Advanced Journal of Microbiology Research Volume 2014
Available online at http://advancedresearchjournals.org/AJMR
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Full Length Research Paper

Cometabolism and immobilized degradation of monochlorobenzoate by *Rhodococcus erythropolis*

Qi Yun, Zhao Lin* and Tan Xin

School of Environmental Science and Engineering,Tianjin University, Tianjin 300072, China.

Accepted 4 July, 2013

The 3-chlorobenzoate degradation pathway by *Rhodococcus erythropolis* strain S-7 was studied. Result affirms our previous work. 3-chlorobenzoate was converted initially to chlorocatechol, and then degraded by modified ortho-pathway. *R. erythropolis* S-7 was able to grow on 3-cholobenzoate as sole growth substrate, but it was unable to grow on any of the other monochlorobenzoate isomers or to degrade these substrates. When glucose was added as grow substrate, strain S-7 can cometabolic degrade 2-chlorobenzoate, but cannot biodegrade 4- chlorobenzoate. Entrapment of strain in four supports(calcium alginate, polyvinyl alcohol, polyvinyl alcohol-calcium alginate and chitosan-calcium alginate) was studied. The surface and internal sturctures of PVA-calcium alginate and chitosan-calcium alginate beads were studied by scanning electron microscopy(SEM). Both SEM and 3-chlorobenzoate degradation results showed that PVA-calcium alginate gel can be used as support to immobilized strain cell.

Key words: monochlorobenzoate*, Rhodococcus erythropolis,* cometabolism, immobilization

INTRODUCTION

Chlorinated aromatic chemicals are used worldwide as pesticides, herbicides, solvents, lubricants, and as precursors for the manufacture of many products, such as polychlorinated biphenyls(PCBs) . The release of these compounds into the environment caused a great concern due to their toxicity to humans and wildlife, their relative persistence in aquatic sediments and soils, and their bioaccumulation in the food chain(Bedard, 2008; Field and Sierra-Alvarez, 2008). Stratford et al. (1996) reported that chlorobenzoate degradation appears to be the rate limiting step in the overall PCB-degradation process. Meanwhile chlorobenzoate constitute a favorable model for studying the biodegradation of halogenated aromatic compounds, and to elucidate the microbial strategies implicated in the release of chlorine substituents (Yi et al., 2000).

Some bacterial can not survive on halogenated aromatics as sole energy and carbon source. Aerobic cometabolism offers a biological method for the removal of the pollutant from the contaminated environment, when the pollutant does not support microbial growth. Using a conventional growth substrate, cell would be expected to prevent the induction of large regulons (Duetz et al., 1994). This approach to bioremediation is particularly appropriate for chlorinated solvents and related compounds where other methods are less likely to result in mineralization of the target compound.

Immobilized cells exhibit many advantages over free cells, including the maintenance of stable, the recovery and reuse. The application of immobilized cells in the biodegradation of toxic compounds received growing attention in the last years.

In a previous work(Qi et al., 2007), we reported *Rhodococcus erythropolis* strain S-7 can utilize 3-chlorobenzoate as the sole carbon and energy source in a range of temperatures from 10 to 30°C with stoichiometric release of chloride ions. In this report, we have affirmed the pathway for degradation, and studied the metabolism of mono-chlorobenzoates by the strain growing in glucose supplemented media. The results obtained give insight in the induction of the pathways for the metabolism of these substrates.

MATERIALS AND METHODS

Bacterial strain and cultural conditions

The 3-chlorobenzoate-degrading strain *Rhodococcus* sp. S-7 was isolated from sewage sludge with 3-chlorobenzoate as the sole

*Corresponding author. E-mail: zhaolin@tju.edu.cn.

source of carbon and energy.

The biodegradation conditions for all of the experiments were controlled at 30°C at 140 rpm. 10 ml samples were taken from each flask at each time interval, clarified by centrifugation at 12,000 r/min for 10 min, filtered through a 0.2 m membrane filters, and held at -20°C until analysis. Chlorocatechol and 3-chlorobenzoate were purchared from Sigma-Aldrich, USA.

Media for cometabolism

Cultures of *Rhodococcus* sp. S-7 were grown either on 300 ml of mineral media in 1000 ml Erlenmeyer flasks or the same medium containing 0.5 g/l glucose (MMG) . Both MM and MMG were supplemented when required with 0.2 g/l 2-chlorobenzoate or 4-chlorobenzoate(99% purity, Sigma-Aldrich, USA). The composi-ition of the mineral media has been described by Kozlovsky et al.(1993). The final pH of the medium was 7.0.

Preparation of crude extracts

Strain grown on nutrient medium were centrifuged at 2,000 r/min for 10 min. The harvested cells were washed twice with MM, and suspended in 10 ml MM medium.

Immobilized cell suspensions

Entrapped in calcium alginate(CA) gel: 3.0 g sodium alginate was diluted with deionized water to 90 ml and heated until dissolved. The solution was cooled down and then mixed with 10 ml crude extracts of *Rhodococcus* sp. S-7 suspension. The final mixture contained about 3%(w/v) sodium alginate. The mixture was extruded as drops into a solution of calcium chlorides(3%, w/v), then immersed for 1 h to form calcium alginate beads. Then frozen at - 20°C for 2 days. There after, the bead were collected and rined with phosphate buffer (pH 7.0, the concentration 0.05 mol/l) and then stored at 4°C until further use.

Entrapped in polyvinyl alcohol-calcium alginate(PVA-CA) gel: 5.0 g PVA and 3.0 g sodium alginate were diluted with deionized water to 90 ml and heated until dissolved. The solution was cooled down and then mixed with 10 ml crude extracts of *Rhodococcus* sp. S-7 suspension. The final mixture contained about 5.0%(w/v) PVA, 3%(w/v) sodium alginate. The mixture was extruded as drops into a solution of calcium chlorides(3%, w/v) using the same sized injector, then immersed for 1 h to form PVA-calcium chloride beads. Then frozen at - 20°C for 2 days. There after, the bead were collected and rined with phosphate buffer (pH 7.0, the concentration 0.05 mol/l) and then stored at 4°C until further use.

Entrapped in chitosan-CA gel: The methods described by Wang and Hu (2007) were used to form chitosan-CA beads, excepted the concertation of chitosan and CA was 0.6%(w/v) and 3%(w/v), respectively.

Analytical methods

Growth was measured by the optical density at 550 nm using Cary 100 UV-Visible Spectrophotometer (Varian, USA).

Residual 3-chlorobenzoate and intermediate were detected by HPLC(Agilent 1100, USA), with a Prontosil 120-5-C18-ace-APS. The mobile phase used was acetonitrile -10 mM phosphate (50:50 v/v). The flow rate was 1 ml/min and detected by UV-absorption at 254 nm.

Scanning electron microscopy (SEM)

To investigate surface and internal macroporous of different immobilized beads, the beads was frozen, fractured in two fractions and dried. After drying, the beads were mounted and gold coated prior to SEM examination.

RESULTS AND DISCUSSION

Confirmation of R. erythropolis S-7 chlorobenzoate biodegradative pathway

Chlorocatechol and 3-chlorobenzoate were detected during the degradation.

The result can be seen in the Figure 1. In the first 24 h, 3-chlorobenzoate was metabolized to chlorocatechol. In the 32 h, chlorocatechol reached the highest, and then decreased. In the 60 h, chlorocatechol can not be detected, neither the 3- chlorobenzoate. The HPLC strongly suggested the formation of chlorocatechol from 3-chlorobenzoate.

In previous work, we proposed R. erythropolis S-7 degradation pathway based on the chloride released rate, enzymes activities and full scan of cell extract results. However chlorocatechol, which is the important intermediate in the degradation pathway, was not detected in previous studies. The chlorocatechol was detected when changed mobile phase. Result affirms our previous work. 3-chlorobenzoate was converted initially to chlorocatechol, and then degraded by modified ortho-pathway.

Cometabolic degradation

R. erythropolis S-7 was able to grow on 3-cholobenzoate as sole growth substrate, but it was unable to grow on any of the other monochlorobenzoate isomers or to degrade these substrates. Other halogenated compounds tested, including dichlorobenzoate isomers neither supported growth of the strain or were they degraded.

Degradation of 2-chlorobenzoate by the strain with or without glucose was tested (Figure 2.).

In the presence of 200 mg/l 2-chlorobenzoate plus 500 mg/l glucose, the growth rate of strain S-7 was slower than in the presence of glucose alone. However, degradation of 2-chlorobenzoate on glucose supplemented media seemed to be more efficient, although a significant amount of substrate remained in the culture. The degradation curve was similar with growth curve. A long lag period of 36 h was necessary for the cells to start growing when the 2-chlorobenzoate was added. When glucose exhausted, the ability of degradation of 2-chlorobenzoate was weaken. Previous workers have shown that the degradation of some xenobiotics can be enhanced by the addition of readily metabolized substrates.

Glucose may afford the energy or reduction equiva-lents(e.g., NAD(P)H), which was required in the aromatic

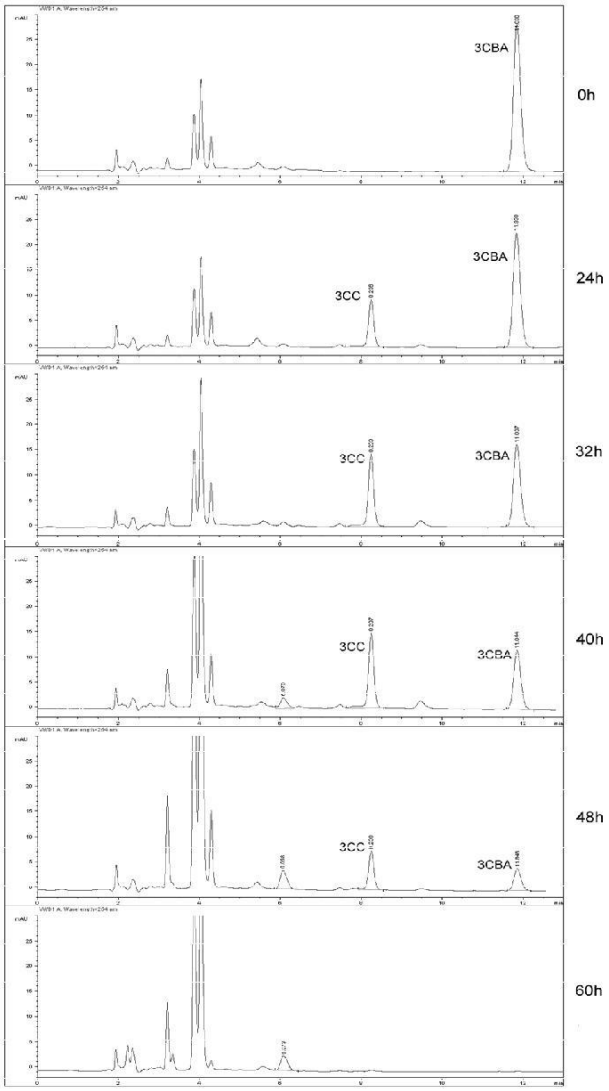


Figure 1. Chromatogram of fermentive broth at different culture time.

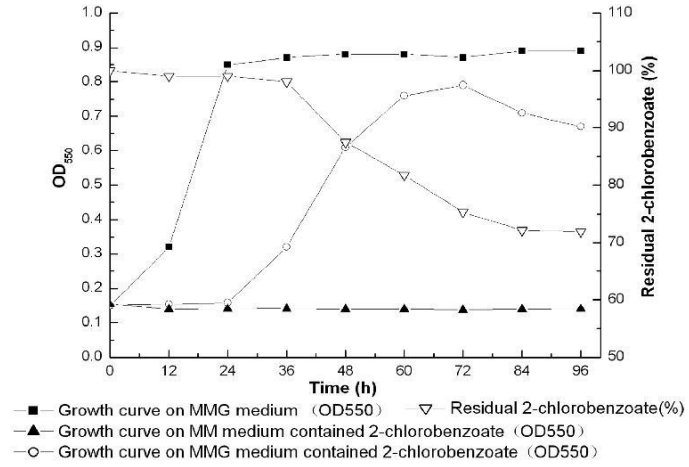


Figure 2. Chlorobenzoate cometabolism with glucose as growth substrate.

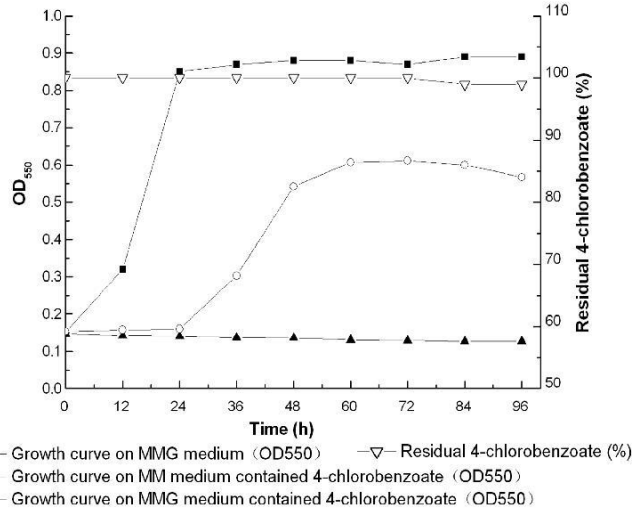


Figure 3. Chlorobenzoate cometabolism with glucose as growth substrate.

-ring hydroxylation(Wang J. and Qian, 1999). Glucose also expected to prevent the induction of large regulons for the catabolism of halogenated compounds by carbon catabolite repression(Duetz et al., 1994).

Such finding may have practical and ecological significance and are useful for improving the efficiency and the stability of some biological treatment processes.

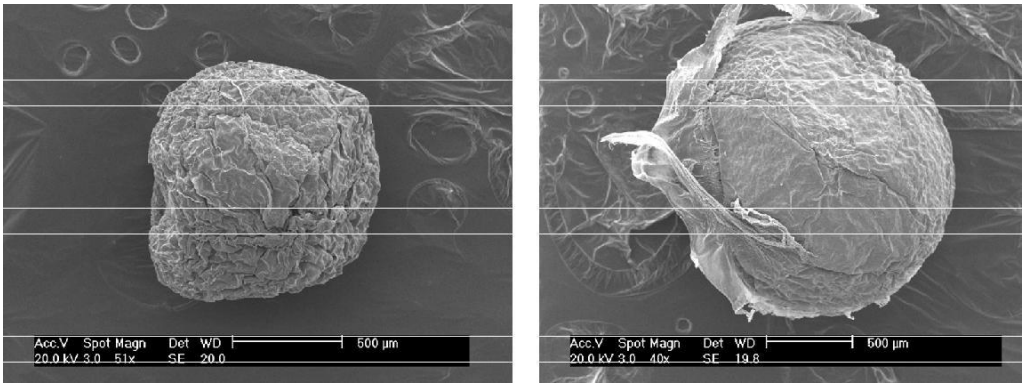
As showed in Figure 3, glucose is only used for cell growth. The 4-chlorobenzoate was not cometabolized, and the addition of glucose did not influence the 4-chlorobenzoate degradation. Failure of R. erythropolis S-7 to degrade 4-chlorobenzoate in the present of glucose indicated that 4-chlorobenzoate degradative enzyme can not induced in glucose grown cell.

The presence of 4-CBA and 2- CBA inhibited the growth of strain S-7 on glucose. 4-CBA had a stronger effect on the growth of strain S-7.

CBA inhibited growth of Pseudomonas has been reported (Vrana et al., 1996). CBA or other similar aromatic compounds, such as 2-chlorophenol and benzoate, may act as energy “uncouplers”. These “uncouplers” negatively affected the growth of the cells. The reduction on growth rates could be one of the first consequences of a disturbance in energy conservation systems (Krayl et al., 2003). 4-CBA (pKa 4.0) may accumulate in the undissociated form in the cytoplasm, and then effect the metabolism of cells (Martínez et al., 2007).

Degradation of 3-chlorobenzoate with different immobilization supports

Entrapment of cells in different supports was studied. Calcium alginate(CA) immobilization was chosen because it entraps whole cells under mild conditions and causes



(a) (b)

Figure 4. Microphotographs of the beads obtained from:(a) PVA-CA; (b) chitosan-CA.

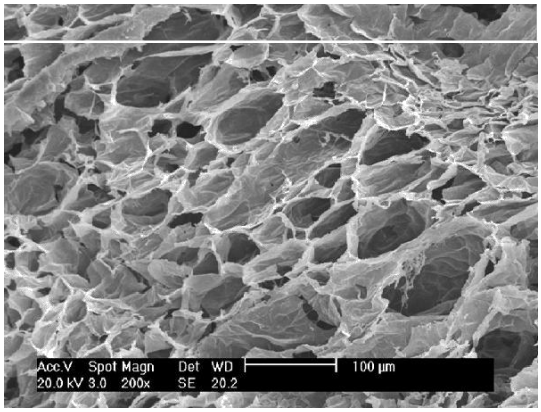


Figure 5(a) . Microphotographs of the internal beads obtained from: PVA-CA.

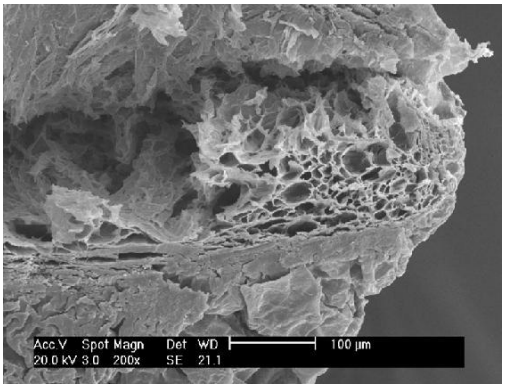


Figure 5(b). Microphotographs of the internal beads obtained from: chitosan-CA.

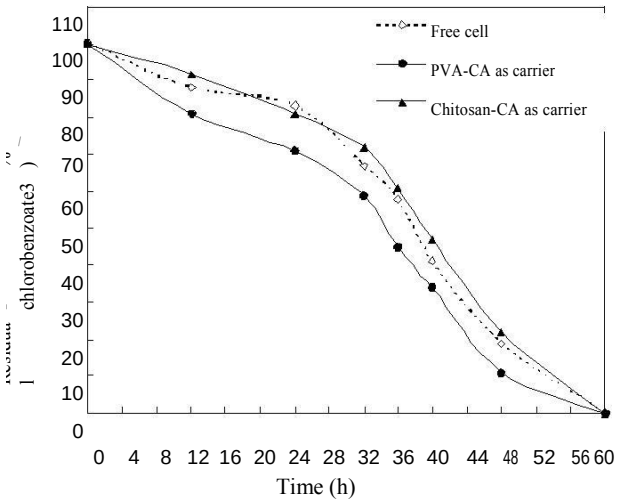


Figure 6. Biodegradation of 3-chlorobenzoate by different immobilized methods.

minimal cell damage. However results showed that when calcium alginate is used alone, intension of the immobi-immobilized cell was not strong enough, which were broken after period of culture time. Using PVA as support, it caused cell disruption. Many attempts had been made to improve the stability of calcium alginate beads. When added PVA to calcium alginate, the immobilized beads have advantages of easy operation of immobilization and high strength.When calcium alginate and chitosan are used as support, the properties of immobilized beads was better than using calcium alginate alone.

PVA-CA and chitosan-CA beads produced were spherical in form. Scanning electron micrographs show that all the samples maintain this spherical form after drying at room temperature (Figure 4).

The internal sturctures of them was different. The internal structure of PVA-CA immobilized bead was loose and finely porous(Figure 5a), which facilitated the diffusion of oxygen and substrate. In contrast, a less homogeneous internal structure was observed in the chitosan–CA beads(Figure 5b).

Degradation result (Figure 6) showed that both immobi-

lized cells and free cell can completely degrade 3-chlorobenzoate within 60 h. Immobilized cells entrapped in the PVA-CA gel showed the highest biodegradation activity. PVA-CA was a better immobilization matrix than chitosan-CA, owing to its greater mechanical strength and improvements in diffusion of oxygen, or 3-chlorobenzoate into the cell. In the case of chitosan-CA bead the chitosan coating membrane prevented the transmission of oxygen and substrate to the organisms as it was encapsulated in the bead. Thus, reduced the bioavailable concentration in the inner spaces of the beads. The biodegradation rate of cells immobilized by chitosan-CA was lower when compared with free cells.

Conclusion

R. erythropolis S-7, which can use 3-chlorobenzoate but not 2-chlorobenzoate or 4-chlorobenzoate as sole carbon source, biodegraded 3-chlorobenzoate initially to chlorocatechol, and then degraded the intermediate through modified ortho-pathway. Strain S-7 can biodegrade 2-chlorobenzoate when glucose was added as grow substrate. However, it can not cometabolic degrade 4-chlorobenzoate with glucose. When used CA as carrier, intension of the immobilized cell was not strong enough. Using PVA alone, the immobilized cell can not form beads. When added PVA to CA, both strength and tenacity were enhanced greatly, and the 3-chlorobenzoate biodegradation rate was faster than the free bacteria. When use CA and chitosan as carrier, although the properties of immobilized ball was better than using CA alone, the degradation rate was a little slower than the free bacteria.

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