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Cyclodextrin-Enhanced Degradation of Toluene and *p*-Toluic Acid by *Pseudomonas putida*

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Degradation of an immiscible aromatic solvent, toluene, and a water-soluble aromatic compound, *p*-toluic acid, by a *Pseudomonas putida* strain in the presence of β-cyclodextrin (β-CD) was investigated. The ability of CDs to interact with hydrophobic organics and form inclusion compounds was exploited in this study to remove or alleviate the toxicities of substrates and consequently to enable or enhance degradation. Liquid toluene was found to be highly toxic to *P. putida*. However, this phase toxicity was removed when crystalline β-CD-complexed toluene was provided as the substrate. The latter was fully degraded at a concentration of up to 10 g/liter. Degradation of toluene vapors was enhanced in the presence of β-CD as a result of reduced molecular toxicity and facilitated absorption of the gaseous substrate. Similarly, β-CD alleviated the inhibitory effect of *p*-toluic acid on *P. putida*. This protective effect of CD was remarkably more prominent when the microbial culture was shock loaded with an otherwise toxic dose of *p*-toluic acid (1.8 g/liter).

Research on microbial degradation of organic compounds has concentrated primarily on degradation mechanisms and metabolic pathways, both in naturally occurring (10) and genetically engineered microorganisms (22). However, it appears that relatively little attention has been given to microbial degradation as a bioprocess. The main goals of such a bioprocess would certainly be degradation of high concentrations of organics and fast kinetics. Indeed, the efficiency of microbial degradation is often seriously impeded by two major obstacles, (i) poor accessibility of lipophilic compounds (liquid or solid) to the microorganism and (ii) toxicity effects of substrates exerted upon the microbe. The first obstacle is expressed not only in the poor aqueous solubility of the hydrophobic compound but also in the slow dissolution rate of the organic liquid or solid into the aqueous environment of the degrading microbe. While this obstacle is likely to slow down degradation, the second may limit or prevent it.

The widely studied degradations of water-insoluble (toluene) and water-soluble (*p*-toluic acid) aromatics (10) constitute good examples of the effects of these two obstacles. Previous attempts to reduce substrate toxicity and increase the efficiency of aromatic-compound biodegradation were made by using activated carbon (20). In recent years, *Pseudomonas putida* isolates claimed to exhibit an unusual tolerance of excess liquid aromatic solvents have been very rare (8, 15, 27). In this study, cyclodextrins (CDs) were employed to significantly alleviate or remove these obstacles from a common *P. putida* strain which does not tolerate liquid toluene.

CDs are cyclic oligomers of 1,4-α-D-linked glucose units (25). The main CDs consist of six, seven, or eight glucose molecules, forming α-, β-, and γ-CDs, respectively. A relevant unique feature of all CD molecules is a cylinder-like shape with an external hydrophilic shell and an apolar internal cavity. A consequence of this structure is the ability to accommodate suitably sized organic guest molecules in the cavity, thus forming inclusion compounds. These inclusion compounds may be either water-soluble complexes, such as the β-CD–*p*-toluic acid complex, or water-insoluble complexes, such as the β-CD–toluene complex. In the latter case, a hydrophobic water-immiscible organic liquid becomes an easily wettable and dispersible crystalline solid (3).

Phase solubility diagrams of organics in CD solutions (25) indicate a definite solubility of the solid complex ($[CD\text{-}G]_s$) according to the following equilibria:

$$[CD\text{-}G]_s = [CD\text{-}G]_{aq} \quad (1)$$

$$[CD\text{-}G]_{aq} = CD_{aq} + G_{aq} \quad (2)$$

where G is the encapsulated guest molecule. The solid β-CD–toluene complex dissolves into the aqueous medium (equation 1) and dissociates to give dissolved CD and toluene molecules (equation 2). Similarly, the already soluble β-CD–*p*-toluic acid complex dissociates into its two components (equation 2). In either case, the liberated aromatic compound is taken up by cells and the dissociation equilibrium is shifted further rightward. In this way, large amounts of toluene and *p*-toluic acid complexed with β-CD can be fully degraded.

CDs have already been used to enhance microbial conversions of either toxic (4) or water-insoluble organic substrates (18). A few studies employing CDs in microbial degradation have also been reported. The first study was reported in the patent literature and involved improved purification of wastewater, supplemented with β-CD, from benzene and phenol (9). Another study was conducted by Olah et al. (21) to demonstrate the detoxifying power of β-CD on industrial wastewater containing pesticides and their degradation products, such as chlorophenols and nitrophenols. Banky et al. (1) employed a β-CD polymer to increase the efficiency of phenol degradation in wastewater by *Candida tropicalis*. In a recent patent (28), β-CD was incorporated into beads of immobilized microbial cells to enhance adsorption and degradation of various substances, such as pesticides and halogenated hydrocarbons. This study was concerned with degradation of β-CD-complexed aromatics by a pure culture of a *P. putida* strain proven to be inert to CDs, thus enabling reuse or recycling of CDs. These aromatics were toluene and *p*-toluic acid, models of insoluble and soluble compounds, respectively.

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MATERIALS AND METHODS

Materials. β-CD was obtained from Roquette Freres (Lille, France). Toluene and *p*-toluic acid were purchased from Frutarom (Haifa, Israel) and Aldrich (Milwaukee, Wis.), respectively. Labelled *p*-toluic acid ($CH_3C_6H_4{}^{14}COOH$) was obtained from Sigma (St. Louis, Mo.).

Maintenance of microorganism. A strain of *P. putida* known to degrade aromatics was obtained from the local collection of the Fermentation Unit of the Hebrew University (Jerusalem, Israel) and maintained on nutrient agar.

Inoculum preparation. This microorganism was cultivated on toluene vapors as the sole carbon and energy source in a basal mineral medium (pH 7.0) which consisted of the following (in grams per liter): $(NH_4)_2SO_4$, 8; KH_2PO_4, 5; $MgSO_4 \cdot 7H_2O$, 0.1; and a stock salt solution (1 ml) prepared according to Bauchop and Elsden (7). A 250-ml shake flask with an air-permeable paper stopper was loaded with 50 ml of sterile medium under magnetic stirring. Toluene vapors were generated by free evaporation from 1 ml of liquid toluene in a small bottle suspended in the upper part of the flask. The medium was seeded with a loopful from the agar slant, and after 48 h of magnetic agitation at room temperature, the bacterial culture served as a source of toluene-grown inoculum.

Degradation of β-CD complexed toluene. Each sterile 100-ml bottle, equipped with a magnetic bar, was loaded with 50 ml of a sterile medium containing weighed amounts of β-CD. Then these β-CD mixtures were heated to ca. 80°C to dissolve all β-CD. Upon addition of known amounts of liquid toluene, bottles were immediately sealed with plastic stoppers to avoid loss of toluene by evaporation. Bottles were magnetically stirred at room temperature for 30 min. The white slurries obtained were aseptically transferred into sterile 250-ml shake flasks, inoculated with a toluene-grown culture (5% [vol/vol]), and incubated in a rotary shaker at 30°C. After 48 h, the microbial slurries which contained free β-CD precipitates (in excess of aqueous solubility) were diluted with a volume of water known to be sufficient to obtain crystal-free bacterial suspensions. Cells were centrifuged, washed two times with distilled water, and then dried at 60°C for weight determinations.

Degradation of toluene vapors. These experiments were performed with magnetically agitated 250-ml flasks at room temperature, as described above for inoculum preparation. Each flask contained 50 ml of a sterile medium with 10 g of β-CD per liter. Identical flasks without β-CD served as controls. All flasks were inoculated with the same toluene-grown culture (5% [vol/vol]). After some time, β-CD-containing flasks turned whitish and turbid as a result of formation of the insoluble β-CD–toluene complex. Intermittently, aliquots (1 ml) were withdrawn and slightly heated with an electric fan for 3 min to drive off toluene and redissolve β-CD. The turbidity of the clear bacterial suspension at 600 nm was subsequently determined.

Respirometry. Cells previously grown in a mineral medium with glucose (2 g/liter) were washed and suspended in the same medium. One milliliter of this bacterial suspension was added to 4 ml of the same medium in a magnetically stirred respirometric cell of a Rank Brothers polagrographic oxygraph, and oxygen readings were immediately recorded as a function of time. The respirometric cell was maintained at 30°C by connecting it to a thermostatted circulating water bath. The oxygen meter was calibrated with air-saturated water and nitrogen-gassed water.

Assays. β-CD was detected and quantified by high-pressure liquid chromatography on a Lichrospher NH_2 (5-μm) column (Merck, Darmstadt, Germany), with an acetonitrile-water (75:25) mixture as eluent (2 ml/min). Aliquots of centrifuged growth media were injected into a chromatograph equipped with an RI detector, and β-CD was eluted after 11.7 min.

The concentration of *p*-toluic acid was spectrophotometrically monitored at a λ_{max} of 241 nm. Mineral media with and without β-CD, as well as the final growth medium, showed virtually no background absorption at this wavelength.

Viability counts were carried out with cells previously agitated in the presence of free and β-CD-complexed toluene. These experiments were done with magnetically agitated 250-ml flasks containing 50 ml of a phosphate buffer (0.05 M [pH 7.2]) and 0.4% (vol/vol) toluene with and without an equimolar amount of β-CD. The agitation was vigorous enough to break the toluene phase (in the absence of β-CD) into minute droplets and to keep these droplets well dispersed in the water phase. Intermittently, stirring was stopped until phase separation and 0.5-ml liquid-free and solid-free aliquots were withdrawn, diluted with sterile buffer, and plated on nutrient agar.

RESULTS

Even though the great majority of *Pseudomonas* species do not hydrolyze starch (12), experiments were performed first to check on the inability of our strain to hydrolyze starch. Thus, while our strain did grow on a starch-beef extract agar plate, no hydrolysis zone was detected with iodine. This strain was equally unable to grow on starch or any of the three natural CDs as the sole carbon and energy source. Furthermore, since some microorganisms have recently been reported to secrete cyclodextrinases (24), our strain was tested and found to be unable to break open any natural CD. Furthermore, the presence of 5 or 15 mM β-CD was shown to have no effect on either the growth profile or respiration rate of *P. putida* in a minimal salt medium containing 2 g of glucose per liter.

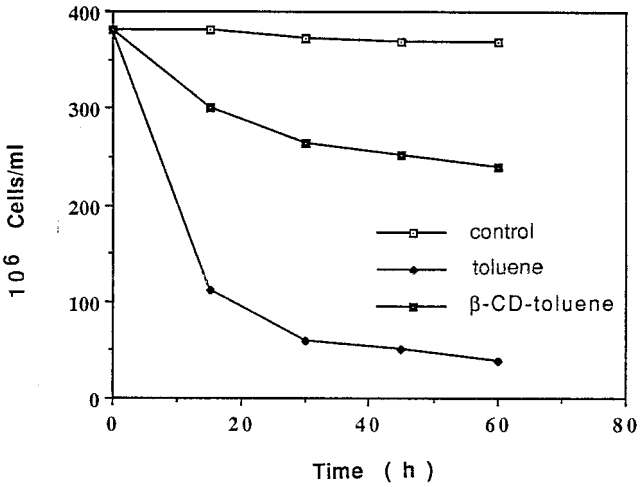

FIG. 1. Viable counts of *P. putida* cell suspensions in phosphate buffer (pH 7.2) agitated without (control) and with either excess liquid toluene (0.4% [vol/vol]) or an equivalent amount of solid β-CD–toluene complex at room temperature.

Toxicities of free and β-CD-complexed toluene to *P. putida*. Respirometric measurements performed on glucose-grown cells in a glucose medium previously saturated with liquid toluene (toluene aqueous solubility at 16°C is 0.47 g/liter [13]) showed a 70% decrease in oxygen uptake rate with respect to a toluene-free control system. In the presence of excess liquid toluene (0.15% [vol/vol]), no oxygen consumption was detected. It is important to note that while the first system consists of cells agitated in a one-liquid phase, the second consists of an identical amount of cells suspended in a stirred water-organic solvent, two-liquid-phase dispersion. Thus, while liquid toluene is toxic, dissolved toluene is inhibitory.

As previously mentioned, the presence of an amount of β-CD equimolar to that of the water-toluene system causes liquid toluene to become a white crystalline solid β-CD–toluene complex. Cells suspended in this water-solid two-phase system maintained a respiration rate which was 57% of that of the control toluene-free cell suspension. Thus, while liquid toluene was totally toxic to our strain, "solidified" toluene was somewhat inhibitory but less inhibitory than free dissolved toluene. The effect of toluene on the viability of bacterial cells in the presence and absence of β-CD is depicted in Fig. 1. During the 60-min agitation, viability remained unaffected, with neither component present. However, excess toluene reduced viable counts by almost 10-fold after 60 min. In the presence of solid β-CD–toluene, viable counts remained fairly high.

Degradation of β-CD-complexed toluene. Figure 2 shows that *P. putida* grows on toluene vapors in a β-CD-free mineral medium. The addition of β-CD (10 g/liter) led to significantly faster and richer growth. When the substrate was free liquid toluene well dispersed in the aqueous medium, bacterial growth was not apparent at all. However, when it was used as a solid complex with β-CD, bacterial growth occurred. Table 1 shows that increasingly larger biomasses were obtained after 48 h from correspondingly larger initial amounts of the solid β-CD–toluene (molar ratio, 1:1) complex, with comparable biomass yields.

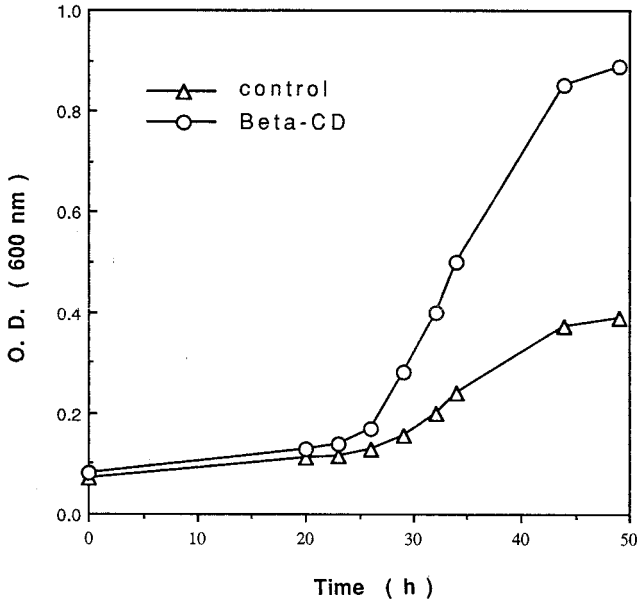

FIG. 2. Growth of *P. putida* on toluene vapors in a mineral salt medium without (control) and with β-CD (10 g/liter) at room temperature. O.D., optical density.

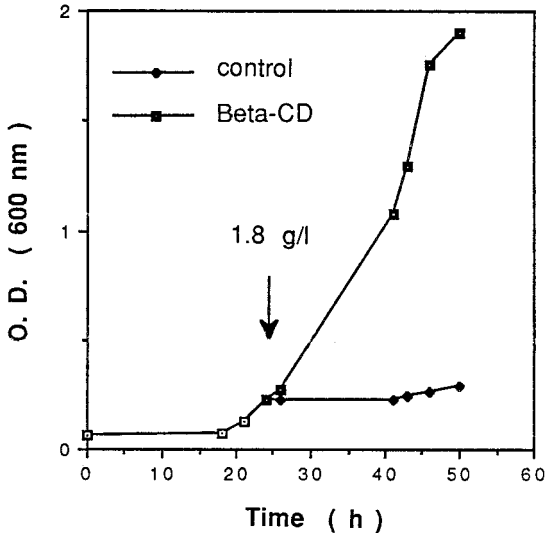

FIG. 3. Growth behavior of *P. putida* on *p*-toluic acid (0.5 g/liter), followed by a shock load of substrate (designated by an arrow) to a final concentration of 1.8 g/liter, without (control) and with an equimolar amount of β-CD in shake flasks inoculated with toluene-grown cells at 30°C. O.D., optical density.

Degradation of β-CD-complexed *p*-toluic acid. This *P. putida* strain was able to grow on *p*-toluic acid dissolved in a minimal salt medium. This growth in a mineral medium constitutes evidence per se for biodegradation of the organic substrate. In this process, spectrophotometrically monitored utilization of the substrate, as well as consumption of gaseous oxygen, was found to parallel bacterial growth. Furthermore, in a growth experiment with radiolabelled *p*-toluic acid ($CH_3C_6H_4{}^{14}COOH$), a material balance was made on the total radiolabel; it was found to partition into trapped CO_2 (80%), biomass (16%), and the final growth medium (4%). Even though the radiolabel was not borne on the aromatic ring (no such compound was commercially available), these results provided additional supporting evidence for *p*-toluic acid degradation.

The effects of β-CD on the behavior of *P. putida* were shown in a shock load experiment (Fig. 3). Initially, 50 ml of mineral medium with 0.5 g of *p*-toluic acid per liter in a 250-ml shake flask was inoculated with 5 ml of cells previously grown on toluene vapors for 24 h. This shake flask culture (200 rpm) was administered at the initial stage of the exponential phase with another load of substrate, thus raising its concentration to 1.8 g/liter, and was then split into halves. The first half was supplemented with an equimolar amount of β-CD (15 g/liter), and the second half, transferred in an identical flask, served as a control. Figure 3 clearly shows that the added β-CD enabled microbial growth to cope with the shock and to proceed smoothly into the exponential phase, in contrast to the totally inhibited control culture.

TABLE 1. Biomasses and biomass yields of *P. putida* growing on β-CD-complexed toluene for 48 h at room temperature

Amt of toluene (g/liter)	Amt of β-CD (g/liter)	Biomass (g [dry wt]/liter)	Biomass yield (g [dry wt]/g of toluene)
4	52	1.43	0.36
6	78	2.00	0.33
8	104	2.26	0.28
10	130	3.44	0.34

DISCUSSION

The mere presence of natural CDs in the degradation medium inevitably introduces interactions with both bacterial cells and organic compounds, such as the substrate (and intermediate by-products), as a result of their complexing abilities. Interactions between CDs and cells may be examined in terms of (i) what cells can do to CDs and (ii) what CDs can do to cells.

Microbial cells may bear or secrete amylolytic enzymes that are capable of hydrolyzing CDs prior to metabolizing degradation saccharides. Therefore, the fate of CDs in the growth medium of our strain needed to be established first. In fact, the proven inertness of our strain toward either CDs or starch comes as no surprise since the genus *Pseudomonas* is a very weak amylolytic genus (12). Thus, the molecular integrities of CDs were preserved, and they in turn maintained their complexing abilities. This inertness of *P. putida* toward CDs is obviously a sine qua non condition for their potential reuse or recycling. Considering what CDs can do to microorganisms, certainly cellular membranes are viewed as the most susceptible site acted upon by CDs (5). Phospholipids, the main constituent of all membranes, form complexes with CDs (17). Thus, CDs can extract membrane components and consequently disrupt the integrity of a membrane (14, 23), ultimately affecting the vitality or viability of cells (5). Nevertheless, natural CDs are generally nontoxic to bacterial cells (6). Indeed, in our system, both growth and respirometric measurements proved β-CD to be neither inhibitory nor toxic to *P. putida* cells.

Toluene is toxic to most microorganisms, although a few, such as some *Pseudomonas* strains, can tolerate or metabolize it (11). The tolerance of these strains depends on whether toluene is provided in the gaseous or liquid state. Vecht et al. (26) cultivated a *P. putida* strain on toluene vapors borne by an airstream entering a fermentor. Our *Pseudomonas* strain neither tolerated nor grew on liquid toluene; this is simply ex-

plained by the deaths of cells agitated with excess toluene (Fig. 1). However, our strain grew on toluene molecules supplied by vapors dissolving into the aqueous medium. This behavior is a very good example of so-called phase toxicity, whereby the mere presence of a dispersed liquid phase exerts toxic effects on microbial cells, i.e., cell wall and membrane disruption (2). In contrast, toxicity caused solely by dissolved solvent molecules is molecular toxicity. A few *P. putida* strains have been reported to exhibit tolerance of toluene phase toxicity. The first one, *P. putida* IH-2000, is capable of growing in a rich medium in shaken test tubes containing 30% (vol/vol) toluene (15, 16). Another one, *P. putida* Idaho, can grow in a rich medium in the presence of up to 50% (vol/vol) toluene, but its growth rates and yields are considerably lower under stronger agitation (8). Recently, *P. putida* S12 was also reported to be capable of growing on acetate in shaken flasks in the presence of 1% (vol/vol) toluene (27). Unfortunately, in all of these studies, no clear and certain distinctions were made between phase and molecular toxicities; the claimed resistance to excess toluene may turn out to be tolerance of molecular toxicity at a solvent saturation concentration maintained by a poorly agitated liquid phase (2). Growth under vigorous interphase mixing of solvent and aqueous medium, at least comparable to that obtained in a homogeneous solvent-saturated aqueous medium, is a test for tolerance of phase toxicity (2). Such growth was not observed with our strain unless the liquid phase was eliminated.

Supplying toluene as vapors is one way of avoiding liquid-phase toxicity. In our experimental setup, free evaporation of toluene was likely to lead after some time to some condensation of vapors to droplets which would then exert liquid-phase toxicity. Such limited condensation may explain the limited growth observed in Fig. 2. However, the presence of β-CD has most likely prevented or considerably reduced the formation of these droplets (via complexation of toluene) and facilitated the absorption of gaseous toluene molecules through the air-water interface. Consequently, enhanced bacterial growth was observed in the presence of 10 g of β-CD per liter. Furthermore, as the encapsulated aromatic molecule was degraded, the freed CD molecule became available for further complexation of the gaseous substrate, thus functioning as a phase transfer catalyst.

Another way of avoiding the liquid phase was made possible by complexation with β-CD, producing solidified toluene. Slurries made up of up to 10 g of toluene per liter and equimolar amounts of β-CD were fully degraded (Table 1). When the complex load corresponded to a subsaturation concentration of β-CD (it has an aqueous solubility of 18.5 g/liter [4]), the initial slurry was transformed upon completion of growth into a clear bacterial suspension with redissolved intact CD. When cells were discarded from this suspension and another batch of toluene was agitated with the supernatant liquid, a new crop of solids soon precipitated. Reinoculation of this slurry led to total degradation of the complexed toluene again. This technique of solidifying a liquid substrate was firstly exploited for cultivating *C. tropicalis* on a β-CD–hexadecane complex (3). Subsequently, this method of delivery was employed to investigate the cytotoxicity of a water-insoluble isoalkane in the form of a solid β-CD complex (19).

Obviously, phase toxicity is nonexistent for a water-soluble substrate such as *p*-toluic acid; inhibitory or toxic effects are exerted at the molecular level. The soluble β-CD–*p*-toluic acid complex reduced the effective concentration of the free substrate, as expected from the equilibrium expressed in equation 2, thus exposing bacterial cells to subinhibitory concentrations. This protective effect of CD complexation was remarkably manifested in the shock load experiment (Fig. 3); the added β-CD enabled the microbial culture to cope with the shock and to proceed smoothly into the exponential phase, in sharp contrast to the totally inhibited control culture.

Finally, if CDs are to find some use in the degradation of pollutants by microbes inert to CDs, an approach which ensures repeated use in a bioreactor would be a reasonable one. First, toxic liquid solvents would be solidified prior to degradation. With regard to gaseous aromatics, one may envisage two methods for their degradation. The first one would to be to directly feed a CD-containing bioreactor with a contaminated airstream. The second, a two-stage method, would be first to absorb aromatic contaminants with a CD solution and subsequently to feed this mixture into a bioreactor. Solvent vapor recovery by CD aqueous solutions has already been reported (25). It is important to note that while water-soluble contaminants are easily absorbed in water, few means are available for absorbing hydrophobic water-immiscible contaminants, such as benzene, toluene, and xylene. In this respect, CDs definitely offer an efficient way of capturing benzene, toluene, and xylene, as well as other heavier aromatics.

REFERENCES

1. **Banky, B., K. Recseg, and D. Novak.** 1985. Application of water soluble beta-cyclodextrin in microbiological decomposition of phenol. Magy. Kem. Lapja **40:**189. (Abstract.)
2. **Bar, R.** 1987. Phase toxicity in water-solvent two-liquid phase microbial system, p. 147–153. *In* C. Laane, M. D. Lilly, and J. Tramper (ed.), Biocatalysis in organic media. Elsevier, Amsterdam.
3. **Bar, R.** 1990. A new cyclodextrin-agar medium for surface cultivation of microbes on lipophilic substrates. Appl. Microbiol. Biotechnol. **32:**470–474.
4. **Bar, R.** 1990. Cyclodextrin-aided microbial transformations of aromatic aldehydes by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **31:**25–28.
5. **Bar, R.** 1994. Interactions between cyclodextrins and microbial cells, p. 27–32. *In* T. Osa (ed.), The proceedings of the 7th International Cyclodextrin Symposium, Tokyo, April 25–28. Business Center for Academic Societies Japan, Tokyo.
6. **Bar, R., and S. Ulitzur.** 1994. Bacterial toxicity of cyclodextrins: luminous *E. coli* as a model. Appl. Microbiol. Biotechnol. **41:**574–577.
7. **Bauchop, T., and S. R. Elsden.** 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. **23:**457–469.
8. **Cruden, D. L., J. H. Wolfram, R. D. Rogers, and D. T. Gibson.** 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. **58:** 2723–2729.
9. **Farkas, P., J. Olah, J. Szejtli, and L. Szente.** 1987. Hungarian patent application 2650/85.
10. **Gibson, D. T. (ed.).** 1984. Microbial degradation of organic compounds. Marcel Dekker, New York.
11. **Heipieper, H. J., F. J. Weber, J. Sikkema, H. Keweloh, and J. A. M. de Bont.** 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. **12:**409–415.
12. **Hendrie, M. S., and J. M. Shewan.** 1979. The identification of pseudomonads, p. 1–14. *In* F. A. Skinner and D. W. Lovelock (ed.), Identification methods for microbiologists. Academic Press, London.
13. **Hodgman, C. D.** 1960. Handbook of chemistry & physics, p. 1247. Chemical Rubber Publishing Co., Cleveland.
14. **Hozbor, D., M. E. Rodriguez, A. Iagares, and O. Yantorno.** 1993. Release of lipopolysaccharide during *Bordetella pertussis* growth. Res. Microbiol. **144:** 201–209.
15. **Inoue, A., and K. Horikoshi.** 1989. A *Pseudomonas* thrives in high concentrations of toluene. Nature (London) **338:**264–266.
16. **Inoue, A., M. Yamamoto, and K. Horikoshi.** 1991. *Pseudomonas putida* which can grow in the presence of toluene. Appl. Environ. Microbiol. **57:**1560–1562.
17. **Irie, T., K. Fukunaga, and J. Pitha.** 1992. Hydroxypropyl cyclodextrins in parenteral use. 1. Lipid dissolution and effects on lipid transfers *in vitro*. J. Pharm. Sci. **81:**521–523.
18. **Jadoun, J., and R. Bar.** 1993. Microbial transformations in a cyclodextrin medium. 3. Oxidation of cholesterol by *Rhodococcus erythropolis*. Appl. Microbiol. Biotechnol. **40:**230–240.
19. **Janz, S., and E. Shacter.** 1991. A new method for delivering alkanes to mammalian cells: preparation and preliminary characterization of an inclusion complex between β-cyclodextrin and pristane (2,6,10,14-tetramethylpentandecane). Toxicology **69:**301–315.
20. **Morsen, A., and H. J. Rehm.** 1987. Degradation of phenol by a mixed culture of *Pseudomonas putida* and *Cryptococcus elinovitii* adsorbed on activated carbon. Appl. Microbiol. Biotechnol. **26:**283–288.

21. **Olah, J., T. Cserhati, and J. Szejtli.** 1988. Beta-cyclodextrin enhanced biological detoxification of industrial wastewaters. Water Res. **22:**1345–1352.
22. **Omenn, G. S., and A. Hollaender (ed.).** 1984. Genetic control of environmental pollutants. Plenum Press, New York.
23. **Perera, V., A. Wardlaw, and J. Freer.** 1987. Release of pertussis and its interaction with outer membrane antigens. J. Gen. Microbiol. **133:**2427–2435.
24. **Saha, B. C., and J. G. Zeikus.** 1992. Cyclodextrin degrading enzymes. Starch **44:**312–315.
25. **Szejtli, J.** 1988. Cyclodextrin technology, p. 1, 393. Kluwer Academic Publishers, Dordrecht, The Netherlands.
26. **Vecht, S. E., M. W. Platt, Z. Er-El, and I. Goldberg.** 1988. The growth of *Pseudomonas putida* on *m*-toluic acid and on toluene in batch and chemostat cultures. Appl. Microbiol. Biotechnol. **27:**587–592.
27. **Weber, F. J., L. P. Ooijkaas, R. M. W. Schemen, S. Hartmans, and J. A. M. de Bont.** 1993. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl. Environ. Microbiol. **59:**3502–3504.
28. **Wildenauer, F. X., R. Franz, R. Menzel, and H. Vetter.** 1992. German patent 4027223. (Chem. abstr. **118:**58248.)