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Substrate inhibition kinetics: Naphthalene degradation by *Pseudomonas putida*

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

Biodegradation of naphthalene by *Pseudomonas putida* was studied in this article. The influence of pH, glucose concentration and inoculum concentrations on the growth and subsequent naphthalene degradation potential of *P. putida* were investigated. The naphthalene degradation and microbial growth was higher at optimum pH (pH 7), glucose concentration (500 mg/L) and inoculum concentration (3%). The non-ionic surfactants were used in the case of very high concentrations of naphthalene (500–2500 mg/L) to investigate the maximum naphthalene tolerance potential of *P. putida*. The surfactants used were Triton X-100 and Tween-80. These surfactants enhanced the availability of naphthalene to the microbes. Results indicated that naphthalene was completely degraded by *P. putida* at an initial naphthalene concentration of 500 mg/L. Further increase in naphthalene concentration decreases the degradation potential of *P. putida*. The inhibition characteristics of substrate were described using four kinetics models (Haldane, Webb, Edward and Aiba). These kinetics models fitted very well and effectively describe the dynamic behavior of naphthalene biodegradation by *P. putida*.

KEYWORDS

biodegradation; PAH; kinetics; *Pseudomonas putida*; surfactant

1. INTRODUCTION

Today's highly industrialized environment is charged with a multitude of potentially toxic chemicals. Polycyclic aromatic hydrocarbons (PAHs) are potentially hazardous compounds that are widely distributed throughout the environment in the form of complex mixtures and congeners. Certain PAHs have been found to be potent mutagens or carcinogens and their activity are strongly dependent upon molecular shape. Lower temperature processes favor formation of PAHs with alkyl side chains, whereas high temperature processes tend to form no substituted PAHs (Donelly and Betowski, 1998). Human exposure to PAHs may occur from activities such as petroleum refining, coke and aluminum production, coal combustion, and wood preservation (Heitkamp et al., 1987). PAHs have high bio-accumulation potentials (Park et al., 1990). Due to the production and usage of PAHs in mass quantities it is impossible to predict the extent of damage to the environment (Dzombak and Luthy, 1984). Heavy PAHs pose an additional problem of being persistent within soil environments (Zappi et al., 1993, Ye et al., 1996).

Contamination of groundwater with PAHs is difficult to remediate as these compounds are volatile and can diffuse rapidly once they enter an aquifer. Techniques for in situ bioremediation of PAH compounds are used to eliminate or reduce contamination levels in an aquifer. A major decomposition process of PAHs in the environment is microbial degradation. The biodegradation of PAHs has been studied using bacteria (Jeffrey et al., 1975; Kiyohara and Nago, 1978; Stringfellow and Aitken 1995; Boonchan et al., 2000; Moody et al., 2001), fungi (Cerniglia and Gibson 1977; Bumpus 1989) and algae (Cerniglia et al., 1980).

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In situ bioremediation refers to the use of natural microbiological processes occurring in the subsurface environment to breakdown complex compounds into simpler, non-toxic compounds without removal of aquifer material. The capacity of microorganisms to degrade specific PAHs in nature depends on the physical and chemical properties of the contaminants, the environment, and the activity of indigenous organisms. The bioavailability of a PAH can be enhanced by adding surfactants which acts as a solubilizing agent (Volkering et al., 1998). Previous studies (Ghoshal and Luthy, 1998; Cuypers et al., 2002) found that the presence of surfactants enhances the biodegradation of PAHs. Naphthalene is the most commonly used low-molecular-weight PAHs in the petroleum contaminated environment. Aerobic bacteria readily metabolize hydrocarbon pollutants (e.g. fuels) entering these systems, and quickly deplete the available oxygen. Most enhanced bioremediation systems involve the addition of oxygen. However, the feasibility of aerobic biodegradation is often limited by its low water solubility. Thus, bioremediation of low-molecular-weight PAHs in the presence of non-ionic surfactants has been the subject of this research.

The aim of this study is to examine the biodegradation of naphthalene using *Pseudomonas putida*. The effects of pH, glucose concentration, inoculum concentration, and concentration of naphthalene on the growth of microbes were examined. Besides, efforts have also been devoted to describe the substrate inhibition using different models and to evaluate the model parameters.

2. MATERIALS AND METHODS

2.1. Bacterial strain and growth media

Pseudomonas putida (MTCC 2445) was purchased from Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacteria were grown in Mineral Salt Medium (MSM) at pH 7.0 which contained per liter: 3 g K_2HPO_4, 3 g KH_2PO_4, 2 g NH_4Cl, 0.05 g $FeSO_4$, 0.005 g $MgSO_4.7H_2O$ and 0.005 g Na_2MoO_4. About 100 mL of MSM was transferred to 250 mL Erlenmeyer conical flask and sterilized at 121°C and 15 lbs for 20 min. In addition to the nutrients, 0.5% glucose was supplemented as co-substrate. Enrichment was carried out by growing the bacteria at room temperature in an orbital shaker at 200 rpm on MSM containing 0.5% glucose and supplemented with naphthalene. In order to acclimatize the *P. putida*, several transfers on the same media with decreasing the glucose concentration and increasing the naphthalene concentration were performed until a stable rate of microbial growth on naphthalene was achieved. During this acclimatization period, inoculation was performed when the cells were in exponential phase (18 h).

2.2. Influence of various parameters on naphthalene degradation

The parameters including the pH, glucose concentration, inoculum concentration, and concentration of naphthalene were tested for investigating their effects on the naphthalene degradation by *P. putida*. For examining the pH effects, 3 mL of bacterial culture at exponential phase was inoculated into 100 mL sterilized MSM medium at pH 5, 6, 7 and 8 containing 500 mg/L glucose and 20 mg/L naphthalene. To examine the glucose concentration (0.1-1%) and inoculum concentration (1–5%), similar incubation was done at fixed naphthalene concentration of 20 mg/L. Samples were withdrawn at regular time intervals to monitor the biomass growth and naphthalene concentrations.

2.3. Role of surfactants on naphthalene degradation

The surfactants were used to enhance the bioavailability of naphthalene to microbes in order to study the effect of high naphthalene concentrations on microbial growth. Control experiments revealed that naphthalene crystals were not dissolved in the medium in the examined naphthalene concentration range of 500 to 2500 mg/L. Two surfactants, viz., Triton X-100 and Tween-80 were used in the present study. The naphthalene concentrations were varied from 500 to 2500 mg/L and 3 mM of Triton X-100 or 1 mM of Tween-80 was added for solubilizing the compound. No glucose was added during experiments. The inoculation and sampling procedures were the same as described in previous sections.

2.4. Instrumental analysis

Free cell concentrations were measured as optical density (OD) at 600 nm using a spectrophotometer (U-3210, Hitachi, Japan). Biomass concentrations were estimated from a correlation between OD and cell weight. Levels of naphthalene were determined by injecting samples into a High Performance Liquid Chromatography (LC-10ATVP, Shimadzu) equipped with a C18 column. The eluent used was a mixture of

acetonitrile and water (80:20). Peaks were detected with a UV detector at 254 nm for naphthalene.

All experiments were repeated three times. The data shown in the corresponding figures in Section 3 were the mean values of the experiments. The average percentage error between the experimental and model predicted values was calculated using

$$\%\,Error = \frac{\sum_{i=1}^{N}(\mu_{exp,i} - \mu_{cal,i}/\mu_{exp,i})}{N} \times 100 \quad (1)$$

where μ_{exp} and μ_{cal} represents experimental and calculated specific growth rates, respectively, and N is the number of measurements.

3. RESULTS AND DISCUSSION

3.1. Influence of pH

Experiments were performed to optimize the effect of pH on the bacterial growth in the presence of naphthalene. The pH was varied between 5.0 and 8.0. The maximum growth occurred at pH 7.0. The growth curve of *P. putida* at different pH is shown in Figure 1. From the growth curves, it was inferred that there was a minimum bacterial growth at acidic pH 5.0. After inoculation of cells into the MSM, the bacterial population remained temporally unchanged for a long period at pH 8.0 and there was no apparent biomass increase. Most heterotrophic bacteria favor a pH near neutral for growth and biodegradation. Microbial activity is usually controlled to a large extent by pH, since it controls microbial enzyme activity, transport process, and nutrient solubility (Wong et al., 2002). The specific growth rate was observed as 0.124, 0.142, 0.207, 0.189 h^{-1} at pH 5, 6, 7 and 8, respectively.

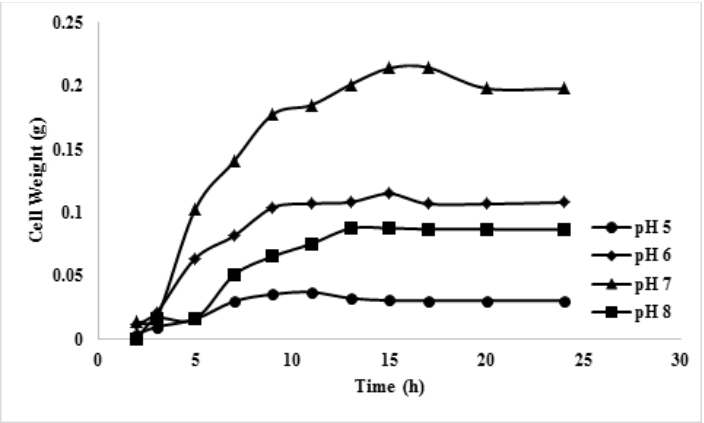

Figure 1. Influence of pH

3.2. Influence of glucose concentration

In the present study, glucose was used as an auxiliary substrate for enzyme production. The bacterium was tested with different concentrations of glucose such as 0.1, 0.2, 0.3, 0.5 and 1% in the presence of naphthalene. There was no inhibition found for the bacterum with the increase in glucose concentration. For *P. putida*, the best growth was observed at 0.5% and 1% glucose concentration. The bacterial growth curve of P. putida for different concentration of glucose is shown in Figure 2. The addition of glucose resulted in a significant increase in the number of bacteria. The specific growth rate was observed as 0.153, 0.149, 0.113, 0.213, and 0.183 h^{-1} at glucose concentrations of 100, 200, 300, 500, and 1000 mg/L, respectively. Also, care should be taken that supply of glucose should not affect the naphthalene degradation ability of *P. putida*. This is due to the assimilation of glucose prior to naphthalene which may inhibit the production of enzymes for naphthalene degradation. A slight decrease in naphthalene degradation was observed at higher naphthalene concentration (1000 mg/L). These results clearly show that growth of bacteria was not associated with naphthalene degradation and the excessive additional carbon source decreased the metabolism of naphthalene. Considering the specific growth rates, 500 mg/L glucose concentration was selected for further studies.

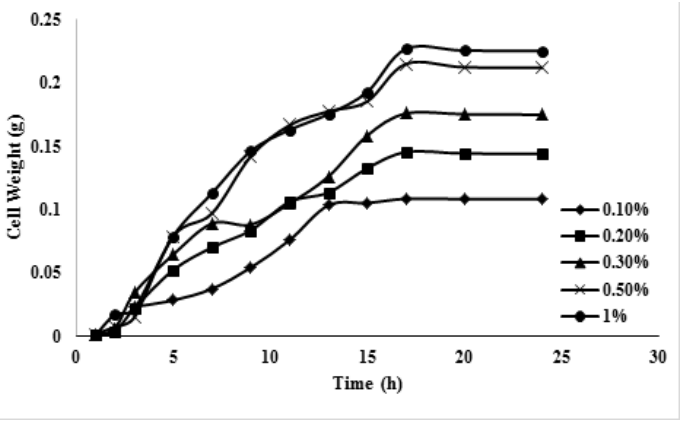

Figure 2. Influence of glucose concentration.

3.3. Influence of inoculum concentration

Several experiments were performed to optimize the inoculum concentration for the microbial growth in the presence of naphthalene and glucose. The inoculum concentrations were varied from 1% to 5%. The growth curves of *P. putida* at different concentration of inoculum are presented in Figure 3. The specific

growth rate was observed as 0.163, 0.133, 0.214, and 0.199 h^{-1} at inoculum concentrations of 1%, 2%, 3%, and 5%, respectively. Of the different inoculum concentrations examined, 3% was chosen as optimal inoculum for microbial growth and biodegradation studies. Inoculum size of the seed microbes is a major factor affecting biodegradation rates. At 1% and 2% inoculum concentration, bacterial multiplication was difficult to bring about extensive biodegradation, since other indigenous microorganisms compete for the limited source of nutrients. Previous studies (Tian et al., 2002) reported that at high cell densities, p-nitro phenol (PNP) was degraded at high rates, while at low cell densities, PNP was not degraded. Also there was a contrary that, an increase in inoculum size or the concentration of cells did not affect pyrene mineralization and could detect only a very slight increase in degradation rates (Heitkamp and Cerniglia, 1988). The 3% inoculum concentration was selected as it completely degraded naphthalene and reduced lag phase.

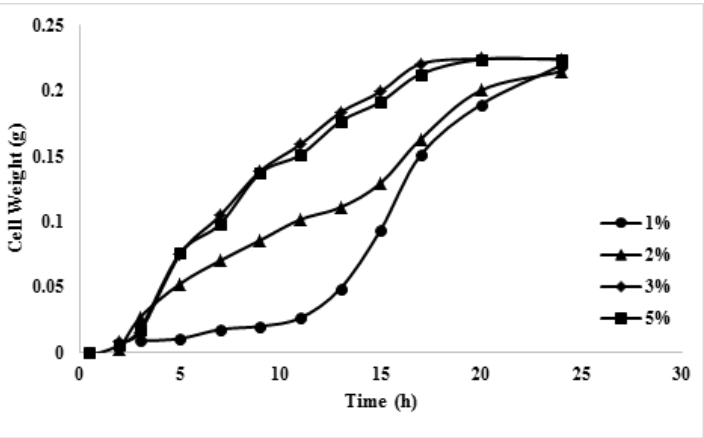

Figure 3. Influence of inoculum concentration.

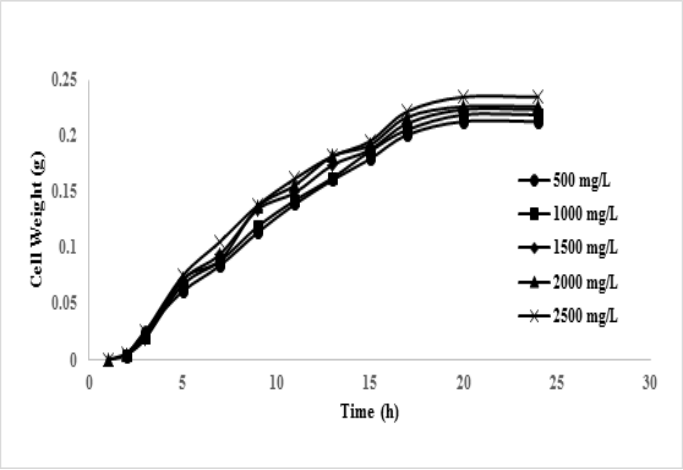

Figure 4. Growth profile of *P. putida* in the presence of naphthalene and Triton X-100.

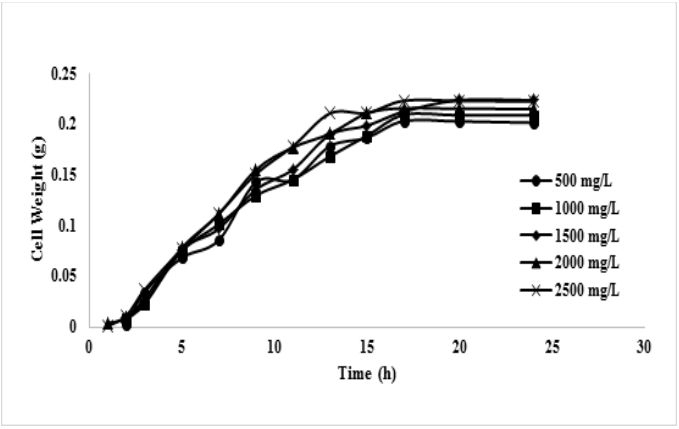

Figure 5. Growth profile of *P. putida* in the presence of naphthalene & Tween-80.

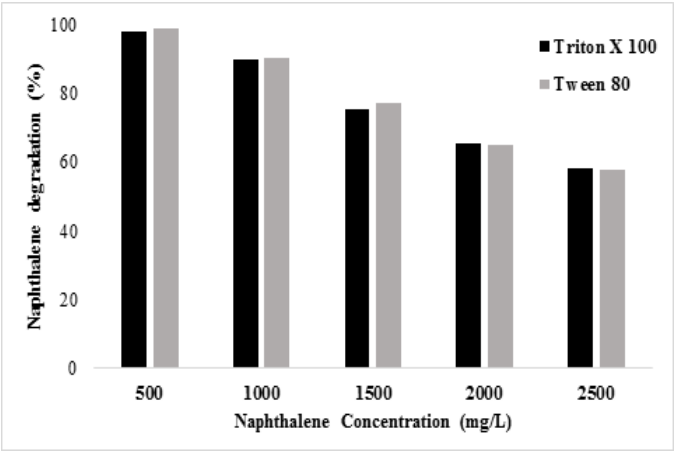

Figure 6. Naphthalene degradation (%) at different naphthalene concentrations in the presence of Triton X-100 and Tween-80.

3.4. Growth of P. putida in presence of surfactants

Surfactants are known to enhance the apparent aqueous solubility of PAHs and may thereby enhance their bioavailability. Therefore experiments were conducted at high naphthalene concentrations (500–2500 mg/L) in the presence of two nonionic surfactants, Triton X-100 and Tween-80. Both surfactants were used above their critical micelle concentration (CMC) limit. It is known that below the CMC the surfactants mainly exist as monomers and did not contribute to the solubility of the PAHs; while above the CMC, the added surfactants form the micelle (the transient aggregate of surfactant molecules), and enhanced the solubility (Kim et al., 2001). Figure 4 and 5 shows the growth curves of *P. putida* at different naphthalene concentrations in the presence of Triton X-100 (3 mM) and Tween-80 (1 mM) respectively. High concentrations of naphthalene had

significant effect on the microbial growth as a relatively high lag phase was observed at all examined conditions. Once the microbes acclimatized to the environment of high naphthalene concentrations, naphthalene was dissolved in the micelle phase and aqueous phase was rapidly biodegraded. It is also worth noting that time required for the microbes to enter stationary phase depended on the surfactant used. In the presence of Tween-80, microbes took 18 h to enter stationary phase compared to more than 20 h by Triton X-100 system. As most of the naphthalene was present in the micelle phase, the microorganism must first degrade the surfactants in order to degrade the naphthalene, i.e., dissolved in the micelle. Therefore microbes can biodegrade naphthalene in micelles that are composed of surfactant molecules. The specific growth rates of Triton X-100 were obtained as 0.169, 0.188, 0.195, 0.189, and 0.174 h^{-1} for 500 mg/L, 1000 mg/L, 1500 mg/L, 2000 mg/L and 2500 mg/L, respectively. Similarly for Tween-80, the growth rates were 0.156, 0.175, 0.182, 0.176 and 0.165 h^{-1}. The naphthalene degradation (%) in both the cases was higher in lower concentration and it reduces gradually at higher concentrations. Nevertheless, degradation was relatively high above 50% till 2500 mg/L (Figure 6). Among the two surfactants, the rate of microbial growth was fast in the case of Tween 80. The ability to tolerate and degrade very high concentrations of naphthalene makes it a potential isolate for naphthalene degradation and can be used to remediate the highly contaminated naphthalene environment. All the added naphthalene crystals were completely dissolved in the systems at the end of experiments. This clearly implies *P. putida* started consuming naphthalene as it gradually enters the micelle phase.

3.5. Growth kinetic study

To describe the substrate biodegradation, it is necessary to identify a suitable kinetic model which relates specific growth rate and naphthalene concentration. For non-inhibitory biomass growth, the Monod kinetic model has been widely reported in literature (Zhuang et al., 2002; Lee et al., 2003). Further development of Monod model has been done to include substrate inhibition because biomass growth is generally observed to be affected by substrate inhibition at high substrate concentrations (Abuhamed et al., 2004; Bai et al., 2007; Singh et al., 2008). The examined kinetic models include,

$$\mu = \frac{\mu_m S}{K_s + S + S^2/K_i} \quad (2)$$

$$\mu = \frac{\mu_m S(1+S/K)}{K_s + S + S^2/K_i} \quad (3)$$

$$\mu = \frac{\mu_m S}{K_s + S + S^2/K_i(1+S/K)} \quad (4)$$

where μ is the specific growth rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}), S is the substrate concentration (mg/L), K_i is the substrate inhibition constant (mg/L) and K is the Webb and Edward model constants (mg/L).

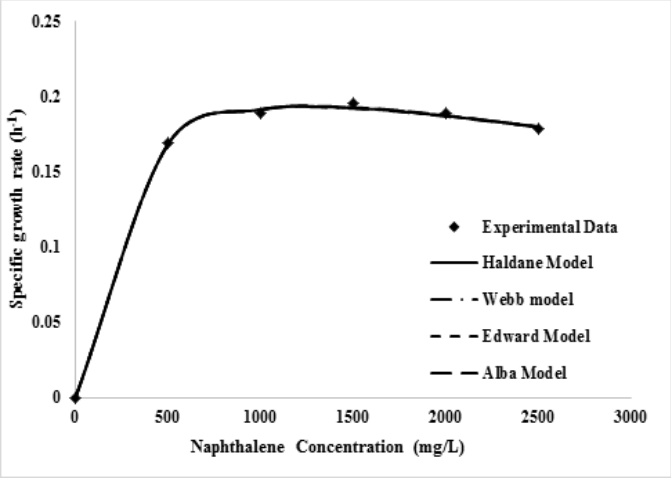

Figure 7. Kinetic plots of naphthalene biodegradation by *P. putida* (Triton X-100).

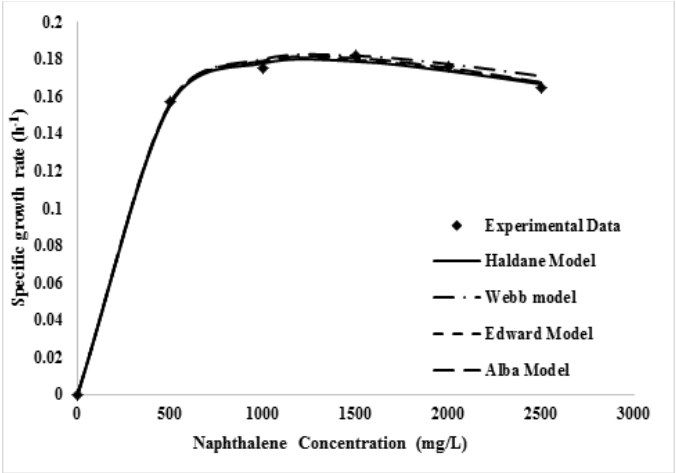

Figure 8. Kinetic plots of naphthalene biodegradation by *P. putida* (Tween 80).

Table 1. Estimated values of parameters for various kinetic models.

Models	Parameters	Triton X-100	Tween-80
Haldane	μ_m (h^{-1})	0.281	0.268
	K_s (mg/L)	290.1	313.7
	K_i (mg/L)	5623	5190
	R^2	0.99	0.998
	Error (%)	0.12	0.32
Webb	μ_m (h^{-1})	0.281	0.269
	K_s (mg/L)	291.2	314.9
	K_i (mg/L)	5460	5450
	K (mg/L)	338500	337800
	R^2	0.996	0.998
	Error (%)	0.13	0.13
Edward	μ_m (h^{-1})	0.281	0.269
	K_s (mg/L)	289.7	313.4
	K_i (mg/L)	5643	5202
	K (mg/L)	1352000	2029000
	R^2	0.996	0.998
	Error (%)	0.06	-0.09
Aiba	μ_m (h^{-1})	0.279	0.268
	K_s (mg/L)	274.3	296.4
	K_i (mg/L)	7460	6998
	R^2	0.99	0.999
	Error (%)	0.04	-0.45

These models were fitted to the experimental data using Sigma Plot non-linear regression, which uses the Marquardt-Levenberg algorithm to determine the parameters that give the best fit between the data and the model equations. The values of kinetic parameters along with correlation coefficients and percentage error values of four substrate inhibition models are presented in Table 1. In general, all models were able to describe the experimental data with very high correlation coefficients and low percentage error values. However, some models showed slight deviation in the values of bio kinetic constants (μ_m, K_s and K_i) probably due to their differences in origin of development. The maximum specific growth rate (μ_m) predicted by all four models lies in the range of 0.26–0.29 h^{-1}. The high value of μ_m indicates that substrate is degraded by microorganism more rapidly (Bailey and Ollis, 1986). The value of K_s, which indicate the affinity of biomass to substrate, lies in the range of 545–575 mg/L for Triton X-100 system; whereas varied in the range of 590-630 mg/L for Tween-80 system. The magnitude of kinetic parameter K_i indicated the inhibition tendency of the substrate. Larger K_i value indicates that biomass has a higher resistance to substrate inhibition (Jegan et al., 2010). This implies that at high value of K_i, inhibitory effect of substrate is low and substrate is less toxic toward microbial growth. Figures 7 and 8 display the curves predicted by the four substrate inhibition models for Triton X-100 and Tween-80.

4. CONCLUSIONS

From the present study on biodegradation of naphthalene, the following conclusions were suggested:

- *P. putida* showed high naphthalene degradation potential.
- The growth and naphthalene degradation potential of *P. putida* were strongly dependent on pH, glucose, and inoculum concentrations. Neutral pH (pH 7), 500 mg/L glucose concentration and 3% inoculum concentration favored maximum microbial growth and corresponding high naphthalene degradation.
- In the presence of surfactants, *P. putida* tolerated as high as 2500 mg/L of naphthalene and exhibited good naphthalene degradation. However, high naphthalene concentration affected the biodegradation ability of *P. putida*.
- High naphthalene concentrations also had serious effect on microbial growth. For Triton X-100 and Tween-80 systems, decrease in cell density was observed after 2000 mg/L of naphthalene in both the systems.
- The substrate inhibition kinetics was described using four models (Haldane, Webb, Edward and Aiba). All models were able to describe the experimental data with very high correlation coefficients and low percentage error values.
- Thus, the results obtained in the study suggested that biodegradation of naphthalene by *P. putida* appeared to be feasible method to remediate naphthalene rich contaminated sites.

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