Chapter 9

Yeasts as Biocatalysts

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9.1 Introduction

When looking at a biological reaction (a reaction due to a microorganism) it appears clearly that it can take place in two different ways:

– A system where the microorganisms are suspended in the medium (here considered as homogeneous): that is the case of the fermentations in winemaking or in brewing, for example.
– A system where the microorganisms are not free in the medium but where they are attached to a support, giving a two-phase system. Such a system is illustrated in nature by biofilms, for example.

In the controlled use of microorganisms in an industrial frame, like alcoholic fermentation, free cells of microorganisms were used first, surely because of tradition, which existed prior to knowledge of the biological nature of the reaction. In oenology, and also in the major part of industrial fermentation processes, this concept of free cells is largely pre-eminent. In some others fields of applications, in contrast, as early as 1960s the purpose was to attach the microorganisms and some industrial applications quickly followed the conceptual developments: electrodes with attached enzymes, bacterial beds in water treatment, adsorbed bacteria for vinegar production, etc.

Thus it appears quite logical to ask:

– Why should the microorganisms be attached?
– How should it be done?
– What interest and what applications are there for the immobilized yeasts in the food industry?

9.1.1 Why Attach the Microorganisms?

In the fermentations in the food industry the main expected interests in the immobilization of microorganisms are first the increase of the reaction rates due to the high cell concentration, second an easier operation in continuous mode and third

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages

the easy separation of the microbial cells at the end of the fermentation step. Moreover it allows the reuse of the catalyst for some cycles of production. As it becomes possible to increase the reaction rates it is also possible to have better control of the reaction. Indeed the reaction rate is the product of the specific rate of a cell and the number of cells. To increase the reaction rate it is possible to increase the specific rate, the number of cells or both together. Increasing the specific rate requires giving the microorganism the best environmental conditions, but these conditions are rarely those encountered in the practice of industrial fermentations (low pH, high sugar concentration, alcohol) where the inhibition or limitation mechanisms generally dominate. In contrast, the sole apparent limitation to the increase of the number of microbial cells is the saturation of the culture medium. However, it is worth noting that the microorganisms may slightly modify their metabolism when submitted to excessive conditions of cellular concentrations.

Also some works deal with the immobilization process in order to allow good control of different microorganisms working together (co-immobilization). This co-immobilization may also concern microorganisms of the same kind (for example, different yeast genus or species), very different microorganisms (yeasts and bacteria, for example) or a microorganism and an enzyme. This co-immobilization may be realized using different processes: adsorption, entrapment or membrane retention.

9.1.2 How to Immobilize the Microbial Cells?

The immobilization of microbial cells may be carried out by different methods which (for most of them) are based on the methods initially proposed for the immobilization of enzymes by Chibata (1979). These methods may be classified in four categories: adsorption, covalent union, inclusion and retention of microbial cells without a support.

Each of these methods will be briefly presented and their applications to the use of yeasts in the food industry will be developed.

9.1.2.1 Adsorption

The method based on the adsorption phenomena of microbial cells on a support is certainly the oldest method used in the food industry (Linko and Linko 1984). The adsorption is the result of electrostatic attractions between the support and the microbial wall, which is charged negatively (Kolot 1980). The supports used can be of very different natures: wood, bricks, PVC, silica, bentonite, fragments of vegetables. The affinity of a microbial cell to a support varies depending on each microorganism/support pair and is very difficult to forecast. Generally yeast cells have better adsorption characteristics than bacterial cells (Navarro 1980).

Adsorption is a reversible phenomenon that depends on the age of the cells, cellular wall composition, pH and ionic composition of the medium. A desorption phenomenon can take place, leading to a hybrid system where the free cells are associated to immobilized cells. However, this desorption can be compensated with the growth of the microorganism cells on the support, which induces a permanent regeneration of the “biocatalytic system”.

In the recent works dealing with the immobilization of yeast cells the supports most used for the different applications in winemaking, brewing, ethanol production, etc. are cellulose and cellulose derivates (Koutinas et al. 1995; Bardi et al. 1996; Viljava and Lommi 2000), (diethylamino)ethyl (DEAE) cellulose (Lommi and Ahvenainen 1990; Linko and Kronlof 1991; Kronlof and Linko 1996; Andersen et al. 2000), fragments of vegetables (apple cuts, Kourkoutas et al. 2002b; dried raisin berries, Tsakiris et al. 2004; cane stalks, Chen 2001; pieces of figs, Bekatorou et al. 2002), gluten pellets (Smogrovicova et al. 1999; Bardi et al. 1997a), wood chips (Linko et al. 1998; Pajunen et al. 2000; Viljava and Lommi 2000), quince (Kourkoutas et al. 2003), minerals such as aluminium (Loukatos et al. 2000), ultraporous fired bricks (Opara and Mann 1988), kissiris (a glassy volcanic rock; Bakoyianis et al. 1993), ceramics (Zhang et al. 1992; Horitsu 1993; Cheng et al. 2000), porous glass (Kronlof and Linko 1992; Breitenbuecher and Mistler 1994; Yamauchi et al. 1994; Kronlof and Linko 1996) and silicon carbide (Masschelein and Andries 1996; Tata et al. 1999) and a flocculent strain has been absorbed into a sponge (Scott and O'Reilly 1995).

The adsorption is obtained by keeping the microbial suspension in contact with the support. After incubation the free cells are eliminated by several washings of the support as explained by Kourkoutas et al. (2002b) for yeast cells onto apple cuts.

As far as the industrial applications are concerned, the main fields are winemaking, brewing and alcohol production. Nevertheless, even though a lot of work has been done, few of the industrial applications are being used at this time.

9.1.2.2 Immobilization by a Covalent Link

In order to avoid the desorption phenomenon it is possible to establish true covalent binding between the microbial cell and the support. This is done using a union agent and the support is then called an "activated" support. Glutaraldehyde is the most commonly used agent, especially when the supports are made of proteins (Phillips and Poon 1988). In this way, the attachment becomes irreversible and the biocatalyst offers great stability. But the union agents are generally highly toxic against the microbial cells and induce a decrease of their activity. That is why this method of immobilization is no longer used for the immobilization of microbial cells, but it remains interesting for the immobilization of enzymes.

9.1.2.3 Inclusion

In this system the microbial cells are incorporated in the matrix of a more or less rigid polymer. These polymers are synthetic, such as polyacrylamide or cellophane, but they also can be made of proteins (gelatine, collagene) or polysaccharides (cellulose, alginate, agar, carragheenans, etc.). Inclusion is a technique that is easy to manage and it leads to products having good stability but weak mechanical resistance. In some cases this weakness may be a problem (mechanical stirring of the reactors, growth of microbial cells into the matrix leading to the breaking of the matrix) and could be a disadvantage for long-term continuous operation as discussed for brewing by Virkajarvi (2001). Also the polymer may be a limiting factor for the free

diffusion of the solutes or gases required (or produced) by the microorganism (Hannoun and Stephanopoulos 1986). It is considered that diffusion is not the limiting step for compounds having a molecular weight of less than 5,000. It is obvious that for use in the food industry the support has to be safe for the consumer (stable and non-toxic support) and is allowed by the regulation in force for the specific use considered. At this time immobilization by inclusion is the most widespread process and thus it is interesting to present the main supports used.

The main materials for inclusion of microbial cells are:

- κ-Carragheenan: This polysaccharide extracted from marine microalgae is commonly used as a food additive. It jellifies when the temperature reduces (room temperature) after the dissolution obtained by heat treatment (60–80°C). Different works dealt with the characterization of the optimal conditions to immobilize the cells into the gel and it was observed that the mechanical strength increased with increasing carragheenan concentration, corresponding to a decreased cell release. To avoid the cell release, which may be a major disadvantage in some cases, Nunez et al. (1990) proposed to treat the beads with $Al(NO_3)_3$; this treatment was shown to be efficient to induce gel hardening but it was observed that cell viability and diffusion were reduced. Recent examples of the use of carragheenan beads with 4.5×10^9 cells mL^{-1} in brewing were given by Mensour et al. (1996) and Pilkington et al. (1999).
- Agar and agarose: Agar is a polymer issued from some marine macroalgae and agarose is obtained from agar by separation and purification. The gelation is induced in the same way as for carragheenan. Even though the procedure to immobilize cells in this polymer is simple, it is not widely used owing to the low mechanical strength that makes this gel unstable compared with alginate or carragheenan.
- Chitosan gel: This polysaccharide is obtained from chitin extracted from crustacean cells. Its gelation occurs by an ionotropic reaction like alginate. It was used quite early but it appeared that acid-soluble chitosan affected cell viability and so that it is not used for cell entrapment, except in some cases to coat alginate beads to avoid cell release.
- Poly(vinyl alcohol) (PVA): PVA can form a gel when treated with UV radiation but this causes cell death. Also it is possible to make a gel by treatment with boric acid, but this acid may be toxic for some microorganisms. It is also possible to induce gelation by some freeze–thaw cycles, and recently Martynenko et al. (2004) proposed a process for champagnizing involving the use of champagne yeasts immobilized into PVA cryogels (PVACs). At this time this polymer does not appear to be used much for cell entrapment.
- Calcium alginate gel: Alginic acid is a complex heteropolysaccharide extracted from some species of algae. Its gelation is obtained by contact with a calcium solution. Its composition varies a lot depending on the source, and the nature of the alginate must be well defined when making the beads. Indeed the rheological properties of the gel depend on the composition of the alginate (sequences and ratios of L-glucuronic acid and D-mannuronic acid; arrangement of the monomers).

At this time, to our knowledge, this polymer is the one most commonly used for cell entrapment in the food industry: for example, calcium alginate beads

have been retained with a capacity of 1.2×10^9 cells mL^{-1} of gel beads (Linko and Linko 1981; Hsu and Bernstein 1985; Patkova et al. 2002). Indeed, this polymer is already allowed by the regulations for some applications in the food industry, such as a binding or thickening material, and also has good mechanical properties. The method is as follows. A solution containing yeast cells and alginate is driven to a solution of calcium chloride. A mechanical device allows the continuous film to be separated into droplets. As soon as the alginate meets the calcium chloride solution it gels quickly giving small spheres (1.5–2-mm diameter) called beads. In these beads the yeast cells are held prisoner in the frame of the alginate gel. The first works dealing with the use of these kinds of beads rapidly showed that the cells entrapped at the surface of the beads were able to multiply and thus to release free cells into the medium. In order to avoid this phenomenon, it was proposed (1980s) to make an external layer of sterile alginate using two concentric pipes. The internal pipe brings the alginate–yeast cell solution, while the external pipe brings the solution of sterile alginate. The critical points are the regularity of the spheres, the continuity of the external layer and the firmness of the gel (depending on the nature and the concentration of the alginate and the residence time in the calcium chloride solution). It is undoubtedly the method of inclusion which presents the most advanced industrial applications, particularly for some steps in winemaking. Recently a method to obtain dry double-layered beads was developed by Proenol (Portugal) and so it is possible to find on the market an industrial product which is easy to carry, with a long storage life (2 years) and easy to use (no problem of adhesion of beads to the walls of pipes or bottles as was the case with the wet beads).

9.1.2.4 Cell Retention Without External Support

It is also possible to increase the microbial cell concentration by using a natural process such as flocculation or by confining the cells to a part of the reactor by way of a membrane. Flocculation is a natural phenomenon resulting in cell aggregation. It involves the setting up of ionic bonds between sites of cell wall and cations of the medium. In some cases, these ionic bonds are strengthened by the production of filaments at the surface of the yeast cells (Teixeira 1988). But not all the cells are able to flocculate and also natural aggregates are often unstable and sensitive to the shear. Nevertheless, this spontaneous mechanism of flocculation is used in the waste-treatment process (activated sludge).

In the field of fermentations for the food industry the main applications at this time concern alcohol production (Zani-floc process in Brazil), some kinds of beers, and sparkling wine making (second fermentation). In the case of confinement, the free cells of the microorganism are kept in a part of the reactor thanks to a membrane or are retained inside a hollow fibre. This device makes it possible to reach a very high cell concentration (more than 100 g dry weight L^{-1}). For sure the membrane is needed to be freely permeable to solutes and gases. The great interest in this process is that it leads to a sterile medium at the end of the fermentation step. Different bench tests were made for alcoholic beverage production using a device

coupling fermentation and ultrafiltration or using a specific hollow fibre device for sparkling wine making (Jallerat et al. 1993) but to our knowledge none of them are being used at the industrial level.

9.1.3 Impact of Immobilization on Cell Physiology and Fermentation Activity

The possible effects of immobilization on cell activity were studied early on and the different authors reported very different conclusions. In fact, physiological reactions of cells vary depending on the method of immobilization used. For example, it is clear that the entrapment or adsorption of cells results in changes in their micro-environment and thus affects their metabolism. Anyway, in all the cases of cell immobilization, the main factor which likely influences the cell behaviour is the mass transfer limitation (Onaka et al. 1985) as it results in gradients of oxygen, substrates and products. Thus, immobilization may influence the cell physiology and activity via a lot of mechanisms which are still poorly characterized and which may act in opposite ways. For sure the effects depend on the immobilized complex size and the type and the concentration of the polymer or the matrix used for the entrapment or the adsorption.

9.1.3.1 Mass Transfer Limitations and Cell Physiology

Different studies led to different conclusions so it is quite difficult to have a clear view. As far as entrapped yeast cells are concerned, it appeared from different studies quoted by Groboillot et al. (1994) that the size of the beads may act as well as the alginate molecular weight, the ratio between guluronic and mannuronic acids (G/M) and the alginate concentration, and, for example, it was observed that the ethanol productivity of immobilized yeast cells increased when the alginate concentration or G/M decreased. This was explained by the fact that a weak gel probably facilitates the transport of substrate and product, thus enhancing the cell activity. It was also observed that mass transfer limitations may be due to cell concentration (or growth). Nevertheless, it seems well accepted by many authors quoted by Martynenko and Gracheva (2003) that respiratory and fermenting activities are higher in immobilized cells than in free cells.

But the use of an immobilized cell system may also be an easy way to reduce glucose inhibition. In most fermentation processes the diffusional limitations are generally recognized as a major disadvantage since they reduce the glucose uptake, but they may also be beneficial for the uptake of other sugars which are repressed by the glucose level. This was proved by Willaert (1999): studying the brewing by immobilized cells in calcium alginate, he established that the glucose concentration was high in the outer layer of the gel matrix but went to zero in the core of the gel. The cells which were located on the surface of the gel consumed most of the available glucose and the maltose (or maltotriose) uptake was repressed because of the glucose content. In contrast, cells located in the core were not repressed by glucose and thus maltose uptake was not repressed. The author suggested that the same mechanism may act for amino acid uptake.

9.1.3.2 Effect of Immobilization on Cell Morphology

Many changes in the morphology of yeast cells entrapped in calcium alginate were described by different authors. The well-documented review of Martynenko and Gracheva (2003) quotes studies dealing with the physiological activity and morphological changes of immobilized cells in the special case of the Champagne process. It was said that the adaptation of yeast cells to immobilization was accompanied by vacuolization. Also the thickness of the cell wall increased and ribosomes became scarcely visible. It was also shown that the specific rate of cell division was very low: the yeasts proliferated at the periphery of the bead, while the number of cells in the core remained constant.

9.1.3.3 Effect of Immobilization on Cell Physiology

Fumi et al. (1994) established that immobilized cells of *Saccharomyces cerevisiae* in alginate beads showed some alterations during alcoholic fermentation: they observed that the percentage of phosphomonoesters with respect to total phosphorous increased from 1.8% for free cells to 30.8% for immobilized ones and that the polyphosphates contents were, respectively, 56.7 and 22.6%. Grego et al. (1994) noticed that the immobilized cells of *S. cerevisiae* exhibited a slightly increased ethanol stress resistance and explained it by the impact of the ethanol stress on the fatty acid composition being smaller in the immobilized cells than in the free cells. For the enzyme activity Sarishvili and Kardash (1980) noticed that yeast cells immobilized on solid supports exhibited a greater activity of some enzymes (NAD or NADP-dependent glutamate dehydrogenase, alcohol dehydrogenase and malate dehydrogenase) than suspended cells and thus they suggested that a process using immobilized cells could be carried out at lower temperatures and shorter times.

9.1.3.4 Immobilization and Effects on the Product

Concerning the composition of the product after the use of immobilized cells it is generally assumed that there are not great differences with the product obtained with suspended cells (Busova et al. 1994). Jallerat et al. (1993) compared the second in-bottle fermentation using free cells or cells retained in a hollow fibre cartridge: they did not note any difference in the time needed for the fermentation and in the organoleptic qualities of the wines. Recently Tsakiris et al. (2004) assumed that the wines obtained using yeast cells immobilized onto raisin berries had the same aromatic profiles as the wines obtained using free cells even though, according to Balli et al. (2003), the glycerol content was slightly higher in wines obtained with immobilized cells on delignified cellulosic material and gluten pellets than in wines obtained with free cells. Studying the fermentation of white wines using different sorts of immobilized yeast cells, Yajima and Yokotsuka (2001) established that the concentrations of undesirable products (methyl alcohol, ethyl acetate, etc.) were lower in wines made using immobilized cells (in alginate beads) than in wines produced with free cells. But Bardi et al. (1997b) observed that the immobilization of yeast cells on delignified cellulosic material or gluten pellets led to a higher production

of ethyl acetate (compared with free cells). The same behaviour (greater ethyl acetate production) was observed with yeast cells immobilized on a kissiris support (Bakoyianis et al. 1993). So it seems obvious that it is difficult to draw a strong conclusion and that the effects of the immobilization on sensory evaluation of the product depend on the field of application and on the sort immobilization process. As an example of this we can quote the work of Ageeva et al. (1985): they noticed that yeast cells immobilized on different clay materials did not behave the same in regard to the volatile product synthesis.

9.2 Immobilized Yeast Cells and Winemaking

In winemaking different possibilities for the use of immobilized yeast cells have been described. Surely this area of application is the one where most work has been done. To analyse these data we can classify them according to the step in winemaking where the immobilized cells act: demalication of must (or wine), alcoholic fermentation and treatment of sluggish or stuck fermentations, in-bottle fermentation in sparkling wine making.

9.2.1 Demalication of Musts or Wines

L-Malic acid is one of the two main acids in musts and its concentration depends on grape variety and climatic conditions. Winemakers often rely on malolactic fermentation (MLF) to deacidify the must and thus to achieve the biological stability of the wine as well as to ensure good organoleptic qualities. MLF is performed by lactic acid bacteria (*Oenococcus oeni*) and many factors such as low pH and sulphur dioxide level could affect these bacteria and, in some cases, this MLF becomes impossible. The yeast *Schizosaccharomyces pombe* has been proposed as an alternative to MLF but it was quickly proved that a too important development of these yeasts leads to some off-flavours. So, some wineries have set up a two-step process: first the must is inoculated with *Schiz. pombe* for the consumption of L-malic acid and in a second step the must is inoculated with a selected strain of *Saccharomyces* in order to achieve the alcoholic fermentation. However, it appeared that the complete elimination of free cells of *Schiz. pombe* was not possible and that the risk of obtaining some off-flavours was always present. So, a process based on the use of immobilized cells of *Schiz. pombe* has been proposed. The first works of Magyar et al. (1987) established clearly the feasibility of the process using cells of *Schiz. pombe* entrapped in alginate beads. Later, Taillandier et al. (1991) and Ciani (1995) analysed a continuous process using immobilized cells of *Schiz. pombe*. But all these experiments were made at a laboratory or pilot scale owing to the impossibility to obtain and to store great quantities of entrapped cells of *Schiz. pombe*. More recently Silva et al. (2002b, 2003) described experiments using cells entrapped in dried double-layered alginate beads (see Sect. 9.1) on a laboratory scale as well on a winery scale. The beads were placed into nylon bags and these bags were poured in the fermentation tank and shaken daily to agitate the cells and improve the diffusion of solutes and the release of carbon dioxide. To stop the deacidification reaction at the desired level it was enough to remove the bags from the tank and to add to the tank the suitable

strain of *S. cerevisiae* to achieve the alcoholic fermentation. It was shown that the process was efficient and led to wines of good quality. Also the reuse of these beads was studied and it was shown that they maintained good activity for at least five cycles. The process developed by the Portuguese company Proenol to produce these beads of entrapped cells of yeasts makes it possible to store them for more than 2 years without any loss of activity. Thus, it can be concluded that this process is now well established and ready to be used in wineries. Some experiments were done using these entrapped cells of *Schiz. pombe* on wines (red or white) after alcoholic fermentation (unpublished data) and the first results were promising.

9.2.2 Alcoholic Fermentation

Many papers deal with the use of immobilized cells of yeasts (generally *S. cerevisiae*) to achieve the alcoholic fermentation of musts (red or white). The main purpose is always to ensure better control of this important step in winemaking: low-temperature fermentations, improvement of organoleptic characteristics, increase of reaction rates, good achievement of sugar consumption, etc.

Gorff (1988) patented a process using yeast cells immobilized on derivatized cellulose and later Divies et al. (1990) patented a process to entrap the yeast cells in calcium alginate beads. The same year Sarishvili et al. (1990) described a "technology for manufacture of dry red wines with immobilized yeast": the cells were immobilized on beech, oak or polyethylene and the authors observed that the quality of wines was improved. Malik et al. (1991) then tried ten different strains immobilized in alginate and noticed a reduction in their acidification potential compared with that of unbound cells. But most of the studies on this subject are due to the Department of the Chemical University of Patras (Greece). As early as 1992 Bakoyianis et al. (1992) published a paper dealing with the use of a psychrophilic and alcohol-resistant yeast strain immobilized on kissiris in a continuous process for making wine at low temperature. Later they showed (Argiriou et al. 1996) that this yeast strain was more efficient if some preservation treatments at 0°C were made. Bardi and Koutinas (1994) described experiments where different supports were tested as well as different conditions of fermentation: immobilization of cells on delignified cellulose and use of them in 55 repeated batch cultures at low (10°C) or room (30°C) temperature: the main result was that the fermentation rates are increased (threefold) compared with those for free cells. Also the stability of the biocatalyst was proven. Bakoyianis et al. (1998) using cells of *S. cerevisiae* immobilized on different supports (alumina, kissiris and alginate) compared the volatile by-products obtained at different temperatures in a continuous process. It was observed that the levels of 1-propanol, isobutyl alcohol and amyl alcohols were less than those synthesized by free cells for all supports and temperatures studied. But the most original studies from this group dealt with the immobilization of yeast cells onto supports such as apple pieces or raisin skins. Kourkoutas et al. (2001, 2002b) proposed using a psychrophilic and alcohol-resistant yeast strain immobilized on apple cuts for speeding up the fermentation. They noticed excellent taste and aroma of the wines produced and concluded that this process could be accepted by the industry for scaling up the winemaking process. Tsakiris et al. (2004) immobilized yeast cells

on dried raisin berries and obtained good stability of the device. The wines were not different from those obtained using free cells.

All these data show clearly that the alcoholic fermentation in winemaking may be realized without any damage by using immobilized cells of the suitable yeast strain, and a batch process as in the continuous process. But the main problem in developing these processes to an industrial scale is linked to the legislation for winemaking. At this time the sole support clearly allowed (for some applications) is calcium alginate gel. Also the continuous process is not allowed for all types of wine, but that is another problem.

9.2.3 Treatment of Stuck and Sluggish Fermentations

Sluggish and stuck fermentations are some of the most challenging problems that can occur during the winemaking process. The causes can be attributed to nutritional deficiencies of the must, the presence of high levels of inhibitory products, inadequate temperature, and residual toxic products. Although the causes are numerous, the main result is the decrease of cell growth, fermentative activity and viability of the yeast population. To try to reinitialize the fermentation an inoculation of activated yeast cells into the fermentation tank is usually carried out, but this procedure is not always efficient. Silva et al. (2002a) suggested the use of yeast cells immobilized into double-layered alginate beads. The results obtained on a laboratory scale as well as at the winery level showed very good efficiency of the device to treat the stuck fermentations. The great success of these immobilized cells can be explained by an adaptation of the cells to high concentrations of alcohol during the immobilization step.

9.2.4 Special Applications

9.2.4.1 Sweet Wine Making

Sweet wines are wines where the fermentation is stopped before the complete utilization of sugars. At this time to stop the fermentation activity of yeast cells in this kind of winemaking strong quantities of sulphur dioxide are employed, but this product has some disadvantages for consumer safety and the aim of the legislation is to reduce its content in wine. The use of immobilized cells was investigated by some researchers in order to find another solution for making sweet wines as it made it possible to stop the reaction by removing the particles (containing the cells) from the medium. Okuda et al. (2001) described an original process: the fermentation was carried out by immobilized yeast cells which were removed from the medium at the desired level of alcohol (or remaining sugar). In order to ensure the microbial stability of the wine they added an antimicrobial substance isolated from paprika seeds. In this way, they obtained a very stable sweet wine with no viable cells. Kourkoutas et al. (2004) suggested producing semi-sweet wines by using cells of *Kluyveromyces marxianus* immobilized on delignified cellulosic material, quince or apple pieces. The fermentation was run at high temperature and 3–4% of alcohol was synthesized. The final alcohol level was obtained by the addition of potable

alcohol to the fermented must. In these conditions it was said that the semi-sweet wine obtained showed good flavour and aroma and may be blended with other products to improve their quality. This idea to use immobilized yeast cells other than *Saccharomyces* was already developed by Crapisi et al. (1996). It was expected that the bad alcohol production activity of this kind of yeast and also its ability to produce aroma could lead to a partially fermented and aromatic beverage. It was established that it was effectively possible to carry out the fermentation by immobilized apiculate yeast species and that the wine bouquet was not affected by the use of these yeast species. Silva et al. (2002c) described a process to obtain sweet wines by using cells of *S. cerevisiae* entrapped in double-layered alginate beads and showed that it was a great way to make such wines. The process was tested in wineries. For sure the must had to be prepared in order to have a very small population of indigenous yeast cells. The beads containing the cells of the selected yeast strain were placed in nylon bags so that they were easy to remove from the tank after they had worked. When the desired level of remaining sugar was reached, the bags were taken out of the tank and the wine was stabilized to prevent the further development of free cells. As a conclusion, it appears clearly that the use of immobilized cells associated with a treatment of stabilization can be an efficient and easy way for making sweet wines with reduced sulphur dioxide contents.

9.2.4.2 Sparkling Wine Making

In sparkling wines according to the traditional method, which is the method used for the Champagne process, the problem is to eliminate yeast cells without taking the wine out of the bottle at the end of the in-bottle fermentation. Traditionally it is made by the so-called operation of *remuage*, which requires special know-how.

Thus, for more than 20 years, other solutions have been sought and tested and there is certainly the field of winemaking, where the possible use of immobilized cells has been investigated the most. For sure the simplest method would be to filter the wine from one bottle to another, but if this is done, the wine is not allowed to be called Champagne.

For the last 20 years, entrapped cells have been tested. All the first experiments were done using homogeneous alginate and cell beads. Obviously, the results were quite bad, for it was always observed that yeast cells were able to escape the bead and to grow in the medium, which resulted in a troubled wine. But as early as the 1990s double-layered alginate beads were tested (Zamorani et al. 1989; Crapisi et al. 1990; Godia et al. 1991). All the studies made clear that the use of entrapped cells in double-layered alginate beads led to a perfectly clear wine and that there was no difference with a wine obtained following the traditional method. Nevertheless it must be noted that these applications remained at a laboratory or a pilot scale, except for a quite large-scale application by Moët et Chandon (France). This was due to the difficulty to produce regular and easy-to-use alginate beads at an industrial level. As an example, using a laboratory apparatus we (in our group in 1992) were only able to make 500 g of wet beads per day. Moreover wet beads were difficult to place in the bottle as they stuck to the walls of pipes. Also a special machine was needed to put a constant and fixed quantity of the beads in the bottles. From this point of

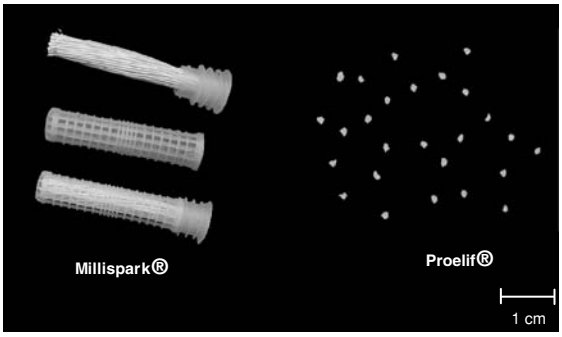

Fig. 9.1. The Millispark device (*left*) and dried double-layered beads (Proelif) (*right*)

view, great progress was made recently (1997) by Proenol (Portugal) and our laboratory working together. So, at this time we are able to produce more than 35 kg of dry beads per hour. These beads are dried so that they can be stored before use for more than 2 years without any loss of activity and also they are easy to put in the bottle thanks to a machine which was developed at the same time.

Another attempt was to place yeast cells in a sort of little cartridge with a membrane or some hollow fibres which separates the cells from the medium: the concept is like a tea bag. This device (called Millispark) developed by Millipore in 1993 (Jallerat et al. 1993) has proven to be very efficient but its development at the industrial level was not possible owing to a too high price.

Figure 9.1 illustrates the Millispark device and dried beads (Proelif) produced by Proenol.

9.3 Ethanol Production

More than 2×10^{10} L of pure alcohol is produced in the world each year and half of that is made in Brazil from sugar cane. It is without doubt the most important fermentation process, and as such the most studied. This alcohol, obtained from the distillation of different kinds of wines, is for the most part used for industrial purposes, such as additives for fuel, solvents for pharmaceutics or food ingredients. The wines to be distilled come from different substrates: sugar (sugar cane or beet root), hydrolysates of starch (maize, wheat or rice), sugar from industrial waste such as lactose from whey or sugars from biomass such as xylose, cellulose or hemicellulose. For sure the main challenge of the fermentation process is in this case to reach the best yield (alcohol produced/sugar used) as well as the highest reaction rates (in order to maximize the use of the fermentation tanks). In contrast to what we observed for the production of drinks, no attention is paid to the organoleptic quality of the wine. The most important thing is to avoid the synthesis of secondary products which can affect the yields and also the distillation process. For these different reasons it is obvious that a lot of studies have been made for 20 years in order to increase the yields, the reaction rates and to minimize the operational costs.

Among these studies a lot deal with the use of immobilized cells. Different processes using different kind of substrates, different kinds of yeasts and also different kinds of apparatus have been described and we will focus here on the most familiar ones or on those developed at the industrial level.

9.3.1 Alcohol from Sugar (Sucrose)

Saccharose (or sucrose) is the main component of sugar cane or sugar beet root. It is extracted from the plant by grinding and water diffusion and the medium obtained contains 120–140 g L^{-1} of sugar. It appears well established that the immobilized cells are more efficient than the free ones: Sree et al. (2000) using a repeated batch fermentation system (*S. cerevisiae* immobilized in alginate beads) noticed that more ethanol was produced by immobilized cells compared with free cells. The maximum amount of ethanol produced by immobilized VS3 cells using 150, 200 and 250 g L^{-1} glucose was 72.5, 93 and 87 g L^{-1} ethanol at 30°C. Using immobilized yeast cells some authors compared the method of immobilization as well as the efficiency of the reaction according to the process used. For example, we can quote the work of Goksungur and Zorlu (2001): they compared the continuous production of ethanol from beet molasses by calcium alginate immobilized *S. cerevisiae* in a packed-bed bioreactor to that obtained in a continuous stirred reactor. They showed that (with a temperature of 30°C and a dilution rate of 0.22 h^{-1}) maximum ethanol (4.62% v/v), yield (0.43 g g^{-1}) and volumetric productivity (10.16 g L^{-1} h^{-1}) were obtained from the beet molasses medium containing 10.90% (w/v) total sugar with 2.0–2.4-mm diameter beads prepared from 2% (w/v) sodium alginate solution. At higher substrate concentrations, substrate was recirculated through the packed-bed bioreactor to increase yields and to decrease residual sugar content. The bioreactor system was operated at a constant dilution rate of 0.22 h^{-1} for 25 days without loss of capacity. In the continuous stirred bioreactor (compared with the packed-bed bioreactor) lower ethanol concentration (3.94% v/v), yield (0.36 g g^{-1}) and productivity (8.67 g L^{-1} h^{-1}) were obtained. Dealing with the continuous fermentation of sugar cane syrup using immobilized yeast cells (*Saccharomyces* sp.) onto chrysotile (fibrous magnesium silicate) in a packed-bed reactor, Wendhausen et al. (2001) showed that the activity of the cells was higher when immobilized, mainly for fermentation of 30–50% w/v glucose solutions. In medium containing 30% w/v glucose, the initial fermentation rate increased 1.2–2.5 times. The yields were in the range 0.41–0.49 g g^{-1} for the immobilized cells and 0.37–0.43 g g^{-1} for the free cells. An average productivity of 20–25 g L^{-1} h^{-1} was obtained in the first 20 days and an average of 16 g L^{-1} h^{-1} was obtained after 50 days of operation. In order to increase the efficiency of immobilized cells, Nagashima et al. (1983) suggested adding some ergosterol and oleic acid to the alginate matrix. In this way they were able to increase the ethanol content of the medium to 57 g L^{-1} instead of 47 g L^{-1} in the same operating conditions but without sterol addition. An example of the use of immobilized cells for ethanol production from molasses on an industrial scale was given by Shi et al. (1995): yeast cells, suspended in the low concentration sodium alginate solution, were immobilized on the fluffy chemical fibre matrix to initiate the associated immobilization. Under factory conditions (four fermentors of 6.5 m^3), the ethanol

production was carried out continuously for 99 days by flowing diluted molasses (16.5–18% w/v sugar), resulting in an ethanol productivity of 6.21 g L^{-1} h^{-1} and an average ethanol concentration in the fermented mash of 9.44% (v/v). It was also proved by Murakami and Kakemoto (2000) that sodium alginate was a better support than κ-carragheenan gel because of its better mechanical strength.

But a natural phenomenon such as flocculation was also used at the industrial level in order to increase the efficiency of the process: Xie et al. (1999) described an industrial plant composed of four air-lift suspended-bed bioreactors in parallel with a total volume of 400 m^3 using cells able to self-flocculate. The process ran for more than 6 months in continuous operation: the effluent contained 70–80 g L^{-1} of ethanol and less than 5 g L^{-1} of residual sugar and an ethanol productivity of 7–8 g L^{-1} h^{-1} was achieved (to be compared with 2–4 g L^{-1} h^{-1} usually observed in classical process).

Studies were also made to appreciate the possible use of yeasts other than *Saccharomyces* and also different immobilization matrices: for example, Gough et al. (1998) analysed the production of ethanol from molasses (140 g L^{-1} sugar) at 45°C using a *Kluyveromyces marxianus* strain immobilized in calcium alginate gels and PVAC. The immobilized cells were used as a biocatalyst in fed-batch reactor systems for prolonged periods. When each system was operated on a fed-batch basis for a prolonged period of time, the average ethanol concentrations produced in the alginate- and the PVAC-immobilized systems were 21 and 45 g L^{-1}, respectively, while the yields remained high (0.41–0.45 g g^{-1}). The results suggested that the PVAC-based immobilization system might provide a more practical alternative to alginate for the production of ethanol by *K. marxianus* IMB3 in continuous or semi-continuous fermentation systems. Love et al. (1998) also tried a mixed matrix made of alginate and kissiris with the same yeast strain and reported good efficiency of the system.

If alginate seems nowadays the most used matrix for immobilization of yeast cells for alcoholic fermentation it must be noted that some different matrices were reported as more efficient. Zhang et al. (1996) suggested that the properties of the ceramic supports compared with those of a calcium alginate gel indicated that the ceramics were the better of the two types of material and had potential for industrial application. Harris and Ghandimathi (1998) immobilized yeast cells of *S. cerevisiae* in a natural rubber coagulum and used them for repeated batch fermentation of molasses. The authors pointed out the fair stability and efficiency of the process and that rubber was inert compared with alginate.

However, the use of a reactor with a very high level of immobilized cells may also have some negative effects and, for example, Yadav et al. (1996) observed that the productivity and the efficiency of a column reactor packed with gel beads might be affected by problems due to gas hold-up and mass transfer effects.

9.3.2 Alcohol Production from Lactose

Lactose is a sugar which may cause environmental damage as it is a major component of whey (waste from the dairy industry). Thus, its use as a substrate for alcohol production was studied early. As *Saccharomyces* sp. are not able to use this substrate the main yeast species used belongs to the genus *Kluyveromyces*. Marwaha

and Kennedy (1985) described a process for the continuous alcohol production from whey permeate using immobilized cell reactor systems. In this process a bioreactor packed with alginate-entrapped *K. marxianus* NCYC179 was used for continuous fermentation of whey permeate to ethanol. A maximum ethanol productivity of 28 g L^{-1} h^{-1} was attained at a dilution rate of 0.42 h^{-1} and 75% lactose consumption (substrate feed rate in the inflowing medium was 200 g L^{-1} lactose). The immobilized cell bioreactor system was operated continuously at a dilution rate of 0.15 h^{-1} for 562 h without any significant change in the efficiency and viability of the entrapped yeast cells (84–81%). More recently, El-Batal et al. (2000) made experiments on whey fermentation by *Kluyveromces* immobilized cells in copolymer carriers produced by radiation polymerization. In this study, yeast cells were immobilized in hydrogel copolymer carriers composed of PVA with various hydrophilic monomers, using a radiation copolymerization technique. Yeast cells were immobilized through adhesion and multiplication of yeast cells themselves by using batch fermentation; the ethanol production was 32.9 g L^{-1}, which was about 4 times higher than that of cells in the free system. Hydrogel copolymer carriers were used in a packed-bed column reactor for the continuous production of ethanol from lactose at different concentrations (50, 100, 150 g L^{-1}). For all lactose feed concentrations, an increase in dilution rates from 0.1 to 0.3 h^{-1} lowered the ethanol concentration in fermented broth, but the volumetric ethanol productivity and the volumetric lactose uptake rate were improved. The fermentation efficiency was lowered with the increase in dilution rate and also at higher lactose concentration in the feed medium, and a maximum of 70.2% was obtained at the lowest lactose concentration, 50 g L^{-1}. More recently, an industrial-scale pilot plant (11,000 L) using kefir yeast immobilized on delignified cellulosic material was described by Athanasiadis et al. (2003): the system showed good operational stability, exhibiting relatively high ethanol yield and ethanol productivity.

9.3.3 Ethanol Production from Starch

Starch is a very abundant substrate but its direct assimilation by yeasts is generally unlikely and thus some pretreatment is often necessary. To avoid this step it is possible to use a specific yeast such as *S. diastaticus* and a process using such a yeast immobilized on wood chip particles was recently described by Razmovski (2000). But it is also possible to use immobilized cells of a good fermenting yeast (*S. cerevisiae*) and immobilized enzymes (glucoamylase) as done by Chithra and Baradarajan (1992) and Giordano et al. (2000): cells and enzyme may be immobilized in separate particles or together in the same particles. However, it appeared that the productivities of these processes were smaller than those for fermentation of glucose-containing solutions.

9.3.4 Ethanol from Other Substrates

Among the substrates having some interest for the production of alcohol, great interest has been devoted to cellulose, hemicellulose and pentoses (xylose). The yeast *Pichia stipitis* appeared as a good species to ferment xylose solutions or hemicellulose

hydrolysates, (Sanroman et al 1994; Liu et al. 2001). *Candida sheateae* was also investigated (Hinfray et al. 1995) and also *Pachysolen tannophilus* (Amin et al. 1988). Chen and Weyman (1989) described a system able to use cellulose directly. In this process, baker's yeast cells were entrapped on glass fibre disks by means of alginate, and the enzymes cellulase and β-glucosidase were precipitated on the yeast cells by tannin. The disks carrying the yeast–enzyme co-immobilizate were installed in a continuous dynamic immobilized bioreactor. Cellulose was added continuously to the bioreactor. In the first few days, the efficiency of the system was good but decreased over the next 5 days to 40%, likely owing to the negative effect of tannin. On this subject of the direct use of cellulose a comprehensive review was made by Chandrakant and Bisaria (1998).

The alcoholic fermentation at a pilot scale from dried sweet potato was investigated by Yu et al. (1994), while Roukas (1994) was interested in the use of carob pod extract as a substrate for the alcoholic fermentation by immobilized cells of *S. cerevisiae* in alginate beads. An interesting substrate may be the Jerusalem artichoke. In this plant the reserves are made of inulin, a polymer of fructose. This polymer may be directly hydrolysed and fermented by *K. marxianus*, and a process using cells immobilized in calcium alginate beads was proposed by Bajpai and Margaritis (1986): the bioreactor was continuously operated with good results (volumetric ethanol productivity of 118 g L^{-1} h^{-1} at a dilution rate of 2.8 h^{-1} and 87% substrate conversion) and its half life was 105 days.

9.3.5 Immobilized Cells and Processes

Because of its economic importance, ethanol production has initiated a lot of studies dealing with the development of specific processes based on the use of immobilized cells.

In order to improve the efficiency of immobilized cell systems, different processes for the fermentation have been analysed for many years. Feng et al. (1989) analysed a continuous fermentation process using *Schiz. pombe* yeast flocs: a suspended-bed bioreactor utilizing air was employed in which the total yeast particles were retained and was allowed to operate over 3 months without interruption. The yeast cell concentration was held at 40 g L^{-1} (dry weight) and a high productivity of 20–24 g L^{-1} h^{-1} was obtained. These *Schizosaccharomyces* yeast flocs may also be used in an immobilized cell reactor separator (ICRS) as described by Dale et al. (1994): an ICRS with gas-phase ethanol product stripping was operated with both sucrose and molasses feeds continuously over 90 days. The feed concentration range was 300–600 g L^{-1}. Using *Saccharomyces* cells, Del Borghi et al. (1985) described a process called rotating biological surface (RBS): a spongy material was employed to trap yeast cells on the disks. In this way, an ethanol productivity of 7.1 g L^{-1} h^{-1} was achieved in the RBS-1CR at a dilution rate of 0.3 h^{-1}.

Many authors developed some apparatus making possible simultaneous bioreaction and separation by a so-called immobilized yeast membrane reactor. Vasudevan et al. (1987) designed a fermentor in which the microbial cells were sandwiched between an ultrafiltration membrane and a reverse osmosis membrane. The ultrafiltration membrane provided free passage for all nutrients which were supplied under

pressure, eliminating diffusional resistance. The reverse osmosis membrane preferentially allowed passage of the product, improving purity and concentration. Ethanol fermentations with *S. cerevisiae* were carried out for 160 h using this reactor with good performances. However, Woehrer (1989) analysed the continuous ethanol production in a three-stage horizontal tank bioreactor (HTR) by yeast cells entrapped in calcium alginate and concluded that "Compared to other continuous ethanol production processes using entrapped yeast cells, the HTR is among the best", and in the same year Shukla et al. (1989) described a novel microporous hollow fibre membrane-based immobilization technique for whole cells making it possible to reach a productivity of 41 g L^{-1} h^{-1} with an initial glucose concentration of 100 g L^{-1} and a yield of 0.45 g g^{-1}. But it is also possible to associate the immobilized yeast cells and a device allowing the elimination of the alcohol (which is a possible inhibitor for the yeast activity). That was done by Shabtai et al. (1991): they developed a system comprising an immobilized yeast reactor producing ethanol, with a membrane pervaporation module for continuously removing and concentrating the ethanol produced. The combined system consisted of two integrated circulation loops: in one, the sugar-containing medium was fed and circulated through a segmented immobilized yeast reactor (the bead matrix was a cross-linked polyacrylamide hydrazide gel coated with calcium alginate), in the other, ethanol-containing medium was circulated through the membrane pervaporation module. Long-term continuous operation (over 40 days) was achieved with a productivity of 20–30 g L^{-1} h^{-1}. As in some cases a possible limitation or inhibition due to a lack of diffusion or to limited escape of carbon dioxide inside the bed of immobilized cells may affect the efficiency of the process, the use of fluidized-bed bioreactors (Busche et al. 1992) or trickled-bed reactors (Jamuna and Ramakrishna 1992) was studied. Ogbonna et al. (2001) presented a study dealing with the scale-up of fuel ethanol production from sugar beet juice using a loofa sponge immobilized bioreactor. They concluded that "by using external loop bioreactor to immobilize the cells (here a flocculent strain of *S. cerevisiae*) uniformly on the loofa sponge beds, efficient large scale ethanol production systems can be constructed". But to our knowledge none of these systems are running on an industrial scale.

Another interesting approach to overcome some technical problems was to use immobilized systems made of a strain of microorganism and something else, such as another strain or species or an enzyme. A study by Andreoni et al. (1983) suggested the utilization of immobilized β-glucosidase enzyme and immobilized growing yeast cells in the ethanol production from municipal solid wastes, and Amin et al. (1983) conducted experiments on the co-immobilization of *S. bayanus* and *Zymomonas mobilis*. A new immobilized biocatalyst called Maxaferm was described later by Noordam et al. (1995) for the continuous production of ethanol from dextrins: the Maxaferm system has been developed for the co-immobilization of enzymes and microorganisms (in this case amyloglucosidase and *S. cerevisiae*).

Recently, Amutha and Gunasekaran (2001) studied the production of ethanol from liquefied cassava starch using co-immobilized cells of *Z. mobilis* and *S. diastaticus*. They noted that the concentration of ethanol produced by immobilized cells was higher than that by free cells of *S. diastaticus* and *Z. mobilis* in mixed-culture fermentation and that in repeated-batch fermentation using co-immobilized cells, the ethanol

concentration increased to 53.5 g L^{-1}. Also, the co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using co-immobilized cells in a packed-bed column reactor exhibited an ethanol productivity of 8.9 g L^{-1} h^{-1}. An original use of immobilized yeast cells and free cells of a fungus was recently studied by Farid et al. (2002): in this paper the authors analysed the alcohol production from starch by mixed cultures of *Aspergillus awamori* and immobilized *S. cerevisiae*. They concluded that repeated batch by this co-culture were successfully used 12 times without a significant loss in alcohol production.

But the co-immobilization of different species of yeasts did not always lead to a better efficiency as shown by Lebeau et al. (1996) when investigating the continuous alcoholic fermentation of a mixture of glucose (35 g L^{-1}) and xylose (15 g L^{-1}) by *S. cerevisiae* and *C. shehatae* co-immobilized in a double-chambered bioreactor.

9.4 Brewing

In the brewing process, fermentation is made up of two steps: the first step or main fermentation consists in the conversion of most of the fermentable sugars into ethanol by a growing and abundant yeast population; in the second step or maturation, the main objective is to decrease the concentration of diacetyl below the taste threshold for organoleptic reasons, diacetyl being a secondary product of main fermentation. A continuous process would be the most interesting from the economic point of view because of the shortened fermentation time, but the major difficulty lies in keeping a biological system aseptic for a relatively long period. However continuous fermentation of beer has long been attractive since as early as the end of the nineteenth century (Virkajarvi and Linko 1999). The emergence of immobilization technology in the 1970s boosted research on the continuous processes. Huge numbers of papers and patents on this subject have been published in the last 30 years. Among them very few led to industrial applications except for beer maturation or alcohol-free beer production. Nevertheless the feasibility of many proposed processes has been demonstrated at the laboratory or pilot scale (Virkajarvi 2001). The main supports used in the brewing process were discussed in Sect. 9.1.2.

9.4.1 Examples of Proposed System Configurations

Because of the different characteristics of primary and secondary fermentations most of the processes either concerned only one of the two fermentations or have several serial reactors (multistage processes). The most difficult fermentation to manage is the primary one, which is more vigorous, and many critical points have to been solved technically: removal of excess biomass and CO_2, sustaining yeast viability, optimization of oxygen feeding, prevention of clogging the reactor, high residence time and prevention of microbial contamination in a continuous run.

9.4.1.1 Main Fermentation

For the main fermentation high amounts of fermentative yeasts are needed. Very few studies have dealt with batch fermentation. Hsu and Bernstein (1985) modified a

conventional fermenting vessel with two screens that hold alginate beads containing fermenting yeast in the vessel. The whole process lasted 7 days but the organoleptic characteristics of the beer were slightly different. Other authors proposed recycling the same biocatalyst 20 (Ju et al. 1986) to 42 times (Pardonova et al. 1982) or for 3 months (Nedovic et al. 1993), shortening the fermentation time to 12–16 h.

In order to get high productivity a continuous process is much more interesting at the industrial level. As early as 1966 some attempts were made at continuous main fermentation by mixing diatomaceous earth and yeasts and passing malt wort through a kieselghur filter with a residence time of 2.5 h, but the bioreactor had a lifetime of only 7 days before clogging. This system was then improved by Baker and Kirsop in 1973 (Virkajarvi 2001) by operating in a tubular reactor containing the mixture, resulting in an increased lifetime, but it still remained insufficient. Moreover, to achieve satisfactory flavour formation and organoleptic qualities a plug flow reactor, packed or fluidized bed, seemed to be more suitable (Yamauchi et al. 1995). An alternative should be a series of continuous stirred-tank reactors (Linko et al. 1998). In these conditions, immobilization by entrapment always led to swelling of the carrier, preventing long-term operation, and immobilization by adsorption was preferred.

Studies on packed-bed reactors seem to be more extensive with different kinds of carriers: ceramics, glass beads, calcium alginate beads, DEAE-cellulose or wood chips. Shindo et al. (1994) experimented with chitosan beads in a fluidized-bed reactor and by optimizing the recycling flow rate in the reactor obtained a life time of 900 h at a flow rate of 40 mL h^{-1}. Later (Pajunen et al. 2000) this kind of system was extrapolated at the pilot scale with a flow rate of 28 L day^{-1} for 50 days and a bed volume of 1,000 L. A similar process was investigated with alginate beads (Wang et al. 1989): 40% (v/v) beads were used and the ratio of recirculation was 5, giving a fermentation time of 14 h.

Another possible technology would be a loop bioreactor containing a tubular matrix carrier made of silicon carbide (Meura-Delta process, see later; (Virkarjarvi and Linko 1999) or one layer of metal fibres which had been sintered. Alternatively the carrier may be a sintered silicon carbide carrier in a multichannel loop reactor design or may comprise several tubes placed concentrically around each other (Arnaut et al. 2001). Complete attenuation was then achieved in a continuous two-stage bioreactor with a hold-up vessel arranged in series. The total residence time including the time in the hold-up vessel was 2.5 days (van de Winkel et al. 1993).

To achieve the high yeast concentration and activity needed in the first fermentation optimal oxygen feeding is necessary. This can be realized using an air-lift or bubble column bioreactor. This was suggested in several works using various carriers such as alginate (Leskosek-Cukalovic and Nedovic 2002) κ-carragheenan (Mensour et al. 1996; Pilkington et al. 1999) or calcium pectinate beads (Yamauchi et al. 1995; Smogrovicova et al. 1998), spent malt grains (Branyik et al. 2004) and DEAE-cellulose (Branyik et al. 2001). As an example, Labbat breweries in Canada produced beer in a 50-L gas-lift reactor containing yeasts entrapped in carragheenan beads with air in the proportion of 2–5% and with a residence time of 20 h (Mensour et al. 1996). With yeasts immobilized in pectate beads at a ratio of 25% (v/v) in the air-lift the residence time was 13 h.

Another strategy consists in using a multistep system, only one step being an aerated reactor: continuous stirred tank or air-lift. Several configurations have been tested: a fluidized-bed reactor and air-lift with a residence time of 12 h (Smogrovicova et al. 1997); a two-stage packed-bed system (Kronlof et al. 1996), in this case the first stage was aerated and considered as a prefermentor, giving a global production of 60–130 L day^{-1} with a residence time of 20–40 h; an aerated continuous stirred-tank reactor followed by a fluidized-bed reactor (Yamauchi and Kashihara 1996).

9.4.1.2 Secondary Fermentation (Maturation)

The use of immobilized yeasts allows the duration of this step to be shortened, compensating the low reaction rates by high catalyst concentrations. In the case where the main fermentation is continuous (with free or immobilized yeasts) the secondary fermentation is run in one or two reactors in series. Several processes have been run at the industrial level with 7–50-m^3 reactors allowing the duration to be reduced from several weeks in the traditional process to 2–3 h. Two kinds of carrier were used for adsorption. The first one, since 1990, was DEAE-cellulose (Pajunen 1996) in an installation with an annual capacity of 1×10^8 L. The continuous production cycles varied between 2 and 8 months before regeneration. The second one was porous glass beads in two-stage column reactors (Virkajarvi and Linko 1999).

9.4.1.3 Examples of Integrated Processes at Laboratory, Pilot or Industrial Scale

A very attractive design for the brewing process would be an integrated one making it possible to carry out both fermentations in a multistage continuous system. At the laboratory level a three-stage packed-bed reactor containing yeast adsorbed on glass beads had been proposed (Yamauchi et al. 1994). A two-stage system has been investigated (Smogrovicova et al. 1999): primary wort fermentation was conducted in a gas-lift reactor using calcium alginate (residence time 12 h), secondary fermentation was conduced in two parallel packed-bed systems using calcium alginate (residence time 57 h) or gluten (residence time 61 h) for yeast immobilization. Another two-step process could consist of a short aerobic prefermentation of the wort in a continuous stirred-tank reactor followed by a packed reactor filled with calcium alginate beads containing yeast cells. In such a configuration no further maturation is needed (Nakanishi et al. 1985).

Finally in spite of numerous studies and trials, beer production using immobilized yeasts has kept the brewing world waiting for a breakthrough, mainly owing to difficulties in controlling the hydrodynamic and temperature stability of the reactor for a long time and only three processes have reached industrial development.

The first one is the Kirin process in Japan developed since the mid-1980s and exploited for about 10 years producing 185,000 L per year (Virkajarvi 2001). It consists in a three-stage system (four reactors) with yeast adsorbed on porous glass beads (Fig. 9.2): the first stage is an aerated stirred-tank reactor for free yeast growth; the second step is made of two packed beds in series with immobilized yeast for the main fermentation; the last step after heat-treatment is also a packed bed for green beer maturation.

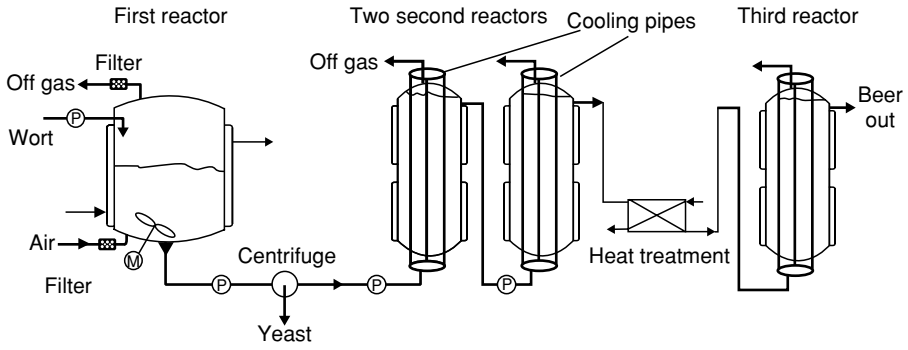

Fig. 9.2. The Kirin process. (Redrawn from Yamauchi et al. 1994)

The second potential industrial process was developed by the VTT technical research centre of Finland (Virkajarvi 2001). It consists of a slightly aerated prefermentor, a buffer tank and a main fermentor (packed-bed reactor containing glass beads); after heat-treatment the green beer enters a second packed-bed reactor for the maturation. The system at a pilot scale was run for 14 months but a problem for longer-term instability and efficiency appeared caused by the decline of yeast viability and plugging. The author proposed solving this problem by substituting glass beads by wood chips. The feasibility at an industrial scale was demonstrated (4×10^6 L per year).

The third semi-industrial process, the Meura-Delta, consists in two loop reactors in series with a matrix of silicon carbide inside for adsorption of yeast (Sect. 9.4.1.1). The first bioreactor is operated at an attenuation of 40% and final attenuation is reached in the second bioreactor. The aeration is arranged by diffusion through plastic tubing in the circulation loop. At least one brewery uses it in Canada with a productivity of 0.5×10^6 –3×10^6 L a year according to the final attenuation.

9.4.2 Alcohol-Free Beer

Traditionally, alcohol-free beers are produced by arrested batch fermentation, vacuum distillation, reverse osmosis, or dialysis. Dealcoholized beers generally lack body and have poor aroma profiles. In order to circumvent these disadvantages, several immobilized yeast cell reactors were developed to produce beer with a final alcohol content below 0.5%. For example, immobilization of yeasts by passive colonization of multichannel silicon carbide membrane carriers with a void volume of 30% and pore sizes ranging from 8 to 100 μm was studied with a view to achieving optimal flavour development (van de Winkel et al. 1991).

Others carriers have been described in the literature. Continuous fermentations with yeasts immobilized on wide-pore sintered glass (Siran) in a fluidized-bed fermentor were carried out at both a laboratory and a pilot plant scale (reactor volumes of 2 and 60 L, respectively) (Aivasidis et al. 1991). Following a colonization phase

at 25–30°C requiring less than 2 weeks, the temperature was lowered to just above 0°C, where the beer fermentation took place. A product containing 0.1–0.3% alcohol (carbohydrate conversion rate of approximately 7%) was obtained after about 2–6 h as compared with 3–4 days with conventional cold/contact fermentation. A system stability of 1 year was observed, with no colonization of wild-type yeast strains from contaminated worts or other sources. Blockages were not observed and yeast or carrier make-ups were not required. The organoleptic properties of the final product corresponded to those of conventionally manufactured low-alcohol beers.

Others processes resort to immobilization on DEAE-cellulose and limited fermentation optimally performed in a packed-bed reactor. This highly controllable system combines short contact times between yeast and wort with the reduction of off-flavours. In some cases this was due to higher activities of hexokinase and pyruvate decarboxylase of immobilized cells compared with those of free cells grown in batch culture (Van Iersel et al. 2000). Using a similar system millions of hectolitres of alcohol-free beer have already been produced. In the reactor a limited fermentation was carried out under strictly anaerobic conditions, very low temperature, relatively high pressure and a short contact time. With the combination of these factors, only a small amount of glucose was metabolized, resulting in a low-alcohol product (less than 0.1% ethanol). In addition, the limited growth under anaerobic conditions stimulated the yeast to restore its redox balance by the reduction of carbonyl compounds (Van Dieren and Bavaria 1996) and simultaneously an increase of ester formation (ethyl acetate and isoamyl acetate) was noted (Van Iersel et al. 1999; Navratil et al. 2002). Nevertheless, in this case, introduction of regular aerobic periods to stimulate yeast growth was recommended to achieve an optimal and constant flavour profile of the alcohol-free beer. A patented method (Lommi et al. 1997) also recommended reactivating the yeast at 2–15°C for 10–30 h. If necessary, the reactor could be regenerated.

During a 12-month pilot-scale project both at Guinness Brewing Worldwide Research Center and at Grolsche Bierbrouwerij Nederland (van de Winkel et al. 1996), a silicon carbide immobilizing carrier system was investigated for the continuous production of alcohol-free beer with 0.05, 0.1 and 0.5% alcohol by volume with a decreased worty flavour and a full beery aroma. The scale-up capability of the immobilized bioreactor system was studied and the operating parameters (dilution rate, fermentation temperature, wort oxygenation, number of bioreactor stages, colonization procedure, cleaning and sterilization procedures) were optimized. From the results it was demonstrated that scaling-up was reproducible and predictable with a single bioreactor stage operating at 10°C with oxygen levels below 1 mg L^{-1} for the production of an acceptable alcohol-free beer.

9.4.3 Miscellaneous

Immobilization of yeasts has been studied for purposes other than conventional fermentation. An oxygen scavenger for beverages has been patented (Edens et al. 1989). It consists of dry yeast immobilized in or on a solid material, e.g. paraffin wax, which allows only very slow penetration by water. Yeast cells were mixed with a slurry of molten paraffin at 95°C. A glass slide was coated with a 0.1-mm layer of

this slurry. The slide was heated at 65°C for 10 min and submerged in air-saturated water. The oxygen concentration of the water was reduced from 7.5 mg L^{-1} to undetectable in 14 days.

For production of diet beer several similar methods have been developed using co-immobilized enzyme (amylase) and yeast (*S. cerevisiae*). This is a way to produce beers with higher attenuation containing less residual sugar. A glucoamylase was bound to living yeast cells, resulting in particle size of approximately 10 μm. The apparent K_m value, the pH and the temperature dependence of the activity and stability of the bound enzyme were significantly different from the characteristics of the free glucoamylase. Compared with the native yeast, the co-immobilizate led to a considerably increased final degree of attenuation when applied for beer wort fermentation or for maturation of beer (Hartmeier and Muecke 1982).

In two others works, brewer's yeast was immobilized in calcium alginate gels. In the first one (Godtfredsen et al. 1981), the immobilized cells were packed in a simple reactor allowing continuous operation. A suitable dextran-coupled amyloglucosidase co-immobilized with brewer's yeast was also applied for production of low-calorie beer in a simple reactor system. In the second work (Juchem et al. 2000), the ability of the beads to reduce the carbohydrate content of the beer was investigated in a repeated batch system and a continuous system using a multistage fluidized-bed bioreactor. In both cases the new technology made possible a significant intensification of the fermentation achieved by a high yeast density coupled with the enzymatic activity.

9.4.4 Influence of Immobilization on the Organoleptic Qualities

Most works report modifications in the minor by-product concentrations for the beer produced by immobilized yeasts. These modifications vary a lot according to the kind of process, batch or continuous (Bardi et al. 1997a), kind of reactor (Yamauchi et al. 1995), fermentation temperature (Bardi et al. 1997a; Bekatourou et al. 2002), dissolved oxygen (Nakanishi et al. 1985; Virkajarvi and Kronlof 1998) and even type of carrier (Smogrovicova and Domeny 1999). Generally succinic acid production was increased (Yamauchi et al. 1995; Shindo et al. 1992, 1993) in connection with an enhanced consumption of isoleucine and acetic acid production was decreased. Most of the time higher alcohols were less concentrated in the final product (Smogrovicova et al. 1998; Smogrovicova and Domeny 1999; Tata et al. 1999) but their concentration could increase for very low temperature (0–7°C) in the batch process (Bardi et al. 1997a); the total nitrogen content was higher (Virkajarvi and Kronlof 1998; Smogrovicova and Domeny 1999), except for immobilization on gluten pellets (Smogrovicova et al. 1999). For production of esters and diacetyl the results are very different according to the studies. It seems that for these metabolites the key parameters, which are the level of yeast activity linked with the sugar flux, the biomass density in the reactor (Smogrovicova et al. 1998) and the redox state of the medium, are strongly dependent on the operating conditions. In fact the organoleptic quality of the beer produced by immobilized yeasts can be controlled by:

– The relative importance of the different stages of a multistage process. For example, yeast metabolism was successfully subdivided into a growth and a

restricted phase through a combination of a continuous stirred-tank reactor and an immobilized yeast packed-bed reactor (Yamauchi et al. 1995). The process control strategy based on the relative intensity of attenuation (proportion of sugar consumption) in the two reactors was optimized with a ratio of 1:2, higher alcohols being mainly produced in the stirred reactor and esters in the packed-bed reactor.
– The level of aeration if the process contains a preaeration step (Virkajarvi and Kronlof 1998).
– The ratio of the biocatalyst volume to the reactor volume (Smogrovicova et al. 1998).

Several authors reported final products with no significant differences from the beer produced in the conventional way (Pardonova et al. 1982; Smogrovicova and Domeny 1999; Umemoto and Mitani 1999; Bekatorou et al. 2002).

9.5 Fruit Wines

As is the case for grape wine, the most frequent technology employed for fruit wine making by immobilized yeasts is entrapment in alginate gel. This method has been studied for:

– The development of a new type of Umeshu (a liqueur made from Japanese apricot fruit, shochu and sugar) (Takatsuji et al. 1992). It was fermented in a reactor containing an immobilized growing yeast strain isolated from Japanese apricot juice. The initial Umeshu juice diluted 2 times with water was circulated through the reactor at 25°C with a dilution rate of 0.13 h^{-1}. Fermented Umeshu with good flavour was manufactured stably by this process for 30 days.
– Fermentation of watermelon juice by a wine yeast (Nakada 1990). The juice with addition of glucose up to 21.1% and adjusted to pH 4 with citric acid flowed through the bioreactor at 0.03 L h^{-1} at 25°C. The alcohol productivity of the bioreactor was 10 g ethanol (L gel)$^{-1}$ h^{-1}. The watermelon wine obtained after 4 days of operation contained 9.3% ethanol and the ratio of isoamyl acetate to isoamyl alcohol (4.47) was high, but formation of ethyl caproate was low compared with the that for wine made by the conventional fermentation method.
– Fermentation of fresh sugar cane juice and fruit juices by mixed yeast strains in a three-stage rhomboid bioreactor. The ethanol concentration in the wine made from cane juice was 9.5% and a mixture of wine yeasts produced a wine containing 10–13% ethanol from mandarin and orange juices at residence times of 6–20 hours for 7 months (Fukushima and Hatakeyama 1983).
– The production of alcoholic beverages from different fruit juices (mango, peach, plum, cherry) containing 8–18% sugar giving 11–12% ethanol (60–84% of the theoretical yield) in batch fermentation by entrapped *S. cerevisiae* (Qureshi and Tamhane 1985).
– Bottle-fermented kiwifruit sparkling wines production by a combination of fermentation using *S. cerevisiae* immobilized in double-layered calcium alginate beads and termination of the fermentation using an antimicrobial substance from paprika seed (PSAS) having strong antimicrobial activity against wine yeasts (Yokotsuka et al. 2004). Secondary alcoholic fermentation in bottles

could be terminated several days after the addition of PSAS to give a concentration of 0.1 g L^{-1} to the fermenting base wine. Beads were easily inserted into the bottles and removed as ice plugs by the conventional disgorging method. The sparkling wines produced had a strong fruity smell and a good balance of sweet and sour flavours.

Entrapment in carragheenan gel has also been proposed for fermentation of ripe Cavendish banana fruit pulp, which contained approximately 126 g L^{-1} total sugars (del Rosario and Pamatong 1985). The volumetric productivity and fermentation efficiency were about 15 g L^{-1} h^{-1} and 94%, respectively. The concentrations of alcohol and residual sugar in the product were 54 and 12.8–14.5 g L^{-1}, respectively.

Adsorption on dicarboxycellulose (Sado et al. 1992) or derivatized cellulose (del Rosario and Pamatong 1985) was tested.

As in grape wine making, the deacidifying yeast *Schiz. pombe* can be useful for malic acid elimination from fruit wines. It has been tested immobilized on oak shavings for apple must amended with red currant must: elimination of 100, 80.7, and 79.8% of initial malic acid, at flow rates of 0.033, 0.079, and 0.092 mL h^{-1}, respectively, was obtained (Czyzycki et al. 1991). Other authors deacidified plum juice by *Schiz. pombe* entrapped in calcium alginate beads (Tachibana et al. 1989) prior to alcoholic fermentation: plum (*Prunus salicina*) juice initially contained 1.2–1.4% malic acid and was batch-treated with 5% of immobilized cells of *Schiz. pombe* (2.1×10^9 cells L^{-1} juice) at 20°C for 3 days to remove 70% of the malic acid. When continuous decomposition of malic acid was carried out using a reactor (2 L) containing 1 L of immobilized cells at 20°C, with a flow rate of 1.5 L day^{-1}, the retention time was 16 h and the concentration of malic acid in the treated juice remained at 27–35 mg L^{-1} for 16 days.

9.6 Cider

For cider production two strategies are possible: the use of yeast alone or the use of co-immobilized yeast and lactic acid bacteria.

For fermentation of pure cultures of yeasts the different methods of immobilization are mainly based on adsorption methods:

- Adsorption on multiple parallel porous ceramic plates. The bioreactors prevented clogging associated with insoluble substances in the sample and gas channelling. Thus, low-fermentation apple juice was possible (Aso et al. 1993).
- Adsorption on an ion-exchange sponge that can have a tailored surface charge was used in high original gravity (1.106) cider fermentation. Continuous circulation of the medium through columns containing weakly basic sponge decreased the batch fermentation time, and increased the final ethanol concentration, possibly aided by sponge-enhanced CO_2 removal from solution (O'Reilly and Scott 1993).
- Adsorption on polyethylene. The use of immobilized yeast at 500×10^6 –600×10^6 cells mL^{-1} for fermentation at 15–20°C improved the quality of fruit wines and decreased the period of fermentation by 3–5 times (Sarishvili et al. 1992).
- Adsorption on foam glass put in a column where the apple juice was circulated with a residence time of 5–6 days. The fermentation was carried out for more

than 3 months at 22°C with no changes in the sensory quality of the product (Bonin and Wzorek 2000).

But entrapment in calcium alginate gel was also carried out for continuous fermentation of apple juice. The average values characterizing the process were as follows: fermentation efficiency, 84.7% of the maximal theoretical yield; ethanol concentration in the mash, 38.9 g L^{-1}; and volumetric productivity, 6.3 g L^{-1} h^{-1} (Dallmann et al. 1987). Alginate gel was shown to be better than pectate gel from an organoleptic point of view (Krasny et al. 1993).

A method for controlling the alcohol and sugar content of cider produced by alginate-entrapped yeast by varying the CO_2 pressure has been patented (Divies and Deschamps 1988). At 0.2 bar of CO_2, the ethanol concentration was 4.6% and the sugar concentration 14.9 g L^{-1} and at 3–5 bar of CO_2, the values were 4.8 and 16.2, respectively. It was possible to produce in the same fermentor a "hard" cider with 3% alcohol and 48 g sugar L^{-1} as well as a "soft" cider with 2% alcohol and 66 g sugar L^{-1}.

For co-immobilization calcium alginate has been proposed for *S. bayanus* and *Leuconostoc oenos* (*Oenococcus oeni*) in a continuous packed-bed bioreactor (Nedovic et al. 2000). The continuous process permitted much faster fermentation compared with the traditional batch process. The flavour formation was also better controlled. By adjusting the flow rate of the feeding substrate through the bioreactor, i.e. its residence time, it was possible to obtain either "soft" or "dry" cider. However, the profile of the volatile compounds in the final product was modified compared with that of the batch process especially for higher alcohols, isoamyl acetate and diacetyl. This modification was due to different physiological states of the yeast in the two processes. Nevertheless, the taste of the cider was acceptable.

A sponge-like material was also used to immobilize both *S. cerevisiae* and *Lactobacillus plantarum* (Scott and O'Reilly 1996). The sponge's open porous network promoted extensive and rapid surface attachment of microorganisms throughout the depth of the material. The matrix surface can also be chemically modified, and basic characteristics enhanced both the initial rate of uptake and also that of final loading (in excess of 10^9 yeast cells g^{-1} sponge and 10^{10} bacterial cells g^{-1} sponge). The flavour of the product was satisfactory.

9.7 Vinegar

For vinegar manufacturing several processes using immobilized yeasts have been proposed using different substrates. Some of them are based on a two-step fermentation, the first one by yeasts and the second one by acetic acid bacteria. For example, continuous production of kiwifruit and persimmon wines in a bioreactor with calcium alginate entrapped yeast cells was studied (Yamashita 2002). When the juice was in the reactor for residence times of 12 and 6 h, the ethanol concentration and the productivity were 11 and 10%, and 7.5 and 13.4 g L^{-1} h^{-1}, respectively. Fermentations of both fruit juices were continued for 50 days without microbial contamination. Continuous production of fruit vinegar using a bioreactor with fixed *Acetobacter aceti* cells on cotton fabrics was then developed. The fabrics were packed into a column and inoculated with *A. aceti*. When the kiwifruit and

persimmon wines fed the column the production rates of acetic acid were 7.4 and 5.2 g L^{-1} h^{-1}, respectively, on the basis of the total column volume with 45 g L^{-1} of the acetic acid in the vinegar. The surface culture using growing cells fixed on the woven cotton fabrics was superior to the submerged culture involving aeration, from the viewpoint of the higher productivity and energy efficiency. Yeast entrapment in calcium alginate beads was also used in view of vinegar production from saccharified rice (Nakajima and Sugiura 1990) or rice flour by repeated batch fermentations (Tamai et al. 1990). When rice flour was used the aim was to reach a high concentration of ethanol (approximately 120 g L^{-1} broth) without residual glucose. This was achieved for a 0.14 L gel L^{-1} packing ratio. The process could be repeated for more than 25 batches with a stable ethanol yield [0.38 g ethanol (g rice flour)$^{-1}$] and cell viability in the gel beads (96%). The ethanol productivity of this system was 3.9 g L^{-1} h^{-1}, which is much higher than that of batch fermentation in vinegar breweries.

A method for manufacturing vinegar from potato comprising (1) liquefaction, (2) saccharification, (3) alcoholic fermentation and (4) acetic acid fermentation was patented (Nagao and Yamamoto 1991). Steps 2 and 3 were combined to reduce microbial contamination in a bioreactor comprising a fermentation chamber packed with beads containing immobilized saccharifying enzymes and yeasts for concomitant reactions.

A process for continuous alcoholic and acetic acid fermentation of onion juice was developed by using yeast and acetic acid bacteria, respectively, immobilized on porous ceramic granules and rings (Takahashi et al. 1993). In continuous vinegar production in multibioreactors, the activities of the immobilized yeast and the acetic acid bacterial cells were not lost during more than 6 months of operation. For acetic acid bacterial cells the method was better than for cells immobilized on calcium alginate, for which a decrease of 50% of initial activity was observed for 3 months of operation. By developing a new cyclic operation, acetic acid yield increased by 16% compared with the yield from steady-state operation. The 4% onion vinegar produced had satisfactory organoleptic properties.

Mixed cultures of immobilized yeasts and another microorganism can be another possible strategy. For this purpose some authors proposed co-immobilizing *Monascus* and *Saccharomyces* in alginate carrier (Wang 1998). Vinegar was prepared by mixed fermentation of glucose mother liquor as the main material. The product ratio of vinegar was 4.5 kg kg^{-1} glucose liquor, and the appearance and flavour were good. Others authors suggested entrapping separately growing yeast cells and *A. aceti* cells in calcium alginate gel as pellets (Sumonpun and Kummun 1989). The immobilized yeast cells converted glucose to ethanol and the immobilized *A. aceti* simultaneously converted ethanol to acetic acid. Preliminary studies showed that a 1:4 ratio of immobilized yeast and immobilized *A. aceti* gave the maximum yield of acetic acid, approximately 3.4% after 22–24 days of cultivation in coconut water medium containing 2% glucose in shake flask culture at 250 rpm.

Finally, immobilized yeasts can also be used for refining fruit vinegar. A method has been patented for apple or apple/Japanese pear (10–30%) vinegar (Panasyuk et al. 1988) which is fed to a packed-bed column containing 300–800 $\times$ 10^6 immobilized cells mL^{-1} at a flow rate of 0.02–0.08 h^{-1} for 12–48 h.

9.8 Dairy Products

As far as dairy products are concerned the different utilizations of immobilized cells can be divided into the treatment of whey for its valorization (except for the production of ethanol presented in Sect. 9.3), the treatment of milk and the production of kefir for manufacturing fermented beverages from milk.

The use of salted whey (a liquid by-product from the dairy industry) was investigated (Mostafa 2001) as a substrate for either acetic acid or glycerol production using two yeast strains (*K. fragilis* and another one isolated from waste whey); pH 8.5 and 32°C were the optimum operating conditions for maximal acetic acid production (25.8 g L^{-1}) and supplementation with peptone and pH 7 for glycerol batch production (13.2 g L^{-1}). The experiments in a membrane cell recycle bioreactor gave better results than those obtained for the immobilized cell batch reactors (18.7 g L^{-1} for glycerol production).

The potential of three lactose metabolizing yeasts, *C. pseudotropicalis*, *S. fragilis* and *K. marxianus*, for the removal of biological oxygen demand and chemical oxygen demand from dairy industry wastewater under unsterilized conditions was evaluated (Marwasha et al. 1988). The most efficient was *C. pseudotropicalis*. Using entrapment procedures in an alginate matrix, 40 g (dry weight) cells L^{-1} was the optimum operational cell density. Nitrogen source supplements further improved the ability of immobilized yeast cells to carry out the treatment. For milk or whey treatment two original and similar methods have been investigated.

An immobilized preparation of whole cell-based catalase was obtained by cross-linking the yeast cells permeabilized with toluene in hen egg white using glutaraldehyde for 2 hours at 4°C. Immobilized cells could be reused for the removal of H_2O_2 from milk (Kubal and D'Souza 2004).

Others authors have developed and characterized a new low-cost enzymatic preparation for milk whey saccharification (Gonzalez Siso and Suarez Doval 1994) consisting of β-galactosidase-rich whole cells of the yeast *K. lactis*, previously cultured on milk whey and immobilized by covalent linkage to corn grits (an inexpensive material). Permeabilization of immobilized cells with ethanol increased the intracellular β-galactosidase activity up to 240-fold, and the cells did not further metabolize the glucose and galactose produced. More than 90% milk whey lactose hydrolysis was achieved in a packed-bed bioreactor at 37°C. In another work (Decleire et al. 1985) whey hydrolysis was compared in column reactors containing whole yeast cells immobilized in calcium alginate or in hen egg white in relation to cell β-galactosidase activity, flow rates, temperature and time. With cells having an activity of 1.3 U mg^{-1} (dry weight) immobilized in calcium alginate, 80% hydrolysis was obtained at 4 and 20°C with flow rates of 0.50 and 1.65 bed vol h^{-1}, respectively; the values were 0.2 and 0.4 bed vol h^{-1} with cells entrapped in hen egg white. When the flow rate was expressed as millilitres per hour per gram of wet yeast, no significant difference was observed between either matrix, and 80% hydrolysis was reached with flow rates of 1.7 and 5 mL h^{-1} (g wet yeast)$^{-1}$, respectively, according to the temperature. The best performance was achieved by the yeast egg white reactor. At 4°C, hydrolysis decreased by 10% after 13 days and by 20% after 17 days. Many more applications of purified immobilized β-galactosidase have been studied.

In the field of dairy products many works deal with kefir. In fact, kefir is made from gelatinous white or yellow particles or granules called "grains" formed on cultured milk. These grains contain the lactic acid bacteria/yeast mixture clumped together with casein and a branched polysaccharide composed of glucose and galactose forming an insoluble matrix. They range from the size of a grain of wheat to that of a hazelnut. The grains, then removed after milk fermentation, as well as the fermented beverage are called kefir.

To remove lactose from milk the use of kefir granules obtained by fermentation, containing both bacteria and yeasts, has also been proposed and patented. They were sterilized at a low temperature, inactivated and coated with a semipermeable film-forming material, yielding an immobilized lactase (Snow Brand Milk Products Co. 1982).

A mixture of wheat flour and sour milk was treated according to the method of the traditional Greek fermented food *trahanas*, and was used as a model cereal-based support (starch–gluten–milk matrix) for co-immobilization of lactic bacteria and yeasts for potential use in food production (Plessas et al. 2005). Cell immobilization was proved by microscopy and by the efficiency of the immobilized biocatalyst for alcoholic and lactic repeated fermentations at various temperatures (5–30°C). The stability of the system was always good, revealing suitability for industrial applications. Finally, respectable amounts of lactic acid and volatile by-products were produced, revealing potential application of the immobilized biocatalyst in fermented food production or use as a food additive, to improve nutritional value, flavour formation or preservation time.

The main application of immobilized kefir microorganisms is the production of fermented beverages from milk or whey. For this purpose the same Greek research group has developed different methods (Athanasiadis et al. 2004) They reported a novel whey-based beverage with acceptable organoleptic properties where various treatments were studied. Kefir yeast immobilized on delignified cellulosic materials (DCM) or gluten pellets were proved to accelerate whey fermentation significantly, with the latter support not being so preferable. Kefir granules seemed to achieve similar fermentation times as DCM. The optimal final pH of the product, indicating the amount of fermented lactose, was suggested to be 4.1 since the profile of the volatile by-products was higher than for other final pH values. The addition of fructose seemed to be beneficial for the volatile content of the product, although its acceptability as determined by a preference panel was similar to that of the control.

This delignified cellulosic-supported biocatalyst was also found to be suitable for batch or continuous modified whey fermentation containing 1% raisin extract and molasses (Kourkoutas et al. 2002a). Batch fermentations were carried out at various pH values, and the effect of temperature on the kinetic parameters, in the range 5–30°C, was examined. At pH 4.7 the shortest fermentation time was obtained. The formation of volatiles indicated that the concentration of amyl alcohols (total content of 2-methyl-1-butanol and 3-methyl-1-butanol) was reduced as the temperature became lower. 1-Propanol and isobutyl alcohol formation also dropped significantly below 15°C. The percentage of ethyl acetate increased as the temperature was reduced. At 5°C the content of total volatiles in the product was only 38% of the volatiles formed during fermentation at 30°C.

For the continuous process, ethanol productivities ranged from 3.6 to 8.3 g L^{-1} day^{-1} (Kourkoutas et al. 2002c). The continuous fermentation bioreactor was operated for 39 days, stored for 18 days at 4°C, and operated again for another 15 days without any diminution of the ethanol productivity. The concentrations of higher alcohols (1-propanol, isobutyl alcohol and amyl alcohols) were still low. The main volatile by-products formed in the continuous process were similar to those observed in alcoholic beverages, particularly ethyl acetate, and the fermented whey had a good aroma. The possibility of using such a process for the production of potable alcohol or a novel, low-alcohol content drink was proposed.

Several lactic acid bacteria (*Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactococcus lactis* subsp. *lactis*), *Enterococcus durans* and yeasts (*S. italicus*, *S. unisporus*), all isolated from kefir-grains from Turkey and Yugoslavia, were immobilized in calcium alginate (Gobbetti and Rossi 1993). A continuous process for the production of a new kefir-like cultured milk was performed at 28°C for 30 days, with dilution rates of 0.03 and 0.06 h^{-1}, respectively, for free and immobilized cells. The pH values were 4.41 and 4.55 and the CO_2 and ethanol concentrations were 0.54 and 0.66 g L^{-1} and 4.50 and 4.58 g.L^{-1}, respectively. The synthesis of aroma compounds (diacetyl and acetoin) was reduced under the conditions of the continuous process, but it increased during storage at 4°C when these compounds reached about the same concentrations as in the traditional kefir. Despite lower values of viability the new cultured milk approached the characteristics of traditional kefir.

9.9 Aroma

In the field of aroma production or aroma enhancement very few applications of immobilized yeasts can be found. They are of two types.

The first one is the production of natural aroma compounds through biocatalysts. 2-Phenylethanol, which has a rose-like odour, can be produced from L-phenylalanine by *S. cerevisiae*. Unfortunately this product inhibits growth even at low concentration. This problem of the inhibitory effect of the product on the yeasts can be tackled by an in situ product removal technique: addition of an ester as a second water-immiscible phase in which partitioning of 2-phenylethanol is very favourable. Thus, the yeast is immobilized in chitosan alginate beads to protect them from the toxic extractant and the production of 2-phenylethanol can be increased compared with production using the conventional method with free cells (Stark et al. 2000).

Another process is based on a simplified double reaction for production of esters which can be added to foods or cosmetics. The first reaction is the production of ethanol by calcium alginate gel immobilized yeast cells and the second reaction is the synthesis of ethyl oleate in presence of oleic acid, in a lipase-catalysed reaction. In this two fluid-phase system the presence of lipase enzyme does not influence the cell growth, the glucose consumption and the concentration of ethanol in the water phase. However, 1 U mL^{-1} lipase in the water phase of the fermentation broth increased threefold the concentration of ethyl oleate in the oleic acid phase (Kiss et al. 1998).

An alternative approach to microbial production of bioflavours, eliminating the need for lengthy product purification, was presented by Kogan and Freeman (1994). It was

based on co-immobilization of precursors for bioflavour generation and microbial cells, traditionally employed for food and beverage processing, within beads made of a food-grade gel matrix. Following incubation under controlled conditions, the bioflavour – or bioflavour mixture – was generated and accumulated within the beads. The flavour-retaining beads might then be employed as a food additive. A feasibility study demonstrated this approach with ethanol production by baker's yeast co-immobilized with glucose medium. Complex bioflavour generation was also demonstrated by baker's yeast co-immobilized with apple juice, generating cider flavours. Beads providing beer taste were also readily made via co-immobilization of brewing yeast with malt.

To efficiently produce a fermented flavoured liquor for improving the taste of bread, a repeated batch fermentation was studied in a bioreactor with the use of immobilized yeast cells (Tamai et al. 1997). It contained about 5% (wt/v) ethanol and ethyl acetate, isoamyl alcohol, isobutyl alcohol as major flavouring component and organic acids with varying concentrations.

The second kind of application is related to the modification of aroma or taste of the product using immobilized yeasts. Several works deal with soy sauce. It can be produced with *Zygosaccharomyces rouxii* and *C. versatilis* immobilized on 4–6-mm porous aluminosilicate glass beads in a two-stage bioreactor (Horitsu et al. 1991). The fermentation time was 6 days. Experiments were also made encapsulating these yeasts in calcium alginate beads, but the fermentation ability was inadequate. A method to produce a soy-sauce-like condiment from a hydrolysed soy sauce raw material by a mixture of immobilized yeasts and immobilized lactic acid bacteria has been patented. *Pediococcus halophilus* and *S. rouxii* were immobilized separately in carragheenan beads (Yamasa Shoyu Co. 1985). The same volume of each kind of bead (60 mL) was then filled in a column (200 mL) and the soy sauce was passed through it in the ascending direction at 28°C for 48 h while flushing with nitrogen gas. A similar process has been tested in order to improve nutritive and organoleptic properties of Worcestershire sauce using immobilized *S. cerevisiae* to increase isoamyl alcohol, ethanol and ethylphenol content, the major aroma components of fermented Worcestershire sauce (Fujimoto et al. 1993).

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