# MICROBIAL DEGRADATION OF CYPERMETHRIN BY IMMOBILIZED CELLS of *Pseudomonas*sp.

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## ABSTRACT

Pyrethroid pesticide cypermethrin is an environmental pollutant because of its widespread uses (cotton, fruits, vegetables), toxicity and persistence. Biodegradation of such chemicals by microorganisms may provide a cost-effective method for their detoxification. An investigation was made to assess the degradation of cypermethrin by immobilized cells of *Pseudomonas* sp. strain CYP-1in various matrices such as, polyurethane foam (PUF), polyacrylamide, sodium alginate and agar. The optimum temperature and pH for the degradation of cypermethrin by immobilized cells of *Pseudomonas*sp were found to be35°Cand 7.0 respectively. The rate of degradation of 10 and 20 mM of cypermethrin by freely suspended cells was compared with that of immobilized cells in batches and semi-continuous with shaken cultures. PUF-immobilized cells showed higher degradation of cypermethrin (10 mM and 20 mM) than freely suspended cells and cells immobilized in other matrices. The PUF-immobilized cells of *Pseudomonas*sp. strain CYP-1retain their degradation capacity. Thus, they can be reused for more than 32 cycles, without losing their degradation capacity. Hence, the PUF-immobilized cells of *pseudomonas* sp. could potentially be used in the bioremediation of cypermethrin contaminated water.

**Key Words: Degradation, Immobilization, Cypermethrin, Polyurethane Foam, *Pseudomonas* sp. Strain CYP-1.**

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## Introduction

Cypermethrin insecticides have been used to control pests in agriculture, forestry, horticulture, public health and for indoor home use for more than 20 years. Because cypermethrin were considered to be a safer alternative to other pesticides organophosphate pesticides (OPs), their applications significantly increased when the use of OPs was banned or

limited. Although cypermethrin has agricultural benefits, its widespread and continuous use is a major problem as they pollute the terrestrial and aquatic environments and affect non-target organisms. Since cypermethrin is not degraded immediately after application and because their residues are detected in soils, there is an urgent need to remediate cypermethrin-polluted environments. Various remediation technologies have been developed for this purpose; however, bioremediation, which involves bioaugmentation and/or biostimulation and is a cost-effective and eco-friendly approach, has emerged as the most advantageous method for cleaning-up pesticide-contaminated water and soils.

There are many reports of degradation of cypermethrin by the free cells of microorganisms (Roberts and Standen, 1981; Jilani and Khan, 2006; Tallur*et al*., 2008; Zhang *et al*., 2010; Lin *et al*., 2011; Chen *et al*., 2012). In addition, the degradation of cypermethrin by physicochemical methods such as oxidation with ozone, photolysis, ultrasonic degradation, Fenton degradation, incineration and adsorption has also been investigated (Segal-Rosenheimer and Dubowski, 2007; Xie *et al*.,2011). However, these methods were found to be more expensive and less-effective than biological systems used for the remediation of toxic pollutants (Yang *et al*., 2011). In nature, cypermethrin may be degraded in several ways, including hydrolysis, volatilization, photolysis, and aerobic degradation by microorganisms. There is a report on the enhanced degradation of cypermethrin by mixed microbial culture (Chen *et al*., 2012). The use of freely suspended cells for the degradation of various toxic/hazardous compounds for industrial applications has a number of disadvantages. It is mostly because of low mechanical strength, low density of cell population and the difficulty in biomass effluent separation (Massa*chuset al*., 2007; Wang and Hu, 2007). Immobilization techniques have now been well established to overcome these problems (Cassidy *et al*., 1996). Immobilized microbial cells have more advantages than freely suspended cells as well as enzymes immobilized in various matrices (Zheng *et al*., 2009). Moreover, the immobilizing materials like polyurethane foam (PUF), agar and sodium alginate are inert, nontoxic to cells, inexpensive and practical (Mulla*et al*., 2012). Use of immobilized cells permit the operation of bioreactors at flow rates that are independent of the microorganisms, tolerate higher concentrations of toxic compounds than do their non-immobilized counterparts (Westmeier*et al*., 1987; Zheng *et al*., 2009). The calcium alginate beads act like a slow release delivery system, where the substrate is slowly

released from the culture medium to the cells for microbial mineralization without a significant impact on the surrounding environment. There are several reports on the potential use of immobilized bacterial cells in different matrices for the degradation of numerous toxic/hazardous chemicals (Chen *et al*., 2002; Tallur*et al*., 2009; Ullah *et al*., 2010; Mulla *et al*., 2012; Ali*et al*., 2013; Mulla*et al*., 2013; Hoskeri*et al*., 2014). However, less information is available on biodegradation of cypermethrin by immobilized cells of microorganisms

Biodegradation, especially microbial degrading, has proven to be a suitable method for insecticide elimination. various studies indicated that microbes play important roles in degrading and detoxifying cypermethrin residues in the environment. Thus far, many reports have described the biodegradation of cypermethrin by various bacteria, including *Ochrobactrumlupini*, *Pseudomonas aeruginosa*, *Streptomyces aureus*, and *Serratia*spp.[ Chen *et al*., 2011, Zhang *et al.*, 2011, Zhang, *et al*., 2010, Lin *et al.*,2011, Toppy, *et al.*, 1991], Among the different genera of bacteria degrading pesticide, the genus *Pseudomonas* has special status to metabolize a broad range of organic compounds, environmental versatility and ability to degrade different xenobiotic compounds including permethrin (Maloney *et al*., 1988), 3-phenoxybenzoate (Chen, *et al.* (2012), β-cyfluthrin (Saikia*et al*., 2005), cypermethrin (Jilani and Khan, 2006), diazinon (Cycoń*et al*., 2009), 4-chloro-2-nitrophenol (Arora and Bae, 2014,),fenpropathrin(Song *et al*., 2015), and β-cypermethrin(Zhang *et al*., 2011).

The objectives of this study were to degradation of cypermethrin by the cells of *Pseudomonas* sp. strain CYP-1 immobilized in sodium alginate (SA), agar, polyacrylamide and polyurethane foam (PUF). The rate of degradation of cypermethrin by the cells immobilized in various matrices was compared with that of freely suspended cells of *Pseudomonas*sp. strain CYP-1 in batches and semi-continuous degradation

## Materials and Methods

### Chemicals

Cypermethrin with 98% purity was purchased from Sigma-Aldrich, Banglore(USA). Sodium alginate, acrylamide, bis-acrylamide, ammonium persulphate with 99% purity were purchased from Himedia, India. Polyurethane foam (PUF) was obtained from local suppliers. All other chemicals used in this study were of analytical grade.

**Microorganism**

*Pseudomonas* sp. strain CYP-1 previously isolated and identified in pollution control research laboratory, Department of Chemical Engineering, Annamalai University, Tamilnadu, India by cypermethrin enrichment culture technique was used in this study (shanmugananthan and mullai, 2010 ; Tallur*et al*., 2008). The organism was maintained on the slants of cypermethrin-mineral salts medium solidified with 2% agar (wt/vol).

**Medium**

Two different media were used in this study. The medium used for precultivation of *Pseudomonas* sp. strain CYP-1 was a mineral salts medium (MSM 1) containing (g $L^{-1}$) $K_2HPO_4$, 6.30; $KH_2PO_4$, 1.82; $NH_4NO_3$, 1.00; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.10; $Na_2MoO_4.2H_2O$, 0.06; $MnSO_4.H_2O$, 0.06 and $FeSO_4.7H_2O$, 0.10 (Tallur*et al*., 2008). The pH of the medium was adjusted to 7.0. One hundred ml aliquots of this medium were transferred into 500 ml Erlenmeyer flask and sterilized by autoclaving at 121 °C for 15 min. Cypermethrin (10 mM) dissolved in ethyl acetate (solvent) was sterilized by membrane filtration, added to the medium and kept on a rotary shaker for solvent evaporation. After two days, the organism was inoculated into the sterilized medium containing cypermethrin (10 mM) and this process was followed for all experiments. The medium (MSM 2) used for cypermethrin degradation studies contained (g$L^{-1}$) $K_2HPO_4$, 6.30; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.20; $NH_4NO_3$, 1.0 and $FeCl_3$, 0.05 (Tallur*et al*., 2009). The pH of the medium was adjusted to 7.0 and sterilized by autoclaving at 121$^oC$ for 15 min. Cypermethrin (10 mM and 20 mM) dissolved in ethyl acetate was added after sterilization of the medium. The cultures were incubated on a rotary shaker (150 rpm) at 30 °C. The bacterial cell concentrations were measured by the plate-count method (Mulla*et al*., 2013).

**Immobilization of whole cells in various matrices**

The cypermethrin-degrading bacteria (*Pseudomonas* sp. strain CYP-1) was harvested during the mid-logarithmic growth phase from 4 L of culture medium. The cell suspension (3 x $10^{12}$cfu/ml) was obtained by centrifugation at 8,000 x g for 10 min at 4 °C and washed thrice with 50 mM phosphate buffer pH 7.0. The washed cells were immobilized in different matrices namely; polyurethane foam (PUF), polyacrylamide, sodium alginate (SA) and agar (Mulla*et al*., 2013).

**Preparation of PUF-entrapment**: The PUF was cut into approximately 5 mm cubes, washed thrice with sterile double distilled water and dried. 100 ml of bacterial cell suspensions (3 x $10^{12}$cfu/ml) were added to 500 ml conical flasks containing sterilized foam cubes (5 g). The content of the flasks was mixed by stirring on a magnetic stirrer for 2 h and then the flasks were kept on a rotary shaker for 1 h at 150 rpm. The flasks were then allowed to stand undisturbed for an additional 4 h. The medium was removed and foam cubes containing the immobilized bacteria were washed with saline. The decanted bacterial suspension and the saline wash were combined and the bacterial population in the mixture was counted by the plate-count method.

**Preparation of sodium alginate (SA) entrapment**: Sodium alginate (4%, wt/vol) was dissolved in boiling water and autoclaved at 121 °C for 15 min. 50 mL of bacterial cell suspension (18 g wet weight/ 50 ml sterilized SA solution)was added to 200 ml sterilized sodium alginate solution (4%, wt/vol) and mixed by stirring on a magnetic stirrer. This sodium alginate cell mixture was extruded drop by drop cold, sterile $CaCl_2$ (0.2 M) solution. Gel beads of approximately 4 mm diameter were obtained. The beads were hardened by suspending in a fresh $CaCl_2$ solution for 7 h with gentle agitation and then frozen at -18 °C for 24 h. Finally, these beads were washed several times with sterile double distilled water and stored at 4 °C for further investigations.

**Preparation of polyacrylamide entrapment**: About 12 g wet cells were suspended in 10 ml distilled water and chilled in ice. To 10 ml of 0.2 M potassium phosphate buffer, (pH 7.0), 2.85 g acrylamide, 0.15 g bisacrylamide and 10 mg ammonium persulphate were added this buffer solution was mixed with the chilled cell suspension followed by the addition of 10 litre of TEMED and poured into 2 or 3 glass Petri dishes. It was then allowed for polymerization for 1 h. The sieved gels were suspended in 100 ml of 0.2 M potassium phosphate buffer pH 7.0 and allowed to settle.

**Preparation of agar entrapment:** 100 mg agar was dissolved in 4.5 ml of 0.9% (wt/vol) sodium chloride by heating at 100°C and then cooled to 40°C. Cell slurry was suspended in 0.9% (wt/vol) sodium chloride solution. 0.5 ml of the cell slurry was added to 4.5 ml of the agar solution and mixed. Immediately, the mixture was poured on a nylon net placed on a glass plate and cooled to 5°C. The membrane was stored in 0.1 M phosphate buffer, pH 7.0.

**Degradation conditions**

**Batch degradation experiments**

The batch degradation experiments were performed to evaluate the degradation of cypermethrin by both freely suspended cells and ysuspended cell cultures, 10ml of exponentially growing cells were inoculated into 90 ml of MSM 2 medium in 500 ml Erlenmeyer flasks along with the same amount of heat-killed cells as controls. The cell concentration was adjusted (3 x $10^{12}$cfu/ml) and different amounts of cypermethrin (10 and 20 mM) were added. For immobilized cells, 12 g wet beads/foam cubes of the various matrices were added to a 500 ml Erlenmeyer flask containing 100 ml of mineral salts medium (MSM 2) with 10 and 20mMofcypermethrin.

The cell counts in the immobilized matrices of the PUF, SA, polyacrylamide and agar were foundtobe1.7x$10^{12}$cfu/gfoamcubes,1.6x$10^{12}$cfu/g,1.8 x $10^{12}$cfu/g and 1.6 x $10^{12}$ cfu/g beads, respectively. Both the freely suspended cell culture and the immobilized cells of *Pseudomonas* sp. strain CYP-1 in various matrices were incubated at 30 °C on a rotary shaker (150 rpm) under identical conditions along with controls. The samples from the culture broth were withdrawn under sterile conditions at different incubation periods and analyzed for residual cypermethrin by HPLC (Metwally*et al*., 1997). The rate of degradation of cypermethrin by freely suspended and PUF-immobilized cells of *Pseudomonas*sp. strain CYP-1 at different pH (4.0-10.0) and temperature (25-45 °C) were measured after 96 h of incubation. The storage stability of both freely suspended cells and PUF immobilized cells were tested up to 60 days at 4°C.

**Semi-continuous degradation**

For establishing the longevity of degrading activity of immobilized cells in various matrices, repeated batch degradations were performed. After each cycle of incubation (96 h/cycle), the spent medium was decanted and beads/foam cubes were washed with sterile water and transferred into fresh MSM2 containing cypermethrin. The degradation process was performed under identical conditions as described above and the residual cypermethrin in the spent medium was analyzed (Metwally*et al*., 1997).

**Analytical methods**

Cypermethrin concentration in the spent medium was determined by High-Performance Liquid Chromatography (HPLC) as described in Metwally*et al*. (1997). At regular intervals, 5

mL samples were withdrawn and centrifuged at 8,000 x g for 10 min. The supernatants were extracted with ethyl acetate and the residue obtained after solvent evaporation was dissolved in methanol and used for HPLC analysis. 10µl of each residual sample of cypermethrin was analyzed by HPLC (Shimadzu, Japan) equipped with SPD-10AVP UV-Detector using shim-pack CLC-C8 column (4.6 x 150 mm) of particle size (5 m) (Phenomenex) and methanol-water (90:10, vol/vol) as mobile phase at the flow rate of 1 ml $min^{-1}$.

**Statistical analysis**

All Experiments were carried out in triplicate and their results are presented as mean standard deviations (SD).

**Results**

**Degradation of cypermethrin of free and immobilized cells of Pseudomonas sp. strain CYP-1 in batch cultures**

The results in the enhanced degradation of 10 and 20 mM of cypermethrin in batch cultures, both by freely suspended cells and immobilized cells of Pseudomonas sp. strain CYP-1 in polyurethane foam (PUF), sodium alginate, polyacrylamide and agar are shown in Figure 1and 2. With the initial concentration of 10 Mmcypertmethrin, the freely suspended cells degraded 9 mM of cypermethrin after 168 h incubation period, whereas immobilized cells degraded the same concentration within 96 h of incubation (Figure 1). But with the increased initial concentration to 20 mM, the rate of degradation freely suspended cells was decreased, whereas immobilized cells degraded the same concentration within 120 h (Figure 1). These results suggest that a higher concentration of cypermethrin was better tolerated and more rapidly degraded by immobilized cells than freely suspend cells.

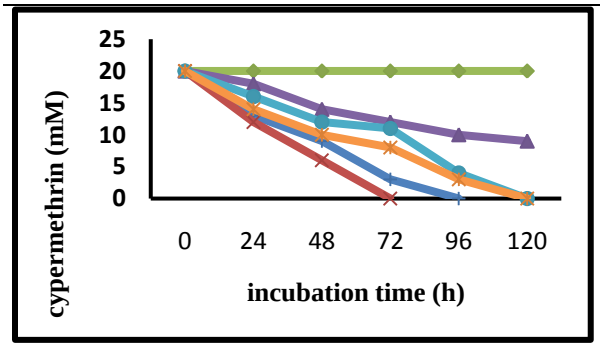


**Figure 1** - Degradation of cypermethrin at 20 mM in batch cultures by cells of *Pseudomonas*sp. strain CYP-1 immobilized on PUF, polyacrylamide, sodium alginate, agar**,** freely suspended cells and uninoculated control. Data values represent means of triplicate and error bars indicate 95% confidence intervals.

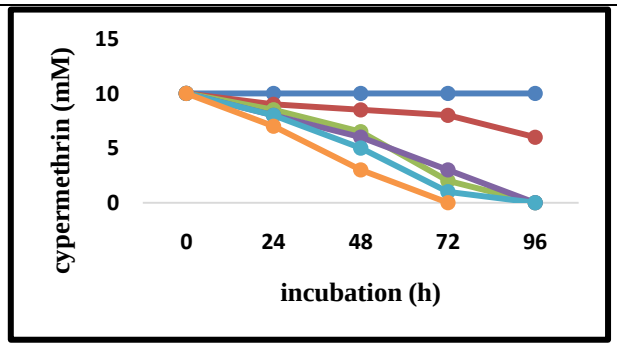


**Figure 2**- Degradation of cypermethrin at 10 mM in batch cultures by cells of *Pseudomonas*sp. strain CYP-1 immobilized on PUF, polyacrylamide, sodium alginate, agar**,** freely suspended cells and uninoculated control. Data values represent means of triplicate and error bars indicate 95% confidence intervals

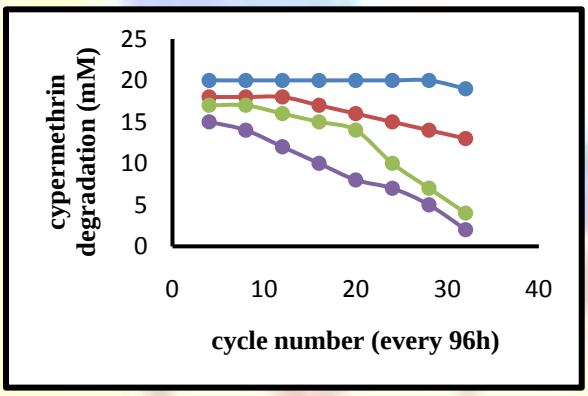


**Figure 3**- Semi-continuous degradation of cypermethrinat 20mM by cells of *Micrococcus* sp. strain CYP-1 immobilized onPUF, polyacrylamide, sodium alginate and agar. Data values represent means of triplicate and error bars indicate 95% confidence intervals.

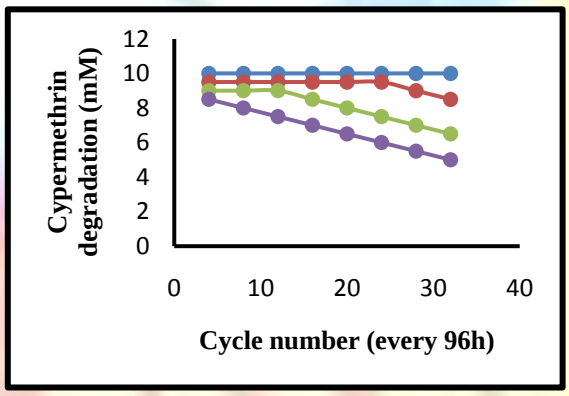


**Figure 4**- Semi-continuous degradation of cypermethrin at 10 mM by cells of *Micrococcus* sp. strain CYP-1 immobilized on, polyacrylamide, sodium alginate and agar. Data values represent means of triplicate and error bars indicate 95% confidence intervals.

**Semi-continuous degradation of cypermethrin by immobilized cells of strain CYP-1**

The results of the semi-continuous degradation of 10 and 20 Mmcypermethrin by cells of Pseudomonas sp. strain CYP-1 immobilized in PUF, alginate, polyacrylamide and agar are shown in Figure 3 and 4. The PUF-immobilized cells can be reused for up to 32 cycles without losing their ability to degrade cypermethrin at the initial concentration of 10 and 20 mM. In contrast, agar, sodium alginate and polyacrylamide immobilized cells could be reused for 16, 20 and 24 cycles, respectively (Figure 3). However, when the initial concentration of cypermethrin was increased to 20 mM, these immobilized cells could be reused with a decreased rate of degradation of cypermethrin (Figure 4). These observations suggest that a lower concentration of cypermethrin (10 mM) could be fed at a much higher frequency than the higher concentration of cypermethrin (20 mM).

**Effect of pH, temperature and storage stability on degradation capacity of PUF-immobilized cells of Pseudomonas sp. strain CYP-1**

The effect of pH on the degradation of cypermethrin by freely suspended cells and PUF immobilized cells shows that variations of the initial pH between 5.0 and 9.0 (Figure 5) had no effect on cypermethrin degradation (Figure 5). In contrast, the freely suspended cells were able to degrade cypermethrin at narrow range pH between 6.5 to 7.5. The effect of temperatures on the degradation of cypermethrin by PUF-immobilized cells showed higher activity at the temperature between 25 and 40 °C (Figure 6). Whereas the freely suspended cells showed activity at the temperaturebetween30and35°C(Figure 6).However, the optimal pH and temperature were found to be 7.0 and 35°C both for PUF-immobilized cells and freely suspended cells in the culture medium. The PUF-immobilized cells can be stored for 60 days at 4 °C without loss of its degradation capacity while the freely suspended cells lost their degrading capacity after 60 days at 4 °C (Figure 7).

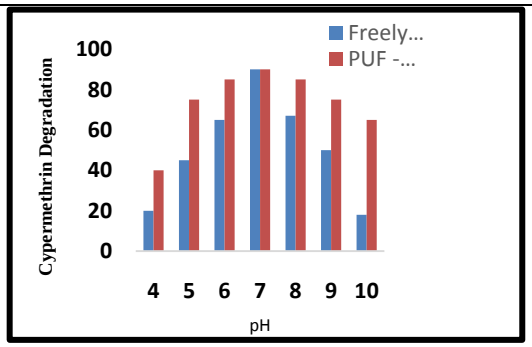


**Figure 5** - Effect of pH on the degradation of cypermethrin (10 mM) by freely suspended cells and PUF-immobilized cells of *Pseudomonas* sp. strain CYP-1. Data values represent means of triplicate and error bars indicate 95% confidence intervals.

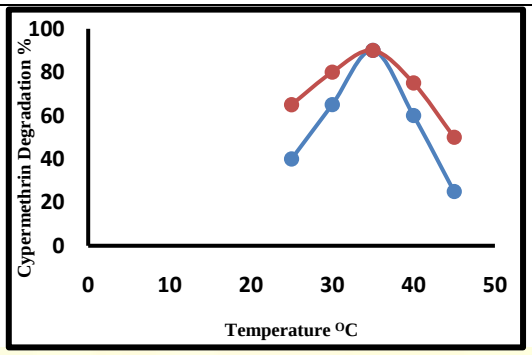


**Figure 6** - Effect of Temperature on the degradation of cypermethrin (10 mM) by freely suspended cells and PUF-immobilized cells of *Pseudomonas* sp. Strain CPN1. Data value represent averages of three replicate determinations

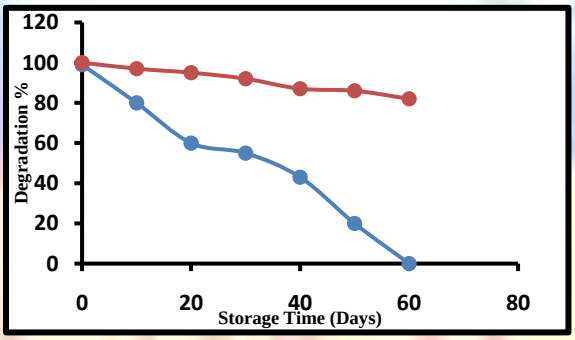


**Figure 7** - Storage stability of freely suspended cells and PUF immobilized cells of *Pseudomonas*sp. strain CYP-1 grown on cypermethrin (10 mM). Data values represent means of triplicate and error bars indicate 95% confidence intervals.

## Discussion

The degradation of cypermethrin by immobilized cells of *Pseudomonas* sp. strain CYP-1 in various matrices such as PUF, sodium alginate, polyacrylamide and agar were compared with

that of freely suspended cells in batches and semi-continuous shaken cultures. The results obtained from cells immobilized in various matrices with batch cultures suggested that the rate of degradation of cypermethrin, even at a higher concentration (20 mM), was much higher than that with freely suspended cells. The enhanced degradation of cypermethrin by immobilized cells may be due to the availability of a high density of cells in or on immobilized matrices. Immobilization of cells may also lead to a stabilization of membrane permeability and protect against the toxicity of high substrate concentration, thus leading to enhanced degradation rate (Cassidy *et al*., 1996).

The results of semi-continuous degradation suggest that the PUF and polyacrylamide immobilized cells retained the cypermethrin degradation capacity for a longer period and they could be reused for 32 and 24 cycles, respectively. When the initial concentration of cypermethrin (10 mM) was increased to 20 mM, the PUF immobilized cells could be reused without losing their degrading capacity. The immobilized cells in other matrices could also be reused but with a decreased rate of degradation at higher concentration (20 mM). The storage stability and activity of cells entrapped in PUF were better than those cells entrapped in other matrices. The alginate and agar-immobilized cells showed lower degradability of cypermethrin with increased cycle numbers. The mechanical instability and gradual cell leakage from these beads decreased the degradation rate with an increasing cycle number (Trevors *et al*., 1992; Tallur*et al*., 2009; Mulla *et al*., 2012). The PUF-immobilized cells showed more tolerance to pH and temperature changes than freely suspended cells. The advantages of PUF in chemical and physical properties compared to other matrices are its high porosity, mechanical strength, stability and adsorbing capacity. PUF is ideal support of cell growth (Romaskevic*et al*., 2006). The cells immobilized in PUF showed a better and faster degradation rate even at a higher initial concentration of substrate. These immobilized cells could be stored for longer periods without losing their degradation ability. Furthermore, the longevity of cells immobilized in PUF and their operational stability is better than those of other matrices determined.

The present study has revealed that the more effective degradation of cypermethrin at higher concentration could be achieved by immobilized cells of *Pseudomonas*sp. strain CYP-1 than freely suspended cells. The immobilized microbial system has an advantage of enhanced rate of degradation, tolerance to higher substrate concentrations and their reusability. Thus, the

immobilized microbial technology provides a highly versatile and cost-effective approach that can be used for the degradation of pesticide-contaminated wastewater.

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