World Journal of Microbiology and Biotechnology 9, 495–502

Review

Potential applications of viable, immobilized fungal cell systems*

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Immobilized cell technology attracts considerable attention because of the many advantages it offers over conventional suspended-cell fermentations. Important advances continue to be made in the potential use of immobilized cells as biocatalysts. This review is mainly devoted to the analysis of recent literature on the applications of immobilized fungal cell systems, ranging from the production or transformation of useful compounds (e.g. organic acids, enzymes, antibiotics, steroids, etc.) to wastewater treatment. The problems and future industrial applications are also discussed.

Key words: Fungi, immobilization, potential applications.

Cell immobilization, one possible method for enzyme immobilization, can be used to overcome the problems and limitations (co-factor regeneration and arrangement of enzyme molecules in ordered clusters) frequently connected with direct enzyme immobilization.

Among various definitions of the 'immobilized cell' concept, the most correct appears to be that proposed by Abbott (1977), in which immobilization is taken as a physical confinement or localization of microorganisms that permits their economic re-use. This definition is sufficiently broad to extend the concept beyond the basic techniques of adsorption and entrapment, to include film-reactor systems in which immobilization is the result of natural adhesion and film growth. It says nothing, however, about the physiological state in which the cells are maintained within the carrier or on the carrier surface. It is, however, of great importance to know the level of cell viability so that the optimum carrier and method of immobilization can be selected and all the possibilities that immobilized-cell technology offers in the field of industrial fermentation can be determined.

Mosbach (1983) described three different levels of viability, based on the biocatalytic capabilities of the immobilized cells: (1) non-viable (giving single enzyme activities); (2) viable but resting (co-factors are not limiting and multi-step enzyme reactions can take place); and (3) viable and growing (all cellular physiological functions are maintained). In this review, which is devoted to the potential applications of immobilized fungal cell systems, only the latter two categories will be considered.

Advantages and Disadvantages of Immobilized Viable Cells

Immobilized living cell systems offers several advantages over conventional suspended-cell cultures:

(1) when immobilized cells are kept in a growing state within or on the surface of inert matrices by a continuous supply of suitable nutrients their biological functions are completely maintained. These cells can be seen as renewable or self-proliferating biocatalysts located in a defined space and protected against unfavourable environmental parameters outside their functional area.

(2) the physico-chemical interactions which take place between carriers and cells often give rise to increased stability of the entrapped cells which may, in turn, lead to increased cell productivity (Baklashova *et al.* 1984; Kopp & Rehm 1984; Li *et al.* 1984; Federici *et al.* 1987, 1990).

(3) the capacity for re-use of immobilized cells, in a resting or growing state, makes repeated or continuous operations possible in various types of

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reactors. In fact, the density of immobilized cells in or on their carriers is higher than that of freely suspended cells, indicating that faster reaction rates should be achieved using the immobilized cells.

(4) wash-out of the cells can be avoided, even at high dilution rates, and the immobilized cells can be easily separated from the reaction system, making the separation of product from biomass easier. The latter is of great importance from an industrial point of view.

There are, however, also several disadvantages in the use of immobilized living cells:

(1) yields of products may be lowered by the consumption of substrates as carbon and energy sources for the maintenance of cell viability or multiplication. However, cell growth can sometimes be restricted, by limitation of specific nutrients, while preserving the desired catalytic activities.

(2) unwanted side-reactions can occur because various metabolic systems, in addition to the desired ones, are found in living cells.

(3) in the case of immobilized growing cells, products may be contaminated by cells leaking from the carriers. Too much growth within the carrier can cause breakage of the carrier itself, although this does not seem to be a real problem when using filamentous fungi because their growth takes place mostly on or immediately below the carrier surface (Baklashova *et al.* 1984; Eikmeyer *et al.* 1984; Kopp & Rehm 1984; Horitsu *et al.* 1985; Petruccioli *et al.* 1987; Federici *et al.* 1991).

Selection of Carrier and Immobilization Method

The selection of a suitable carrier and immobilization method is of great importance if high performance is to be achieved in immobilized cell systems. It is clear that a direct relationship exists between the efficiency of a reaction and the physico-chemical features of a carrier. However, it is still difficult to find the optimal carrier for a given reaction except by trial and error (Tanaka & Nakajima 1990).

Almost all the methods employed for the immobilization of enzymes can be utilized for the immobilization of whole cells, although, generally, more care must be taken with the cells to prevent inactivation of the required metabolic activity. Of the various methods currently available, adsorption and entrapment appear to be the most extensively used techniques for filamentous fungi (Anderson 1983; Powell 1990; Tanaka & Nakajima 1990). However, the mildest method of immobilizing fungal mycelia is probably by natural adhesion and subsequent surface growth, as occurs in rotating disc fermenters (Anderson & Blain 1980; Anderson *et al.* 1981; Ju & Wang 1984, 1986).

Application of Immobilized Fungal Cell Systems

Most studies on cell immobilization have used bacteria and yeasts. However, use of filamentous fungi has recently increased because these fungi can produce (or transform) many compounds of commercial interest, including organic acids, enzymes, antibiotics and steroids.

Organic Acids

Organic acids, including citric, itaconic and lactic acids, are largely used in the food, chemical and pharmaceutical industries. They can be synthesized from carbohydrates by immobilized, growing fungal cells (Table 1).

Production of citric acid from sucrose by growing cells of *Aspergillus niger* immobilized on various carriers has been extensively studied (Vajia *et al.* 1982; Eikmeyer *et al.* 1984; Horitsu *et al.* 1985; Tsay & To 1987; Lee *et al.* 1989; Vassilev & Vassileva 1990; Roukas 1991). Vajia *et al.* (1982) used an air-lift completely-stirred tank reactor to study the

Table 1. Production of organic acids by immobilized growing fungal cells.

Organic acid	Organism	Support material	Reference
Citric acid	*Aspergillus niger*	Ca-alginate	Vajia *et al.* (1982); Tsay & To (1987); Roukas (1991)
		κ-Carrageenan	Eikmeyer *et al.* (1984)
		Polyacrylamide	Horitsu *et al.* (1985)
		Polyurethane	Lee *et al.* (1989); Vassilev & Vassileva (1990)
Itaconic acid	*Aspergillus terreus*	Polyacrylamide	Horitsu *et al.* (1983)
		Ca-alginate	Kautola *et al.* (1985)
		Polyurethane	Kautola *et al.* (1989); Vassilev *et al.* (1992)
Lactic acid	*Rhizopus oryzae*	Ca-alginate	Hang *et al.* (1989)
Gluconic acid	*Aspergillus niger*	Glass	Heinrich & Rehm (1982)
Fumaric acid	*Rhizopus arrhizus*	Polyurethane	Kautola & Linko (1989);
		Ca-alginate	Petruccioli *et al.* (1992)

continuous production of citric acid by Ca-alginate entrapped cells of *A. niger* and obtained a maximum production rate of 70 mg/g mycelium.h, with an overall efficiency of 40%. Greater efficiencies (45 to 48%) were obtained by Roukas (1991) with the same organism but in a repeated-batch process. It is interesting that, according to Roukas (1991), a shake-culture would be a better fermentation system than a bioreactor for citric acid production. Very high production rates (about 96 mg/h per 80 g gels) were obtained by Horitsu *et al.* (1985) after 8 days of continuous cultivation of polyacrylamide-entrapped growing cells of *A. niger* in a two-stage bioreactor; the half-life of the immobilized cell system was approximately 96 days.

Itaconic acid can be easily synthesized using immobilized cells of *Aspergillus terreus*. Kautola *et al.* (1985, 1989, 1990, 1991) studied the production of this acid by *A. terreus* immobilized on various supports, including Ca-alginate, agar, polyurethane foam, nylon and celite, with xylose and sucrose as carbon sources. The highest productivity (0.33 g itaconic acid/day.g of carrier) was obtained on glucose in a packed-bed bioreactor which was continuously operated for 4.5 months (Vassilev *et al.* 1992). The remarkable stability of the biocatalytic activity of this immobilized fungal cell system indicates considerable potential for transfer to commercial production.

Production of Enzymes

The large majority of enzymes currently utilized in food and other technological processes are produced aerobically by fermentation of free cells in submerged cultures. However, the production of some, mainly carbohydrate-hydrolysing and proteolytic enzymes, has been studied using immobilized growing fungal cells (Table 2).

Federici *et al.* (1987, 1990) and Gallo Federici *et al.* (1990) investigated the production of glucoamylase by Ca-alginate immobilized cells of *Aureobasidium pullulans* in shake-culture in repeated-batch processes and in a fluidized-bed reactor operated either semi-continuously or continuously. The volumetric productivity increased from 6.2 U/l.h for the free cells to 21.3, 53.3 and 71.0 U/l.h for the immobilized cells in shake-culture and in the fluidized-bed reactor operated semi-continuously and continuously, respectively.

High levels of glucoamylase and α-amylase activity were obtained, in shake-culture in repeated-batch processes, by Li *et al.* (1984) and Linko *et al.* (1988) with Ca-alginate immobilized cells of *A. niger*.

Of great interest are the results of studies on the production of cellulase by growing cells of *Trichoderma reesei* immobilized on various supports (Frein *et al.* 1982; Taniguki *et al.* 1983; Kumakura *et al.* 1984a,b). The industrial conversion of cellulose into mono- and oligo-saccharides and, eventually, into microbial biomass, through enzymatic hydrolysis, is mainly limited by economic problems, particularly the cost of cellulase. This cost could be reduced by the use of immobilized cellulolytic cell systems. Frein *et al.* (1982), for instance, obtained relatively high volumetric productivities (26 U filter paper activity/l.h, 600 U carboxy-methylcellulase/l.h and 9 U β-glucosidase/l.h) in a continuously-operated column bioreactor with growing cells of *T. reesei* immobilized in a 4% κ-carrageenan gel.

In spite of their considerable commercial interest, relatively little work has been carried out on the use of immobilized fungal cell systems for the production of acid and alkaline proteases. Aleksieva *et al.* (1991) recently immobilized the mycelium of *Humicola lutea* in poly-2-hydroxyethyl-methacrylate for the production of acid proteinases in repeated-batch processes and obtained a volumetric productivity of 70 U proteinase/l.h.

Production of Antibiotics

Presently, the production of antibiotics by fermentation is almost exclusively based on batch processes in which the cells are induced to produce the desired antibiotic after

Table 2. Production of enzymes by immobilized growing fungal cells.

Enzyme	Organism	Support material	Reference
Glucoamylase	*Aureobasidium pullulans*	Ca-alginate	Federici *et al.* (1987, 1990); Gallo Federici *et al.* (1990)
	Aspergillus phoenicus	Ca-alginate	Kuek (1991)
Glucoamylase, α-amylase	*Aspergillus niger*	Ca-alginate	Li *et al.* (1984); Linko *et al.* (1988)
Cellulase	*Penicillium funiculosum*	Polyurethane	Linko *et al.* (1988)
	Talaromyces emersonii	Ca-alginate	McHale (1988)
	Trichoderma reesei	κ-Carrageenan	Frein *et al.* (1982)
		Poly (2-hydroxyethylmethacrylate)	Kumakara *et al.* (1984a, b)
		Nylon	Taniguki *et al.* (1983)
Acid protease	*Humicola lutea*	Poly (2-hydroxyethylmethacrylate)	Aleksieva *et al.* (1991)
Alkaline protease	*Conidiobolus* sp.	Various	Sutar *et al.* (1986)
Pectinase	*Aspergillus awamori*	Not specified	Blieva & Belkhodjaeva (1990)
Lignin peroxidase	*Phanerochaete chrysosporium*	Nylon	Linko (1988); Linko *et al.* (1988)
		Glass (Rashing)	Jäger & Wandrey (1990)

Table 3. Production of antibiotics by immobilized growing fungal cells.

Antibiotic	Organism	Support material	Reference
Penicillin G	*Penicillium chrysogenum*	κ-Carrageenan	Deo *et al.* (1983, 1984); Jones *et al.* (1986)
		Ca-alginate	El-Sayed & Rehm (1987)
		Celite	Keshavarz *et al.* (1990); Lilly *et al.* (1990)
^{14}C-Penicillin G	*Penicillium chrysogenum*	Ca-alginate	Kurzatkowski *et al.* (1984)
Patulin	*Penicillium urticae*	κ-Carrageenan	Jones *et al.* (1983); Deo & Gaucher (1985)
Cyclosporin A	*Tolypocladium inflatum*	κ-Carrageenan	Foster *et al.* (1983)
6-APA	*Pleurotus ostreatus*	Chitosan	Kluge *et al.* (1982)

exponential growth has stopped. Continuous production processes are very difficult when organisms forming mycelia, such as actinomycetes and filamentous fungi, are employed. Several studies, however, have shown the feasibility of continuous production processes by using immobilized living cells that guarantee higher cell densities within the bioreactors and an easier control of the process operations (Deo *et al.* 1983; Foster *et al.* 1983; Deo & Gaucher 1985; Jones *et al.* 1986; El-Sayed & Rehm 1987; Lilly *et al.* 1990) (Table 3).

Immobilized mycelia of *Penicillium chrysogenum* have been used for the production of penicillin G (Deo *et al.* 1983; Kurzatkowski *et al.* 1984; Jones *et al.* 1986; El-Sayed & Rehm 1987; Lilly *et al.* 1990; Keshavarz *et al.* 1990). Lilly *et al.* (1990) and Keshavarz *et al.* (1990), in particular, have shown the feasibility of continuous antibiotic production with low cell growth using a temperature-sensitive mutant of the fungus in a 320 l (working volume) air-lift tower reactor in which the fermentation was carried out for >500 h. Immobilization on celite effectively avoided wash-out of poorly-growing cells from the reactor.

The production of cyclosporin A by carrageenan-entrapped *Tolypocladium inflatum* in an air-lift reactor with an external circulating loop was reported by Foster *et al.* (1983). The antibiotic, recovered by a single ethyl acetate extraction, was essentially free of contaminating media and other microbial by-products.

Finally, 6-aminopenicillanic acid was produced by a specific enzymatic cleavage reaction from penicillin-V using chitosan-immobilized cells of *Pleurotus ostreatus* (Kluge *et al.* 1982). Compared with free cells, the immobilization led to a 10-fold increase in the half-life of the biocatalyst.

Biotransformation of Steroids

Use of immobilized-cell technology for the biotransformation of steroids has attracted considerable attention (Mahato & Mukherejee 1984; Tanaka & Nakajima 1990). Several immobilized fungal cell systems have been studied for hydroxylation processes, which involve complex reactions characterized by activation of molecular oxygen and a continuous supply of reducing power (Table 4). A good example of their application is the conversion of the Reichstein compound S (cortexolone) to prednisolone via cortisol by the immobilized fungus *Curvularia lunata* employed in conjunction with an immobilized bacterium, *Corynebacterium simplex* (Mosbach & Larsson 1970). The process, which was further improved by immobilizing spores of *C. lunata* in preference to vegetative mycelium (Ohlson *et al.* 1980), appears to be the first genuine immobilized

Table 4. Transformation of steroids by immobilized fungal cells.

Substrate	Reaction	Organism	Support material	Reference
Cortexolone	11β-Hydroxylation	*Curvularia lunata*	Ca-alginate	Ohlson *et al.* (1980); Sukhodoloskaya *et al.* (1990); Ghanem *et al.* (1992)
			Photo-crosslinked resin	Sonomoto *et al.* (1981, 1983)
Progesterone	11α-Hydroxylation	*Rhizopus nigricans*	Agar	Maddox *et al.* (1981)
		Rhizopus stolonifer	Photo-crosslinked resin	Sonomoto *et al.* (1982)
		Aspergillus ochraceus	Polyacrylamide	Bihari *et al.* (1984)
			Celite	Broad *et al.* (1984)
		Aspergillus phoenicus	κ-Carrageenan, Ca-alginate, albumin	Kim *et al.* (1982)
	15α-Hydroxylation	*Aspergillus phoenicus*	κ-Carrageenan, Ca-alginate, albumin	Kim *et al.* (1982)
Androstane	Dehydrogenation	*Rhizopus* sp.	Various	Fukui *et al.* (1980)
		Aspergillus sp.	Various	Fukui *et al.* (1980)

Table 5. Production of alkaloids, gibberellic acid and methyl-ketone.

Product	Organism	Support material	Reference
Clavine alkaloids	*Clàviceps purpurea*	Ca-alginate	Kopp & Rehm (1984); Kopp (1987)
		Ca-alginate, pectate, κ-carrageenan	Kren (1990)
	Claviceps fusiformis	Ca-alginate, pectate, κ-carrageenan	Kren (1990)
Ergot alkaloids	*Claviceps purpurea*	Ca-alginate	Kopp & Rehm (1984); Lohmeyer *et al.* (1990)
		Ca-alginate, pectate, κ-carrageenan	Kren (1990)
	Claviceps purpurea (protoplasts)	Ca-alginate	Komel *et al.* (1985)
Lysergic acid derivatives	*Claviceps paspali*	Ca-alginate	Pertot *et al.* (1988)
		Ca-alginate, pectate, κ-carrageenan	Kren (1990)
Roquefortine	*Penicillium roqueforti*	Ca-alginate	Kusch & Rehm (1986)
Gibberellic acid	*Gibberella fujikuroi*	κ-Carrageenan	Jones & Pharis (1987)
		Ca-alginate, agar, κ-carrageenan	Kumar & Lonsane (1988)
		Ca-alginate	Nava Saucedo *et al.* (1989)
	Fusarium moniliforme	Ca-alginate	Kahlon & Malhotra (1986)
Methyl-ketone	*Penicillium roqueforti*	Ca-alginate	Larroche *et al.* (1989)

fungal cell system developed to an industrial, though limited, scale (Cheetham 1980).

Production of Other Compounds

Ergot alkaloids are fungal secondary metabolites of considerable pharmaceutical interest due to their broad spectrum of therapeutic applications. Since their chemical synthesis is too costly, industrial production is mainly based on the saprophytic culturing of *Claviceps* mycelia. However, cultivation in large fermenters with conventional batch processes presents many difficulties as the organisms are sensitive to mechanical stress and have a strong tendency to degenerate (Kopp 1987). Immobilization of the fungal mycelium can overcome these problems and stabilize alkaloid production (Table 5).

Kopp & Rehm (1984) and Kopp (1987) reported semi-continuous production of alkaloids by Ca-alginate immobilized mycelium of *Claviceps purpurea* which retained high catalytic activity for over 200 days (16 cycles). In contrast, free cells lost their production capacity after only 60 days of cultivation. Limitation of phosphate was extremely important to achieve long fermentations and alkaloid production (Lohmeyer *et al.* 1990). Also, the morphological development of the immobilized fungus appears to be of great importance. According to Pertot *et al.* (1988), the immobilization of *Claviceps paspali* mycelium in Ca-alginate would shift the fungal metabolic activities towards secondary metabolism because of the physically restricted growth and morphological differentiation of the mycelium into arthrosporoid-like cells.

Gibberellic acid, a potent plant growth promoter, is currently produced in submerged culture using free mycelium of *Gibberella fujikuroi* (Pitel *et al.* 1971). In recent times, however, a number of studies have been reported on the use of immobilized growing cells of *G. fujikuroi* (Jones & Pharis 1987; Kumar & Lonsane 1988; Nava Saucedo *et al.* 1989) and *Fusarium moniliforme* (Khalon & Malhotra 1986) for batch- and semi-continuous production of gibberellic acid.

Wastewater Treatment

The well-known film-forming properties of fungi have been utilized to develop fixed-bed reactors in which mycelial films develop naturally by adhesion and subsequent surface growth. The rotating disc reactor seems to provide an exceptionally mild method of immobilizing mycelia (Anderson & Blain 1980) and a reactor of this type was employed in the productive processing of effluents containing low amounts of carbohydrates (Anderson *et al.* 1981).

Numerous studies have also been carried out on the continuous biotreatment of phenolic wastes with immobilized fungal cell systems. Takahashi *et al.* (1981) employed the yeast-like fungus *Aureobasidium pullulans* adhered to fibrous asbestos in a glass-column reactor and Anselmo *et al.* (1985) and Anselmo & Novais (1992) used *Fusarium flocciferum* immobilized on polyurethane foam.

Another application of potential interest is the removal of colour from kraft-mill effluents using *Coriolus versicolor* mycelia immobilized in beads of Ca-alginate; in the presence of glucose, such treatment results in 80% decolorization within 3 days (Livernoche *et al.* 1983).

Conclusions

The number of studies carried out on the immobilization of filamentous fungi in the last 10 to 15 years is undoubtedly remarkable. As a consequence, considerable experimental know-how is now available which should really contribute

to innovative industrial fermentation processes based on fungi. However, in spite of the many potential advantages of immobilized fungal cell systems, the industrial world still shows a certain reluctance towards their utilization. The need for expensive plant reconversions, the requirement of new and more complex process controls and problems of sterility maintenance (Kristiansen & Bu'Lock 1980; Stafford 1986) appear to be the major difficulties preventing the transfer of immobilization technology to a commercial scale. Further and more thorough studies of process scale-up, as well as the development of other and more adequate immobilization methods, should overcome most of these problems. The results obtained by Ju & Wang (1986), in the production of itaconic acid by *A. terreus* immobilized in a porous disc reactor, and Lilly *et al.* (1990), in the production of penicillin G by *P. chrysogenum* cells adsorbed to celite, appear particularly promising. However, only economic criteria will ultimately dictate whether or not immobilized fungal cell systems become commercial (Nava Saucedo *et al.* 1989).

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(Received in revised form 26 April 1993; accepted 29 April 1993)