Chapter 6
State of the Art in Immobilized/Encapsulated Cell Technology in Fermentation Processes

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6.1 Introduction

The process of sugar conversion from wort or malt into alcohol, carbon dioxide, and other components catabolized by yeast enzymes is called the alcohol fermentation process. In beverage production, it is of great importance to achieve a particular balance between different secondary metabolites. High productivity is another demand of the beverage industry. Immobilization of cells provides high cell densities leading to higher volumetric productivities, and as a consequence, reduces essential bioreactor sizes (decreased capital costs) and shortens residence times. Immobilized cell technology (ICT) coupled with continuous mode of fermentation offers additional benefits, like ease of biomass separation and recovery, simplification of process design, lower risk of microbial contamination of the pitching yeast population, greater efficiency in utilization of carbohydrates, and better use of equipment and potential savings. However, continuous fermentation processes have not been commercially successful due to many practical problems, such as increased risk of contamination not only during fermentation but also during storage of wort in supplementary holding tanks, which are usually required for batches upstream and downstream fermentation processes; in addition, there are variations in beverage flavor and poor understanding of the fermentation kinetics

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under continuous conditions. Over the last 30 years, ICT for alcoholic beverage production has been extensively investigated and some systems have already reached commercial exploitation. Intensification of a particular fermentation process using ICT can generally be industrialized if the acquired new characteristics result in a more economic system and the new technology can be readily scaled up. ICT processes have been designed for different stages in the beer fermentation process: wort acidification, primary fermentation, and bioflavoring during secondary fermentation; these fermentation processes are used in the production of alcohol-free or low-alcohol beers (Brányik et al. 2005; Nedovic et al. 2005a; Willaert and Nedovic 2006), as well as wine (Divies and Cachon 2005) and cider (Nedovic et al. 2000; Durieux et al. 2005). The most challenging complex application in fermentation processes is the combined main (ethanol fermentation) and secondary fermentation (maturation) processes.

Traditional beer fermentation technology uses freely suspended yeast cells to ferment wort in a non-stirred batch reactor. The traditional primary fermentation for lager beer takes approximately 7 days with a subsequent secondary fermentation (maturation) of several weeks. The resulting beer has a well-balanced flavor profile. Nowadays, large breweries use a selected specific yeast strain and elevated temperatures to accelerate production. This enables the production of finished lager beer in 12–15 days. ICT is able to produce lager beer in a much shorter time period (usually 1–3 days). A major difficulty is to achieve the correct balance of sensory compounds to create an acceptable flavor profile in such a short time frame. ICT for beer production can only be introduced successfully on an industrial scale if the flavor profile can be controlled and fine-tuned.

Cider and wine production also involves complex processes that imply transformation of apple juice in the case of cider, or grape juice in the case of wine, by activity of both yeast and lactic acid bacteria (LAB) to accomplish alcoholic and malolactic fermentations (MLFs). The traditional process consists of natural fermentation via autochthon yeasts and bacteria associated with the fruit or the cellar equipment. This natural process is very unpredictable in terms of desirable flavor compounds formation. The development of starter cultures enabled the use of selected strains and the control of cider production to achieve high and uniform quality, through several successive steps: pretreatment, alcoholic fermentation of sugars into ethanol proceeded by yeast strains, and malolactic fermentation (MLF), that is, bacterial conversion of L-malic into L-lactic acid and carbon dioxide (needed to reduce acidity). Spontaneous MLF of cider begins within a few hours if the temperature of the juice rises above 10°C. This process is usually very slow. It requires 2–3 weeks to accomplish the main fermentation and several months for the maturation. There is a risk of spontaneous fermentation by indigenous microbial flora and it is difficult to control the flavor formation. The initiation of MLF appears to be the main limiting factor in cider and wine production. MLF can occur several weeks after alcoholic fermentation but there is no guarantee it will occur, which is an unfavorable milieu for growth of microorganisms (ethanol $> 10\%$; pH < 3.0–3.5; temperature < 15°C). ICT offers a new alternative to better control of the microbiology that defines the final product. In addition, this new technology

significantly reduces consumption of time, facilitating MLF simultaneously with alcoholic fermentation or at the end of this process.

Key parameters of this technology are the selection of carrier materials and the method of immobilization together with the bioreactor design. Determination of these parameters is directed by operational conditions such as temperature, pH, substrate composition, and fluid dynamics, wherein special attention should be paid to mass transfer properties since limited nutrient supply can result in changes in yeast metabolism, leading to inadequate flavor of the final product.

6.2 Carrier Selection and Design

Cell immobilization can be classified into four categories based on the mechanism of cell localization and the nature of support material: (i) attachment to the support surface, which can be spontaneous or induced by linking agents; (ii) entrapment within a porous matrix; (iii) containment behind or within a barrier; and (iv) self-aggregation, naturally or artificially induced. Various supports and immobilization techniques have been proposed and tested for application in brewing and wine- and cider-making. Those that fulfill the following prerequisites are preferable:

- High surface-to-volume ratio of the immobilization support to achieve high cell loading capacity
- Simple procedure and non-harsh conditions under way during immobilization
- Mechanical stability (compression, abrasion) and chemical stability of the immobilization support
- Sterilization capability and regeneration of the immobilization support
- Cost-effectiveness of the support and immobilization process
- Suitability for conventional reactor systems
- Acceptance of immobilization support by consumers and avoidance of negative effects on final product (e.g., off-flavor formations)
- Retention of immobilized cell viability
- Avoidance of negative effects of cell immobilization on biological and metabolic activity of immobilized cells
- Easy separation of carriers with immobilized cells from media
- Wide choice of yeast
- Compounds approved for food applications

Table 6.1 Summarizes most of the carrier materials and bioreactors used in fermentation processes for alcoholic beverages.

6.2.1 Immobilization on Solid Carrier Surfaces

Cell immobilization by adsorption to a support material is a very popular method, because it is simple, easy to carry out, cheap, and fast. Microorganisms adsorb

Table 6.1 Carrier materials and reactor types for selected fermentation processes using immobilized cells

Carrier material	Reactor type	Type of fermentation	Product	Reference
Apple pieces	Fixed-bed	AF	Wine	Kourkoutas et al. 2001, 2002
γ-Alumina	Fixed-bed	AF	Wine	Bakoyianis et al. 1997; Loukatos et al. 2000
Ca-alginate beads	Fixed-bed	AF	Beer	Ryder and Masschelein 1985; White and Portno 1979; Onaka et al. 1985; Ryder and Masschelein 1985;
Ca-alginate beads	Gas-lift	AF	Beer	Nedovic et al. 1993, 1996, 2004, 2005a
Ca-alginate beads		AF	Sparkling wine	Fumi et al. 1987; Fumi et al. 1988
Ca-alginate beads	Fixed-bed	AF	Wine	Ferraro et al. 2000
Ca-alginate beads	Fixed-bed	AF and MLF	Cider	Simon et al. 1996
Ca-alginate beads	Fixed-bed	MLF	Cider	Cabranes et al. 1998
Ca-alginate beads	Fixed-bed	AF and MLF	Cider	Nedovic et al. 2000
Ca-alginate beads	Shaken flasks	MLF	Cider	Herrero et al. 2001
Ca-alginate beads	Fixed-bed	Maturation	Beer	Shindo et al. 1994
Ca-alginate beads	Gas-lift	AF	Beer	Smogrovicová et al. 1997; Smogrovicová and Dömény 1999
Ca-alginate beads	Shaken flasks	MLF	Wine	Kosseva et al. 1998
Ca-alginate beads	Shaken flasks	MLF	Wine	Kosseva and Kennedy 2004
κ-Carrageenan beads	Gas-lift	AF	Beer	Mensour et al. 1996, Mensour et al. 1997; Decamps et al. 2004
Ceramic beads	Fixed-bed	AF	Beer	Inoue 1995
Corncobs	Gas-lift	AF	Beer	Brányik et al. 2006
Chitosan	Shaken flasks	MLF	Wine	Kosseva et al. 1998
Chitosan	Fluidized-bed	AF	Beer	Unemoto et al. 1998; Maeba et al. 2000
DEAE-cellulose	Fixed-bed	AF	Beer	Kronlöf et al. 1989; Andersen et al. 1999
DEAE-cellulose	Fixed-bed	Maturation	Beer	Pajunen and Grönqvist 1994
DEAE-cellulose	Fixed-bed		Acidified wort	Pittner et al. 1993
DEAE-cellulose	Fixed-bed	MLF	Wine	Maicas, Pardo, and Ferrer 2001
DEAE-cellulose	Fixed-bed		Alcohol-free beer	Collin et al. 1991; Lommi 1990
Delignified cellulosic material	Fixed-bed	AF	Wine	Bardi and Koutinas 1994; Iconomou et al. 1996; Iconomopoulou et al. 2003
Delignified cellulosic material	Fixed-bed	MLF	Wine	Agouridis et al. 2005
Gluten pellets	Fixed-bed	AF	Beer	Bardi et al. 1997
Gluten pellets	Gas-lift (external-loop)	AF	Beer	Manojlovic et al. 2008
Gluten pellets	Fixed-bed	AF	Wine	Bardi et al. 1996; Iconomopoulou et al. 2002
Gluten pellets	Fixed-bed and MFBT	AF	Wine	Sipsas et al. 2009
Kieselguhr (diatomaceous earth)	Fixed-bed	AF	Beer	Narziss and Hellich 1971; Moll et al. 1973; Virkajärvi and Pohjala 2000
Kissiris	Fixed-bed	AF	Wine	Bakoyianis et al. 1992
	"in the bottle"	Maturation	Beer	Lemonnier and Duteurtre 1989;

(*continued*)

Table 6.1 (continued)

Carrier material	Reactor type	Type of fermentation	Product	Reference
Microfiltration membranes				
Microfiltration ceramic membranes	Membrane reactor	MLF	Cider	Lovitt et al. 2006
Orange pieces	Fixed-bed	AF	Wine	Plessas et al. 2007
Pear pieces	Fixed-bed	AF	Wine	Mallios et al. 2004
PVA beads	Gas-lift	AF	Beer	Smogrovicová et al. 2001
PVA beads	Fixed-bed	Maturation	Beer	Smogrovicová et al. 2001
PVA beads	Champagne bottles	AF	Champagne	Martynenko et al. 2004
PVA/alginate beads	MFBT	AF	Beer	Manojlovic et al. 2007
PVA Lentikats®	Gas-lift	AF	Beer	Smogrovicová et al. 2001; Bezbradica et al. 2007
Polyvinyl chloride granules	Fixed-bed	AF	Beer	Moll et al. 1973
Porous glass beads	Fixed-bed	AF	Beer	Virkajärvi and Krönlof 1998; Virkajärvi and Pohjala 2000
Porous glass beads	Fixed-bed	Maturation	Beer	Linko et al. 1993; Aivasidis 1996
Porous glass beads	Fixed-bed		Alcohol-free beer	Aivasidis et al. 1991
Quince pieces	Fixed-bed	AF	Wine	Kourkoutas et al. 2003
Raisin berries	Fixed-bed	AF	Wine	Tsakiris et al. 2004a, 2004b
Self-aggregation using super-flocculent yeast	Stirred-tank reactors	AF	Beer	Coutts 1957; Linko et al. 1997
Self-aggregation using yeast biocapsules	Erlenmeyer flasks	AF	Wine	Peinado et al. 2006; Peinado et al. 2005
Silicon carbide rods	Monolith reactor	AF	Beer	Van De Winkel et al. 1993; Andries et al. 1996
Silicon carbide rods	Monolith reactor		Alcohol-free beer	Van De Winkel et al. 1991
Spent grains	Gas-lift	AF	Beer	Brányik et al. 2002, 2004
Spent grains	Fixed-bed	AF	Beer	Kopsahelis et al. 2007
Spent grains	Fixed-bed	AF	Wine	Mallouchos et al. 2007
Sponge-like material	Fixed-bed	AF and MLF	Cider	Scott and O'Reilly 1996
Stainless-steel fiber cloth	Gas-lift	AF	Beer	Verbelen et al. 2006
Stainless-steel wire spheres	Fluidized-bed	AF	Beer	Cross and Mavituna 1987
Wood chips (Aspen)	Fixed-bed	AF	Beer	Pajunen et al. 2001
Wood chips (Beech)	Fixed-bed	AF	Beer	Linko et al. 1997; Kronlöf and Virkajärvi 1999
Watermelon pieces	Fixed-bed	AF	Wine	Veeranjaneya Reddy et al. 2008

PVA polyvinyl alcohol, *AF* alcoholic fermentation, *MLF* malolactic fermentation, *MFBT* multi-stage fixed-bed tower

spontaneously on a wide variety of organic and inorganic support materials. Binding of cells occurs through interactions such as Van der Waals forces, ionic bonds, hydrogen bridges, or covalent interactions. Microbial cells exhibit a dipolar character and behave as cations or anions depending on the cell type and environmental conditions, such as pH of the solution. The thickness of cell film usually ranges from one layer of cells to 1 mm or more. The strength with which the cells are bonded to the carriers as well as the depth of the biofilm varies from one system to another. Cell detachment and relocation readily occur, followed by establishment of equilibrium between adsorbed and freely suspended cells. Various rigid organic and inorganic support materials have been used in alcohol fermentation processes. Inorganic materials are cheap and abundant. Among the inorganic types, porous glass beads have been used successfully for primary beer fermentation (Tata et al. 1999) and beer maturation (Yamauchi et al. 1995), kissiris (a porous volcanic mineral found in Greece, similar to granite, containing 70% SiO_2,13% Al_2O_3, and other inorganic oxides) for wine (Kana et al. 1989), ethanol production (Bakoyianis et al. 1992), γ-alumina for wine-making (Kana et al. 1989; Loukatos et al. 2000), and stainless-steel wire spheres for ethanol production (Bekers et al. 1999). Various organic materials are suitable for immobilization in beverage production, such as diethylaminoethyl (DEAE) cellulose, delignified cellulosic material, wood, sawdust, delignified sawdust, gluten pellets, and spent grains, aimed at applications in packed-bed reactors. A micrograph of yeast cells immobilized on wood chips is shown in Fig. 6.1. Solid materials like glass and cellulose have been treated with polycations, chitosan, or other chemicals to obtain preformed carriers with enhanced adsorption ability (Norton and D'Amore 1994). In recent years, special attention has been paid to usage of fruit pieces, since they are of food-grade purity, and are easily accepted by consumers. Apple (Kourkoutas et al. 2001, 2002), quince (Kourkoutas et al. 2003), pear (Mallios et al. 2004), raisin berries (Tsakiris et al. 2004a, 2004b), grape skin (Mallouchos et al. 2002), orange (Plessas et al. 2007),

Fig. 6.1 Scanning electron microscope (*SEM*) photo of yeast cells immobilized on wood chips

and watermelon (Veeranjaneya Reddy et al. 2008) have been used so far as support materials for cells involved in fermentation processes.

6.2.2 Entrapment Within Porous Matrix

Entrapment involves containment of living cells within a network, which permits the diffusion of substrates and products, thereby making possible the growth and maintenance of active cells. Natural polysaccharides (e.g., alginate, pectate, carrageenan, chitosan, agar, polygalacturonic acid), synthetic polymers (polyvinyl chloride, polyvinyl alcohol (PVA) lens-shaped Lentikats, polyacrylamide), and proteins (gelatin, collagen) can be gelled into hydrophilic matrices under mild conditions, thus allowing cell entrapment with minimal loss of viability. Very high biomass loadings can be achieved, since gel systems are characterized by very high porosities (95–98%) and the cells are protected from fluid shear. Cell growth in the porous matrix depends on diffusion limitations imposed by the porosity of the matrix and the available interstitial space, which decreases in time with cell propagation and the accumulation of biomass. Available literature shows that the effective oxygen penetration rate varies in the range 0.08–010 mm in carrageenan beads (Huang et al. 1990) and 0.1–0.15 mm in alginate beads (Ogbonna et al. 1991). Cells that are growing can cause stress expressed in volumetric deformation and also partial disintegration of the hydrogel. It opens a new space for cell growth inside the matrix. This particular part of the process causes the mechanical transformation of the network. There are only a few reports on the relaxation effects of hydrogel caused by cell growth (a recent one is from Pajic-Lijakovic et al. 2007). Gels are mostly used in the form of spherical beads with diameters ranging from about 0.3 to 5 mm. Smaller particles show better mass transfer properties of nutrients and metabolic products. Moreover, reduction in bead size lowers the shear forces and may increase their long-term stability. However, small beads have larger surface-to-volume ratio compared to big particles and therefore can be more easily harmed by swelling or by exposure to oppositely charged ions (Strand et al. 2002). In addition, smaller spheres are more fragile to internal stresses caused by cell proliferation and expansion of cell colonies. Numerous techniques for bead production have been developed up to now and are available to achieve the desired size of capsules (Nedovic and Willaert 2004; Prusse et al. 2008). The use of synthetic hydrogels may allow design of a carrier/matrix with preferred characteristics. LentiKats® (specially designed particles made from PVA) have a specific lenticular shape (Fig. 6.2) due to which they combine the advantages of small (good diffusion properties) and large (easy retention and removal) beads. Production of LentiKats® particles is based on the usage of the specially designed LentiKats® Printer in lab and industrial scales. LentiKats® particles with immobilized yeast have been successfully used for beer fermentation performed in a gas-lift bioreactor (Bezbradica et al. 2007) and in cider production (Durieux et al. 2002). The design of synthetic materials that could balance the opposite demands of high open-pore

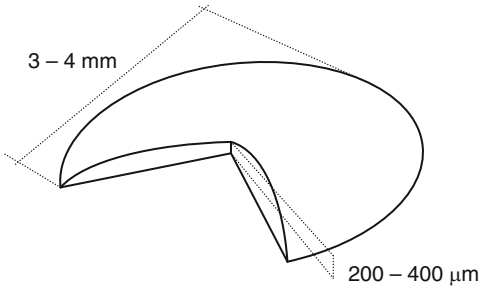

Fig. 6.2 LentiKats® hydrogel particle based on polyvinyl alcohol (*PVA*)

structure, and at the same time, provide good protection to cells against washout is a challenge for researchers involved in polymer science. One such attempt was the design of a synthetic double-layer hydrogel, where the core was made of hydroxyethylcellulose cryogel and then coated with a layer of poly(ethylene oxide) (Manojlovic et al. 2009).

A disadvantage of gels is the limited mechanical stability under conditions of rapid cell growth, excessive CO_2 production, or prolonged exposure to phosphates during the maturation process. Several methods have been proposed for reinforcement of gel structures. For example, alginate gel can be strengthened by reaction with polyethyleneimine, gluteraldehyde cross-linking, addition of silica, genepin, and PVA, or by partial drying of the gel (Willaert and Baron 1996). The major drawback in these systems is mass transfer limitation. However, understanding of mass transfer phenomena within entrapment matrices may allow one to simultaneously provide different conditions at the carrier surface and in the interior, which could be attractive for co-immobilization of different cell types performing consecutive processes. For example, gels with varying degrees of anisotropy, with respect to polymer concentration, can be formed by controlling the kinetics of the gel formation. Simply by adjusting the concentration of alginate and the cross-linking ions, the distribution of the polymer in the gel can be controlled; alginate beads with a capsular structure have been made without adding polycations or any other non-gelling polymer (Thu et al. 2000). Another way is to create an external layer of another polymer around the hydrogel core. Double-layer beads solve the problem of escaping of cells out of beads when the system also contains (besides immobilized cells) free ones. However, microencapsulation is generally too expensive to be used in the beverage industry.

Porous preformed supports can be inoculated directly from the bulk medium. In these systems, cells are not completely separated from the effluent, similarly as in the adsorption method. Cell immobilization occurs by attachment to the internal surfaces, self-aggregation, and retention in dead-end pockets within the material (Baron and Willaert 2004). Ideally, the colonized porous particles should retain some void spaces for flow so that mass transport of substrates and products can be achieved by both molecular diffusion and convection. Consequently, mass transport limitations are less stringent under optimal conditions as compared to gel entrapment methods. However, when high cell densities are reached, convection is no longer possible and the particles behave as dense cell agglomerates with high diffusion limitations.

As compared to gel particles, preformed carriers provide better mechanical properties and higher resistances to compression and disintegration.

6.2.3 Cell Aggregation

Cell immobilization by self-aggregation is based on formation of cell clumps or floccules, which can be naturally occurring as in the case of flocculent yeast strains, or induced by addition of artificial flocculating agents or cross-linkers. It is the simplest and the least expensive immobilization method. However, interactions among cells are not easily controlled and cell aggregates are very sensitive to conditions in fermentors, including pH, dissolved oxygen, and medium composition. The flocculation of *Saccharomyces cerevisiae* is determined by the adhesin protein family. The adhesin proteins are encoded by the genes *FLO1*, *FLO5*, *FLO9*, and *FLO10* (Verstrepen et al. 2003a, 2004). These proteins are called flocculins (Caro et al. 1997) because they promote cell–cell adhesion forming multicellular clumps that settle out of solution. The structure, location, and activity of flocculins are well described in a recent report from Van Mulders et al. (2009). The ethanol productivity achieved by flocculated cells was almost double that of a freely suspended yeast cell system (Xu et al. 2005). It was found that the floc size distribution influenced the effectiveness of glucose uptake and ethanol production (Ge et al. 2006). An interesting approach has been recently proposed by Peinado and coworkers (2005, 2006): a filamentous fungus and a flor yeast under adequate conditions form a cluster that looks like a hollow biocapsule; here mycelium creates walls around the biocapsule and the yeast is entrapped in an inner space. The yeast biocapsules were successfully used in must (wine) fermentation.

6.2.4 Containment Behind a Membrane Barrier

Cell immobilization behind or within a porous barrier includes systems with cells contained in a compartment separated by a preformed membrane such as hollow fiber and flat membrane modules. Micromembrane technology, like microencapsulation, is generally too expensive to be used in beverage production. Moreover, mass transfer limitations are relatively high (Lebeau et al. 1997), and membrane biofouling caused by cell growth often occurs (Gryta 2002).

6.3 Bioreactor Design

Selecting the appropriate reactor type or configuration for an immobilized cell system is related to a number of important factors, such as carrier design, supply and removal of gases and solutes in the liquid phase, as well as removal of excess

biomass formed, investment and operation costs, operation mode, maintaining sterile conditions, heat and mass transfer rates, and others. In fermentors, immobilized cells can be either mixed with suspended carriers or localized on carrier particles/surfaces, which are then fixed or in movement. The operation mode of immobilized cell reactors can be batch, fed-batch, or continuous. Continuous operation eliminates the unproductive time in batch and fed-batch processes associated with filling, emptying, cleaning and disinfection/sterilization, and start-up phase of the fermentation. Therefore, it provides a higher productivity compared to the "old-fashion" batch method of processing. With respect to sterility, reactors that can be directly inoculated with cells or cell-aggregates (e.g., membrane modules or reactors packed with preformed porous carriers) are more desirable compared to reactors that require transfer of a biocatalyst from the immobilization equipment to the reactor (e.g., fermentors using cells entrapped in gel systems). The bioreactor should be designed and the hydrodynamic conditions optimized to provide easy access to nutrient media, optimum mass transfer from flowing media to the support interior, controlled yeast growth, low shear experienced by cells, simple scale-up, controlled oxygenation, complete attenuation and desired flavor profile, consistent product quality, and low risk of contamination.

Most of the studies have been on packed-bed (fixed-bed) bioreactors. Packed-bed bioreactors are characterized by a simple design consisting of a single column packed with biocatalysts. The liquid flow is close to the plug flow regime and causes low shear rates (Obradovic et al. 2004). The main disadvantages of packed-bed fermentors are high mass transfer restrictions, accumulation of carbon dioxide, non-uniform temperature profiles, flow channeling, and stagnant zones. Therefore, the first trials conducted on using a packed-bed configuration for primary beer fermentation on an industrial scale gave unsatisfactory results with respect to product quality. Afterwards, packed-bed reactors were selected for production of alcohol-free or low-alcohol beers and for enhanced flavor maturation using immobilized cells. In these applications, conditions are anaerobic and yeast growth is limited. Immobilization of cells can be by adsorption (e.g., DEAE-cellulose beads) or by a combination of adsorption and entrapment (e.g., porous glass beads). These carrier materials need to be mechanically strong to withstand the high pressures in packed-bed reactors. However, the use of mechanically week materials (e.g., hydrogels) can be limited to lower bed heights and liquid flow rates due to possible compression of beads. In order to eliminate some of the drawbacks of the packed-bed configuration, a modification of a packed-bed fermentor, that is, the "multistage fixed-bed tower" (MFBT), has been proposed for beer production (Manojlovic et al. 2007) and wine-making (Sipsas et al. 2009). It consists of a vertical cylindrical tank with five packed sections containing freeze-dried immobilized cells. A relatively small (5000–10000 L) MFBT bioreactor (Fig. 6.3) (Koutinas et al. 1997; Loukatos et al. 2000) was proposed for industrialization of immobilized cells in wine-making. Handling of the support at this scale could be performed without any problems, and cell immobilization could be carried out in the bioreactor. The application of the MFBT bioreactor on an industrial scale eliminates insufficient mass transfer and enables support

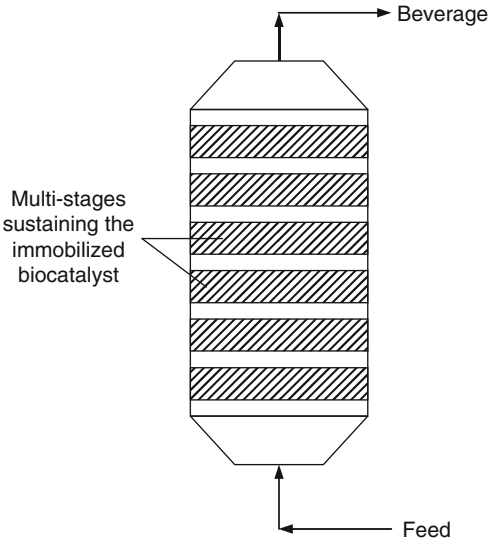

Fig. 6.3 Multistage fixed-bed tower (*MFBT*) bioreactor (Adapted from Sipsas et al. 2009)

division, especially when mechanically unstable supports are used to minimize high pressure effects, which may result in support destruction and reduction of fermentation activity (Kourkoutas et al. 2009). Experiments concerning long-term storage of the immobilized biocatalysts (Kourkoutas et al. 2003) are very promising, since the preparation of new biocatalysts, emptying and filling of the bioreactor, could be avoided when industrial production is halted. Taking into consideration the above discussion of technical problems, the scale-up of the proposed technology seems feasible.

Different approaches for the adaptation of bioreactors containing immobilized cells were investigated in order to correct the final beverage quality. One was to establish biocatalyst movement or circulation for the purpose of speeding up the transfer of nutrients and metabolic products through the fermenting medium, as in fluidized-bed, stirred-tank, and gas-lift bioreactors. In the fluidized-bed bioreactors, particles with immobilized cells are fluidized in the liquid up-flow, while gas can be optionally supplied. As a consequence of particle fluidization, moderate local mixing is established, which provides better mass and heat distribution with more uniform liquid flow throughout the reactor volume, as compared to packed-bed reactors. It is difficult to maintain low-density particles in fluidization and to prevent their washout. Particle movements and collisions in the fluidized state result in moderate shear stresses and abrasion, creating a need for relatively mechanically stable supports (Obradovic et al. 2004). The scaling up of fluidized-bed bioreactors addresses problems due to the difficulties in controlling the bed expansion and may encounter hydrodynamic problems. In stirred tank reactors high aeration resulted in a less balanced aroma profile of the final product. Beers produced in fluidized and stirred-tank fermentors had high concentrations of diacetyl and low concentrations of higher alcohols and esters (Okabe et al. 1992; Mensour et al. 1997).

Gas-lift reactors are especially attractive since they apply pneumatic agitation with no mechanical devices. They are based on liquid circulation, which can be effectively tuned to achieve an adequate flow regime and optimal external mass transfer. This bioreactor concept was introduced in beer fermentation studies by a Serbian group in 1993 (Nedovic et al. 1993). Internal loop configuration (Fig. 6.4) has been investigated in lab- and pilot-scale production mainly for beer fermentation (Nedovic et al. 1993, 2004, 2005a; Mensour et al. 1997), while the external loop design (Fig. 6.5) has been recently tested for alcoholic fermentation in lab-scale beer production (Manojlovic et al. 2008). Efficient mixing and low shear rates make gas-lift reactors suitable for all types of low-density immobilization materials (Mensour et al. 1997; Obradovic et al. 2004).

The design of membrane reactors is relatively complex and expensive, mainly due to the high cost of the membrane material. Membrane reactors provide simultaneous bioconversion and product separation. A special design of a multichannel loop bioreactor has been developed by the Belgian company, Meura (Tournai), for production of lager, ale, and acidified wort (Masschelein et al. 1994). Yeast cells are

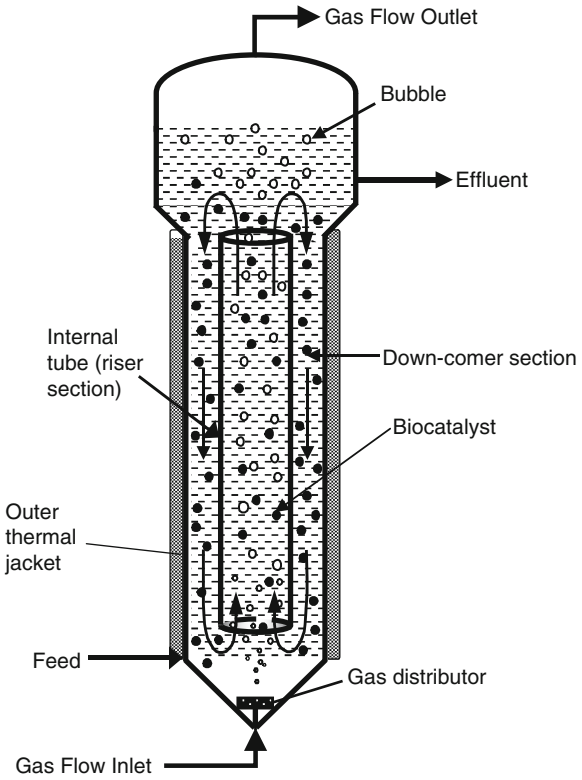

Fig. 6.4 Gas-lift bioreactor internal loop configuration with an immobilized biocatalyst (Adopted from Nedovic et al. 1993)

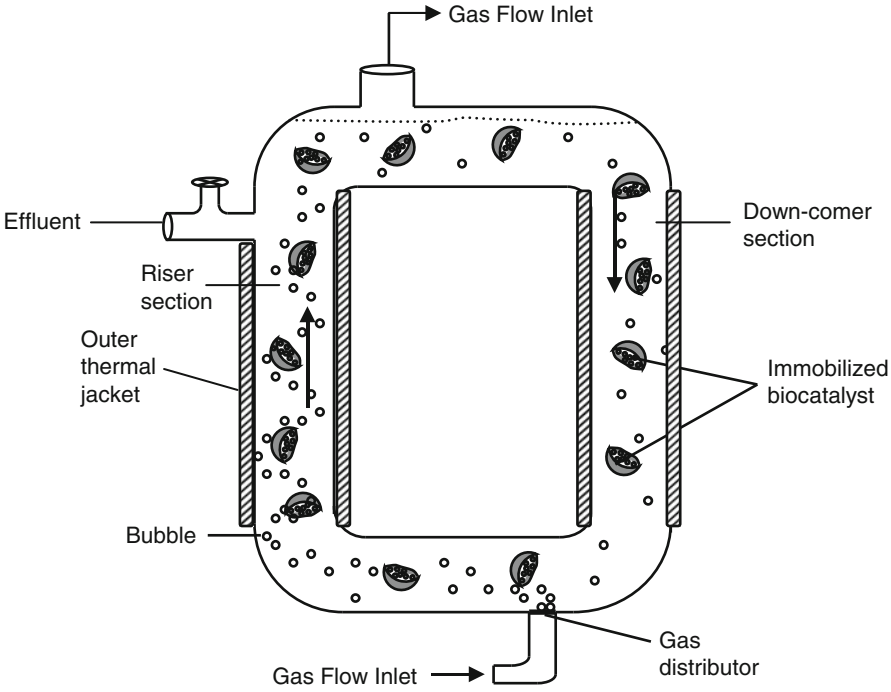

Fig. 6.5 Gas-lift bioreactor external loop configuration with an immobilized biocatalyst (Adapted from Manojlovic et al. 2008)

immobilized in porous sintered silicon carbide rods perforated with 19 or 37 channels for fluid flow. This immobilization method can be regarded as containment behind a preformed barrier, and as entrapment in a porous preformed support. Continuous beer fermentation technology using yeast flocculation and cell recycling has been successfully exploited over almost 40 years by Dominion Breweries in New Zealand (Coutts 1957; Van de Winkel and De Vuyst 1997).

Selected yeasts entrapped in micro-filtration membranes have been developed and used in wine production. On-market available "Millispark" cartridges (Millipore) were used for secondary fermentation of sparkling wine in bottles (Lemonnier and Duteurtre 1989). In dry wine production, a single-vessel membrane bioreactor was found unsuitable for continuous fermentation, as high levels of unfermented sugars were reported (Takaya et al. 2002). However, a double-vessel continuous membrane configuration resulted in a sugar content lower than 4 g/L, which was considered satisfactory for dry wine-making. Additionally, wine productivity was 28 times higher compared to traditional batch systems.

In a recent study, the approach of splitting cell propagation and cell maturation was applied in a pilot-scale membrane bioreactor with ceramic membrane modules to perform MLF of media containing ethanol (Lovitt et al. 2006). Herein, the overall productivity of both process rate and longevity was successfully increased.

6.4 Impact of Immobilization on Flavor Formation

Although ICT offers a number of benefits, it has so far found limited application in the fermentation industry. A major difficulty is to achieve the correct balance of volatile compounds to create an acceptable flavor profile of alcoholic beverages. Immobilized cells appear to have modified physiology compared to the physiology of free cells. The nutrient uptake and synthesis patterns of metabolites such as fusel alcohols, esters, and carbonyl compounds change upon immobilization; the following paragraphs describe the impact of immobilization on flavor formation.

6.4.1 Influence of ICT on Higher Alcohol Production

Higher alcohols (also called "fusel alcohols") are produced by yeast cells and represent the major fraction of the volatile compounds. Higher alcohols can be classified as aliphatic [n-propanol, isobutanol, 2-methyl butanol (or active amyl alcohol), 3-methyl butanol (or isoamyl alcohol)], and aromatic (2-phenyl ethanol, tyrosol, tryptophol). Aliphatic higher alcohols contribute to the "alcoholic" or "solvent" aroma of a beverage, and produce a warm mouthfeel. The aromatic alcohol 2-phenyl ethanol has a sweet scent and is a positive contribution to the aroma, whereas the aroma of tyrosol and tryptophol are undesirable. Higher alcohols are synthesized by yeast during fermentation via the catabolic (Ehrlich) and anabolic pathway (amino acid metabolism) (Ehrlich 1904).

Catabolism of the branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenylalanine, tyrosine, and trytophan), and sulfur-containing amino acid (methionine) leads to the formation of fusel acids and fusel alcohols. Firstly, the yeast cells use amino acids from the wort to produce the corresponding α-keto acids via a transamination reaction. The excess oxoacids are subsequently decarboxylated into aldehydes and further reduced (by alcohol dehydrogenase) to higher alcohols. The simplified Ehrlich pathway is shown in Fig. 6.6. The genes encoding each step of the process are quoted in a recent review by Hazelwood et al. (2008).

In the anabolic pathway, the higher alcohols are synthesized from α-keto acids during the synthesis of amino acids from the carbohydrate source. Both pathways may take place during the same fermentation in the traditional batch process with a switch from the degradative route to the biosynthetic route, occurring when the amino acids in the substrate have been metabolized or missed. The pathway choice depends on the individual higher alcohol and on the level of available amino acids. The importance of the anabolic pathway increases during the later stage of a conventional batch fermentation as wort amino acids are depleted, as well as in cider production where the apple juice contains only small amounts of amino acids.

Conditions that promote yeast cell growth – such as high levels of nutrients (amino acids, oxygen, lipids, zinc), increased temperature, and agitation – stimulate

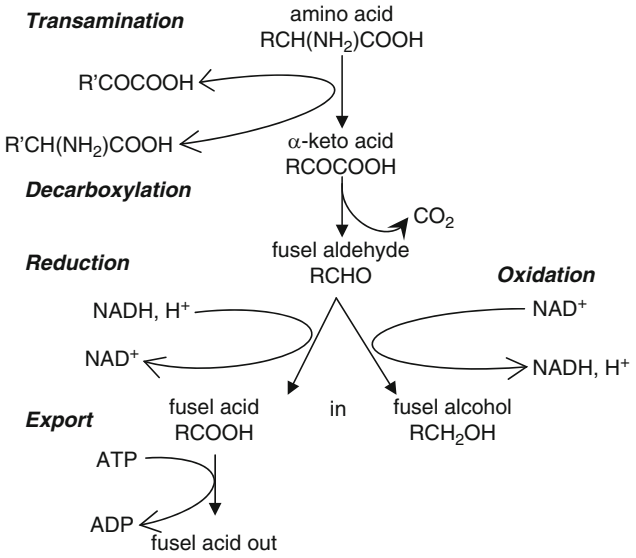

Fig. 6.6 The Ehrlich pathway (Adapted from Hazelwood et al. 2008)

the production of higher alcohols (Landaud et al. 2001). On the other hand, conditions that restrict yeast growth – such as lower temperature and higher (CO_2) pressure – reduce the extent of higher alcohol production (Renger et al. 1992).

In immobilized systems with enhanced or similar free amino nitrogen (FAN) uptake levels, the formation of higher alcohols was higher or equal to batch systems (Shen et al. 2003). A decrease of higher alcohol production in beer upon cell immobilization, compared to free-cell fermentation, has been frequently reported and nicely summarized by Willaert and Nedovic (2006). This decrease has been attributed to the limited cellular growth in immobilized cell systems, leading to poor nitrogen removal. Similarly, in the case of cider production, in a continuous fermentation system with *Saccharomyces bayanus* co-immobilized with *Oenococcus oeni* in alginate beads, production of fusel alcohols was several times lower compared to synthesis during batch fermentation process with suspended cells (Nedovic et al. 2000). The anabolic flux limitation of yeast cells in the pseudo-stationary phase was proposed to justify the lower concentration of fusel alcohols. It was also found that the behavior of cells adsorbed on the carrier surface was similar to that of free cells, but significantly different from entrapped cells (Smogrovicová and Dömeny 1999). New technologies have introduced some new inclusion carriers, with adjusted shape and size to overcome internal mass transfer restrictions, which give similar higher alcohol concentrations, compared to a conventional process (Nedovic et al. 2005b).

It has been demonstrated that mass (i.e., amino acids) transfer rates in the fermenting medium or, in other words, the external mass transfer properties, also

influence higher alcohol synthesis. Thus, in fluidized-bed and gas-lift bioreactors the rate of amino acid uptake increased with the superficial velocity of the fluid (Cop et al. 1989; Masschelein et al. 1994; Nedovic et al. 1996; Aivasidis et al. 1991).

6.4.2 Ester Production in ICT Systems

Esters constitute a major group of desirable flavor compounds. Among the esters formed, the most significant in fermented beverages are ethyl acetate (fruity, solvent-like), isoamyl acetate (pear drops), isobutyl acetate (banana-like), ethyl hexanoate (apple-like), and 2-phenyl acetate (honey, fruity, flowery). They are formed by yeast during fermentation in a reaction between the alcohols, fatty acids, co-enzyme A (CoASH), and an ester synthesizing enzyme. Actually, the formation of esters occurs in two steps: (1) fatty acids that have undergone a previous activation by CoASH form acyl-CoA and (2) alcohols become esterified by reacting with acyl-CoA to the corresponding ester under the action of alcohol acetyl transferase (Peddie 1990). Because ethanol is the dominant alcohol in fermenting beverages, ethyl acetate (produced from acetyl-CoA and ethanol) is the dominant ester. It has been shown that the main factor controlling ester biosynthesis is the expression level of the *ATF1* gene, which encodes alcohol acetyl transferase I (Lilly et al. 2000; Verstrepen et al. 2003b). *ATF1* gene expression is repressed by oxygen and unsaturated fatty acids (Fujii et al. 1997; Fujiwara et al. 1998). The ester production rate is influenced by many factors, such as temperature, specific growth rate, pitching rate, top pressure, oxygen availability, as well as fermenting medium composition (Willaert and Nedovic 2006; Verbelen et al. 2009).

In some immobilized processes low ester concentrations are found, while in others ester synthesis is increased upon cell immobilization. Low ester content is related to the low cellular metabolic activities in these systems. In a study of continuous fermentation in cider, concentration of isoamylacetate was two times lower compared to concentration achieved in a control batch fermentation process with suspended cells, as a result of isoamylalcohol availability (Nedovic et al. 2000). On the other hand, due to mass transfer limitations, oxygen concentration in an immobilization matrix is low, causing reduced cellular growth, so that the cellular acetyl-CoA pool is more available for ester synthesis instead of channeling for fatty acid biosynthesis. Thus, the anaerobic conditions and the absence of substantial levels of unsaturated fatty acids limit cell growth during production and stimulate formation of acetate esters. For example, this occurred during the production of alcohol-free beer in a packed-bed reactor with surface-attached cells on DEAE-cellulose beads (Van Iersel et al. 1999). In another study, a 22% increase in ester concentration upon cell immobilization on stainless-steel fiber cloth was explained by a significant rise in the expression level of *AFT1* in the immobilized cells, leading to enhanced ester concentrations in the final fermented product (Shen et al. 2003).

6.4.3 Carbonyl Compounds Production in ICT Systems

The most important carbonyl compounds formed in beverage fermentation are acetaldehydes, diacetyl, and 2,3-pentanedione. Aldehydes, having very low flavor thresholds, tend to be considered as off-flavors (e.g., acetaldehyde causes a green leaf-like flavor in beer). As intermediates in the formation of ethanol and higher alcohols from amino acids and sugar, the conditions favoring alcohol production also generate the formation of small quantities of aldehydes. These may be excreted but can be reabsorbed and reduced by yeast to the corresponding alcohol during the later stages of fermentation (acetaldehyde is normally reduced to ethanol). The most extensively studied carbonyl compound is diacetyl, which makes an important contribution to the flavor of cider, red wine, beer, and some distilled products such as whisky and rum. Although its presence may contribute to the correct flavor, especially in cider and red wine, excessive production can lead to off-flavors, particularly in the case of beer. Diacetyl and 2,3-pentanedione are side products of amino acid synthesis in yeast. They are produced by the spontaneous oxidative decarboxylation of the corresponding acetohydroxy acids, α-acetolactate, and α-acetohydroxybutyrate, which are metabolite intermediates of the common biosynthetic pathways of valine and isoleucine. Acetohydroxy acids are firstly excreted from the yeast cells to the surrounding medium where they are transformed chemically into diacetyl and 2,3-pentanedione. The formation and subsequent reassimilation of actohydroxy acids by the yeast and degradation of diacetyl are shown schematically in Fig. 6.7. Due to the coupling of acetohydroxy acids with the anabolic metabolism of yeast, they are produced only in the earlier phases of batch fermentation. In the later stages of fermentation, actively metabolizing yeast cells are able to reduce diacetyl and 2,3-pentanedione to acetoin and butane-2,3-dione, and 2,3-butanediol, respectively. Hence, the balance between rate of formation and rate of degradation determines the final concentrations of diacetyl and 2,3-pentanedione in beverages. In cider, in addition to the formation of vicinal diketones by yeast, a part of diacetyl present is also produced by bacteria from pyruvic acid. Thus, *Oenococcus oeni* is able to produce diacetyl directly by the activity of the diacetyl synthetase without any excretion of precursors in the fermenting medium.

In the case of diacetyl content in beer, different phenomena upon immobilization have been reported. In most cases, the production of diacetyl by immobilized cells is much higher than for free cells. In addition, the production of vicinal diketones can be controlled by the initial yeast cell concentration in Ca-alginate beads (Shindo et al. 1994). This has been explained by an increased expression of the acetohydroxy acid synthetase gene during the growth of the yeast cells in the carrier (Shindo et al. 1994). In a recirculation bioreactor system with continuous sugar feed, the concentration of 2,3-pentanedione was two to four times larger than the diacetyl concentration due to a more intensive pentanedione pathway (Pajunen et al. 2001). In an air-lift reactor with spent grains as the immobilization matrix, the total diacetyl concentration largely varied depending on the operational conditions and decreased with increasing aeration and temperature (Brányik et al. 2004).

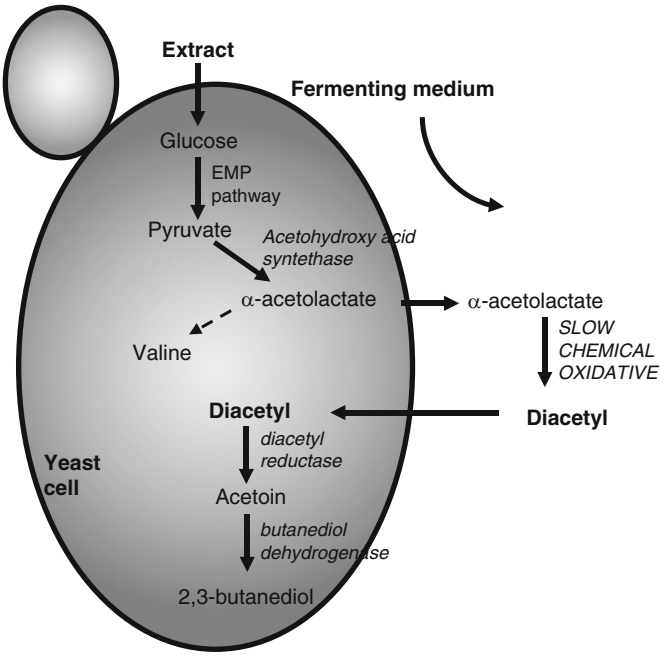

Fig. 6.7 Schematic presentation showing diacetyl formation, reassimilation, and removal (Adapted from Willaert and Nedovic 2006)

After a maturation period of 10 days at 4°C, the concentration of diacetyl was reduced below its flavor threshold (Brányik et al. 2002). The concentrations of vicinal diketones were around 20 times higher in a continuous fermentation of cider with yeast immobilized in Lentikats at a sugar attenuation of 95% compared with those obtained in a batch process (Durieux et al. 2002). In another study with alginate beads used for cider production in the same continuous system, diacetyl concentration was increased two times compared to concentration achieved in a fermentation process with suspended cells (Nedovic et al. 2000). The larger concentration of diacetyl in the immobilized systems was explained by the diffusion mass transfer effect that prevents transfer of diacetyl from the medium to the immobilized yeast after chemical oxidative decarboxylation of α-acetolactate in the medium. Addition of the missing enzyme α-acetolactate decarboxylase (commercially available) to the wort may also lead to increased formation of diacetyl (Hanneman 2002).

The drawback in using alginate is biomass leakage due to local overpressure in the beads generated by carbon dioxide production. Nedovic and coworkers (2004, 2005a) suggested that free yeast issued from the continuous reactor could be used for diacetyl uptake in a maturation tank. Optimization of the operational parameters in a gas-lift bioreactor with alginate microbeads as yeast carriers can provide low concentrations of diacetyl. One way to decrease the diacetyl level in a continuous

system is to prolong the residence time during fermentation and/or maturation process (Andersen et al. 1999; Nedovic et al. 2000). Also the use of genetically modified yeast enables reduction of the diacetyl level (Hammond 1995).

6.4.4 *Secondary Fermentation Using ICT*

The maturation of green beer is needed primarily to reduce the level of diacetyl (an unwanted aroma compound in beer). This vicinal diketone has a very low threshold (0.08–0.15 ppm) in beer (Wainwright 1973). The traditional maturation process lasts for 3–4 weeks at a low temperature and low yeast concentration. However, using ICT, this period could be reduced to 2 h. So far, two continuous maturation systems have been implemented industrially. The first one is a packed-bed bioreactor with DEAE-cellulose granules (later replaced by cheaper aspen wood chips) used at Sinebrychoff Brewery (Finland); it has a capacity of 1 million hectoliters/year (Yamauchi et al. 1995; Virkajärvi 2002). Another system was developed by Alfa Laval and Schott Engineering (Mensour et al. 1997) based on porous glass beads (Dillenhofer and Ronn 1996). The Alfa Laval system for secondary fermentation of beer is shown in Fig. 6.8. This system has been implemented in several breweries in Finland, Belgium, and Germany. The German company Brau & Brunnen purchased and installed a 30,000 hL/year pilot-scale Alfa Laval

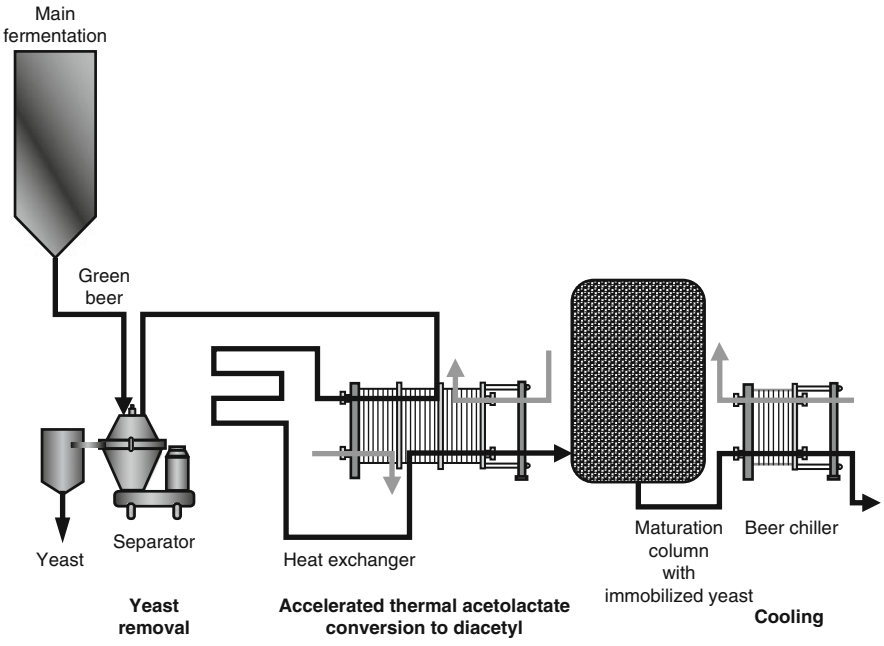

Fig. 6.8 Process flow sheet for secondary fermentation of beer using the Alfa Laval system

maturation system in 1996 (Mensour et al. 1997). The same system was implemented in a medium-sized German brewery as well (Schäff/Treuchtlingen) (Back et al. 1998). The beers obtained overall yielded good analytical and sensorial results.

6.4.5 Malolactic Fermentation in ICT Systems

MLF is a very important process in the maturation of alcoholic beverages, particularly wines and ciders. The fermentation is catalyzed by a number of LAB that can gain a competitive advantage by metabolizing malic acid to lactic acid. MLF is generally recognized as an important manufacturing step: (1) it reduces the acidity of wine or cider, (2) it stabilizes the product with respect to microbial spoilages through the bacteriostatic effect of the lactic acid produced and consumption of residual substrates, and (3) it contributes to the flavor complexity of wine/cider by producing compounds such as acetaldehyde, acetic acid, ethyl acetate, ethyl lactate, diacetyl, acetoin, and 2,3-butanediol. The MLF process, therefore, not only affects the flavor of the beverage, but also stimulates growth or enhances resistance to the extreme environment found in wines and ciders, for example, low pH and high alcohol concentration. Whether MLF should be encouraged or discouraged in wine-making depends on the quality of ripe grapes and the desired level of certain flavorful by-products.

The growth of LAB, that is, *O. oeni* and *Lactobacillis brevis*, has been investigated in this context. The initiation of MLF appears to be the main limiting factor in cider and wine production. Several strategies have been suggested to sustain and accomplish MLF in wine and cider: the use of enzymatic reactors, recombinant yeast strains, and cell-recycle bioreactors (Durieux et al. 2005). The immobilization of LAB for controlling MLF provides increased tolerance of malolactic bacteria and acceleration of the MLF process. Different immobilization supports have been used so far to immobilize *O. oeni*, such as calcium alginate, kappa-carrageenan, cellulose sponge, and polyacrylamide (reviewed by Kourkoutas et al. 2009).

The immobilization of *O. oeni* enabled accomplishment of MLF simultaneously with the alcoholic fermentation or at the end of this fermentation. The alcoholic fermentation was preceded either by free (Cabranes et al. 1998) or co-immobilized yeast cells in batch (Scott and O'Reilly 1996) and continuous (Nedovic et al. 2000) bioreactor systems. Yeast growth and ethanol production were not affected by the presence of immobilized *O. oeni*. The final organoleptic profile of cider depended on the fermentation temperature and the carrier used.

Thus, in the case of alginate beads, malic acid was fully metabolized at 18°C, while a residual amount of this acid was detected at 12°C (Cabranes et al. 1998). Acetic acid produced at the end of fermentation at 18°C was doubled compared to that obtained by free *O. oeni*. In another study, using Lentikats as an alternative to alginate beads in a continuous system, the largest malic acid attenuation was achieved at temperatures between 25°C and 30°C (Durieux et al. 2000). Surprisingly,

the malic acid conversion was possible even at very acidic pH (down to pH 2.3), while with free cells the MLF did not occur below 3.9. A modification of the cell physiology and the immobilized cell microenvironment, characterized by pH gradient inside the matrix, was proposed to explain the improved performance of *O. oeni* at acidic pH by respectively allowing generation of enough adenosine triphosphate (ATP) to maintain cytoplasmic pH without any perturbation of the MLF and by restoring favorable pH in the direct environment of the cells. In continuous systems, deacidification levels can be easily adjusted as a function of the residence time (Nedovic et al. 2000). In those systems, the production of soft or dry cider is possible by controlling the feeding flow rates.

6.5 Conclusion

In this chapter, an overview of ICT applications in fermentation processes aimed at alcoholic beverage production was presented. The objective was to analyze and assess data on the impact of immobilization technologies on viable microbial cells in the alcoholic and malolactic fermentation of beer, wine, and cider. ICT is well established for flavor maturation and the production of alcohol-free and low-alcohol beer on an industrial scale. In addition, several primary beer and wine fermentation processes based on ICT have been developed on a pilot and an industrial scale. However, the issues of mass transfer limitations and process control still need to be resolved in order to obtain a beverage of consistent quality. Selecting a suitable carrier and bioreactor system is a challenge and many issues should be taken into account, such as maintenance of cell viability during production, product quality, safety and stability during processing and storage, and investment and operating costs. In particular, assessment of industrial feasibility of the immobilization fermentation technology is mandatory for cost-effective, large-scale applications.

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