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Stable Degradation of Benzoate by *Klebsiella oxytoca* C302 Immobilized in Alginate and Polyurethane

Jun-Ho Kim[1], Won-Hwa Jeong[2], T. B. Karegoudar[3], and Chi-Kyung Kim[1]*

[1] Department of Microbiology and Biotechnology, and Biotechnology Research Institute, Chungbuk National University, Cheongju 361-763, Korea
[2] R & D Center, Boryung Pharmaceutical Co., Jincheon 365-800, Korea
[3] Department of Biochemistry, Gulbarga University, Gulbarga 585-106, India

Abstract Benzoate produced from the degradative pathways of various aromatic chemicals is generally recognized as a pollutant compound. However, various bacterial strains isolated as benzoate degraders have exhibited certain limits to their functions, including a loss of viability and degradability when cultivated in a broth medium for a longer time. Accordingly, immobilization techniques have been utilized to overcome such problems, and the current study examined the use of alginate and polyurethane for immobilizing *Klebsiella oxytoca* C302 to extend its viability and degradability of benzoate. The organism was well encapsulated by both matrices and the immobilized cells showed a high stability as regards their viability and degradability of 2 mM benzoate in a MM2 broth medium during cultivation for longer than 60 h in a semicontinuous batch system.

Keywords: benzoate degradation, immobilization, alginate, polyurethane, *Klebsiella oxytoca* C302

INTRODUCTION

Benzoate can be produced by many different kinds of microorganisms through the *meta*- or *ortho*-cleavage of biphenyl and polyaromatic hydrocarbons. In addition, it has also been reported that the compound can be produced from toluene through the consecutive oxidation of methyl groups and dehydrogenation of benzylalcohol [1]. Chlorobenzoate can be produced from polychlorinated biphenyls (PCBs) by bacteria [2] and several fungal strains [3,4].

However, the benzoate and chlorobenzoate produced from biphenyl and PCBs are dead-end toxic compounds [5]. This phenomenon has also been reported in *Pseudomonas* sp. P20, which actively degrades 4-chlorobiphenyl to produce 4-chlorobenzoate [6]. As such, these kinds of aromatic hydrocarbons have been categorized as serious environmental pollutants [7].

Meanwhile, a variety of microorganisms have been isolated as potential degraders of benzoate [8,9] and 4-chlorobenzoate [10,11]. In particular, *Klebsiella oxytoca* C302 is capable of degrading benzoate to produce 4-hydroxybenzoate [8], which can be further catabolized into catechol, then protocatechuate, and finally utilized as a carbon and energy source. However, the viability and degradability of these microorganisms decrease when they are incubated as free cells in a liquid medium for longer than one day.

Therefore, to maintain the bacterial degradation of pollutant chemicals and production of useful metabolites and avoid the problems of handling and cell separation, immobilization techniques have been introduced using different encapsulation matrices, such as alginate, agar, polyacrylamide-hydroxide, and polyurethane foam [12].

Immobilized cells are extensively used in the bioremediation of toxic pollutant chemicals, including phenol [13], 4-chlorobenzoate [14], naphthalene [15], and polychlorinated biphenyls [16]. Recently, Manohar *et al.* [15] demonstrated the enhanced degradation of naphthalene by cells of *Pseudomonas* sp. strain NGK1 immobilized in alginate, agar, polyacrylamide, and polyurethane foam. They reported that free cells only degraded a maximum of 30 mM naphthalene after 4 days of incubation with 50 mM naphthalene, whereas polyurethane-immobilized cells degraded all the 50 mM initial naphthalene after 6 days of incubation. Furthermore, foam-immobilized cells were used 45 times over a period of 90 days without losing their naphthalene-degrading activity [17].

Accordingly, the current study investigated the benzoate-degrading activity of *Klebsiella oxytoca* C302 cells immobilized in alginate and polyurethane compared to that of free cells at the same concentration of 2.0 mM benzoate.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

Experiments were performed with a pure culture of

* **Corresponding author**
Tel: +82-43-261-2300 Fax: +82-43-264-6900
e-mail: environ@trut.chungbuk.ac.kr

Klebsiella oxytoca C302, isolated as a benzoate and catechol degrader [8]. The bacterium was cultivated in an MM2 broth medium containing 1 μM $FeSO_4 \cdot 7H_2O$, 1 mM $(NH_4)_2SO_4 \cdot 7H_2O$, 100 μM $CaCl_2 \cdot 2H_2O$, 8.5 mM NaCl, and a 10 mM phosphate buffer. The pH of the medium was adjusted to 7.0, as reported previously [8]. 250 mL aliquots of the medium in 500 mL Erlenmeyer flasks were autoclaved, then supplemented with 2.0 mM benzoate. The bacterial cells in the cultures were enumerated periodically during the cultivation on Luria-Bertani (LB) agar plates after incubation at 30°C for 48 h.

Cell Growth and Benzoate Degradation

The organism was grown in an MM2 broth medium containing different concentrations of benzoate at 30°C, then the cell density was measured at 660 nm during cultivation. The degradation of benzoate was examined using a growing cell assay, as described by Arensdorf and Focht [18]. Cells grown in an LB broth for 12 h at 30°C were harvested by centrifugation at 8,000 × g for 10 min. The cells were then washed twice with a 10 mM phosphate buffer (pH 7.2) and suspended in the MM2 broth. 2.0 mM benzoate was added to the suspensions, which were then incubated at 30°C for an appropriate time. The benzoate in the suspensions was examined at 225 nm using a UV-visible spectrophotometer.

Immobilization of Cells

Klebsiella oxytoca C302 cells harvested during the exponential growth phase were immobilized in either alginated or polyurethane foam, as described by Manohar *et al.* [15]. Fifty mL of the cell suspension was added to 200 mL of a sterilized alginate solution (4% w/v) and mixed on a magnetic stirrer. The mixture was extruded drop by drop into a cold sterile 0.2 M $CaCl_2$ solution.

The polyurethane foam was cut into approximately 2 mm cubes. The bacterial cell suspension was then added to a 500 mL conical flask containing the sterilized foam cubes and the contents mixed by a magnetic stirrer for 2 h. Thereafter, the foam cubes containing the immobilized cells were washed with saline.

Batch Cultivation

Batch cultivations for the degradation of 2.0 mM benzoate were performed using both freely suspended cells and cells immobilized in alginate or polyurethane foam cubes [15]. For the freely suspended cells, 100 mL of the MM2 broth in 250 mL flasks was inoculated with cells at about $10^5 \sim 10^6$ cells/mL, then 2.0 mM benzoate was added. The degradation process was carried out at 30°C on a rotary shaker at 150 rpm for the desired incubation period. After 26 h of incubation, an additional 2 mM benzoate was added and incubated under identical conditions to compare the results with the first culture.

Table 1. Degradation of benzoates and catecholic compounds by *Klebsiella oxytoca* C302

Aromatics	Degradation
Benzoate	+++
2-chlorobenzoate	+
3-chlorobenzoate	+
4-chlorobnezoate	-
2,4-dichlorobenzoate	-
3,4-dichlorobenzoate	-
Catechol	+++
2-chlorocatechol	-
3-chlorocatechol	-
4-chlorocatechol	-

For the immobilized cells, 25 g of wet beads of the respective entrapment matrices was added to 250 mL flasks containing 10 mL of the MM2 medium and 2.0 mM benzoate. The entrapped cell cultures in both matrices were incubated under the same conditions as in the freely suspended cell culture.

Semicontinuous Batch Cultivation

Repeated batch cultivations were carried out to determine the long-term stability of the *K. oxytoca* C302 cells immobilized in the different matrices as regards the degradation of 2.0 mM benzoate [17]. Every 6 h during the incubation period, the spent medium was decanted and the beads washed with sterile saline and transferred into a fresh MM2 medium containing benzoate. Then, the cultivation was continued in the same way as before.

RESULTS AND DISCUSSION

Degradation of Benzoate by *K. oxytoca* C302

Klebsiella oxytoca C302 has been previously reported to be a bacterial degrader of benzoate, catechol, 4-hydroxybenzoate, and protocatechuate [8]. The chlorinated benzoates and catechols were not degraded by strain C302, as shown in Tabel 1, even though strain C302 is known as a benzoate and catechol degrader. This means that the organism can only degrade non-halogenated monocyclic aromatic compounds using benzoate monooxygenase and catechol dioxygenase, as reported in other bacterial strains by Kim *et al.* [8] and Mohamed *et al.* [9].

The degradation of benzoate was carried out by the cells in the MM2 broth medium containing benzoate at various initial concentrations (0.5 to 4.0 mM) with incubation at 30°C. The cell growth in the medium was also measured during the benzoate degradation experiment. The results are given in Figure 1. An initial benzoate concentration of 2.0 mM or lower was completely

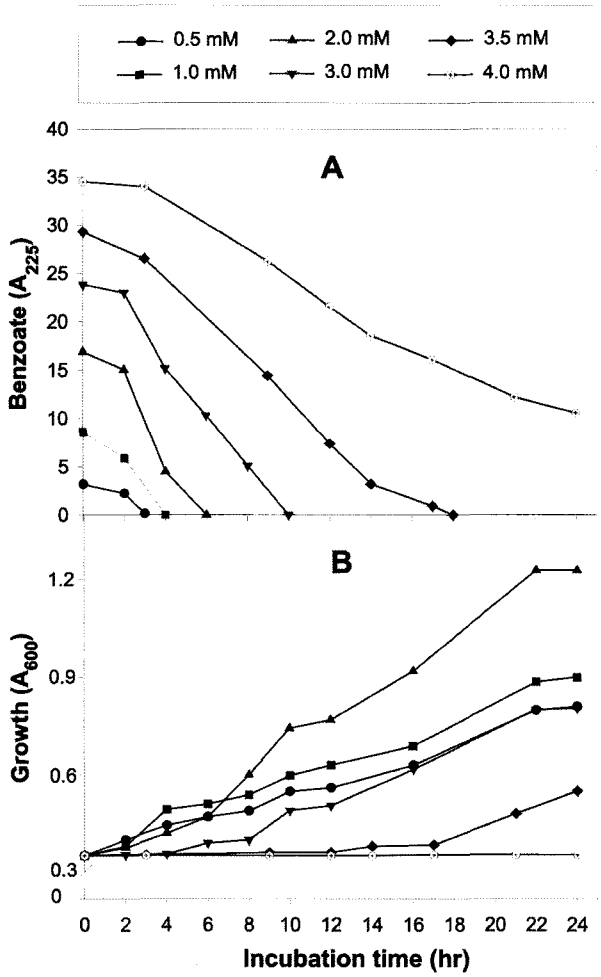

Fig. 1. Degradation of benzoate (A) by *K. oxytoca* C302 at different concentrations and growth of *K. oxytoca* C302 (B).

degraded within 6 h of incubation. Benzoate concentrations of 3.0 mM or higher were also degraded by the cells, however, higher concentrations took longer (Fig. 1A). The cells showed a gradual increase in growth at all the benzoate concentrations as a function of the incubation time, as seen in Fig. 1B. The most effective benzoate degradation by the organism was a 2.0 mM concentration within 22 h of incubation, while exhibiting maximum cell growth. Therefore, the degradation of benzoate by the immobilized cells was carried out at a concentration of 2.0 mM benzoate in the MM2 medium.

Effects of Incubation Conditions on Benzoate Degradation

The effects of the temperature and pH of the medium on the benzoate degradation and cell growth were examined in the MM2 broth containing 2.0 mM benzoate after 24 h of incubation. The benzoate degradation and cell growth exhibited a maximum value at 30°C and pH

Table 2. Effects of temperature and pH on degradation of benzoate by *K. oxytoca* C302 after 24h incubation in MM2 broth medium containing 2.0 mM benzoate and its growth

Environmental factor		Relative degradation (%)	Relative growth (%)
Temp (°C)	20	56	60
	30	100	100
	37	87	91
pH	5.0	61	65
	7.0	100	100
	9.0	12	18

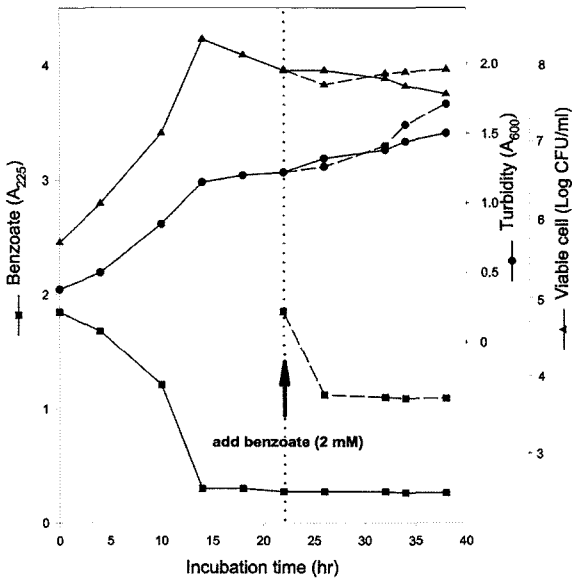

Fig. 2. Degradation of benzoate and growth of *K. oxytoca* C302 in MM2 broth medium supplemented with 2 mM benzoate. An additional 2 mM benzoate was added to the original culture after 22 h.

7.0, as shown in Table 2. When the incubation time in the MM2 broth medium containing 2.0 mM benzoate was extended over 20 h, the cells exhibited no further growth and their viability was decreased (Fig. 2). When an additional 2.0 mM benzoate was added to the original culture and incubated under the same conditions, the rate of benzoate degradation was remarkably lower, in spite of the same number of viable cells (Fig. 2). This kind of decreased cell viability and lower catabolic activity has previously been observed in batch cultivations of bacteria [12,17,19], thereby motivating the immobilization of bacteria with various types of matrices for the degradation of pollutant chemicals and production of useful metabolites.

Benzoate Degradation by Immobilized Cells and Their Viability.

The *Klebsiella oxytoca* C302 cells were immobilized in

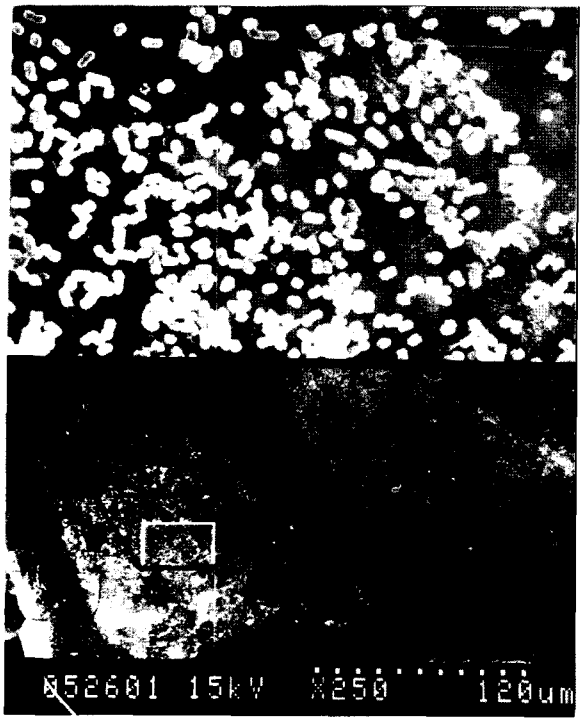

Fig. 3. Electron micrographs of *K. oxytoca* C302 cells immobilized in polyurethane. The upper part is the enlarged picture of the rectangle in the lower part.

both alginate and polyurethane foam, as described by Manobar *et al.* [17]. The cells immobilized on the surface of a polyurethane foam cube are shown by electron micrographs in Fig. 3. The cells immobilized in the polyurethane were not detached from the foam even after three mild washes with a fresh medium.

The immobilized cells were used for benzoate degradation in batch and semicontinuous batch systems. The cells immobilized in alginate and polyurethane were compared with the freely suspended cells as regards their degradation of benzoate and viability in the MM2 broth containing 2.0 mM benzoate (Fig. 4). The freely suspended cells only maintained their degradability and viability when the cells collected from the original 6-h culture were inoculated in the amount of $10^5 \sim 10^6$ cells/mL into a fresh medium containing 2.0 mM benzoate. However, the cells in the old culture lost their degradability and viability after 6 h of incubation (Fig. 4A). In contrast, the cells immobilized in either alginate (Fig. 4B) or polyurethane (Fig. 4C) exhibited a very stable degradability and viability when they were transferred into a fresh medium containing 2.0 mM benzoate and cultivated under the same conditions. In this case, the inoculum size was irrelevant, as the number of cells in the matrices was sufficient to maintain the maximum rate of benzoate degradation in the fresh medium.

Alginate has also been proved as a good immobilizing matrix for other bacterial strains that degrade pollutant chemicals, such as phenol [13], naphthalene [15], PCBs

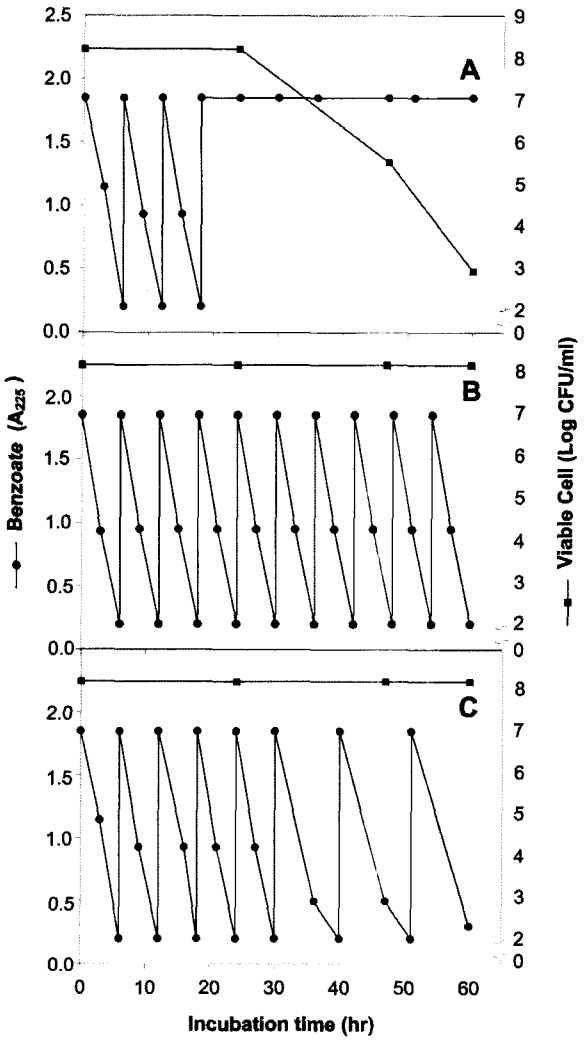

Fig. 4. Degradation of 2 mM benzoate in MM2 broth medium and viability of *K. oxytoca* C302 free cells (A) and cells immobilized in alginate (B) and polyurethane (C) in semicontinuous batch culture system.

[16], and aromatic compounds [20]. In particular, polyurethane foam has been reported as an excellent matrix for the immobilization of bacterial cells for the degradation of naphthalene [17].

Accordingly, the current results proved that alginate and polyurethane are very good matrices for the immobilization of *Klebsiella oxytoca* C302 cells as regards extending their degradation of benzoate and viability in a broth cultivation.

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