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Methods of immobilization of microbial cells

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Contents

Introduction

There is a need at the present time to consider viable alternatives to fossil resources for the production of fuels and chemicals. One such alternative is the utilization of microbial metabolism. Metabolic pathways of microbial cells may be changed by manipulating growth conditions so as to enhance (or reduce) the production of metabolites that may be useful as fuels or chemical sources [1]. Continuous systems are more appropriate than batch systems for maintaining a constant microbial cell growth milieu. An advanced continuous bioreactor for the culture of (and metabolite production by) microbial cells requires that the cells be retained in the reactor; otherwise, cell washout occurs. This immobilization of the microbial cells is accomplished through their attachment to, or entrapment by, water-insoluble materials that can be either organic or inorganic in nature (Fig. 1). The resulting immobilized cells are denser than they are in the free state and hence are retained by the reactor. Several methods exist for achieving the desired cell immobilization.

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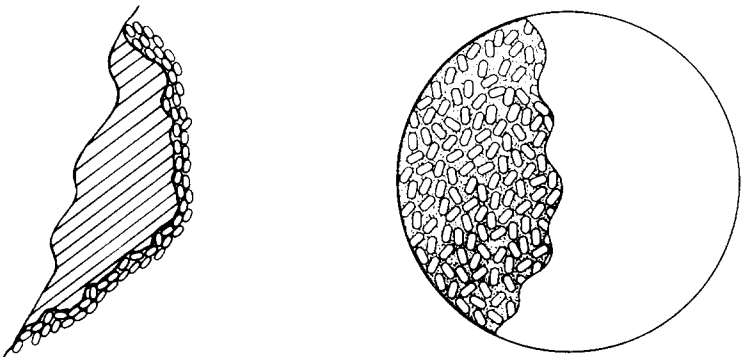

Fig. 1. Two primary types of cellular immobilization.

Flocculation of cells

When microbial cells aggregate or flocculate naturally to form dense cellular clusters, this is, of course, the simplest means of immobilization. Strandberg et al. [2] have described continuous ethanol production by a flocculent strain of *Zymomonas mobilis.* The method of immobilization and utilization is given in Table 1. The so called 'floc' can be used for the continuous production of ethanol from glucose in the bioreactor configurations shown in Fig. 2. It is clear that the immobilized cells will be retained in the bioreactors; only after 2 to 3 weeks are sufficient flocculent cells produced to result in their appearance in the effluent stream.

Artificial methods of forming cell 'flocs' by chemical crosslinking with agents such as glutaraldehyde have also been reported. However, such methods may have adverse effects on the metabolic activity of the cells [3].

TABLE 1

IMMOBILIZATION OF *Z. MOBILIS* BY FLOCCULATION

1. Culture *Z. mobilis* NRRL B-12526 (flocculating strain) in a liquid medium containing 5% (w/v) glucose and 0.5% (w/v) yeast extract at 28 °C without agitation.
2. After 48 h growth (visible flocculation) the cultures can be stored at 4 °C.
3. Culture the flocculent 2 – 3 days under the same conditions 48 – 72 h prior to inoculation of the bioreactor.
4. Inoculate a sterile bioreactor with 10 – 20 ml of the cultured flocculant and continue culture for 2 days prior to continuous operation.

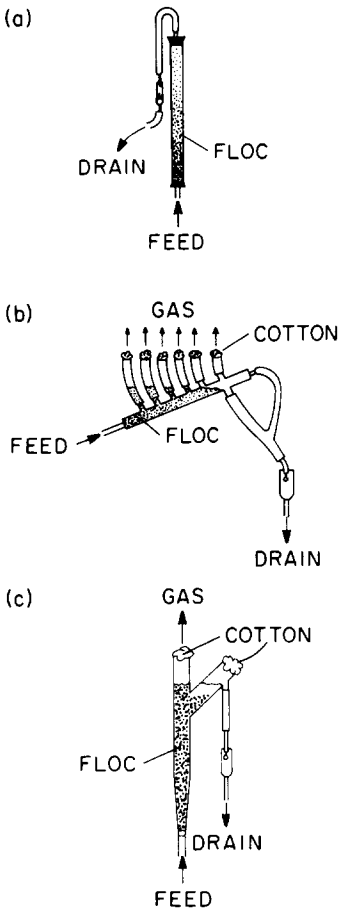

Fig. 2. Bioreactor configurations for the retention of cells of flocculent *Zymomonas mobilis* NRRL B-12526. (a) Vertical, straight column; (b) angled, gas-venting column; (c) vertical, tapered column with angled side arm. All columns are ~ 30 cm long, with 2.54 cm i.d. and 3.5 cm o.d. Reproduced, with permission, from ref. 2.

Adsorption of cells

Adsorption of cells is generally considered to be one of the easiest methods of immobilization, and this is certainly the case when microbial cells adhere naturally to the surfaces of insoluble materials. A photograph showing the adherence of a mixed population of bacterial cells (fixed-film) to inert ceramic Raschig rings packed in a continuous-flow bioreactor is seen in Fig. 3. The fixed film that attaches to the support material after inoculation of the bioreactor with municipal sludge can be used in the treatment of municipal wastewater [4]. Other fixed-film bioreactors can be used for the treatment of various wastewaters. For example, a fluidized-bed bioreactor (Fig. 4)

Fig. 3. Fixed films of bacterial cells attached to 2.5 cm diam. inert Raschig rings used for municipal wastewater treatment.

containing a mixed population of bacterial cells attached to anthracite coal particles (30 to 60 mesh) has been used for the treatment of coal gasification wastewater [5]. A downflow stationary fixed-film bioreactor in which active biomass attaches itself to stationary rough surfaces to form vertical channels in the reactor has also been shown to be effective in the treatment of acetic acid-based synthetic wastewater [6]. As a rule, the microbial population (inoculant) is circulated through the bioreactor until immobilization is achieved. The biomass grows on the support material and is retained in the reactor, with the total retention depending upon the loading capacity of the system.

One of the disadvantages of using fixed-film bioreactors is the time required to build up a microbial film sufficient for effecting a bioprocess. In contrast, the adsorption of microbial cells to ion exchange materials is rapid. DEAE-cellulose is one such material that has been shown to adsorb cells of both *Acetobacter aceti* [7] and *Z. mobilis* [8]. The basic immobilization technique is given in Table 2. The major disadvantage of this method is that a change in the fermentation medium conditions (such as ionic strength, pH, and stirring rates) can dramatically alter the degree of cell adsorption [8]. Also, ion exchange polymers generally possess poor binding capacity ($\sim$10 to 50 mg/g) for microbial cells.

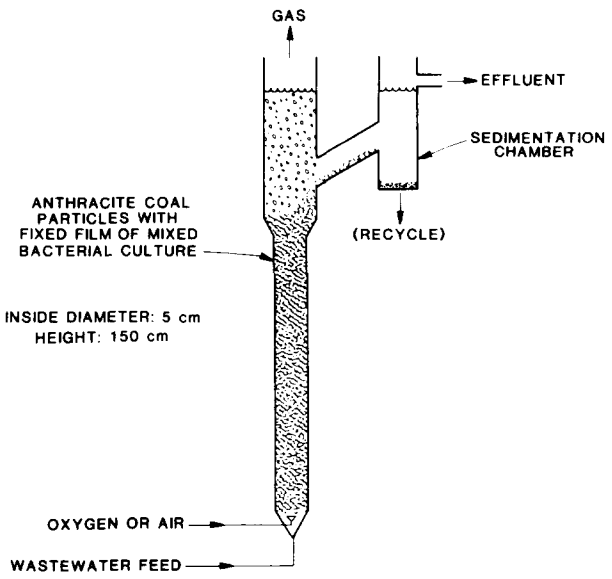

Fig. 4. Fluidized-bed bioreactor for coal gasification wastewater treatment contains a mixed population of bacterial cells attached to anthracite coal particles.

TABLE 2

IMMOBILIZATION OF MICROBIAL CELLS BY ADSORPTION TO DEAE-CELLULOSE

1. Equilibrate a given quantity of DEAE-cellulose in fermentation media at approximately pH 6.0 and an ionic strength of 50 mM, at room temperature overnight.
2. Filter the support and add a given amount of the wet ion exchanger (e.g., 1 g) to 50 ml of fermentation media containing 0.1[a] g of cells.
3. Mix gently by end-to-end rotation for 30 min.
4. Filter the mixture using an appropriate glass sinter and wash the ion exchange particles thoroughly in deionized water suspending the particles each time to allow for removal of unknown cells.

[a] Value can be altered depending on the saturating binding curve.

Immobilization by gel entrapment

This method of immobilization involves the 'trapping' of microbial cells in the interstices of naturally occurring gel polymers such as alginate or carrageenan, or within synthetic polymeric networks formed from monomeric precursors such as acrylamide and hydroxyethylmethacrylate. The simplest, easiest, and safest method for microbial cell entrapment utilizes the naturally occurring gel polymers, and a practical approach has been described previously [9]. Methods for the entrapment of cells in polyacrylamide, alginate, carrageenan, and agar are outlined in Tables 3–6.

Typically, in gel bead formation, syringe needles are used to exude the soft gel

TABLE 3

ENTRAPMENT OF CELLS IN CROSS-LINKED AND PREPOLYMERIZED LINEAR POLYACRYLAMIDES

1. Materials required:
   Washed cells
   0.2 M potassium phosphate buffer, pH 7.0
   Acrylamide
   N,N'-methylene-*bis*-acrylamide (bisacrylamide)
   Tetramethylethylenediamine (TEMED)
   Ammonium persulphate
   Scalpel
   Coarse sieve
   Glass petri dishes
2. Suspend about 5 g wet weight of cells in 10 ml of distilled water and chill in ice.
3. Chill 10 ml of 0.2 M potassium phosphate buffer, pH 7.0, in ice.
4. Add to the buffer:
   2.85 g acrylamide
   0.15 g bisacrylamide
   10 mg ammonium persulphate. Mix to dissolve these solids.
5. Immediately mix the chilled buffer solution with the chilled cell suspension, pour into 2 or 3 glass petri dishes and cover.
6. Allow polymerization to proceed for 1 h.
7. Suspend the sieved gel in 100 ml of 0.2 M potassium phosphate buffer, pH 7.0, allow to settle and then decant the fines.

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TABLE 4

ENTRAPMENT OF CELLS IN CALCIUM ALGINATE GELS

1. Materials required:
   Sodium alginate solution (4%, w/v)[a]
   0.2 M $CaCl_2$
   Cell slurry (~ 10 – 30 g dry weight/100 ml)
   Syringe (10 ml) fitted with a wide bore needle approximately 1 mm diameter for droplet formation or a 10 ml pipette with an opening of about 3 mm diameter
   Magnetic stirrer
2. Mix equal volumes (50 ml is suitable for preliminary work) of sodium alginate solution and of cell slurry gently together.
3. Extrude the mixture dropwise via a 10 ml syringe from a height of about 20 cm into an excess of 0.2 M $CaCl_2$[b]. One liter of $CaCl_2$ solution is a suitable volume for 100 ml of mixture.
4. Leave the beads of calcium alginate entrapped cells to harden in the $CaCl_2$ solution for about 20 min.

[a] Preparation of the sodium alginate solution may give problems because the dry powder, being hygroscopic, may form clumps. Accordingly, it is recommended that the powder be added to the water while being stirred and that stirring is continued for a further hour to ensure dissolution. The solution is then left to stand for about 30 min to allow air bubbles to escape. Removal of air bubbles is essential since otherwise they will be entrapped in beads causing these to float and so create problems in use of the beads.

[b] If desired, the gels can be formed into slabs or cubes by pouring the sodium alginate/cell mixture into the appropriate mould and overlaying with a solution of calcium chloride (0.2 – 0.5 M). When formed, the slabs or cubes can be hardened further by incubation with an excess of 0.2 M calcium chloride. (Reprinted, with permission, from Ref. 9.)

TABLE 5

ENTRAPMENT OF CELLS IN κ-CARRAGEENAN[a]

1. Materials required:
   κ-carrageenan (4%, w/v) in 0.9% (w/v) NaCl. Prepare this by heating at 60 °C to dissolve the polysaccharide and then maintain the solution at 40 °C.
   KCl (2% w/v) kept at room temperature (18 – 20 °C).
   Cell slurry (~ 10 – 30 mg dry weight/100 ml).
2. Mix the warm carrageenan solution with the cell slurry at a ratio of 9:1. While still at 40 °C, add the mixture dropwise, using a syringe, to the KCl solution.
3. Leave the beads so formed to harden in the KCl solution for 20 min.

[a] The extrusion and bead preparation procedures are essentially similar to those for preparation of calcium alginate beads (Table 4).
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TABLE 6

ENTRAPMENT OF CELLS IN AGAR

1. Materials required:
   Agar solution: dissolve 100 mg agar in 4.5 ml of 0.9% (w/v) NaCl by heating at 100 °C and then cooling to 50 °C.
   Cell slurry (10 – 30 mg dry wt/100 ml) suspended in 0.9% (w/v) NaCl solution.
   Nylon (100 mesh) net on a glass plate.
   0.1 M sodium phosphate buffer, pH 7.0.
2. Add 0.5 ml of the cell slurry to 4.5 ml of the agar solution at 50 °C and mix. Cast the mixture onto the nylon net and cool to 5 °C. Store the membrane which forms in 0.1 M solution phosphate buffer, pH 7.0, until required.

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dropwise into the hardening agent, resulting in the formation of beads 2 to 5 mm in diameter. Recently, an apparatus was described [10] that will produce gel beads in the 0.5- to 3.0-mm diam. size range (shown in Fig. 5). A pressurized, stirred reservoir delivers the gel solution (including microbes and other additives) to a small nozzle such as a syringe needle, which is vibrated at a given frequency to produce a stream of monodispersed gel droplets. The frequency of vibration must be 'tuned' to the jet diameter as well as the velocity and fluid properties of gel solution. The beads when formed will vary little (< 10%) in their diameter (Table 7).

Biocatalyst gel beads, 1 mm diam. made using this apparatus, are shown in Fig. 6. The gel solution contains ~10 wt% *Z. mobilis* cells and iron oxide (Fe_2O_3) particles (to control density and reduce cell leakage from the beads). These gel beads are fixed in 0.1 M $CaCl_2$ (for alginate) and 0.3 M KCl (for carrageenan) and then 'cured' in the salt solution for 12 to 16 h prior to their use. They have been used successfully in a fluidized-bed bioreactor for ethanol production from an industrial glucose feedstock [11].

Microbial cells and enzymes can also be coentrapped within gel spheres. Coentrapment may be necessary if the microorganism does not utilize a particular

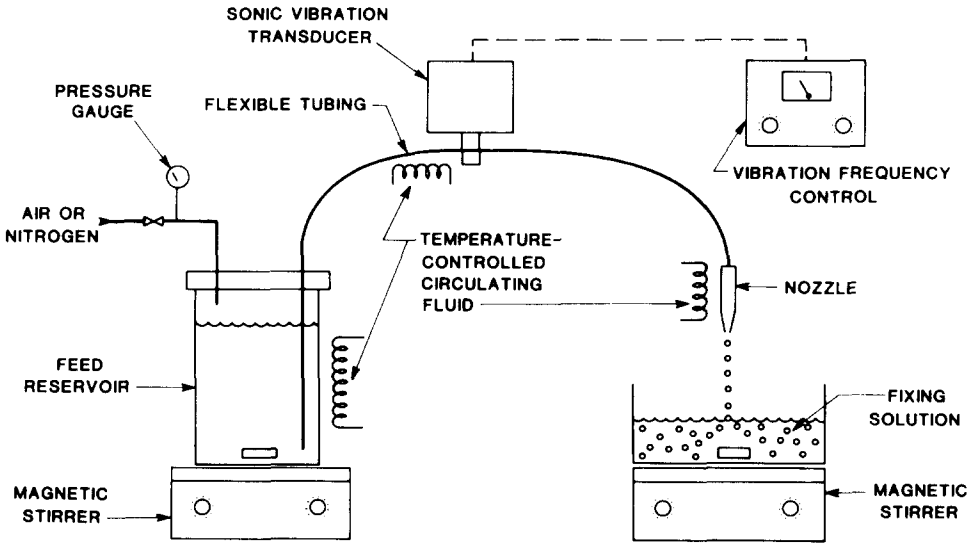

Fig. 5. Experimental setup for the production of monodispersed biocatalyst beads. (Reproduced, with permission, from ref. 10.)

TABLE 7

TYPICAL BIOCATALYST BEAD DIMENSIONS AS A FUNCTION OF OPERATING CONDITIONS

Material	Nozzle size (mm)	Flow rate (ml/s)	Vibrational frequency (Hz)	Bead size	
				Average (mm)	S.D. (mm)
4% κ-carrageenan	0.25	0.09	0	1.96	0.40
4% κ-carrageenan	0.25	0.10	190	1.96	0.16
4% κ-carrageenan	0.25	0.10	300[a]	1.18	0.05
4% κ-carrageenan	0.35	0.19	325[a]	1.30	0.11
4% κ-carrageenan	0.78	0.37	250[a]	2.33	0.34
4% κ-carrageenan and 3% Fe_2O_3	0.78	0.30	260[a]	1.65	0.13
4% κ-carrageenan and 10% *Z. mobilis*	0.25	0.11	330[a]	1.08	0.09

[a] Frequency at apparent maximum stream stability as indicated under stroboscopic light. (Reprinted, with permission, from Ref. 10.)

substrate directly. For example, although *Z. mobilis* does not ferment cellobiose to ethanol, a system incorporating β-glucosidase coentrapped with *Z. mobilis* in calcium alginate (Table 8) is able to convert cellobiose to ethanol in both batch and continuous-flow fermentation systems [12, 13]. Mixed cultures of microorganisms can also be coimmobilized. In one study, *Aspergillus awamori* and *Z. mobilis* were coimmobilized in calcium alginate gel beads for the conversion of starch to ethanol [14].

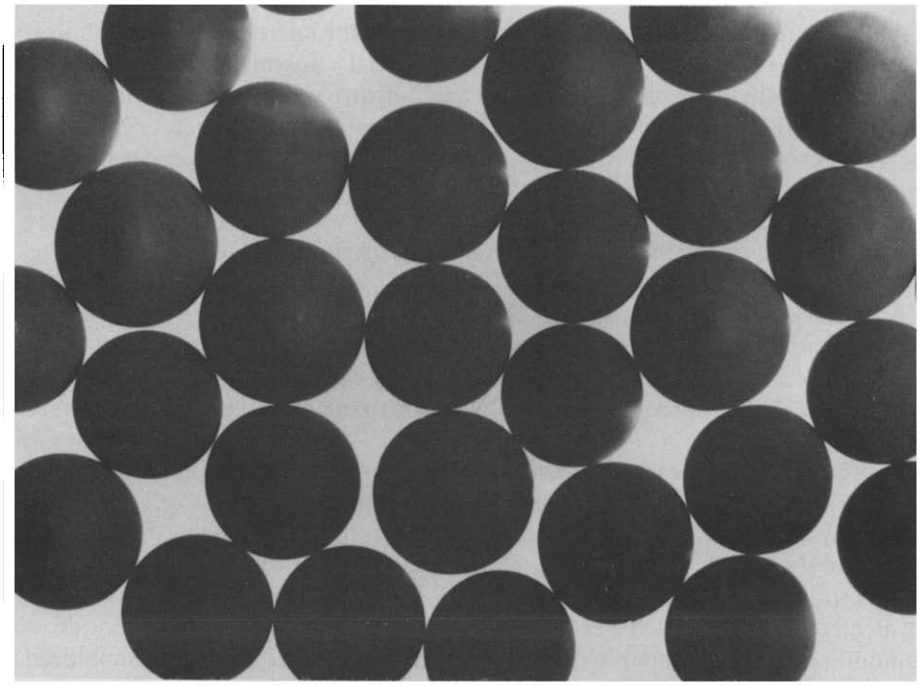

Fig. 6. Biocatalyst beads (nominal 1-mm diam.) made with κ-carrageenan and including iron oxide particles, to control density, and ~10% *Z. mobilis*, for conversion of sugars to alcohol.

TABLE 8

CO-ENTRAPMENT OF YEAST AND IMMOBILIZED β-GLUCOSIDASE IN CALCIUM ALGINATE

1. Materials required:
   1 g of swollen CNBr-activated Sepharose-4B (Pharmacia Ltd.)
   2.8 mg of partially purified β-glucosidase[a] dissolved in 5 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl.
   1 M ethanolamine-HCl, pH 8.0
   1 M NaCl, 0.1 M sodium acetate buffer, pH 4.0
   1 M NaCl, 0.1 M borate buffer, pH 8.0
   The reagents needed for the entrapment of cells in calcium alginate gels (Table 3).
2. Immobilize the β-glucosidase using the following method: to 1 g of swollen CNBr-activated Sepharose-4B[b], add 2.8 mg of β-glucosidase in the solution above and gently mix for 2 h.
3. Separate the support material to which the enzyme is now covalently linked, from the supernatant by decantation or vacuum filtration and incubate in 1 M ethanolamine-HCl, pH 8.0 for 2 h.
4. Wash the system alternately with 1 M NaCl, 0.1 M sodium acetate, pH 4.0 and with 1 M NaCl, 0.1 M borate buffer, pH 8.0 until the washings are free of enzyme activity.
5. Prepare a sodium alginate/yeast cell mixture as described in Table 3.
6. Add the immobilized β-glucosidase to this mixture to a maximum of 10%.
7. Bring about co-entrapment by formation of a calcium alginate gel as described in Table 3.

[a] β-glucosidase partially purified from the culture filtrate of *Talaromyces emersonii* [12].

[b] As an alternative to this procedure, the enzyme can be immobilized on concanavalin A-Sepharose [13].

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Although the most frequently used gel materials for microbial cell immobilization are alginate and carrageenan, other artificial polymers have been used. Methoxypolyethyleneglycol methacrylate and 2-hydroxyethylacrylate monomers have been polymerized by radiation, swollen, and then incubated with yeast cells that attach themselves to the internal surfaces of the gel [15]. Such gels have greater mechanical and chemical stability than those occurring naturally.

Immobilization of microbial cells by metal-link/chelation processes

A partial explanation of this immobilization method seems to be the formation of covalent bonds between the gelatinous hydrous metal oxides used as support materials and suitable ligands contributed by proteins and carbohydrates present on the cell surface [16]. Table 9 describes the immobilization of microbial cells on a hydrous metal oxide support.

Hydrous titanium(IV) and zirconium(IV) oxides seem to have minimal effects on the activity of enzymes immobilized to these supports, and yeast cells immobilized in this way have remained viable, according to measurements of their external invertase activity in the periplastic space [17]. The cells also appear to be quite firmly attached to the surface of the metal oxide [16]. Cells of *Arthrobacter globiformis* have also been covalently bound to silica gel after activation with chromium(III) chloride. such immobilized cells are capable of steroid transformation [18]. Cells immobilized on transition-metal-activated inorganic supports appear to be operationally stable [19].

Recommendations

Immobilized cells are likely to have increased application in the future for the production of fuels, pharmaceuticals, and other chemical products. Appropriate bioreactors necessary to accomplish these biotransformations must be developed [20]. The use of immobilized mixed cultures is likely to become important to adjust the production rates of both the unwanted and desired end products [21, 22]. Recently, a method has been described for the increased production of L-aspartic acid by a strain

TABLE 9

IMMOBILIZATION OF MICROBIAL CELLS ON HYDROUS METAL OXIDE

1. To a 15% w/v titanium (IV) chloride solution in 15% w/v HCl (or a 0.65 M zirconium (IV) chloride solution in 1.0 M HCl) slowly add a 2.0 M ammonia solution until neutrality (pH 7.0).
2. Wash the precipitate with 3×5.0 ml of a 0.9% w/v saline solution to remove ammonium ions.
3. To the hydrous metal oxide prepared above add a suspension of *Escherichia coli* cells ($A_{600}^{1.0\ cm}$: 0.216) in 10 ml of 0.9% w/v saline. Agitate gently for 5 min at room temperature.
4. Allow the mixture to stand at room temperature and the suspension to settle out, leaving a clear supernatant ($A_{600}^{1.0\ cm}$: 0.222). (Note this liquid is practically devoid of microorganisms as shown by microscopy.)
5. Consolidate the immobilized cell preparation by centrifugation at low speed and remove the supernatant.

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of *E. coli* in which the fumarase activity was almost completely eliminated [23]. These cells, immobilized in carrageenan, can be used for the continuous production of L-aspartic acid.

The generation of stable, inexpensive, and nontoxic support materials for microbial cell immobilization is essential to bioreactor development. The required use of ions, toxic cross-linking agents, and radiation for gel formation and stability are definite disadvantages to the use of gel entrapment of cells [24, 25]. The production of stable, maintenance-free, gel materials should be a major goal.

Summary

There is a large potential for the use of immobilized microbial cells for the production of fuels and chemicals in continuous bioreactor systems. Immobilization of microbial cells can be achieved by natural attachment to solid surfaces, ionic adsorption to appropriate carriers, entrapment in naturally occurring and synthetic polymeric gels and by covalent bonding to transition-metal-activated inorganic supports (including hydrous transition metal oxides). Practical aspects of these immobilization methodologies are described, and specific examples are given.

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