying this substance (Ferhen et al. 2002). Thus, *Pseudomonas aeruginosa* will have different potential of degradation for different phenolic compounds. Keeping this in mind, studies here have been devoted to biochemical kinetics determination. The effect of concentration and time on the removal behavior has been investigated and discussion on the results obtained has been presented subsequently.

4.2 THEORY

4.2.1 Microbial Kinetics

The determination of biochemical kinetic constants is essential as it helps in understanding the capacity of the microorganisms for the degradation and the operation of biological treatment process. There are several kinetic models available to represent the degradation data. These models have been mainly taken from the enzyme kinetics since the enzyme is the key element in the biological reaction for the transformation of organic compounds into metabolic products and cell mass. The kinetics of microbial growth, substrate utilization and calculations involved in their determination has been dealt herewith as explained below.

In a batch reactor, as the substrate is consumed with time four distinct phases of microbial growths are observed. These are lag phase, exponential phase, stationary/constant rate phase and death or

decay phase (Bellgardt 2000). During lag phase, microbes adapt themselves to the new environment and substrate. After the end of lag phase, the microbes start growing by fission process in an exponential manner and during this period, substrate is consumed for the production of cell mass. The kinetics of this phase may be written as follows:

$$\frac{dx}{dt} = \mu x \qquad (4.1)$$

In this equation, the specific growth rate μ is a function of the limiting substrate concentration, S in the liquid medium, and x is the concentration of bacterial mass present at any time. The most commonly used functional relationships are those given by Monod and Haldane. These relationships are analogous to Michaelis- Menten and Briggs- Haldane enzyme kinetics expressions (Bailey and Ollis 1986).

The slope of the substrate or product concentration versus time curve is determined from the data. Typically initial rate data are used to determine the slope, since the reaction conditions including enzyme and substrate concentrations are best known at time zero.

After the experimental rate data are obtained the second logical step is to correlate the data mathematically. Enzymes act as catalyst in biological transformations. Therefore, the concentration of enzyme initially present remains the same throughout the reaction. Also depending on the nature and amount of substrate involved in the reaction period, the enzyme catalyzes the reactions in the specific manner. The substrate binds to a specific region of the enzyme called the active site, where a reaction occurs and products are released. The notions of active site and the enzyme substrate complex are universally accepted as the starting point for most theories of enzyme action. Based on these specific interactions and using mass action law for kinetics of molecular events, equations to correlate the rate of reaction have been developed.

Various enzyme substrate interactions have been proposed in the literature (Blanch and Clark 1996). One that has been used in deriving Michaelis-Menten enzyme kinetics is as follows:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \qquad (4.2)$$

$$ES \xrightarrow{k_2} P + E \qquad (4.3)$$

It is clear from the chemical equations that the enzyme E and the substrate S combine to form a complex ES which then dissociates into product P and free enzyme. Though this is considered to be the over simplification of the actual reaction mechanism, yet this has been found reasonable in the derivation of the enzyme kinetics model.

Assuming that the Eq. (4.2) is in equilibrium state, applying mass-action for kinetics, one gets

$$\frac{[S][E]}{[ES]} = \frac{k_1}{k_{-1}} = K_m = \text{Dissociation constant} \qquad (4.4)$$

Further, it is assumed that the decomposition of the complex ES to product P and free enzyme E, is irreversible, then

$$v = \frac{dp}{dt} = k_2 [ES] \qquad (4.5)$$

Also the mass of enzyme is conserved, therefore

$$[E]+[ES]=[E_0] \qquad (4.6)$$

After mathematical manipulations with the equations above and putting $V_{max}= k_2 [E_0]$ one obtains

$$v = \frac{v_{max} S}{K_m + S} \qquad (4.7)$$

The Eq. (4.7) is called as Michaelis-Menten enzyme kinetics equation. This equation reflects the three salient features of the enzyme-catalyzed reactions.

I. At relatively low values of concentration the rate of reaction is first order in concentration of substrate.
II. As the concentration of substrate is increased, the rate of reaction becomes zero.
III. The rate of reaction is proportional to the total amount of enzyme present.

The parameter $v_{max.}$ is called the maximum or limiting velocity and K_m is known as the Michaelis constant.

Though Michaelis-Menten equation has been found to represent many enzyme catalyzed reactions, this may not describe all such reactions. Certain extensions and modifications have been required in order to incorporate the various enzymes and reaction conditions. Another possibility of enzyme-substrate interaction is that two substrate molecules bind to the enzyme instead of one as given in the development of Michaelis-Menten type enzyme-catalyzed reactions. The following reaction steps have represented this type of reaction:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \tag{4.8}$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES_2 \tag{4.9}$$

ES_2 obtained as a result of binding of S with ES is non-reactive intermediate and the slow step is

$$ES \xrightarrow{k} P + E \tag{4.10}$$

After certain mathematical manipulation and using the necessary condition of conservation of mass of enzyme initially present, one gets

$$v = \frac{k[E_0]}{1 + \frac{K_1}{S} + \frac{S}{K_2}} \tag{4.11}$$

where $K_1 = \frac{k_{-1}}{k_1}$, $K_2 = \frac{k_{-2}}{k_1}$, & $v_{max} = k[E_0]$

Reaction rate as given by Eq. (4.11) reflects a particular pattern in change of reaction rate with the concentration of substrate. With increase in the concentration of substrate, the reaction rate passes through a maximum (Fig. 4.1). From this point onward, the reaction rate diminishes with the increase in the substrate concentration. This type of kinetics has important implications on the behavior of biochemical reactors.

4.2.2 Microbial Growth Kinetics in Batch Reactor

In batch reactor operation, nothing is added or removed from the reactor after seeding the liquid medium with an inoculum of living cells. The concentration of nutrients, cells, and product change with time and concentration of biomass increases. The material balance on the biomass results in the following differential equation (Bailey and Ollis 1986).

$$\frac{dx}{dt} = \mu x \tag{4.12}$$

Two types of behavior have been observed with respect to the change of value of specific growth rate with increasing substrate concentration analogous to enzyme catalyzed response kinetics. When the value of specific growth rate keeps on increasing with the increase in substrate concentration and approaches a maximum value, this is called non-inhibitory behavior. In contrast, for inhibitory type behavior, the value of specific growth rate passes through a maximum and then falls down. (Fig. 4.1).

4.2.2.1 Non-Inhibitory Growth Kinetics

For non-inhibitory substrates, Monod (1949) proposed a functional relationship between the specific growth rate, μ and concentration of the limiting substrate; this was similar to the Langmuir adsorption isotherm and the Michaelis- Menten equation for enzyme-catalyzed reaction kinetics. Several authors (Garcia et al., 1997, Kus and Weismann, 1998 and Kumaran and Parachuri, 1997) have used the Monod relationship for a variety of organisms for degradation of various compounds including phenol both in aerobic and anaerobic environments. According to Monod, specific growth rate, μ may be stated as

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{4.13}$$

In the above equation, μ_{max} is the maximum achievable growth rate $S >> K_s$. K_s is called half saturation coefficient and is equal to the value of substrate concentration at which the specific growth rate reaches half the value of the maximum specific growth rate.

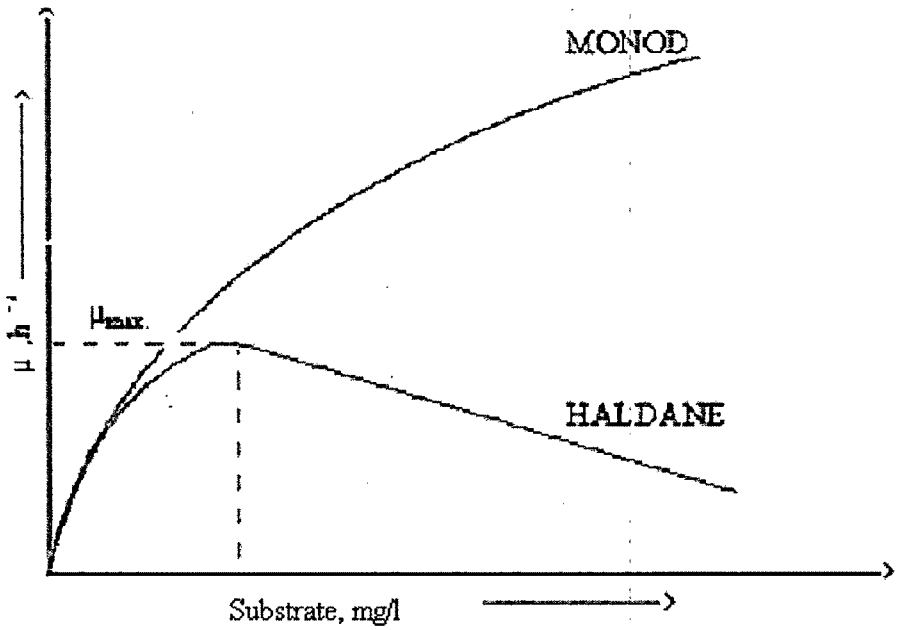

Figure 4.1. Biokinetic models

The knowledge of K_s value may be useful in defining the regions where specific growth rate is strongly dependant on substrate concentration and where the specific growth rate is more or less independent of substrate concentration.

4.2.2.2 Inhibitory Growth Kinetics

Several inhibitory growth kinetic models have been proposed and reviewed by Edward (1970) for phenol degradation studies. However, Rozich et al. (1984, 1985) compared several values of μ and S for several types of seeds to assess the inherent variation of the growth model constants for heterogeneous populations, metabolizing phenol and concluded that Haldane's function equation which contain three fittable constants produces relatively simple predictive equations when incorporated into reactor mass balance equations and it should be selected as growth rate functions.

In addition to this, Haldane's model has mostly been utilized in describing the growth kinetics of microorganisms on phenolic compounds (Yang and Humphrey 1975, Kotturi et al. 1991). This is also similar to the enzyme catalyzed reaction kinetics for inhibitory type of substrate. Hence, the Haldane's model equation (1930) is selected for present study (Eq. 4.14).

$$\mu = \frac{\mu_{max} S}{K_s + S + \frac{S^2}{K_i}} \tag{4.14}$$

The value of inhibitory coefficient, K_i reflects the degree of inhibition caused by a substrate. The higher the value, the less will be the degree of severeness of inhibition. In the limiting cases, when the value of K_i reaches infinity, the Eq. (4.14) becomes the well-known growth kinetic equation of Monod for non-inhibitory type of substrates.

4.2.3 Kinetics of Substrate Utilization

Substrate utilization and biomass growth are related phenomenon, as the later is the result of the consumption of the substrate. The kinetics of substrate utilized in a batch reactor may be as follows:

$$-r_s = \frac{ds}{dt} = \frac{\mu x}{Y} \tag{4.15}$$

where r_s represents the rate of substrate utilization; x is the active biomass present at an instant of time, Y is the yield coefficient.

4.2.4 Kinetics of Endogenous and Maintenance Metabolism

After the substrate is consumed fully by the cell population present at that instant of time, biomass undergoes constant rate period that is the phase when population achieves its maximum size. And finally the number of cells start decreasing and this phase is called endogenous or death phase. During this phase, cells die and consume the product of endogenous metabolism. The kinetics of this phase has been represented in a manner similar to the exponential growth phase with the exception that the endogenous kinetic rate coefficient, k_d considered as constant. Therefore, the kinetics of death phase may be stated as

$$\frac{dx}{dt} = -k_d x \tag{4.16}$$

where k_d is termed as endogenous death rate coefficient. Its effect on the kinetics is that it reduces the specific growth rate.

4.2.5 Yield Coefficient

As the substrate is metabolized, it is converted into some products and the cell mass itself. Though the molecular structure of the cell is complex and it is difficult to write a chemical formula for the composition of the cell. The law of conservation of matter has been observed even in these biochemical reactions similar to the chemical reactions of compounds of known composition. Accordingly, a certain amount of the cell mass is produced for an amount of the substrate consumed. Mathematically this may be stated as

$$Y = \frac{\Delta X}{-\Delta S} \tag{4.17}$$

The negative sign has been put before ΔS to indicate that an increase in cell mass is accompanied by a decrease in substrate concentration. Generally, this is considered a constant quantity for a substrate microbe system under a given physical condition (Pirt 1975).

4.3 EXPERIMENTAL

4.3.1 Material

4.3.1.1 Chemicals

Different phenolic compounds namely phenol, catechol and 3- aminophenol used were of AR grade and supplied by Sisco Research Lab, Mumbai and Qualigens Ltd., Mumbai.

4.3.1.2 Stock Solutions

Stock solutions of all the phenols of 5000 ppm (0.5 g in 100 ml of distilled water) concentration were prepared and stored in amber glass bottles.

4.3.1.3 Minimal Medium (Basal Salt Medium)

Bushnell Hass Broth (magnesium sulfate 0.2 g/l, calcium chloride 0.02 g/1, mono potassium hydrogen phosphate 1.0 g/1, di potassium hydrogen phosphate 1.0 g/l, ammonium nitrate 1.0 g/1, and ferric chloride 0.05 g/1) of Hi Media Lab. Ltd, Mumbai was used. The pH of the medium was not adjusted as it was found to be 7.1. Sterilization was done in autoclave at steam pressure of 1.05 kgf/cm^2 for 15 min.

4.3.1.4 Microorganism

A pure culture of *Pseudomonas aeruginosa* (ATCC 9027) was procured from Food and Drug Administration (FDA) laboratory, Baroda.

4.3.1.5 Storage of Culture

Since the history of the culture is not known, it cannot be used directly in degradation studies. Therefore these cultures were transferred to Antibiotic assay medium No.1 (Seed Agar) of Hi Media Lab. Pvt. Ltd., Mumbai. The cultures are grown on solid seed agar petri dishes and slants (Fig. 4.2). Composition of storage medium is given in the Table 4.1. Liquid medium was prepared by dissolving appropriate quantities (30.5 g in 1 liter) agar in hot distilled water in a 250 ml conical flask (100 ml working volume). 5 ml of medium was transferred in test tubes. The liquid medium in conical flask and test tubes were autoclaved for 15 min at 1.05 kg/cm^2. While hot, this was put in slanted form and poured in petri dishes. On cooling, the liquid medium with agar solidified. It was considered as ready for use. The loop-ful of culture from the slant was spread on the agar plate. The above steps were performed in a laminar airflow bench over the burner flame. Later on, the test tubes and petri dishes were transferred to incubator, which was maintained at 30°C temperature. After 24 h, colonial growth on slants and plates were observed.

4.3.1.6 Stock Culture

A loop-ful of the culture obtained in the previous step, was spread on a newly prepared slant. This slant was kept in Incubator at 30°C for 24 h. After the growth was observed, this slant was transferred to the refrigerator. This was denoted as sub culture, which was used to initiate the acclimatization experiments.

4.3.1.7 Inoculum

Inoculum for use in experiments was developed by pouring 0.1% saline water into test tube of sub culture slant. This solution was inserted into minimal medium amended with 0.5% dextrose in Erlenmeyer flasks of 250 ml, with a working volume of 100 ml. After appreciable growth appeared, a small portion of broth of medium was used as inoculum for 25 ppm initial concentration of phenols. 5 ml broth of this solution obtained after complete degradation of 25 ppm concentration of phenolic solution was used as inoculum for the solution of 50 ppm initial concentration. Similarly for higher concentration solution of phenols inoculums were taken.

Table 4.1. Medium for storage

Nutrient	Concentration g/1
Peptic digest of animal tissue	6.0
Casein enzymatic hydrolysate	4.0
Beef Extract	1.5
Yeast Extract	3.0
Dextrose	1.0
Agar	15.0
Final pH at 25 °C	6.6± 0.2

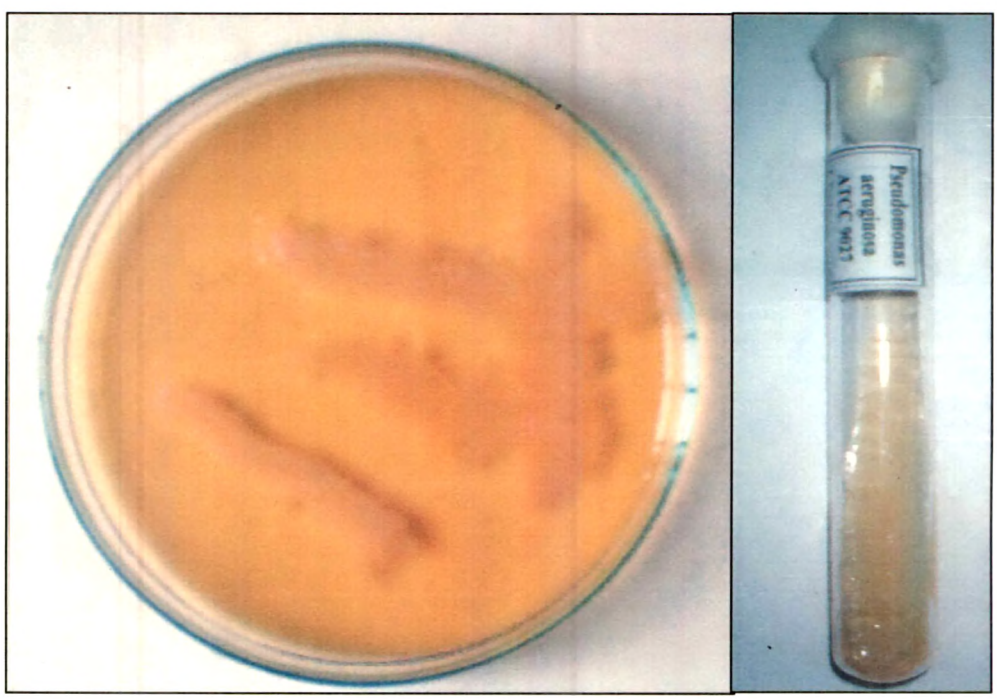

Figure 4.2. Adapted *Psuedomonas aeruginosa* (ATCC 9027) in storage medium

For kinetic studies, the initial concentration of 800 mg/l of phenol, 600 mg/l of catechol and 500 mg/l of 3-aminophenol was used. A certain volume of this broth was used as inoculum when the entire phenol, 3-aminophenol or catechol was completely metabolized. Sometimes, culture viability was also checked microscopically or by streak plate technique. Inoculums were always transferred in laminar airflow bench over the burner flame to avoid contamination.

4.3.2 Methods

4.3.2.1 Phenol Estimation

4- Amino antipyrine colorimetric method (5530 D APHA/ AWWA) has been used for estimation using visible spectrophotometer (Systronic make VISIMAX 167). Calibration curve for all the three phenolic compounds using minimal medium as control were prepared (Appendix A: Figs. A-1, A-2 and A-6).

4.3.2.2 Measurement of Growth of Bacterial culture

Samples (5 ml) were withdrawn at regular time intervals and were centrifuged at around 8000 rpm for 15 to 20 min. at room temperature. The supernatant layer was used to measure phenol concentration. The pellet was re-suspended in 5 ml of distilled water and the growth was determined by measuring the absorbance of bacterial culture at 660 nm wavelength against distilled water as reference using, Systronic make visible spectrophotometer (Model Visimax-Visiscan-167).

The dry weight of biomass present in the suspension was determined by filtering through 0.22 micron Pall membrane filter, and drying the membrane filter for 12 hours at 60°C. Then the calibration curve was prepared by plotting dry weight of biomass per liter against optical densities of the suspension. The curves are given in Appendix A (Figs. A-7, A-8 and A-9).

Pour plate technique with serial dilution for viable count of cell mass was used sometimes to check the growth and purity of bacterial strain.

4.3.2.3 Kinetics Experiments

Substrate utilization and bacterial growth kinetics studies were carried out in 250 ml Erlenmeyer flasks. The working volume in each flask was 100 ml. Initial concentrations were varied from 25 to 800 mg /l in case of phenol, 25 to 600 mg/l for catechol and 25 to 500 mg/l for 3-aminophenol.

All the experiments have been performed in orbital incubator cum shaker of Scigenics make, Saksham Technologies, Mumbai at 30°C.

4.4 RESULTS AND DISCUSSION

4.4.1 Acclimatization of Culture

Phenols being toxic compounds, degrading bacteria are required to be adapted to the phenolic environment. This is very important in cases where these bacteria are to be used to degrade aqueous solutions of intermediate to high concentrations of phenols. In the present research work, the phenolic compounds at low to intermediate concentration (500-800 mg/l) have been used. Therefore, the acclimatization procedure was initiated for the phenolic compounds. The degradation of phenol by bacterial strain *Pseudomonas sp* is available in literature [Table 2.4-Chapter 2]. However, studies on the biodegradation of phenol, 3-aminophenol and catechol for determination of bio-kinetic constants on the strain, *P. aeruginosa* have not been reported. Hence it was decided to work with this strain, for removal of these three phenols.

Initially, the bacterial strains used were received as gift from Sarvajanik Institute of Computer Education (SICE), Microbiology, Surat and Postgraduate Department of Biosciences, Sardar Patel University(SPU), Vallabh Vidyanagar. Adaptation was tried with strain from SICE, Surat for an initial phenol concentration of 50 ppm for more than 15 days, bacterial growth was observed, but there was no degradation of phenol in minimal growth medium, hence it could not be used.

Bacterial consortium gifted from SPU, V.V. Nagar was tested for further studies. The consortium has the ability to degrade reactive dyes. One of the bacterial cultures present in the consortium was *Pseudomonas aeruginosa.* In adaptation experiments with isolated *P. aeruginosa* strain from consortium, first 1% of inoculum was added to growth medium amended with 50 ppm of phenol. Degradation was not obtained in 48 h of incubation. Hence, the initial inoculum volume was increased to 5 %. Importance of inoculum size is very important in growth studies as indicated by Andrews and Trapaso (1984). He concluded that not only inhibitory effect of the phenol but also the size of the inoculum might affect the duration. With the increase in inoculum size 50 ppm phenol was degraded in 116 h, re-spiking of phenol (50 ppm) has been done. The second aliquot of 50 ppm was degraded in 84 h. This was treated as acclimatized culture and was subsequently used as an inoculum in medium containing 50 ppm of phenol and further acclimatization was done till 300 ppm phenol. Degradation of 400 ppm of phenol was not observed for more than 20 days.

Finally, the strain, *P. aeruginosa* (ATCC 9027) obtained from FDA, Baroda has been used to remove phenols in desirable concentration range. Since, the history of culture was not known for phenols, the acclimatization process was very much essential for all the three phenols.

The acclimatization of the culture was performed in similar manner as for other strain in 250 ml Erlenmeyer flask. This was started by first transferring a 0.1 % saline water containing bacteria from subculture slant to conical flask having BSM solution. To avoid problems due to contamination and non-growth, the bacteria was transferred to twelve flasks, four for each compound. After 72 h, significant bacterial growth was observed; the synthetic medium turned turbid. The stock solutions of phenol, catechol and 3-aminophenol were added to the flasks so as to give 100 mg/l concentration of the phenolic compounds in the BSM solution. It was observed during adaptation studies that when catechol is added to the BSM solution, its color changes from light pinkish to brownish to characteristic blackish. The samples were taken from these broths to determine degradation and growth rate. The phenolic compounds were added till the cumulative concentration of the compound reached 500 mg/1 in case of phenol, catechol, and 3-aminophenol. Degradation of phenol, catechol and 3-aminophenol was complete. By this time, the culture would have acclimatized to these compounds. Therefore, it was planned to prepare 12 new flasks; 4 for each phenol in BSM solution. The inoculum of 5 ml size was transferred from the previous step.

From the previous step wherein culture could degrade phenol and catechol up to 500 mg/l inoculum was taken for addition to the flasks containing phenol, catechol and 3-aminophenol at the final concentration of 600 mg/l. For these studies, three flasks for each concentration were kept. In case of 3-aminophenol, the degradation did not start even in 30 days and solution turned black in 5 days indicating culture started dying. Hence it was concluded that it is not possible to degrade 3-aminophenol beyond the initial concentration of 500 mg/l. However, for phenol and catechol, the degradation up to 800 mg/l and 600 mg/l respectively was possible. In case of phenol well acclimatized culture exposed to 1000 mg/l could not even start degradation in 23 days. It was also observed that culture started dying above 600 mg/l of catechol concentration. Before starting the kinetics study, re-spiking was done by growing this acclimatized bacterial strain to 800 mg /l of phenol, 500 mg/l of 3-aminophenol and 600 mg/ l of catechol as initial concentration.

Preliminary study with other target compounds like 4-aminophenol and resorcinol has also been tried. Surprisingly, *P. aeruginosa* could acclimatize up to 1.2% (i.e. 12000 mg/l) of 4- aminophenol in 33 days, and strain could not grow on resorcinol even at 50 mg/l. Both the compounds were not studied further. Different behavior and potential of the strain *P. aeruginosa* indicated during adaptation reveals different degradative pathways for different phenols.This finding has also been reported by Kumar et al. (2004) using strain *P. putida.*

4.4.2 Effect of Initial Concentration

Batch experiments were conducted to examine the effect of initial concentration on the degradation behavior of phenol, 3-aminophenol and catechol using bacterial strain *P. aeruginosa* at 30°C. As stated earlier the initial concentration of phenol, 3-aminophenol and catechol were varied from 25 to 800 mg/l, 25 to 500 mg/l and 25 to 600 mg/l respectively. In all these experiments, the concentration of substrate and biomass in liquid medium were recorded till the substrate initially present was fully consumed. The results of these studies are plotted in Figs. 4.3 to 4.13 for phenol, Figs. 4.14 to 4.21 for 3-aminophenol and Figs. 4.22 to 4.30 for catechol at selected concentrations. In all these experiments, the concentration of substrate and biomass in liquid medium were recorded till the substrates initially present were fully consumed. The results of batch experiments showed that the initial concentration of phenol, 3-aminophenol and catechol concentration of 800, 500 and 600 mg/l respectively could be fully degraded in 136, 160 and 85 h. As expected it is clear from figures that higher the concentration of the phenol, more the time it takes to be fully consumed. Although, well-acclimatized inoculum was used in these experiments, the lag phase was observed. The lag phase of as high as one week have been reported to occur during degradation of phenol at initial concentration of 700 mg/l using well-acclimatized *P. putida* in literature by Hill and Robinson (1975).

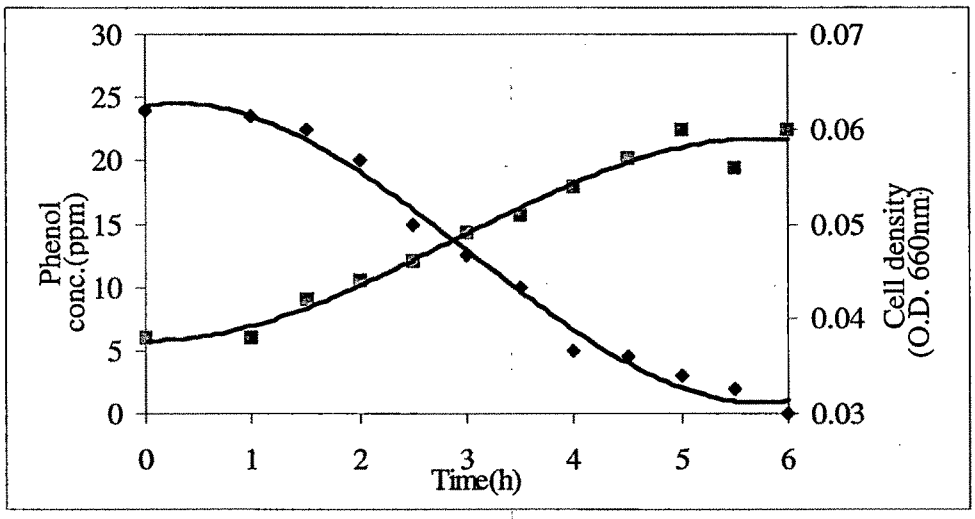

Figure 4.3. Phenol degradation by *P. aeruginosa* in Batch reactor
[Initial conc.= 25 ppm, Temp.= 30°C, pH=7]

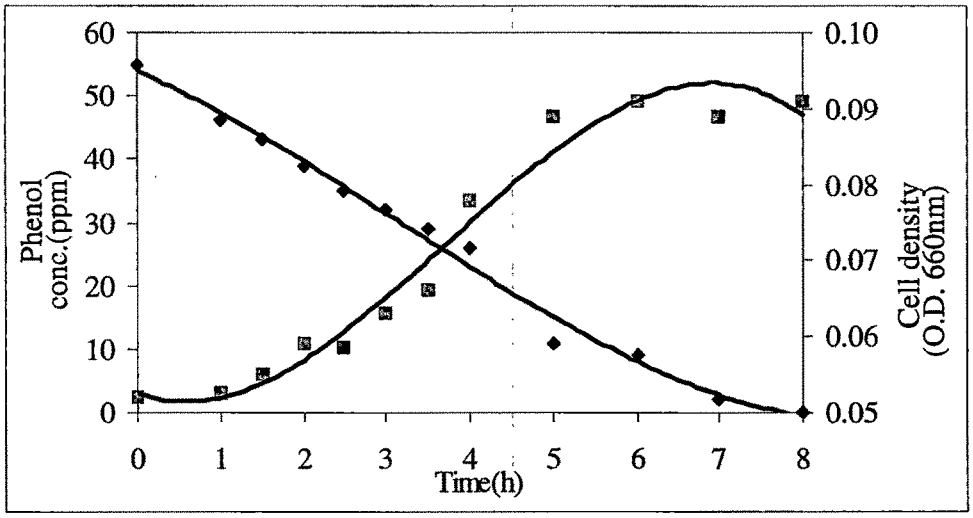

Figure 4.4. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 50 ppm, Temp.= 30 °C, pH=7]

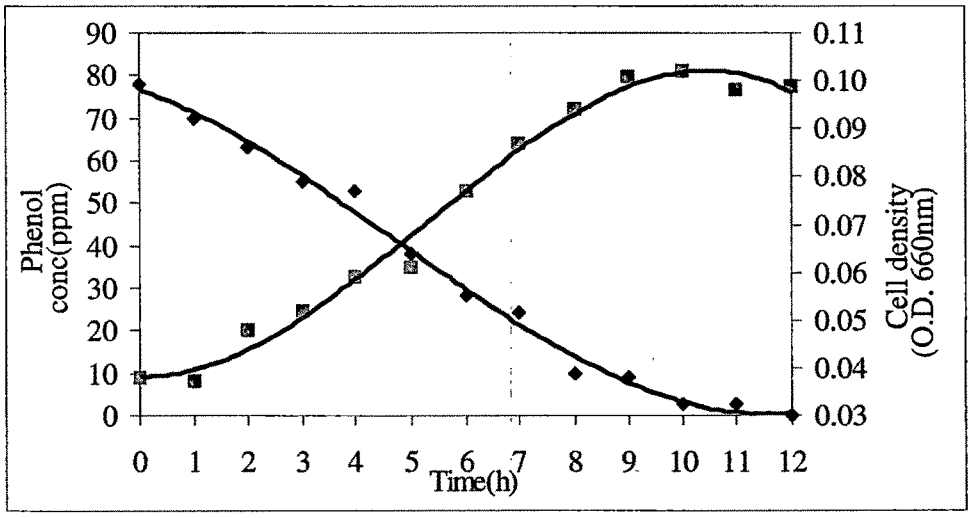

Figure 4.5. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 75 ppm, Temp.= 30 °C, pH=7]

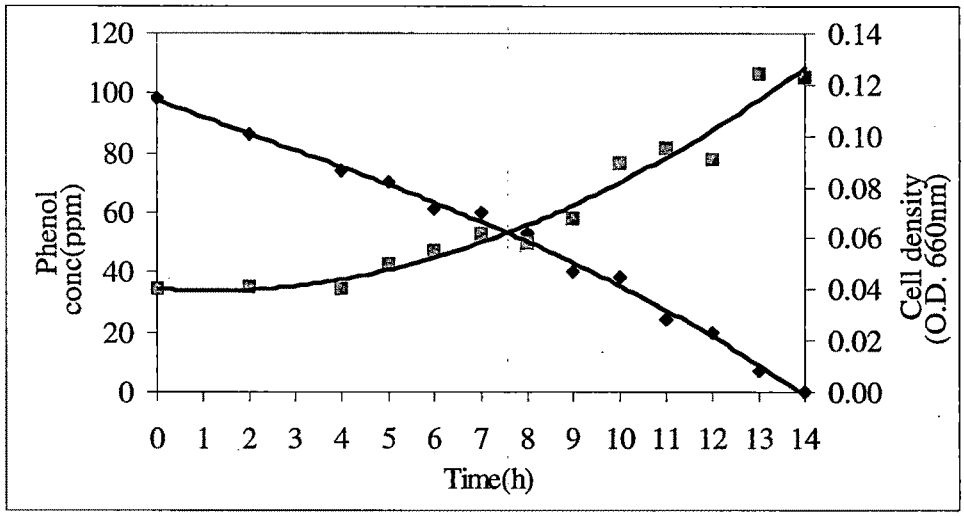

Figure 4.6. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 100 ppm, Temp.= 30 °C, pH=7]

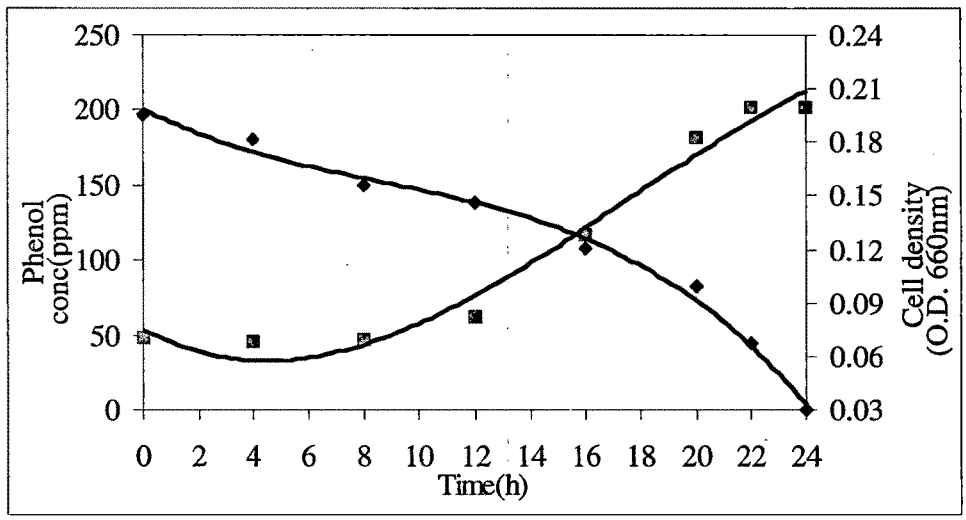

Figure 4.7. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 200 ppm, Temp.= 30 °C, pH=7]

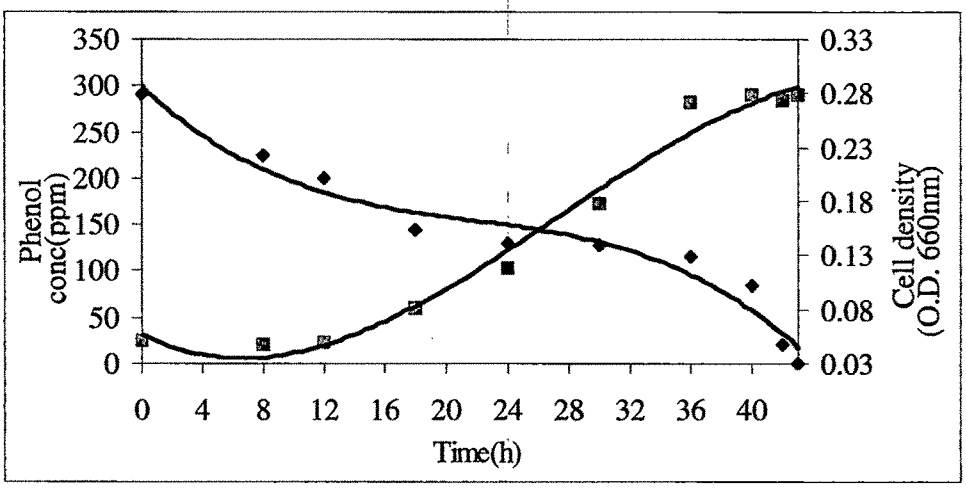

Figure 4.8. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 300 ppm, Temp.= 30 °C, pH=7]

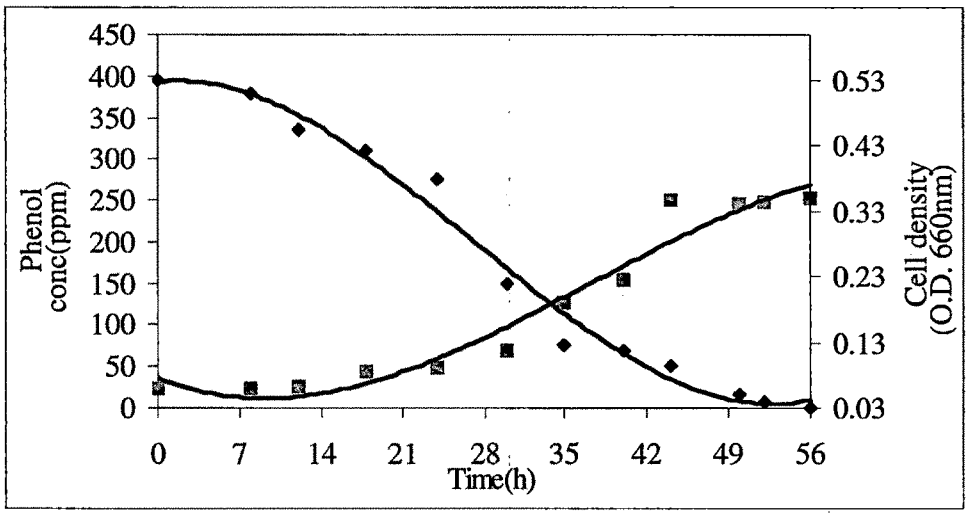

Figure 4.9. Phenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 400 ppm, Temp.= 30 °C, pH=7]

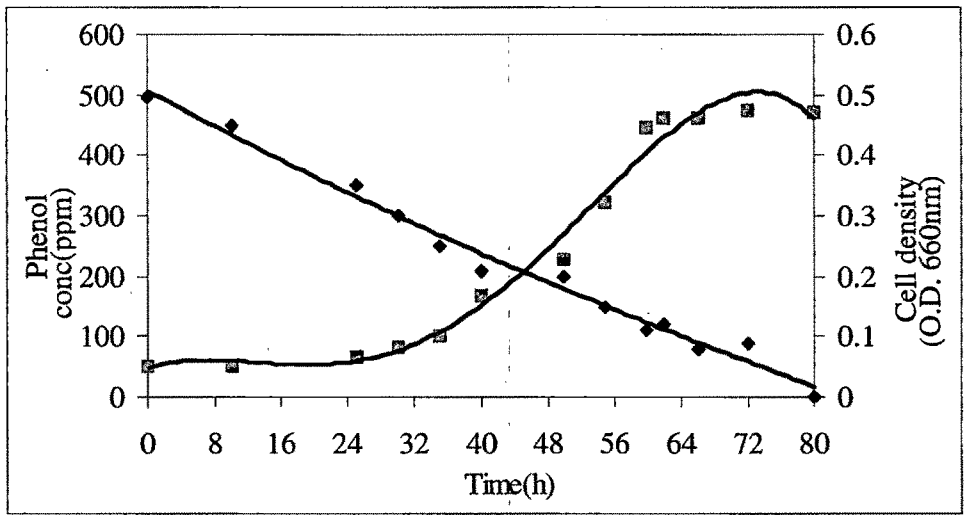

Figure 4.10. Phenol degradation by *P.aeruginosa* in batch reactor

[Initial conc.= 500 ppm, Temp.= 30°C, pH=7]

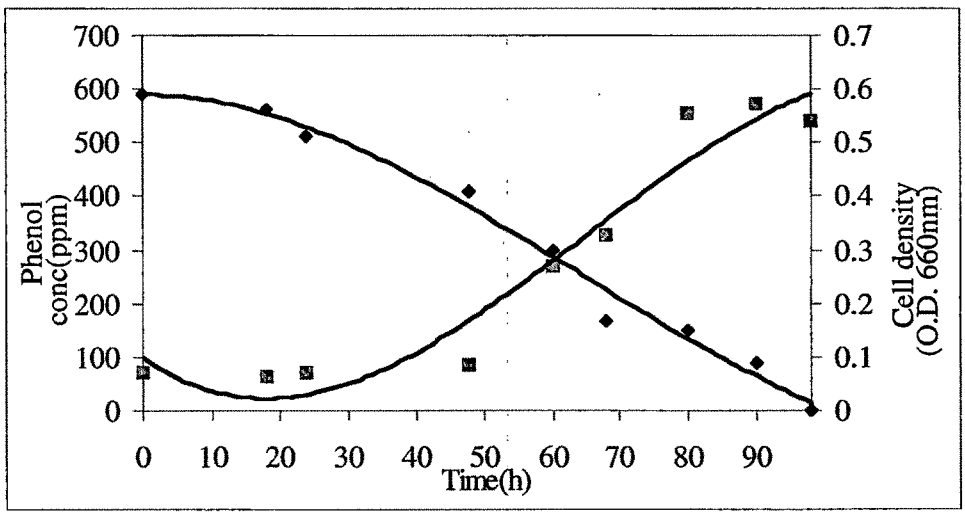

Figure 4.11. Phenol degradation by *P.aeruginosa* in batch reactor

[Initial conc.= 600 ppm, Temp.= 30 °C, pH=7]

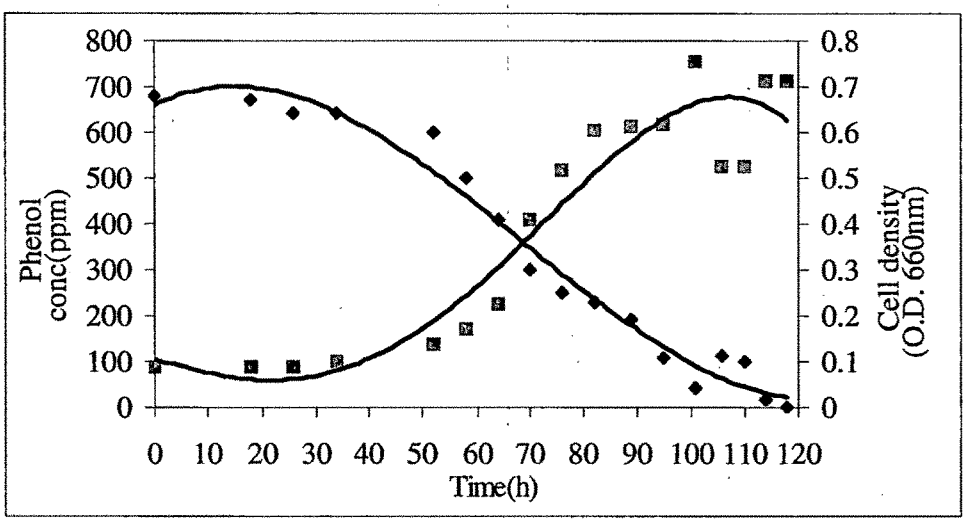

Figure 4.12. Phenol degradation by *P.aeruginosa* in batch reactor

[Initial conc.= 700 ppm, Temp.= 30 °C, pH=7]

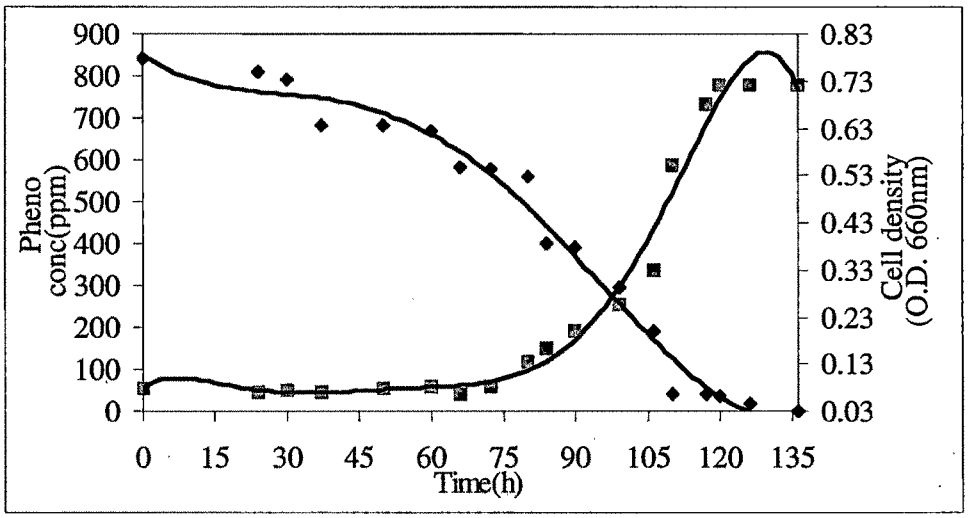

Figure 4.13. Phenol degradation by *P.aeruginosa* in batch reactor

[Initial conc.= 800 ppm, Temp.= 30 °C, pH=7]

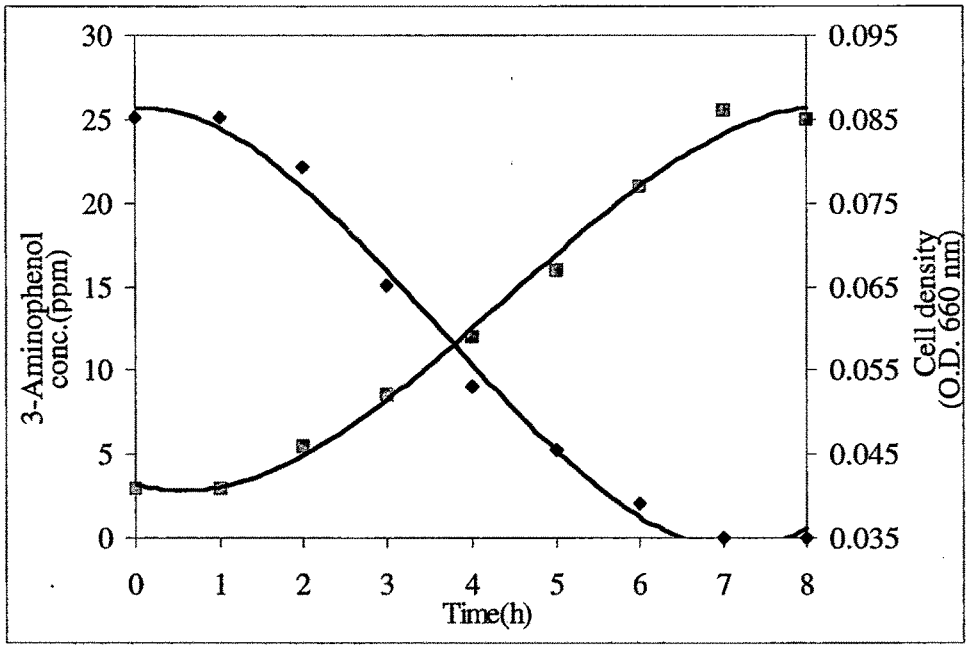

Figure 4.14. 3-Aminophenol degradation by P.aeruginosa in batch reactor
[Initial conc.= 25 ppm, Temp.= 30 °C, pH=7]

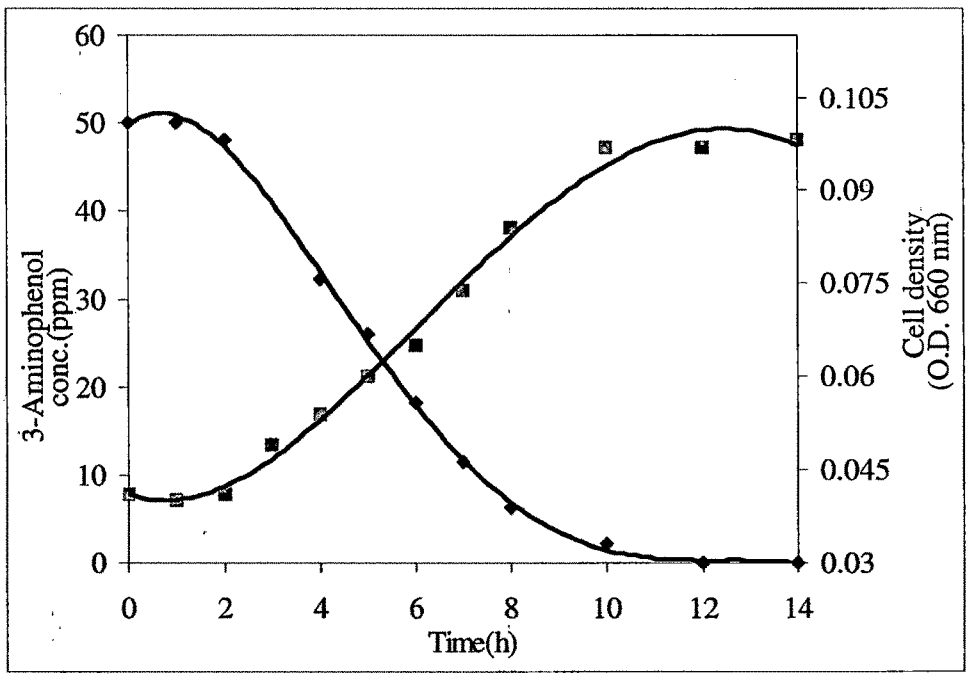

Figure 4.15. 3-Aminophenol degradation by P.aeruginosa in batch reactor
[Initial conc.= 50 ppm, Temp.= 30 °C, pH=7]

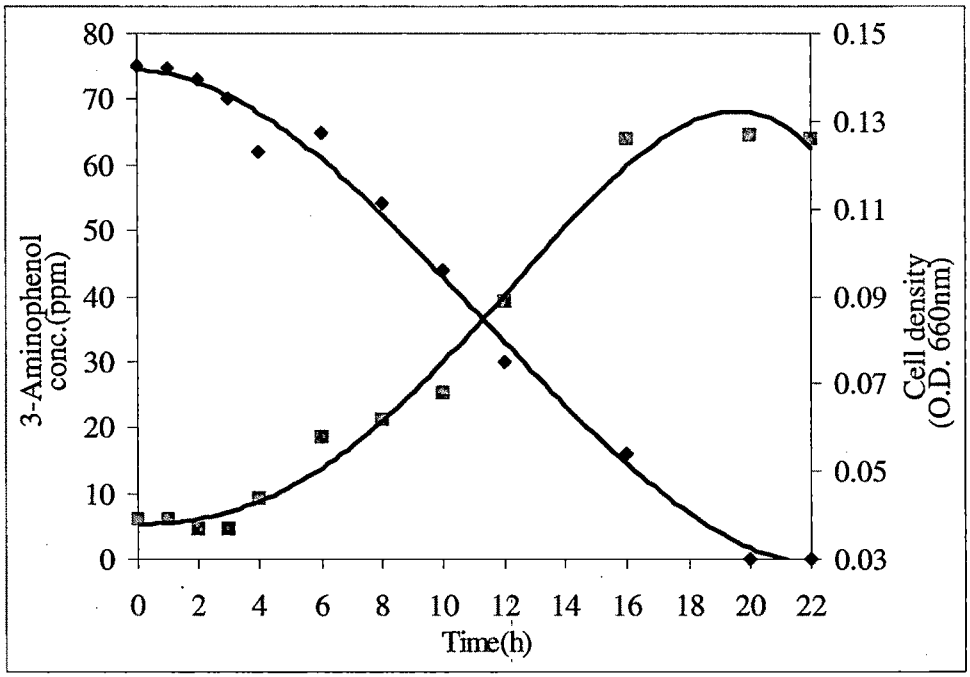

Figure 4.16. -Aminophenol degradation by P.aeruginosa in batch reactor
[Initial conc.= 75 ppm, Temp.= 30 °C, pH=7]

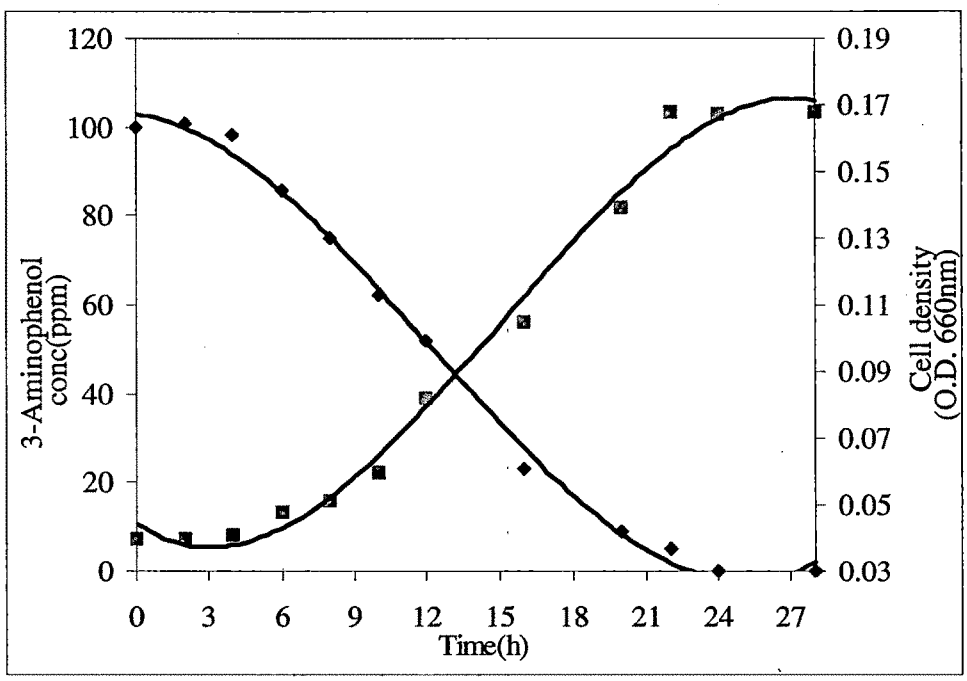

Figure 4.17. 3-Aminophenol degradation by P.aeruginosa in batch reactor
[Initial conc.= 100 ppm, Temp.= 30 °C, pH=7]

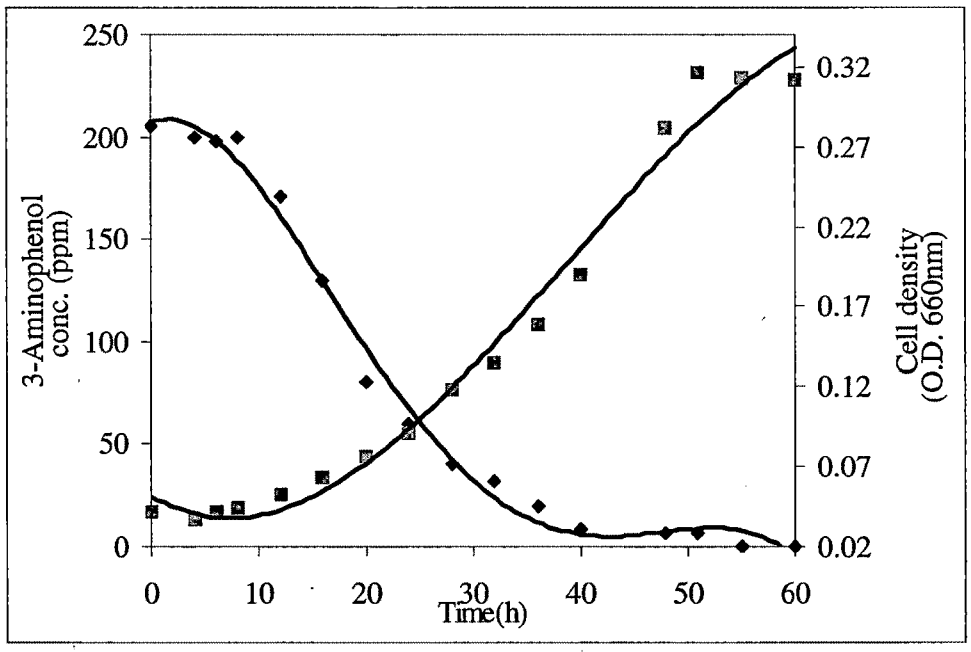

Figure 4.18-Aminophenol degradation by P.aeruginosa in batch reactor
[Initial conc.= 200 ppm, Temp.= 30 °C, pH=7]

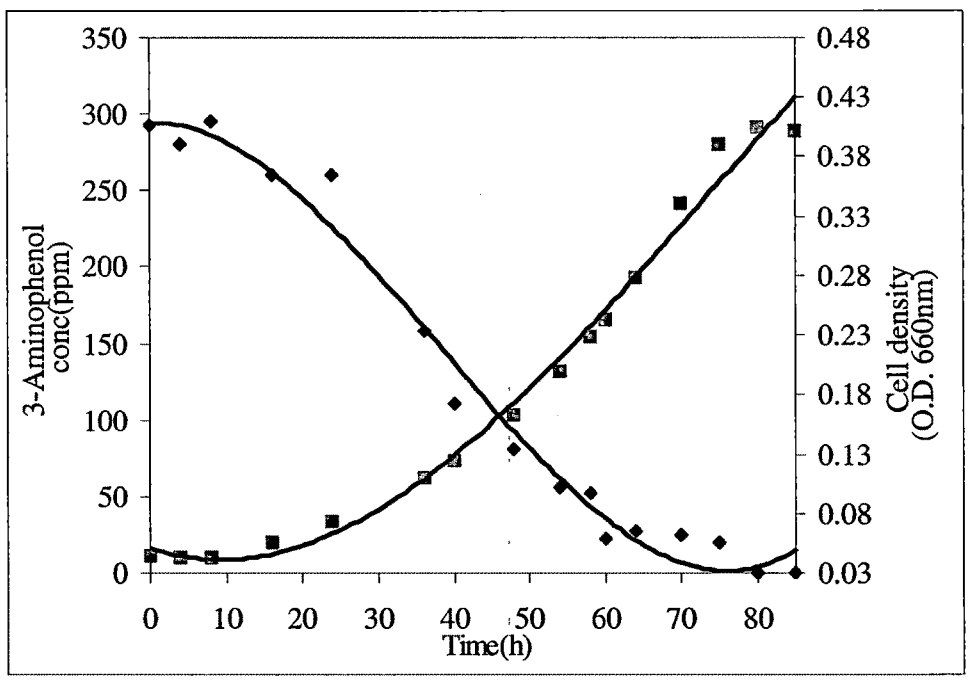

Figure 4.19. 3-Aminophenol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 300 ppm, Temp.= 30 °C, pH=7]

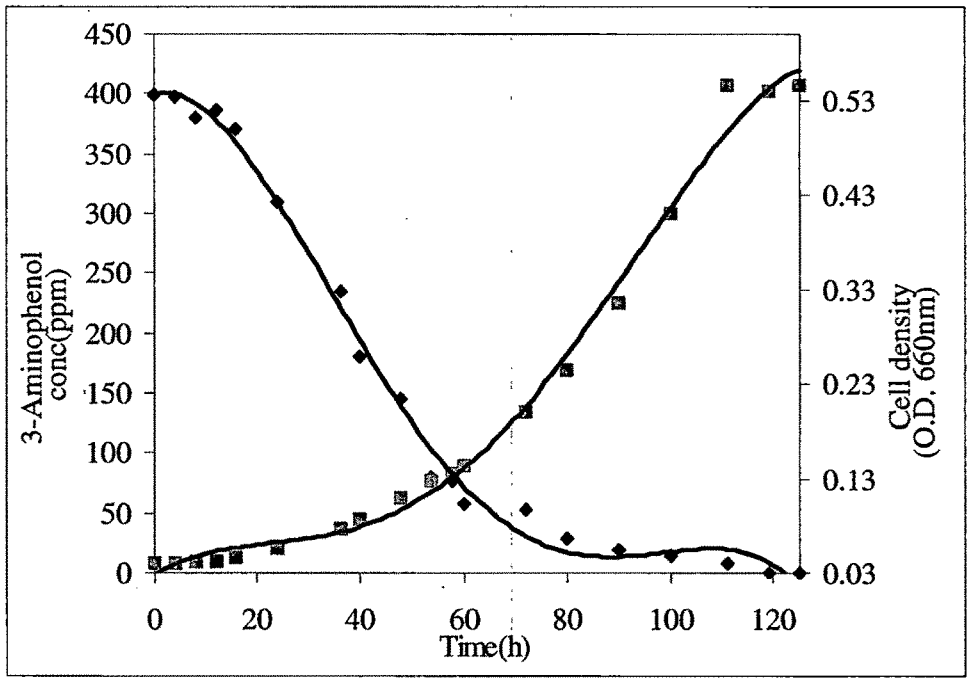

Figure 4.20. 3-Aminophenol degradation by *P.aeruginosa* in batch reactor [Initial conc.= 400 ppm, Temp.= 30 °C, pH=7]

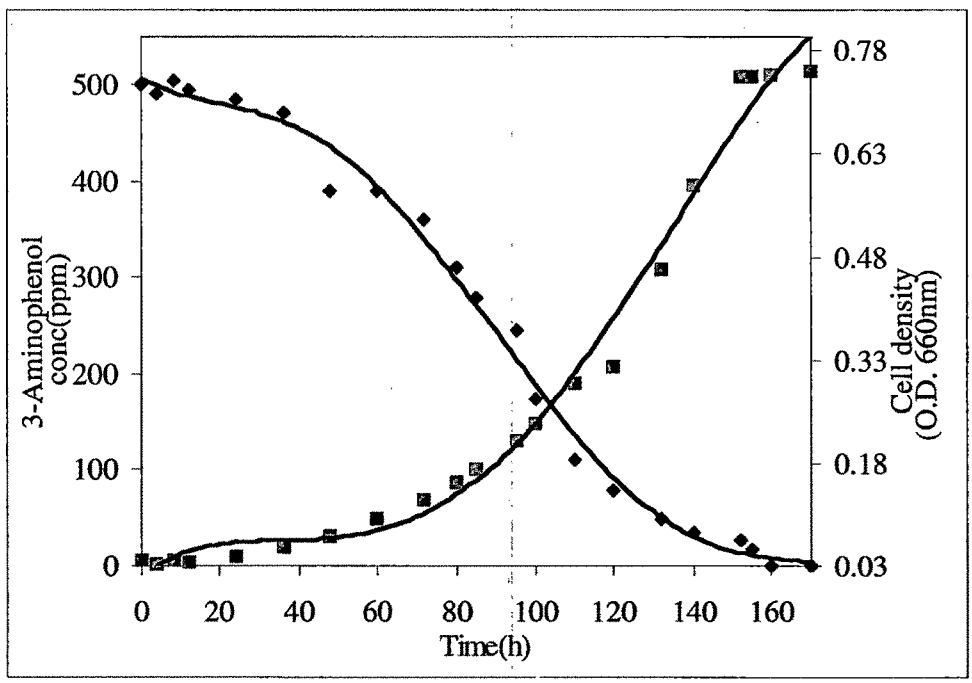

Figure 4.21. 3-Aminophenol degradation by *P.aeruginosa* in batch reactor [Initial conc.= 500 ppm, Temp.= 30 °C, pH=7]

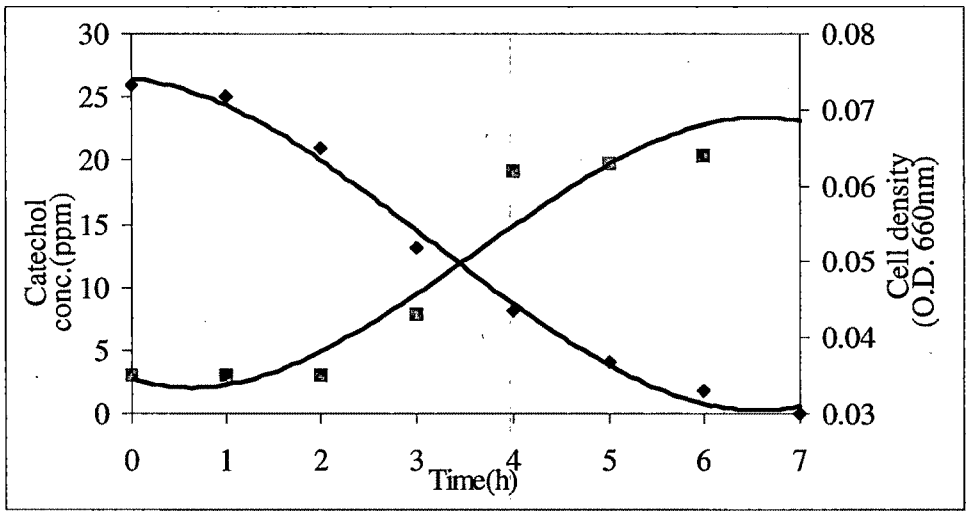

Figure 4.22. Catechol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 25 ppm, Temp.= 30 °C, pH=7]

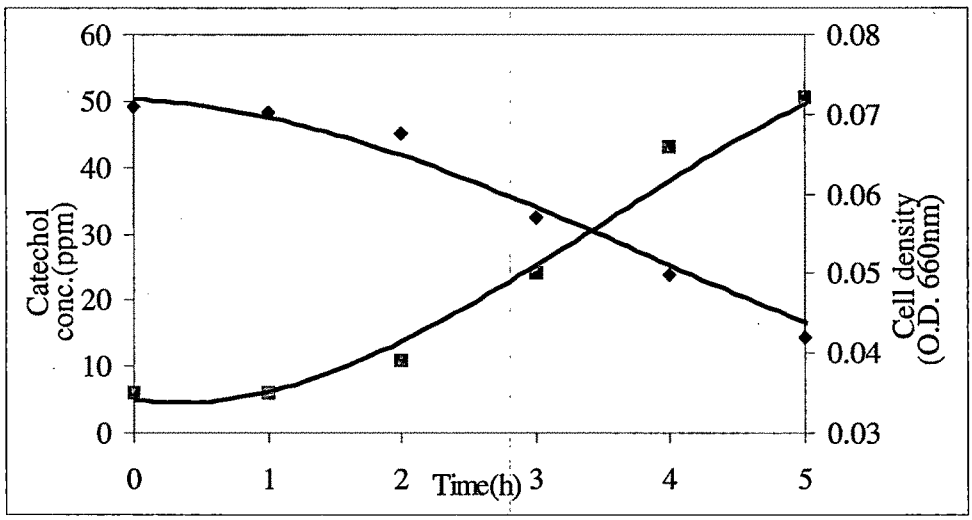

Figure 4.23. Catechol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 50 ppm, Temp. = 30 °C, pH=7]

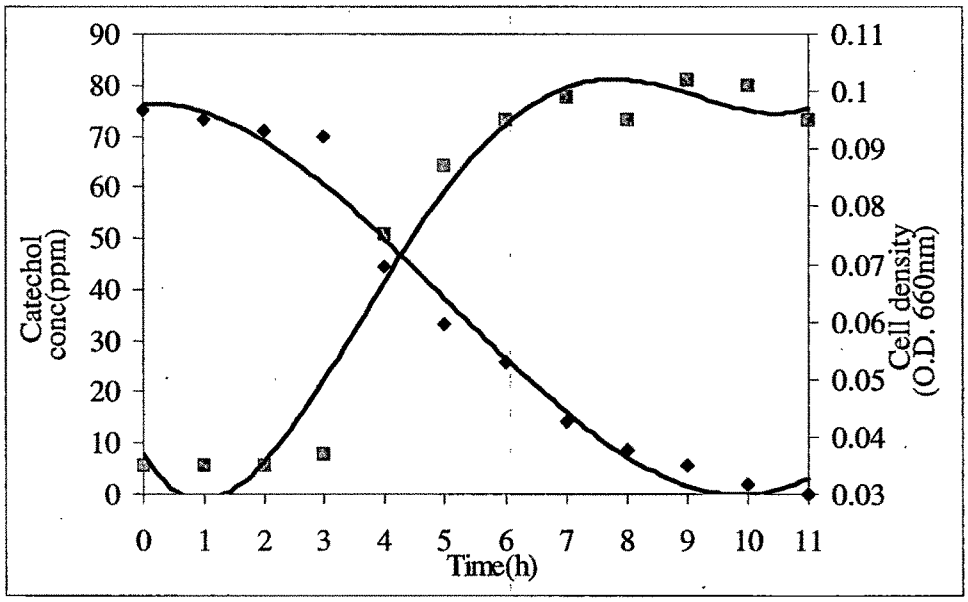

Figure 4.24. Catechol degradation by *P.aeruginosa* in batch reactor
[Initial conc.= 75 ppm, Temp.= 30 °C, pH=7]

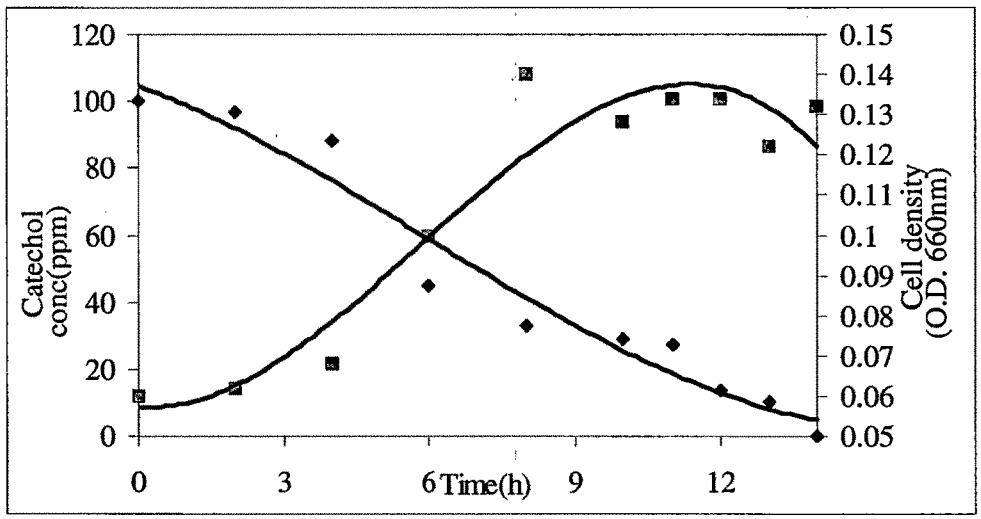

Figure 4.25. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 100 ppm, Temp.= 30 °C, pH=7]

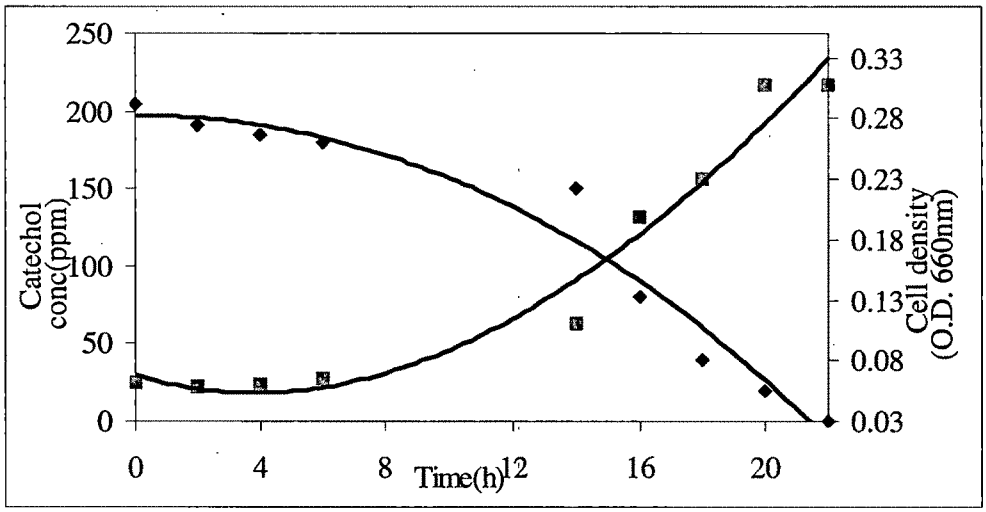

Figure 4.26. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 200 ppm, Temp.= 30 °C, pH=7]

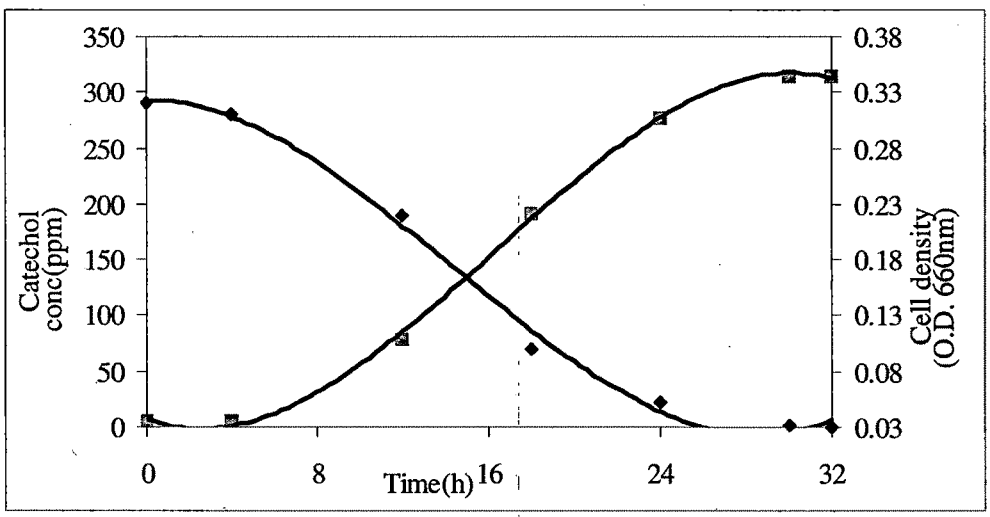

Figure 4.27. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 300 ppm, Temp.= 30 °C, pH=7]

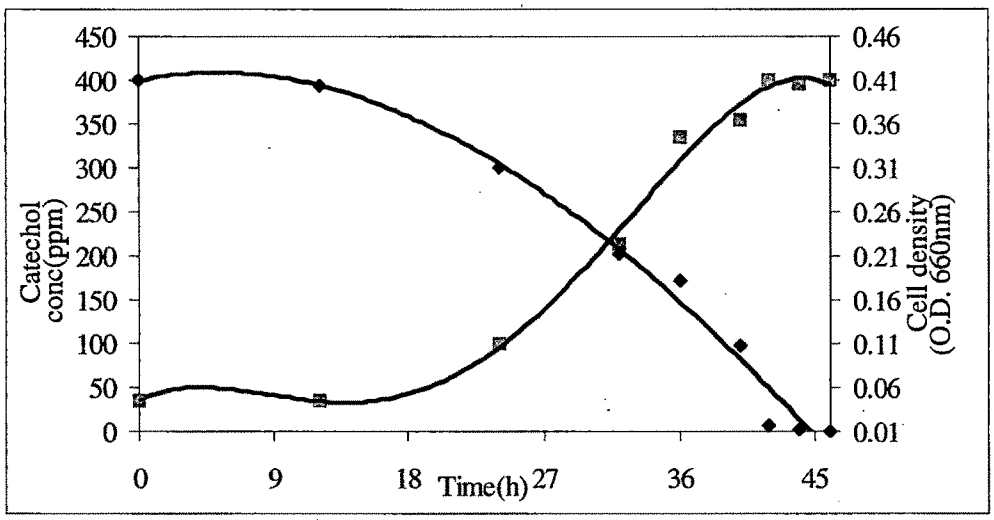

Figure 4.28. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 400 ppm, Temp.= 30 °C, pH=7]

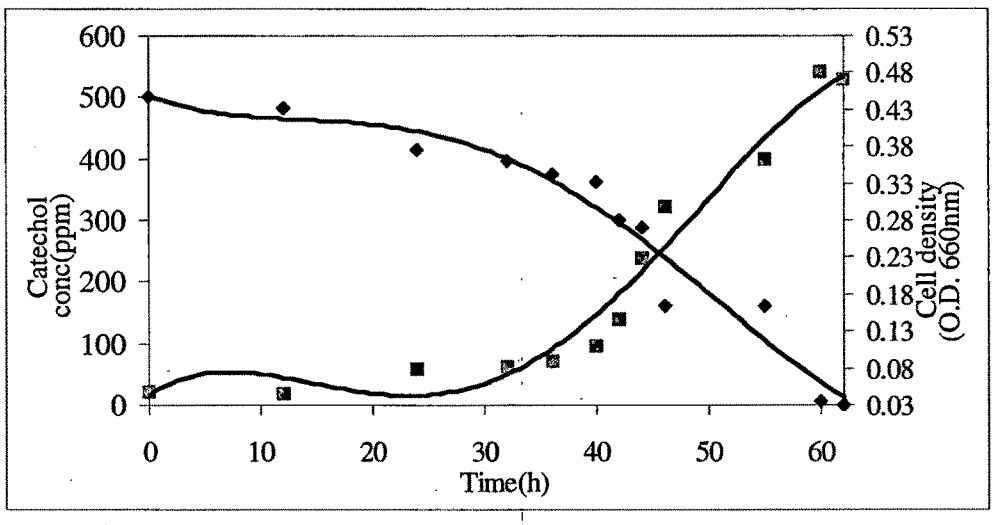

Figure 4.29. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 500 ppm, Temp.= 30 °C, pH=7]

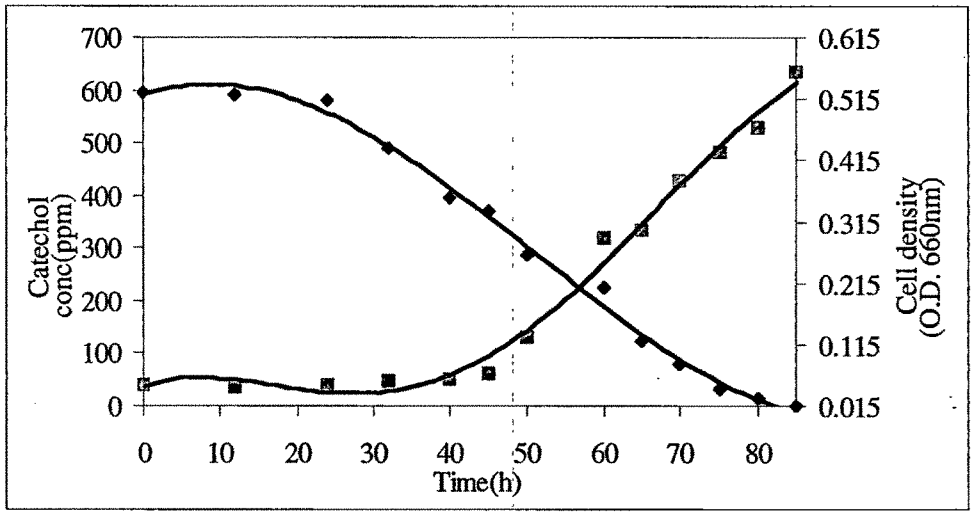

Figure 4.30. Catechol degradation by P.aeruginosa in batch reactor
[Initial conc.= 600 ppm, Temp.= 30 °C, pH=7]

The influence of phenols concentration on the duration of the lag phase is shown in Figs. 4.31, 4.32 and 4.33 for phenol, catechol and 3-aminophenol respectively. It is observed that the length of lag phase increases exponentially with initial concentration of phenols. The exponential lag phase behavior has also been reported in literature by Monteiro et al. (2000) for phenol removal. However, Vijayagopal (2005) has reported linear lag phase behavior for degradation of phenol.

At higher concentrations, it was observed from Figs. 4.12, 4.21 and 4.27 that the rate of substrate removal becomes relatively less near the end of the substrate degradation curves. There are two possible reasons for this:

(i) The deficiency in the availability of oxygen as these experiments were done in cotton plugged conical flasks of 250 ml with 100 ml working volume. In this case there were two sources of oxygen: first, the air in headspace and second, the oxygen dissolved in the liquid medium.

(ii) This may be due to the fall in pH of the solution. Lallai and Mura (1989) have reported this observation for phenol metabolization by mixed culture composed of *Pseudomonadaceae, Vibrionaceae* etc.

Thus, low concentration of oxygen and low pH affect the kinetics of substrate consumption. In microbial growth curve after the lag phase as well as exponential phase, drop in microbial growth rate may be due to lower oxygen concentration in solution (Yang and Humphrey 1975).

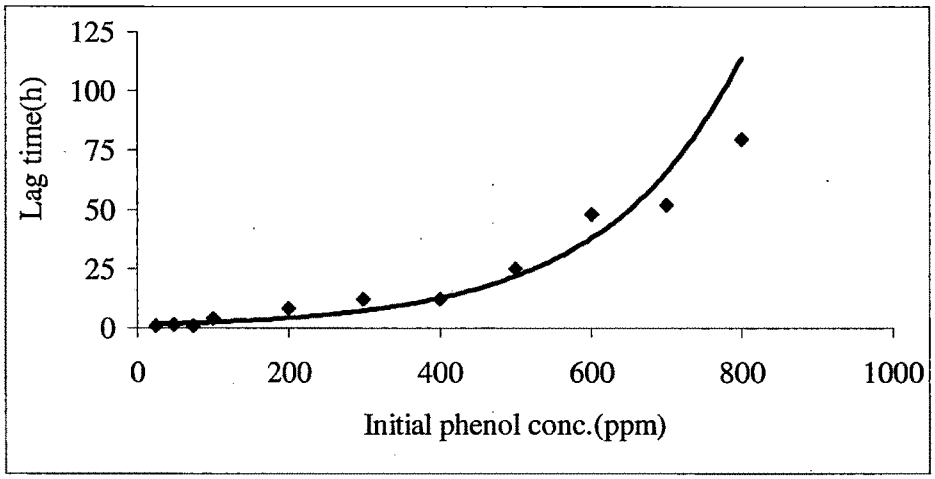

Figure 4.31. Behavior of time lag in biodegradation of phenol using *P. aeruginosa*

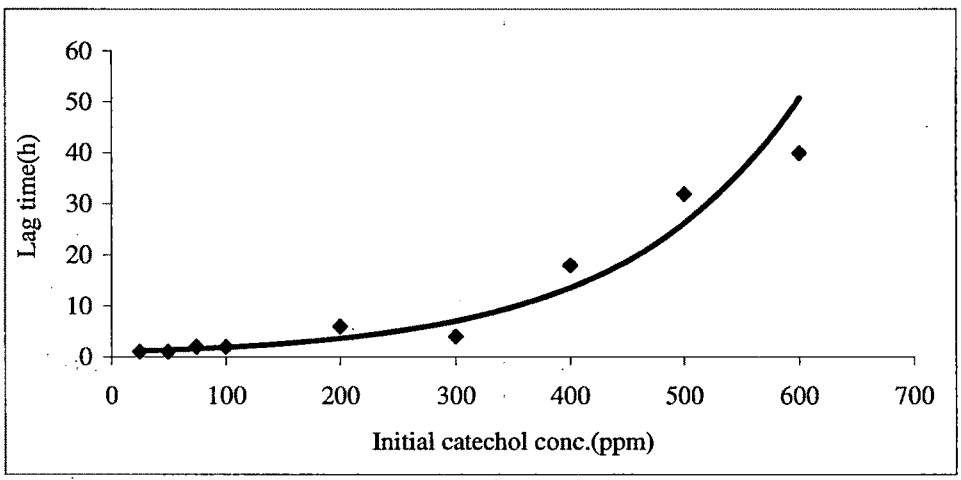

Figure 4.32. Behavior of time lag in biodegradation of catechol using *P. aeruginosa*

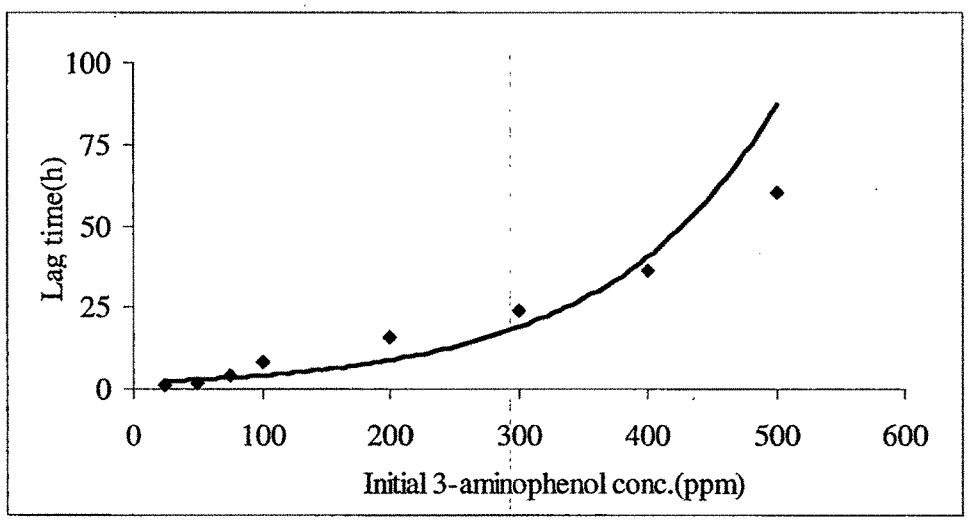

Figure 4.33. Behavior of time lag in biodegradation of 3-aminophenol using *P. aeruginosa*

4.4.3 Growth Kinetics

The analysis of the batch growth data for microbes metabolizing inhibitory carbon sources like phenols requires careful plotting of the semi logarithmic plot of biomass concentration versus time in order to determine the actual specific growth rate exhibited by the microorganisms for a particular initial substrate concentration. After a short lag phase, all phenols act as the limiting substrate and the cultures grow exponentially. The data from exponential region has been taken for specific growth rate calculations. Such plots have earlier been used for the determination of specific growth rate for different initial concentration of phenols (Yang and Humphrey 1975, Livingston and Chase 1989, Kumar 2004).

In the present study also, the specific growth rate, μ for that particular initial phenolic concentration was calculated from the slope of the ln(optical density) versus time plots for all concentrations. Fig. 4.34 shows the typical graphs of ln(optical density) against time for phenol. Since same method is used for catechol and 3-aminophenol to determine specific growth rates, graphs for these systems have not been shown here. The experimental values of specific growth rate μ derived from initial concentration have been plotted against initial constant concentration of phenols to determine kinetic constants using different kinetic models. The parameters of kinetic model of Haldane were determined by fitting the model to the experimental data using non-linear regression technique. However, it should be noted that there are almost infinite number of numerical combinations of the constants, which can provide equally adequate fits to the data, since all the parameters are obtained from the single curve fitting operation.

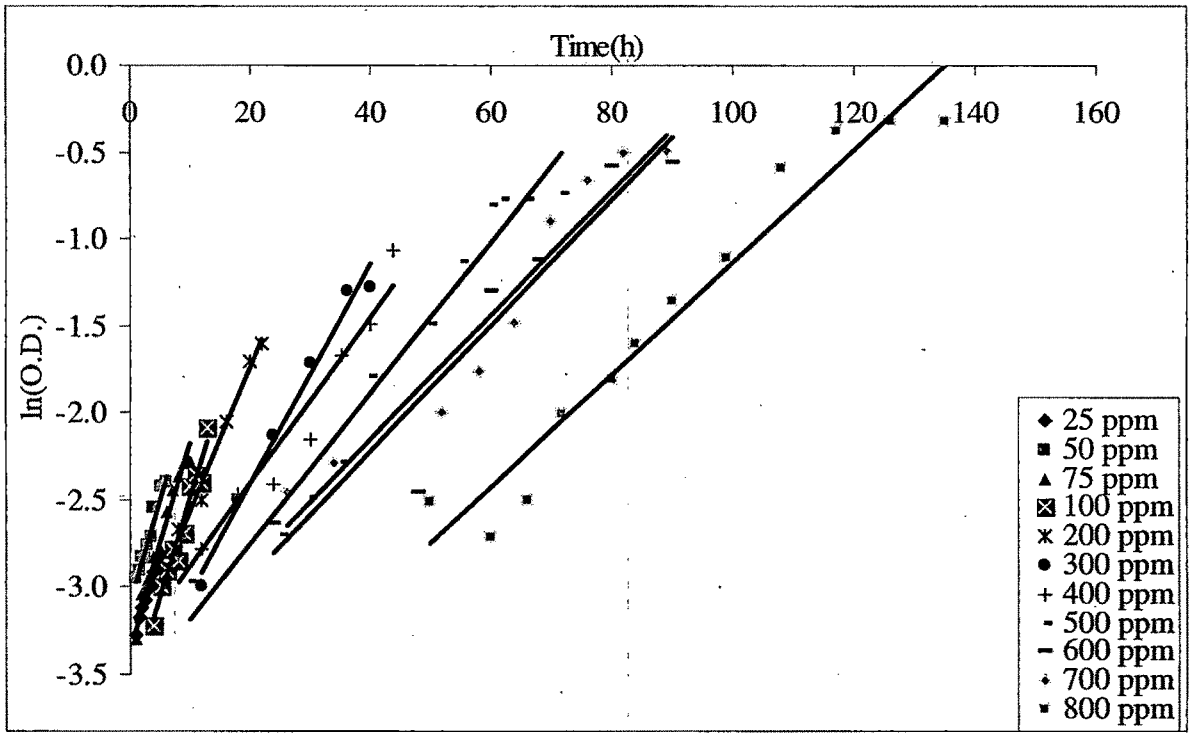

Figure 4.34. Determination of specific growth rate in the region of exponential phase of batch growth curve [Phenol/ *P. aeruginosa* system]

Trendline equation and correlation coefficient values for Fig. 4.34				
Co	25 ppm	50 ppm	75 ppm	100 ppm
y=	0.1085x - 3.35	0.1217x – 3.093	0.1133x - 3.309	0.1112x - 3.6142
R^2=	0.992	0.952	0.965	0.944
Co	200 ppm	300 ppm	400 ppm	500 ppm
y=	0.0816x – 3.3815	0.0634x - 3.679	0.0472x – 3.35	0.0433x-3.62
R^2=	0.982	0.984	0.944	0.961
Co	600 ppm	700 ppm	800 ppm	
y=	0.0363x-3.68	0.0354x-3.56	0.0323x-4.36	
R^2=	0.902	0.93	0.948	

Consequently, a unique set of values will not be produced unless an independent evaluation of one of the parameters is obtained or unless numerical limits are placed on the input estimates and computed outputs from the parameter generated in the curve fitting algorithm. Range for the numerical limits can be discerned by utilizing both practical engineering insight and observation from previous work with non-inhibitory substrates. Hence, biomass growth on phenol has been modeled both by inhibitory and non-inhibitory type of substrate kinetics. In the present study, specific growth rate data from low concentration region were fitted to Monod's model and those from high concentration region were fitted to Linearized Haldane's model. Figs. 4.35 and 4.38 show the plots to evaluate Monod's kinetic parameters and Figs. 4.36, 4.39 and 4.41 show the plots to determine parameters of Linearized Haldane's model. Table 4.2 presents the parameter values

obtained using these models. The data of full concentration range cannot be correlated by either of the models in the case of phenol and catechol. However for 3-aminophenol, linearized Haldane model could represent the complete range of concentration. The values obtained by these expressions have been used as a base for the values of parameters for inhibitory kinetic models.

Based on these parameters, the inhibitory model of Haldane was fitted to data using SPSS version 8.0 for Windows. In this procedure of data fitting, two criteria namely maximum percentage deviation and correlation coefficient (R^2) are met to get parameter values. Percentage deviation was calculated using Eq.(4.18).

$$\text{Percentage deviation} = \frac{\mu_{experimental} - \mu_{predicted}}{\mu_{experimental}} \times 100 \qquad (4.18)$$

Figs. 4.37, 4.40 and 4.42, show comparison of experimentally obtained specific growth rate, μ with those predicted by the model. This is evident from the figures that the growth kinetics of phenol, 3-aminophenol and catechol could be represented by Haldane's growth kinetic model very well. In the case of all the phenols, the maximum deviation was less than 10% and the coefficient of regression, R^2 in all the three cases was found to be more than 0.99. The values of the growth kinetics parameters obtained for phenol, 3-aminophenol and catechol are given in Table 4.3. These expressions and values clearly indicate that μ_{max} obtained from Monod's equation and Linearized Haldane's model cannot be true as it is less than that obtained by Haldane's model. Since μ_{max} is rate constant, calculated when the culture is grown under non-limiting growth conditions, the value should then be one that is determined as the highest value. Therefore, μ_{max}, K_s and K_i calculated by Haldane's model should be taken as correct values.

The calculated values of the parameters were compared with the values available in literature compiled in Table 2.4 in Chapter 2. The maximum specific growth rates and the half saturation coefficients are within the range for phenol / mixed culture system [0.131 to 0.363 $h^{-1}$and 5 to 226 mg/l, D'Adamo et al. (1984)]. The substrate inhibition coefficients are also within the range when compared with different systems [Ki =54.1 mg/l, Monterio (2000), Ki=129.79, Kumar (2004)].

Though the calculated parameters are well within the range, these values (μ_{max}, K_s, and K_i) in combination do not match exactly with other systems. The possible reasons for the difference in values of kinetic parameters obtained and available in literature are described below:

- Kotturi et al (1991) noted that the half saturation coefficient is influential on the growth kinetics in the low concentration region and at the same time the small changes in the biomass and substrate concentrations cannot be measured accurately in batch reactors. Therefore, this may be one of the possible reasons for the difference in values of kinetic coefficients.

- Pawlowsky (1972, 1973) found that system coefficients are depending on correlating method for data fitting. Pawlowsky (1972) using batch cultivation of phenol found μ_{max} = 0.27 h^{-1}, K_i= 1190 mg/l and K_s=19.1 mg/l, by linearization of data using Haldane model. For the same system and model, Pawlowsky and Howell (1973) reported μ_{max} = 0.223 h^{-1}, K_i= 934.5 mg/l and K_s=19.1 mg/l.

The engineering significance of the kinetic constants is that, when hydraulic shock is affected beyond μ_{max}, there is every possibility of the biomass being washed out. Similarly, when organic shock is made beyond the K_i value the rate of degradation of substrate is retarded, resulting in substrate build up which also reduces treatment efficiency and functioning of treatment plants. Based on this, it can be concluded that catechol can withstand higher hydraulic shock compared to phenol and 3-aminophenol, where as 3-aminophenol is more toxic/inhibitory than phenol and catechol.

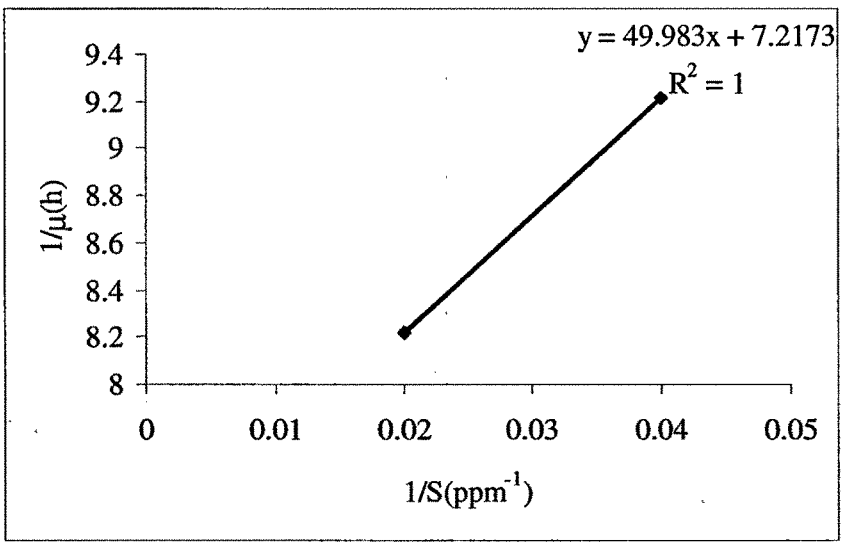

Figure 4.35. Monod's growth kinetic model fitted to batch growth data to evaluate μ_{max} and Ks [Phenol/ *P. aeruginosa* system, Conc. Range 0-50 ppm]

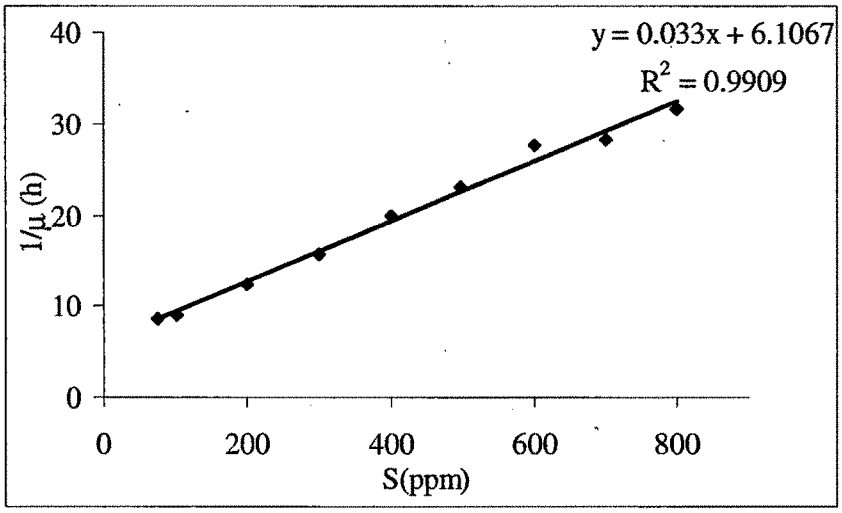

Figure 4.36 Linearized Haldane's growth kinetic model fitted to batch growth data to evaluate μ_{max} and Ki [Phenol/ *P. aeruginosa* system, Conc. Range 50-800 ppm]

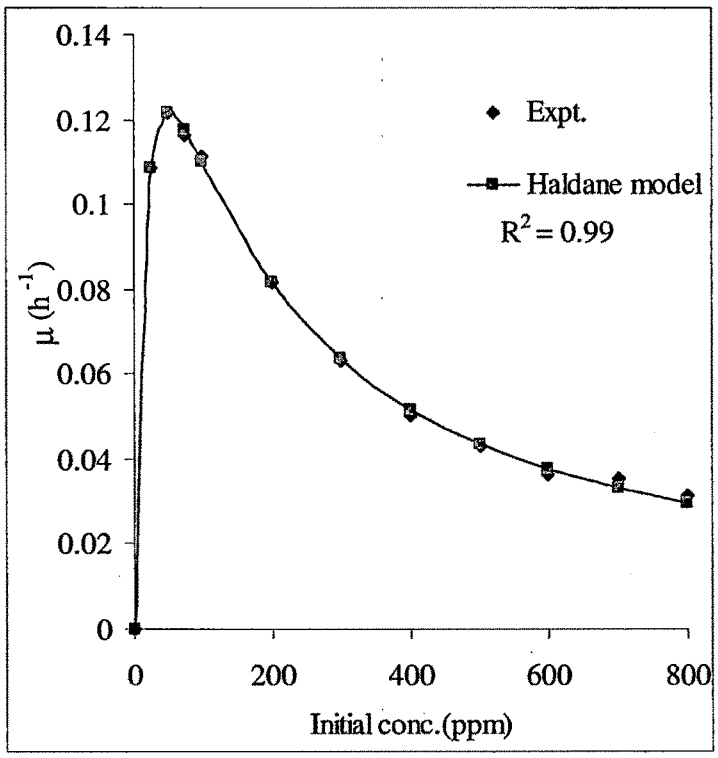

Figure 4.37. Haldane's kinetic model fitted to batch growth data to evaluate μ_{max}, K_s and K_i [Phenol/ *P. aeruginosa* system, Conc. Range 0-800 ppm]

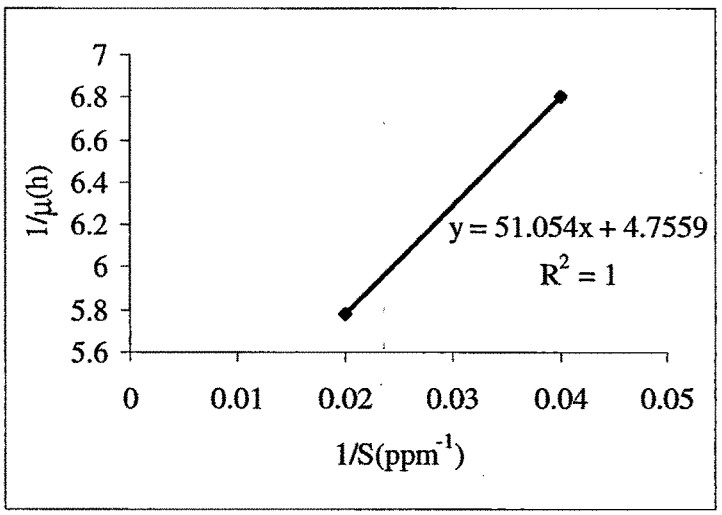

Figure 4.38. Monod's growth kinetic model fitted to batch growth data to evaluate μ_{max} and K_s [Catechol / *P. aeruginosa* system, Conc. Range 0-50 ppm]

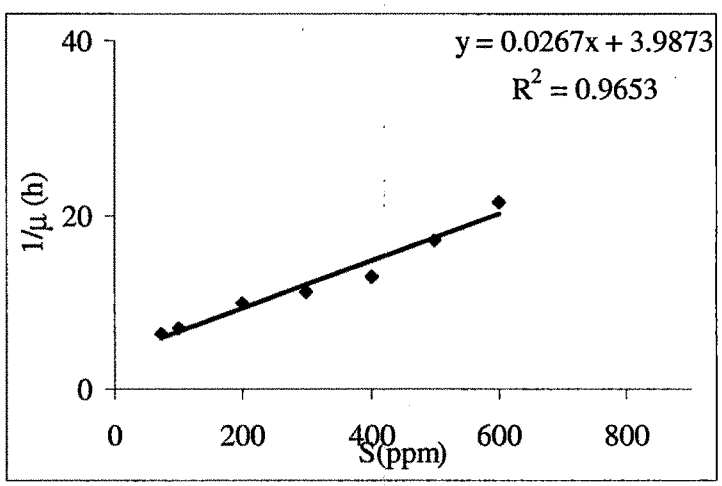

Figure 4.39. Linearized Haldane's growth kinetic model fitted to batch growth data to evaluate μ_{max} and K_i
[Catechol / *P. aeruginosa* system, Conc. Range 50-600 ppm]

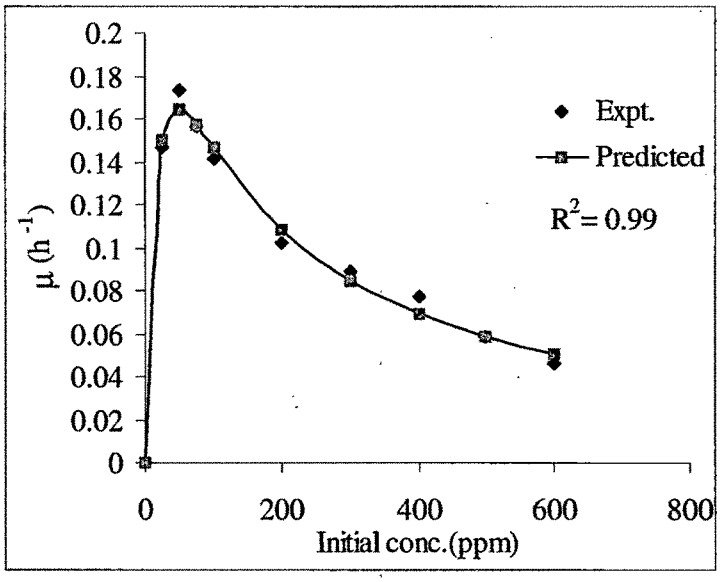

Figure 4.40. Haldane's kinetic model fitted to batch growth data to evaluate μ_{max}, K_s and K_i
[Catechol/ *P. aeruginosa* system, Conc. Range 0-600 ppm]

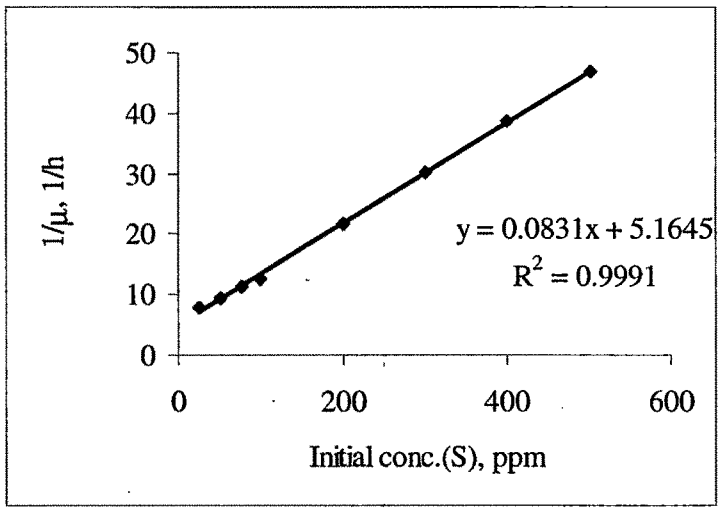

Figure 4.41. Linearized Haldane's growth kinetic model fitted to batch growth data to evaluate μ_{max} and K_i
[3-Aminophenol / *P. aeruginosa* system, Conc. Range 0-500 ppm]

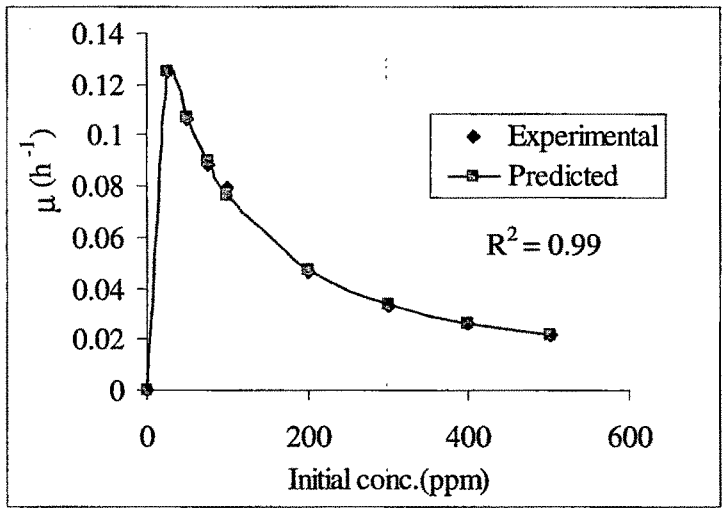

Figure 4.42. Haldane's kinetic model fitted to batch growth data to evaluate μ_{max}, K_s and K_i [3-aminophenol / *P. aeruginosa* system, Conc. Range 0-500 ppm]

Table 4.2. Growth kinetic parameter values of Monod's and linearized Haldane's model for biodegradation of phenol, 3-aminophenol and catechol using *P. aeruginosa*

Compound	Monod's model		Linearized Haldane's model	
	μ_{max}(h^{-1})	K_s(mg/l)	μ_{max}(h^{-1})	K_i(mg/l)
Phenol	0.139	6.93	0.164	185.05
Catechol	0.21	10.74	0.251	149.34
3-Aminophenol	-	-	0.194	62.15

Table 4.3. Haldane's kinetic parameter values for biodegradation of phenol, 3-aminophenol and catechol using *P. aeruginosa*

Compound	Haldane's model				
	μ_{max} (h^{-1})	K_s (mg/l)	K_i (mg/l)	Maximum % deviation	R^2
Phenol	0.221	20.89	124.6	6.21	0.996
Catechol	0.286	17.77	129.28	10.25	0.991
3-Aminophenol	0.228	8.73	52.39	4.03	0.999

4.4.4 Endogenous or Decay Coefficient

The batch kinetics experiments were stopped when the phenol/catechol/3-aminophenol was fully consumed at all concentrations except at 400 mg/l of phenol, 300 mg/l of 3-aminophenol and 200 mg/l of catechol. The biomass growth was further monitored for another 10 days for catechol, 12 days for phenol and 17 days for 3-aminophenol in order to calculate endogenous or decay coefficients. The selection of these particular concentrations was arbitrary rather than intentional since the endogenous or decay coefficient is not a function of substrate but a constant entity. Therefore, assuming the value of k_d will not change with different initial concentration in batch kinetics studies. Figs. 4.43, 4.45 and 4.47 show the batch growth curves extended up to endogenous region to calculate the endogenous or decay rate coefficient. These three curves are typical of those obtained in batch studies. After stationary phase, a phase of negative growth is followed. Here, some of the cells of the population are used for maintenance of others. The absorbance data from this region were plotted as ln(optical density) versus time graph (Figs. 4.44, 4.46 and 4.48). A linear relationship with negative slope value was obtained. The negative slope of this gives decay coefficient.

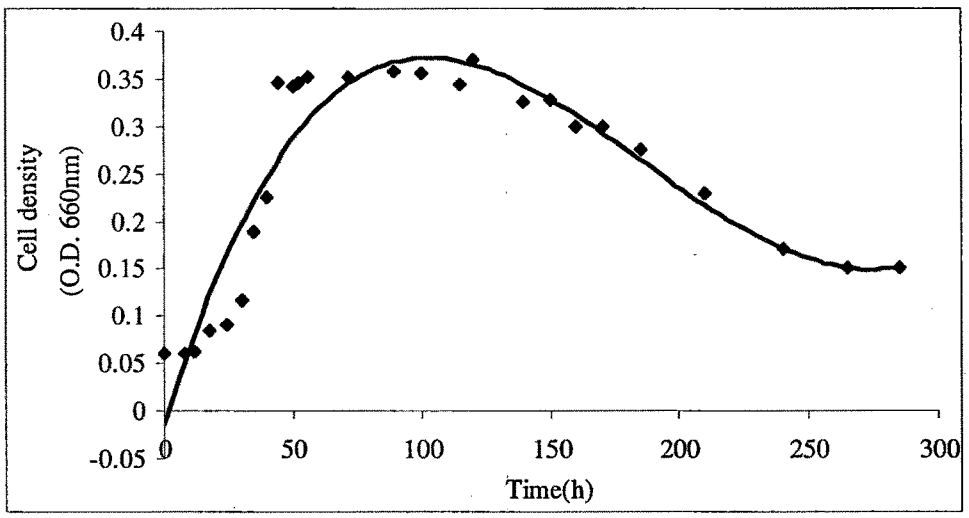

Figure 4.43. Complete batch growth cycle for Phenol/ *P. aeruginosa* system

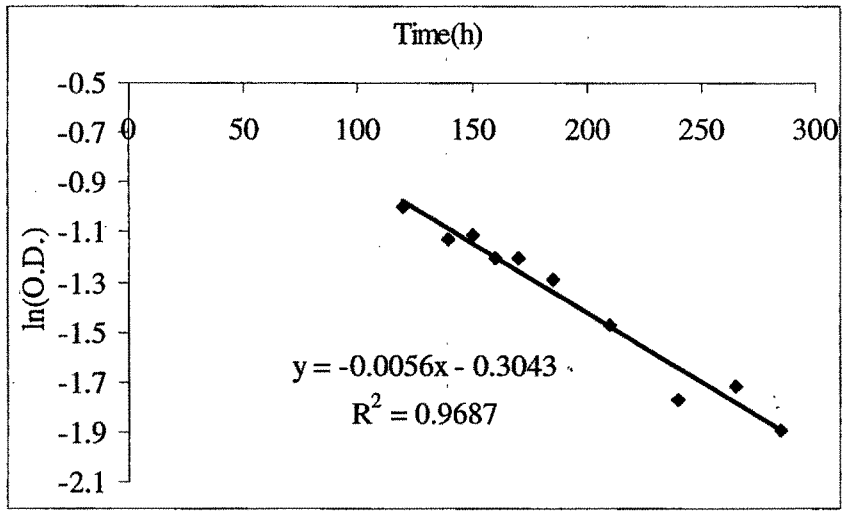

Figure 4.44. Decay coefficient K_d evaluation for Phenol/ *P. aeruginosa* system using decay phase batch kinetic experimental studies

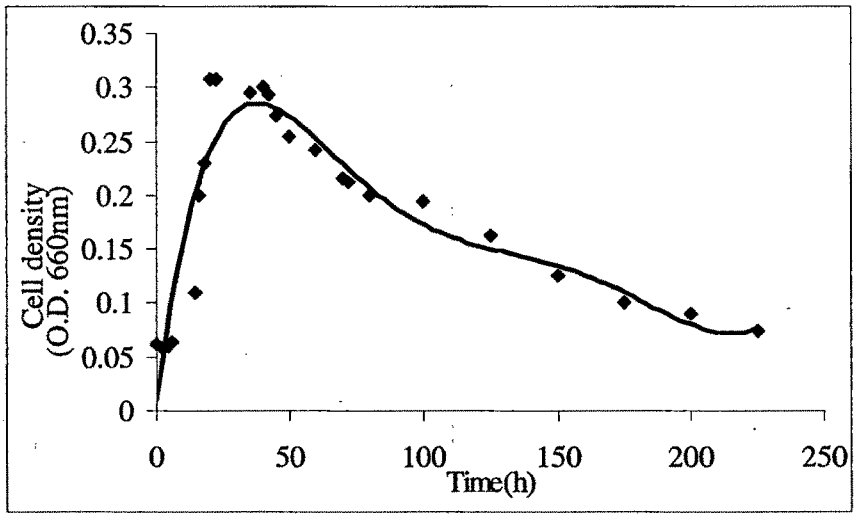

Figure 4.45. Complete batch growth cycle for Catechol/ *P. Aeruginosa* system

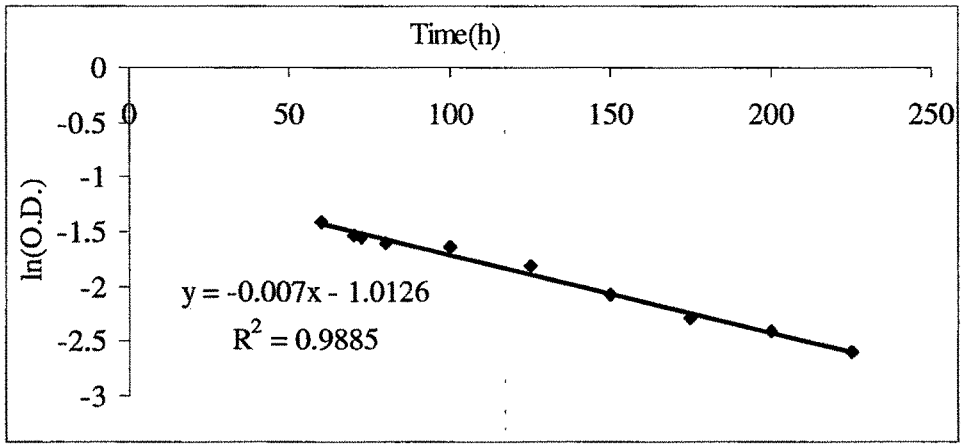

Figure 4.46. Decay coefficient K_d evaluation for Catechol/ P. aeruginosa system using decay phase batch kinetic experimental studies

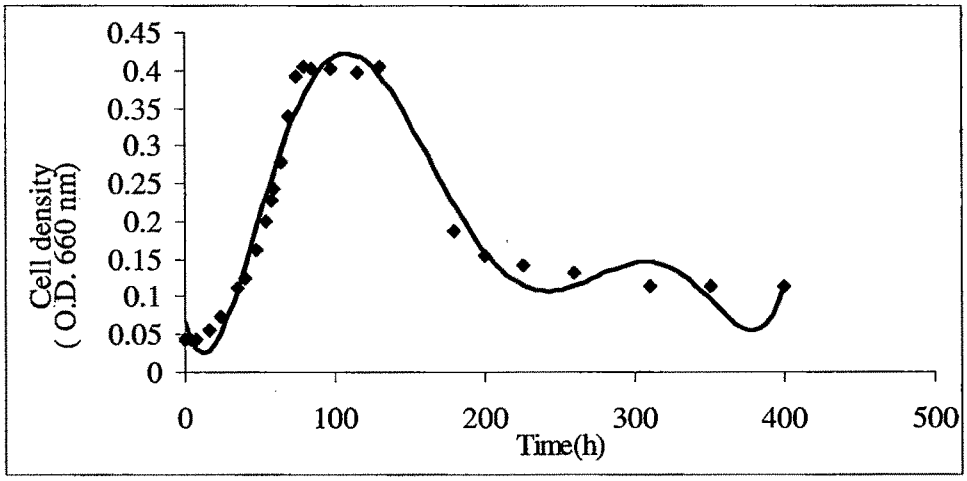

Figure 4.47. Complete batch growth cycle for 3-Aminophenol/ *P. Aeruginosa* system

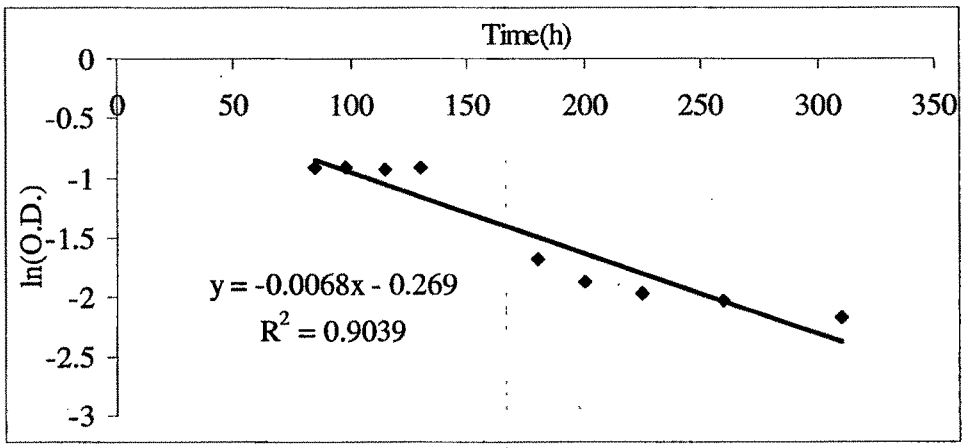

Figure 4.48. Decay coefficient K_d evaluation for 3-Aminophenol/ *P. aeruginosa* system using decay phase batch kinetic experimental studies

The values of the decay rate coefficient are 0.0056, 0.007 and 0.0068 h^{-1} for phenol, 3-aminophenol and catechol respectively. The value of endogenous or decay rate coefficient for phenol degradation was available only in two research papers. Kumaran and Parachuri (1997) have reported a value of 0.005 hr^{-1} for phenol degradation by a mixed culture and Kumar et al.(2004) have reported a value of 0.0056 and 0.0067 h^{-1} phenol and catechol degradation by *P. putida*. The values obtained in the present study are very much in agreement with these values of decay coefficient. The decay coefficient appears in the material balance equation of treatment processes based on biological degradation. The value of the specific growth is reduced by as much as the value of decay coefficient. Therefore the process efficiency is reduced. For example in case of continuous flow mixed biological reactor, the wash out condition will occur at lower dilution rates. This implies that the continuous flow mixed biological reactor treating influents containing mainly catechol and/or 3-aminophenol should be operated at less dilution rate than when treating influent containing phenol.

4.4.5 Yield Coefficient

To calculate the yield coefficient of phenol, 3-aminophenol and catechol, the results of all initial concentrations of batch experiments were used. These studies were carried out till the phenol or 3-aminophenol or catechol initially present was fully consumed. The value of biomass present just at the end of exponential phase was used in calculating the biomass produced as a result of consumption of substrate. By doing this, any discrepancy that may arise due to the effect of maintenance requirements on substrate consumption and biomass produced, may be avoided. The yield coefficient was than determined by performing linear regression on the Eq. (4.17).

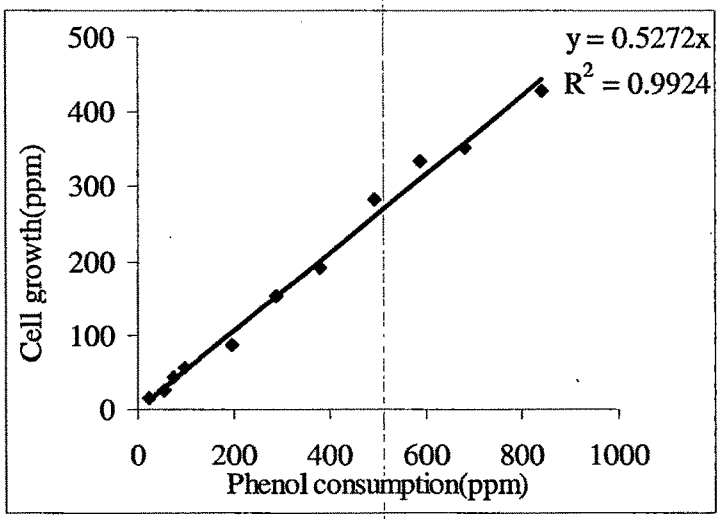

Figure 4.49. Yield coefficient calculation for Phenol/ *P. aeruginosa* system.

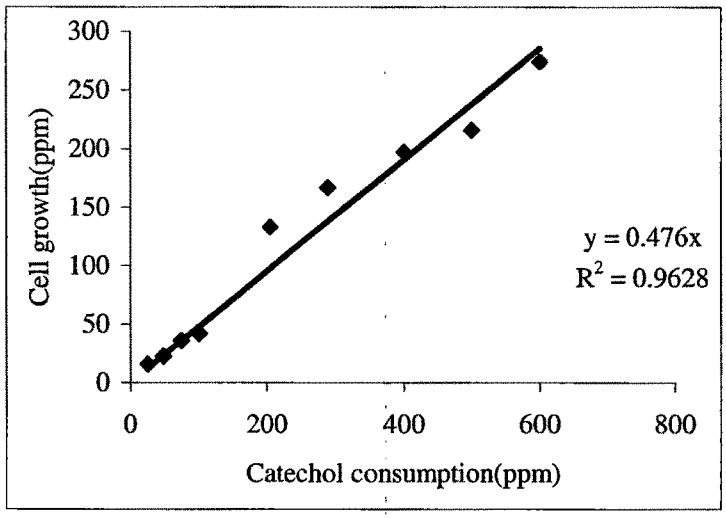

Figure 4.50. Yield coefficient calculation for Catechol/ *P. aeruginosa* system

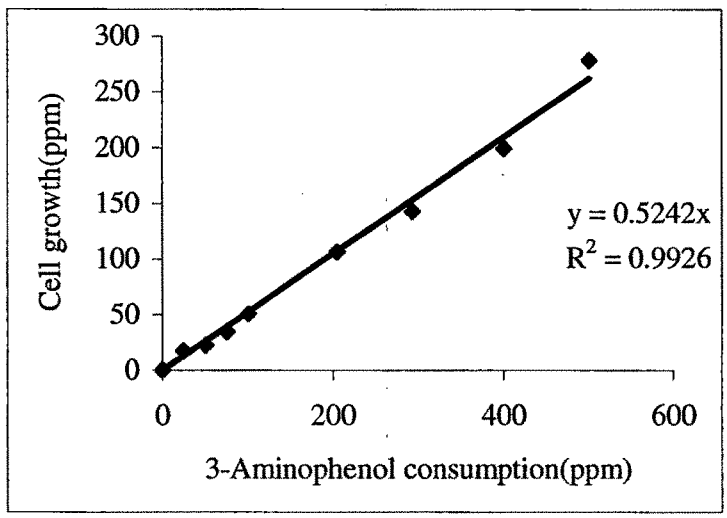

Figure 4.51. Yield coefficient calculation for 3-Aminophenol/ *P. aeruginosa* system

Figures 4.49, 4.50 and 4.51 show the plots used to determine yield coefficients for phenol, 3-aminophenol and catechol respectively. There is scatter in data in the different regions of the plot; it may be due to experimental error. However, in all the cases, the coefficient of regression, R^2 is more than 0.9. The value of yield coefficient obtained for all the phenols [0.5272, 0.5242 and 0.476 mg/mg for phenol, 3-aminophenol and catechol respectively] falls within the range reported in the literature [Y= 0.6 ± 0.12 mg /mg, Livingston and Chase (1989), Y=0.65± 0.15 mg/mg, Kumar (2004)]. There is a minor difference in the yield coefficients of all the phenols studied. The value of yield coefficient; phenol > 3-aminophenol> catechol is observed. Carbon to molecular weight ratio of phenol is higher than that of aminophenol and catechol and nitrogenous source in 3-aminophenol is the possible reason for higher yield coefficient. The influence of a readily degradable ancillary

carbon source on performance of pure culture is reported also by Murialdo (2003). Hence, these may be possible reasons for the difference in the values of the yield coefficients of the three phenols, though all are being consumed by *P. aeruginosa* in the same environmental conditions.

4.5 CONCLUDING REMARKS

In this chapter, an experimental procedure to conduct bacteriological studies pertaining to the degradation of phenol and its derivatives using *P. aeruginosa* (ATCC 9027) have been studied.
In acclimatization experiments, *P. aeruginosa* (ATCC 9027) could be acclimatized to 3-aminophenol up to 500 mg/l and catechol up to 600 mg /1 concentration, at higher concentration culture started dying. In case of catechol, the solution turned from pinkish brown to blackish. This blackish solution has been used for further experimentation. In case of phenol, P. aeruginosa could adapt to 800 mg/l concentration, however, well acclimatized culture exposed to 1000 mg/l could not even start degradation in 23 days. Resorcinol could not be metabolized by the bacterial strain *P. aeruginosa* whereas this strain could adapt to 12000 mg/l of 4-aminophenol solution. Adaptation of *P. aeruginosa* to such high concentration of 4-aminophenol solution shows that exploring/searching different potent microorganisms present in nature may make biodegradation technique ecologically and economically feasible treatment for phenolic waste.

The results of batch experiments showed that the initial concentration of phenol, 3-aminophenol and catechol concentration of 800, 500 and 600 mg/l respectively could be fully degraded in 136, 160 and 85 h. Even well acclimatized culture (up to 800 mg/1) showed lag phase. The higher the initial concentration of the phenol, the longer the lag phase was observed. It was observed that lag phase increased exponentially with increase in initial concentration of substrate.
Data taken from the initial part of the exponential phase of growth curve for all the batch experiments were used in determining the specific growth rate at that initial concentration of phenol. The initial concentration of compound versus specific growth rate data were than fitted to Haldane growth kinetics model. The data could be fitted within maximum 10 % deviation. The values of parameters of Haldane's model were found within the range given in the literature. The value of decay coefficient was found to be in the order:
catechol $\cong$ 3-aminophenol > phenol,
whereas the value of yield coefficient was found to be in the order:
catechol < 3-aminophenol < phenol.

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