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Biodegradation kinetics of *o*-cresol by *Pseudomonas putida* DSM 548 (pJP4) and *o*-cresol removal in a batch-recirculation bioreactor system

Yasin Kaymaz[1] · Anıl Babaoğlu[1] · Nurdan Kasikara Pazarlioglu[1] ✉

1 Ege University, Faculty of Science, Biochemistry Department, Bornova-Izmir, Turkey

✉ Corresponding author: nurdan.pazarlioglu@ege.edu.tr
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Abstract The biodegradation kinetics of *o*-cresol was examined by acclimatized *P. putida* DSM 548 (pJP4) in batch experiments at varying initial *o*-cresol concentrations (from 50 to 500 mg/L). The kinetic parameters of *o*-cresol aerobic biodegradation were estimated by using the Haldane substrate inhibition equation. The biodegradation kinetics of *o*-cresol was investigated. In batch culture reactors, the Maximum specific growth rate (μ_{max}), Monod constant (K_s) and the inhibition constant (K_i) were established as 0.519 h^{-1}, 223.84 mg/L and 130.883 mg/L, respectively. *o*-cresol biodegradation in a batch-recirculation bioreactor system by immobilized *P. putida* was also studied. The recycled packed bed reactor system, which was composed of Ca-alginate beads and pumice on which cells immobilized, has been performed to determine possible stability for further developments.

Keywords: biodegradation, cell immobilization, *o*-cresol, *Pseudomonas putida,* substrate inhibition kinetics

INTRODUCTION

An enormous amount of organic compounds are released into the waste stream of various industries. Among them, the cresols are highly toxic compounds. *O*-cresol is an isomeric phenol with methyl substituent in the orto position relative to the hydroxyl group. *O*-cresol has a wide variety of usages comprising as disinfectants, fumigants in dyes and odors, in photographic developers, in pesticides which are used extensively in agricultural methods, etc. (UNEP, 2006).

Several animal studies suggest that cresols may promote tumor growth. United States Environmental Protection Agency (USEPA) has classified cresols within the C: Possible human carcinogens (USEPA, 2010). In addition to being highly toxic and potentially carcinogenic, according to the Agency for Toxic Substances and Disease Registry (ATSDR) cresols cause, even at very low concentrations, adverse effects on the nervous system, cardiovascular system, lungs, kidney and liver resulting in central nervous system depression (ATSDR, 2008).

Early pioneer studies were based on physicochemical absorption of organic pollutants (Asakawa et al. 1985). Biodegradation has rapidly become the most effective modality for complete mineralization of organic pollutants. In the natural environment, the microorganisms perform a major role in the biodegradation of toxic chemicals. Phenol is the most widely focused compound in biodegradation studies (Essam et al. 2010), even though a few of them consider cresol derivatives as a mixture of phenolic pollutants (Kar et al. 1997; Zlateva et al. 2005). Several microorganisms utilize cresol as a sole carbon source, despite of its toxicity (Maeda et al. 2005). These microorganisms may be fungi (Santos and Linardi, 2004) or algae, but the most practicable one is bacteria; especially Pseudomonas genus, because of its growth rate and efficiency, most studies on degradation of phenolic compounds have been carried out using bacteria (Kumar et al. 2004).

Although the biodegradation of phenolic wastes has been extensively studied with various types of microorganisms (El-Naas et al. 2008; Massalha et al. 2010), it is hard to find those in which present data is sufficient to illuminate the kinetic properties of potential microorganisms (Bergauer et al. 2005) and that are based on *o*-cresol as a sole carbon source (Vasu, 2008; Seo et al. 2009). In this study, we tried to demonstrate that *o*-cresol, one of the substantially toxic compounds, can be exploited by *Pseudomonas putida* which is strengthened with transformation of pJP4 from *Ralstonia eutropha*.

MATERIALS AND METHODS

Microorganism

The organism, *Pseudomonas putida* DSM 548 (pJP4), was maintained by weekly subculturing with 2% (w/v) nutrient agar slants that were stored in a refrigerator at 4ºC and the organism was activated by incubation at 28ºC, for 24 hrs. *o*-cresol biodegradation was performed by using recombinant *P. putida*.

The plasmid pJP4 was purified from *Ralstonia eutropha* JMP134 (formerly *Alcaligenes*) and the transformation of the related plasmid was executed chemically with 0.1 $MCaCl_2$ solutions in our work previously (unpublished data). *Pseudomonas putida* DSM 548 and *Ralstonia eutropha* were obtained from the DSMZ (Deuthsche Sammlung Von Mikroorganismen und Zellkulturen GmbH, Braunschweig), Germany.

Medium and culture conditions

The bacteria were grown on nutrient agar (NA), OXOID CM3. For adaptation experiments of the cells to *o*-cresol, which was also used as a sole carbon source, a simple minimal salts medium (MSM) was used (Mamma et al. 2004). Cells were grown in a synthetic mineral salt medium containing (g/L) NH_4NO_3 1.0, $(NH_4)_2SO_4$ 0.5, NaCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, KH_2PO_4 0.5, K_2HPO_4 1.5, $CaCl_2{\cdot}2H_2O$ 0.014, $FeSO_4{\cdot}7H_2O$ 0.01 and 1 ml of trace mineral solution containing (g/L) $FeSO_4{\cdot}7H_2O$ 1.0, $MnSO_4{\cdot}H_2O$ 1.0, $NaMoO_4{\cdot}2H_2O$ 0.25, H_3BO_3 0.1, $CuSO_4{\cdot}7H_2O$ 0.25, $ZnSO_4{\cdot}7H_2O$ 0.25, NH_4NO_3 0.1, $Co(NO_3)_2{\cdot}6H_2O$, 0.5, $NiSO_4{\cdot}6H_2O$ 0.01 and 5 ml conc. H_2SO_4 (final pH 6.9). The growth substrate *o*-cresol was added at various concentrations after adaptation as sole carbon source. The inoculum was prepared by transferring a cell suspension from the stock culture to 250 ml Erlenmeyer flasks containing 50 ml of the above medium. The cultures were grown on a rotary shaker (150 rev/min) at 28ºC up to 24 hrs.

O-cresol determination

O-cresol determinations were performed using a spectrometric method employing 4-aminoantipyrine (4-AAP) as a colour reagent. The method is based on the reaction between *o*-cresol and 4-AAP in the presence of ferricyanide at pH 10 to form a coloured antipyrine dye. The absorbance of the dye was measured at 505 nm (Pazarlıoğlu and Telefoncu, 2005).

Cell growth determinations

The biomass was determined turbidometrically at 560 nm and converted to dry cell weight with a standard conversion curve (Abuhamed et al. 2003).

Batch kinetic studies

The amount of *o*-cresol degraded and the kinetics of the process were studied in batch experiments at various initial concentrations of *o*-cresol. The initial *o*-cresol concentrations varied between 50 and 500 mg of cresol per liter.

Cell immobilization

Immobilization was carried out for about 3 hrs in flasks, which contained various sterilized (120ºC, 20 min) adsorbents (1 g) on a shaker at a controlled speed (60 rpm). The initial cell numbers were similar

for all adsorbents. The number of adsorbed cells of *P. putida* was estimated by determining the difference in cell numbers before and after immobilization.

Pumice particles were treated with 3 M HCl and then 0.5 M and finally washed with distilled water and dried at room temperature. Acid washed pumice particles were silanized in toluene. Pumice (10 g) was added to 40 ml of a 3-aminopropyl-triethoxysilane (γ-APTS) solution (10% (v/v)) in toluene and refluxed for 18 hrs. The support material was washed with toluene and acetone and dried in an oven. In order to prepare Zr-activated pumice particles, acid washed pumice was treated with 0.65 M $ZrOCl_2$ in 1.0 M HCl. The mixture was dried in an oven for 48 hrs at 55ºC and then washed three times with distilled water (Pazarlioğlu and Telefoncu, 2005).

Batch-recirculation bioreactor system

P. putida cells were suspended in 50 ml MSM and percolated through a bed comprised of 5.0 g of support material in a glass column (Figure 1). Figure 1 schematically shows the key components: A small packed bed of particles and a stirred tank. The cell suspension was re-circulated at 28ºC with a peristaltic pump. The oxidation of *o*-cresol occurred within the packed bed, with the addition of fresh feed and oxygen and the mixing of reagents taking place in the stirred tank.

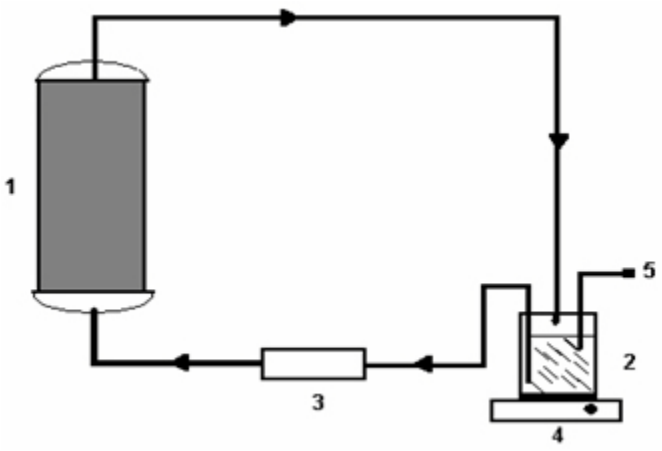

Fig. 1 Batch recirculation system operated bioreactor: (1) reactor, (2) feed reservoir, (3) peristaltic pump, (4) magnetic stirrer, (5) sampling.

RESULTS AND DISCUSSION

Adaptation of *P. putida* to *o*-cresol

The activated culture was inoculated into 50 ml of MSM which contains glucose 250 mg/L and was incubated at 28ºC, 150 rpm. After 24 hrs, cells were transferred into (glucose 225 mg/L + *O*-cresol 25 mg/L) MSM as 2% inoculate and this culture was inoculated in flasks which include gradually more *o*-cresol concentrations and also gradually less glucose concentrations in MSM and finally tertiary cultures of *P. putida* were used for the next *o*-cresol biodegradation studies.

The oxidation potential of *P. putida* is significantly dependent on the conditions of adaptation. The metabolic activity is influenced not only by the concentration of substrate during the growth of the cells, but also by the character of variations of its concentration during cultivation.

The most prominent novel accomplishment of our study is the adaptation of *P. putida* to *o*-cresol which is a highly toxic compound in order to have it utilized as a sole carbon source. This acclimatization achievement has brought our biodegradation system to perfection without any necessity of expensive

carbon sources, such as glucose. Toluene degradation pathway enzymes and monooxygenases encoded naturally by *P. putida* genome plays impressive role in adaptation process (Jahng and Wood, 1994; Parales et al. 2008). Acclimatization of the cells during the adaptation process is represented in Figure 2.

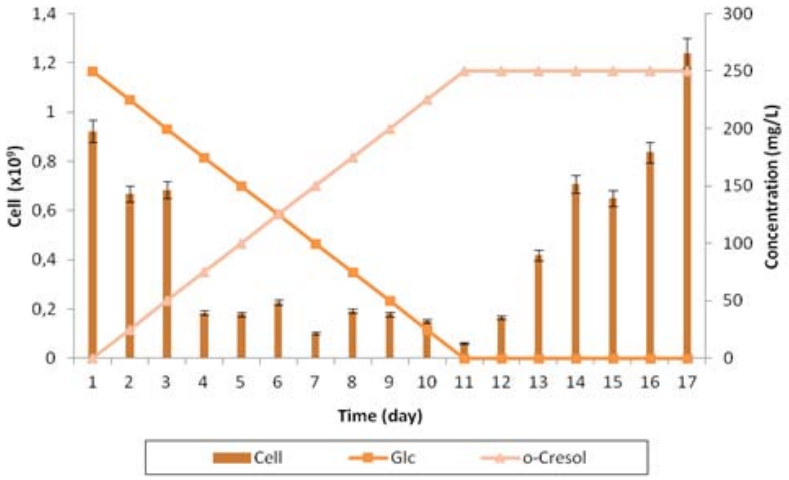

Fig. 2 Adaptation process of *P. putida.*

Cell density decreased while decreasing glucose concentrations in the first 11 days. However, cell growth gradually increased after *o*-cresol became the sole carbon source in the culture medium.

Effect of initial *o*-cresol concentration

Owing to their simplicity, shake-flask experiments were conducted to examine the effect of the initial concentration on the degradation behaviour of *o*-cresol using the bacterial strain *P. putida* 548 (pJP4) at 28ºC. The batch system consists of 50 ml of a sterile medium with *o*-cresol as the sole carbon source at various concentrations. The degradation of *o*-cresol was found to be efficient.

All of the varied initial concentrations of *o*-cresol were consumed by *P. putida* (pJP4). One of the most significant achievements that our study has is the efficiency of time needed for biodegradation process which has been developed. While some of the studies indicate that they have reached the degradation rate of 99% in 2.5 months of continuous reactor process (Perron and Wekander, 2004), we have accomplished the same degradation rate in 150 min-450 min depending on the initial *o*-cresol concentration varying from 50 mg/L to 500 mg/L as seen in Figure 3.

In all concentration levels, cells entered the stationary stage right after consuming most of the carbon source, *o*-cresol, as expected. All experiments sustained until cell growth reached a bit after the stationary phase. Specific growth rates were determined from the exponential phases when the substrate has been consumed at the fastest rate. These specific growth rates that reflect the affinity of cells to the carbon source were used in the growth kinetics estimation studies. Figure 4 shows that the cell growth at the different initial concentrations of *o*-cresol.

Growth kinetics

In order to evaluate growth kinetics, the biomass growth data from different initial *o*-cresol degradation batch experiments were plotted on a semi-logarithmic graph. The plot of specific growth rate versus the

initial substrate concentration was composed with using 'Curve Expert 1.3' software. Figure 5 shows a typical trend that the value of specific growth rate (μ) increases with the increase in initial *o*-cresol concentration up to a certain concentration level, then this rate starts decreasing with the increase in the concentration. This suggests that *o*-cresol is an inhibitory type of substrates.

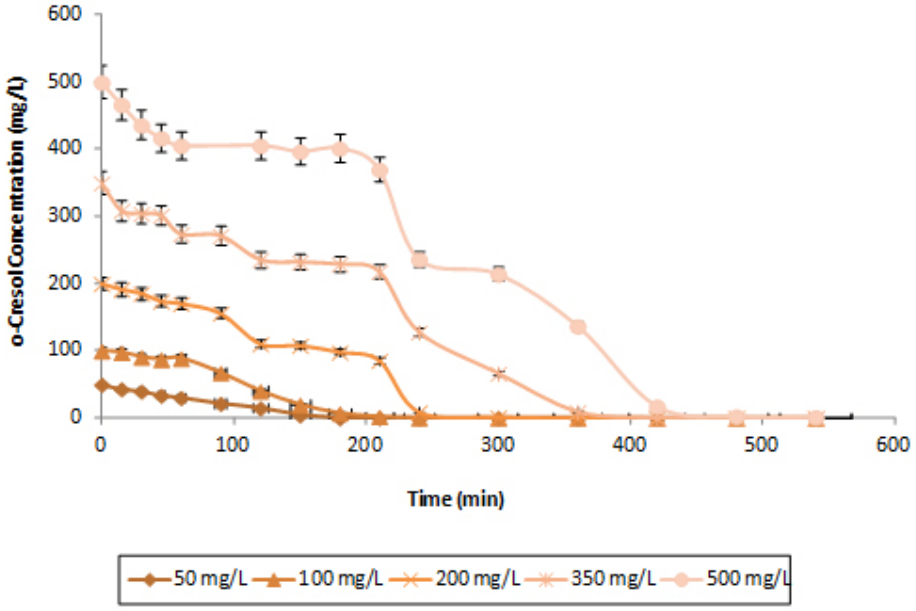

Fig. 3 Biodegradation of *o*-cresol at different initial concentrations of *o*-cresol.

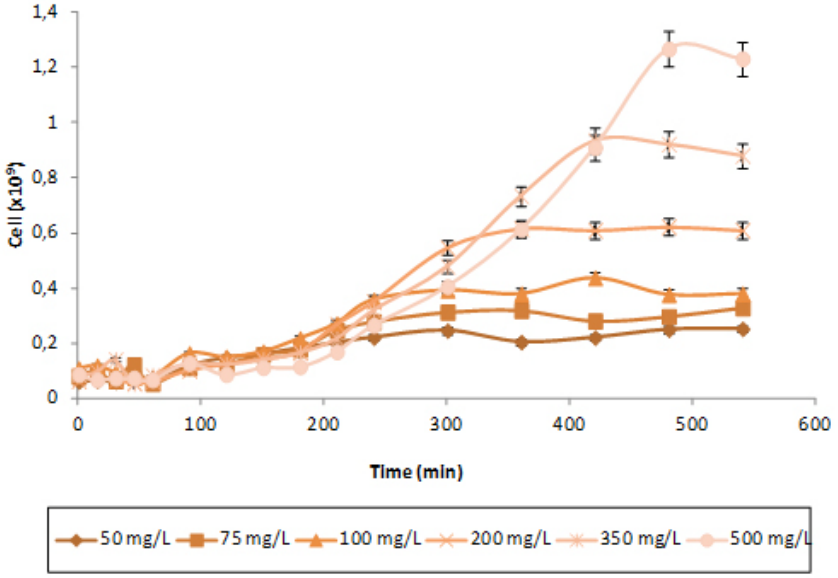

Fig. 4 Cell growth at different initial concentrations of *o*-cresol.

Substrate inhibition is a feature of phenol metabolism for different microorganisms at different concentration levels as suggested by Bajaj et al. (2009). Furthermore, Chung et al. (2003) indicated that *P. putida* cannot tolerate the toxicity of phenol at high concentrations of between 8.5-10.6 mM (800-1000 mg/L). The toxicity of phenol at high concentrations level above 1,500 mg/L could inhibit the related metabolism of degradation resulting in a lower efficiency of free cells to degrade phenol (Saravanan et al. 2009).

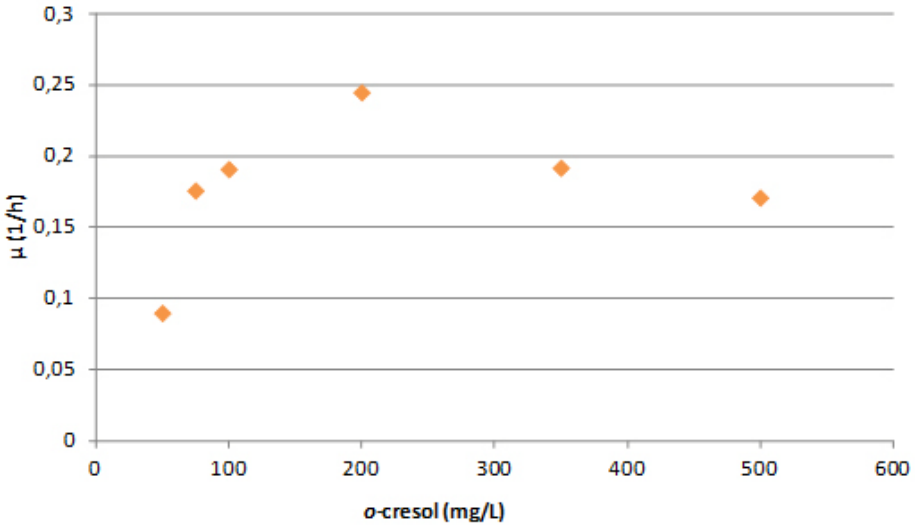

Fig. 5 Haldane's growth model fitted to results of batch growth experimental data to determine kinetic parameters.

Haldane's growth model was selected due to its mathematical simplicity and the wide acceptance for representing the growth kinetics of inhibitory substrates. The Haldane's inhibitory growth kinetics equation is shown in **Equation 1**:

$$\mu_g = \frac{\mu_{max} S}{K_s + S + (S^2 / K_i)}$$

[Equation 1]

In this expression, S is the substrate concentration; μ, the specific growth rate; μ_{max}, the maximum specific growth rate; K_S, the Monod half-saturation constant and K_i is the substrate inhibition constant. K_S is that value of the limiting nutrient concentration at which the specific growth rate is half of its maximum value. At higher substrate concentrations, $S >> K_s$, the above equation reduces to the following.

$$\mu_g = \frac{\mu_{max} S}{S + (S^2 / K_i)}$$

[Equation 2]

Specific growth rate is higher at low concentrations of *o*-cresol than at high concentrations because substrate inhibition began to appear after the *o*-cresol concentration of 200 mg/L. The values of the growth kinetics parameters obtained for *o*-cresol are given in Table 1.

Table 1. Haldane's growth kinetics parameter values for degradation of *o*-cresol using *P. putida* 548 (pJP4).

	Haldane's model		
Compound	μ_{max} (h^{-1})	K_i (mg/l)	K_s (mg/l)
o-cresol	0.519	130.883	223.84

The kinetic parameters μ_{max}, K_s, and K_i were determined as 0.519 h^{-1}, 223.84 mg/L and 130.883 mg/L, respectively. The specific growth rate that we achieved is remarkable as we compare it to the similar studies (Gallego et al. 2008). The kinetic parameters gathered from batch culture experiments indicate that *o*-cresol is preferred carbon source compared to phenol (Bajaj et al. 2009). Phenol degradation studies have revealed that Haldane constants are consistent to *o*-cresol inhibition kinetic as we show here. Information about the kinetics of *o*-cresol biodegradation is useful for optimal design and operation of aerobic biological treatment reactors.

Cell immobilization

A large variety of carriers (or supports) are used for cell immobilization. Degradation of phenol has also been performed using immobilization methods (Chen et al. 2002). This study examined the ability of carriers as support material to promote the attachment of *P. putida* under controlled culture conditions, and its adsorption of the carriers was estimated by determining the difference in cell numbers before and after immobilization. Pumice, granular silica gel, amberlite IRA-938 and exhausted perlite were used as support materials. As a comparison for absorption rates of different support materials and ca-alginate beads can be seen in Figure 6. Pumice was also activated by $ZrOCl_2$ and the cell adsorption ratio was 50% (Figure 7). Pumice was chosen as a support material rather than the amberlite because it is less expensive. The next recycled system studies were conducted on the usage of $ZrOCl_2$ activated pumice.

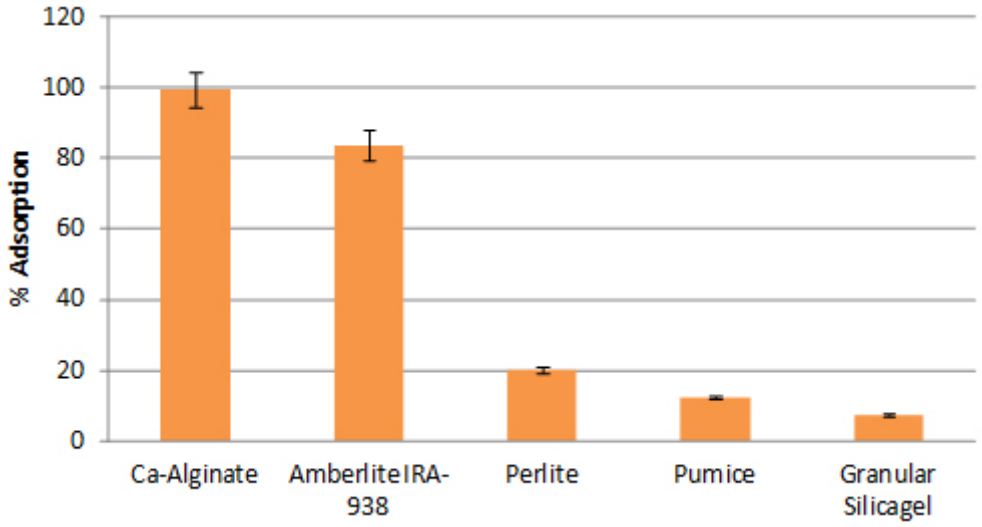

Fig. 6 Cell adsorptions of various adsorbents.

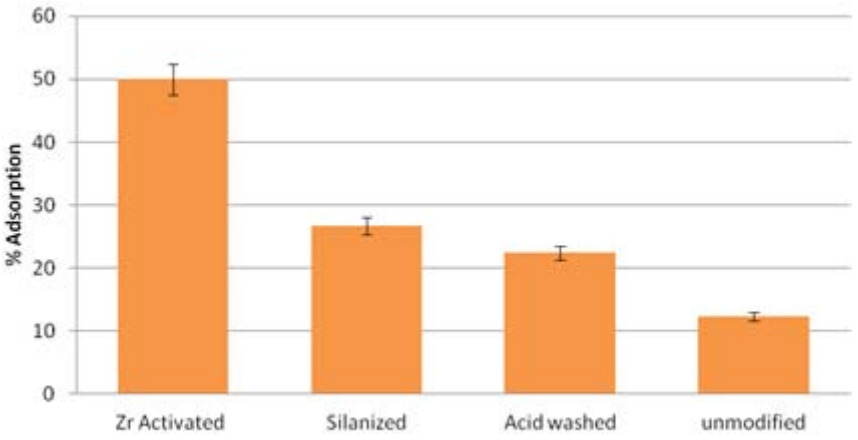

Fig. 7 Cell adsorptions of different treated pumices.

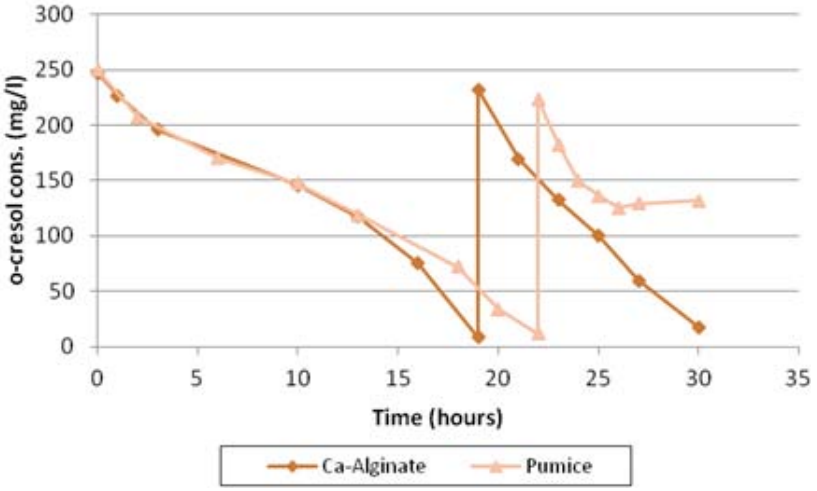

Fig. 8 *o*-cresol biodegradation comparison between packed bed reactor cycles.

Recycled packed bed reactor

The recycled packed bed reactor was examined for estimated facilities of biodegradation (Figure 8). Bioreactor operating conditions: Flow rate (F); 1.5 ml/min, Retention time; 55 sec, Carrier amount; 5.0 g, Dilution rate (D); 0.2 min^{-1}.

Continuous measurement of *o*-cresol concentration allowed us to estimate the consumption of sole carbon source. Henry's law coefficient of *o*-cresol is 0.1 Pam^3/mol and *o*-cresol essentially non-strippable (Alley, 2006). This information allows us to make an inference that the most of the consumption is because of the biodegradation. The packed bed reactor, which was composed of Ca-alginate beads in which carried immobilized cells, lasted at least two cycles as well as the other packed bed reactor with full of cell immobilized pumice particles. The degradation times of Ca-alginate and pumice systems were approximately 18 hrs and 22 hrs, respectively. After the second cycle results showed that performance of the degradation began to decrease. Substrate diffusion limitations in the

reactor might cause inadequate supply of oxygen or nutrients to the cells. This incompetent environment produced cell death. The packed bed reactor with Ca-alginate beads was more stable than pumice. Deformation of particles and detachment of cells from the surface of pumice were the main causes for this result.

CONCLUDING REMARKS

Most of the recent kinetic studies are performed by using phenol, whereas in our approach we have used *o*-cresol which is also a phenolic compound and has a very effective toxicity. The biodegradation kinetics of *o*-cresol was examined in batch experiments at varying initial *o*-cresol concentrations. At high substrate concentrations *o*-cresol was toxic to the microbial activities and as a result the *o*-cresol biodegradation. The kinetic parameters of *o*-cresol biodegradation by *P. putida* were estimated using the Haldane substrate inhibition equation.

The novel approach of our study is the elimination of costs that may arise during temperature fixation and from expensive carbon sources needed for basic cell maintenance. We provide a strong demonstration of feasibility of *Pseudomonas putida* that has been exposed to acclimatization process for biodegradation of *o*-cresol.

As a conclusion, we offer several cell immobilization options for more effective and recurrent systems as we demonstrated in a toy model. In the light of results that were gathered from our recycled packed bed reactor, large scale wastewater treatment systems can be established.

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