



Anaerobic Biodegradation of Phenol: Comparative Study of Free and Immobilized Growth

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(Received: July 19, 2011; Accepted: August 15, 2011)

Abstract: Biodegradation of phenol using adapted free and immobilized mixed cultures were investigated. The culture was grown under anaerobic condition at room temperature of about 25°C and initial pH of 7.0. The initial concentration of phenol was in the range of 70 to 1000 mg/l. The culture was able to degrade phenol effectively up to 700 mg/l. The immobilized cells were able to remove phenol at concentration of 100 to 700 mg/l in a slightly shorter time period. At phenol concentration of 1000 mg/l, the removal efficiency was enhanced from 10 to about 40% in the presence of immobilized cells. The biodegradation rate of phenol improved when immobilized cells were applied. Maximum biodegradation rate happened at phenol concentration of 700 mg/l which was 2.13 and 2.65 mg/l.h for free and immobilized cells, respectively. Monod and Haldane models were used to estimate the growth kinetics. Monod model was unable to predict efficiently the growth kinetics. However, Haldane model was correlated favorably with experimental data; with a correlation coefficient of $R^2 = 0.962$. Haldane kinetic parameters, μ_m , K_s and K_i were 0.038 h⁻¹, 18.87 and 339 mg/l, respectively.

Key words: Biodegradation • Phenol • Mixed culture • Immobilization • kinetics

INTRODUCTION

Phenol and its derivatives are known as major organic pollutants. These compounds occur in the environment either naturally or artificially [1]. The main sources of phenol release into the environment are effluents of industries such as petroleum refineries, steel plants, pulp and paper, pharmaceuticals, manufacturing of synthetic chemicals, coal conversion, coke oven and etc. [2-4]. Phenol concentrations of up to 17,500 mg/l in industrial wastewaters have been reported [3]. Phenolic compounds are lethal to human being, plants, aquatic life and microorganisms even at low concentrations. Due to the high toxicity, US Environmental Protection Agency (U.S. EPA) has listed phenol as priority organic pollutant. WHO has designated a permissible concentration of 1 µg/l phenol in drinking water [5-6].

Due to the high toxicity of phenol, treatment of phenolic wastes is a necessity before discharging them to the receiving body of waters. A number of physical,

chemical and biological methods have been employed to treat phenolic wastes [7-8]. Biological treatments play a significant role in wastewater management. Organic pollutants are efficiently deteriorated in bioprocesses with cost effective and environmental friendly approaches. Also, these technologies are able to mineralize toxic hazardous contaminant [8-17]. Phenol removal via biological method is the most favored process because of the possibility of complete mineralization of phenol [18]. Biological treatments can be conducted either aerobically or anaerobically. Anaerobic biological process is the most advantageous due to the low energy requirement, low production of bio-solids and biogas production which is considered as an innovative energy source [19].

Despite substrate inhibition, many types of microorganisms are able to utilize phenol as the sole carbon and energy source. Toxic intermediates may be produced during biodegradation of phenol using pure culture. This problem may be resolved by implementation

of mixed microbial consortium which has wide spectrum of metabolic activity [20-21]. One of the important and effective techniques which has been employed for protecting the microbial population against inhibition is immobilization of biomass [3, 18]. The method has the potential of providing a unique microstructure which is suitable for growth as well as convenient separation and reutilization of biomass [22]. Gonzalez *et al.* [8, 23] applied calcium-alginate gel beads of pure strain of *Pseudomonas putida* for degrading phenol in a fluidized bed reactor (FBR). Over 98% of phenol with concentration of 250-2000 mg/l was removed by immobilized bacteria. Najafpour *et al.* [24] reported that high ethanol concentration may not inhibit ethanol fermentation of sugar in a continuous immobilized cell reactor (ICR) by *Saccharomyces cerevisiae*. Substrate and product inhibition was not apparent up to 150 g/l glucose in ICR while in batch system substantial substrate inhibition was occurred as 50 g/l glucose. Immobilization of organism in gel matrices of sodium alginate enhanced its potential to utilize concentrated substrate uptake and higher rate of ethanol formation was achieved. The free community of *Alcaligenes* sp. could degrade 99% of phenol within 32 h under aerobic condition while the same percentage of phenol removal was achieved by immobilized population of this strain within 20 h. Continuous treatment of phenolic wastewater with immobilized *Alcaligenes* sp. in an up-flow packed bed reactor resulted in 99% phenol and 92% chemical oxygen demand (COD) removal [25]. The inoculated biofilm from pig slurry on biolite® support could eliminate more than 99% of phenol and TOC (total organic carbon) in an anaerobic FBR [3].

The about studies on anaerobic phenol biodegradation by immobilized cells are scant. In the present research, the effect of immobilization of the culture on anaerobic phenol removal was investigated. A comparative study was conducted to evaluate free and immobilized cells behavior for phenol biodegradation. A mixed microbial consortium was employed to carry out the batch experiments over a wide range of phenol concentrations. The mixed culture was strictly acclimatized to phenol for a long duration. Growth kinetics parameters were defined for the best understanding of the culture tolerance in phenol uptake.

MATERIALS AND METHODS

Microorganism and Growth Medium: A mixed culture was isolated from the effluents of two industries including coke oven, Isfahan, Iran and pulp and paper, Sari, Iran

along with activated sludge collected from wastewater treatment facility of the latter. The mixed culture was employed for anaerobic biodegradation of phenol.

The culture was cultivated and enriched in 100 ml of a rich growth medium. The growth medium composed of (in mg/l): Phenol (Sharlau, Spain), 70-1000; yeast extract, 800; K_2HPO_4 , 522.54; KH_2PO_4 , 408.27; NH_4Cl , 200; $NaCl$, 200; KCl , 200; $CaCl_2 \cdot 2H_2O$, 150; $MgCl_2$, 100; $MgSO_4 \cdot 7H_2O$, 5; $FeSO_4 \cdot 7H_2O$, 10; $CoCl_2 \cdot 2H_2O$, 0.2; $NiCl_2 \cdot 2H_2O$, 0.2; $ZnCl_2$, 0.2; $CuCl_2 \cdot 2H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$, 0.2; $NaMoO_4 \cdot 2H_2O$, 0.5; H_3BO_3 , 0.2 (pH = 7.0). All chemicals were analytical grade supplied by Merck, Germany. All media was sterilized in an autoclave at 121°C and 15 psig for 20 min. The experiments were conducted at room temperature of about 25°C. The culture was strictly pre-adapted to phenol for about 5 months. During acclimation period, stepwise increase in phenol concentration from 70 to 1000 mg/l was implemented. When phenol removal efficiency of 95% was observed, microorganisms were transferred to a fresh medium containing higher phenol concentrations. Inocula concentration was about 10% (v/v).

Immobilization Protocol: The microorganisms were entrapped and immobilized in calcium-alginate gel beads. Immobilization procedure was performed by adding sterilized sodium alginate (2% w/v) mixed with cell suspension to sterilized calcium chloride solution (6% w/v). To prepare sodium alginate (SIGMA) solution, 10 g of powder was added to 500 ml of distilled water. Calcium chloride (Sharlau, Spain) solution was separately prepared by adding 60 g of anhydrous granulated form to 1 l of distilled water. A peristaltic pump and a small silicon tube with internal diameter of 1.1mm were used to immobilize cells. Spherical bio-particle beads with average diameter of 3-4mm were obtained.

Batch Biodegradation Assay: Batch experiments on phenol degradation using free cells were performed in 250 ml Erlenmeyer flasks containing 100 ml of growth medium. Phenol concentration was varied in the range of 70 to 1000 mg/l. Freshly grown seed culture was inoculated to media. Inocula concentration was 10% (v/v). The experiments were conducted under anaerobic condition by placing flasks in anaerobic jar.

To evaluate the capability of immobilized cells for phenol degradation, 100 ml of growth medium and bio-particle beads were dispensed in serum bottles. Initial phenol concentration was varied in the range of 100 to 1000 mg/l. The bottles were continuously stirred in an orbital shaker at 200 rpm and a temperature of about 25°C.

During 14 days of experiments, samples were collected, centrifuged and analyzed for biomass and remaining phenol concentrations.

Analytical Procedures: Biomass concentration was defined by measuring absorbance at 600 nm using a spectrophotometer (UNICO, 2100, USA) which was subsequently correlated to standard curve. For measuring undegraded phenol concentration, samples were centrifuged in a micro centrifuge (HERMLE Z 233 M-2, Germany) at 10000 rpm for 5 min. Direct photometric method was used for determination of undegraded phenol concentration. In this method, a red coloration resulted by rapid condensation of 4-aminoantipyrene (Fisher scientific, UK) followed by oxidation with alkaline potassium ferricyanide (Merck, Germany) can be detected by spectrophotometer. The procedure was developed in accordance to standard method [26].

Kinetic Concepts: For the best comprehension of microbial tolerance in phenol uptake, the growth kinetics for phenol biodegradation was investigated. Monod equation is a simple model which predicts cell growth on a non-inhibitory growth-limiting substrate:

$$\mu = \frac{\mu_m S}{K_s + S} \quad (1)$$

Where μ represents the specific growth rate (1/h), S is the substrate concentration (mg/l), μ_m is the maximum specific growth rate (1/h), K_s is the half-saturation substrate constant (mg/l). K_s is described by substrate concentration in which the specific growth rate (μ) is equal to half of the maximum specific growth rate (μ_m). However, this model is not capable to predict substrate inhibition. If phenol can be regarded as a non-inhibitory substrate for an acclimated culture, Monod equation may be satisfactorily used to calculate kinetic constants of the growth. In order to consider toxicity effect of the substrate, Haldane or Andrew's equation, a modification Monod model is used:

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

Which K_i is substrate inhibition coefficient (mg/l). It demonstrates cell sensitivity to substrate inhibition. Haldane model simplifies to Monod equation for very large inhibition coefficients [6]. The experimental specific

growth rates were obtained on the basis of Malthus law for various phenol concentrations:

$$\mu = \frac{dX}{X dt} \quad (3)$$

The parameter X stands for concentrations of biomass. It can be defined on the basis of either cell dry weight (mg/l) or optical density of the culture at a specific wavelength, e. g. 600 nm.

Effect of initial phenol concentration on its degradation was identified by calculating phenol biodegradation rate. The slopes of phenol degradation curves were determined as bio rates.

RESULT AND DISCUSSION

Biodegradation of Phenol by Free Cells: Figure 1 shows the profiles of phenol biodegradation by suspension of the mixed culture. Initial phenol concentration was varied in the range of 70 to 1000 mg/l.

Phenol degradation profiles with respect to time exhibited similar trends except for phenol concentration of 1000 mg/l. Since the organisms were strictly adapted to high phenol concentration, the degradation of phenol up to initial concentration of 700 mg/l was completely accomplished within short period of time. Time interval for degradation of low phenol concentrations e.g. 70 and 100 mg/l was approximately 2-3 days. As phenol concentration increased to 300 and 500 mg/l, the degradation period was slightly prolonged. Complete removal of 300 and 500 mg/l phenol was achieved in 7 and 10 days, respectively. Initial phenol concentration of 700 mg/l was entirely degraded in 12 days. Although long duration was required for the removal of high

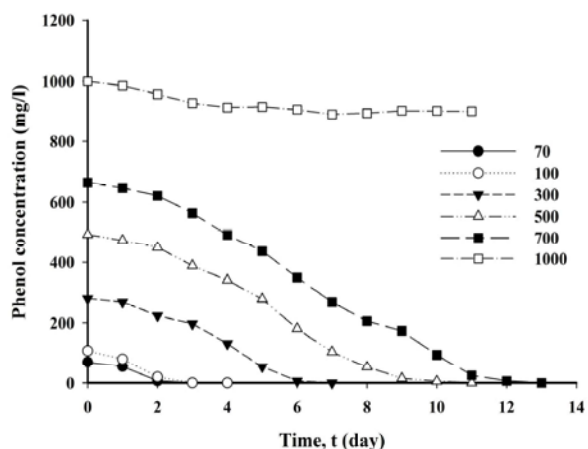


Fig. 1: Time profiles of phenol biodegradation by free cells

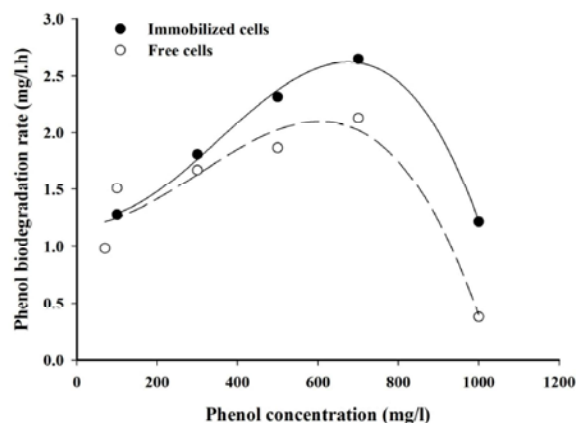


Fig. 2: Effect of the initial phenol concentrations on biodegradation rate

concentrations of phenol; the biodegradation rate increased with an increment in phenol concentration (Figure 2). Maximum biodegradation rate was about 2.13 mg/l.h, which happened at 700 mg/l phenol. The rate was declined at the concentrations of phenol below and above 700 mg/l. This indicates the effect of initial concentration of inhibitory substrate on its biodegradation rate. Though, decrease in the phenol degradation rate at initial concentration of 500 mg/l was not considerable. At high phenol concentration of 1000 mg/l, the culture was not able to degrade phenol significantly; the concentration of phenol in medium decreased to 901 mg/l during the entire period of experiment. That was due to the intensive inhibition imposed by 1000 mg/l phenol; in spite of the fact that a long acclimation period had been performed. The degradation rate at this concentration of phenol was very low (0.37 mg/l.h). For anaerobic degradation of phenol, Shinoda *et al.* [27] reported the biodegradation rate of 1.8 mg/l.h by *Azoarcus* sp. The rate was increased to 2 mg/l.h while the species of *Magnetosprilium* sp. was used for phenol degradation. Mixed methanogenic culture was able to degrade phenol with degradation rate of about 1-4 mg/l.h in the experiment conducted by Karlsson *et al.* [28]. Evidently, the anaerobic degradation rate of phenol is lower than the reported data in aerobic researches. Saravanan *et al.* [29] investigated aerobic phenol degradation with a mixed culture that resulted in phenol biodegradation rate of 10-15.7 mg/l.h. The organism *Acinetobacter* sp. was able to degrade the phenol concentration of 100-1500 mg/l aerobically with the rate of about 11-24 mg/l.h.

Figure 3 depicts the removal efficiency for phenol concentration in the range of 70-1000 mg/l. More than 96% of phenol with initial concentration of 70-700 mg/l

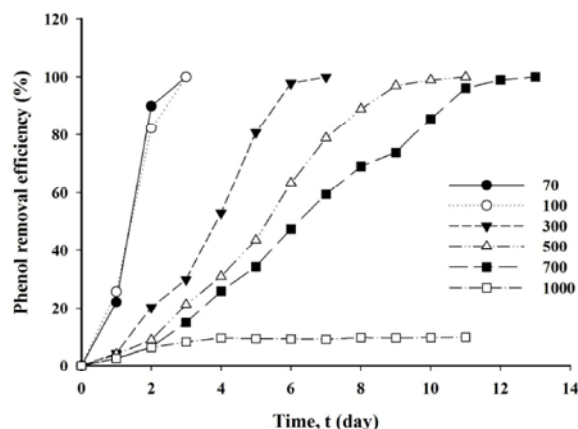


Fig. 3: Phenol removal efficiency with respect to the initial phenol concentrations using free cells

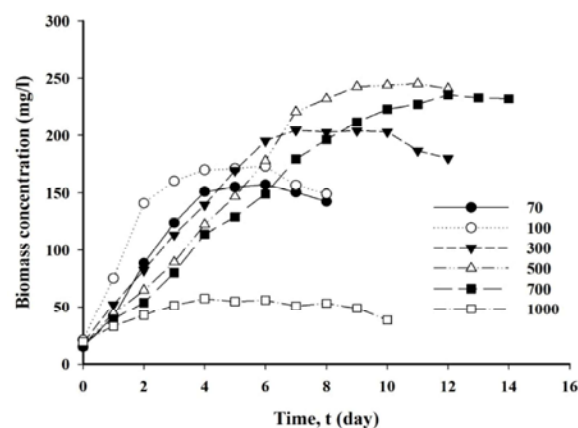


Fig. 4: Culture growth with defined initial phenol concentrations

was efficiently removed within 3 to 11 days. As initial phenol concentration increased, appropriate removal efficiencies were obtained in a prolonged durations. Further increase in phenol concentration resulted in a low removal efficiency; for instance 10% for 1000 mg/l phenol.

Growth Kinetics in Phenol Biodegradation: Culture growth curves as cell dry weight with respect to time are presented in Figure 4. Although it has been frequently reported that phenol has significant inhibitory effect on growth of microorganisms [29], for the culture growth no lag phase was observed. That may be due to the long acclimation period which was carried out for the culture in order to have potential growth on phenol and utilize it as sole carbon/energy source. Maximum biomass concentration was obtained for phenol concentration of 500 mg/l.

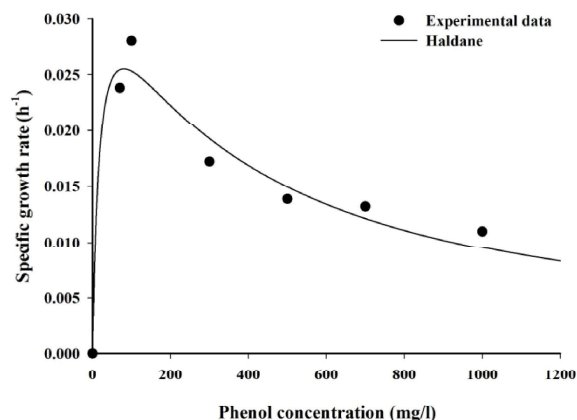


Fig. 5: Specific growth rate at various phenol concentrations

Table 1: Kinetic parameters obtained by Monod and Haldane models

Models	μ_m (h ⁻¹)	K_s (mg/l)	K_i (mg/l)	R ²
Monod	0.018	-	-	0.558
Haldane	0.038	18.87	339	0.962

Simultaneous exponential growth of the culture happened along with phenol removal. When phenol concentration drastically reduced in the media, no more growth was observed and stationary phase of the growth was reached. Phenol concentration of 500 mg/l led to the maximum biomass growth of about 243 mg/l within 9 days. Subsequently, the growth of the culture diminished as the initial concentration of phenol increased. By increasing the phenol concentration from 500 to 700 mg/l, the obtained maximum biomass concentration slightly decreased to 236 mg/l. Cell growth was severely hindered at phenol concentration of 1000 mg/l.

The specific growth rates are shown in Figure 5. The rate noticeably increased to a maximum value at phenol concentration of 100 mg/l. Further increase in phenol concentration led to an intense decrease in the specific growth rate. At high phenol concentrations, the substrate inhibition severely imposed adverse effect on the growth of the culture. The lowest value of specific growth rate (0.011 h⁻¹) was obtained at phenol concentration of 1000 mg/l.

Monod and Haldane models were fitted to the experimental data. Table 1 presents the best fit values of kinetic parameters. The equations were solved by Sigma Plot 11.0 software using a non-linear least-squares regression method. As it is evident, Monod equation was not an efficient model to predict the growth behavior of organisms which utilized phenol as the carbon source. The value of K_s could not be obtained

by this model and correlation coefficient (R²) was far from real value (0.558). That means, the model could not follow the trend of the experimental data depicted in Figure 5. Prolonging the acclimation period at high concentrations of phenol may cause this model to be able to predict the behavior of the culture for phenol biodegradation.

The substrate inhibition was figured out from the correlated Haldane equation with the experimental growth rates (R² = 0.962). Haldane kinetic parameters, μ_m , K_s and K_i were 0.038 h⁻¹, 18.87 and 339 mg/l, respectively. The maximum specific growth rate, μ_m of 0.038 h⁻¹ was low compared to the values obtained in aerobic studies. Maximum specific growth rate of about 0.3 h⁻¹ has been reported for phenol-degrading mixed culture under aerobic condition [29-30]. This approves the fact that growth is slow in anaerobic system. However, the value of μ_m in this study was higher than data reported for anaerobic phenol degradation. Bakhshi *et al.* [31] achieved μ_m of 0.031 h⁻¹ for anaerobic biodegradation of 300-1000 mg/l phenol by a pure strain of *Pseudomonas putida*. Suidan *et al.* [32] have reported μ_m of 0.027 h⁻¹ for an anaerobic mixed culture. The value of K_s and K_i are within the range of reported data in literature [29-30, 32-34]. The parameter K_s shows the low affinity of microbial population to substrate. In the present study, the value of K_s was lower than 63.9 mg/l which was attained by Bakshi *et al.* [31]; although Suidan *et al.* [32] reported very low K_s value of 0.03 mg/l. The inhibition coefficient, K_i of 339 mg/l described the relatively high inhibitory effect of phenol on the growth of the mixed culture. The value of K_i is not high enough for reducing Haldane equation to Monod model. This was well indicated by the low correlation coefficient obtained by Monod model.

Biodegradation of Phenol by Immobilized Cells:

Figure 6 depicts the phenol degradation profiles of immobilized cells for initial phenol concentrations of 100-1000 mg/l.

Adapting immobilized cells in biodegradation experiments led to slightly shorter time interval for the removal of 100-700 mg/l phenol. It was observed that phenol concentrations of 300, 500 and 700 mg/l were completely degraded within 5, 8 and 10 days, respectively. With further increase in phenol concentration to 1000 mg/l, the residual phenol in the media decreased to 692 mg/l within the time of experiment. Removal efficiency of more than 95% was obtained for initial phenol concentration of 300-700 mg/l within 5-8 days (Figure 7).

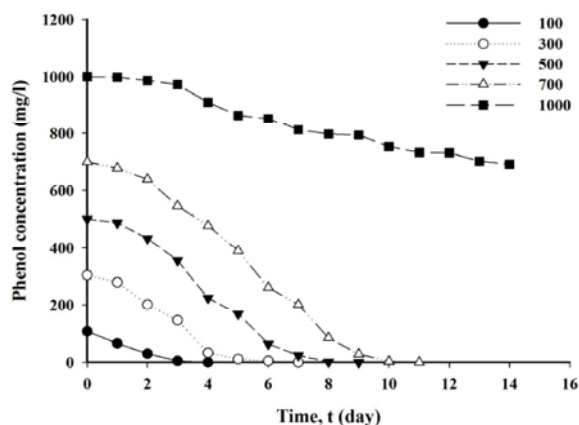


Fig. 6: Time profiles of phenol biodegradation by immobilized cells

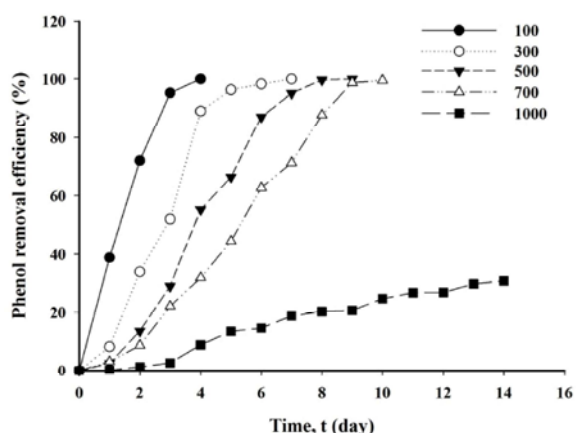


Fig. 7: Phenol removal efficiency with respect to the initial phenol concentrations using immobilized cells

For phenol concentration of 1000 mg/l, the removal efficiency in immobilized reactor increased to about 40%. It seems that the tolerance of immobilized organisms against substrate inhibition has increased. In consequence, their capacity for phenol uptake was satisfactorily improved.

For the low concentrations of phenol, the rate of degradation did not considerably promote by the immobilized cells (Figure 2). However, it was obvious that the immobilization of the cells accelerated the degradation rate at high phenol concentrations. For phenol concentration of 300 and 500 mg/l, the rate of biodegradation increased to 1.8 and 2.31mg/l.h, respectively. Maximum phenol biodegradation rate of 2.65 mg/l.h was achieved at the initial phenol concentration of 700 mg/l.

CONCLUSION

Anaerobic biodegradation of phenol for the initial concentrations of 70 to 1000 mg/l was investigated using adapted free and immobilized mixed cultures. The free cells successfully degraded phenol concentrations of 70-700 mg/l with removal efficiencies of more than 96%. Intense substrate inhibition adversely affected the biodegradation process at the phenol concentration of 1000 mg/l; the residual phenol in the medium reached to 901 mg/l within the entire time of experiment. Adaptation of immobilized cells led to slightly shorter time for complete phenol removal in the range of 100-700 mg/l. At the phenol concentrations of 1000 mg/l, immobilized culture could improve phenol removal efficiency to about 40%. The biodegradation rate was significantly enhanced at high concentrations of phenol. Due to the substrate inhibition of phenol, Monod model was incapable to predict the cell growth over the stated concentration range. Though, Haldane model was adequately correlated to the experimental data ($R^2 = 0.962$).

ACKNOWLEDGMENT

The authors are grateful for the facilities provided to carry out the present research in the Biotechnology Research Center, Babol Noshirvani University of Technology.

REFERENCE

1. Arutchelvan, V., V. Kanakasabai, S. Nagarajan and V. Muralikrishnan, 2005. Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *J. Hazardous Materials*, 127(1-3): 238-243.
2. Pishgar, R., G. Najafpour, N. Mousavi, Z. Bakhshi and T. Taghizade, 2011. Biodegradation of phenol in an anaerobic batch reactor using mixed culture in presence of supplementary substrate. in *WasteSafe* pp: 23.
3. Carbajo, J., K. Boltes and P. Leton, 2010. Treatment of phenol in an anaerobic fluidized bed reactor (AFBR): continuous and batch regime. *Biodegradation*, 21(4): 603-613.
4. Fang, H., D. Liang, T. Zhang and Y. Liu, 2006. Anaerobic treatment of phenol in wastewater under thermophilic condition. *Water Res.*, 40(3): 427-434.

5. Yan, J., W. Jianping, B. Jing, W. Daoquan and H. Zongding, 2006. Phenol biodegradation by the yeast *Candida tropicalis* in the presence of m-cresol. *Biochemical Engineering J.*, 29(3): 227-234.
6. Nuhoglu, A. and B. Yalcin, 2005. Modelling of phenol removal in a batch reactor. *Process Biochemistry*, 40(3-4): 1233-1239.
7. Carlo, R. and A. Laura, 2008. Technologies for the removal of phenol from fluid streams: a short review of recent developments. *J. Hazardous Materials*, 160(2-3): 265-288.
8. Gonzalez, G., G. Herrera, M. García and M. Pena, 2001. Biodegradation of phenolic industrial wastewater in a fluidized bed bioreactor with immobilized cells of *Pseudomonas putida*. *Bioresource technology*, 80(2): 137-142.
9. Asadi, M., G.D. Najafpour, B.A. Hashemiyeh and M. Mohammadi, 2009. Removal of Acetone from Contaminated Air in Biofilter using *Pseudomonas putida*. *American-Eurasian J. Agric. & Environ. Sci.*, 5(5): 712-719.
10. De Oliveira, L.L., R.B. Costa, D.Y. Okada, D.V. Vich, I.C.S. Duarte, E.L. Silva and M.B.A. Varesche, 2010. Anaerobic degradation of linear alkylbenzene sulfonate (LAS) in fluidized bed reactor by microbial consortia in different support materials. *Bioresource Technol.*, 101(14): 5112-5122.
11. Khodadoust, A.P., J.A. Wagner, M.T. Suidan and R.C. Brenner, 1997. Anaerobic treatment of PCP in fluidized-bed GAC bioreactors. *Water Res.*, 31(7): 1776-1786.
12. En, S. and G. Demirer, 2003. Anaerobic treatment of real textile wastewater with a fluidized bed reactor. *Water Res.*, 37(8): 1868-1878.
13. Najafpour, G., M. Komeili, M. Tajallipour and M. Asadi, 2010. Bioconversion of Cheese Whey to Methane in an Upflow Anaerobic Packed Bed Bioreactor. *Chemical and Biochemical Engineering Quarterly*, 24(1).
14. Hosseini, B., N.G. Darzi, M. Sadeghpour and M. Asadi, 2008. The effect of the sludge recycle ratio in an activated sludge system for the treatment of Amol's industrial park wastewater. *Chemical Industry and Chemical Engineering Quarterly*, 14(3): 173-180.
15. Khademi, M., G. Najafpour, B.N. Nia, A. Zinatizadeh and R.R. Kalantary, 2009. Biological Treatment of Antibiotic Plant Effluent in an UASFF Bioreactor.
16. Zinatizadeh, A., A. Mohamed, G. Najafpour, M. Hasnain Isa and H. Nasrollahzadeh, 2006. Kinetic evaluation of palm oil mill effluent digestion in a high rate up-flow anaerobic sludge fixed film bioreactor. *Process Biochemistry*, 41(5): 1038-1046.
17. Ebrahimi, A., M. Asadi and G. Najafpour, 2008. Dairy Wastewater Treatment Using Three-stage Rotating Biological Contactor (NRBC). *International J. Engineering-Transactions B: Applications*, 22(2): 107.
18. El-Naas, M., S. Al-Muhtaseb and S. Makhlof, 2009. Biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel. *J. Hazardous Materials*, 164(2-3): 720-725.
19. Saghafi, S., Z. Bakhshi, G.D. Najafpour, E. Kariminezhad and H.A. Rad, 2010. Biodegradation of Toluene and Xylene in an UAPB Bioreactor with Fixed Film of *Pseudomonas putida*.
20. Monteiro, A., R. Boaventura and A. Rodrigues, 2000. Phenol biodegradation by *Pseudomonas putida* DSM 548 in a batch reactor. *Biochemical engineering J.*, 6(1): 45-49.
21. Arutchelvan, V., V. Kanakasabai, R. Elangovan, S. Nagarajan and V. Muralikrishnan, 2006. Kinetics of high strength phenol degradation using *Bacillus brevis*. *J. Hazardous Materials*, 129(1-3): 216-222.
22. Rittmann, B. and P. McCarty, 2001. *Environmental biotechnology: principles and applications*. McGraw-Hill New York.
23. Gonzalez, G., M. Herrera, M. García and M. Pena, 2001. Biodegradation of phenol in a continuous process: comparative study of stirred tank and fluidized-bed bioreactors. *Bioresource technol.*, 76(3): 245-251.
24. Najafpour, G., H. Younesi and K. Syahidah Ku Ismail, 2004. Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. *Bioresource Technol.*, 92(3): 251-260.
25. Nair, I.C., K. Jayachandran and S. Shashidhar, 2007. Treatment of paper factory effluent using a phenol degrading *Alcaligenes sp.* under free and immobilized conditions. *Bioresource Technol.*, 98(3): 714-716.
26. Eaton, A. and M. Franson, 2005. *Standard methods for the examination of water & wastewater*. Amer Public Health Assn.
27. Shinoda, Y., Y. Sakai, M. Ue, A. Hiraishi and N. Kato, 2000. Isolation and characterization of a new denitrifying spirillum capable of anaerobic degradation of phenol. *Applied and environmental Microbiol.*, 66(4): 1286.

28. Karlsson, A., J. Ejlertsson, D. Nezirevic and B. Svensson, 1999. Degradation of phenol under meso-and thermophilic, anaerobic conditions. *Anaerobe*, 5(1): 25-35.
29. Saravanan, P., K. Pakshirajan and P. Saha, 2008. Growth kinetics of an indigenous mixed microbial consortium during phenol degradation in a batch reactor. *Bioresource Technol.*, 99(1): 205-209.
30. Bajaj, M., C. Gallert and J. Winter, 2009. Phenol degradation kinetics of an aerobic mixed culture. *Biochemical Engineering J.*, 46(2): 205-209.
31. Bakhshi, Z., G. Najafpour, E. Kariminezhad, R. Pishgar, N. Mousavi and T. Taghizade, 2011. Growth kinetic models for phenol biodegradation in a batch culture of *Pseudomonas putida* *Environmental Technology*.
32. Suidan, M.T., I.N. Najm, J.T. Pfeffer and Y.T. Wang, 1988. Anaerobic biodegradation of phenol: Inhibition kinetics and system stability. *J. Environmental Engineering*, 114(6): 1359-1376.
33. Eismann, F., P. Kuschek and U. Stottmeister, 1997. Microbial phenol degradation of organic compounds in natural systems: Temperature-inhibition relationships. *Environmental Science and Pollution Res.*, 4(4): 203-207.
34. Ho, K., B. Lin, Y. Chen and D. Lee, 2009. Biodegradation of phenol using *Corynebacterium* sp. DJ1 aerobic granules. *Bioresource Technol.*, 100(21): 5051-5055.