Chapter 11
Bioprocess Intensification of Beer Fermentation Using Immobilised Cells

Pieter J. Verbelen, Viktor A. Nedović, Verica Manojlović, Freddy R. Delvaux, Ida Laskošek-Čukalović, Branko Bugarski, and Ronnie Willaert

11.1 Introduction

Beer production with immobilised yeast has been the subject of research for approximately 30 years but has so far found limited application in the brewing industry, due to engineering problems, unrealised cost advantages, microbial contaminations and an unbalanced beer flavor (Linko et al. 1998; Bránýik et al. 2005; Willaert and Nedović 2006). The ultimate aim of this research is the production of beer of desired quality within 1–3 days. Traditional beer fermentation systems use freely suspended yeast cells to ferment wort in an unstirred batch reactor. The primary fermentation takes approximately 7 days with a subsequent secondary fermentation (maturation) of several weeks. A batch culture system employing immobilization could benefit from an increased rate of fermentation. However, it appears that in terms of increasing productivity, a continuous fermentation system with immobilization would be the best method (Verbelen et al. 2006). An important issue of the research area is whether beer can be produced by immobilised yeast in continuous culture with the same characteristic as the traditional method.

P.J. Verbelen and F.R. Delvaux
Centre for Malting and Brewing Science, Faculty of Bioscience Engineering KU Leuven, Kasteelpark Arenberg 22, 3001 Heverlee, Belgium

V.A. Nedović (✉) and I. Laskošek-Čukalović
Department of Food Technology and Biochemistry, University of Belgrade, Nemanjina 6, PO Box 127, 11081 Belgrade-Zemun, Serbia and Montenegro
e-mail: vnedovic@agrif.bg.ac.rs

V. Manojlović and B. Bugarski
Department of Chemical Engineering,University of Belgrade, Karnegijeva 4, 11000 Belgrade, Republic of Serbia

R. Willaert (✉)
Structural Biology Brussels, Vrije Universiteit Brussel, Flanders Institute for Biotechnology, Pleinlaan 2, B-1050 Brussel, Belgium
e-mail: ronnie.willaert@vib-vub.be

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In beer production, as opposed to a process such as bio-ethanol production, the goal is to achieve a particular balance of different secondary metabolites rather than the attainment of high yields of one product. Any alterations of the fermentation procedure can thus have serious implications on the flavor profile. At present, only beer maturation and alcohol-free beer production are obtained by means of commercial-scale immobilised yeast reactors, because in these processes no real yeast growth is required. Immobilised cell physiology control and fine-tuning of the flavor compounds formation during long-term fermentation processes remain the major challenges for successful application of immobilised cell technology on an industrial scale. The key factors for the implementation of this technology on an industrial level are carrier materials, immobilization technology and bioreactor design.

The purpose of this chapter is to summarise and discuss the main cell immobilization methods, process requirements, available carrier materials and bioreactor designs aimed for better yeast physiology control and fine-tuning of the flavor formation during beer fermentation process. Further, it will provide an overview on the latest important breakthroughs, accomplished in understanding of the effects of immobilization on yeast physiology, metabolism and fermentation behaviour.

11.2 Carrier Materials and Design

11.2.1 Cell Immobilization Methods and Carrier Materials

Generally, immobilised cell systems can be classified into four categories based on the physical mechanism of cell localisation and the nature of the support mechanisms: “attachment to a surface,” “entrapment within a porous matrix,” “containment

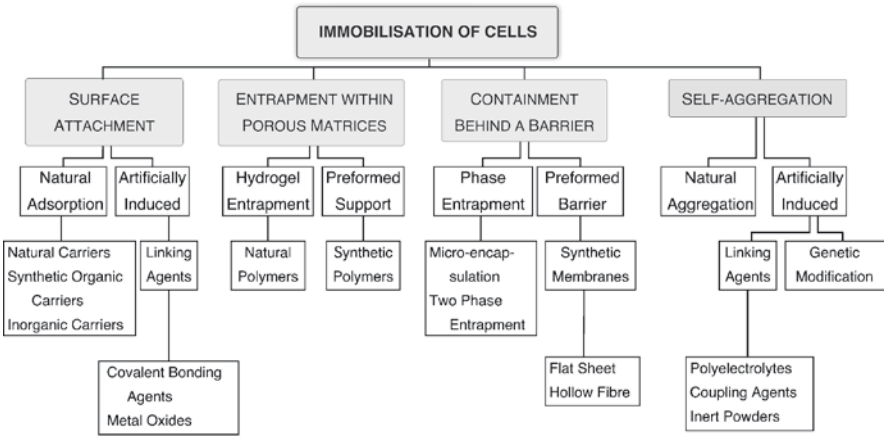

Fig. 11.1 Classification of immobilised cell systems according to the physical localisation and the nature of the micro-environment (Willaert and Baron 1996)

behind a barrier" and "self aggregation" (Karel et al. 1985; Willaert and Baron 1996) (Fig. 11.1). For beverage (and other food) applications, special attention must be paid to the selection of approved, food grade compounds or to the prevention of any leakages of undesired compounds into the beverages.

Cell immobilization by adsorption to a support material is a very popular method, because it is a simple, cheap and fast method. Micro-organisms adsorb spontaneously on a wide variety of organic and inorganic supports. 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. Furthermore, cell physiology has a significant influence on the strength of the adhesion. Various rigid support materials are available mainly aimed at applications in packed-bed reactors. Diethylaminoethyl (DEAE)–cellulose supports have been successfully used on industrial scale for the production of alcohol-free beer and maturation of green beer (Lommi 1990). It is an inert, non-dissolving cellulose matrix, which has a non-uniform granular shape. Yeast cells are immobilised by ionic attraction. Compared to porous supports, the biomass loading capacity of DEAE–cellulose is considerably lower. Other selected materials are presented in Table 11.1.

Cell immobilization in porous matrices can be performed by two different basic methods. In the first method, indicated as gel entrapment, the porous matrix is synthesised in situ around the cells to be immobilised. In the second method, cells are allowed to move into a preformed porous matrix. Generally, both methods provide cell protection from the fluid shear and higher cell densities as compared to surface immobilization are reached. A drawback of these systems can be mass transfer limitations. 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 (Willaert et al. 1999).

Over the last 30 years, most of the research concerning the immobilization of living microbial cells was focused on gel entrapment. Natural polysaccharides (e.g., alginate, chitosan, pectate and carrageenan), synthetic polymers (e.g., polyvinylalcohol, PVA) and proteins (e.g., gelatin, collagen) can be gelled into hydrophilic matrices under mild conditions, allowing cell entrapment with minimal loss of viability. As a result, very high biomass loadings can be achieved. Gels are mostly used in the form of spherical beads with diameters ranging from about 0.3 to 5 mm. However, a disadvantage of gels is their limited mechanical stability. It has been frequently observed that the gel structure is easily destroyed by the growth of the cells and carbon dioxide production. Moreover, calcium alginate gels are weakened in the presence of phosphates (which is present in wort). Long-term use of calcium alginate beads in continuous production (maturation) of beer resulted in loss of bead 3D-structure due to high phosphate contents in the wort/beer. However, several methods have been proposed for reinforcement of gel structures. For example, calcium alginate gel can be strengthened by reaction with polyethyleneimine, glutaraldehyde

Table 11.1 Carrier materials and reactor types for some selected beer fermentation processes using immobilised cells

Carrier material	Reactor type	Reference
Flavor maturation		
Calcium alginate beads	Fixed bed	Shindo et al. (1994)
DEAE-cellulose beads	Fixed bed	Pajunen and Grönqvist (1994)
Polyvinyl alcohol beads	Fixed bed	Smogrovicová et al. (2001)
Porous glass beads	Fixed bed	Linko et al. (1993), Aivasidis (1996)
Alcohol-free beer		
DEAE-cellulose beads	Fixed bed	Collin et al. (1991), Lommi (1990)
Porous glass beads	Fixed bed	Aivasidis et al. (1991)
Silicon carbide rods	Monolith reactor	Van De Winkel et al. (1991)
Acidified wort		
DEAE-cellulose beads	Fixed bed	Pittner et al. (1993)
Main fermentation		
Calcium alginate beads	Gas lift	White and Portno (1979), Onaka et al. (1985), Ryder and Masschelein (1985), Nedovic et al. (1993), Nedovic et al. (1997)
Calcium alginate beads	Fixed bed	Ryder and Masschelein (1985)
Calcium pectate beads	Gas lift	Smogrovicová et al. (1997), Smogrovicová and Dömény (1999)
κ-Carrageenan beads	Gas lift	Mensour et al. (1996), Mensour et al. (1997), Decamps et al. (2004)
Ceramic beads	Fixed bed	Inoue (1995)
Corncobs	Gas lift	Brányik et al. (2006a)
DEAE-cellulose beads	Fixed bed	Kronlöf et al. (1989), Andersen et al. (1999)
Gluten pellets	Fixed bed	Bardi et al. (1997)
Gluten pellets	Gas-lift (external-loop)	Manojlović et al. (2008)
Kieselguhr (diatomaceous earth)	Fixed bed	Narziss and Hellich (1971), Moll et al. (1973), Virkajärvi and Pohjala (2000)
Polyvinyl alcohol beads	Gas lift	Smogrovicová et al. (2001)
Polyvinyl alcohol Lentikats®	Gas lift	Smogrovicová et al. (2001), Bezbradica et al. (2007), Bugarski & Nedović (2007)
Polyvinyl chloride granules	Fixed bed	Moll et al. (1973)
Porous glass beads	Fixed bed	Virkajärvi and Krönlof (1998), Virkajärvi and Pohjala (2000)
Porous chitosan beads	Fluidised bed	Unemoto et al. (1998), Maeba et al. (2000)
Self-aggregation using super-flocculent yeast	Continuous stirred tank reactors	Coutts (1957), Linko et al. (1997)
Silicon carbide rods	Monolith reactor	Van De Winkel et al. (1993); Andries et al. (1996)
Spent grains	Gas lift	Brányik et al. (2002, 2004a)
Stainless steel fibre cloth	Gas lift	Verbelen et al. (2006)
Stainless steel wire spheres	Fluidised bed	Cross and Mavituna (1987)
Wood chips Aspen	Fixed bed	Pajunen et al. (2001)
Wood chips Beech	Fixed bed	Linko et al. (1997), Kronlöf and Virkajärvi (1999)

cross-linking, addition of silica, genepin and polyvinylalcohol, or by partial drying of the gel (Willaert and Baron 1996). For beer production, a food grade reinforcement method should be used. Gel or polymeric matrix beads (usually spherical in shape) could be further coated with an outer layer to create (micro)capsules. In those, the solid core may also be dissolved within the capsule to create the liquid media for cells. However, micro-encapsulation is generally too expensive to be used in beer production and only suited for non-growing cells (Raymond et al. 2004). Another approach is to contain cells within a compartment separated by a preformed membrane such as hollow fibre and flat membrane modules. Entrapment behind preformed membranes represents a gentle immobilization method since no chemical agents or harsh conditions are employed. Usually polymeric microfiltration or ultrafiltration membranes were used, although other types of membranes were also investigated, such as ceramic, silicone or ion exchange membranes. Mass transfer through the membrane is dependent on the pore size and structure as well as on the hydrophobicity/hydrophilicity and surface charge.

Unlike the in situ formed gels, preformed porous 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 (Baron and Willaert 2004; Mavituna 2004). Cell immobilization occurs by attachment to the internal surfaces, self-aggregation and retention in dead-end pockets within the carrier material. Ideally, the colonised porous particles should retain some void spaces for flow so that mass transport of substrates and products could be achieved by both molecular diffusion and convection, avoiding mass transfer limitations. However, fluid flow within the support can be realised only if cell adhesion is not very strong so that excessive biomass could be washed out from the matrix. When high cell densities are obtained, convection is no longer possible and the particles behave as dense cell agglomerates with high diffusion limitations. Yet the cell densities represented per unit of support volume are lower than those achievable by gel entrapment since the preformed porous matrix material takes up significant volume fraction. As compared to the in situ gel particles, preformed porous carriers provide better mechanical properties and higher resistances to compression and disintegration.

Cell immobilization behind or within a porous barrier includes systems with a barrier formed around cells such as microcapsules, and systems with cells contained within a compartment separated by a preformed membrane such as hollow fibre and flat membrane modules. However, micro-encapsulation is generally too expensive to be used in beer production and only suited for non-growing cells (Raymond et al. 2004).

Cell immobilization by self-aggregation is based on the formation of cell clumps or floccules, which can be naturally occurring as in the case of flocculent yeast strains (or can be induced by addition of flocculating agents). It is the simplest and the least expensive immobilization method.

Yeast flocculation is a reversible, asexual and calcium dependent process in which cells adhere to form flocs consisting of thousands of cells (Stratford 1989; Bony et al. 1997; Jin and Speers 1999). Many fungi contain a family of cell wall

glycoproteins (called "adhesines") that confer unique adhesion properties (Teunissen and Steensma 1995; Guo et al. 2000; Hoyer 2001; Sheppard et al. 2004). These molecules are required for the interactions of fungal cells with each other (flocculation and filamentation) (Teunissen and Steensma 1995; Lo and Dranginis 1998; Guo et al. 2000; Viyas et al. 2003), with inert surfaces such as agar and plastic (Gaur and Klotz 1997; Lo and Dranginis 1998; Reynolds and Fink 2001; Li and Palecek 2003) and with mammalian tissues/cells (Cormack et al. 1999; Staab et al. 1999; Fu et al. 2002; Li and Palecek 2003). They are also crucial for the formation of fungal biofilms (Baillie and Douglas 1999; Reynolds and Fink 2001; Green et al. 2004). The adhesin proteins in *S. cerevisiae* are encoded by genes including *FLO1*, *FLO5*, *FLO9* and *FLO10* (Verstrepen et al. 2004). These proteins are called flocculins (Caro et al. 1997), because these proteins promote cell–cell adhesion to form multicellular clumps that sediment out of solution. The *FLO1*, *FLO5*, *FLO9* and *FLO10* genes share considerable sequence homology. The member proteins of the adhesin family have a modular configuration that consists of three domains (A, B and C) and an N-terminal secretory sequence that must be removed as the protein moves through the secretory pathway to the plasma membrane (Hoyer et al. 1998). The N-terminal domain (A) is involved in sugar recognition (Kobayashi et al. 1998). The adhesins undergo several post-translational modifications. They move from the endoplasmic reticulum (ER), through the Golgi and pass through the plasma membrane and find their final destination in the cell wall where they are anchored by a glycosyl phosphatidylinositol (GPI) (Teunissen et al. 1993; Bidard et al. 1994; Hoyer et al. 1998; Bony et al. 1997). The GPI anchor is added to the C-terminus in the ER and mannose residues are added to the many serine and threonine residues in domain B in the Golgi (Udenfriend and Kodukula 1995; Bony et al. 1997; Frieman and Cormack 2003; De Groot et al. 2003). The *FLO1* gene product (Flo1p) has been localised at the cell surface by immunofluorescent microscopy (Bidard et al. 1995). The amount of Flo proteins in flocculent strains increased during batch yeast growth and the Flo1p availability at the cell surface determined the flocculation degree of the yeast. Flo proteins are polarly incorporated into the cell wall at the bud tip and the mother–daughter neck junction (Bony et al. 1997). The transcriptional activity of the flocculation genes is influenced by the nutritional status of the yeast cells as well as other stress factors (Verstrepen et al. 2003a). This implies that during beer fermentation, flocculation is affected by numerous parameters such as nutrient conditions, dissolved oxygen, pH, fermentation temperature, and yeast handling and storage conditions.

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; Pilkington et al. 1998; Van de Winkel and De Vuyst 1997). This fermentation system consists of a hold-up vessel followed by two stirred tank fermentors for the primary fermentation. Subsequently, the flocculent yeast cells are separated from the green beer in a conical settler by gravity. Yeast is then recycled back into the hold-up vessel to increase the cell density and to achieve better control of the fermentation rates.

11.2.2 Carrier Selection

Various cell immobilization carrier materials have been tested and used for beer production. Selection criteria are summarised in Table 11.2. Depending on the particular application, reactor type and operational conditions, some selection criteria will be more appropriate. Examples of selected carrier materials for particular applications are tabulated in Table 11.1.

In one of the first ICT process for continuous beer fermentation, kieselguhr (diatomaceous earth) was selected as carrier material (Narziss and Hellich 1971; Moll et al. 1973). Later on, alginate hydrogel encapsulation became popular (e.g., White and Portno 1979; Hsu and Bernstein 1985; Onaka et al. 1985; Curin et al. 1987; Shindo et al. 1994; Nedovic et al. 1997; Nedovic et al. 2001; Nedovic et al. 2004). In addition, some new hydrogel materials were introduced, like κ-carrageenan, pectate gels and polyvinyl alcohol (PVA) (e.g., Mensour et al. 1996; Pilkington et al. 1999; Smogrovicová and Dömény 1999; Raymond et al. 2004; Nedovic et al. 2005a). The main advantage of the hydrogel entrapment method is the attainment of extremely high cell loadings and – consequently – providing high fermentation rates. However, in some cases cell proliferation and activity can be limited by low mass transfer rates within the matrices. The reduced cell growth in

Table 11.2 Selection criteria for yeast cell immobilization carrier materials for beer production (Nedovic et al. 2005b)

High cell mass loading capacity
Easy access to nutrient media
Simple and gentle immobilization procedure
Immobilization compounds approved for food applications
High surface area-to-volume ratio
Optimum mass transfer distance from flowing media to centre of support
Mechanical stability (compression, abrasion)
Chemical stability
Highly flexible: rapid start-up after shut-down
Sterilisable and reusable
Suitable for conventional reactor systems
Low shear experienced by cells
Easy separation of cells and carrier from media
Readily up-scalable
Economically feasible (low capital and operating costs)
Desired flavor profile and consistent product
Complete attenuation
Controlled oxygenation
Control of contamination
Controlled yeast growth
Wide choice of yeast

immobilised conditions can result in an insufficient free amino nitrogen consumption and as a consequence an unbalanced flavor profile of the final beer (Curin et al. 1987; Hayes et al. 1991). Recently, cheap carrier materials have been investigated since the use of these materials avoids the costly regeneration of used immobilization matrices. Wood chips showed good performance and the use of this material reduced the total investment cost by one third compared to more expensive carriers (Kronlöf et al. 2000).

An alternative preformed support material, based on spent grains, has been proposed recently for yeast cell immobilization for primary beer fermentation in an air-lift bioreactor (Brányik et al. 2001; Brányik et al. 2002; Brányik et al. 2004a; Brányik et al. 2004b; Brányik et al. 2006a). This is an interesting carrier for cell immobilization, since spent grains are a waste by-product from the brewing process. Recently, corncobs were also investigated as carrier material (Brányik et al. 2006a).

11.3 Bioreactor Design

In bioreactors for beverage production, immobilised cells are either mixed with suspended carriers or fixed on carrier particles or large surfaces. Beer production is based on the utilisation of yeast cells, while the acidification of wort employs lactic acid bacteria. Reactor configuration is related to the choice of cell carrier and various modifications and combinations of stirred tank, packed-bed, fluidised-bed, gas-lift and membrane reactors were proposed for different phases in beer production (Table 11.1). Critical issues related to the selection of reactor type and configuration are supply and removal of gases and solutes in the liquid phase and removal of excess biomass formed.

Most studies have been made in packed-bed bioreactor. Packed-bed bioreactors are characterised by a simple design, consisting of a column, which is packed with biocatalyst. The liquid flow is close to plug regime causing low shear rates. This enables usage of various, even fragile materials for cell immobilization (Obradovic et al. 2004). In packed-bed fermentors, high mass transfer restrictions, accumulation of carbon dioxide, non-uniform temperature profiles, flow channelling and stagnant zones were observed during primary fermentation. Therefore, different approaches for the adaptation of immobilised systems were investigated in order to correct the final beer quality. Packed-bed reactors have been selected for the production of alcohol-free or low-alcohol beers and for enhanced flavor maturation using immobilised cells. In these applications, conditions are anaerobic and yeast growth is limited. Preformed carrier materials are selected. Immobilization of cells can be by adsorption (e.g., DEAE-cellulose beads) or 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, such as hydrogels, can be limited to lower bed heights and liquid flow rates due to possible compression of beads.

In the fluidised-bed bioreactors, particles with immobilised cells are fluidised in the liquid up-flow, while gas can be optionally supplied. As a consequence of particle fluidisation, moderate local mixing is established providing 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 fluidisation and prevent their washout. Particle movements and collisions in the fluidised state result in moderate shear stresses and abrasion, creating a need for relatively mechanically stable supports. The scaling up of fluidised-bed bioreactors meets problems due to the difficulties in controlling the bed expansion and may encounter hydrodynamic problems. In stirred tank reactors high aeration resulted in less balanced aroma profile of the final product. Beers produced in fluidised and stirred-tank fermentors had high concentrations of diacetyl, and low 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. Gas-lift reactors can be constructed as internal loop or external loop configuration. In general, the flow in the riser and down-comer section can be described as plug flow with axial dispersion (Obradovic et al. 2004). A gas-lift reactor that was introduced in beer fermentation studies by a Serbian group (Nedovic et al. 1993) retains the advantages of fluidised-beds, such as high loading of solids and good mass transfer properties and it is particularly suitable for applications with low-density carriers (Nedovic et al. 1997, 2004, 2005a, b). Efficient mixing and low shear rates make gas-lift reactors suitable for all types of immobilization materials.

As compared to conventional reactor types, the design of membrane reactors is relatively more complex and more 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 used by the Belgian company Meura (Tournai) for production of lager and ale, and acidified wort (Masschelein et al. 1994). Yeast cells are immobilised 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.

11.4 The Impact of Immobilised Yeast Cell Systems on Beer Flavor

An overview of the flavor formation of various systems for the primary fermentation of beer demonstrates that flavor formation is dependent on the bioreactor system and carrier material (Willaert and Nedovic 2006). The mechanisms of the formation of these compounds in beer fermentation by freely suspended cell systems and changes that usually occur when immobilised systems are used, are explained in the following paragraphs.

11.4.1 Secondary Fermentation Using Immobilised Yeast Cells

One of the objectives of the maturation of green beer is the removal of diacetyl, an unwanted aroma compound. This vicinal diketone has a very low threshold (0.08–0.15 ppm) and imparts a buttery aroma to the beer (Wainwright 1973). During the primary fermentation, diacetyl is produced as a by-product in the synthesis pathway of isoleucine, leucine and valine (ILV pathway) (Fig. 11.2). Because the formation of α-acetolactate is related to the amino acid metabolism, more α-acetolactate will be produced with increasing yeast growth. Because brewer's yeast does not possess α-acetolactate decarboxylase activity, α-acetolactate is excreted from the cell and non-enzymatically converted to diacetyl by an oxidative decarboxylation. This step is the rate-limiting step and proceeds faster at high temperature and lower pH. Subsequently, diacetyl is re-assimilated in the yeast cell, which possesses the necessary enzymes (reductases) to reduce it to the flavor inactive acetoin and further to 2,3-butanediol (Bamforth and Kanauchi 2004). The reduction can occur fast when sufficient yeast is present.

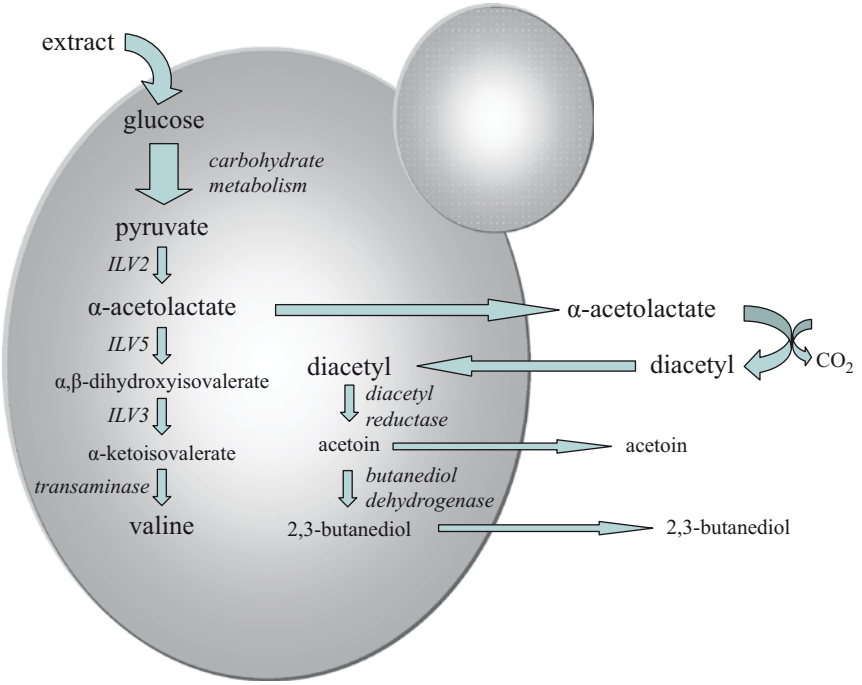

Fig. 11.2 Schematic presentation of the formation, re-assimilation and reduction of diacetyl. The grey oval shape represent a yeast cell, and the smaller circle its daughter cell

The traditional maturation process is performed at a low temperature and low yeast concentration, resulting in a very long maturation period of 3–4 weeks. Nowadays, several strategies have been developed to accelerate the diacetyl removal (Willaert 2007). However, using immobilised cell technology, this period could be further reduced to a few hours. Two continuous maturation systems have been implemented industrially so far: one at Sinebrychoff Brewery (Finland, capacity: 1 million hl per year) and another system, developed by Alfa Laval and Schott Engineering (Mensour et al. 1997). They are both composed of a separator (to prevent growing yeast cells in the next stages), an anaerobic heat treatment unit (to accelerate the chemical conversion of α-acetolactate to diacetyl, but also the partial directly conversion to acetoin), and a packed-bed reactor with yeast immobilised on DEAE-cellulose granules or porous glass beads (to reduce the remaining diacetyl), respectively (Yamauchi et al. 1995). Later on, the DEAE-cellulose carriers were replaced by cheaper wood chips (Virkajärvi 2002). Recently, the heat treatment has been replaced by an enzymatic transformation in a fixed-bed reactor, in which the α-acetolactate decarboxylase is immobilised in special multilayer capsules, followed by the reduction of diacetyl by yeast in a second packed-bed reactor (Nitzsche et al. 2001).

11.4.2 Alcohol-free or Low-alcohol Beer Using Immobilised Yeast Cells

The main objective during fermentation of alcohol-free beer is the reduction of wort carbonyl flavors by the activity of alcohol dehydrogenases of yeast, without the formation of alcohol (Van Iersel et al. 1999). Traditionally, alcohol-free beer is being produced by arrested batch fermentations. An alternative method is the removal of alcohol by using membrane, distillation or vacuum evaporation processes, although these have the disadvantage that the production cost is increased. Controlled ethanol production for low alcohol or alcohol-free beers has been successfully achieved by partial fermentation using immobilization reactors. The reduction of the wort aldehydes can be efficiently achieved by a short-contact time with the immobilised yeast cells. The process is performed at a low temperature to avoid undesirable cell growth and ethanol production and to maintain a good long-term yeast viability (Van Dieren 1995). A disadvantage of this process is the low production of desirable esters. Bavaria Brewery (the Netherlands) is using a packed-bed immobilised yeast bioreactor with a production capacity of 150,000 hl alcohol-free beer per annum (Mensour et al. 1997).

11.4.3 Primary Fermentation with Immobilised Yeast

During the main fermentation of beer, not only ethanol is being produced, but also a complex mixture of flavor-active secondary metabolites, of which the higher (or

fusel) alcohols and esters are the most important. In addition, diacetyl and some sulphury compounds can cause off-flavors. Since this complex flavor profile is closely related to the amino acid metabolism and consequently to the growth of the yeast cells, differences in the growth metabolic state between freely suspended and immobilised yeast cell systems are most probably responsible for the majority of alterations in the beer flavor. For that reason, it is important that the physiological and metabolic state of the yeast in conventional batch systems is mimicked as much as possible during the continuous fermentation with immobilised yeast. In the continuous mode of operation, cells are not exposed to significant alterations of the environment, influencing the metabolism of the cells and consequently the flavor. Hence, the microbial population of continuous systems lacks the different growth phases of a batch culture. To imitate the batch process as much as possible, plug-flow reactors or a series of reactors can be used. As can be assumed, both the continuous mode of operation and the immobilization of yeast cells can influence the beer flavor.

11.4.3.1 Nutrient Uptake

An increase of glucose uptake and ethanol formation during glucose fermentation using immobilization systems is often stated (Navarro and Durand 1977; Doran and Bailey 1986; Galazzo and Bailey 1990). In conventional beer fermentations, a sequential uptake of the wort sugars is observed. First glucose, sucrose and fructose are taken up. After the uptake of glucose, the main wort sugar maltose (60–70%) is assimilated. Finally, maltotriose is slowly taken up (Hammond 1995). In immobilised yeast systems, alterations in the sugar uptake can occur, which can result in an altered beer taste (Van De Winkel et al. 1993; Willaert et al. 1999).

In a normal batch fermentation, the amino acids of the wort are categorised based on the sequential uptake pattern (Jones and Pierce 1964). Amino acids of Group A (arginine, asparagine, aspartic acid, glutamic acid, lysine, serine and threonine) are immediately taken up, while the uptake of amino acids of Group B (histidine, isoleucine, leucine, methionine and valine) is delayed. Amino acids of Group C (alanine, glycine, phenylalanine, tyrosine and tryptophan) are only taken up when Group A amino acids are depleted in the medium. Proline (Group D) is not utilised by the yeast. In immobilised yeast systems, the uptake pattern often differs from that of the traditional pattern (Ryder and Masschelein 1985; Shen et al. 2003a). Because amino acid metabolism is closely associated with flavor compounds, altered nitrogen uptake can have significant effects on beer flavor. Less uptake of amino acids were reported in a continuous packed-bed reactor (Ryder and Masschelein 1985). This was circumvented by using a fluidised bed reactor, suggesting that mass transfer limitations affected the nitrogen uptake (Cop et al. 1989). In a batch reactor with yeast immobilised on stainless steel fibre cloth, the final utilisation of Group A, B and C amino acids was more complete than in the standard free cell system (Shen et al. 2003a). In the system, described by Pajunen et al. (2001), little FAN ("*Free Amino Nitrogen*") was taken up by the cells, due to non-growth conditions, causing a high beer pH. Dunbar et al. (1988) reported that a

continuous free cell system was characterised by a different uptake pattern than that proposed by Jones and Pierce (1964) for batch fermentations.

11.4.3.2 Higher Alcohols

The most important higher alcohols are isoamyl alcohol (3-methyl-1-butanol) and 2-phenyl ethanol which can be found around their threshold concentrations (60–70 and 25–125 ppm, respectively) in lager beer. Other aliphatic alcohols can also contribute to the alcoholic aroma of the beer (Meilgaard 1975). The aromatic alcohol 2-phenylethanol has a sweet rose-like aroma and is believed to mask the dimethyl sulphide (DMS) perception (Hegarty et al. 1995). Higher alcohols are synthesised by yeast during the fermentation via the catabolic (Ehrlich) or anabolic pathways (Genevois) of the amino acid metabolism (Chen 1978). In both pathways α-keto acids are formed, which are decarboxylated to aldehydes and further reduced (by an alcohol dehydrogenase) to higher alcohols. Higher alcohols are mainly produced during the active growth phase of the fermentation and are therefore influenced by each factor affecting the yeast growth, such as high levels of nutrients (amino acids, oxygen, lipids, zinc), increased temperature and agitation (Landaud et al. 2001).

The influence of immobilised cell systems on the production of higher alcohols differs from system to system. At the same degree of attenuation in batch and immobilised systems, the differences can be most probably ascribed to different levels of amino acid utilisation and yeast growth. In immobilised systems with enhanced or similar FAN uptake levels, the formation of higher alcohols was higher or equal to batch systems (Kronlöf et al. 1989; Shen et al. 2003a). Decreased higher alcohol production rates by immobilised as opposed to free-cell systems were attributed to limited cellular growth. The use of entrapped yeast was most of the time associated with FAN uptake limitations (Ryder and Masschelein 1985; Smogrovicová and Dömény 1999). However, new technologies introduced some new inclusion carriers with adjusted shape and size to overcome internal mass transfer restrictions, which gave similar higher alcohol concentrations compared to a conventional process (Nedovic et al. 2005a).

There is a trend of increased propanol yields, compared to isobutanol (Fig. 11.3). According to Pajunen et al. (2001), the overproduction was the result of a more active α-ketobutyrate pathway, which could also explain the increased concentrations of the vicinal diketone 2,3-pentanedione. Alternatively, continuous inflow of the amino acid threonine could be causing the stimulation of propanol production, as threonine is the precursor of propanol in the catabolic pathway (Chen 1978).

11.4.3.3 Esters

Esters are the most important flavor compounds in beer, since their low flavor thresholds can be crossed in lager beers. The major esters in beer are ethyl acetate (solvent-like, threshold: 33 ppm), isoamyl acetate (banana, threshold: 1.4 ppm), ethyl caproate (apple, threshold: 0.2 ppm), ethyl caprylate (apple, threshold: 0.9 ppm)

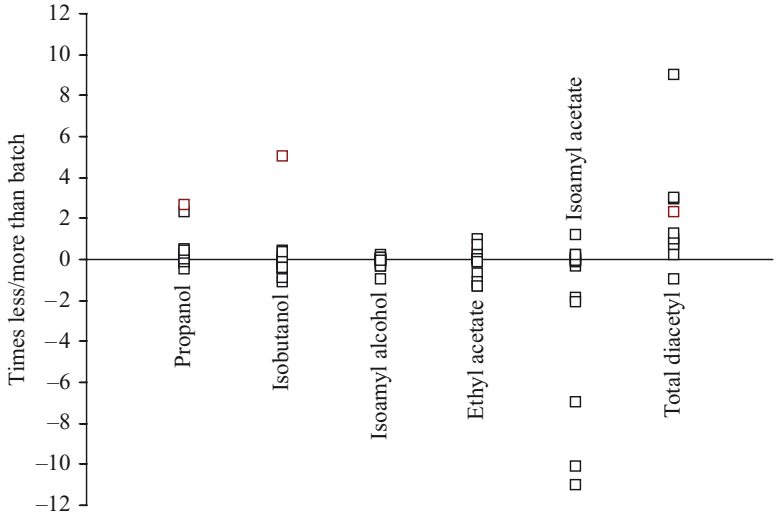

Fig. 11.3 Multiples of compound concentrations and their distribution in continuous beer fermentation systems using immobilised yeast systems compared to corresponding conventional, batch beer fermentation systems [data based on Brányik et al. (2006b), with permission]

and phenylethyl acetate (rosey, threshold: 3 ppm) (Meilgaard 1975). They are desirable components of beer when present in appropriate quantities. Esters are produced by yeast during the growth phase (60%) and the stationary phase (40%). They are formed by the condensation reaction between acetyl/acyl-CoA and higher alcohols catalysed by the alcohol acyltransferases of the yeast (Peddie 1990) (Fig. 11.4). Furthermore, it has been shown that the balance between ester-synthesising enzymes and esterases, which hydrolyse esters, might be important for the net rate of ester accumulation (Fukuda et al. 1998). Fundamentally, two factors determine the rate of ester formation: the availability of the two substrates (acetyl/acyl-CoA and fusel alcohols) and the activity of the enzymes. Ester formation is therefore influenced by temperature, hydrostatic pressure, growth rate, dissolved oxygen, fatty acids, carbon source and nitrogen concentration (Fig. 11.4, for a review, see Verstrepen et al. 2003b).

Immobilised yeast systems give variable amounts of esters, depending on the type of the system and operational conditions. However, the overall tendency in most continuous systems indicates a somewhat reduced ester formation (Fig. 11.4). It can be suggested that the most important factor affecting the ester formation is the oxygen availability during the fermentation, because immobilization itself stimulates the expression of *ATF1* (Shen et al. 2003b). Low oxygen concentrations, due to mass transfer limitations, trigger the formation of esters, by a direct effect upon the gene expression and by an indirect effect on substrate availability

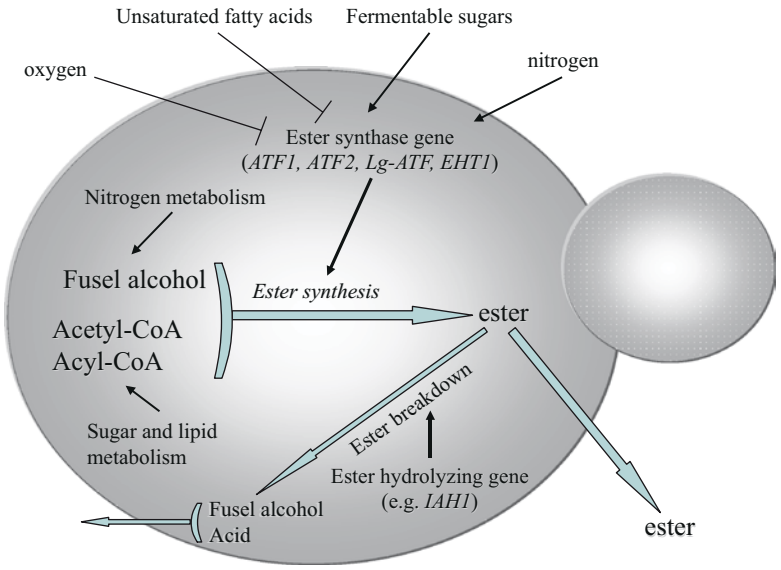

Fig. 11.4 Biochemical formation of esters (Verstrepen et al. 2003b)

(more acetyl-CoA is available for ester synthesis instead of fatty acid synthesis) (Masschelein et al. 1985; Van Iersel et al. 1999; Shen et al. 2003a; Shen et al. 2003b). High oxygen concentrations, due to continuous aeration of the wort in the reactor, result in poor ester formation (Virkajärvi et al. 1999; Wackerbauer et al. 2003).

Generally, the ester synthesis is a sensitive process, which is rather difficult to control, due to numerous influencing factors involved. The selection of an optimal yeast strain or the use of genetically modified strains could ameliorate the ester profile of beer, made by immobilised cell technology.

11.4.3.4 Diacetyl

In most cases, the amount of diacetyl formed by immobilised systems is much higher than in young beers of traditional free cell systems (Ryder and Masschelein 1985; Kronlöf et al. 1989; Van De Winkel et al. 1993; Brányik et al. 2006a). This has been explained by the following:

– Increased expression of Ilv2 enzyme (acetohydroxy acid synthetase) due to immobilization, resulting in increased α-acetolactate concentrations (Shindo et al. 1994).
– Alterations in the amino acid metabolism, caused by cell immobilization itself (Ryder and Masschelein 1985; Shindo et al. 1993; Shen et al. 2003a).

- Enhanced uptake of amino acids, due to rapid yeast growth, causing valine depletion and anabolic formation of the amino acid, resulting in increased α-acetolactate concentrations. The excess yeast growth is often a result of over-aeration (Brányik et al. 2006a).
- Continuous inlet of amino acids of Group A, which inhibit the uptake of valine (Dufour and Devreux 1986).
- Short residence times, meaning that the slow oxidative decarboxylation from α-acetolactate to diacetyl and thus the reduction of diacetyl by the yeast is incomplete (Okabe et al. 1994).
- Increasing the concentration of immobilised cells and prolonging the residence time leads to lower diacetyl concentrations (Shindo et al. 1994; Pajunen et al. 2001; Brányik et al. 2006a).

However, without lowering the fermentation rate, other strategies are possible to suppress these excessive diacetyl concentrations during continuous fermentation:

- Addition of the missing enzyme α-acetolactate decarboxylase, which is commercial available, to the wort (Hanneman 2002).
- Optimization of FAN-content, especially the valine concentration, of the wort (Pajunen et al. 2001; Petersen et al. 2004).
- The use of genetically modified yeast, encoding the α-acetolactate decarboxylase or overexpressing the *ILV5* gene, encoding the acetohydroxy acid reductoisomerase enzyme in the rate-limiting step of the ILV-pathway (Linko and Kronlöf 1991; Hammond 1995).

11.4.3.5 Control Strategies

The optimization of temperature, wort gravity, feed volume and wort composition seems to be an important tool for the control of the flavor-active compounds formation in immobilised beer fermentation systems (Table 11.3) [for a recent review of these effects, see Willaert and Nedovic (2006)]. Many researchers have concluded that the optimization of aeration during continuous fermentation is essential for the quality of the final beer (Virkajärvi et al. 1999). Oxygen is needed for the formation of unsaturated fatty acids and sterols, needed for growth (Depraetere et al. 2003). However, excess oxygen will lead to low ester production and to excessive diacetyl, acetaldehyde and fusel alcohol formation (Okabe et al. 1992; Wackerbauer et al. 2003; Brányik et al. 2004a). It is possible to adjust the flavor of the produced beer by ensuring the adequate amount of dissolved oxygen by sparging with a mixture of air, nitrogen or carbon dioxide (Kronlöf and Linko 1992; Brányik et al. 2004a). However, it remains difficult to predict the right amount of oxygen, because the oxygen availability to the immobilised yeast cells is dependent of external and internal mass transfer limitations.

Finally, the reactor design, the carrier and yeast strain can also have a dominant effect on flavor formation (Cop et al. 1989; Linko, et al. 1997; Smogrovicová and Dömény 1999; Tata et al. 1999; Virkajärvi and Pohjala 2000).

Table 11.3 Some operational parameters and their impact on flavor in immobilised beer fermentation systems

Parameter	Impact on flavor	Reference
Temperature	Increased diacetyl, higher alcohols and esters concentrations	Smogrovicová and Dömény (1999)
Wort gravity	Increased acetaldehyde; similar higher alcohols and esters	Virkajärvi et al. (2002)
Feed volume	Controlling attenuation, enhancing diacetyl removal	Pajunen et al. (2001)
Wort composition	Optimal FAN levels lower diacetyl concentration	Pajunen et al. (2001); Petersen et al. (2004)
Dissolved oxygen	Increased concentrations of diacetyl, acetaldehyde and higher alcohols; decreased esters levels	Kronlöf and Linko (1992)

11.5 Conclusions

The main benefit of immobilised cell technology (ICT) in the beverage industries is rendering high-productivity continuous fermentation feasible. Although research on immobilised cells is now approximately 30 years old, difficulties encountered in pilot-plant and full industrial-scale fermentation processes have not been solved yet. In fact, engineering problems linked to choice of the carrier and reactor design are complicated by the effects of immobilization on the flavor profile of the final product. Incomplete knowledge of the effects of immobilization on the physiology of brewers' yeast will lead to incomplete and partially empirical use of immobilised cell technology for processes based on alcoholic fermentation. These processes are very complex, linked to various side reactions important for flavor formation and final product quality.

Ongoing basic research is continuing to explore new materials as potential carriers for microbial cells and to identify and characterise changes in cell physiology and metabolism upon immobilization. Mass transfer limitations and process control are still issues that have to be resolved in order to obtain consistent quality of the beer. The assessment of the industrial feasibility of the immobilised fermentation technology is mandatory for providing cost-effective, large-scale applications. Since scale up is not always that easy and the beer quality should be as desired, piloting is required to fine tune the specifications. Manipulating yeast strains that have been genetically modified to develop an ability to produce excessive amounts of one and/or lower amounts of another flavor compound is another direction in the area of fermentation technology development.

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