



Immobilized cell systems for batch and continuous winemaking

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The Major challenges in the food industry are the development of healthier, safer and environmental friendly products. To achieve these objectives, it is essential to develop advanced technologies to make the production processes economically attractive. The use of immobilized cell systems has been widely applied in the production of several products. However, in winemaking, it has only been studied to prove its applicability. The studies targeting the production of wine using these innovative fermentation systems aim to overcome limitations related to the product quality, the operational costs associated with the material used as support and the immobilization process itself.

Introduction

Wine is a well-known ancient beverage spread all over the world. It had an important role in the old civilizations and reached our days with no less importance. The grapes were used in the ancient times, as confirmed by the finding of an installation for winemaking in the territory of Armenia dating to around 4000 BC (Barnard, Dooley, Areshian, Gasparyan, & Faull, 2011). Eastern Europe is considered to be the birthplace of the vine, more specifically the area between and below the Black Sea and the Caspian Sea. In 2011, according to statistics of the International Organization of Vine and Wine (OIV, 2012), 7.6×10^6 ha of vines, allowed the production of 267×10^8 L of wine around the

world. This amount is changing every year, depending on various circumstances like occasionally unfavorable climate conditions.

The two main processes associated with wine production are the alcoholic and malolactic fermentations. Traditionally, the wine fermentation technology uses free yeast biomass suspended into the must that ferments in an unstirred batch reactor during long periods of time, making the fermentation a very time-consuming stage of the process. In the last decades new methods have been under study, in order to improve the fermentation performance and productivity, namely the use of immobilized yeast cells which speed up the fermentation process. By doing so, labor requirements are diminished, thus simplifying time-consuming procedures which can help to reduce costs. Continuous winemaking technology with immobilized cells is still under study to demonstrate its application in industrial processes. However, its economic benefits are the basis of a research area aimed at studying and implementing continuous fermenters.

Traditional winemaking

Traditionally, the process of winemaking includes several steps (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). The first stage of the wine production is related with the preparation of the grape juice – must – which includes: harvesting of grapes, crushing, maceration (in the case of rosé and red wines), pressing and must clarification (Fig. 1). Before alcoholic fermentation the must usually needs some specific preparation, depending on its initial characteristic and on the desired characteristics of the resulting wine. Sometimes the total must acidity may need adjustment of either increasing (acid addition, using tartaric acid) or decreasing (acidity reduction, using for example CaCO_3). The addition of enzymes (pectinases) accelerates particle sedimentation and help for the clarification of the grape must; glycosidases may be used to enhance varietal flavor of white wines (Rensburg & Pretorius, 2000). Sulfur dioxide (SO_2) is added to the grape must to prevent oxidation and growth of wild yeasts and bacteria. Selected dry yeasts are usually used as fermentation starters, as this ease the control of the fermentation and can bring to the final product specific aroma compounds (Grainger & Tattersall, 2005). The fermentation of red wines is accompanied by the maceration, the process of extraction of color (and other relevant compounds) from

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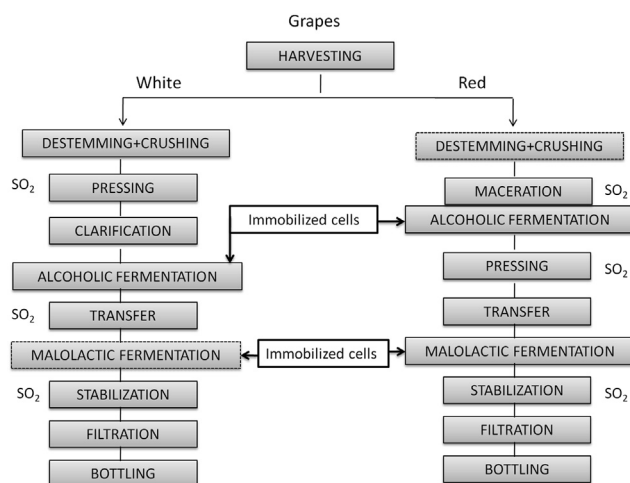


Fig. 1. Winemaking technology and the possibility of using immobilized cells.

the grape pomace (mainly skins). The length and the intensity of the macerations are very important and depend on the grape variety and on the desired type of wine (Grainger & Tattersall, 2005; Ribéreau-Gayon *et al.*, 2006).

When the must is ready, the next phase begins with the conversion of sugar into ethanol, *i.e.* alcoholic fermentation occurs, followed by malolactic fermentation (if desired), maturation, stabilization and bottling.

Although both alcoholic and malolactic fermentations are usually conducted with free suspended cells, the implementation of immobilized cell systems could be also considered in production of wines.

In some cases, before the alcoholic fermentation, a deacidification of the grape must by means of so called malo-alcoholic fermentation may be carried out (Silva *et al.*, 2003; Yokotsuka, Otaki, Naitoh, & Tanaka, 1993). Here, malic acid is transformed into ethanol, thus decreasing the acidity of the grape must. However, malo-alcoholic fermentation is used mostly in laboratory studies and not in traditional winemaking. Immobilized cell systems are commonly used.

Alcoholic fermentation

Alcoholic fermentation (AF) is the primary fermentation during winemaking. Throughout the AF the fermentable sugars of the must, mainly glucose and fructose, are transformed to ethanol and carbon dioxide, according to the Equation (1):



The fermentation process is much more complex than this simplified equation and several other compounds are formed during different chemical and biochemical reactions along the fermentation evolution. The main compounds formed are ethanol and glycerol but other compounds, in much lower concentrations, are also formed,

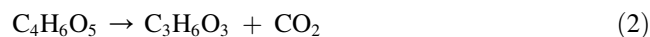
contributing for the global taste and aroma of the wine. They belong to several chemical families: organic acids, higher alcohols, aldehydes, volatile fatty acids, ethyl esters and acetates, etc. (Oliveira, Oliveira, Baumes, & Maia, 2008). Although varietal volatile compounds may define the typical characteristics, the volatile compounds formed at this step represent quantitatively the biggest contribution to the wine aroma (Oliveira *et al.*, 2008; Vilanova & Oliveira, 2012).

Traditionally, the fermentation of the must starts spontaneously by the action of the yeast that naturally covers the surface of the grapes. Most of the strains of that yeast biomass are not tolerant to ethanol and for this reason, during a spontaneous fermentation there is a succession of organisms that prevails throughout the process.

Even though *Saccharomyces cerevisiae* is present on the grapes and in the fresh must in low percentages, it is considered to be the principal “fermenting” yeast during AF (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). However, during winemaking, other genera of yeast are present and can contribute, positively or negatively, to the final quality of the wine. Yeasts found in must or wine belong mainly to the genera: *Candida*, *Dekkera*, *Hanseniaspora*, *Issatchenkia*, *Metschenikowia*, *Pichia*, *Saccharomyces*, *Saccharomyces*, *Schizosaccharomyces* and *Zygosaccharomyces* (Fugelsang & Edwards, 2007).

Malolactic fermentation

The malolactic fermentation (MLF) is a secondary fermentation in which L-malic acid is transformed into L-lactic acid and carbon dioxide. In summary, the process can be explained with the simplified equation:



The main consequences of the MLF are the decreasing of the wine acidity and a subtle modification of the aroma *i.e.* may bring favorable organoleptic properties to the wines (Ribéreau-Gayon *et al.*, 2006). In terms of acid conversion, the fermentation of 1 g of malic acid per liter reduces the total acidity, expressed as tartaric acid, by approximately 0.6 g L⁻¹ (Ribéreau-Gayon *et al.*, 2006).

Normally, MLF starts when AF has finished and involves the growth of particular lactic acid bacteria such as: *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Hornsey, 2007). MLF is a time consuming and difficult to control process. It is strongly influenced by environmental conditions and the process is often extended in time, or in the worst scenario, it can fail completely (Hornsey, 2007). *Oenococcus oeni* is the main bacterial species found in wine during MLF as it is the most adapted to high concentrations of ethanol and low pH values (Ribéreau-Gayon *et al.*, 2006). The ability for spontaneous MLF is dependent on the grape region, vineyard and year. The start and completing of MLF depends on environmental conditions such as pH, temperature, ethanol, nutrients, sulfur dioxide and wine flora.

Immobilized cell systems

Cells can be kept inside of bioreactors in suspension (free cells) or immobilized in various supports. There are four main immobilization techniques for yeast cells (Fig. 2): attachment to a surface, entrapment within a porous matrix, cell aggregation (flocculation) and containment behind barriers (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004; Verbelen, De Schutter, Delvaux, Verstrepen, & Delvaux, 2006).

The attachment to a surface (Fig. 2A) can be done by natural adsorption, electrostatic forces or covalent binding, with cross-linking agents (Margaritis & Kilonzo, 2005). The attachment of cells to an organic or inorganic support may be obtained also by creating chemical bonds (covalent) between cells and the support using cross-linking agents. However, this immobilization procedure is generally incompatible with cell viability, since the cross-linking agents are highly toxic for the microbial cells decreasing their activity (Junter & Jouenne, 2004; Strehaiano, Ramon-Portugal, & Taillandier, 2006). As consequence, this method of immobilization is no longer used for microbial cells but still remains suitable for the immobilization of enzymes (Strehaiano et al., 2006). The adsorption of cells on different types of support is a natural process. The surface of the immobilization support is important in the process of adsorption of cells as rough surfaces allows the cell retention into the support's cavities (Brányik, Vicente, Oliveira, & Teixeira, 2004; Genisheva, Mussatto, Oliveira, & Teixeira, 2011). This immobilization technique is often used as it is an easy and natural process that takes place spontaneously. However, there is no barrier between

the liquid and the immobilized cell and the cells can be easily detached from the support. Normally, the equilibrium between free and immobilized cells is established at some point of the cell growth. The detachment of cells depends on the age of the cell, cellular wall composition, pH and ionic composition of the medium. However, desorption is compensated with the growth of new cells on the support (Strehaiano et al., 2006). The natural adsorption technique is advantageous over other types of immobilization as the oxygen transfer is good and no scale-up drawback exists (Ory, Romero, & Cantero, 2004). In the last years, natural adsorption is the most used technique for yeast cell immobilization and further applied in winemaking (Kandylis, Dimitrellou, & Koutinas, 2012; Kandylis, Drouza, Bekatorou, & Koutinas, 2010, Kandylis, Goula, & Koutinas, 2012; Torresi, Frangipane, & Anelli, 2011; Tsakiris et al., 2006).

Entrapment within a porous matrix (Fig. 2B) can be performed by two approaches: a) the cells are introduced in a porous material and, after growing, their mobility is restricted by the presence of other cells and by the matrix; b) a solid matrix is synthesized *in situ* around the cells. The cells are incorporated in the matrix of a more or less rigid polymer. The polymers are synthetic such as polyacrylamide, or can be made from proteins (gelatin, collagens) and polysaccharides (cellulose, alginate, agar, and carrageenan). This technique can be expensive, time consuming and short reactor life (Jackson, 2003), with serious drawbacks such as diffusion limitations of nutrients, metabolites and oxygen, instability of the gel beads and detachment of cells, as well as limit cell division (Jackson, 2003; Kregiel,

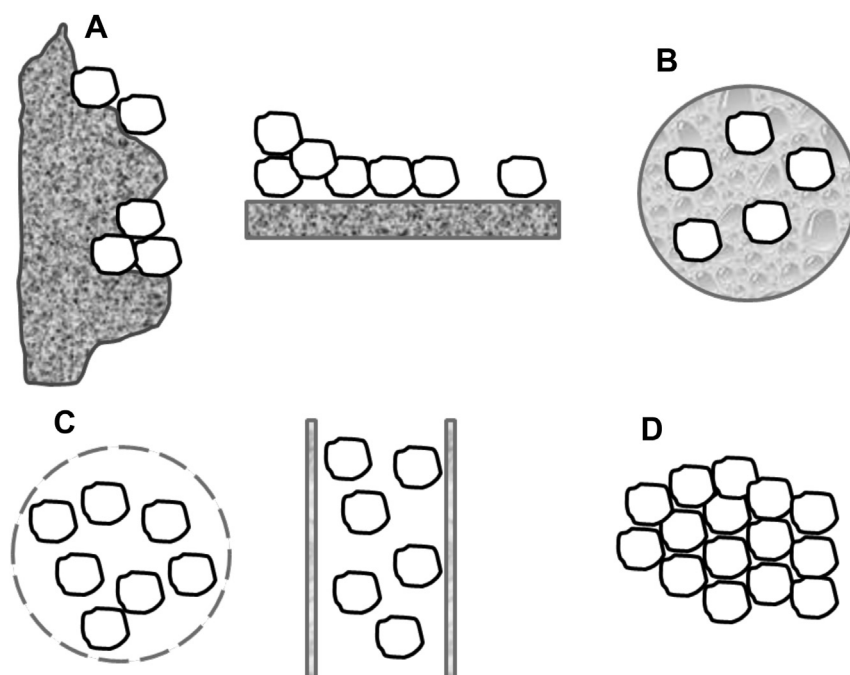


Fig. 2. Immobilization techniques. A. attachment to a surface; B. entrapment within a porous matrix; C. containment behind barriers; D. cell aggregation (flocculation).

Berlowska, & Ambroziak, 2013). Calcium alginate gel is the most commonly used material for cell entrapment in the food industry (Strehaiano *et al.*, 2006). The cells on the surfaces of the alginate beads can be released from the beads and because of this fact, it was proposed in the 80's to make an external layer of sterile alginate and produce double layer alginate beads.

Containment behind a barrier (Fig. 2C) can be achieved by two main methods: entrapment of the cells in microcapsules and by the use of microporous membrane filters (hollow fiber) or by cell immobilization onto an interaction surface of two immiscible liquids (Kourkoutas *et al.*, 2004; Verbelen *et al.*, 2006). The method based on the entrapment of cells in microcapsule or encapsulations, consists firstly in entrapping the cells in a spherical gel and posterior coating with a polymer such as polyethyleneimine. Then, the gel is dissolved but the cells are left in suspension, contained behind the polymer barrier. The microporous membranes filters are normally made of polymers, *e.g.* polyvinylchloride or polypropylene (Margaritis & Kilonzo, 2005). The containment of the cells behind a barrier allows very high cell concentrations. For this reason, the membranes used should be freely permeable to nutrients and products released during the fermentation (Strehaiano *et al.*, 2006), as well as mechanically resistant. This method of immobilization is normally used when a cell free product is needed. The main disadvantages are related to mass transfer limitations and the possibility of membrane fouling caused by the cell growth (Gryta, 2002).

Cell aggregation or flocculation (Fig. 2D) can occur naturally or by using artificial flocculating agents. It is a complex process connected with the expression of flocculation genes such as FLO1, FLO5, FLO8 and FLO11 (Verstrepen, Derdelinckx, Verachtert, & Delvaux, 2003). Yeast flocculation is an attractive method because of its simplicity and low costs (Verbelen *et al.*, 2006). The flocculation depends on various parameters such as pH, nutrients, dissolved oxygen, medium composition and fermentation conditions (temperature and agitation) as well as the age of the cell (Jim & Speers, 1998; Verstrepen *et al.*, 2003). An important issue for the success of this system is the selection of a proper yeast strain and fermentation system. In food industry, the main applications of the flocculation are the alcohol production, some kind of beers and sparkling wines (secondary fermentation). The flocculation is very important for the brewing industry as it is an effective, environmentally friendly, easy and without costs method to separate the yeast cells from the green beer at the end of the fermentation (Verstrepen *et al.*, 2003). The flocculation of the yeast is a very important characteristic also in the traditional making of sparkling wines (Torresi *et al.*, 2011).

Types of supports

It is of the highest importance the selection of the immobilization support for further implementation in the food industry. The support must be easily accepted by the

consumer and its selection depends on the process in which it will be applied as well as the process conditions. The support can be used in their natural form or submitted to some treatment to modify the surface in contact with the biomass (Genisheva *et al.*, 2011).

Several works have been published with inorganic supports like *kissiris* (volcanic rock) and γ -alumina. Inorganic supports are thought to be more attractive than organic supports due to their low cost, abundance in nature, reusability and are environmentally friendly. Studies with *kissiris* and γ -alumina demonstrated increased fermentation rates and ethanol productivity at ambient and low temperatures (Bakoyianis, Koutinas, Agelopoulos, & Kanellaki, 1997). Even though the wines produced with an inorganic support had improved aroma, these supports turn undesirable for winemaking because of the mineral residues left in the final product. A comparative study on *kissiris*, γ -alumina and calcium alginate as potential supports for cells immobilization, demonstrated that calcium alginate had the best results in winemaking, by representing a more stable environment for the entrapped yeast cells. At the same time, it was the most expensive and time consuming material. The cheapest and the more abundant support mentioned above is the *kissiris*, followed by γ -alumina (Bakoyianis *et al.*, 1997).

Organic supports from natural sources have received higher attention for wine production. Parts of fruits are the most common support used for batch or continuous winemaking. Wines were produced using apple cuts (Kourkoutas, Koutinas, Kanellaki, Banat, & Marchant, 2002), quince (Kourkoutas, Koutinas, *et al.*, 2002), watermelon (Reddy, Reddy, Reddy, & Reddy, 2008), dry raisin berries (Tsakiris *et al.*, 2006), pear (Mallios *et al.*, 2004) and others. Even though the aforementioned fruits, apple, quince and dry raisin berries are appropriate for winemaking, their cultivation, availability and cost are limited for industrialization (Reddy *et al.*, 2008). Lately, whole grains of wheat, corn and barley were used for cell immobilization (Kandyilis, Dimitrellou, *et al.*, 2012; Kandyilis *et al.*, 2010; Kandyilis, Mantzari, *et al.*, 2012). These natural products are interesting in terms of compatibility with the final product and it is expected that they will not interfere or will bring positively changes to it. Moreover, the natural origin of these supports induces an easier acceptance by the consumer. The use of residues from the wine industry like grape skins (Genisheva, Mota, Mussatto, Oliveira, & Teixeira, 2014; Genisheva, Vilanova, Mussatto, Teixeira, & Oliveira, 2014; Mallouchos *et al.*, 2002) and grape pomace (Genisheva, Macedo, Mussatto, Teixeira, & Oliveira, 2012) is a very good approach, as these residues are parts of the vine itself. Another natural material widely used as support for immobilization is the delignified cellulosic material. The cellulosic material is alcohol resistant giving high operational stability in alcoholic fermentation. Moreover, it is a solid with low market value that does not release any contaminants into the final product (Iconomou, Kanellaki, Voliotis, Agelopoulos, & Koutinas, 1995).

Polysaccharides are originated from renewable sources such as algae, plants and selected microbial strains, and are normally considered to be more economically profitable over the synthetic polymers (Coviello, Matricardi, Marianecchi, & Alhaique, 2007). Polysaccharides are a class of polymers with a complex structure bringing a large variety of composition and properties. One of the most widely known and used polysaccharide is the alginate; it can be extracted from marine brown algae or produced by bacteria. It is considered to be one of the best matrices to entrap whole microbial cells, because gelification is carried out under very mild conditions. Moreover, a large amount of cells can be immobilized, the substrates and products can easily cross the support and cell leakage is small (Spettoli, Bottacin, Nuti, & Zamorani, 1982). To prevent the cell leakage from the beads new approaches were used such as the technique of coating alginate beads (Crapisi, Pasini, Spettoli, Borin, & Versini, 1992) or using beads with double-layers (Yokotsuka, Yajima, & Matsudo, 1997). Another well-known polysaccharide is carrageenan. It is obtained by extraction of certain species of red seaweeds. There are different types of carrageenan depending on the degree of sulfation (normally between 15% and 40%), identified traditionally by a Greek prefix (Coviello et al., 2007). From the three commercially most important carrageenans, ι -(mono-sulfate), κ -(di-sulfate), and λ -carrageenan (three-sulfate), κ -carrageenan is the one already used as support material for wine production (Crapisi, Nuti, Zamorani, & Spettoli, 1987).

When choosing a proper support for cell immobilization, some aspects must be considered, like price of the material, easiness of regeneration, cell load, type of immobilization, stability, rigidity, sterilization, possibility to use in different reactor designs and approval for food use (Virkejärvi & Linko, 1999).

Initially, the selection of supports for cell immobilization in wine fermentation was on the basis of its price and availability or abundance in nature. In the recent years the selection of the support is connected with its acceptance from the consumer. The more naturally and close to the human diet support, the better.

Advantages and disadvantages

The use of immobilized cells for winemaking have advantages improved productivity with high volumetric reaction rates and high specific product yields (Bakoyianis et al., 1997; Genisheva et al., 2012). Immobilized cell systems (ICS) have the capability to regenerate their biocatalyst activity after storage for 1 month (Genisheva, Mussatto, Oliveira, & Teixeira, 2013; Genisheva, Vilanova, et al., 2014) or 6 months (Sipsas et al., 2009). The possibility to reutilize the ICS can bring down the cost of the wine production (Genisheva, Vilanova, et al., 2014). Moreover, the high volumetric reaction rates make possible the use of smaller fermentation facilities, which can reduce the capital and the running cost of the process. According to Genisheva,

Vilanova, et al. (2014), immobilized cells were able to carry out the complete alcoholic fermentation in 4 d against the 7 d needed for the traditional free cells system. The use of ICS simplifies the removing of the microbial cells from the final product (Genisheva et al., 2012); moreover, it may be adapted to a continuous process of winemaking (Genisheva, Mota, et al., 2014; Sipsas et al., 2009). It was also proved the greater tolerance of the immobilized cells to the inhibitory substances in winemaking like SO₂ and ethanol (Crapisi et al., 1987; Genisheva, Vilanova, et al., 2014). Additionally, immobilized cells were able to conduct wine fermentation in the presence of 54.4 mg L⁻¹ of free SO₂, while the free cell fermentation did not started at this condition (Genisheva et al., 2013). ICS have biological stability at prolonged operation times, like continuous winemaking, and can keep the cell activity for long terms (Genisheva et al., 2013; Genisheva, Vilanova, et al., 2014). The ICS help for the better control and conduction of the fermentation processes, especially for malolactic fermentation of wine (Genisheva et al., 2012; Genisheva, Mota, et al., 2014).

When using immobilized cell systems some disadvantages must also be considered such as mechanical stability of the matrix used to immobilize microbial cells or loss of activity on prolonged operation.

Wine production with immobilized cells

Immobilization technology is used in various fermentation processes. Immobilized cells were used for bioethanol production (Rakin, Mojovic, Nikolic, Vukasinovic, & Nedovic, 2009), cider production (Scott & ÓReilly, 1996), vinegar production (Ory et al., 2004) and brewing (Brányik et al., 2004a) as well as for winemaking (Table 1). Not many works were published for alcoholic fermentation of grape must with immobilized systems and little are for malolactic fermentation of wine.

In our days, the induction of alcoholic fermentation and malolactic fermentation is done with starter cultures of cells, *i.e.* pure culture of cells isolated and developed for conducting wine fermentations. Most fermenters used in the winemaking industry are of a batch type, *i.e.* separate lots (batches) and are individually fermented till conclusion of the process (Jackson, 2008). Some industries adopted continuous methods, because of its advantages in controlling the yeast population and activity, keeping them in their maximum (Verbelen et al., 2006). The environmental conditions of continuous fermentations are favorable for the yeast growth, thus the biomass concentration is approximately two times larger than traditional winemaking (Genisheva, Vilanova, et al., 2014; Jackson, 2008). One of the most important characteristic of the continuous process is the high volumetric productivity (Verbelen et al., 2006) but, despite of its potential advantages, it is only profitable when working all year-round (Jackson, 2008; Ribéreau-Gayon et al., 2006). Immobilized cell systems emerged as a technique that provides also large amounts

Table 1. Immobilization type, supports, mode of operation, microorganisms and bioreactor operation conditions used in winemaking.

Fermentation type	Grape/Wine type	Microorganism	Support	Immobilization type	Operation mode	Bioreactor/conditions	Reference
Alcoholic	White	<i>S. cerevisiae</i>	Ca-alginate, single double layer	Entrapment	BATCH	10 °C–40 °C	Yajima & Yokotsuka, 2001
Alcoholic	White	<i>S. cerevisiae</i> (Montrachet522)	κ-carrageenan	Entrapment	Continuous	tapered packed bed column, 13 °C	Uematsu et al., 1988
Alcoholic	Synthetic medium	<i>S. cerevisiae</i> (AXAZ-1)	Delignified spent grains	Thermal dried, Attachment	Batch	15 °C	Tsaousi, Koutinas, Bekatorou, & Loukatos, 2010
Alcoholic	Red	<i>S. cerevisiae</i> Uvaferme299	Raisins	Attachment	Batch	packed bed reactor 6 °C–30 °C	Tsakiris et al., 2004
Alcoholic	–	<i>S. cerevisiae</i> (AXAZ-1)	Freeze-dried gluten pellets	Entrapment	Batch continuous	multi-stage fixed bed reactor, packed bed, 5 °C–30 °C	Sipsas et al., 2009
Alcoholic	White	<i>S. cerevisiae</i>	kissiris, γ-alumina, Ca-alginate	Attachment, entrapment	Batch, continuous	two linked glass tower reactors 7 °C to 20 °C	Bakoyianis et al., 1997
Alcoholic	Red	<i>S. cerevisiae</i> CFTRI (101)	Watermelon	Attachment	Batch	15 °C to 35 °C	Reddy et al., 2008
Alcoholic	Red	<i>S. cerevisiae</i> CFTRI (101)	guava	attachment	batch	15 °C to 35 °C	Reddy, Reddy, & Reddy, 2006
Alcoholic	Synthetic medium	<i>S. cerevisiae</i>	Orange peel	Attachment	Batch	15 °C to 30 °C	Plessas et al., 2007
Alcoholic	Synthetic medium	<i>S. cerevisiae</i> (AXAZ-1)	Brewer's spent grains	Attachment	Batch	10 °C to 25 °C	Mallouchos, Loukatos, Bekatorou, Koutinas, & Komaitis, 2007
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Delignified cellulosic material, gluten pellets	attachment	Batch	10 °C to 20 °C	Mallouchos et al., 2003
Alcoholic	–	<i>S. cerevisiae</i> (AXAZ-1)	Grape skin	Attachment	batch	10 °C to 25 °C	Mallouchos et al., 2002
Alcoholic	White	<i>S. cerevisiae</i>	γ-alumina	Attachment	Batch, continuous	multi-stage fixed bed reactor, packed bed reactor, 7 °C–27 °C	Loukatos et al., 2000
Alcoholic	–	<i>S. cerevisiae</i> (AXAZ-1)	Quince	Attachment	Batch, continuous	packed bed reactor, 5 °C–30 °C	Kourkoutas, Koutinas, et al., 2002
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Pear	Attachment	Batch, continuous	packed bed reactor, 5 °C to 30 °C	Mallios et al., 2004
Alcoholic	–	<i>S. cerevisiae</i> (AXAZ-1)	Apple cuts	Attachment	Batch, continuous	packed bed reactor, 5 °C–30 °C	Kourkoutas et al., 2002
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Delignified cellulosic material	Attachment	Batch	packed bed reactor, 0 °C to 30 °C	Bardi & Koutinas, 1994
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	corn starch gel	entrapment	batch	2 °C–30 °C	Kandyliis, Goula, & Koutinas, 2008
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Wheat grains	Attachment	Batch	5 °C to 30 °C	Kandyliis et al., 2010
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Corn grains	attachment	batch	5 °C–30 °C	Kandyliis, Mantzari, et al., 2012
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Barley grains	Attachment	Batch	5 °C–30 °C	Kandyliis, Dimitrellou, et al., 2012
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Gluten pellets	Attachment	Batch	packed bed reactor, 5 °C–30 °C	Iconomopoulou, Psarianos, Kanellaki, & Koutinas, 2002
Alcoholic	White	<i>S. cerevisiae</i> (AXAZ-1)	Delignified cellulosic material	Attachment	Batch, continuous	packed bed reactor, 25 °C	Iconomou et al., 1995
Alcoholic, malo-alcoholic	White, red	<i>Schiz. pombe</i>	Ca-alginate	Entrapment	Continuous	multi-stage packed-bed reactor, 25 °C	Ogbonna et al., 1989
Malo-alcoholic	White	<i>S. cerevisiae</i> <i>Schiz. pombe</i>	Ca-alginate, double layer	Entrapment	Batch	20 °C	Silva et al., 2003

Alcoholic, malolactic	White	<i>S. cerevisiae</i>	grape skin	attachment	continuous	packed bed reactor, 20 °C	Genisheva, Mota, et al., 2014
Bottle	White	<i>S. cerevisiae</i>	Ca-alginate, double layer	Entrapment	Batch	15 °C or 25 °C	Yokotsuka et al., 1997
Bottle	White	<i>S. cerevisiae</i>	Ca-alginate	Entrapment	Batch	11 °C to 14 °C	Fumi et al., 1988
Malolactic	Red	<i>O. oeni</i>	Positively charged fibrous sponge	Attachment	Batch	28 °C	Maicas et al., 2001
Malolactic	White	<i>O. oeni</i>	Corn cobs, grape skins, grape stems	Attachment	Batch	25 °C	Genisheva et al., 2013
Malolactic	Red	<i>L. oenos</i> ^a	Ca-alginate	Entrapment	Batch	Stirred, 20 °C	Spettoli et al., 1982
Malolactic	White	<i>Lactobacillus</i>	κ -carrageenan	Entrapment	Continuous	Column, 7 °C–40 °C	Crapisi et al., 1987
Malolactic	White	<i>O. oeni</i> (ATCC 23279)	Delignified cellulosic material	Attachment	Batch	Glass cylinder, 27 °C	Agouridis et al., 2008
Malolactic	White	<i>L. casei</i> (ATCC 393)	Delignified cellulosic material	Attachment	Batch	Glass cylinder, 20 °C	Agouridis et al., 2005
Malolactic	Synthetic medium	<i>L. oenos</i> ^a PSU-1	κ -carrageenan	Entrapment	Batch	25 °C	McCord & Ryu, 1985
Malolactic	White	<i>L. casei</i>	Pectate gel, modified chitosan beads	Entrapment, attachment	Batch	Shaking, 20 °C, 25 °C and 36 °C	Kosseva et al., 1998
Malolactic	Red	<i>Issatchenkia orientalis</i> (KMBL 5774)	Mixture of oakcharcoal and sodium alginate	Entrapment	Batch	Shaking, 30 °C	Hong et al., 2010

^a *Leuconostoc oenos* was reclassified by Dicks et al. (1995) to *Oenococcus oeni*.

of cells but is more economic than the free cells continuous winemaking (Jackson, 2008). Immobilized cell systems (ICS) give the possibility to produce new styles of beverages, with low alcohol content and very aromatic, and facilitate the conduction of fermentations where convenient removal of yeast cells is desired like the champagne method (Diviès & Cachon, 2005).

For implementation of the immobilized systems in industrial wine production, it is important to identify a suitable support for cell immobilization that is of food-grade purity. Moreover, the support should be abundant, of low cost, without interfering negatively, in the sensory characteristics of the final product (Kourkoutas et al., 2002).

During the process of winemaking, immobilized cell systems can be used in the alcoholic, malo-alcoholic and malolactic fermentations as well as for production of sparkling wines (Table 1).

Alcoholic fermentation

Alcoholic fermentation is the process where the immobilized cell systems are mainly used in winemaking, and the most used microorganism is *S. cerevisiae*. According to the tendencies of local wines protection locally isolated strains of *S. cerevisiae* are often used (Genisheva et al., 2012; Kandyliis, Dimitrellou, et al., 2012; Kandyliis et al., 2010; Kandyliis, Mantzari, et al., 2012).

Bakoyianis et al. (1997) used three different supports for the immobilization of an alcohol-resistant strain of *S. cerevisiae*. Yeast cells were immobilized on *kissiris*, γ -alumina and calcium alginate and further applied for wine production at different temperatures. From the three solid supported biocatalysts, calcium alginate presented the highest fermentation rates and ethanol productivity ($15.5 \text{ g L}^{-1} \text{ d}^{-1}$) at low temperatures (7 °C). *Kissiris* is considered to be a good option for immobilization as it is abundant in nature, environmentally friendly and can be easily regenerated. The use of γ -alumina in winemaking implies an additional step in the process, i.e. the removal of the aluminum from the produced wine (Loukatos et al., 2000).

Natural supports of food-grade purity like delignified cellulosic material (Bardi & Koutinas, 1994) and gluten pellets (Bardi, Bakoyianis, Koutinas, & Kanellaki, 1996) were used successfully for winemaking at ambient and low temperatures (from 0 °C to 30 °C). This ICS caused about a three-fold increase of the fermentation rate when compared with free cells; moreover, the ethanol productivity and daily wine production were higher. For each temperature, ethanol and wine productivities for immobilized cells were higher than those for free cells. For example at 15 °C ethanol and wine productivities for free cell fermentation were $68.5 \text{ g L}^{-1} \text{ d}^{-1}$ and $7 \text{ g L}^{-1} \text{ d}^{-1}$, respectively, while for fermentations with immobilized cells were $400 \text{ g L}^{-1} \text{ d}^{-1}$ and $35.2 \text{ g L}^{-1} \text{ d}^{-1}$, respectively. Sipsas et al. (2009) also used yeast cells immobilized on gluten pallets, which were subsequently freeze-dried. The system showed high operational stability, even after storage for 6 months at 4 °C and produced wines with an improved quality.

In order to find a suitable support for immobilization that corresponds to the prerequisites of food-grade purity together with consumer acceptance, researchers proposed pieces of fruits or whole grains. Yeast cells immobilized on orange peel showed to be a suitable biocatalyst for commercial applications (Plessas et al., 2007). This ICS was used for alcoholic fermentation at different temperatures (15 °C–30 °C) resulting in high ethanol productivity (173 g L⁻¹ d⁻¹, at 15 °C and 128 g L⁻¹ d⁻¹, at 30 °C) and low fermentation times (8.1 h, at 15 °C and 9.8 h at 30 °C). Reddy et al. (2008) used watermelon pieces as immobilization support for winemaking at different temperatures (15 °C–30 °C). This ICS improved the fermentation rates, the viability and vitality of the immobilized yeast cells. Fermentation time with immobilized cells (63 h) was twice faster than fermentation time with free cells (120 h). Ethanol productivity in fermentations with immobilized cells (39.1 g L⁻¹ d⁻¹) was almost three times higher compared with free cells (15.1 g L⁻¹ d⁻¹). The produced wines were found to be with good taste and with improved quality. The main drawback of this system was the significant loss of watermelon volume; however, after the seventh or eighth batch the watermelon pieces volume stayed constant. The study carried out by Kourkoutas, Komaitis, Koutinas, and Kanellaki (2001) with apple cuts as support materials, also observed an important decrease of the immobilized support during the first batches. Nevertheless the immobilized cells were able to produce wines at low temperatures (0 °C, 5 °C and 10 °C) and kept their biocatalyst activity for 7 months. Tsakiris, Sipsas, Bekatorou, Mallouchos, and Koutinas (2004) used yeast cells immobilized on raisin for the production of red wine at different temperatures. Kandyliis et al. (2010); Kandyliis, Dimitrellou, et al. (2012) and Kandyliis, P., Mantzari, et al., (2012) used whole grains of wheat, corn and barley as support materials for yeast immobilization. The resulting wines had improved aromatic profiles when compared to fermentations with free cells. Genisheva et al. (2012), Genisheva, Mota, et al. (2014) and Genisheva, Vilanova, et al. (2014) used grape pomace and grape skins, agro-industrial wastes, as supports for yeast cell immobilization. The wines produced with free and immobilized cells on grape pomace were not found different according to the 35 panelists that made the sensory evaluation of the wines.

Immobilization of cells promotes alcoholic fermentations especially at low temperatures. In wine fermentation with immobilized cells is often that the total concentration of higher alcohols decreased when the fermentation temperature is lower (Kandyliis, Dimitrellou, et al., 2012; Kandyliis et al., 2010; Kandyliis, Mantzari, et al., 2012; Loukatos et al., 2000; Plessas et al., 2007; Sipsas et al., 2009). In contrast the total concentration of volatile compounds may increase or decrease with the temperature dropped, probably depending on the type of reactor used for the fermentation. For example according to Loukatos et al. (2000), the total volatile compounds decreased with

16% when the temperature of fermentation was lowered from 30 °C to 16 °C, using multistage fixed batch tower reactor. Nevertheless, Sipsas et al. (2009) did not observe decrease in total volatile compounds when the temperature was decreased from 20 °C to 10 °C, using packed bed and multiple fixed bed reactors.

Malo-alcoholic fermentation

The fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of malo-alcoholic fermentation. However, the use of *Schiz. pombe* in vinification may be unsuitable as this yeast can produce undesirable off-flavors in the wines (Yokotsuka et al., 1993). During the malo-alcoholic fermentation malic acid is directly transformed into ethanol. Malo-alcoholic fermentation with immobilized *Schiz. pombe* cells, even though is not a perfect alternative to the malolactic fermentation, can improve the acid harmony of wines with high acidity (Magyar & Panyik, 1989).

Schiz. pombe cells are normally immobilized in Calcium alginate beads (Ciani, 1995; Magyar & Panyik, 1989) or fibers (Yokotsuka et al., 1993). This ICS can be used for deacidification of grape must before alcoholic fermentation (Silva et al., 2003; Yokotsuka et al., 1993) or wines (Ciani, 1995; Magyar & Panyik, 1989), by degrading malic acid into ethanol. In some cases the *Schiz. pombe* immobilized cells were still active after 20 months of storage; moreover, the alginate beads with entrapped cells could be recycled up to five times without cell leakage (Silva et al., 2003). Sometimes the resulting wines had small amounts of sediments and little distinct off-flavor (Yokotsuka et al., 1993). However, most of the authors concluded that wines obtained by this method had better organoleptic quality than the wines without previous deacidification (Silva et al., 2003), and no off-flavor or off-taste were detected (Ciani, 1995; Magyar & Panyik, 1989).

The existing published works on the use of immobilized cells in winemaking are mostly for the conducting of primary alcoholic fermentation. The use of immobilized cells in secondary fermentations, namely, malolactic fermentation is more functional. The uses of immobilized cells in malolactic fermentation helps for its fast commence and proper conduction, as malolactic fermentation is difficult to predict and carry out.

Malolactic fermentation

Normally the supports used for conducting malolactic fermentation (MLF) in wines are from organic origin. The bacterial cells used in immobilized cell systems for MLF are *O. oeni* or species of *Lactobacillus*.

Leuconostoc oenos (today known as *O. oeni*) cells were immobilized on calcium alginate gels to be used for conducting MLF in red wines (Spettoli et al., 1982). Even though this ICS showed high reaction yields and small number of cells leaked from the gel (0.1%), the operational activity of the system declined gradually with time (after 17 d).

Crapisi et al. (1987) used *Lactobacillus* cells immobilized on κ -carrageenan gel for controlling and conducting MLF. The conversion ratio of malic acid was 53.9%, and the sensory properties of the wine stayed unchanged.

Calcium pectate gel and chemically modified chitosan beads were used as supports for immobilization of *Lactobacillus casei* (Kosseva, Beschkov, Kennedy, & Lloyd, 1998). Repeated batch fermentations were carried out with different wine samples and at different temperatures (35 °C, 25 °C and 20 °C). The temperature was found to be the main factor affecting the rates of the MLF. The best fermentation rates were recorded for assays conducted at 25 °C, where malic acid decreased 30% within 1 h. The degradation rate of malic acid using immobilized cells was twice as high as that obtained with free cells. These ICS were found to be with potential for industrial application as they showed long term operational stability; calcium gel beads were stable for 6 months and chitosan beads for 2 months. In another study, Kosseva and Kennedy (2004) demonstrated that encapsulated *L. casei*, in a pectate gel, carried out fermentation at high ethanol concentrations (12% vol. to 13% vol.) increasing the fermentation rates.

However, the encapsulation method has mass transfer limitations for nutrients that lead to inactivation, or even death, of the cells in the center. Therefore, a new immobilization support was proposed: a fibrous sponge which is cellulose based (Maicas, Pardo, & Ferrer, 2001). The surface of the sponge can be modified and ionized. Maicas et al. (2001) showed that the positively charged sponge immobilized the highest amounts of *O. oeni* cells and used this ICS for MLF in red wines. Although the results were better than assays performed with free cells, a decrease of the activity of the immobilized cells was detected after 4 to 6 repeated batches. The main reason was considered to be the diminished viability of cells after long exposure to ethanol.

Agouridis, Bekatorou, Nigam, and Kanellaki (2005) also used a cellulosic material for the immobilization of *L. casei* and conducted MLF at 27 °C. Once again with the repeated batch fermentation (more than 1 month) the malolactic activity of the immobilized cells decreased. Nevertheless, the authors concluded that the delignified cellulosic material (DCM) is a promising support for MLF, but more research is required for improving some parameters. In another study, the DCM was used for the immobilization of *O. oeni*, strain that is highly resistant to ethanol (Agouridis, Kopsahelis, Plessas, Koutinas, & Kanellaki, 2008). In this study the authors demonstrated a good operational stability of the ICS during all 11 repeated batch fermentations. The malic acid degradation could be maintained stable within an average value of 54.0%. This result is comparable with results obtained by Maicas et al. (2001), 50.0%, and Crapisi et al. (1987), 53.9%.

Genisheva et al. (2013) used natural residues for support of *O. oeni* cells for malolactic fermentation of white wine. All three materials (corn cobs, grape skins and grape stems)

showed mechanical stability and long operational activities (respectively 150 d, 192 d and 174 d). The malic acid conversion in fermentation assays of 30 d was as follows: 100% for assays with cell immobilized on corn cobs, 75% in assays with cells immobilized on grape skins and 83% in assays with cells immobilized on grape stems.

Sparkling wines

In the traditional production of sparkling wines, lees removal is a very laborious and time-consuming process and the use of immobilized yeasts has been investigated in order to diminish and simplify the riddling and disgorging procedures. Among the available immobilization techniques, encapsulation in polysaccharide gels such as alginate is the most widely used.

Immobilized *S. cerevisiae* cells on calcium alginate were used for sparkling wine production (Fumi, Trioli, Colombi, & Colagrande, 1988). Cells were released from the beads but with little influence on the clarity of the wine, according to the tasters. However, there were not found differences between the wines obtained with immobilized cells and wines obtained by the traditional method in terms of the main components: ethanol, organic acids and higher alcohols.

For preventing cellular leakage from the beads, Crapisi et al. (1992) used coated alginate beads and were able to obtain a biologically stable sparkling wine. Sparkling wines produced with free and immobilized cells were not found different in terms of aromatic compounds.

Yokotsuka et al. (1997) used *S. cerevisiae* cells immobilized in double-layer gel beads or strands for the bottle-fermentation. The beads were easily inserted in the bottle and simply removed in ice plugs during disgorging. The produced sparkling wine was clear and similar in taste and bouquet to that made using free yeast cells. Moreover, with the increase of the amount of beads the calcium content in the sparkling wine also increased.

Reactors used with immobilized cells

Reactors operating with immobilized cells have higher productivity and operational stability, as well as easier downstream processing. Another attractive advantage of the immobilized cell bioreactors, compared to the existing free cell fermenters, is the faster fermentation time. Because of this and other benefits, the immobilized cell bioreactors have been applied in many industrial processes, including beverage production. Choosing the proper reactor for use with immobilized cell systems depends on the type of immobilization, the type of the used support, mass transfer requirements and conditions of the process. For example, it is of a big importance the resistance of the immobilized cell system to the shear forces as well as the size of the support. According to the type of immobilization procedure and support used, an appropriated reactor must be designed.

Batch and fed-batch reactors

The biological reactors mostly used in the industries can have different operation modes: batch or continuous. Batch reactor is a “closed reactor”, *i.e.* once inoculated, no further inputs of nutrients or outputs of products occur. In this type of reactors the velocity of cell growth tends to zero. It is one of the most used reactors in a big variety of industrial processes. The batch reactor can be stirred or not stirred.

The fed-batch reactor is a variance intermediate between batch and continuous reactors. It is an “open reactor” like the continuous, but operates on an unsteady-state basis like the batch reactor. The main characteristic of the fed-batch system is to control the inflow of the growth limiting nutrients, leading to high cell densities in the bioreactor. The controlled addition of nutrients affects the growth rate of the cells and helps to avoid formation of site metabolites.

The use of immobilized cell systems are, however, more attractive for using in continuous reactors.

Continuous reactors

The continuous reactor is an “open reactor” where there is a constant inflow of nutrients and outflow of product (Fig. 3). The main characteristic of the continuous reactor is the possibility of reaching a dynamic equilibrium, *i.e.* the system operates on steady-state basis. Continuous reactors are used widely in the food, pharmaceutical and chemical industries. For continuous production the most used reactors are the multiphase reactors, including packed bed reactor, fluidized bed reactor, bubble column and air-lift reactor (Verbelen *et al.*, 2006). The multiphase reactors include three phases: solid (the support), liquid (the medium) and gas (air, or other). In the case of wine production an inert gas like N_2 or CO_2 may be used instead of air to avoid must/wine oxidation.

Packed bed reactor

Packed bed (Fig. 3A) or also known as fixed bed reactor is extensively used in the chemical, petrochemical and biotechnology industries (Larachi, Cassanello, Laurent, Midoux, & Wild, 1997). In this reactor type the immobilized cells are packed inside the reactor and a co-current of gas and fermentation media is passed upflow (flooded bed reactor) or downflow (trickle-bed reactor). Despite its simplicity, during the operation of a packed bed reactor the following drawbacks can take place: channeling, fouling, mass transfer limitations, difficulties in CO_2 evacuation and compression of some support materials (Verbelen *et al.*, 2006).

Fluidized bed reactor

In fluidized bed reactor (Fig. 3B) the cells are attached and grow onto an inert support and the fermentation liquid is fed as at an upstream flow above the “minimum fluidization velocity” that guarantees the fluidization of the support particles. In this system there is a vigorous mixing of gas,

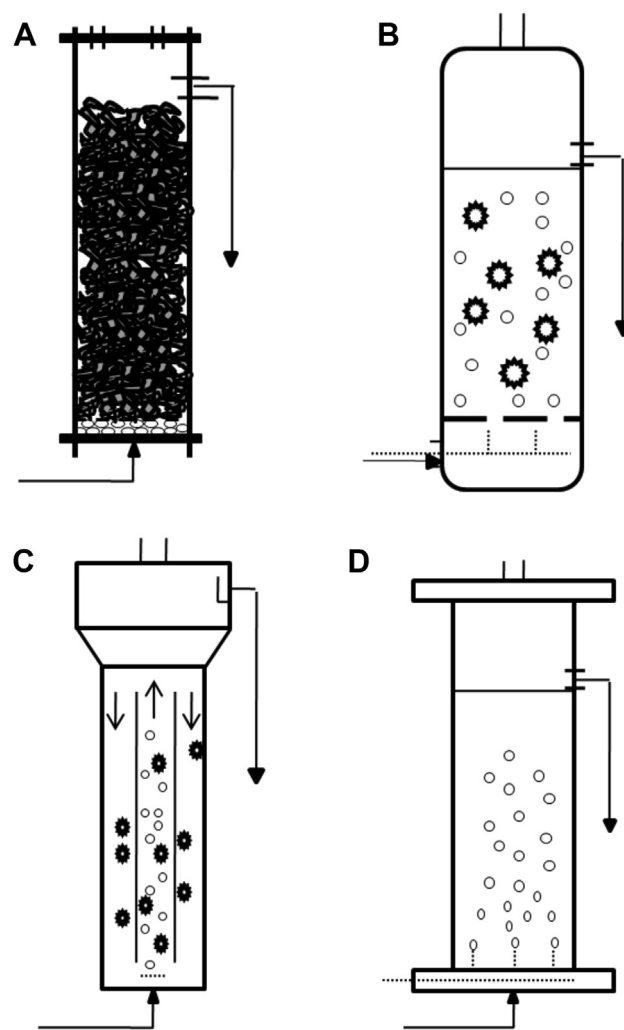


Fig. 3. Immobilized cell reactors working in continuous mode. A. Packed bed reactor; B. Fluidized bed reactor; C. Air-lift reactor; D. Bubble column reactor.

liquids and solids by the upstream flow. When using this reactor, it is important to take in consideration the specific weight of the support used for cell immobilization, as too light support will result in wash-out (Verbelen *et al.*, 2006). The main advantages of this reactor type are high biomass concentration and surface area, good transfer of nutrients, high substrate utilization rates, low pressure drop across the bed and good process control (Nicolella, Felice, & Rovatti, 1997).

Air-lift reactor

In air-lift reactor (Fig. 3C) the mixing of the liquid is provided by the injection of gas. There are two types of air-lift reactors – “internal” and “external” loop. The air-lift consists of two tubes linked together on the top and on the bottom. In one of the tubes (riser), the air is injected at the bottom, while normally in the other tube no air is injected (downcomer). The loop liquid circulation is caused by the density differences between the riser and the

downcomer. The ideal cell support for air-lift reactor should have a low enough terminal settling velocity to be suspended by the upflowing gas and liquid streams. When comparing air-lift reactor to bubble column reactor or stirred tank reactor shear stress is mild and constant throughout the reactor. These types of reactors are economical as the aeration requests low-energy input, can be easily scaled up and used commercially (Couvert, Roustan, & Chatellier, 1999).

Bubble column reactor

In a bubble column reactor (Fig. 3D), gas is injected in the bottom of the reactor through a gas distributor. Kulkarni (2008) compared the motion of the bubbles to that of a swarm. Moreover, the gas phase moves homogeneously or heterogeneously in a continuous liquid phase. The homogeneous gas regime occurs when the superficial gas velocity is lower than 5 cm/s. The size and the concentration of the bubbles in this type of regime are uniform. The heterogeneous gas regime occurs at high superficial gas velocity. The characteristic of this type of regime is the presence of radial hold-up profiles, originating intense liquid circulation (Joshi, 2001). Bubble column reactors are commonly used in the chemical industry as they are simple in construction and operation, have high mass and heat transfer rates, without any moving parts, are compact and have low operation and maintenance costs (Kantarci, Borak, & Ulgen, 2005). Nevertheless, this type of reactor presents some disadvantages such as local flow, turbulence and gas hold up and complex hydrodynamics.

All these reactors were used at different production processes. The most used reactors with immobilized cells, for vinegar production, in laboratory conditions, are the packed bed reactor, the batch reactor and the fluidized bed reactor (Ory et al., 2004). The main reactor types used for continuous beer production with immobilized cells are the packed bed reactor, the air-lift reactor and the fluidized bed reactor (Brányik, Vicente, Dostálek, & Teixeira, 2004; Verbelen et al., 2006). There are limited reports for continuous production of cider with immobilized supports. Herrero, Laca, García, and Díaz (2001) produced cider continuously in Erlenmeyer flasks with cells entrapped in alginate beads. Nedovic et al. (2000) reported the successful used of continuous packed bed reactor in cider production.

Immobilized cell reactors used in winemaking

Table 1 presents the applications of ICS and biological reactors in winemaking in the last 25 years. As it can be seen, a larger amount of works is made with winemaking in batch mode than in continuous mode. Moreover, most of the works are about alcoholic fermentation and less are about malolactic fermentation. It can be seen that for continuous production of wine the most used reactor type is the packed bed reactor. In the subsequent paragraphs some examples of batch and continuous winemaking are described.

Sipsas et al. (2009) used a multi-stage fixed bed tower reactor (MFBT) for winemaking in batch and in continuous. The MFBT operated at low temperatures (5 °C) and showed significant operational stability. Moreover the MFBT resulted in higher ethanol productivity (24.2 g L⁻¹ d⁻¹ at 20 °C) of wines compared with packed bed reactor (PB) (19.8 g L⁻¹ d⁻¹ at 20 °C). Nevertheless, the analyzed volatile compounds of the produced wines in MFBT and in PB reactors did not show significant differences. Sensory evaluation of the wines produced in continuous mode in MFBT reactor revealed a predominant acid note when compared to wines produced in PB reactor. Moreover, the MFBT reactor resulted in higher alcohol productivity compared to fermentations carried out in PB reactor.

Tsakiris et al. (2004) used a 1.5 L tower glass reactor for batch production of red wine with cells immobilized on black and golden raisins berries. The fermentations were carried out with 300 mL of grape must and 100 g of immobilized support, at temperatures between 6 °C and 30 °C. The fermentation times for the different temperatures were as follows: 35 h–40 h at 30 °C; 4 d at 22 °C and; 8 d at 6 °C. The sensory evaluation of the produced wines showed that the tasters preferred wines produced with immobilized cells rather than wines produced with free cells.

Kourkoutas et al., (2002) and Kourkoutas, Koutinas, et al., 2002 carried out continuous and batch fermentations of wines in a glass tower reactor with a total volume of 2 L. The volume of the grape must used in the experiments was 720 mL and the immobilized support added (apple or quince cuts) was around 1.2 kg. The batch fermentations with cells immobilized on apple cuts resulted in wines with high ethanol concentrations. The operation stability of the continuous system was for 71 d at least. Wine productivities in continuous mode of operation were much higher than in the repeated batch fermentations.

Uematsu, Fong, and Ryu (1988) found extremely difficult to operate and control the conventional cylindrical type packed bed reactor. As a result they modified the system and used a tapered (conical) packed bed reactor for continuous wine production. ICS had improved fermentation performance compared to free cell fermentations. The new design bioreactor gave satisfactory results as well as operational stability for 2–3 months.

Bakoyianis et al. (1997) used three different support materials (kissiris, γ -alumina and calcium alginate) for batch and continuous winemaking. For the batch fermentations a 500 mL glass tower reactor was used, with 300 mL of grape must and the weight of each immobilized support was calculated so that the concentration of the immobilized cells is the same for all assays. The pilot plant for the continuous fermentations consisted of two glass reactors (each of 1.5 L total volume and 1.0 L liquid volume) linked together so that the outlet of the first reactor was the inlet of the second reactor. Here, the ethanol production was found to be 4–10 fold higher compared to batch fermentations.

The three continuous systems were operated for 80 d without loose of operational activity.

In general, there are less published studies about malolactic fermentation of wines conducted with immobilized cells. In 1998, Kosseva and co-workers (Kosseva et al., 1998) published a work about conducting malolactic fermentation in Chardonnay wines with immobilized cells on two different materials (calcium pectate gel and chemically modified chitosan beads). Repeated batch fermentations were carried out at shake flask at different temperatures (Table 1). The degradation of malic acid was 30% for 1 h, twice higher compared with free cell assays.

Agouridis et al. (2008) used a 1 L glass tower reactor for repeated batch malolactic fermentations. The average value of the malic acid degradation was 54% and stayed stable for the 11 successive batch fermentations. Nevertheless, the average concentrations of produced lactic (0.98 g L^{-1}) and acetic acids (0.39 g L^{-1}) were low.

Continuous winemaking

Continuous fermentation process is a solution for reducing production costs as well as improving the process efficiency and ethanol yield (Ribéreau-Gayon et al. 2006). Continuous processes are preferred in most fields of industry because of the great economic advantages. The effort of implementing a continuous process is not always successful (Virkajärvi & Linko, 1999) as there are some major issues linked to a process in continuous like keeping the system aseptic for long periods of time (at least for several months). If a system is contaminated and there is a need of stopping the process and making a new immobilization this increases the costs of the process and slows down the production. According to Ribéreau-Gayon et al. (2006), it is recommended the use of higher concentrations of SO_2 in the continuous fermenters, to avoid contaminations. In winemaking, the continuous fermentation system must be able to respond to another important issue that is the inhibitory effect of the formed products over the growth of the microorganisms (Virkajärvi & Linko, 1999). However, when immobilized cells are used this problem is softened as immobilized cells are more tolerant to the inhibitory effect of products like ethanol (Genisheva et al., 2012; Genisheva, Vilanova, et al., 2014).

The main advantages of the continuous processes are (Clement, Perez, Mouret, Sablayrolles, & Camarasa, 2011; Genisheva, Mota, et al. 2014; Ribéreau-Gayon et al., 2006): higher conversion rates; faster fermentation rates; improved product consistency; reduced product losses; environmental advantages; easier process control and; less fluctuation in product quality.

Wine productivity in continuous winemaking with immobilized cells was found to be three to six folds higher than those obtained by natural fermentation (Iconomou et al., 1995). Nevertheless, continuous fermentations systems, common in other industries, are rarely used in the wine industry (Sipsas et al., 2009). Continuous fermentations with immobilized cells are very beneficial as it links

high cell density with high flow rates that results in short residential times (Ribéreau-Gayon et al., 2006; Verbelen et al., 2006). The supports to be used for immobilization and further implementation for continuous winemaking should complete more prerequisites than low cost, abundance and food-grade purity. Besides the ones referred, it should also have the ability for long term storage, should have high resistance and stability and should not damage the quality of the final product (Genisheva et al., 2012; Genisheva, Vilanova, et al., 2014; Sipsas et al., 2009).

Sipsas et al. (2009) produced wine in a continuous mode in a multi-stage fixed bed tower (MFBT) reactor, at different temperatures. The authors concluded that the continuous mode of operation and the fermentation temperatures affected the concentrations of ethyl acetate, amyl alcohols and methanol. Wine produced with immobilized cells in continuous mode of operation had higher amount of residual sugars (11.6 g L^{-1}) and lower sugar conversion rates (94.3%) compared with batch fermentation (5.5 g L^{-1} and 97.3%, respectively). Reddy et al. (2008) used immobilized yeast cells on watermelon pieces in a continuous winemaking for 100 d at $20 \text{ }^\circ\text{C}$, where the cells remained 90%–95% viable.

Apple cuts (Kourkoutas et al., 2002) and quince pieces (Kourkoutas, Koutinas, et al., 2002) were found to be suitable for wine production in continuous mode. Both ICS worked for 95 d and 46 d, respectively, without diminishing of the ethanol productivity. These systems were appropriate for working at low temperatures ($5 \text{ }^\circ\text{C}$) and the produced wines demonstrated improved quality compared to other commercial wines and distinctive flavor profiles, even though an increase of the total acidity was observed, especially when apple cuts were used. Sensory test demonstrated that the wine produced in continuous mode with cells immobilized on quince had a pleasant and soft aroma, as well as fruity taste. Moreover, the use of quince pieces as immobilization support can be considered more appropriate as the residual sugar (1.6 g L^{-1}) content in the produced wine was much lower compared to the residual sugar content when apple cuts (11.7 g L^{-1}) were used.

Continuous MLF was also studied by Crapisi et al. (1987) using *Lactobacillus* cells immobilized on *k*-carrageenan gel. The ICS functioned for 46 d at temperatures between $7 \text{ }^\circ\text{C}$ and $40 \text{ }^\circ\text{C}$. The lowest (1.8 g L^{-1}) and the highest (4.2 g L^{-1}) values of produced lactic acid were obtained at $7 \text{ }^\circ\text{C}$ and $30 \text{ }^\circ\text{C}$, respectively.

Delignified cellulosic material was used as a support for cell immobilization and further applied in continuous process of winemaking (Iconomou et al., 1995). Wine productivity was six fold higher than in a traditional process. The ICS had an operational stability for 2 months. The alcoholic content of the produced wine was in the range of 9.4% and 11.2%; however, the residual sugar content was still high in the range of 19.6 g L^{-1} and 52.3 g L^{-1} .

Genisheva, Mota, et al. (2014), for the first time demonstrated an integrated continuous process of winemaking including sequential alcoholic and malolactic fermentations.

The cells were immobilized on grape skins and packed in a packed bed reactor. The system worked for one month without contaminations and without losing its biological activity. The results showed that continuous winemaking with immobilized cells was much more efficient compared to a batch winemaking with immobilized cells (4 times more efficient in the case of alcoholic fermentation and 17 times in the case of malolactic fermentation). Genisheva, Mota, et al. (2014) and Genisheva, Vilanova, et al. (2014) confirmed that 1 L of wine was produced with immobilized cells in batch mode of operation for 4 d, while in continuous mode for 24 h.

Wine production with immobilized cells, accordingly to the existing data, is mostly carried out in the batch mode of operation. The main reason is associated to seasonality of the raw-materials, the grapes, and therefore the winemaking process. However, the secondary alcoholic fermentation (e.g. for sparkling wines) and malolactic fermentation may be conducted all year around. Moreover, primary alcoholic fermentation could be produced, at any time, from preserved musts (high SO₂ doses, low temperature). Accordingly, complementary work should be carried out for better understanding the operation with immobilized cells in continuous mode of operation, and therefore to evaluate the possibility of implementing such a process in the winemaking industry.

Conclusions

Until now, the use of immobilized cells in winemaking is mostly limited to investigation purposes. Nevertheless, in the last decades is noticeable the increased number of works about the use of immobilized cells for winemaking. The focus is on the search of natural support for cell immobilization, which will not interfere negatively to the quality of the final product. Most of the published data is about batch alcoholic fermentation. Few researches exist on malolactic fermentation of wine with immobilized cells. Moreover, most of the published work refers to a batch processes of fermentation. The successful implementation of continuous immobilized cells fermentation processes on wine production demands more investigation to be done on the final quality of the wine produced.

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