

IMMOBILIZATION OF PHENOL DEGRADER *PSEUDOMONAS* sp IN REPEATED BATCH CULTURE USING BIOCERAMIC AND SPONGE AS SUPPORT MATERIALS

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Abstract. The performance of two types of inert support, namely bioceramic and sponge to immobilize a locally isolated phenol degrader *Pseudomonas* sp. in a packed column was investigated in repeated batch culture. Prior to this, our study indicated that immobilization had doubled the tolerance limit of the cells towards phenol from 1000 ppm (in the suspended culture), to 2000 ppm. For the same volume, the bioceramic managed to trap bacterial cells 1.8 times greater than the sponge did. As a result, it was able to remove 100% of 1000 ppm 600-ml phenol fed at a rate of 2.5 ml/min within 24 hours, and the phenol removal capacity was sustained in the next six consecutive batches. Cells entrapped in sponge however, managed to remove around 90% phenol in five batches. Despite lower performance, at large scales, the use of sponge for cell entrapment offers some merits such as lightness, and easily available at cheaper cost.

Keywords: Immobilization, phenol, *Pseudomonas* sp, bioceramic, repeated batch

Abstrak. Prestasi dua jenis penyokong yang lengai iaitu bioseramik dan span untuk menyekat gerak bakteria pengurai fenol *Pseudomonas* sp di dalam turus terpadat telah dikaji dalam kultur kelompok berulang. Sebelum ini, kajian kami menunjukkan bahawa penyekat gerakan telah menggandakan had daya tahan mikrob terhadap kepekatan fenol dari 1000 ppm (dalam kultur ampaian) ke 2000 ppm. Pada isipadu yang sama, bioseramik berupaya memerangkap sel bakteria 1.8 kali lebih banyak daripada span. Oleh itu, ia berupaya mengurai 100% fenol yang berkepekatan 1000 ppm dalam masa 24 jam pada kadar alir suapan 2.5 ml/min, dan mengulangi prestasi yang sama dalam enam kelompok berturut-turut seterusnya. Namun demikian, sel yang terperangkap dalam span hanya berupaya mengurai 90% fenol dalam lima kelompok. Walaupun prestasinya lebih rendah, penggunaan span untuk memerangkap sel dalam skala yang besar memberikan beberapa kebaikan seperti ringan dan senang diperoleh pada harga yang lebih murah.

Kata kunci: Tersekat gerak, fenol, *Pseudomonas* sp, bioseramik, kultur kelompok berulang

1.0 INTRODUCTION

Phenolic compounds are well-known components in a wide spectrum of wastewaters including those from steel industries, coal conversion processes, coking plant, petroleum refineries, pharmaceutical and textile [1-3]. The accumulation of these compounds has resulted in environmental contamination and contributing to many deleterious effects on living systems.

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Various methods of treatment are available for the removal of phenol from wastewaters such as chlorination, ozonation, adsorption, solvent extraction flocculation, and biological treatment. Currently, biological method has been widely used as a low-cost alternative and offered the possibility of complete mineralization of organic compounds [4].

Many aerobic bacteria are capable of using aromatic compounds as their sole carbon source and energy [2]. The biological degradation is accomplished through benzene ring cleavage mediated by intracellular enzymatic reaction [5]. The microorganisms normally used in phenol biodegradation include *Pseudomonas* sp. [1, 5-7], *Candida tropicalis* [8] *Azotobacter* sp. [9], *Rhodococcus* sp. [1], *Alcaligenes* sp. [10] and *Acinetobacter* sp. [11].

The choice of initial phenol concentration in phenol removal system is of great concern [2, 8, 12]. At certain level of concentration, phenol has an inhibitory effect on microbial growth [13] and may cause cellular lysis [14]. Immobilization of microorganisms was carried out to protect the microorganisms from being damaged as well as to maintain continuous cell growth and phenol degradation [12]. Thus, the maximum tolerance level of our isolated microbe was investigated in both suspended cell and immobilized system.

Immobilization of microorganisms on inert supports was widely used in bioprocess wastewater treatment. The system could provide several advantages over freely suspended cells such as simple reuse of the biomass, production of high cell concentration, protection of cells against phenol toxicity, easier liquid-solid separation and minimal clogging in continuous-flow systems [1, 15]. Various materials have been used as matrix for immobilization of cells such as Calcium alginate [12], chitin and cellulose derivatives [16], polyurethane foam [17] and nylon sponge [18], ceramic [1] and silica-based particle [7]. Sponge and Bioceramic were chosen as support or carrier in this study due to their porous nature. In addition, sponge is easily available and cheap while bioceramic is resistant to organic solvents and is of high mechanical strength. Their ability to trap the cells, and hence enhance phenol degradation was evaluated in this study.

2.0 MATERIALS AND METHODS

2.1 Media and Growth Condition

Pseudomonas sp. was isolated from a local residential wastewater treatment plant, periodically sub-cultured on nutrient agar (NA), and maintained at 4°C. The bacteria was grown on Ramsay medium supplemented with NH_4NO_3 , 2.0g/l; KH_2PO_4 , 0.5 g/l; K_2HPO_4 , 1.0 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g/l; KCl, 0.1 g/l; and yeast extract, 0.06 g/l [19]. The medium was autoclaved at 121°C for 15 min. 0.3 g/l phenol was separately sterilized by membrane filter (Whatman, 0.2 μm) before aseptically added into sterile Ramsay medium. The culture was grown on 140 rpm of

shaker (Infors AG, Switzerland) at 30°C and was harvested at exponential phase which occurred between 15 to 18 hours of cultivation. Cell density was estimated by optical density measurement at 600 nm using UV- spectrophotometer [1]. Based on the dry weight calibration curve, 1 unit of an optical density corresponded to approximately 1 g/L of cell. Cell dry weight calibration curve was prepared by plotting the graph of optical density versus cell dry weight.

2.2 Standard Curve for Phenol

Phenol was calorimetrically assayed by using Folin-Ciocalteu reagent [20]. Samples containing 2 ml phenol were added with 0.3 ml of 200g/l sodium carbonate to give an alkaline condition and 0.1 ml of Folin-Ciocalteu reagent. The mixture was incubated for 60 minutes at 30°C to allow complete reaction and adsorbance was measured at 750 nm. A standard calibration curve was prepared with concentration ranging from 0 - 0.01 g/l to determine the phenol concentration of sample.

2.3 Suspended Cell Cultivation

Active culture of *Pseudomonas* sp. taken at late log phase (10 % v/v) was used to inoculate 100-ml of Ramsay medium in 250 ml-shake flasks. Each flask was supplemented with different phenol concentration ranging from 200-2500 ppm. The medium was placed on a shaker at 140 rpm and incubated at 30°C until phenol was completely depleted.

2.4 Packed Reactor Set Up

The full grown culture was first inoculated into a fresh Ramsay medium (10 %, v/v) and circulated using a peristaltic pump through a 7 cm internal diameter and 40 cm length packed column with approximately 850 cm³ support. The support was a crushed bioceramic (500 g) that was made of hwang-to and oyster shells (Haejoong, Korea) or synthetic sponge 1 × 1 × 1 cm cubes (5 g). The batch degradation was performed by passing 600-ml phenol at constant rate of 2.5 ml/min through the column. This gave a total of six passes in 24 hours. The concentration of phenol tested was varied from 250-2000 ppm to study the microbe's tolerance level. In batch degradation studies, the whole experiment was repeated with fresh 600-ml 1000 ppm phenol solution. The experimental set up is illustrated in Figure 1.

3.0 RESULTS AND DISCUSSION

The tolerance limits towards a series of phenol levels of *Pseudomonas* sp was firstly compared between the suspended and immobilized batch cultures. The aim was to benchmark the maximum tolerance level of our isolate with the published data

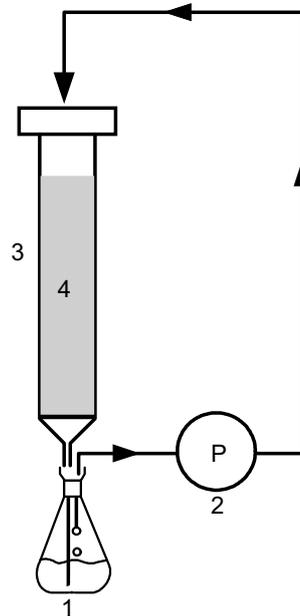


Figure 1 Experimental set up (1) Feed reservoir, (2) Peristaltic pump, (3) Packed column, (4) Support

(Table 1). Secondly, the performance of two inert supports, namely bioceramic and sponge were investigated in repeated batch cultures. The experiment aimed at comparing the performance of the supports to entrap and provide a conducive environment that favor phenol degradation by *Pseudomonas* sp.

The ability of microorganisms in degrading phenol was limited by its sensitivity to phenol toxicity. Phenol has an inhibitory effect on microbial growth at a certain level of concentration [13] and can possibly cause cellular lysis [14]. Investigation on

Table 1 Reported studies on maximum phenol concentrations in batch cultures

Author	Microorganism	System	Max concentration (ppm)
This study (2006)	<i>Pseudomonas</i> sp	Imm cell	2000
		Free cell	1000
Gonzalez <i>et al.</i> [23]	<i>Pseudomonas putida</i>	Imm. cell	2000
Chen <i>et al.</i> [8]	<i>Candida tropicalis</i>	Imm. cell	1500
		Free cell	1000
Hannaford and Kuek [2]	<i>Pseudomonas putida</i>	Imm. cell	1200
Prieto <i>et al.</i> [1]	<i>Rhodococcus erythropolis</i>	Imm. cell	1000
Kumaran and Paruchuri [24]	<i>Acinetobacter</i> sp	Free cell	500
Okaygun <i>et al.</i> [25]	<i>Klebsiella</i> sp	Free cell	230

varying initial concentration on the degradation kinetic may determine the microbe's final tolerance level. The exposure on toxic wastes in microorganism's habitat nature strongly influences their tolerance on phenol. Suspended cell and immobilized cell cultures were employed to investigate the final tolerance level of the microbe. The range of concentrations tested was 250 to 2000 ppm. The suspended cell system was carried out in shake flasks while immobilization system in packed reactor using bioceramic as support material. Results in Figure 2 show that complete phenol removal was witnessed between 250-1000 ppm in both cultures. Nevertheless, the degradation activity by the suspended culture was completely inhibited above 1000 ppm concentration. Substrate inhibition is a characteristic of toxic substrate metabolism that could inhibit related metabolism of degradation. The toxicity exerted by high concentration causes loss of cytoplasmic membrane integrity [21]. This finally resulted in disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function, and subsequent cell death.

Meanwhile, the immobilized cultures in this study showed excellent phenol degradation even at 2000 ppm. This is probably due to the protective role of the support material against the toxicity of phenol [8]. In addition, the microorganisms were resistant to washout and phenol toxicity because they were held on a stationary carrier surface [22]. However, the degradation activities were reduced beyond 60 hours of cultivation (Figure 2) due to phenol toxicity at higher concentrations. Although the rate of phenol degradation in immobilized culture at 2000 ppm initial phenol concentration significantly declined, we predict that degradation activity would still occur beyond this concentration.

Comparison of degradation rate (ppm phenol/hour) between suspended and immobilized cultures may be clearly seen through a histogram as demonstrated in Figure 3. The degradation rate is defined based on the relationship between the rate

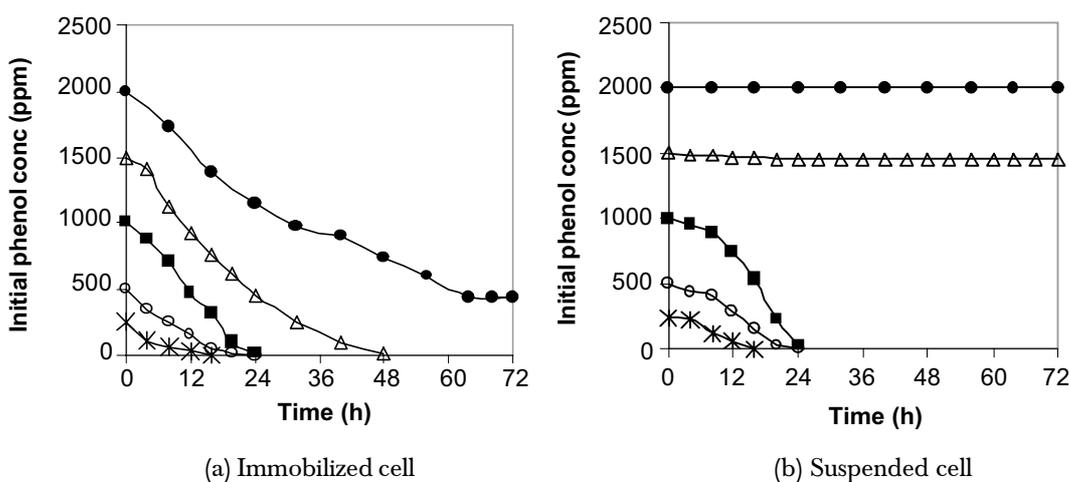


Figure 2 Phenol residues in liquid medium

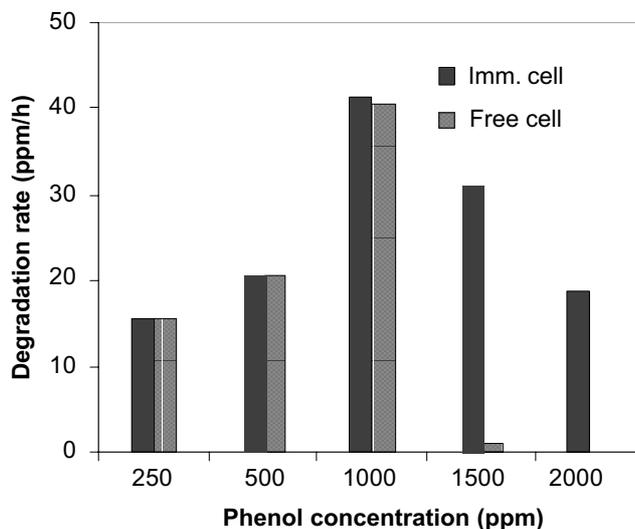


Figure 3 Phenol degradation rate at different initial phenol concentrations

of substrate consumption and the biomass growth. It is clearly shown that immobilization has extended the volumetric degradation rate limit of the microbe. Data in Table 1 summarizes the published reports on phenol degradation. In general, the immobilized cultures demonstrated higher tolerance to phenol compared to suspended cultures. Most importantly, the outcome of this study has indicated that the performance of our isolated microbe exceeded most of the published data. Hence, the likelihood of employing this isolate in the pilot or real scale is potentially high. Immobilization of viable cells could provide a better environment for reaction and alter physiological features in metabolism such as enhanced enzyme induction [8]. Moreover, immobilization system also provides high biomass concentrations due to the formation of biofilm.

The support material or carrier in immobilization system may affect the phenol degradation performance. Excellent support material is characterized by its ability to trap high number of bacteria. Bioceramic and sponge were chosen as carrier due to their inert and highly porous nature. Their ability to adsorb cells was firstly investigated. The biomass was resuspended in distilled water to avoid microbial growth before it was circulated through a packed column which contained 500 g of bioceramic, or 5 g of sponge. Results in Figure 4 show that the cell concentration was gradually increasing until it reached the equilibrium state after four hours in both carriers. The results revealed that bioceramic had better adsorptive ability, i.e. 0.543 g/l cell as compared to 0.301 g/l in sponge. That means bioceramic has 1.8 times greater loading capacity than the sponge.

Their performance in batch degradation was evaluated by passing a 600-ml 1000 ppm phenol solution at a constant rate of 2.5 ml/min through a packed reactor. This gave a total of six cycles in 24 hours. The whole experiment was then repeated with

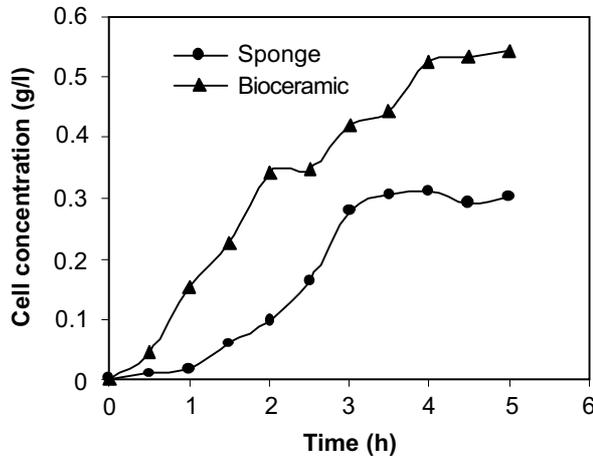


Figure 4 Concentration of cell entrapped in sponge and bioceramic

fresh 600-ml 1000 ppm phenol solution until the degradation rate started to decline. Results in Figure 5 indicated that *Pseudomonas* sp. entrapped in bioceramic resulted in 100% phenol removal up to seven consecutive batches before experiencing the first decline in the eighth batch. The experiment using sponge demonstrated 90% phenol removal in five batches in a row. The superiority of bioceramic over sponge was most probably due to the adsorptive ability of bioceramic, as discussed previously in Figure 4. Bioceramic has a lot of micro-pores that provide high surface area for cells attachment. Moreover, rapid formation of biofilm on the carrier was observed and studied by Xiangchun *et al.* [26]. It is also important to note that in large scale immobilization, despite lower removal capability and mechanically weaker, sponge is much lighter. This is extremely crucial for large scale mobility, as well as maintenance. Moreover, it is easily available at low cost.

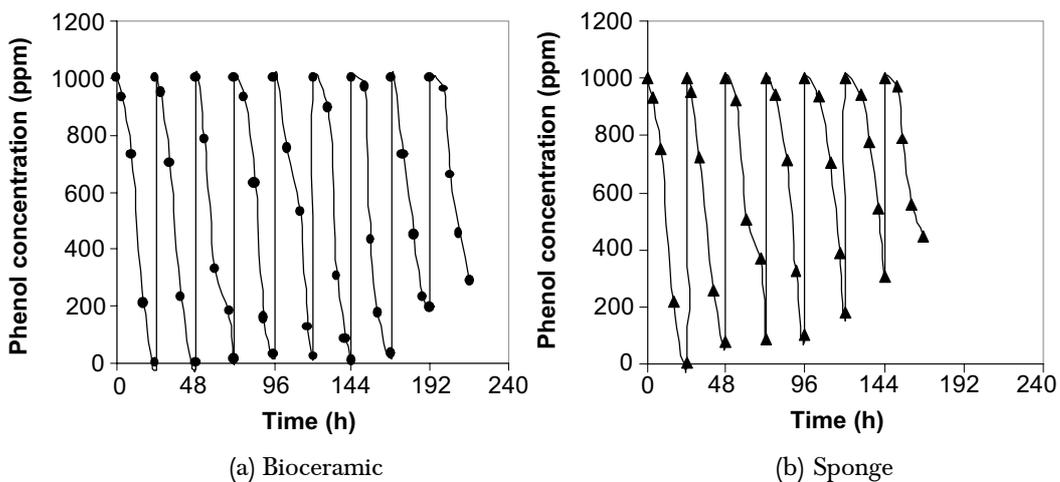


Figure 5 Phenol concentration profile in repeated-batch system

4.0 CONCLUSION

In this study, immobilization resulted in denser culture, and doubled the tolerance limit of *Pseudomonas* sp. culture towards phenol from 1000 ppm in suspended culture, to more than 2000 ppm. This also proved that locally isolated phenol degrader *Pseudomonas* sp. performed at par or somewhat better than some of other phenol degraders reported in the literature. The high surface area per unit volume of bioceramic provided loading capacity which was 1.8 times greater than that of the sponge. Culture entrapped in bioceramic managed to repeat the 100% of phenol removal in 24 hour in seven consecutive batches in comparison to only around 90% removal achieved by culture immobilized in sponge. Despite this, merits offered by sponge for large scale applications such as lightness and availability at cheap price could not be ignored.

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REFERENCES

- [1] Prieto, M. B., A. Hidalgo, J. L. Serra, and M. J. Lama. 2002. Degradation of Phenol by *Rhodococcus erythropolis* UPV-1 Immobilized on Biolite in a Packed-bed Reactor. *Journal of Biotechnology*. 97: 1-11.
- [2] Hannaford, A. M. and C. Kuek. 1999. Aerobic Batch Degradation of Phenol Using Immobilized *Pseudomonas putida*. *Journal of Industrial Microbiology and Biotechnology*. 22: 121-126.
- [3] Nuhoglu, A. and B. Yalcin. 2004. Modeling of Phenol Removal in a Batch Reactor. *Process Biochemistry*. 1-7.
- [4] Collins, L. D. and A. J. Daugulis. 1997. Characterization and Optimization of a Two-phase Partitioning Bioreactor for the Biodegradation of Phenol. *Applied Microbial Biotechnology*. 48: 18-22.
- [5] Kumar, A., K. Shashi, and K. Surendra. 2004. Biodegradation Kinetics of Phenol and Catechol Using *Pseudomonas putida* 1194. *Biochemical Engineering Journal*. 22: 151-159.
- [6] Wang, S. J. and K. C. Loh. 1999. Modelling the Role of Metabolic Intermediates in Kinetic of Phenol Biodegradation. *Enzyme and Microbial Technology*. 25: 177-184.
- [7] Nurdan, K. P. and T. Azmi. 2004. Biodegradation of Phenol by *Pseudomonas putida* Immobilized on Activated Pumice Particles. *Process Biochemistry*. 48: 1807-1814.
- [8] Chen, K. C., Y. H. Lin, W. H. Chen, and Y. C. Liu. 2002. Degradation of Phenol by PAA-immobilized *Candida tropicalis*. *Enzyme and Microbial Technology*. 31: 490-497.
- [9] Hughes, E. J. and R. C. Bayly. 1983. Control of Catechol *meta* Cleavage Pathway in *Ralstonia eutropha*. *Journal Bacteriology*. 154: 1363-1370.
- [10] Valenzuela, J., U. Bumann, R. Cespedes, L. Padila, and B. Gonzalez. 1997. Degradation of Chlorophenols by *Alcaligenes eutrophus* JMP134 (pJP4) in Bleached Kraft Mill Effluent. *Applied Environment Microbiology*. 63: 227-32.
- [11] Hao, O. J., M. H. Kim, E. A. Seager, and H. Kim. 2002. Kinetics of Phenol and Chlorophenol Utilization by *Acinetobacter* Species. *Chemosphere*. 46: 797-807.
- [12] Chung, T. P., H. Y. Tseng, and R. S. Juang. 2003. Mass Transfer Effect and Intermediate Detection for Phenol Degradation in Immobilized *Pseudomonas putida* Systems. *Process Biochemistry*. 38: 1497-1507.

- [13] Monteiro, A. M. G., A. R. Boaventura, and A. E. Rodrigues. 2000. Phenol Biodegradation by *Pseudomonas putida* DSM 548 in a Batch Reactor. *Biochemistry Engineering Journal*. 6: 45-49.
- [14] Ruiz-Ordaz, N., J. Ruiz-Lagunez, J. H. Castanon-Gonzales, E. Hernandez-Mansamo, E. Cristiani-Urbina, and J. Galindez-mayer. 2001. Phenol Biodegradation Using a Repeated Batch Culture of *Candida tropicalis* in a Multistage Bubble Column. *Revista Latinoamericana de Microbiology*. 43: 19-25.
- [15] Couto, S. R., M. A. Sanroman, D. Hofer, and D. M. Gubitz. 2004. Stainless Steel Sponge: a Novel Carrier for the Immobilization of the White-rot Fungus *Trametes hirsuta* for Decolourization of Textile Dyes. *Bioresource Technology*. 95: 67-72
- [16] Arica, M. Y., F. A. Sharif, N. G. Alaeddinoglu, N. Hasirci, and V. Hasirci. 1993. Covalent Entrapment of *Aspergillus niger* on pHEMA Membrane: Application to Continuous-flow Reactors. *Journal Chemical Technology Biotechnology*. 58: 281-285.
- [17] Nakamura, Y., T. Sawada, M. G. Sungusi, F. Kobayashi, M. Kuwahara, and H. Ito. 1997. Lignin Peroxidase Production by *Phanerochaete chrysosporium*. *Journal Chemical Engineering*. 30: 1-6.
- [18] Haapala, A. and S. Linko. 1993. Production of *Phanerochaete chrysosporium* Lignin Peroxidase under Various Culture Conditions. *Applied Microbiology Biotechnology*. 40: 494-498.
- [19] Ramsay, B. A, D. G. Cooper, A. Margaritis, and J. E. Zajic. 1983. *Rhodochorous* Bacteria: Biosurfactant Production and Demulsifying. *Microbial Enhanced Oil Recovery*. 61-65.
- [20] Box, J. D. 1981. Investigation of the Folin-ciocalteau Phenol Peagent for the Determination of Polyphenolic Substances in Natural Waters. *Water Research*. 17: 511-524.
- [21] Keweloh, H., G. Weyrauch, and H. J. Heipieper. 1990. Phenol Induced Membrane Changes in Free and Immobilized *Escherichia coli*. *Applied Microbial Biotechnology*. 33: 66-71.
- [22] Kim, J. H., K. K. Oh, S. T. Lee, S. W. Kim, and S. I. Hong. 2001. Biodegradation of Phenol and Chlorophenols with Defined Mixed Culture in Shake-flasks and a Packed Bed Reactor. *Process Biochemistry*. 37: 1367-1373.
- [23] Gonzalez, G., G. Herrera, M. T. Garcia, and M. Pena. 2001. Biodegradation of Phenolic Industrial Wastewater in a Fluidized Bed Bioreactor with Immobilized Cells of *Pseudomonas putida*. *Bioresource Technology*. 80: 137-142.
- [24] Kumaran, P. and Y. L. Paruchuri. 1997. Kinetics of Phenol Biotransformation. *Water Resource*. 31: 11-22.
- [25] Okaygun, M. S., L. A. Green, and A. Akgerman. 1992. Effects of Consecutive Pulsing of an Inhibitory Substrate on Biodegradation Kinetics. *Environment Science Technology*. 26: 1746-1752.
- [26] Xiangchun, Q., S. Hanchang, Z. Yongming, W. Jianlong, and Y. Qian. 2003. Biodegradation of 2,4-dichlorophenol in an Air-lift Honeycomb-like Ceramic Reactor. *Process Biochemistry*. 38: 1545-1551.