Malate degradation by Schizosaccharomyces yeasts included in alginate beads

Patricia Taillandier, Jean-Pierre Riba, Pierre Strehaiano

Malate degradation by Schizosaccharomyces yeasts included in alginate beads

P. Taillandier, J. P. Riba, P. Strehaiano, Toulouse, France

Abstract. Schizosaccharomyces yeasts can be used for deacidifica tion of grape musts. To this aim, we studied malic acid degradation by yeasts included in double layer alginate beads in a bubble column reactor. Use of immobilized micro organisms allowed a continuous process with high dilution rates giving a deacidification capacity of 0.032 g of malate/hour/dm^3/g of beads. The pneumatic agitation was very convenient in this case.

List of symbols

D	h^{-1}	Dilution rate for continuous culture
τ	h	Residence time for continuous culture
dM/dt	$kg/(m^3 \cdot h)$	Rate of degradation of malic acid
dS/dt	$kg/(m^3 \cdot h)$	Rate of consumption of glucose
μ_{max}	h^{-1}	Maximal specific rate of growth

1 Introduction

The Schizosaccharomyces yeasts are of great interest for the field of enology, but are not very well known. The main characteristic of these yeasts is their ability to convert malic acid from grape musts into ethanol and CO_2 (to obtain high quality wines, malate removing is often necessary). In the same time, glucose is fermented into ethanol; the yield of conversion is similar to the one observed with classical vinification yeasts (species Saccharomyces cerevisiae). Among the 4 species of Schizosaccharomyces, the species pombe seems to be the most efficient one for malate degradation [1].

In collaboration with a winery in the area of Jurançon (Pyrénées-Atlantiques, France), we have carried out a complete study about the kinetic and metabolic behaviour of Schizosaccharomyces pombe [2]. A part of this study dealing with the use of S. pombe included in double layer alginate beads is presented in this paper.

This way of immobilization in simple alginate layer is the most widely used for yeasts cells, specially in the case of alcoholic fermentation [3].

In a preliminary study on included cells, we observed the influence of 2 parameters: the concentration of yeasts into the gel, the concentration of the beads in the liquid phase. Then, fermentations using alginate beads were conducted in a bubble column reactor in semi-continuous and continuous runs.

2 Materials and methods

2.1 Yeast strain

The Schizosaccharomyces pombe strain used was the strain G2 isolated by the Institut Coopératif du Vin (ICV, Montpellier, France).

2.2 Culture medium

A synthetic medium consisting of the following components (in kg/m^3) was chosen: glucose 100; malate 8; yeast extract 4; KH_2PO_4 5; $MgSO_4$ 0.4; asparagine 4; pH 3. With this medium, the growth and deacidification kinetics were similar to those obtained with white grape musts [4].

2.3 Culture conditions

For all experiments, temperature was regulated at 30 °C and aeration rate in reactors was 0.15 VVM.

The preliminary study was carried out in Erlenmeyers flasks containing 150 cm^3 of medium, with magnetic stirring at 100 min^{-1}. The bubble column reactor was a glass cylinder of 2.33 dm^3 total volume (diameter = 7.5 cm; height = 46 cm). Agitation and aeration were achieved by microbubbles of air arriving at the bottom of the column. For continuous cultures, fresh medium was supplied by a peristaltic pump at a constant rate at the bottom of the reactor while fermented medium was removed at the top.

2.4 Immobilization technique

Cells were grown in a standard medium for 24 h and then centrifugated at 4000 min^{-1} for 20 min. After that they were washed twice with 0.9% saline solution. A suspension of cells at the appropriate concentration was mixed with the same volume of an autoclaved 4% sodium alginate solution.

To make beads, a system consisting of 2 concentric needles was used. The central needle was connected with the solution of alginate and cells (concentration of alginate = 2%), while the external needle was connected with a 2% sodium alginate solution. These 2 solutions were added dropwise to a stirred 170 kg/m^3 $CaCl_2$ solution forming double-layer beads of 2 mm diameter. The beads were incubated in $CaCl_2$ for 30 min before being washed with distilled water.

2.5 Analytical methods

Biomass determination

In the liquid phase, cells concentration and viability were determined by microscope counting after vital staining with methylene blue.

Glucose, ethanol, malic acid determination

Glucose and malic acid were measured by HPLC using a I-300 INTERACTION column for organic acid analysis and a GC 801 INTERACTION guard column. The elution solvant was a 0.05 N H_2SO_4 solution drived with a WATERS pump at a flow rate of 0.4 cm^3/min. The detector was a differential SPECTRA-PHYSICS refractometer.

Ethanol was quantified by gas chromatography using isopropanol as internal standard and a porapak-Q column.

3 Results

A continuous culture of free cells was first achieved with dilution rates (D) varying from 0.035 to 0.25 h^{-1}. To get an adequate deacidification, at least 90% of malic acid has to be eliminated. Such results could be observed only if the residence time (τ) was greater than 10 h ($D < 0.1$ h^{-1}). This bad efficiency of the process is due to the slow kinetics of the yeast. So, in order to define a continuous process for malate elimination from grape musts it was necessary to have recourse to immobilized micro-organisms.

3.1 Preliminary study

Variable capacity of the inclusion matrix is one of the main advantages of micro-organisms inclusion. Indeed, biomass concentrations of up to $1 \cdot 10^9$ cells per cm^3 of alginate can be immobilized if necessary [5]. We measured the malic acid (Fig. 1) and the glucose (Fig. 2) consumption rates for different concentrations of beads in the liquid phase and different concentrations of cells into the gel. These experiments were performed in Erlenmeyer flasks using the synthetic medium.

It can be observed that deacidification activity increases with cells density into the beads. From 50 kg/m^3 of beads in the medium, degradation rates are kept constant for a same kind of beads.

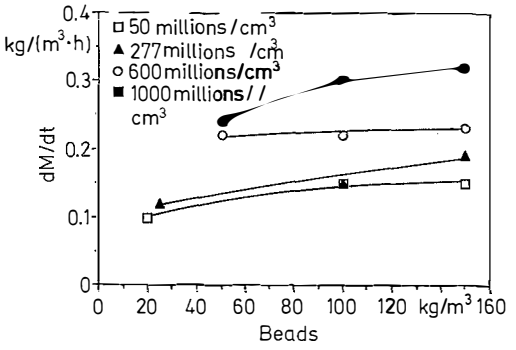

Fig. 1. Rates of consumption of malic acid as a function of: the concentration of the cells in the alginate beads (in millions of cells per cm^3 of internal layer); the concentration of the beads in the medium (in kg/m^3)

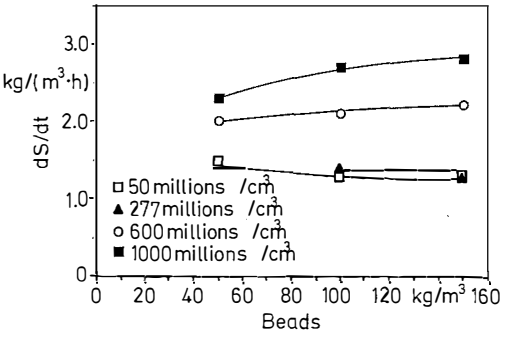

Fig. 2. Rates of consumption of sugar as a function of: the concentration of the cells in the alginate beads (in millions of cells per cm^3 of internal layer); the concentration of the beads in the medium (in kg/m^3)

3.2 Experiments in the bubble column reactor

This type of reactor seemed more convenient for cultivation of yeasts included in alginate beads, since the agitation resulting from air flow was very mild. The second advantage of inclusion of micro-organisms is re-usability of the biocatalyst.

We studied two kinds of fermentations in a bubble column: semi-continuous and continuous.

Semi-continuous run

It consisted in repeated batches, always keeping beads into the reactor and changing the medium when malic acid was exhausted. As shown in Fig. 3, four repeated batches were realized with 90 kg/m^3 of beads containing $1 \cdot 10^9$ yeasts per cm^3 of alginate (internal layer).

We can note for both substrates, sugar and acid, increasing consumption rates for each batch until the third. Afterwards these rates keep constant. This well known phenomena might be caused by cell growth inside the gel [6–9]. It could be verified by counting cells inside the gel after dissolution of beads, before and after fermentation. Thus a "pseudo-stationary" state of the system was established at the third cycle when the density of the biomass in the beads was maximum.

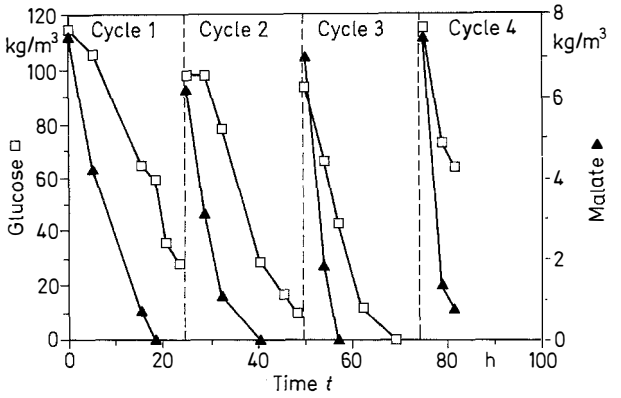

Fig. 3. Semi-continuous run of beads in the bubble column: consumption of sugar and malate

Table 1. Residual concentrations of sugar and malate during the stationary states of the continuous culture

D [h^{-1}]	τ [h]	Glucose [kg/m^3]	Malate [kg/m^3]
0.24	4.2	36	0.5
0.28	3.6	43	0.9

Continuous run

A continuous fermentation was carried out with 100 kg/m^3 of beads containing $1 \cdot 10^9$ cells per cm^3 of alginate internal layer. Dilution rates driving to an adequate deacidification (at least 90%) were studied. Data are shown in Table 1.

In this case, a residence time of about 4 h could be used, whereas with the free cells continuous process the same deacidification level was obtained for $\tau = 12$ h. The higher efficiency of the included yeasts process could be attributed partly to the free cells released from the beads to the liquid phase during fermentation, and partly to the high amount of biomass brought by the biocatalyst. Its deacidification capacity was equal to 3.2 g of malate per hour and dm^3 of liquid broth, equal to 0.032 g of malate/g of beads/hour/dm^3 of liquid broth.

In the case of the semi-continuous process, the deacidification capacity was only 0.02 g of malate/g of beads/h/dm^3 of liquid broth.

4 Discussion

First, we must underline the originality of our inclusion method consisting of adding a sterile alginate layer around beads. The interest of this double layer is to limit the release of the cells in the outside medium. This characteristic can be determining for some applications like sparkling wine elaboration, but is not very important in the case of must deacidification by S. pombe. In this case, culture conditions were in favour of rapid growth and thus allowed cell leakage. But in conditions of the wine-making process, Schizosaccharomyces yeasts would not be able to develop.

The increase of the consumption rates observed when the concentration of the cells in the beads was higher can be explained by the following hypothesis. Inside the alginate beads, only cells on the periphery of the gel are active. Substrates cannot reach the center of the bead either because their diffusion is limited [5, 10], or because they are completely consumed by the cells on the periphery [11]. So, when the total concentration of yeasts in the gel increases, the percentage of active cells is also increased.

Micro-organisms immobilization always allows higher productivities for two reasons:

- The system can work continuously with $D > \mu_{max}$ of the cells;
- Very important amounts of biomass can be employed, thus the reaction rates are increased.

These characteristics were well illustrated with fermentations in the bubble column in comparison with results obtained with free cells.

Despite the double alginate layer, cells releasing from the beads into the liquid phase sometimes appeared. During repeated batches, it happened at the end of the third cycle. In the continuous run, the concentration of free cells at stationary state was equal to $45 \cdot 10^6$ cells per cm^3. This is in agreement with some authors reporting that newly released cells got higher specific growth rate than free cells [6, 12, 13]. As we told before, in the context of grape musts deacidification by S. pombe, cells release was not negative. On the opposite, it could improve the global activity of the system [13].

For continuous utilization, the bubble column worked with mixing conditions near the one of a plug flow reactor, which contributed to enhance the efficiency of the system.

5 Conclusion

Inclusion of micro-organisms in alginate beads widely used in biotechnology found a new application in our experiments. Indeed, this technique allows the total control of grape musts deacidification by S. pombe. Inoculation with an important amount of deacidifying yeasts is possible, because their immobilisation drives to a rapid deacidification and at the same time solves the problem of the implantation of these micro-organisms.

Experiments on grape musts not shown here were carried out in the winery in tanks of 100 dm^3 non-aerated and non agitated. They gave interesting results: the deacidification was rapid and intense; beads were resistant, easy to manipulate and no cells release was noted.

Bubble column reactors present some advantages: they are easy to build and to extrapolate, the energy required is smaller compared to mechanically stirred fermentors. For fermentations with cells included in alginate beads, the pneumatic agitation was preferable, because it creates less breakages in the gel.

Acknowledgements

The authors gratefully acknowledge the financial support of the Direction Générale de l'Alimentation – Ministère de l'Agriculture.

References

1. Lodder, J.: The yeasts. A taxonomic study. 2nd Edition, 733 755. North Holland, Pub. Co: Amsterdam, London, 1970
2. Taillandier, P.; Riba, J. P.; Strehaiano, P.: Malate utilisation by Schizosaccharomyces pombe. Biotech. Lett. 10, 7 (1988) 469 472
3. Margaritis, A.; Merchant, F. J. A.: Advances in ethanol produc tion using immobilized cells system. CRC Crit. Rev. Biotechnol. 1, 4 (1984) 339 393
4. Auriol, P.; Tulasi, S.; Goma, G.; Strehaiano, P.: Désacidification par Schizosaccharomyces. Etude sur les aptitudes de différentes souches. Approche cinétique sur la dégradation de l'acide malique. Rev. Fr. Oenol. 108 (1987) 37 42
5. Ogbonna, J. C; Amanoy, Y.; Nakamura, K.: Elucidation of optimal conditions for immobilization of viable cells using Ca alginate. J. Ferm. Bioeng. 67, 2 (1989) 92–96
6. Spetolli, P.; Bottacin, A.; Nuti, M. P.; Zamorani, A.: Immobi lization of Leuconostoc oenos ML 34 in Ca-alginate gels and its application to wine technology. Am. J. Enol. Vitic. 30, 1 (1982) 45 48
7. Tanaka, T.; Yamamoto, K.; Towprayoon, S.; Nakajima, H.; Sonomoto, K.; Yokozeki, K.; Kubota, K.; Tanaka, A.: Continu ous production of 1 serine by immobilized growing Coryne bacterium glycinophylum cells. Appl. Microbiol. Biotechnol. 30 (1989) 564–568
8. Zache, G.; Rhem, H. J.: Degradation of phenol by a co-immobi lized entrapped mixed culture. Appl. Microbiol. Biotechnol. 30 (1989) 426–432
9. Yang, H. D.; Hamanci, H.; Woodams, E. E.: Production of 1 lactic acid by Rhizopus oryzae immobilized in Ca alginate gels. Biotech. Lett. 11, 2 (1989) 119 120
10. Hannoun, J. M.; Stephanopoulos, G.: Diffusion coefficients of glucose and ethanol in cell-free and cell occupied Ca-alginate membrane. Biotech. Bioeng. 28 (1989) 829 835
11. Gosmann, B.; Rhem, H. J.: Influence of growth behavior and physiology of alginate entrapped micro organism on the oxygen consumption. Appl. Microbiol. Biotechnol. 29 (1988) 554–559
12. Santos-Rosa, F.; Ganvan, F.; Vega, J. M.: Biological viability of Chlamidomonas reinhartii cells entrapped in alginate beads for ammonium photoproduction. J. Biotechnol. (1989) 209 220
13. Mori, A.; Matsumoto, N.; Imai, C.: Growth behaviour of immo bilized acetic acid bacteria. Biotech. Lett. 11, 3 (1989) 183 188

P. Taillandier
J. P. Riba
P. Strehaiano
ENSIGC
Laboratoire de Génie pour les Bio-Industries
Chemin de la Loge
F 31078 Toulouse
France