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Immobilization of Cells for Application in the Food Industry

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ABSTRACT: Immobilization of cells offers advantages to the food process industries, including enhanced fermentation productivity and cell stability and reduced downstream processing costs due to facilitated cell recovery and recycle. This article summarizes the varied immobilization methodologies, including adsorption, entrapment, covalent binding, and microencapsulation. Examples of interest to the food industry are provided, together with a review of the physiological effects of immobilization. Topics in process engineering include immobilized cell bioreactor configurations and the scale-up potential of the various immobilization techniques.

KEY WORDS: cell immobilization, adsorption, covalent binding, carrageenan, alginate, microencapsulation, mass transfer, continuous stirred tank reactor, packed bed reactor, scale-up.

I. INTRODUCTION

Numerous techniques for the immobilization of cells have been developed in the last 2 decades as shown in Table 1. Biotechnology in the food industry involves a large spectrum of cells in fermentation processes, including bacteria, yeast, fungi, plant, and potentially animal cells. Immobilization often mimics what occurs naturally when cells grow on surfaces or within natural structures. Industrial benefits to immobilization include enhanced fermentation productivity and cell stability and lower cost of downstream processing because cells are easily recovered and recycled. Cell immobilization may also protect cells against shear damage. Other applications in food production may include the immobilization of flavors, fragrances, pigments, and antioxidants as examples but will not be discussed in this review.

Surface binding and entrapment techniques are commonly used to immobilize cells. Binding involves cell attachment to a surface by ionic interactions or covalent binding, or cell to cell adhesion that may be natural or induced by chemical agents. Adsorption is a simple and inexpensive procedure, but cell leakage is often observed. Entrapped cells are captive within a protective matrix structure or a capsule, reducing cell release but often resulting in mass transfer limitations.

The most important advantage of cell immobilization is enhancement of productivity via continuous operation and reuse of cells. Thus, a variety of immobilized cell bioreactors have been investigated to optimize the conditions for fermentation. Cell immobilization is used industrially, but further application will depend on the development of immobilization procedures that can be readily scaled-up.

The purpose of this article is to review immobilization techniques of interest to the food industry, to consider the effects of immobilization on

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TABLE 1
Examples of Immobilization Techniques and Products Formed by Cells of Interest to the Food Industry

Culture	Immobilization technique	Product	Ref.
Escherichia coli	Polyacrylamide gel	L-malate L-aspartate	Chibata, 1976
Yeast	Gelatin cross-linked by oxidized starch	Wine	Parascandola, 1992
Lavandula vera	Carrageenan/agar	Pigments	Nakajima, 1985
Leuconostoc oenos	Carrageenan with silica gel	L-malate to L-lactate	Spettoli, 1987
Lactobacillus sp.			
Bifidobacterium longum	Gellan gum	L-Lactic acid	Camelin, 1993
Corynebacterium glutamicum	Polyvinyl alcohol	L-Lysine	Velizarov, 1992
Yeast	Alginate with alginate coat	Sparkling wine	Godia, 1991
Saccharomyces cerevisiae	Polyacrylamide-coated alginate	Ethanol	Ruggeri, 1991
Bifidobacteria	Cellulose acetate phthalate membranes	Cells	Rao, 1989
Acetic acid bacteria	Carrageenan	Acetic acid	Mori, 1989
Saccharomyces cerevisiae	Porous ceramics	Ethanol	Demuyakor, 1992
Lactobacillus casei	Carrageenan and locust bean gum	Yogurt	Lacroix, 1990
Zymomonas mobilis	Alginate	Ethanol	Melick, 1987
E. coli	Carrageenan	L-Aspartic acid	Seko, 1986
Alcaligenes faecalis	Polyacrylamide	β-Glucosidase	Wheatley, 1983
Zymomonas mobilis	Birch wood chips	Ethanol	Gencer, 1980
Brevibacterium flavum	Collagen	Glutamic acid	Constantinides, 1981
Propionibacterium acidipropionici	Spiral wound matrix	Propionic acid	Lewis, 1992
Saccharomyces carlsbergensis	Alginate	Ethanol	Tzeng, 1991
Clostridium thermosaccharo-lyticum	Porour perlag beads	Ethanol	Kennard, 1991
Recombinant yeast	Agarose	L-Fumarate to L-malate	Neufeld, 1991
Aspergillus oryzae	Alginate	Kojic acid	Kwak, 1992
Bacillus amyloliquefaciens	Alginate	α-Amylase	Argarikos, 1992
Recombinant yeast	Alginate	β-Glucanase	Cahill, 1990
Saccharomyces cerevisiae	Cross-linked gelatin	Ethanol	Doran, 1986
Yeast	Alginate	Beer	Shindo, 1993

cell physiology and fermentation activity, to consider various bioreactor configurations for immobilized cell systems, and to examine scale-up of immobilization procedures.

II. PROCEDURES AND TECHNIQUES OF IMMOBILIZATION

A. Adsorption Techniques and Covalent Binding

Adsorption and covalent binding may take place between the cells and an appropriate surface, or from cell to cell. Part of the cell surface is directly in contact with the medium in contrast to encapsulation or entrapment techniques. A large fraction of the cells are captive and mass transfer limitation may be present.

1. Adsorption Techniques

Cells may be naturally attached to a surface, such as acetic acid bacteria on wood chips in vinegar production. The cells are attached to the support by electrostatic interactions (Van der Walls forces, ionic, and hydrogen bonds) thus experience similar conditions to those in suspension culture. Growth results in a biofilm on the support surface and mass transfer limitations take place within this biofilm. Carriers include wood chips, sand, ion-exchange resins, and organic materials such as cellulose and activated carbon (Kennedy and Cabral, 1983; Klein and Vorlop, 1983; Gonçalves et al., 1992; Andrews and Fonta, 1989). In order to increase cell loading, porous carriers are often used, permitting cells to attach to the internal surface structure (Iwasaki et al., 1992, 1993). This type of immobilization is performed directly in the fermentor by introducing both inoculum and carrier to the medium. The carrier is washed to remove nonadhering cells before commencing fermentation. Thus, the procedures are simple and inexpensive and the conditions gentle on the cells. The disadvantage lies in the vulnerability of the weakly bound biofilm to detachment from the support.

Natural adsorption as an immobilization method is widely used for wastewater treatment, including within the food industry (Huang et al., 1992). However, adsorption techniques are not as common in food production (Bakoyianis et al., 1992; Jain et al., 1991).

2. Covalent Binding

Stronger cell-support binding reducing cell loss is possible via covalent attachment to a support material. Direct linkage of the cells to an activated support is possible by chemical modification of the carrier (Kennedy and Cabral, 1983; Klein and Vorlop, 1985; Kozlyak et al., 1992). Modified supports used have included cross-linked gelatin (Doran and Bailey, 1986), cross-linked chitosan beads (Shinonaga et al., 1992), and ceramics pretreated with polyethylenimine (Guoqiang et al., 1992). Porous materials enhance the surface area available for cell binding (Ahmad and Johri, 1992). The disadvantage compared with adhesion on an inert support is the risk of cell damage because the cell membrane is involved in covalent binding to the support (Doran and Bailey, 1986).

3. Cell to Cell Attachment

Floculation is a natural phenomenon resulting in cell aggregation. Not all the cells floculate and natural cell aggregates are generally unstable and sensitive to shear. Thus, aggregates are promoted by addition of cross-linking agents (Klein and Vorlop, 1985; Kennedy and Cabral, 1983).

B. Entrapment Techniques

Polyacrylamide gel (Mosbach and Mosbach, 1966) was the first matrix material used to immobilize cells (Mattiasson, 1983). Monomer toxicity and reactive polymerization reduces cell viability. According to Siess and Divies (1981), about 30% of *Lactococcus casei* were destroyed during immobilization. However, cell growth regenerated a stable population in the biocatalyst beads.

This procedure was used in industrial production of L-malic and L-aspartic acid (Chibata et al., 1976). Klein and Vorlop (1985) described other polymers used to entrap whole cells, such as polymethylacrilamide and polyhydroxyethylmethacrylate. Synthetic polymers obtained by polycondensation, such as epoxy resins, and polyurethane were tested for whole cell immobilization, but generally the toxicity of the reagent largely reduced the proportion of viable cells (Klein and Wagner, 1983; Klein and Vorlop, 1985). Polymer precipitation (Klein and Wagner, 1983) has been used to entrap whole cells in a network of cellulose triacetate, cellulose and Eudragit as examples. In this case, coagulation occurs by phase separation induced by changing physicochemical parameters. This method involves solvents that are quite toxic.

The covalent cross-linking of natural polymers such as collagen (Vieth and Venkatsubramanian, 1979), albumin (Petre et al., 1978; Drioli, 1982), or gelatin (Bachman et al., 1981) results in a network formation due to chemical cross-linking of polymer by a bifunctional reagent. Similar results in terms of cell viability were obtained for the polymerization techniques because of the toxicity of the cross-linking agent. However, living yeast cells were immobilized within gelatin cross-linked by oxidized starch that has no antimicrobial activity (De Alteriis et al., 1990). This technique offers interesting advantages for wine production because of mechanical properties and chemical stability of the matrix (Parascandola et al., 1992).

The tendency in the food industry is more toward the use of nontoxic polymers that tend to form gel-like structures such as proteins, polysaccharides, and polyvinyl alcohols.

Regarding proteins, Mattiasson (1983) describes applications for collagen. Gelatin also forms a gel at reduced temperature (Klein and Vorlop, 1985). Poor mechanical properties of the gels may be improved by cross-linking. Polyvinyl alcohol, although a nontoxic synthetic polymer, may induce loss of viability.

The advantage of polysaccharides such as κ-carrageenan, calcium alginate, agar, agarose, chitosan, and gellan gum lies in their biocompatibility and acceptability in food applications. Gelification is induced by temperature changes in the case of agar, agarose, κ-carrageenan, and gellan gum. An ionotropic gelation due to ionic cross-linking of polyionic chains with multivalent counterions occurs with alginate, chitosan, carboxymethyl-cellulose, and carboxy-guar gum.

Enhanced mechanical and chemical stability of gels formed by various mixed polymers has been investigated (De Smet Poncelet et al., 1993). The matrix structure may be a membrane or a gel layer (Johansen and Flink, 1986b; Mitani et al., 1984). Because matrix entrapment often results in mass transfer limitations, beads are often preferred over films because of the enhanced surface area with spherical geometries. The gel may be formed in molds, partitioned into pieces of 2 to 5 mm (Cheetham et al., 1979b; Siess and Divies, 1981), or pressed through a sieve to generate small particles.

Beads are typically formed in a two-step procedure involving dispersion and hardening. The dispersion can be performed either by extrusion or by emulsification. In the extrusion method, the cell/polymer suspension is extruded through a needle, generating spherical droplets that fall into a hardening solution. The emulsion technique involves the dispersion of the aqueous phase consisting of the cell/polymer suspension into an organic phase, resulting in a water in oil emulsion. The dispersed aqueous droplets are hardened by cooling or by addition of the gelling agent or cross-linking agent in the case of polyacrylamide gels (Nilsson et al., 1983). The emulsion technique results in smaller diameter beads than the droplet method and is better suited to scaled-up applications.

1. κ-Carrageenan

κ-carrageenan is a natural polysaccharide extracted from marine macroalgae and is commonly used as a food additive. Takata et al. (1977a) were the first to use carrageenan for cell immobilization. High temperatures (60 to 80°C) are needed to dissolve the polymer at concentrations ranging from 2 to 5% (Klein and Vorlop, 1985) in physiological saline (0.9% NaCl). Addition of the cell slurry to the heat-sterilized polymer solution

is performed at 40 to 45°C. The maximum cell concentration in the carrageenan solution is about 3×10^{-2} mg dry weight/ml (Tosa et al., 1979). Higher cell densities can be achieved by growth of cells within the matrix. Gelation occurs by cooling to room temperature. Potassium ions are generally used for stabilization of the gel and to prevent swelling or to induce gelation.

Tosa et al. (1979) compared the influence of different gelation conditions on gel strength. Monovalent ions such as K^+, Rb^+, Cs^+, and NH_4^+ resulted in stronger gels compared with mono-, bi-, or trivalent metal ions. Stronger gels were also obtained by supplementing the polymer with locust bean gum at a ratio of 2:1 (Takata et al., 1977b; Miles et al., 1987). Oscillatory shear experiments and compressional tests on carrageenan/locust bean gum mixed gels demonstrated that elasticity of the mixed gel decreased in a supplemented whey permeate medium (Arnaud et al., 1989). The experiments suggested that a minimum critical locust bean gum complementation and an optimum KCl treatment resulted in high rigidity and important damping properties. However, KCl was shown to have an inhibitory effect on some lactic acid bacteria such as *S. thermophilus* or *L. bulgaris* (Audet et al., 1988), thus the hardening time in KCl was reduced. The rheological properties were less affected by cell growth than by physicochemical interactions between gel and whey permeate-supplemented medium (Arnaud et al., 1989). Carrageenan rheological properties appeared to be more sensitive to LBS medium (Lactobacillus selection medium) used to produce biomass with *Lactococcus casei.* As this strain is not inhibited by KCl, bead integrity was maintained on LBS medium by addition of KCl to the fermentation broth (Lacroix et al., 1990). Moon and Parulekar (1991) investigated the physical properties of carrageenan used to immobilize *Bacillus firmus.* The mechanical strength increased with increasing carrageenan concentration, corresponding to decreased cell release. The optimum concentration for strength and reduced cell leakage was 4%.

Cell release was also observed by Audet et al. (1989). Cell leakage may be of interest when continuous inoculation is desired. However, for biomass production, cell release is a disadvantage. Nunez et al. (1990) investigated bead treatment with $Al(NO_3)_3$ to enhance cell retention. The treatment induced gel hardening, but diffusion and cell viability was also reduced.

Block and membrane structures may be prepared, as well as beads by the droplet method, or by the emulsification technique. The droplet method consists in adding dropwise the warm cell/polymer suspension (3 to 4% w/v) to a KCl solution (2%) at room temperature (Tosa et al., 1979). By the emulsion technique (Audet et al., 1988; Audet et al., 1989; Audet and Lacroix, 1989) the cell/polymer suspension is emulsified in sterile vegetable oil in a thermostated reactor. The emulsion is then cooled to room temperature by circulating tapwater in a reactor jacket, resulting in the gelation of carrageenan droplets. The gel beads are then harvested in sterile 0.3 *M* KCl for 2 h. Oil is removed and beads are sieved to obtain the desired size distribution. Depending on the operational conditions of the process, the size can vary between 250 µm and 4 mm (Audet and Lacroix, 1989). This procedure has been used successfully to immobilize lactic acid bacteria for batch (Lacroix et al., 1990) and continuous fermentation (Audet et al., 1992).

Carrageenan has been used to immobilize plant cells. Entrapment of *Lavandula vera* for production of pigments was performed (Nakajima et al., 1985), but low growth rates and productivities were obtained compared with immobilization within alginate gels. Beaumont et al. (1989) demonstrated improved protein production and cell viability in carrageenan than in alginate with immobilized *Apium graveolens.*

Carrageenan has been used for monolactic fermentation in wine instead of alginate, which tends to disrupt with phosphate present (Spettoli et al., 1987). *Leuconostoc oenos* or *Lactobacillus* immobilized in carrageenan with and without silica gel exhibited operational stability in continuous fermentation. Silica gel increased the half-life of immobilized *L. oenos* and induced a higher conversion rate of L-malic acid to L-lactic acid. Silica gel probably increased gel porosity, resulting in better cell activity. Mattiasson (1983) noted that activity and viability of cells immobilized in carrageenan are generally similar to that of free cells.

2. Calcium Alginate Gel

Alginic acid is a heteropolysaccharide of L-guluronic acid and D-mannuronic acid extracted from various species of algae. Depending on the source, the composition and the sequence in L-guluronic acid and D-mannuronic acid varies widely. The monomers are arranged in a pattern of blocks along the chain, with homopolymeric regions interspersed with regions of alternating structure (Smidsrod et al., 1972).

After processing, alginates are available as water-soluble sodium alginates. When the water-soluble carboxylic polyelectrolyte is mixed with multivalent counterions (Ca^{2+}, Al^{3+}, Zn^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, or Fe^{3+}), gelation occurs. Water-soluble Na-alginate forms a Ca-alginate gel in $CaCl_2$ solution.

Alginate beads are formed by droplet or emulsification methods (Figure 1). A 2 to 4% Na-alginate solution is sterilized by filtration prior to introducing the cell suspension (Smidsrod and Skjak-Braek, 1990). Gel beads are produced by dropping the cell/polymer suspension through a syringe needle (0.22 to 1 mm i.d.), to free-fall approximately 20 cm into a $CaCl_2$ (20 to 100 m*M*) hardening solution.

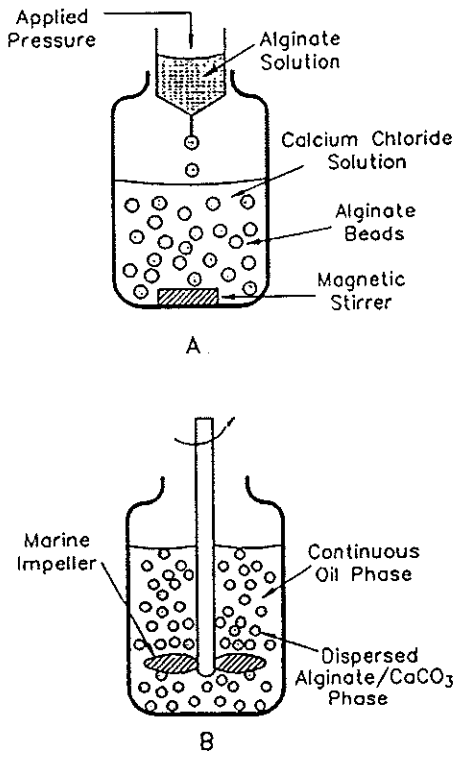

FIGURE 1. Production of alginate beads by (A) extrusion of sodium alginate sol containing cells into calcium chloride solution; (B) emulsification/internal gelation by solubilization of coencapsulated calcium carbonate.

The emulsification process involves the dispersion of the cell/polymer suspension into a vegetable oil. The cell/alginate droplets are gelled following addition of $CaCl_2$ solution (Nilsson et al., 1983) or by internal gelation (Poncelet et al., 1993). In the later case, an insoluble calcium vector such as the carbonate salt is added prior to emulsion formation. The liberation of Ca^{2+} is initiated by the addition of an oil-soluble acid such as acetic acid, reducing the alginate pH from 7.5 to approximately 6.5. Following gelation, the beads are partitioned into water and washed to remove residual oil. The technique is referred to as emulsification/internal gelation.

Ionotropic gelation is reversible by immersing the beads in citrate (50 m*M*) or phosphate buffer at pH 7 (Smidsrod and Skjak-Braek, 1990), resulting in liquefaction of the beads.

Various physicochemical properties and different operational conditions during bead formation influence the strength, size, and stability of the gel. Homoguluronic sequences of the polymer are mainly responsible of the gelation in the presence of Ca^{2+} (Smidsrod and Haug, 1972). The monomeric composition, block structure, and molecular size of the alginate strongly affect the mechanical and swelling properties of the alginate beads (Martinsen et al., 1989). Reduction of the mechanical properties are observed for alginate with low viscosity or molecular weight, and below a certain critical molecular weight the gel-forming abilities of alginates are reduced (Smidsrod, 1990; Johansen and Flink, 1986a). Jamuna et al. (1992) optimized the sodium alginate, calcium chloride and cell concentration, and curing time to obtain better strength associated with good immobilized yeast cell productivity. The size and the shape of the bead depend on the viscosity of the Na-alginate solution, which is related to the molecular weight, and on the distance between the syringe and the $CaCl_2$ collecting solution (Smidsrod and Skjak-Braek, 1990).

The stability of alginate beads is quite low in the presence of chelating agents such as phosphate, lactate, and citrate due to shared affinity for calcium, destabilizing the gel (Smidsrod and Skjak-Braek, 1990). Thus, stability problems were encountered during lactic acid fermentation (Roy et al., 1987). Yeast extract also contains chelating agents (Birnbaum et al., 1981). Some authors proposed using ions with better affinity for alginate such as Al^{3+}, Sr^{2+}, and Ba^{2+} to strengthen the gel, but these alternatives may be unacceptable for food applications because of potential toxicity. Other ions such as Pb^{2+} or Cu^{2+} are more efficient but are also more toxic. Stabilizing Ca-alginate gel with trivalent ions such as Al^{3+} was successful, and yeast immobilized in such gels demonstrated good viability (Rochefort et al., 1986).

Johansen and Flink (1986a) used an internal gelation method by adding tricalcium citrate to Na-alginate solution containing cells. The gelation was performed in molds, and the gels were then cut into small pieces. Higher strength was obtained with this technique. Another technique to enhance gel stability involves supplementing fermentation medium with calcium (Martinsen et al., 1989; Champagne et al., 1993).

3. Agar and Agarose

Agar is a natural polymeric complex extracted from various species of marine macroalgae. Agarose is obtained from agar by separation and purification. Agar gelation is induced by temperature decrease similar to that of carrageenan. Immobilization of cells in agar or agarose gel is obtained by dissolving the polysaccharide in water (1 to 4%) at 100°C (between 90 and 100°C for agarose), then cooling to 50°C (30 to 50°C for agarose) for mixing with cells. Finally, gelification occurs when the gel is cooled to ambient temperature. The gelification is performed using molds and further disruption (Holcberg and Margalith, 1981) by droplet methods or the emulsion technique similar to that employed for carrageenan beads (Neufeld et al., 1991; Nilsson et al., 1983).

This immobilization technique is only suitable for cells able to tolerate the higher temperatures to which they would be subject prior to gelation. Agarose with a low gelling temperature (15°C) is available and has been used to immobilize islets of Langerhans (Dupuy et al., 1986). The mechanical strength of agar and agarose is rather weak and makes the gel unstable compared with alginate or carrageenan. However, the procedure is simple and does not require ionic gelification or stabilization of the gel.

Agar was used to immobilize *Clostridium butyricum* (Matsunaga et al., 1978), yeast (Holcberg and Margalith, 1981), and *Lactobacillus casei* (Tuli et al., 1985). Plant cell entrapment was performed successfully with agarose for *Catharanthus roseus* in ajmalicine isomer production (Felix et al., 1981; Brodelius, 1983). Immobilization of *Lavandula vera* (plant cells) for pigment production was performed (Nakajima et al., 1985) in agar. In this case, cell release was significant and mechanical strength of agar gel was insufficient for repeated use of the entrapped cells.

4. Chitosan Gel

Chitosan like alginate forms a gel by ionotropic gelation. Chitosan is a linear polysaccharide obtained by deacetylation of chitin extracted from crustacean shells. Chitosan can be dissolved in dilute acid at pH under 6. Chitosan, a polycation with amine groups, can be cross-linked by cations or polycations (poly-phosphates, $[Fe(CN)_6]^{4-}$, $[Fe(CN)_6]^{3-}$, polyaldehydrocarbonic acid; Klein and Vorlop, 1983). The procedure to obtain chitosan beads is similar to that used for alginate beads. The cell slurry is introduced to a chitosan-acetate solution (final chitosan concentration of 1.6 to 2.4% (w/v) and added dropwise into a gently stirred 1.5% (w/v) sodium-tripoly-phosphate solution at pH 5.5 (Vorlop and Klein, 1981b). Further hardening involves placing the beads in sodium tripolyphosphate solution of pH 8.5 for 30 min. This polymer was used to immobilize *E. coli* (Vorlop and Klein, 1981) and for plant cell entrapment (Knorr et al., 1985). Chitosan appeared to be advantageous as it enhances the permeability of plant cells with an elicitor in secondary plant metabolite production of oxalate and proteases

(Knorr and Teutonico, 1986). However, chitosan exhibited inhibitory effects on different types of cells, lactic acid bacteria (Groboillot et al., 1993) and yeast (Ralston et al., 1964). Beaumont and Knorr (1987) demonstrated that acid-soluble chitosan affected cell viability. Beaumont et al. (1989) showed that different plant cell suspensions treated with water-soluble chitosan exhibited normal viability and enhanced protein release in the medium compared with control free cell suspension. Furthermore, water-soluble chitosan allowed release of intact pigment, whereas acid-soluble chitosan treatment resulted in product degradation (Beaumont et al., 1989; Knorr et al., 1990). To overcome viability problems with chitosan, this polymer was used for cell immobilization to obtain coacervate capsules or to coat alginate beads.

5. Gellan Gum

Gellan gum is a natural nontoxic extracellular anionic polysaccharide secreted by *Pseudomonas elodea*. Gellan gum is a linear unbranched homopolymer with tetrasaccharide-repeating units consisting of two beta-D-glucose, one beta-D-guluronic acid, and one alpha-L-rhamnose residue (O'Neil et al., 1983). Decreasing temperature induces gel formation that is stabilized by cations. The gelation occurs around 35 to 50°C. Gellan gels are cross-linked by a large variety of ions, including H^+, in contrast to alginate, which reacts with specific ions (Grasdalen and Smidsrod, 1987). Thus it is difficult to predict the rheological properties of the gel containing cells, as the complex ion composition introduced with the inoculum reacts with the polysaccharide (Camelin et al., 1993). However, divalent ions such as Ca^{2+} and Mg^{2+} exhibited better efficiency than monovalent ions such as Na^+ or K^+ (Camelin et al., 1993).

Deacetylated gellan gum is preferred because of better thermal stability and resistance to enzymes (Kang et al., 1982; Norton and Lacroix, 1990; Camelin et al., 1993). Rheological properties of this gel were similar at 60°C and room temperature (Norton and Lacroix, 1990). Thus, this polymer is suitable for thermophilic fermentation. As is the case with carrageenan and alginate, deacetylated gellan gum gel is affected by chelating ions. When the gel was hardened with calcium ions, lactate, citrate, phosphate, or acetate induced decreases of rheological properties (Norton and Lacroix, 1990). In order to decrease the gelling temperature to immobilize mesophilic microorganisms, sodium citrate and sodium metaphosphate, which are chelatants, were added to polysaccharide solution (Camelin et al., 1993). These chelating compounds always decrease the rheological properties of the gels. However, gellan gel at concentrations in the range 1.5 to 2.5% exhibited higher strength than mixed gel formed with 2.75% carrageenan/0.25% locust bean gum.

The mold, droplet, or emulsion method can be used to form the gel (Camelin et al., 1993; Norton and Lacroix, 1990). Continuous fermentation of *Bifidobacterium longum* immobilized in gellan gum beads was reported; however, supplementation of the fermentation medium was necessary to maintain high gel mechanical properties because of the production of lactic acid, which acts as a chelating agent (Camelin et al., 1993).

6. Polyvinyl Alcohol (PVA)

Polyvinyl-alcohol can form a gel when cross-linked with ultraviolet radiation. Several hours of irradiation are needed to obtain hardening, inducing cell death. PVA gel is also obtained with boric acid (Wu and Wisecarver, 1992), which may be toxic to some microorganisms. Ariga et al. (1987) investigated PVA beads hardened by iterative freezing and thawing to immobilize activated sludge. Gel strength increased with the number of freeze-thaw cycles to seven iterations. The addition of cryoprotectants such as glycerol and skim milk helped stabilize activity. Velizarov et al. (1992) demonstrated by measuring the level of intracellular ATP content that *Corynebacterium glutamicum* were partially deactivated during immobilization in PVA-cryogel. In this case, nongrowing cells were an advantage in achieving better conversion rates of glucose to lysine. Good lysine productivity was observed with immobi-

lized cells compared with growing or nongrowing cells in suspension.

7. Coated Beads

Cell leakage from an entrapment matrix is generally observed due to the gel structure and the growth of cells. Except for some applications where cell release is used as inoculum such as in yogurt production, cell leakage represents a drawback for efficient fermentation with immobilized cells. To avoid this phenomenon, improvement in the mechanical strength of the matrix was conducted (Martinsen et al., 1989; Johansen and Flink, 1986; Champagne et al., 1992). However, increased gel strength may induce lower performance because of a higher mass transfer limitation. Thus, various techniques of coating were investigated. Coating was performed to avoid cell release but also to increase mechanical and chemical stability. Overgaard et al. (1991) produced alginate beads coated with a chitosan film. The beads were obtained by dropping alginate solution in a mixture of $CaCl_2$ and chitosan. The coating of the gel bead by a free cell gel layer was performed to reduce cell leakage (Tanaka et al., 1989). Cell-free alginate coating of alginate beads containing immobilize yeast for sparkling wine production (Godia et al., 1991) or Lactococci (Prevost and Divies, 1992), reduced cell release totally or partially. However, Champagne et al. (1992) showed that two-layer calcium alginate beads or poly-L-lysine coating was not sufficient to reduce cell release during five successive fermentations of Lactococci in milk. Periodic dipping of beads in ethanol between fermentations results in death of cells near the surface of the beads, maintaining acidification activity and low cell release. Recently, *E. coli* was immobilized in agar coated with a microporous membrane to prevent cell leakage (Jouenne et al., 1993). However, the efficiency decreased for high cell loading and high growth rate.

Alginate beads coated with polyacrylamide resin (Eudragit RL100) were used by Ruggeri et al. (1991). After gelification, the beads were coated by Eudragit dissolved in ethanol either by spray or by immersion. In both cases, the coating involved ethanol evaporation. This procedure did not affect metabolic activity of the entrapped *S. cerevisiae*, and the glucose diffusivity was the same with and without polyacrylamide film. Cell release was reduced and mechanical properties improved by the coating. Larroche and Gros (1989) used this technique to immobilize spores of *Penicillium roquefortii*.

C. Microencapsulation

Microencapsulation is used largely in the pharmaceutical or medical fields for drug and enzyme immobilization (Klein et al., 1985; Chang et al., 1976). However, microencapsulation shows promise for cell immobilization in overcoming drawbacks encountered with gel entrapment. A microcapsule consists of a semipermeable, spherical, thin, and strong membrane surrounding a liquid core, with a diameter varying from a few microns to 1 mm. A capsule exhibits the same properties except for the larger diameter (0.5 to 4 mm). Consequently, because of the absence of a solid or gelled core and small diameter, mass transfer limitations should be reduced. The membrane serves as a barrier to cell release and may minimize phage contamination. Smaller diameter microcapsules exhibit increased mechanical strength (Poncelet et al., 1989) and are not sensitive to chelating agents if the membrane does not involve ionic cross-linking.

1. Encapsulation by Ionic Cross-Linking

The coacervation or ionic cross-linking of anionic and cationic polysaccharides/polyelectrolytes around a liquid core (Rha et al., 1985; McKnight et al., 1988; Hsu and Chu, 1992) may be exemplified by alginate cross-linked with chitosan, resulting in membrane formation. Typically, alginate beads are coated with chitosan with subsequent liquefaction of the alginate core. The membrane is formed by suspending the alginate beads in a chitosan solution (McKnight et al., 1988). This technique was largely applied with

poly-L-lysine or polyethyleneimine instead of chitosan for immobilization of islets of Langerhans (Lim and Sun, 1980). Poly-L-lysine films are also coated with an alginate layer before core liquefaction in order to avoid toxic effects of polycations and to increase the capsule strength.

Capsules may also be obtained by dropping liquid alginate containing cells directly into a chitosan solution. Because of the high molecular weight of chitosan (1 to 3 million), only alginate at the surface of the droplet is gelled, preventing any further penetration of chitosan (Rha et al., 1985). An alternative technique resulted in capsules consisting of a liquid chitosan core and a membrane of chitosan cross-linked by alginate. Braun et al. (1985, 1986, 1987) obtained strong capsules exhibiting high biocompatibility using cellulose sulfate cross-linked in a solution of poly(dimethyl diallyl ammonium chloride).

Generally, the core consists of the anionic polymer that is more suitable for cell viability, including carrageenan, carboxymethyl-cellulose (CMC), alginate, dextran sulfate, or a mixture of these polymer (Rha, 1985; Hsu and Chu, 1992). Rha (1985) proposed anionic polymers such as hyaluronic acid, xanthan, furcelleran, and sulfonated organic polymer. The cationic polymer may be poly-L-lysine (PLL), polyethyleneimine (PEI), chitosan, DEAE-dextran, and poly(vinylamine) (Hsu and Chu, 1992; Wang et al., 1992a, 1992b).

The mechanical stability of such capsules depends on the type, concentration, and molecular weight of polymers used. For example, PLL appeared to be stronger than PEI and DEAE-dextran to coat alginate, alginate/CMC, or alginate/dextran sulfate (Hsu and Chu, 1992). Shimi et al. (1991) showed how the molecular weight of the cationic polymer affected the thickness of the coated membrane: low-molecular-weight molecules can diffuse further into the anionic gel, resulting in a thicker but more porous membrane (Hwang et al., 1986; Huguet et al., 1993). Higher-molecular-weight cationic polymer induced thinner membrane but lower permeability to proteins.

Phase separation-coacervation methods have been used by Rao et al. (1989) to encapsulate Bifidobacteria within enteric cellulose acetate phthalate membranes. This method was originally developed (Maharaj et al. 1984) for the encapsulation of high-molecular-weight biological materials such as viral antigen and concanavalin A.

III. IMPACT OF IMMOBILIZATION ON CELL PHYSIOLOGY AND FERMENTATION ACTIVITY

Physiological reactions of cells will vary depending on the method of immobilization used. For example, direct cross-linking of cells chemically modifies the cell membrane affecting cell metabolism and growth, adsorbed cells may have different membrane properties, cell accumulation in a biofilm induces transfer limitations, and entrapment techniques result in changes to the physicochemical properties of the microenvironment, influencing cell metabolism. Typical changes to the microenvironment of the immobilized cell include the presence of ionic charges, altered water activity, modified surface tension, and cell confinement. The main factor likely influencing cell behavior is the mass transfer limitation, resulting in gradients of oxygen, substrate, and product. These changes may enhance or reduce cell metabolism; thus, immobilization may influence cell physiology and performance via a number of mechanisms that are often poorly characterized and possibly operating in opposite directions.

A. Effect of Immobilization on Fermentation Parameters

Contradictory results between different studies are evident as seen in Table 2. Differences in growth/activity of the culture are often explained in terms of mass transfer limitations, depending on bead size, concentration, and type of polymer or matrix structure and the hydrodynamic characteristics of the fermentation. Some of the factors influencing the growth/activity of immobilized cells are examined in the following sections.

1. Effect of Mass Transfer Limitations on Cell Physiology

Confinement due to immobilization induces a different environment for immobilized cells.

TABLE 2
Impact of Immobilization on Growth/Activity of Various Cell Lines

Culture	Support/matrix	Product/property	Effect	Ref.
Pseudomonas fluorescens	Polyvinyldene polyethylene or glass	Glucose assimilation	Enhanced by 2- to 5-fold	Fletcher, 1986
Aspergillus oryzae	Alginate	Kojic acid (flavor enhancer)	Lower growth, production, yield	Kwak and Rhee, 1992
Bacillus amyloliquefaciens	Alginate	α-Amylase	Productivity reduced 20%	Argarikos, 1992
Saccharomyces	Alginate	Biomass, ethanol	Growth rates reduced 20%, ethanol production increased 4-fold; yields were similar	Hannoun and Stephanopoulous, 1990
S. bayanus	Carrageenan	Biomass, ethanol	Increased ethanol, cell yields; reduced glucose uptake; reduced amount of byproduct	Taipa et al., 1993
S. carlsbergensis	Carrageenan	Ethanol	Higher productivity	Wada et al. 1980
S. cerevisiae	Alginate	Ethanol, glycerol	Higher productivity by 2-fold	Galazzo and Bailey, 1989
Streptococcus salivarius Lactococcus lactis	Carrageenan locust bean gum	Lactic acid	Similar production	Audet et al., 1989
L. delbrueckii	Carrageenan locust bean gum	Lactic acid	Reduced production	Audet et al., 1989
Recombinant yeast	Alginate	β-Glucanase	Higher productivity	Cahill et al., 1989
Yeast	Cross-linked to silica	Ethanol	Glycolytic flow and growth similar for free and immobilized cells	Navarro and Durand, 1977
Saccharomyces cerevisiae	Cross-linked gelatin	Ethanol	Increased DNA content and reduced double stranded RNA compared with free cells; higher fermentation capacity, although growth rate decreased by 45%	Doron and Bailley, 1986
Yeast	Alginate	Continuous ethanol fermentation	Increased enzyme, carbohydrate levels, glycolytic flow decreased by 60% compared with free cells; higher glycogen levels were characteristic of resting cells	Simon et al., 1990
Saccharomyces cerevisiae	Agarose	L-Fumarate to L-malate	Reduced activity with increasing bead diameter	Neufeld et al., 1991
Yeast	Alginate	Beer	Production of succinic acid due to increased isoleucine consumption; reduced ester accumulation compared with conventional brewing	Shindo et al., 1993

Rouxhet et al. (1990) established a simple mass transfer model through a matrix of immobilized cells at steady state. Model results obtained fit experimental data for the rate of oxygen uptake by yeast cells in a silica hydrogel. This model showed that for cells with a diameter of 5 μm oxygen is supplied over a thickness of about 60 μm or 12 cellular strata. Shinmyo et al. (1982) reported that *Bacillus amyloliquefaciens* immobilized in carrageenan were oxygen deficient, resulting in reduced cell growth and improved production of β-amylase. Mass transfer limitations affect nutrient or oxygen uptake but also removal of product, resulting in product accumulation around the immobilized cell. Many models have been developed to predict the effect of product inhibition due to mass transfer limitation on productivity and cell viability within gel beads (Luong, 1985; Monbouquette et al., 1990; and Sayles and Ollis, 1990).

The influence of bead size on fermentation kinetics is often observed and related to mass transfer limitation. Vives et al. (1993) did not observe any difference between suspended yeasts and those immobilized in 1-mm-diameter beads. Hannoun and Stephanopoulos (1990) did observe differences in activity following immobilization in 2.4-mm-diameter beads within a 0.4-mm-thick membrane. With lactic acid bacteria, similar kinetics between free and immobilized cells were reported in beads ranging from 0.5 to 1 mm. Larger diameter beads (1 to 2 mm) resulted in lower lactic acid production, sugar consumption, and growth (Audet et al. 1989).

The properties of the polymer used to form the gel affect the fermentation kinetics by modifying mass transfer characteristics of the final matrix. For yeast immobilized within alginate cubes, Johansen and Flink (1986b) studied the influence of alginate molecular weight, the ratio between guluronic and mannuronic acid (G/M ratio) and alginate concentration on the gel strength, gel stability during fermentation, fermentation rate, and ethanol productivity. When either alginate concentration or G/M ratio decreased or when the molecular weight was very low (viscosity of 1% alginate solution around 5 cp), the strength of the beads decreased and the fermentation rate as ethanol productivity of immobilized yeast cells increased. The alginate properties resulting in weaker gels, probably facilitating a higher degree of substrate and product transport through the gel network, enhancing fermentation performance. However, Gossmann and Rehm (1986) found that increasing alginate concentration had only a small influence on the oxygen diffusion within the gel.

The cell density within the matrix can also affect the mass transfer properties of the beads. An optimum cell content for maximum product yield was found for alginate entrapped *S. cerevisiae* (Axelsson, 1988). Epoxidation of propene by mycobacteria was used by Smith et al. (1990) as a model system to compare physiology of immobilized and free cells. When the cell loading within alginate beads was maintained at a low level, kinetics of the epoxidation reaction was similar for immobilized and free bacteria. A high cell loading likely induced mass transfer limitation affecting reaction kinetics of immobilized cells.

Mass transfer limitations may also be due to cell growth. It was demonstrated for different types of cells immobilized within alginate beads of 2.3 mm diameter that with increasing cell concentration the specific respiration rate decreased (Gossman and Rehm, 1986). In fact, with increasing cell concentration oxygen was consumed faster than it could diffuse into the beads; thus, oxygen diffusion became the limiting factor.

Mass transfer limitations exhibit some advantages, such as when immobilization reduces diffusion of a repressor of cell activity. Fortin and Vuillemard (1990) showed that protease production by *Myxococcus xanthus* is inhibited by end-products of proteolysis. Immobilized cells were less sensitive to this inhibition and produced six times more protease than free cells.

A variety of hypotheses have been proposed to explain the modified physiological state of immobilized cells. According to Mattiasson and Hahn-Hägerdal (1982), transfer limitations result in decreased water activity and oxygen or substrate deficiency, the entrapped cells sustaining a high maintenance metabolism. Reduced growth results in improved yields for reactions involving maintenance metabolism.

Most of the time, growth is observed within the matrix even if diffusion problems are present. As low-molecular-weight compounds easily diffuse through alginate gel, Axelsson and Pearson

(1988) interpreted the matrix influence as a space limitation affecting cell growth rather than a mass transfer effect. However, Karel and Robertson (1989) did not agree with this assumption, as microscopic evidence suggests that growing cells maintain a normal shape and a reasonable fraction of extracellular volume within the matrix continues to be found in the growth region. No inhibitory effect of crowding was observed on cell growth as cell concentrations can reach extremely high values in the growing region.

2. Effect of Soluble Components of the Matrix

Smith et al. (1990) reported that, whereas cell physiology was altered by immobilization in alginate, the same effect may be obtained with free cells incubated with soluble sodium alginate. Higher ethanol productivity was obtained by immobilized *Saccharomyces bayanus* in carrageenan than by free cells (Brito et al., 1990). A similar performance was observed when free cells were cultured in a medium containing empty carrageenan gel beads or celite particles (Vieira et al., 1989). The effect was at least partially due to media supplementation with components in the matrix, such as Ca^{2+}, which increases yeast ethanol tolerance.

Saucedo et al. (1989a) observed that viability of immobilized mycelia under starvation conditions was longer than that of free mycelial culture. It was postulated that mycelia can metabolize alginate with glycolytic enzymes.

Hahn-Hägerdal et al. (1985) demonstrated that some water-soluble polymers such as dextran and polyethylene glycol enhanced ethanol production with yeast by modifying the cell membrane. Alginate or the other immobilizing polymers may similarly affect cell physiology. Diefenbach et al. (1992) observed that fatty acid impurities in alginate probably result in a modified fatty acid pattern for immobilized cells compared with free cells.

3. Cell Distribution within the Gel and Immobilized Cell Morphology

Various methods have been used to observe cells or estimate cell density within the matrix, including microscopic techniques, bead sectioning, or dissolution of the beads in the case of alginate. Cells have been observed to grow within small cavities of the gel (Barbotin et al., 1990). It is generally observed that growth of cells is not uniform throughout the gel particle but often occurs in the surface region (Audet et al., 1988; Wada et al., 1980; Gosmann and Rehm, 1986; Godia et al., 1987b). Because growth depends on diffusion of nutrients and products, a majority of *E. coli* cells were located in a 150-μm-thick peripheral layer (Barbotin et al., 1990). By using scanning or transmission electron microscopy, cell density may be determined as a function of location (Burrill et al., 1983). Jamuna and Ramakrishna (1990) showed that yeast cell growth mainly occurred in the periphery of the 4-mm-diameter bead, with some growth also evident within the core. Rapid growth near the gel surface induces a decrease in the rigidity and mechanical strength of the gel; consequently, cavities near the surface rupture releasing cells (Barbotin et al., 1990), perhaps indicating an optimal diameter of 300 μm to minimize mass transfer limitations. Cell release was enhanced with high dilution rate during continuous fermentation with immobilized acetic acid bacteria in carrageenan (Mori et al., 1989). Newly released cells from the gel maintained extremely high growth rates and respiratory activity for a few generations.

Inoculum size affects spatial distribution of cells within the matrix (Berry et al., 1988). When a small inoculum was used (4.7×10^3 cell/ml), the number of cells in the gel increased to 2×10^{10} cells/ml at stationary phase with the cells forming large microcolonies within the beads. The use of a higher inoculum density resulted in growth limited to the outer layer of the beads achieving a population of 3.8×10^{10} cells/ml (Barbotin et al., 1990).

Morphological changes of immobilized cells were observed using electron microscopy. Simon et al. (1990) showed that the total volume of the entrapped cells is increased compared with free cells. For *Gibberella fujikuroi*, mycelial growth within the beads was affected by a physical barrier imposed by the alginate network structure, resulting in zigzag-shaped mycelia (Saucedo et al., 1989b). Immobilized mycelium exhibited a

rough external cell wall compared with a smooth cell observed with nonimmobilized hyphea.

B. Modification of Cell Metabolism

1. Type of Immobilization

The type of immobilization, whether adsorption or entrapment, has different effects on cell physiology. For example, during ethanol fermentation with *S. cerevisiae* (Sroka and Rzedowski, 1991), the amount of byproduct produced depended on the type of support; cells immobilized on porous glass produced low levels of byproduct in contrast to high levels produced by cells immobilized on foamed polystyrene.

Saucedo et al. (1989b) observed that the type and concentration of the support (polyurethane, carrageenan, or alginate) affected the production of secondary metabolites, growth, and viability of immobilized *G. fujikuroi* under starvation conditions.

2. Tolerance to Toxic Compounds

The tolerance of immobilized cells to toxic compounds such as alcohols (Holcberg and Margalith, 1981) is often enhanced, possibly because of the cell membrane. The proportion of the main saturated fatty acid, palmitic acid (C16:0) of *E. coli* increases strongly after immobilization. Changes in the lipid-protein ratio of the membranes were also observed in immobilized cells (Keweloh et al., 1990). The proportion of saturated fatty acids, and therefore the degree of saturation, was higher in immobilized cells because of the mode of growth that corresponded to the late exponential phase of free cells. It was also shown that the uptake of saturated fatty acids present in commercial alginates, and incorporation into membrane lipids increased the phenol tolerance of growing *E. coli* by modifying membrane permeability (Keweloh et al. 1990).

3. Secondary Metabolite Production

Plant cells produce important secondary metabolites with applications as drugs, flavors, and fragrances. Positive and negative effects of immobilization on secondary metabolite production were reported by Bringi and Shuler (1990). As secondary metabolites are produced by different pathways that depend largely on environmental conditions, immobilization often affects production. The matrix may act an elicitor, resulting in enhanced product concentration in the fermentation medium.

4. Internal and Optimum pH

During fermentation with *Saccharomyces bayanus* entrapped in carrageenan (Taipa et al., 1993) or *S. cerevisiae* immobilized in porous ceramics (Demuyakor and Ohta, 1992), the medium pH was less acidic than in free cell culture. Furthermore, ^{31}P NMR was used to show that immobilized cells maintain a higher cytoplasmic pH during glucose fermentation, whereas freely suspended cells were unable to maintain an efficient pumping of protons to the exterior of the cell under conditions of strong acidification of the external medium following contact with glucose. In the case of free cells, acidification of the cytoplasm is enhanced with increasing ethanol concentration of the medium (Loureiro-Dias and Santos, 1990). The authors interpreted the modified behavior of immobilized cells, resulting in increased ethanol productivity, as a protective effect of immobilization.

Different effects of immobilization were observed on the cytoplasmic pH of *S. cerevisiae* immobilized in alginate (Galazzo and Bailey, 1989), where immobilization resulted in a lower pH of the cell cytoplasm. In these studies, growth was undetected within the beads during the pH monitoring. Better conversion of glucose to ethanol was observed for immobilized cells compared with free cells.

The protective effect of immobilization was also observed for *S. cerevisiae* immobilized in alginate. External pH levels between 2.5 and 6.2 had no influence on fermentation parameters in contrast with suspended cells that demonstrate an optimum at pH 4 (Buzas et al., 1989). Improved tolerance to perturbations in the external pH was also observed for fermentation of dairy waste by

Candida pseudotropicalis; however, the optimum pH was unaffected (Marwaha et al. 1989). The optimum pH was also not modified for *Corynebacterium glutamicum* immobilized in carrageenan (Kim et al., 1982).

5. Cell Distribution and Metabolism

Heterogeneous distribution of cells within the immobilization matrix is often observed and results in altered metabolic states of the cell depending on location. Variation in cell physiology with location is generally related to mass transfer limitations. Simon et al. (1990) found that the proportion of C16 and C18 fatty acids in the cell membrane in immobilized cells varied with the location of the cells in the beads. Amounts at the surface and at the center of the beads are similar to levels measured in aerobic and anaerobic culture, respectively. Scanning microphotometry and microfluorimetry revealed that relative RNA content per cell varied with the radial position of cells within the beads. RNA levels have been shown to vary approximately linearly with specific growth rate for a number of species (Harder and Roels, 1982). The specific RNA levels of cells in the rapidly growing colonies of the outer cell layer were two times higher than the values obtained for cells of the much smaller colonies found in the bead center.

Similar results were obtained by using ^{35}S pulse labeling combined with autoradiography to quantify protein synthesis and degradation by cells immobilized in a porous wall of a hollow-fiber reactor (Karel and Robertson, 1989). Generally, in the core of the fiber, cells are in a resting, starving phase or are nonviable. Karel et al. (1989) explained the dynamics of growing and starving cells by the regulation of the internal pressure. Growing cells have an elevated internal pressure. In the presence of mechanical constraints such as the growth region of a gel, cells can exert forces with similar magnitudes to that of the constraint. Starving cells are unable to maintain their volume when high pressure is exerted, resulting in cell compression in the core of the matrix.

6. Plasmid Stability

As a consequence of cell captivity within the immobilization matrix, immobilized recombinant cell populations exhibit better plasmid stability than suspended cells. Plasmids that replicate at rates slower than the growth of the host cell are eventually depleted because of the progressive dilution of plasmid-bearing microorganisms. The usual strategy to overcome this disadvantage is to impose a positive counterselection by cloning antibiotic resistance into the plasmid. Thus, only cells with plasmids can survive and multiply in the presence of the antibiotic. Immobilization of cells with plasmids significantly enhanced the volumetric productivities due to high cell concentrations (Marin Iniesta et al., 1988; Sode et al., 1988). Immobilization resulted in maintenance of plasmid expression as a function of the generation number. The improvement of plasmid stability was caused by restricted growth in the gel beads that prevented plasmid loss. A limited number of cell divisions occur within each clone of cells before the clone is released from the gel bead. This low number of generations does not permit the apparition of plasmid-free cells. Thus, the number of cells without plasmids is reduced with immobilization (Nasri et al., 1987; de Taxis du Poët et al., 1986, 1987).

Plasmid stability is also improved for cells attached to cross-linked gelatine beads (Walls and Gainer, 1989). Because medium composition affects the plasmid copy number, under conditions where plasmid instability was increased with free cells in nutrient depleted medium, immobilization improved plasmid maintenance (Sayadi et al., 1989). Improved overall plasmid stability could be due to an evolution of the outer cell population in relation to those within the core maintaining better plasmid stability. This hypothesis seems to be confirmed, as the percentage of plasmid-containing cells was higher in the core of the beads (50%) compared with 40% in the periphery and 28% for released cells (Cahill et al., 1990). Briasco and Thomas (1990) also observed that plasmid stability tended to decrease for cells near the bead surface. Heterogeneity of the distribution of cells containing plasmids is increased with fermentation time and

corresponds to heterogeneity of cell growth rates within the gel.

IV. IMMOBILIZED CELL BIOREACTORS

Various reactors have been used with immobilized cells. The choice of reactor is related to the type of immobilization, to the metabolism of cells, and to the mass transfer requirement. For example, the resistance of the matrix to shear stress, the size of the beads that affects the mass transfer properties, or the oxygen requirements of the cells may determine the type of reactor. Typical reactors used for immobilized cells (Figure 2) are the continuous stirred tank (CSTR), fluidized bed (FBR), packed bed (PBR), and air-lift reactor (ALR). The FBR provides a compromise between good mixing conditions (CSTR) and low shear stress (PBR) on the immobilized biocatalyst.

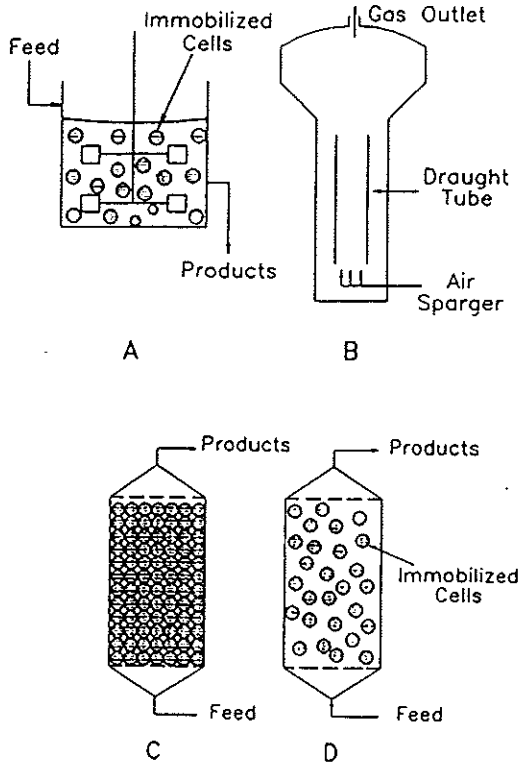

FIGURE 2. Immobilized cell bioreactor configurations. (A) Continuous stirred tank reactor; (B) air-lift reactor; (C) packed bed reactor; (D) fluidized bed reactor.

Many industrial, pilot, or laboratory-scale fermentations are typically run in batch mode. However, continuous fermentation with immobilized cells may enhance productivity because cell separation, recycle, or growth are not required to maintain high cell density in the reactor, enhancing productivity and minimizing risk of contamination. When the cells are immobilized, elevated dilution rates can be achieved without wash-out, boosting higher substrate concentrations, increasing reaction rate. Ansemle and Tedder (1987) reported better performance during continuous production of ethanol with immobilized yeast compared with batch mode.

The performance of a bioreactor using immobilized cells cannot be expressed by the intrinsic volumetric productivity, as the total biomass is difficult to determine. Thus, the volumetric productivity is used, either by volume of liquid or volume of reactor.

A. Continuous Stirred Tank Reactor (CSTR)

The CSTR provides complete mixing by the use of impellers mounted on a rotating shaft. The liquid components of the reactor are homogeneous in composition, similar to the concentration of the outflow. With immobilized cells, high fluid velocities are needed to achieve a constant supply of substrate and product removal. However, high stirring rates necessary to obtain well-mixed conditions may result in abrasion damage to the immobilized catalyst because of high shear forces at the impeller surface. Nevertheless, the CSTR offers the best mixing characteristics and oxygen transfer.

The substrate concentrations in a CSTR are typically lower than the packed bed and fluidized bed reactor, resulting in lower average reaction rates. However, a lower substrate concentration may be advantageous for inhibited cell culture. Lacroix et al. (1990) produced yogurt with cells immobilized in carrageenan and locust bean gum in a CSTR. It was observed that 4 to 5% of the beads was destroyed by shear, even if the medium was supplemented with KCl.

B. Packed Bed Reactor (PBR)

In a PBR, the biocatalyst particles are packed into a column through which the substrate solution passes. If the fluid velocity profile is perfectly flat, the PBR operates as a plug-flow reactor that is an ideal behavior. This type of reactor has been largely used for ethanol production (Godia et al. 1987b). PBRs with immobilized cell aggregates have also been used on an industrial scale (Chibata et al., 1979).

Productivity of the PBR for a given biocatalyst depends on the type of immobilization, whether by entrapment or surface attachment. Higher cell loadings are often achieved by entrapment, resulting in improved productivity (Anselme and Tedder, 1987). Anselme and Tedder screened carriers for surface immobilization demonstrating that fire brick resulted in the best reactor performance. Particle size for cell attachment had a strong influence on productivity with an optimum diameter of 910 μm.

PBRs have the advantage of simplicity of operation and low cost. Anaerobic conditions are easily maintained if needed and may be improved by sparging the substrate solution with nitrogen.

High rates of reaction may be achieved throughout the length of the PBR. Lower regions receiving highly concentrated substrate medium exhibited an almost complete substrate conversion (Godia et al., 1987a) in contrast to higher levels in the column. In ethanol fermentations, high ethanol concentrations in the upper portions of the column result in product inhibition. Activity within the lower regions are affected by substrate inhibition. Daugulis and Swaine (1987) modeled the effect of product and substrate inhibition on ethanol productivity in a PBR. The glucose and ethanol profiles in a PBR were predicted by Melick et al. (1987). The proposed model for ethanol production by *Zymomonas mobilis* immobilized in Ca-alginate combined simultaneous diffusion and reaction kinetics. As a consequence of substrate and product variation along the column (increasing ethanol concentration and decreasing nutrients with increasing column height), the cell viability in the reactor varied as a function of axial distance along the bioreactor. Godia et al. (1987a) found a 40% decrease in viability from bottom to top for immobilized yeast in carrageenan for ethanol fermentation. An empirical equation was established by the authors to describe the distribution of viable cells in the reactor. Anselme and Tedder (1987) measured the cell dry mass per unit of reactor volume as a function of position in a four-chamber reactor. A large variation was observed with reactor height. Carrageenan immobilized yeast in a tapered column PBR (Hamamci and Ryu, 1987) varied from about 50% viability at the bottom to about 30% at the top of the reactor. An uneven decay of immobilized cell viability in the axial direction of a PBR was also observed in the case of L-aspartic production from fumarate and ammonia by carrageenan immobilized *E. coli* due to the exothermic reaction. To overcome this problem, Seko et al. (1986) used a PBR equipped with a horizontal heat-exchange tube bundle.

The PBR does not offer any advantage for aerobic culture, as oxygenation of the substrate may not be sufficient to oxygenate the entire reactor because of rapid oxygen depletion. The PBR is more commonly used for anaerobic fermentations, including the production of ethanol (Lee and Maddox, 1986).

The rate of external mass transfer of substrate and product in a PBR is typically less than that of a CSTR; thus, reaction rate may be limited by mass transfer. Comparisons were made between a laboratory-scale stirred vessel and a PBR for β-glucosidase activity of polyacrylamide-immobilized *Alcaligenes faecalis* (Wheatley and Philips, 1983). At high substrate concentrations, no difference was observed on the substrate conversion, but a low substrate concentration resulted in lower performance caused by external diffusion limitation. However, Cheetham et al. (1979a) showed that a rapid diffusion of sucrose into pellets was achieved when the fractional voidage of the column was as low as 0.1. Godia et al. (1987c), using a steady state fermentor model, including intrinsic fermentation kinetics, dispersion from plug flow in the fermentor, external mass transfer in the gel beads, and simultaneous internal diffusion and reaction, showed that the external transfer was not the rate-limiting factor.

Gas accumulation during the process, such as occurs when CO_2 is produced during ethanol

fermentation, may induce dead space in the reactor. Resultant channeling of fluids may affect substrate conversion (Ghose and Bandyopadhyay, 1980). Gas build-up may also result in back-mixing, resulting in deviation from ideal plug flow behavior.

High-pressure drops in the PBR may result in bed compaction. According to Cheetham et al. (1979), a packed column of alginate beads exhibits high resistance to hydrostatic compression. These results were confirmed by constant strain rate and stress-relaxation measurements of the compressibility of unstressed columns of pellets (Cheetham, 1979a). Seko et al. (1986) used a pilot reactor with a bed height of 2 m loaded with 1.32 m^3 of *E. coli* immobilized in carrageenan. Compaction was not observed during L-aspartic acid production. Compaction of bed particles was reported by Furasaki et al. (1982) in the case of gel beads and Ramakrishna et al. (1988) observed compaction of the bed for a small column (50 mm i.d. × 50 cm bed height) packed with alginate immobilized yeast. The compaction appeared more significant in the case of a larger column (100 mm i.d. × 100 cm bed height). The compression of the bed induced lower reaction rates by reducing void fraction in the bed (Ramakrishna et al., 1988).

Bed compaction also depends on the type of bead. When attachment on an inert support by self-immobilization or by covalent binding is used, the compaction of the particles is less of a problem because of the stronger resistance to compression of the support. Gencer and Mutharasan (1980) produced ethanol with *Zymomonas mobilis* immobilized on birch wood chips. Compaction resulted in a damaged matrix and enhanced cell release. Cell growth and accumulating biomass tended to plug the PBR after extended operation (Anselme and Tedder, 1987), reducing reactor productivity.

Sieve plate baffles were incorporated in the reactor designed by Ramakrishna et al. (1988) in order to reduce compaction problems. CO_2 hold-up and increased pressure drop in the reactor resulted in the design of a tapered column PRB in which the cross-sectional area increased with height, resulting in better gas removal and reduced pressure drop (Hamamci and Ryu, 1987). Colak and Hamamci (1991) showed that the angle (0 to 4°) of the tapered column containing yeast immobilized in carrageenan did not affect the ethanol productivity for different packed volumes. The smallest angle that allowed stable operation without any build-up was 2°. Margaritis et al. (1985) and Lee and Maddox (1986) improved the performance of the PBR by fitting a cylindrical steel mesh to the inside of the column that was then operated at an angle of 10° to the horizontal with upward flow of feed medium, facilitating gas removal.

A PBR based on Couette Taylor flow was developed in order to provide a vigorous local mixing with low shear and to maintain an axial concentration profile in the reactor (Baron and Van Cappellen, 1989). This reactor consisted of an inner rotating cylinder, concentric to the outer cylinder that was stationary. A limited axial flow coupled with a moderate angular velocity resulted in a well-mixed vortex within which biocatalyst particles remain stationary. Anaerobic fermentation with alginate or polyurethane immobilized *Saccharomyces cerevisiae* was successfully performed in both horizontal and vertical reactors. The gas was well evacuated from the fluid except in the case of the vertical configuration using large beads (1 mm). This system was also applied for plant cell culture by Thomas and Janes (1987). In order to solve the oxygenation problem, Constantinides used a spirally wound packed bed reactor for glutamic acid production by collagen bound whole cells of *Brevibacterium flavum.* This reactor is a compromise between PBR and the CSTR because of the presence of agitation. A spiral wound matrix packed in the reactor was also proposed to avoid clogging and loss of productivity by accumulation of dead cells (Lewis and Yang, 1992). The fibrous matrix is hydrophilic and water absorbent. The system provides a large surface area for cell attachment. Mass transfer limitations are minimized by using a thin matrix layer. Vertical gaps among spiral wound layers of the matrix allow excess cell biomass to fall to the bottom of the reactor and CO_2 removal from the top with minimum pressure drop. This reactor was used for continuous production of propionic acid by *Propionibacterium acidipropionici.* A constant productivity was achieved during 4 months without clogging or degeneration.

C. Fluidized Bed Reactor (FBR)

FBRs provide conditions that are intermediate to those of the CSTR and PBR. Mixing is better than in the PBR and lower levels of shear are encountered compared with the CSTR. A FBR consists in a column in which the biocatalyst particles are maintained in motion by a continuous flow of the substrate solution. The pressure drop of the fluid flow supports the weight of the bed. The FBR offers higher productivity than the CSTR because liquid approximates plug flow similar to the PBR. However, the FBR is more advantageous for fermentation with substrate inhibition than the PBR because of the mixing caused by fluid flow. In contrast with the PBR, the FBR facilitates solid-fluid mixing, gas removal, and minimizes pressure drop. FBRs can expand to accommodate growing biomass, so they are less sensitive to plugging. This type of reactor is more convenient for cultures for which oxygenation is needed than a PBR as substrate may be supplemented with air sparging (bubble column). Compared with the CSTR, the FBR has the advantage of reduced chance of contamination because of the absence of a drive shaft seal.

Andrews and Prezezdziecki (1987) developed an effectiveness factor correlation for flocs, solid spherical supports, porous supports, and adsorbent supports in a FBR. The FBR was shown to exhibit operational and scale-up difficulties. Axial back-mixing was present, resulting in loss of efficiency in the case of product inhibition. CO_2 build-up inducing channeling and fluidization irregularities were significantly reduced compared with the PBR.

To achieve good fluidization characteristics, the density difference between the biocatalyst particles and the substrate solution should be as high as possible. Hydrated hydrocolloid gels have a similar density to the substrate solution. Thus, fluid velocity is often reduced to avoid expanding the bed, resulting in wash-out conditions. Changes in bead density are observed with time because of cell growth and gas formation inside the beads.

A multistage reactor may be used to reduce back-mixing in continuous ethanol fermentation in a FBR. Each stage is separated by a mesh sieve plate and the solid and liquid phases are well mixed. Good interphase mass transfer is possible with sufficient gas release to avoid channeling (Tzeng et al., 1991). Ethanol conversion in an 8-stage bioreactor with alginate immobilized *S. carlsbergensis* was simulated, taking into account complete mixing flow behavior at each stage, free-cell kinetics, liquid-solid mass transfer and intraparticulate diffusion (Tzeng et al., 1991). Good agreement was obtained between experimental and predicted results, except for the later stages when high flow rate was used, resulting in high hold-up and liquid-solid mass transfer limitation. Good production rates in the first three stages were obtained at the steady state due to high glucose and low ethanol concentration, resulting in negligible inhibition. Thus, multistage operation is advantageous to localize the high inhibitory product concentration to the top of the reactor because of reduced back-mixing (Keay et al., 1990).

In order to break up channels, Sisak et al. (1990) introduced impellers consisting of perforated discs and propellers fixed in an alternating arrangement on a common shaft in a FBR. Polyacrylamide beads were stable for 60 d.

The loss of immobilized cells by wash-out from the top of the column can be avoided by a mesh protection of the outlet or the provision of overflow weirs or expanded upper regions of the column where flow rate is reduced (Davison and Scott, 1992).

The problem of low bead density was dealt with by addition of Fe_2O_3 to carrageenan (Scott, 1987). Silica gel was incorporated together with immobilized yeast to enhance the specific gravity and facilitate CO_2 liberation (Fukushima et al., 1988; Shabtai et al., 1991).

FBR operation with continuous product removal was used to reduce product inhibition (Davison, 1989; Bajpai et al., 1990). Busche and Allen (1989) and Roffler et al. (1988) incorporated liquid extraction of product in a multistage FBR that improved the performance. Limitations due to solvent selectivity and toxicity were encountered. For solvent removal, membrane pervaporation coupled with a multistage FBR overcame the effects of product inhibition. Davison and Scott (1992) investigated a solid adsorbent for *in situ* product removal in a FBR. Two types of solid particles, including the biocatalyst and

the solid adsorbent formed the biparticle FBR. Selective removal of the solid absorbent from the fermentor was accomplished by stratification on the basis of size and density. The heavier particles (absorbent) were added continuously as a slurry to the top of the column and removed from the bottom below the liquid feed inlet by a central tube connected to a pump. This resulted in countercurrent flow of the adsorbent with respect to the stable fluidized bed of biocatalyst particles. Activated carbon was tested, but it demonstrated low adsorption for lactic acid and glucose during fermentation with *Lactobacillus.*

D. Air (Gas)-Lift Reactor

In an air-lift reactor (ALR), gas bubbles carry the liquid, resulting in a reduction in liquid bulk density. Gas escapes from the top and the liquid cascades down a separate portion of the reactor (downcomer). The reactor typically consists of two concentric tubes. Gas sparging induces liquid upflow in the inner draft tube. The external tube forming the column may incorporate a conical section at the bottom to prevent sedimentation of solid particles. An external loop system may replace the inner draft tube for the recirculation of liquid in some bioreactors.

Agitation in the ALR due to gas flow results in low shear with efficient mixing and mass transfer. The size of the draft tube influences the hydrodynamics of the fermentor, such as the gas holdup (Kennard and Janekeh, 1991).

The ALR was used for attached biofilm in order to reduce abrasion problems and cell desorption (Kargi and Cervoni, 1983). Stability of alginate beads (2.1 mm) was also improved by using an ALR for transformation of steroids by *Arthrobacter* (Kloosterman and Lilly, 1985). The rate of bead loss was 50% less than in a CSTR. Anaerobic fermentations were also performed in an ALR, using sparged nitrogen (Bu'Lock et al., 1984). Kennard and Janekeh (1991) carried out ethanol fermentation with *Clostridium thermosaccharolyticum* immobilized on porous perlag beads.

V. SCALE-UP OF BEAD PRODUCTION

Among all the immobilization methods described, significant scale-up problems are encountered. The droplet extrusion method that is commonly used on a laboratory scale to produce gel beads presents serious difficulties for large-scale production. The cell/polymer suspension is pumped through a needle at a rate low enough to prevent jet formation. The production capacity is limited by the speed of droplet formation at the tip of the needle. Thus, the production capacity may only be enhanced by increasing the number of needles. The other limitation to this popular procedure is the large diameter of the resultant beads, typically 2 to 3 mm. Diameters higher than 1 mm often induce significant mass transfer limitations (Ryu et al., 1984) and heterogeneous cell distribution within the beads. Smaller diameters would be a preferred alternative for better productivity of the immobilized cell and for increased mechanical strength (Poncelet and Neufeld, 1989). Different procedures derived from the extrusion technique or using centrifugal force are reported in the literature, allowing smaller beads at higher production rates. Other techniques based on forming a dispersed droplet phase within an immiscible fluid have emerged as suitable for larger-scale production of small beads. The present limitation for scaled-up applications is a desire to form monodispersed beads in order to improve the performance of the immobilized cell fermentor. Spray drying technology may be an interesting alternative if very small diameter, dry bead preparations are desired. Cells would need to tolerate the high levels of shear and drying conditions encountered.

A. Extrusion Under Gravity Force

When a droplet is formed at the tip of the needle, it falls into a hardening solution under gravity force. Pressure is applied to extrude the viscous polysaccharide solution through the narrow-bore needle. According to Poncelet et al. (1993), the droplet diameter is mainly influenced by the needle diameter and is limited to diameters greater than 1 mm. The bead diameter also depends

on the polymer viscosity. In the case of alginate, the bead size is also related to the alginate composition, as the degree of shrinkage during gel formation is dependent on the guluronic composition (Smidsrod and Skjak-Braek, 1990). The size dispersion of the beads obtained by simple extrusion results in a typical relative standard deviation of approximately 20%.

Scale-up of this procedure is performed by using a large number of needles. Vorlop and Klein (1981a) described a system of 42 outlets with a capacity of 3 to 5 kg of biocatalyst particles (bead diameter of 3 mm) produced per hour.

B. Extrusion Under Coaxial Liquid or Air Jet

Polysaccharide solution containing cells may be extruded through a needle, facilitated by a concentric air or liquid flow around the needle outlet to detach the droplet. With the coaxial air jet, the bead diameter depends mainly on the air jet velocity. A high gas flow rate induces a high shear rate at the droplet surface promoting early release of the droplet, resulting in a smaller diameter (Rehg et al., 1986). Equations predicting drop size vs. coaxial airjet velocity are reported (Rehg et al., 1986; Poncelet et al., 1993). The diameter also depends on the polymer viscosity and the needle diameter (Su et al., 1989). For liquid jets, the bead diameter is inversely proportional to the flow velocity (Poncelet et al., 1993). Regarding size dispersion, higher relative standard deviations were observed when higher air flow rate was used corresponding to smaller beads (Poncelet et al., 1993; Su et al., 1989).

Vorlop and Klein (1981) reported that the production capacity of a 42 outlet system decreased for smaller bead formation. Rehg et al. (1986) used a multiple syringe/needle system with a coaxial air jet. The authors encountered problems of needle plugging at alginate concentration greater than 1%. Depending on the experimental conditions, the bead size ranged from 0.4 to 1.6 mm, with a relative standard deviation of 13%.

A small-scale production of agarose beads was attempted with coaxial liquid flow by Dupuy et al. (1986).

C. Extrusion Under Electrostatic Potential

The cell/polymer suspension may be extruded through a capillary subjected to high static potential between the capillary and the hardening solution, inducing droplet separation from the dispensing outlet. Alternatively, the electrical potential may be applied between the capillary and a stainless steel ring placed beneath the capillary outlet (Hommel et al., 1988). Increasing potential causes a decrease in the droplet size and an increase in drop frequency, yielding droplets with diameters to several hundred micrometers. Hommel et al. (1988) proposed replacing the continuous voltage by very short pulses (1 to 6 ms) with a controlled frequency (10 to 100/s) in order to obtain beads in the range of 100 to 200 μm. Bugarski et al. (1993) demonstrated alginate microbead production (<150 μm) by electrostatic spraying. In this case, pulsed voltage was not necessary. A multineedle (20 needles) device was used to process 0.7 l/h of 400 μm diameter alginate beads (Bugarski et al., in press).

D. Droplet Formation by Jet Break-Up

In this technique, the polymer/cell suspension exits from a vibrating nozzle as a jet, causing breakup of the jet. Increased bead production rates are possible with small-diameter beads. Correlations were proposed to predict the required frequency for a desired bead diameter (Haas, 1987; Hulst et al., 1985; Poncelet et al., 1993). Hulst et al. (1985) proposed a vibrating nozzle technique that combined coaxial airflow with needle vibration. Scott (1987) used a sonic-vibrating transducer applied to the external nozzle surface or to the flexible tubing providing polymer solution to the nozzle. The frequency was adjusted empirically until droplet formation was stable using a coupled stroboscopic light source to visualize the droplet. A suitable frequency range between 300 and 450/s was observed (Hulst et al., 1985). The optimum frequency of 400/s resulted in minimal size dispersion. The beads were deformed at high velocity with certain nozzle sizes, and increasing velocity resulted in larger standard deviations. A

maximum volumetric flow rate of 24 l/h allowed formation of spherical beads. For Hulst et al. (1985) and Scott (1987) the bead size ranged between 1 to 2 mm (standard deviation of 4 to 14%) depending on nozzle diameter, polymer solution flow rate, and vibrational frequency. Droulez et al. (1989) optimized conditions to produce beads with mean diameter of 0.62, 1.07, or 1.38 mm.

Beads obtained without vibration compared with those produced with high frequency of vibration resulted in means and relative standard deviations of 2 mm (20%) and 1.18 mm (4%), respectively, demonstrating the effectiveness of this procedure to reduce the bead diameter and size distribution (Scott, 1987). Nir et al. (1990) prepared beads with a diameter of 30 μm using a similar technique.

Jet breakup in producing carrageenan beads (Hunik and Tramper, 1993) was performed at a maximum flow rate of 27.6 l/h to obtain 2.1-mm beads with a relative standard deviation of 6%. Correlations were developed to estimate the local viscosity in order to predict the droplet diameter and optimum breakup frequency. Good agreement was obtained between calculated and experimental results. It was demonstrated that the viability and activity of plant and yeast cells were not influenced by the nozzle vibration (Hulst et al., 1985; Scott, 1987). Droulez et al. (1989) immobilized *K. fragilis* within 1 mm alginate beads for continuous fermentation of ethanol from lactose as growth-limiting substrate. Wijffels et al. (1989) produced 1-mm-diameter alginate beads for immobilization of *Nitrobacter agilis* for continuous fermentation in an air-lift-loop reactor.

E. Emulsion Technique

Beads and microcapsules may be formed using emulsification to disperse the cell/polymer suspension. The reactors used are cylindrical vessels equipped with a marine (Poncelet et al., 1992; Audet and Lacroix, 1989) or a grid impeller (Poncelet de Smet, 1990; Poncelet et al. 1993; Groboillot et al. 1993; Hyndman et al., 1992). The grid impeller design induces smaller droplets with a more homogeneous size distribution (Poncelet et al., 1992; Poncelet De Smet, 1990). However, to produce alginate beads, the marine impeller was more suitable because the beads tended to accumulate on the grid. Baffles can be added in the vessel to improve mixing conditions. Poncelet et al. (1993) proposed a static mixer as an alternative shear device, producing emulsions on a continuous basis.

Parameters influencing particle diameter and size distribution include rotational speed of the impeller (Audet and Lacroix, 1989; Castillo et al., 1992; Nilsson et al., 1983), polymer concentration, aqueous and organic phase viscosity (Groboillot, et al., 1993), surfactant concentration (Poncelet de Smet, 1993), and alginate composition (Poncelet et al., 1992). In the case of alginate beads formed by emulsification/internal gelation, the use of calcium citrate as the calcium vector for high guluronic acid alginate results in larger diameter beads (800 μm). The use of calcium carbonate with high guluronic acid alginate leads to the formation of smaller beads (less than 300 μm) (Poncelet et al., 1993). The presence of different compounds in either the aqueous or organic phase may influence the final bead size by affecting surface tensions. For example, variations in the pH of the aqueous phase significantly modifies PEI microcapsule size (Poncelet et al., 1994).

Depending on the conditions, the mean diameter of carrageenan beads varied from 150 μm to 5 mm (Audet and Lacroix, 1989) and from 100 μm to 1 mm for carrageenan, gelatin, or agar beads (Castillo, 1992). Alginate beads obtained by internal gelation ranged from 70 to 800 μm (Poncelet, 1993). Nilsson et al. (1983) obtained agarose, agar, carrageenan, alginate, fibrin, or polyacrylamide beads with a diameter of 500 to 1000 μm. According to Poncelet et al. (1993), the usual relative standard deviation for beads or microcapsules obtained by emulsification range between 30 and 70%. The standard deviation decreases with increasing impeller speed corresponding to decreasing mean diameter and to better mixing conditions (Audet and Lacroix, 1989; Poncelet De Smet et al., 1990).

For industrial-scale applications, the emulsification technique shows promise. Small turbine reactors (100 ml) were used to prepare beads in

15 min (Audet and Lacroix, 1989; Poncelet et al., 1992). Industrial-scale emulsification is routinely conducted in the food industry and may be easily applied to bead or microcapsule production.

F. Rotating Nozzle-Ring

Matulovic et al. (1986) and Duteurtre et al. (1986) proposed a new procedure to produce small-diameter beads in large quantities. The technique is based on extrusion of the cell/polymer suspension through a rotating nozzle ring. The polymer is forced under pressure through a vertical hollow shaft equipped with a nozzle head. Shaft rotation ejects the droplet from the different nozzles into a hardening solution forming around the nozzle head as a vortex (Matulovic et al., 1986) or a slanting flow maintained by a draught tube and a motor (Siemann et al., 1990).

The bead diameter depends on the nozzle internal diameter, distance between two nozzles, rotational speed, and polymer solution viscosity (Siemann et al., 1990). The production capacity is affected by the number of nozzles on the head, the speed of the shaft, and the pressure applied (Matulovic et al., 1986). The standard deviation was around 15% and the proportion of deformed beads was about 5% for nozzle diameters ranging from 0.1 to 0.5 mm (Siemann et al., 1990). However, the surface tension of the hardening solution strongly influenced the proportion of deformed beads and the standard deviation. The production capacity decreased with nozzle diameter; with nozzle diameters of 0.25 and 0.8 mm, the productivities were 0.1 and 1 g/h per nozzle, respectively. The productivity was not significantly enhanced by increasing the nozzle number, and elevated air pressure for extrusion resulted in a higher proportion of deformed beads. The production rate of beads with a diameter of 250 µm (standard deviation of 8.5 and percentage of deformed beads of 5%) was optimized at 2.8 kg/h with a pressure of 25 mbar.

This technique was applied for immobilization of *Pseudomonas* sp. in chitosan or carrageenan, and the productivity varied between 2 and 24 kg/h of beads with a diameter of 0.5 or 1 mm, depending on the conditions (Matulovic et al., 1986). Results were not presented on immobilized cell viability or activity. Bead formation with highly viscous polymer is possible. As the length of the nozzles is determined only by the thickness of the nozzle ring wall (e.g., 1 mm), nozzle clogging occurred rarely.

G. Rotating Flat Disc Atomizer

Rotating atomizers were shown to be suitable for production of uniform droplets with viscous liquids (Dombrowski and Lloyd, 1974; Kamyiya and Kayano; 1972, Matsumoto et al., 1974). Ogbonna et al. (1989) and Begin et al. (1991) used the technique to produce alginate beads. The atomization system consisted of a variable speed motor on which a flat disc is mounted. The cell/polymer suspension is dropped on top of the rotating disc and disintegrates into droplets that fall into the hardening solution. In order to achieve a uniform liquid sheet on the disc, the solution was passed through a distributor consisting of a chamber with a liquid entrance and a stationary tube to prevent destabilizing effects produced by the shaft rotation on solution flow (Bégin et al., 1991). The hardening solution is either in a very large vessel or a vortex of $CaCl_2$ solution is formed to receive droplets produced by the rotating disc (Ogbonna et al., 1989).

Several atomization mechanisms were observed depending on operational parameters such as liquid flow rate, disc diameter, and rotational speed. Drop formation occurs either directly at the disc periphery or by ligament breakup into droplets. At high flow rate and rotational speed, thick ligaments are formed at the periphery of the disc. The direct drop disintegration mode results in bimodal bead size distribution, while the ligament mode gave unimodal distributions. In the ligament mode, beads with a diameter ranging from 1 to 3 mm were produced. According to Ogbonna et al. (1989), 2% alginate beads with a diameter of 100 to 600 µm were produced with a standard deviation of 40%. The mean diameter depended on volumetric flow rate of the polymer suspension, rotational speed of the disc, and disc diameter (Bégin et al.). Higher flow rates re-

sulted in larger bead diameters (Ogbonna et al., 1989). Physical properties of the cell/polymer suspension also influenced the bead diameter. Ogbonna et al. (1989) and Bégin et al. (1991) proposed equations to predict the mean volumetric bead diameter. When a vortex is employed for the collecting solution, the surface tension of this solution largely affected the bead shape, as was observed previously for beads obtained with a rotating nozzle (Siemann et al., 1990). The surface tension was decreased by adding surfactant resulting in formation of spherical beads (Ogbonna et al., 1989). This apparatus permitted bead production rates of 60 to 120 l/h. Productivity could be enhanced by using several discs on the same shaft. The advantage of this technique compared with the rotating nozzle ring is that plugging is avoided and viscous polymer solutions may be handled. The ligament mode is thought to result in reduced shear conditions. Sieving of the beads is often required to obtain a narrow size distribution.

VI. CONCLUSIONS

The purpose of this article was to survey cell immobilization techniques applicable to the food industries, the impact of immobilization on cell growth and metabolism as it relates to productivity, bioreactors suitable for containing immobilized cells, and procedures that show promise for large-scale immobilization. While the volume of published literature in this field is considerable, the present application of immobilized cell technology within the food industry is limited. Food industry endorsement of immobilized cell technology will depend on:

1. Low-cost immobilization procedures adaptable to large-scale production
2. Control of bead properties, including diameter, size distribution, stability, and mechanical strength
3. Reduction of cell release often encountered with gel immobilization
4. Immobilized cell preparations demonstrating sustainable cell viability and activity, particularly under demanding process conditions

Interesting future research developments will include a closer examination of the extent to which immobilized cells are protected from phage contamination and the potential for maintaining high levels of cell viability and activity in dried bead preparations.

REFERENCES

Ahmad, S. and Johri, B. N., Immobilization of *Rhodococcus equi* DSM 89-133 onto porous celite beads for cholesterol side-chain cleavage, *Appl. Microbiol. Biotechnol.*, 37, 468, 1992.

Andrews, G. F. and Fonta, J. P., *Appl. Biochem. Biotechnol.*, 20, 375, 1989.

Andrews, G., Fluidized-bed bioreactors. *Biotechnol. Genet. Eng. Rev.*, 6, 151, 1988.

Andrews, G. F. and Przezdziecki, J., Design of fluidized-bed fermentors. *Biotechnol. Bioeng.*, 28, 802, 1987.

Anselme, M. J. and Tedder, D. W., Characteristics of immobilized yeast reactors producing ethanol from glucose, *Biotechnol. Bioeng.*, 30, 736, 1987.

Argarikos, G., Thayanithy, K., and Wase, D. A. J., Effect of immobilization on the production of α-amylase by an industrial strain of *Bacillus amyloliquefaciens*, *J. Chem. Tech. Biotechnol.*, 53, 33, 1992.

Ariga, O., Takagi, H., Nishizawa, H., and Sano, Y., Immobilization of microorganisms with PVA hardened by iterative freezing and thawing, *J. Ferment. Technol.*, 65, 651, 1987.

Arnaud, J. P., Choplin, L., and Lacroix, C., Rheological behavior of kappa-carrageenan/locust bean gum mixed gels, *J. Text. Stud.*, 19, 419, 1989.

Arters, D. C. and Fan, L.-S., Experimental methods and correlation of solid-liquid mass transfer in fluidized beds, *Chem. Eng. Sci.*, 45, 965, 1990.

Audet, P., Paquin, C., and Lacroix, C., Immobilized growing lactic acid bacteria with K-carrageenan-locust bean gum gel, *Appl. Microbiol. Biotechnol.*, 29, 11, 1988.

Audet, P. and Lacroix, C., Two-phase dispersion process for the production of biopolymer gel beads: effects of various parameters on bead size and their distribution. *Process Biochem.*, 24, 217, 1989.

Audet, P., Paquin, C., and Lacroix, C., Sugar utilization and acid production by free and entrapped cells of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *lactis* in a whey permeate medium, *Appl. Environ. Microbiol.*, 55, 185, 1989.

Audet, P., Lacroix, C., and Paquin, C., Continuous fermentation of a supplemented whey permeate medium

with immobilized *Streptococcus salivarius* subsp. thermophilus. *Int. Dairy J.*, 2, 1, 1992.

Axelsson, A. and Persson, B., Mass transfer and alginate beads, diffusivity of molecules with small molecular weight. *Appl. Biochem. Biotechnol.*, 18, 231, 1988.

Axelsson, A., Optimization of cell loading within alginate beads for maximum product yield. *Appl. Biochem. Biotechnol.*, 18, 91, 1988.

Bachman, S., Gabricka, L., and Gasyna, Z., *Starch*, 33, 63, 1981.

Bajpai, R., Thompson, J. E., and Davison, B. H., FBR, with extractive fermentation to remove butanol. *Appl. Biochem. Biotech.*, 24, 25, 1990.

Bakoyianis, V., Kanellaki, M., Kaliafas, A., and Koutinas, A. A., Low-temperature wine making by immobilized cells on mineral kissiris. *J. Agric. Food Chem.*, 40, 1293, 1992.

Barbotin, J. N., Nava Saucedo, J. E., and Thomasset, B., Morphological observations on immobilized cells. in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Baron, G. V. and Van Cappellen, P., A novel bioreactor for immobilized cell systems, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Beaumont, M. and Knorr, D., Effects of immobilizing agents and procedures on viability of cultured celery (*Apium Graveolens*) cells, *Biotechnol. Lett.*, 9, 377, 1987.

Beaumont, M., Pandya, Y., and Knorr, D., Chitosan immobilization and permeabilization of cultured *Apium Graveolens, Chenopodium Rubrum* and *Docus Carota* cells, *Food Biotechnol.*, 3, 71, 1989.

Bégin, A., Castaigne, F., and Goulet, J., Production of alginate beads by a rotative atomizer, *Biotechnol. Techniques*, 5, 459, 1991.

Berry, F., Sayadi, S., Nasri, M., Barbotin, J. N., and Thomas D., Effects of growing conditions of recombinant *E. coli*, in carrageenan gel beads on biomass production and plasmid stability, *Biotechnol. Lett.*, 10, 619, 1988.

Birnbaum, S., Pendleton, R., Larsson, P. O., and Mosbach, K., Covalent stabilization of alginate gel for the entrapment of living whole cells, *Biotechnol. Lett.*, 3, 393, 1981.

Black, G. M., Webb, C., Matthews, T. M., and Atkinson, T., Practical reactor systems for yeast cell immobilization using biomass support particles, *Biotechnol. Bioeng.*, 26, 134, 1984.

Boyaval, P., Lebrun, A., and Goulet, J., Etude de l'immobilisation de *Lactobacillus helveticus* dans des billes d'alginate de calcium, *Le Lait.*, 65, 185, 1985.

Braun, K., Besch, W., Jahr, H., Dautzenberg, L. F., and Hahn, H. J., The encapsulation of pancreatic islets. Investigations of insulin secretion and content in vitro, *Biomed. Biochim. Acta*, 44, 143, 1985.

Braun, K., Kauert, C., Dunger, A., and Hahn, H. J., Allogeneic transplantation of microencapsulated islets, *Transplant. Proc.*, 19, 931, 1987.

Braun, K., Besch, W., Lucke, S., and Hahn, H. J., The long-term culture of encapsulated islets, *Exp. Clin. Endocrinol.*, 87, 313, 1986.

Briasco, C. A., Barbotin, J. N., and Thomas, D., Spatial distribution of viable cell concentration and plasmid stability in gel-immobilized recombinant *E. coli*, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Bringi, V. and Shuler, M. L., A framework for understanding the effects of immobilization on plant cells: differentiation of tracheary elements in tobacco, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Brito, L. C., Vieira, A. M., Leitao, J. G., Sa-Correia, I., Novai, J. M., and Cabral, J. M. S., Effect of the aqueous soluble components of the immobilization matrix on ethanol and microbial exopolysaccharides production, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Brodelius, P., Production of biochemicals with immobilized plant cells: possibilities and problems, *Ann. N.Y. Acad. Sci.*, 413, 383, 1983.

Bugarski, B., Smith, J., Wu, J., and Goosen, M. F. A., Methods for animal cell immobilization using electrostatic droplet generation, *Biotechnol. Tech.*, 7, 677, 1993.

Bugarski, B., Poncelet, D., Neufeld, R. J., Li, Q., Vunjak, G., and Goosen, M. F. A., Electrostatic droplet generation: investigation of mechanism of polymer droplet formation, *AIChE J.*, in press.

Bu'Lock, J. D., Comberback, M. D., and Ghommidh, C., A study of continuous ethanol production using a highly flocculent yeast in the gas lift tower fermenter, *Chem. Eng. J.*, 29(1), B9, 1984.

Burrill, H. N., Bell, L. E., Greenfield, P. F., and Do, D. D., Analysis of distributed growth of *Saccharomyces cerevisiae* cells immobilized in polyacrylamide gel. *Appl. Environ. Microbiol.*, 46, 716, 1983.

Busche, R. M. and Allen, R. M., Technoeconomics of butanol extractive fermentation in a multiphase fluidized bed bioreactor, *Appl. Biochem. Biotechnol.*, 20/21, 357, 1989.

Buzas, Z., Dallmann, K., and Szajani, B., Influence of pH on the growth and ethanol production of free and immobilized *Saccharomyces cerevisiae* cells, *Biotechnol. Bioeng.*, 34, 882, 1989.

Cahill, G., Walsh, P. K., and Ryan, T. P., Studies on the production of β-glucanase by free and immobilized recombinant yeast cells, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Camelin, I., Lacroix, C., Paquin, C., Prevost, H., Cachon, R., and Divies, C., Effect of chelants on gellan gum rheological properties and setting temperature for immobilization of living bifidobacteria, *Biotechnol. Prog.*, 9, 291, 1993.

Castillo, E., Ramirez, D., Casas, L., and Lopez-Munguia, A., A two phase method to produce gel beads, *Appl. Biochem. Biotechnol.*, 34/35, 477, 1992.

Champagne, C. P., Baillargeon-Côté, C., and Goulet, J., Whey fermentation by immobilized cells of *Propionibacterium shermanii*, *J. Appl. Bacteriol.*, 66, 175, 1989.

Champagne, C. P., Gaudy, C., Poncelet, D., and Neufeld, R. J., *Lactococcus lactis* release from calcium alginate beads, *Appl. Environ. Microbiol.*, 58, 1429, 1992.

Champagne, C. P., Girard, F., and Rodriguez, N., Production of concentrated suspensions of thermophilic lactic acid bacteria in calcium-alginate beads, *Int. Dairy J.*, 3, 257, 1993.

Chang, T. M. S., Enzymes immobilized by microencapsulation within spherical ultrathin polymeric membranes, *J. Macromol. Sci. Chem.*, A10, 245, 1976.

Chattaway, T., Goma, G., and Renaud, P. Y., Modelling ethanol and secondary inhibitions of ethanol fermentation in a multistage reactor, *Biotechnol. Bioeng.*, 32, 271, 1988.

Cheetham, P. S. J., Physical studies on the mechanical stability of columns of calcium alginate gel pellets containing entrapped microbial cells, *Enzyme Microb. Technol.*, 1, 183, 1979a.

Cheetham, P. S. J., Blunt, K. W., and Bucke, C., Physical studies on cell immobilization using calcium alginate gels, *Biotechnol. Bioeng.*, 21, 2155, 1979b.

Chibata, I. and Tosa, T., *Appl. Biochem. Bioeng.*, 1, 329, 1976.

Chibata, I., Tosa, T., and Sato, T., Production of L-aspartic acid by microbial cells entrapped in polyacrylamide gels, *Methods Enzymol.*, 44, 739, 1976.

Chibata, I., Tosa, T., and Sato, T., *Microbial Technology*, 2nd ed., Peppler, H. J. and Perlman, D., Ed., Academic Press, San Diego, 1979.

Cho, G. H., Choi, C. Y., Choi, Y. D., and Han, M. H., Ethanol production by immobilized yeast and its CO_2 gas effects in a packed bed reactor, *J. Chem. Tech. Biotechnol.*, 32, 959, 1982.

Colak, T. and Hamamci, H., The optimum taper angle for ethanol fermentation in a packed-bed column reactor, *J. Chem. Tech. Biotechnol.*, 52, 445, 1991.

Constantinides, A., Bhatia, D., and Vieth, W. R., Immobilization of *Brevibacterium flavum* cells on collagen for the production of glutamic acid in a recycle reactor, *Biotechnol. Bioeng.*, 23, 899, 1981.

Daugulis, A. J. and Swaine, D. E., Examination of substrate and product inhibition kinetics on the production of ethanol by suspended and immobilized cell reactors, *Biotechnol. Bioeng.*, 29, 639, 1987.

Davison, B. H., Dispersion and holdup in a three-phase fluidized bed, *Appl. Biochem. Biotechnol.*, 20/21, 449, 1989.

Davison, B. H. and Scott, C. D., A proposed biparticle fluidized-bed for lactic acid fermentation and simultaneous adsorption, *Biotechnol. Bioeng.*, 39, 365, 1992.

De Alteriis, E., Parascandola, P., and Scardi, V., Oxidized starch as a hardening agent in the gelatin-immobilization of living yeast cells, *Starch/Stärke*, 42, 57, 1990.

Demuyakor, B. and Ohta, Y., Promotive action of ceramics on yeast ethanol production, and its relationship to pH, glycerol and alcohol dehydrogenase activity, *Appl. Microbiol. Biotechnol.*, 36, 717, 1992.

De Taxis du Poet, P., Dhulster, P., Barbotin, J. N., and Thomas, D., Plasmid inheritability and biomass production: comparison between free and immobilized cell cultures of *Escherichia coli* BZ18(pTG201) without selection pressure, *J. Bacteriol.*, 165, 871, 1986.

De Taxis du Poet, P., Arcand, Y., Bernier, R., Jr., Barbotin, J.-N., and Thomas, D., Plasmid stability in immobilized and free recombinant *Escherichia coli* JM105(pKK233-200): importance of oxygen diffusion, growth rate, and plasmid copy number, *Appl. Environ. Microbiol.*, 53, 1548, 1987.

Diefenbach, R., Keweloth, H., and Rehm, H. J., Fatty acid impurities in alginate influence the phenol tolerance of immobilized *Escherichia coli*, *Appl. Microbiol. Biotechnol.*, 36, 530, 1992.

Dombrowski, N. and Lloyd, T. L., *Chem. Eng. J.*, 8, 63, 1974.

Doran, P. M. and Bailley, J. E., Effects of immobilization on growth fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin, *Biotech. Bioeng.*, 28, 73, 1986.

Doran, P. M. and Bailley, J. E., Effects of immobilization on the nature of glycolytic oscillations in yeast, *Biotech. Bioeng.*, 29, 892, 1987.

Drioli, E., Iorio, G., De Rosa, M., Gambacorta, A., and Nicolaus, B., *J. Memb. Sci.*, 11, 365, 1982.

Dror, Y., Cohen, O., and Freeman, A., *Enzyme Microb. Technol.*, 10, 273, 1988.

Droulez, C., Marison, I., and von Stockar, U., Comparison of ethanol production by *Kluyveromyces fragilis* immobilized in calcium alginate beads with suspension culture, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Dupuy, B., Gin, H., Baquey, C., and Ducassou, D., *Innov. Tech. Biol. Med.*, 7, 628, 1986.

Duteurtre, K. B., Ors, P., Charpentier, M., and Midoux, N., Patent no. 84 14994, France.

Felix, H., Brodelius, P., and Mosbach, K., Enzyme activities of the primary and secondary metabolism of simultaneously permeabilized and immobilized plant cells, *Anal. Biochem.*, 116, 462, 1981.

Fletcher, M., Measurement of glucose utilization by *Pseudomonas fluorescens* that are free-living and that attached to surfaces, *Appl. Environ. Microbiol.*, 52, 672, 1986.

Flink, J. M. and Johansen, A., *Biotechnol. Lett.*, 7, 765, 1985.

Fortin, C. and Vuillemard, J. C., Effect of immobilization in calcium alginate beads on regulation of protease production by *Myxococcus xanthus* cells, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Fukushima, Y., Okamura, K., Imai, K., and Motai, H., A new immobilization technique of whole cells and enzymes with colloidal silica and alginate, *Biotechnol. Bioeng.*, 32, 584, 1988.

Furusaki, S., Engineering aspects of immobilized biocatalysts, *J. Chem. Eng. Jpn.*, 21, 219, 1988.

Furasaki, S., Okamura, Y., and Miyauchi, T., *J. Chem. Eng. Jpn.*, 15, 148, 1982.

Galazzo, J. L. and Bailey, J. E., *In vivo* nuclear magnetic resonance of immobilization effects on glucose metabolism of yeast *Saccharomyces cerevisiae*, *Biotechnol. Bioeng.*, 33, 1283, 1989.

Gencer, M. A. and Mutharasan, R., Ethanol fermentation in a yeast immobilized tubular fermentor, *Biotechnol. Bioeng.*, 25, 2243, 1983.

Ghose, T. K. and Bandyopaddhyay, K. K., Rapid ethanol fermentation in immobilized cell reactor, *Biotechnol. Bioeng.*, 22, 1489, 1980.

Godia, F., Casas, C., and Sola, C., A survey of continuous ethanol fermentation systems using immobilized cells, *Process Biochem.*, 22, 43, 1987a.

Godia, F., Casas, C., Castellano, B., and Sola, C., Immobilized cells behaviour of carrageenan entrapped yeast during continuous ethanol fermentation, *Appl. Microbiol. Biotechnol.*, 26, 342, 1987b.

Godia, F., Casas, C., and Sola, C., Mathematical modelization of a packed-bed reactor performance with immobilized yeast for ethanol fermentation, *Biotechnol. Bioeng.*, 30, 836, 1987c.

Godia, F., Casas, C., and Sola, C., Application of immobilized yeast cells to sparkling wine fermentation, *Biotechnol. Prog.*, 7, 468, 1991.

Gonçalves, L. M. D., Barreto, M. T. O., Xavier, A. M. B. R., Carrondo, M. J. T., and Klein, J., Inert supports for lactic acid fermentation — a technological assessment, *Appl. Microbiol. Biotechnol.*, 38, 305, 1992.

Gosmann, B. and Rehm, H. J., Oxygen uptake of microorganisms entrapped in Ca-alginate, *Appl. Micro. Biotechnol.*, 23, 163, 1986.

Grasdalen, H. and Smidsrod, O., *Carbohydr. Polymer*, 7, 371, 1987.

Groboillot, A. F., Champagne, C. P., Darling, G. D., Poncelet, D., and Neufeld, R. J., Membrane formation by interfacial cross-linking of chitosan for microencapsulation of *Lactococcus lactis*, *Biotechnol. Bioeng.*, 42, 1157, 1993.

Guoqiang, D., Kaul, R., and Mattiason, B., Evaluation of alginate-immobilized *Lactobacillus casei* for lactate production, *Appl. Microbiol. Biotechnol.*, 36, 309, 1991.

Guoqiang, D., Kaul, R., and Mattiason, B., Immobilization of *Lactobacillus casei* cells to ceramic material pretreated with polyethylenimine, *Appl. Microbiol. Biotechnol.*, 37, 305, 1992.

Haas, P. A., Turbulent dispersion of aqueous drops in organic liquids, *AIChE J.*, 33, 987, 1987.

Hackel, U., Klein, J., Megnet, R., and Wagner, F., Immobilisation of microbial cells in polymeric matrices, *Eur. J. Appl. Microbiol.*, 1, 291, 1975.

Hahn-Hägerdal, B., Jönsson, B., and Lohmeier-Vogel, E., *Appl. Microbiol. Biotechnol.*, 21, 173, 1985.

Hahn-Hägerdal, B., Physiological aspects of immobilized cells: a general overview, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Hamamci, H. and Ryu, D. D. Y., Performance of tapered column packed-bed bioreactor for ethanol production, *Biotechnol. Bioeng.*, 29, 994, 1987.

Hannoun, B. J. M. and Stephanopoulos, G., Diffusion coefficients of glucose and ethanol in cell-free and cell-occupied calcium alginate membranes, *Biotechnol. Bioeng.*, 28, 829, 1986.

Hannoun, B. J. M. and Stephanopoulos, G., Intrinsic growth and fermentation rates of alginate-entrapped *Saccharomyces cerevisiae*, *Biotechnol. Prog.*, 6, 341, 1990.

Harder, A. and Roels, J. A., *Adv. Biochem. Eng.*, 21, 55, 1982.

Holcberg, I. B. and Margalith, P., Alcoholic fermentation by immobilized yeast at high sugar concentrations, *Eur. J. Appl. Microbiol. Biotechnol.*, 13, 133, 1981.

Hommel, M., Sun, A. H., and Goosen, M. F. A., Droplet Generation Technique, Canadian patent no. 1,241,598, 1988.

Hsu, Y. L. and Chu, I. M., Poly(ethylenimine)-reinforced liquid-core capsules for the cultivation of hybridoma cells, *Biotech. Bioeng.*, 40, 1300, 1992.

Huang, J., Hao, O. J., Al-Ghusain, I. A., Chen, H. M., Kim, M. H., Phull, K. K., and Davis, A. P., Biological fixed film systems, *Wastewater Treatment*, 64, 359, 1992.

Huguet, M.-L., Groboillot, A., Neufeld, R. J., Poncelet, D., and Dellacherie, E., Hemoglobin encapsulation in chitosan/calcium alginate beads, in Proceedings of Bioencapsulation III Workshop, Brussels, Oct. 20 to 22, 1993.

Hulst, A. C., Tramper, J., Vant Riet, and Westerbeek, J. M. M., A new technique for the production of immobilized biocatalyst in large quantities, *Biotechnol. Bioeng.*, 27, 870, 1985.

Hunik, J. H. and Tramper, J., Large-scale production of kappa-carrageenan droplets for gel-bead production: theoretical and practical limitations of size and production rate, *Biotechnol. Prog.*, 9, 186, 1993.

Hwang, C., Rha, C. K., and Sinskey, A. J., Encapsulation with chitosan: transmembrane diffusion of proteins in capsules, in *Chitin in Nature and Technology*, Muzzarelli, R., Jeuniaux, C., and Gooday, G. W., Eds., Plenum Press, New York, 1986, 389.

Hyndman, C. L., Groboillot, A., Poncelet, D., Champagne, C., and Neufeld, R. J., Immobilization of

Lactococcus lactic susp. *cremoris* with cross-linked gelatin microcapsules, *J. Chem. Tech. Biotechnol.*, 1992.

Iwasaki, K., Nakajima, M., and Sasahara, H., Porous alumina beads for immobilization of lactic bacteria and its application for repeated-batch fermentation in soy sauce production, *J. Ferm. Bioeng.*, 73, 375, 1992.

Iwasaki, K., Nakajima, M., and Sasahara, H., Rapid continuous lactic acid fermentation by immobilised lactic acid bacteria for soy sauce production, *Process Biochem.*, 28, 39, 1993.

Jain, D. K., Tyagi, R. D., Kluepfel, D., and Agbebavi, T. J., *Process Biochem.*, 26, 217, 1991.

Jain, V. K., Toran-Diaz, I., and Baratti, J., *Biotechnol. Bioeng.*, 27, 273, 1985a.

Jain, V. K., Toran-Diaz, I., and Baratti, J., *Biotechnol. Bioeng.*, 27, 723, 1985b.

Jamuna, R., Sai, P. S. T., Vora, S., and Ramakrishna, S. V., Optimization of criticals parameters for immobilization of yeast cells to alginate gel matrix, *J. Ferment. Bioeng.*, 73, 319, 1992.

Jamuna, R. and Ramakrishna, S. V., Physiological behavior of *Saccharomyces cerevisiae* in gel matrix, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Johansen, A. and Flink, J. M., A new principle for immobilized yeast reactors based on internal gelation of alginate, *Biotechnol. Lett.*, 8, 121, 1986a.

Johansen, A. and Flink, J. M., Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells, *Enzyme Microbiol. Technol.*, 8, 737, 1986b.

Jouenne, T., Bonato, H., Mignot, L., and Junter, G. H., Cell immobilization in composite agar layer microporous membrane structures: growth kinetics of gel-entrapped cultures and cell leakage limitation by a microporous membrane, *Appl. Microbiol. Biotechnol.*, 38, 478, 1993.

Kamyiya, T. and Kanaya, A., Film type disintegration by rotating disc, *J. Chem. Eng. Jpn.*, 5, 174, 1972.

Kang, K. S., Veeder, G. T., Mirrasoul, P. J., Kaneko, T., and Cottrell, I. W., *Appl. Environ. Microbiol.*, 43, 1086, 1982.

Karel, S. F., Libicki, S. B., and Robertson, C. R., The immobilization of whole cells: engineering principles, *Chem. Eng. Sci.*, 40, 1321, 1985.

Karel, S. F. and Robertson, C. R., Cell mass synthesis and degradation by immobilized *Escherichia coli*, *Biotechnol. Bioeng.*, 34, 337, 1989.

Karel, S. F., Salmon, P. M., Steward, P. S., and Robertson, C. R., Reaction and diffusion in immobilized cells: fact and fantasy, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Karkare, S. B., Venkatasubramanian, K., and Vieth, W. R., Design and operating strategies for immobilized living cell reactor system. I. Biosynthesis of candicidin, *Ann. N.Y. Acad. Sci.*, 469, 83, 1986.

Kargi, F. and Cervoni, T. D., *Biotechnol. Lett.*, 5, 33, 1983.

Kautula, H., Vassilev, N., and Linko, Y. Y., *Biotechnol. Lett.*, 11, 313, 1989.

Keay, L., Eberhardt, J. J., Allen, B. R., Scott, C. D., and Davison, B. H., Improved production of ethanol and *N*-butanol in immobilized cell bioreactors, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Keim, C. R. and Venkatasubramanian, K., *Trends Biotechnol.*, 7, 22, 1989.

Kennedy, J. F. and Cabral, J. M. S., Immobilized living cells and their applications, in *Applied Biochemistry and Bioengineering*, Vol. 4, Chibata, I. and Wingard, L. B., Jr., Eds., Academic Press, New York, 1983, 189.

Kennard, M. and Janekeh, M., Two and three-phase mixing in a concentric draft tube gas-lift fermentor, *Biotechnol. Bioeng.*, 38, 1261, 1991.

Ketel, D. H., Hulst, A. C., Gruppen, H., Breteler, H., and Tramper, J., Effects of immobilization and environmental stress on growth and production of non-polar metabolites of *Tagates minuta* cells, *Enzyme Microbiol. Technol.*, 9, 303, 1987.

Keweloh, H., Heipieper, H. J., and Rehm, H. J., Phenol tolerance of immobilized bacteria, in *Physiology of Immobilized Cells*, de Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Kim, H. S. and Ryu, D. D. Y., Continuous glutamate production using an immobilized whole-cell system, *Biotechnol. Bioeng.*, 24, 2167, 1982.

Klein, H. E., Sundstrom, D. W., and Shim, D., Immobilization of enzymes by microencapsulation, in *Immobilized Cells and Enzymes. A Practical Approach*, Woodward, J., Ed., IRL Press, Oxford, 1985, 49.

Klein, J. and Wagner, F., Methods for the immobilization of microbial cells, in *Applied Biochemistry and Bioengineering*, Vol. 4, Chibata, I. and Wingard, L. B., Jr., Eds., Academic Press, New York, 1983, 11.

Klein, J. and Vorlop, K. D., Immobilized cells, catalyst preparation and reaction performance, in *Foundations of Biochemicals Engineering Kinetics and Thermodynamics in Biological Systems, ACS Symposium, Series No. 207*, Blanch, H., Papoutsakis, E. T., and Stephanopoulos, G., Eds., American Chemical Society, Washington, D.C., 1983.

Klein, J. and Vorlop, K. D., Immobilization techniques-cells, in *Comprehensive Biotechnology*, Vol. 2, Moo-Young, M., Cooney, C. L., and Humphrey, A. E., Eds., Pergamon Press, Oxford, N.Y., 1985.

Kloosterman, L. and Lilly, M. D., *Biotechnol. Lett.*, 7, 25, 1985.

Knorr, D., Miazga, S. M., and Teutonico, R. A., Immobilization and permeabilization of cultured plant cells, *Food Technol.*, 39, 135, 1985.

Knorr, D. and Teutonico, R. A., Chitosan immobilization and permeabilization of *Amaranthus tricolor* cells, *J. Agric. Food Chem.*, 34, 96, 1986.

Knorr, D., Immobilization of microbial and cultured plant cells, in *Food Biotechnology*, Knorr, D., Ed., Marcel Dekker, New York, 1987.

Knorr, D., Beaumont, M. D., Caster, C. S., Deurnenburg, H., Gross, B., Pandya, Y., and Romagnoli, L. G., Plant tissue culture for the production of naturally derived ingredients, *Food Technol.*, 44, 71, 1990.

Kolyak, E. I., Yakimov, M. M., Utkin, I. B., Roghozhin, I. S., Solomon, Z. G., and Bezborodov, A. M., Physicochemical basis of cell immobilization by the absorption method, *Appl. Biochem. Microbiol.*, 27, 595, 1992.

Kwak, M. Y. and Rhee, J. S., Cultivation characteristics of immobilized *Aspergillus oryzae* for kojic acid production, *Biotechnol. Bioeng.*, 39, 903, 1992.

Lacroix, C., Paquin, C., and Arnaud, J. P., Batch fermentation with entrapped growing cells of *Lactobacillus casei*, *Appl. Microbiol. Biotechnol.*, 32, 403, 1990.

Larroche, C. and Gros, J. B., Batch and continuous 2-heptanone production by Ca-alginate/eudragit RL entrapped spores of *Penicillium roqueforti*, *Biotechnol. Bioeng.*, 34, 30, 1989.

Lee, H. K. and Maddox, I. S., Continuous production of 2,3-butanediol from whey permeate using *Klebsiella pneumoniae* immobilized in calcium alginate, *Enzyme Microbiol. Technol.*, 8, 409, 1986.

Lewis, V. P. and Yang, S-T., Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed-bed bioreactor, *Biotechnol. Bioeng.*, 40, 465, 1992.

Lim, F. and Sun, A. M., Microencapsulated islets as bioartificial endocrine pancreas, *Science*, 210, 908, 1980.

Linko, Y. Y. and Linko, P., *Biotechnol. Lett.*, 3, 21, 1991.

Linko, P. and Linko, Y. Y., *Crit. Rev. Biotechnol.*, 1, 289, 1984.

Luong, J. H. T., Cell immobilization in kappa-carrageenan for ethanol production, *Biotechnol. Bioeng.*, 27, 1652, 1985.

Loureiro-Dias, M. C. and Santos, H., Effects of ethanol on *Saccharomyces cerevisiae* as monitored by *in vivo* ^{31}P and ^{13}C nuclear magnetic resonance, *Arch. Microbiol.*, 153, 384, 1990.

Maharaj, I., Nairn, J. G., and Campbell, J. B., Simple rapid method for the preparation of enteric-coated microspheres, *J. Pharm. Sci.*, 73, 39, 1984.

Margaritis, A. and Merchant, F. J. A., *Crit. Rev. Biotechnol.*, 1, 339, 1984.

Margaritis, A., Promod, K., Bajpai, P., and Wallace, R., *Biotechnol. Lett.*, 7, 601, 1985.

Marin-Iniesta, F., Nasri, M., Dhulster, P., Barbotin, J. N., and Thomas, D., Influence of oxygen supply on the stability of recombinant plasmid pTG201 in immobilized *E. coli* cells, *Appl. Microbiol. Biotechnol.*, 28, 455, 1988.

Martinsen, A., Skjak-Braek, G., and Smidsrod, O., Alginate as immobilization material. I. Correlation between chemical and physical properties of alginate gel beads, *Biotechnol. Bioeng.*, 33, 79, 1989.

Marwaha, S. S., Kennedy, J. F., Khanna, P. K., Tewari, H. K., and Redhu, A., Comparative investigations on the physiological plane, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Matsunaga, T., Karube, I., and Suzuki, S., *Anal. Chim. Acta*, 99, 233, 1978.

Matsumoto, S., Saito, Y., and Takashima, Y., Phenomenal transition of liquid atomization from discs, *J. Chem. Eng.*, 7, 13, 1974.

Matsumoto, S., Kobayashi, H., and Takashima, Y., Production of monodispersed capsules, *J. Microencapsul.*, 3, 25, 1986.

Mattiasson, B., Immobilization methods, in *Immobilized Cells and Organelles*, Vol. 1, Mattiasson, B., Ed., CRC Press, Inc., Boca Raton, FL, 1983, 3.

Mattiasson, B. and Hahn-Hägerdal, B., Microenvironmental effects on metabolic behavior of immobilized cells: a hypothesis, *Eur. J. Appl. Microbiol. Biotechnol.*, 16, 52, 1982.

Mattiasson, B., Larsson, M., and Hahn-Hägerdal, B., Metabolic behavior of immobilized cells — effects of some microenvironmental factors, in *Enzyme Engineering*, Vol. 7, Laskin, A. J., Tsao, G. T., and Wingard, L. B., Eds., 1984.

Matulovic, U., Rasch, D., and Wagner, F., New equipment for the scaled up production of small spherical biocatalysts, *Biotechnol. Lett.*, 8, 485, 1986.

McKnight, C. A., Goosen, M. F. A., Penney, C., and Sun, D., Synthesis of chitosan-alginate microcapsules membranes, *J. Bioact. Comp. Polym.*, 3, 334, 1988.

Melick, M. R., Karim, M. N., Linden, J. C., Dale, B. E., and Mihaltz, P., Mathematical modeling of ethanol production by immobilized *Zymomonas mobilis* in a packed bed fermentor, *Biotechnol. Bioeng.*, 29, 370, 1987.

Miles, M. J., Morris, V. J., and Carroll, V., Caroub gum K-carrageenan mixed gels: mechanical properties and X-ray fiber diffraction studies, *Macromolecules*, 17, 2443, 1987.

Min, Lee, G. and Palsson, B. O., Immobilization can improve the stability of hybridoma antibody productivity in serum-free media, *Biotechnol. Bioeng.*, 36, 1049, 1990.

Mitani, Y., Nishizawa, Y., and Nagai, S., *J. Ferm. Technol.*, 62, 249, 1984.

Monbouquette, H. G., Sayles, G. D., and Ollis, D. F., Immobilized cell biocatalyst activation and pseudo-steady-state behaviour: model and experiment, *Biotechnol. Bioeng.*, 35, 609, 1990.

Moon, S. H. and Parulekar, S. J., Characterization of κ-carrageenan gels used for immobilization of *Bacillus firmus*, *Biotechnol. Prog.*, 7, 516, 1991.

Mori, A., Matsumoto, N., and Imai, C., Growth behaviour of immobilized acetic acid bacteria, *Biotechnol. Lett.*, 11, 183, 1989.

Morikawa, Y., Karube, I., and Suzuki, S., Continuous production of bacitracin by immobilized living whole

cells of *Bacillus* sp., *Biotechnol. Bioeng.*, 22, 1015, 1980.

Morin, N., Bernier-Cardou, M., and Champagne, C. P., Production of concentrated *Lactococcus lactis* subsp. *cremoris* suspensions in calcium alginate beads, *Appl. Environ. Microbiol.*, 58, 545, 1992.

Mosbach, K. and Mosbach, R., Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their application in column techniques, *Acta Chem. Scand.*, 20, 2807, 1966.

Nakajima, H., Sonomoto, K., Usui, N., Fumihiko, S., Yamada, Y., Tanaka, A., and Fukui, S., Entrapment of lavandula vera cells and production of pigments by entrapped cells, *J. Biotechnol.*, 2, 107, 1985.

Nasri, M., Sayadi, S., Barbotin, J. N., and Thomas, D., The use of immobilization of whole living cells to increase stability of recombinant plasmids in *Escherichia coli*, *J. Biotechnol.*, 6, 147, 1987.

Nasri, M., Sayadi, S., Barbotin, J. N., Dhulster, P., and Thomas, D., Influence of immobilization on the stability of pTG201 recombinant plasmid in some strains of *Escherichia coli*, *Appl. Environ. Microbiol.*, 53, 740, 1987.

Navarro, J. M. and Durand, G., *Eur. J. Appl. Microbiol.*, 4, 243, 1977.

Neufeld, R. J., Peleg, Y., Rokem, J. S., Pines, O., and Goldberg, I., L-Malic acid formation by immobilized *Saccharomyces cerevisiae* amplified for fumarase, *Enzyme Microbiol. Technol.*, 13, 991, 1991.

Nilsson, K., Birnbaum, S., Flygare, S., Linse, L., Shröder, U., Jeppson, U., Larsson, P. O., Mosbach, K., and Brodelius, P., A general method for the immobilization of cells with preserved viability, *Eur. J. Appl. Microbiol. Biotechnol.*, 17, 319, 1983.

Nir, R., Lamed, R., Gueta, L., and Sahar, E., Single cell entrapment and microcolony development within uniform microspheres amenable to flow cytometry, *Appl. Environ. Microbiol.*, 56, 2870, 1990.

Norton, S. and Lacroix, C., Gellan gum gel as entrapment matrix for high temperature fermentation processes: a rheological study, *Biotechnol. Tech.*, 4, 351, 1990.

Nunez, M. T., Chamy, R., Lema, J. M., and Sanroman, A., Enhancement of the cell retention for immobilized yeasts in K-carrageenan, in *Physiology of Immobilized Cells*, DeBont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Elsevier Science Publishers, Amsterdam, 1990.

Ogbonna, J. C., Matsumura, M., Yamagoata, T., Sakuma, H., and Kataoka, H., Production of microgel beads by a rotating disc atomizer, *J. Ferment. Bioeng.*, 68, 40, 1989.

Ollis, D. F., Monbouquette, H., Kuhn, R., and Peretti, S. W., Scanning microphotometry and microfluorimetry of immobilized cells: an emerging quantitative tool, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

O'Neil, M. A., Selverdran, R. R., and Morris, V. J., *Carbohydr. Res.*, 124, 123, 1983.

Overgaard, S., Scharer, J. M., Moo-Young, M., and Bols, N. C., Immobilization of hybridoma cells in chitosan alginate beads, *Can. J. Chem. Eng.*, 69, 439, 1991.

Parascandola, P., De Alteriis, E., Farris, G. A., Budroni, M., and Scardi, V., Behaviour of grap must ferment *Saccharomyces cerevisiae* immobilized within insolubilized gelatin, *J. Ferm. Bioeng.*, 74, 123, 1992.

Passarinho, P. C. V. L., Vieira, A. M. S., Cabral, J. M. S., Novais, J. M., and Kennedy, J. F., Effect of carrier matrix on fermentative production of ethanol by surface immobilised yeast cells, *J. Chem. Technol. Biotechnol.*, 44, 183.

Petre, D., Noel, C., and Thomas, D., A new method for cell immobilization, *Biotechnol. Bioeng.*, 20, 127, 1978.

Pines, G. and Freeman, A., *Eur. J. Appl. Microbiol. Biotechnol.*, 16, 75, 1982.

Poncelet, D. and Neufeld, R. J., Shear breakage of nylon membrane microcapsules in a turbine reactor, *Biotech. Bioeng.*, 33, 95, 1989.

Poncelet, D., Poncelet de Smet, B., Beaulieu, C., and Neufeld, R. J., Scale-up of gel bead and microcapsule production in cell immobilization, in *Fundamentals of Animal Cell Encapsulation and Immobilization*, Goosen, M. F. A., Ed., CRC Press Inc., Boca Raton, FL, 1993.

Poncelet de Smet, B., Poncelet, D., and Neufeld, R. J., Emerging techniques, materials, and applications in cell immobilization, in *Fundamentals of Animal Cell Encapsulation and Immobilization*, Goosen, M. F. A., Ed., CRC Press Inc., Boca Raton, FL, 1993.

Poncelet, D., Alexakis, T., and Neufeld, R. J., Microencapsulation within cross-linked polyethyleneimine membranes, *J. Microencap.*, 11, 31, 1994.

Poncelet De Smet, B., Poncelet, D., and Neufeld, R. J., Preparation of hemolysatefilled hexamethylene sebacamide microcapsules with controlled diameter, *Can. J. Chem. Eng.*, 68, 443, 1990.

Poncelet, D., Beaulieu, C., Hallo, J. P., Fournier, A., and Neufeld, R. J., Production of alginate beads by emulsification/internal gelation. I. Methodology, *Appl. Microbiol. Biotechnol.*, in press.

Prevost, H. and Divies, C., Cream fermentation by a mixed culture of lactococci entrapped in two-layer calcium alginate gel beads, *Biotechnol. Lett.*, 14, 583, 1992.

Ralston, G. B., Tracey, M. V., and Wrench, P. M., The inhibition of fermentation in baker's yeast by chitosan, *Biochim. Biophys. Acta*, 93, 652, 1964.

Ramakrishna, S. V., Sreedharan, V. P., and Prema, P., Continuous ethanol production with immobilized yeast cells in a packed bed reactor, in *Bioreactor Immobilized Enzymes and Cells, Fundamentals and Applications*, Moo-Young, M., Ed., Elsevier Applied Science, New York, 1988.

Rao, A. V., Shiwnarain, N., and Maharaj, I., Survival of microencapsulated *Bifidobacterium pseudologum* in simulated gastric and intestinal juice, *Can. Inst. Food Sci. Technol. J.*, 22, 345, 1989.

Rehg, T., Dorger, C., and Chau, P. C., Application of an atomizer in producing small alginate beads for cell immobilization, *Biotechnol. Lett.*, 8, 111, 1986.

Rha, C. K., Process for Encapsulation and Encapsulated Active Material System, Eur. Patent 152-898-A2, 1985.

Rha, C. K., Rodriguez-Sanchez, D., and Kienzle-Sterzer, C., Novel applications of chitosan, in *Biotechnology of Marine Polysaccharides*, Colwell, R. R., Pariser, E. R., and Sinskey, A. J., Eds., Hemisphere Publishing Corporation, Washington, D.C., 1985.

Rochefort, W. E., Rehg, T., and Chau, P. C., Trivalent cation stabilization of alginate gel for cell immobilization, *Biotechnol. Lett.*, 8, 115, 1986.

Roffler, S. R., Blanch, H. W., and Wilke, C. R., *In situ* extractive fermentation of acetone and butanol, *Biotechnol. Bioeng.*, 31, 135, 1988.

Roy, D., Goulet, J., and LeDuy, A., Continuous production of lactic acid from whey permeate by free and calcium alginate entrapped *Lactobacillus helveticus*, *J. Dairy Sci.*, 70, 506, 1987.

Rouxhet, P. G. and Mozes, N., The microenvironement of immobilized cells: critical assessment of the influence of surfaces and local concentrations, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Ruggeri, B., Sassi, G., Specchia, V., Bosco, F., and Marzona, M., Alginate beads coated with polyacrylamide resin: potential as a biocatalyst, *Process Biochem.*, 26, 331, 1991.

Ryu, D., Kim, H. S., and Taguchi, H., Intrinsic fermentation kinetic parameters of immobilized yeast cells, *J. Ferment. Technol.*, 62, 255, 1984.

Saucedo, N. J. E., Barbotin, J. N., and Thomas, D., Physiological and morphological modifications in immobilized *Giberella fujikuroi* mycelia, *Appl. Environ. Microbiol.*, 55, 2377, 1989a.

Saucedo, N. J. E., Barbotin, J. N., Velut, M., and Thomas, D., Ultrastructural examination of *Giberella fujikuroi* mycelia: effect of immobilization in calcium alginate beads, *Can. J. Microbiol.*, 35, 1118, 1989b.

Sayadi, S., Nasri, M., Barbotin, J. N., and Thomas, D., Effect of environmental growth conditions on plasmid stability, plasmid copy number, and catechol 2,3-dioxygenase activity in free and *Escherichia coli* cells, *Biotechnol. Bioeng.*, 33, 801, 1989.

Sayles, G. D. and Ollis, D. F., Periodic operation of immobilized cell systems: analysis, *Biotechnol. Bioeng.*, 34, 160, 1989.

Sayles, G. D. and Ollis, D. F., Product inhibition influence on immobilized cell biocatalyst performance, *Biotechnol. Prog.*, 6, 153, 1990.

Scott, C. D., Techniques for producing monodispersed biocatalyst beads for use in columnar bioreactors, *Ann. N.Y. Acad. Sci.*, 501, 487, 1987.

Seko, H., Takeuchi, S., Yajima, K., Senuma, M., and Tosa, T., L-Aspartic acid production using immobilized *E. coli* cells in a packed bed reactor. Design of reactor and its optimal operation, *Ann. N.Y. Acad. Sci.*, 469, 540, 1986.

Shabtai, Y., Chaimovitz, S., Freeman, A., Katchalski-Katzir, E., Linder, C., Nemas, M., Perry, M., and Kedem, O., Continuous ethanol production by immobilized yeast reactor coupled with membrane pervaporation unit, *Biotechnol. Bioeng.*, 38, 869, 1991.

Shimi, S. M., Newman, E. L., Hopwood, D., and Cushieri, A., Semi-permeable microcapsules for cell culture: ultra structural characterization, *J. Microencapsul.*, 8, 307, 1991.

Shindo, S., Sahara, H., and Koshino, S., Relationship of production of succinic acid and methyl citric acid pathway during alcohol fermentation with immobilized yeast, *Biotechnol. Lett.*, 15, 51, 1993.

Shindo, S., Murakami, J., and Koshino, S., Control of acetate ester fermentation during alcohol fermentation with immobilized yeast, *J. Ferm. Bioeng.*, 73, 370, 1992.

Shinmyo, A., Kimura, H., and Okada, H., Physiology of alpha-amylase production by immobilized *Bacillus amyloliquefaciens*, *Eur. J. Appl. Microbiol. Biotechnol.*, 14, 7, 1982.

Shinonaga, M. A., Kawamura, Y., and Yamane, T., Immobilization of yeast cells with cross-linked chitosan beads, *J. Ferm. Bioeng.*, 74, 90, 1992.

Siemann, M., Muller-Hurtig, R., and Wagner, F., Characterization of the rotating nozzle-ring technique for the production of small spherical biocatalysts, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Siess, M. H. and Divies, C., Behaviour of *Saccharomyces cerevisiae* cells entrapped in a polyacrylamide gel and performing alcoholic fermentation, *Eur J. Appl. Microbiol. Biotechnol.*, 12, 10, 1981.

Silbiger, E. and Freeman, A., *Biotechnol. Bioeng.*, 30, 675, 1987.

Simon, J. P., *Belg. J. Food Chem. Biotechnol.*, 44, 6, 1989.

Simon, J. P., *Belg. J. Food Chem. Biotechnol.*, 44, 183, 1989.

Simon, J. P., Benoot, T., Defroyennes, J. P., Deckers, B., Dekegel, D., and Vandegans, J., Physiology and morphology of Ca-alginate entrapped *Saccharomyces cerevisiae*, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1990.

Sisak, C., Boross, L., and Szajani, B., Stirred fluidised-bed reactor developed for low-density biocatalyst supports, *Biotechnol. Lett.*, 4, 15, 1990.

Smidsrod, O., Haug, A., and Lian, B., Properties of poly (1,4-hexuronates) in the gel state. Evaluation of a method for the determination of stiffness, *Acta Chem. Scand.*, 26, 71, 1972.

Smidsrod, O. and Haug, A., *Acta Chem. Scand.*, 26, 2063, 1972.

Smidsrod, O. and Skjak-Braek, G., Alginate as immobilization matrix for cells, *TIBTECH*, 8, 71, 1990.

Smith, M. R., de Haan, A., and Bont, J. A. M., The physiology of epoxyalkane-producing immobilized bacterial cells, in *Physiology of Immobilized Cells*,

De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Sode, K., Morita, T., Peterhans, A., Meussdoerffer, F., Mosbach, K., and Karube, I., Continuous production of alpha-peptide using immobilized recombinant yeast cells, *J. Biotechnol.*, 8, 113, 1988.

Spettoli, P., Nuti, M. P., Crapisi, A., and Zamorani, A., Technological improvement of malolactic fermentation in wine by immobilized microbial cells in a continuous flow reactor, *Ann. N.Y. Acad. Sci.*, 501, 386, 1987.

Sroka, W. and Rzedowski, W., The effects of yeast cell immobilization on the proportion of selected by-products of ethanol fermentation, *Biotechnology Letters*, 13, 879, 1991.

Stewart, P. S. and Robertson, C. R., Product inhibition of immobilized *Escherichia coli* arising from mass transfer limitation, *Appl. Environ. Microbiol.*, 54, 2464, 1988.

Su, H., Bajpai, R., and Preckshot, W., Characterization of alginate beads formed by a two fluid annular atomizer, *Appl. Biochem. Biotechnol.*, 20/21, 561, 1989.

Taipa, M. A., Cabral, J. M. S., and Santos, H., Comparison of glucose fermentation by suspended and gel-entrapped yeast cells: an *in vivo* nuclear magnetic resonance study, *Biotechnol. Bioeng.*, 41, 647, 1993.

Takata, L., Tosa, T., and Chibata, I., *Biochim. Biophys. Acta*, 483, 193, 1977a.

Takata, L., Tosa, T., and Chibata, I., *J. Solid-Phase Biochem.*, 2, 225, 1977b.

Tampion, J. and Tampion, M. D., Reactor design and operation, in *Immobilized Cells: Principles and Applications*, Cambridge University Press, New York, 1987.

Tanaka, H., Irie, S., and Ochi, H., A novel immobilization method for prevention of cell leakage from gel matrix, *J. Ferm. Bioeng.*, 68, 216, 1989.

Tanaka, A., Tosa, T., and Kobayashi, T., *Industrial Application of Immobilized Biocatalysts*, Marcel Dekker, New York, 1992.

Thomas, N. H. and Janes, D. A., Fluid dynamic considerations in airlift and annuler vortex bioreactors for plant cell culture, *Ann. N.Y. Acad. Sci.*, 506, 171, 1987.

Tosa, T., Sato, T., Mori, T., Yamamoto, K., Takata, I., Nishida, Y., and Chibata, I., Immobilization of enzymes and microbial cells using carrageenan as matrix, *Biotechnol. Bioeng.*, 21, 1697, 1979.

Tuli, A., Sethi, R. P., Khanna, P. K., and Marwaha, S. S., Lactic acid production from whey permeate by immobilized *Lactobacillus casei*, *Enzyme Microbiol. Technol.*, 7, 164, 1985.

Tzeng, J. W., Fan, L. S., Gan, Y. R., and Hu, T. T., Ethanol fermentation using immobilized cells in a multistage fluidized bed bioreactor, *Biotechnol. Bioeng.*, 38, 1253, 1991.

Vega, J. L., Clausen, E. C., and Gaddy, J. L., Maximizing productivity in an immobilized cell reactor, *Ann. N.Y. Acad. Sci.*, 506, 208, 1987.

Velizarov, S. G., Rainina, E. I., Sinitsyn, A. P., Varfolomeyev, S. D., Lozinsky, V. I., and Zubou, A. L., Production of L-lysine by free and PVA-cryogel immobilized *Corynebacterium glutamicum* cells, *Biotechnol. Lett.*, 14, 291, 1992.

Venkatasubramanian, K. and Karkare, S. B., Process engineering considerations in the development of immobilized living cell systems, in *Immobilized Cells and Organelles*, Vol. 2, Mattiasson, B., Ed., CRC Press, Boca Raton, FL, 1983.

Venkatasubramanian, K., Karkare, S. B., and Vieth, W. R., Chemical engineering analysis of immobilized-cell systems, in *Immobilized Microbial Cells*, Wingard, L. B., Katchalski-Katzir, E., and Goldstein, L., Eds., *Appl. Biochem. Bioeng.*, 4, 312, 1983.

Venkatasubramanian, K. and Vieth, W. R., *Prog. Ind. Microbiol.*, 15, 61, 1979.

Vieira, A. M. S., Sa-Correia, I., Novais, J. M., and Cabral, J. M. S., Could the improvements in the alcoholic fermentation of high glucose concentrations by yeast immobilization be explained by media supplementation, *Biotechnol. Lett.*, 11, 137, 1989.

Vieth, W. R. and Venkatasubramanian, K., Immobilized microbial cells in complex biocatalysts, in *Immobilized Microbial Cells*, Vol. 106, American Chemical Society, Washington, D.C., 1979.

Vives, C., Casas, C., Godia, F., and Sola, C., Determination of the intrinsic fermentation kinetics of *Sac charomyces cerevisiae* cells immobilized in Cal-alginate beads and observations on their growth, *Appl. Microbiol. Biotechnol.*, 38, 467, 1993.

Vlaev, S. D. and Valeva, M., An extension of the pressure-test technique for immobilized biocatalyst preparations, *Biotechnol. Bioeng.*, 39, 361, 1992.

Vorlop, K. D. and Klein, J., New developments in the field of cell immobilization. Formation of biocatalysts by ionotropic gelation, in *Enzyme Technology*, Vol. 3, Rotenburger Ferm. Symp., Lafferty, R. M., Ed., Springer-Verlag, Heidelberg, 1981a, 219.

Vorlop, K. D. and Klein, J., Formation of spherical chitosan biocatalysts by ionotropic gelation, *Biotechnol. Lett.*, 3, 9, 1981b.

Wada, M., Kato, J., and Chibata, I., Continuous production of ethanol using immobilized growth yeast cells, *Eur. J. Appl. Microbiol. Biotechnol.*, 10, 275, 1980.

Walls, E. L. and Gainer, J. L., Retention of plasmid bearing cells by immobilization, *Biotechnol. Bioeng.*, 34, 717, 1989.

Wang, F. F., Shaw, J. F., Wu, C. R., and Wang, Y. J., Microencapsulation with poly(vinyl amine) and alginate, *Biotechnol. Tech.*, 6, 185, 1992.

Wang, F. F., Wu, C. R., and Wang, Y. J., Preparation and application of poly(vinylamine)/alginate microcapsules to culturing of a mouse Erythroleukemia cell line, *Biotechnol. Bioeng.*, 40, 1115, 1992.

Wheatley, M. A. and Philips, C. R., The influence of internal and external diffusional limitations on the observed kinetics of immobilized whole bacterial cells with cell-associated beta-glucosidase activity, *Biotechnol. Lett.*, 5, 79, 1983.

White, F. H. and Portno, A. D., Continuous fermentation by immobilized brewing yeast. *J. Inst. Brew (London)*, 84, 228, 1978.

Wijffels, R. H., de Gooijer, C. D., Kortekaas, S., and Tramper, J., *Nitrobacter agilis* immobilized in carrageenan: oxygen consumption and biomass density, in *Physiology of Immobilized Cells*, De Bont, J. A. M., Visser, J., Mattiasson, B., and Tramper, J., Eds., Elsevier Science Publishers, Amsterdam, 1989.

Wu, K. Y. A. and Wisecarver, K. D., Cell immobilization using PVA crosslinked with boric acid, *Biotechnol. Bioeng.*, 39, 447, 1992.