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REVIEW

Immobilized yeast cell systems for continuous fermentation applications

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Abstract In several yeast-related industries, continuous fermentation systems offer important economical advantages in comparison with traditional systems. Fermentation rates are significantly improved, especially when continuous fermentation is combined with cell immobilization techniques to increase the yeast concentration in the fermentor. Hence the technique holds a great promise for the efficient production of fermented beverages, such as beer, wine and cider as well as bio-ethanol. However, there are some important pitfalls, and few industrial-scale continuous systems have been implemented. Here, we first review the various cell immobilization techniques and reactor setups. Then, the impact of immobilization on cell physiology and fermentation performance is discussed. In a last part, we focus on the practical use of continuous fermentation and cell immobilization systems for beer production.

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Introduction

Traditional fermentation systems use freely suspended yeast cells in a batch bioreactor. The reactor is filled with unfermented medium and the whole reactor volume (''batch'') is gradually fermented and subsequently removed from the reactor. By contrast, continuous fermentation systems have a continuous flow of unfermented medium into the fermentor and a corresponding continuous flow of fermented product out of the system. In its simplest one-reactor incarnation, the continuous fermentor operates at steady-state with a content that is equal to the finished product that flows out of the system. A relatively slow inflow and little internal heterogeneity between the points of in- and outflow in the reactor are needed to avoid direct mixing of the unfermented inflow and the finished product. Alternatively, a cascade of interconnected separate fermentors can be used to avoid a direct flow of unfermented medium into the near-finished product.

Continuous fermentation offers important advantages, such as higher conversion rates, faster fermentation rates, improved product

consistency, reduced product losses and environmental advantages. An important aspect of continuous fermentation is the high volumetric efficiency, which is usually obtained by increased yeast cell concentrations in the reactor compared to traditional batch systems. Immobilizing yeast cells on several support types can provide high cell densities in the bioreactor, which, in combination with high flow rates, leads to short residence times. These economic benefits are the driving force for a global research effort aimed at studying and implementing continuous fermentors. The first continuous fermentation system appeared in the 1960s, but few systems grew up to industrial scale, which is indicative of the many technical and qualitative pitfalls associated with this technology.

Various immobilization methods are available to researchers and the nature of the application often dictates the choice. A thorough knowledge of the influence of immobilization on the fermentation performance and the parameters affecting the immobilization is vital to fine-tune the continuous process and reach an acceptable product quality. Here, we review these complex parameters (including yeast immobilization, yeast physiology and reactor design) and we critically discuss their advantages and disadvantages for continuous fermentation. In a last part, we focus in greater detail on a concrete example of continuous fermentation for beer production.

Immobilization materials and methods

Generally, four categories of immobilization techniques can be distinguished, based on the physical mechanism of cell localization and the nature of support mechanisms: ''attachment to a surface'', ''entrapment within a porous matrix'', ''containment behind a barrier'' and ''self-aggregation'' (Karel et al. 1985) (Fig. 1).

An industrial carrier for food fermentations should be inexpensive, stable, reusable, nontoxic and should allow for high yeast cell concentrations with minimal internal mass transfer limitations.

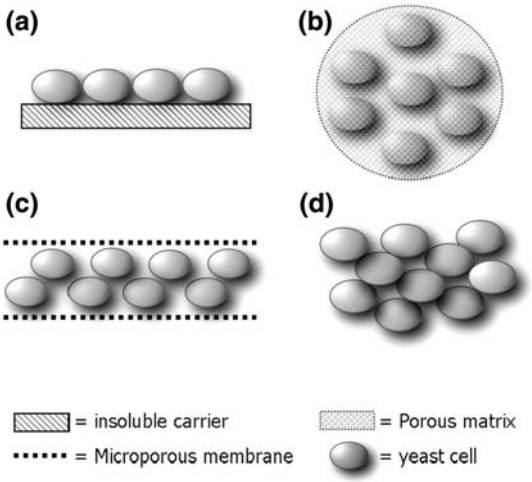

Fig. 1 Basic methods of yeast immobilization: (**a**) attachment to a surface, (**b**) entrapment within a porous matrix, (**c**) containment behind a barrier and (**d**) self-aggregation

Surface attachment of yeast cells

In this type of immobilization, yeast cells are allowed to attach to a solid support. Many different carrier materials are being used (Willaert 2006). Cellular attachment to the carrier can be induced using linking agents (such as metal oxides, glutaraldehyde or aminosilanes). However, for the production of beverages and ethanol, natural adhesion is often preferred over the use of (potentially harmful or unstable) inducers. Natural immobilization is very simple and the conditions are mild, but cell loadings are usually not as high as those obtained in systems in which the cells are entrapped (see further). Moreover, as there are no barriers between the cells and the solution, cell detachment and relocation is possible.

While the natural adhesion of yeast cells to substrates remains somewhat mysterious, several mechanisms have been proposed (for a review, see Verstrepen and Klis 2006). The adhesion phenomenon could, for example, be conferred by electrostatic, ionic (Lewis acid/base) and hydrophobic (Lifshitz–van der Waals) interactions (Oliveira 1997), but retention within carrier cavities and yeast flocculation can also play an important role in the immobilization process on preformed, roughly shaped carriers (Brányik et al.

2004b). Hence, the physicochemical properties of the yeast cell wall and the carrier, such as hydrophobicity, charge, electron-donor and electron-acceptor properties, should be considered when designing new immobilization carriers.

Entrapment within porous matrices

The second major category of yeast immobilization is entrapment within porous matrices. Two methods of entrapment exist. In the first, cells are allowed to diffuse into a preformed porous matrix. After the cells begin to grow, their mobility is hindered by the presence of other cells and the matrix and they are thus effectively entrapped (Baron and Willaert 2004). Attachment on the surface of this materials is also possible (Mistler and Breitenbücher 1995). Sponge, sintered glass, ceramics, silicon carbide, polyurethane foam, chitosan and stainless steel fibres are commonly used materials (Masschelein 1994; Scott and O'Reilly 1995; Tata et al. 1999; Shen et al. 2003b).

In the second method, the porous matrix is synthesized in situ around the cells. Most often, natural and synthetic polymeric hydrogels such as Ca-alginate, κ-carrageenan, agar, polyurethane, polystyrene and polyvinylalcohol are being used (Ramakrishna and Prakasham 1999). These polymeric beads are usually spherical with diameters ranging from 0.3 to 3 mm. Although high biomass loadings can be obtained, gel entrapment has received less attention in the fermentation industry because of several drawbacks, such as diffusion limitations of nutrients, metabolites and oxygen due to the gel matrix and the high cell densities in the gel beads, the chemical and physical instability of the gel and the non-regenerability of the beads, making this immobilization type rather expensive.

Recently, attempts are made to solve most of these drawbacks by the introduction of new techniques that are able to adjust the size (microbeads) and shape (lenticular shape) of the hydrogels (Nedovic et al. 2005a).

Containment behind a barrier

Containment of yeast cells behind a barrier can be attained either by the use of microporous membrane filters or by entrapment of cells in microcapsules. This type of immobilization is most suited when a cell free product is required, or when high molecular weight products need to be separated from the effluent. Inherent problems of this technique are mass transfer limitations and possible membrane fouling caused by cell growth (Lebeau et al. 1998). This type of immobilization is attractive in terms of productivity, but it seems that the cost/benefit ratio for low-added-value fermentations like beer will remain unfavorable as long as high-performance membranes remain expensive. Several research groups have nevertheless investigated their use for the production of ethanol (Inloes et al. 1983; Kargupta et al. 1998; O'Brien et al. 2000).

Yeast flocculation

The common brewer's yeast, *Saccharomyces cerevisiae*, has the natural ability to adhere to inert surfaces as well as other yeast cells, the latter process called ''flocculation''. Yeast flocculation is a reversible, asexual and calcium dependent process in which cells adhere to form flocs consisting of thousands of cells (Bony et al. 1997). It involves lectin-like proteins, which stick out of the yeast cell wall and selectively bind mannose residues present on the cell walls of adjacent yeast cells (Kobayashi et al. 1998). Yeast flocculation is a complex process that depends on the expression of several specific genes such as *FLO1*, *FLO5*, *FLO8* and *Lg-FLO1*. Other genes, such as *FLO11*, confer adhesion to inert substrates and the formation of biofilms on nutrient sources (Verstrepen and Klis 2006).

Because of their macroscopic size and their mass, the yeast flocs rapidly sediment from the fermenting medium, thus providing a natural immobilization of the cells. The use of flocculating yeast is very attractive, due to its simplicity and low cost. However, things are more complex than they may seem. Flocculation is affected by numerous parameters, such as nutrient conditions, agitation, Ca^{2+}-concentration, pH, fermentation temperature, yeast handling and storage conditions (Verstrepen et al. 2003; Sampermans et al. 2005). Hence, the fermentation medium itself, and more specifically

the content of glucose, sucrose and nitrogen compounds may influence the success of immobilization (Verstrepen et al. 2004). However these parameters have not yet been systematically studied and it is hard to predict the impact of the medium on cell adhesion. Above all, flocculation is a strain-specific phenomenon (Jin and Speers 1998). The ability of yeast cells to flocculate is of considerable importance for the brewing industry, as it provides an effective and simple way to separate most of the yeast cells from green beer at the end of fermentation. Therefore, strong and complete flocculation is a desirable property for every brewer's yeast. However, the yeast cells should not flocculate before the wort is completely attenuated. Such premature flocculation causes sluggish, so-called ''hanging'', fermentations, which might lead to beers with severe off-flavors (Verstrepen et al. 2003). The growing interest in flocculation bioreactors, because of the prospect of high cell densities in continuous processes, further intensifies the need for controlling yeast flocculation (Xu et al. 2005). In this case, constitutive flocculent yeast strains (by genetic engineering) are desired, because normal strains only flocculate in the stationary phase and thereby the exponentially growing cells would be washed out (Domingues et al. 2000).

Furthermore, yeast flocculation has an important influence on active immobilization on different carriers; strongly flocculent cells seem to accumulate faster on the carrier (Linko et al. 1998; van Iersel et al. 1998). Hence, an appropriate choice of yeast strain is important for the success of certain immobilization methods, as cells that fail to adhere may simply be washed out of the system.

Reactor configuration

Generally, in continuous immobilized yeast fermentation systems, 5 types of bioreactors are being used, which are depicted schematically in Fig. 2. The bioreactors contain three phases: solid (the carrier or aggregate), liquid (the medium) and gas (air, oxygen or other gas feeds). A more detailed review is given elsewhere (Baron et al. 1996; Obradovic et al. 2004). The choice of bioreactor is related to the type of immobilization, to the metabolism of cells, and to the mass and heat transfer requirements.

In a packed bed reactor (Fig. 2A), the fermenting medium is passed either upward or downward through the reactor which is packed with immobilized yeast. This type of reactor has the advantages of simplicity and the ability of realizing a plug flow. Theoretically, maintenance of ideal plug flow conditions would allow the various stages of a batch fermentation to be mimicked. This is especially useful when a balanced flavor profile must be formed during the fermentation. In practice, these ideal conditions are difficult to achieve and, in addition, fixed bed reactors are prone to channeling, mass transfer limitations, difficulties in CO_2-evacuation, compression of some carrier materials and fouling.

In a fluidized bed reactor (Fig. 2B), intensive mixing of gas, liquid and solids occurs by recirculating the fermenting substrate, resulting in less abrasion of the carrier particles compared with stirred reactors. A flow of liquid is directed through the particles at velocities above the 'minimum fluidization velocity'. It is important to consider the density of immobilized cell beads when designing a fluidized bed system, because a low density difference between the carriers and the medium could cause wash-out. On the other hand, a large difference could raise the pumping costs.

In airlift (Fig. 2C) and bubble column (Fig. 2D) reactors, the circulation is performed by gas injection. An airlift reactor gives more vigorous circulation for the same air flow than a bubble column reactor, due to the cooling and the internal draft tube, which creates a 'rising' zone in the centre of the reactor and a 'downcoming' zone on the outside. With this type of reactor, only immobilizing particles with a density close to that of the liquid are chosen, such as hydrogels or cell aggregates.

Stirred tank reactors (Fig. 2D) are provided with a means of increasing mass transfer rates by forced agitation, although care must be taken to ensure that the support is not damaged and that the yeast cells don't suffer too much from shear stress. Impellers, such as a helical ribbon, screw or

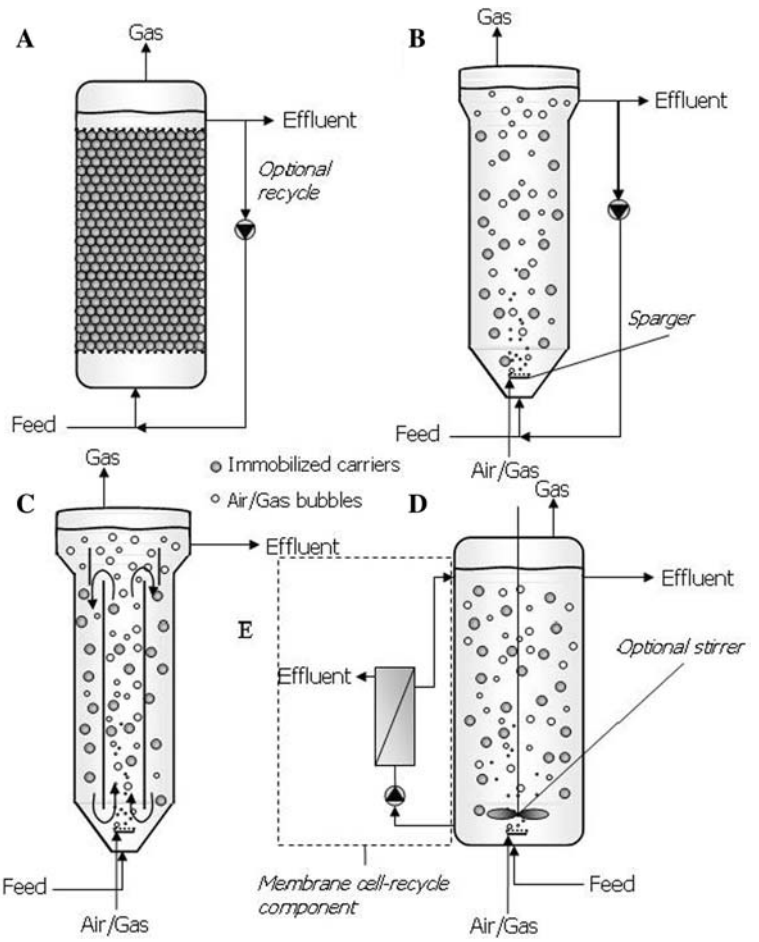

Fig. 2 Five common types of immobilized cell bioreactors: (**A**) packed bed reactor; (**B**) fluidized bed reactor; (**C**) gas lift reactor; (**D**) bubble column reactor (or stirred reactor if stirred); and (**E**) membrane cell-recycle reactor

anchor, are preferred over turbines or propellers for their more gentle stirring profile.

In Fig. 2E, a typical membrane bioreactor is depicted. In the case of ethanol production, pervaporation is often applied as membrane separation process, in which ethanol is continuously removed as vapor (Mulder 1996; O'Brien and Craig 1996).

Effect of immobilization on yeast cells

There is a considerable body of evidence suggesting that the physiology of immobilized yeast differs from that of free cells. Some of these alterations have beneficial effects on the yeast fermentation performance but many others are disadvantageous. Since *S. cerevisiae* cells are found attached to each other or to a surface in their natural habitat (e.g. a grape), immobilization may be regarded as the natural growth form, which may offer protection from certain stresses (Verstrepen and Klis 2006). The negative responses relate to the stress-factors that immobilization imposes on yeast cells, of whom the effects of restricted mass transfer are the most important.

Entrapment in gel beads and inert carriers show similar diffusional limitation characteristics. Adsorption encounters only the external mass transfer barrier (between bulk medium and carrier) without internal (within the carrier) mass transfer limitations. The internal mass transfer properties are determined by the chemical-mechanical characteristics of the immobilizing matrix, namely: size, porosity and texture. An important parameter in the evaluation of the internal mass transfer is the Thiele modulus,

which is defined as the ratio of the rate at which substrate is consumed over the rate at which new substrate is supplied by the diffusion process (Willaert et al. 1996). The main issue considering external mass transfer is the choice between packed bed reactor and a fluidized or agitated reactor. One should be careful in concluding that immobilized yeast activity is intrinsically altered, since it is difficult to remove the effect of mass transfer limitations. To solve this problem, yeast cells can be attached as a monolayer (Doran and Bailey 1986; Shen et al. 2003b).

A comparison between the metabolic activity of immobilized and free cells shows an activation of the yeast energetic metabolism and an increase in both storage (glycogen and trehalose) and structural polysaccharides (glucan and mannan) (Doran and Bailey 1986; Galazzo and Bailey 1990; Jirku et al. 2000). The increase in ploidy and protein content in immobilized cells, led Doran and Bailey (1986) to hypothesize that due to the dense cell packing within the gel, cell budding and replication, but not DNA and polysaccharide production, was prevented. However, the increase in storage polysaccharides could also be explained by the interaction between glucose uptake and the activity of the enzyme phosphofructokinase (Galazzo and Bailey 1990).

The comparison of the growth rate has given more discordant results. Reported data have described an increase, static or decreased growth rate (Norton and D'Amore 1994).

Immobilized cells are considered to be more tolerant against ethanol than freely suspended yeast cells. According to Hilge-Rotmann and Rehm (1990), the increased saturation of the fatty acid content of immobilized yeast (due to altered osmotic conditions in the microenvironment of the cells) is correlated to this increase in tolerance. Indeed, immobilized yeast cells show a higher proportion of saturated fatty acids compared to free cells (Norton et al. 1995; van Iersel et al. 1999; Jirku et al. 2003; Shen et al. 2003b). Norton et al. (1995) as well as Shen et al. (2003b) stated that the matrix provides a protective environment against ethanol toxicity, so that resuspended yeast cells showed no increased ethanol tolerance. The results of the expression profile of stress-related genes, *HSP12* and *SSA3*, confirmed that immobilized cells in general were under less stressful conditions than free cells, possibly because of the protective environment. In addition, this particular microenvironment might activate the cAMP/PKA pathway, which in turn affects several targets, for example *ATF1*, resulting in an increase in ester formation (Shen et al. 2003a; Verstrepen et al. 2004). In any case, immobilization has a major influence on the plasma membrane properties of the yeast, which can cause modifications of some solute transport systems (Shen et al. 2003b).

The enhanced fermentation properties of immobilized cell systems could also be explained by the CO_2 nucleation effect of the matrix (Scott and O'Reilly 1995; Shen et al. 2004).

A further characteristic of the immediate environment may be a decreased water activity (a_w), which represents another stress that immobilized yeast has to cope with. The yeast cells react to this lower a_w by excreting osmoprotective metabolites, such as glycerol and proline (Shen et al. 2003b).

Finally, our knowledge about the physiology and metabolic activity of immobilized cells could increase significantly with the application of recent technologies in proteomics and the measurement of genome-wide gene expression (Junter et al. 2002; Higgins et al. 2003; Stoughton 2005).

Immobilized cell technology in the production of beer

Beer production is traditionally a batch process. This method dates back to the ancient Egyptians and is intensively optimized in the last century by the transformation of open fermentation vessels to closed cilindroconical tanks, capable of fermenting large quantities of wort with a significant improvement in product quality and hygiene. In the 1960s, the interest in continuous beer fermentation rose intensively, giving birth to a series of systems (Boulton and Quain 2001). However, these continuous beer fermentation processes never became commercially successful due to many practical problems, such as increased risk of contamination (mainly because of

the necessity to store wort in supplementary holding tanks), variations in beer flavor, complex system design and a lack of flexibility. Only the continuous beer production process of Dominion Breweries in New Zealand by Morton Coutts has been implemented successfully (Coutts 1966; Dunbar et al. 1988). In the 1970s there was a revival in the development of new continuous beer fermentation systems, owing to the development of immobilized cell technology. The main advantages of using immobilized cells for the production of beer are enhanced volumetric productivities, improved cell stability, easier implementation of continuous operation, improved operational control, facilitated cell recovery and reuse, and simplified downstream processing (Nedovic et al. 2005b). Narziss and Hellich developed one of the first immobilization systems for the production of beer. Their ''biobrew bioreactor'' consisted of a Kieselguhr filter, characterized by short residence times, although the flavor profile was not satisfactory (Narziss and Hellich 1971; Narziss 1997).

At present, only beer maturation and alcohol-free beer production are obtained by means of commercial-scale immobilized yeast reactors (Mensour et al. 1997). In these processes, no real yeast growth and flavor formation is required. During secondary fermentation, diacetyl, which has a buttery taste, is being reduced to the relative flavor-inactive acetoin and 2,3-butanediol by yeast cells. This vicinal diketone is formed during the main fermentation by an extracellular oxidative decarboxylation of α-acetolactate, which leaks from the isoleucine-valine pathway (Wainwright 1973). 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), a heat treatment unit (to accelerate the chemical conversion of α-acetolactate to diacetyl and acetoin), and a packed bed reactor with yeast immobilized on DEAE-cellulose granules or porous glass beads (to reduce the remaining diacetyl) respectively. 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 immobilized in special multilayer capsules, followed by the reduction of diacetyl by yeast in a second packed bed reactor (Nitzsche et al. 2001).

The main objective during fermentation of alcohol-free beer is the reduction of wort carbonyl flavors by yeast, without the formation of alcohol, and therefore it resembles the secondary fermentation. Traditionally, alcohol-free beer is being produced by arrested batch fermentations. Keeping the yeast cell in optimal steady-state conditions at low temperature leads to a more complete reduction of the wort carbonyls with a minimum of alcohol formation. Bavaria (the Netherlands) is using a packed bed immobilized yeast bioreactor with a production capacity of 150,000 hl alcohol-free beer per annum (van Dieren 1995).

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 (which contribute to the positive alcoholic and fruity flavors of the beer) are the most important. In addition, the vicinal diketones and some sulphury compounds can cause off-flavors. Because this complex flavor profile is closely related to the amino acid metabolism and thus the growth of the yeast cells, differences in the growth metabolic state between freely suspended and immobilized 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 immobilized yeast. The optimization of aeration and temperature seems to be an important tool for the control of flavor-active compounds (Smogrovicová and Dömény 1999; Virkajärvi et al. 1999; Brányik et al. 2004a). Alternatively, the use of genetically modified yeast strains with flavor profiles tailored to counteract the off-flavors observed in continuous systems may also provide a solution (Verstrepen and Pretorius 1990).

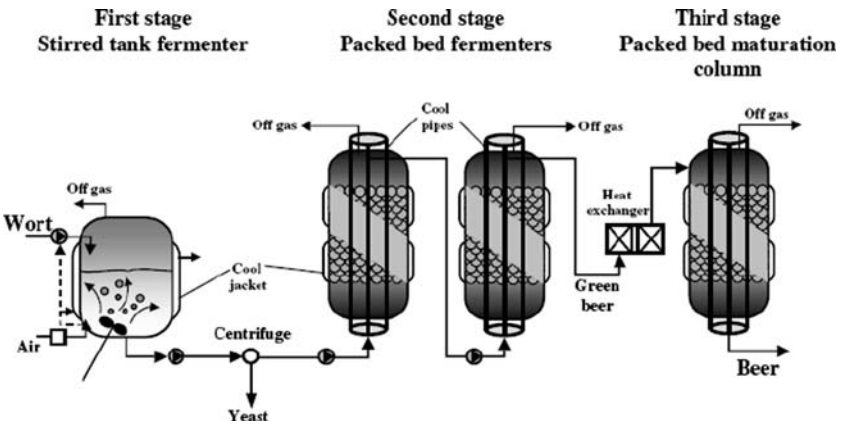

Fig. 3 Kirin's three stage fermentor system for continuous beer fermentation (adapted from Inoue 1995)

As an example, the multistage system of the Japanese brewery Kirin (10 0 hl pilot plant) is depicted in Fig. 3. During its development the following assumption was made: ''if the physiological state of yeast during fermentation is the same, the same quality beer will be produced'' (Inoue 1995). Therefore, the first stage consists of an aerated stirred tank reactor for yeast growth with a desirable free amino nitrogen consumption. As a consequence, higher alcohols are formed and a drop in pH occurs. Between the first and second reactor, the yeast is centrifuged to prevent that oxygenated, growing yeast is introduced in the second stage. In the packed bed reactors, mainly esters and ethanol are produced anaerobically. Ca-alginate gel beads were initially selected as carrier material to immobilize the yeast cells, but they were later on replaced by ceramic beads, because of longer lasting yeast viability. To improve the cooling capacity during scaling-up, cooling pipes were inserted in the packed bed reactors. In the third stage, which is preceded by a heat treatment step and is analogue to the former stage, beer maturation takes place. This is necessary, as the remaining concentration of vicinal diketones is usually higher in immobilized cell systems (possible because of the short residence times, so that insufficient diacetyl reduction takes place). Beer has been produced in this process within three to five days.

In Table 1, the most promising laboratory and pilot scale systems for continuous main beer fermentation in the future, are summarized.

Table 1 Promising immobilized yeast systems for main beer fermentation

Immobilization method	Immobilization material	Process/Bioreactor type	Reference
Entrapment	κ-carrageenan beads	Gas lift	Decamps et al. (2004)
Entrapment	Alginate microbeads	Gas lift	Nedovic et al. (2005a)
Entrapment	Lens shaped PVA[a] particles	Gas lift	Smogrovicová et al. (2001)
Adsorption	Spent grains	Gas lift	Brányik et al. (2004a)
Adsorption	Wood chips	Packed bed (2 stages)	Virkajärvi (2001)
Adsorption	Wood chips	Packed bed (1 stage, with recirculation)	Tapani et al. (2003)
Adsorption	SS[b] fibre cloth	Gas lift	D.P. Schutter and P.J. Verbelen (unpublished results)
Adsorption	Gluten pellets	Fluidized bed	Bardi et al. (1997)
Self-aggregation	Super-flocculent yeast	Perforated, aerated reactor	Linko et al. (1997)

[a]PVA = Polyvinyl alcohol

[b]SS = Stainless steel

Conclusions and future perspectives

Despite numerous potential advantages, continuous fermentation with immobilized yeast has still not been applied on industrial scale, because of unrealized cost advantages, several engineering problems and altered yeast physiological and metabolic properties, influencing the flavor of the beverage or the fermentation performance. Moreover, the carrier cost is a determining factor in the feasibility of the immobilized system. Driven by the recent energy crisis, intensified research aimed at developing improved reactors, a better understanding of the physiology of immobilized cell systems and the immobilization mechanisms, together with the search for novel, innovative and cheap carrier materials, should enable a more general implementation of this promising technology.

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References

Bardi EP, Koutinas AA, Kanellaki M (1997) Room and low temperature brewing with yeast immobilized on gluten pellets. Proc Biochem 32:691–696

Baron GV, Willaert RG (2004) Cell immobilization in preformed porous matrices. In: Nedovic V, Willaert R (eds) Fundamentals of cell immobilisation biotechnology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 229–244

Baron GV, Willaert RG, De Backer L (1996) Immobilised cell reactors. In: Willaert RG, Baron GV, De Backer L (eds) Immobilised living cell systems: modelling and experimental methods. John Wiley & Sons, Chichester, England, pp. 67–95

Bony M, Thines-Sempoux D, Barre P, Blondin B (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. J Bacteriol 179:4929–4936

Boulton C, Quain D (2001) Brewing yeast and fermentation. Blackwell Science Ltd, Oxford

Brányik T, Vicente A, Cruz JMM, Teixeira JA (2004a) Continuous primary fermentation of beer with yeast immobilized on spent grains – the effect of operational conditions. J Am Soc Brew Chem 62:29–34

Brányik T, Vicente A, Oliveira R, Teixeira JA (2004b) Physicochemical surface properties of brewing yeast influencing their immobilization onto spent grains in a continuous reactor. Biotechnol Bioeng 88:84–93

Coutts MW (1966) The many facets of continuous fermentation. In: Proceedings of the 9th Convention of the Institute of Brewing. Australië & New Zealand Section, Auckland, pp 1–7

Decamps C, Norton S, Poncelet D, Neufeld RJ (2004) Continuous pilot plant-scale immobilization of yeast in (-carrageenan gel beads. AIChE J 50:1599–1605

Domingues L, Vicente AA, Lima N, Teixeira JA (2000) Applications of yeast flocculation in biotechnological processes. Biotechnol Bioprocess Eng 5:288–305

Doran PM, Bailey JE (1986) Effects of immobilization on growth, fermentation properties, and molecular composition of *Saccharomyces cerevisiae* attached to gelatin. Biotechnol Bioeng 28:73–87

Dunbar J, Campbell SI, Banks DJ, Warren DR (1988) Metabolic aspects of a commercial continuous fermentation system. In: Proceedings of the 20th Convention of the Institute of Brewing, 8th–13th May, Brisbane, pp 151–158

Galazzo JL, Bailey JE (1990) Fermentation pathway kinetics and metabolic flux control in suspended and immobilized *Saccharomyces cerevisiae*. Enzyme Microb Technol 12:162–172

Higgins VJ, Beckhouse AG, Oliver AD, Rogers P, Dawes IW (2003) Yeast genome-wide expression analysis identifies a strong ergosterol and oxidative stress response during the initial stages of an industrial lager fermentation. Appl Environ Microbiol 69:4777–4787

Hilge-Rotmann B, Rehm H-J (1990) Relationship between fermentation capability and fatty acid composition of free and immobilized *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 34:502–508

Inloes DS, Taylor DP, Cohen SN, Michaels AS, Robertson CR (1983) Ethanol production by *Saccharomyces cerevisiae* immobilized in hollow-fiber membrane bioreactors. Appl Environ Microbiol 46:264–278

Inoue T (1995) Development of a two-stage immobilized yeast fermentation system for continuous beer brewing. In: Proceedings of the 25th European Brewery Convention. IRL Press, Oxford, Brussels, pp 25–36, ISBN 0-19-963614-1

Jin Y-L, Speers AR (1998) Flocculation of *Saccharomyces cerevisiae*. Food Res Int 31:421–440

Jirku V, Masak J, Cejkova A (2000) Yeast cell attachment: a tool modulating wall composition and resistance to 5-bromo-6-azauracil. Enzyme Microb Technol 26:808–811

Jirku V, Masak J, Cejkova A (2003) The potential of functional changes in attached biomass. Adv Environ Res 7:635–639

Junter G-A, Coquet L, Vilain S, Jouenne T (2002) Immobilized-cell physiology: current data and the potentialities of proteomics. Enzyme Microb Technol 31:201–212

Karel SF, Libicki SB, Robertson CR (1985) The immobilization of whole cells: Engineering principles. Chem Eng Sci 40:1321–1354

Kargupta K, Datta S, Sanyal SK (1998) Analysis of the performance of a continuous membrane bioreactor with cell recycling during ethanol fermentation. Biochem Eng J 1:31–37

Kobayashi O, Hayashi N, Kuroki R, Sone H (1998) Region of Flo1 proteins responsible for sugar recognition. J Bacteriol 180:6503–6510

Lebeau T, Jouenne T, Junter G-A (1998) Diffusion of sugars and alcohols through composite membrane structures immobilizing viable yeast cells. Enzyme Microb Technol 22:434–438

Linko M, Haikara A, Ritala A, Penttilä M (1998) Recent advances in the malting and brewing industry. J Biotechnol 65:85–98

Linko M, Virkajärvi I, Pohjala N, Lindborg K, Kronlöf J, Pajunen E (1997) Main fermentation with immobilized yeast – a breakthrough? In: Proceedings of the 26th European Brewery Convention. IRL Press, Oxford, Maastricht, pp 385–394. ISBN 0-19-963690-7

Masschelein CA (1994) State-of-the-art and future developments in fermentation. J Am Soc Brew Chem 52:28–35

Mensour N, Margaritis A, Briens CL, Pilkington H, Russell I (1997) New developments in the brewing industry using immobilised yeast cell bioreactor systems. J Inst Brew 103:363–370

Mistler M, Breitenbücher K (1995) Continuous fermentation of beer with yeast immobilized on porous glass carriers. Brewers' Digest July:48–51

Mulder M (1996) Basic principles of membrane technology, 2nd edn. Kluwer Academic Publishers, Dordrecht, The Netherlands

Narziss L (1997) Global brewing technology – a look over the fence. Brauwelt Int 1:16–21

Narziss L, Hellich P (1971) Ein Beitrag zur wesentlichen Beschleunigung der Gärung und Reifung des Bieres. Brauwelt 111:1491–1500

Nedovic VA, Cukalovic IL, Bezbradica D, Obradovic B, Bugarski B (2005a) New porous matrices and procedures for yeast cell immobilisation for primary beer fermentation. In: Proceedings of the 30th European Brewery Convention. Prague, pp 401–413, ISBN 90-70143-23-2

Nedovic VA, Willaert R, Leskosek-Cukalovic I, Obradovic B, Bugarski B (2005b) Beer production using immobilised cells. In: Nedovic V, Willaert R (eds) Applications of cell immobilisation biotechnology. Springer, Dordrecht, The Netherlands, pp 259–273

Nitzsche F, Höhn G, Meyer-Pittroff R, Berger S, Pommersheim R (2001) A new way for immobilized yeast systems: secondary fermentation without heat treatment. In: Proceedings of the 28th European Brewery Convention. Budapest, pp 486–494, ISBN 90-70143-21-6

Norton S, D'Amore T (1994) Physiological effects of yeast immobilization: applications for brewing. Enzyme Microb Technol 16:365–375

Norton S, Watson K, D'Amore T (1995) Ethanol tolerance of immobilized brewer's yeast cells. Appl Microbiol Biotechnol 43:18–24

Obradovic B, Nedovic VA, Bugarski B, Willaert RG, Vunjak-Novakovic G (2004) Immobilised cell bioreactors. In: Nedovic V, Willaert R (eds) Fundamentals of cell immobilisation biotechnology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 411–436

O'Brien DJ, Craig JCJ (1996) Ethanol production in a continuous fermentation/membrane pervaporation system. Appl Microbiol Biotechnol 44:699–704

O'Brien DJ, Roth LH, McAloon AJ (2000) Ethanol production by continuous fermentation–pervaporation: a preliminary economic analysis. J Membr Sci 166:105–111

Oliveira R (1997) Understanding adhesion: a means for preventing fouling. Exp Therm Fluid Sci 14:316–322

Ramakrishna SV, Prakasham RS (1999) Microbial fermentations with immobilized cells. Curr Sci 77:87–100

Sampermans S, Mortier J, Soares EV (2005) Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients. J Appl Microbiol 98:525–531

Scott JA, O'Reilly AM (1995) Use of a flexible sponge matrix to immobilize yeast for beer fermentation. J Am Soc Brew Chem 53:67–71

Shen H-Y, De Schrijver S, Moonjai N, Verstrepen KJ, Delvaux F, Delvaux FR (2004) Effects of CO_2 on the formation of flavour volatiles during fermentation with immobilised brewer's yeast. Appl Microbiol Biotechnol 64:636–643

Shen H-Y, Moonjai N, Verstrepen KJ, Delvaux F, Delvaux FR (2003a) Immobilization of *Saccharomyces cerevisiae* induces changes in the gene expression levels of *HSP12*, *SSA3* and *ATF1* during beer fermentation. J Am Soc Brew Chem 61:175–181

Shen H-Y, Moonjai N, Verstrepen KJ, Delvaux FR (2003b) Impact of attachment immobilization on yeast physiology and fermentation performance. J Am Soc Brew Chem 61:79–87

Smogrovicová D, Dömény Z (1999) Beer volatile by-product formation at different fermentation temperature using immobilised yeasts. Process Biochem 34:785–794

Smogrovicová D, Dömény Z, Navrátil M, Dvorák P (2001) Continuous beer fermentation using polyvinyl alcohol entrapped yeast. In: Proceedings of the 28th European Brewery Convention. Budapest, pp 540–548, ISBN 90-70143-21-6

Stoughton RB (2005) Applications of DNA microarrays in biology. Annu Rev Biochem 74:53–82

Tapani K, Soininen-Tengvall P, Berg H, Ranta B, Pajunen E (2003) Continuous primary fermentation of beer with immobilised yeast. In: Smart KA (ed) Brewing yeast fermentation performance, 2nd edn. Blackwell Science, Oxford, pp 293–301

Tata M, Bower P, Bromberg S, Duncombe D, Fehring J, Lau V, Ryder D, Stassi P (1999) Immobilized yeast bioreactor systems for continuous beer fermentation. Biotechnol Prog 15:105–113

van Dieren B (1995) Yeast metabolism and the production of alcohol-free beer. In: EBC Symposium ''Immobilized Yeast Applications in the Brewery Industry'',

Monograph XXIV, Oct 1995, Espoo, Finland: Verlag Hans Carl Getränke-Fachverlag, pp 66–76, ISBN 3-418-00749-X

van Iersel MFM, Meersman E, Arntz M, Rombouts FM, Abee T (1998) Effect of environmental conditions on flocculation and immobilization of brewer's yeast during production of alcohol-free beer. J Inst Brew 104:131–136

van Iersel MFM, van Dieren B, Rombouts FM, Abee T (1999) Flavor formation and cell physiology during the production of alcohol-free beer with immobilized *Saccharomyces cerevisiae*. Enzyme Microb Technol 24:407–411

Verstrepen KJ, Derdelinckx G, Verachtert H, Delvaux FR (2003) Yeast flocculation: what brewers should know. Appl Microbiol Biotechnol 61:197–205

Verstrepen KJ, Iserentant D, Malcorps P, Derdelinckx G, Van Dijck P, Winderickx J, Pretorius IS, Thevelein JM, Delvaux FR (2004) Glucose and sucrose: hazardous fast-food for industrial yeast? Trends Biotechnol 22:531–537

Verstrepen KJ, Klis FM (2006) Flocculation, adhesion and biofilm formation in yeasts. Mol Microbiol 60:5–15

Verstrepen KJ, Pretorius IS (2006) The development of superior yeast strains for the food and beverage industry: challenges, opportunities and potential benefits. In: Querol A, Fleet G (eds) The yeast handbook, volume 8: yeasts in food and beverages. Springer-Verlag, Heidelberg, Germany, pp 399–444

Virkajärvi I (2001) Feasibility of continuous main fermentation of beer using immobilized yeast. PhD thesis, Helsinki University of Technology, Espoo

Virkajärvi I (2002) Some developments in immobilized fermentation of beer during the last 3 0 years. Brauwelt Int 20:100–105

Virkajärvi I, Lindborg K, Kronlöf J, Pajunen E (1999) Effects of aeration on flavor compounds in immobilized primary fermentation. Mon Schr Brauwiss 52:9–12, 25–28

Wainwright T (1973) Diacetyl – a review. J Inst Brew 79:451–470

Willaert RG (2006) Cell immobilisation and its applications in biotechnology: current trends and future prospects. In: El-Mansi EMT, Bryce CFA (eds) Fermentation microbiology and biotechnology, 2nd edn. Taylor and Francis (in press)

Willaert RG, De Backer L, Baron GV (1996) Mass transfer in immobilised cell systems. In: Willaert RG, Baron GV, De Backer L (eds) Immobilised living cell systems: modelling and experimental methods. John Wiley & Sons, Chichester, England, pp 21–45

Xu TJ, Zhao XQ, Bai FW (2005) Continuous ethanol production using self-flocculating yeast in a cascade of fermentors. Enzyme Microb Technol 37:634–640