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Review Article

Immobilization technologies and support materials suitable in alcohol beverages production: a review

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Abstract

Various supports and immobilization techniques have been proposed and tested for application in wine-making, cider-making, brewing, distillates, potable alcohol and novel beverages production. Immobilization applications suitable for use by these alcohol-related industries are described together with an evaluation of their potential future impact, which is also highlighted and assessed. Topics in process engineering including immobilized cell bioreactor configurations and the scale-up potential of the various immobilization supports and techniques are also discussed.

Keywords: Immobilization; Biocatalysts; Wine-making; Cider-making; Brewing; Potable alcohol; Malolactic fermentation; Bioreactors; Distillates

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1. Introduction

An upsurge of interest in cell immobilization for alcoholic beverages and potable alcohol production has been taking place recently. This is mainly due to the numerous advantages that cell immobilization offers including enhanced fermentation productivity, feasibility of continuous processing, cell stability and lower costs of recovery and recycling and downstream processing (Margaritis and Merchant, 1984; Stewart and Russel, 1986). Cell immobilization may also protect cells against shear force. Industrial use of immobilized cells is still limited however further application will depend on the development of immobilization procedures that can be readily scaled-up.

The overall objective of this review, hence, is to analyse and assess data available in the literature on supports and techniques of viable cell immobilization for application in alcoholic beverages production.

1.1. Cell immobilization supports and techniques

Whole cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity" (Karel et al., 1985). Immobilization often mimics what occurs naturally when cells grow on surfaces or within natural structures. Many microorganisms own the ability to adhere to different kinds of surfaces in nature.

Numerous biotechnological processes are advantaged by immobilization techniques and therefore several such techniques and support materials have been proposed. These techniques can be divided into four major categories based on the physical mechanism employed (Fig. 1): (a) attachment or adsorption on solid carrier surfaces, (b) entrapment within a porous matrix, (c) self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced), and (d) cell containment behind barriers (Pilkington et al., 1998).

1.1.1. Immobilization on solid carrier surfaces

Cell immobilization on a solid carrier is carried out by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier. The thickness of cell film usually ranges from one layer of cells to 1 mm or more. Systems using immobilized cells on a surface are popular due to the relative ease of carrying out this type of immobilization. The strength with which the cells are bonded to the carrier as well as depth of the biofilm often varies and is not readily determined. As there are no barriers between the cells and the solution, cell detachment and relocation is possible with potential establishment of equilibrium between adsorbed and freely suspended cells. Examples of solid carriers used in this type of immobilization are cellulosic materials (DEAE-cellulose, wood, sawdust, delignified sawdust), inorganic materials (polygorskite, montmorilonite, hydromica, porous porcelain, porous glass), etc. Solid materials like glass or cellulose can also be treated with polycations, chitosan or other chemicals (pre-formed carriers) to enhance their adsorption ability (Norton and D'Amore, 1994; Navarro and Durand, 1977).

1.1.2. Entrapment within a porous matrix

In this type of immobilization, the cells are either allowed to penetrate into the porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed in situ into a culture of cells. Both entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into the surrounding medium, while still allowing mass transfer of nutrients and metabolites.

Characteristic examples of this type of immobilization are the entrapment into polysaccharide gels like alginates, κ-carrageenan, agar, chitosan and polygalacturonic acid or other polymeric matrixes like gelatin, collagen and polyvinyl alcohol (Norton and D'Amore, 1994; Park and Chang, 2000). Cell growth in the porous matrix depends on diffusion limitations imposed by the porosity of the material and later by the impact of

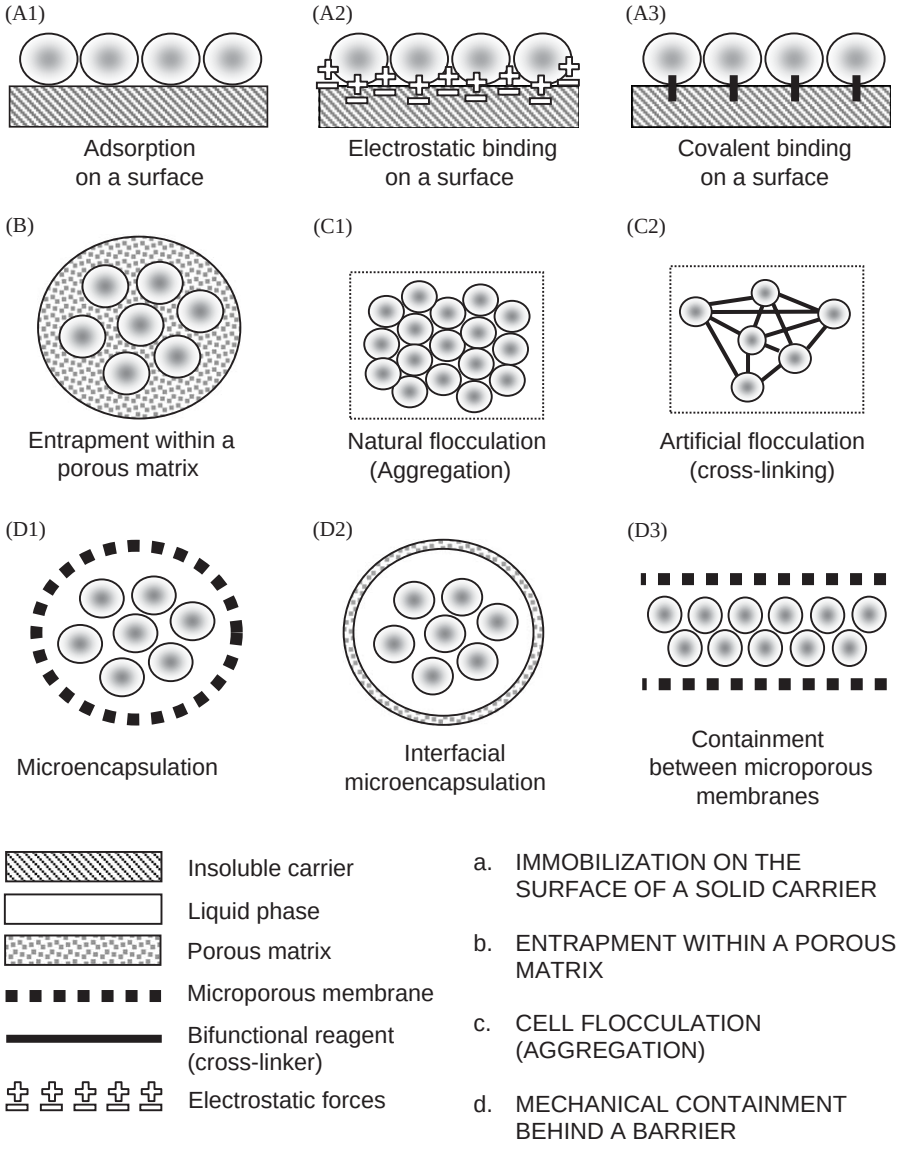

Fig. 1. Basic methods of cell immobilization.

accumulating biomass. Effective oxygen penetration range, for example, has been estimated to be 0.08–0.10 mm in carrageenan beads (Huang et al., 1990) and 0.1–0.15 mm in alginate beads (Ogbonna et al., 1991). A non-homogeneous cell population pattern may therefore develop and cells near the surface may behave differently compared to the partially starved cells inside the beads (Freeman and Lilly, 1998).

One of the problems of cell entrapment within a porous matrix such as polysaccharide gel is the ability of cells located on the outer surface of the beads to multiply and be released from the inclusion bead. This leads to a system comprising of immobilized and free cells. To avoid this problem double layer beads have been developed where the hydrogel beads with an internal core that contains the cells and an external layer, which prevents the cells from the core to escape (Tanaka et al., 1989; Taillandier et al., 1994; Ramon-Portugal et al., 2003).

The enumeration of biomass entrapped in a gel matrix is critical for application of biotechnological processes using viable immobilized cells. Such methods usually are gravimetric or include determination of proteins, DNA, NADH, which is expressed as biomass concentration. Navratil et al. (2000) proposed a luminometric method (ATP determination) as a reliable, accurate and rapid method for estimating active biomass of brewing, wine-making and ethanol-production yeast strains, immobilized in commonly used ionotropic hydrogels like alginates, calcium pectate and κ-carrageenan.

1.1.3. Cell flocculation (Aggregation)

Cell flocculation has been defined by many authors as an aggregation of cells to form a larger unit or the

property of cells in suspensions to adhere in clumps and sediment rapidly (Jin and Speers, 1998). Flocculation can be considered as an immobilization technique as the large size of the aggregates makes their potential use in reactors possible. Such reactors include packed-bed, fluidized-bed and continuous stirred-tank reactors. The ability to form aggregates is mainly observed in moulds, fungi and plant cells. Artificial flocculating agents or cross-linkers however can be used to enhance aggregation in cell cultures that do not naturally flocculate. Yeast flocculation is a property of major importance for the brewing industry as it affects fermentation productivity and beer quality in addition to yeast removal and recovery. It is affected by many factors including cell wall composition, pH, dissolved oxygen and medium composition. The importance of flocculation properties of *Saccharomyces cerevisiae* for alcoholic beverage production and mechanisms and factors affecting it has been reported by Jin and Speers (1998).

1.1.4. Mechanical containment behind a barrier

Containment of cells behind a barrier can be attained either by use of microporous membrane filters or by entrapment of cells in a microcapsule or by cell immobilization on to an interaction surface of two immiscible liquids. This type of immobilization is ideal when cell free product and minimum transfer of compounds are required (Park and Chang, 2000). Membrane bioreactor technology is widely used in cell recycling and continuous processes (Lebeau et al., 1997; Kargupta et al., 1998). Selected yeasts confined by micro-filtration membranes have been developed and are available for wine-making in the market. An example is the "Millispark" cartridge, which was developed by Millipore for secondary fermentation of sparkling wine into the bottle (Lemonnier and Duteurtre, 1989; Ramon-Portugal et al., 2003). The major disadvantages of cells immobilization between microporous membranes are mass transfer limitations (Lebeau et al., 1998) and possible membrane biofouling caused by cell growth (Gryta, 2002).

1.2. Prerequisites for cell immobilization

A carrier is suitable for cell immobilization for use in the production of alcoholic beverages, when the following prerequisites are satisfied (Freeman, 1984; Martin, 1991):

1. The carrier should have a big surface, with functional groups for cells to adhere to.
2. The carrier must be easy to handle and regenerate.
3. Cell viability and operational stability of the immobilized biocatalyst must be high and retained for longer times.
4. The biological activity of the immobilized cells should not be adversely affected by the immobilization process.
5. The porosity of the support should be uniform and controllable, allowing free exchange of substrates, products, cofactors and gases.
6. The carrier should retain good mechanical, chemical, thermal and biological stability and not be easily degraded by enzymes, solvents, pressure changes or shearing forces.
7. The carrier and immobilization technique should be easy, cost effective and amenable to scale-up.
8. The carrier must be of food grade purity, not affect product quality by remaining residues and readily accepted by consumers.

1.3. Effect of immobilization on microbial cells

Alterations in cell growth, physiology and metabolic activity may be induced by cell immobilization, of both yeast and bacteria species. Various reviews discussed the reasons for the altered metabolic behaviour of immobilized cells (Melzoch et al., 1994; Norton and D'Amore, 1994; Walsh and Malone, 1995).

It has been generally observed that it is difficult to predict the type and magnitude of metabolic changes possible through immobilization. A number of parameters have been considered responsible for these alterations, such as mass transfer limitations by diffusion (Webb et al., 1986), disturbances in the growth pattern (Doran and Bailey, 1986), surface tension and osmotic pressure effects (Vijayalakshmi et al., 1979), reduced water activity (Mattiasson et al., 1984), cell-to-cell communication (Shuler, 1985), changes in the cell morphology (Shirai et al., 1988), altered membrane permeability (Brodelius and Nilsson, 1983) and media components availability (Chen et al., 1990).

Comparative studies on immobilized and free cells reported effects on activation of yeast energetic metabolism, increase in storage polysaccharides, altered growth rates, increased substrate uptake and product yield, lower yield of fermentation by-products, higher intracellular pH values, increased tolerance against toxic and inhibitory compounds and increased invertase activity (Norton and D'Amore, 1994). Among these effects the following are of prominence:

1.3.1. Effects on growth and physiology

Melzoch et al. (1994) observed differences in the morphology and shape between free and immobilized on alginates *S. cerevisiae* cells, which were attributed to insufficient space for growth in the support. The immobilized cells showed increased viability and activity when stored at low temperature for long periods.

In the fermentation of wort by *S. cerevisiae* cells immobilized in calcium alginate gel beads, in

packed- and fluidized-bed bioreactor configurations, the cells exhibited altered growth behaviour compared to free cells, where the striking feature was the decrease of growth rate when the proportion of yeast increased into the gel beads (Ryder and Masschelein, 1985).

Adsorption of yeast to various solid surfaces has been reported to affect the intrinsic growth rate of the cells, which either increased (Bandyopadhyay and Ghose, 1982) or decreased (Doran and Bailey, 1986).

Jamai et al. (2001) reported that cells of *Candida tropicalis* and *S. cerevisiae* immobilized in Ca-alginate show insignificant morphological alterations although their metabolic activity was affected. Comparing their results to other works using different gel matrices, they suggested that it is the microenvironment inside the beads that affects physiology and metabolic behaviour and not the nature of the gel matrix.

1.3.2. Effects on metabolic activity

Buzas et al. (1989) observed that optimum pH for fermentation using free *S. cerevisiae* cells was 4.0, while fermentative activity for immobilized cells in alginates was independent of pH. Intracellular pH measurements in free and alginate immobilized *S. cerevisiae* cells were 6.9 and 6.8, respectively (Galazzo and Bailey, 1990). The reduced intracellular pH value in the immobilized cells resulted to an increased enzyme activity and therefore productivity. The reduced intracellular pH was attributed to increased permeability of cytoplasmatic membrane to protons, which led to higher consumption of ATP causing increased glucolytic activity and glucose uptake.

Adsorption of *S. carlsbergensis* onto porous glass beads increased the yield of ethanol on glucose and decreased the carbon dioxide yield (Navarro and Durand, 1977). Similar observations were reported with immobilized *S. cerevisiae* cells on ceramics (Demuyakor and Ohta, 1992). *S. cerevisiae* cells entrapped in gelatine were also found to contain higher concentrations of polysaccharides, DNA and RNA compared to free cells (Hilge-Rotmann and Rehm, 1990).

Aspergillus niger immobilized in Ca-alginate gel beads showed increased synthesis of antioxidant enzymes compared to free cells (Angelova et al., 2000). *Candida tropicalis* and *S. cerevisiae* cells immobilized in Ca-alginate demonstrated different mathematical patterns when the relationship of growth rate, respiration rate and ethanol tolerance were compared (Jamai et al., 2001).

1.3.3. Effects on stress tolerance

Norton and D'Amore (1994) discussed the increased ethanol tolerance of immobilized yeast cells and suggested that this phenomenon 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. Similar high tolerance was reported by Dale et al. (1994) for immobilized *Kluyveromyces marxianus* yeast cells. Several other reports highlighted increased productivity in immobilized thermotolerant *K. marxianus* IMB3 (Nolan et al. 1994; Barron et al., 1996; Brady et al., 1997, Love et al., 1998). 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 (Norton and D'Amore, 1994).

Finally, yeast and bacillus cells immobilized in various polymer matrices showed enhanced viability and thermal stability in freezing and freeze-drying

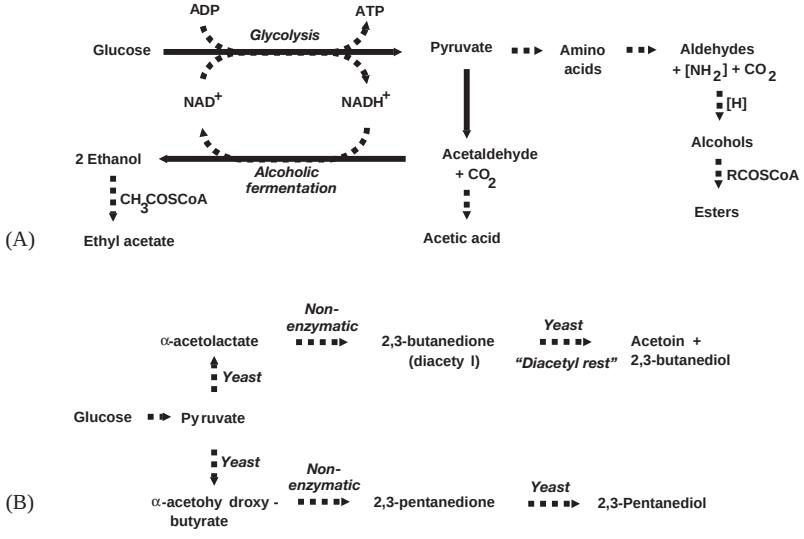

Fig. 2. Mechanisms showing pathways involved in (A) formation of the major flavour compounds during alcoholic fermentation and (B) formation and reduction of vicinal diketones.

conditions compared to free cells (Kearney et al., 1990; Lodato et al., 1999).

1.3.4. Effects on flavour formation

Yeast metabolic activity, especially amino acid metabolism, has a crucial contribution to flavour, because it is linked to the production of compounds such as vicinal diketones, esters, higher alcohols, aldehydes, fatty acids and sulphur compounds (Fig. 2A) (Russell and Stewart, 1992; Masschelein et al., 1994).

Increased ester and decreased fusel alcohol formation observed in immobilized cells fermentations, as well as the ratio of esters to alcohols have the greatest impact on beverage technology (Cop et al., 1989; Bardi and Koutinas, 1994; Mallouchos et al., 2003). The use of immobilization for the removal of diacetyl and therefore controlling flavour and reducing maturation time and production costs in brewing has been reported as a promising technological application (Linko et al., 1998; Smogrovicova and Domeny, 1999).

1.4. Advantages of immobilized cells over free cell systems

The use of immobilized systems for alcoholic beverages production offers many advantages over conventional free cells fermentations including:

1. Prolonged activity and stability of the biocatalyst. The immobilization support may act as a protective agent against physicochemical effects of pH, temperature, solvents or even heavy metals.
2. Higher cell densities per unit bioreactor volume, which leads to high volumetric productivity, shorter fermentation times and elimination of non-productive cell growth phases.
3. Increased substrate uptake and yield improvement.
4. Feasibility of continuous processing.
5. Increased tolerance to high substrate concentration and reduced end product inhibition.
6. Feasibility of low-temperature fermentation leading to improved product quality.
7. Easier product recovery through reduction of separation and filtration requirements, thus reducing cost for equipment and energy demands.
8. Regeneration and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.
9. Reduction of risk of microbial contamination due to high cell densities and fermentation activity.
10. Ability to use smaller bioreactors with simplified process designs and therefore lower capital costs.
11. Reduction of maturation times for some products.

The above advantages become obvious through some of the most recent examples of research on immobilization techniques and their application in the production of alcoholic beverages like wine, beer, distillates and potable alcohol production. The various supports used for cell immobilization are classified as organic, inorganic, natural supports and membrane systems. Natural supports are mainly of food grade purity and are used with minimum or no pre-treatment such as wood, sawdust, pieces of fruit etc. On the other hand organic materials are synthetically made (like plastic) or extracted from natural sources by more complex processes (like polymeric hydrogels) regardless of their food grade purity.

2. Immobilization supports and techniques applied to alcoholic beverage production

2.1. Immobilization for wine-making

Cell immobilization for wine-making is a rapidly expanding research area, although applications of this technology at industrial scale are limited. The purpose for using such technique is to improve alcohol productivity and overall product aroma, taste and quality. For successful industrial application of this technology the proposed supports must ideally be of food grade quality, abundant in nature and cost effective.

Many such supports for yeast immobilization in wine-making have been proposed (Colagrande et al., 1994; Divies et al., 1994). These supports are mostly natural organic polysaccharides or inorganic material abundant in nature. They may be used without much modification or after minor treatment to alter their properties (porosity, surface charges, etc.), others can be commercially synthesized. Examples of supports and techniques proposed in wine-making in the recent years include the following.

2.1.1. Inorganic supports for cell immobilization in wine-making

Mineral kissiris (a cheap, porous volcanic mineral found in Greece, which contains mainly 70% SiO_2) was used for yeast immobilization for batch and continuous low-temperature wine-making (Bakoyianis et al., 1992; 1993). The biocatalyst was found to be suitable for low-temperature fermentation, with productivity at 5°C equal to that at 22–25°C of traditional wine-making. The produced wines had an improved aroma with higher ethyl acetate content and lower higher alcohols on total volatiles. Argiriou et al. (1996) found that successive preservations at 0°C of cells of a strain of *S. cerevisiae* immobilized on mineral kissiris increased both ethanol productivity and the biocatalytic stability, which was retained for about 2.5 years, in both batch and continuous processes.

Loukatos et al. (2000) used porous γ-alumina as support for cell immobilization for continuous

wine-making. In the same study a method for removing Al from the produced wines was also proposed due to increased levels in the final product.

Many considered inorganic supports advantageous compared to organic supports because they are usually abundant in cheap and although they improved fermentation productivity and in most cases wine aroma, they were considered to be undesirable for wine-making due to high concentrations of mineral residues found in the product. Apart from safety, consumer acceptance is a main issue that should be taken into account before applying immobilization on such supports for beverage production.

Nevertheless, their use in cell immobilization can be considered as promising for use in distillates or potable alcohol production, since mineral residues do not distil. Ogbonna et al. (1989) tested immobilization of *S. cerevisiae* and *Schizosaccharomyces pombe* on glass pellets covered with a membrane of alginates for batch and continuous wine-making and produced wines similar to those produced by free cells. Many other inorganic supports such as polygorskite, montmorilonite, hydromica, porous porcelain, pumice stone and glass beads were studied for yeast immobilization, but had mainly little advantages in wine-making (Ageeva et al., 1985; Hamdy et al., 1990; Colagrande et al., 1994).

2.1.2. *Organic supports for cell immobilization in wine-making*

Organic materials usually proposed as supports of immobilization in beverage production are mainly polymeric materials, such as polysaccharides and are widely found in nature as constituents of cell walls of plants, crustaceans or insects, etc. The most commonly used polysaccharides for cell immobilization and wine-making, are alginates, cellulose, carrageenan, agar, pectic acid and chitosan. The most prevalent alginates are found in the cell wall of brown algae (*Phaeophycota*). They are heteropolysaccharides made up of mannuronic acid and guluronic acid units, of different ratios, depending on the source species. Sodium, calcium and barium salts of alginates have been extensively used for cell entrapment but calcium alginate gels are considered more suitable for alcoholic fermentation (Colagrande et al., 1994). Otsuka (1980) reported the use of cellulose covered with Ca-alginate as immobilization support for continuous wine-making. In order to immobilize yeast cells on DEAE-cellulose, researchers covered it with an anion-exchange resin and used it for wine-making (Lommi and Advenainen, 1990).

Ciani and Ferraro (1996) carried out fermentation tests using *Candida stellata* immobilized on Ca-alginate, alone or in combination with *S. cerevisiae* to enhance glycerol formation in wine. The immobilized cells exhibited about a 30- and a 2-fold improvement in fermentation rate (g of CO_2/day) compared to free cells of *C. stellata* and *S. cerevisiae*, respectively. These differences were attributed to higher biomass concentrations and elimination of competition between the two micro-organisms. The immobilized *C. stellata* cells produced a 2-fold increase in ethanol content and a strong reduction in acetaldehyde and acetoin production compared to free cells. The process using immobilized *C. stellata* cells was characterized as an interesting perspective to enhance glycerol content in wine. Ferraro et al. (2000) used immobilized *C. stellata* cells on Ca-alginate with an inoculum of *S. cerevisiae* cells at pilot scale and under real vinification conditions (non-sterile environment) in order to evaluate and control the must's wild microflora. The activity of the wild microflora was not completely repressed but the produced wines had an interesting flavour profile.

Suzzi et al. (1996) immobilized highly flocculent strains of *S. cerevisiae* in Ca-alginate beads to optimize primary must fermentation with cell-recycle batch process. Variability in formation of secondary products of fermentation was observed and the system was proposed for application in the wine industry.

Nevertheless, the use of alginates and polysaccharide hydrogels in general did not offer a good industrial choice because of their high cost and low chemical and mechanical stability that leads to cell and residues release in the wine. Most efforts were made for the application of alginate gels for secondary fermentation in the bottle in order to improve sparkling wine technology. For example, Busova et al. (1994), used four different immobilization techniques for *S. bayanus* cells, based on Ca-alginates, for sparkling wine production in the bottle for easy clarification and removal of cells. Similar techniques that imitate the traditional Champagne method have also been reported and are the only cases to have been commercially applied in wine-making processes (Fumi et al., 1988; Colagrande et al., 1994).

Finally, the investigation of the suitability of hydrogels for enzyme immobilization in wine-making has also been reported. Supports such as chitin, chitosan, and diethyl-amino-ethyl chitosan have been used for α-L-rhamno-pyranosidase immobilization. This enzyme is commonly used for increasing the aroma of wines, musts, fruit juices and beverages through the breakage of the glycosidic linkages of rhamnose with other compounds, including precursors of the aromatic components present in glycosidic form. DEAE-chitosan with attachment of the enzyme by a bifunctional agent (carbodiimide) was proposed as the most suitable for wine-making (Spagna et al., 2001).

Silva et al. (2002a) used whole cells of *S. cerevisiae* encapsulated in Ca-alginate gel for the treatment of sluggish and stuck fermentation in wine-making in several French and Portuguese wineries. The immobilized cells were used with success and had better results

than the traditional method that uses free cells. Under real vinification conditions they achieved a consumption rate of $2.8\,g\,L^{-1}$ per day of reducing sugar with a concentration of 5 million of viable cells mL^{-1} and with no increase in volatile acidity or off-flavour development.

2.1.3. Membrane systems for cell immobilization in wine-making

Applications of membrane technology in wine-making are limited. Takaya et al. (2002) studied the efficiency of two membrane bioreactor systems for continuous dry wine-making. Their first system was a single-vessel bioreactor in which cells where entrapped by a cross-flow type microfilter and the second configuration included two vessels; one operated as a continuous stirred tank reactor and the other was the membrane bioreactor. The single-vessel system was found unsuitable for dry wine-making due to high residual sugar concentrations, while the double-vessel was suitable and had 28 times higher productivity than a batch system.

2.1.4. Natural supports for cell immobilization in wine-making

The use of natural supports for cell immobilization such as delignified cellulosic materials (DCM) (Bardi and Koutinas, 1994) and gluten pellets (GP) (Bardi et al., 1996a, c, 1997) proved to be effective at both room and low-temperature wine-making. These supports showed a significant increase in fermentation rates compared to free cells. The produced wines had less higher-alcohol contents and increased ethyl acetate concentrations on total volatiles. Therefore, the produced wines had an improved organoleptic quality and a distinct fruity aroma (Mallouchos et al., 2003). They concluded that the combined effects of temperature and immobilization produced wines with a dominating fruity character compared to free cells, due to the better ratio of esters to alcohols and intermediate acidity.

DCM and GP are supports of food grade purity, very cheap, abundant and easy to prepare industrially. They can be easily accepted by consumers and compared to other natural supports like fruit pieces, they present longer operational stability. The possible commercialization of yeast cells immobilized on GP and DCM was evaluated through freeze-drying experiments (Iconomopoulou et al., 2000; Bekatorou et al., 2001a). No protecting medium was needed for freezing and freeze-drying of the immobilized biocatalysts (*S. cerevisiae* cells immobilized on DCM or GP), which retained viability and showed high productivity and stability for glucose fermentation. The freeze-dried biocatalysts produced wines of similar quality to those produced by fresh immobilized cells and of improved quality compared to free cells (Iconomopoulou et al., 2002, 2003). In general they had lower fermentation times and long operational stabilities and were suitable for low-temperature wine-making. The ability of the freeze-dried cells to be stored for long periods without loss of cell viability or fermentation ability makes them attractive for industrial use. Fruit pieces were also introduced as supports for yeast immobilization for wine-making due to ease in the immobilization techniques needed. Apple (Kourkoutas et al., 2001) and quince (Kourkoutas et al., 2003a) pieces were considered cheap, abundant supports of food grade purity of immobilization and led to a product with improved sensory characteristics. They were also found suitable for continuous processes (Kourkoutas et al., 2002a, 2003b).

Finally, Mallouchos et al. (2002) produced wine with *S. cerevisiae* cells immobilized on grape skins for obvious reasons of ease of application. They report increased productivity using this support compared to free cells and a positive influence on wine aroma. The support was suitable for wine-making and was proposed for further investigation for use in more integrated processes including main and secondary (malolactic) fermentation of wine. Table 1 presents the average values of the major volatile compounds in wines produced by free and immobilized cells on various supports and at various temperatures.

2.2. Immobilization in malolactic fermentation

Malolactic fermentation (MLF) is a secondary process that occurs in red wines, or wines with high acidities, during the maturation period. Although MLF is generally used for dry red wines, it can also be desirable for some dry white wines like Chardonnay, Sauvignon Blanc and Pinot Gris, but it is not recommended for sweeter wines, like Riesling, Gewürztraminer and Muscat.

During MLF L-malic acid is converted to L-lactic acid and carbon dioxide (Fig. 3) by lactic acid bacteria, predominately of the genera *Oenococcus*, *Lactobacillus* and *Pediococcus*. Most lactic acid bacteria convert malic acid to lactic acid with an intermediate formation of pyruvic acid, while *Oenococcus oeni* posseses the malolactic enzyme to directly convert malic acid in one step reaction (Lonvaud-Funel and Straaser de Saad, 1982; Salou et al., 1994). Yeasts like *Schizosaccharomyces pombe* and *Saccharomyces* strains can convert malic acid through maloethanolic fermentation (Fig. 3) (Redzepovic et al., 2003). Lactic acid as a mono-acid, is 'less acidic' than malic acid and as a consequence of this reaction, the total acidity of wine decreases (deacidification or demalication) leading to improvement in the organoleptic properties and biologic stability of the wines. In addition from several other components of wine, the production of by-products, mainly acetaldehyde, acetic acid, ethyl acetate, diacetyl and higher

Table 1
Ranges of the major volatile by-products in wine and beer produced by free and immobilized cells (average values obtained from various references in the literature for fermentations in the range 15–25°C for wine and 10–20°C for beer

Immobilization support	Ethyl acetate ($mg\,l^{-1}$)	Amyl alcohols ($mg\,l^{-1}$)	Acetaldehyde ($mg\,l^{-1}$)	Propanol-1 ($mg\,l^{-1}$)	Isobutyl alcohol ($mg\,l^{-1}$)	Methanol ($mg\,l^{-1}$)	Diacetyl ($mg\,l^{-1}$)
Wine (15–25°C)							
Free cells	50–80	130–160	20–25	35–50	20–35	75–90	—
Delignified cellulosic material	100–115	120–150	25–30	30–40	20–30	75–95	—
Gluten pellets	60–170	210–360	35–80	25–60	30–50	30–90	—
Apple pieces	50–120	180–300	5–40	5–20	15–30	10–90	—
Quince pieces	40–100	150–290	10–30	10–25	10–25	10–40	—
Mineral kissiris	80–140	120–150	15–30	30–50	20–30	75–110	—
γ-alumina	70–110	90–130	15–50	25–40	15–25	60–105	—
Alginates	90–130	120–140	5–20	30–40	20–50	65–100	—
Beer (10–20°C)							
Free cells	5–20	15–70	5–15	5–10	15–59	40–65	0.30–1.10
Delignified cellulosic material	10–40	35–80	5–20	10–25	5–20	20–50	0.20–0.40
Gluten pellets	25–85	100–180	5–40	20–40	30–50	10–35	0.20–0.05
Dried figs	50–85	60–130	10–20	15–40	15–30	60–85	0.30–0.50
DEAE-cellulose	15–20	90–100	3–5	5–10	50–60	NA	0.20
Ca-pectate	15–20	65–80	3–5	9–10	45–55	NA	0.10
κ-carrageenan	10–20	60–75	4–5	8–9	40–50	NA	0.09

NA = not available.

alcohols, is considered to affect wine flavour positively. After MLF the wine's flavour profile is characterized as being more smooth, round and complex. The improved stability of the product after MLF is due to the reduced possibility of microbial spoilage, by inhibition of microbial growth due to the formation of lactic acid and the consumption of reducing residual sugars (Nedovic et al., 2000; Lonvaud-Funel, 1995). Without controlled MLF, wine not adequately preserved with SO_2, might go through the process in the bottle by the wild bacterial microflora, which would transform residual sugars into lactic acid, acetic acid and other by-products, causing undesirable wine turbidity and development of off-flavours. MLF is sensitive to SO_2, ethanol concentration, residual glucose, lysosyme, biotin and thiamine contents, phenolic compounds, the presence of bacteriophages, low temperatures and low pH values (Vivas et al., 1997; Lonvaud-Funel, 1999; Gindreau and Lonvaud-Funel, 1999; Redzepovic et al., 2003). MLF is not always desired, for example in wines with very low acidities it may reduce further the acidity and affect both flavour and biological stability (Lonvaud-Funel, 1995; Versari et al., 1999). Suitable, genetically modified micro-organisms to conduct MLF have been proposed, but difficulty of expressing the malolactic gene in host cells and fully understanding the mechanism of the malolactic reaction was reported (Lonvaud-Funel, 1995). Engineered *S. cerevisiae* for example has been used successfully for efficient malate degradation, by cloning and expressing the *S. pombe* malate permease gene with either the *S. pombe* malic enzyme or the *L. lactis* malolactic gene (Volschenk et al., 1997, 2001). The use of immobilization technology, however is thought to be a safer alternative. Controlled MLF by the use of selected immobilized lactic acid bacteria is desired for the following reasons:

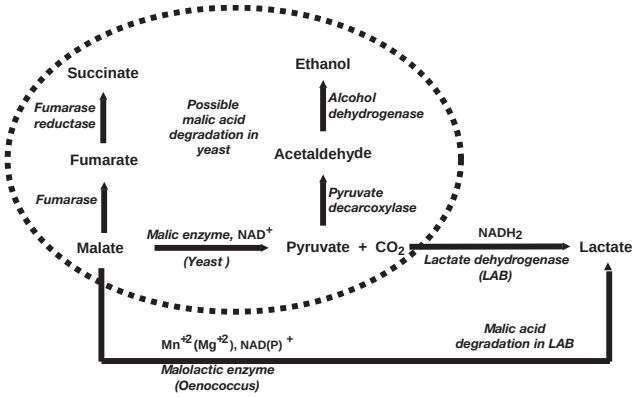

Fig. 3. Mechanisms showing pathways involved in malolactic fermentation by yeast and lactic acid bacteria.

1. Natural MLF takes a long time and growth limitations of lactic acid microflora affect and depend on the physicochemical properties and nutritional composition of wine, for example fatty acids and ethanol may inhibit lactic acid bacteria growth. Therefore, immobilization techniques aim to increase the tolerance of the MLF bacteria.
2. The development of desired flavour by using selected cultures of bacteria.
3. The acceleration of MLF by higher cell densities achieved by immobilization techniques.

4. The feasibility of application and commercialization of the process by lyophilized and immobilized cultures.
5. The reuse of cell for MLF and the application of continuous processes (Lonvaud-Funel, 1995; Kosseva et al., 1998; Maicas et al., 2001).

Attempts to use both immobilized bacteria and enzymes in order to convert malic acid started in the seventies. Almost all of the first efforts included immobilization of *Leuconostoc oenos* in alginate gels (Colagrande et al., 1994). At that point considerations on the effect on product quality were limiting factors for proposing the application of such techniques in wine-making at industrial scale. Later works investigated the use of polyacrylamide, κ-carrageenan, silica gel, pactate gels, chitosan and even oak chips as immobilization supports for various *Lactobacillus* sp. with satisfactory results (Kosseva et al., 1998; Maicas, 2001).

More recently the use of immobilized *O. oeni* (previously classified as *L. oenos*) was extensively studied for use in MLF of wine or cider, because it exhibits higher tolerance to inhibition effects of ethanol, low pH and SO_2 (Versari et al., 1999).

Iorio et al. (1985) carried out a kinetic analysis on the behaviour of malic enzyme under immobilization conditions using three different immobilization techniques including (1) within a polymeric membrane via cross-linking reaction, (2) within polyurethane foams, (3) within a gel-like membrane formed on the active side of capillary UF membranes. This was to define the best rector system suitable for practical applications. The last system was found most suitable.

As an alternative to MLF, the use of *Schizosaccharomyces* strains has been proposed for the deacidification of grape must, by the conversion of malic acid to ethanol. Taillandier et al. (1994) used *S. pombe* immobilized in double layer alginate beads, for the continuous deacidification of grape musts. The double layer method was used in order to eliminate cell release from the beads. A fluidized laboratory scale column bioreactor was employed, which was considered as more suitable for the process than fixed-bed systems. High productivities were obtained (1.5–2.4 $g\,L^{-1}\,h^{-1}$ of malic acid consumed) and the system was stable for 6 weeks. This method was proposed for large-scale deacidification of must.

The use of immobilized *L. casei* cells in Ca-pectate gel and chemically modified chitosan beads (commercial products) for MLF in Chardonnay wine was studied by Kosseva et al. (1998). Malic acid degradation rate by immobilized cells was twice that in free cells. Possible industrial application of the immobilized biocatalysts was advocated by the high operational stability of Ca-pectate gel and chemically modified chitosan beads, which was 6 and 2 months, respectively. The immobilized cells were also not inhibited by SO_2.

Maicas et al. (2001) assessed the possibility of employing *O.* cells immobilized on positively charged cellulose sponge for MLF of wine. The effects of surface charges in the immobilization material, pH and media composition used for cell suspension were reported. Chemical treatment responsible for the positive charge to the sponges, gave the highest cell loadings and subsequent resistance to removal. The use of a semi-continuous system permitted a high-efficiency malic acid conversion in at least four subsequent batch fermentations. The use of alternative technologies including immobilized cells to develop MLF in wine has been review by Maicas (2001). The various techniques, immobilization supports, and malolactic bacteria and scale-up efforts were discussed.

Understanding the nature and factors (environmental, nutritional) affecting MLF, which is a very complex process, is crucial for optimising wine technology. Also important is understanding and evaluating its effect on flavour formation. Therefore, suitable training as well as availability of pure commercial cultures, design of suitable bioreactors and optimization of MLF conditions are necessary in order to apply immobilization techniques in full scale.

2.3. Immobilization in brewing

Beer production requires fermentation times of 6–7 days and large-scale fermentation and storage capacities. Yeast cell immobilization technology can provide the brewing industry with a reduction in processing time without negatively affecting product quality, while high cell densities in the bioreactor can result in a faster fermentation and higher productivity leading to a significant reduction in cost.

Research in the last 30 years has focused on immobilization techniques in order to apply continuous processes in brewing and reduce maturation time, while many efforts have been made to produce alcohol-free beer. These advances have been reviewed by many authors (Ryder and Masschelein, 1985; Stewart and Russel, 1986; Russell and Stewart, 1992; Norton and D'Amore, 1994; Masschelein et al., 1994; Linko et al., 1998; Pilkington et al., 1998).

2.3.1. Alcohol free beer

In order to meet the increasing demand for alcohol-free beer over the last decade, several methods have been developed including alcohol removal from the product or limited fermentation of wort. In the case of limited fermentation, production is most efficient when immobilized cells are employed (Masschelein et al., 1994; Norton and D'Amore, 1994; Pilkington et al., 1998). Such systems have already been successfully applied.

Van Iersel et al. (1995) used a system for production of non-alcohol beer by limited fermentation with

immobilized *S. cerevisiae* in a packed-bed reactor. Limited metabolism was achieved by low temperatures and anaerobic conditions leading to ethanol contents lower than 0.08%, while these conditions stimulated the production of esters and alcohols. Flavour formation and cell physiology during alcohol-free beer production using immobilized *S. cerevisiae* on a granular material consisting of polystyrene coated with DEAE-cellulose was also investigated by Van Iersel et al. (1999). The production of alcohol-free beer was carried out by limited fermentation in a controlled down-flow packed-bed reactor system. Changes in immobilized yeast physiology were monitored and optimal flavour profile was achieved by introduction of regular aerobic periods to stimulate yeast growth. Temperature variations were used in order to control growth rate and flavour formation. The system was characterized as highly controllable, with reduction of off-flavours below threshold values, the activities of hexokinase and pyruvate decarboxylase of immobilized cells were higher and a higher glucose flux was observed with enhanced production of main fermentation products indicating the reduction in the flux of sugar for biomass production (Van Iersel et al., 2000).

2.3.2. Maturation and aroma control

Beer is produced through a number of complex biochemical (starch and protein enzymic degradation) and technological processes (malting, mashing, fermentation, maturation) which affect the flavour of the final product. Yet, the raw material and the production process do not contribute to the final flavour as much as the yeast metabolism does. Russell and Stewart (1992) reported that more than 600 flavour compounds present in beer are produced during the fermentation process. Carbon dioxide, ethanol and glycerol are the major compounds that control the overall effect of the other flavour constituents, which belong to the groups of higher alcohols, esters, vicinal diketones, aldehydes, sulphur compounds and fatty acids. Amino acid metabolism is a key to the formation of the above compounds and since it is affected by immobilization technology as mentioned above, this technology has become interesting for controlling or altering flavour leading to the production of beers with characteristic flavour profiles (Table 1).

Most of the flavour compounds are produced during the main fermentation of beer while only few but very important changes occur during the maturation (lagering) stage. One of the key compounds in beer maturation is diacetyl, a compound with an undesired butter flavour, which is formed from α-acetolactate by a slow non-enzymatic reaction (Fig. 2B). Diacetyl forms slowly at low temperatures, as is the lagering temperature (0°C). The oxidative non-enzymatic conversion of α-acetolactate to diacetyl and the conversion of diacetyl to acetoin through yeast metabolism are the main reactions occurring during beer maturation. These reactions are time-consuming and rate-limiting factors for the brewing process since they are occurring at very low temperatures in order to avoid obstruction of yeast metabolism and product quality (Yamauchi et al., 1995a). Traditional maturation lasts for about 1 month, requiring large storage capacities and energy, which makes it less cost effective. Rapid maturation of beer has been attempted by employing immobilization techniques in batch and continuous processes and has potential industrial application (Pajunen et al., 1989; Yamauchi et al., 1995a, b). Another advantage of immobilization was the lack of the need for filtration in beers produced since the concentration of free cells was very low Centenera et al. (1989).

2.3.3. Organic supports for cell immobilization in brewing

Several organic materials were used as immobilization supports for the production of beer by batch or continuous fermentation, such as polyethylene film or polyethylene rings, alginate gel beads, hollow PVA gel beads, diatoms, PVC and plastic. Alginate gels were the most extensively tested supports for brewer's yeast immobilization (Pardonova et al., 1982; Onaka et al., 1985). A two-stage reactor system was proposed for continuous secondary fermentation of wort in laboratory scale using immobilized yeast (Domeny et al., 1998). The first stage included an up-flow gas-lift bioreactor for main fermentation, and the second stage used column packed-beds reactors with yeast entrapped in three different polysaccharide hydrogels (Ca-alginate, Ca-pectate and κ-carrageenan). All three carriers were found suitable for continuous secondary fermentation of green beers produced by continuous main fermentation. In another study, it was found that beers produced by bottom-fermented *S. cerevisiae* yeast entrapped in Ca-pectate and κ-carrageenan contained lower amounts of diacetyl and higher alcohols at all studied temperatures from 5°C to 20°C (Smogrovicova and Domeny, 1999). The characteristics of beers produced by yeast adsorbed on DEAE-cellulose was similar to those produced by free cells. Diacetyl concentration in beers produced by immobilized yeast on calcium pectate and κ-carrageenan decreased as fermentation temperature increased. The opposite was observed in beers produced by immobilized on DEAE-cellulose and free cells.

Patkova et al. (2000) used Ca-alginate entrapped yeast and fermented successfully high gravity wort within 8 days, which was half the time needed for fermentation by free yeast. They also observed that when the original wort gravity was increased, the specific rate of ethanol production remained constant and the viability did not fall bellow 95% of living cells, confirming protection of cell against osmotic stress by gel.

Smogrovicova et al. (1998) studied the influence of immobilized yeasts on fermentation parameters and beer quality using a continuous gas-lift bioreactor system with *S. uvarum* entrapped in Ca-pectate beads. The higher the amount of biomass, the shorter the fermentation time. The produced beer had a suitable flavour, with low level of diacetyl, an optimum ratio of the higher alcohols to esters content and maximum specific rate of saccharide utilization.

2.3.4. Inorganic supports for cell immobilization in brewing

A few inorganic materials have been used as immobilization supports for brewing including porous ceramics, diatomaceous silica (kieselghur), porous bricks pieces and porous glass. Porous spherical glass beads were used as immobilization support of yeast in attempts to apply immobilized cells in continuous processes for rapid maturation of green beer (Yamauchi et al., 1995a, b). In similar experiments Tata et al. (1999) used porous glass beads in fluidized-bed reactors connected in series for continuous fermentation of high gravity worts. They also used a two-reactor system with silicon carbide cartridges with immobilized yeast inserted in the reactors for beer production. The fermentation times were reduced by half compared to the conventional batch processes, and the proposed technology was demonstrated as feasible for application in brewing if combined with a heat treatment system to reduce the relatively high diketone contents.

2.3.5. Natural supports for cell immobilization in brewing

As in the case of wine-making the biocatalysts prepared by immobilization of a cryotolerant strain of *S. cerevisiae* on DCM and GP were found suitable for batch and continuous fermentation of wort at low temperatures. The immobilized yeast showed important operational stability without a decrease of its activity, even at low temperatures (below 5°C). Batch fermentations at various temperatures were faster than those of free cells and those usual in commercial brewing, while beer produced from the immobilized yeast contained lower amounts of diacetyl and polyphenol as well as bitterness and pH compared to beer produced by free cells. The fruity aroma of beers obtained at low temperatures was attributed to an increase in ethyl acetate and a decrease in amyl alcohols contents (Bardi et al., 1996b). Higher alcohols were reduced at low temperatures, whereas beer flavour remained stable for the long period of 10 months storage. The use of immobilized biocatalysts were found to be advantageous for brewing and their possible commercialization in freeze-dried form was evaluated (Bekatorou et al. 2001b, 2002a). They retained their viability during a long fermentation period (13 and 14 months for DCM and GP supported biocatalyst, respectively). The produced beers were clear and had low concentrations of free cells and lower polyphenol and diacetyl contents compared to beers produced by free cells fermentations. Therefore, the long-term stable composition of the produced beer, the possibility to produce at low temperatures, the fine clarity and improved aroma of the green beer just after the end of main fermentation, the abundance and low cost of gluten and sawdust their food grade purity were considered as factors encouraging use in full-scale brewing process based on freeze-dried immobilized cells on DCM and GP.

Bekatorou et al. (2002b) immobilized strain AXAZ-1 *S. cerevisiae* on dried figs to test in brewing. Repeated fermentations of wort at low and room temperatures (3–20°C) resulted in significant reduction of the fermentation time. The produced green beers had a fine clarity, and were sweet, smooth with a special fruity fig-like aroma and taste clearly distinct from other commercial products including those produced by yeast immobilized on DCM and GP. This is type of immobilization was considered interesting for areas were figs or other fruits are abundant. The safety, low cost and consumer acceptance of these types of supports are unquestionable.

2.4. Immobilization in cider-making

Cider production is a complex process which combines two successive fermentations: (i) the alcoholic fermentation converting sugars to ethanol carried out by various yeasts, (ii) the malolactic fermentation converting L-malic acid to L-lactic acid, which occurs during maturation by lactic acid bacteria. Traditional cider is produced by mechanical pressing of apples for juice extraction followed by natural fermentation (alcoholic and malolactic) by the apple wild microflora. This technology leads to an unstable product of variable quality. Recent studies have been focusing on the use of selected starter cultures and novel technologies in order to improve cider quality. The major part of the research concerning the malolactic fermentation of cider and therefore immobilization techniques were considered to increase productivity, accelerate maturation and improve cider quality and stability. There are limited reports on using immobilized cells in cider-making. A sponge-like material was used to immobilize both *S. cerevisiae* and *L. plantarum* for carrying out fermentation and partial maturation of cider (Scott and O'Reilly, 1996). Fermentations carried out with immobilized yeast and sequential addition of lactic acid bacteria, achieved both enhanced rate of fermentation and flavour development. The sponge's open porous network promoted extensive attachment of the micro-organisms, and the material was suitable for yeast and bacteria immobilization and accelerated cider

production and maturation, which normally take 2–3 weeks and 8 weeks, respectively.

Another system for cider production was tested by Simon et al. (1996), consisting of an immobilized *S. bayanus* fixed bed bioreactor coupled to a second fixed immobilized *L. oenos* bed bioreactor. This system produced both dry and sweet ciders through control of dilution rate of the bioreactor and could also be used for producing other fruit wines.

Cider with negligible amounts of residual sugars and variations on the flavour profile was obtained with *S. cerevisiae* and *L. oenos* immobilized in alginate beads, depending on temperatures and the type of inoculation (Cabranes et al., 1998). In this study, malic acid metabolism and acetaldehyde production were affected by temperature, while ethyl acetate and methanol production were mainly influenced by the type of *L. oenos* inoculation.

Co-immobilization of *S. bayanus* and *L. oenos* in Ca-alginate gel was used to test simultaneous alcoholic and malolactic fermentation of apple juice for cider production in a continuous packed-bed reactor (Nedovic et al., 2000). Compared to the traditional cider-making they achieved rapid alcoholic and malolactic fermentation and improved flavour with a reduction of higher alcohols, isoamylacetate and diacetyl formation. These changes were attributed to altered metabolism of immobilized cells. Soft and dry cider was obtained by adjusting feeding flow rates. Likewise, cells of *O. oeni*, isolated from the cellar of a cider industry, were immobilized in alginate beads and used as starter culture for malolactic fermentation of cider (Herrero et al., 2001). The immobilized system had higher alcohol productivity and the rates of malic acid consumption were similar to those obtained using free cells while no significant differences of volatile by-products were observed. The immobilized cells produced lower acetic acid and ethyl acetate.

The use of immobilized malolactic bacteria for secondary fermentation of cider or co-immobilized bacteria and yeasts for both main and secondary fermentation, has proven in all cases to be efficient as far as fermentation rates are concerned, however further research for application to full-scale production of ciders with improved and controlled flavour profiles is needed.

2.5. Immobilization in potable alcohol and distillates production

The requirement for ethanol as an additive in the beverage industries has been steadily increasing and so is the pursuit of immobilized microbial cell systems for ethanol fermentation. Research on potable alcohol production usually focuses on volatile by-products formation, since these constituents are critical parameters for distillates and alcoholic beverages quality. The nature of the immobilization carrier does not affect ethanol composition as in the case of wine-making or brewing, since non-volatile constituents are not distilled. The requirement for food grade purity is not essential due to the employment of a distillation step. Immobilized yeasts strains such as *S. cerevisiae*, *S. diastaticus*, *K. marxianus* and *Candida* sp., and bacteria like *Zymomonas mobilis* have all been used for ethanol production (Table 2).

3. Bioreactor configurations and scale-up efforts involving immobilization

Bioreactor design is one the most important factors in applying immobilization technologies in alcoholic beverage production. The majority of efforts and applications in industrial or semi-industrial scale concern beer and ethanol (fuel or potable) production. Limited are the references for scaling-up efforts for wine-making, probably due to the strongly traditional character of the product. Nevertheless, various bioreactor configurations, at lab-scale, employing immobilized cells in batch or continuous processes have been proposed for wine, beer, cider and ethanol production. Many of these configurations have been characterized as promising for scale up and industrial application. Table 2 lists some of the most important reports in alcoholic beverage production in the past 15 years and gives some information on the bioreactor configurations used in each case.

3.1. Bioreactors and scale-up efforts in brewing

Scale-up efforts based on immobilized cells for brewing have been reported by many authors (Baker and Kirsop, 1973; Masschelein et al., 1994; Shindo et al., 1994; Norton and D'Amore, 1994; Yamauchi et al., 1995a, b; Andries et al., 1996; Mensour et al., 1997) and reviewed by Virkajarvi (2001). The first attempts to apply immobilization techniques in the brewing industry in the early 1970s used packed-bed configurations with brewer's yeast mixed with diatomaceous earth (kieselguhr) forming porous biomass beds. This was soon abandoned due to many technical disadvantages, need for addition of viable yeast cells, low operational stability, decreased foam and high vicinal diketones content in the product. These disadvantages led to the concept of using immobilized cell bioreactors for secondary fermentation (lagering or maturation). Therefore, in full industrial scale the use of immobilized cells mainly developed for secondary fermentation and for alcohol-free beer (Van Iersel et al., 1995, 2000), while only small-scale installations were reported for the main fermentation of beer (Mensour et al., 1997).

Table 2
Summary of the main immobilization supports and techniques proposed for alcoholic beverages production

Micro-organism	Immobilization support/ technique	Type of fermentation	Process/bioreactor	Substrate/product	Reference
S. cerevisiae+*S. cerevisiae f.r. bayanus*	Alginate beads	Secondary AF	10^9 immob. cells l^{-1} wine; 12–14°C	Wine/sparkling wine	Fumi et al., 1987
S. cerevisiae	Mineral kissiris	AF	Batch-stationary; 300 ml, 30°C	Glucose; raisin extracts/ ethanol	Kana et al., 1989a
S. cerevisiae	γ-alumina pallets	AF	Batch-stationary; 110 ml, 30°C	Glucose; raisin extracts/ ethanol	Kana et al., 1989b
S. pombe	Double-layer alginate beads	MLF	FBR; continuous; 580 ml; 25°C	Grape must/de-acidified grape must	Taillandier et al., 1994
S. cerevisiae	Microfiltration membranes	Secondary AF	Millispark cartridge; ''in the bottle''	Wine/sparkling wine	Lemonnier and Duteurtre, 1989; Ramon-Portugal et al., 2003
S. cerevisiae	Delignified cellulosic material	AF	Batch-stationary; PBR; 500 ml; 0–30°C	Glucose; grape must/ ethanol; wine	Bardi and Koutinas, 1994
S. cerevisiae	Mineral kissiris	AF	Two PBRs; continuous; 1500 ml; 5–16°C	Grape must/wine	Bakoyianis et al., 1992
S. cerevisiae	Mineral kissiris	AF	Industrial-scale pilot-plant; multistage fixed-bed tower reactor; 7,000L-100,000L; 30°C	Molasses/ethanol	Bakoyianis and Koutinas, 1996; Koutinas et al., 1997
S. cerevisiae	Porous, spherical glass beads	Secondary AF	Pilot-scale; up-flow tubular PBR; 500 L; 0–60°C	Green beer/mature beer	Yamauchi et al., 1995a, b
S. cerevisiae	Wood blocks	AF	Vertical PBR; continuous; 100 ml; 33°C	Glucose-fructose mixtures/ ethanol; fructose enr. syrup	Guenette and Duvnjak, 1996
S. cerevisiae+*L. plantarum*	Sponge-like, neutral, acidic and basic cross-linked cellulose	Post-primary AF; MLF	Up-flow, PBR; continuous; 2000 ml; 20°C	Fresh cider/mature cider	Scott and O'Reilly, 1996
S. cerevisiae	Delignified cellulosic material	AF	Batch-stationary: 400 ml; continuous: 2100 ml; 0–30°C	Wort/beer	Bardi et al., 1996a
S. cerevisiae	Gluten	AF	Batch-stationary: 400 ml; continuous:, 2170 ml; 0–30°C	Wort/beer	Bardi et al., 1997
S. cerevisiae+*Candida brassicae*	*Luffa cylindrica* sponge; chitosan	AF	PBR; continuous; 1500 ml; 30°C	Glucose/ethanol	Ogbonna et al., 1997
S. cerevisiae+*Candida shehatae*	Agar layer; microporous membrane filters	AF	Two-chambered reactor; batch; symmetrical and asymmetrical aeration; 30°C	Glucose; xylose/ethanol	Lebeau et al., 1997
L. casei	Calcium pectate; modified chitosan	MLF	Batch-shaken flasks; 100–170 ml; 20–36°C	Wine/wine	Kosseva et al., 1998
S. cerevisiae	Calcium pectate; κ-carrageenan; DEAE-cellulose	AF	Batch-stationary: 320–400 ml; 5–20°C; Continuous: up-flow, GLR; 482 ml; 15°C	Wort/beer	Smogrovicova and Domeny, 1998, 1999

S. cerevisiae	Stainless steel wire spheres; untreated and modified	AF	Batch-stationary; 75 ml; 30°C	Sucrose/ethanol	Bekers et al., 1999
S. cerevisiae	Pre-polymeric matrix (epoxy resin + diamino-polyethylene oxide)	AF	PBR; continuous; 200 ml; effect of pH and temperature	Glucose/ethanol	Jirku, 1999
S. bayanus+L. oenos	Alginate gel	AF; MLF	Up-flow, PBR; continuous; 2500 ml; 30°C	Apple juice/cider	Nedovic et al., 2000
S. pastorianus	Porous cellulose carriers; modified and non-modified	AF	Batch-shaken flasks; 150 ml, 30°C	Glucose/ethanol	Sakurai et al., 2000
C. stellata	Alginate beads	AF	Pilot-scale reactor; batch; 100 l; 20°C	Grape must/wine	Ferraro et al., 2000
O. oeni	Alginate beads	MLF	Batch-shaken flasks; 100 ml; 22°C	Cider/cider	Herrero et al., 2001
S. cerevisiae	Gluten pellets	AF	PBR; batch; 400 ml; 0–15°C	Glucose; wort/ethanol; beer	Bekatorou et al., 2001a, b
Z. mobilis	Stainless steel wire spheres; Al_2O_3 granules	AF	Batch-stationary; 70 ml; 30°C	Sucrose/levan; ethanol	Bekers et al., 2001
S. cerevisiae	Grape skins	AF	Batch-stationary; 800 ml; 5–25°C	Grape must/wine	Mallouchos et al., 2002
S. cerevisiae	Apple pieces	AF	PBR; batch: 300 ml, 1–25°C; continuous: 720 ml, 5–30°C	Grape must/wine	Kourkoutas et al., 2001, 2002a
S. cerevisiae	Delignified cellulosic material	AF	PBR; batch; 400 ml; 0–15°C	Wort/beer	Bekatorou et al., 2002a
S. cerevisiae	Dried figs	AF	PBR; batch; 600–1000 ml; 3–30°C	Glucose; wort/ethanol; beer	Bekatorou et al., 2002b
S. cerevisiae	Cross-flow microfilters	Cell growth; AF	Single/double vessel MBs; 500 ml; 25°C	Glucose; grape must/ biomass; wine	Takaya et al., 2002
K. marxianus	Delignified cellulosic material	High-temp. AF	PBR; batch-stationary; 250 ml; 37–50°C	Lactose; whey/ethanol	Kourkoutas et al., 2002b
S. cerevisiae	Gluten pellets	Low-temp. AF	PBR; batch; 300 ml; 5–30°C	Grape must/wine	Iconomopoulou et al., 2002
S. cerevisiae	Ca-alginate	Stuck AF	Fermentation tanks; commercial scale	Wine	Silva et al., 2002a
S. cerevisiae	Ca-alginate	Secondary AF	"In the bottle"	Wine/sparkling wine	Silva et al., 2002b, 2003
S. cerevisiae	Quince pieces	Low-temp. AF	PBR; continuous; 700 ml; 5–30°C	Grape must/wine	Kourkoutas et al., 2003b
S. cerevisiae	Acrylamide-sodium acrylate copolymer hydrogels	AF	Batch-rotary shaking; 30°C	Glucose; molasses/ethanol	Oztop et al., 2003

PBR = Packed Bed Reactor; FBL = Fluidized Bed Reactor; GLR = Gas-Lift Reactor; MB = Membrane reactor; AF = Alcoholic Fermentation, MLF = Malolactic Fermentation.

Alginates were the second support to be tested in large-scale increasing operational stability compared to kieselguhr, reducing maturation time, simplifying clarification and lowering investment cost. They were however found unsuitable due to lack of mechanical stability, poor regenerating properties and flavour alteration. For the above reasons alginates were replaced with DEAE-cellulose and porous glass beads.

In brewing with immobilized yeast cells, reactor design has a leading role not only in system selection but also in system efficiency. Packed-bed systems used for primary fermentation resulted to lower amino acid concentrations in beers compared to fluidized-bed reactors (Cop et al., 1989; Aivasidis et al., 1991). Also, insufficient mass transfer, for example of nutrients to yeast and removal of fermentation by-products, was considered the main factor for the unbalanced flavour profile in beer produced using immobilized yeast packed-bed reactors. It was therefore important to assess and improve mass transfer in immobilized cell bioreactors. Immobilized fluidized-bed reactors appears to be the most promising, while in stirred-tank reactors high aeration resulted in less balanced aroma profile of the final product. The resultant beers had high concentrations of diacetyl, and low of higher alcohols and esters (Okabe et al., 1992; Mensour et al., 1997). Fluidized-beds are suitable for support particles that are significantly denser than fermentation media. These systems improved contact between the immobilized yeast and the substrate, which increases the productivity and permits shorter residence times. Mixing however may also cause abrasion of the support material, thus affecting the efficiency of the system.

The latest developments in brewing processes with immobilized cells indicate that by the use of different reactor and system designs improvement can be accomplished. Among the proposed systems are the two-stage multi-channel loop-reactor system by Andries et al. (1996); the fluidized-bed reactor with wort recirculation by Shindo et al. (1994); the multi-stage reactor system by Inoue (Yamauchi et al., 1995b) and the gas-lift bioreactor system by Nedovic et al. (1996). Tata et al. (1999) used two different systems consisting of two rectors connected in series. The first was a two fluidized-bed system of yeast immobilized on porous glass beads and the second consisted of two rectors in which silicon carbide cartridges with immobilized *S. cerevisiae* were inserted. Application of these systems for continuous beer production was feasible in terms of flavour formation, fermentation kinetics and process economics.

An integrated design of fermentation systems which we believe can be used in the brewing industry for continuous rapid main and secondary fermentation is presented in Fig. 4. Most of the reported applications describe main fermentation systems including one or two bioreactors connected in series.

3.2. Bioreactors in wine and cider-making

There are few attempts for applying immobilized cell technologies in wine and cider-making at large scale (Colagrande et al., 1994; Divies et al., 1994). Most of these efforts concern the production of sparkling wine, secondary (MLF) fermentation of wine, and cider production with spontaneous main and secondary (MLF) fermentation. Batch-stationary, and in fewer cases continuous, packed-bed or fluidized-bed systems are the usual configurations proposed at the majority of research efforts, which concern lab scale processes (Table 2). An integrated design of fermentation systems, which we believe, can be used for wine and cider-making is presented in Fig. 5. This integrated system for continuous wine or cider-making as described by the above workers may include a system of heat exchangers and immobilized cell reactors for premature deacidification (malolactic fermentation) of must or apple juice (Taillandier et al., 1994). The main fermentation step

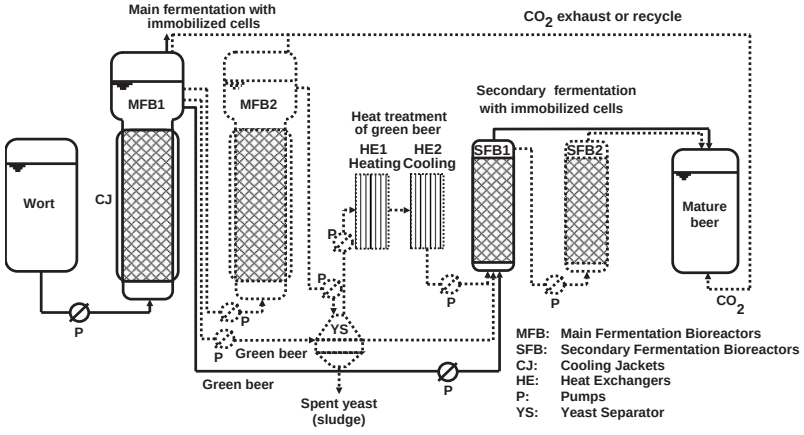

Fig. 4. An integrated review of the technology using immobilized cell bioreactors for continuous rapid main and secondary fermentation (maturation) of beer, in industrial or semi-industrial scale (dot lines: the steps are optional or are reported to be used in various combinations).

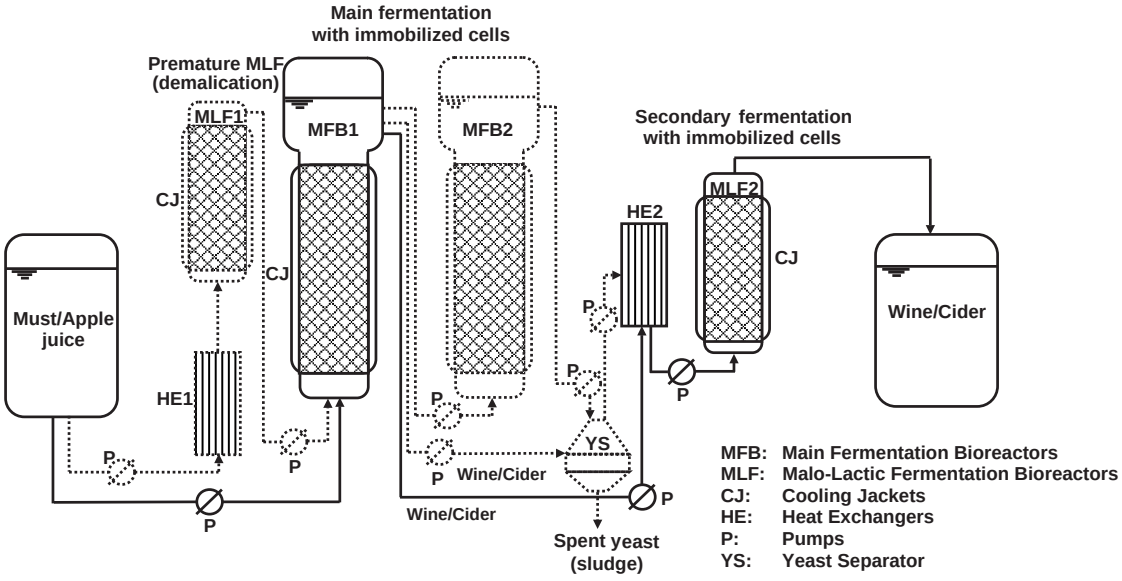

Fig. 5. An ideal continuously operating system with three simultaneous functions: (a) deacidification of apple juice or must by malolactic fermentation with immobilized cells, prior to alcoholic fermentation, (b) alcoholic fermentation with one or two bioreactors in series and (c) post-alcoholic malolactic fermentation with immobilized cells.

could involve one or two packed-bed bioreactors connected in series. A sludge and yeast removal step could also be involved. The amount of spent yeast depends on whether the type of immobilization allows cell release.

Maturation (MLF) of wine and cider can take place as it occurs in a traditional process, in a secondary fermentation step. Heat exchangers for suitable temperature control and immobilized cell reactors (one or two connected in series) containing selected MLF cultures may be involved in this stage. For continuous sparkling wine-making the final product containers is sealed under pressure.

3.3. Bioreactors in ethanol production

Various bioreactor configurations employing immobilized cells for potable or fuel ethanol production were reported. In the majority of these studies the common configurations used are the batch-stationary or continuous packed-bed systems (Table 2), and the efficiency of various processing types like batch, continuous, packed-bed, fluidized-bed, stirred-tank reactors for ethanol production employing immobilized cells is assessed. A comparison between various types of bioreactor configurations for ethanol production like gel entrapment systems, carrier binding, membrane and biofilm reactors with respect to product concentrations, productivities, diffusion limitations, product inhibition, and process costs have be reviewed (Vega et al., 1988). In all cases contamination was one of the main reasons for hindering application of continuous processes in industrial scale (Virkajarvi et al., 2001).

Bakoyianis and Koutinas (1996) described the development of an industrial-scale, multistage fixed-bed tower bioreactor using the promoter mineral kissiris for industrial alcohol production using free cells. Pilot-plant operations were carried out in a 70001 total working volume bioreactor and was operated in batch mode, firstly as a one-stage and consequently as a two-stage fixed-bed system. Operational stability of the process was excellent for a long period and the support was easily regenerated by washing with hot water. The fermented product was directly pumped into the distillation unit. The process was estimated to require 30% less energy and 10–20% less capital. Scale-up at industrial scale of the previous system was achieved with a pilot-plant of a multi-stage fixed bed bioreactor with 100,0001 capacity (Koutinas et al., 1997).

Ogbonna et al. (1997) found the use of loofa (*Luffa cylindrica*) sponge, for yeast immobilization efficient for ethanol production in the packed-bed bioreactor, and attempted a scale-up of the method for ethanol production from sugar beet juice. They used a fixed column reactor with cylindrical loofa sponges and an external loop reactor for circulating the broth during immobilization they achieved uniform distribution of cells in the beds, and managed to scale-up the method efficiently. Although their Initial goal was to make a cost-effective method for fuel ethanol their process was found interesting for other applications as well, like the production of secondary metabolites of high value.

It should be noted that commercial preparations of immobilized cells on the basis of polyvinyl-alcohol carrier (LentiKats®) are available in the market of Central and Eastern Europe for use in the brewing,

distilling, wine industry as well as for other applications. Also various preparations of selected immobilized yeasts on alginates (Silva et al., 2003, 2002b), or confined by micro-filtration membranes, like the ''Millispark'' cartridge (Lemonnier and Duteurtre, 1989), are available for sparkling wine production for secondary fermentation in the bottle according to the traditional Champagne method.

The commercial utilizations of immobilized cell methods, traditional and novel, including immobilized yeast on alginate beads, double layer alginate beads, the Millispark cartridge and agglomerated yeast preparations, have been discussed (Ramon-Portugal et al., 2003) and an economical and comparative analysis of their use for bottle fermentation is also available (Tita et al., 2003).

4. Conclusions

Available literature shows the high number of immobilization supports proposed by various researchers for alcoholic beverages production. The advantages associated with the production of potable alcohol using immobilized cell systems (increased rates of productivity, reduced risk of contamination, biocatalyst recycling, rapid product separation and ease with which the product may be recovered) are well established. However, attention should be also focus on the improvement of quality of the products. Therefore, efforts should be concentrated on cheap, abundant, non-destructive and food-grade purity immobilization supports, which will improve quality and give a distinctive aroma profile and a fine taste to the final product.

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