# Deacidification of grape musts by *Schizosaccharomyces* entrapped in alginate beads: a continuous-fluidized-bed process

P. Taillandier*, M.L. Cazottes and P. Strehaiano

*Laboratoire de Génie Chimique, URA CNRS 192, Ecole Nationale Supérieure d'Ingénieurs de Génie Chimique, 18 chemin de la Loge, 31078 Toulouse Cedex (France)*

**Abstract**

One possible biological method for wine deacidification is elimination of malic acid from grape musts by *Schizosaccharomyces* yeasts prior to alcoholic fermentation. In this work we propose a new continuous process with yeasts included in double-layer alginate beads. The beads were retained in a fluidized-bed column reactor. In order to avoid $CO_2$ retention, a stirred flask was added to the classical experimental set-up. The feasibility of the process has been demonstrated and its performance has been evaluated: 1.5–2.4 g $l^{-1}$ $h^{-1}$ of malic acid consumed for 60% of beads in the column.

## 1. Introduction

Removing malic acid from wines is often necessary in order to improve their organoleptic quality and their biological stability. The method generally used throughout the world is the malolactic fermentation (MLF) due to lactic acid bacteria but this method is not always well controlled by winemakers [1–4]. For several decades, microbiologists have been suggesting deacidification by *Schizosaccharomyces* (*SHZ*) yeasts as an alternative to MLF since these yeasts are able to convert malic acid into ethanol with great efficiency [5, 6]. We have been collaborating with a winery in the South of France (Jurançon area, Pyrénées-Atlantiques) for several years. In this winery, for dry white wines, malic acid elimination from grape musts is achieved by *SHZ pombe* during batch fermentations prior to alcoholic fermentation by *Saccharomyces* yeasts. Our first observations on this subject were reported elsewhere [7, 8].

In other respects, in the field of alcoholic beverages, the use of micro-organisms entrapped in alginate beads has been widely proposed [1, 9–13] since this polymer is very suitable for food technology. In addition, immobilization of micro-organisms allows the development of continuous processes [12, 14–16].

*Author to whom correspondence should be addressed.

This work deals with a new process developed in the laboratory scale for continuous deacidification of grape musts by immobilizing yeasts in double-layer alginate beads. A double-layer bead consists of an internal layer of alginate and cells coated by an external layer of sterile alginate. The interest of the double layer is to limit the release of yeasts by the beads.

## 2. Materials and methods

### *2.1. Micro-organism*

The yeast strain used was the *SHZ pombe* G2 isolated on grape must and provided by the Institut Coopératif du Vin (Montpellier, France).

### *2.2. Culture media*

#### *2.2.1. Synthetic media*

This medium was used for biomass growth before immobilization in alginate beads and for the experiment with free cells. Its composition was as follows in tap water (g $l^{-1}$): glucose, 50; yeast extract, 4; asparagine, 4; $KH_2PO_4$, 5; $MgSO_4$, 0.4; malic acid, 8. Its pH was 3.

#### *2.2.2. Natural media*

This medium was pasteurized concentrated grape must. Before continuous fermentations by entrapped *SHZ* it was diluted with sterile water in such a way that the sugar concentration $S_0$ was 100 g $l^{-1}$. It

was completed with malic acid of concentration $M_0$ up to 9 g $l^{-1}$.

*2.3. Immobilization technique*

Cells were grown in a synthetic medium for 24 h at 30 °C and then centrifuged at 4000 rev $min^{-1}$ for 20 min. The immobilization procedure in 2% alginate beads was described elsewhere [17]. The diameter of the double-layer beads obtained was 2.5 nm and their cells loading was $5 \times 10^8$ yeast cells per gram of beads (equal to 20 mg of biomass per gram of beads).

*2.4. Comparison between free and entrapped cells*

These experiments were carried out in Erlenmeyer flasks containing 500 ml of synthetic medium. The medium was magnetically stirred and maintained thermostatically at 30 °C. Each experiment was duplicated.

*2.5. Continuous deacidification process*

*2.5.1. Reactor design*

The device (Fig. 1) consisted of a glass column reactor of total volume 0.58 l (diameter, 0.075 m; height, 0.15 m) connected to an agitated flask of working volume 1 l. Beads were retained into the column between two perforated plates. Thanks to a peristaltic pump the medium was circulating throughout the system passing through the column from the bottom to the top and fluidizing the bed of beads; the flow rate of this pump was designated at the fluidization rate $F$ (l $h^{-1}$). The percentage of fluidization is defined as the ratio of the difference

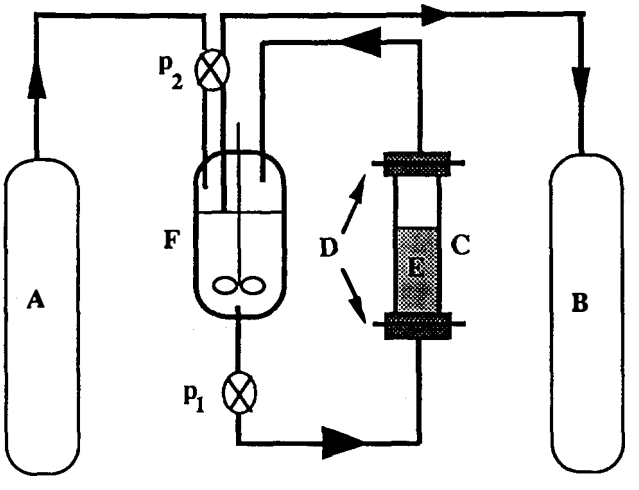


Fig. 1. Schematic drawing of the continuous demalication device: A, fresh medium storage tank; B, exit for fermented medium; C, column reactor; D, perforated plates; E, bed of beads; $p_1$, peristaltic pump for bed fluidization; $p_2$, peristaltic pumps for feeding and effluent; F, agitated flask for $CO_2$ release.

between the height of the fluidized bed and the height of the fixed bed to the height of the fixed bed. At the same time, two other peristaltic pumps were respectively supplying fresh medium to the agitated flask and removing fermented medium from there at the same rate $Q$ (l $h^{-1}$). The dilution rate $D$ ($h^{-1}$) is defined as the ratio of feeding rate $Q$ to total liquid volume $V_L$ (l). The residence time $\tau$ (h) is the inverse of the dilution rate.

*2.5.2. Operating conditions*

The device was sterilized by autoclaving at 120 °C. Its total working volume (column, flask and connections) was 2 l. The column was filled with 60% (apparent volume of beads/column volume) of alginate beads (corresponding to 280 g of beads). Diluted grape must was then added. The total liquid volume $V_L$ was 1.7 l. The system temperature was kept thermostatically at 25 °C by a water jacket surrounding the column.

*2.6. Analytical methods*

The sugar and malic acid concentrations were assayed using the enzymatic tests of Boehringer. The total concentration of free cells was estimated by counting under a microscope.

## 3. Results and discussion

*3.1. Reactor design and operation*

We first assayed fermentations by *SHZ* immobilized in alginate beds in a bubble column reactor [17]. Although these experiments gave good results for both deacidification rate and alginate bead resistance, an industrial process for winemaking without the use of continuous gas flow would be better. These observations led us to conceive a new process. Thought alginate gel is rather strong, mechanical agitation cannot be used for a long time without inducing gel breakage [18]. This is why most of the processes involving yeasts included in alginate beads consist of a column reactor homogenized by liquid circulation. Two kinds of device are found: fixed bed or fluidized bed. The latter seems the more efficient for alcoholic fermentation and it is more widely used in this field [9, 14, 19–22]. Nevertheless, one of the main problems encountered with this device is the $CO_2$ retention in the column [15, 23–25]. According to Nagashima [9] two designs of column reactor exist: height/diameter = 7–10 or height/diameter = 1.5–2.

During preliminary studies we found that the second kind of fluidized-bed column geometry containing 60% of beads (apparent volume of beads/

column volume) according to the literature [15] showed the least gas retention. In order to solve completely the problem of $CO_2$ retention we brought about some modifications to the classical fluidized-bed column reactor by adding an agitated flask, so allowing $CO_2$ release.

The objective of these experiments was to evaluate the performance of the continuous process for complete demalication of highly acidic grape musts.

Several dilution rates $D$ were tested for a constant fluidization rate $F = 21$ l h $^{-1}$. For this value, at steady state, the malic acid and sugar concentrations were the same in the column and in the stirred flask. This was possible because $F$ was 40–70 times greater than $Q$ according to recommendations of Radovich [26].

When the dilution rate was modified, the new steady state was reached after about 5 times the residence time. Each steady state was maintained for at least 10 times the residence time and each run was duplicated or triplicated. During each steady state three samples were taken daily from the column and also from the stirred flask in order to check the homogeneity of the liquid phase in the system.

### 3.2. *Performance of the continuous process for deacidification*

Three steady states were produced. For each steady state the average value of the malic acid consumed was calculated. Productivity was defined as the product of $D$ by the amount of malic acid consumed. Table 1 summarizes the results.

For the studied values of $D$, 86.7%–96.7% of the malic acid initially present in the grape must were consumed. The must initially contained 9 g l $^{-1}$ of malic acid. This concentration is very high and is rarely encountered. Acidic grape must usually contains 7–8 g l $^{-1}$ of malic acid. So we can consider that the aim of this work, namely complete deacidification, was achieved. According to the characteristics of the grape must the winemaker could easily control the extent of deacidification by adjusting the feeding rate $Q$.

The maximum specific growth rate $\mu_{max}$ of the strain of *SHZ pombe* is equal to 0.24 h $^{-1}$ [8]. Compared with continuous fermentation of free cells, the immobilization of yeasts allowed high values of $D$ (greater than $\mu_{max}$) and high biomass concentration at the same time, and so high productivities for continuous operation. The system was shown to be very stable as the same beads were employed for 6 weeks.

### 3.3. *Consumption of sugars*

The results for sugar consumption can be found in Table 2.

Figure 2 shows a comparison between consumed malic acid and consumed sugars as a function of dilution rate. Whereas the variation of $D$ has little effect on the malic acid consumed, the amount of consumed sugars strongly decreased when $D$ was raised. These data corroborate the hypothesis that sugar metabolism and malic acid metabolism are not strictly linked together [7, 27]. The amount of consumed sugars remained small, especially when the dilution rate was high. Thus after deacidification by *SHZ* treatment there is enough sugar left for the alcoholic fermentation by *Saccharomyces*.

### 3.4. *Influence of F*

The influence of the fluidization rate was then studied for $D = 0.26$ h $^{-1}$. $F$ was increased to 48 l h $^{-1}$; the percentage of fluidization then became equal to 30%. At steady state, the malic acid and sugar concentrations were the same as for the former value of $F$, indicating that for 10% fluidization the mixing of the liquid phase was enough and the transfer between the liquid phase and the beads was good [26].

### 3.5. *Comparison of kinetic data with free and entrapped cells*

This experiment was performed in order to determine whether the diffusion of substrates in the alginate gel was limited as suggested by some authors [28, 29].

The concentration of yeast cells in the continuous process is equivalent to $82 \times 10^9$ cells per litre of liquid medium. We started the batch fermentations in flasks with the same concentration of beads.

TABLE 1. Influence of dilution rate on the amount of consumed malic acid for the different steady stages[a]

| Dilution rate $D$ ($h^{-1}$) | Consumed malic acid (g $l^{-1}$) | Consumed malic acid (%) |
|---|---|---|
| 0.17 | 8.7 | 96.7 |
| 0.26 | 8.1 | 90 |
| 0.31 | 7.8 | 86.7 |

[a] $M_0 = 9$ g $l^{-1}$.

TABLE 2. Influence of dilution rate on the amount of consumed sugars for the different steady stages[a]

| Dilution rate $D$ ($h^{-1}$) | Consumed sugars (g l $^{1}$) | Consumed sugars (%) |
|---|---|---|
| 0.17 | 48 | 48 |
| 0.26 | 28 | 28 |
| 0.31 | 14 | 14 |

[a] $S_0 = 100$ g $l^{-1}$.

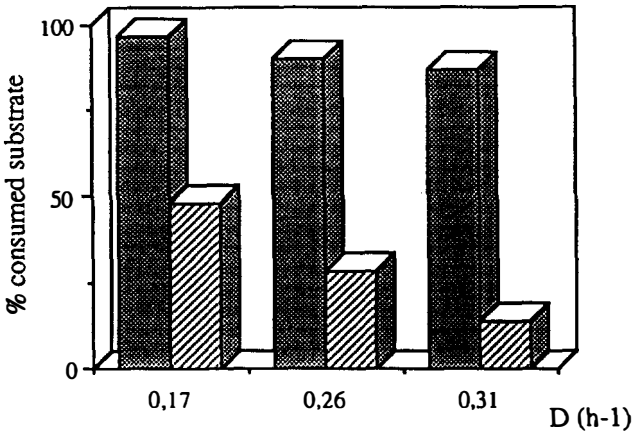


Fig. 2. Influence of the dilution rate on the percentage of consumed substrate from grape must: left column, malic acid; right column, sugars.

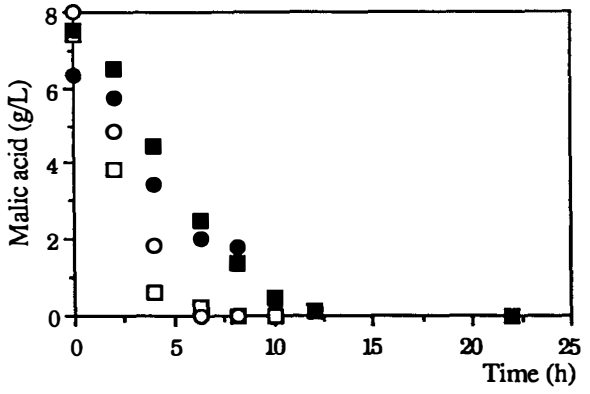


Fig. 3. Comparison of the demalication kinetics on a synthetic medium for free *SHZ* (□, ○) and for included *SHZ* (■, ●).

During the experiment with free cells, the growth of cells could not be avoided. We inoculated the medium with such an amount of free cells that the average concentration of yeast during fermentation was equal to the total entrapped biomass concentration. The initial concentration was $40 \times 10^9$ cells per millilitre and the final concentration was $120 \times 10^9$ cells per millilitre.

The kinetics of malic acid consumption during duplicated experiments is shown in Fig. 3.

The mean deacidification rate was calculated to be 0.6 g $l^{-1}$ h $^{1}$ for entrapped cells and 1.2 g l $^{1}$ h $^{1}$ for free cells. Assuming that the biomass concentration was the same in both cases we can conclude that diffusion of the substrates in the alginate beads was limited. Some explanations for this phenomenon are proposed in the literature. This diffusion limitation for large molecules could be due to an inhomogeneous concentration of alginate within the beads [30]. According to some authors [18, 31, 32] it can be attributed to a cell density gradient in the beads because of the cell growth in the gel after immobilization. The cells singly or in aggregates then occupy the originally empty pores in the gel beads, leading to larger diffusional resistance [31] or building up a diffusion barrier [32]. For the same alginate concentration, limitation of diffusion also depends on the bead diameter. The intraparticle diffusion limitation represented by the Thiele modulus increases with increasing bead diameter [33]. The overall diffusion rate is lowered when the bead diameter is raised from 1 to 3 mm [17].

Despite these observations the continuous process with entrapped yeasts remains much more efficient than free-cell fermentations for two reasons: more biomass can be retained in the system and higher flow rates ($D$ greater than $\mu_{max}$) can be used in continuous operation.

## 4. Conclusion

In the field of winemaking, *SHZ* yeasts are able to achieve the removal of malic acid before the alcoholic fermentation. At present this deacidification method is industrially employed in free-cell batch fermentations, giving a productivity for malic acid consumption of 0.05 g l $^{1}$ h $^{1}$ [7]. The aim of this study was to develop an experimental set-up on a laboratory scale for continuous grape must deacidification and to evaluate its performance. This aim has been achieved. The method of immobilization chosen was inclusion in double-layer alginate beads. For the process proposed we obtained high productivity (1.5–2.4 g l $^{1}$ h $^{1}$ of malic acid consumed according to dilution rate). It can be easily adapted to the malic acid concentration of grape must for its complete deacidification: $D$ has to be adjusted to $M_0$. The reactor can be considered as a homogeneous one and no problem of $CO_2$ retention occurred. The system was shown to be stable for 6 weeks.

## Acknowledgments

This research was partly supported by a grant from Onivins and Conseil Régional de Midi-Pyrénées.

## References

1 C.R. Davis, D. Wibowo and R. Eschenbruch, *Am. J. Enol. Vitic.*, *36* (4) (1985) 290.
2 S. Lafon-Lafourcade, A. Lonvaud Funel and E. Carre, *Antonie Van Leeuwenhoek*, *49* (1983) 349.
3 A. Lonvaud Funel, Les levains malolactique, in *Les Acquisitions Récentes en Microbiologie du Vin*, Lavoisier, Paris, 1992, pp. 103–110.
4 F. Radler, Malolactic fermentation and the effect of antimicrobial compounds on lactic acid bacteria, in *Les Acquisitions Récentes en Microbiologie du Vin*, Lavoisier, Paris, 1992, pp. 35 42.
5 I. Benda, *Proc. Colloque International d'Oenologie d'Arc et Senans, Doubs, Vignes Vins*, (1973) 31.
6 R.D. Beelman and J.F. Gallander, in C. Chichester (ed.), *Advances in Food Research*, Academic Press, New York, 1979, pp. 1–53.
7 P. Taillandier and P. Strehaiano, *Biotechnol. Lett.*, *10* (7) (1988) 469.
8 P. Taillandier, *Thesis*, Institut National Polytechnique de Toulouse, 1990.
9 M. Nagashima, Progress in ethanol production with yeasts, in H. Verachtert and R. De Mot (eds.), *Biotechnology and Biocatalyst*, Dekker, New York, 1990, pp. 57–84.
10 C.R. Phillips and Y.C. Poon, *Immobilization of Cells*, Springer, Berlin, 1988.
11 P. Spetolli, A. Bottacin, M.P. Nuti and A. Zamorani, *Am. J. Enol. Vitic.*, *33* (1) (1982) 1.
12 F. Godia, C. Casas and C. Sola, *Process Biochem.*, (April 1987) 43.
13 P. Strehaiano, L'immobilisation des microorganismes: application en œnologie, in *Les Acquisitions Récentes en Microbiologie du Vin*, Lavoisier, Paris, 1992, pp. 89 102.
14 P. Linko and Y.Y. Linko, Industrial applications of immobilized cells, *CRC Crit. Rev. Biotechnol.*, *1* (4) (1984) 289–339.
15 M. Nagashima, M. Azuma, S. Noguchi, K. Inuzuka and H. Samejima, *Biotechnol. Bioeng.*, *26* (1984) 992.
16 P. Taillandier, J.P. Riba and P. Strehaiano, *Bioprocess Eng.*, *7* (1991) 141.
17 S.V. Ramakrishna, P. Prema and P.S.T. Sai, *Bioprocess Eng.*, *6* (1991) 117.
18 J.C. Ogbonna, Y. Amano and K. Nakamura, *J. Ferm. Bioeng.*, *67* (2) (1989) 92.
19 G.H. Black, C. Webb, T.M. Matthews and B. Atkinson, *Biotechnol. Bioeng.*, *26* (1983) 134.
20 G.H. Cho, Y.D. Choi, C.V. Choi and M.H. Han, *Biotechnol. Lett.*, *3* (1987) 667.
21 A. Margaritis and F.J.A. Merchant, *CRC Crit. Rev. Biotechnol.*, *1* (4) (1984) 339.
22 E. Melin and W.K. Shieh, *Chem. Eng. J. B*, *50* (1992) 17.
23 H. Hamanci and D.D.Y. Ryu, *Biotechnol. Bioeng.*, *29* (1986) 994.
24 W. Woeher, *Bioprocess Eng.*, *4* (1989) 35.
25 A. Converti, M. Del Borghi and C. Sommavira, *Chem. Eng. J. B*, *47* (1991) 1.
26 J.M. Radovich, *Biotechnol. Adv.*, *3* (1985) 1.
27 I. Magyar and I. Panyik, *Am. J. Enol. Vitic.*, *40* (4) (1989) 233.
28 B.A. Westrin and A. Axelsson, *Biotechnol. Bioeng.*, *38* (1990) 439.
29 B. Ruggeri, A. Gianetto, S. Sicardi and V. Specchia, *Chem. Eng. J.*, *46* (1991) 21.
30 A. Martisen, I. Storro and G. Skjak-Braek, *Biotechnol. Bioeng.*, *39* (1991) 186.
31 H.T. Pu and R.Y.K. Yang, *Biotechnol. Bioeng.*, *32* (1988) 891.
32 B. Gosamann and H.G. Rehm, *Appl. Microbiol. Biotechnol.*, *29* (1988) 554.
33 C. Vives, C. Casas, F. Godia and C. Sola, *Appl. Microbiol. Biotechnol.*, *38* (1993) 467.

## Appendix A: Nomenclature

| | |
|---|---|
| $D$ | dilution rate $Q/V_L$ ($h^{-1}$) |
| $F$ | fluidization rate ($l\ h^{-1}$) |
| $M_0$ | initial concentration of malic acid in the medium ($g\ l^{-1}$) |
| $S_0$ | initial concentration of sugars in the medium ($g\ l^{-1}$) |
| $Q$ | feeding rate ($l\ h^{-1}$) |
| $V_L$ | total liquid volume in the system (l) |

*Greek letters*

| | |
|---|---|
| $\mu_{max}$ | maximal specific growth rate of the yeast ($h^{-1}$) |
| $\tau$ | residence time $1/D$ (h) |