Aroma formation by immobilized yeast cells in fermentation processes

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Keywords: Flavour, yeast, immobilization, fermentation, higher alcohol, ester, carbonyl

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/YEA.3042

Abstract

Immobilized cell technology has shown a significant promotional effect on the fermentation of alcoholic beverages such as beer, wine and cider. However, genetic, morphological and physiological alterations occurring in immobilized yeast cells impact on aroma formation during fermentation processes. The focus of this review is exploitation of existing knowledge on biochemistry and the biological role of flavour production in yeast for the biotechnological production of aroma compounds of industrial importance by means of immobilized yeast. Various types of carrier materials and immobilization methods proposed for application in beer, wine, fruit wine, cider and mead production are presented. Engineering aspects with special emphasis on immobilized cell bioreactor design, operation and scale-up potential are also discussed. Ultimately, examples of products with improved quality properties within the alcoholic beverages are addressed together with identification and description of the future perspectives and scope for cell immobilization in fermentation processes.

Introduction

The unique flavour profile of fermented alcoholic beverages, such as beer and wine, can be attributed to the biochemical activities within the yeast cell during fermentation (Lodolo *et al.*, 2008). The aroma compounds produced by yeast are the intermediates in pathways leading from the catabolism of medium components (sugars, nitrogenous compounds and sulphur compounds) to the synthesis of components needed for yeast growth (amino acids, proteins, nucleic acids, lipids, etc.) (Lambrechts and Pretorius, 2000; Lodolo *et al.*, 2008). Alcohols (ethanol, higher alcohols), esters (acetate esters, medium chain fatty acid esters), organic acids (medium chain fatty acids), carbonyl compounds (acetaldehyde, vicinal diketones) and sulphur compounds (hydrogen sulphide, sulphur dioxide, dimethyl sulphide) are the main flavour-active compounds produced by yeast during fermentation (Dufour *et al.*, 2003). Figure 1 shows the formation of the major flavour groups. Relative concentrations of these by-products of fermentation can be influenced by the choice of yeast, nutritional factors, and the environmental conditions of the fermentation (Buglass, 2010a). High volumetric productivities of aroma and other metabolites can be achieved with high volumetric cell densities by packing the cells in a small defined volume, either by entrapment within a carrier matrix or adsorption on the surface of a porous material. This approach is known as immobilized cell technology (ICT) and has been widely investigated from the middle of the last century and designed for different stages in fermentations of alcoholic beverages such as beer, wine and cider. However, immobilized yeast cells' morphology, growth and physiology can be changed relative to free cells and this influences bioflavour formation during fermentation processes. This review article provides fundamentals on biochemistry of flavour production in yeast and gives information on some of biological roles which these volatiles have in nature. It also gives an overview of the current scientific knowledge on biotechnological generation of aroma compounds by means of immobilized yeast. Technological applications of such processes are presented through beer, wine, fruit wine, mead and cider production.

Biochemistry of flavour production in yeast

Biochemical background of yeast-derived higher alcohols

The term higher alcohols refers to compounds which have more than two carbon atoms and with a higher molecular weight and boiling point than ethanol. They are quantitatively the

largest group of aroma compounds in alcoholic beverages, but due to the relatively high threshold values (10-600 mg/l), the presence of specific alcohols is often not very pronounced. However, it is certain that higher alcohols do contribute to the overall aroma of fermented beverages, mainly because of the synergistic matrix effect (Verstrepen *et al.*, 2003d). Excessive concentrations of higher alcohols (exceeding 400 mg/l) can result in a strong, pungent smell and taste, whereas optimal levels (below 300 mg/l) impart fruity characters (Bartowsky and Pretorius, 2009). Apart from this direct flavour effect, higher alcohols are also extremely important because they are one of the two substrates for volatile ester formation (Verstrepen *et al.*, 2003a).

The higher alcohols are formed during fermentation by two routes, the anabolic Genevois pathway and the catabolic Ehrlich pathway. The first pathway involves synthesis from carbohydrates via pyruvate, whereas the second involves production of by-products of amino acid metabolism (Ehrlich pathway) (Dufour *et al.*, 2003; Lodolo *et al.*, 2008; Branyik *et al.*, 2008). The Ehrlich pathway involves the conversion of branched-chain amino acids (such as valine, leucine, isoleucine, methionine, and phenylalanine) to higher alcohols via three enzymatic steps (transamination, decarboxylation and reduction) (Äyräpää, 1968). These amino acids are taken up by yeast slowly in a sequential manner throughout the fermentation time. After the initial transamination reaction, the resulting α-keto acid cannot be redirected into central carbon metabolism. Before α-keto acids are released into the growth medium, yeast cells convert them into fusel alcohols or acids via the Ehrlich pathway (Hazelwood *et al.*, 2008). The relative contributions of the two pathways depend on the level of amino acids present in the fermentation medium. At low levels of amino acids, the biosynthetic pathway predominates, whereas at high levels of amino acids, the Ehrlich pathway becomes dominant (Dufour *et al.*, 2003). The metabolic purpose for higher alcohol formation is not clear yet. According to Verstrepen *et al.*, (2003b), the formation of higher alcohols could serve as an additional route to fine-tune the redox balance of the cell. The presence of higher alcohols in the growth medium exerts a certain signal function, promoting pseudohyphyal growth. While this signalling function of higher alcohols is not yet completely understood, it has been demonstrated that some higher alcohols inhibit translation initiation. It is therefore possible that, apart from NAD recycling, higher alcohol formation may also function as a low-nitrogen signal. Similarly, Lodolo *et al.*, (2008) stated that higher alcohol formation may appear wasteful, as in the case of glycerol, but these metabolites form part of the overall cellular redox balance. Other possible roles of higher alcohols are discussed further below.

Biochemical background of yeast-derived esters

Volatile esters are extremely important for the flavour profile of fermented beverages such as beer and wine in contrast to their low amounts in these drinks (Peddie, 1990; Verstrepen *et al.*, 2003d). They are responsible for the desirable fruity, candy and perfume-like aromas of fermented beverages (Dufour *et al.*, 2003). The most important aroma-active esters consist of two groups. The first group includes acetate esters (the acid residue of these esters is acetate). The most important flavour-active acetate esters in fermented beverages are ethyl acetate (solvent-like aroma), isoamyl acetate (fruit, banana, pear) and phenylethyl acetate (floral, roses, honey). The second group is the so-called ethyl esters or medium-chain fatty acid esters (MCFA esters). In MCFA esters the alcohol residue is ethanol, while the acid part usually is a medium-chain fatty acid (C6-C10). The group of MCFA esters includes ethyl caproate (ethyl hexanoate; C6 fatty acid), ethyl caprylate (ethyl octanoate; C8 fatty acid) and ethyl caprate (ethyl decanoate; C10 fatty acid). These ethyl esters have a characteristic sour apple flavour (Verstrepen *et al.*, 2003d).

Volatile esters are produced by an enzyme-catalyzed condensation reaction between acyl-CoA and an alcohol (Nordstrom, 1964; Verstepen *et al.*, 2003d). The reaction requires energy provided by the thioester linkage of the ac(et)yl-CoA molecule (Verstrepen *et al.*, 2003c). Several different enzymes take part in the formation of esters, and the best characterized are the alcohol acetyl transferases I and II (AATase I and II; EC 2.3.1.84) which are encoded by the genes *ATF1* and *ATF2*, respectively (Malcorps and Dufuor, 1992). It has also been shown that the balance between ester-synthesizing enzymes and ester-hydrolyzing enzymes (esterases, such as Iah1p) might be important for the net rate of ester accumulation (Fukuda *et al.*, 1998). Basically, two factors are important for the rate of ester formation: (1) the concentration of the two substrates, acyl-CoA and fusel alcohol, and (2) the total activity of the enzymes involved in the formation and breakdown of the respective ester. Hence, all parameters that affect substrate concentrations or enzyme activities will influence ester production (Verstrepen *et al.*, 2003d). The physiological role of aroma-active ester synthesis remains unknown. However, three different hypotheses have previously been suggested. According to the first hypothesis, ester formation regenerates free CoA accumulated under anaerobic conditions; the second proposes ester formation as a detoxification mechanism for free medium-chain fatty acids; the last hypothesis suggests that it is possible that certain

esters of long-chain hydroxy fatty acids could serve as fatty acid analogues (Lambrechts and Pretorius, 2000; Verstrepen *et al.*, 2003c).

Biochemical background of yeast-derived carbonyl compounds

Carbonyl compounds (aldehydes and ketones) contain a functional group composed of a carbon atom double-bonded to an oxygen atom (Lodolo *et al.*, 2008). Of these, acetaldehyde and the vicinal diketones or VDKs (diacetyl (2,3-butanedione) and 2,3-pentanedione) are the most important because of their low sensory threshold values (Lambrechts and Pretorius, 2000).

Short-chain, volatile aldehydes are important for the flavour of a number of foods and beverages, , contributing flavour characteristics ranging from "apple-like" to "citrus-like" to "nutty" depending on the chemical structure. Among these, acetaldehyde is the major component, constituting more than 90% of the total aldehyde content in wines and spirits (Lambrechts and Pretorius, 2000). Acetaldehyde is also a precursor metabolite for acetate, acetoin, and ethanol synthesis. At low levels this compound imparts a pleasant fruity aroma to wine and other beverages, but at higher concentrations this turns into a pungent irritating odour reminiscent of green grass or apples (Styger *et al.*, 2011).

Together with the keto-acids, the short-chain aliphatic aldehydes are the key compounds in the biochemical reaction involving the production of higher alcohols from amino acids and sugars by yeast. They are formed in the yeast cell and then transferred to the medium (Lambrechts and Pretorius, 2000). Acetaldehyde is a by-product of fermentative glycolysis, namely the decarboxylation of pyruvate. It is mostly formed during the active growth phase of yeast, when metabolic flux reaches its maximum. During the later fermentation phase, acetaldehyde formation drops, and some of the acetaldehyde that was previously excreted is again taken up and further reduced to ethanol (Verstrepen *et al.*, 2003a). During fermentation, the most rapid accumulation of acetaldehyde occurs when the rate of carbon dissimilation is at its maximum, after which it falls to a low level at the end of fermentation and then slowly increases over time. Acetaldehyde concentration is also yeast-strain-dependent (Bartowsky and Pretorius, 2009).

VDKs originate from the chemical decomposition of two acids, α-acetolactate and α-acetohydroxybutyrate, respectively. It can be expected that the higher the production of the α-

acetohydroxyacids, the higher the levels of VDK (Krogerus and Gibson, 2013a). These acids are intermediates in the synthesis of valine and isoleucine, respectively. The amounts and profile of α-acetohydroxyacids produced during fermentation are influenced by yeast strain, medium composition, and fermentation conditions (Dufour *et al.*, 2003; Branyik *et al.*, 2008; Gibson *et al.*, 2014). It is also suggested that VDK formation is linked to amino acid metabolism. Wort deficient in valine results in elevated diacetyl levels and worts deficient in leucine result in increased 2,3-pentanedione (Lodolo *et al.*, 2008; Krogerus and Gibson, 2013b).

Yeast immobilization and the role of volatile compounds

Yeast immobilization in nature

While considered an archetypal unicellular eukaryote, the yeast *Saccharomyces cerevisiae* can engage in various multicellular modes of existence wherein the cells cooperate to fully utilize available resources or maximize their chances of survival. Social behaviour may explain why immobilized yeast show improved resistance to stress and consequently improved performance relative to free cells. Cooperative tactics include flocculation, adhesion, filament formation and biofilm formation (Honigberg, 2011). Some of these behaviours are industrially beneficial and have been co-opted for our own benefit. Other social behaviours have a negative impact on human society and considerable effort is required to prevent their occurrence or mitigate their effects. To better understand and control these processes it is perhaps instructive to consider their role in the natural world. The classic example of yeast social behaviour is flocculation during alcoholic fermentation. This involves the reversible, non-sexual, Ca^{+}-dependent aggregation of individual yeast cells to form large flocs (Verstrepen *et al.*, 2003e; Soares, 2011, Vidgren and Londesborough, 2011). Flocculation is mediated mainly by the genes *FLO1*, *FLO5*, *FLO9*, *FLO10* that encode adhesins, or flocculins– proteins that extend from the yeast cell wall and bind to mannose residues on the walls of adjacent cells (Douglas *et al.*, 2007). In brewing and some other alcoholic fermentations, flocculation has an important role in clarification of the product, but from a yeast cell's point of view the most important benefit of such behaviour is avoidance of stressful conditions. Flocculation is normally triggered by sugar depletion and not necessarily induced by stress *per se* (Claro *et al.*, 2007). However, yeast cells capable of flocculating or those contained within flocs appear to be more resistant to a number of stresses including ethanol, peroxide, high temperature or antibiotic exposure (Lei *et al.*, 2007; Smukalla *et al.*,

2008). Flocculation apparently represents a community behaviour in which aggregated cells are physically protected from stress by an outer layer of sacrificial cells. This behaviour is only likely to be of benefit when sufficient numbers of cells are present to create large flocs, which have a high mass to surface ratio, with the risk of an individual cell being located in the protective layer being relatively small (Lei *et al.,* 2007). An analogous stress avoidance mechanism is encapsulation of cells in alginate or similar for immobilized yeast fermentation (Sun *et al.,* 2007a,b). However, cells adhering to carrier materials such as glass beads, wood chips etc. may still be directly exposed to the stresses prevalent during fermentation.

In recent years another yeast social behaviour, biofilm formation, has attracted interest due its potential for biofouling in industrial processes. Biofilm formation involves adherence to a foreign surface followed by the formation of a colony or mat. These mats may develop structurally, forming fluffy or wrinkled colonies (Granek *et al.,* 2013; Šťoviček *et al.,* 2010) and even forming stalk-like protuberances up to three centimetres in length (Scherz *et al.,* 2001). In most organisms, biofilm formation typically involves production of an extracellular matrix (ECM), though the evidence for this is less strong for yeast than for bacteria. Several studies have observed ECM-like material in yeast biofilms (Kuthan *et al.,* 2003; Zara *et al.,* 2009) though it is not clear to what extent this ECM protects yeast against stress (Beauvais *et al.,* 2009). Regardless of the protective role of yeast ECM, cells embedded in biofilms are, like those contained within flocs, protected to some extent from the stresses present in the extracellular environment (Jirku, 1999; Tristezza *et al.,* 2010). The mechanisms that contribute to stress tolerance of biofilms probably also operate in the case of immobilized yeast fermentations, which are characterized by reduced stress sensitivity (and hence greater productivity) and which will be discussed in greater detail later.

Molecular control of immobilization

The yeast Flo11 protein has been strongly implicated in biofilm formation (Van Mulders *et al.,* 2009; Zara *et al.,* 2009), colony structure (Šťoviček *et al.,* 2010) and ECM production (Karunanithi *et al.,* 2010), but how exactly it operates is not clear. Its function may be determined by its hydrophobic nature to some extent (Purevdorj-Gage *et al.,* 2007; Van Mulders *et al.,* 2009). There is likewise little known about the factors that determine the strain-to-strain variation in the Flo11 phenotype (Bayly *et al.,* 2005; Douglas *et al.,* 2007). Flocculation and adhesion are controlled quite differently at the genetic level. The *FLO11* gene in particular is responsible for adhesion to surfaces (as well as for invasive growth and

formation of pseudohyphae), though many other genes are likely to be involved (Vandenbosch *et al.*, 2013). The gene belongs to the FLO family but is the most diverged gene within the group and only rarely has a significant influence on flocculation (Guo *et al.*, 2000; Bayly *et al.*, 2005). In contrast to flocculation, the main environmental stimulus for yeast cell adhesion appears to be amino acid starvation (Braus *et al.*, 2003; Kleinschmidt *et al.*, 2005; Valerius *et al.*, 2007), which influences cells even in the presence of sufficient ammonium and glucose, compounds which normally prevent adhesion (Braus *et al.*, 2003).

Biological function of yeast volatiles

Recent work has demonstrated that *FLO11* is controlled by a quorum sensing (QS) mechanism and may be directly influenced by yeast-derived ethyl alcohol and higher alcohols. QS molecules are hormone-like molecules produced by the yeast population that trigger a phenotypic response when the concentration rises above a threshold concentration. This mechanism allows yeast populations to adapt to their environment in a way that takes population density into account (Sprague and Winans, 2006). This mechanism has been widely studied in bacteria but QS in fungi has received attention only in recent years, with a focus in particular on morphological switching from hyphal to yeast-like forms in dimorphic pathogenic fungi (Kügler *et al.*, 2000). Interestingly, higher alcohols (and ethanol) are known to act as QS molecules. In an early investigation into QS in *S. cerevisiae* by Chen and Fink (2006), it was found that the aromatic alcohols produced by a yeast population greatly stimulated *FLO11* and had a major influence on phenotypic expression. In this case the focus was on development of pseudohyphae rather than biofilm formation, though aromatic alcohols are likely to participate in both cases as they are related phenotypic traits. Phenylethanol and tryptophol derived from phenylalanine and tryptophan, respectively, were found to induce *FLO11* transcription. Tryptophol also induced expression of *ARO9* and *ARO10*, genes responsible for production of fusel aldehydes via transamination and decarboxylation in the Ehrlich pathway. High cell densities and a correspondingly high concentration of specific fusel alcohol concentrations can therefore potentially act to promote both biofilm formation and production of precursors of higher alcohols and esters. Quorum sensing molecules such as phenylethanol and tyrosol have been shown to promote adhesion to surfaces by other yeast species including *Debaryomyces hansenii* (Gori *et al.*, 2011), while other QS molecules such as farnesol produced by *S. boulardii* have been found to inhibit adherence of other yeast (Krasowska *et al.*, 2009). Phenylethanol has been found to support

adhesion and biofilm formation of *Kloeckera apiculata* on citrus fruit, seemingly through up-regulation of the yeast's *FLO* genes (Liu *et al.*, 2014). It may be speculated that the high cell densities that are found in immobilized yeast reactors promote the production of QS molecules such as phenylethanol, which in turn promote yeast adhesion, biofilm formation and improved productivity.

As outlined above, higher alcohols are produced in yeast through amino acid synthesis or degradation via the Ehrlich pathway, the first step of which involves a transamination reaction that produces an α-keto acid. This α-keto acid has no role in central carbon metabolism and the subsequent decarboxylation and reduction reactions serve to produce a volatile compound that can be readily removed from the cell via passive diffusion (Hazelwood *et al.*, 2008). Higher alcohol production is therefore primarily an excretory mechanism that is necessary to maintain metabolic function of the cell. However, as shown above, higher alcohols can have other functions such as regulation of social activity and control of resources in competitive environments. In recent years, studies have also highlighted the importance of higher alcohols in mediating interactions with insects. Such interactions may be critical for dispersal of the non-airborne *Saccharomyces* yeast (Nout and Bartelt, 1998, Lorenzo *et al.*, 1999; Becher *et al.*, 2012; Cha *et al.*, 2012; Stefanini *et al.*, 2012; Witzgall *et al.*, 2012; Palanca *et al.*, 2013). It is clear that yeast volatiles can impact on yeast cells' propensity to remain in a free or immobile state. We will show further that artificial methods of immobilization can have a corresponding effect on volatile production. The nature and magnitude of the effect will depend on the specific strategy used for immobilization.

Design strategies for immobilization

Methods for immobilization

There are four basic types of yeast cell immobilization that are classified by the mechanism of cell localization and the nature of support material. The simplest involve immobilization on a support surface or flocculation of yeast cells. The third type of immobilization is mechanical containment behind a barrier. Finally, the most investigated in the last few decades is entrapment in a porous matrix.

Cell immobilization on a solid carrier has been defined as an adsorption of yeast cells to some support material by covalent bonding between the cell membrane and the carrier or by electrostatic forces. The strength and depth of bonds between a carrier material and yeast cells may differ from one system to another. In general, they depend on the nature of the support material, cell physiology, and environmental conditions. This type of immobilization has been widely applied due to the ease of carrying out the process and cheapness of the carrier materials used such as cellulosic and inorganic materials.

Flocculated cells can be used in reactors such as packed-bed or fluidized-bed or even continuous stirred-tank reactors (Kourkoutas *et al.,* 2004). Yeast flocculation is a very important phenomenon in the brewing industry and affects fermentation productivity, quality as well as yeast removal and recovery. This kind of immobilization is the simplest and cheapest one, but it is easily affected by many factors such as wall composition of cells, medium, pH, and dissolved oxygen (Kourkoutas *et al.,* 2004; Nedović *et al.,* 2005).

There are several types of mechanical containment of cells behind a barrier. The most common are entrapment of cells in a microcapsule and use of microporous or ultraporous membrane filters. This type of immobilization is desirable when minimum transfer of compounds or cell free products is required (Park and Chang, 2000). Selected yeasts confined by microfiltration membranes have been successfully used in wine-production. Beside expense, the main disadvantages of immobilization between membranes are mass transfer limitations (Lebeau *et al.*, 1998) and possible membrane biofouling caused by cell growth (Gryta, 2002). Commonly, micro- and ultra-filtration membranes have been employed, but also silicon, ceramic, and other membranes. Entrapment in a porous matrix is achieved by inclusion of yeast cells within the matrix of a porous material. In this way cell diffusion is prevented, and simultaneously, transfer of nutrients and metabolites is enabled through pores of the matrix. The main disadvantage of this type of cell immobilization is related to propagation of cells located on surface of the beads and their easy release. In order to circumvent this, double layer beads have been developed (Taillandier *et al.*, 1994). Typical examples of materials used for matrix entrapment are polysaccharide polymers and proteins (Norton and D'Amore, 1994; Park and Chang, 2000).

Carrier materials

A major criterion for successful application of cell immobilization for bioflavour production is the choice of a suitable carrier material, since a number of factors should be taken into account from safety, legality and stability to product quality and operating costs. Various materials have been tested at laboratory and pilot-plant scale, resulting in either improved or unbalanced flavour profiles. With reference to the immobilization technique applied, the selected material should conform to a number of requirements that are summarized in Table 1. As regards their chemical composition, origin or immobilization technique applied, carrier materials can be categorized into either organic, inorganic and natural supports or those where cells are adsorbed on solid surfaces through various types of interactions, i.e. Van der Waals forces, ionic bonds, hydrogen bridges or covalent interactions, or entrapped within a porous matrix, which allows the diffusion of nutrients and products (Kourkoutas *et al.*, 2010; Nedović *et al.*, 2010)

Natural or synthetic polymers, such as polysaccharides and proteins or polyvinyl alcohol, have been extensively investigated for cell immobilization, most likely due to their ability to gel under mild conditions and form spherical beads. Among them, alginates, pectins, chitosan, *κ*-carrageenan and gelatin are the most widely used biopolymers, as they easily form a highly flexible, biocompatible and non-toxic gel matrix (without the use of organic solvents and at room temperature) that protects the cells against inhibitory substances and contamination, favouring at the same time better substrate utilization and enhancing stability, flavour productivity and efficiency (Nedović *et al.*, 2010). Calcium alginate or pectate capsules can be prepared by either external or internal ionic gelation, with the first one being the most widely applied, though in both cases a source of Ca^{+2} is required. Beads of a relatively large size (1 to 3 mm) can be produced by the dropwise extrusion of the polymer/cell suspension mixture through a nozzle to a calcium chloride solution under gentle stirring. Nevertheless, the application of electrostatic forces to disrupt the liquid surface at the capillary/needle tip has led to a charged stream of small-diameter droplets and finally to a significant size reduction (to 0.3 mm) and uniformity (Nedović *et al.*, 2001).

The application of yeast cell systems encapsulated in biopolymer matrices in the brewing, wine, cider, and mead-making industries has been examined for many years. In all cases the aim was the production of a well-balanced alcoholic beverage with respect to aroma, taste

and overall quality. Kosseva *et al.*, (1998, 2004) used *Lactobacillus casei* cells immobilized in calcium pectate gel or chemically modified chitosan beads in order to study the kinetics of malolactic fermentation in chardonnay wine. Immobilised yeast fermentation has been also successfully applied in sparkling wine production. The use of sodium alginate and *κ*-carrageenan-entrapped yeast cells led to the production of rosé sparkling wine with sensory characteristics similar to those of the traditional products as well as to faster fermentation rates and lower cost since the removal of beads (riddling) from the bottles was much easier (Tataridis *et al.*, 2005). Likewise, yeasts immobilized on a support of gellan gum remained included in beads, which led to the production of clear sparkling wine and elimination of riddling stages (Mantaluta *et al.*, 2011). Yeast immobilized in sodium alginate beads was also found to be a suitable biocatalyst in the fermentation of diluted honey for mead production (Pereira *et al.*, 2014), in pomegranate wine making at ambient temperatures (Sevda and Rodrigues, 2011) as well as in wine made from the tropical fruit cagaita (Oliveira *et al.*, 2011). Additionally, according to Andrade Neves *et al.*, (2014), fermentation with yeast cells immobilized in calcium alginate could be associated with the thermovinification technique for the production of acceptable young wines from cabernet sauvignon and pinot noir grape varieties. Alginate beads have been also used in green beer production in a fluidized-bed bioreactor operated by means of a circulating wort system (Wang *et al.*, 1989) as well as for stout beer production (Almonacid *et al.*, 2012). Lager brewing yeasts, encapsulated in alginate/chitosan matrix, have been found to produce beers comparable to conventional ones with higher levels of total higher alcohols and esters and slightly lower amounts of aldehydes at different original wort gravities (Naydenova *et al.*, 2013). The co-immobilization of *S. bayanus* and *Leuconostoc oenos* in a calcium alginate matrix led to better flavour formation control and acceptable taste of the final cider beverage (Nedovic *et al.*, 2000). In order to provide protection for yeast cells against *D*-limonene during orange peel hydrolysate fermentation, Lalou *et al.*, (2013) exploited the use of yeast cells immobilized in sodium alginate beads. Correspondingly, Lee *et al.*, (1998) tried four different immobilization media (i.e. *κ*-carrageenan, chitosan, agarose, calcium alginate) for the yeast *Sporidiobolus salmonicolor* to overcome the toxicity of ricinoleic acid during *γ*-decalactone production (peach-like aroma). Furthermore, the biotransformation of isoeugenol for the production of vanilla metabolites (vanillin, vanillic acid and ferulic acid) was found to be more effective in immobilized cell cultures of *Capsicum frutescens*, something that was further enhanced by the addition of *β*-cyclodextrin and fungal elicitor (Ramachandra and Ravishankar, 1999).

Natural supports, such as delignified cellulosic material, gluten pellets, brewer's spent grains, fruit pieces, etc. represent another type of material examined for yeast cell immobilization. Their low cost (they usually need the least or no pre-treatment), abundance and food-grade nature have made them an attractive way to improve the aroma profile of many products (e.g. wine, beer). Delignified cellulosic materials and gluten pellets have proved to be effective during both room-temperature and low-temperature winemaking (Bardi *et al.*, 1996a; Mallouchos *et al.*, 2003; Sipsas *et al.*, 2009). Accordingly, their use in brewing, either in fresh or freeze-dried form, was suitable for batch and continuous fermentation of wort at low temperatures, while beer produced contained lower amounts of diacetyl and polyphenol compared to beer produced by free cells (Bardi *et al.*, 1996b, c; 1997; Bekatorou *et al.*, 2001, 2002a). Additionally, cells immobilized to DEAE-cellulose have been successfully applied for the production of alcohol-free beer (Van Iersel *et al.*, 2000), while other cheap alternative carriers, such as spent grains and corncobs, have been also tested for beer production by high gravity batch and continuous processes at different temperatures (Dragone *et al.*, 2008; Silva *et al.*, 2008)

During the last decade, pieces of various fruits (e.g. apple, quince, pear, fig, raisin berries, grape berries, grape stems and skins, orange and watermelon) have been also used as support materials for cells involved in fermentation processes. Rapid fermentations, great stability, suitability for continuous processes as well as enhanced product flavour characteristics have been reported when yeast cells immobilized on apple, quince, pears and orange peel pieces were employed in wine-making (Kourkoutas *et al.*, 2001, 2003a, b; Mallios *et al.*, 2004; Plessas *et al.*, 2007). Similar results were obtained when *S. cerevisiae* cells immobilized on guava and watermelon pieces were considered by Reddy *et al.*, (2006, 2008) as novel biocatalysts for wine production. Likewise, the production of green beer by yeast cells immobilized on dried figs resulted in a sweet, smooth product with a special fruity, fig-like aroma and taste clearly different from other commercial products (Bekatorou *et al.*, 2002b). A different approach includes the use of wine industry wastes, such as grape skins, stems or pomace, as well as raisins and grape berries. Mallouchos *et al.*, (2002), who investigated grape skins as a natural support for yeast immobilization, reported increased productivity and a positive impact on wine aroma, while in another work, grape pomace, the solid waste resulting from grapes pressing, and grape stems were studied as a means of yeast cell immobilization by natural adsorption for white wine production (Genisheva *et al.*, 2012; 2014). Freeze-dried grape berries from two varieties were also assessed as yeast support

matrices for the fermentation of honey. Supplementation with the freeze-dried particles significantly affected the fermentation since increased fermentation rate and ethanol concentration and decreased volatile acidity of the produced meads were observed (Sroka *et al.*, 2013).

A number of inorganic materials, such as porous ceramics, porous glass, polyurethane foam etc., have been proposed as yeast cell carrier materials. The effective adhesion of yeasts on these materials has been applied in many fermentation processes. The use of three carrier materials (i.e. porous glass beads, DEAE-cellulose, diatomaceous earth) for immobilized primary fermentation of beer affected aroma composition of green beers suggesting at the same time that the choice of carrier material should be based on the yeast strain used and the product's desired characteristics (Virkajarvi and Pohjala, 2000). Kregiel *et al.,* (2012), who studied the influence of immobilization conditions on cell attachment to two different ceramic surfaces: hydroxylapatite and chamotte tablets came to similar conclusions. Furthermore, in order yeast cell adhesion efficiency be enhanced, chamotte surface was covered by using different organosilanes and tested for proadhesive properties using industrial brewery yeast strains in different physiological states (Berlowska *et al.*, 2013a; Kregiel & Berlowska, 2014; Kregiel, 2014). The use of *S. cerevisiae* and *Schizosaccharomyces pombe* cells immobilized on glass pellets covered with an alginate film has been found to produce wines with similar characteristics to those produced by free cells (Ogbonna *et al.,* 1989). Porous spherical glass beads have been also tried as a yeast immobilization support in continuous processes for rapid maturation of green beer or fermentation of high gravity worts (via Kourkoutas *et al.*, 2004). Virkajarvi *et al.*, (2002),aiming to find process parameters that facilitate very high gravity brewing, used porous glass beads as the carrier for wort fermentation. In a more recent work, cubes of white foam glass were employed as an immobilization medium for a wine yeast strain of *S. bayanus* in order to study the effect of continuous fermentation of high-sugar fruit must (i.e. apple), supplemented with magnesium ions, on the viability and morphology of yeast (Bonin and Skwira, 2008). The use of a kissiris (i.e. a porous volcanic mineral containing mainly 70% SiO_2) supported biocatalyst was also found to perform well in repeated batch alcoholic fermentations of raisin extract (Kana *et al.*, 1992) as well as for batch and continuous low-temperature wine-making (Bakoyianis *et al.*, 1992; 1993) retaining at the same time its biocatalytic activity for about 2.5 years. In similar studies, γ-alumina, in the form of porous

cylindrical pellets, was also tested as immobilization support for wine making (Kana *et al.*, 1992; Loukatos *et al.*, 2000).

Comparison of immobilized vs suspended yeast cells

Effects on morphology, growth and physiology

Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization. Many studies have discussed these issues (Melzoch *et al.*, 1994; Nortonand D'Amore, 1994; Walsh and Malone, 1995; Willaert and Nedović, 2006; Kregiel *et al.*, 2013). In general, the kinetic properties of immobilized *S. cerevisiae* are different from those of suspended yeast. Immobilized yeast cells have higher glucose flux, i.e. consume glucose faster than suspended cells and, consequently, more substrate is channeled to biomass and ethanol. Increased, static, and decreased growth rates have been reported for immobilized yeast, but in most cases, a very limited or no cell growth have been observed. Pajic-Lijakovic *et al.* (2006) assigned a lower specific growth rate in encapsulated yeast cells to oxygen diffusion problems. Due to the decrease in specific growth rate, amino acid metabolism also decreases and concentrations of oxo-acids in the fermentation medium increase. It has been seen that growth of immobilized cells largely depends on growing conditions Shaking conditions promote cell growth in comparison to stationary conditions (Ali *et al*., 2014). An increase of the storage polysaccharide glycogen and structural polysaccharides, and an increase of ploidy for immobilized cells are also side-effects of immobilization (Norton and D'Amore, 1994). Cells immobilized in calcium alginate beads can be stored for a long time before application or between cultivations, even longer than 1 year, without loss in their glycolytic activity and viability (Melzoch *et al.*, 1994). Moreover, immobilized cells had reduced activity of some enzymes and ATP content in comparison to free cells. Kregiel *et al.* (2013) determined reduced activity of succinate dehydrogenase and pyruvate decarboxylase when yeast was immobilized in foamed alginate gels. Similarly, Berlowska *et al.* (2013b) reported reduced activity of the same two enzymes for six brewing yeast strains immobilized on a chamotte carrier. As a consequence of these physiological changes, the metabolic activity become changed, so that concentrations of the main aromatic compounds are changed in comparison to those obtained by free cells. There have been some recent trials to model accumulation of major yeast metabolites produced by free and immobilized cells (Vassilev *et al.*, 2013) and the effect of the fermentation temperature on immobilized cell mass and original wort extract (OE) on fermentation dynamics (Naydenova *et al.*, 2014).

These models are useful for developing a control strategy for a fermentation process to obtain beverages with different organoleptic profile. The diffierences between free and immobilizes cells have been rarely evaluated at the genetic level. In the recent study of Nagarjan *et al.* (2014), immobilized cells exhibited a stable pattern of gene expression that differed markedly from growing or starving planktonic cells, highly expressing genes in glycolysis, cell wall remodeling, and stress resistance, but decreasing transcription of genes in the tricarboxylic acid cycle and genes that regulate the cell cycle, including master cyclins CDC28 and CLN1.

Ethanol tolerance

Immobilized cells, depending on the carrier used for immobilization, show various modifications in physiology, morphology, biochemical composition and metabolic activity. Doran and Bailey (1986) demonstrated that yeast cells immobilized on glutaraldehyde cross-linked, gelatin-coated glass beads showed a pattern of DNA, RNA, protein and structural polysaccharides (glucan and mannan) contents significantly different from that of freely suspended cells. In comparison with free cells, they also had a higher content of glycogen and trehalose. Immobilization also causes changes in the proteome of a cell, and the level of gene expression, and has a significant impact on the quantitative composition and organization of the cytoplasmic membrane and cell wall structures (Norton *et al.*, 1995; Jirku, 1999; Parascandola *et al.,* 1997; Junter *et al.,* 2002). These alterations have a profound impact on cell stress resistance.

According to Hilge-Rotmann and Rehm (1991), the increased saturation of the fatty acid content of immobilized yeast correlates positively with improved fermentation rates obtained with the immobilized cells. This enhanced saturation of fatty acid composition in immobilized cells may be due to altered osmotic conditions in the microenvironment of the cells. The authors found that yeast cells immobilized by entrapment in calcium-alginate beads or by adsorption on sintered glass contained significantly higher percentages of saturated fatty acyl residues, especially of palmitic acid (C16:0), and a decreased amount of oleic acid (C18:1) compared with free cells.

A higher proportion of saturated fatty acids in immobilized yeast cells compared to free cells was confirmed also by Ciesarová *et al.,* (1996a), Van Iersel *et al.,* (1999), Jirku *et al.,* (2003), and Shen *et al.,* (2003a). In accordance with a higher proportion of saturated fatty acids in immobilized yeast cells, they are considered to be more tolerant against ethanol than freely

suspended yeast cells. Some reports also suggest that increased fatty acid saturation facilitates the excretion of endogenous ethanol into the fermentation medium (Jirku, 1999; Hilge-Rotmann and Rehm 1991). The increased resistance of immobilized cells to acid stress (Krisch and Szajáni, 1997; Taipa *et al.*, 1993; Hansen *et al.*, 2002) and organic solvent stress (Qun *et al.*, 2002; Desimone *et al.*, 2003) is connected to changes in structural features affecting immobilized cells' permeability, namely the composition and organization of the cell wall and the plasma membrane (Parascandola *et al.*, 1997).

Adverse environmental conditions in immobilized cells structures, i.e. high osmotic pressure (Hilge-Rotmann and Rehm, 1991) and nutrient limitations or/and mechanical stress (Parascandola *et al.*, 1997) have been put forward to try to explain these modifications in immobilized cell wall permeability. Nevertheless, Jirku (1999) advocated more potent signals than transient microenvironmental stimuli, since salt stress did not alter the cell attachment response (Junter *et al.*, 2002).

Norton *et al.* (1995) compared the stress resistance of free and κ-carrageenan-immobilized yeast cells. Results demonstrated a significant increase in yeast tolerance to ethanol with immobilized cells as compared to free cells, while no marked difference in heat resistance was observed. When entrapped cells were released by mechanical disruption of the gel beads and submitted to the same ethanol stress, they exhibited a lower survival rate than entrapped cells, but a similar or slightly higher survival rate than free cells. It was concluded that the increased ethanol tolerance of immobilized yeast cells can be attributed to cell encapsulation by a protective layer of gel material or to modified fatty acid concentration in cell membranes due to oxygen diffusion limitations. They also reported the partial removal of substrate inhibition by cell immobilization, as well as the fact that entrapped cells returned to normal physiological behaviour as soon as they were released. Osmotic stress caused by the immobilization techniques was found to lead to an intracellular production of pressure-regulating compounds such as polyols, which lead to decreased water activity and consequently higher tolerance to toxic compounds. Kirsh and Szajáni (1997) have found that when *S. cerevisiae* cells were immobilized by adsorption on pre-formed cellulose beads or by etrapment in calcium alginate and were treated with 20% ethanol (lethal for free yeast cells), 62 or 72%, respectively, of the immobilized cells survived. Cells released from the carrier showed an intermediate survival (20–60%). In addition, 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.

Sun *et al.,* (2007b) reported the influence of the microenvironment in alginate–chitosan–alginate microcapsules on the physiology and stress tolerance of *S. cerevisiae*. Cells cultivated in alginate–chitosan–alginate with a liquid core showed a nearly two-fold increase in the intracellular glycerol content, trehalose content, and superoxide dismutase activity, all of which are stress tolerance agents. Solid-core microcapsules did not cause significant physiological change. In accordance with physiological modification after being challenged with osmotic stress (NaCl), oxidative stress (H_2O_2), ethanol stress, and heat shock stress, the cell survival in liquid-core microcapsules was increased. Cells released from these microcapsules were more resistant to hyperosmotic stress, oxidative stress, and heat shock stress than cells liberated from solid core microcapsules. However, the microcapsules with a solid core protected the cells from damage under ethanol stress. It was found that the resistance of liquid core microcapsules to hyperosmotic stress, oxidative stress, and heat shock stress mainly depended on the protective effect of the microcapsule's microenvironment. The physical barrier of the liquid core constituted by the alginate–chitosan membrane and liquid alginate matrix separated the cells from the effects of oxidative stress and ethanol stress. The significant tolerance against ethanol stress of solid-core microcapsules was attributed to the physical barrier, which consists of a solid alginate–calcium matrix and an alginate–chitosan membrane.

Similar results were found when comparing yeast cells encapsulated in calcium alginate micro-gel beads or in alginate–chitosan–alginate microcapsules with liquid core and solid core after osmotic shock induced by NaCl solution. The liquid core gave rise to the highest survival rate of encapsulated cells or cells released from the microcapsule. It was demonstrated that microcapsules with a liquid core were able to induce the highest stress response and stress tolerance of cells, which was adapted during culture, while sold-core microencapsulation failed. The theoretical analysis revealed that it was the liquid alginate matrix in microcapsules that played a central role in adaptation to hyper-osmotic stress. This finding provides a very useful guideline to cell encapsulation (Sun *et al.*, 2007a).

Šmogrovičová (2014) compared the influence of immobilization on fatty acid composition of yeast lipids during fermentation. Increasing ethanol decreased the relative percentage of saturated fatty acids more in free and on DEAE-cellulose immobilized cells than in the yeast

entrapped in calcium pectate, calcium alginate or κ-carrageenan. A lower unsaturation index correlated with an increased rate of fermentation and ethanol tolerance of yeasts entrapped in gels. The specific rates of ethanol production of free yeast cells and cells immobilized on DEAE-cellulose were very similar at all concentrations of wort and were reduced, as compared to yeast cells immobilized in calcium pectate or calcium alginate. The specific rate of ethanol production of yeast immobilized in calcium pectate or in calcium alginate in 24 % wort was at the level of the specific rate of ethanol production of free yeast and yeast adsorbed on DEAE-cellulose in wort of only 16 % concentration, and at the level of the specific rate of ethanol production of yeast immobilized in κ-carrageenan in 20 % wort. Krisch and Szajáni (1997) reported also greater ethanol tolerance of entrapped *S. cerevisiae* cells relative to cells adsorbed on cellulose. Similarly there was a difference between resistance to acetic acid stress of *S. cerevisiae* and *Acetobacter aceti* cells immobilized by adsorption on cellulose and by entrapment in calcium alginate.

Ciesarová *et al.,* (1998) have observed that the production of carbon dioxide by yeast immobilized in calcium alginate and calcium pectate gel beads was approximately 2.5-times higher than by the free yeast at 5 and 10 % of ethanol. A 4-fold increase of carbon dioxide production was observed at 15 % ethanol. Entrapment in calcium-containing carriers (alginate, pectate) resulted in enhanced activities of yeasts compared to the κ-carrageenan carrier. The protective effect of Ca ions resulting from increased membrane stability was found to prevent the release of cytoplasmic compounds. Calcium ions increase plasma membrane stability either by decreasing the ethanol-induced passive protons influx or stabilizing the ATP-ase activity inhibited by ethanol. However, any positive effect due to calcium supplementation on yeast growth is compromised by its antagonistic effect on magnesium uptake, as yeast cells have also an absolute requirement for magnesium, which acts as a cofactor for many enzymes and is necessary particularly for enzymes involved in glycolysis (Saltukoglu and Slaughter, 1983; Alexandre *et al.,* 1993; Ciesarová *et al.,* 1996b; Walker *et al.,* 1996; Walker, 2004; Gibson, 2011).

Mechanical stress tolerance

In contrast to the tremendous knowledge on the genetics of *S. cerevisiae* very little is known about its response to mechanical stress. The response of a cell to applied forces is dependent on several factors, including the strength and elasticity of individual molecules composing

the cell wall, the three-dimensional arrangement of individual molecules, and genetic factors programming composition and assembly. Cell mechanical properties have been investigated by using micropipette aspiration, osmotic swelling/shrinking, cell poking, cell compression and atomic force microscopy techniques (Zahalak *et al.*, 1990; Mashmoushy, 1998). The finding used to predict when applied stresses (e.g. because of fluid flow) will lead to wall rupture is mainly empirical. Smith *et al.* (2000a) developed a micromanipulation technique to measure the force required to burst single cells. They determined the average surface modulus of the *S. cerevisiae* cell wall to be 11.1 ± 0.6 N/m and 12.9 ± 0.7 N/m in exponential and stationary phases, respectively. Similarly, in another study the wall surface modulus for a commercially available baker's yeast (Fermipan, Gist-Brocades, Delft, The Netherlands) was found to be 11.4 ± 0.4 N/m, while the wall strain at cell breakage of 75% was also determined (Smith *et al.*, 2000b).

Immobilized cells are protected from mechanical stress to some extent. The extent of protection is determined by the interplay of several factors, including the type of immobilization (entrapment *versus* surface adsorption), support material and agitation rate (i.e. rate of shear). Sufficient agitation is desirable so that the thickness of the liquid film surrounding each carrier particle and consequently external mass transfer resistance is minimized, thus facilitating the transfer of molecules (nutrients from the bulk medium to the carrier and metabolites diffusing in the opposite direction). In general, the rate of shear on the particles in a reactor will increase as agitation is increased. However, for a given agitation rate, different reactor designs may differ in rate of shear; bioreactors with mechanical stirring induce the highest shear rate for example. If shear rates are too high, biomass may be lost by detachment from adsorption matrices, or particles may break in case of matrix-based immobilized cells or cell aggregates may be disrupted.

Swollen hydrogels are known to be weak materials which exhibit poor mechanical properties. The compression modulus of alginate hydrogels ranges from less than 1 kPa to greater than 1000 kPa, the shear modulus has values in the range of 0.02–40 kPa, and tensile modulus values in the range 10-55 kPa (Drury *et al.*, 2004). They depend on the polymer composition, cross-linking density, the conditions under which the polymer is formed and cross-linking density. The geometry of the sample also plays a role. Alginate (0.9- 1.5 wt.%) discs (8 mm thick and 30 mm in diameter) had a compressive modulus below 100 kPa at $CaCl_2$ concentrations up to 680 mM (Nunamaker *et al.*, 2011). The values of the Young's modulus

for slightly smaller empty beads (0.8-0.9 mm) were found to be in the same range (1-20 kPa) depending on the composition (Lekka *et al.*, 2004). Alginate hydrogel is much weaker in comparison to others used for yeast immobilization, e.g. PVA. PVA samples (with 75-80 % water content, cylindrical in shape, nominally 6mm in diameter and 6mm in height) expressed the compressive modulus from 1 -18 MPa over a strain range of 10-60% and a compressive failure of the hydrogels occurred between 45 and 60% strain (Stammen *et al.*, 2001).

Despite a significant number of publications dedicated to rheological response of hydrogel matrices to stresses generated by compression, shear and tension, only a few of those actually refer to hydrogels with cells within. In general, the presence of cells leads to the weakening of the gel structures. This was confirmed for different cell-hydrogel systems. Thus, the shear modulus of millimetric alginate beads entrapping cells (hepatocytes with 15-30 μm in size) was found to be smaller than for empty beads (3 kPa *vs.* 11 kPa), but it increased with the duration of stay in the fluidized bed bioreactor (David *et al.*, 2006). It has been shown that the stress, strain and energy at failure of yeast-filled alginate samples depend on the initial cell content (Junter *et al.*, 2009a,b; Krouwel *et al.*, 1982). Yeast cells are relatively large microparticles, with an average diameter of 5μm (Junter and Vinet, 2009). They behave like an elastic material with a Young's modulus in the range of 1–2MPa (Svaldo-Lanero *et al.*, 2006), i.e., they are noticeably more rigid that the crude alginate gel matrix. It has been noticed that Ca-alginate microbeads became deformed in the course of cell propagation. Hydrogel deformation is a complex process influenced by relaxation of the expanded polymer network, and forces generated by cell growth inside the bead and interactions between solvent, network parts and cells. It is likely that electrostatic interactions between alginate chains and cells (as both are negatively charged at pH and ionic strengths characteristic for fermentations) are negligible in the view of behaviour under mechanical stress. It is interesting how mechanical deformations affect cell number increase during cultivation and *vice versa*. This interference was the subject of interest of Pajic-Lijakovic *et al.* (2007a; 2008) who investigated yeast cell growth within the Ca-alginate microbead during air-lift bioreactor cultivation. They discovered that after some critical time, the growth rate of cell colonies decreased drastically, but then suddenly increased again despite all experimental conditions being the same. This was interpreted as disintegration of the gel network and opening of new free space for growth of cell clusters. This particular effect causes the mechanical transformation of the network. These complex phenomena have been modeled

using the thermodynamical free energy formalism (Pajic-Lijakovic *et al.*, 2007a) without considerations of the relaxation effects. In another study (Plavsic *et al.*, 2010a, b) the self-organization of cells into clusters within a polymer matrix has been considered; in particular the existence of scaling laws for cell colony growth, related to their self-assembly and response to polymer hydrogel micro-environment constraints was analyzed as a function of rate of cluster density increase. The disintegration produces additional electrostatic repulsions between relatively stiff chains of poly-electrolytes as alginate. On the other hand, the attractive forces of the network segments tend to keep the structural integrity and cause the damping of energy dissipation. Pajic-Lijakovic *et al.*, (2007b) developed a mathematical model to describe the mechanism of Ca-alginate microbead deformation induced by cell propagation. The model comprised effects of different nature during different stages of the process. The comparison of the model developed with experimental values of isotropic volumetric beads deformations indicated a high impact of partial decomposition, i.e. plastic response of polymer network due to cell growth.

Bioreactor systems for ICT

Continuous processing coupled with ICT

Traditional beer fermentation and maturation processes use open fermenters and lagering tanks. During the past decades they were replaced by large-volume cylindroconical tanks in many breweries. Another promising approach for continuous brewing is based on immobilized cell technology. The main economic advantages of continuous, immobilized cell fermentation are the possibility to use very short fermentation times and to minimize the downstream processing (filling, cleaning, standby). The increased productivity results from several times higher cell concentration which is provided by immobilization of the biomass. Thus, it is possible to produce lager beer in a very short time period, usually 1–3 days, while traditional processes may take as long as several weeks. Furthermore, employing immobilized yeasts allows the use of yeast strains regardless of their flocculation characteristics. Moreover, regeneration of large amounts of carrier may be unnecessary, when a cheap, replaceable carrier, such as wood chips, spent grains or corncobs, are used (Kronlöf *et al.*, 2000; Brányik *et al.*, 2006).

However, immobilized cell technology has still found only a limited number of industrial applications. The reasons include engineering problems (excess biomass and problems with

carbon-dioxide removal, optimization of operating conditions, clogging and channeling of the reactor, risk of contamination), unbalanced beer flavour (altered cell physiology, cell aging), and high cost claims (carrier price, complex and unstable operation). Continuous fermentation with immobilized brewing yeast induces modifications in cell physiology due to the continuous mode of reactor operation, internal and external mass transfer limitations, aging of immobilized biomass. Continuous fermentation has been considered as an alternative to traditional batch fermentation since the late 19th century (Kleber, 1987) but did not see commercial application until the 1956 (Coutts, 1956; Geiger and Compton, 1957) when the first continuous (free-cell) fermentation process at industrial scale was derived from collaborative research between Dominion Breweries and New Zealand Breweries. Portno (1978), in an assessment of the relative merits of continuous fermentation, lists the main criteria that must be met for this approach to be successful. Of these, possibly the most important is maintenance of a high yeast cell concentration to facilitate the rapid conversion of wort to beer. High concentrations of cells in suspension necessitated fermenter designs which incorporated steps to separate and re-circulate yeast (for review see Maule, 1986; Boulton and Quain, 2001), thereby adding a degree of complexity not found in traditional batch fermentations. This complexity was one of the reasons that the industrial batch fermentation process was never seriously challenged by continuous fermentation with free cells. The first attempt to introduce immobilized systems into the brewing industry was made in 1970s after a failure to produce beer continuously using free cells (Narziss and Hellich, 1971). A combination of continuous fermentation and immobilized biomass removes the washout limitation of continuous operation with free cells and results in a higher productivity (Masschelein *et al.*, 1994; Linko *et al.*, 1997). The microbial population of the continuous systems lacks gradual changing of the external environment; instead it is exposed to a steady-state continuous operation. Continuous systems will mimic batch fermentations either in tubular reactors with plug-flow (Pajunen *et al.*, 1989) or in a series of agitated reactors (Verbelen *et al.*, 2006). Therefore, complete continuous beer fermentation is conducted in a series of two or more fermentation vessels, combining agitated vessels and plug-flow-like packed-bed reactors, where the correct balance of flavour compounds in beer is achieved by controlling the temperature, dissolved oxygen, and other substrate levels in the reactors (Branyik *et al.*, 2005; Virkajarvi *et al.*, 1998; Yamauchi, 1994a,b; Šmogrovičová *et al.*, 1999). It has been shown that the four-stage configuration had a better ability to reproduce batch fermentation characteristics of winemaking than the two-stage set-up (Clement *et al.*, 2011). A multi-stage continuous fermentation system enables decoupling of different phases

of fermentation, the growth phase (maintained in the first tank) and the stationary phase (non-proliferating cells kept in the later tanks). This system enabled increases productivity and optimized the production of higher alcohols and esters (Loukatos *et al*., 2003; Sipsas *et al*., 2009; Yamauchi *et al*., 1995). Optimization of the process conducted in a multi-stage continuous fermentation system requires detailed understanding of biokinetics and of the bioreactor configuration. Here, the key role is design of bioreactor wherein yeast cultivation takes place. The best oxygen transfer is achieved under strong agitation maintained in stirred reactors. The addition of some organic substances (e.g. perfluorocarbon, n-dodecane) which have higher oxygen solubility than water (thus referred as oxygen vectors) may enhance oxygen transfer in a bioreactor which is operated under low agitation conditions (Jia *et al.*, 1997).

The major challenge for a successful application of ICT at the industrial scale is the control and fine-tuning of the flavour profile during a combined primary and secondary fermentation, since many parameters can influence flavour formation of alcoholic beverages. Despite extensive research carried out in the last few decades, immobilized fermentation has not yet managed to outperform traditional batch technology. An industrial breakthrough in favour of continuous brewing using immobilized yeast could be expected only upon achieving the following process characteristics: simple design, low investment costs (application of cheap carrier materials), flexible operation, effective process control and good product quality (Šmogrovičová, 2008).

Bioreactor design for ICT

Various bioreactor configurations, at lab-scale, employing immobilized cells in batch or continuous processes have been proposed for fermentation processes. Here we will describe some of the most important configurations with respect to the influence they have on the end-products of yeast metabolism. However there is only limited literature on scaling-up efforts in yeast applications. Transferring a fermentation process from a lab-scale unit to a commercial one is a challenge due to the difficulty in assessing the factors affecting the scale-up process during the cultivation. Thus, many large-scale fermentation processes give a lower yield than is expected in the laboratory. Namely, extracellular changes induced by the drop of hydrodynamic efficiency in a large-scale production have several impacts at the level of the physiology of microorganisms, from metabolic shift to specific gene expression (stress response) (Lejeune *et al.,* 2013). The empirical criteria for scale-up are related to transport

process criteria without consideration of cell kinetics. Scale-up estimates have been performed based on geometric similarity, agitator tip speed, gassed power per unit volume and mixing time (Junker, 2004).

Stirred-tank bioreactors

A stirred-tank reactor has mechanical stirring turbines or propellers that can blow in or disperse air (Fig. 2a). The stirrer (propeller or turbine) creates turbulence to distribute the air evenly. Aeration is important actually at the early stage of the process for cells rapid propagation. When the height/diameter ratio is greater than 1.4 it is essential to have multiple stirrers. By selecting the optimum stirring conditions (depending on the volumetric fraction of the biocatalyst, biocatalyst geometry/size and impeller types) it is possible to achieve very efficient mixing without or with minimum loss of biocatalyst physical integrity even when the hydrogels (known as weak) have been selected as a matrix (Galaction *et al*., 2009a; 2009b; 2010; Lupasteanu *et al*., 2008; Cascaval *et al*., 2010). Arifin *et al.* (2011) developed the so-called continuous-closed-gas-loop bioreactor (CCGLB) system for bioconversion of geraniol into citronellol by free *S. cerevisiae*. The CCGLB system consisted of a stirred-tank bioreactor coupled with the pumps/reservoirs system for recycling of the gas (highly volatile substrate for biotransformation). Since gas phase promotes diffusion and reduces mass transfer limitation in the liquid phase, the process resulted in high productivity.

Packed bed bioreactors

Packed-bed or fixed-bed bioreactors (Figure 2b). have been widely used since the 1970s due to their simple design and operational control. The first version of this type was with diatomaceous earth (kieselguhr) porous beds for brewer's yeast immobilization (Kourkoutas *et al.,* 2004). Many other types of inorganic and organic supports in packed bed bioreactors have been tested since then. The most recent applictions include fruit peaces (Genisheva *et al*., 2014a), spent grains (Kopsahelis *et al*., 2012), calcium alginate (Sritrakul *et al*, 2007), starchy materials such as corn grains (Kandylis et al., 2012b), wheat (Kandylis *et al*., 2010a, b), corn starch gel (Kandylis *et al*., 2008) and potatoes (Kandylis and Koutinas, 2008), delignified cellulosic materials (Koutinas *et al*., 2012) and composite biocatalysts such as tubular delignified cellulosic material (DCM) coated with starch gel (Servetas *et al*., 2013). Packed-bed systems used for primary fermentation resulted in lower amino acid concentrations in beers compared to some other reactor types (Kourkoutas *et al*., 2004). The

main reason for the unbalanced flavour profile in beverages produced using immobilized yeast packed-bed reactors is insufficient mass transfer of nutrients to yeast and removal of fermentation by-products. Other engineering problems are linked to compacting of the bed, inter- and intra-particle gas entrapment, liquid-phase channeling, disintegration of the biocatalyst, deviations of the plug-flow model, etc. One trial in design improvement involved a pulsing device connected to a conventional packed-bed reactor (Rota *et al.*, 1994; 1995). This configuration mimics the plug-flow model by allowing the introduction of a square wave disturbance. The productivity was increased up to 20%, but operational control was rather difficult as for each flow rate and initial substrate concentration, there was a particular pulsation frequency which produces optimal results. Another approach to the design of a packed-bed is a multi-stage bed in which the biocatalyst is divided in several sections. It has been used in a horizontal fermentor containing five replaceable immobilized plates (Ogbonna *et al.*, 1989) or vertical one containing kissiris (Bakoyianis and Koutinas, 1996; Koutinas *et al.*, 1997), gluten pellets (Sipsas *et al.*, 2009), glass beads (Shindo *et al*., 2001), zeolite (Shindo *et al*., 2001) or hydrogel beads (Manojlovic *et al.*, 2007) as support for yeast. Such a bioreactor design is considered advantageous over typical packed bed configurations, as the pressures to which biocatalyst is exposed are dramatically reduced, and thus its destruction is avoided. Rotaru *et al.* (2011) proposed the basket type of bioreactors with the biocatalyst particles being fixed in an annular cylindrical bed, which was rotating. Owing to this design, the mechanical lysis of the biocatalysts (yeast in alginate beads) was avoided, but the substrate and product accumulation inside the basket bed occured during the fermentation process.

There are a number studies showing that cell immobilization causes unbalanced beverage flavour as a consequence of altered yeast metabolism (see recent reviews of Genisheva *et al*., 2014a) Some reports claim satisfactory physicochemical quality of the beverages. Thus, Genisheva *et al.* (2014b) developed an integrated winemaking process – including sequential alcoholic and malolactic fermentations operated with yeast immobilized either on grape stems or on grape skins, and bacterial cells (*Oenococcus oeni*) immobilized on grape skins. The flavour profile was good and both processes were more effcient in comparison to those with free cells, but only in the batch mode of operation. However, there are also some reports claiming that apart from faster fermentations and increased productivity, immobilized cell technology is beneficial with respect to aroma formation. Thus Kandylis *et al.* (2010a) showed that packed bed systems (both laboratory scale and 80 L bioreactor) with cells

immobilized on whole wheat grains, increased the formation of esters and produced wines with improved aromatic profile compared to those with free cells. This result has been ascribed to usage of starchy supports of wheat origin, which may behave as catalysts or promoters of the enzymes involved in the process (Kandylis and Koutinas, 2008; Kandylis *et al*., 2008). Sensory evaluation of the wine scored high (score of ~7.8 out of 10.0) when using multiple in-alginate immobilized cells of two specific yeast cultures and a malolactic *Lactobacillus* for wine production in packed near-horizontal columns (15º angle) (Aaron *et al*., 2004). *Fluidized-bed bioreactor*

In the 1960s fluidized bed bioreactors (Figure 2c). were used for the first time for continuous brewing of beer at industrial scale. The first fermenters contained fluidized beds of flocs of a specially chosen strain of *S. cerevisiae*. Fluidization of biocatalyst particles provides moderate local mixing and better mass distribution through the reactor volume compared to a static type of processing such as packed bed fermenters. Fluidized-beds are suitable for support particles that are significantly denser than fermentation media, e.g. porous glass beads used (Tata *et al.,* 1999). Hydrogel particles (density close to that of fermenting liquid) have also been fluidized (Aivasidis *et al.,* 1991; Ryder and Masschelein, 1985). Low velocities required for fluidization of very light particles are quite difficult to attain. The processing in a fluidized-bed bioreactor provides increased ethanol productivity and permits shorter residence times as compared with the traditional batch systems and other continuous reactor configurations (Davison and Scott, 1988; Gilson and Thomas, 1993). The potential problems during processing are particle floatation due to carbon-dioxide hold-up and insufficient mass transfer while the main difficulty in operation control is related to maintaining bed expansion (Nedović *et al.,* 2010). A magnetically-stabilized fluidized bed reactor coupled with yeast cells immobilized in particles made of Ca-alginate-magnetic powder mixture have been shown many times to be efficient for alcohol production by yeast (Terranova and Burns, 1991; Gilson and Thomas, 1993; Ivanova *et al.,* 1996; Liu *et al.,* 2009; Brady *et al.,* 2004; Webb *et al.,* 1996; Sakai *et al.,* 1994; Larsson and Mosbach, 1979; Hu and Wu, 1987). Even modelling of a complex three-phase fluidized bed bioreactor has been done for ethanol production using immobilized yeast in a gas–liquid–solid three-phase bioreactor (Sheikhi *et al,* 2012). Despite this, according to our knowledge it has not been tested yet for beverages.

Gas-lift bioreactors

The continuous closed-loop gas-lift bioreactor systems have been tested on free yeast-induced aroma production (Mihal *et al*., 2012a;b). Free-yeast bioreactor systems can be very complex, since a microfiltration module (connected to the bioreactor) is needed for the biomass removal coupled with an an extraction unit for removal of the product (Mihal *et al.*, 2012a;b). Gas-lift bioreactors with immobilized yeast (Figure 2d) have been used in fermentations for beverage productions since 1996 (Nedovic *et al.*, 1996). They were developed from the loop configuration which was used for the primary fermentation of beer in industry by Meura-Delta (Pilkington *et al.*, 1998). In this first system yeast cells were immobilized on porous rod matrices containing numerous internal channels; the fermentation medium flowed in a loop from the bottom of the fermenter, through both the internal channels and around the carrier for contact with the immobilized yeast, to the top of the reactor with external recycle. In gas-lift type of reactor liquid circulation is performed by gas injection. A gas-lift reactor provides an adequate amount of agitation at a reasonable shear rate. This type of reactor is characterized by low power consumption, optimal liquid mixing, heat and mass transfer and low particle abrasion. This makes gas lift systems very attractive for large scale industrial operations. Only immobilizing particles with a density close to that of the fermenting medium are chosen, such as hydrogels, Ca-alginate beads (Nedovic *et al.*, 1996), Ca-pectate (Šmogrovičová 1997; 1998), PVA (Bezbradica *et al.*, 2007), spent grains (Mota *et al.*, 2010) or cell aggregates (Sousa *et al.*, 1994a; 1994b). Internal loop (Nedovic *et al.*, 1996, Šmogrovičová 1997; 1998) and external loop configurations (Manojlovic *et al.*, 2008) have been used for brewing of beer at both, laboratory- and pilot-scales.

Membrane bioreactors

When a solute has to be continually removed from a fermentation tank such a setup is called a submerged membrane bioreactor or simply just a membrane bioreactor (Figure 2e). In this bioreactor the biocatalyst is separated from the medium by a membrane that cannot be penetrated by the cells. In contrast to more conventional membrane filtration systems, which are often operated at constant pressure, membrane bioreactors are often operated at constant flux, controlled by a suction pump. The pump creates a lowered pressure on the permeate side, thereby inducing a pressure driving force which is often relatively low. When operating at constant flux, the transport towards the membrane surface is kept constant which might be advantageous in order to handle and control fouling problems. Valadez-Blanco *et al*. (2008)

applied membrane bioreactor with nanofiltration membranes (for removal of organic solvents) for biotransformations of geraniol to R-citronellol by baker's yeast. Gao and Fleet (1995) described an efficient membrane bioreactor system used for continuous malolactic fermentation in wine. Takaya *et al.*, (2002) showed that the double-vessel membrane bioreactor had the productivity of dry wine 28 times higher than that in the batch fermentation. The membrane bioreactor is convenient for studying the microbial interactions between two microorganisms, which are kept in a homogenous liquid phase but physically separated by a membrane. Thus, Nehme *et al.* (2010) used a membrane bioreactor to evaluate the impact of the co-culture (*S. cerevisiae–O. oeni*) on the output of malolactic fermentation.

Case studies: brewing

Immobilized yeast in brewing

Beer fermentation is traditionally a batch process using freely suspended yeast cells in an unstirred batch reactor, and is the most time-consuming step in the production of beer. Immobilized cell systems offer many advantages, such as a faster fermentation rate, increased volumetric productivity, and possibility of continuous operation. Therefore, immobilization technology has been attracting the attention of the fermentation industry for over forty years and has been utilized for a number of purposes including continuous primary fermentation, low alcohol beer production and secondary maturation, with varying degrees of success.

Immobilized cell technology is able to produce lager beer in a very short time period, usually 1–3 days; however, a major difficulty is to achieve the proper balance of sensory compounds to create an acceptable flavour profile in such a short time frame. Therefore, only a limited number of beer fermentation, maturation and alcohol-free beer production processes have found their way into the industry (Narziss, 1997; Virkajärvi and Linko, 1999; Brányik *et al.*, 2005; Verbelen *et al.*, 2006; Willaert and Nedović, 2006).

Immobilized systems for beer fermentation

The primary fermentation of beer gives rise to not only ethanol, but also a complex mixture of flavour-active secondary metabolites, of which the higher alcohols, esters and vicinal diketones (diacetyl and 2,3-pentanedione) are the most important for a well-balanced flavour profile. Early studies on primary beer fermentations with immobilized yeast cells reported

lower concentrations of higher alcohols and esters due to a low metabolic activity of bound yeast.

A disadvantage of immobilization for primary beer fermentation is the lack of temporal heterogeneity that typifies batch fermentation and is crucial for normal flavour development. In batch fermentations the yeast cells take up amino acids in a particular order with preferred amino acids such as glutamine and aspartic acid taken up first, followed by less-preferred amino acids such as alanine and glycine that are taken up only towards the end of fermentation (or not at all) (Jones and Pierce, 1964; Gibson *et al.,* 2009). This temporal heterogeneity may not be found in simple continuous systems (depending on flow rates and mixing) where yeast fed constantly with fresh wort might only utilize the preferred amino acids. The situation is compounded by the fact that immobilized yeast systems are characterized by reduced growth and consequently an overall low and atypical amino acid uptake (Doran and Bailey, 1986; Ryder and Masschelein, 1985; Šmogrovičová and Dömény, 1999; Shen *et al.,* 2003a). Amino acid uptake directly influences higher alcohol and ester production. In particular, utilization of branched-chain amino acids such as valine, leucine and isoleucine is likely to influence the production of the corresponding higher alcohols isobutanol, isoamyl alcohol and amyl alcohol (Äyräpää, 1971; Schultess and Ettlinger, 1978), though less direct effects have also been observed (Lei *et al.,* 2013; Procopio *et al.,* 2013) Beers produced with immobilized yeast in continuous fermentation systems rarely match the flavour profiles of beers produced by batch fermentation, particularly due to lower levels of aroma compounds (Narziss and Hellich, 1971; 1972; Hsu and Bernstein, 1985).

Acceptable beer flavours may only be possible where immobilized yeast reactors are coupled with fermenters containing free yeast in suspension, with the possibility therefore of maintaining nutrient gradients typical of the batch fermentation process. Such a system has been applied at the Kirin brewery in Japan to produce beer on a commercial scale (Yamauchi *et al.,* 1994a, b; 1995). Yamauchi and colleagues (1995) observed that yeast in continuously stirred tank reactors produced greater levels of higher alcohols, while yeast in packed bed reactors mainly produced esters. Modification of flow rates through the different reactors could therefore be used to control beer flavour profile. Additionally, volatile flavour profile is controlled via oxygenation, with aeration typically promoting production of higher alcohols, presumably due to greater metabolic activity and growth. (Andries *et al.,* 1997; Virkajärvi and Kronlöf, 1998; Virkajärvi *et al.,* 1999). Increased aeration or oxygenation results in a

lower level of esters. This is apparently due to a direct effect on activity of the *ATF1* and *ATF2* genes that encode acetyltransferases and are responsible for synthesis of acetate esters (Fujii *et al.*, 1997; Fujiwara *et al.*, 1998; 1999). Low production of aroma compounds due to reduced growth may also be corrected by increasing fermentation temperature. This approach has been shown to increase concentrations of higher alcohols and to some extent esters during fermentation with yeast entrapped in calcium pectate and κ-carrageenan (Šmogrovičová and Dömény 1999). Dragone and colleagues (2008) also noted an increase in production of higher alcohols by immobilized yeast during continuous fermentation of all-malt wort at higher temperatures but also reported a concomitant decrease in ester concentration. Alternatively, the use of genetically modified yeast strains with flavour profiles tailored to counteract the off-flavours observed in continuous systems may also provide a solution (Verstrepen and Pretorius, 2006).

Combinations of immobilized yeast reactors and suspended yeast reactors have been employed at pilot or industrial-scale at various locations around the world (Mensour *et al.*, 1997). Continuous fermentation systems employing immobilized yeast have only rarely been in operation for significant periods. Even when beer flavours are successfully matched with those of batch fermented beers, operational issues can limit the usefulness of continuous fermentations systems. The main benefit of such systems, i.e. continuous production of beer with little 'down-time' can also be a disadvantage as such systems are quite inflexible compared to batch fermentations and when problems do occur the long restart times can negate any advantages gained during normal operation. As such, immobilized yeast are increasingly of interest for specialized applications such as secondary fermentation (beer maturation) or low-alcohol brewing rather than mainstream beer production (Brányik *et al.*, 2012).

Secondary fermentation in brewing

In addition to the unbalanced ester and higher alcohol profiles observed during primary beer fermentation with immobilized yeast, excessive amounts of vicinal diketones that are responsible for an undesirable buttery flavour are also typical. The basic aim of secondary fermentation of lager beer is reduction of by-products, mainly diacetyl but also sulphur compounds and other volatiles at low temperature. At this stage, fermentation occurs only at

a very limited rate, and no yeast growth and flavour formation is required. Therefore, immobilized yeast systems may be suitable for secondary fermentation.

Diacetyl is formed during primary fermentation by extracellular oxidative decarboxylation of its precursor α-acetolactate, and is subsequently re-assimilated and reduced by yeast to the relatively flavour-neutral acetoin and 2,3-butanediol (Krogerus and Gibson, 2013a). The reduction of diacetyl occurs at the end of the conventional main fermentation and continues during maturation. During secondary fermentation with immobilized yeast, the cells are capable of rapidly reducing diacetyl, but as the rate limiting step is the non-enzymatic conversion of α-acetolactate to diacetyl, the process is too slow to be feasible. This problem can be overcome by heat-treatment of beer after primary fermentation. During the heat treatment (10 minutes at 80–90 °C), α-acetolactate contained in the unconditioned beer is converted into diacetyl (and partly into acetoin). Total conversion of α-acetolactate directly to acetoin by heat treatment is not possible. The diacetyl formed can be rapidly reduced to acetoin by yeast, preferably immobilized in a continuously operated bioreactor (Narziss, 1997; Baker and Kirsop, 1973; Pajunen, 1995).

Combining the main and secondary fermentation is a particularly challenging and complex task. The most successful continuous maturation systems, which have been developed at VTT, the Technical Research Centre of Finland and implemented industrially, include one at the Sinebrychoff Brewery in Finland with a capacity of 1 million hl per year, and a system developed by Alfa Laval and Schott Engineering (Mensour *et al.,* 1997; Nitzsche *et al.,* 2001; Virkajarvi, 2002). In 1997, VTT built a pilot plant for primary fermentation and combined the primary and secondary fermentation processes to form a complete fermentation block producing quality beer in less than two days (Kronlöf *et al.,* 2000).

Low-alcohol beer

Numerous methods are available for the production of low alcohol and non-alcoholic beers. The strategies available for producing low levels of alcohol during fermentation include modification of the malt mashing process to reduce the levels of fermentable sugars, use of non-conventional yeast that produce low levels of alcohol, and limited fermentation where process conditions and fermentation times are modified to reduce yield (Brányik *et al.,* 2012). In the latter example the aim is to achieve limited fermentation but still generate flavours comparable to fully fermented beers. In particular the removal of ‘worty’ aldehyde flavours is

critical in producing low alcohol beers that mimic regular beers in sensorial properties (Perpète and Collin, 1999). Removal of these aldehydes along with the sweet-tasting sugars glucose, fructose and sucrose can occur quite rapidly but require fast separation of wort and yeast, or alternatively a rapid reduction in yeast metabolic activity, to avoid unwanted ethanol production. Continuous fermentation using immobilized yeast cells is a strategy that allows accurate control of wort contact time as well as facilitating control of yeast physiology through temperature control and has considerable potential for industrial production of low alcohol beers (Debourg *et al.,* 1994; van Iersel *et al.,* 2000). As ever, the control of flavour profile is the main issue limiting wide-spread application of this system. It has been observed however that those process conditions, e.g. temperature and aeration that can be used to control higher alcohol and ester production during regular continuous fermentation can also be applied when the desired product is a low alcohol beer (van Iersel *et al.,* 1999; Lehnert *et al.,* 2008). The relatively low levels of aroma compounds frequently associated with limited fermentations may be overcome by appropriate yeast strain selection or alternatively through modification of an existing strain to promote volatile production (Strejc *et al.,* 2013). Downstream modification of flavour profile is also an option (Daenen *et al.,* 2009).

Industrial applications of ICT in brewing

Bio-Brew Bioreactor for primary beer fermentation

Narziss and Hellich (1971) were pioneers in the use of immobilized yeast for beer fermentation. Their continuous reactor for primary fermentation called Bio-Brew was very simple – a kieselguhr filter filled with a mixture of kieselguhr and yeast. The residence time was only 2.5 hours, but the concentration of α-acetolactate and vicinal diketones in the green beer was very high. Therefore, not only maturation but also the addition of viable yeast was necessary. The reduction of vicinal diketones together with cold beer lagering increased the production time to 7 days. The final beer had a lower amount of higher alcohols and esters due to a reduced amino nitrogen consumption, too high pH, and very poor foam stability. Furthermore, the lifetime of the bioreactor was only 7 to 10 days before clogging. Probably the most serious problem was the high concentration of α-acetolactate in the beer leaving the bioreactor (Narziss, 1997). The process developed by Narziss and Hellich (1971) was further optimised by Dembowski *et al.,* (1993). An aerobic reactor was installed immediately upstream of the bio-brew reactor, the beer flow through the filter was optimised and a cooling plate was installed in the filter reactor to control the temperature. This resulted in increased

yeast viability in the reactor and improved sensory quality of the beer. However, the concentration of the low molecular weight nitrogenous substances in the beer remained too high. Overall, the Bio-Brew experiments were not successful.

Baker and Kirsop system for primary and secondary beer fermentation

Baker and Kirsop (1993) improved the Bio-Brew system, and modified it for primary and secondary fermentation. They were the first to report the heat treatment of beer for rapid conversion of α-acetolactate to diacetyl and the subsequent removal of diacetyl by immobilized yeast. The system consisted of a yeast plug formed with kieselguhr in a tubular reactor for main fermentation and a heating unit, cooling coil and a smaller reactor for secondary fermentation. Once again, the changing flavour of the beer was a problem. Immobilization of yeast by mixing it with diatomaceous earth was not ideal for beer production; however, these early experiments were valuable for further development, although they did not lead directly to industrial applications.

Kirin Brewery Company system for primary and secondary beer fermentation

A research group at the Kirin Brewery Company in Japan published their process in 1985 (Inoue 1995). The process consisted of a three-stage bioreactor system with immobilized yeast for rapid lager beer fermentation (Yamauchi *et al.*, 1994a). The first reactor was an aerated, continuously stirred tank for growing yeast. The yeasts were then removed in a centrifuge, and the green beer obtained was fed into a packed-bed reactor, in which the main fermentation was completed. The next step was the conversion of α-acetolactate into diacetyl and partly directly to acetoin in a subsequent heat treatment. Finally, the beer matured in another packed-bed reactor with immobilized yeast (*Fig. 3*). The total residence time varied between 72 and 96 hours.

Entrapment in alginate beads was first used for immobilization, but was replaced by porous glass beads developed by Kirin because of decreasing fermenting capacity, insufficient mechanical strength, and swelling of the carrier leading to clogging of the bioreactor and prevention of long-term operation. Other disadvantages attributed to alginate beads were heat

lability and poor regeneration ability for repeated use (Yamauchi *et al.,* 1994a). Aseptic filling of the reactors was also challenging with alginate beads. Kirin scaled up the system to a small commercial-scale production (1850 hl per year); later, the brewing was stopped due to diminished demand and the limited number of products. Other reasons for not maintaining the technology included: a slow start-up of the system (2 weeks), high energy costs because of the heat treatment prior to the third bioreactor, and beer losses in the centrifugation step (Inoue, 1995).

Labatt Breweries system for primary and secondary beer fermentation

A research group at Labatt Breweries (InBev) in Canada used κ-carrageenan beads for yeast immobilization and a gas-lift fluidised bed reactor. The small bead size (0.2 to 1.4 mm) together with a fluidised bed design was claimed to solve problems such as insufficient amino acid consumption leading to an unbalanced flavour profile. The cell growth was controlled by air and carbon dioxide feeds into the bioreactor, allowing growth of the yeast. Most of the improvements in beer quality were attributed to a better mass transfer (Šmogrovičová, 2008).

The beer produced in the 50-litre gas-lift bioreactor with air proportions between 2 and 5 % and with a residence time of 20 hours was judged by a taste panel to be acceptable, but not a perfect match to the traditionally produced control (Mensour, 1997).

Meura Delta system – combination of immobilized and free-cell stages

The company Meura Delta in Belgium used a sintered tubular silicon carbide matrix carrier and a loop reactor. The matrix was 900 mm long and 25 mm in diameter and had 19 channels, each 2.5 mm in diameter. The pore size of the matrix varied from 30 μm (near the surface) to 150 μm in the core of the material (Van De Winkel *et al.,* 1993).

For the main fermentation of lager beer, two similar bioreactors were used in a series. The first reactor was operated at an apparent attenuation of 40 %, and the final attenuation was reached in the second bioreactor. The residence time was 22 hours per stage. The productivity reported for one matrix at 15 °C was 6.6 hl beer per year. The beer quality was said to resemble that of conventional batch-fermented beer, although the amount of higher alcohols was somewhat lower and the amounts of esters were higher. This system has been adjusted for the production of top-fermented beer at semi-industrial scale (Andries *et al.,* 2000). The

second immobilized yeast loop bioreactor was replaced by a cylindrical conical tank with free cells. This tank was equipped with a circulation loop. Thus, the system was a combination of immobilized and free cell stages (*Fig. 4*). The advantages included an improved productivity and decreased investment cost compared with the totally immobilized system. The immobilized bioreactor supplied the second stage continuously with free, viable cells. The beer was similar to the one produced traditionally. Lastly, the Aubel brewery is using the Meura Delta system with 500 matrices for the production of top fermented beer (Mensour, 1997); there are also reports of at least one microbrewery in Canada utilising the same system. The loop reactor can also be used for production of alcohol-free beer and for maturation of lager beer.

VTT system for primary and secondary beer fermentation

VTT, Technical Research Centre of Finland has successfully been using immobilized yeast for maturation of beer since 1984 (Andries *et al.*, 2000). Their investigations led to the industrial application at Sinebrychoff's Helsinki brewery in 1990, and later at Sinebrychoff's Kerava brewery and at Hartwall Plc's Lahti brewery. In the Hartwall Lahti brewery, immobilized yeast has been used at the full production scale, i.e., 300,000 hl per year. The main fermentation was conventional. The yeast was then removed almost entirely by a separator to avoid off-flavour formation and technical problems in the subsequent heat treatment. Entry of air into the separator must be prevented completely. Oxidation during the heat treatment would lead to the formation of carbonyl compounds, thus giving the beer a stale flavour. A typical heat treatment is 10 minutes at 80–90 °C, the beer is then cooled to 10–15 °C, which is a suitable temperature for the continuous bioreactor. A residence time of 2 hours or even less is sufficient to reduce all diacetyl to acetoin (Grönqvist *et al.*, 1993). The carrier in one of these industrial reactors was DEAE-cellulose with addition of titanium oxide and polystyrene (Pajunen 1995), and porous glass was used in the other (Hyttinen *et al.*, 1995). The reactors are operated either by downflow or upflow. There are several other options for carriers to be used in the continuous secondary fermentation process, such as tubular silicon carbide units (Andries *et al.*, 2000) and wood chips (Kronlöf *et al.*, 2000). Because of technical difficulties, such as clogging of the reactors, DEAE-cellulose, which was successful for the secondary fermentation, was replaced by porous glass beads for the main fermentation. In the latter case, the flavour formation was also satisfactory and stable. The residence time in the packed reactors was 30 hours. A supply of oxygen can be used to

control ester formation. A feed mixture of air and nitrogen or carbon dioxide (2.5–5 % air) was fed to a pre-reactor at a rate of 0.1 gas volume per carrier volume per minute. This led to a good balance of flavour compounds, good yeast viability, and sufficient uptake of free amino nitrogen. Long-term experiments at the bench scale have confirmed that the system is stable and produces beer that is essentially identical to commercial beer produced from the same wort (Virkajärvi and Linko 1999, Virkajärvi *et al.*, 1999).

In 1997, VTT designed a primary two-stage fermentation system (packed-bed bioreactors) with the capacity of 600 litres per day (200,000 litres per year) at the Hartwall brewery. Later, the unit was extended to include a continuous secondary fermentation system with the same capacity. Wood chips were used as the carrier material, which reduced the total investment cost by one third compared with other, more expensive carriers. The results showed that, in only 40 h, the beer composition and flavour were very similar to those of the beer produced by the traditional batch process (Kronlöf *et al.*, 2000).

Sinebrychoff Brewery and Alfa Laval and Schott Engineering systems for secondary beer fermentation

Alfa Laval and Schott Engineering and Sinebrychoff Brewery in Finland developed systems for secondary beer fermentation with the capacity of 1 million hl per year. Both systems were composed of a heat treatment unit and a packed-bed reactor with yeast immobilized on DEAE-cellulose granules or porous glass beads, respectively. Later, the DEAE-cellulose carrier was replaced by cheaper wood chips. The heat treatment was replaced by an enzymatic transformation in a fixed bed reactor, in which the α-acetolactate decarboxylase was immobilized in special multilayer capsules, followed by the reduction of diacetyl by yeast in a second packed-bed reactor. During the fermentation process, the concentration of carbon dioxide was controlled in a fixed-bed reactor. This way, forced circulation of fermenting beer was established, channelling and carbon dioxide accumulation were avoided and mass and heat transfer were enhanced. The carbon dioxide formed was kept dissolved and removed from the beer without foaming problems (Nitzsche *et al.*, 2001; Virkajärvi, 2002).

Sapporo Breweries system for primary and secondary beer fermentation

Sapporo Breweries Ltd. in Japan have developed a fluidised-bed reactor with yeast immobilized in chitosan beads for both primary and secondary fermentation. The fermentation was carried out at 11 °C with a feed rate of 40 ml per hour using 11 °P wort. The fermentation system could be run for 900 hours without any damage to the beads or a decline in the fermentation efficiency. The wort was treated with glucoamylase to increase the glucose concentration, which led to increased acetate ester formation. The beer was similar in its flavour profile to conventionally produced beer. The process was scaled up to 80 litres in a repeated batch mode (Maeba, 2000).

Bavaria continuous alcohol-free beer production

Bavaria in the Netherlands developed a packed-bed DEAE-cellulose immobilized yeast bioreactor working at a low temperature, with a short residence time and production capacity of 150,000 hl alcohol-free beer per year (Van Dieren, 1995). The low temperature not only helped keep the viability of the yeast high over long time periods, but also restricted the yeast growth, which reduced the risk of clogging the reactor.

Case studies: wine making

The use of immobilized microbial cells in wine making has been reviewed in previous years (Kourkoutas *et al.*, 2004; Kourkoutas *et al.*, 2010; Nedović *et al.*, 2010; 2011, 2013). All research efforts attempt to apply immobilization technology to provide technical and economic advantages. In the major studies on immobilization of microbial cells for wine making so far, thetechnology has been shown to offer many advantages such as high cell density and high ethanol yield and volumetric productivity, reuse of biocatalysts in continuous operation bioreactor systems, avoidance of microbial contamination, physical and chemical protection of the cells and ability to perform low-temperature fermentation. Considering profitability together with consumer acceptance and safety issues, scientific evidence suggests that the choice of the support and bioreactor design for application at industrial scale is crucial.

Among the supports that have been the subject of research papers (discussed in detail earlier), certain food-grade natural materials seem to meet the prerequisites outlined previously and

result in overall improvement of the sensory characteristics of the final product by promoting aroma formation during the fermentation process. Examples of supports used are starchy materials such as barley (Kandylis *et al.*, 2012a), corn grains (Kandylis *et al.*, 2012b), wheat (Kandylis *et al.*, 2010a, b), corn starch gel (Kandylis *et al.*, 2008) and potatoes (Kandylis and Koutinas, 2008), gluten pellets (Bardi *et al.*, 1996a), fruit pieces (e.g. apple, quince, pear, papaya) (Kourkoutas *et al.*, 2010; Maragatham and Panneerselvam, 2011), delignified cellulosic materials (Koutinas *et al.*, 2012), grape pomace (Genisheva *et al.*, 2012), brewer's spent grains (Mallouchos *et al.*, 2007; Kopsahelis *et al.*, 2012), sugarcane pieces (Reddy *et al.*, 2011) and cork pieces (Tsakiris *et al.*, 2010). The advantages of immobilization technology in wine making become obvious through some of the most recent examples of research.

The use of barley and corn grains (Kandylis *et al.*, 2012a, b) as supports for yeast immobilization was found to be efficient during both ambient and low-temperature fermentation processes. These systems showed good operational stability during repeated batch fermentation of grape must even at extremely low temperature (5 ^{o}C). The fruity aroma of the product obtained with immobilized cells was attributed to the higher concentrations of ethyl acetate and other fruity esters-ethyl hexanoate, ethyl octanoate, ethyl dodecanoate, 2-phenylethyl acetate and ethyl-9 decenoate compared to the respective values in the product from the free-cell system. Also, a reduction of higher alcohols with decreasing temperature (from 25°C to 5°C) was more pronounced in the case of immobilized cells, improving the organoleptic quality of the respective product. The above results verify and extend previous research during the last 20 years dealing with the screening of a large number of natural materials. All findings indicate the combined positive impact of low temperature and immobilized cells on the fruity character of the final products due to the improved ratio of esters to alcohols. This has been partially attributed to the fact that immobilization induces changes in the expression levels of genes that encode key enzymes involved in acetate ester formation such as alcohol acetyltransferases encoded by the genes *ATF1*and *ATF2* (Shen *et al.*, 2003a). In addition, immobilization supports have been shown to assist in reducing dissolved CO_2 level in the fermentation medium by providing nucleation sites for CO_2 bubble formation. Under controlled dissolved CO_2 level, the uptake of branched-chain amino acids by yeast is enhanced, resulting in increased production of both higher alcohols and esters. The increase of esters as a consequence of surplus acetyl-CoA and higher alcohols via efficient metabolism of assimilable carbon and nitrogen at controlled CO_2 level should be

also pointed out. The milder effect of gas stripping on the reduction of volatile ester concentrations in the case of the immobilized-cell system may also explain the improved ratio of esters to alcohols in the derived products (Shen *et al.*, 2004).

During recent years, the use of osmotolerant *S. cerevisiae* strains entrapped in biocapsules with walls composed of mycelium of the fungus *Penicillium chrysogenum* (López de Lerma *et al.*, 2012; García-Martínez *et al.*, 2013) was found to be advantageous for sweet wine production via partial fermentation of raisin must, by overcoming the process limitation related to the growth and fermentation difficulties of yeast cells under osmotic stress. These studies, comparing the wines with those obtained with free yeasts and with the traditionally produced ones that avoid fermentation by adding wine alcohol, identified the following findings. The enhanced production of compounds related to the osmoregulatory system in the yeast (i.e. glycerol, acetaldehyde, acetoin, and butanediol) clearly differentiates wines produced traditionally and by fermentation (García-Martínez *et al.*, 2013). Also, the increase of volatiles with the greatest impact on wine aroma, ethyl hexanoate, ethyl octanoate, 4-butyrolactone, isoamyl alcohols, acetaldehyde, ethyl acetate, 2,3-butanediol and 2-phenylethanol- resulted in an increased complexity of wine aroma. The sensory profile of wines produced with immobilized yeasts was appreciated due to the secondary components, the ones typical of the grape variety and also the improved acidity–sweetness balance. The yeast biocapsules have been also proposed as low-cost, natural, and suitable biocatalyst for the production of sparkling wine with improved enological characteristics and lower calcium ion content, compared to that produced by yeast immobilized in Ca-alginate beads (Puig-Pujol *et al.*, 2013). Considering also technological limitations of the latter, such as the mechanical instability in high-capacity bioreactors (Kourkoutas *et al.*, 2010), the yeast biocapsules may be a good alternative for the application of immobilized cell technology in the industrial production of sparkling wines.

Gonzalez-Pombo *et al.*, (2011, 2013) contributed to the research on wine aroma enhancement by exploitation of immobilized enzymes for the controlled hydrolysis of glycosidic flavour precursors. The latter is expected to allow a rapid release of terpenes in young wines with a concomitant reservation of a portion of bound flavour-active compounds to be liberated with time. Specifically, treatment of Muscat white wine with glycosidases from *Issatchenkia terricola* (Gonzalez-Pombo *et al.*, 2011) or *Aspergillus niger* (Gonzalez-Pombo *et al.*, 2013) immobilized on epoxy-activated acrylic beads (Eupergit C) enhanced the release of

monoterpenes (α-terpineol, geraniol, linalool oxides) and norisoprenoids (vomifoliol and 3-oxo-α-ionol), with regard to the respective levels in untreated wine. The fruity and floral character of these products was positively received during sensory analysis. The above findings, along with the increased stability of the biocatalyst, offer many advantages for industrial application. Also, the fact that the product obtained with an immobilized biocatalyst is enzyme-free is expected to be more acceptable to customers.

Several studies promote the use of immobilized microbial cells for the biological de-acidification of wines to improve the organoleptic characteristics of products. For example, Genisheva *et al.,* (2013) evaluated the efficiency of immobilized lactic acid bacteria *O. oeni* on corn cobs, grape skins and grape stems for implementation of malolactic fermentation of white wine. In this study, the protection of immobilized cells against the inhibitory effect of ethanol and SO_2 was shown. In the case of corn cob biocatalyst, previous adaptation to the SO_2 during storage in wine at 25 ^{o}C for 27 d ensured complete malic acid degradation in a young wine with a high dose of free SO_2 (30 mg/l). In a similar fermentation experiment, the combined effect of SO_2 and storage negatively influenced the malic acid degradation ability of immobilized cells on grape skins and grape stems (75% and 83% conversion, respectively). All systems showed an operational stability of at least 5 months. For industrialization of the process, the same research group proposed a continuous winemaking process involving sequential alcoholic and malolactic fermentations with immobilized *S. cerevisiae* on grape stems or on grape skins, and *O. oeni* on grape skins, respectively, implemented in distinct packed bed reactors (Genisheva *et al.,* 2014a). The proposed integrated continuous process proved to be much more efficient than the batch processes conducted with free or immobilized cells. In the final products, isoamyl acetate together with the ethyl esters ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate were present in concentrations above their perception thresholds, resulting in wines with sweet and fruity flavours. *S. cerevisiae* cells immobilized on grape pomace resulted in a fast and efficient process, especially when large amounts of SO_2 were present in the must, while the wines obtained showed, in general, higher concentrations of ethanol, major volatile compounds (i.e. acetaldehyde, ethyl acetate, higher alcohols, etc.) and minor volatile compounds (i.e. isoamyl acetate, ethyl hexanoate, *E-3*-hexen-1-ol, 2-phenylethyl acetate *β*-damascenone, etc.) and a higher colour intensity compared with the wines produced with the free cells (Genisheva *et al.,* 2012).

In another study, co-immobilization of *S. cerevisiae* and *O. oeni* on wheat starch gel and tubular delignified cellulosic material (DCM), respectively, was evaluated for simultaneous alcoholic and malolactic wine fermentations (Servetas *et al.*, 2013). The biocatalyst was effective for simultaneous low temperature (10 °C) alcoholic and malolactic wine fermentation. The combined positive impact of low temperature and immobilized cells on the fruity character of the final products due to the improved ratio of esters to alcohols was more pronounced with the use of DCM/starch gel composite biocatalyst compared with DCM and starch gel biocatalysts separately.

Vilela *et al.,* (2013) used cells of the commercial strain *S. cerevisiae* S26 immobilized in double-layer alginate–chitosan beads for the deacidification of white wine with volatile acidity higher than 1.44 g/l acetic acid. The immobilized cells caused a rapid decrease of the volatile acidity (28 and 62% within 72 and 168 h, respectively) without affecting ethanol concentration considerably (a decrease of 0.7 % only). This technology was also found to be efficient for the reduction of acetic acid in matrices with high sugar content highlighting its prospective use for grape must deacidification to overcome stuck fermentations. Also, its potential application to reduce the volatile acidity of red wines was considered very attractive.

Considering industrialization issues, Kandylis *et al.,* (2010a) studied wheat-grain-supported biocatalyst efficiency for winemaking in a scale-up system of 80 l. According to the results, the fermentative ability of the biocatalyst was not negatively affected by the scale-up process, even at extremely low temperatures (2 °C), while the final products had an improved aromatic profile compared to free cells. The potential for using immobilized cell technology for commercial production of wine appears also in a recent work of Kopsahelis *et al.,* (2012), who pointed out the successful use of *S. cerevisiae* AXAZ-1 with both psychrophilic and thermotolerant behaviour immobilized on brewer's spent grains for the production of wine in a multi-stage fixed bed tower bioreactor at temperature ranging from 5°C to 40°C. This bioreactor is a modified packed bed bioreactor consisting of a vertical cylindrical tank with five packed sections containing immobilized cells. Due to the avoidance of high pressures that may result in support destruction, this bioreactor has been proposed for batch and continuous winemaking processes with high alcohol productivity and operational stability of the biocatalyst (Sipsas *et al.,* 2009). Kopsahelis *et al.,* (2012) reported increased ethanol yield and productivity at high temperatures using this support compared to a well-known

thermotolerant strain of *Kluyveromyces marxianus* for wine production and a positive influence on wine aroma. The latter was correlated with the presence of ethyl acetate, 3-methyl-butyl acetate, ethyl hexanoate, ethyl octanoate and 2-phenylethyl acetate, hexanoic, octanoic and decanoic acid (C_6–C_{10} acids at concentrations below 4.0 mg/l), and 2-phenylethanol, even at high fermentation temperatures. The biocatalyst showed also high operational stability, even in abrupt temperature changes. The latter is expected to create economical and technical advantages by eliminating the need for cooling/heating facilities.

The possibility of long storage of thermally dried immobilized yeast on delignified brewer's spent grains (Tsaousi *et al.*, 2010; 2011), freeze-dried wheat (Kandylis *et al.*, 2010b), gluten pellets and delignified cellulosic material (Kourkoutas *et al.*, 2010) without any loss of cell viability and fermentation activity and, most importantly, capable of producing wines with similar organoleptic characteristics to those of fresh cultures emphasises the commercial potential for industrial application.

Based on the findings presented above, there is evidence suggesting that immobilization of microbial cells using different methods and supports can improve cell metabolism even under extreme alcoholic fermentation conditions (i.e. low and high temperatures, high sugar content) and thus, the efficiency of the process and the quality of the final products. It is also obvious that both enzymes and whole cells immobilized on appropriate supports can be used to improve the organoleptic characteristics of young wines upon the completion of alcoholic fermentation via biological deacidification or controlled liberation of flavour-active compounds. The long-term storage of immobilized cells, as well as processes and bioreactor designs that can be readily scaled-up will promote industrialization of immobilized technology in wine-making.

Sensory quality

Most of the published results relating to aroma formation by free or immobilized cells in wine production have been based on chemical analyses such as gas chromatography. Chemical analyses of volatile compounds and their profiles give important information about the potential odour-active compounds. However, when an untrained consumer panel or a trained analytical sensory panel samples evaluates the samples, the contribution of volatile compounds is connected with actual sensory quality, e.g. aroma perceived with the sense of smell.

Some studies have compared the sensory quality of wine produced by free and immobilized cells using a trained panel. Based on the triangle sensory testing there is a significant difference in sensory properties between white wines produced by immobilized and free cells (Genisheva *et al.,* 2012, Tsakiris *et al.,* 2004, Mallios *et al.,* 2004). However, without descriptive analyses and sensory profiling of the wine samples, it is difficult to estimate the direction of the difference or, moreover, the contribution of difference with regard to the acceptability or pleasantness of wines. When sweet muscat wine was studied both in difference testing and descriptive analyses (Gonzalez-Pombo *et al.,* 2014), it was found that the higher concentration of terpenes increased the intensity of fruity and floral flavour. In their study, enzyme-treated wine was more fruity and floral than control wine. In another study (Kourkoutas *et al.,* 2004) semi-sweet wines had a stronger flavour and aroma compared to control wines. However, neither of these studies (Gonzalez-Pombo *et al.,* 2014, Kourkoutas *et al.,* 2004b) reported the influence of those sensory attributes on the pleasantness of wine. Consumers were asked to evaluate the pleasantness of red wine samples produced by immobilized cells (Tsakiris *et al.,* 2004). Although the scores for liking were slightly higher with wines produced by immobilized cells compared with free cells, the difference was not statistically different. They found that the temperature of the production process was also important. Wines produced at lower temperature were preferred by consumers.

In the future researchers should give more attention to actual sensory quality evaluated in a sensory laboratory with trained panel or consumers, together with instrumental analyses, when evaluating the quality of the final products of fermentation processes. This kind of knowledge supports development of acceptable products for consumers before they are placed on the market.

Case studies: cider fermentation

Cider is a fermented alcoholic beverage made from apple juice. The total production rate of cider in Europe in 2010 was about 14 million hectoliters (Association of the Cider and Fruit Wine Industry of the European Union). Apart from ethanol (1.2-8.5 % (v/v)) it contains many by-products of yeast and bacterial metabolism. Yeast strains are used in primary fermentation, while the later stage, malolactic fermentation, is performed by employing malolactic bacteria. Natural fermentation is still the main method of cider fermentation in many countries. In this kind of processing many types of yeast strains participate. Thus, in

1990, Cabranes *et al.,* identified as many as 560 yeast species in cider plants only in Asturias, northern Spain which has an annual cider production of about 80 million liters (data from 2010). Diverse yeast microflora and variable composition of apple must are the main reasons for many varieties in organoleptic profiles of cider. The most dominant yeast type used for the alcoholic fermentation of apple must is *Saccharomyces* sp. It provides more neutral sensorial feel in comparison to aroma of ciders produced with some other species. For example, the *Hanseniaspora* sp. strain gives "fruity" sensory notes to cider due to the presence of esters such as ethyl acetate and phenyl ethyl acetate (De Aruda Pietrowski *et al.,* 2012). The type of cider microorganisms is an important issue not only for formation of desirable bioflavours but also with respect to off-flavour defects, such as volatile phenols usually associated with "animal", "horsey", "leather", "phenolic" or "spicy" aromatic notes. Buron *et al.,* (2011) performed an extensive investigation for screening of representative cider yeasts and bacteria (47 yeast strains and 16 bacterial strains) for volatile phenol-production ability. Interestingly, when components were determined in ciders of the same remaining fructose concentration produced with twelve different yeast strains (70-day fermentations carried out at 8°C), the only significant effect of the yeast strain was on the amounts of glucose and ethanol in sweeter cider (fructose 34 g/l), or on the amounts of glucose, acetic add, isobutanol and amyl alcohols in dryer ciders (fructose 17 g/l) (Leguerinel *et al.,* 1989). The trends in food fermentation are focused on the isolation of proper wild-type strains from traditional products to be used as starter cultures, with the aim of conducting industrial production processes without losing their unique flavour and product characteristics. The use of starter cultures in cider fermentation might allow cider makers to produce a uniformly high quality product to be maintained during successive processes and seasons. However, as far as we know, starter cultures of lactic acid bacteria have not been yet industrially employed in Europe (as occurs in the wine industry) but only a few smaller companies in the USA and Canada put in practice this regime (Buglass, 2010b). Apart from spontaneous malolactic fermentation and the one brought about by addition of starter cultures, processes involving high cell concentrations have been described in the literature (Zhang and Lovitt, 2006).

Cider fermentation is performed at a temperature of 4-16 ºC. The process starts with a concentrated inoculum (about 10^6 yeasts per ml) and growth is virtually completed (at 10^7 yeasts per ml), on the third day of the process. When produced in the conventional way using free cell systems, the cider is ready to drink after a fermentation period of five weeks to three

months. During the prolonged fermentation a loss of vitality (energy-yielding capabilities) (Lloyd and Hayes, 1995) and viability (reproductive capacity) (Dinsdale *et al.,* 1999) occur due to increasing concentrations of ethanol and more toxic products (e.g. 2-phenylethanol, propan-1-ol, butan-2-ol and hexan-1-ol) (Willetts *et al.*, 1997). It is the synergism of certain compounds (e.g. ethanol, higher alkanol and aryl-alcohol) that induces membrane-associated lesions with deleterious effects in yeast rather than simple summary impact of individual effects (Seward *et al.,* 1996).

The reduction of acidity by bacteria inducing malolactic fermentation is recognized as a significant phase for cider production. This stage of processing is also important for stabilization of cider with respect to microbial spoilages through the bacteriostatic effect of the lactic acid produced. Besides, malolactic fermentation contributes to the flavour complexity of cider by producing compounds such as acetaldehyde, acetic acid, ethyl acetate, ethyl lactate, diacetyl, acetoin, and 2,3-butanediol. Actually, it is believed that about 160 components are present in cider, but many of them have not been identified yet. The major volatile compounds in ciders are alcohols, esters, fatty acids, carbonyls and acetals. Of these, ethanol, 1-butanol, 1-hexanol, 3-methylbutyl acetate, 2-phenylethyl acetate, butyl acetate, and hexanoic acid are typically dominant. Terpenes and phenolic derivatives have also been identified, but to a lesser extent. The specific bitterness and astringency of cider is associated with procyanidins (Lea *et al.,* 1974). The level of ethyl carbamate, which is considered as a contaminant ester (carcinogenic and mutagenic effects is confirmed in animals), is 55 ppb (Cairns *et al.*, 1987).

Malolactic fermentation is performed via lactic acid bacteria belonging to various genera and species but *O. oeni* is the predominant organism associated. The process is conducted at a temperature of 10-30 ºC. There are many factors, nutritional and physicochemical, that affect the growth and metabolism of lactic acid bacteria during malolactic fermentation. However, these factors are rather difficult to control. There are two ways to carry out the process; either malolactic fermentation proceeds after alcoholic fermentation reaches attenuation or both fermentations occur simultaneously. Temperature had a more important effect on the levels of certain volatile compounds when the simultaneous inoculation method was used. Thus, Herrero *et al.,* (2006) observed that when fermentation temperature increased from 15 to 22°C, using the simultaneous method, the final concentrations of ethyl acetate and some of the higher alcohols decreased, while others maintained similar levels. In the sequential

inoculation model, after completion of the alcoholic fermentation at 15°C, the same increase in the temperature of the malolactic fermentation showed no statistically significant differences in the profiles of the volatile compounds tested (ethyl acetate, 2-methyl-1-propanol, 1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol). Thus, malolactic fermentation could be conducted at 22°C, favouring malic acid degradation, without losses in the major volatile compounds, in relation to the levels measured at the lower temperature; at even more elevated temperatures (27 °C) excessive acetic acid was developed (Herrero *et al.,* 1999).

When performed separately, alcoholic and malolactic fermentations can be conducted in a bi-reactor system of which one fermentor vessel contains immobilized yeast and the other one immobilized bacteria (Simon *et al.,* 1996). Another way is to start the process with yeast for the first few days, followed by sequential addition of bacteria that subsequently co-immobilize with the yeast. Timing of bacteria addition is important since it may influence the organoleptic character of the final cider (Scott *et al.,* 1996). The approach based on co-immobilization of yeast and bacteria within the same porous matrix allows a complete fermentation of apple juice to cider in one integrated system. The time order of the two fermentations (simultaneous versus sequential) affects bioflavour formation. For example, the accumulation of ethyl acetate was stimulated by simultaneous inoculation of yeast and lactic acid (Cabranes *et al.,* 1998). The continuous processing coupled with microbial co-immobilization enables drastic reduction of fermentation duration compared to that of the traditional batch process, i.e. increases in the volumetric productivity of the bioreactor (Nedovic *et al*., 2000). In view of the volumetric productivity, the LentiKats (lens-shape PVA support) tubular bioreactor gave better performance than continuous reactor with *O. oeni* immobilized in alginate beads; the specific malic acid consumption increased by a factor of 4.6 due to the increase of the ratio of external surface to volume, allowing better mass transfer (Durieux *et al.,* 2000). A simple adjustment of residence time, by changing the flow rate of substrate through the fluidized bed bioreactor, enables better control of flavour formation and the production of either “soft” cider (high residual sugar concentration) or “dry” cider (without residual sugar) (Nedovic *et al.,* 2000). Immobilized cell technology offers the possibility of separating the malic acid bioconversion step from the cell propagation steps. Namely, hostile conditions in the cider (acidic pH of 3.5 or lower, presence of other inhibitors and ethanol up to 13% v/v) limit the specific growth rate of the starter culture. Alternatively, propagation of *O. oeni* can be accomplished in more convenient

conditions in a medium which allows a rapid growth rate in a separate bioreactor before encapsulation and then used as a biocatalyst in the adverse conditions presented in cider.

Unlike beer brewing and winemaking where immobilized cell technology has been quite well explored, cider production by immobilized biocatalysts has been the subject of few scientific publications; and none in recent years. Even the existing reports diverge considerably when discussing the sensory impact of immobilization on the flavour formation. Thus, 'finger print' analysis of the bioflavour (formed by capillary gas chromatography) revealed the same profile of components extracted from the cider regardless of their physical state (freely suspended or co-immobilized with natural precursors for bioflavour production within bilayer millimeter-size hydrogel beads) (Kogan and Freeman, 1994). However, Herrero *et al.,* (2001) determined that with alginate-immobilized *O. oeni* lower ethanoic acid content, lower ethyl ethanoate level and higher concentration of alcohols (propan-1-ol, 2-methylpropan-1-ol and butan-1-ol) were produced than with free cells under the same conditions. In contrast, when simultenous alcoholic and malolactic fermentation were conducted by *S. bayanus* and *L. oenos* co-immobilized in the same alginate matrix, the level of higher alcohols (propanol, isobutanol, isoamyl alcohol) was several timels lower compared with the batch process (Nedović *et al.,* 2000). In the same study, the concentrations of ethylacetate and ethylhexanoate were similar for both of the configurations, but the concentration of isoamylacetate was two times lower in the continuous process as a result of the isoamylalcohol availability.

The excesive formation of carbonyl compounds such as acetaldehyde, diacetyl, and 2,3-pentanedione is a frequent side-effect of yeast immobilization. The production of acetaldehyde is temperature-sensitive; thus, at a fermentation temperature of 12 °C it was about eight times higher than at 18 °C (Cabranes *et al.,* 1998). The most extensively studied carbonyl compound is diacetyl, the presence of which is considered essential for the correct flavour especially in cider. However, when produced in high amounts it can lead to off-flavouring. High diacetyl concentration is a result of the slow rate of decarboxylation of α-acetolactate to diacetyl (considered a rate limiting step in the traditional batch process) and the diffusion barrier in the immobilized reactor that prevents diffusion of diacetyl from medium to immobilized yeast cells (considered a classical drawback of the immobilized systems). In addition to the formation of vicinal diketones by yeast, some diacetyl present in

cider is produced by lactic acid bacteria from pyruvic acid directly by the activity of the diacetyl synthetase without any excretion of precursors in the fermenting medium.

The authors believe that full-scale immoblized cell technology of cider production could eventually give improved and controlled flavour profiles of this beverage. However, much work has to be done in collecting and understanding all the effects on bioflavour formation triggered by immobilization.

Case studies: fruit wine fermentation

During the last years, fruit wines have gained the interest of the consumers and the beverage industry worldwide since these products serve useful functions in the human diet as they increase satisfaction and improve digestion and absorption of food (Reddy *et al.*, 2012). Moreover, scientific findings support their health-promoting properties due to the presence of important nutrients and phytochemicals such as phenolic compounds, carotenoids, essential elements and vitamins (Duarte *et al.*, 2010; Mena *et al.*, 2012; Reddy *et al.*, 2012). Thus, the content of polyphenols is in the range of 335 to 1830 mgGAE/l for strawberry and black currant wines, respectively (Heinonen *et al.*, 1998). From an economical point of view, fruit wines can significantly contribute to the profitable utilization of fruit surplus as well as secondary quality and over-ripe fruits, thus reducing post-harvest losses. In European and Asian countries fruit winemaking involves the exploitation of raw materials available in each region. For example, India, the largest producer of fruits in the world, has already invested in exploitation of many tropical fruits (e.g. guava, banana, pineapple, pomegranate, mango, and melon) as raw materials for wine production (Reddy *et al.*, 2012).

Vinification of fruit closely resembles that of grape wine, the main differences being the pre-fermentative steps, especially the adjustment of fruit juice composition. Most fruits give juice with poor balance of sugars and acids. Since the efficiency of the fermentation process for fruit wine production depends on fruit composition, readjustment of juice components by the addition of adjuncts - sweetening materials such as sugar or syrup, acids, yeast nutrients, pectic enzymes, sodium or potassium metabisulphite - is considered critical to produce a balanced table wine (McKay *et al.*, 2011; Reddy *et al.*, 2012). Infusion techniques with water (hot or cold infusion), followed by the addition of adjuncts, is an alternative approach to overcome low yield of juice or unbalanced levels of sugars and acids (McKay *et al.*, 2011).

Considering the strong variation in fruit composition and the fact that yeast responds differently in various environments, it is important to select yeast strains for fermentation performance that will result in good quality wine. Yeast metabolic activity strongly influences the sensory profile of the final product and content of volatile compounds. In the investigation of Duarte *et al.*, (2010) among sixteen strains of *S. cerevisiae* and *S. bayanus* evaluated for their potential to ferment raspberry pulp, only three of them, namely CAT-1, *S. bayanus* CBS 1505 and UFLA FW 15, were pre-selected for their ability to produce beverages with particular sensory profiles. Of the above strains, UFLA FW 15 would be recommended as the most appropriate starter culture for raspberry wine since the beverage was characterised by pleasant odours correlated with the presence of ethyl butyrate (papaya, apple, fruity, and perfumed) (135.9 μg/l), 3-methylbutyl acetate (banana) (1927.0 μg/l), 3-mercapto-1-hexanol (passion fruit and grapefruit) (3.9 μg/l), α-ionol (lemon-sweet and violet) (74.7 μg/l) and β-ionone (flowery, violet-like; artificial raspberry; floral, perfume, raspberry) (43.7 μg/l). Yet, different metabolic activities of a specific yeast strain may be expected when fermenting the juice of different fruits. This statement is strengthened by the results obtained by Duarte *et al.*, (2010) concerning acetaldehyde content (9900 μg/l) in the raspberry wine produced with free cells of CAT-1 that strongly differ from the respective value reported by Oliveira *et al.*, (2011), who determined very low levels of this compound (1607 μg/l) in cagaita wines produced by the same strain in a free-cell system.

Apart from the *de novo* synthesis of flavour-active compounds produced during fermentation of the juice, many of the characteristic ones that contribute to the aroma of the beverage derive from the fruit type. This is more pronounced when no heat treatment (e.g. hot water infusion) takes place during the winemaking process. This is the case of (*Z*)-3-hexen-1-ol, α-ionol and β-ionone, indicative of the aroma of raspberries, in raspberry wine (Duarte *et al.*, 2010) and limonene, a flavour component of clementine (*Citrus reticula* Blanco) wine (Selli *et al.*, 2004).

Taking into account both improved fermentation efficiency and fruit wine quality and stability, the use of immobilized cells in the making of fruit wine could be of high interest. Despite the fact that there are only a limited number of reports on the application of immobilization technology in fruit wine production, the research highlights several advantages compared to free cell systems. Oliveira *et al.*, (2011) carried out a comparative study of the production of fruit wine from cagaita (*Eugenia dysenterica* DC) by two strains of

S. cerevisiae (UFLA CA11 and CAT-1), both in free and immobilized in Ca-alginate form. For both yeast strains, the immobilized cells retained high fermentation activity and exhibited much shorter fermentation duration and lower residual sugars than the respective values for free cell systems. Regarding flavour profile of the beverages the immobilization process had opposing effects in the two yeast strains UFLA CA11 and CAT-1. The immobilization of UFLA CA11 cells resulted in cagaita wine with lower contents of alcohols, ethyl esters, volatile fatty acids and aldehydes than in the wine produced by free UFLACA11 cells. This trend was reversed when CAT-1 cells were used, as higher levels of the above compounds were found in the cagaita wine produced by immobilized CAT-1 cells. These differences stress the fact that a lot of work is still needed in regulation and optimization of metabolic activities of immobilized yeast cells with regard to aroma formation in fruit wines.

The use of immobilized yeast in alginate gel beads was found to be advantageous for pomegranate and mango winemaking (Sritrakul *et al.*, 2007; Sevda and Rodrigues, 2011). In the first case, after establishing the optimum process parameters (concentrations of alginate, cell loading and bead diameter) for manufacturing the immobilized cells of *S. cerevisiae* NCIM 3095 on sodium alginate, improvement in fermentation performance (shorter fermentation time and higher sugar update rate) compared to that of free cells was observed. Also, improved flavour of the final product from immobilized cells was attributed to the restricted synthesis of volatile acids. This immobilized biocatalyst was characterized as an interesting tool to be applied in continuous processes and fluidized bed bioreactor systems for pomegranate wine production. In the experiments of Sritrakul *et al.,* (2007) a glass three-column packed-bed bioreactor was used for the production of mango wine by immobilized *S. cerevisiae* in Ca-alginate beads, in continuous operation mode (35% bead volume packed in the columns, dilution rate of 0.5 d^{-1}). According to the results, the system was stable for at least 60 days of operation, reaching an average ethanol concentration and ethanol productivity of 12.8 % (v/v) and 50.6 g/l/d, respectively. From all volatile compounds detected, acetaldehyde, diethyl succinate, ethyl acetate, ethyl butyrate, isoamyl alcohol, 1-hexanol, ethyl decanoate and caproic acid were found to contribute mostly to the aroma of the beverage.

The system of Reddy *et al.,* (2005), where watermelon-rind-immobilized yeast biocatalyst was used for the preparation of mango wine follows the trend of using natural supports for cell immobilization in grape wine making and in brewing (Kourkoutas *et al.,* 2004). Using

this system, cell viability and metabolism was not much affected and also the fermentation rate was increased. The produced wine had an overall improved quality with a fine fruity character as compared to the one produced from free yeast due to good balance between aroma compounds (methanol, ethyl acetate, propanol-1, isobutanol and amyl alcohols).

Another system for mango wine production, using immobilized yeast cells in natural materials, was evaluated by Varakumar *et al.,* (2012), where yeast-mango peel immobilized biocatalyst was used. This system showed good operational stability during repeated batch fermentation of mango juice even at low temperature (15 ^{o}C). The fruity aroma of the beverage obtained with immobilized cells was attributed to the presence of ethyl acetate at appropriate levels (< 30 mg/l), and a decrease of higher alcohols (< 330 mg/l) compared to the respective value in the beverage from free-cell fermentation. The reduction of the amyl alcohol content with the decrease in temperature (from 30°C to 15°C) was more pronounced in the case of fermentation batches with immobilized cells (from 262 to 147 and from 240 to 184 mg/l in the immobilized and free cell system, respectively). The increased glycerol concentration in the wines produced by immobilized yeast on mango peel could be attributed to the nature of the supports, immobilization, and yeast strain. In addition to the improved quality of the wines produced by the immobilized system the low cost, high accessibility and abundance, and food grade status of this biocatalyst makes it a possible material for the production of other fermented beverages (Varakumar *et al.*, 2012).

Apart from productivity and aroma profile, some components of important biological value also become affected by immobilization. Thus, Đorđević *et al.,* (2012) determined that, beside temperature, immobilization of yeast in alginate beads had direct influence on the total polyphenol content and antioxidative power of raspberry wine. Similarly, in the study of Kalušević *et al.,* (2012) the higher total phenol content of raspberry wine was achieved with immobilized yeast (1900 mgGAE/l) compared to that of wine produced in the free-cell system (1360 mgGAE/l), while the presence of selected yeast cells *versus* unselected yeast population had no significant influence.

It is obvious that basic research on the exploitation of immobilized yeast cells for the production of fruit wines is rather limited and non-systematic. However, approaches using immobilized yeast cells have proven to be very promising for application in the production of fruit wines as far as fermentation efficiency and the quality of the beverages are concerned. Systematic research is needed to direct efficient selection of yeast strains and support

materials so that industrial-scale production of fruit wines with improved and controlled aroma formation can be achieved.

Case studies: honey fermentation

Mead, also referred to as honey wine or honey beer, is a traditional alcoholic beverage containing from 9 to 18 % ethanol (v/v), produced by the fermentation of diluted honey with possible addition of spices, herbal extracts, fruit juices, etc. Mead fermentation takes a long time, often several months. The fermentation rate is dependent especially on honey variety, yeast strain, yeast nutrition and control of pH. The production of mead is usually performed by free yeast cells, usually of *Saccharomyces* genera, in a batch fermentation process following by maturation (Mendes-Ferreira *et al.,* 2010; Ramalhosa *et al.,* 2011; Šmogrovičová *et al.,* 2012), however, a few papers deal with mead production using immobilized yeast cells.

Qureshi and Tamhane (1986) produced mead by immobilized cells of *Hansenula anomala* in calcium alginate gels. Continuously operated column reactors enabled the quick production of matured mead by a single culture and the elimination of the traditionally used long aging periods. Navrátil *et al.,* (2001) showed that *S. cerevisiae* immobilized in calcium pectate gel optimally fermented honey mash to mead in continuous fermentation using a two-column system.

Šmogrovičová *et al.,* (2012) compared the aroma profile of Slovak and South African meads. The meads from Slovakia were produced using batch fermentation of acacia honey, cherry floral honey or honeydew forest apian honey, while the meads from South Africa were produced by continuous fermentation of wild natural plants of Eastern Cape apian honey using immobilized yeast. Therefore, it was a very interesting observation that, with the exception of one unidentified compound present in one of the South African meads, all main volatile aroma compounds were very similar in abundance in all the samples from both of the countries. Ethyl acetate represented the main component of all volatile compounds across all the samples tested, with a significantly higher concentration in the Slovak meads (from 46.36 to 60.03 mg/l) compared to the South African ones (16.35 and 16.97 mg/l). Higher alcohols were more prevalent in South African meads.

In the work of Pereira *et al.,* (2014), the potential for application of immobilized yeast cells on single-layer Ca-alginate or double-layer alginate–chitosan for mead production was assessed. The meads produced either with entrapped or free cells were evaluated in terms of quality and aroma profile. Although meads obtained with entrapped yeast cells presented less ethanol and glycerol and more acetic acid, they contained larger amounts of volatile compounds. Immobilized cells produced meads with more compounds with fruity characteristics, such as ethyl octanoate and ethyl hexanoate; however the concentrations of undesirable compounds in such meads were also higher. The effect of immobilization on the aroma profile was important, but the strain contribution was also of major importance. Thus, the sensory analysis of the final product gives an important insight on the overall quality.

Exploitation of agro-industrial residues

Agro-industrial wastes and by-products are generated in large amounts. Efforts directed at their valorization aim to develop environmentally and economically sustainable protocols and technologies, addressing at the same time the wellbeing requirements of modern society. This could be achieved through their conversion into functional and health-benefiting food ingredients (e.g. antioxidants, vitamins, flavours, amino acids, biopolymers) by means of biological, chemical, physical or tailored biotechnological processes. Through this type of approach it is envisaged that the ever-increasing demand for food with enhanced nutritional value and quality characteristics would be met while the production of processing residues would be minimized.

The utilization of non-conventional media as substrates in bioprocessing and their influence on aroma compound production has been studied by many researchers (Rodríguez Couto and Sanromán, 2006; Bicas *et al.,* 2010; Mussato *et al.,* 2012; Mantzouridou and Paraskevopolou, 2013). Many successful procedures have been developed using a great variety of agro-industrial residues (e.g., cassava bagasse, sugarcane bagasse, apple pomace, soybean, coffee husk, orange peel), in most of which the residue had a double action during the fermentation process, i.e.as physical support as well as source of nutrients. In this respect, coffee-derived wastes, enhanced or not with leucine, have been shown to produce strong pineapple and banana aromas by *Ceratocystis fimbriata* during fermentation (Pandey *et al.,* 2000a; Soares *et al.,* 2000). Sugarcane and cassava bagasse alone or in admixture with other residues, such as apple pomace, soybean, wheat bran and giant palm bran and supplemented or not with amino acids, have been found to constitute a valuable substrate for the *de novo* synthesis of fruity

aroma compounds (mainly esters and alcohols) by various microorganisms (*C. frimbriata, Rhizopus oryzae, K. marxianus*) (Bramorski *et al.*, 1998a, b; Christen *et al.*, 1997, 2000; Medeiros *et al.*, 2000; Pandey *et al.*, 2000b, c). Cereal or maize bran, sugar beet pulp and rice bran oil could also serve as a ferulic acid source, which is the precursor of vanillin, for the production of vanillin by biotransformation (Bicas *et al.*, 2010). More recently, the treatment of mixed solid and liquid food industry wastes, i.e. cheese whey, molasses, brewer's spent grains, malt spent rootlets, orange and potato pulp, using selected *S. cerevisiae* and *K. marxianus* strains and the natural mixed culture *kefir*, led to the production of a significant amount of the aroma compound ε-pinene (Aggelopoulos *et al.*, 2014). Additionally, Rossi *et al.*, (2009) found that citric pulp supplemented with soya bran, sugarcane molasses and mineral saline solution was an adequate substrate for aroma production (especially isoamyl acetate) by *C. fimbriata*, while sugar beet molasses fermented by *Williopsis saturnus* var. *saturnus* has also appeared to be an alternative way for the obtainment of production of natural banana flavour (Yilmaztekin *et al.*, 2008; 2009; 2013).

The ability of citrus wastes to produce aroma-active compounds by means of environmentally-friendly biotechnological processes has been also stressed by other researchers (Mantzouridou and Paraskevopoulou, 2013; Lalou *et al.*, 2013). Orange processing residue remaining after squeezing oranges for juice is considered an ideal substrate for microbial processes as a result of its favoured composition (rich in sugars, organic acids, proteins, polysaccharides, etc.). More specifically, orange peel was found to stimulate the *de novo* synthesis of six volatile esters with fruit aroma, i.e. isoamyl acetate, phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate, by using a commercial wine yeast strain (*S. cerevisiae*) under static fermentation conditions (Mantzouridou and Paraskevopoulou, 2013). The positive effect of orange peel on ester production was largely enhanced following conversion of polysaccharide-rich fractions (i.e. pectin and cellulose) into simple sugars upon acid hydrolysis of orange peel along with yeast cell immobilization in sodium alginate beads (Lalou *et al.*, 2013). This approach contributed greatly to the cells' resistance to substrate toxicity caused by D-limonene and other yeast hydrolysis by-products (such as carboxylic compounds, furans and phenolic compounds), which were expected to negatively affect bioprocess performance. The increased ability of volatile ester synthesis was accompanied by better growth performance of immobilized cells in comparison to freely suspended yeast cells suggesting increased survival of encapsulated yeast cells in the toxic hydrolysate. Besides, the unequivocal beneficial effect and the

economic feasibility of cell immobilization was further strengthened by the tendency of the bioflavour mixture to be accumulated within the alginate micro-beads as well as by the capability to perform repeated batch fermentations of hydrolysate after six consecutive cycles of a total period of 240 h (Figure 5) (Lalou *et al.*, 2013).

Conclusions

Currently available literature shows the potential of yeast cell immobilization as an important tool in the food sector for carrying out fermentation processes characterized by high cell density and volumetric productivity of target products, recycling of biocatalysts, continuous mode of reactor operation, reduced risk of microbial contamination, physical and chemical protection of the cells with consequent economical profits. However, the successful development and use of immobilized cells in microbial processes is not a straightforward process since a number of technological challenges exist. The major challenge for a successful application of immobilized cells technology at the industrial scale is the control and fine-tuning of the flavour profile. Microenvironmental changes around the immobilized cells may influence cellular responses with a concomitant impact on flavour formation. Careful selection of carrier material and immobilization method with consideration of safety, legality and stability, product quality and operating costs is vital. Among the production systems that have been the subject of research papers, certain ones seem to meet the above prerequisites and result in overall improvement of the sensory characteristics of the final products (e.g. beer, wine and cider) by promoting aroma formation during the fermentation process. Nevertheless, immobilized yeast fermentation processes at industrial scale are rather limited. For the promotion of industrial application of immobilized cell systems future research should focus on the long-term storage of immobilized cells, as well as on the development of processes and bioreactor designs that are simple and flexible, have low investment costs and can be readily scaled-up.

Acknowledgements

This work was supported by the COST Action FA0907 "Yeast flavour production – New biocatalysts and novel molecular mechanisms (BIOFLAVOUR)".

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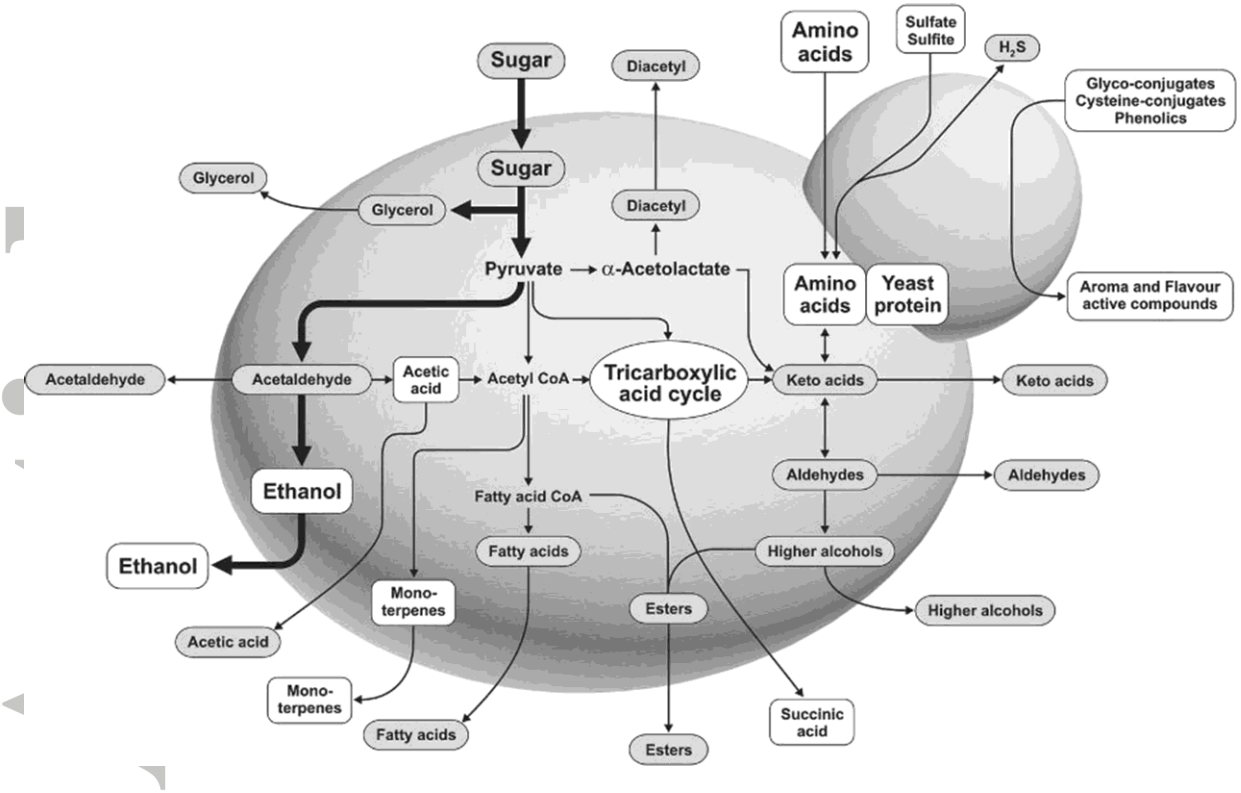

Figure 1. Formation of the major flavour groups during fermentation (with permission from Bartowsky and Pretorius, 2009)

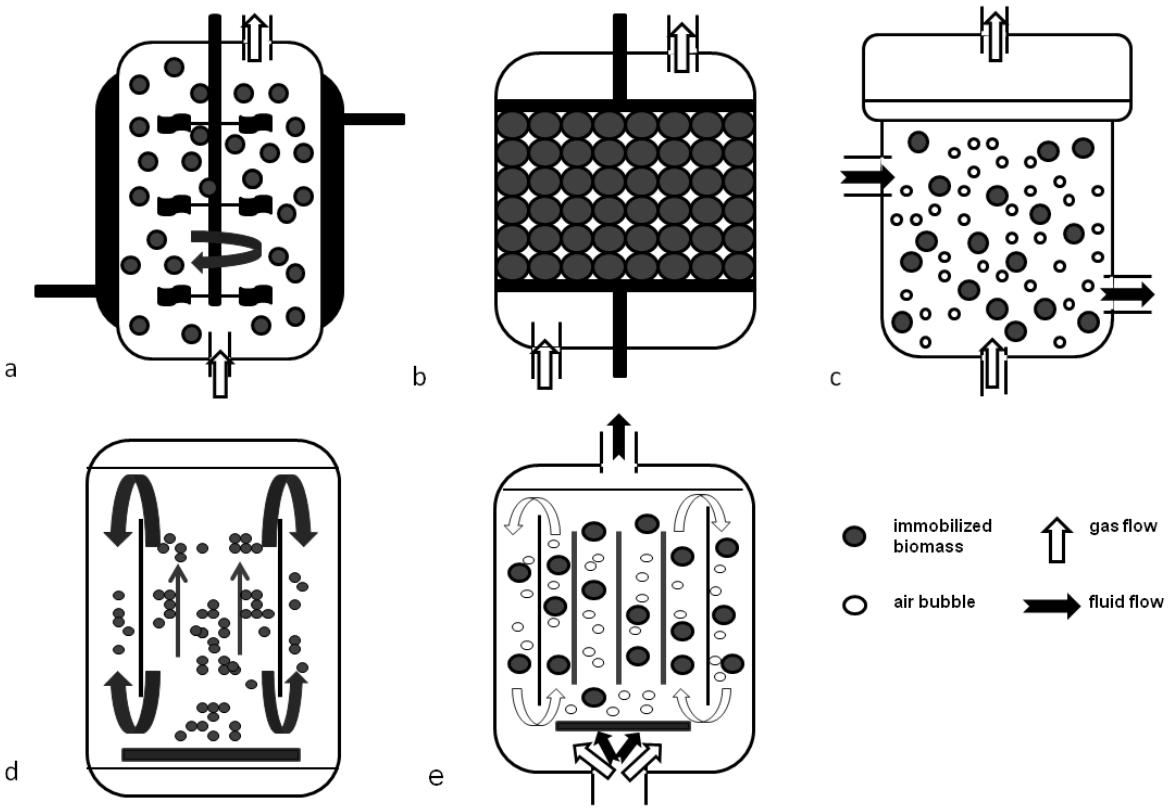

Figure. 2 Basic design of fermenters used in ICT: a) stirred tank fermenter; b) packed bed fermenter; c) fluidized bed fermenter; d) air-lift fermenter with internal loop; e) membrane fermenter.

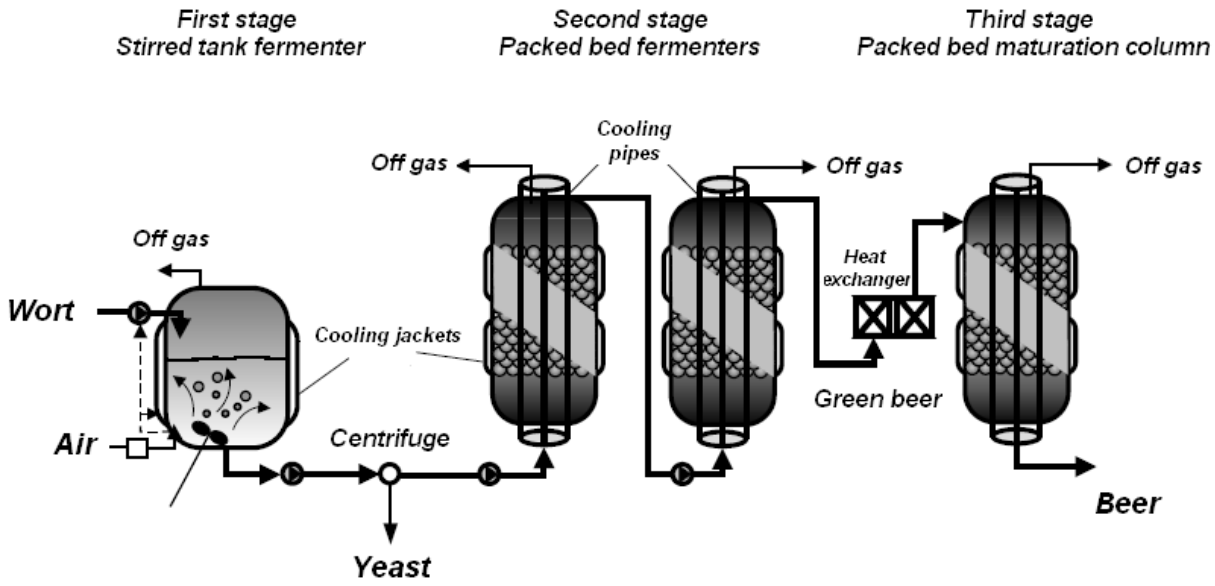

Figure. 3 Kirin's three stage fermenter system for continuous fermentation (adapted from Inoue, 1995).

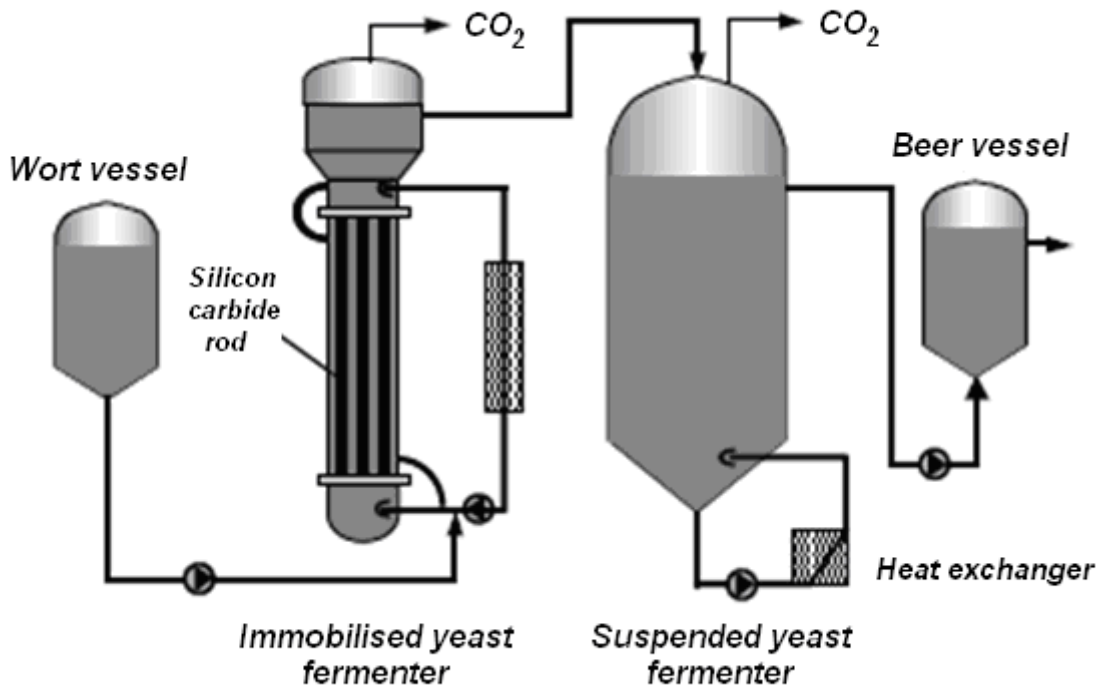

Figure. 4 Silicon carbide cartridge loop fermenter (adapted from Andries *et al*., 2000).

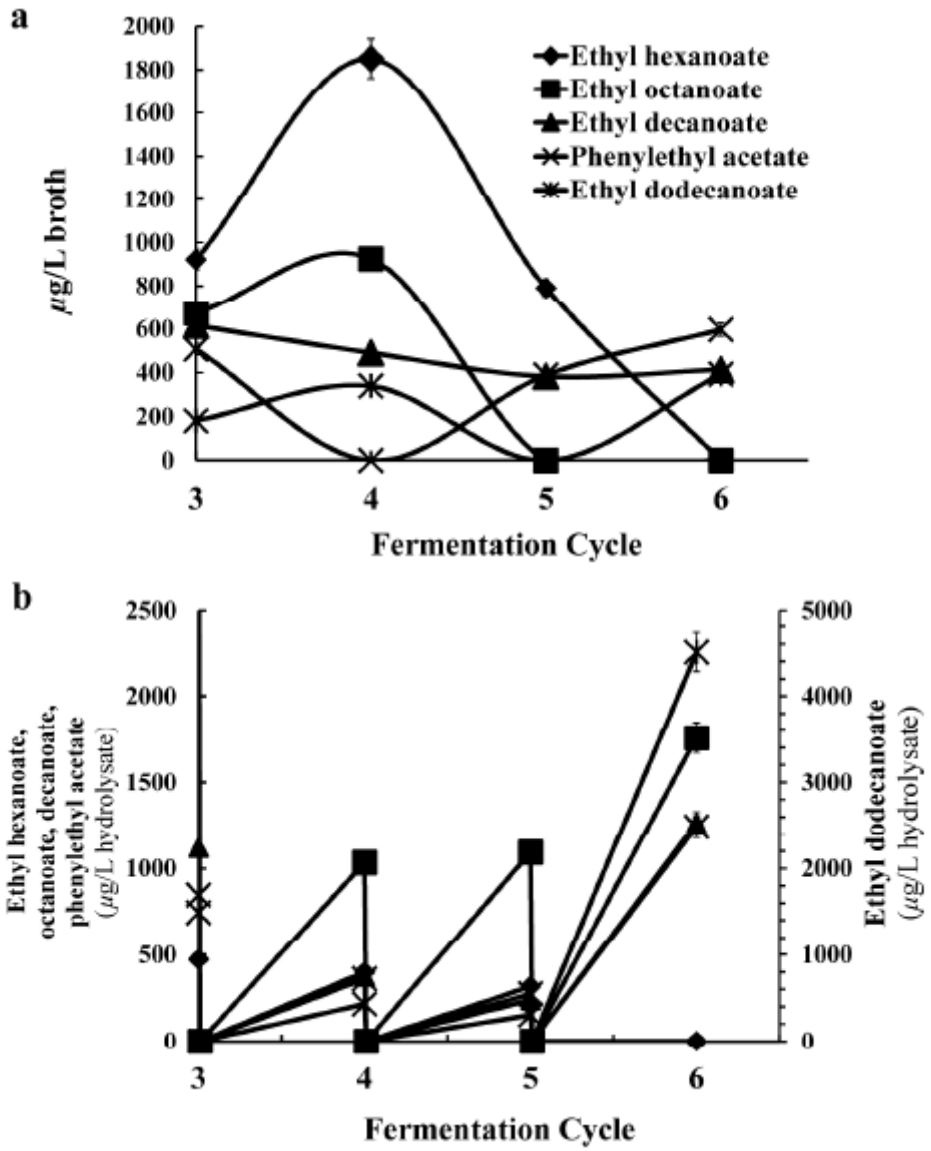

Figure. 5 Kinetics of volatile ester production (a) inside the beads and (b) in the liquid medium by S. cerevisiae immobilized cells in orange peel hydrolysate (with permission from Lalou *et al.*, 2013).

Table1. Requirements for yeast cell immobilization carrier materials for bioflavour production.

	Requirement
1	High cell loading capacity
2	Easy immobilization procedure under non-severe conditions
3	Mechanical and chemical stability
4	Accessibility of nutrients
5	Sterilization capability
6	Regeneration capability
7	Low cost
8	Easy scale-up
9	Suitable for conventional reactor systems
10	Desired flavour profile and control of off-flavour formation
11	Retention of immobilised cell viability
12	Maintenance of biological and metabolic activity of immobilised cells
13	Easy separation (of carrier and cells) from media
14	Controlled yeast growth and oxygenation
15	Non-toxic (approved for food applications)
16	Easy-handling