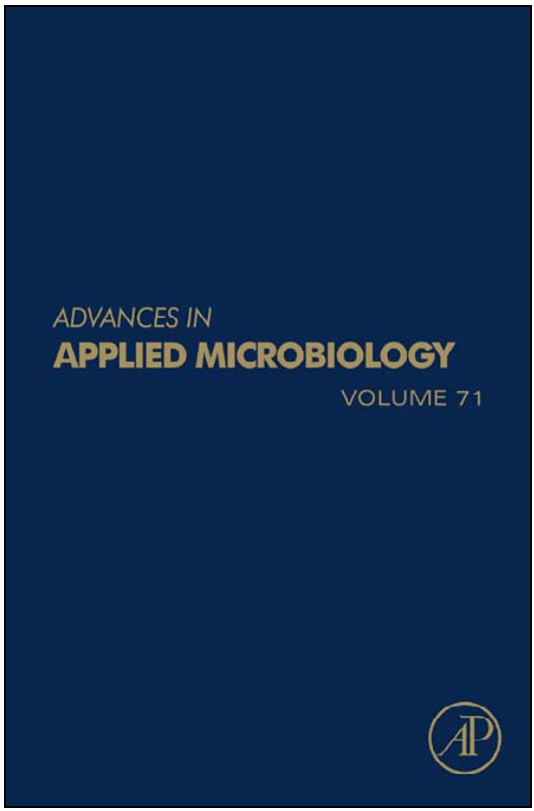

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CHAPTER 5

Cell Immobilization for Production of Lactic Acid: Biofilms Do It Naturally

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Abstract

Interest in natural cell immobilization or biofilms for lactic acid fermentation has developed considerably over the last few decades. Many studies report the benefits associated with biofilms as industrial methods for food production and for wastewater treatment, since the formation represents a protective means of microbial growth offering survival advantages to cells in toxic environments. The formation of biofilms is a natural process in which microbial cells adsorb to a support without chemicals or polymers that entrap the cells and is dependent on the reactor environment, microorganism, and characteristics of the support. These unique characteristics enable biofilms to cause chronic infections, disease, food spoilage, and devastating effects as in microbial corrosion. Their distinct resistance to toxicity, high biomass potential, and improved stability over cells in suspension make biofilms a good tool for improving the industrial economics of biological lactic acid production. Lactic acid bacteria and specific filamentous fungi are the main sources of biological lactic acid. Over the past two decades, studies have focused on improving the lactic acid volumetric productivity through reactor design development, new support materials, and improvements in microbial production strains. To illustrate the operational designs applied to the natural immobilization of lactic acid producing microorganisms, this chapter presents the results of a search for optimum parameters and how they are affected by the physical, chemical, and biological variables of the process. We will place particular emphasis upon the relationship between lactic acid productivity attained by various types of reactors, supports, media formulations, and lactic acid producing microorganisms.

I. INTRODUCTION

The first mention of biocomplexity in the form of a biofilm was in the dental plaque and visualized at the onset of microbiology by Leeuwenhoeck (1683) following the development of the first microscope. He described

them as different forms of ''animalculi'' adhering to his teeth. Biofilms are a complex society of interacting microbiological communities that attach to various materials at the solid–liquid interface (Branda *et al.*, 2005; Davey and O'toole, 2000). Remarkably, biofilms can have enhanced resistance to solvents and toxins when compared to their suspension counterparts (Anderson and O'toole, 2008; Hall-Stoodley *et al.*, 2004; O'Toole *et al.*, 2000). This distinction is observed when pathogenic microbes forming microbial biofilms exhibit enhanced resistance to antimicrobial agents and cause chronic infections and persistent disease (Brady *et al.*, 2008; Bryers, 2008; Costerton *et al.*, 1999; Fux *et al.*, 2005; Hall-Stoodley *et al.*, 2004; O'Toole *et al.*, 2000; Spormann, 2008). Biofilms commonly cause food spoilage (Kubota *et al.*, 2008), devastating corrosion that can wear away or block water pipes, and common problems associated with surfaces exposed to water, such as growth on ship hulls causing drag (Little and Wagner, 1997; Spormann, 2008). One of the latest developments in biofilm formation has taken place in the production of probiotics with increased resistance to environmental stress, such as more resilient *Bifidobacterium* or *Lactobacillus*. Factors encountered by these organisms during production and consumption also include adaptation to conditions encountered in the gastrointestinal ecosystem. Therefore, biofilm formed on the surface of food particles leads to the development of cultures with improved capacity to survive and function during production and passage through unfavorable conditions within the digestive tract (Cinquin *et al.*, 2004; Lacroix and Yildirim, 2007; Macfarlane *et al.*, 1997; Probert and Gibson, 2002).

Apart from their natural environments, where biofilms are widely distributed, they have found an application in biotechnology as an immobilization method. Today, natural immobilization as an industrial application is widely used for the treatment of wastewater, desulfurization of gas, and food production (Kornaros and Lyberatos, 2006; Lazarova *et al.*, 2000; Linko and Linko, 1984; Majumder and Gupta, 2003; Nicolella *et al.*, 2000), as well as for converting agriculturally derived materials into alcohols and organic acids such as acetic acid, ethanol, and butanol (Crueger and Crueger, 1990; Demirci *et al.*, 1997; El-Mansi and Ward, 2007; Ho *et al.*, 1997; Qureshi *et al.*, 2004, 2005; Wang and Chen, 2009).

Biofilm as a technology applied to production allows high cell density processes, improves cell stability, enables continuous operation, and reduces downstream processing needed to separate product from cells. From an industrial standpoint, cell immobilization is becoming one of the most useful methods for increasing catalyst concentration in bioreactors and, as a result, the rate of product generation. Biofilm-based processes also present economic and environmental advantages such as the reduction in waste and side products and the low cost of immobilization materials. While operating immobilized cell reactors, the biological catalyst is kept fixed in a natural or artificial matrix and substrates and

products continuously flow in the mobile phase. The production rates can be accelerated by increasing cell density in the bioreactor or through a lengthy process of selection and development of hyperproductive cells (Davidson *et al.*, 1995; Demirci and Pometo, 1992).

As shown in the following lines, immobilized cells exhibit protective features against toxic substances and other adverse conditions, in addition to an increase in plasmid stability and increased metabolic activity with respect to their suspension cell planktonic counterparts (Anderson and O'toole, 2008; Cassidy *et al.*, 1996; Groboillot *et al.*, 1994). A number of studies have also exploited biofilm processes without cellular degeneration or contamination over several months, strengthening biofilm's potential as a feasible industrial application (Cho *et al.*, 1996; Hekmat *et al.*, 2007; Lewis and Yang, 1992; Qureshi *et al.*, 2005; Xia *et al.*, 2005). Problems with biofilm-based processes have included excessive biomass growth and cell shedding. Nutrient limitations and flow rate adjustments have been used to control cell growth while maintaining an active productive state. Technical improvements incorporate enhanced reactor designs and supports that can sustain controlled biofilm formation and growth (Bruno-Bárcena, 1997; Bruno-Bárcena *et al.*, 2001; Huang *et al.*, 1998; Qureshi *et al.*, 2005). Ultimately, development of new strains through recombinant technology and/or screening with the aim of finding microbial strains suitable for long-term production are viable options for solving the above-mentioned problems. This review examines lactic acid production using biofilm reactors. Special emphasis is placed on reactor design, support type, microorganism employed, nutrient source, and long-term stability. The overall aim is to present a viable technology suitable for biological lactic acid production.

II. BIOFILM FORMATION

When cells come in contact with wet surfaces, they show an adherent phenotype which results in colonization of the surface, for example, cells growing in suspension tend to adhere to the wall of the container or to any solid support immersed in the liquid. If the material is porous, there is an increase in the surface area available for cells to bind to the external and internal surface structures. This type of cellular dynamic is known as immobilized cells naturally attached to a surface, or biofilm growth. Cell attachment is a critical step in the multilayered process of biofilm generation. By attaching to organic and/or inorganic materials, cell growth results in microcolonies on their surfaces and, ultimately, mature biofilms are formed. An important factor throughout biofilm formation is the production of polymers that allows many individual adherent cells (sessile cells) to bond together, providing structure to the microcolonies

during formation and growth. To trigger this process it is postulated that cells can produce and sense molecules, allowing the whole population to initiate a concerted action once a critical concentration (corresponding to a particular population density) of the molecule has been reached, a phenomenon known as quorum-sensing. AI-2 is suggested to be a universal bacterial signaling molecule synthesized by the LuxS, which forms an integral part of the activated methyl cycle. Moreover, previous reports have shown that the well-documented probiotic strain *Lactobacillus rhamnosus* GG, a human isolate, produces AI-2-like molecules (Lebeer *et al.*, 2007). In fact, many potentially probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* strains possess a *luxS* homologue (Altermann *et al.*, 2005; Azcarate-Peril *et al.*, 2008; Buck *et al.*, 2009) and can produce AI-2. The role of *luxS* in the adhesion of *L. acidophilus* and *L. rhamnosus* GG to surfaces has been investigated. The same genomic organization of the *luxS* gene has been found in *L. acidophilus*, *L. casei*, and *L. gasseri* (Buck *et al.*, 2009; Rodionov *et al.*, 2004)

Functional biofilm formation and growth generally involve the following series of events:

- *Absorption to surfaces*. The ability of microorganisms to adsorb to exposed surfaces is dependent on the characteristics of the support, the microorganism, and the environment. Rough surface textures and electrostatic forces on the surface (Van der Waals forces as well as ionic and hydrogen bonds), have been suggested to promote cell settlement and biofilm growth (Goller and Romeo, 2008). Cell adhesion is also dependent on the physiological state of the microorganism, mass transport phenomena, and response to chemical concentration gradients such as motile attachment using flagella or chemotaxis (Annachhatre and Bhamidimarri, 1992; Gjaltema *et al.*, 1994; Hekmat *et al.*, 2007; Li *et al.*, 2007; Pedraza *et al.*, 2009; Ragout *et al.*, 1996). The hydrodynamic conditions of the medium can also favor adherence to the surface by minimizing cell shearing. Many microorganisms react to excessive turbulence and shearing forces by inducing a global genetic response that causes a complete modification of cell surface components including flagella, fimbriae, pili, capsule, and other cell-wall polysaccharides (Lawrence *et al.*, 1995).
- *Polysaccharide production*. Once the cells are in contact with the surface and the conditions are adequate, biofilm formation is further strengthened by cell growth and synthesis of extracellular polymeric substances (EPS) which can consist of special combinations of polysaccharides, proteins, carbohydrates, DNA, and lipids, depending on the microorganisms and environmental conditions (Karatan and Watnick, 2009; Steinberger and Holden, 2004; Sutherland, 2001). The polysaccharide will eventually build bridges between cells providing a type of support

that can overcome electrostatic repulsion allowing negatively charged cells to adhere to both positively and negatively charged surfaces (Little and Wagner, 1997).

- *Biofilm maturing process*. As maturation progresses, the heterogeneous distribution of cells and the diffusion limitations within the organization form a differentiated biofilm with unique characteristics that affect cell physiology and fermentation activities (Stewart and Franklin, 2008; Werner *et al.*, 2004; Xu *et al.*, 1998). Biofilm populations often contain cells resistant to toxic compounds and specialized in aerobic or anaerobic growth, fixation to surfaces, and food scavenging, as well as cells that synthesize protective films. The specific subpopulations have been suggested to form channels used for substrate delivery, waste removal, and product delivery. This architecture of circulatory channels within the biofilm is strongly dependent on its structure and the hydrodynamic conditions (sheer forces) applied (Beyenal and Lewandowski, 2001; Costerton *et al.*, 1995; De Beer and Stoodley, 1995; De Beer *et al.*, 1994; O'Toole *et al.*, 2000).
- *Planktonic cell dispersal*. Following maturation, planktonic cells detach and/or disperse from the mature biofilms. Factors such as nutrient availability and cell density have been suggested to trigger biofilm dispersal (Spormann, 2008; Stoodley *et al.*, 2002). Detachment of planktonic cells is often seen as a mechanism of dispersion to colonize new surfaces (Costerton *et al.*, 1999; Hall-Stoodley *et al.*, 2004; Stoodley *et al.*, 2002). These mechanisms have been suggested to be quorum-sensing-regulated swarming phenotypes, or flagella-driven movement of differentiated swarmer cells (hyperflagellated, elongated, multinucleated) by which bacteria can spread as biofilm over surfaces (Daniels *et al.*, 2004). Studies with immobilized reactors working in two-stage systems have shown that the phenomena of physical detachment may also be due in great measure to shear forces that can separate pieces of biofilm in the flow. The detached pieces are in fact capable of efficient colonization on new surfaces (Bruno-Bárcena *et al.*, 1999).

III. APPLICATION OF BIOFILMS TO LACTIC ACID PRODUCTION

Lactic acid (2-hydroxypropanoic acid) is a chiral molecule with two optical enantiomers, L(+) and D(–), widely used by the pharmaceutical, plastics, food, and cosmetic industries (VickRoy, 1985). Lactic acid is either produced by chemical synthesis or microbial fermentation. To meet the growing demand for lactic acid, a number of different strategies have been pursued to improve productivity, accelerate

production rates, and reduce cost. Lactic acid production rates have been improved through increased cell density, use of systems such as multiple fiber reactors (Vick Roy *et al.*, 1982), cell recycling (Ohleyer *et al.*, 1985; Vick Roy *et al.*, 1983), cell entrapment in polymers such as κ-carrageenate or calcium alginate (Audet *et al.*, 1988; Boyaval and Goulet, 1988; Guoqiang *et al.*, 1991; Salter and Kell, 1991; Smidsrod and Skjakbraek, 1990), strain development (Demirci and Pometo, 1992), as well as cell immobilization on activated inert supports (Avnir *et al.*, 2006; Guoqiang *et al.*, 1992; Senthuran *et al.*, 1997). Others have analyzed the influence of support characteristics (Demirci *et al.*, 1993a,b; Gonçalves *et al.*, 1992) and also used strains with the ability to form biofilms to generate lactic acid (Bruno-Bárcena *et al.*, 1999; Demirci and Pometo, 1995; Krischke *et al.*, 1991).

Although there is abundant literature on lactic acid production by immobilized cells, most report poor productivity or low final metabolite concentrations (Norton and Vuillemard, 1994). Generally, production will depend on many parameters that influence the dynamics of the bioprocess including characteristics of the microorganism, reactor configurations, carbon source, nitrogen source, support type, and quantity and health of the immobilized biomass. Moreover, a major challenge continues to be the lactic acid inhibition of cell growth during production and accumulation (Friedman and Gaden, 1970).

IV. HOST ORGANISMS

The lactic acid bacteria group includes more than 125 species and subspecies (http://www.bacterio.cict.fr/). While a number of organisms capable of forming biofilms have been tested for metabolite production, homofermentive *L. rhamnosus* and *L. casei* have been well studied and are currently the most widely used species for lactic acid production. Since the description of a chemostat selection method to obtain an adhesive phenotype from an originally nonadherent strain of *Sreptococcus salivarius* subsp. *thermophilus* CRL 412 (Ragout *et al.*, 1996), only a small number of strains able to form biofilms have been tested for production: *L. rhamnosus* (RS 93), capable of forming biofilms, derived from *L. rhamnosus* DSM 20021 (Ragout *et al.*, 1995) and *L. casei* ADNOX95 (Culture collection of PROIMI, Tucumán, Argentina) an offspring of *L. casei* subsp. *casei* CRL 686 (Centro de Referencia para Lactobacilos, Tucumán, Argentina) (Bruno-Bárcena, 1997). Other biofilm formers were also recently reported for *Lactobacillus delbrueckii* NCIM 2365 on polyurethane foam (Rangaswamy and Ramakrishna, 2008) and *Lactobacillus casei* subsp. *rhamnosus* (ATCC 11443) (Table 5.1).

TABLE 5.1 Biofilm-forming strains used for production of lactic acid

Strain	Reference
Lactobacillus casei subsp. *casei* (DSM 20244)	Krischke *et al.* (1991)
Lactobacillus delbrueckii NRRL B445 renamed *L. rhamnosus*	Gonçalves *et al.* (1992)
Lactobacillus helveticus (ATCC 15009)	Silva and Yang (1995)
Lactobacillus rhamnosus RS 93	Ragout *et al.* (1995)
Streptococcus salivarius subsp. *thermophilus* CRL 412	Ragout *et al.* (1996)
Lactobacillus casei (ADNOX95)	Bruno-Bárcena *et al.* (1998, 1999)
Lactobacillus casei (ATCC 393)	Daraktchiev *et al.* (1997)
Lactobacillus casei subsp. *rhamnosus* (ATTC11443)	Ho *et al.* (1997), Cotton *et al.* (2001), Velazquez *et al.* (2001), Demirci *et al.* (2003)
Lactobacillus delbrueckii (NCIM 2365)	Rangaswamy and Ramakrishna (2008)

A. Optical purity of the product

Lactic acid bacteria strains usually generate a racemic mixture of L(+) and D(−) isomers of lactic acid. However, it is common to find organisms that are able to produce each isomer independently (Garvie, 1980). Microbial production has the advantage of being able to use strain selection to obtain an optically pure product, whereas synthetic production always results in a racemic mixture of lactic acid. The production of L(+)-lactate isomer is of significant importance to mammals since it is the only form that can be assimilated. Thus, optically pure L(+) lactic acid is the preferred and recommended choice for food industries (FAO/WHO, 1974). Optically pure L(+)-lactate is also required in the biomedical industry (Lipinsky, 1981; Tsai and Moon, 1998) and for manufacturing polymers such as polylactic acid (PLA) degradable plastics (Rincones *et al.*, 2009).

Since 1919 (Orla-Jensen, 1919), the type of isomer and its racemic proportion in the fermented broth have been used as a taxonomic characteristic. Current methods of lactate detection allow the quantification of millimolar amounts of each isomer previously impossible to detect (Gawehn and Bergmeyer, 1974). As a result of this improved detection method, species formerly assigned as producers of only one isomer have been recataloged. Some relevant cases have been *L. casei* ssp., previously

considered to exclusively produce L(+) lactate, and *L. delbrueckii* ssp. *bulgaricus* cataloged as a D(–) lactate exclusive producer (Dellaglio and Torriani, 1985; Ragout *et al.*, 1989).

It is well established that the biochemistry behind the generation of each lactate isomer from pyruvate requires the action of stereospecific enzymes called NAD-dependent lactate dehydrogenases (nLDHs EC 1.1.1.27 and EC 1.1.1.28). L(+) nLDHs are highly homologous proteins in amino acid sequence, diverse in size and behavior, and the allosteric group requires the action of additional molecules such as fructose 1, 6 diphosphate and Mn^{+2} to modulate its function. D(–) nLDHs are enzymes with diverse evolutionary origins. They are more closely related to the family of D-isocaproate dehydrogenases with high levels homology that indicate recent evolutionary divergence (Bhowmik and Steele, 1994; Delcour *et al.*, 1993; Taguchi and Ohta, 1999). D(–) nLDHs enzymes do not catalyze the oxidation of pyruvate *in vivo*; hence, cells also possess a different group of stereospecific enzymes named NAD-independent lactate dehydrogenases (iLDHs). However, in the lactic acid bacteria group iLDHs are not active when lactate is being actively generated. The last family of enzymes that can affect the L(+) and D(–) racemic ratio are the lactate racemases, though these enzymes are not frequently found in lactic acid bacteria (Garvie, 1980).

It has been suggested that the ratio of isomers generated by *L. delbrueckii* ssp. *bulgaricus* may be a function of nutrient availability and influenced by the initial concentration of carbon and energy source (Ragout *et al.*, 1989). This possible nutrient–isomer connection was later tested by evaluating the consequence of initial glucose concentration on isomer proportion from *L. casei* CRL686 (Bruno-Bárcena, 1997) (Fig. 5.1). The linearly of the plot clearly indicated that the isomer proportion was independent of the initial glucose concentration. These results suggest the isomer ratio may be organism-specific and differences in response to glucose limitations may require significant product profile studies before being placed in production. Speculation has also focused on whether the duration of production influences the behavior of the microorganism in a manner that favors creation of one lactate isomer over the other (Bruno-Bárcena, 1997). This was the case for artificially immobilized *L. rhamnosus*, where the proportion of L(+)-lactate could be correlated to the cells' age (Hjörleifsdottir *et al.*, 1990), and in two-stage chemostat, *L. helveticus* fermentation time and growth rate varied the fraction of L(+)-lactate from 55% to 70% (Aeschlimann *et al.*, 1990). The fraction of the L(+) isomer also increased with the dilution rate in continuous processes with cell recycling (Aeschlimann and Vonstockar, 1991). In contrast, the product and biomass yields remained constant and the ratio of L(+)- and D(–)-lactate isomers were not affected by the dilution rate or production time using continuous culture in chemostat operations with *L. coryniformis* and

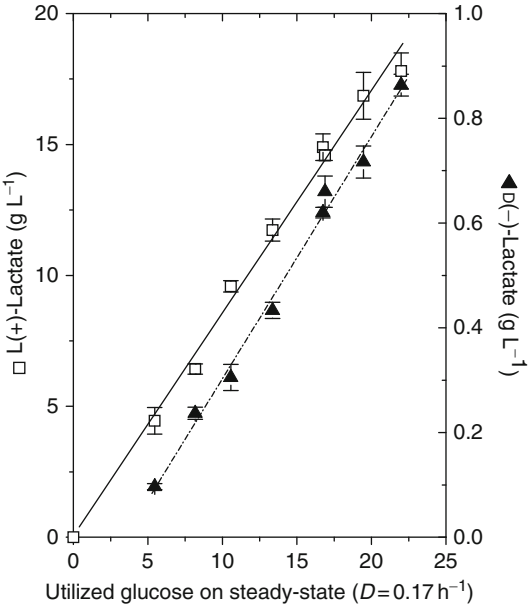

FIGURE 5.1 Lactic acid isomer concentrations generated by *Lactobacillus casei* CRL 686 during steady-state fermentation (D = 0.17 h^{-1}) at 37 °C in BFRS medium as a function of the glucose concentration.

L. casei (González-Vara *et al.*, 1996). However, the percentage of the D(–) fraction almost linearly increased from 4% to 18% during the long, 8-day stationary phase in a batch process of *Lactobacillus case* ssp. *rhamnosus* (Hjörleifsdottir *et al.*, 1990). Therefore, the isomer ratio attained will be strain-specific and must be taken into consideration for the production process. Clearly, young cells produce more L(+)-lactate then older ones, and consistently high L(+)-lactate production can be correlated with elevated growth rate. Production values evaluated as OP% = 100 × {L(+) − D(−)}/{L(+) + D(−)} presented later in this review reveal that biofilm age (with natural adhered cells) does not appear to influence the final optical purity. However, batch cultures of cells in suspension and artificially immobilized cells will eventually show the symptoms of age caused by nutrient depletion or confined growth. One of the most beneficial steps for increasing total product output is related to the ability to prolong the duration of the process. This has been demonstrated with extended production cycles from regenerated natural adherent biomass that provides a constant stream of new cells and also counteracts the variable physiology of isomer production observed in conventional processes.

V. CELL IMMOBILIZATION

Cell attachment has been explored with a variety of support materials including wood chips (Krischke *et al.*, 1991), porous bricks and cotton cloth (Gonçalves *et al.*, 1992), glass and ceramics (Guoqiang *et al.*, 1992), foam (Dong *et al.*, 1996), and plastic composite supports (PCS) (Demirci and Pometo, 1995; Demirci *et al.*, 1993a,b; Guoqiang *et al.*, 1992; Ho *et al.*, 1997; Velazquez *et al.*, 2001). A number of supports have also been evaluated for biofilm formation for lactic acid production (Demirci *et al.*, 1993b). Cell immobilization has not been limited to chemical cross-linking and polymer entrapment; for example, when used in conjunction with natural biofilm former strains, those strains can entrap non-natural biofilm formers. Among different strains tested as helpers for entrapment good results were obtained using *Pseudomonas fragi*, *Streptomyces viridosporus*, and *Thermoactinomyces vulgaris* (Demirci *et al.*, 1993a).

Other common supports have included polyurethane foams (PUFs) (Rangaswamy and Ramakrishna, 2008). Testing support materials for biofilm reactors have benefited lactic acid production methods: increasing lactic acid production rates, minimizing lag phase, rising tolerance to high glucose concentrations, reducing requirement for micronutrients, increasing cell density (Demirci *et al.*, 2003; Velazquez *et al.*, 2001), and reducing the toxic effects from solvent leakage (Demirci *et al.*, 2003). Characteristics of some common supports are displayed in Table 5.2.

A. Cell immobilization by entrapment and attachment

The cell immobilization method selected can influence cell physiology, metabolism, and distribution of cells in the matrix, resulting in variable outcomes. If cell immobilization is accomplished by entrapment or cross-linking to a support, there is generally good initial performance, but the cells decrease in viability and productivity over time and must be replaced (Guoqiang *et al.*, 1991, 1992). Furthermore, covalent binding to a support has the major disadvantages of high cost and low yield due to exposure of the cells to toxic reagents or severe reaction conditions (Tanka and Kawamoto, 1999). Cell entrapment with calcium alginate or k-carrageenan beads have been used to increase cell density; nonetheless, industrial applications are not feasible because of bead disintegration, slow leakage of cells during lengthy continuous operation, and mass transfer limitations (Audet *et al.*, 1988; Boyaval and Goulet, 1988). Shrinkage and reduced strength of the entrapment during lactic acid fermentation has been attributed to the displacement of Ca^{+2} ions by lactate ions in alginate beads (Roy *et al.*, 1987).

TABLE 5.2 Characteristics of supports used for microorganism's immobilization

Supports	Form	Material	Average pore diameter (μm)	Porosity (%)	Density (g cm^{-3})	Reference
Poraver®[a]	Beads	Recycled glass	<200	49	0.225	Bruno-Bárcena (1997), Bruno-Bárcena *et al.* (1999), Gonçalves *et al.* (1992), Ragout *et al.* (1995)
	Beads	Sintered glass	60–300	55–60	2.4	Krischke *et al.* (1991), Gonçalves *et al.* (1992)
Rings	Raschig	Sintered glass	60–100	60		Gonçalves *et al.* (1992)
Bi86	Irregular particles	Ceramic	22	70	3.21	Gonçalves *et al.* (1992)
Plastic carbon support (PCS)	Disk	Polypropilene and organic[b]				Cotton *et al.* (2001), Demirci *et al.* (2003), Ho *et al.* (1997), Velazquez *et al.* (2001)
Polyurethane	Foam		Not reticulated	91		Bruno-Bárcena (1997)
Cotton fibers			Not reticulated			Silva and Yang (1995)

[a] Dennert Shaumglass GmbH, 8439 Postbauer-Heng, Gewerbebiet Ost, FRG.
[b] 50% (w/w) polypropylene, 35% (w/w) ground soybean hulls, 5% (w/w) yeast extract, 5% (w/w) soybean flour, and 5% (w/w) bovine albumin.

B. Natural cell attachment

Cell adhesion and adsorption is common in natural systems, though strains and species differ in their ability to adhere to surfaces. Adsorption through electrostatic interaction to solid supports can be achieved by treating either the microbial cells or the support matrix with cations (Thonart *et al.*, 1982). Also, adsorption on inert supports allows bacteria to form biofilms, although the properties of the attachment surface do impact biofilm formation. For example, rough surfaces can enhance biofilm formation because of increased surface area for cells to attach and decreased shear forces near the surface. Shear forces will also be low within pores of porous material, providing a protective environment for cells to attach and grow. Hydrophobicity and ionic charge of the surface material also increase biofilm formation. Numerous studies using continuous processes and diverse cellular supports have focused on increasing adhesion properties of the support matrix. Such adhesive properties can also be varied to create a more functional three-dimensional structure for greater ability to control cell concentration and the mass-transfer properties in the reactor. One method used for increasing biofilm reactor productivity is to increase the support attachment surface area by growing biofilms on porous support materials or plastic composite supports. An earlier report studied a PCS packed-bed biofilm reactor for lactic acid production from rich media and polypropylene chips blended with various agricultural materials as support for biofilms with pure and mixed cultures (Demirci and Pometo, 1995). It was theorized that the agricultural material blended in the with polypropylene forming the PCS probably stimulated biofilm formation on the support surface by serving as a carbon and/or nitrogen source, increasing adsorption of the microorganism to the surface or creating a favorable surface energy.

C. Free and adhered biomass

Since the complex biology of biofilm formation tends to vary among organisms, of greatest concern is the possibility of cell attachment fluctuations due to the dynamic nature of biofilms. For this reason, long-term studies must be carried out with each particular strain to evaluate its benefit to the process. Estimates of free and adhered biomass should be evaluated at the end of assays. Table 5.3 shows that the percentages of free cells with respect to total cell number in Poraver®- and polyurethane-filled reactors for strains RS93 and ADNOX95 performed at two dilution rates. The values for Poraver-filled reactors were 5.8% for strain RS93 at $D = 0.3\ h^{-1}$ and 3.18% at $D = 0.55\ h^{-1}$. For strain ADNOX95, the values were 4.87% at $D = 0.3\ h^{-1}$ and 3.36% at $D = 0.5\ h^{-1}$. In the case of the polyurethane-filled reactors, the percentages were 2.29% at $D = 0.3\ h^{-1}$

TABLE 5.3 Biomass values for planktonic and sessile cells in two up-flow packed-bed reactors inoculated with two biofilm forming-*Lactobacillus* strains

	Planktonic cells (g l^{-1})		Sessile cells (g l^{-1})
Strain	$D = 0.3\ h^{-1}$	$D = 0.55\ h^{-1}$	Final
Reactor filled with Poraver®			
RS93	4.0	2.2	69.1
ADNOX95	4.2	2.9	86.2
Reactor filled with polyurethane foam			
RS93	4.0	2.9	174
ADNOX95	4.9	3.3	174

Values are the means of at least two independent determinations. Free biomass was measured by dry weight determination: Cells were centrifuged at 5000 × g for 10 min, washed with distilled water, and dried at 105 °C. The immobilized biomass was determined by weight differences of a measured volume of the matrix dried at 105 and 550 °C.

and 1.66% at $D = 0.55\ h^{-1}$ for strain RS93; in the case of strain ADNOX95, the percentages were 2.81% at $D = 0.3\ h^{-1}$ and 1.9% at $D = 0.5\ h^{-1}$. Adhered biomass in polyurethane-filled reactors was 174 g l^{-1} for both strains and there were no significant differences between the two strains in the case of Poraver®-filled reactors. Poraver® beads and polyurethane foam supports showed adsorption after 7 days of incubation and continued to improve with time, indicated by the decrease in the percentage of free cells, even with decreasing feed rates. Cell adhesion to both supports was very high (as shown by the sessile/planktonic ratio), indicating that the production kinetics of the system was primarily controlled by the sessile cells in both packed reactors. It is important to consider that, due to the substantial structural differences between the supports, the performance of the cells attached to Poraver® was substantially enhanced compared to those attached to polyurethane foam. The same behavior in specific productivity reduction could be obtained by decreasing the Poraver® bead size, which resulted in higher biomass accumulation. Therefore, very small bead sizes (<200 μm) can become a double-edged sword (Gonçalves *et al.*, 1992). The reason for this phenomenon is that at a given dilution rate the velocity of media or ascensional velocity increases as the bead size decreases due to the reduced active volume or the space between the biomass-covered beads. This increase in velocity can result in preferential flow paths, higher biomass sheering, lower assimilation of nutrients and, consequently, lower volumetric product formation.

VI. CULTIVATION MEDIA

Nutrient availability in the culture medium can obviously affect the specific growth rate and impact biofilm formation, thereby altering substrate conversion and productivity (Aeschlimann and von Stockar, 1990). Homolactic bacteria are demanding in terms of their nutritional requirements; consequently, a great number of the lactic acid fermentation processes have been conducted with nutrient-rich media which favor biofilm generation. The high cost of rich media is of particular concern for moving lactic acid production to industrial levels when considering downstream processing and production separations. In order to reduce downstream processing to a minimum, the preferred compound choice should be feedstock low in nitrogen, low effluent cell mass production, and negligible amounts of residual substrate or other biological products (Córdoba *et al.*, 1999; Raya-Tonetti *et al.*, 1999; Senthuran *et al.*, 2004). Nitrogen sources used in lactic acid fermentation include steep liquor, malt sprout extract, casein hydrolysate, whey permeates, and yeast extract. The most common effective media supplement has traditionally been yeast extract; however, yeast extract is expensive and, when its concentration exceeds 1% in the medium, the optical purity of lactic acid in the fermented broth can be reduced by the D(–)-lactic acid inherently found in yeast extract (Bruno-Bárcena *et al.*, 1998, 1999). Previous studies in chemostat carried out for media optimization showed that *L. casei* subsp. *casei* CRL 686 could result in the same biomass yields in medium BRFS as in MRS (Bruno-Bárcena *et al.*, 1998). Growth of the strain RS93 was compared in BRFS and MRS media using the R3 reactor design, (Fig. 5.2) filled with Poraver® beads. Maximal productivity values were comparable to those obtained by Ragout *et al.* (1995), 8.7 and 14.9 $g\,l^{-1}h^{-1}$, using GS medium (Guoqiang *et al.*, 1992) (Fig. 5.3). Similar tendencies were observed in volumetric productivity with both culture media at similar dilution rates, demonstrating that both media are suitable for growth and production of biofilms. The simplicity of medium BFRS makes it more than adequate for downstream processing (Córdoba *et al.*, 1999; Raya-Tonetti *et al.*, 1999), and its low cost makes BFRS medium more cost effective for the continuous production of lactic acid.

Another feedstock for lactic acid production of particular interest is whey, a byproduct from cheese production. Lactose (4–5%) is the major component of whey solids in addition to minerals, proteins, and water-soluble vitamins (Marwaha and Kennedy, 1988; Tuli *et al.*, 1985). Whey permeate, along with added supplements, can provide the complex nutrients demanded by *L. casei* (Table 5.2). However, *L. casei* cells in batch fermentation are unable to utilize untreated or unsupplemented whey,

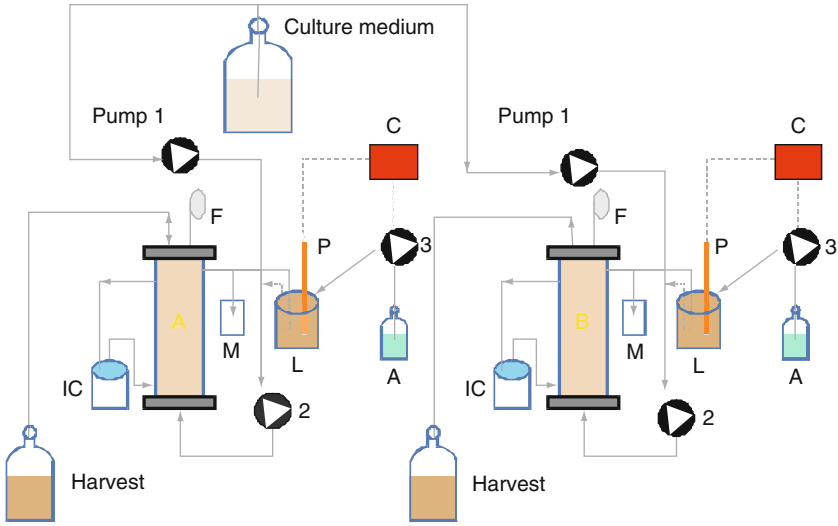

FIGURE 5.2 Schematic diagram of two up-flow packed-bed reactors (R3). A, alkali; 1, feed pump for complete medium; 2, recycling pump; 3, alkali pump; C, pH controller; F, air filter; IC, heat exchanger; L, external pH controller device; M, sampling port; R, reactor; P, pH electrode.

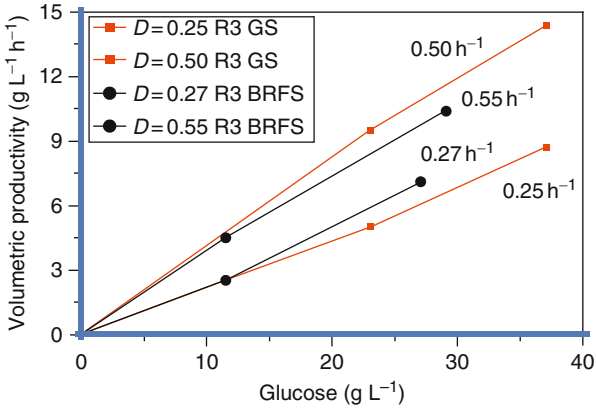

FIGURE 5.3 Volumetric productivities as a function of glucose concentration of *L. rhamnosus* RS93, at two dilution rates in different culture media using Poraver® as support and (R3) reactor design. In each case, the reactors were inoculated with 6% (v/v) of an overnight culture.

leading to poor productivity and incomplete sugar utilization. Alternatively, whey hydrolysates can serve as an inexpensive nitrogen base and carbon source. Nevertheless, pretreatment of whey with protease or supplementation contributes to the overall cost of lactic acid production and is not desirable for large-scale industrial processes. Lactic acid fermentation in unsupplemented whey medium has been reported for lactic acid bacteria. *L. helveticus* immobilized to a continuous fibrous bed reactor resulted in productivity of 5 g l^{-1} h^{-1} with total lactose conversion in unsupplemented acid whey; productivity was further improved by supplementing with yeast extract, indicating that substrate utilization was incomplete (Krischke *et al.*, 1991; Senthuran *et al.*, 1997; Silva and Yang, 1995). Media components were also used to investigate the performance of immobilized *L. rhamnosus* on a sintered glass support in a continuous recycle tubular reactor at different substrate concentrations. The observed volumetric productivity increased when the substrate concentration was raised from 50 to 100 g l^{-1} while complete conversion occurred only when 50 g l^{-1} of glucose or very low dilution rates (>0.1 h^{-1}) were used (Gonçalves *et al.*, 1992). Bear in mind that industrial processes require high substrate conversion rates because unused carbon sources such as glucose can complicate extraction and final purification of lactic acid.

VII. BIOFILM APPARATUS AND OPERATION

The popularity of lactic acid production over the past two decades has led to the development of a number of different reactor configurations designed especially to improve volumetric productivity. Reactor productivity is directly correlated with the reaction rate, which can be improved by increasing cell biomass to a point where volumetric productivity remains positively correlated to high substrate utilization. In this context, high cell concentrations are achieved by adsorption to supports naturally, without chemicals or artificial entrapment. Biofilm reactors allow continuous fermentation by providing a faster flow of fresh media to the cells, eliminating nutrient limitations, and increasing cell surface/volume ratios. Compared to conventional free cell systems, biofilm reactors show improved productivity, simplify separation of cells from products, and operate at high dilution rates without cell washout. Likewise, biofilm reactors are extremely adequate for microaerophilic or anaerobic processes though there is a loss in stability in processes where gas generation occurs. Important parameters considered in reactor designs have been volumetric productivity, cell attachment, and biofilm stability. The media compositions, cell adsorption supports, and microorganisms have been important components in studying new reactor designs.

A number of biofilm reactor configurations have been tested for lactic acid production including batch reactors (BRs), continuous-stirred tank rectors (CSTRs), fluidized-bed reactors (FBRs), and packed-bed reactors (PBRs).

Batch biofilm reactors are not rationally used for industrial fermentation procedures since nutrients are provided by emptying and refilling the reactor rather than by continuous flow, increasing downtime and reducing productivity. However, batch reactors have been used to quickly evaluate new conditions before moving to other reactor designs. The performances of repeated batch fermentations were evaluated with and without polypropylene composite supports (PCS) consisting of polypropylene, soybean hulls, soybean flour, yeast extract, dried bovine albumin, and mineral salts. In repeated immobilized batch cultures, PCS were shown to perform better for L-lactic acid production than polypropylene alone. In those experiments, cultures of *Streptomyces viridosporus* T7A ATCC 39115 formed a biofilm that was utilized to entrap *Lactobacillus casei* subsp. *rhamnosus* ATCC 11443 (Demirci and Pometo, 1995). Suspended culture and repeated fed-batch cultures with PCS biofilm were used to evaluate control of yeast contamination with nystatin (Velazquez *et al.*, 2001). Chemical mutagenesis was used to develop enhanced strains of *Lactobacillus* with faster growth rates and higher product yields in various media modifications in batch fermentations and continuous fermentations (Demirci and Pometo, 1992).

CSTRs attempt to achieve perfect mixing of the reactor contents with more efficient control of the temperature and pH than in high-density systems. Unlike the PBR, the support cannot be packed into the bioreactor; however, fibrous types of bed supports can be used for adsorption of cells. This system is well suited for reactions in which substrate inhibition is problematic and stable productivity is crucial. Examples of continuous biofilm reactors used to study lactic acid production include the evaluation of PCS tubes to improve the growth of *L. casei* (Cotton *et al.*, 2001). This type of reactor was used with PCS tubes fixed to the agitator shaft to favor biofilm generation, whereas the biofilm thickness on the PCS tubes was controlled by the agitation speed. More recently, two reactor systems composed of a stirred-tank bioreactor coupled to a packed-bed biofilm column operating in continuous mode were used to develop a functional biofilm of *L. delbrueckii* (Rangaswamy and Ramakrishna, 2008).

The *PBR* is most commonly used for lactic acid fermentation. The support can be readily packed into a column and the feed can be moved across the bed of adhered cells. The substrates are usually fed from the bottom and the product is collected from the top of the reactor. The R3 reactor has been evaluated for long-term activity and enhanced tolerance to toxins and these characteristics were used to evaluate the production of lactic acid and performance using foam glass beads (Poraver®) and

polyurethane foam (Fig. 5.2). This type of reactor design has low contamination potential, operational simplicity, and low cost. Examples of PBRs include the two-stage, two-stream immobilized upflow PBR system used to obtain high productivity and high lactic acid concentrations. This operational system keeps production costs down by using a simplified medium while maintaining overall yeast extract concentration at 5 g l^{-1} (Bruno-Bárcena *et al.*, 1999).

In *FBRs* the biofilm surrounding the adsorbent particles are kept in motion by a continuous flow of the substrate, offering good solid–fluid mixing with minimal pressure drops. Biofilm surrounding the adsorbent particles also allows long operation times compared to PBRs and CSTRs. Unlike PBRs, the media is recycled and less biofilm formation and blockage is observed. The use of FBRs allows the testing and use of feed without pretreatment. For example, FBRs and CSTRs were used to evaluate culture media consisting of whey permeate and various supplements, which enabled exponential growth of *L. casei* (Krischke *et al.*, 1991).

A. Comparison between productivities of *L. rhamnosus* cells immobilized through natural adhesion to different supports in an identical reactor design

Figure 5.2 provides an example of reactor configurations applied to biofilm lactic acid production processes in the form of two immobilized PBRs operated in parallel (R3 reactor). As an example of operational similarity, lactic acid production by natural biofilm systems using strains from different origins have been summarized in Table 5.4. The parallel reactors A and B shown in Fig. 5.2 were inoculated with *L. casei* RS93 and *L. casei* ADNOX95, respectively. Those homofermentative adherent variants were previously obtained from *L. casei* subsp. *casei* CRL 686 and *L. rhamnosus* DSM 20021, respectively. Reactors were run in parallel for each strain and support type used. The recirculation rate was adjusted to a 50:1 ratio to ensure that pH levels are maintained and nutrient gradients do not develop inside the reactors. The dilution rate was increased stepwise to test different steady states. After a month of operation at the highest rate, the initial dilution rate was reset to check the system's stability. This series of assays analyzed the ability of the strains to adhere to a support and to produce lactic acid (considering kinetic and yield parameters) (Table 5.1). For reactors filled with Poraver®, the results showed that glucose conversions ranged from 61.1% to 100% in the reactor inoculated with strain RS93 and between 85% and 96.8% when using strain ADNOX95. Similar yields were obtained with both strains. Strains RS93 and ADNOX95 showed similar productivity results though strain ADNOX95 presented a slightly higher value (11.9 g $l^{-1}h^{-1}$ for $D = 0.55$ h^{-1}) associated with a higher conversion rate. At the highest

TABLE 5.4 Continuous fermentation at 42 °C using BFRS medium with two adhesive *Lactobacillus* strains in different supports

Strains	*L. rhamnosus* RS93				*L. casei* ADNOX95			
Dilution rate (h^{-1})	$Y_{p/s}$[a] (%)	Glucose used (%)	Productivity ($g\ l^{-1}h^{-1}$)	OP (%)	$Y_{p/s}$ (%)	Glucose used (%)	Productivity ($g\ l^{-1}\ h^{-1}$)	OP (%)
Poraver®								
0.30	91.4	95.3	7.1	85	91.9	95.4	8.3	88
0.36	87.0	100	8.3	86	85.5	96.8	7.8	82
0.40	100	63.2	8.1	86	100	72.2	8.1	83
0.48	86.0	77.9	10.3	86	100	88.3	10.7	89
0.55	83.0	61.1	10.4	87	88.0	85.0	11.9	88
0.70	89.7	31.9	7.4	86	98.0	37.3	8.4	86
Polyurethane-foam								
0.30	96.4	74.2	7.7	91	97.1	84	7.1	88
0.36	79.9	91.8	9.1	93	81.1	81.2	8.9	91
0.40	96.7	71.4	9.2	91	97.9	87.8	11.3	91
0.48	100.0	48.6	8.3	84	80.0	44.2	4.7	87
0.55	98.0	46.3	7.7	85	94.1	41	5.1	89

[a] Yield as lactic acid ($g\ l^{-1}$) per used glucose ($g\ l^{-1}$).

dilution rate ($D = 0.70\ h^{-1}$) tested, both strains showed reduced glucose consumption and lactic acid production. On the other hand, strain ADNOX95 showed more efficient glucose conversion at $D = 0.55\ h^{-1}$, though no significant differences were found in productivity. The results for reactors filled with polyurethane foam are shown in Table 5.4. Maximal productivity values were obtained at $D = 0.40\ h^{-1}$, 9.2 g $l^{-1}h^{-1}$ for strain RS93 and 11.3 g $l^{-1}h^{-1}$ for strain ADNOX95. Strain ADNOX95 was also more efficient at converting glucose than with RS93. However, at a dilution rate of 0.55 h^{-1} glucose consumption and productivity decreased significantly, with final product yields in the range of 94–98%. The polyurethane support accumulated more biomass (174 g l^{-1} active volume) than Poraver®, though the Poraver®-filled reactor resulted in increased productivity (11.9 g l^{-1} h^{-1}) using 27 g l^{-1} initial glucose concentration. Biofilms that adhered to polyurethane and Poraver® supports proved to be very effective and suitable for lactic acid production. Among the two strains tested, Poraver® performed better than polyurethane in its effectiveness for the production of lactic acid.

B. Comparison between productivities of *L. rhamnosus* cells immobilized with polyethyleneimine (PEI) or by natural adhesion in different reactor designs

In the R3 reactor shown in Fig. 5.2, the medium flows through the bottom of the reactor and goes out through the top with continuous recirculation at a constant ascensional velocity (1.5 cm min^{-1}). This design permits the elimination of the planktonic biomass, and the generated shear eliminates weakly adhered biomass fragments. A steady state between the adhered self-proliferating microorganisms and the pieces of biofilm that break off due to shear and continuous recycling of the medium inside the reactor is generated. This balance allows management of recycling rate, residence time, and optimal addition of nutrients for easier and more practical control of the process. The R3 biofilm reactor with Poraver® is designed to increase cell density, productivity, and yield for continuous lactic acid fermentations over long periods. These bioreactors can be employed to economically produce lactic acid and can be operated for months or even years, reducing tear-down and reassembly costs. The geometry of Poraver® achieves spatial control over biofilm development by naturally controlling biofilm thickness in addition to an established positive effect on cell density, production rates, and yields.

With the purpose of illustrating the relationship between different reactor designs filled with Poraver®, the volumetric productivity with respect to initial concentration of glucose in each assay was analyzed. The results obtained by Guoqiang *et al*. (1992) using *L. rhamnosus* DSM 20021 immobilized with PEI are compared with those obtained by

Ragout *et al*. (1995) using the adhesive offspring strain *L. rhamnosus* RS93 growing in identical culture media (Fig. 5.4). The productivity of the strain immobilized with PEI reached 2 g l^{-1} h^{-1} at a dilution rate of 0.25 h^{-1}, with an initial glucose concentration of 30 g l^{-1}, and at $D = 0.5$ h^{-1} the productivity reached 3.3 g l^{-1} h^{-1} at the same sugar concentration.

The adhesive variant RS93 grown in a PBR (R2), differing from R3 by holding a larger recirculation circuit, exhibits productivities with a direct relationship to the initial glucose concentration at the different dilution rates assayed (Fig. 5.4). Operating the R3 with a smaller recirculation circuit (Fig. 5.2) showed better performance than R2, and productivities were even larger under the same conditions (14.9 g l^{-1} h^{-1}, $D = 0.5$ h^{-1} and 37 g l^{-1} initial glucose) (Bruno-Bárcena, 1997). These results demonstrate that the reactor using the biofilm-forming strain shows enhanced performance over systems using chemically induced adhesion, given that productivity values are larger at the same initial glucose concentration. The results also indicate that minimizing recirculation volumes allows for more efficient cell biotransformation supposedly by increasing the cells' contact time with media components.

VIII. LACTIC ACID VOLUMETRIC PRODUCTIVITY VERSUS CONCENTRATION

Compared with completely mixed systems where homogeneous substrate concentration could be assumed, in immobilized cell reactors the substrate concentration on the biofilm surface is not identical to the concentration in the mobile phase, and consequently mass transfer events

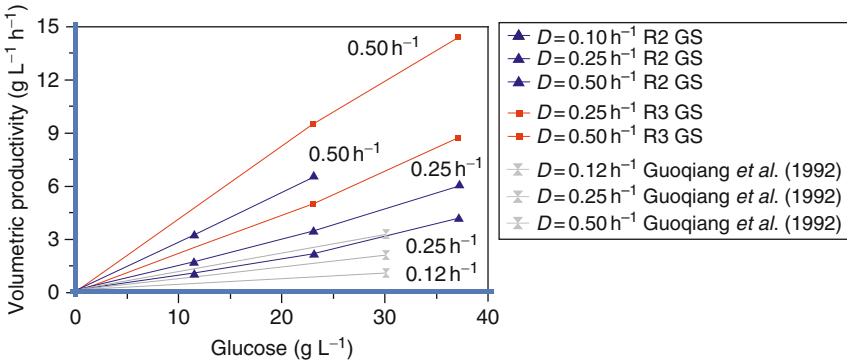

FIGURE 5.4 Productivities obtained by different authors with different reactor designs and methods of immobilization using a microorganism of the same origin and identical culture medium.

can occur on the biofilm surface even if the general environmental conditions in liquid phase may not be favorable. Compared to a fully agitated reactor, the biomass is denser in the biofilm where distance is minimal facilitating accelerated conversion rates. In the differentiated biofilm, with naturally adhered cells, there is continuous replacement of biomass allowing for constant regeneration of the biofilm along the operating time, resulting in long periods of stable performance. Regeneration is achieved by operating the system in such a way that optimal mixing of the inflow feed (new and recirculation currents) is ensured, thus allowing for real steady-state values of product yield accompanied of high substrate conversions and high product concentration. Kinetic and yield parameters for lactic acid production by different operational systems have been summarized in Table 5.5.

Productivity is often used to correlate or compare lactic acid production between diverse processes. Achieving high lactic acid concentrations inversely affects productivity, since product and substrate inhibition simultaneously act to drive the production values downward. Therefore, as lactic acid builds up in the broth, the rate of carbon utilization is reduced which forces a proportional reduction in the dilution rates in order to maintain high volumetric concentrations (Bruno-Bárcena *et al.*, 1999; Senthuran *et al.*, 2004).

Dilution rates in continuous systems can also influence the adherence of a microorganism, consequently altering productivity and yields. Higher dilution rates and increased agitation have resulted in increased suspended EPS, while production rates increased, and percent yields can be adversely affected in FBRs (Krischke *et al.*, 1991) and PCS continuous reactors (Cotton *et al.*, 2001). The effectiveness of using PCS tubes has been tested in continuous lactic acid fermentation where biofilm thickness is controlled by agitation. The production rate, yield, and cell density of a reactor with PCS tubes was compared to those a reactor with polypropylene tubes at increasing dilution rates. At a dilution rate of 0.4 h^{-1} the PCS tube biofilm reactor demonstrated productivity of 5.08 and 8.95 g l^{-1} h^{-1} with yields of 68.89% and 70.7%, while the polypropylene control reactor showed productivity of 5.75 g l^{-1} h^{-1} and 69.55% yield. At double the dilution rate, productivity of the PCS biofilm reactor increased slightly to 9.88 g $l^{-1}h^{-1}$; however, percentage yields dropped to 52.38% and 58.61% (Cotton *et al.*, 2001). Other studies have also noticed that high productivity appears to be coupled to incomplete use of substrate leading to lower conversion and decreased yields. Reduced yields were reported for higher dilution rates when continuous fermentations in stirred-tank and FBRs with sintered glass beads were compared. In the stirred reactor, 100% substrate conversion at 0.22 h^{-1} dilution rate could be obtained. At 0.4 h^{-1} dilution rate in the FBR, productivity of 10 g l^{-1} h^{-1} with 93% yield could be obtained, while further increasing the dilution rate to

TABLE 5.5 Comparison between different systems used for lactic acid production as function medium enrichment coefficients

System	Nitrogen source	Sugar	Sugar utilization (%)	Lactic acid ($g\ l^{-1}$)	Lactic acid productivity ($g\ l^{-1}\ h^{-1}$)	Productivity/ nitrogen source[a]	Organism	Reference
Batch								
Repeated batch Biofilm reactors	Y extract, 10 $g\ l^{-1}$	Glucose 80 $g\ l^{-1}$	89	55	0.75	0.075	*Streptomices viridospurus L. casei*	Demirci and Pometo (1995)
Immobilized alginate (batch)	Y extract, 10 $g\ l^{-1}$	Glucose 30 $g\ l^{-1}$	99.2	25	1.60	0.160	*L. casei*	Guoqiang *et al.* (1991)
Continuous								
Chemostat	Y extract, 5 $g\ l^{-1}$; whey retenate 50 $g\ l^{-1}$	Lactose 40 $g\ l^{-1}$	100	33	5.5 ($D = 0.22\ h^{-1}$)	0.100	*L. casei*	Krischke *et al.* (1991)
Chemostat	Y extract, 30 $g\ l^{-1}$	Glucose 40 $g\ l^{-1}$	70	29	14.43 ($D = 0.467\ h^{-1}$)	0.481	*L. casei*	González-Vara *et al.* (1996)
Single chemostat	Y extract, 10 $g\ l^{-1}$	Glucose 22 $g\ l^{-1}$	100	18.6	3.2 ($D = 0.17\ h^{-1}$)	0.320	*L. casei*	Bruno-Bárcena *et al.* (1999)
Single chemostat	Y extract, 10 $g\ l^{-1}$; whey permeate 60 $g\ l^{-1}$	Lactose 45 $g\ l^{-1}$[b]	51[b]	20.6[b]	8.27 ($D = 0.4\ h^{-1}$)	0.118	*L. helveticus*	Aeschlimann *et al.* (1990)

Two chemostats	Overall Y extract 5 g l^{-1}	Glucose 71 g l^{-1}	100	48.5	2.42 (D = 0.06 h^{-1})[c]	0.480	*L. casei*	Bruno-Bárcena *et al.* (1999)
Two chemostats	Y extract 10 g l^{-1}; whey permeate 60 g l^{-1}	Lactose 45 g l^{-1}[b]	59[b]	18.2[b]	7.64 (D = 0.42 h^{-1})	0.109	*L. helveticus*	Aeschlimann *et al.* (1990)
Fluidized bed	Y extract 5 g l^{-1}; whey retenate 50 g l^{-1}	Lactose 40 g l^{-1}	50	13	13.5 (D = 1.0 h^{-1})	0.245	*L. casei*	Krischke *et al.* (1991)
Immobilized stirred tank	Y extract 10 g l^{-1}	Glucose 30 g l^{-1}	99.2[b]	[b]28	5.2 (D = 0.18 h^{-1})	0.520	*L. casei*	Guoqiang *et al.* (1992)
Packed bed	Y extract 10 g l^{-1}	Glucose 12 g l^{-1}	90[b]	9.5[b]	1.17 (D = 0.11 h^{-1})[c]	0.120	*L. casei*	Guoqiang *et al.* (1992)
Single packed bed	Y extract 10 g l^{-1}	Glucose 26 g l^{-1}	96.8	22	7.8 (D = 0.36 h^{-1})	0.780	*L. casei*	Bruno-Bárcena *et al.* (1999)
Two packed beds	Overall Y extract 5 g l^{-1}	Glucose 92 g l^{-1} Glucose 95 g l^{-1}	89.5 58.7	57.6 37.4	5.76 (D = 0.15 h^{-1})[c] 9.72 (D = 0.40 h^{-1})	1.150 1.940	*L. casei*	Bruno-Bárcena *et al.* (1999)
Packed bed	Y extract, 15 g l^{-1}	Glucose 100 g l^{-1}	75[b]	51.4[b]	20.1 (D = 0.39 h^{-1})	1.300	*L. delbrueckii*	Gonçalves *et al.* (1992)

(*continued*)

TABLE 5.5 (*continued*)

System	Nitrogen source	Sugar	Sugar utilization (%)	Lactic acid (g l^{-1})	Lactic acid productivity (g l^{-1} h^{-1})	Productivity/ nitrogen source[a]	Organism	Reference
Repeated batch Biofilm reactor	Y extract 8 g l^{-1} plus PCS	Glucose 100 g l^{-1}		60	4.26	0.540[d]	*L. casei*	Ho *et al.* (1997)
Repeated fed batch fermentation	Y extract 7 g l^{-1} plus PCS	Glucose 80 g l^{-1}		146	4.2	0.600[d]	*L. casei.*	Velazquez *et al.* (2001)
Continuous stirred tank bioreactor	Y extract 5 g l^{-1} plus PCS	Glucose 40 g l^{-1}	70.7	12.34	8.95 ($D = 0.4$ h^{-1})	N/D[d]	*L. casei*	Cotton *et al.* (2001)
Continuous fibrous bed immobilized cell bioreactor system	Acid whey plus PCS Not reported	Lactose 86.2 g l^{-1}	76	50.8	4.8	N/D[d]	*L. helveticus*	Silva and Yang (1995)
Stirred-tank reactor coupled to a packed-bed biofilm column	Not reported	Molasses (10% sugar)	90–95	60	5 ($D = 0.0833$ h^{-1})	N/D[d]	*L. delbrueckii*	Rangaswamy and Ramakrishna (2008)

[a] Medium enrichment coefficients (MEC). Calculated utilizing nitrogenous source evaluated from data of methodology in reference cited.
[b] Values obtained from published results.
[c] Values of productivity corresponding to the highest sugar conversion.
[d] Total nitrogen source not reported.

1.0 h^{-1} produced higher productivity of 13.5 g l^{-1} h^{-1}, the yield dropped to 50% (Krischke *et al.*, 1991).

A satisfactory agreement between dilution rates and experimental productivity predictions must be demonstrated since high concentrations of lactic acid could be achieved only with high sugar conversion at the expense of reducing lactic acid volumetric productivity. Higher dilution rates can result in changes in the carbon flow in *Lactobacillus*. Moreover, maintenance of the glycolytic activity in non-growing, immobilized cells can lead to increases in lactic acid concentration and reduction in the amount of nitrogen source needed, generating lower amounts of impurities than single-state process and lowering the quantity of cell mass for disposal. Immobilization supports for microbial cells have been tested to reduce or eliminate inhibition caused by high concentrations of substrate or product and also enhance productivity. PCS-containing biofilm reactors have shown minimal lag phase, increased cell tolerance to high glucose concentration, increased cell density, reduced requirement for micronutrients, and higher lactic acid production rates (Ho *et al.*, 1997; Velazquez *et al.*, 2001). The performance of PCS was characterized in suspended-cell bioreactors and repeated fed-batch biofilm lactic acid fermentations containing *L. casei* in the presence and absence of the antibiotic nystatin for controlling yeast contamination while optimizing yeast extract and glucose concentrations. Fed-batch reactors containing PCS supports demonstrated productivity of 2.45 g l^{-1} h^{-1}; however, lactic acid productivities and glucose consumption rate decreased with each glucose pulse (Velazquez *et al.*, 2001). The reduction in lactic acid productivity following each glucose pulse was thought to be caused by a dilution of the yeast extract concentration, by addition of ammonium hydroxide to control pH, or by increased lactic acid concentration (Gonçalves *et al.*, 1991; Velazquez *et al.*, 2001). Otherwise, repeated fed-batch fermentation containing PCS operated more efficiently than suspended-cell bioreactors by requiring less yeast extract to produce up to 146 g l^{-1} of lactic acid with 7 g of yeast extract (Velazquez *et al.*, 2001). Ho *et al.*, 1997 studied lactic acid production from three bioreactors containing PCS and compared the data to those in a bioreactor with suspended cells as a control. They found PCS bioreactors shortened the lag time up to sixfold, increased productivity from 2.78 g l^{-1} h^{-1} in the control to 3.26 g l^{-1} h^{-1} in the PCS bioreactors, and required reduced complex nutrient addition by lowering yeast extract in the medium and increasing starting glucose concentration. However, inhibition of substrate utilization by high starting glucose concentrations was observed, as also reported by Gonçalves *et al.* (1992). Results for typical lactic acid percentage yield for *L. casei* are between 70% and 72% (Cotton *et al.*, 2001; Ho *et al.*, 1997). Yields below 69% are thought to represent a physiological shift to overproduction of EPS and a reduction in lactic acid production (Cotton *et al.*, 2001; Ho *et al.*, 1997).

The values shown in Table 5.5 allow comparisons between datasets from lactic acid production processes under optimal conditions between reports in the literature, highlighting the uppermost lactate (g l^{-1}) producing condition within each study.

A. How much will cells pay for a good nitrogen source?

Theoretically, adding more yeast extract should increase biomass leading to higher volumetric lactic acid produced. However, yeast extract is a high-priced commodity and is major factor in costly lactic acid fermentations. Since yeast extract is a major expenditure, an unbiased method for comparing the efficiency of lactic acid production for diverse bioreactor operating conditions was accomplished by calculating productivity from a given amount of nitrogen source (productivity/gram nitrogen source), designated as medium enrichment coefficient (MEC) (Table 5.5). Scanning the lactic acid concentration and MEC values, it is clear that the naturally immobilized systems yielded lactic acid concentrations that parallel with productivity values when the results are analyzed in terms of the amount nitrogen source added. The amount of nitrogen source supplied for two-stage systems, chemostat, and PBRs is accompanied by substantially high substrate consumption and lactate concentration, and comparable with the high concentration values reported. However, many of the continuous fermentations with very high reported productivities were usually accomplished by adding high amounts yeast extract, reducing the benefits by escalating fermentation costs. Naturally immobilized PBRs and CSTRs produced the highest MEC values, while covalently immobilized or entrapped cells produced low MEC values and are comparable to batch and chemostat processes. Early kinetic studies on lactose utilization in acidogenic biofilms suggested that immobilization changes the physiological properties of the microorganisms resulting in decreased substrate utilization compared to free cells. This decrease in substrate utilization may be due to decreased cell surface area for substrate consumption and considerable internal mass transfer resistance inside the biofilm in addition to differences in apparent substrate affinity of the adsorbed cells (Jeffrey and Paul, 1986; Yu and Pinder, 1993).

Biofilm bioreactor designs that minimize diffusion limitations and inhibition by substrate, thereby improving productivity in reduced nitrogen media components, have been implemented (R3 design reactor Fig. 5.2) (Bruno-Bárcena, 1997; Bruno-Bárcena *et al.*, 1999) and tested in a two-stage, two-stream immobilized up-flow Poraver® PBRs with two mixed feed stock streams. The design predictions were in good agreement with the data reported. The results suggest that yeast extract concentrations can be maintained at low levels while still achieving high productivity and high lactic acid concentrations with no significant amount of

free cells, as compared to productivity obtained from nonadherent free cells. By using reactors operating in cascade for the two-stage, two-stream immobilized up-flow PBR, the overall nitrogen source can be reduced and simple medium recipes could be still simplified even further reducing downstream processing costs by the reduction in impurities.

IX. CONCLUSIONS

The advantages of microbial production of lactic acid over chemical synthesis from petroleum-based products are utilization of renewable carbon sources and the ability to mainly produce the L- or D-isomer of lactic acid. To make fermentation cost-competitive with chemical synthesis, improvements have been made that decrease production costs while increasing lactic acid productivity and product concentration. Production rates have been enhanced by increasing cell densities using various supports and advancing natural biofilm adsorption by strain development in addition to augmenting existing bioreactor designs.

Conventional artificial methods for cell immobilization involved cross-linking or entrapment within polymers. However, these artificial immobilization methods require additional intricate preparation steps, lack a universally applicable technology, limited mass transfer through the immobilization matrix, and reduced viability of the entrapped organism. Natural immobilization offers the best possibility for continuous fermentation that significantly increases product output and thus reduces cost when compared to artificial cell immobilization systems. Biofilms naturally adapt to their own matrix and show long-term viability as well as enhanced tolerance to toxins, allowing for continuous processing. Product inhibition continues to be a bottleneck in lactic acid fermentation processes, and has been addressed with bioreactor design modifications such as cell recycling thereby increasing productivity. Future modifications may also include developments in product removal in conjunction with the fermentation process. Consequently, industrial demand for technologies ensuring microbial stability remains strong, since high cell survival rather than constant replenishment continues to be economically important.

A comparison between reactor designs, organisms, and support matrices employed during the past two decades was presented. Numerous studies using continuous processes and various cellular supports with the aim of increasing cell concentration in the reactor have been researched. Ultimately, understanding the relationships between the support, strain, and productivity will allow for a more systematic tailoring of the process. It is clear that increases in cell density vastly improve reactor volumetric productivity, and further improvements should focus on

increasing specific cell productivity. As a final point, we would like to highlight that the use of naturally adherent strains in an adequate reactor design resulted in the highest efficiency reported, reinforcing the era that first began by examining longer operational times and high cell densities through constant biomass regeneration of naturally adherent microorganisms.

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