IMMOBILIZED YEASTS: ACTUAL OENOLOGIC UTILIZATIONS

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INTRODUCTION

The biological reactions observation (realized by a micro-organism) puts in evidence that these reactions can occur in two different systems:
-A system in which the micro-organisms are in suspension in the liquid phase (which seems to be homogeneous): it is the example of the fermentations in oenology\wine-making or in bread making.
-A system in which the micro-organisms are not free, but appear fixed to a support thereby creating a system of two distinct phases: the depuration procedure with bacterial beds may be considered an example.

In oenology, traditionally, the concept of free micro-organism largely dominates. On the other hand, in other areas we have since the 60's, tried to fix the "catalyzers" of the reaction and industrial applications of this process soon have been developed: adsorbed bacteria's involved in vinegar production, bacterial discs to depuration processes…
Before developing some oenological applications with immobilized micro-organisms, we will remember which the interest of such a process is and define which different existing technical possibilities are.

Why should we immobilize the micro-organisms?
The immobilization (or confinement) of micro-organisms have several advantages:

- The micro-organisms can be reused after a working/functioning period (see the application in the desacidification of the musts).
- The micro-organisms are not dispersed in a medium turning easier the recuperation at the end of culture (see the application in the foaming process)
It is possible to activate an important microbial population without the growing phase, which allows us increasing the reaction speeds (see in the application in the treatment of stuck fermentations)

How can we immobilize micro-organisms?
The immobilization or confining of micro-organisms can be done through different methods, which had been mostly proposed to the enzymes by Chibata (1979).
-The inclusion: in this method the microbial cells are embodied in a rigid polymer matrix; the most used is the calcium alginate (Margaritis et Merchant, 1984). This procedure (adapted to the realization of the double layer dry spheres) will be later developed.
-The adsorption: in this method the fixation of micro-organisms to the support is related to a weak connection between the microbial cell walls and the support (wood, pozzolana…) One of the major inconveniences of this technique is the disadsorption risk, for example, during the death of the micro-organisms.
-The retention of micro-organisms without support. These terminologies rejoin:
- Systems that are a result of the spontaneous agglomeration (or provoked) of microbial cells. The capacity to flocculation of certain strain yeasts was, for example, rehearsed in the second fermentation of sparkling wines.

- The procedures that aim in confining the micro-organisms in a part or the reactor through a physical barrier, generally a micro filtration membrane. In oenology, the interesting realization was the cartage "Milispark" developed by Milipore to the second fermentation of sparkling wine. (Lemonnier et Duteurtre,1989).

In this work, we will only present results with importance at the winery level. They originate from works done since more than ten years ago, both by the (UMR CNRS 5503) laboratory and by Proenol, Lda (Oporto). These realizations refer to the "prise de mousse" both in the traditional and ancestral methods, the desacidification of the musts or wines by the *Schizosaccharomyces pombe*, the treatment of stuck fermentations and the control of sweet fortified wine fermentation.

PRODUCTION OF ENCAPSULATED YEASTS

Whatever it is the origin of the yeasts (*Saccharomyces or Schizosaccharomyces*) the producing procedure of the spheres of encapsulated cells is the same.
The immobilization of yeasts in alginate gel in a double layer is realized following the protocol proposed by Tanaca and Coll (1989). The biomass from the yeasts is embodied in an (4%) alginate solution. The mixture is bombed into the internal tube of a gear composed by two concentric tubes and, at the same time, a solution of sterile alginato (4%) is conducted to an external tube. The alginates flux is shacked in a way that the drops fall in a calcium chloride solution which originates the alginate jellification: the alginates spheres are composed by an internal nodule which arrests the yeasts and by an external and continual sterile alginate layer which opposes to the yeasts cells exit. These "beads" (usually called like this due to its spherical geometry) are next accommodated under an inert atmosphere and preserved at a 4ºC until the utilisation moment. Then the "beads" are rehydrated under the guidelines of the protocol specified by the producer according to the planned application.
By comparing with the preceding examples, usually involved at a laboratory level, but rarely developed in experimental installations at an industrial level, these process offers the advantage of supplying the dry "beads" and by consequence stabilized as time goes on, (as the ADY), easy to manipulate and to dose (necessary for the foaming).

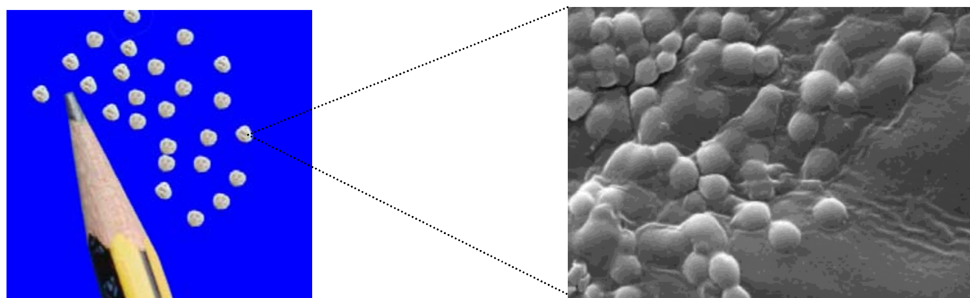


***Figure 1**. The encapsulated yeasts in a sodium alginate gel present a spherical form of 2 mm of diameter (**A**). Electronic microscope photo of the inside of a bead (**B**)*

OENOLOGICAL APLICATIONS

Foaming

In this case the introduction of an amount of encapsulated yeasts can replace the traditional addiction of free yeasts in order to realize the second fermentation or "prise de mousse" (foaming). Before the beginning of the encapsulated yeasts application, the base wine should be stabilized, in what refers to tartaric precipitations, and sterile in order to avoid the yeasts or bacteria development which the traditional "remuage" may allow to eliminate.
Since the encapsulated yeasts (*S. cerevisiae*, commercial yeasts, Proelif®) are retained inside the solid support, the "remuage" process is done in a few seconds by the simple act of turning the bottle upside down (picture 2.A). Therefore, the fast sediment/deposit of the yeasts can be obtained after the sugar total consumption (traditional method) or just after the residual sugar concentration reaches a certain value (ancestral method); this evolution is monitorized by the

pressure measurement (picture 2.B). The “degorgement” happens next through the classical method.
This application is nowadays the more significative one in the industrial elaboration plan, since Proenol made 8 million bottles in 2002. Since these encapsulated yeasts are available under the dehydrated form, we can expect a huge development of this procedure.

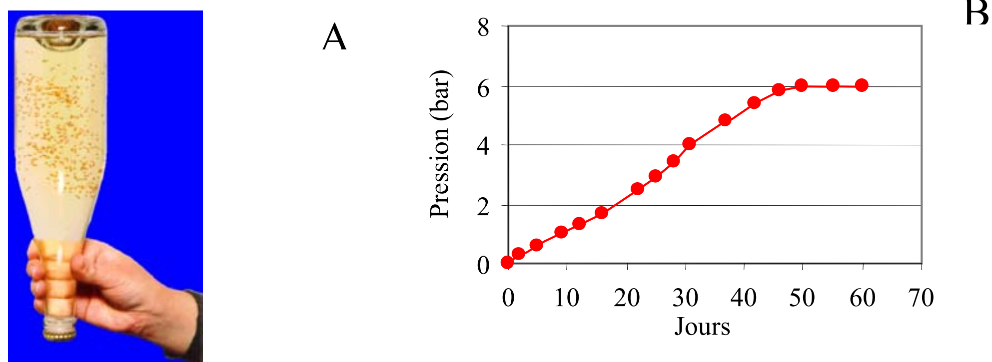


***Figure 2**. The «remuage» is done in a few seconds by turning upside down the bottle **(A)**. Pressure evolution in the bottle, using the dried encapsulated yeasts **(B)**.*

The musts desacidification

The yeast *Schizosaccharomyces* (*Shz*.) *pombe, which* has the capacity of turning malic acid into ethanol, has been proposed as an alternative to malolactic fermentation (MLF) (Ciani, 1995; Dharmadhikari et Wilker, 1998). However, some authors underline the fact that we can obtain wine with inferior organoleptic characteristics in comparation to the wines that result from traditional fermentations due to the excessive growth of this yeast (Bidan et coll.,1974). With the objective of minimizing this inconvenience, the realization of desacidification /vinification process was proposed in two phases:
In the first phase the grape must is inseminated by Shz. pombe and just as soon as the degradation of the malic acid reaches the wanted level, then the Shz. Pombe population is eliminated from the must by filtration or by pasteurization. In a second phase, a commercial strain of S. cerevisiae is adicionated for the realization of the alcoholic fermentation. Good results had been obtained with these protocols at a laboratory scale and with micro-vinifications, but the escape to the real conditions is difficult. The utilization of wine encapsulated yeasts Shz *pombe* (strain G2 IVC) was therefore considered because of its easy separation after the malic total or partial degradation in the grape must. (Magyar et Panyik, 1989; Taillandier et Strehaiano, 1991; Yokotsuka et coll., 1993).

The first works done in a laboratory show that this *Shz. pombe* strain is able to consume the malic acid until high concentrations (20g/L), and that these consume can occur during the growing phase or in the stationary phase with or without the simultaneous sugar consume (picture 3A). It is, in fact, possible to use the desacidificant capacities of this yeast both in the must before the alcoholic fermentation and in the must at the end of the alcoholic fermentation; for instance in the cases in which the malo-lactic fermentation does not happen. The desacidification activity realized by the *Shz. Pombe* encapsulated yeast (Promalic®) has been tested at a concentration of 5 million cells per ml of grape must. The picture 3B shows three desacidification successive cycles at a laboratory scale; at the end of each cycle the *Shz pombe* “beads” had been taken and drained off before its re-utilization in the next cycle. During the first cycle the malic acid consume profile, in what refers to the contact time, shows that in order to consume 9 gr/l of malic acid it takes only 75 hours at a temperature of 20ºC. During the second cycle, in order to consume the same malic acid quantity the contact time has considerably decreased.

Preservation trials show that the encapsulated yeasts can be preserved at least 20 months after their immobilization without losing their demalicating activity. This preservation time can be compared with the one of the active dried yeasts.

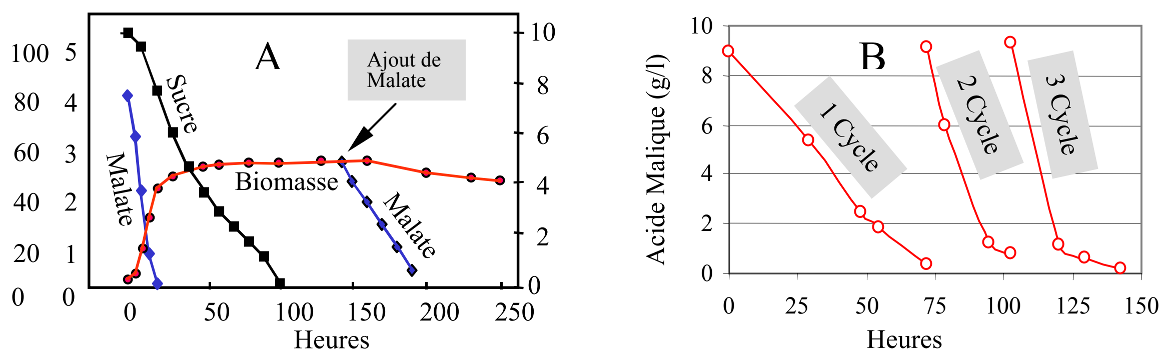
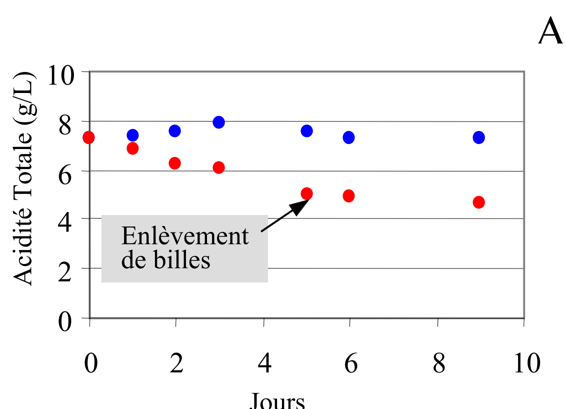


Figure 3. *Consumption profile of the malic acid according to the growth phase and the residual sugars concentration (**A**). Evolution throughout time of the residual malic acid concentration for 3 successive cycles of demalication using encapsulated Shz. pombe (**B**).*

Trials under real winemaking conditions have been carried out on volumes of 300 to 1000 litres, on white and red musts, in France and in Portugal. The balls containing the yeasts are initially placed in Nylon bags that allow the free spread of the must while preventing the balls to get into the medium (see fig. 4A and 4B). These bags are introduced (after balls reactivation) in the fermentation vat and the malic acid consumption can be for instance evaluated by the follow up of the total acidity or of the pH. When the chosen indicator reaches the desired value, the bags are withdrawn from the medium and a yeast addition is done according to the usual protocol. In those trials, only a partial reduction was wished. The fig. 5A shows one of the results obtained as far as the total acidity reduction during the treatment is concerned, in comparison with a control vinification.
The presence of aromatic defaults has sometimes been quoted when *Schizosaccharomyces* has been used (Bidan & coll., 1974). In this study, 9 volatile compounds have been dosed in white wines made of desacidified musts and in the control wines. Besides, a tasting trial has been made on the treated wines and on their respective control wines. A triangular trial has first been carried out and then the wines have been submitted to a tasting setting up 12 organoleptic descriptors. The results to this trial show a significant difference between the wines treated with *Shz. Pombe* and with the control wines (non desacidified). The results of the second step show a better organoleptic balance for the treated wines compared with the control ones. As a whole, the desacidified wines have been perceived as more balanced, with more persistence and a better structure. As far as the olfactory evaluation is concerned, the treated wines got better marks; those wines are characterized by “floral, citric and tropical” descriptors. As an example, fig. 5B shows the organoleptic profiles of a white wine (treated with the balls) and of its control: the reduction of the “acid” perception and the gain over the above mentioned descriptors can be seen clearly.

Figure 4. *The encapsulated yeasts are placed in Nylon bags, which length has been adapted to the fermentation vat height. A ballast is fixed at the inferior extremity of the bags in order to avoid them from floating. The bags are hung up to the superior part of the fermentation vat (A). Before being introduced into the fermentation vat, the bags containing the balls are rehydrated during 30 minutes in a solution sugared with 40g/L of saccharose in order to reactivate the yeasts (B)*



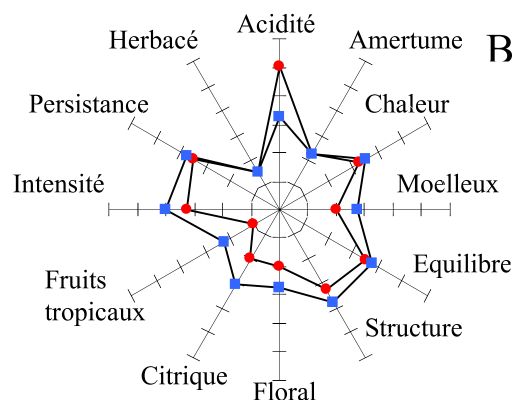


Figure 5. *Evolution of the total acidity (H2SO4) during a white vinification using encapsulated Shz. Pombe yeasts and free S. cerevisiae* ● *in comparison with a control made with free S. cerevisiae only* ● *(**A**). Comparison of two organoleptic profiles of a white wine made from a must treated with encapsulated Shz pombe* ■ *and of its control wine* ● *(**B**).*

Treatment of stuck fermentations

Whatever the cause of a stuck fermentation might be (Bisson, 1999; Alexandre and Charpentier, 1998; Ribereau-Gayon, 1999), in some cases, the curative protocols are not efficient. The use of included micro-organisms can be a solution to that because of the easy control over the micro-organism's activity it allows: managing of quantities, managing of the action period and possibility of reusing them.

The application protocol (Silva and coll., 2002) first consists of re-hydrating and activating the S. cerevisiae encapsulated yeast (Prorestart®). Thus, the bags containing the balls are immersed during 3 hours in a water solution which composition in g/L is as follows: saccharose 80, Fermaid 0,4 (Lallemand S.A., Canada). For each kg of balls, 5 litres of this re-hydration/ activation solution are used. Then, an acclimation step consists of immersing the balls in a solution made of 70% of the previous solution and of 30% of the wine to be treated. The duration of this stage is of 5 hours. Then the bags are drained in order to eliminate the

excess of the reactivating solution and finally the bags are introduced into the stuck alcoholic fermentation vat.

Fig. 6A shows the evolution of the reductive sugars after the active cells of encapsulated *S. cerevisiae 1118* have been introduced in order to treat the stuck fermentation in red. The wine volume (Cabernet Sauvignon grape variety) treated for this example is of 325hL. During the whole treatment, the fermentation vat temperature has been kept at 20°C. When the bags containing *S. cerevisiae* have been introduced into the vat, a fermentative activity has been immediately noted. In this case, the sugars consumption rate reaches a maximum value of 2,8g/L of sugars consumed on each day of treatment. The sugars consumption rate progressively diminishes till it reaches its minimum value after 6 days of treatment. Fig. 6B shows the treatment of a white wine in stuck fermentation at 14g/L of reductive sugars.

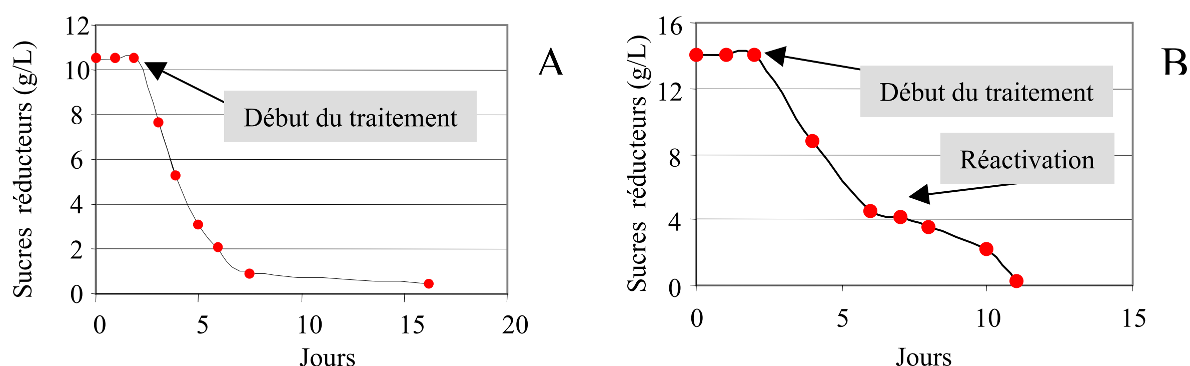


Figure 6. *Evolution of the residual sugars concentration after a stuck fermentation has been treated using encapsulated S. cerevisiae into calcium alginate balls in a red vinification in a volume of 325 hL (**A**). White vinification in a volume of 22 hL (**B**).*

Sweet wines winemaking

The benefit of being able to withdraw the immobilized cells at a previously well-set sugars concentration has been exploited in this study for the production of sweet wines. Indeed, in the white wines winemaking which have a high residual sugars content, the alcoholic fermentation has to be rigorously stopped when the residual sugars concentration reaches a pre-set value, according to the desired characteristic of the final product and which is of 50 to 100 g/L. Commonly, to stuck the alcoholic fermentation at the desired time is obtained through the cooling and/or the racking sheltered from air, filtration and then fortification with sulphur dioxide (SO2 L = 50-60 mg/L). The included yeasts offer the possibility of withdrawing the micro-organisms and stopping thus the fermentation at the desired time, without being obliged to use an inhibitor.

We present here as an illustration, the results obtained under real sweet winemaking conditions using the encapsulated *S cerevisiae* yeasts in order to ensure the alcoholic fermentation.

Fig. 7 shows the evolution curves of the ethanol and residual sugars concentrations. The temperature during the fermentation has been measured but not controlled. The average temperature has been of around 16°C. On the 20th day, fresh must has been added to the fermentation vat and disturbed the kinetic profile, but without stopping the fermentation that went up to 49g/L of residual sugars (very close from the 50g/L that are wished by the enologist). At this very moment, the bags are withdrawn from the tank. Fig. 7 shows that after the balls withdrawal, the residual sugars concentration has not changed, which indicates that there has been no fermentative activity from eventual indigenous yeasts or by bacteria: no change in the ethanol nor in the volatile acidity has been noted (Silva and coll., 2002). The values of these variables remained constant during 15 days after the withdrawal of the immobilized yeasts.

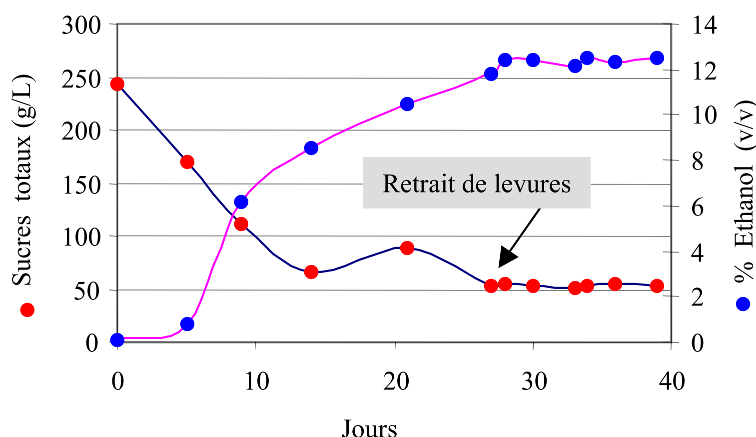


Figure 7. *Evolution of the residual sugars consumption, of the ethanol production during the vinification of a sweet white wine by immobilized S. cerevisiae yeasts.*

CONCLUSION

The studies that have been carried out for more than 10 years about the formulation of included yeasts have led to the creation of those double-coat dried balls. The practician has at his disposal an efficient technical tool, stable and easy to use. The main applications of those formulations concern up to today the foaming, the must desacidification by *Schizosaccharomyces* yeasts and the curative treatment of the stuck fermentations. Another interesting use concerns the managing of the liqueur-like wines, in which case there's always the problem of being able to stop the fermentation at the right time.

The additional research that is being currently led turns on:

- The application of Shizosaccharomyces included yeasts for the musts desacidification at the end of the alcoholic fermentation and which MLF does not happen. On this matter, some positive preliminary results have been obtained in laboratories. The validation in wineries is currently being carried out.
- The encapsulation of specific yeasts from which a partial activity in the must is desirable, for example aromatic yeasts.
- The encapsulation of lactic bacteria
- The encapsulation of specific enzymes

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