Chapter 14
Encapsulates for Food Bioconversions and Metabolite Production

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14.1 Introduction

The control of production costs in the food industry must be very strict as a result of the relatively low added value of food products. Since a wide variety of enzymes and/or cells are employed in the food industry for starch processing, cheese making, food preservation, lipid hydrolysis and other applications, immobilization of the cells and/or enzymes has been recognized as an attractive approach to improving food processes while minimizing costs. This is due to the fact that biocatalyst immobilization allows for easier separation/purification of the product and reutilization of the biocatalyst. The advantages of the use of immobilized systems are many, and they have a special relevance in the area of food technology, especially because industrial processes using immobilized biosystems are usually characterized by lower capital/energy costs and better logistics. The main applications of immobilization, related to the major processes of food bioconversions and metabolite production, will be described and discussed in this chapter.

Immobilization of cells permits higher cell densities to be attained in bioreactors, and thus results in higher enzyme productivity (Krisch and Szajani 1996). The immobilization of either cells or enzyme to achieve the enzymatic process makes re-utilization and continuous operation possible, and also precludes the need to separate the active biocatalyst from the surrounding medium following the process. Encapsulation of whole cells instead of pure enzyme often leads to better enzyme stability as well as cofactor regeneration. These advantages can drastically reduce

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production costs, thereby enabling large-scale application of cells. Enzymes, and cells producing the enzymes, have been immobilized by various methods, such as gel entrapment, physical absorption and covalent attachment on various supports (Katchalski-Katzir 1993).

The selection of the immobilization method and support depends on the biocatalyst (enzyme or microorganism) and on the bioprocess itself (aerobic or anaerobic, production of small soluble metabolites or macromolecules). Hydrogels, such as calcium-alginate, κ-carrageenan, calcium-pectate, sepharose, dextrans and others are frequently used for cell immobilization by entrapment (Santoyo et al. 1996). Their interaction with the biomass and possible effects on the cell morphology and metabolism are relatively well known and diffusion coefficients for small organic molecules in hydrogels are relatively high. Immobilization of whole cells can protect them, and the associated enzyme activities, from shear forces resulting in higher viability and bioconversion rates, particularly for continuous and long-term applications. Although biomass is efficiently retained with the entrapment technique, the immobilization of proteins (enzymes) in hydrogels is often difficult due to leaching (exodiffusion) resulting from the relatively small spatial molecular volume when compared with the microparticle porosity. Hydrogels, and the production of beads under aseptic conditions, are costly, and may limit their use on an industrial scale. A major problem in using hydrogel beads is their chemical and mechanical stability in culture media over prolonged operational times. Cell growth can clog the pores of hydrogels, or cause rupture of the material. Stability of the beads may be increased by cross-linking, for example, with glutaraldehyde or complexation with poly-L-lysine (Leng et al. 2006). This, however, introduces additional mass transfer resistance, and may also introduce cytotoxicity issues. Entrapped cells are normally grown in packed-bed, fluidized-bed, gas-lift, or stirred tank reactors (Bodalo-Santoyo et al. 1999; Hernandez et al. 2001; Talabardon et al. 2000).

Surface immobilization by adsorption has the advantage of being very simple to carry out. In order to increase cell loads and make them comparable to entrapment techniques, highly porous materials, such as pretreated cellulose, are frequently used. (Tisnadjaja et al. 1996). A disadvantage of this method is the relatively low interaction between the support and the biocatalyst, resulting in biomaterial release to the surroundings. However, when using adsorbed cells, there is no additional mass transfer barrier as in the case of the entrapped or encapsulated cells. A serious problem in using cells immobilized by adsorption in submerged cultures is the mechanical damage to the cells caused by the abrasion between the carrier particles. This may be avoided by the use of solid state fermentations in which the cells are immobilized on a solid carrier.

Covalent binding, between a functional group of the biocatalyst and the support, is a technique used for immobilization of enzymes (Bodalo-Santoyo et al. 1999). Many different types of support are used, including cotton cloth, chitosan, alginate, silica hydrogel and agarose to name but a few. The release of enzyme to the surroundings is thus drastically reduced compared to physical adsorption techniques, which extends the duration of the process and also reduces the cost since the loss of biocatalyst is reduced.

14.2 Reactor Design for Immobilized Biocatalysts

Immobilized biocatalysts are generally quite stable, they are indeed frequently more stable than non-immobilized forms. This observation has enabled the design and construction of reactors that can be adapted to immobilized cells/enzyme (Fig. 14.1).

Current reactors for submerged immobilized biocatalysts include stirred-tank, packed-bed, fluidized-bed, gas-lift and stirred-tank reactors with a recycling device

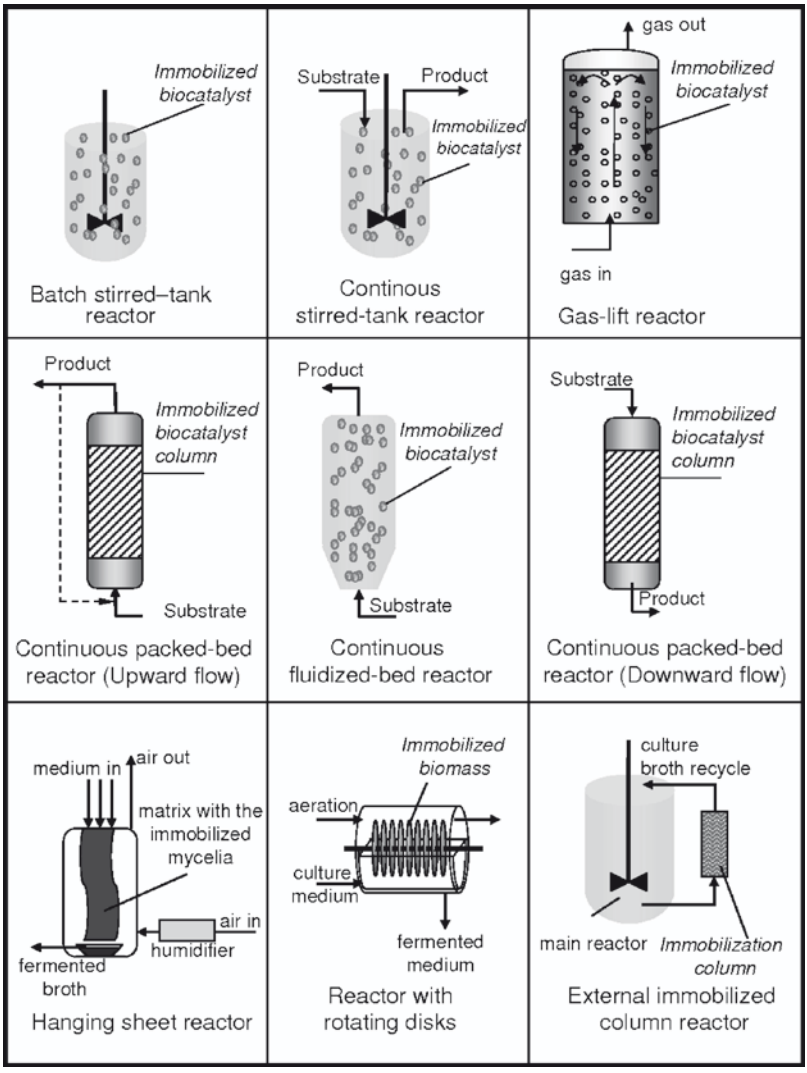

Fig. 14.1 Bioreactor design used with immobilized biocatalyst

operating in batch, fed-batch continuous and perfusion (continuous with cell recycle such as by the incorporation of a microfiltration/ultrafiltration membrane) modes (Katchalski-Katzir 1993). Common reactors for solid state fermentation include packed-bed reactors, hanging sheet reactors, rotating disk and external recirculation column reactors.

Stirred tank reactors are commonly used for processes with cells entrapped in hydrogels or adsorbed on the surface of solid porous beads for both aerobic and anaerobic processes. They provide good mixing conditions but are limited by the rheological properties of the immobilization support suspension in the culture medium, as well as by relatively high shear stress and abrasion that may damage the immobilization support and the cells growing on their surface.

Packed-bed reactors consist of columns filled with the fixed layer of biocatalyst immobilized by entrapment or by adsorption. These reactors provide high cell densities and high volumetric productivities. The shear stresses are low, which makes the culture of filamentous fungi possible. However, a major problem with packed-bed columns is due to mass and heat transfer limitations, which may reduce the performance of the reactor significantly. Oxygen supply may also be difficult in packed-bed reactors. The use of feed that contains insoluble matter, as well as cell growth, may lead to clogging of the reactor. Reactor maintenance between operation cycles is complicated.

Fluidized-bed reactors are a modification of packed-bed reactors, in which the energy needed for mixing is provided by the flow of medium introduced at the base of the reactor. Through this way, shear stresses are significantly reduced compared to mechanically stirred reactors. Fluidized-bed operation is normally used for hydrogel-entrapped cell cultures, although the abrasion of cells on the surface of the solid beads may pose a problem. However, as the density of hydrogels is only slightly higher than that of water, the maximum applicable velocity is low, which in turn limits the efficiency of mixing and mass transfer.

An improvement to fluidized-bed reactors can be achieved using gas-lift operation to achieve microparticle fluidization. Such reactors are normally cylindrical with an aspect ratio of between 6 and 12, and may incorporate an inner draft tube. A gas (air or oxygen in the case of aerobic cultures) is introduced at the bottom of the reactor within the draft tube. The resulting reduction in medium density, combined with the gas velocity, results in the medium rising, and entrains the movement of the microparticles. De-gassing at the liquid/headspace interface results in an increase in fluid density and gravitation-induced downward flow. This results in better mixing conditions than in the equivalent fluidized-bed systems, although scale-up of gas-lift reactors may be complex.

Hanging-sheet reactors consist of a sheet of fabric with immobilized cells hanging in a vertical chamber. The substrate is introduced at the top of the reactor and moves down through the action of gravity. This set-up provides a good oxygen supply and low shear stress (little mechanical damage to the cells), and is therefore very useful for aerobic bioprocesses with filamentous fungi. The medium viscosity is very much improved compared to submerged fungal cultures since the cells are mostly retained in the solid phase. High product concentrations can be achieved. However, this reactor design is susceptible to high concentration and temperature gradients.

Local temperature gradients caused by the heat transfer limitations and the heat released by cell metabolism may cause the drying of mycelium, even when water-saturated air is supplied. Together, these problems may result in a significant reduction in volumetric productivity.

Rotating disk reactors are an improvement of the hanging sheet systems. The reactor is normally a horizontal cylinder with disks (or drums) attached to a central rotating axis. The reactor is partially filled with nutritive medium, so that the mycelium immobilized on the rotating element is periodically submerged in the medium and exposed to the air (or oxygen).

External immobilized column reactors with re-circulation consist of two connecting vessels of different dimensions filled with culture medium which is continuously re-circulated through the system. The smaller vessel is filled with the immobilization support, which is generally a cellulose fiber material. The operation begins with a batch culture of cells in the larger vessel, until the desired biomass concentration is reached. At this point, the recirculation pump is turned on and the broth is forced through the immobilization column, where cells are retained by adsorption. This design allows for long-term operation, since the immobilized biomass is constantly renewed by the free cells growing in the larger vessel. Immobilization is straightforward and the reactor can be operated in batch or continuous mode. As a result of continuous flow of culture broth through the immobilization column, clogging may be a problem for long-term operation.

These relatively new types of reactor have the following advantages over other methods (Katchalski-Katzir 1993): (1) they allow high biocatalyst loading; (2) they prolong enzyme activity or cell life; (3) they have the ability to be used in recycle mode; (4) they can be used with high flow rates; (5) they operate with reduced capital and operating costs; (6) they have reduced energy requirements; (7) they produce less waste products; (8) they generally have high yields; (9) they are readily scalable.

Furthermore, automatization of the process leads to considerable reduction of operating costs, mainly through reduced enzyme levels and labor costs resulting from the higher production yields achieved.

The choice of reactor is generally defined by the process under consideration. Thus, batch processes might involve direct enzyme addition to the substrate (e.g., whole milk), since they will not be recycled, whereas continuous processes (e.g., glucose production) will require microencapsulates which are physically robust, from which the enzyme does not leach over the duration of use, in which the enzyme is stable under the conditions of pH, temperature and ionic strength employed and which can be operated at the required scale. Pre-requisites are that the enzyme be adsorbed or immobilized throughout the microencapsulate matrix, that the loading density is as high as possible, that the porosity of the microencapsulates allows both substrates and products to diffuse readily through them to avoid undue mass transfer limitations and product inhibition. Thus, the choice and scale of reactor will depend on (1) how much enzyme is required to carry out the process in the desired time (process kinetics); (2) the mechanical stability of the microencapsulates, since there are significant shear forces associated with stirred tank reactors and certain types of fluidized-beds. For packed-bed applications the flow rate,

and thus kinetics, will be dictated by the pressure drop across the packed-bed, consequently elastic hydrogel matrices are less suited to these types of reactors; (3) the density of the microencapsulates, since hydrogels have a specific density only slightly higher than that of the reaction medium in most cases, therefore would be unsuitable for use in fluidized-beds unless ceramic or inert inorganic particles are co-immobilized to achieve a density which avoids wash-out; (4) whether the process is batch, fed-batch or continuous, with or without enzyme recycle; (5) whether purified enzymes or whole cells are used. In general, purified enzymes would be used for continuous processes to avoid excessive cost associated with enzyme consumption; (6) the productivity required. Generally continuous processes provide much higher rates of substrate conversion than batch or fed-batch processes; however, this must be counter-balanced by the requirement for sufficient enzyme concentration and residence time in the reactor to allow complete conversion, while avoiding product accumulation to inhibitory levels.

14.3 Sugar Conversions

In everyday terminology, sugar refers to sucrose, also referred to as "table sugar", "saccharose" or "dextrose", and is mainly used to alter the flavor (sweetening) and properties (preservation, texture) of food and beverages. Commercially produced table sugar is derived from sugarcane or sugar beet. More specifically, the term sugar refers to carbohydrates which may be mono-, di- or poly-saccharides.

Monosaccharides are the simplest carbohydrates, with the general chemical structure $(CHO)_n$. They consist of a single sugar moiety, are generally colorless, water soluble and crystalline solids, some of them having a sweet taste. Monosaccharides are classified by the number of carbon atoms they contain, the major families being pentoses and hexoses. In the food industry, the most common monosaccharides are glucose, fructose and galactose, which are hexoses. Monosaccharides are the building blocks of disaccharides such as sucrose (common sugar) or lactose (milk sugar) and polysaccharides (such as cellulose and starch).

14.3.1 Glucose Production

Glucose, in crystalline form or in syrups, is largely required in the food industry to sweeten beverages (Wang et al. 2007) or to adjust the physico-chemical properties of food products, especially for confectionary. Commercially, glucose is generally produced through enzymatic hydrolysis of starch. Many crops are used as a source of starch, such as maize, rice, wheat and potato. The first step for starch hydrolysis is a heat treatment (1–2 h at 100°C), in the presence of amylase to allow starch solubilization and to lead to the formation of oligosaccharides containing 5–10 glucose units. After heating, a second enzyme is added to hydrolyze the oligosaccharides

into glucose units. This second step is termed "saccharification" (Rebroš et al. 2006), and the most commonly used enzyme is glucoamylase.

Another possibility to produce glucose is through the enzymatic hydrolysis of sucrose to glucose and fructose through the action of invertase, a relatively expensive enzyme compared to amylases and glucoamylases (Altinok et al. 2006). Immobilization of amylase, glucoamylase and invertase enzymes has been shown to be essential for the economic production of glucose, particularly glucose syrups, since (1) the enzyme may be re-used a number of times or used continuously; (2) it is relatively easy to separate the product from the reaction medium; (3) it is relatively easy to recover the enzyme; and (4) there is generally an improvement in enzyme stability (Rebroš et al. 2006; Wang et al. 2007).

Industrial processes using such immobilized biosystems are generally characterized by lower capital and energy costs (Rebroš et al. 2006). Due to the low added value of glucose for use in the food industry, the minimization of costs is essential (Karaa et al. 2005). In the case of glucose production, the enzymes may be immobilized to increase process efficiency. Moreover, immobilization has been reported to lead to changes in the pH and temperature profiles of the enzymes and to enhance pH and thermo-stability (Brijak 2003). The methods generally used for immobilization of the enzymes, including adsorption, entrapment, chemical cross-linking and covalent attachment, are non-exhaustively listed in the Table 14.1.

Table 14.1 Enzyme immobilization techniques for the production of glucose

Technique	Principle	References
Adsorption	Montmorillonite clay support	Sanjay and Sugunan (2007)
	Zirconia support	Reshmi et al. (2007)
	Alumina support	Reshmi et al. (2006)
Entrapment	Calcium-alginate beads	Ertan et al. (2007) Konsoula and Liakopoulou-Kyriakides (2006) Dey et al. (2003)
	Chitosan-alginate	Sankalia et al. (2007)
	Acrylated epoxidized soybean resin	Kahraman et al. (2006)
	Polyacrylamaide gels	Raviyan et al. (2003)
	Agar gels	Stefanova et al. (1998)
Cross-linking	Cross-linking with glutaraldehyde	Hamerska-Dudra et al. (2007)
	Chemically activated poly (2-hydroxyethyl methacrylate) beads	Altinok et al. (2006)
	Bonding between enzyme ester groups and *N*-(acryloxy)-succinimide	Chen et al. (1998)
	Amide bonds between amino groups of protein and phtaloyl chloride functionalized glass beads	Kahraman et al. (2007)
	Condensation of the epoxy groups of glycidyl methacrylate with amino groups of enzyme	Danisman et al. (2004)

Adsorption is a relatively easy and fast process; however, it often results in significant enzyme leaching and loss. This may be overcome by covalently linking the enzyme and support. However, care must be taken to achieve high enzyme loading without significant loss of activity.

The main problem with enzyme entrapment in a matrix is due to diffusional limitations, mainly diffusion of substrate to the enzyme, or of the product from the matrix to the surrounding. This results from the hydrolytic enzymes being subject to feedback (competitive) inhibition by the products (glucose) of the reaction. Thus, while entrapment allows for protection of the enzymes, allowing constant activity over long periods of time, it is essential to use small microparticle sizes with excellent fluid dynamics within the reactor to achieve high conversion rates.

Thus, the nature of the polymer matrix is generally less important than the requirement for small, monodisperse microencapsulates which are mechanically robust, such that they can be employed in a wide range of reactors such as stirred-tank, packed- and fluidized-beds at high liquid velocities. The matrix must provide the chemically defined support necessary to adsorb or immobilize the enzyme to achieve the desired loading density (specific activity, units/gram or volume of microencapsulate), while providing a high porosity and low mass transfer resistance (high rates of diffusion of substrates and products). Adsorption onto inorganic support matrices (Table 14.1) generally provides lower enzyme loadings than other techniques, due to the limited surface available, but has the advantage of having a high specific density which allows them to be used in a wide range of reactors due to their physical stability (Sanjay and Sugunan 2007; Reshmi et al. 2007). Enzyme desorption can be a problem when used for continuous processes, and the presence of enzyme on the surface of the particles makes them susceptible to shear forces. Entrapment can have the advantage of physically retaining the enzyme within a defined porosity matrix; however, it can be difficult to produce microencapsulates with high enzyme loadings due to the exodiffusion of the enzyme from encapsulates during the production process (Ertan et al. 2007). For example, when using alginate, the enzyme would be mixed with the sodium alginate solution and extruded into a calcium chloride bath. The rate of calcium complexation, and therefore gelation, of the alginate is rapid but even so >50% of the enzyme added will exodiffuse and not be retained within the calcium alginate microencapsulates. Another difficulty is related to the mechanical properties since hydrogels deform and change properties and porosity as a function of pH and ionic strength, resulting in abrasion and increased porosity, and leading to enzyme loss.

Chemical cross-linking to microencapsulates is best suited for long-term mechanical strength, such as required for continuous processes, with high enzyme loading (Hamerska-Dudra et al. 2007; Chen et al. 1998). These have the lowest loss of enzyme through exodiffusion, providing that a suitable immobilization chemistry can be found which minimizes activity loss, through enzyme denaturation.

14.3.2 Fructose Production

Fructose, an isomer of glucose, has a sweet taste similar to cane sugar, but with a "fruity aroma". It is naturally found in many foods, such as fruits, honey, some vegetables (beets, sweet potatoes, onions, etc.). Fructose is the sweetest naturally occurring sugar, and is estimated to be twice as sweet as glucose, and 50% more than sucrose. This sugar has the property to enhance flavor, color and product stability, and is thus widely used instead of sucrose for the sweetening of foods and beverages. Moreover, fructose has a Generally Recognized As Safe (GRAS) status, and is thus a safe alternative sweetener, which is of great advantage to the food industry (Singh et al. 2007), particularly due to its beneficial role for diabetics and its low cariogenic nature. As a result the demand is ever increasing, and efficient production requires development.

There are two principle processes for fructose production. The first involves the enzymatic hydrolysis of sucrose to glucose and fructose through the action of invertase, followed by the isomerization of glucose into fructose using glucose isomerase (Singh et al. 2007). The latter is the final step in the production of High Fructose Syrups, which is traditionally performed in packed-bed reactors using immobilized glucose isomerase (Brijak 2003; Salehi et al. 2005). Once again the main advantages of using immobilized glucose amylase are that they allow reuse of the enzyme and continuous operation and while avoiding enzyme contamination of the processed product. Moreover, the pH and temperature dependence of immobilized glucose isomerase is lowered, leading to more stable and controlled processes.

The second common way to produce fructose is through the degradation of inulin, a reserve carbohydrate found in the roots and tubers of many plants. Inulin is a polyfructan consisting of linear chains of β-2,1-linked D-fructofuranose terminating at the reducing end with a glucose residue. The enzymatic degradation of inulin molecules by inulinase allows the production of syrups containing more than 75% D-fructose (Nakamura et al. 1995). To allow the continuous production of fructose from inulin, the use of inulinase in an immobilized form in packed-bed column reactors is used. As with processes using amylases and glucoamylases, the immobilization methods are numerous and based on similar techniques, such as adsorption on a macroporous support (Gupta et al. 1992), cross-linking or covalent bond formation between the support and the enzyme (Brijak 2003; Nakamura et al. 1995; Singh et al. 2007), or entrapment in a polymer matrix (Gill et al. 2006).

14.3.3 Lactose Production

The main carbohydrate present in milk is lactose, a disaccharide composed of one galactose and one glucose unit. Lactose is poorly soluble, and not very sweet, and is also not easily digested by a significant fraction of the population. Lactose

intolerance is caused by low levels of β-D-galactosidase (commonly named lactase) in the digestive system, and the consequent inability to digest this disaccharide.

Lactase is used to hydrolyze the lactose present in milk, in order to obtain glucose and galactose, which may find many applications in the food industry. Lactose is known to readily crystallize, particularly at concentrations exceeding 20–25%, which limits certain processes in the dairy industry. As a consequence, the treatment of milk (or milk products) with this enzyme reduces the lactose content, thereby avoiding the solubility and/or crystallization problems, while simultaneously increasing the sweetness. Moreover, it is known that cheese ripening is accelerated using hydrolyzed milk (Panesar et al. 2006). Finally, treatment of milk with lactase enables consumption of milk products by lactose-intolerant people. Lactose in milk and cheese whey may also be hydrolyzed using the same enzyme, thus converting whey into a sweet syrup which can then be used in the confectionary, dairy, baking and soft drink industries, in a similar way to glucose and fructose syrups obtained from sucrose or starch. Hydrolysis of whey lactose has the added advantage of converting a low-grade waste product from the dairy industry, having a BOD in the range 30,000–50,000 mg/L to a higher added value product. More recently, the enzymatic hydrolysis of whey lactose has also been proposed for the production of bioethanol.

β-D-galactosidase (lactase) can be obtained from a wide variety of sources, such as microorganisms, plants and animals. Generally, microorganisms are preferred, since they are easier to handle, and offer a higher production yield. Commercially available lactases generally originate from yeasts, fungi or bacteria (Mahoney 1997), with the optimal conditions of enzyme activity highly dependant on the type of microorganism. The difficulty and expense related to the release of active enzyme in high yield from cells and the cost of purification are major constraints affecting further industrial applications.

To overcome this problem, immobilization and encapsulation offer several ways to improve the process economics: (1) by immobilization of cells producing lactase in a bioreactor to achieve higher enzyme yields; (2) by immobilization of lactase for the hydrolysis of lactose to high yields and at high rates; (3) by immobilization of whole cells (containing lactase) to directly achieve lactose hydrolysis at reduced costs, compared to the use of purified enzyme.

For example, in the simplest process, β-D-galactosidase is added directly to whole milk, and when the hydrolysis of lactose has attained the desired level, the enzyme is deactivated by heat treatment. Since the enzyme is denatured and cannot be re-used, the resulting operating costs are very high. One solution is the hydrolysis of skimmed milk with immobilized lactase followed by the addition of cream to adjust the fat content (Hirohara et al. 1981).

Lactase and cells producing this enzyme have been immobilized by various methods, such as gel entrapment, physical absorption and covalent attachment on various supports. Successful applications based on physical adsorption of lactase (or cells producing lactase) have been designed, using different kinds of carrier, such as cotton cloth (Sharma and Yamazaki 1984), chitosan (Champluvier et al. 1988), polyvinyl chloride (Bakken and Hill 1990) and silica (Bakken and Hill 1992).

The advantage of immobilizing whole cells instead of pure lactase for the direct hydrolysis of lactose is that enzyme activity is conserved, and beads are mechanically stable and protect cells in the reactor where the reaction takes place. This is generally achieved through entrapment of microorganisms in a gel matrix.

β-D-galactosidases can be used in many different applications in order to hydrolyze lactose in milk, whey and whey permeate. The choice of the technology, immobilization method, support, enzyme or cell strain depends on the conditions (pH, temperature, continuous vs. batch) under which the process is undertaken and is similar to that described for glucose production in Sect. 14.3.1.

14.4 Use of Immobilized Cells for the Production of Organic Acids

14.4.1 Lactic Acid

Lactic acid is extensively used in the food industry as a preservative, antioxidant or acidifier. In recent years, it became interesting as a primary material for the production of biodegradable plastics. Although it is possible to produce by chemical synthesis, over 95% of the world lactic acid production is through biotechnological processes. The use of microorganisms for the production of lactic acid has the advantage that stereochemically pure D- or L-lactic acid may be produced. Lactic acid is readily produced using lactic acid bacteria, which readily ferment lactose present in significant amounts in whey, a by-product of the cheese industry. The use of immobilized *Lactobacillus casei*, *Lactobacillus delbrueckii* and *Rhizopus oryzae* for lactic fermentation has been reported.

Lactobacillus casei, immobilized in calcium-pectate beads (Panesar et al., 2007), or PEI coated porous glass (Poraver) beads (Senthuran et al. 1999), was used for fermentation of whey or enriched whey permeates. Due to the good stability at low pH, calcium-pectate gels can be used as a carrier for lactic acid production over prolonged periods of time. In a laboratory, batch reactor up to 33 g/L of lactate was produced with 95% yield over 30 h per batch. As a result of the high stability of the beads, up to 16 batch cultures could be performed, with virtually no loss of conversion yields or rates (Panesar et al. 2007).

Lactic acid production by *Lactobacillus casei* immobilized by adsorption on Poraver beads has been reported using an external column reactor. Both freely suspended cells and adsorbed cells in the external column are active and contribute to the bioconversion of lactose. The high volumetric productivity was shown to be the direct result of the action of the immobilized cells, rather than an eventual increase in suspended cell density compared to submerged cultures. Lactate concentrations of up to 90 g/L were attained using this system, during a fermentation time of about 30 h (Senthuran et al. 1999).

Lactobacillus delbrueckii can be used for lactic acid production from glucose. Cells entrapped in calcium-alginate beads have been used for the conversion of corn cob residue, a cheap and abundant residue from xylose production. This residue is a

porous cellulosic material that can be hydrolyzed enzymatically to yield a glucose solution. Addition of wheat bran hydrolyzate improves culture performance. After 48 h of batch culture up to 49 g/L of lactate with 95% conversion could be achieved, and 12 successive batches were possible. In a continuous culture with immobilized cells an increase in dilution rate increased the productivity, but reduced the yield and the residual concentration. It was suggested that a compromise dilution rate of 0.13 h^{-1} could be used to give a 92.4% yield and 44.2 g/L lactate concentration. A simultaneous saccharification and fermentation process can be used to avoid the feedback inhibition of enzymes used for cellulose hydrolysis. Enzymatic breakdown of cellulose is compatible with the lactic acid fermentation in terms of pH and temperature, and authors propose two coupled reactors with re-circulation. The hydrolysis product is pumped from the enzymatic reactor through a filter to the immobilized cell reactor for the lactic acid fermentation. In this way 55.6 g/L lactic acid concentration is achieved in 60 h, with a yield from cellulose of 91%. This could be improved by performing a fed-batch fermentation involving multiple additions of cellulose substrate and hydrolytic enzymes. In this way, 108 g/L lactate could be produced in 90 h with 88% yield from cellulose.

The use of another cheap cellulosic material, called loofa sponge or vegetable sponge, as a support for the immobilization of filamentous fungi for lactic acid production has been described. The material is cut into pieces, washed and autoclaved, then used for the immobilization of *Rhizopus oryzae*. This is achieved by inoculating the reactor with spores and cultivating the mycelium during 24 h. The fermentation was conducted in shake flasks. Growth on enriched rice starch hydrolysate medium yielded 81 g/L of product in 10 h. The immobilized mycelium could be reused several times, with the reactor performance falling from cycle to cycle. After ten cycles, volumetric productivity fell by half, and the final lactic acid concentration to about 20 g/L (Ganguly et al. 2007).

14.4.2 Citric Acid

Citric acid is a common food additive especially in soft drinks, mainly as an acidifier, but also as a preservative and an antioxidant. It is often used as part of a formula for effervescent tablets. Nowadays it is produced in biotechnological processes, most frequently using *Aspergillus niger* strains, although *Yarrowia lypolitica* has been identified as a potential producer of citric acid in high concentrations from glycerol, a by-product from biodiesel production.

Citric acid production using *Aspergillus niger* entrapped in calcium-alginate beads (Bayraktar and Mehmetoglu 2000; Demirel et al. 2005), polyacrylamide gel (Horitsu et al. 1985; Wang and Liu 1996), adsorbed on cellulose materials (Sakurai et al. 1997; Sankpal et al. 2001; Tisnadjaja et al. 1996), or adsorbed on polyurethane (Lee et al. 1989; Ricciardi et al. 1997; Wang 2000) has been reported. Alginate entrapment of fungi for citric acid production is normally done by suspending conidiaspores in Na-alginate and extruding this suspension into a $CaCl_2$ solution

(Bayraktar and Mehmetoglu 2000; Demirel et al. 2005; Gupta and Sharma 1994; Hamamci et al. 1988; Tsay and To 1987). Fermentation using entrapped filamentous fungi proved to be difficult because of filamentous growth and clogging of the pores of the gel, but also because of the physical damage to the beads caused by the growth (Demirel et al. 2005). In addition citric acid is often used as a dissolving agent for alginate gels as a result of its ability to form stable chelates with Ca^{2+} ions, which results in reduction of bead stability during the fermentation. Addition of silicone oil to the fermentation enhances O_2 supply thus increasing productivity, but the re-usability of alginate immobilized mycelia is reduced even further (Ates et al. 2002). Generally speaking, very low productivity and citric acid concentrations could be achieved using alginate beads with 2–3 g/L formed after 4 days, with practically no re-usability of the beads (Bayraktar and Mehmetoglu, 2000; Demirel et al. 2005).

Gupta and Sharma (1994) described a double horizontal reactor with combined submerged Ca-alginate immobilized and surface stabilized *A. niger* cells. The set-up allowed very good productivities and yields on sugarcane molasses but, due to complex operation, was not implemented industrially.

The use of a rotating disk reactor with *A. niger* immobilized on polyurethane foam disks allowed eight batches with no loss of activity (Wang 2000), and production of up to 85 g/L in 96 h with about 72% product yield.

Sankpal et al. (2001) investigated different set-ups and modes of operation of bioreactors containing *A. niger* mycelium immobilized on a woven cotton or in submerged culture. Immobilized cell culture was performed in a hanging sheet reactor with a spirally wound woven cotton placed in a cylindrical chamber with a constant supply of humidified air, and the nutritive medium dripped on the top of the mycelium support. The nutritive medium used in the experiments was either rich medium containing sucrose as the main carbon source or sugar cane juice. Immobilization increased the volumetric productivity and yield as compared to submerged cultures. Continuous mode of operation increased productivity, even further while maintaining the high citric acid yield. Four repeated batches with no reduction of performance yielded 60 g/L citric acid in each 9 h cycle. However, as biomass increases, the specific productivity is reduced. When operated in continuous mode, a residence time of 20 h resulted in nearly 100% conversion of the 50 g/L sucrose, to yield 65 g/L citrate in the exiting medium (the product is somewhat concentrated due to evaporative concentration). Sugar cane medium can be used, but some technical difficulties related to irregular and thick mycelium growth causing mass transfer limitations were noted (Sankpal et al. 2001).

Due to its ability to convert both galactose and glucose to citric acid, the yeast *Candida guilliermondii* has been used for the production of citric acid from whey hydrolyzates. Tisnadjaja et al. (1996) demonstrated the use of sawdust as a cheap material for adsorption of yeast cells and operation in a bubble column reactor. They used rich medium with glucose as carbon source, and attained much higher volumetric productivities, but lower yields and specific productivity, compared to suspension cultures. This may have been the result of a low culture pH. The final citrate concentration was lower than 10 g/L.

Adsorption on synthetic porous cellulose beads, and cultivation on rich medium containing glucose in submerged culture in shake flasks resulted in 60 g/L citric acid, but only 50% yield. In addition, re-usability was poor, with less than half the citric acid concentration achieved after the second of two consecutive 8-day batch runs (Sakurai et al. 1997).

14.4.3 Gluconic Acid

Gluconic acid is a product of the oxidation of glucose, which is used extensively as a pH regulator in food industry. *Aspergillus niger*, grown on low nitrogen-containing medium, has been reported to convert glucose to gluconic acid. Sankpal et al. (1999) described a system for continuous gluconic acid production using hanging sheet reactors with the mycelium immobilized on a woven cotton cloth hung in a chamber with controlled humidity, temperature and air flow. The defined culture medium, containing glucose as carbon source, was dripped onto the top of the cloth. A tenfold increase in productivity, compared to a submerged culture, with a final product concentration of up to 125–140 g/L, was reported. Falling conversion rates and yield could be partially overcome by increasing the residence time progressively. It was noted that when aerated, mycelium growth was uneven resulting in an uneven nutrient and air supply and severe reduction of the overall volumetric productivity. If a pure oxygen stream was used instead of air, mycelium grows evenly and continuous operation of up to 60 days was possible. The inherent problem of pH, concentration and temperature gradients, which might reduce reactor performance, could be solved by careful process design and optimization.

14.4.4 Acetic Acid and Other Carboxylic Acids

Acetic acid is one of the most important organic acids with broad use in the food industry, as acidifying additive and/or preservative. Although most of the market demand for acetic acid is satisfied by chemical synthesis, all of the acetic acid used in the food industry must be of biological origin and is produced using acetogenic bacteria. Of the other major carboxylic acids, propionic acid is used as a food preservative in the form of sodium salt, while butyric acid is used as a flavoring additive, either in free or in ester forms.

The laboratory-scale production of acetic acid using entrapped *Acetobacter aceti* cells in calcium-alginate beads and cells adsorbed on cellulose beads has been reported (Krisch and Szajani 1996). After 70 h of culture on rich medium with glucose and ethanol as substrates, up to 30–34 g/L could be attained using both immobilization systems, which is comparable to free cell fermentations. It was also shown that immobilization improved culture tolerance to suboptimal temperatures and pH, although no data were reported for long-term operation.

The anaerobic homofermentative bacterium *Clostridium formicoaceticum* is capable of converting fructose from corn syrup and other fructose containing

substrates to acetic acid in high yields and with good specific productivity. External fibrous-bed columns containing spirally wound terry cloth were used in an experimental set-up for adsorption of *Clostridium formicoaceticum*. A comparison of batch, fed-batch and continuous modes of operation revealed that cell immobilization gave higher yields, volumetric productivities and acetate concentrations. Up to 78 g/L of acetate could be achieved with fed-batch fermentations using such immobilized cells (Huang et al. 1998).

An external column reactor set-up was also used for a mixed-culture fermentation of whey permeate using *Clostridium thermolacticum* and *Clostridium thermoautotrophicum* co-immobilized by adsorption on cotton fabric (Talabardon et al. 2000). Being a heterofermentative bacterium, *Clostridium thermolacticum* is capable of converting lactose almost entirely to lactic acid under growth-limiting conditions. *Clostridium thermoautothropicum* is a homoacetogen that can assimilate lactate and produce acetic acid. The yields and productivity of the co-immobilized co-culture were relatively low with only 25 g/L acetate produced. The authors suggest that process optimization might improve performance, with the use of cheap substrates rendering the process economically viable even with lower yields. Both of the microorganisms used are sporogenous bacteria, which means that, theoretically, the process could be stopped and re-continued depending on the availability of whey supply (Talabardon et al. 2000). A similarly conceived co-culture of homolactic *Lactobacillus lactis* and homoacetogenic *Clostridium formicoaceticum* has been used for the conversion of whey lactose or corn meal hydrolyzate to acetic acid with almost 100% yield and 76 g/L of product (Huang et al. 2002).

Huang et al. (2002) used a fibrous-bed reactor for the fermentation of hydrolyzed corn meal for the production of acetic, propionic and butyric acids. *P. acidipropionici* was used for propionic acid production, and *Clostridium tyrobutylicum* for butyric acid production. Propionic acid could be produced on enriched whey, glucose medium and corn syrup hydrolyzate, with immobilization increasing volumetric productivity, while maintaining the yield resulting in up to 40 g/L propionate. Butyric acid could also be produced on glucose medium and pentose-containing corn meal hydrolyzate with immobilized *Clostridium tyrobutylicum* giving higher yields compared to free cell fermentations, with significantly increased volumetric productivity, reactor performance and with a final concentration of up to 45 g/L (Huang et al. 2002; Zhu et al. 2002). All three processes could be operated continuously for several months, even with corn meal hydrolyzate, which has a high content of insoluble material. No column fouling was observed, probably as a result of the way that the cotton fabric was packed, with sufficient space between the winding layers for the insoluble particles to pass through.

14.5 Amino Acids

Amino acids are the building blocks of proteins, being one of the three largest groups of nutrients. The daily nutritional requirement for amino acids is met by the intake of proteins which are hydrolyzed during digestion. However, some amino

acids have important applications in the food industry, such as the artificial sweetener aspartame (aspartyl-phenylalanine-1-methyl ester) which is produced from aspartic acid and phenylalanine. Sodium glutamate, or mixtures of amino acids, is often used as flavor enhancers. Depending on the use and on the particular amino acid, production of amino acids is normally done by (1) enzymatic conversion of precursors to the desired product, for example, L-Asp production from fumaric acid using the aspartase from *Escherichia coli*; (2) hydrolysis of readily available and cheap proteins followed by extraction of the desired amino acid; (3) fermentation methods; or (4) chemical methods (DL-methionine is produced by chemical synthesis from raw materials such as acrolein, hydrocyanic acid and methyl mercaptan).

14.5.1 Production of Amino Acids Using Immobilized Whole Cells

Chao et al. (1999) immobilized recombinant *Escherichia coli,* which overproduces aspartase, aspartate aminotransferase or aromatic amino acid aminotransferase, in calcium-alginate beads and used these immobilized cells for the simultaneous production of phenylalanine and aspartic acid from phenylpyruvic acid, fumaric acid and ammonia. The beads were treated with glutaraldehyde and hexamethylenediamine in order to enhance their mechanical resistance. Fermentation in shake flasks resulted in product yields that were comparable to those of free cell cultures though a reduced specific productivity was observed, indicating mass transfer limitations or loss of viability. Immobilized preparations could be re-used for up to six cycles with virtually no activity loss for either of the products (Chao et al. 1999). Longer duration cultures led to rupture of the beads. However, long time operation using recombinant organisms was also limited by the genetic instability of the recombinant strain.

Recombinant *Escherichia coli* expressing transaminase, for the production of L-Phe from phenylpyruvic acid, was immobilized in κ-carrageenan beads as a whole cell catalyst for the production of L-phenylalanine from L-aspartic acid and phenylpyruvate (Leng et al. 2006). The treatment of cells with 0.05% glutaraldehyde prior to immobilization enhanced enzyme activity and operational stability. The gel cubes, containing immobilized cells, were used in packed-bed reactors operating in both batch and continuous modes. A conversion yield of about 90% was obtained in batch mode, falling to 88% after ten repeated batches with the same immobilized cells. When operated in continuous mode with a residence time of 10 h, 20.6 g/L of product could be obtained. After 40 days of continuous operation the conversion was still higher than 88%. A mutant Pseudomonas strain, selected for low L-cystein desulfhydrase activity, was entrapped in calcium-alginate beads and used to produce L-cystein by the enzymatic conversion of the chemically synthesized precursor, DL-2-amino thiazoline 4-carboxylic acid. The presence of sorbitol in the feed increased the stability of the enzymes. The developed process comprised two packed-bead columns in series, followed by an ion-exchange column for separation of L-cystein. The residual L-cystein degrading enzyme was responsible for 23% product loss. In the presence of 40% sorbitol in the feed at 37°C, 60% of the conversion was maintained after 1,000 h of continuous cultivation (Ryu et al. 1997).

Calcium-alginate bead entrapment of a *Pseudomonas* sp. which expresses aminoacylase was used for the production of L-alanine by the enzymatic, stereospecific deacetylation of a chemically produced an *N*-acyle derivative. The production process must be performed under non-growing conditions otherwise cells used the *N*-acyle alanine for biomass formation, thus reducing the product yield. Cells were immobilized in κ-carrageenan beads (Santoyo et al. 1998), or calcium-alginate beads cross-linked with glutaraldehyde (Hernandez et al. 2001; Santoyo et al. 1996). Total conversion was attained after approximately 5 h with up to 4 g/L of L-alanine produced in batch mode using κ-carrageenan beads. However, less than 6 h of continuous operation was possible with κ-carrageenan beads because of physical damage to the beads. Experiments with alginate showed lower conversion, probably because the process was not optimized. Continuous cultures with alginate beads were possible; however, after 48 h only 82% of the activity was conserved due to loss of enzyme activity, rather than cell leakage or damage to the beads. Pretreatment of the alginate beads with up to 20 mM glutaraldehyde solution increased the half-life of the enzymatic activity, and even resulted in increased activity, while higher glutaraldehyde concentrations were found to inhibit the enzyme.

The possibility to use cyanobacteria (a mutant of *Anabaena* Sp-287) immobilized in calcium alginate beads for the overproduction of D-Ala and L-His has also been investigated (Thomas and Shanmugasundaram 1992). The use of microorganisms capable of fixing atmospheric nitrogen and releasing either NH_3 or amino acids would be expected to make the process more cost effective. However, technical difficulties, such as correct illumination, still hinder the use of photosynthetic microorganisms in industrial biotechnology.

Tryptophan production from serine and indole using whole recombinant tryptophanase-overproducing *Escherichia coli* cells immobilized in κ-carrageenan beads has been described (Mateus et al. 2004). The reaction was conducted in a simple stirred vessel and up to 10 mM L-tryptophan could be obtained in about 2 h, with almost 100% conversion.

14.5.2 Amino Acid Production Using Immobilized Enzymes

N-acetyl-amino acid racemase and L- or D-aminoacylase can be used for the separation of amino acid stereoisomers. Chemically synthesized *N*-acetyl derivatives of an amino acid can be stereoselectively deacetylated and the produced L- or D-amino acid readily separated from the precursor using standard chromatographic methods. The remaining *N*-acetyl derivative is racemized using the racemase enzyme, and the process is repeated. *N*-acetyl-amino acid racemase and aminoacylase have been immobilized on a number of supports (Tokuyama and Hatano 1996; Tsai et al. 1992). Very good yields and operational stabilities were obtained with these immobilized preparations, with half-lives of 10–30 days and the initial yield of close to 100%.

Bodalo-Santoyo et al. (1999) described a reactor with fluidized- and packed-beds, containing enzymes immobilized by covalent linkage on controlled pore glyceryl glass for separation of D,L-valine from chemically produced *N*-acyl valine,

using enantio-specific hydrolase, the unhydrolyzed D-isomer chemically racemized. The system could be operated in packed-bed and fluidized-bed modes, although conversion dropped after 10 h.

Oikawa et al. (1999) described a system with two packed-bed columns containing immobilized enzymes (glutamate racemase and L-glutamate oxidase) for the production of D-glutamate from L-glutamate. The enzymes were immobilized on Chitopearl 2505 and 2506 by adsorption followed by glutaraldehyde cross-linking. The process was divided into two phases (and two columns) such that both enzymes could operate under optimal conditions. As a result there was negligible loss of activity after 10 days of continuous operation (Oikawa et al. 1999).

Co-immobilization of D-amino acid oxidase and catalase on glutaraldehyde-agarose beads for the production of α-ketoglutaric acid (AKG) from D,L-alanine, or for separation of alanine enantiomers have been described (Buto et al. 1994; Fernandez-Lafuente et al. 1998). Catalase was used to remove the H_2O_2 and prevent oxidative damage to the system. A maximum AKG yield of 86% was obtained, due to part of the AKG formed being converted to the oxidation product phenyl acetic acid. Immobilization clearly increased the stability of the enzymes, with virtually no loss in activity after re-using the beads 20 times. Proteases may be immobilized in agarose gels (Pedroche et al. 2006, 2004) for protein digestion and production of hydrolyzates for food supplements and for medical purposes.

14.6 Hydrolysis of Triglycerides

14.6.1 Lipases in Food and Flavor Making

As described previously, the use of enzymes to improve traditional chemical processes for food manufacture has been extensively studied and developed in the past few years, due to the technological and economical advantage they offer. Most lipase-mediated food processes embody biocatalysis of lipase, especially fats and oil (Jachmanian et al. 1997). During storage, important changes arise from the hydrolysis of triglycerides catalyzed by lipases, leading to the formation of fatty acids. Esters of short chain fatty acids are extremely important aroma compounds, and are hence important compounds for the characteristic flavor of products. Moreover, it is often desirable to alter the fatty acid composition of naturally occurring triglycerides in fat with the required melting characteristics.

The modification of fats and other lipids may be achieved through (1) hydrolysis, (2) esterification, (3) inter or trans-esterification. All of these reactions are catalyzed by lipases (Wisdom et al. 1984). Inter-esterification processes consist in promoting an uncontrolled migration of fatty acid groups between the triglyceride molecules (Mukherjee and Kiewitt 1996). This process allows the formation of fats suitable for use in emulsions, margarine, artificial creams and ice-creams (Benjamin and Pandey 1998). A known application of this technology is the production of a

cocoa-butter substitute from palm oil, where palmitoyl moieties are replaced by stearoyl groups (Katchalski-Katzir 1993). The esterification process allows the formation of short and medium chain esters, which are known to be flavor molecules, which are of great use for food flavoring.

Fermentation is induced by microorganisms, which also have lipolytic activity. As a consequence fermented food from fruits, vegetable, cereals, oilseed and meat is also affected by this process. The addition of lipase to these preparations can also be an alternative to alter the taste of these products, especially in terms of acidity (Katchalski-Katzir 1993).

The cost of microbial production of lipase is often prohibitive. Hence, processes that do not require the presence of lipase in the final product are more economic if the lipase is immobilized, and therefore re-cycled a number of times. The choice of the support for lipase immobilization is of central importance since it determines the enzymatic activity. Since lipase acts on organic products, the types of support are different than for reactions taking place in aqueous conditions. Several applications use diatomaceous earth (diatomite) as a support for lipase immobilization, while others use porous silica-based supports (Wisdom et al. 1984). The method used to immobilize the enzyme usually involves precipitation of the lipase from solution onto the support surface. Precipitation is induced by addition of chilled acetone as precipitant. Thus the enzyme remains associated with the support and is unable to dissolve in either the organic solvent, water or aqueous solution. The immobilized enzyme can then be re-used, allowing the process to run continuously, which significantly decreases the production costs.

14.7 Conclusions

Immobilization of both cells and enzymes is an important tool in the food industry for carrying out specific bioconversions and for the formation of metabolites. Indeed as a result of the increasing need for so-called – white and green biotechnologies – increasing numbers of bioconversions and/or molecular synthesis in the food industry are carried out using natural biocatalysts. In the case of products such as yoghurt, cheese and the like, whole microbial cells may be added to the food material during processing, since they are ultimately found in the product. On the other hand, many situations arise where purified enzymes are used to carry out specific reactions within the process. Since enzymes are relatively expensive and by their nature subject to loss of activity and denaturation under many of the environmental conditions found in food processing, the ability to encapsulate the enzymes has resulted in a significant stimulation to their use. This is due to encapsulated enzymes enabling (1) the design of continuous processes with increased productivity; (2) re-use of the enzymes over many weeks or indeed months, resulting in lower enzyme consumption; (3) increased stability and activity of enzymes for bioconversions, which allows extended use; (4) higher enzyme and cell densities which result in the maintenance of viability/activity for metabolite

production; (5) physical protection of the enzymes and cells from mechanical shear forces found in reactor conditions.

In order to achieve this, enzymes and cells may be adsorbed onto the surface of particles, where the particles have a higher specific density than hydrogels, and thereby may be used in fluidized-bed systems, and where the loading density is limited by the available surface area. In cases where the enzymes have a relatively large molecular weight, or where whole cells are used, and require protection from the denaturing forces found under the process conditions used, then entrapment in a range of polymers is most suitable. However, these polymers have relatively high porosity and low mechanical stability and cannot be used in packed-bed operation at high flow rates or where enzyme leaching is a problem. Where significant stability is required it is essential to covalently cross-link the enzymes to the polymers in such a way as to retain activity over extended periods. However, the range of polymers, reactor types and immobilization methods and chemistries means that it is now possible to tailor the encapsulation technique to almost any process in order to get the optimum activity, stability and cost.

References

Altinok H, Aksoy S, Tumturk H, Hasirci N (2006) Covalent immobilization of invertase on chemically activated poly(2-hydroxyethyl methacrylate) microbeads. Russ Chem Bull 66:1860–1864

Ates S, Dingil N, Bayraktar E, Mehmetoglu U (2002) Enhancement of citric acid production by immobilized and freely suspended Aspergillus niger using silicone oil. Process Biochem 38:433–436

Bakken A, Hill CJ (1990) Use of a novel immobilized beta galactosidase reactor to hydrolyze the lactose constituent of skim milk. Biotechnol Bioeng 36:293–309

Bakken A, Hill CJ (1992) Hydrolysis of lactose in skim milk by immobilized beta galactosidase. Biotechnol Bioeng 39:408–417

Bayraktar E, Mehmetoglu U (2000) Production of citric acid using immobilized conidia of Aspergillus niger. Appl Biochem Biotechnol 87:117–125

Benjamin S, Pandey A (1998) Candida rugosa lipases: Molecular biology and versatility in biotechnology. Yeast 14:1069–1087

Bodalo-Santoyo A, Gomez-Carrasco JL, Gomez-Gomez E, Bastida-Rodriquez J, Maximo-Martin MF, Hidalgo-Montesinos AM (1999) Production of optically pure L-valine in fluidized and packed bed reactors with immobilized L-aminoacylase. J Chem Technol Biotechnol 74:403–408

Brijak J (2003) Alpha-amylase and beta-amylase immobilisation on acrylic carriers. Biochem Eng J 16:347–355

Buto S, Pollegioni L, Dangiuro L, Pilone MS (1994) Evaluation of D-amino-acid oxidase from Rhodotorula gracilis for the production of alpha-keto acids – a reactor system. Biotechnol Bioeng 44:1288–1294

Champluvier B, Kamp B, Rouxhet P (1988) Immobilization of beta galactosidase retained in yeast: Adhesion of the cells on a support. Appl Microbiol Biotechnol 27:464–469

Chao YP, Fu HY, Lo TE, Chen PT, Wang JJ (1999) One-step production of D-*p*-hydroxyphenylglycine by recombinant Escherichia coli strains. Biotechnol Prog 15:1039–1045

Chen JP, Sun YM, Chu DH (1998) Immobilization of alpha-amylase to a composite temperature-sensitive membrane for starch hydrolysis. Biotechnol Prog 14:473–478

Danisman T, Tan S, Kacar Y, Ergene A (2004) Covalent immobilization of invertase on microporous pHEMA-GMA membrane. Food Chem 85:431–466

Demirel G, Yaykasli KO, Yasar A (2005) The production of citric acid by using immobilized Aspergillus niger A-9 and investigation of its various effects. Food Chem 89:393–396
Dey G, Singh B, Banerjee R (2003) Immobilization of alpha-amylase produced by Bacillus circulans GRS 313. Braz Arch Biol Technol 46:167–176
Ertan F, Yagar H, Balkan B (2007) Optimization of alpha-amylase immobilization in calcium alginate beads. Prep biochem biotechnol 37:195–204
Fernandez-Lafuente R, Rodriguez V, Guisan JM (1998) The coimmobilization of D-amino acid oxidase and catalase enables the quantitative transformation of D-amino acids (D-phenylalanine) into alpha-keto acids (phenylpyruvic acid). Enzyme Microb Technol 23:28–33
Ganguly R, Dwivedi P, Singh RP (2007) Production of lactic acid with loofa sponge immobilized Rhizopus oryzae RBU2–10. Bioresour Technol 98:1246–1251
Gill P, Manhas R, Singh P (2006) Hydrolysis of inulin by immobilized thermostable extracellular exoinulinase from Aspergillus fumigatus. J Food Eng 76:369–375
Gupta S, Kaur M, Kaur N, Singh RP (1992) A comparison of properties of inulases of Fusarium-oxysporum immobilized on various supports. J Chem Technol Biotechnol 53:293–296
Gupta S, Sharma CB (1994) Continuous production of citric-acid from sugarcane molasses using a combination of submerged immobilized and surface-stabilized cultures of Aspergillus-niger-kcu-520. Biotechnol Lett 16:599–604
Hamamci H, Gursoy G, Hang YD (1988) Production of citric-acid using immobilized dried reactivated Aspergillus-niger. Abstracts of Papers of the American Chemical Society 196, 92-Mbtd.
Hamerska-Dudra A, Bryjaka J, Trochimczuka A (2007) Immobilization of glucoamylase and trypsin on crosslinked thermosensitive carriers. Enzyme Microb Technol 41:197–204
Hernandez H, Asanza ML, Alcaraz I, Hidalgo AM, Maximo MF, Bastida J (2001) Production of optically pure L-alanine in batch and continuous stirred tank reactors using immobilized Pseudomonas sp. Biotechnol Lett 23:887–891
Hirohara H, Yamamoto H, Kawano E, Nagase T (1981) Continuous hydrolysis of lactose in skim milk and acid whey by immobilized lactase of Aspergillus oryzae. Enzyme Eng 6:295
Horitsu H, Adachi S, Takahashi Y, Kawai K, Kawano Y (1985) Production of citric-acid by Aspergillus-niger immobilized in polyacrylamide gels. Appl Microbiol Biotechnol 22:8–12
Huang YL, Mann K, Novak JM, Yang ST (1998) Acetic acid production from fructose by Clostridium formicoaceticum immobilized in a fibrous-bed bioreactor. Biotechnol Prog 14:800–806
Huang YL, Wu ZT, Zhang LK, Cheung CM, Yang ST (2002) Production of carboxylic acids from hydrolyzed corn meal by immobilized cell fermentation in a fibrous-bed bioreactor. Bioresour Technol 82:51–59
Jachmanian I, Schulte E, Mukherjee K (1997) Substrate selectivity in esterification of less common fatty acids catalysed by lipases from different sources. Appl Microbiol Biotechnol 44:563–567
Kahraman M, Bayramoglu G, Kayaman-Apohan N, Gungor A (2007) alpha-Amylase immobilization on functionalized glass beads by covalent attachment. Food Chem 104:1385–1392
Kahraman M, Kayaman-Apohan N, Ogan A, Gungor A (2006) Optimization of alpha-amylase immobilization in calcium alginate beads. J appl polym sci 100:4757–4761
Karaa A, Osmana B, Yavuzb H, Beşirlia N, Denizlib A (2005) Immobilization of alpha-amylase on Cu2+ chelated poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) matrix via adsorption. Reactive funct polym 62:61–68
Katchalski-Katzir E (1993) Immobilized enzyme-learning from past successes and failures. Trends Biotechnol 11:471–478
Konsoula Z, Liakopoulou-Kyriakides M (2006) Starch hydrolysis by the action of an entrapped in alginate capsules alpha-amylase from Bacillus subtilis. Process Biochem 41:343–349
Krisch J, Szajani B (1996) Effects of immobilization on biomass production and acetic acid fermentation of Acetobacter aceti as a function of temperature and pH. Biotechnol Lett 18:393–396
Lee YH, Lee CW, Chang HN (1989) Citric-acid production by Aspergillus-niger immobilized on polyurethane foam. Appl Microbiol Biotechnol 30:141–143
Leng Y, Zheng P, Sun ZH (2006) Continuous production of L-phenylalanine from phenylpyruvic acid and L-aspartic acid by immobilized recombinant Escherichia coli SW0209–52. Process Biochem 41:1669–1672

Mahoney R (1997) Lactose: Enzymatic modification. In: Fox F (ed) Adv Dairy chem, vol 3. Chapman & Hall, London, pp 77–125

Mateus DMR, Alves SS, Da Fonseca MMR (2004) Kinetics of L-tryptophan production from indole and L-serine catalyzed by whole cells with tryptophanase activity. J Biosci Bioeng 97:289–293

Mukherjee K, Kiewitt I (1996) Enrichment of very long chain mono-unsaturated fatty acids by lipase-catalysed hydrolysis and transesterification. Appl Microbiol Biotechnol 44:557–562

Nakamura T, Ogata A, Shitara A, Nakamura A, Ohta K (1995) Continuous production of fructose syrups from inulin by immobilized inulinase from Aspergillus-Niger Mutant-817. J Ferment eng 80:164–169

Oikawa T, Watanabe M, Makiura H, Kusakabe H, Yamade K, Soda K (1999) Production of D-glutamate from L-glutamate with glutamate racemase and L-glutamate oxidase. Biosci Biotechnol Biochem 63:2168–2173

Panesar P, Panesa RR, Singh R, Kennedy J, Kumar H (2006) Microbial production, immobilization and applications of β-D-galactosidase. J Chem Technol Biotechnol 81:530–543

Panesar PS, Kennedy JF, Knill CJ, Kosseva MR (2007) Applicability of pectate-entrapped Lactobacillus casei cells for L(+) lactic acid production from whey. Appl Microbiol Biotechnol 74:35–42

Pedroche J, Yust MD, Lqari H, Megias C, Giron-Calle J, Alaiz M, Vioque J, Millan F (2006) Production of Brassica carinata protein hydrolyzates with a high Fischer's ratio using immobilized proteases. J Agric Food Chem 54:7621–7627

Pedroche J, Yust MM, Lqari H, Giron-Calle J, Vioque J, Alaiz M, Millan F (2004) Production and characterization of casein hydrolysates with a high amino acid Fischer's ratio using immobilized proteases. Int Dairy J 14:527–533

Raviyan P, Tang J, Rasco B (2003) Thermal stability of alpha-amylase from Aspergillus oryzae entrapped in polyacrylamide gel. J Agric Food Chem 51:5462–5466

Rebroš M, Rosenberga M, Mlichováa Z, Krištofíkováa L, Palucha M (2006) A simple entrapment of glucoamylase into LentiKats® as an efficient catalyst for maltodextrin hydrolysis. Enzyme Microb Technol 39:800–804

Reshmi R, Sanjay G, Sugunan S (2006) Enhanced activity and stability of alpha-amylase immobilized on alumina. Catal Commun 7:460–465

Reshmi R, Sanjay G, Sugunan S (2007) Immobilization of alpha-amylase on zirconia: A heterogeneous biocatalyst for starch hydrolysis. Catal Commun 8:393–399

Ricciardi A, Parente E, Volpe F, Clementi F (1997) Citric acid production front glucose by Aspergillus niger immobilized in polyurethane foam. Ann Microbiol Enzimol 47:63–76

Ryu OH, Ju JY, Shin CS (1997) Continuous L-cysteine production using immobilized cell reactors and product extractors. Process Biochem 32:201–209

Sakurai A, Itoh M, Sakakibara M, Saito H, Fujita M (1997) Citric acid production by Aspergillus niger immobilised on porous cellulose beads. J Chem Technol Biotechnol 70:157–162

Salehi Z, Sohrabi M, Kaghazchi T, Bonakdarpour B (2005) Application of down flow jet loop bioreactors in implementation and kinetic determination of solid–liquid enzyme reactions. Process Biochem 40:2455–2460

Sanjay G, Sugunan S (2007) Enzymes immobilized on montmorillonite K 10: Effect of adsorption and grafting on the surface properties and the enzyme activity. Appl Clay Sci 35:62–75

Sankalia M, Mashru R, Sankalia J, Sutariya V (2007) Reversed chitosan-alginate polyelectrolyte complex for stability improvement of alpha-amylase: Optimization and physicochemical characterization. Eur J Pharm Biopharm 65:215–232

Sankpal NV, Joshi AP, Kulkarni BD (2001) Citric acid production by Aspergillus niger immobilized on cellulose microfibrils: Influence of morphology and fermenter conditions on productivity. Process Biochem 36:1129–1139

Sankpal NV, Joshi AP, Sutar II, Kulkarni BD (1999) Continuous production of gluconic acid by Aspergillus niger immobilized on a cellulosic support: Study of low pH fermentative behaviour of Aspergillus niger. Process Biochem 35:317–325

Santoyo AB, Rodriguez JB, Carrasco JLG, Gomez EG, Rojo IA, Teruel LMA (1996) Immobilization of Pseudomonas sp BA2 by entrapment in calcium alginate and its application for the production of L-alanine. Enzyme Microb Technol 19:176–180

Santoyo AB, Rodriguez JB, Carrasco JLG, Gomez EG, Rojo IA, Teruel MLA (1998) Production of optically pure L-alanine by immobilized Pseudomonas sp. BA2 cells. J Chem Technol Biotechnol 73:197–202

Senthuran A, Senthuran V, Hatti-Kaul R, Mattiasson B (1999) Lactic acid production by immobilized Lactobacillus casei in recycle batch reactor: A step towards optimization. J Biotechnol 73:61–70

Sharma S, Yamazaki H (1984) Preparation of hydrophobic cotton cloth. Biotechnol Lett 6:301–306

Singh R, Dhaliwal R, Puri M (2007) Production of high fructose syrup from Asparagus inulin using immobilized exoinulinase from Kluyveromyces marxianus YS-1. J Ind Microbiol Biotechnol 34:649–655

Stefanova M, Tonkova A, Dobreva E, Spasova D (1998) Agar gel immobilization of Bacillus brevis cells for production of thermostable alpha-amylase. Folia Microbiol 43:42–46

Talabardon M, Schwitzguebel JP, Peringer P, Yang ST (2000) Acetic acid production from lactose by an anaerobic thermophilic coculture immobilized in a fibrous-bed bioreactor. Biotechnol Prog 16:1008–1017

Thomas SP, Shanmugasundaram S (1992) Amino-Acid Overproduction by Analog Resistant Mutants of the Nitrogen-Fixing Cyanobacterium Anabaena Sp-287. Appl Biochem Biotechnol 37:111–121

Tisnadjaja D, Gutierrez NA, Maddox IS (1996) Citric acid production in a bubble-column reactor using cells of the yeast Candida guilliermondii immobilized by adsorption onto sawdust. Enzyme Microb Technol 19:343–347

Tokuyama S, Hatano K (1996) Overexpression of the gene for *N*-acylamino acid racemase from Amycolatopsis sp TS-1–60 in Escherichia coli and continuous production of optically active methionine by a bioreactor. Appl Microbiol Biotechnol 44:774–777

Tsai YC, Lin CS, Tseng TH, Lee H, Wang YJ (1992) Production and immobilization of d-aminoacylase of Alcaligenes-faecalis da1 for optical resolution of *N*-acyl-DL-amino acids. Enzyme Microb Technol 14:384–389

Tsay SS, To KY (1987) Citric-acid production using immobilized conidia of Aspergillus-niger tmb-2022. Biotechnol Bioeng 29:297–304

Wang F, Guoa C, Liua H, Liu C (2007) Reversible immobilization of glucoamylase by metal affinity adsorption on magnetic chelator particles. J Mol Catal B Enzym 48:1–7

Wang JL (2000) Production of citric acid by immobilized Aspergillus niger using a rotating biological contactor (RBC). Bioresour Technol 75:245–247

Wang JL, Liu P (1996) Comparison of citric acid production by Aspergillus niger immobilized in gels and cryogels of polyacrylamide. J Ind Microbiol 16:351–353

Wisdom R, Funnill P, Lilly M (1984) Enzymic interesterification of fats: Factors influencing the choice of support for immobilized lipase. Enzyme Microb Technol 6:443–446

Zhu Y, Wu ZT, Yang ST (2002) Butyric acid production from acid hydrolysate of corn fibre by Clostridium tyrobutyricum in a fibrous-bed bioreactor. Process Biochem 38:657–666