Appl Microbiol Biotechnol (1989) 31:383-389

Applied and Microbiology Biotechnology

Protection of bacteria against toxicity of phenol by immobilization in calcium alginate

Heribert Keweloh, Hermann-Josef Heipieper, and Hans-Jürgen Rehm

Institut für Mikrobiologie, Universität Münster, Corrensstrasse 3, D-4400 Münster, Federal Republic of Germany

Summary. The antibacterial activity of phenol was determined by measuring inhibition of exponentially growing free and immobilized cells of *Escherichia coli*, *Pseudomonas putida* and *Staphylococcus aureus*. Immobilization of microorganisms in calcium alginate beads reduced the growth inhibition caused by bacteriostatic concentrations of phenol. The increase in phenol tolerance occurred at different culture conditions and growth rates of the cells. The strength of the effect, however, was found to correlate with the formation of colonies in the gel matrix. Dissolution of gel beads led to a substantial loss of the protection against phenol of immobilized-grown cells.

Introduction

In biological waste water treatment, immobilization of microorganisms has a great tradition. Adsorption of organisms on activated carbon is one possibility which has the advantage that microbial resistance to compounds like phenol is increased because the pollutant itself is bound and so removed from the water phase (Ehrhardt and Rehm 1985).

The entrapment of cells in alginate is another promising method for microbial degradation of toxic substances (Westmeier and Rehm 1985). These biocatalysts are also favourable for investigation of the physiology of immobilized microorganisms. The immobilization method is not toxic to the cells, and the dissolution of gel particles, and thus the liberation of the immobilized cells, is easy and rapid (Vorlop and Klein 1983).

Offprint requests to: H.-J. Rehm

The degradation of phenol by *Pseudomonas putida*, immobilized in calcium alginate, was examined by Bettmann and Rehm (1984). The authors found that these cells showed better degradation rates than free cells. Moreover, the immobilized bacteria could be exposed to higher phenol concentrations without loss of cell viability. The reasons for this are unknown. We examined the phenol tolerance of *P. putida* in a medium with glucose as carbon source to prevent the degradation-dependent change in phenol concentration. Further we studied the phenol tolerance of free and immobilized cells of *Escherichia coli* and *Staphylococcus aureus* which are examples of well-investigated microorganisms.

Materials and methods

Microorganisms. Escherichia coli K-12 strain Ymc (lac^-, supF, λ^s) and *Staphylococcus aureus* (no 257) were derived from the strain collection of our institute. *Pseudomonas putida* P8 was isolated from a phenol-contaminated waste water (Bettmann and Rehm 1984).

Media. The minimal medium contained (per l): $(NH_4)_2SO_4$, 1.06 g; $MgSO_4$, 0.1 g; KCl, 0.74 g; K_2HPO_4, 2 g; $FeSO_4$, 1.6 mg, and 1 ml of a trace element solution (Pfennig and Lippert 1966). The pH was set to 7.5. Glucose (2 g/l) or sodium succinate (5.4 g/l) were added as carbon sources before inoculation. The medium was supplemented with sodium citrate (0.3 g/l) in the case of *E. coli*, with yeast extract (0.1 g/l) for *P. putida*, and with thiamine (0.1 mg/l) and yeast extract (0.2 g/l) for *S. aureus*. The complex medium was Standard I nutrient broth (Merck, Darmstadt, FRG).

Culture conditions. Batch cultures of free and immobilized bacteria were grown in a gyratory shaker at 30° C for *E. coli* and *P. putida*, and at 35° C for *S. aureus*. Free cells were inoculated from overnight cultures in 50 ml medium. Immobilized cells in 40 ml alginate were suspended in 160 ml medium which was concentrated to compensate for the increase in volume by the alginate gel particles.

Immobilization. Alginate (Manugel DJX) was obtained from Alginate Industries, Hamburg, FRG. The entrapment of cells was carried out according to the method of Eikmeier and Rehm (1987). Bacteria of a preculture were suspended in sodium alginate by stirring for 15 min to obtain a 2% alginate suspension. The mixture was extruded through a thin needle (30 × 0.65 mm) into a 1% $CaCl_2$ solution, and left to harden for 1 h. The free Ca^{2+} was then washed out twice with saline, followed by transferring the gel beads into growth medium.

Dissolution of biocatalysts. The alginate beads were separated from the medium and washed with 0.05 *M* potassium phosphate buffer, pH 7.0, for some minutes. The gel particles were dissolved by shaking at 30° C in 0.05 *M* sodium hexametaphosphate, which rapidly (8–10 min) liberated the cells without loss of viability.

Determination of growth rate and minimal inhibitory concentration. Cell growth was monitored by measuring the optical density ($OD_{560\,nm}$) with a Hitachi 101 spectrophotometer (Tokyo). In some experiments, the total and the viable cell number were also determined to verify the OD measurements. The cell density of immobilized bacteria, determined after dilution with sodium hexametaphosphate for gel dissolution, was calculated for the volume of the biocatalysts. Phenol was added at approximately equal cell concentrations for free and immobilized cells in experiments that were compared. With regard to immobilized cells the phenol concentrations were adjusted to the total volume of liquid and gel phase. Growth rates after phenol addition were calculated from the exponential phase of reduced growth. The growth rates were set in relation to that of an unpoisoned control; this was done in every experiment.

The minimal inhibitory concentration was defined as the phenol concentration that completely blocked growth. It was detected in a series of tests with concentration steps of 0.25 g/l. This concentration was always identical to the minimal concentration that reduced the vial count determined at 4 h after toxification in comparison to the value obtained at the moment of phenol addition.

Scanning electron microscopy (SEM). The samples were fixed for 1 h in a solution of glutaraldehyde (2% in 0.15 *M* Sörensen buffer, pH 7.0), followed by dehydration in 30%, 50%, 70%, 80%, 90% and 96% ethanol (each step for 1 h), and critical point drying. In a "sputter coating" process the particles were covered with a gold layer to increase their electrical conductivity. The micrographs were made with a Hitachi scanning electron microscope (type S 450).

Results

Phenol toxicity of free and immobilized cells of Escherichia coli

Escherichia coli K-12 was immobilized in alginate spheres with a diameter of about 3 mm. Compared with free cells, these cells showed a slightly but significantly increased growth rate in a minimal medium with glucose as growth substrate. After addition of phenol to the medium a reduction of the concentration corresponding to the total volume of the alginate beads was established.

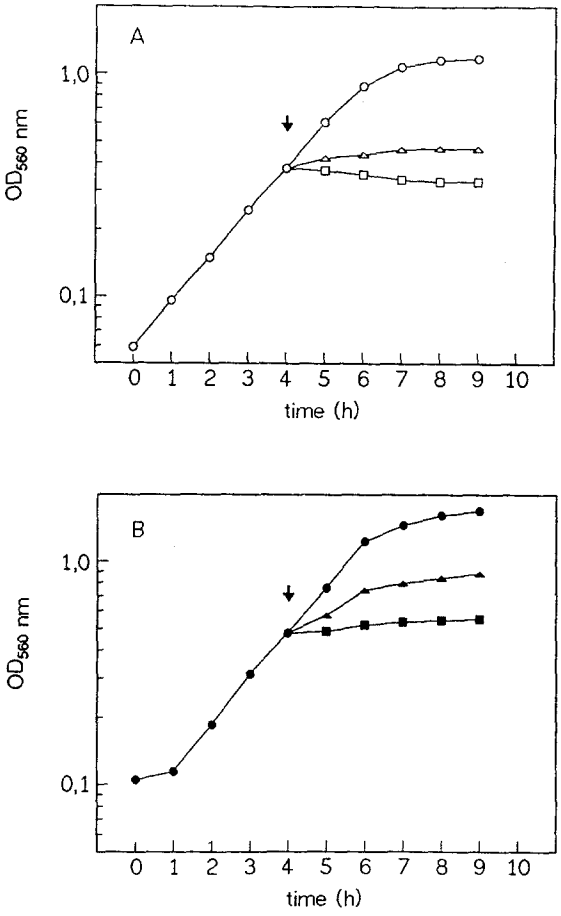

Fig. 1A, B. Growth inhibition of *Escherichia coli* by phenol. Free (**A**) and calcium alginate immobilized cells (**B**) were grown in minimal medium with glucose as growth substrate. Phenol was added at the time indicated by the *arrow* at a concentration of 1 g/l (△, ▲) or 2 g/l (□, ■). Control cells without phenol: ○, ●

The quantity of phenol dilution was in accordance with an even distribution of phenol in the liquid and in the gel phase as previously demonstrated by other authors (e.g. Hering 1987). Therefore we could conclude that the phenol concentration inside the gel was equal to the concentration outside.

When bacteriostatic concentrations of phenol were added to cells after 4 h of exponential growth, the bacteria continued to grow at a reduced rate for 2–4 h and then entered the stationary phase at a lower cell density. Free cells were incubated under the same conditions with phenol. Figure 1 shows that 1 g/l phenol inhibited the growth of suspended cells 3–4 times more strongly than the growth of immobilized cells. This reduced phenol toxicity of immobilized bacteria was found for all bacteriostatic concentrations tested (Fig. 2). When 2 g/l phenol was ad-

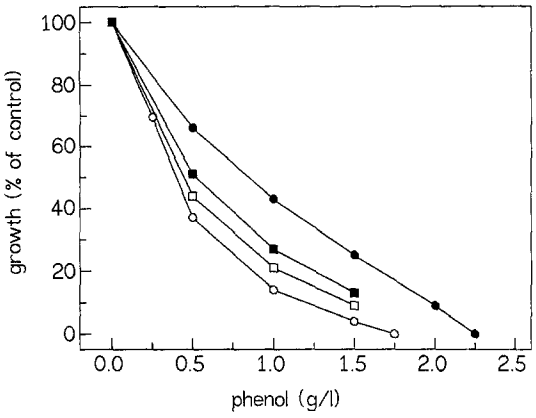

Fig. 2. Effect of phenol on growth of free and immobilized *E. coli* in minimal medium with glucose. Growth inhibition of immobilized grown cells was measured before (●) and after dissolution of the gel followed by incubation of the liberated cells in growth medium (■). The free cells were measured as usual (○) or immobilized and liberated immediately afterwards before toxicity determination (□)

ded to cells, only the immobilized bacteria were still able to grow (Fig. 1). This shows that the minimal inhibitory concentration was different: the immobilized cells tolerated about 0.5 g/l phenol more than the free cells (Figs. 2 and 3). The use of higher concentrations led to a drastic reduction of the viable cell number for immobilized and for free cells. The bactericidal action of phenol is apparently also influenced by the immobilization of the microorganisms.

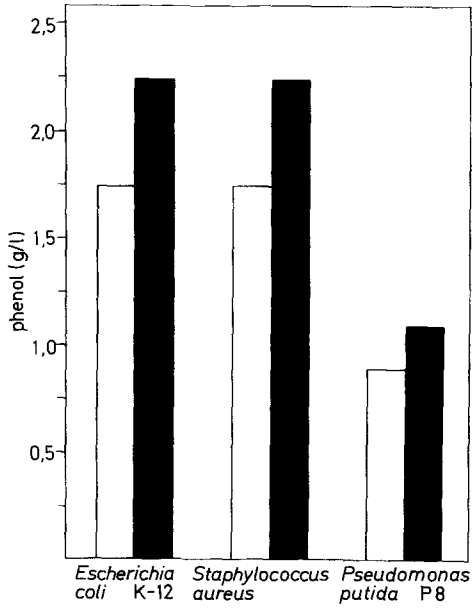

Fig. 3. Minimal inhibitory phenol concentrations of different bacteria. Free (□) and immobilized cells (■) were grown in minimal medium with glucose

Minimal inhibitory concentrations of S. aureus and P. putida

The growth inhibition induced by phenol was also examined for two other bacteria in the free and in the immobilized state. *Staphylococcus aureus* was chosen because this species is often used in toxicity studies. Besides, it is a Gram-positive organism which has a markedly different structure of the cell envelope. Nevertheless, immobilization also increased the phenol tolerance of *S. aureus* cells. Figure 3 shows that the minimal phenol concentration that completely blocked growth was, as for *E. coli*, higher in the case of immobilized cells. At all other bacteriostatic concentrations tested, immobilization into the alginate gel was advantageous.

Free and also immobilized-grown cells of *P. putida* P8 were more sensitive to phenol than the first two species. This is surprising because the strain is able to use this substance as a substrate for growth. When grown with glucose as carbon and energy source (Fig. 3), the minimal inhibiting concentration of phenol was higher for immobilized cells, as observed for the other bacteria.

Variations in growth conditions for Escherichia coli

Apparently, the culture conditions can be changed to a great extent without loss of protection by immobilization in alginate. The increased phenol tolerance of immobilized *E. coli* cells was also apparent when glucose was replaced by succinate in the minimal medium (Fig. 4). The phe-

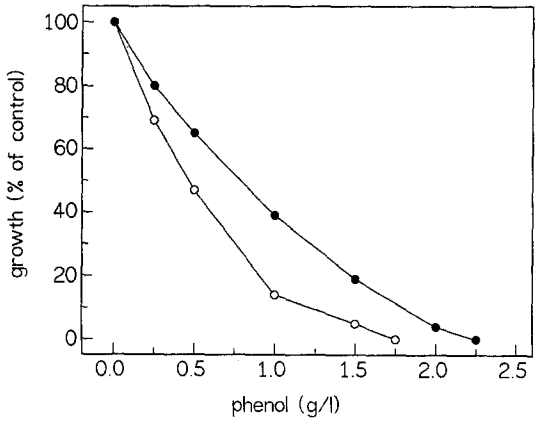

Fig. 4. Growth inhibition of *E. coli* by phenol in minimal medium with succinate. The different phenol concentrations were added to cultures of free (○) and immobilized cells (●) after 3-4 h of exponential growth. The growth rate after the addition was set in relation to a control without phenol

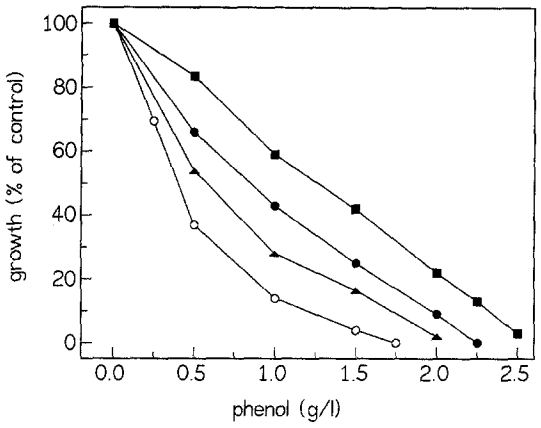

Fig. 5. Effect of different cell numbers in the gel microcolonies on growth inhibition by phenol. Free (○) and immobilized cells *(closed symbols)* of *E. coli* were grown in minimal medium with glucose. Phenol was added at a total cell concentration of $4.8–5.5 \times 10^8 \times ml^{-1}$. ●, biocatalysts seeded with 2.2×10^6 cells per gel bead (grown for 4 h), ■, biocatalysts seeded with 1.0×10^5 cells per gel bead (grown for 8 h), ▲, biocatalysts seeded with 7.0×10^6 cells per gel bead (grown for 2 h)

nol concentrations that completely blocked growth showed the same difference as glucose. In these experiments the growth rates of the non-inhibited free and immobilized cells were decreased to about 70% compared with the growth rates in the glucose-containing medium.

The increase in growth rate with the use of a complex medium did not change the protection against phenol reached by immobilization in alginate (for details see Heipieper 1988). However, in this case low phenol concentrations induced lower inhibition of growth than in minimal medium, probably because of adsorption of the phenol to lipophilic constituents in the medium. Also the difference between free and immobilized cells was smaller.

Cultivations with calcium alginate biocatalysts are carried out in media of low phosphate concentrations to maintain the stability of the gel particles. However, it is known that the toxicity of phenol may depend on the pH of the environment (Hamilton 1971). Therefore, the buffering capacity of the gel matrix could cause the decreased sensitivity of the cells inside. Thus we studied the effect of increasing the phosphate concentration in the medium (Heipieper 1988); the results showed that the influence of buffer capacity was negligible and did not give an explanation for the cell protection by immobilization.

Significance of formation of cell colonies in the gel matrix

A partial loss of the protection against phenol occurred when the alginate beads were seeded with a higher cell concentration and received the phenol addition after 2 h instead of 4 h of growth (Fig. 5). Also cells were examined that grew for a longer time (8 h) inside gel beads which had a smaller inoculum. Although the total cell density at the time of poisoning was as high as in the other experiments, larger colonies could develop during the growth period. The cells in this experiment had an even higher phenol tolerance (Fig. 5). These results demonstrate that the response of immobilized cells to phenol is independent of the total cell concentration in the gel. However, the number of generations of the immobilized cells and, apparently, the formation of cell aggregates are important for increased phenol tolerance. The

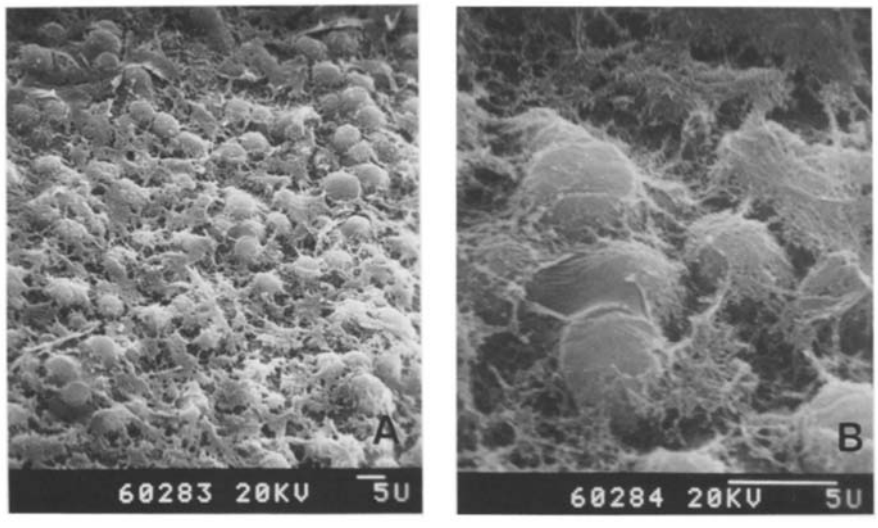

Fig. 6 A, B. Scanning electron micrographs of *E. coli* immobilized in calcium alginate. The general view (**A**) and details (**B**) of the gel surface show the microcolonies at the moment of phenol addition as described in Fig. 1

more cells are located closely together in the microcolonies, the less is the damage caused by phenol.

Figure 6 shows that distinct multicellular arrangements were indeed formed by *E. coli* after growing for 4 h inside the gel. As described for other immobilized aerobic microorganisms (Bettmann and Rehm 1984), the microcolonies were confined to the outer area of the beads probably because of oxygen limitation. A smooth extracellular layer is apparently built by the colony, but we cannot exclude that this is an artefact of sample preparation for SEM.

If the organization of cells in a microcolony is important for phenol tolerance, it can be expected that the increase in tolerance drops after the dissolution of the cell aggregations. Therefore cells were grown for 4 h as usual in the immobilized state. Before determination of phenol tolerance, the alginate beads were dissolved and thus immobilized-grown bacteria were tested as suspended cells. They were compared with cells that grew for the same period of time in a free mode but were immobilized shortly before phenol addition and dissolved immediately after. Thus the influence of the immobilization procedure and of the dissolution of beads on cell physiology and phenol tolerance was eliminated. The results of this experiment show that shortly (1 h) after separation of the cells much of the tolerance gained was lost (Fig. 1). The growth inhibition induced by phenol was only to the extent that was reached by the control cells. These cells, however, showed a significantly higher phenol tolerance than cells that had never come in contact with alginate.

Discussion

Phenol degradation of *P. putida* immobilized in alginate was studied by Bettmann and Rehm (1984). They observed that immobilized cells tolerated higher phenol concentrations than free cells. This was also found in our studies with bacteria lacking the potential for phenol degradation. Therefore, the tolerance enhancement is rather a general characteristic of immobilization and not specific for phenol-degrading organisms. The effect is also apparently independent of culture conditions and growth rates. Moreover, a protection against more toxic phenol derivatives like chlorinated phenols by immobilization of microorganisms can be concluded from the studies of Westmeier and Rehm (1985). Considering that, a new great advantage of these biocatalysts is evident in the treatment of waste waters which are contaminated with substances of antimicrobial activity.

The phenol-degrading strain *P. putida* P8 was obviously more sensitive to phenol compared with the other bacteria tested. This surprising fact contradicts specific mechanisms of phenol-degrading bacteria to increase resistance. Karabit et al. (1985) also found a clearly higher phenol sensitivity for a *P. aeruginosa* strain than for *E. coli* and *S. aureus*. An explanation for this is, perhaps, the observation that lipopolysaccharide-deficient mutants have different sensitivities to phenols (Russell et al. 1987). The outer membrane of the Gram-negative bacteria may act as an obstruction for the access of these substances to the cell.

Many methods to determine minimal inhibitory concentrations rely on the control of the supposed endpoint of growth behaviour. In contrast to this, the continuous monitoring of the response of exponentially growing cells to a toxic agent is superior because it gives more and time-dependent information. This method of toxicity examination guarantees that only the reaction of growing and not of resting cells is recorded. The transition of cells from resting to growing is a more complex system, in which the toxic effects of phenol and the adaptive response of the cell are more difficult to study.

Owing to our method of toxicity examination, the advantage of immobilization could be elucidated: only when cell aggregates developed in the gel matrix was significant protection against phenol obtained. The size of the microcolonies apparently determines the extent of phenol tolerance. It is possible to suppose that the external cells of the colonies in the gel bind the phenol and so protect internal bacteria which continue to multiply without any inhibition. This different cell behaviour contradicts the fact that no lag phase was observed after phenol addition to exponentially growing cells. This was even true for the experiment in which the high-loaded biocatalysts could develop only very small colonies and so the protection from phenol was poor (Fig. 5).

It is known that in colonies the cells can be markedly different in morphology compared with free-grown cells. Shapiro (1987) found in *E. coli* colonies sharply delineated zones of bacterial populations differentiated from each other with respect to cell sizes, cell shapes and patterns of multicellular alignment. He suggested that the different cells have specialized tasks in the building of the colony. Furthermore he reported that extracellular material is deposited. This was also found in SEM studies of *P. putida* (Shapiro 1985); the

colonies of this bacterium are surrounded by a "membrane" of extracellular material.

In this work we observed an envelope surrounding the microcolonies formed by *E. coli*. Similar extracellular structures can be found with other bacterial strains immobilized and grown in alginate (unpublished results). Studies to elucidate this phenomenon are in progress. At the moment we do not know the function of this structure but it could have a significance in phenol tolerance. Possibilities for that are the binding of the toxic agent or the inhibition of diffusion into the colony. It can be excluded that the alginate matrix itself is a diffusion barrier for compounds of the size of phenol (Tanaka et al. 1984).

Mattiasson et al. (1984) proposed that a reduction of water activity is concerned in the change in physiology of immobilized cells. Our experiments show that it is not the different environment inside the gel particles, but the reduced water activity or other changed conditions in the microcolonies that influence the physiology of the cells. Shirai et al. (1988) found an increased oxygen uptake rate of growing hybridoma cells immobilized in calcium alginate. This effect correlated with the formation of colonies. The authors concluded also that close cell contact is a prerequisite to change in cell physiology. The similarities of these and our observations are striking although rather different cell types were used. However, common to both is that life without contact to other cells is not the usual form of existence in nature. This is surely also true for many other microorganisms. The rather artificial conditions of growing *E. coli* in a free state are apparently unfavourable for cell biosynthesis, demonstrated by a reduction of the growth rate, and also a disadvantage in the defence against toxic agents.

Considering the importance of gradients across the cytoplasmic membrane of bacteria, e.g. in the transport of solutes or cellular energetics, it is reasonable to assume that a very close cell-to-cell contact influences the dynamics of the membrane. Moreover, antimicrobial action of most phenols in bacteriostatic concentrations is probably caused by the injury of membrane function (Hamilton 1971; Davidson and Branen 1981). Some chloro- and nitrophenols are known as uncoupling agents of oxidative phosphorylation (Hugo 1978). However, phenol itself does not apparently cause the retardation of growth by inhibition of membrane-dependent energy production. This can be concluded from the fact that the replacement of glucose by succinate did not enhance the inhibitory action of phenol (Fig. 4). In this case energy production of *E. coli* relies solely on oxidative and not partly on substrate-level phosphorylation.

We conclude from our experiments that close cell-to-cell contact mainly influences the dynamic and not the structural aspects of the membrane. Shortly after dissolution of the gel particles, the liberated bacteria responded with similar sensitivity to phenol as cells grown in the free state.

The control cells of the last-mentioned experiment ran through the same immobilization and dissolution procedure to detect phenol-independent influences, e.g. of the dissolution agent. Unexpectedly, these cells were more resistant to phenol than cells that had never come in contact with alginate and hexametaphosphate. We have evidence that impurities in the alginate were responsible for this phenomenon and, at present, we are trying to elucidate it.

Acknowledgements. We thank Dr. Pfautsch, Institut für Medizinische Physik, Münster, FRG, for taking the scanning electron micrographs. This work was supported by a grant from the AIF (Arbeitsgemeinschaft Industrieller Forschungsvereinigungen).

References

Bettmann H, Rehm HJ (1984) Degradation of phenol by polymer entrapped microorganisms. Appl Microbiol Biotechnol 20:285–290

Davidson PM, Branen AL (1981) Antimicrobial activity of non-halogenated phenolic compounds. J Food Prot 44:623–632

Ehrhardt H, Rehm HJ (1985) Phenol degradation by microorganisms adsorbed on activated carbon. Appl Microbiol Biotechnol 21:32–36

Eikmeier H, Rehm HJ (1987) Stability of calcium-alginate during citric acid production of immobilized *Aspergillus niger*. Appl Microbiol Biotechnol 26:105–111

Hamilton WA (1971) Membrane active antibacterial compounds. In: Hugo WB (ed) Inhibition and destruction of the microbial cell. Academic Press, London, pp 77–93

Heipieper HJ (1988) Toxische Wirkung von Phenol auf freie und in Ca-Alginat immobilisierte Bakterien. Diploma thesis, Institut für Mikrobiologie, Universität Münster

Hering L (1987) Phenolabbau mit in Alginat immobilisierten Mikroorganismen in einem Modellboden. Diploma thesis, Institut für Mikrobiologie, Universität Münster

Hugo WB (1978) Membrane-active antimicrobial drugs — a reappraisal of their mode of action in the light of the chemiosmotic theory. Int J Pharm 1:127–131

Karabit MS, Juneskans OT, Lundgren P (1985) Studies on the evaluation of preservative efficacy I. The determination of antimicrobial characteristics of phenol. Acta Pharm Suec 22:281–290

Mattiasson B, Larsson M, Hahn-Hägerdahl B (1984) Metabolic behavior of immobilized cells — effects of some microenvironmental factors. Ann N Y Acad Sci Enz Eng 7:475–478

Pfennig N, Lippert KD (1966) Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch Mikrobiol 55:245–256

Russell AD, Furr JR, Pugh WJ (1987) Sequential loss of outer membrane lipopolysaccharide and sensitivity of *Escherichia coli* to anti-bacterial agents. Int J Pharm 35:227–233

Shirai Y, Hashimoto K, Yamaji H, Kawahara H (1988) Oxygen uptake rate of immobilized growing hybridoma cells. Appl Microbiol Biotechnol 29:113–118

Shapiro JA (1985) Scanning electron microscope study of *Pseudomonas putida* colonies. J Bacteriol 164:1171–1181

Shapiro JA (1987) Organization of developing *Escherichia coli* colonies viewed by scanning electron microscopy. J Bacteriol 169:142–156

Tanaka H, Matsumara M, Veliky IA (1984) Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26:53–58

Vorlop KD, Klein J (1983) New developments in the field of cell immobilization. Formation of biocatalysts by ionotropic gelation. In: Lafferty R (ed) Enzyme technology. Springer, Berlin, Heidelberg, New York, pp 219–235

Westmeier F, Rehm HJ (1985) Biodegradation of 4-chlorophenol by entrapped *Alcaligenes* sp. A 7-2. Appl Microbiol Biotechnol 22:301–305

Received 15 December, 1988/Accepted 10 April, 1989