Strategies for the enhancement of malolactic fermentation in the new climate conditions

J.Vila-Crespo[1], J.M. Rodriguez-Nogales[2], E. Fernández-Fernández[2] and M.C. Hernanz-Moral[1]

[1]Area of Microbiology, University of Valladolid, Technical High School of Agronomic Ingeneering, Av. Madrid 44, 34071 Palencia, Spain
[2]Area of Food Technology, University of Valladolid, Technical High School of Agronomic Ingeneering, Av. Madrid 44, 34071 Palencia, Spain

Malolactic fermentation of wines involves not only the conversion of malate into lactate, but also several biotransformations in the wine composition that can affect positively the sensory characteristics of the wine. This secondary fermentation is held by lactic acid bacteria coming naturally from the grapes, although the wine composition not always constitutes the best medium for the lactic acid bacteria development. Nowadays, because of the climate change, the conditions are getting worse for the growing and metabolism of these bacteria: the increasing of the alcoholic content and the reduction of the nutrient content of the wines make life quite difficult for these microorganisms. For this reason, several strategies are being developed in order to enhance the wine malolactic fermentation, most of them focussing in the bacteria themselves. This document deals about the different biotechnological ways that are being used to try to solve this oenological problem.

Keywords: malolactic fermentation, lactic acid bacteria

1. Introduction

1.1. Malolactic fermentation

Winemaking process includes two main phases carried out by a complex mix of microorganisms: alcoholic fermentation (developed by yeasts) and malolactic fermentation (developed by bacteria). Several studies have focussed on the microbial succession taking place along the spontaneous must fermentation [1].

Lactic acid bacteria are naturally present in the grape surfaces and can represent quite important populations in the grape must [2]. These microorganisms play a double role in winemaking: they are responsible for the development of malolactic fermentation and, in some cases; they can make the wine go off. Lactic acid bacteria isolated from wine belong to the genera *Oenococcus, Lactobacillus, Pediococcus* and *Leuconostoc*, they are microaerophila and able to grow under the anaerobia conditions of a fermenting wine. These bacteria ferment hexoses mainly into lactate (homofermentative) or into a mix of lactate, ethanol or acetate and carbon dioxide (heterofermentative) [3].

Malolactic fermentation (MLF) consists in the biological transformation of malic acid into lactic acid and carbon dioxide, and the consequent reduction in acidity and increasing in pH, accompanied by several sensory changes in the wine. Several secondary compounds producing important changes in both the wine quality and composition are also synthesized. Besides, MLF provides microbiological stability to the final wine.

MLF is nowadays considered essential in every red and some white winemaking [2]. The increasing oenological interest in the process and its implications in the quality of the wine make it a subject for new researchal studies focussing in the different parameters affecting the MLF development.

In the other hand, the main advantage and potential danger of the metabolism of lactic acid bacteria is the possible synthesis of some compounds that could affect the food security, like the biogenic amines and the etil carbamate [4,5]. Lactic acid bacteria are able to develop MLF under quite different conditions, nevertheless, several components of the wine can induce stress on them, like ethanol, acidic pH, phenolic compounds, sulphur dioxide, etc. These components do have an inhibitory effect on the bacterial growing and on the length of MLF [3].

MLF is generally supposed to take place spontaneously in the wine after the alcoholic fermentation (AF) but it can start during the AF or it can suffer a delay if the conditions are not so good for the lactic acid bacteria. This fact makes it unforeseeable, so the control of the wine gets essential to avoid the growing of any undesirable microorganism, like acetic bacteria. There are two main ways to control MLF: the control and enhancement of the different factors influencing the process (ampelographic, physical, chemical or of any other origin), and the addition of bacterial inoculums.

Some researchal studies have focussed on the application of different molecular techniques for the microbial identification of lactic acid bacteria, like cellular fatty acids analysis [6], whole-cell protein analysis [7], DNA-DNA hybridisation studies [8], specific-specific PCR [9], and restriction analysis of the amplified 16S-rDNA (PCR-ARDRA) [10].

1.2. How climate change affects must and wine composition

Wine is a complex food in which composition take part more than 1000 different components. Most of these components come naturally from the grape [11]. For this reason, well ripe grapes are essential for the winemaking of a good quality wine. The ripening of the grapes in a single bunch is heterogeneous, so having a good idea of the average composition of the harvest is very important. The different parts of the grapes – skin, pulp and seeds – also provide different components for the future wine. Pulp contribution consists mainly in the majority components of the grape, like water, glucose and fructose sugars (that will turn into ethanol by the yeast metabolic action) and organic acids as malic and tartaric. The big amount of components provided by the skin contributes significantly to the variety characteristics. Phenolic compounds like hidroxicinamic acids, antocians and tannins, together with aromatic compounds of different chemical characteristics like isoprenoids, thiols and metoxipirazines can be found in the skins. While the seeds contribute to the tannin composition of the red wines mainly [12].

Taking all this into account, the diversity and complexity of the wines depend on the development and ripening conditions of the grapes. So that, the variety is not the only parameter that influences the wine complexity, and the external aspects, like the climate change, do play an important role in the differences between wines from different harvests.

The increasing use of fossil combustible and the changes in the use of the soil are the main factors that have contributed, and still contribute, to the emission of the greenhouse effect gases to the atmosphere – like carbon dioxide, methane and nitrogen dioxide-. The increasing of these gases has raised the atmospheric ability for the retention of the infrared radiation coming from the earth surface, which global temperature has also risen. This bigger retention produces in the air a heat exceeding coming from the earth radiation that, in normal circumstances, would have been irradiated to the space again. For this reason, these gases have produced an amplified greenhouse effect by enlarging the atmospheric ability to retain the radiant energy. This energy turns into heat and causes the so called global warming [13].

The main characteristics of the climate change are the following:

- Increasing in the global average temperature
- Changes in the pressure centres behaviour and in the rain pattern
- Melting of the patches of ice and the glaciers and reduction of the mantles of snow
- Increasing of the temperature and acidity of the oceans

Because of that, the climate change implies changes in the annual average temperature and fluctuations in the rain schedule. Talking about viticulture, different historical records of productivity and climate show the strong relationship existing amongst the optimum grape growing areas and the changes in the climate of the different regions. In general terms, the base climate defines the style of the wine that can be produced in a specific region. In each region, the climate variability stamps important differences in the quality of the musts when comparing the harvests of several years. Taking into account the influence of the climate change into the variability and into the base climate conditions, the climate change itself can really influence the personality of the regional wines [14].

In climatic terms, it is considered the existence of optimum areas for the growing of every specific grape variety [15]. If we relate production parameters and quality of the wine together with the climatic parameters, some aspects can be established:

- A no-balanced wine, with low levels of sugar and immature odours is obtained in a too cold region. The wine quality parameters are below the optimum in this case.
- A no-balanced wine, with low levels of acidity and too matured odours is obtained in a too warm region. The wine quality parameters are also below the optimum.
- On the contrary, a well balanced wine, with the right level of sugar and showing the aromatic profile of the variety is obtained in a region with the ideal climatic parameters. The production values and the quality of the wine are the optimum under these climatic conditions because of the fragile and delicate climatic balance.

As a whole, both the impact of the climate change and the challenge that the production of quality wine constitutes, related to the climate change and the climatic alterations during the harvesting period will impact, mainly, in the faster plant growing and the imbalance of the ripening. For example, if in a region nowadays the ripening period allows the grape to reach a good level of sugar, with the right acidity and the optimum odour content for a specific grape variety, then the winemaking in that region produces well balanced wines. In a place warmer than the optimum, the vineyard will experience all the phenological changes much more quickly, so the grape will reach its maturity with high levels of sugar and, as the oenologist waits for the optimum odours development, the acidity of the grape will fall down. So the wine will not reach its optimum. This problem can be solved by different adjustments in the cellar. Nevertheless, these alterations can increase the alcoholic content of the wines in some regions. For example, [16] discovered that the alcoholic content of the Riesling wine in Alsace had increased around 2.5% (in volume) along the last 30 years and that it seemed to be correlated with the increasing of the temperature during the harvest and with an earlier phenology. [17] resume the last trends in the Australian wine composition and, in spite of not considering the influence of the increase of temperature in Australia [18, 19], they show the increasing in the alcoholic content from 12.3% to 13.9% in red

wines and from 12.2% to 13.2% in white wines from 1984 to 2001. In the wines from Napa Valley, the average alcoholic content have increased from 12.5% to 14.8% from 1971 to 2001, while the acidity has decreased considerably as the pH has increased. While this study says that this increasing in the alcoholic content is due to the change in preference of the wine tasters (now choosing more bodied varieties) and to the economy of the harvest systems, two recent researchers show that the variability and the climate change are the ultimate responsible for, at least, the 50% of the increase in the alcoholic content [20].

Besides the alterations in the characteristics of the wines, one of the most significant problems due to the increasing of the alcoholic content is the alteration in the ageing flair of the wines with such a high alcoholic content. Finally, the harvests that take place at the beginning of summer, in one of the warmest time of the growing period, will bring grapes which ripening will take place in a hot weather, so these grapes will get dry if the vineyard are not water more frequently [13].

Last, among the more significant changes produced by the climate change resides the different ripening of the harvest, there is the reduction in productivity, the dryness of the grapes, or the changes that affect the illnesses and the insects affecting the vineyards. In consequence, different musts and wines, less balanced, will be obtained. To get the odours and the mature poliphenols from the musts, oenologists must work with grapes of high sugar content, giving quite alcoholic and low acidic wines. These kinds of wines are heavy and sensible to oxidation phenomena, and if the oenologists choose earlier harvests, they will get more fresh and light but with less odour complexity wines.

2. Strategies for the enhancement of malolactic fermentation

2.1. Selected starter cultures

As it has been told before, MLF can take place spontaneously in wines. Nevertheless, its not predictable evolution has turned the use of malolactic starters into a quite useful practice in winemaking [21, 22]. *Oenococcus oeni*, the predominant organism associated with MLF, is an acidophilic bacterium and is able to grow in wine at pH 3.5 or lower in the presence of ethanol and sulphite. For this reason, this bacterium has traditionally been chosen as a commercial starter culture for MLF in winemaking [23]. However, as MLF takes place in a highly alcoholic environment by means of the climate change, slow growth and poor yields are frequently encountered when starter cultures are used. During recent years several technologies have been proposed to induce MLF in wines by using malolactic bacteria, principally *O. oeni* and *Lactobacillus* sp, as starters [24].

The use of starter cultures to induce MLF can in some cases ensure more rapid fermentation, reduce potential spoilage by other LAB, reduce potential interference by bacteriophages [25] and, furthermore, allow the control and selection of the strain responsible for the MLF and its contribution to the wine quality [26, 27].

Some researchers have focussed their studies in the selection of LAB strains able to carry out effective MLF [28, 29, 30]. Several parameters affecting the bacterial activity have been considered for the selection of the different starters, among the most important are: ethanol, pH, fermentation temperature and sulphur dioxide [30, 31, 32, 33, 34, 35, 36, 37].

One of the effects of the climate change is the increasing of the sugar content, and so the increasing also in the alcoholic content of the wine. The inhibitory effect of ethanol can be so important that some authors [38] have focussed their studies in this aspect trying different strategies to attain a good MLF. One of the pointed solutions is the inoculation of yeasts with acclimatised bacteria before AF to obtain a complete MLF in high–alcoholic wines. These same authors point at the acclimatisation of the bacteria as a crucial step for having a successful MLF.

Recent studies have even used a multi-strain bacterial inoculation technique, trying to emulate spontaneous inoculation under winery conditions with the aim of letting the best adapted strain or strains to lead the process [39].

2.2. Yeast-bacteria co-inoculation

As it has been told, the inhibitory effect of ethanol and acidity, so frequently found in wines, make quite difficult or slow down the development of (MLF) in many occasions [40, 41]. Any delay in the MLF can turn into a big problem, for the wine is kept under risky conditions that may enhance the development of some other alterating microorganisms. In the other hand, a long lasting MLF implies not only a non-efficient use of the fermentation tanks, which are occupied along the post-harvest period, but also a delay in the wine marketing.

Although MLF can take place spontaneously because of the natural presence of LAB in the must, in the last years the addition of selected strains of LAB from *O. oeni* species is very common. The aim of this addition is to assure the right development of the MLF process while reducing the problems derived from spontaneous MLF (sensory deviations, biogenic amines production, etc). Anyway, the best moment to do the bacterial addition is still under discussion. Traditionally, an addition at the end of the (AF) when all the sugars have been metabolized by the yeasts has been recommended. This recommendation tried to avoid both a high acetic acid and D-lactic acid production and the risk of sluggish or stuck fermentations.

More recent literature talks about the possibility of a simultaneous inoculation (co-inoculation) of yeast and bacteria into the must [23]. These authors think that the bacteria can in this way get use and grow better in the presence of ethanol and with a big amount of nutrients at their disposal, and that these conditions will not necessarily lead to an excessive acetic acid production [42]. In this case, the interactions between the chosen yeasts and bacteria must be taken into account, so the control of a low pH level [28, 43-48]. Some researchers advise an earlier inoculation (before the total ending of the AF), because the bigger amount of free sulphur dioxide combining with the carbonilic compounds coming from the yeasts growing phase, and the lower alcoholic content (non reaching toxic levels) would suppose an important advantage [49]. When the metabolism of the sugars has not finished, different compounds produced by the yeasts reach their maximum level, so the main problem of the earlier inoculation of the bacteria is the possible existence of an inhibitory effect of the metabolites from the yeasts on them [50].

The microbiological stability of the wine seem to be another advantage of the yeasts and bacteria co-inoculation, for the earlier MLF avoids the maintaining of the wine under dangerous conditions without sulphur dioxide for long lasting periods and exposed to the development of different contaminant microorganisms, like the undesirable acetic bacteria [3].

On the contrary, the quick development of the MLF when inoculating bacteria is opposed to some traditional winemaking techniques in several regions, in which MLF used to take place spontaneously when the wine temperature reached the necessary values for the bacteria development. This gap of time between the two fermentations leads to a better stability of the colour of the wine [54]. The initial stop of the spontaneous development followed by an induction by inoculating the selected strain of bacteria after a time could be a way to deal with the problem.

In spite of the considerable interest of the co-inoculation technique, it is not usually employed by the oenologists, because of the lack of knowledge about it, the absence of many researchal data, and the scare of the loose of quality that bacteria could produce when growing in a must. Nevertheless, more recent researchal studies focussing on the use of this technique in cold climate with white musts of low pH levels and a potential high content in alcohol, have found no evidence of any negative impact of the simultaneous development of MLF and AF neither in the kinetic of the fermentation nor in the characteristics of the wine [51]. On the contrary, these studies suggest microbiological and technological advantages when applying this schedule for fermentation. Other researchers [52] using this co-inoculation technique for the winemaking of red musts in template climate, show the reduction of the total winemaking process period and the better control of the MLF that this technique may achieve.

Our researchal group has been working on this subject for two harvests (2007 and 2008) focussing on the influence of yeasts-bacteria co-inoculation in the MLF of Tinta del País wines [53-55].

The conclusions of our studies are the following:

- *Saccharomyces cerevisiae* yeasts were not affected by the simultaneous inoculation of bacteria during the AF. These yeasts were able to finish the AF at the same time than the non-inoculated or traditional wines.
- Referring to the MLF development, there was a significant difference among the wines, for the coinoculated fermentations were much faster than the traditional ones. This fact can mean an important advantage, because if the wine is finished in a shorter period of time, it will be less time exposed to the possible development of undesirable microorganisms like acetic bacteria or the scaring *Brettanomyces.*
- By the other hand, the chemical analyses of the coinoculated wines showed less acidity and less alcoholic content, which could lead to a better acceptance by the consumers. However, the colour parameters were better in the traditional wines.

2.3. Immobilized cells

A cellular population of about 10^6-10^7 cfu/ml is needed in the cellular growing phase in order to have the amount of bacteria enough to convert malic acid into lactic acid during MLF. When MLF takes place in a very alcoholic and acidic medium, like wine, bacterial growing is very slow and MLF can last for weeks or even months, as it has been reported [23]. Taking this into account, the inoculation of the wine with a big LAB population increases significantly the possibility of having a fast and complete MLF [56]. In this sense, the use of the classic cell immobilization techniques and the membrane bioreactor systems (MBR) allows a better conduction of MLF with a big amount of bacteria whilst reducing the fermentative length. The main advantage in the use of the immobilized cells to conduct MLF compared with the systems using high populations of non-growing cells is the possibility of recycling.

The applications of immobilized cells in food technology have extended along the last 25 years, mainly in the production of alcoholic drinks. This technology has shown to be a good biotechnological tool for the winemaking industry, especially in the conduction of MLF, for it allows a reduction in the duration of fermentation without affecting the quality of the final product.

Cell immobilization was defined as 'the physical confinement or localization of intact cells to a certain region of space with preservative of some desired catalytic activity' [57]. Taking into account the relation between the biocatalyst and the support, cell immobilization techniques have been classified in different categories: i) immobilization on the surface of a solid carrier by physical adsorption due to electrostatic forces or by covalent binding between the cell

membrane and the carrier; ii) entrapment with a porous matrix; iii) self-aggregation by flocculation or with cross-linking agents, and iv) cell containment behind barriers (microencapsulation and containment between microporous membranes) [58].

Some of the reasons that support the use of immobilized cells for the development of MLF are the following: i) the duration of MLF depend on the physical-chemical and nutritive properties of the wine, for these affect the development of the microbiota responsible of MLF. So, the cell immobilization techniques allow the increasing in tolerance of these microorganisms; ii) it allows the conduction of MLF with selected immobilized microorganisms; iii) enhanced MLF productivity by higher cell densities; iv) feasibility of continuous processing, v) lower cost of recovery and recycling, and vi) downstream processing [59, 23].

In spite of the published studies, neither standard immobilization techniques nor supports have been generically established for the different kind of cells, on the contrary, an individual study for each concrete application is needed. Different materials have been tested as cell immobilization supports for the development of MLF, like polyacrylamide [60], k-carrageenan [61, 62], calcium pectate gel [63], chitosan [63], cellulose sponge [64], calcium alginate [65-68], oak chips [69, 70], and delignified cellulosic material [71, 72], nevertheless, alginate gel and k-carrageenan were the most extensively tested support for lactic bacteria immobilized. Related to the immobilization techniques applied to the induction of MLF in wine, two of the main immobilization categories have been tried (entrapment and immobilization on the surface of a solid carrier) [24].

Almost all of the effort included immobilization of *O. oeni*, although several trials have been developed with some other kind of LAB like *Lactobacillus brevis* [66], *Lactobacillus fructivorans* [66], *Lactobacillus casei* [72], *Lactobacillus delbrueckii* [65] Some other researchal studies focus on the use of immobilized yeasts able to metabolize malic acid, like *Issatchenkia orientales* [70] and *Schizosaccharomyces pombe* [73].

Some authors have realized preliminary studies on induction of MLF with immobilized LAB in specific media supplemented with malic acid or in synthetic wines. [60] used a culture medium supplemented with 5 g/l malic acid to test the fermentative ability of *O. oeni* immobilized in k-carrageenan gel (10^9-10^{10} cfu/ml gel), obtaining a 100% performance after 84 h. Other authors immobilized *O. oeni* in polyacrylamide gel at 13% (wet w/v) cell concentration obtaining conversion rates of about 71% in a buffer solution of malic acid (2.3-4.5 g/l) after 10 min of bioconversion [61]. [67] designed a fluidized bed reactor with *O. oeni* or *Lactobacillus* sp. immobilized in calcium alginate gel. Using this method, conversions of 100% were achieved in a synthetic wine with 5.5 g/l of malic acid after 11.7 h of bioreactor operation. [72] used *O. oeni* cells immobilized by adsorption in oak chips in a continuous bioreactor. Very good conversions of malic acid (320-440 mg/kg chips/h) were obtained working on synthetic wines with pH 3.15-3.53 and alcoholic content 10-13%.

Even though all the mentioned reasearchal studies focus on the ability of the immobilized microorganisms for the malic acid bioconversion along MLF, new research working on non synthetic media, like wine, is needed. [62] studied the conversion ability of malic acid in a red wine by *O. oeni* immobilized in positively-charged cellulose sponge. The effect of surface charges in the immobilization material was evaluated, as well as the pH and the composition of the media where the cells were suspended. Better immobilization abilities were found when the support was positively charged ($2 \cdot 10^9$ ufc/g), though pH effect in the rate 3.5-5.5 did not affect the cell percentage of immobilization. Conversions of 100% malic acid in a Monastrell wine (pH 3.5; 11% alcoholic content; 3.5 g/l malic acid) were obtained after the first fermentation cycle (conversion period of 24 h), descending to 60% when the support was used in a second cycle. By the other hand, the ability of *L. brevis*, *L. fructivorans* y *O. oeni* immobilized in alginatum gel to conduct MLF in a red wine (9 g/l malic acid) in a continuous stirred tank reactor was compared. *L. brevis* got the better results in terms of efficacy and stability, reaching bioconversions of 75% with a retention time of 2 h [66]. A continuous stirred tank reactor with packed columns of beads was also used to study the efficacy of *O. oeni* ML34 and *Lactobacillus* sp. 48 immobilized in k-carragenan gel [68]. Bioconversions of 77-85% were reached with retention time of 0.64 vol/h in white wine with 2.27 g/l of malic acid. More recently the ability of *O. oeni* cells immobilized on delignified cellulosic material (DCM) for malolactic fermentation of wine was studied [71]. Results obtained by these authors show that DCM is a right support for the immobilization of *O.oeni*, for it allowed the consecution of a conversion average of 53% in red wines (pH = 3.5; 12% (v/v); 2 g/l malic acid) after 11 reutilization cycles with the same support. [70] have immobilized a yeast strain of *Issatchenkia orientales* (KMBL 5774) in oriental oak charcoal and alginate obtaining a high conversion rate for malic acid (92%) in a Korean wine with a high initial content of malic acid (8.96 g/l).

These results present the use of immobilized microorganisms in solid supports like an alternative for the conduction of MLF in wines, for it allows the reutilization of the biocatalyst, a configuration of MLF in continuous, high rates of conversion in wines with high alcoholic content and low pH, and a better control of MLF. Nevertheless, the disadvantages of this alternative include diffusion problems of substrates and products, possibility of microbial contamination of the reactor, loss of activity on prolonged operation, and the liberation of cells into the wine [23].

2.4. Membrane bioreactor systems (MBR)

Membrane bioreactors have been reviewed previously [74-77]. They are alternative approaches to classical methods of immobilizing biocatalysts such as enzymes, microorganisms and antibodies, which are suspended in solution and

compartmentalized by a membrane in a reaction vessel or immobilized within the membrane matrix itself. Immobilized whole cell membrane bioreactors have also been tested successfully [79].

The retention of the biocatalyst (cell or enzyme) by a semi permeable membrane that allows the pass of the reaction products is the main objective for an MBR. To attain this, the biocatalyst must be confined in a specific region of the reactor, where the transformation of the substrate will take place. The biocatalyst is normally retained in a free or an immobilized way in the membrane surface, in this kind of bioreactors. In the first method, the system might consist of a traditional stirred tank reactor combined with a membrane-separation unit, where the immobilization must achieve its confinement by molecular exclusion or electrostatic repulsion. In the second method, the membrane acts as a support for the catalyst and as a separation unit. The retention of the undissolved biocatalyst by the membrane may be direct or through an intermediate linked by chemical binding, adsorption or electrostatic attraction [77].

Membrane bioreactors offer several advantages instead of conventional bioreactors. Some of them are the following: i) the possibility of continuous work, and so the intensive use of the cells, increasing the process productivity; ii) it allows the continuous and selective elimination of the products from the reaction media, so in those processes where the chemical equilibrium affects the performance of the reaction, it provokes the equilibrium displacement towards the synthesis of the products; iii) working with high density of biocatalyst is possible, what implies the reduction of the operation time and the increasing of the process productivity; iv) MBRs can also be easily scaled up; v) the problems of transference of mass which generate lots of problems in those enzymatic reactions where substrates and products of high molecular weight are implied get minimized [74]. The disadvantages in the use of these systems are related to the fall in the efficacy due to the deficiencies in the transference of mass, and the fall in the biocatalyst activity.

There are a short number of researchal studies about the application of MBR for MLF. [79] designed a MBR with *O. oeni* cells retained in a free state inside a membrane of 0.45 μm and 240 cm^2 of area, with a microbial population of 10^{10} cfu/cm^3. The bioreactor worked at a flow rate of 0.36 dm^3/h reaching malic acid conversions over 95% with white and red wine as substrate. Other authors have studied the behaviour of an MBR for MLF in a synthetic cider with malic acid rates of 2-5 g/ml and high concentrations of *O. oeni* (10^{11} cfu/ml). They obtained high conversions of malic acid (over 85%) in alcoholic contents of 5-10% (v/v) and low retention times (less than 3 hours) [23]. A recent study has used a MBR to evaluate the impact of the co-culture of *S. cerevisiae-O. oeni* on the output of malolactic fermentation using a synthetic grape juice medium. For the co-culture, both fermentations were conducted together by inoculating yeast and bacteria into membrane bioreactor at the same time. The MBR was a good tool for studying the microbial interactions between these microorganisms, which are kept in a homogenous liquid phase but physically separated by a membrane made of polysulfone hollow fibres of 0.1 μm porosity [80].

In spite of the high advantages that a MBR configuration offers related to the possibility of reusing the biocatalyst with high rates of conversion of malic acid in low retention times, the acceptance of this technology will depend on its impact in the sensory characteristics of the final product. Something to consider in the design of a MBR is the right selection of the limit of membrane molecular weight cut-off for the cells to remain kept by the system instead of getting out of it. This aspect implies the use of porous sizes of less than 0.45 μm which can provoke a decrease in the sensory quality of the wine. An alternative could be the retention of the undissolved biocatalyst on the membrane of the MBR, which could allow the use of membranes with a bigger porous size.

2.5. Use of bacterial enzymes and genetically modified yeasts

Taking into account the problems derived from the use of LAB in the different ways mentioned before, the searching of another solution has been tackled by many authors.

We must realized that current viticultural practices and vinification processes are essentially protocols for favouring the activities of certain enzymes while discouraging the activities of others, that is what is done when using any selected wine yeast or bacteria strain characterized by desirable physiological and hence enzymatic properties [81, 82].

The major function of LAB is the conversion of L-malic acid to L-lactic acid during the MLF. Most of these bacteria decarboxylate L-malic acid into L-lactic acid and carbon dioxide in a reaction catalyzed by the malolactic enzyme without the release of intermediates. Nevertheless, bacterial behaviour is unpredictable in wine and, consequently, techniques that facilitate the efficient and complete malolactic conversion have been sought. Such techniques aim to separate this central enzyme-driven conversion from the often problematic growth of the source LAB in the wine. Examples from the beverage and food industries include bioreactor systems comprising enzymes and cofactors [83, 84, 85].

Some other researchal studies have focussed on the possibility of *S. cerevisiae* developing MLF. In fact, *S. cerevisiae* can not degrade malic acid efficiently due to the lack of a malate transporter and the low substrate affinity of its malic enzyme, so there have been different trials to introduce the necessary genetical information in the yeast. [86, 87] introduced efficient pathways for malate degradation in *S. cerevisiae* by cloning and expressing the *Schizosaccharomyces pombe* malate permease *(mae1)* gene with either the *S. pombe* malic enzyme *(mae2)* or *Lactococcus lactis* malolactic *(mleS)* gene in this yeast. The so obtained recombinant strain was able to degrade 8 g/l of malic acid in a glycerol-ethanol medium within 7 days, and fermented 4.5 g/l of malate in a synthetic grape must within 4 days. Later on [87] constructed a genetically stable industrial strain of *S. cerevisiae* by integrating a linear cassette containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *O. oeni* malolactic gene (*mleA*) under

control of the *S. cerevisiae PGK1* promoter and terminator sequences into the *URA3* locus of industrial wine yeast. The malolactic yeast strain, ML01, fully decarboxylated 5.5 g/l of malate in Chardonnay grape must during the alcoholic fermentation. This modified microorganism is able to develop MLF and AF simultaneously. One of the objectives of using this kind of genetically modified yeasts is also the prevention of the formation of noxious biogenic amines produced by lactic acid bacteria in wine.

Anyway, whether achieved via such recombinant methods or via bioconversions with cells or enzyme preparations, the potential benefits of enhanced application of malolactic enzyme warrant further research. Identification of a malolactic enzyme that is more resilient under wine conditions and improved delivery systems is of foremost importance.

Conclusions

Biotechnology provides different tools for the overcoming of the oenological problems. Several ways are open to try to avoid the possible lack of quality in wines derived from the climate change. What must be considered by the researchers are the real necessities of the oenological sector in the different regions. In this sense, more deep studies must be done to improve the handling of the so-mentioned biotechnological tools in order to make them easy to use at the cellar.

References

[1] Fleet, G.H., Lafon-Lafourcade, S. and Ribéreau-Gayon, P. Evolution of yeast and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol*. 1984; 48: 1034-1038.
[2] Lonvaud-Funel, A. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*, 1999; 76: 317-331.
[3] Ribéreau-Gayon, P., Dubourdieu, D., Donéche, and B. Lonvaud, A. *Tratado de Enología. 1. Microbiología del vino*. Vinificaciones. Edición Hemisferio Sur. Paris 2003; 151-155.
[4] Isabel, L., Santamaría, P., Tenorio, C., Garito, P., Gutierrez, A.R., and López, R. Evaluation of lysozyme to control vinification process and histamine production in Rioja wines. *J. Microbiol Biotechnol*. 2009; 19(9): 1005-1012.
[5] Araque, I., Gil, J., Carreté, R., Bordons A., and Reguant C. Detection of arc Genes related with the Ethyl Carbamate Precursors in wine lactic acid bacteria. *J. Agric. Food Chem*. 2009; 57 (5): 1841–1847.
[6] Tracey, R. P. and Britz, T.j.: Cellular fatty acid composition of *Leuconostoc oenos*. *J. Appl. Bacteriol*. 1998; 66: 445-456.
[7] Dicks, L.M.T., Van Vuuren, H. J.J., Dellaglio, F. Taxonomic *Leuconostoc* species particularly *L. Oenos* as revealed by numerical analysis of total soluble cell protein patterns, DNA base composition and DNA-DNA hybridization. *Int. J. Sys. Bacteriol*. 1990; 40: 83-91.
[8] Sohier, D. and Lonvaud-Funel, A. Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiol*. 1998; 15: 391-397.
[9] Sohier, D., Coulon, J. and Lonvaud-Funel, A. Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis. Int. J. Syst. Bacteriol*. 1999; 49: 1075-1081.
[10] Sato, H., Yanagida, F., Shinohara, T. and Yokotsuka, K. Restriction fragment length polymorphism análisis of 16S rRNA genes in lactic acid bacteria isolated from red wine. *J. Biosc. Bioeng*. 2000; 90: 335-337.
[11] Conde, C.; Silva, P.; Fontes, N.; Dias, A.C.R.; Tavares, R.M.; Sousa, M.J.; Agasse, A.; Delrot, S. and Gerós, H. Biochemical changes throughout grape berry development and fruit and wine quality. *Food.* 2007; 1 (1): 1-22.
[12] Kennedy, J.A.; Matthews, M.A. and Waterhouse, A.L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry.* . 2000; 55 (1): 77-85.
[13] Jones, G.V., White, M.A., Cooper, O.R. and Storchmann, K. Climate change and global wine quality. *Climatic Change*. 2005; 73: 319-343.
[14] Jones, G. V. and Hellman, E. Site Assessment: in "*Oregon Viticulture*" Hellman, E. (ed.), 5th Edition, Oregon State University Press, Corvallis, Oregon. 2003; 44–50.
[15] Jones, G. V., White, M. A., Cooper, O. R. and Storchmann, K-H. Climate and wine: Quality issues in a warmer world. *Proceedings of the Vineyard Data Quantification Society's 10th OEonometrics Meeting*. 2004; Dijon, France, May.
[16] Duchêne, E. and Schneider, C. Grapevine and climatic changes: a glance at the situation in Alsace. *Agron. Sustain. Dev.* 2005; 24: 93-99.
[17] Godden, P. and Gishen, M. Trends in the composition of Australian wine. *The Australian and New Zealand Wine Industry Journal*. 2005; 20 (5): 21-46.
[18] McInnes, K.L., Whetton, P.H., Webb, L. and Hennessy, K.J. Climate change projections for Australian viticultural regions. *The Australian and New Zealand Grapegrower and Winemaker.* 2003; Febr: 40-47.
[19] Webb, L.B, Whetton, P.H. and Barlow, E.W.R. Impact on Australian Viticulture from Greenhouse Induced Temperature Change. In: Zerger, A. y Argent, R.M. (eds.) *MODSIM 2005 International Congress on Modelling and Simulation.* Modelling and Simulation Society of Australia and New Zealand. 2005; Dic: 170-176.
[20] Jones, G.V. and Goodrich, G.B. Influences of climate variability on wine regions in the western USA and on wine quality in the Napa Valley. *Climate Research*. 2008; 35 (3): 241-254.
[21] Davis C.R., Wibowo D., Lee T.H. and Fleet G.H. Properties of wine lactic acid bacteria; their potential oenological significance. *Am. J. Enol. Vitic*. 1998; 39: 137-142.
[22] Bauer R. and Dicks L.M.T. Control of malolactic fermentation in wine. A review. *S. Afr. J. Enol. Vitic.* 2004; 25(2): 74-87.

[23] Zhang D. and Lovitt R.W. Strategies for enhanced malolactic fermentation in wine and cider maturation. *J. Chem. Technol.* Biotechnol. 2006; 81: 1130-1140.
[24] Maicas S. The use of alternative Technologies to develop malolactic fermentation in wine. *Appl. Microbiol. Biotechnol.* 2001; 56: 35-39.
[25] Sozzi T., Gnaegi F., D'Amico N. and Hose H. Difficultées de fermentation malolactique du vin dues a des bacteriophages de *Leuconostoc oenos. Rev. Suisse Vitic. Arbor. Hort*. 1982; 14: 17-23.
[26] Orduña M de R., Patchett M.L., Liu S.Q. and Pilone G.J. Growth and arginine metabolisme of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. *Appl. Environ. Microbiol*. 2001; 67: 1657-1662.
[27] Bayon M.A., G-Alegria E., Polo M.C., Tenorio C., Martin-Alvarez P.J., Calvo de la Banda H.T., Ruiz-Larrea F. and Moreno-Arribas M.V. Wine volatile and amino acid composition alter malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. J. Agric. Food Chem. 2005; 53: 8729-8735.
[28] Costello P.J., Henschke P.A. and Markides A.J. Standardized methodology for testing malolactic bacteria and wine yeast compatibility. *Aust. J. Grape Wine Res*. 2003; 9: 127-137.
[29] Coucheney F., Desroche N., Bou M., Tourdot-Marechal R., Dulau L. and Guzzo J. A new approach for selection of *Oenococcus oeni* strains in order to produce malolactic starters. *Int. J. Food Microbiol*. 2005; 105(3): 463-470.
[30] Carrete R., Reguant C., Rozes N., Constanti M. and Bordons A. Analysis of *Oenococcus oeni* strains in similated microvinifications with some stress compounds. *Am. J. Enol. Vitic*. 2006; 57(3): 356-362.
[31] Guzzo J., Coucheney F., Pierre F., Fortier L., Delmas F. Dives C. and Tourdot-Marechal R. Acidophilic bahavour of the malolactic bacterium *Oenococcus oeni. Sci. Alim*. 2002; 22(1-2): 107-111.
[32] Teixeira H., Gonçalves M.G., Rozes N., Ramos A. and San Romao M.V. Lactobacillic acid accumulation in the plasma membrane of *Oenococcus oeni:* a response to ethanol stress? *Microb. Ecol*. 2002; 43(1): 146-153.
[33] Rosi I., Fia G. and Canuti V. Influence of different pH values and inoculation time on the growth and malolactic activity of a strain of *Oenococcus oeni. Aust. J. Grape Wine Res*. 2003; 9: 194-199.
[34] Liu J.W.R. and Gallander J.F. Effect of pH and sulphur dioxide on the rate of malolactic fermentation in red table wines. *Am. J. Enol. Vitic*.1983; 34(1): 44-46.
[35] Reguant C., Carrete R., Constanti M., and Bordons A. Population dynamics of *Oenococcus oeni* strains in a new winery and the effect of sulphur dioxide and yeast strain. *FEMS Microbiol. Lett*. 2005; 246(1): 111-117.
[36] Carrete R., Vidal M.T., Bordons A. and Constanti M. Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni. FEMS Microbiol. Lett*. 2006; 211(2): 155-159.
[37] Ribéreau-Gayon P., Dubourdieu D., Doneche B. and Lonvaud-Funel A. *Handbook of enology: the microbiology of wine and vinification.* 2nd ed. Wiley and Sons, Chichester, UK; 2006.
[38] Zapparoli G., Tosi E., Azzolini M., Vagnoli P. and Krieger S. Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. *S. Afr. J. Enol. Vitic*. 2009; 30(1): 49-55.
[39] Guzzoni R., Poznanski E., Conterno L., Vagnoli P., Krieger-Weber S. and Cavazza A. Selection of a new highly resistant strain for malolactic fermentation under difficult conditions. *S. Afr. J. Enol. Vitic*. 2009; 30(2): 133-141.
[40] Wibowo, D., Fleet, G.H., Lee, T.H. and Eschenbruch, R.E. (1988). Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos. J. Appl. Bacteriol*. 1988; 64:421-428.
[41] Vaillant, H.P., Formisyn and Gerbaux, V. Malolactic fermentation of wine: study of the influence of some physico-chemical factors by experimental design assays*. J. Appl. Bacteriol*. 1995; 79:640-653.
[42] Beelman, R.B. and Kunkee, R.E. Inducing simultaneous malolactic – alcoholic fermentation in red table wines. *Proc. Aust. Soc. Vitic. Oenol. Sem. On malolactic fermentation.* 1985; 97-112.
[43] King, S.W. and Beelman, R.B. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice wine system. *Am. J. Enol. Vitic*. 1986; 37(1): 53-60.
[44] Larsen, J.T., Nielsen, J.C., Kramp, B., Richelieu, M., Bjerring, P., Riisager, M.J., Arneborg, N. and Edwards, C.G. Impact of different strains of *Saccharomyces cerevisiae* on malolactic fermentation by *Oenococcus oeni*. *Am. J. Enol. And Vitic*. 2003; 54(4): 246-251.
[45] Zapparoli, G., Torriani, S., Malacrino, P., Suzzi, G. and Dellaglio, F. (2003). Interactions between *Saccharomyces* and *Oenococcus oeni* strains from Amarone wine affect malolactic fermentation and wine composition. *Vitis* 2003; 42[2): 107-108.
[46] Comitini, F., Ferreti, R., Clementi, F., Mannazzu, I. and Ciani, M. Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: preliminary characterization of a yeast proteinaceous compound active against *Oenococcus oeni. J. Appl. Microbiol*. 2005; 99(1): 105-111.
[47] Reguant, C., Carrete, R., Ferrer, N. and Bordons, A. Molecular analysis of *Oenococcus oeni* population dynamics and the effect of aeration and temperature during alcoholic fermentation on malolactic fermentation. *Int. J. of Food Sci. and Tech*. 2005; 40(4): 451-459.
[48] Comitini, F. and Ciani, M. The inhibitory activity of wine yeast starter on malolactic bacteria. *Annals of Microbiol*. 2007; 57(1): 61-66.
[49] Gerbaux, V., Briffox, C., Dumont, A. and Krieger, S. Influence of inoculation with malolactic bacteria on volatile phenols in wines. *Am. J. Enol. Vitic.*2009; 60 (2): 233-235.
[50] Santamaría, P.; Tenorio, C.; Gariño, P. and López, R. Ensayos de inoculación de bacterias lácticas. *Revista técnica de la Conserjería de agricultura y desarrollo económico.* 2006; 34: 30-34.
[51] Jussier, D.; Morneau, A.D. and Mira. R. Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. *Appl.Env. Microbio.* 2006; 72 (1): 221-227.
[52] Massera, A., Soria, A., Catania, C., Krieger, S. and Combina, M. Simultaneous inoculation of Malbec (Vitis vinifera) musts with yeast and bacteria: Effects on fermentation performance, sensory and sanitary attributes of wines. *Food Tech. and Biotech.* 2009; 47 (2): 192-201.

[53] Fernández-Fernández, E., Rodriguez-Nogales, J.M., Vila-Crespo, J., Yuste-Bombín, J. and Felipe-Fuentes, E. Influencia de la coinoculación levaduras-bacterias en el desarrollo de la fermentación maloláctica. *Actas X Congreso Nacional de investigación enológica*. 2009; 281-283.
[54] Sánchez, M. *Influencia de la coinoculación bacteria-levadura en la elaboración de vinos tintos.* 2009; Proyecto Fin de Carrera. Universidad de Valladolid.
[55] Felipe, E. *Influencia de la coinoculación bacteria-levadura en el desarrollo de la fermentación maloláctica*. Proyecto Fin de Carrera. 2009; Universidad de Valladolid.
[56] Maicas S., Gonzalez-Cabo P., Ferrer S., and Pardo,I. Production of *Oenococcus oeni* biomass to induce malolactic fermentation in wine by control of pH and substrate addition. *Biotechn. Lett*. 1999; 21(4): 349-353.
[57] Karel S.F., Libicki S.B. and Robertson C.R. The immobilization of whole cells. *Eng. Pr. Chem. Eng. Sci*. 1985; 40(8): 1321-1354.
[58] Pilkington P.H., Margaritis A., Mensour NnA. and Russell I. Fundamentals of immobilised yeast cells for continuous beer fermentation: A review. *J. Inst. Brew*. 1998; 104(1): 19-31.
[59] Kourkoutas Y., Bekatorou A., Banat I.Mm, Marchant R. and Koutinas AA. Immobilization technologies and support materials suitable in alcohol beverages production: a review. *Food Microbiol*. 2004; 21(4): 377-397.
[60] Rossi J. and Clementi F. L-Malic acid catabolism by polyacrylamide-gel entrapped Leuconostoc oenos. *Am. J. Enol. Vitic*. 1984; 35(2): 100-102.
[61] McCord J.D. and Ryu D.D.Y. Development of malolactic fermentation process using immobilized whole cells and enzymes. *Am. J. Enol. Vitic*. 1985; 36(3): 214-218.
[62] Spettoli P., Nuti M.P., Crapisi A. and Zamorani A., Technological improvement of malolactic fermentation in wine by immobilized microbial cells in a continuous-flow reactor. *Am. J. Enol. Vitic* 1987; 501: 386-389.
[63] Kosseva M., Beschkov V., Kennedy J.F. and Lloyd L.L. Malolactic fermentation in Chardonnay wine by immobilised *Lactobacillus casei* cell. *Process Biochem*. 1998; 33(8): 793-797.
[64] Maicas S., Pardo I. and Ferrer S. The potential of positively-charged cellulose sponge for malolactic fermentation of wine, using *Oenococcus oeni. Enz. Microb. Tech*. 2001; 28(4-5): 415-419.
[65] Aaron R.T., Davis R.C., Hamdy M.K. and Toledo R.T. Continuous alcohol/malolactic fermentation of grape must in a bioreactor system using immobilized cells. *J. of Rapid Methods and Autom. In Microbiol*. 2004; 12(2): 127-148.
[66] Crapisi A., Spettoli P., Nuti MP., Zamorani A. Comparative traits of *Lactobacillus brevis-Lact. Fructivorans* and *Leuconostoc oenos* immobilized cells for the control of malolactic fermentation in wine. *J. Appl. Bacteriol*. 1987; 63(6): 513-521.
[67] Naouri P., Bernet N., Chagnaud P., Arnaud A., Galzy P. and Rios G. Bioconversion of L-malic acid into L-lactic acid using a high compacting multiphasic reactor (HCMR). *J. of Chem. Tech. and Biotech*. 1991; 51(1): 81-95.
[68] Spettoli P., Bottacin A., Nuti M.P., Zamorani A. Immobilization of *Leuconostoc oenos* ML 34 in calcium alginate gels and its application to wine technology. *Am. J. Enol. Vitic.* 1982; 33: 1-5.
[69] Janssen, D.E., Maddox, I.S., and Mawson A.J. An immobilized cell reactor for the malolactic fermentation of wine. *Aust. New Zealand Wine Ind. J*. 1993; 8: 161–165.
[70] Hong, S. K., Lee, H. J., Park, H. J., Hong, Y. A., Rhee, I. K, Lee, W. H., Choi, S. W, Lee, O. S. and Park, H. Degradation of malic acid in wine by immobilized *Issatchenkia orientalis* cells with oriental oak charcoal and alginate. *Lett. in Appli. Microbiol*. 2010; 50(5): 522-529.
[71] Agouridis, N., Kopsahelis, N., Plessas, S., Koutinas, A.A. and Kanellaki, M. *Oenococcus oeni* cells immobilized on delignified cellulosic material for malolactic fermentation of wine. *Bioresource Technol*. 2008; 99(18): 9017-9020.
[72] Agouridis, N., Bekatorou, A., Nigam, P. and Kanellaki M. Malolactic fermentation in wine with *Lactobacillus casei* cells immobilized on delignified cellulosic material . *J. Agric. Food Chem*. 2005; 53(7): 2546-2551.
[73] Taillandier, P., Cazottes, M.L. and Strehaiano, P. Deacidification of grape musts by a *Schizosaccharomyces* entrapped in alginate beads. A continuous-fluidized-bed process. *Chem. Eng. J. Biochem. Eng. J*. 1994; 55(1-2): B29-B33.
[74] Prazeres, D.M.F. and Cabral J.M.S. Enzymatic membrane bioreactors and their applications. Enz. Microbiol. Tech. 1994; 16(9):738-750.
[75] Giorno L, and Drioli E. Biocatalytic membrane reactors: applications and perspectives. *Tib. Tech*. 2000;18:339–349.
[76] Giorno L, De Bartolo L, and Drioli E. Membrane bioreactors for biotechnology and medical applications. Bhattacharyya D, Butterfield DA, editors. *New insights into membrane science and technology: polymeric and biofunctional membranes*. Elsevier; 2003. Chap 9.
[77] Charcosset,C. Membrane processes in biotechnology: An overview. *Biotechnology Advances* 2006; 24:482–492.
[78] Belfort G., and Heath C.A. New developments in membrane bioreactors. In: Crespo JG, Böddeker KW, editors. *Membrane processes in separation and purification*. NATO ASI Series E: Applied Science Dordrecht: Kluwer Academic Publishers; 1993. p. 127–49.
[79] Gao C. and Fleet, G. Degradation of malic and tartaric-acids by high-density cell-suspensions of wine yeasts. Food Microbiol. 1995; 12 (1): 65-71.
[80] Nehme, N., Mathieu, F. and Taillandier, P. Impact of the co-culture of *Saccharomyces cerevisiae-Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. *Food Microbiol*. 2010; 27 (1):150-157.
[81] Lambrechts, M. G., and Pretorius, I.S. Yeast and its importance to wine aroma: a review. *S. Afr. J. Enol. Vitic*. 2000; 21:97-129.
[82] Rainieri, S., and Pretorius, I.S. Selection and improvement of wine yeasts. *Ann. Microbiol*. 2000; 50:15-31.
[83] Formisyn, P., Vaillant, H., Lantreibecq, F. and Bourgois, J. Development of an enzymatic reactor for initiating malolactic fermentation in wine. *Am. J. Enol. Vitic*.1997; 48:345-351.
[84] McCord, J. D., and D. D. Y. Ryu. Development of a malolactic fermentation process using immobilized whole cells and enzymes. *Am. J. Enol. Vitic.* 1985; 36:214-218.

[85] Vaillant, H., and Formisyn, P. Purification of the malolactic enzyme from a *Leuconostoc oenos* strain and use in a membrane reactor for achieving the malolactic fermentation of wine. *Biotechnol. Appl. Biochem.* 1996; 24:217-223.
[86] Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud-Funel, A., Denayrolles, N., Subden, R.E. and Van Vuuren H.J.J.. Malolactic fermentation in grape must by a genetically engineered strain of *Saccharomyces cerevisiae*. *Am. J. Enol. Vitic*. 1997;48:193-197.
[87] Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M., Van Vuuren, H.J.J. Engineering pathways for malate degradation in *Saccharomyces cerevisiae. Nature Biotechnology.* 1997; 15: 253 – 257.
[88] Husnik, J.I., Volschenk, H., Bauer, J., Colavizza, D., Luo, Z.and Van Vuuren, H.J.J. Metabolic engineering of malolactic wine yeast. *Metabolic Engineering.* 2006; 8(4): 315-323.