Journal of Industrial Microbiology & Biotechnology (1999) 22, 48–51

http://www.stockton-press.co.uk/jim

Simultaneous and sequential fermentations with yeast and lactic acid bacteria in apple juice

M Herrero, C de la Roza, LA Garcia and M Díaz

Department of Chemical Engineering and Environmental Technology, University of Oviedo, Spain

A complex substrate, reconstituted concentrated apple juice, was used for testing the principal processes during yeast and malolactic bacteria fermentations. Interactions between microorganisms were studied based on two controlled inoculation procedures, and at different fermentation temperatures. Temperature had a more important effect on yeast growth than the presence of malolactic bacteria in the medium. Acceleration of the death phase of the bacterial population was detected at increased temperatures. In all cases, malic acid degradation was affected by the fermentation temperature. When experiments were carried out with simultaneous inoculation, acidification of the medium took place at both temperatures tested (15°C and 22°C), that was not observed when the malolactic bacteria were inoculated after completion of alcoholic fermentation by yeasts.

Keywords: alcoholic fermentation; malolactic fermentation; apple juice

Introduction

Alcoholic and malolactic fermentations are the main processes that take place in wine and cider making. Yeasts transform sugars to ethanol, but at the same time play an important role in organic acid production and volatile end-products in alcoholic beverages. The malolactic fermentation remains an imperfectly controlled process, since many nutritional and physico-chemical factors affect the growth and metabolism of lactic acid bacteria. Some of them depend on yeast strain used, providing different amounts of amino acids, peptides and vitamins available in must as growth factors for lactic acid bacteria development, or the presence of products of yeast metabolism which act as inhibitors, as for example, fatty acids and ethanol. Interactions between yeasts and lactic acid bacteria in wine or grape juice, and synthetic media have been previously studied [1,7,9].

The use of starter cultures in cider fermentation, both yeast and malolactic cultures, would allow one to maintain uniformity in the final product during successive processes and seasons. Although the use of starters to control industrial fermentations is well established for yeasts in the brewing and wine industry, it has not been widely adopted in cider making. Several lyophilized starter cultures for malolactic fermentation are available on the market, but are only useful for wine fermentations. Commercial starter cultures have a great advantage over traditional inoculation methods. Nevertheless, previous studies reported that indigenous malolactic bacteria were more adapted to musts and yielded better results in malic acid degradation [4,6]. Then, it was interesting to isolate and select an indigenous strain able to perform the malolactic fermentation in this complex and variable media.

Correspondence: M Díaz, Department of Chemical Engineering and Environmental Technology, Faculty of Chemistry, University of Oviedo, C/Julián Clavería s/n, 33071, Oviedo, Spain
Received 4 August 1998; accepted 9 December 1998

Malolactic bacteria can be inoculated at different stages: simultaneously with the yeast inoculum, or once the alcoholic fermentation has been completed. The first method might have some advantages for bacterial development, due to the lower ethanol levels and higher sugar content in musts, but may produce a delay in bacterial growth and malic acid degradation [5]. With the second method, production of d-lactate and acetate from sugars is avoided, and essential nutrients are available for bacteria as a result of yeast excretion and autolysis [1]. It seemed convenient to test both methods for the strains used under operating conditions, with the aim of selecting the optimal procedure to perform a controlled inoculation in industrial cider production.

In this work, a laboratory study using reconstituted concentrated apple juice sterilized by microfiltration was carried out to follow the interactions between a *Saccharomyces cerevisiae* yeast strain and an indigenous malolactic bacterium, at different inoculation times and fermentation temperatures, in order to establish the optimum conditions to induce these processes in a rapid and reproducible manner applicable to the cider industry.

Materials and methods

Microorganisms

A commercial active dry yeast strain of *Saccharomyces cerevisiae* was used. The malolactic bacterium (strain Lc2) was previously isolated in the cellar of the cider industry Escanciador, SA (Villaviciosa, Asturias, Spain), and was identified as a *Leuconostoc oenos* strain, which was selected on the basis of its ability to degrade malic acid.

Experimental conditions

Concentrated apple juice, supplied by an industrial cider factory, was reconstituted with distilled water (1 : 6), with a final density of approximately 1060 g L^{-1}. The juice was sterilized in a tangential flow filtration device (Filtron Omegacell 150™, Northborough, MA, USA) connected to

a peristaltic pump, using polyethersulfone membranes (0.33 μm pore diameter).

Fermentations were carried out in pre-sterilized 250-ml Erlenmeyer flasks containing 100 ml of the culture medium and placed on an orbital shaker (New Brunswick, G25, Edison, NJ, USA), at 100 rpm at the assay temperature.

An active dried preparation of yeast was rehydrated in sterile apple juice and grown under aerobic conditions at 250 rpm, 28°C, for 18 h. The apple must was then inoculated with yeast at a final concentration of 10^6 CFU ml^{-1} (colony forming units per ml). Malolactic bacteria were grown in apple juice, prepared as previously described, supplemented with yeast extract 0.5% (w/v), incubated at 30°C, without shaking due to the microaerophilic nature of this bacterium, for 6 days until the stationary phase was reached. To start the malolactic transformation, a high cell inoculum was used, adjusted to 10^7 CFU ml^{-1} in the fermentation medium (must or cider). In this way, the malic acid degradation phase was uncoupled from the bacterial growth phase. In sequential inoculation fermentations, once the alcoholic fermentation was completed and must density reached approximately 1005 g per litre as a result of yeast sugar metabolism, the malolactic bacterium inoculum was added to the flasks that was incubated at the assay temperatures and at the same conditions.

Microbiological populations

Populations of yeast and bacteria were followed by counting viable cells. Serial dilutions were done in saline solution, plating duplicates of statistically significant dilutions on selective media: YPG for yeast, and MRS (Biokar, Beauvais, France) supplemented with antibiotics to inhibit yeast growth (100 ppm cycloheximide) and 25 ppm 8-OH-quinoline) for malolactic bacteria. YPG plates were incubated at 30°C for 48 h; MRS plates, for 5 days at the same temperature.

Sample preparation and analytical methods

Apple juice and cider samples were filtered through 0.45-μm pore size membranes. Density of the culture medium (must or cider) without cells was measured by picnometry (giving the relative weight of a defined volume). The pH meter employed was a Crison (Barcelona, Spain) micropH 2001 model. l-Malic acid was determined by enzymatic assays (Boehringer Mannheim, Mannheim, Germany). Carbohydrates and polyalcohols were analysed by HPLC (Waters, Milford, MA, USA, Alliance 2690), with a differential refractometer (Waters 410). A Spherisorb-NH2 analytical column (20 × 0.4 cm, 5 μm, Teknokroma, Barcelona, Spain) was used under the following conditions: column temperature 30°C, mobile phase 80/20 acetonitrile/H_2O, 0.9 ml min^{-1}, detector temperature 45°C and volume injection 10 μl.

Results and discussion

Alcoholic fermentation by yeast in sequential inoculation model

When sequential inoculation was used, the temperature for alcoholic fermentation by yeast was fixed at 15°C, a low but non-restrictive temperature for the yeast fermentation, since a higher fermentation temperature would lead to formation of undesirable volatile end-products due to yeast metabolism. Under these conditions, the alcohol fermentation was completed in 11 days.

Density of the culture medium, viable cells (Figure 1a), and evolution of major sugars were followed during this phase (Figure 1b). The pH of the medium showed no significant variation, since values near the initial pH of apple must (3.5) were obtained.

S. cerevisiae viable cells reached stationary phase at 10^7 CFU ml^{-1}, thus increasing one logarithmic unit from the inoculation level. The yeast strain used was able to metabolize malic acid. Yeast may either break down or form malate during fermentations [11], and *S. cerevisiae* metabolized 3–45% of the malic acid in grape juice [7,10]. In duplicate fermentation experiments, using the same juice, the mean amount of malic acid degraded by the yeast strain at the completion of alcoholic fermentation was 0.65 g L^{-1} (data not shown).

Glucose is the first sugar consumed during wine and cider fermentation. In Figure 1b, apparently sucrose is the first sugar depleted, due to its hydrolysis under acidic conditions, yielding glucose and fructose.

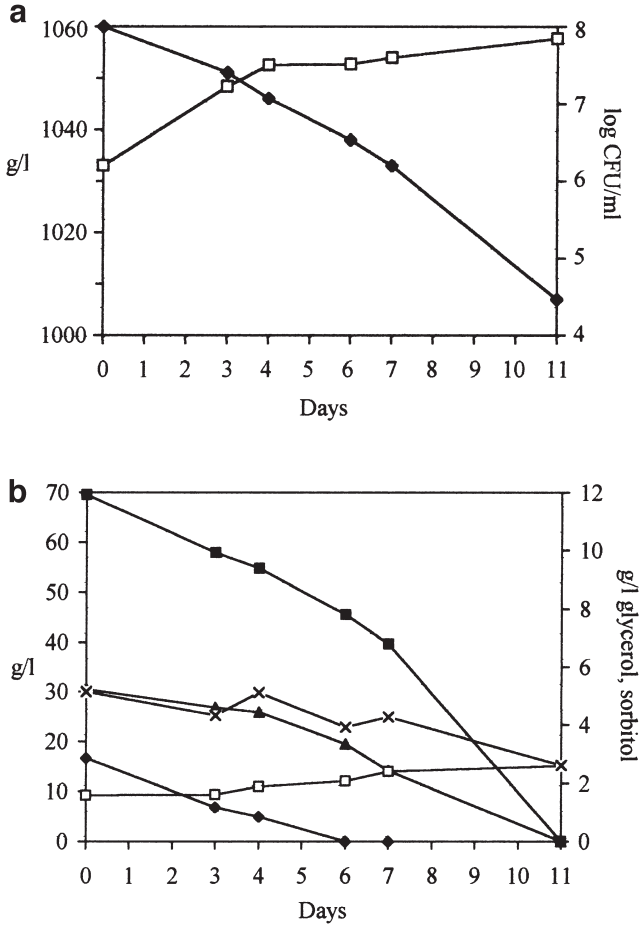

Figure 1 Fermentation by yeast at 15°C. (a) Change of must density (solid symbols) and growth of *S. cerevisiae* (open symbols). (b) Change of major sugars: fructose -■-, glucose -▲-, sucrose -◆-, glycerol -□- and sorbitol -×-.

Alcoholic fermentation with simultaneous inoculation of yeast and malolactic bacteria

Two different fermentation temperatures were assayed: 15°C, the same temperature used in the model described above, and 22°C, near the optimum growth temperature for *Leuconostoc* and a more permissive temperature for the activity of the malolactic enzyme. The variation in different parameters tested at both temperatures is shown in Figure 2.

As expected, yeast metabolized sugars more rapidly at 22°C than at 15°C, as indicated by the measurement of the density of the culture medium (Figure 2a) and evolution of sugars (Figure 2b and c). At 22°C, the alcoholic fermentation is completed in 3–4 days; at 15°C, the presence of malolactic bacteria in must delayed this phase only 2 days. At 22°C (Figure 2d), stationary phase was reached two logarithmic units above the inoculum level. Based on these observations, temperature has a more important effect over yeast growth than the presence of malolactic bacteria in the medium, although a slight delay in the initial sugar fermentation rate could be detected at 15°C. In previously reported work [7], *S. cerevisiae* growth in wine was unaffected by the presence of *L. oenos*, an observation contrary to other published results [2] which indicate that yeast growth and alcoholic fermentation rate were inhibited by lactic acid bacteria.

The number of malolactic bacteria (Figure 2d) maintained approximately the inoculation level. At 22°C, the malolactic population began to decline from day 10. At 22°C, the pH increased from the initial value until approx. 0.4 units at days 7–9, which indicates that malolactic transformation would be almost complete at that time, followed by an acidification phase (approx. 0.3 pH units) probably caused by acetic acid formation, that may be related to the decrease of viable malolactic cells. The pH values varied slightly at 15°C until day 12, when again an increase in pH was detected (data not shown).

Malolactic fermentation with simultaneous inoculation of yeast and malolactic bacteria

Malic acid degradation was determined at both temperatures assayed (data not shown). At the higher temperature, malic acid degradation was complete in 9 days while 33 days were needed at the lower fermentation temperature. In both cases, malic degradation began during alcohol fermentation by yeast.

A 0.4 pH unit variation revealed the existence of malolactic transformation. At 15°C, this maximum level was reached on days 16–17, when approximately 2/3 of the total malic acid was consumed, followed by a pH decrease (approx 0.3 pH units; data not shown). Greater acetic acid formation was reported in cider production after simultaneous inoculation of lactic bacteria and yeast [3].

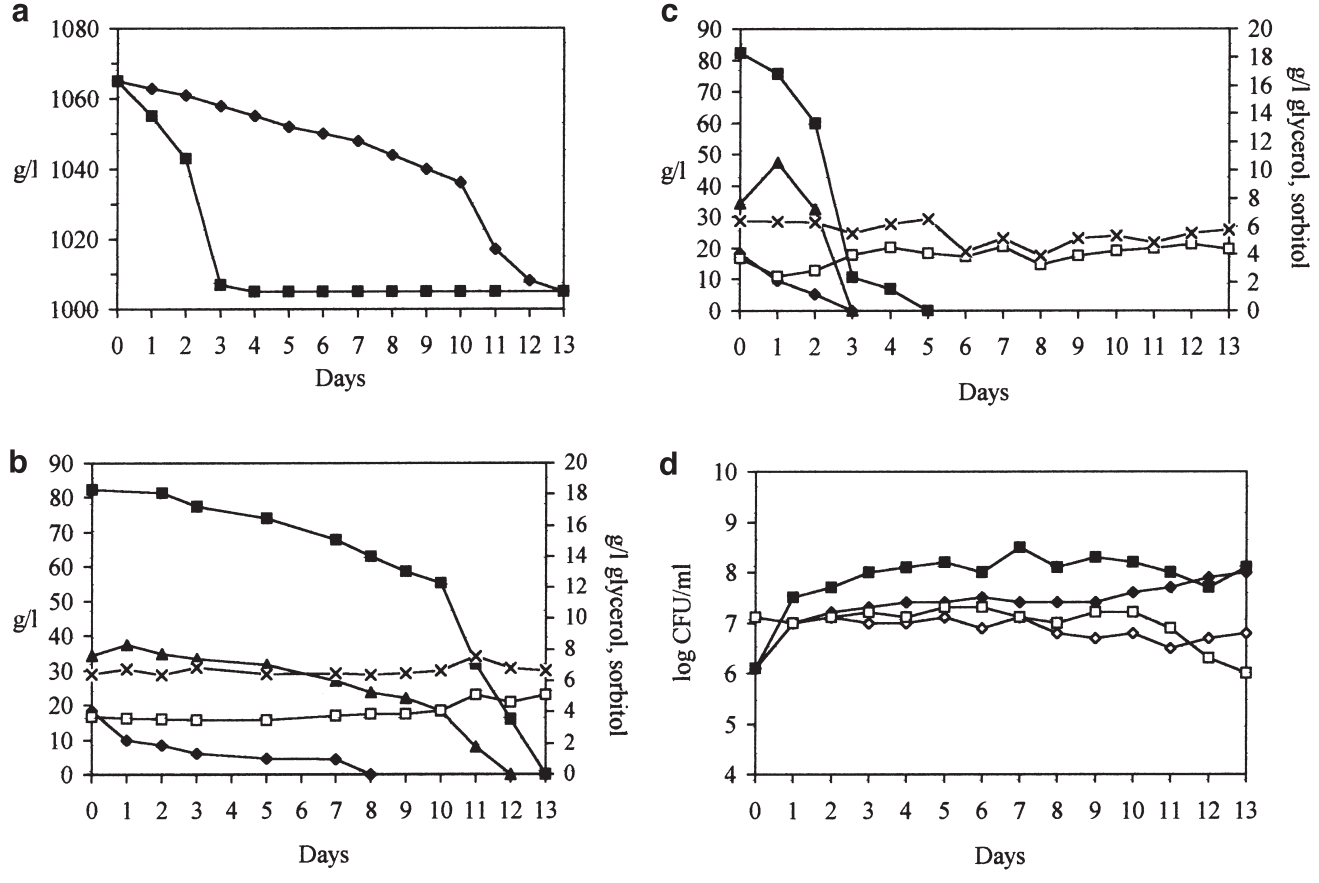

Figure 2 Alcoholic fermentation with simultaneous inoculation of yeast and malolactic bacteria. (a) Must density at 15°C (◆) and 22°C (■); major sugar consumption at 15°C (b) and 22°C (c): fructose -■-, glucose -▲-, sucrose -◆-, glycerol -□- and sorbitol -×-; (d) viable cells: *S. cerevisiae* (solid symbols) and *L. oenos* (open symbols), at 15°C (◆ ◇) and 22°C (■ □).

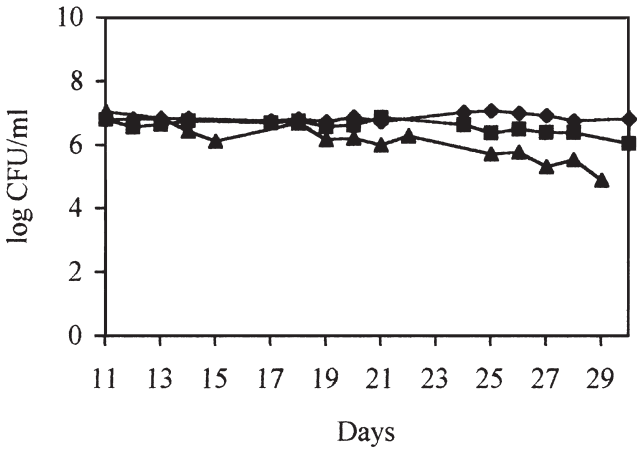

Figure 3 Malolactic bacteria viable cells in the sequential inoculation model at 15°C (◆), 22°C (■) and 27°C (▲).

The yeast population remained nearly constant at 15°C throughout the 38-day fermentation; fermentation at the higher temperature accelerated yeast death. Similar results were obtained for the bacterial population: the death phase began from day 27 at 15°C, or from day 10 at 22°C (data not shown).

Malolactic fermentation after sequential inoculation

Once the alcohol fermentation (at 15°C) was finished, malolactic bacterial inoculum was added and flasks were incubated at 27°C, 22°C or 15°C for 20 more days. Yeast populations remained at stationary phase and showed no differences at the three temperatures (results not shown), but the number of viable bacterial cells was significantly affected since acceleration of the death phase was detected at increasing temperatures (Figure 3).

There was an important effect of temperature fermentation on malic acid degradation: at 15°C, malic acid in the medium was consumed slower than at higher temperatures, taking 15 days from the inoculation of malolactic bacteria (results not shown). Based on these observations, 22°C was selected as the optimal temperature for malic acid degradation, since 9 days after inoculation of malolactic bacteria, 99% of malic acid was degraded while only 90% was metabolized at 27°C. It was previously reported that the optimal temperature for malolactic fermentation in wine is 20–25°C [8].

After bacterial inoculation, only glycerol and sorbitol could be detected. No significant differences were observed in the evolution of these compounds under the assayed temperatures (data not shown).

Conclusions

Sequential inoculation of malolactic bacteria was the most favourable, since acidification caused by malolactic bacteria when major sugars were available in must was observed, as well as a delay in initial sugar consumption rate during alcoholic fermentation. Low temperature fermentation (15°C) slowed down malic acid degradation, and the optimal temperature for malic acid transformation was near 22°C. It should be pointed out the different initial malic acid content in the two concentrated apple juices employed in this work (3.6 g L^{-1}, pH 3.5 and 6 g L^{-1}, pH 3.4; the latter juice being used only in the simultaneous fermentation experiments). This situation clearly reflects the lack of uniformity in raw material that should be overcome by industries. At the same time, the results obtained highlight the ability of the indigenous selected strain to perform the malolactic fermentation even at high malic acid concentrations.

Acknowledgements

This work was financially supported by the following Asturian cider industries: Sidra Escanciador, SA, Valle, Ballina y Fernández, SA, Sidra Mayador, SA and Industrias Zarracina, SA, Asturias, Spain and by FICYT (Foundation for Scientific and Technical Research, Asturias, Spain).

References

1 Beelman RB, RM Keen, MJ Banner and SW King. 1982. Interactions between wine yeasts and malolactic bacteria under wine conditions. Dev Ind Microbiol 23: 107–121.
2 Boidron AM. 1969. Sur deux causes d'inhibition des levadures par les bactéries lactiques. CR Acad Sci Paris, pp 922–924.
3 Cabranes C and JJ Mangas. 1996. Controlled production of cider by induction of alcoholic fermentation and malolactic conversion. J Inst Brew 102: 103–109.
4 Davis CR, DJ Wibowo, R Eschenbruch, TH Lee and GH Fleet. 1985. Practical implications of malolactic fermentation: a review. Am J Enol Vitic 36: 290–301.
5 Gallander JF. 1979. Effect of time of bacterial inoculation on the stimulation of malolactic fermentation. Am J Enol Vitic 30: 157–159.
6 Henick-Kling T, WE Sandine and DA Heatherbell. 1989. Evaluation of malolactic bacteria isolated from Oregon wines. Appl Environ Microbiol 55: 2010–2016.
7 King SW and RB Beelman. 1986. Metabolic interactions between *Saccharomyces* and *Leuconostoc oenos* in a model grape juice/wine system. Am J Enol Vitic 37: 53–60.
8 Lafon-Lafourcade S. 1983. Wine and brandy. In: Biotechnology (Reed G, ed),Vol 5, pp 81–163, Verlag-Chemie, Heidelberg.
9 Lemaresquier H. 1987. Inter-relationships between strains of *Saccharomyces cerevisiae* from the Champagne area and lactic acid bacteria. Lett Appl Microbiol 4: 91–94.
10 Rankine BC. 1966. Decomposition of l-malic acid by wine yeasts. J Sci Food Agric 17: 312–316.
11 Whiting GC. 1976. Organic acid metabolism of yeast during fermentation of alcoholic beverages. A review. J Inst Brew 82: 84–92.