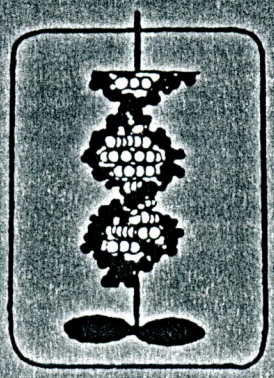
FROM GENES TO BIOPRODUCTS



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BIOTECHNOLOGY OF THE MALOLACTIC FERMENTATION IN WINEMAKING IN THE UTIEL-REQUENA REGION

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The studies on biotechnological applications of lactic acid bacteria (LAB) have been recently developed. These works have been leaded by dairy industries, whereas in winemaking these applications are less developed. Only in the recent years improvement in genetics, development of immobilized systems, or studies in enzymology, have been subject of interest to wine biotechnologists.

LAB are important microorganisms in winemaking. They affect the wine quality because they are responsible for malolactic fermentation (MLF). MLF is an enzymatic process that converts the L-malic acid in L-lactic acid and CO_2. Besides this, LAB are well known for their ability to produce flavor compounds. Some of them, such as diacetil, acetoin and 2,3-butanediol, are of considerable importance in the flavor profiles of wines (1).

The application of the new biotechnological techniques to improve wine quality is the subject of our work. We focused this approach under the following schedule:

a) Isolation of strains from musts and wines in cellars.
b) Study of the dynamics of LAB species developing in must fermentation, and which of them conducted the MLF.
c) Controlling the MLF by:
    1.- inoculation of selected strains.
    2.- development of genetic methods to improve malolactic activity of selected strains.

d) Determination of the role of MLF in the overall metabolism of LAB.

The main results from the two first points are followed. The strains isolated in fresh musts belonged to *Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus hilgardii*, *Lactobacillus plantarum* and *Leuconostoc paramesenteroides*. Throughout the alcoholic fermentation, only *Lactobacillus fructivorans*, *L. hilgardii* and *Leuconostoc oenos* remained, being this last species which conducted the MLF in wines of Utiel-Requena region (2).

The third level of this research was the control of the MLF. Selection of LAB were performed on the basis of their high malolactic activity, and resistance to wine conditions such as low pH and temperature, and high SO_2 and ethanol contents. Degradation of tartaric, fumaric and citric acids were also tested in synthetic medium, and also the ability to grow in wine at pH below 3.5. Three strains belonging to *L. oenos* species and one homofermentative lactobacillus, not yet identified, were well adapted. to grow and perform MLF under enological conditions (2).

An applied result from this research came from the comparative inoculation at pilot plant level of one selected strain of *L. oenos* (M42) and the commercial malolactic starter VINIFLORA LP (Boll Etablishements, Arpajon, France). It was found that M42 was the most suitable strain to perform MLF in musts and wines from Utiel-Requena region, for its better properties such as greater malolactic activity and the better taste that it conferred to wine.

Studies on direct inoculation from synthetic medium into wine revealed a sudden and rapid death of *L. oenos*. So, precultural conditions were investigated to reduce this initial loss of viability. It was observed that successive passages through wine reduced in more than three magnitude orders the inoculum level needed to perform

the MLF. Also the minimal pH value at which the MLF could be induced was lowered, and the fermentation rates were accelerated.

Our work about genetics for the improvement of the MLF has been focused on the cloning of the malolactic enzyme gene from *L. oenos*. We have approached this problem from two sides: the isolation of MLF$^-$ mutants, and the development of a cloning vector. Regarding the MLF$^-$ mutants, we have first designed a new system for the isolation of these mutants. Previously described methods were based on the differentiation of these mutants on solid media, but there was not a selective process to isolate them directly (3,4). Our medium (MMM) is composed on mineral salts, sugars and malic acid, and has a high initial pH value. The growth of a normal strain is inhibited because of the raise of the pH when the MLF is accomplished. But a MLF$^-$ mutant will not raise the pH, and even the fermentation of the sugars will decrease it; so it will grow under these conditions. This system let us to inoculate up to 10^6 viable cells per plate, and we have got MLF$^-$ mutants from *L. oenos* M42 with a frequency of 2.5×10^{-5}. We have verified these strains with chromatographic and enzymatic tests. This method can provide hundreds of MLF$^-$ mutants per experiment and in a single step, which reduces considerably the work needed for the isolation of these mutants.

Leuconostoc oenos is probably the species of LAB with less developed genetic systems: there are not described methods for transfer of genetic material, and neither cloning vectors. So we have been working on the search of plasmids in this organism, and the transfer of foreign vectors from other species. We have tested eleven strains of *L. oenos* by three different methods (5-7), and we found only one that harbored one plasmid. This agrees with other references about the few extrachromosomal material found in *L. oenos* (6,8). We are now characterizing this plasmid in order to construct cloning vectors for this species.

About the transfer of other vectors, it has been described the conjugative mobilization in *Leuconostoc*, but not yet in *L. oenos*. We have used the pVA797 plasmid, a derivative of pIP501 which can be transferred to a broad spectrum of LAB. We transferred it from *Streptococcus sanguis* to *Lactococcus lactis*, and from this species to *Leuconostoc oenos*. The frequencies are low, and we have observed the production of deletions in the pVA797 plasmid; however, these derivatives are stable in *L. oenos* and they express efficiently the selection marker.

The role of MLF in the metabolism of LAB is unclear. Many hypothesis have been suggested:

1) MLF would confer a physiological advantage but would not yield utilizable energy (9), because of the pH increase in acidic environments.
2) Malate is converted to pyruvate that would serve as a substrate for growth by a secondary pathway (10).
3) The decarboxilation of L-malic acid could give the cell an energetic advantage by increasing internal pH (11).

With all of these proposals in mind our work was oriented to determine the role of MLF. In this way we have studied the survival of LAB at extreme low pH values in presence or in absence of malate. *Lactobacillus plantarum* CECT 220 was chosen for the following investigations.

Initial assessments of the acid sensitivity of malate degradation by this microorganism revealed that malate can be catabolized at pH values below the minimum for growth or glycolysis (respectively 2.3, 3.1 and 2.7).

Results in viability experiments showed that at every pH value tested, cells remained viable longer if incubated with L-malic acid than in its absence. At the same time, measurements of L-malic acid degradation were assayed showing that

at pH 3.0 a more rapid degradation of malate is observed than at pH 5.5. This result is in accordance with Henick-Kling (11) who pointed out that the rates of malic acid degradation decreased as the buffer pH increased. These results indicate that L-malic acid metabolism can provide a protection to the cells in some way.

In order to clarify in which way this protection occurs, measurements of pHi were performed. Assays were carried out at pH 3.0 and 5.5. At pH 3.0 a greater gradient of pH (ΔpH) was obtained when cells were cultured in presence of L-malic acid. Thus, metabolism of malate can provide a pHi which is high enough for the functioning of intracellular enzymes. This could be the reason for the longer viability of cells incubated with L-malic acid. At pH 5.5 no ΔpH was detected neither in cells cultured with L-malic acid nor when incubated without it. Perhaps, this result could be explained by the fact of the lower malate degradation rate at this pH value. Nevertheless, further work on this matter is needed.

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