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Environmental applications of immobilized microbial cells: a review

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Immobilized microbial cells have been used extensively in various industrial and scientific endeavours. However, immobilized cells have not been used widely for environmental applications. This review examines many of the scientific and technical aspects involved in using immobilized microbial cells in environmental applications, with a particular focus on cells encapsulated in biopolymer gels. Some advantages and limitations of using immobilized cells in bioreactor studies are also discussed.

Keywords: alginate; bacteria; biodegradation; bioremediation; κ-carrageenan; encapsulation; immobilization; microorganisms; soil

Introduction

Immobilized microbial enzymes, organelles, and cells have been used in a variety of scientific and industrial applications. The economic importance of immobilization has resulted in considerable research for industrial applications. Immobilization technology has been used extensively in commercial bioreactor fermentations [151]. Reviews on the use of immobilized organelles [131,132] and enzymes [138–140] have addressed the importance of these respective technologies. Immobilized cells have also been used in bioreactors, and production of useful compounds such as amino acids, organic acids, antibiotics, steroids and enzymes using immobilized cells have been reviewed by Brodelius and Vandamme [24] and Tanaka and Nakajima [199]. Various whole cell techniques [5,103,104,106, 131,132,233] and the many applications [5,39,180,199] possible have been examined. The commercial success of these processes illustrates the value of using immobilization technology under controlled conditions.

Immobilization is a general term that describes many different forms of cell attachment or entrapment. These different forms include flocculation, adsorption on surfaces, covalent bonding to carriers, cross-linking of cells, encapsulation in a polymer-gel and entrapment in a matrix (Figure 1). The advantages of various methods of cell immobilization for bioreactor systems have been addressed in several reviews [44,104,106,107,108,109,198,233]. One method that has emerged as successful in the laboratory and useful in commercial applications is the encapsulation of cells in a polymer gel-matrix. Results from bioreactor studies have demonstrated that encapsulated cells have advantages over free cells under numerous conditions. For example, increased metabolic activity and metabolite production [64,178], protection from toxic substances [48,100,101,223,236], and increased plasmid stability [17,145,146] of encapsulated cells compared to free cells have all been observed.

Increased awareness of the profound effects of environmental problems, such as toxic waste sites and aspects of pesticide usage, has stimulated investigations of technologies which avoid, reduce or eliminate these problems. The use of immobilized cells has been investigated as an alternate technology for environmental applications. For example, the use of immobilized cells in agriculture [16,95,196,207], biocontrol [11], pesticide application [38] and pollutant biodegradation in contaminated soil or groundwater [18,88,122,153–155,176,226,238] have all been examined. However, most of the investigations were performed at the laboratory level, and applications of immobilized cells in the open environment have yet to be realized.

Many different microbial species have been encapsulated in various matrices for different applications. Fungal strains have been encapsulated for biocontrol [11,61] and biodegradation [7]. Encapsulated microalgae have been investigated for metal sequestration [62] and wastewater nutrient removal [37]. The use of encapsulated bacteria for metal uptake [166,232] and degradation of toxic compounds (Table 1) has been explored. Encapsulated thermophilic [96], methanogenic [178], and other anaerobic bacteria [98] have also been investigated for potential commercial uses. The variety of microorganisms which have been encapsulated successfully and the wide range of applications which have been explored attest to the utility and versatility of this technology and its potential for use in the environment. However, encapsulation of microorganisms in a gel-matrix as a carrier for environmental applications, such as the *in situ* bioremediation of contaminated soil or groundwater, is still in the early developmental stage.

This paper will review immobilization of microorganisms for pollutant biodegradation, with an emphasis on applying this technology for *in situ* bioremediation of chemically contaminated soil.

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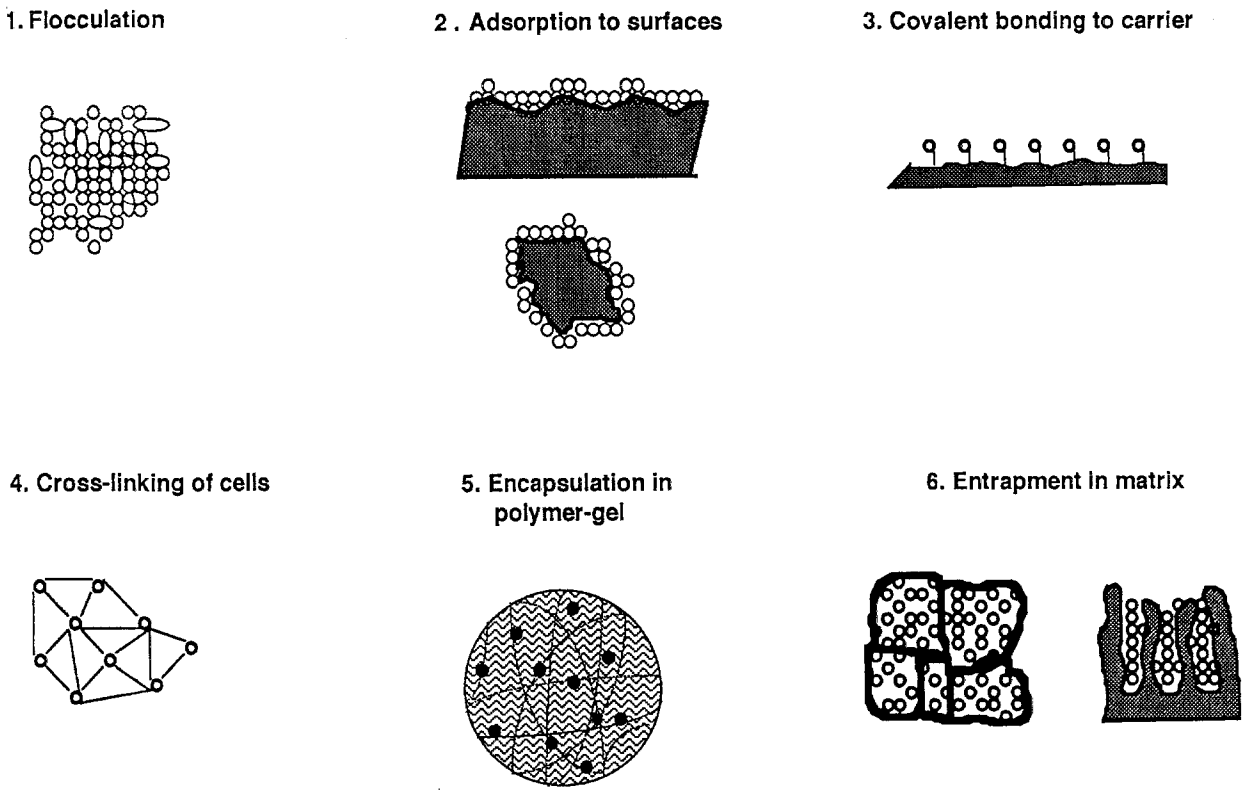

Figure 1 Methods for immobilization of viable microbial cells.

Uses of microorganisms in soil and their application

Microbial inoculants have been investigated for soil applications such as enhancement of symbiotic or associative N_2 fixation, biological control of soil-borne plant pathogens, biological control of frost injury and biodegradation of xenobiotic compounds [212]. Microorganisms have been introduced into soils as liquid suspensions or adsorbed to different carriers such as peat, charcoal, vermiculite and other organic particulate matter [190]. Formulations can contain microorganisms in a usable form and optimize the efficacy, stability, safety and ease of application [173]. Liquid cell-suspensions may be more easily contaminated, more difficult to contain in case of an accidental spill, and may have greater potential for off-site dispersal during application than dry carrier/cell formulations.

Once applied, the success of a bioremediation procedure depends on establishment of microorganisms and on expression of the necessary degradative genes [43,169]. Various methods of microbial introduction into soil have been reviewed [212]. The authors cited examples of variations in survival observed with inoculation into soil from a progressive decline in bacterial numbers to improved survival of rhizobia with the addition of clay, and they suggested results have been variable because soil is a highly heterogeneous and unpredictable environment for introduced bacteria. Both abiotic and biotic factors play critical roles in determining microbial survival. Soil moisture content, temperature, pH, texture, oxygen availability, rate of oxygen diffusion and nutrient availability have been suggested as abiotic factors controlling survival of introduced bacteria in soil [213]. Biological factors include predation by protozoans, a lower level of starvation resistance of the introduced bacteria [2], and lack of suitable soil niches for extended cell survival [212].

The use of carriers for microorganisms has been proposed as a means with which to overcome some of the problems associated with microbial survival in soil after inoculation. The advantages and disadvantages of various solid carriers for use in soil have been reviewed [207]. The authors suggested that an effective and safe carrier for soil-applied microorganisms should be non-toxic and non-polluting, consistent in quality, have a long shelf life, allow sufficient cell activity and cell density, and permit accurate release of bacteria into the target site. Encapsulation emerged as one of the best candidates because it provides many features which may aid in the introduction and establishment of microorganisms. McLoughlin [133] suggested that microenvironments in the bead may initially protect cells from the soil macroenvironment. Microorganisms are released after adaptation to prevailing environmental conditions. This may enable cells to overcome the numerous changing conditions in soil and increase microbial survival. He also suggested that immobilizing cells by encapsulation in a gel-matrix offers a stable, defined, consistent, protective environment, without the immediate release of large number of cells, where cells can survive and metabolic activity can be maintained for extended periods of time.

Carrier materials

Both natural and synthetic polymers have been used for microbial encapsulation. Algal polysaccharides such as agar, agarose, alginate and carrageenan are classified as natural polymers, whereas polyacrylamide, polystyrene and polyurethane are synthetic polymers [108]. Encapsulation

Table 1 Examples of solid carriers used to immobilize microbial cells for use in biodegradation

Compounds degraded or reactions	Microorganisms	Carriers	References
Acrylamide	*Pseudomonas* sp *Xanthomonas* sp	alginate	[148]
3-Chloroaniline	*Pseudomonas acidovorans* CA28	alginate	[58]
3-Chlorobenzoate dehalogenation	*Pseudomonas* sp B13	alginate	[175]
4-Chloro-2-nitrophenol	Mixed culture	alginate	[20]
2-Chloroethanol	*Pseudomonas putida* US2	granulated Lecaton-particles	[162]
Chlorophenols	Mixed culture	alginate	[115]
Chlorophenol	*Rhodococcus* spp	polyurethane	[209]
Chlorophenols	Activated sludge	celite R-633 microcarriers	[183]
Chlorinated phenols	Several strains	glass cellulose chitin	[168]
4-Chlorophenol	*Alcaligenes* sp A 7-2	alginate granulated Lecaton-particles	[223, 224]
4-Chlorophenol	*Alcaligenes* sp A 7-2	granular clay	[14]
p-Cresol	*Pseudomonas* sp	alginate	[153]
p-Cresol	*Pesudomonas* sp	alginate polyurethane	[155]
Cyanuric acid	*Pseudomonas* sp NRRL B-12228	granular clay	[56]
DDT	Mixed culture	alginate	[22]
Dechlorination of spent sulphite bleach effluents	*Streptomycetes* spp	polyurethane	[238]
Dichloroacetic acid	*Xanthobacter autotrophicus*	alginate	[81]
Glyphosate	Mixed culture	diatomaceous earth pellets	[75]
Hydrocarbons	*Candida parapsilosis*	granular clay	[161]
Inorganic cyanides	*Pseudomonas putida*	agar alginate κ-carrageenan	[34]
p-Nitrophenol	Mixed culture (*Pseudomonas* spp)	diatomaceous earth biocarrier	[82]
PAHs	Mixed culture	granular clay slag of lava	[226]
Pentachlorophenol	*Flavobacterium* sp	alginate	[153]
Pentachlorophenol	*Flavobacterium* sp	polyurethane	[154]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	alginate alginate and activated carbon	[122]
Pentachlorophenol	*Phanerochaete chrysosporium*	alginate	[121]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	alginate and activated carbon	[186]
Pentachlorophenol	*Flavobacterium* sp	polyurethane	[88]
Pentachlorophenol	*Flavobacterium* spp	polyurethane	[193]
Pentachlorophenol	Mixed culture	anaerobic granules	[234]
Phenol	*Candida*	alginate	[73]
Phenol	*Pseudomonas* sp	alginate polyacrylamide-hydrazide	[18]
Phenol	*Fusarium flocciferum*	agar alginate κ-carrageenan polyurethane	[7]
Phenol	*Candida* sp *Pseudomonas* sp	activated carbon	[51]
Phenol	Methanogenic consortium	agar	[48]
Phenol	Mixed culture	alginate chitosan/alginate	[236]
Phenol	Mixed culture	cellulose triacetate	[235]
Phenol	*Fusarium flocciferum*	polyurethane	[6]
Phenol	*Pseudomonas putida* P8 and *Cryptococcus elinovii*	activated carbon	[137]
Phenol	*Pseudomonas putida* P8	activated carbon	[52]
Phenol(s)	*Pseudomonas putida* P8	polyacrylamide-hydrazide	[19]
Pyridine	*Pimelobacter* sp	alginate	[116]
Raw surfactants/industrial waste	*Pseudomonas* C12B	polyacrylamide	[201, 202]
Sodium cyanide	*Pseudomonas putida*	alginate	[12]
Sodium dodecyl sulfate	*Pseudomonas* C12B	polyacrylamide	[225]
Substituted phenols	Various degraders	granular activated carbon	[191]
n-Valeric acid	*Alcaligenes denitrificans*	alginate	[32]
Volatile fatty acids	*Alcaligenes denitrificans*	polyacrylamide	[31]

of cells into micro-beads made from polyurethane have been investigated for use in groundwater remediation, with the authors citing the small size and strength of the polyurethane important for success of *in situ* applications, although the fate of the beads after successful clean-up was not addressed [87,193]. Criteria for gel-beads to be used for soil applications are different from those required for controlled bioreactor systems. Although the long-term stability and small pore sizes afforded by synthetic polymers can be effective for use in bioreactors, they may not be desirable in environmental applications. For example, with polyacrylamide, careful handling is required due to pre-polymer toxicity, and the stability of polyacrylamide beads in soil may not be environmentally acceptable [207]. As well, cell integrity and activity can be impaired during polymerization of the synthetic matrices [66], which in the case of polyacrylamide, generates heat and free radicals [35]. Despite this, a *Rhizobium* sp encapsulated in polyacrylamide infected legumes as effectively as free cells [46]. Polymer pore size may be a critical parameter in matrix selection for a particular process. The small pore size afforded by polyacrylamide encapsulation may be useful for applications in bioreactors because of the minimal leakage of proteins and cells from the matrix [200]. Larger pores are created when encapsulating cells with natural polymers, such as algal polysaccharides. This allows some cell leakage and bead degradation to occur within months [16,211]. Cell release and subsequent establishment in the soil matrix are advantageous in a soil application.

Natural polymers may provide improved bacterial survival after dessication and during storage. For example, with an initial inoculum of log 9.0 CFU ml^{-1} *Rhizobium* sp, Jung *et al* [95] recovered log 6.7 CFU ml^{-1} from dried alginate beads compared to log 1.5 CFU ml^{-1} from dried polyacrylamide beads after 150 days in storage at 28°C.

In general, non-toxic, natural polymers are recommended for use in soil. Encapsulation of cells in biodegradable gel matrices such as alginate or κ-carrageenan can be a safe and effective method for introducing bacteria into soil. Encapsulation processes are not stressful to cells, aseptic conditions minimize contamination, and the carriers are biodegradable and non-toxic. They also provide a slow release of microorganisms over time, while protecting cells until beads are degraded [16].

Characteristics of algal polysaccharides: alginate and κ-carrageenan

Alginate is produced by brown algae, principally *Macrocystis pyrifera*, but also by *Laminaria digitata*, *L. hyperborea* and *Eklonia cava*. Extracellular alginate is also produced by certain bacteria such as *Azotobacter vinelandii* and several pseudomonads [59,60]. Alginates are linear polymers of β(1,4)-D-mannuronic acid and α(1,4)-L-guluronic acid monomers. Different algae produce alginates that vary in monomer composition, arrangement and chain length. Therefore, the properties of alginates are variable. When exposed to Ca^{+2} ions, a cross-linking network is formed by the bonding of Ca^{+2} ions with polyguluronic portions of the polymer strands, a process known as ionic gelation. The best gel formers are those with high guluronic acid content because the guluronic acid units bind Ca^{+2} much more strongly than the mannuronic acid units. The gelation process is temperature-independent, thereby allowing a working temperature range of between 0 and 80°C. Typical alginate concentrations used in encapsulation vary between 1 and 8% (w/v) of sodium alginate in water. The carrier/cell suspension is extruded dropwise into a 0.05–2.0% $CaCl_2$ solution. Ionic bonding between interacting strands of polymers and Ca^{+2} ions occurs immediately on contact with the solution, and the resulting polymer network has been depicted as an 'egg-crate' matrix, in which the cells are entrapped [71]. The traditional method of extrusion with a syringe equipped with a 21-gauge needle results in wet gel beads of about 2–3 mm in diameter. Lancy and Tuovinen [114] encapsulated *Thiobacillus ferroxidans* cells in 1% (w/v) alginate, and calculated that with an average bead diameter of 3 mm, the average surface area was 28.3 mm^2, average volume was 14.1 μl^3, and the beads contained an average of 74% water. Beads, however, can be prepared in a broad range of particle sizes, typically 0.5–3.5 mm in diameter. Very small particles are also possible with special devices to obtain sizes of between 2 and 120 μm [103,194]. Complete gelling of alginate beads without cells was reported to be less than 30 min [67]; gelling time increased with cell addition prior to gelation, but decreased with increasing $CaCl_2$ concentration up to 1 M, and with increasing temperature over the range of 0 to 50°C. After gelation, encapsulated cells can be utilized as is, or placed in a nutrient solution to encourage additional cell growth inside the gel-matrix prior to use. Alternatively, beads can be dried after either process and stored until use. The alginate encapsulation process is mild and generally suitable for many different types of microorganisms.

Carrageenan is produced by red algae, principally *Chondrus crispus, Eucheuma cottonii*, *Gigartina stellata* and *G. radula*. Guisely [71] suggested that carrageenan affords the greatest diversity with respect to molecular structure and range of properties compared to the other natural polymers. Three types of carrageenan are produced and labelled by the Greek suffixes ι, λ, and κ. All carrageenans feature a common backbone of alternating β(1,3)-D-galactose and α(1,4)-D-galactose. The carrageenans differ in the number and site of sulfonation on both sugars. For the carrageenans, gelation is primarily temperature-dependent, and further strengthening of the polymer network occurs in the presence of K^+, or Al^{+3} ions as they interact with the sulfate residues on the polymer strands [71]. κ-Carrageenan, composed mainly of linear chains of alternating 3-0-substituted β-D-galactopyranose-4-sulfate and 4-0-substituted 3,6-anhydro-α-D-galactopyranose units, is often used in encapsulation processes because of its firmer gelling potential. Comparisons using different microorganisms and enzymatic reactions have demonstrated that gelation with K^+ ions resulted in the best performance [104], and solutions of 0.1–0.3 M KCl are traditionally used. Typically, 2–5% (w/v) κ-carrageenan in physiological saline is warmed to 70–80°C and maintained at 42°C. The cell suspension is also warmed to 40–50°C, added to the κ-carrageenan solution and extruded dropwise into cold KCl solution for gel formation. The main disadvantage of the carrageenan encapsulation system is that heating cells may

cause irreversible inactivation of some enzymes [103]. Despite this, many different types of microbial cells have been successfully encapsulated in this polymer and used for various applications in bioreactors.

Addition of amendments to formulations

Both alginate and κ-carrageenan carriers can be used either in their pure forms or with additional amendments which may enhance effectiveness. Amendments such as locust bean gum [8,10], or carob bean gum [28] have been incorporated for improved bead strength and stability in bioreactor systems. This level of stability is not required for soil applications, so different amendments have been investigated to provide a protective and/or nutrient source. Clay [61,95] and skim milk powder [99], or a combination of the two [29,119,206,211] are effective when used with alginate and κ-carrageenan carriers.

Clay

Addition of clay to carrier formulations can increase cell survival and act as a bulking agent providing additional mechanical strength. The effects of clays on bacterial survival in soil have been reviewed by England *et al* [55] and Stotzky [195]. In laboratory studies, certain clays offer advantages to bacterial survival in soil, but not all clays provide the same effect. For example, addition of montmorillonite clay to a sandy soil protected *Rhizobium trifolii* cells from heat at 70°C, while kaolinite did not [127]. This was interpreted as formation of a protective clay envelope around cells which modified rates of water flow into and out of the cells during drying and rewetting [126,128]. Bentonite clay by itself increases survival of rhizobia cells introduced into soil [78,79]. Clays can influence microbial events by modifying the physicochemical characteristics of microbial habitats, attenuating growth and metabolism of the microbial populations [195]. Clay-adsorbed cell systems have shown increased oxygen consumption by factors of 1.4 to 6.7 over free cells [124]. This indicates increased biological activity.

Studies in which clay has been incorporated in beads have demonstrated that survival results are not the same for all types of clay, or for all microorganisms interacting with various clays. For example, Jung *et al* [95] compared survival of two *Rhizobium japonicum* strains encapsulated in clay-amended alginate beads which had been dried and stored for 100 days. Cell numbers of *R. japonicum* strain 135 remained unchanged for 100 days when the alginate-*Rhizobium* slurry was amended with 5% kaolinite. However, this pattern was not observed for *R. japonicum* strain 138 whose numbers dropped from log 9.3 CFU ml^{-1} to log 1.2 ml^{-1} after 100 days storage. These results were similar to those of Bushby and Marshall [26] who reported that montmorillonite clay protected some bacterial strains (such as *Rhizobium leguminosarum* and *R. trifolii*) but not others (such as *R. lupini* and *R. japonicum*) from dessication. Therefore, the best combination of clay type and microorganism needs to be determined, and such a combination may provide additional benefits for microbial persistence. For example, *Pseudomonas fluorescens* R2f cells encapsulated in alginate beads amended with 3% (w/v) bentonite clay resulted in significantly higher cell survival (log 7 CFU g^{-1} dry soil) compared with free cells (log 3.5 CFU g^{-1} dry soil) or cells encapsulated in alginate alone (log 6 CFU g^{-1} dry soil) after 84 days in an agricultural soil from an initial level of log 8 CFU g^{-1} dry soil [211].

The ability to use existing mechanical equipment for inoculum handling and application is an attractive feature of these carriers. Formulations containing clay were judged to be easier to process, better suited for field applications, and more commercially feasible than those without clay [61,219]. Entrapping ectomycorrhizal mycelium in alginate and adding clay material to the gel produced a suitable inoculum for use in forestry compared with a vermiculite-peat mixture or mycelia alone [118]. The inoculum was tested for growth and mycorrhizal development of Douglas-fir and Norway spruce seedlings in a nursery. The authors observed significant increases in growth of both species over 6 months using alginate/clay-entrapped inoculum compared to peat or vermiculite with inoculum.

One potential problem with adding clay to the encapsulation formulation is the provision of charged surfaces which can bind heavy metals. Stotzky [195] suggested that clays may bind some heavy metal ions, reducing their bioavailability and potential toxicity in soil. However, clay-amended beads may concentrate metals to concentrations that are lethal to cells encapsulated within. Toxic metals may exchange with divalent cation cross-linkers in the alginate matrix [62], or metals may bind to beads due to the high cation exchange capacity (CEC) of clays. Wessolek and Fahrenhorst [222] mixed a modified alumina-silicate (Bergenite) into a soil contaminated with Zn and Cd to test metal binding to this clay-like substance. Their simulation model predicted immobilization of Zn and Cd to the Bergenite in the solid phase and also predicted this immobilization could last for 80 years. Although these predictions resulted from a simulation and heavy metal content varies for individual soils, this is an area that requires further research.

Skim milk as an amendment

Addition of skim milk to bead formulations provides a nutrient source which may increase activity, growth and/or survival. For example, increased persistence of bacteria was observed with skim milk-amended and alginate-encapsulated bacteria after they were dried [11,57]. This subject is further discussed in a review by Lievense and van't Riet [120]. Extrapolating from these results, the addition of skim milk may provide increased survival of encapsulated cells during drying and wetting cycles which occur in soil.

Pseudomonas fluorescens R2f cells encapsulated in alginate beads amended with skim milk showed improved survival in soil compared to both free cells and cells encapsulated in alginate beads only [211]. Sometimes, combined amendments in beads can result in further increases in bacterial survival. For example, Van Elsas *et al* [211] reported that a log 8 CFU g^{-1} dry soil *P. fluorescens* R2f cells encapsulated in alginate amended with 3% (w/v) skim milk powder and 3% (w/v) bentonite clay maintained cell numbers at log 8 CFU g^{-1} dry soil in soil columns over 84 days. In the same study, the number of cells in unamended alginate dropped to log 6 CFU g^{-1} dry soil, cells encapsulated in alginate with clay only dropped to log 6.5 CFU g^{-1} dry soil

and cells encapsulated in alginate amended with skim milk dropped to log 7 CFU g^{-1} dry soil. Hekman *et al* [83] reported similar findings using an initial inoculum of log 7.2 CFU g^{-1} dry soil *P. fluorescens* R2f cells in soil columns. Cell numbers in CFU g^{-1} dry soil after 45 days were: unamended alginate, log 7.34; 3% (w/v) skim milk-amended alginate, log 7.82; 3% (w/v) bentonite clay-amended alginate, log 6.33; and 3% (w/v) skim milk and 3% (w/v) bentonite clay-amended alginate, log 8.72.

Use of alginate and κ-carrageenan as carriers

Alginate has been more widely used and studied than κ-carrageenan due to the mild conditions (eg ambient temperature) of the encapsulation process [187]. In particular, alginate has been used extensively in bioreactors (Table 1). Studies in soil demonstrated increased survival of *P. fluorescens* R2f cells encapsulated in alginate compared with free cells [206,211].

The elevated temperatures (35–55°C) necessary to maintain the appropriate viscosity of κ-carrageenan during cell encapsulation may be stressful to some microbial species and some enzymes may be inactivated. Special commercial types of κ-carrageenan eg Genugel X-8086 and X-0909 (Copenhagen Pectin, Denmark) remain viscous at 20°C, but K^+, Ca^{2+} and NH_4^+ ions have to be excluded from the solution, and cell loadings higher than 20% (g wet cells per 100 g wet catalyst) are difficult to obtain. Because of these limitations, temperatures above 35°C are recommended for encapsulating cells even in these specialized κ-carrageenan formulations [105]. A number of studies have demonstrated the effectiveness of κ-carrageenan encapsulation of microorganisms for use in bioreactors [203,218,227, Table 1], but few studies have investigated its use with microorganisms targeted for soil applications.

κ-Carrageenan is usually less expensive than alginate which can substantially reduce costs in a large-scale environmental application. Little information is available on altering encapsulation conditions to enable use of standard κ-carrageenan at temperatures below 30°C. Research in our laboratory to overcome this problem indicated that a formulation of 1% (w/v) κ-carrageenan amended with clay and skim milk allows encapsulation to occur at 30°C or lower [29]. Alginate and κ-carrageenan amended with 5% (w/v) clay and 1% (w/v) skim milk were used as encapsulation materials for *Pseudomonas aeruginosa* UG2Lr cells. After drying the κ-carrageenan beads, and storing them at 4°C in the dark, survival in κ-carrageenan was significantly better than in alginate in the first 3 months, and similar to but not significantly different over a year.

κ-Carrageenan may be a useful encapsulation carrier for bioremediation purposes in soil for reasons other than potential cost reduction. Dry κ-carrageenan beads swell to about 25 times their volume when placed in an aqueous solution and the polymer network is still chemically stable [106]. However, dry alginate beads do not swell in an aqueous medium. This may physically limit potential cell growth inside the beads, and may cause extra stress and physical pressures as the cells multiply. For example, Stewart and Robertson [192] studied *Escherichia coli* cells encapsulated in alginate and observed that when cells were physically stressed, the porosity of the bead decreased, cell shape was distorted, cell size was reduced and dewatering of encapsulated cells was possible due to the compression generated by cell growth. The porosity of alginate beads may also be significantly reduced by drying the beads partially. If beads are composed of an alginate high in guluronic acid, they will re-swell only slightly in water with reduced average pore size [187]. Only when alginate beads come in contact with a calcium sequestrant such as phosphate will they weaken or dissolve [71]. Even though the stability of κ-carrageenan beads is temperature dependent, Nguyen and Luong [149] suggested that bead pore size is unlikely to be affected between 10 and 25°C since the diffusivity of glucose remains unchanged in this temperature range. Further research will help to establish the value of using κ-carrageenan as an encapsulation medium for microbial cells intended for environmental applications.

Advantages of using encapsulated bacterial cells for soil applications

The use of encapsulated cells for soil applications provides a number of advantages over free cells or other carriers such as peat, vermiculite or charcoal. These are outlined in Table 2 and discussed further below.

Formulations must be carefully designed to maintain viability and activity during extended storage and distribution. Immobilization under aseptic conditions allows entrapment of only the cells of choice. Cells adsorbed onto activated carbon and kept at 4°C kept their degradation capacity for up to 12 months [137]. Dried beads can be stored for up to 3 years at 4°C [29,141], and the bead structure provides protection against contamination during storage, transport and application [95]. Alginate carrier formulations prolonged storage life and provided protection from mechanical abrasion during transport and application [11]. Existing mechanical equipment can be used for the application of inocula, and since no bioaerosol formation

Table 2 Advantages and limitations of using encapsulation for soil applications

Advantages

1) Reduced possibility of inoculum contamination during storage, transport and application
2) Reduced possibility of off-site drift during application
3) Beads are non-toxic, biodegradable and non-polluting
4) Can be produced in large quantities, stored for extended periods as dried beads and used with existing mechanical application equipment
5) Provides protection from biotic and abiotic environmental stresses leading to increased microbial survival
6) Increased metabolic activity of encapsulated cells
7) Slow cell release with reduced cell movement through soil from water flow-induced transport
8) Increased plasmid stability

Limitations

1) Gas and solute diffusion may be restricted
2) Reduced oxygen consumption rates of encapsulated cells may occur
3) Cell morphological or metabolic alterations may have a detrimental effect
4) Effects of changes in water activity may limit effectiveness
5) Cells may not establish colonies outside of beads. Repeat applications of beads may be required.

occurs, the possibility of drift off-site is minimized. The importance of bacterial containment, detection and survival in the environment was reviewed by Jackman *et al* [93]. These areas are of prime concern, particularly when considering the use of genetically-engineered microorganisms (GEMs). Accidental spills of encapsulated cells in dried beads can likely be contained and cleaned up more easily than a liquid cell-suspension spill.

Potential for commercial production of large bead quantities

For a large scale application of encapsulated microorganisms to a contaminated soil site, large quantities of uniform carrier must be prepared. Research has been conducted on large scale production of beads necessary for environmental applications. A number of methods have been explored to scale-up bead production. For example, a resonance nozzle technique allowed production capacity of uniform alginate beads to increase by two orders of magnitude to 24 L h^{-1} over the conventional procedure while maintaining viability of encapsulated yeast (*Saccharomyces cerevisiae*) and plant (*Haplopappus gracilis*) cells [90]. A production rate of 1.05 dm^3 h^{-1} of alginate micro-beads (500-μm diameter) has been achieved using a rotating disk atomizer which disperses the aqueous alginate solution in air [157]. Stormo and Crawford [194] used a low-pressure ultrasonic nozzle apparatus to produce consistent beads of very small sizes quickly. The beads ranged from 2 to 50 μm in diameter. A maximum production rate of 27.6 dm^3 h^{-1} of uniform (2-mm diameter) κ-carrageenan beads was obtained by breaking up a capillary jet of κ-carrageenan solution with sinusoidal vibrations [91]. Poncelet *et al* [167] designed a parallel plate electrostatic droplet generator to produce consistent microbeads from 480 μm to 750 μm in diameter depending on needle size. They observed that there was no detectable effect of the applied potential on viability of *Spodoptera frugiperda*.

Any of these techniques allow large-scale production of encapsulated cells, and the variety of techniques also allow alternatives for particular applications.

Protection of encapsulated microbial cells

The protective features of the carriers are the basis for their potential value for applications in soil. Results of inoculating free cells into agricultural soils have been variable, with both successes and failures being described. Generally, a slow decline of introduced bacteria has been observed [208]. Encapsulation provides a microenvironment for the cells different from the macroenvironment, and this may minimize some of the detrimental effects. The improvement in survival of encapsulated cells over free cells in soil that has been observed is a good indication of the potential value of encapsulated cells for environmental applications. For example, Van Elsas *et al* [211] reported that free *P. fluorescens* R2f cells decreased from log 7.3 to log 3.6 CFU g^{-1} dry soil in a loamy sand over 84 days. Cells encapsulated in alginate with or without adjuvants either maintained initial cell levels, or decreased by only one log unit. Trevors *et al* [206] observed similar results using the same microorganism and the same soil. They reported that free cells decreased from log 7.8 to log 4.1 CFU g^{-1} dry soil after 63 days, whereas alginate-encapsulated cells (with or without adjuvants) maintained a level of log 8 CFU g^{-1} dry soil throughout the experiment. Using the same microorganism and soil, Hekman *et al* [83] observed no significant reduction over 45 days in the number of encapsulated cells whereas that of free cells decreased significantly from log 7.16 to log 5.33 CFU g^{-1} dry soil. Leung *et al* [119] reported that κ-carrageenan encapsulated *Pseudomonas aeruginosa* UG2Lr cell numbers increased 190-fold to log 8.78 CFU g^{-1} dry soil after 1 week in a forest soil and remained at that level for 3 weeks. Free cells declined steadily from log 9 to log 4.94 CFU g^{-1} dry soil. Weir *et al* [220] reported an increase of alginate/clay/skim milk-encapsulated *P. aeruginosa* UG2Lr cells in a sandy loam from log 6.85 to log 7.7 CFU g^{-1} dry soil after 98 days whereas free cells declined from log 9 to log 4 CFU g^{-1} dry soil. The increase in bacterial survival observed may be the result of protection of the cells from environmental abiotic stress, predation and/or toxicity, and will be discussed in the next three sections.

Protection of encapsulated microbial cells from environmental stress

Encapsulation may improve cell survival and provide a means for establishment of cells introduced into a site [206,211] by protecting cells from various environmental stresses. The bead can be viewed as a natural soil aggregate containing microbial microcolonies both on its surface and in its interior. It has been suggested that free cells may lose their viability more readily than those sorbed to soil particles [77]. The physical soil environment is heterogeneous and changing environmental conditions can result in various alterations of the soil. Encapsulation provides not only protection, but a more stable microenvironment for the entrapped microbial cells. For example, Trevors *et al* [206] demonstrated that a population of *P. fluorescens* R2f cells encapsulated in alginate remained stable after one drying/rewetting cycle in soil, whereas free cells were reduced by about log 2 CFU g^{-1} dry soil. Subsequent soil drying/rewetting cycles did not result in as large a difference. Kearney *et al* [99] suggested that protection during dehydration and bead rehydration may occur because the diffusional properties and limited volume of beads control the rate and volume of fluids entering and exiting beads. Less chance of osmotic shock is possible because an instantaneous removal or influx of water does not occur. The addition of skim milk or clay as an amendment to beads may provide further protection during drying and rewetting cycles [77,206].

The protective effect of encapsulation during freeze/thaw cycles was demonstrated in our research using a new κ-carrageenan formulation. Survival of free and κ-carrageenan-encapsulated *P. aeruginosa* UG2Lr cells in an acidic forest soil was monitored for 3 weeks, after which time the soil was frozen and thawed three times in succession. Survival of encapsulated UG2Lr cells was significantly higher by four orders of magnitude than free cells in both sterile and non-sterile soil, demonstrating that encapsulation protected cells from freezing and thawing cycles [119].

Encapsulation appears to minimize the effects of some

environmental parameters, resulting in a more stable environment. For example, alginate-encapsulated *Gluconobacter oxydans* cells maintained consistent oxidative activity with variations in pH and temperature during oxidation of glycerol to dihydroxyacetone while decreases were observed with free cells [4]. Buzas *et al* [27] reported that fermentation capacity of *Saccharomyces cerevisiae* cells was dependent on pH, with an optimal value of pH 4, whereas the fermentation capacity of alginate-encapsulated cells was independent of H^+ concentration between pH 2.5 and 6.2. Soil may contain small pores with variable pH. We hypothesize that beads and amendments such as skim milk powder may buffer against changes in pH, making conditions more stable for survival and activity of cells in beads in the soil environment.

Protection from predation

Predation of soil bacteria by protozoans has been cited as a factor for a decrease in cell numbers after their introduction into soil [42,72]. Effects of protozoans on bacterial survival in soil has been reviewed by England *et al* [55] who concluded that protozoa play an important role in determining microbial survival and activity. There is little evidence to demonstrate the protection from predation afforded by encapsulation of microbial cells. However, if we examine the similarity between a soil aggregate and a biopolymer bead, there may be some credence to the idea of protection from predation. Vargas and Hattori [216] observed that *Aerobacter aerogenes* cell numbers decreased in the presence of *Colopoda* sp in the outer 1–2 mm of a soil aggregate, whereas no reduction in cell number was found in the inner zone after the same period of exposure. England *et al* [55] suggested that spatial location within aggregates influenced susceptibility of bacterial cells to predation. It was suggested that physical restrictions do not allow predator access. For example, protective microhabitats with pore sizes less than 6 μm significantly reduced predation, as demonstrated by addition of bentonite clay, which effectively reduced soil pore sizes [80]. Pore sizes in this range do not allow grazing by protozoan and other predators due to size restrictions. It has also been suggested that in soil aggregates, pores 2–6 μm in diameter may be the most favourable microhabitats of bacteria [77], and capillary water is also held in pores of such sizes. Encapsulation may provide a microhabitat to protect cells in a similar manner. The matrices created by alginate and κ-carrageenan encapsulation are in this size range, and electron micrographs of the interior of κ-carrageenan beads indicate the pore size to be about 4–5 μm in diameter (Figure 2). Similarly, pore sizes ranging from 0.1 to 5 μm have been observed with alginate beads [30,110,144,215]. However, Danso *et al* [42] suggested that protection from protozoan predation may not be the result of microhabitats because they had observed similar bacterial survival in sterile pond water as in soil.

Protection of alginate-encapsulated *P. fluorescens* R2f cells from the bacteriophage ΦR2f in soil resulted in increased survival compared to free cells, although cells released from beads were still susceptible to phage attack [188]. It should be noted that in a contaminated soil, protozoans and other predators may be limited in numbers due to pollutant toxicity.

Protection from toxicity

Persistence and activity of microorganisms added to a contaminated soil may be adversely affected by the presence of toxic compounds. Providenti *et al* [169] reviewed factors which can limit microbial degradation of pollutants in natural environments. Effects of various environmental factors, limited availability of contaminants, possible metabolic limitations, and the susceptibility of microorganisms to toxins or predators were discussed. Limitations may occur if the microorganisms are unable to move through contaminated soil sites [68]. Immobilization of microbial cells has been observed to confer protection from toxicity of compounds exterior to the immobilized cells, and phenol has been used for the majority of these studies. Bettmann and Rehm [18] investigated degradation of the highly toxic phenol by free and alginate-encapsulated *Pseudomonas* sp cells. Encapsulated cells degraded phenol up to a concentration of 3 g L^{-1}, which was double the maximum concentration degraded by free cells. The authors postulated two mechanisms for their observations: (a) that cell growth into microcolonies inside the beads may protect the cells, or (b) the colonies around the outside edge of the beads form a phenol diffusion barrier to protect cells inside the bead.

Dwyer *et al* [48] studied anaerobic phenol degradation by a methanogenic consortium encapsulated in agar and observed protection against phenol at concentrations which inhibited methane production by free cells. At a phenol concentration of 0.5 mg ml^{-1}, both free and encapsulated cells produced about 0.5 mmol CH_4. However, at 2.0 mg phenol ml^{-1}, activity of free cells was almost completely inhibited and less than 0.05 mmol CH_4 was produced, whereas cells immobilized in agar produced 0.25 mmol of CH_4. Zache and Rehm [236] also observed a protective effect from phenol toxicity by encapsulation of a defined mixed culture of *Pseudomonas putida* P8 and *Cryptococcus elinovii* H1 cells in alginate. Encapsulated cells completely degraded phenol at 3.5 mg ml^{-1}, which was more than twice the maximum phenol concentration degraded by free cells.

Keweloh *et al* [100] supported the view of Bettmann and Rehm that allowing growth of microcolonies inside the bead resulted in protection from phenol toxicity. *Pseudomonas putida* (a phenol-degrading bacterium), *Escherichia coli* and *Staphylococcus aureus* cells were tested for their tolerance to phenol both as free cells, and when encapsulated in alginate beads. Cells were first grown to mid-log phase in a minimal media with 2 g L^{-1} glucose. Phenol was then added, and growth measured. At 2 mg phenol ml^{-1}, only the immobilized cells were able to grow. The authors concluded that the number of generations of immobilized cells and the size of microcolonies determined the phenol tolerance level. Studies on cell growth within beads have shown that microcolonies proliferate inside the matrix [36,165,192,217,231]. By testing bacteria that were unable to degrade phenol, Keweloh *et al* [100] concluded that increased tolerance to phenol is a characteristic of immobilization and not specific for phenol-degrading bacteria. The authors also observed an envelope surrounding the microcolonies formed by encapsulated *E. coli*. The envelope was

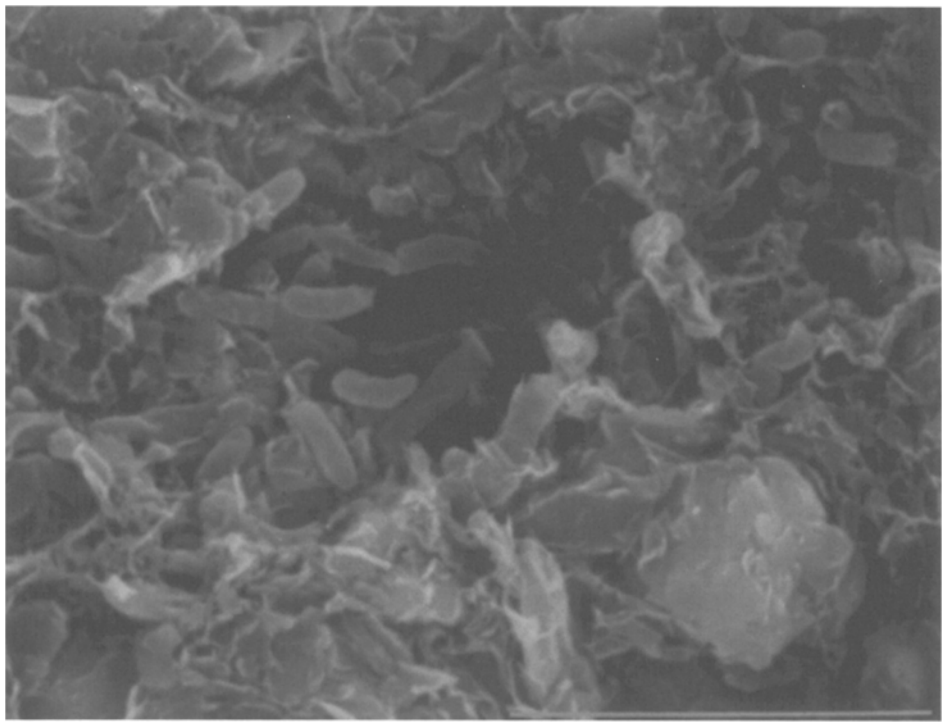

Figure 2 *Pseudomonas aeruginosa* UG2Lr cells encapsulated in κ-carrageenan (bar = 0.5 μm).

not present with free cells. This lent evidence to the view that morphology of cells in colonies can be markedly different from non-encapsulated cells. They suggested this envelope may bind toxic agents, or inhibit diffusion of the toxic agent into the colony. Subsequent work by Keweloh *et al* [101] suggested that phenol tolerance may result from alterations in the cell's membrane. Higher protein-to-lipid ratios in membranes of immobilized cells were observed compared to free cells, with a different protein pattern in the outer membrane.

Diefenbach *et al* [45] treated commercial alginate with chloroform or ethanol to remove fatty acid impurities, and compared phenol tolerance of *E. coli* cells encapsulated in raw commercial alginate with cells encapsulated into the treated alginate. They observed decreased tolerance of the cells encapsulated in the treated alginate to phenol; and a change occurred in the fatty acid profile of the *E. coli* plasma membrane, with increased palmitic acid content and the appearance of oleic acid, which cannot be synthesized by *E. coli*. Gas chromatographic analysis of the chloroform-extracted alginate demonstrated that the palmitic and oleic acid fatty acid impurities were in agreement with the alteration observed in the plasma membrane composition of the encapsulated cells. The authors suggested that these impurities in the alginate were incorporated into membranes of immobilized cells. A strengthened membrane would result since incorporation of the saturated fatty acids found would have a stiffening effect on the cell membrane, providing protection against the effects of phenol [45].

Immobilization appears to protect cells from levels of compounds other than phenols, which normally result in toxic or inhibitory effects on free cells. For example, *Saccharomyces bayanus* cells encapsulated in κ-carrageenan were protected from toxic effects of oleic acid used as a solvent for extractive fermentation of ethanol, where small organic acids can inhibit cell growth in these systems [15]. Protection of *Alcaligenes* sp A 7-2 against normally toxic levels of 4-chlorophenol was attributed to encapsulation in alginate [223]. Protection of *Saccharomyces cerevisiae* cells from twice the normally toxic level of alcohol has also been observed with cells immobilized to beet chips [41] or encapsulated in alginate, κ-carrageenan or polyacrylamide matrices [85,150]. Toxicity reduction may also be due to adsorption of the toxic compound to the carrier, reducing overall toxicity levels. Mörsen and Rehm [137] suggested this may be the mechanism in the degradation of up to 17 g L^{-1} phenol by *Pseudomonas putida* P8 cells adsorbed onto activated carbon. Hu *et al* [88] also suggested this mechanism in the degradation of up to 700 ppm pentachlorophenol (PCP) using *Flavobacterium* sp cells encapsulated in polyurethane beads, but this was not observed for alginate-encapsulated cells which only degraded up to 250 ppm PCP.

This protective effect allows degradation of concentrations of substrates which would be lethal to free cells as observed with the PCP example above, and with immobilized *Pseudomonas* sp in an activated sludge reactor which exhibited a threshold for acute toxicity of between 2100 and 2500 mg L^{-1}, where the lethal dose for *Escherichia coli* has been reported as 100 mg L^{-1} [82]. Encapsulated cells retained activity and effectiveness at high substrate concentrations which would inhibit free cells, for example 10000 ppm surfactant [202], and ten times the phenol concentration toxic to free cells [51]. The use of encapsulated cells would extend the range of contaminated sites when using microbial inocula as a remediation strategy, and increase the potential effectiveness of this strategy compared with the use of free cells.

Increased metabolic activity of encapsulated cells

Beads provide not only a protective environment, but also an increased surface area for cell attachment. Surface attachment of cells improves the activity, growth and survival rates of microorganisms [64]. Factors which may affect cell activity on surfaces include: the concentration of substrate and ions; change in pH and/or Eh; change in inhibitor concentrations; and the release of active metabolites or ions from cells [77]. In a review on the influence of interfaces on microbial activity, van Loosdrecht *et al* [214] indicated that there was no conclusive evidence for a direct influence of adhesion on cellular metabolism. They suggest that effect(s) observed can be attributed to indirect mechanisms, such as modification of the surrounding area.

There are demonstrated increases of metabolic activity of encapsulated cells compared to free cells. For example, alginate-encapsulated *S. cerevisiae* cells produced 80% more ethanol compared to free cells [65] and gelatin-immobilized *S. cerevisiae* cells produced 40–50% more ethanol than free cells [47]. Bailliez *et al* [13] reported increases of 20–25% in hydrocarbon production from alginate-encapsulated *Botryococcus braunii* cells compared with free cells. Barros *et al* [15] observed that κ-carrageenan-encapsulated *Saccharomyces bayanus* completely fermented a solution of 300 g L^{-1} of glucose although free cells cannot convert more than 200 g L^{-1}. Alginate-encapsulated *Methanosarcina barkeri* cells maintained the ability to convert methanol to methane for a longer period of time than free cells [178]. Pertot *et al* [164] reported that alkaloid production was seven times higher for alginate-encapsulated *Claviceps paspali* than free cells and suggested that immobilization shifts from growth towards secondary metabolism. A 1.4- to 6.7-fold increase in specific oxygen consumption rate was observed in *S. cerevisiae* cells adsorbed to clay supports compared to free cells [124]. Gadkari [64] used a mixed nitrifying population to study the effect of alginate and encapsulation on the rate of nitrification. He noted that when sterile alginate beads were added to the medium with the free cells, the ammonium oxidation was completed in 5–7 days, half the time necessary for free cells to complete the process. In this treatment, there was a lag of 1 or 2 days for ammonium oxidation to begin. When alginate-encapsulated cells were used, no lag period was observed and ammonium oxidation was completed in half the time of the control cells. These results indicate that higher efficiencies are observed when cells are attached to, or are sheltered by a solid particle.

In another study, Smith *et al* [189] used ethene-grown *Mycobacterium* sp E3 which was capable of transforming propene to 1,2-epoxypropane. Using alginate-encapsulated cells with beads less than 1 mm in diameter, a system free from diffusional limitations was established. They observed a 1.5-fold increase in 1,2-epoxypropane production by encapsulated cells compared to free cells. When alginate or calcium was added to free cell suspensions in amounts similar to those found in encapsulated cells, a 1.2- to 2-fold increase in 1,2-epoxypropane production was observed. These results led the authors to conclude that the presence of alginate and calcium, rather than the encapsulation matrix, was responsible for the increase in 1,2-epoxypropane production.

The increased metabolic activity observed with immobilized cells occur in many of the systems studied. While the precise mechanism(s) responsible for this effect is not known, immobilized cells may provide a method to increase or maximize bioremediation activity in the environment.

Release and transport of cells in soil

Persistence and stability of the bead have to be maintained to allow establishment of introduced microorganisms. Therefore, photostability, rainfastness and rate of degradation of beads in the soil must be addressed in formulations [173]. There is little information on the release of cells from beads in soil, particularly in chemically contaminated soils. It is unknown if cells released from beads will perform their intended task(s) in a small proportion of the total soil volume. They may end up becoming microsites of high microbial numbers and activity, surrounded by areas of lower activity, unless cells are dispersed by water or beads are applied at a high density per unit soil [207]. Ideally the beads should protect cells, release them slowly over a period of time and allow establishment of cells in as large an area as possible from the beads. The variety of algal polysaccharides available, and alterations possible in the encapsulation process allow some flexibility with which to produce beads that release active cells [16,133].

It is well known that cells are released from beads, and considerable research has been undertaken to minimize this phenomenon for bioreactor applications. For example, actively-growing entrapped *E. coli* cells can exert high pressure (3 atm) on the bead surface, allowing them to disrupt the surface and be released [192]. Lancy and Tuovinen [114] measured the protein content of alginate beads containing *Thiobacillus ferrooxidans* cells and monitored column effluents for cell release in a bioreactor. They observed an increase from 30 to 50 μg protein per bead and cell numbers in the effluent increased from non-detectable levels to 1.2×10^5 cells ml^{-1} over a 14-week period. This led them to conclude that bacteria had multiplied in the beads and were being released from them. It has been suggested by others that cells are released after the matrix space in the beads has been occupied [74], and that cell division, not loss of initially entrapped cells is the major cause of cell leakage [35]. Bashan [16] observed that *Azospirillum brasilense* cells were released at densities of 10^5–10^6 CFU g^{-1} beads per 24 h, and the beads amended with skim milk were degraded almost completely over 4 weeks. However, using alginate alone, only slight degradation on bead edges was observed after 4 weeks. Van Elsas *et al* [211] did not detect any beads with or without amendments after 30 days in a loamy sand.

Once released from the beads in a contaminated soil, establishment of microbial colonies outside the bead will increase the soil area for potential biodegradation. Free microorganisms can be transported through soil and a number of examples, along with factors affecting this movement, were reviewed by Abu-Ashour *et al* [1]. Studies on the release and transport of cells from beads in soil have shown that 15% of *P. fluorescens* R2f cells encapsulated in bentonite/skim milk-amended alginate beads were able to be transported from the beads [83] and 11.5% colonized

plant roots over a distance of 1 cm in soil [206]. Hekman *et al* [83] studied water-mediated transport of free and encapsulated cells in soil microcosms and observed reduced transport of encapsulated cells compared with free cells. The results suggested that encapsulated cells may be highly localized in the soil, thereby reducing the chances of contacting flowing water. This may keep introduced cells in the contaminated surface areas and prevent them from moving into deeper soil levels or groundwater. Some researchers indicate that immobilized cells may be useful for treatment of contaminated surface soil if cells are released from the encapsulation matrix to inoculate the soil [210, 218]. Further study of cell release and transport will be necessary to determine the practicality and effectiveness of using encapsulated cells in environmental applications.

Plasmid stability

Many genes encoding degradative enzymes are carried on plasmids. Therefore, increased plasmid stability would be an advantage when using microorganisms for *in situ* soil bioremediation. Plasmid stability in immobilized genetically-engineered cells has been reviewed recently [111]. Increased bacterial plasmid stability has been observed with encapsulated recombinant cells. For example, two groups of researchers have consistently observed increased plasmid stability in *E. coli* cells containing the pTG201 plasmid encapsulated in κ-carrageenan. The plasmid pTG201 is an Ap^RTc^S *E. coli* cloning vector derivative of pBR322 carrying the *xyl*E gene from *Pseudomonas putida* under transcriptional control of the λP_R and cI857 repressor. Nasri *et al* [145] used three different *E. coli* hosts and observed an increase in stability of the pTG201 plasmid in encapsulated cells compared to free cells in free continuous culture in the absence of antibiotic selection. They concluded that increased stability effects were not due to plasmid transfer between the immobilized cells or due to an increase in immobilized cell plasmid copy numbers. Nasri *et al* [146] reported no plasmid-free segregants in κ-carrageenan-encapsulated *E. coli* harbouring pTG201 after 240 generations of operation in continuous culture with no antibiotic selective pressure. High stability of plasmid pTG201 and pTG206 were also observed for at least 200 generations of *E. coli* cells encapsulated in κ-carrageenan and grown in LB broth supplemented with 0.1 M KCl [17]. Sayadi *et al* [177] observed high plasmid stability with the same cells and encapsulation procedure over 140 generations at temperatures of 31, 34 or 37°C with no selection pressure. For example, after 140 generations at 37°C, immobilized cells contained 88% of plasmid-bearing cells whereas free cells contained approximately 40% plasmid-bearing cells. The precise mechanism(s) that confer plasmid stability in encapsulated cells is not understood. However, the absence of competition between P^+ and P^- cells within the matrix or a significant reduction of the rate of elimination of less fit organisms by modifying selective wash-out pressure in bioreactors has been postulated [111]. Influences of oxygen supply have been investigated and in all cases plasmid stability of κ-carrageenan-encapsulated cells was higher when compared with free cells and was highest at the 100% oxygen-saturation level [86,89,125]. Plasmid stability is a feature which can influence the effectiveness of microbial bioremediation of contaminated soils, and the potential use of genetically-modified microorganisms for this purpose. Encapsulation may provide a method to maintain plasmid stability and increase the chance of success for bioremediation.

Limitations

Although there are numerous advantages in applying microbial encapsulation technology for soil applications, there are also limitations. These are listed in Table 2 and discussed further in this review.

Diffusion of substrates in immobilized cells

The matrix created by encapsulation may restrict diffusion of gases and solutes into beads, particularly into the central interior area (Figure 3). The thin liquid film which can surround the bead in soil may play a role in restricting gas diffusion [158]. Oxygen is the major substrate which appears to be limited [71]. The internal structure of the beads is not homogeneous, and may be affected by many factors such as time of gelation, carrier concentration and type, concentration and pH of the ionotropic cation solution, viscosity, internal particles and temperature [147]. Lack of homogeneity in beads will affect results of diffusion studies and must be kept in mind when attempting to interpret and extrapolate results [71]. Variations in the methods, diffusants, and gel concentrations used by different researchers [71] makes direct comparisons difficult, and have produced conflicting results in mass transfer studies [181].

For example, diffusion rates of solutes such as glucose in gels have been reported to be lower than in water [142, 149], attributed to a reduction in the available volume by the polymer network, and an obstructing effect on the solute. However, others found no difference in diffusion rates [63,200], even when a variety of substrates were tested [202]. It is also noteworthy that the gel-matrix is negatively charged, and the influence of electrostatic forces on ionic compounds should be considered [129,200], but little information is available in this area.

Substrate diffusion is thought to be further decreased in three ways: by increasing cell density within beads, by increasing carrier concentration, and by increasing gel particle size [158,159]. The majority of cells that produce microcolonies initially grow homogeneously in the bead, but ultimately result in growth close to the bead's outer surface [54,92,230], which may be a factor in limiting substrate diffusion. However, Gosmann and Rehm [69] suggested it was not possible to generalize about the oxygen supply of microorganisms in alginate because the supply was dependent on the physiological status of the entrapped cells. The authors suggested that beads which had biomass concentrated at the gel surface had the highest effectiveness factor of oxygen uptake and therefore would be best suited to aerobic processes.

The difficulty in establishing a particular effect is that evidence exists to support substrate diffusional decrease with the addition of microorganisms [3,17,63,70,197], whereas other researchers have seen no effect on the diffusion rate [35,65,76]. Wijffels *et al* [228] investigated the effect of initial biomass concentration on the growth of

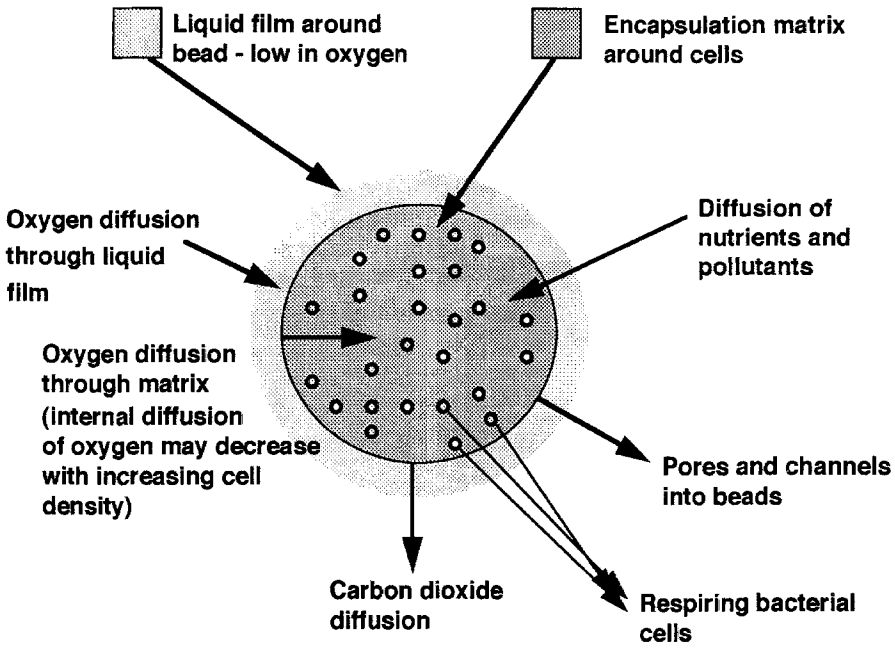

Figure 3 Schematic representation of oxygen diffusion into bead, oxygen consumption by respiring cells, and carbon dioxide evolution and diffusion from bead.

Nitrosomonas europea immobilized in κ-carrageenan beads and discovered that by increasing inoculum size, effects of diffusion limitation over micro-colonies can be avoided. Results from some studies suggest the diffusion rate is decreased by increased carrier concentration [35,63,70,76,149]. However, no diffusional limitations have been reported as well [97,178,204]. Increased particle size (2.0 to 4.0-mm diameter) has resulted in decreased diffusion in some cases [4,36], but has not been observed in other studies [150], in particular, not in a study done in soil [205].

If diffusion of dissolved gases into the beads is reduced [71], oxygen-limited conditions in the bead centre may result in reduced activity [158], analogous to the hypothesis that oxygen diffusion into soil aggregates may be a limiting factor for bacterial activity [77]. Oxygen gradients have been identified using microelectrodes in single beads [21,86,89,125], but Muller *et al* [143] questioned these results by demonstrating through microscopic observations and photography that artifacts and diffusional barriers were being created by unidirectional movement of the electrode into the bead, and suggested that earlier experiments may have to be re-evaluated.

Aspects of the encapsulation process itself, such as the temperature of the preparation of encapsulated cell beads and the presence of ions, particularly K^+, can contribute to lower oxygen uptake rates (OURs) of encapsulated cells [179]; specific OURs of κ-carrageenan-encapsulated *Streptomyces clavuligerus* cells were 50% of the rates of free cells. Gelling time to attain maximum hardness of κ-carrageenan beads was 40 min in 1 M KCl, but after this time the OUR of the encapsulated cells had fallen to 20% of the rate of encapsulated cells exposed to 1 M KCl for 2 min. The OUR of κ-carrageenan-encapsulated cells exposed for 1 h at 1 M KCl was almost zero. This indicates that the encapsulation process itself plays an integral role in determining detrimental effects on cells, and can be adjusted to minimize those effects.

Some researchers are creatively exploring alternatives and adjusting existing technology to overcome possible oxygen limitations. Ogbonna *et al* [156] reviewed the three main strategies used: oxygenation of bulk medium, *in situ* oxygen generators incorporated in the matrix and reduction of bead size. They concluded that using beads 0.2–1.2 mm in diameter is most likely the most effective alternative, providing more uniform cell growth and increased efficiency due to a reduced nutrient diffusion barrier. Stormo and Crawford [194] used micro beads 2–50 μm in diameter to minimize the possibility of oxygen limitation. They claimed further advantages of high cell loadings (>50%, w/v) and cells as active as free cells based on the ability to mineralize pentachlorophenol (PCP).

A novel idea for using standard size beads is to combine aerobic bacteria on the outer bead edge with facultatively aerobic bacteria in the central portion to take advantage of the difference in oxygen availability in these bead regions. This approach has been used in studies on synchronous reductive and oxidative degradation of xenobiotics by immobilized microorganisms [21], and may prove to have utility in environmental applications. A more extensive discussion of co-immobilization of mixed cultures has been done by O'Reilly and Scott [152].

For soil applications, optimization of pore sizes to provide protection without adverse effects on diffusion of substrates is necessary to maximize effectiveness, and consideration must be given to the different options available. Scott *et al* [181] suggested the pore structure of alginate gels was significantly different from that of κ-carrageenan, resulting in reduced diffusivity of glucose through alginate. The authors observed a significant decrease in the glucose diffusion coefficient from 6.6×10^6 to 5.0×10^6 $cm^2\ s^{-1}$ over a range of 1–3% in alginate, but observed no signifi-

cant difference in the coefficient over a range of 1–4% in κ-carrageenan. The factors which affected solute diffusion were the concentration of the polysaccharide, the encapsulation method and the addition of amendments, all of which may have resulted in reduced pore sizes.

Hu *et al* [87] successfully used polyurethane foams and suggested that a combination of wide porosity range and good mechanical properties of polyurethane potentially eliminate diffusional barriers, but these non-biodegradable carriers may be more applicable to bioreactor processing than to environmental releases.

Adjustments of the formulation processes may improve carrier effectiveness in soil. In our research program, the new formulation uses less carrier material (1% κ-carrageenan), less hardening time in the salt solutions (30 min), and processing was carried out at ambient temperature (20–22°C) [29]. These conditions may reduce adverse diffusional effects on encapsulated cells and are areas for further study.

Encapsulation can provide a stabilizing effect on cells with respect to diffusivity and temperature. For example, temperatures between 0 and 80°C do not affect the alginate pore sizes, and the diffusivity of κ-carrageenan gel remains unchanged below 25°C [149], temperature ranges generally found in soils in temperate climates. Wijffels *et al* [229] observed the nitrification process of encapsulated *Nitrosomonas europea* and *Nitrobacter agilis* was insensitive to temperature changes from 5 to 30°C as compared to free cells. Adlercreutz *et al* [3] observed that *Gluconobacter oxydans* cells encapsulated in alginate oxidized glycerol at similar rates between 25 and 50°C, while oxidative activity in free cells decreased rapidly between these temperatures.

Effects of immobilization on the mass transfer of substrates are varied, and may depend on a number of factors in the immobilization process. Further study, perhaps with new non-invasive techniques, may provide more information on whether oxygen diffusion may be limiting to encapsulated microbial cell activity.

The mass transfer characteristics in a soil system differ from those in bioreactor systems, and although there is a lack of data using encapsulated cells in soil studies to establish any diffusional effect, it may be possible to optimize the encapsulation process to minimize diffusional problems.

Alterations in cell morphology

Altered cell morphology of encapsulated cells has been observed in some instances which may or may not result in physiological changes as well. For example, immobilized *Claviceps fusiformis* cells underwent significant morphological changes during the semi-continuous production of alkaloids over a period of 550 days [109]. Micropellet formation within alginate-encapsulated, but not free cells of *Streptomyces aureofasciens* have also been noted [123]. Doran and Bailey [47] observed a number of changes in *S. cerevisiae* cells attached to gelatin. The mean size of immobilized cells were about two-thirds that of suspended cells, the immobilized cells contained only about one-fourth the amount of stable double-stranded RNA found in suspended cells, immobilized cells contained almost four times more polysaccharide than suspended cells and the average DNA content of immobilized cells was 3.8 times that of suspended cells. Steric constraints of colonies enclosed in pores were suggested as a reason for the morphological differences observed in alginate-encapsulated *Botryococcus braunii* cells compared to free cells [13].

The increase in metabolic activity which has been observed in encapsulated cells may result from altered cell morphology. For example, membrane permeability of immobilized cells has been postulated as an explanation for observed changes in metabolic activity by Marcipar *et al* [124] and Mattiasson [132] who observed increased oxygen consumption of immobilized *Saccharomyces cerevisiae* cells, and by Galazzo and Bailey [65] who recorded a decrease in the intracellular pH and an increase in the glucose uptake rates of alginate-encapsulated *S. cerevisiae* cells. Pertot *et al* [164] observed thick-walled, swollen *Claviceps paspali* cells with granular cytoplasm and vacuoles when encapsulated in alginate which had never been seen with free cells. They suggested that this cell differentiation may have resulted from limited diffusion of oxygen and nutrients causing unfavourable environmental conditions in the beads, but proposed the idea that cell differentiation may be responsible for prolonged vitality and metabolic activity of the cells.

However, not all researchers agree that morphological changes are due to encapsulation. For example, Keweloh *et al* [100] observed an envelope surrounding microcolonies formed by encapsulated *E. coli* cells. They suggested that formation of the envelope may be due to formation of microcolonies and not a result of encapsulation since the morphology of cells in colonies can be markedly different than free-grown cells. Morphological changes are not seen in all cases. For example, alginate-encapsulated cyanobacteria *Anabaena* ATCC 27893 were observed to be morphologically normal over a period of 792 h [144]. There is also controversy over the influences of immobilization on microbial metabolic activity [189], and the effect may be species-specific.

Low water activity and/or oxygen deficiency of the bead microenvironment were suggested as reasons for altered properties of immobilized cells [132]. Shreve and Vogel [185] investigated the influence of immobilization on cellular growth and substrate utilization. They used a 2,4-D-degrading *Pseudomonas cepacia* strain DBO131 and toluene-degrading *Pseudomonas* sp strain K 3-2 in a system designed to measure effects on growth and substrate utilization. No difference in substrate utilization between immobilized and free cells was observed for either strain, and no growth rate difference was observed between free and immobilized *P. cepacia* cells. The slower growth rate observed for immobilized *Pseudomonas* sp strain K 3-2 was attributed to the formation of a diffusible intermediate during toluene degradation. They concluded that much of the previously observed changes in metabolic activities upon immobilization may be due to changes in the physical or chemical environment, such as localized water activity, pH, temperature, ion concentrations, nutrient and waste product concentration and oxygen and nutrient diffusion. Keweloh *et al* [100] also indicated that it is not the effects on the beads, but rather reduced water activity or oxygen

deficiency in microcolonies that influence cell physiology. They concluded that close cell-to-cell contact influences the dynamic and not the structural aspects of the membrane since after the dissolution of the beads, the liberated bacteria were as sensitive to phenol as cells grown in the free state. Shirai *et al* [184] observed an increased oxygen uptake rate of growing alginate-encapsulated hybridoma cells correlating with the formation of colonies. They also concluded that close cell contact can influence changes in cell physiology.

Therefore, while some morphological and physiological changes occur in encapsulated cells, they may be a result of other limitations such as diffusion, and may prove to be beneficial in improving activity. If limitations are reduced or removed, the cellular changes might also be minimized, but it must be stressed that changes may be species-specific and investigative testing may be necessary to determine any potential benefits or drawbacks. Further information and discussion of these alterations can be found in a review by Rehm and Omar [172], who also concluded that more investigation is required to explain the observed effects.

Growth rates of immobilized microbial cells

Growth responses of encapsulated cells may vary depending on the initial inoculum size used, and the actual cell position within the bead. When low cell densities (8×10^6 cells g^{-1} of beads) were used, the growth rate of alginate-encapsulated *Saccharomyces cerevisiae* cells was similar to that of free cells in suspension, and growth may have occurred initially throughout the bead [217]. With larger initial cell densities, the majority of cell growth appeared to occur at or close to the bead surface [33], limited to the outer 50–150 μm of the beads [53,218]. Champagne *et al* [33] also observed growth of *Lactococcus lactis* when a lower level (1×10^9 CFU ml^{-1}) of inoculum was used for immobilization, but not with a higher (2×10^{10} CFU ml^{-1}) inoculum level. Berry *et al* [17] observed large *E. coli* colonies near the surface and in the centre of κ-carrageenan beads when very low (4.7×10^3 CFU ml^{-1} of gel bead) inoculum densities were allowed to grow. Chen and Huang [36] studied *Trichosporon cutaneum* cells encapsulated in alginate beads ranging from 0.6 to 4.0 mm in diameter which had been incubated in a growth medium for 24 h after encapsulation. In beads 1.5 mm in diameter or less, cells were more homogeneously distributed throughout the bead, whereas above 2 mm in diameter, larger cell populations were observed near the surface. In another study [231] on-line microscopy was used as a non-invasive technique to observe growth of a *S. cerevisiae* microcolony inside alginate. Cells of the growing microcolony pushed the surrounding gel away, leading to dense packing of cells in fluid, and not in the alginate gel.

In general, cell crowding may affect cell growth [97]. Lag times and growth rates can be affected, but the nature of the effect depends on the immobilization material, cell loading and/or type of cells used. Galazzo and Bailey [65] observed no lag during growth of *S. cerevisiae* cells encapsulated in alginate beads, while a 6-h lag was observed with free cells. However, growth rate in beads was observed to be slower ($\mu = 0.25\ h^{-1}$) as compared to free cells ($\mu = 0.41\ h^{-1}$). Other researchers have observed reduced growth rates in encapsulated cells as well [12,44,82,109,117]. However, Zhang *et al* [237] observed similar specific growth rates between free and alginate-encapsulated *E. coli* cells. No difference in lag time was observed with alginate-encapsulated *T. cutaneum* cells (3 h) compared with free cells (10 h). However, the doubling time of cells in the beads was faster (3 h) compared with free cells (4 h). Chevalier and de la Noue [37] observed similar growth rates between microalgae entrapped in κ-carrageenan and free cells. Using scanning microfluorimetry and cellular RNA content as an indicator of cell growth, Monbouquette and Ollis [136] observed that alginate-encapsulated *Zymomonas mobilus* cells in a single bead exhibited specific growth rate variations from resting to maximal. Thus, there is no consistent effect of encapsulation on growth rate. Further studies in this area are necessary to determine if altered growth rates from encapsulation may affect physiological functions of the cells.

Role of water activity in microbial encapsulation

Dry formulations allow easy handling, distribution and application of the encapsulated microorganisms. Excluding water slows cell metabolic rates, prevents nutrient depletion, prevents accumulation of toxic metabolites and slows denaturation of proteins [173]. However, drying of the gel-beads after encapsulation reduces viable cell numbers to about 1% of the original cell count [16,29,95,163]. Paul *et al* [163] suggested the extent of survival is dependent on a number of parameters such as the strain, carrier composition, method used, and the drying conditions. After the initial drying period, however, survival appears to stabilize for periods of up to 3 years [29,141,163]. The ability to store encapsulated cells for long periods of time is advantageous for potential commercial use.

Water activity may play an important role in survival. Mugnier and Jung [141] studied the relationship between water activity and survival of cells of *Rhizobium*, *Agrobacterium*, *Arthrobacter*, *Penicillium* and *Saccharomyces* spp encapsulated in xanthan gum. Survival was not adversely affected by the extreme dehydration of the cells, but was adversely affected by the appearance of water of hydration. Solutes can diffuse in this water which, by an osmotic effect, destroys the cells. Dried xanthan gum-entrapped cells maintained viability at their initial level of $10^{10}\ g^{-1}$ dry polymer at 28°C for more than 3 years, but only when inocula were held at an a_w of less than 0.069. The authors suggested that below this level there may be a monolayer of water surrounding and bound to the bead so solutes are not mobile. Between a_w values of 0.069 and 0.083, solute concentration and mobility allow water/solutes to reach cell surfaces and destroy cells by an osmotic effect. When a_w is greater than 0.083, the aqueous solution surrounding cells is sufficiently dilute to remove the adverse conditions. However, they found that water activity alone was not causing the deleterious effect, as variations in survival occurred depending on the nutrient sources used to grow the microorganisms, with higher molecular weight compounds (C_6 to C_{12}) having a protective effect. These results differ from those reported by Paul *et al* [163] who found that water activity in the range 0–0.55 had no effect on viability of alginate-entrapped *Azospirillum lipoferum* during storage

for more than 150 days. These authors suggested that the difference between their results and those of Mugnier and Jung [141] may be due to differences in experimental conditions, the carrier materials used and the physiological state of the cells.

Little is known about the effect of water activity on encapsulated cells introduced to soil. Rattray *et al* [171] investigated effects of water activity on free genetically-engineered *E. coli* cells introduced into soil. They found increasing water stress had an immediate inhibitory effect on microbial activity and resulted in significant reductions in long-term survival. Both alginate and κ-carrageenan have a high affinity for water. The effect of water activity on microbial cells encapsulated in either matrix and introduced into soil still needs to be investigated.

Colonization of beads by soil fungi

Since both alginate and κ-carrageenan can be utilized as nutrient sources by fungi, the possibility exists that beads may be colonized by indigenous soil fungi which outcompete bacterial cells inside and on the surface of beads. Electron micrographs we prepared indicate the presence of fungal mycelia on the outer κ-carrageenan bead surface after incubation in non-sterile soil for 4 weeks (Figure 4). Eikmeier *et al* [53] observed that when an abundant nitrogen source was available (0.5 g L^{-1} NH_4NO_3), *Aspergillus niger* encapsulated into alginate and κ-carrageenan formed a dense layer on the surface of the beads, limiting diffusion into the beads and causing a large decrease in cell growth inside the beads. However, complex formation between the polysaccharide and clays in beads may not allow complete availability of the biopolymer. More studies are needed to determine if fungi residing on bead surfaces can affect the physiology of encapsulated cells.

Use of immobilized microbial cells for the degradation of xenobiotic compounds

There have been numerous studies on the degradation of various toxic organic compounds using immobilized microbial cells in bioreactors (Table 1). The number of studies completed, the variety of microorganisms used and the number of compounds degraded attest to its effectiveness in a closed system.

We have studied the ability of encapsulated cells to mineralize pentachlorophenol (PCP) in broth. Four bacterial strains capable of PCP degradation were compared, using both free cells and cells encapsulated in our new milder κ-carrageenan formulation [29]. The four strains included *Flavobacterium* sp ATCC 39723, originally isolated by Saber and Crawford [174] and shown to be able to mineralize up to 150 ppm PCP; *Sphingomonas* RA2 originally isolated and designated as a *Pseudomonas* sp by Radehaus and Schmidt [170] also capable of mineralizing up to 150 ppm PCP; and two novel *Pseudomonas* strains isolated and characterized in our laboratory and capable of mineralizing over 100 ppm PCP at rates faster than either the *Flavobacterium* or the *Sphingomonas* sp (unpublished results). No statistically significant difference was observed in the percent of $^{14}CO_2$ evolved from [^{14}C]PCP by encapsulated and free cells (Figure 5). This illustrates the mildness of the encapsulation process for a variety of cell types and the ability of the encapsulated cells to maintain degradative activity comparable to free cells. Although encapsulated cells can be effective in broth and in bioreactors, efficient degradation of pollutants in soil is desired if cells are to be used to treat dispersed contaminants in the open environment.

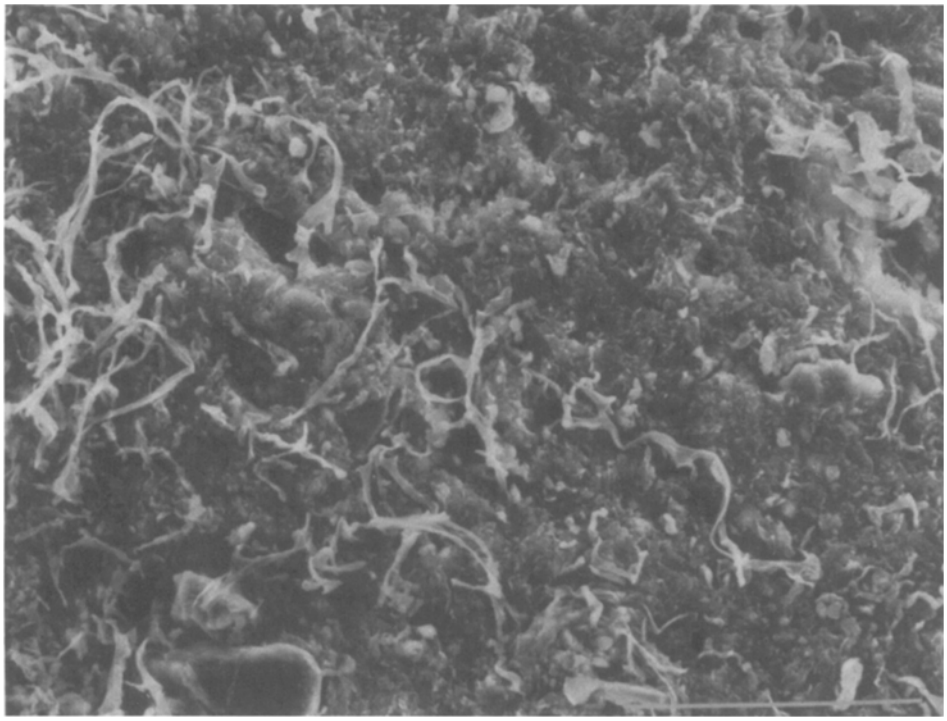

Figure 4 Fungal mycelia on the surface of κ-carrageenan bead after 4 weeks in non-sterile soil (bar = 1.0 μm).

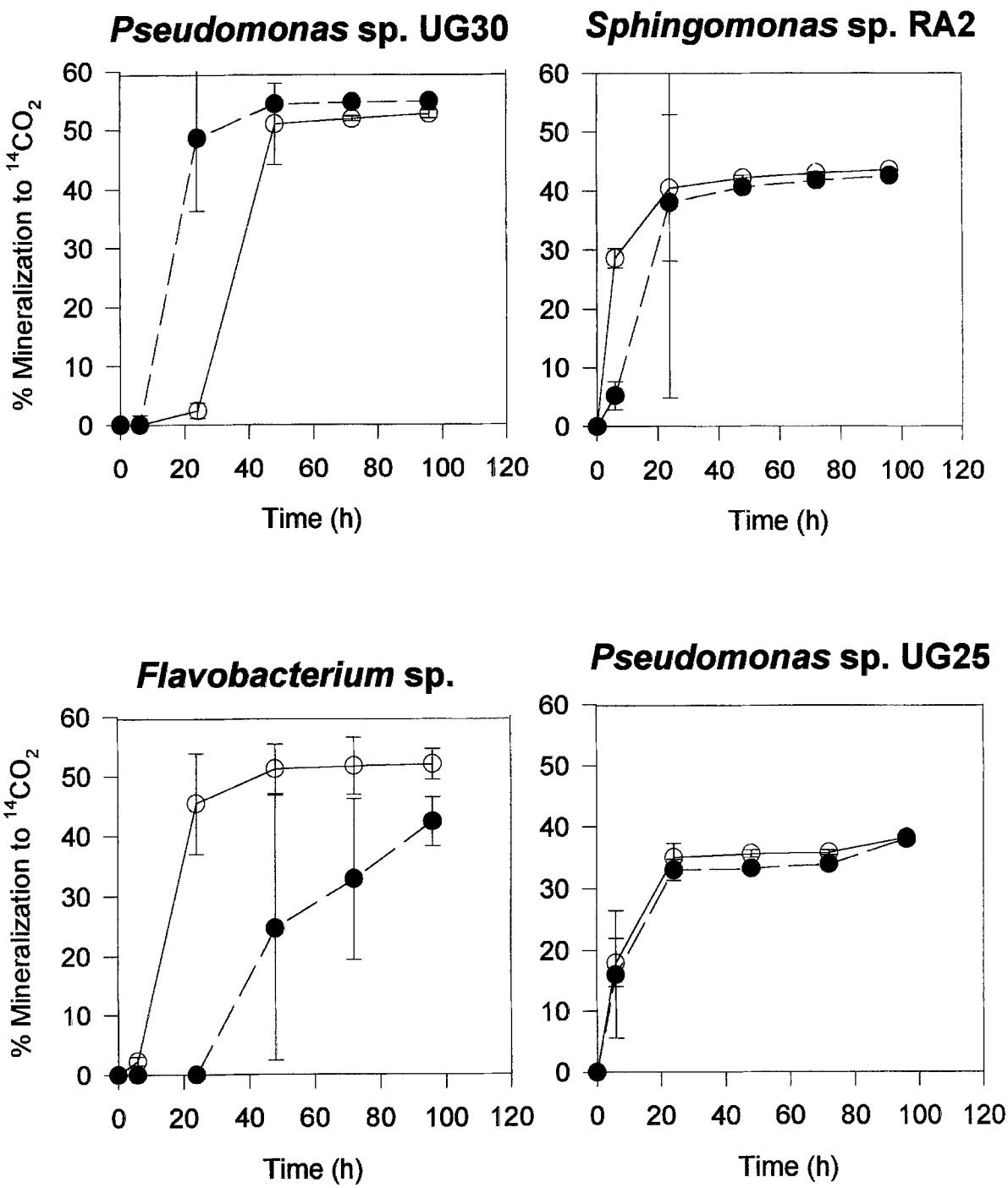

Figure 5 Mineralization of 100 ppm pentachlorophenol by four species of free (○) and encapsulated (●) bacterial cells.

Degradation of toxic organic compounds in soil by introduced microorganisms

Successful degradation of toxic compounds in soil by free bacterial cells has been demonstrated by a number of researchers. Table 3 provides examples of pollutants degraded by microbial inocula added to soils. Many introductions of microorganisms into soils to perform a particular degradative function have not met with success. As discussed earlier, the use of a carrier may improve the success rate by overcoming some of the problems that inocula introduced into the environment have encountered.

In cases where remediation was successful in contaminated soil, the rate and extent of pollutant removal could be highly variable depending on factors such as soil type, temperature, moisture level and initial inoculation density [40]. The fastest mineralization rates usually occur with the highest inoculation levels (10^8 CFU g^{-1} soil) although rates were strain-dependent [40,84,94]. In many cases it was demonstrated that degradation of pollutants in soil could be enhanced by addition of bacteria under optimal conditions [14,84,94,210], but studies using non-sterile, contaminated field soil also resulted in pollutant degradation by intro-

Table 3 Examples of pollutants successfully degraded by microbial inocula introduced into soil

Compound degraded	Microorganism	System	References
Atrazine	Microbial consortium	soil	[9]
Chlorobenzoate	*Pseudomonas aeruginosa* JB2 *P. putida* P111	soil	[84]
Chlorobenzenes	*P. aeruginosa* RH01	soil slurry	[25]
Chlorophenol	Mixed culture	composting contaminated soil	[210]
4-Chlorophenol	*Alcaligenes* sp A 7-2	sandy soil	[14]
2,4-Dichlorophenoxyacetic acid	*Alcaligenes eutrophus* *Pseudomonas cepacia*	soil	[94]
Nitroaromatic compounds	Various microorganisms	soil	[130]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	soil	[50]
Pentachlorophenol	*Rhodococcus chlorophenolicus* *(Mycobacterium)*	heavily polluted soil	[135]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	sand	[49]
Pentachlorophenol	*Arthrobacter* sp ATCC 33790	clay soil	
Pentachlorophenol	*Rhodococcus chlorophenolicus*	soil	[23]
Pentachlorophenol	*Flavobacterium* sp ATCC 39723	contaminated soil	[40]
Pentachlorophenol	*Flavobacterium* sp ATCC 39723	soil	[182]
Pentachlorophenol	*Phanerochaete* spp	soil	[112]
Pentachlorophenol	*Phanerochaete* sp	soil	[113]
2,4,5-Trichlorophenoxyacetic acid	*Pseudomonas cepacia*	soil	[102]

duced bacteria [40,135,182,210], and fungi [112, 113].

The results from these studies provide an indication of the potential use of microorganisms for soil remediation. The possibility of commercial application in the future may depend in part on an effective and safe carrier.

Application of immobilization technology for use in bioremediation of contaminated aquifers

Although this review focuses more on the remediation of contaminated soils, there is work being done to examine the potential for the use of encapsulated cells to remediate contaminated aquifers. Stormo and Crawford [194] investigated *in situ* aquifer bioremediation using microencapsulation of *Flavobacterium* sp ATCC 39723 in agarose-gel beads 2–50 μm in diameter. The authors used microencapsulated cells at 5×10^7 cells per gram of aquifer material and reported no significant difference in PCP degradation between free and encapsulated cells. They also determined survivability of cells under conditions of starvation, predation and no water flow and observed that microencapsulated cells in the non-sterile treatments after both 13 and 24 months were the only ones to exhibit PCP degradation after enrichment to 100 ppm.

Application of immobilization technology for use in bioremediation of contaminated soil

Investigations using immobilization of microbial cells for biodegradation of contaminated soils are few in number, but promising in their results. Omar and Rehm [160] observed that *Candida parapsilosis* and *Penicillium frequetans* effectively degraded *n*-alkanes when immobilized on granular clay in columns. The authors observed residuals of C_{12} to C_{18} alkanes from 13.4 to 32.3% of original levels with immobilized cells, whereas using cells in aquifer sand resulted in residuals between 85.9 and 98.9%. They suggested the degradation potential in soil may be enhanced by inoculating with immobilized microorganisms. Meusel and Rehm [134] used a packed-bed fermenter filled with sand to imitate soil systems and found that immobilized and free *Xanthobacter autotrophicus* cells completely degraded up to 20 mM of dichloroacetic acid in the sand. Wiesel *et al* [226] observed that a mixed bacterial culture immobilized on granular clay exhibited good growth, and demonstrated equivalent degradation potential of polyaromatic hydrocarbons (PAHs) compared to freely suspended cells in their model soil system. Balfanz and Rehm [14] used *Alcaligenes* sp A 7-2 immobilized on granular clay in a packed-bed fermenter with sandy soil to degrade 70.3 mg L^{-1} 4-chlorophenol. The authors also observed that in semi-continuous culture the degradation rate of the free cells slightly increased, from 2.4 to 2.6 mg L^{-1} h^{-1} during fermentation, but with immobilized cells, the degradation rate continuously increased from 3.1 to 10 mg L^{-1} h^{-1}. Overmeyer and Rehm [162] observed degradation of up to 25 mM 2-chloroethanol using *Pseudomonas putida* US2 cells immobilized on lecaton granulate in a packed-bed fermenter with sand to simulate soil conditions.

Encapsulated cells have also been investigated for biodegradation in soil. For example, Salkinoja-Salonen *et al* [176] observed that alginate-immobilized *Rhodococcus chlorophenolicus* inoculum retained its PCP-mineralizing activity in soil more effectively than the free cell inoculation. Co-immobilization using living cells and activated carbon in alginate has also demonstrated effectiveness in degrading PCP in solution, soil extract and sand [121]. Although Weir *et al* [221] observed no degradation of phenanthrene using log 8 CFU g^{-1} dry soil alginate-clay-skim milk-encapsulated *Pseudomonas aeruginosa* UG14Lr cells in creosote-contaminated soil slurries, survival of encapsulated cells was higher after 30 days with log 2 CFU g^{-1} dry soil recovered. Free cells, however, were not detected after 18 days.

Summary

Immobilization/encapsulation of microorganisms is effective for many applications in closed, controlled bioreactor systems. The encapsulation procedure is simple, applicable to a range of microorganisms without detrimental effects, and a variety of carriers allow choices for various applications. Industrial processes involving microorganisms have become more efficient and cost-effective with the application of immobilization technology. Considerable research on immobilized microorganisms has been done to optimize the microbial processes. Even with the large body of literature available, there are still questions and mechanisms which need further elucidation. Diffusion of substrates through the immobilizing matrix, alterations in physiology or morphology of immobilized cells, cell growth rates and the role of water activity are areas where there are wide variations in results, and therefore it is difficult to make generalizations. Improvements in investigative techniques and standardization of methods between studies may provide a clearer understanding of these significant areas, and may provide the means for consistent success. While a successful process in the lab under controlled conditions does not imply similar success in an uncontrolled environment, it is a good starting point to focus on addressing potential problems. One major reason for considering the use of encapsulation technology for environmental purposes is that the encapsulation process itself adds a modicum of control, potentially becoming a miniature bioreactor in the environment [133]. Applications of free microorganisms directly into the environment to enhance biological processes or remediate contaminated areas have achieved mixed results, with both successes and failures reported. Advances in genetic engineering may further enhance the effectiveness of microorganisms to perform specific tasks. The combination of improved microbial metabolism with the benefits of immobilization, such as increased metabolic activity, increased plasmid stability, and protection from environmental stresses and toxicity observed in bioreactor studies, may optimize the effectiveness of environmental inocula application. The ease of storage, transportation and application of encapsulated cells provides additional benefits for commercial purposes, and existing mechanical equipment can be utilized. Biosafety features that limit contamination and bioaerosol formation are important requirements for public and environmental health.

Environmental applications of microbial inocula would be less harmful to the environment than chemical treatment or physical removal of soil or water off-site, and may provide cost reductions if it can be proven to be effective. The application of immobilization technology to this area is in its preliminary stages, but the results seen so far are promising. More research is needed to establish the potential effectiveness of immobilization technology for use in the environment, particularly in the areas of microbial survival, establishment, transport and ability to mineralize different pollutants in varied soil systems.

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References

1 Abu-Ashour J, DM Joy, H Lee, HR Whiteley and S Zelin. 1994. Transport of microorganisms through soil. Water Air Soil Pollut 75: 141–158.
2 Acea MJ, CR Moore and M Alexander. 1988. Survival and growth of bacteria introduced into soil. Soil Biol Biochem 20: 509–515.
3 Adlercreutz P. 1985. Oxygen supply to immobilized cells. PhD Thesis for Department of Biotechnology, University of Lund, Lund, Sweden.
4 Adlercreutz P, O Holst and B Mattiasson. 1985. Characterization of *Gluconobacter oxydans* immobilized in calcium alginate. Appl Microbiol Biotechnol 22: 1–7.
5 Akin C. 1987. Biocatalysis with immobilized cells. Biotechnol Genet Eng Rev 5: 319–367.
6 Anselmo AM and JN Novais. 1992. Biological treatment of phenolic wastes: comparison between free and immobilized cell systems. Biotechnol Lett 14: 239–244.
7 Anselmo AM, M Mateus, JMS Cabral and JN Novais. 1985. Degradation of phenol by immobilized cells of *Fusarium flocciferum*. Biotechnol Lett 7: 889–894.
8 Arnaud JF and C Lacroix. 1991. Diffusion of lactose in κ-carrageenan/locust bean gum gel beads with or without entrapped growing lactic acid bacteria. Biotechnol Bioeng 38: 1041–1049.
9 Assaf NA and RF Turco. 1994. Accelerated biodegradation of atrazine by a microbial consortium is possible in culture and soil. Biodegradation 5: 29–35.
10 Audet P, C Paquin and C Lacroix. 1988. Immobilized growing lactic acid bacteria with κ-carrageenan–locust bean gum gel. Appl Microbiol Biotechnol 29: 11–18.
11 Axtell RC and DR Guzman. 1987. Encapsulation of the mosquito fungal pathogen *Lagenidium giganteum* (oomycetes:lagenidiales) in calcium alginate. J Am Mosq Control Assoc 3: 450–459.
12 Babu GRV, JH Wolfram and KD Chapatwala. 1992. Conversion of sodium cyanide to carbon dioxide and ammonia by immobilized cells of *Pseudomonas putida*. J Ind Microbiol 9: 235–238.
13 Bailliez C, C Largeau and E Casadevall. 1985. Growth and hydrocarbon production of *Botryococcus braunii* immobilized in calcium alginate gel. Appl Microbiol Biotechnol 23: 99–105.
14 Balfanz J and HJ Rehm. 1991. Biodegradation of 4-chlorophenol by adsorptive immobilized *Alcaligenes* sp A 7-2 in soil. Appl Microbiol Biotechnol 35: 662–668.
15 Barros MRA, JMS Cabral and JM Novais. 1987. Production of ethanol by immobilized *Saccharomyces bayanus* in an extractive fermentation system. Biotechnol Bioeng 29: 1097–1104.
16 Bashan Y. 1986. Alginate beads as synthetic inoculation carriers for slow release of bacteria that affect plant growth. Appl Environ Microbiol 51: 1089–1098.
17 Berry F, S Sayadi, M Nasri, J-N Barbotin and D Thomas. 1988. Effect of growing conditions of recombinant *E. coli* in carrageenan gel beads upon biomass production and plasmid stability. Biotechnol Lett 10: 619–624.
18 Bettmann H and HJ Rehm. 1984. Degradation of phenol by polymer entrapped microorganisms. Appl Microbiol Biotechnol 20: 285–290.
19 Bettmann H and HJ Rehm. 1985. Continuous degradation of phenol(s) by *Pseudomonas putida* P8 entrapped in polyacrylamide-hydrazide. Appl Microbiol Biotechnol 22: 389–393.
20 Beunink J and HJ Rehm. 1990. Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl Microbiol Biotechnol 34: 108–115.
21 Beunink J, H Baumgartl, W Zimelka and HJ Rehm. 1989. Determination of oxygen gradients in single Ca-alginate beads by means of oxygen-microelectrodes. Experientia 45: 1041–1047.
22 Beunink J and HJ Rehm. 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl Microbiol Biotechnol 29: 72–80.
23 Briglia M, PJM Middeldorp and M Salkinoja-Salonen. 1994. Min-

eralization performance of *Rhodococcus chlorophenolicus* strain PCP-1 in contaminated soil simulating on site conditions. Soil Biol Biochem 26: 377–385.
24 Brodelius P and J Vandamme. 1987. Immobilized cell systems. In: Biotechnology: A Comprehensive Treatise in Eight Volumes. Vol 7a (Kennedy JF, ed), VCH Verlagsgesellschaft mbH, Germany.
25 Brunsbach FR and W Reineke. 1994. Degradation of chlorobenzenes in soil slurry by a specialized organism. Appl Microbiol Biotechnol 42: 415–420.
26 Bushby HVA and KC Marshall. 1977. Water status of rhizobia in relation to their susceptibility to desiccation and to their protection by montmorillonite. J Gen Microbiol 99: 19–27.
27 Buzas Z, K Dallmann and B Szafani. 1989. Influence of pH on the growth and ethanol production of free and immobilized *Saccharomyces cerevisiae* cells. Biotechnol Bioeng 34: 882–884.
28 Casas LT, F Dominguez and E Brito. 1990. Characterization and optimization of a new immobilized system of κ-carrageenan through interaction with carob bean gum and polyols. J Ferment Bioeng 69: 98–101.
29 Cassidy MB, KT Leung, H Lee and JT Trevors. 1995. Survival of *lac-lux* marked *Pseudomonas aeruginosa* UG2Lr cells encapsulated in κ-carrageenan and alginate. J Microbiol Meth 23: 281–290.
30 Casson D and AN Emery. 1987. On the elimination of artefactual effects in assessing the structure of calcium alginate cell immobilization gels. Enzyme Microb Technol 9: 102–106.
31 Caunt P. 1987. Immobilization of cells in a linear polyacrylamide matrix: degradation of a mixture of volatile fatty acids by *Alcaligenes denitrificans*. Biotechnol Lett 9: 47–48.
32 Caunt P and HA Chase. 1987. Degradation of *n*-valeric acid by alginate-entrapped *Alcaligenes denitrificans*. Appl Microbiol Biotechnol 25: 453–458.
33 Champagne CP, C Gaudy, D Poncelet and RJ Neufeld. 1992. *Lactococcus lactis* release from calcium alginate beads. Appl Environ Microbiol 58: 1429–1434.
34 Chapatwala KD, GRV Babu and JH Wolfram. 1993. Screening of encapsulated microbial cells for the degradation of inorganic cyanides. J Ind Microbiol 11: 69–72.
35 Cheetham PSJ, KW Blunt and C Bucke. 1979. Physical studies on cell immobilization using calcium alginate gels. Biotechnol Bioeng 21: 2155–2168.
36 Chen KC and CT Huang. 1988. Effects of the growth of *Trichosporon cutaneum* in calcium alginate gel beads upon bead structure and oxygen transfer characteristics. Enzyme Microbiol Technol 10: 284–292.
37 Chevalier P and J de la Noue. 1985. Wastewater nutrient removal with microalgae immobilized in carrageenan. Enzyme Microbiol Technol 7: 621–624.
38 Connick WJ. 1982. Controlled release of the herbicides 2,4-D and dichlobenil from alginate gels. J Appl Poly Sci 27: 3341–3348.
39 Coughlan MP and MPJ Kierstan. 1988. Preparation and applications of immobilized microorganisms: a survey of recent reports. J Microbiol Meth 8: 51–90.
40 Crawford RL and WW Mohn. 1985. Microbiological removal of pentachlorophenol from soil using a *Flavobacterium*. Enzyme Microbiol Technol 7: 617–620.
41 Curtain CC. 1986. Understanding and avoiding ethanol inhibition. Trends Biotech 4: 110.
42 Danso SKA, SO Keya and M Alexander. 1975. Protozoa and the decline of *Rhizobium* populations added to soil. Can J Microbiol 21: 884–895.
43 Daubaras D and AM Chakrabarty. 1992. The environment, microbes and bioremediation: microbial activities modulated by the environment. Biodegradation 3: 125–135.
44 Dervakos GA and C Webb. 1991. On the merits of viable-cell immobilisation. Biotechnol Adv 9: 559–612.
45 Diefenbach R, H Keweloh and HJ Rehm. 1992. Fatty acid impurities in alginate influence the phenol tolerance of immobilized *Escherichia coli*. Appl Microbiol Biotechnol 36: 530–534.
46 Dommergues YR, HG Diem and C Divies. 1979. Polyacrylamide-entrapped *Rhizobium* as an inoculant for legumes. Appl Environ Microbiol 37: 779–781.
47 Doran PM and JE Bailey. 1986. Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. Biotechnol Bioeng 28: 73–87.
48 Dwyer DF, ML Krumme, SA Boyd and JM Tiedje. 1986. Kinetics of phenol biodegradation by an immobilized methanogenic consortium. Appl Environ Microbiol 52: 345–351.
49 Edgehill RU. 1994. Pentachlorophenol removal from slightly acidic mineral salts, commercial sand, and clay soil by recovered *Arthrobacter* strain ATCC 33790. Appl Microbiol Biotechnol 41: 142–148.
50 Edgehill RU and RK Finn. 1983. Microbial treatment of soil to remove pentachlorophenol. Appl Environ Microbiol 45: 1122–1125.
51 Ehrhardt HM and HJ Rehm. 1985. Phenol degradation by microorganisms adsorbed on activated carbon. Appl Microbiol Biotechnol 21: 32–36.
52 Ehrhardt HM and HJ Rehm. 1989. Semicontinuous and continuous degradation of phenol by *Pseudomonas putida* P8 adsorbed on activated carbon. Appl Microbiol Biotechnol 30: 312–317.
53 Eikmeier H, F Westmeier and HJ Rehm. 1984. Morphological development of *Aspergillus niger* immobilized in Ca-alginate and κ-carrageenan. Appl Microbiol Biotechnol 19: 53–57.
54 Enfors SO and B Mattiasson. 1983. Oxygenation of processes involving immobilized cells. In: Immobilized Cells and Organelles, Vol 2 (Mattiasson B, ed), pp 41–60, CRC Press, Boca Raton, FL, USA.
55 England LS, H Lee and JT Trevors. 1993. Bacterial survival in soil: effect of clays and protozoa. Soil Biol Biochem 25: 525–531.
56 Ernst C and H-J Rehm. 1995. Development of a continuous system for the degradation of a cyanuric acid by adsorbed *Pseudomonas* sp NRRL B-12228. Appl Microbiol Biotechnol 43: 150–155.
57 Fages J 1990. An optimized process for manufacturing an Azospirillum inoculant for crops. Appl Microbiol Biotechnol 32: 473–478.
58 Ferschl A, M Loidl, G Ditzelmuller, C Hinteregger and F Streichsbier. 1991. Continuous degradation of 3-chloroaniline by calcium-alginate-entrapped cells of *Pseudomonas acidovorans* CA28: influence of additional substrates. Appl Microbiol Biotechnol 35: 544–550.
59 Fett WF, SF Osman, L Fishman and TS Siebles III. 1986. Alginate production by plant-pathogenic pseudomonads. Appl Environ Microbiol 52: 466–473.
60 Fett WF and C Wijey. 1995. Yields of alginates produced by fluorescent pseudomonads in batch culture. J Ind Microbiol 14: 412–415.
61 Fravel DR, JJ Marois, RD Lumsden and WJ Connick Jr. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75: 774–777.
62 Fry IV and RJ Mehlhorn. 1994. Polyurethane and alginate-immobilized algal biomass for the removal of aqueous toxic metals. In: Emerging Technology for Bioremediation of Metals (Means JL and RE Hinchee, eds), pp 130–134, CRC Press, Florida, USA.
63 Furui M and K Yamashita. 1985. Diffusion coefficients of solutes in immobilized cell catalysts. J Ferment Technol 63: 167–185.
64 Gadkari D. 1990. Nitrification in the presence of soil particles, sand, alginate beads and agar strands. Soil Biol Biochem 22: 17–21.
65 Galazzo JL and JE Bailey. 1990. Growing *Saccharomyces cerevisiae* in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. Biotechnol Bioeng 36: 417–426.
66 Gianfreda L, P Parascandola and V Scardi. 1980. A new method of whole microbiol cell immobilization. Appl Microbiol Biotechnol 11: 6–7.
67 Gilson DD, A Thomas and FR Hawkes. 1990. Gelling mechanism of alginate beads with and without immobilized yeast. Proc Biochem 25: 104–108.
68 Goldstein RM, LM Mallory and M Alexander. 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl Environ Microbiol 50: 977–983.
69 Gosmann B and HJ Rehm. 1988. Influence of growth behaviour and physiology of alginate-entrapped microorganisms on the oxygen consumption. Appl Microbiol Biotechnol 29: 554–559.
70 Gosmann B and HJ Rehm. 1986. Oxygen uptake of microorganisms entrapped in Ca-alginate. Appl Microbiol Biotechnol 23: 163–167.
71 Guiseley KB. 1989. Chemical and physical properties of algal polysaccharides used for cell immobilization. Enzyme Microbiol Technol 11: 706–716.
72 Habte M and M Alexander. 1977. Further evidence for the regulation of bacterial populations in soil by protozoa. Arch Microbiol 13: 181–183.
73 Hackel U, J Klein, R Megnet and F Wagner. 1975. Immobilization of microbial cells in polymeric matrices. Appl Microbiol Biotechnol 1: 291–293.

74 Hahn-Hagerdal B. 1989. Physiological aspects of immobilized cells: a general overview. In: Physiology of Immobilized Cells (Bont JAM, J Visser, B Mattiasson and J Tramper, eds), pp 481–486, Elsevier Science Publishers, Amsterdam, The Netherlands.

75 Hallas LE, WJ Adams and MA Heitkamp. 1992. Glyphosate degradation by immobilized bacteria: field studies with industrial wastewater effluent. Appl Environ Microbiol 58: 1215–1219.

76 Hannoun BJM and G Stephanopoulos. 1986. Diffusion coefficients of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes. Biotechnol Bioeng 28: 829–835.

77 Hattori T and R Hattori. 1976. The physical environment in soil microbiology: an attempt to extend principles of microbiology to soil microorganisms. CRC Crit Rev Microbiol 4: 423–461.

78 Heijnen CE, JD van Elsas, PJ Kuikman and JA van Veen. 1988. Dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil; the effect of bentonite clay on predation by protozoa. Soil Biol Biochem 20: 483–488.

79 Heijnen CE, CH Hok-A-Hin and JA van Veen. 1992. Improvements to the use of bentonite clay as a protective agent, increasing survival levels of bacteria introduced into soil. Soil Biol Biochem 24: 533–538.

80 Heijnen CE and JA van Veen. 1990. A determination of protective microhabitats for bacteria introduced into soil. FEMS Microbiol Ecol 85: 73–80.

81 Heinze U and HJ Rehm. 1993. Biodegradation of dichloroacetic acid by entrapped and adsorptive immobilized *Xanthobacter autotrophicus* GJ10. Appl Microbiol Biotechnol 40: 158–164.

82 Heitkamp MA, V Camel, TJ Reuter and WJ Adams. 1990. Biodegradation of *p*-nitrophenol in an aqueous waste stream by immobilized bacteria. Appl Environ Microbiol 56: 2967–2973.

83 Hekman WE, CE Heijnen, JT Trevors and JD Van Elsas. 1994. Water flow induced transport of *Pseudomonas fluorescens* cells through soil columns as affected by inoculant treatment. FEMS Microbiol Ecol 13: 313–324.

84 Hickey WJ, DB Searles and DD Focht. 1993. Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. Appl Environ Microbiol 59: 1194–1200.

85 Holcberg IB and P Margalith. 1981. Alcoholic fermentation by immobilized yeast at high sugar concentration. Eur J Appl Microbiol Biotechnol 13: 133–140.

86 Hooijmans CM, CA Briasco, J Huang, BGM Geraats, J-N Barbotin, D Thomas and KChAM Luyben. 1990. Measurement of oxygen concentration gradients in gel-immobilized recombinant *Escherichia coli*. Appl Microbiol Biotechnol 33: 611–618.

87 Hu Z-C, RA Korus and KE Stormo. 1993. Characterization of immobilized enzymes in polyurethane foams in a dynamic bed reactor. Appl Microbiol Biotechnol 39: 289–295.

88 Hu Z-C, RA Korus, WE Levinson and RL Crawford. 1994. Adsorption and biodegradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium*. Environ Sci Technol 28: 491–496.

89 Huang J, CM Hooijmans, CA Briasco, BGM Geraats, KChAM Luyben, D Thomas and J-N Barbotin. 1990. Effect of free-cell growth parameters on oxygen concentration profiles in gel-immobilized recombinant *Escherichia coli*. Appl Microbiol Biotechnol 33: 619–623.

90 Hulst AC, J Tramper, K van't Riet and JMM Westerbeek. 1985. A new technique for the production of immobilized biocatalyst in large quantities. Biotechnol Bioeng 27: 870–876.

91 Hunik JH and J Tramper. 1993. Large-scale production of κ-carrageenan droplets for gel-bead production: theoretical and practical limitations of size and production rate. Biotechnol Prog 9: 186–192.

92 Hunik JH, MP van den Hoogen, W de Boer, M Smit and J Tramper. 1993. Quantitative determination of the spatial distribution of *Nitrosomonas europaea* and *Nitrobacter agilis* cells immobilized in κ-carrageenan gel beads by a specific fluorescent-antibody labelling technique. Appl Environ Microbiol 59: 1951–1954.

93 Jackman SC, H Lee and JT Trevors. 1992. Survival, detection and containment of bacteria. Microb Rel 1: 125–154.

94 Jacobsen CS and JC Pedersen. 1992. Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) in soil inoculated with *Pseudomonas cepacia* DBO1 (pRO101), *Alcaligenes eutrophus* AEO106 (pRO101) and *Alcaligenes eutrophus* JMP134(pJP4): effects of inoculation level and substrate concentration. Biodegradation 2: 253–263.

95 Jung G, J Mugnier, HG Diem and YR Dommergues. 1982. Polymer-entrapped rhizobium as an inoculant for legumes. Plant and Soil 65: 219–231.

96 Kanasawud P, S Hjorleifsdottir, O Holst and B Mattiasson. 1989. Studies on immobilization of the thermophilic bacterium *Thermus aquaticus* YT-1 by entrapment in various matrices. Appl Microbiol Biotechnol 31: 228–233.

97 Karel ST and CR Robertson. 1989. Autoradiographic determination of mass-transfer limitations in immobilized cell reactors. Biotechnol Bioeng 34: 320–336.

98 Karsten G and H Simon. 1993. Immobilization of *Proteus vulgaris* for the reduction of 2-oxo acids with hydrogen gas or formate to D-2-hydroxy acids. Appl Microbiol Biotechnol 38: 441–446.

99 Kearney L, M Upton and A McLoughlin. 1990. Enhancing the viability of *Lactobacillus plantarum* inoculum by immobilizing the cells in calcium-alginate beads incorporating cryoprotectants. Appl Environ Microbiol 56: 3112–3116.

100 Keweloh H, HJ Heipieper and HJ Rehm. 1989. Protection of bacteria against toxicity of phenol by immobilization in calcium alginate. Appl Microbiol Biotechnol 31: 383–389.

101 Keweloh H, G Weyrauch and HJ Rehm. 1990. Phenol-induced membrane changes in free and immobilized *E. coli*. Appl Microbiol Biotechnol 33: 66–71.

102 Kilbane JJ, DK Chatterjee and AM Chakrabarty. 1983. Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia*. Appl Environ Microbiol 45: 1697–1700.

103 Klein J and KD Vorlop. 1985a. Immobilization of whole cells. In: Biotechnology Focus. 1 Fundamentals, Applications, Information (Finn RK, P Prave, M Schlingmenn, W Crueger, K Esser, R Thauer and F Wagner, eds), Hanser Publishers, New York, USA.

104 Klein J and KD Vorlop. 1985b. Immobilization techniques—cells. In: Comprehensive Biotechnology Vol 2. The Principles of Biotechnology: Engineering Considerations (Moo-Young M, CL Cooney and AE Humphrey, eds), Pergamon Press, Oxford, UK.

105 Klein J and KD Vorlop. 1984. Kinetic aspects of process development for penicillin G cleavage with immobilized cell biocatalysts. German Chem Eng 7: 233–240.

106 Klein J and F Wagner. 1983. Methods for the immobilization of microbial cells. In: Applied Biochemistry and Bioengineering Volume 4: Immobilized Microbial Cells (Chibata I and LB Wingard Jr, eds), Academic Press, NY, USA.

107 Kolot FB. 1988. Immobilized Microbial Systems: Principles, Techniques, and Industrial Applications. Robert E Krieger Publishing Co, Malabar, Florida, USA.

108 Kolot FB. 1981. Microbial carriers—strategy for selection. Proc Biochem 5: 2–9.

109 Kren V, J Ludvik, O Kofronova, J Kozova and Z Rehacek. 1987. Physiological activity of immobilised cells of *Claviceps fusiformis* during long-term semicontinuous cultivation. Appl Microbiol Biotechnol 26: 219–226.

110 Kuek C and TM Armitage. 1985. Scanning electron microscopic examination of calcium alginate beads immobilizing growing mycelia of *Aspergillus phoenicus*. Enzyme Microb Technol 7: 121–125.

111 Kumar PKR and K Schugerl. 1990. Immobilization of genetically engineered cells: a new strategy for higher stability. J Biotechnol 14: 255–272.

112 Lamar RT and DM Dietrich. 1990. *In situ* depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl Environ Microbiol 56: 3093–3100.

113 Lamar RT, MW Davis, DM Dietrich and JA Glaser. 1994. Treatment of a pentachlorophenol- and creosote-contaminated soil using the lignin-degrading fungus *Phanerochaete sordida*: a field demonstration. Soil Biol Biochem 26: 1603–1611.

114 Lancy ED and OH Tuovinen. 1984. Ferrous ion oxidation by *Thiobacillus ferrooxidans* immobilized in calcium alginate. Appl Microbiol Biotechnol 20: 94–99.

115 Lee CM, CJ Lu and MS Chuang. 1994. Effects of immobilized cells on the biodegradation of chlorinated phenols. Water Sci Technol 30: 87–90.

116 Lee ST, SK Rhee and GM Lee. 1994. Biodegradation of pyridine by freely suspended and immobilized *Pimelobacter* sp. Appl Microbiol Biotechnol 41: 652–657.

117 Lefebvre J and J-C Vincent. 1995. Diffusion-reaction-growth coupling in gel-immobilized cell systems: model and experiment. Enzyme Microb Technol 17: 276–284.

118 Le Tacon F, G Jung, J Mugnier, P Michelot and C Mauperin. 1985. Efficiency in a forest nursery of an ectomycorrhizal fungus inoculum produced in a fermenter and entrapped in polymeric gels. Can J Bot 63: 1664–1668.
119 Leung K, MB Cassidy, S Holmes, H Lee and JT Trevors. 1994. Detection of κ-carrageenan-encapsulated *Pseudomonas aeruginosa* UG2Lr by polymerase chain reaction (PCR) and non-selective plating in a forest soil. FEMS Microbiol Ecol 16: 71–82.
120 Lievense LC and K van't Riet. 1993. Convective drying of bacteria. II. Factors influencing survival. Adv Biochem Eng/Biotechnol 50: 71–89.
121 Lin JE, HY Wang and RF Hickey. 1991. Use of coimmobilized biological systems to degrade toxic organic compounds. Biotechnol Bioeng 38: 273–279.
122 Lin JE and HY Wang. 1991. Degradation of pentachlorophenol by non-immobilized, immobilized and co-immobilized *Arthrobacter* cells. J Ferment Bioeng 72: 311–314.
123 Mahmoud W and HJ Rehm. 1986. Morphological examination of immobilized *Streptomyces aureofaciens* during chlortetracycline fermentation. Appl Microbiol Biotechnol 23: 305–310.
124 Marcipar A, N Cochet, L Brackenridge and JM Lebeault. 1979. Immobilization of yeasts on ceramic supports. Biotechnol Lett 1: 65–70.
125 Marin-Iniesta F, M Nasri, P Dhulster, J-N Barbotin and D Thomas. 1988. Influence of oxygen supply on the stability of recombinant plasmid pTG201 in immobilized *E. coli* cells. Appl Microbiol Biotechnol 28: 455–462.
126 Marshall KC. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J Gen Microbiol 68: 290–295.
127 Marshall KC and FJ Rogers. 1963. Influence of fine particle material on survival of *Rhizobium trifolii* in sandy soil. Nature 198: 410–412.
128 Marshall KC. 1968. Interaction between colloidal montmorillonite and cells of *Rhizobium* species with different ionogenic surfaces. Biochim Biophys Acta 156: 179–184.
129 Martinsen A, I Storro and G Skjak-Braek. 1992. Alginate as immobilization material: III diffusional properties. Biotechnol Bioeng 39: 186–194.
130 Marvin-Sikkema FD and JAM de Bont. 1994. Degradation of nitroaromatic compounds by microorganisms. Appl Microbiol Biotechnol 42: 499–507.
131 Mattiasson B. 1983a. Immobilization methods. In: Immobilized Cells and Organelles. Vol I (Mattiasson B, ed), pp 3–25, CRC Press, Boca Raton, FL, USA.
132 Mattiasson B. 1983b. Immobilized viable cells. In: Immobilized Cells and Organelles, Vol II (Mattiasson B, ed), pp 23–40, CRC Press, Boca Raton, FL, USA.
133 McLoughlin AJ. 1994. Controlled release of immobilized cells as a strategy to regulate ecological competence of inocula. Adv Biochem Eng/Biotechnol 51: 1–45.
134 Meusel M and HJ Rehm. 1993. Biodegradation of dichloroacetic acid by freely suspended and adsorptive immobilized *Xanthobacter autotrophicus* GJ10 in soil. Appl Microbiol Biotechnol 40: 165–171.
135 Middeldorp PJM, M Briglia and MS Salkinoja-Salonen. 1990. Biodegradation of pentachlorophenol in natural soil by inoculated *Rhodococcus chlorophenolicus*. Microb Ecol 20: 123–139.
136 Monbouquette HG and DF Ollis. 1988. Scanning microfluorimetry of Ca-alginate immobilized *Zymomonas mobilis*. Bio/Technology 6: 1077–1079.
137 Mörsen A and HJ Rehm. 1987. Degradation of phenol by a mixed culture of *Pseudomonas putida* and *Cryptococcus elinovii* adsorbed on activated carbon. Appl Microbiol Biotechnol 26: 283–288.
138 Mosbach K (ed). 1987a. Immobilized enzymes and cells. Part B. Methods in Enzymology. Vol 135. Academic Press, London, UK.
139 Mosbach K (ed). 1987b. Immobilized enzymes and cells. Part C. Methods in Enzymology. Vol 136. Academic Press, London, UK.
140 Mosbach K (ed). 1987c. Immobilized enzymes and cells. Part D. Methods in Enzymology. Vol 137. Academic Press, London, UK.
141 Mugnier J and G Jung. 1985. Survival of bacteria and fungi in relation to water activity and the solvent properties of water in biopolymer gels. Appl Environ Microbiol 50: 108–114.
142 Muhr AH and JMV Blanshard. 1982. Diffusion in gels. Polymer 23: 1012–1026.
143 Muller W, A Winnefeld, O Kohls, T Scheper, W Zimelka and H Baumgartl. 1994. Real and pseudo oxygen gradients in Ca-alginate beads monitored during polargraphic Po_2-measurements using Pt-needle microelectrodes. Biotechnol Bioeng 44: 617–625.
144 Musgrave SC, NW Kerby, GA Codd and WDP Stewart. 1983. Structural features of calcium alginate entrapped cyanobacteria modified for ammonia production. Eur J Appl Microbiol Biotechnol 17: 133–136.
145 Nasri M, S Sayadi, J-N Barbotin, P Dhulster and D Thomas. 1987a. Influence of immobilization on the stability of pTG201 recombinant plasmid in some strains of *Escherichia coli*. Appl Environ Microbiol 53: 740–744.
146 Nasri M, S Sayadi, J-N Barbotin and D Thomas. 1987b. The use of the immobilization of whole living cells to increase stability of recombinant plasmids in *Escherichia coli*. J Biotechnol 6: 147–157.
147 Nava Saucedo JE, B Audras, S Jan, CE Bazinet and J-N Barbotin. 1994. Factors affecting densities, distribution and growth patterns of cells inside immobilization supports. FEMS Microbiol Rev 14: 93–98.
148 Nawaz MS, W Franklin and CE Cerniglia. 1992. Degradation of acrylamide by immobilized cells of a *Pseudomonas* sp and *Xanthomonas maltophilia*. Can J Microbiol 39: 207–212.
149 Nguyen AL and JHT Luong. 1986 Diffusion in κ-carrageenan gel beads. Biotechnol Bioeng 28: 1261–1267.
150 Norton S, K Watson and T D'Amore. 1995. Ethanol tolerance of immobilized brewers' yeast cells. Appl Microbiol Biotechnol 43: 18–24.
151 Nunez MJ and JM Lema. 1987. Cell immobilization: application to alcohol production. Enzyme Microbiol Technol 9: 642–651.
152 O'Reilly AM and JA Scott. 1995. Defined coimmobilization of mixed microorganism cultures. Enzyme Microb Technol 17: 636–646.
153 O'Reilly KT, R Kadakia, RA Korus and RL Crawford. 1988. Utilization of immobilized-bacteria to degrade aromatic compounds common to wood-treatment wastewaters. Water Sci Technol 20: 95–100.
154 O'Reilly KT and RL Crawford. 1989a. Degradation of pentachlorophenol by polyurethane-immobilized *Flavobacterium* cells. Appl Environ Microbiol 55: 2113–2118.
155 O'Reilly KT and RL Crawford. 1989b. Kinetics of *p*-cresol degradation by an immobilized *Pseudomonas* sp. Appl Environ Microbiol 55: 866–870.
156 Ogbonna JC, M Matsumura and H Kataoka. 1991. Effective oxygenation of immobilized cells through reduction in bead diameters: a review. Proc Biochem 26: 109–121.
157 Ogbonna JC, M Matsumura, T Yamagata, H Sakuma and H Kataoka. 1989. Production of micro-gel beads by a rotating disk atomizer. J Ferm Bioeng 68: 40–48.
158 Omar SH. 1993a. Oxygen diffusion through gels employed for immobilization. 1. In the absence of microorganisms. Appl Microbiol Biotechnol 40: 1–6.
159 Omar SH. 1993b. Oxygen diffusion through gels employed for immobilization. 2. In the presence of microorganisms. Appl Microbiol Biotechnol 40: 173–181.
160 Omar SH and HJ Rehm. 1988. Degradation of *n*-alkanes by *Candida parapsilosis* and *Penicillium frequentans* immobilized on granular clay and aquifer sand. Appl Microbiol Biotechnol 28: 103–108.
161 Omar SH, U Budecker and HJ Rehm. 1990. Degradation of oily sludge from a flotation unit by free and immobilized microorganisms. Appl Microbiol Biotechnol 34: 259–263.
162 Overmeyer C and H-J Rehm. 1995. Biodegradation of 2-chloroethanol by freely suspended and adsorbed immobilized *Pseudomonas putida* US2 in soil. Appl Microbiol Biotechnol 43: 143–149.
163 Paul E, J Fages, P Blanc, G Goma and A Pareilleux. 1993. Survival of alginate-entrapped cells of *Azospirillum lipoferum* during rehydration and storage in relation to water properties. Appl Microbiol Biotechnol 40: 34–39.
164 Pertot E, D Rozman, S Milicic and H Socic. 1988. Morphological differentiation of immobilized *Claviceps paspali* mycelium during semi-continuous cultivation. Appl Microbiol Biotechnol 28: 209–231.
165 Pidoux M, MF Pilet and V Ripoche. 1992. Growth performances of *Lactobacillus hilgardii* immobilized in dextran gel and in continuous fermentation. World J Microbiol Biotechnol 8: 393–398.
166 Pilar Pons M and M Carmen Fuste. 1993. Uranium uptake by immobilized cells of *Pseudomonas* strain EPS 5028. Appl Microbiol Biotechnol 39: 661–665.
167 Poncelet D, B Bugarski, BG Amsden, J Zhu, R Neufeld and MFA Goosen. 1994. A parallel plate electrostatic droplet generator: para-

meters affecting microbead size. Appl Microbiol Biotechnol 42: 251–255.
168 Portier RJ and K Fujisaki. 1986. Continuous biodegradation and detoxification of chlorinated phenols using immobilized bacteria. Toxicity Assessment 1: 501–513.
169 Providenti MA, H Lee and JT Trevors. 1993. Selected factors limiting the microbial degradation of recalcitrant compounds. J Ind Microbiol 12: 379–395.
170 Radehaus PM and SK Schmidt. 1992. Characterization of a novel *Pseudomonas* sp that mineralizes high concentrations of pentachlorophenol. Appl Environ Microbiol 58: 2879–2885.
171 Rattray EAS, JI Prosser, LA Glover and K Killham. 1992. Matric potential in relation to survival and activity of a genetically modified microbial inoculum in soil. Soil Biol Biochem 24: 421–425.
172 Rehm H-J and SH Omar. 1993. Special morphological and metabolic behaviour of immobilized microorganisms. In: Biotechnology. Volume 1. Biological Fundamentals (Sahm H, ed), pp 223–248, VCH Verlagsgesellschaft mbH, Weinheim, FRG.
173 Rhodes DJ. 1993. Formulation of biological control agents. In: Exploitation of Microorganisms (Jones DG, ed), pp 411–439, Chapman and Hall, London, UK.
174 Saber DL and RL Crawford. 1985. Isolation and characterization of *Flavobacterium* strains that degrade pentachlorophenol. Appl Environ Microbiol 50: 1512–1518.
175 Sahasrabudhe SR, AJ Modi and VV Modi. 1988. Dehalogenation of 3-chlorobenzoate by immobilized *Pseudomonas* sp B13 cells. Biotechnol Bioeng 31: 889–893.
176 Salkinoja-Salonen MS, PJM Middeldorp, M Briglia, R Valo, M Haggblom and A McBain. 1989. Clean up of old industrial sites. In: Advances in Applied Biotechnology Vol 4 (Kamely D, A Chakrabarty and G Omenn, eds), pp 344–367, Gulf Publishing Company, Houston, TX, USA.
177 Sayadi S, M Nasri, F Berry, J-N Barbotin and D Thomas. 1987. Effect of temperature on the stability of plasmid pTG201 and productivity of *xylE* gene product in recombinant *Escherichia coli*: development of a two-stage chemostat with free and immobilized cells. J Gen Microbiol 133: 1901–1908.
178 Scherer P, M Kluge, J Klein and H Sahm. 1981. Immobilization of the methanogenic bacterium *Methodsanosarcina barkeri*. Biotechnol Bioeng 23: 1057–1065.
179 Scott RI, SJ Wills and C Bucke. 1987. Oxygen uptake by κ-carrageenan entrapped *Streptomyces clavuligerus*. Enzyme Microbiol Technol 10: 151–155.
180 Scott CD. 1987. Immobilized cells: a review of recent literature. Enzyme Microb Technol 9: 66–73.
181 Scott CD, CA Woodward and JE Thompson. 1989. Solute diffusion in biocatalyst gel beads containing biocatalysts and other additives. Enzyme Microbiol Technol 11: 258–263.
182 Seech AG, JT Trevors and TL Bulman. 1991. Biodegradation of pentachlorophenol in soil: the response to physical, chemical, and biological treatments. Can J Microbiol 37: 440–444.
183 Shieh WK, JA Puhakka, E Melin and T Tuhkanen. 1990. Immobilized-cell degradation of chlorophenols. J Environ Eng 116: 683–697.
184 Shirai Y, K Hashimoto, H Yamaji and H Kawahara. 1988. Oxygen uptake rate of immobilized growing hybridoma cells. Appl Microbiol Biotechnol 29: 113–118.
185 Shreve GS and TM Vogel. 1993. Comparison of substrate utilization and growth kinetics between immobilized and suspended *Pseudomonas* cells. Biotechnol Bioeng 41: 370–379.
186 Siahpush AR, JE Lin and HY Wang. 1991. Effect of adsorbents on degradation of toxic organic compounds by coimmobilized systems. Biotechnol Bioeng 39: 619–628.
187 Smidsrod O and G Skjak-Braek. 1990. Alginate as immobilization matrix for cells. Trends Biotechnol 8: 71–78.
188 Smit E, H Lee, JT Trevors and JD Van Elsas. 1996. Interaction between a genetically engineered *Pseudomonas fluorescens* and bacteriophage ΦR2f in soil: effect of nutrients, alginate encapsulation and rhizosphere. Microb Ecol (in press).
189 Smith MR, A de Haan and JAM de Bont. 1993. The effect of calcium alginate entrapment on the physiology of *Mycobacterium* sp strain E3. Appl Microbiol Biotechnol 38: 642–648.
190 Sparrow SD and GE Ham. 1983. Survival of *Rhizobium phaseoli* in six carrier materials. Agron J 75: 181–184.
191 Speitel Jr GE, C-J Lu, M Turakhia and X-J Zhu. 1989. Biodegradation of trace concentration of substituted phenols in granular activated carbon columns. Environ Sci Technol 23: 68–74.
192 Stewart PS and CR Robertson. 1989. Microbial growth in a fixed volume: studies with entrapped *Escherichia coli*. Appl Microbiol Biotechnol 30: 34–40.
193 Stormo KE and RL Crawford. 1994. Pentachlorophenol degradation by microencapsulated flavobacteria and their enhanced survival for *in situ* aquifer bioremediation. In: Applied Biotechnology for Site Remediation (Hinchee RE, DB Anderson, SB Metting and GD Sayler, eds), pp 422–427, Lewis Publishers, Ann Arbor, MI.
194 Stormo KE and RL Crawford. 1992. Preparation of encapsulated microbial cells for environmental applications. Appl Environ Microbiol 58: 727–730.
195 Stotzky G. 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. In: Interactions of Soil Minerals with Natural Organics and Microbes. pp 305–428, Soil Science Society of America Special Publication No 17, Madison, WI, USA.
196 Strullu DG and C Plenchette. 1991. The entrapment of *Glomus* sp in alginate beads and their use in inoculum. Mycol Res 95: 1194–1196.
197 Sun Y, S Furusaki, A Yamauchi and K Ichimura. 1989. Diffusivity of oxygen into carriers entrapping whole cells. Biotechnol Bioeng 34: 55–58.
198 Tampion J and MD Tampion. 1987. Immobilized Cells: Principles and Applications. Cambridge University Press, Cambridge, UK.
199 Tanaka A and H Nakajima. 1990. Application of immobilized growing cells. Adv Biochem Eng/Biotechnol 42: 97–131.
200 Tanaka H, M Matsumura and IA Veliky. 1984. Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26: 53–58.
201 Thomas ORT and GF White. 1990. Immobilization of the surfactant-degrading bacterium *Pseudomonas* C12B in polyacrylamide gel beads: I. Effect of immobilization on the primary and ultimate biodegradation of SDS, and redistribution of bacteria within beads during use. Enzyme Microbiol Technol 12: 697–705.
202 Thomas ORT and GF White. 1991. Immobilization of the surfactant-degrading bacterium *Pseudomonas* C12B in polyacrylamide gel. III. Biodegradation specificity for raw surfactants and industrial wastes. Enzyme Microbiol Technol 13: 338–343.
203 Tosa T, T Sato, T Mori, K Yamamoto, I Takata, Y Nishida and I Chibata. 1979. Immobilization of enzymes and microbial cells using carrageenan as matrix. Biotechnol Bioeng 21: 1697–1709.
204 Tramper J, KChAM Luyben and WJJ van den Tweel. 1983. Kinetic aspects of glucose oxidation by *Gluconobacter oxydans* cells immobilized in ca-alginate. Eur J Appl Microbiol 17: 13–18.
205 Trevors JT. 1991. Respiratory activity of alginate-encapsulated *Pseudomonas fluorescens* cells introduced into soil. J Microbiol Meth 14: 11–20.
206 Trevors JT, JD Van Elsas, H Lee and AC Wolters. 1993. Survival of alginate encapsulated *Pseudomonas fluorescens* cells in soil. Appl Microbiol Biotechnol 39: 637–643.
207 Trevors JT, JD Van Elsas, H Lee and LS Van Overbeek. 1992. Use of alginate and other carriers for encapsulation of microbial cells for use in soil. Microb Rel 1: 61–69.
208 Trevors JT, JD Van Elsas, LS Van Overbeek and ME Starodub. 1990. Transport of a genetically engineered *Pseudomonas fluorescens* strain through a soil microcosm. Appl Environ Microbiol 56: 401–408.
209 Valo RJ, MH Haggblom and M Salkinoja-Salonen. 1990. Bioremediation of chlorophenol containing simulated groundwater by immobilized bacteria. Wat Res 24: 253–258.
210 Valo R and M Salkinoja-Salonen. 1986. Bioreclamation of chlorophenol-contaminated soil by composting. Appl Microbiol Biotechnol 25: 68–75.
211 Van Elsas JD, JT Trevors, D Jain, AC Wolters, CE Heijnen and LS Van Overbeek. 1992. Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. Biol Fertil Soils 14: 14–22.
212 Van Elsas JD and CE Heijnen. 1990. Methods for the introduction of bacteria into soil: a review. Biol Fertil Soils 10: 127–133.
213 Van Elsas JD, AF Dijkstra, JM Govaert and JA Van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol Ecol 38: 151–160.
214 Van Loosdrecht MCM, J Lyklema, W Norde and AJB Zehnder. 1990.

Influence of interfaces on microbial activity. Microbiol Rev 54: 75–87.
215 Van Neerven ARW, RH Wijffels and AJB Zehnder. 1990. Scanning electron microscopy of immobilized bacteria in gel beads: a comparative study of fixation methods. J Microbiol Methods 11: 157–168.
216 Vargas R and T Hattori. 1986. Protozoan predation of bacterial cells in soil aggregates. FEMS Microbiol Ecol 38: 233–242.
217 Vives C, C Casas, F Godia and C Sola. 1993. Determination of the intrinsic fermentation kinetics of *Saccharomyces cerevisiae* cells immobilized in ca-alginate beads and observations on their growth. Appl Microbiol Biotechnol 38: 467–472.
218 Wada M, J Kato and I Chibata. 1979. A new immobilization of microbial cells. Appl Microbiol Biotechnol 8: 241–247.
219 Walker HL and WJ Connick Jr. 1983. Sodium alginate for the production and formulation of mycoherbicides. Weed Sci 31: 333–338.
220 Weir SC, H Lee and JT Trevors. 1995a. Survival of free and alginate-encapsulated *Pseudomonas aeruginosa* UG2Lr in soil treated with disinfectants. J Appl Bacteriol (in press).
221 Weir SC, SP Dupuis, MA Providenti, H Lee and JT Trevors. 1995b. Nutrient-enhanced survival of and phenanthrene mineralization by alginate-encapsulated and free *Pseudomonas* sp UG14Lr in creosote-contaminated soil slurries. Appl Microbiol Biotechnol 43: 946–951.
222 Wessolek G and C Fahrenhorst. 1994. Immobilization of heavy metals in a polluted soil of a sewage farm by application of a modified alumino-silicate: a laboratory and numerical displacement study. Soil Technol 7: 221–232.
223 Westmeier F and HJ Rehm. 1985. Biodegradation of 4-chlorophenol by entrapped *Alcaligenes* sp A 7-2. Appl Microbiol Biotechnol 22: 301–305.
224 Westmeier F and HJ Rehm. 1987. Degradation of 4-chlorophenol in municipal wastewater by adsorptive immobilized *Alcaligenes* sp A 7-2. Appl Microbiol Biotechnol 26: 78–83.
225 White GF and ORT Thomas. 1990. Immobilization of the surfactant-degrading bacterium *Pseudomonas* C12B in polaycrylamide gel. II. Optimizing SDS-degrading activity and stability. Enzyme Microb Technol 12: 969–975.
226 Wiesel I, SM Wubker and HJ Rehm. 1993. Degradation of polycyclic aromatic hydrocarbons by an immobilized mixed bacterial culture. Appl Microbiol Biotechnol 39: 110–116.
227 Wijffels RH, GC Schukking and J Tramper. 1990. Characterization of a denitrifying bacterium immobilized in carrageenan. Appl Microbiol Biotechnol 34: 399–403.
228 Wijffels RH, AW Schepers, M Smit, CD de Gooijer and J Tramper. 1994. Effect of initial biomass concentration on the growth of immobilized *Nitrosomonas europaea.* Appl Microbiol Biotechnol 42: 153–157.
229 Wijffels RH, G Englund, JH Hunik, EJTM Leenen, A Bakketun, A Gunther, JM Obon de Castro and J Tramper. 1995. Effects of diffusion limitation on immobilized nitrifying microorganisms at low temperatures. Biotechnol Bioeng 45: 1–9.
230 Wijffels RH and J Tramper. 1989. Performance of growing *Nitrosomonas europaea* cells immobilized in κ-carrageenan. Appl Microbiol Biotechnol 32: 108–112.
231 Willaert R and G Baron. 1993. Growth kinetics of gel-immobilized yeast cells studied by on-line microscopy. Appl Microbiol Biotechnol 39: 347–352.
232 Wong PK, KC Lam and CM So. 1993. Removal and recovery of Cu(II) from industrial effluent by immobilized cells of *Pseudomonas putida* II-11. Appl Microbiol Biotechnol 39: 127–131.
233 Woodward J. 1988. Methods of immobilization of microbial cells. J Microbiol Meth 8: 91–102.
234 Wu WM, L Bhatnagar and JG Zeikus. 1993. Performance of anaerobic granules for degradation of pentachlorophenol. Appl Environ Microbiol 59: 389–397.
235 Yang PY and TS See. 1991. Packed entrapped mixed microbial cell process for removal of phenol and its compounds. J Environ Sci Health 8: 1491–1512.
236 Zache G and HJ Rehm. 1989. Degradation of phenol by a coimmobilized entrapped mixed culture. Appl Microbiol Biotechnol 30: 426–432.
237 Zhang X, S Bury, D DiBiasio and JE Miller. 1989. Effects of immobilization on growth, substrate consumption, β-galactosidase induction, and byproduct formation in *Escherichia coli.* J Ind Microbiol 4: 239–246.
238 Zhou W, B Winter and W Zimmermann. 1993. Dechlorination of high-molecular-mass compounds in spent sulphite bleach effluents by free and immobilized cells of streptomycetes. Appl Microbiol Biotechnol 38: 418–423.