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Malolactic fermentation of wines with immobilised lactic acid bacteria – Influence of concentration, type of support material and storage conditions

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ABSTRACT

Corn cobs, grape skins and grape stems were evaluated as support materials for immobilization of the lactic acid bacteria *Oenococcus oeni*. The support materials with immobilized cells were further used in malolactic fermentation (MLF) of white wine. Viability of using the immobilized supports was evaluated in consecutive batch fermentations under different conditions of temperature, ethanol and SO_2. Additionally, the possibility of storage and operational stability of the immobilized supports was also studied. All the three supports presented large potential for immobilization of *O. oeni* cells. The consecutive batches of MLF were successfully conducted for a total period of around 5 months with the possibility of storage of the biocatalyst for 30 d in wine at 25 °C.

1. Introduction

The two main fermentation processes in winemaking are alcoholic fermentation (AF) conducted by yeasts that transform sugars into ethanol and carbon dioxide, and malolactic fermentation (MLF) carried out by lactic acid bacteria that convert malic acid to lactic acid and carbon dioxide (Diviès & Cachon, 2005). MLF is a secondary fermentation that usually occurs during storage of young wines several weeks after the AF. MLF normally occurs spontaneously and is a very slow and unpredictable process that can undergo for weeks and even months, and not always give a satisfactory result (Bauer & Dicks, 2004). The wine presents unfavourable conditions for the growth of microorganisms so, even when the wine is inoculated with selected starters, there is no guarantee that the MLF will occur (Diviès & Cachon, 2005; Herrero, García, & Díaz, 2003).

The implementation of MLF is very important for wines produced in cold regions as it reduces the acidity, brings biological stability and may improve the organoleptic characteristics of the product (Diviès & Cachon, 2005; Kosseva, Beschkov, Kennedy, & Lloyd, 1998). MLF determines the final quality of red and white wines and of some sparkling wines, being especially crucial for the specific organoleptic profile of Chardonnay, Burgundy white wines and Bordeaux red wines (Bauer & Dicks, 2004). In the Portuguese *Vinho Verde* wines, which are young wines, the MLF is often desirable as it partially decreases the acidity and increases the pH. A low value of pH in wines brings instability of the volatile compounds and, consequently, MLF at a suitable extent may help to preserve the aromatic characteristics of *Vinho Verde*.

In recent years, immobilized lactic acid bacteria were used for implementation of MLF in wines. According to Vila-Crespo, Rodriguez-Nogales, Fernandéz-Fernandéz, and Hernanz-Moral (2010), immobilized cell system is one of the strategies for the enhancement of malolactic fermentation in the changed climate conditions. Moreover, immobilized cell systems showed to be a good tool for the winemaking industry. Nevertheless deeper studies on this area must be done in order to ease the handling of the process and the use of this tool at the cellar (Vila-Crespo et al., 2010). Two main immobilization methods have been employed: encapsulation of the bacteria cells (Crapisi, Nuti, Zamorani, & Spettoli, 1987; Kosseva & Kennedy, 2004; Kosseva et al., 1998; Spetolli, Buttacin, Nuti, & Zamorani, 1982) and attachment/adsorption onto a support (Agouridis, Kopsahelis, Plessas, Koutinas, & Kanellaki, 2008; Maicas, Pardo, & Ferrer, 2001). The use of immobilized bacteria during MLF helps to accelerate the process and also simplifies the control of its extension. However, the material to be used as immobilization support must be carefully chosen in order to not negatively affect the final product, and should also be cheap, abundant in nature, and of food grade purity.

In this work, the lactic acid bacteria *Oenococcus oeni* was immobilized on three different natural materials (namely corn cobs, grape skins and grape stems) and used to induce malolactic fermentation in white wine. A simple, fast and effective method for immobilization of bacteria cells was used. Additionally, the viability of the biocatalyst after periods of storage in different environments and temperatures was evaluated. The resistance of

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the immobilized lactic acid bacterium against the inhibitory effect of high concentration of SO_2 was also determined.

2. Materials and methods

2.1. Inoculum preparation

A commercial strain of *O. oeni* (Uvaferm® Alpha, Lallemand) was the bacterial strain used in the experiments. The inoculum was prepared by cultivation of the bacteria in 500 ml Erlenmeyer flasks containing 200 ml of MRS Broth medium (Cultimed, Panreac, Barcelona). Cells were cultivated under static conditions, at 28 °C for 48 h, being subsequently recovered by centrifugation (7000 min^{-1}, 15 min), washed with distilled water and re-suspended in the fermentation medium to obtain an initial concentration of 1 g/l (dry weight).

2.2. Support materials for cell immobilization

Grape skin, grape stem and corn cobs were used, separately, as support materials for the bacterium immobilization and in two different concentrations, 10 g/l and 30 g/l. Grape skin and grape stem, were supplied by a local wine-making industry and the corn cobs were obtained from local farmers. Before use, the support materials were washed with distilled water and dried at 60 °C until constant weight. For further use as immobilization supports, the materials were cut and prepared according to Genisheva, Mussatto, Oliveira, and Teixeira (2011). Finally the supports were sterilized at 121 °C for 20 min.

2.3. Cell immobilization

Fermentation runs were performed in complex culture medium with the following composition (g/l): glucose (15), yeast extract (4.0), meat extract (8.0), bacteriological peptone (10.0), $MgSO_4$ (0.2), $MnSO_4$ (0.05), sodium acetate (5.0), tween 80 (1.0), di-potassium hydrogen phosphate (2.0), di-ammonium hydrogen citrate (2.0) and malic acid (5.0). The assays were carried out in 500 ml Erlenmeyer flasks containing 200 ml of medium and 2 g (or 7 g) of the support material. The flasks were statically incubated at 28 °C for 10 h. Fermentations were carried out in duplicate, and samples were taken periodically for estimation of biomass, glucose and malic acid consumption, and lactic acid production.

2.4. Malolactic fermentations

MLF was conducted in white wine produced in laboratory conditions. Fig. 1 is a schematic representation of the assays of malolactic fermentation carried out in the present study. Fermentation runs F6 and F7 were supplemented with sulphur dioxide in the concentration of 30 mg/l. All the assays were carried out in 500 ml Erlenmeyer flasks containing 200 ml of white wine and 7 g of each previously immobilized support. The flasks were statically incubated at 25 °C for 17 days (except for the first and second fermentations (Fig. 1)). Fermentations were carried out in duplicate, and samples were taken periodically for estimation of glucose, fructose and malic acid consumption, and lactic acid production. Before fermentations F5 and F7 the immobilized supports were stored as shown in Fig. 1.

2.5. Determination of immobilized biomass

The concentration of immobilized cells was determined at the end of the cell immobilization assays. Part of the immobilized material was taken aseptically from the fermentation flask and then placed in 200 ml Erlenmeyer flasks containing 20 ml of distilled water. Subsequently, the sample of biocatalyst was autoclaved for 20 min at 121 °C. The autoclaved support was separated from the liquid using a strainer and left to dry at 60 °C till constant weight. The total volatile suspended solids were calculated according to Clesceri, Greenberg, and Trussel (1989). Corrections of the weight of volatile suspended solids for the losses of support itself were carried out by blank experiments using support without immobilized cells.

Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to an analytical curve (dry weight × optical density).

2.6. HPLC analysis

Glucose, fructose and organic acids (malic and lactic) concentrations were determined by High Performance Liquid Chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 °C. A 5 mmol/L H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 ml/min.

2.7. Fermentation parameters

The concentration of cells immobilized on the support ($C_{i,biom}$, mg/g) was calculated as the ratio of cell mass immobilized on the support to the support mass. The concentration of immobilized cells in the assay (C_i, g/l) was calculated as the ratio of cell mass immobilized on the support to the volume of fermentation medium. The concentration of free cells in the assay (C_{free}, g/l) was calculated as the ratio of cell mass to the volume of fermentation medium. Mass immobilization efficiency (Y_i, %) was defined as the ratio between immobilized cells and total formed cells (free + immobilized, X_t, g/l). The cell yield factor ($Y_{X/S}$, g/g) was defined as the ratio between the mass concentrations of total formed cells and the malic acid consumed. The concentration of the consumed malic acid ($C_{mal.ac}$, g/l) was calculated as the ratio of the grams of consumed malic acid per litre fermentation medium The concentration of produced lactic acid ($C_{lac.ac}$, g/l), was calculated at the 8th hour of the fermentation for immobilization. Lactic acid productivity (Q_p, g/(L h)) was defined as the ratio between lactic acid mass concentration and the fermentation time. Malic acid conversion (%) was calculated as the ratio between the mass concentration of the consumed malic acid and initial malic acid mass concentration.

2.8. Statistical analysis

The results were analyzed by ANOVA using FAUANL software (Olivares, 1994). Tuckey's test was used to detect significant differences between samples.

3. Results and discussion

3.1. Cell immobilization

The support materials used in the present work were chosen taking into account their nature, abundance and cost values, as well as their suitability to be used as support material for yeast cells immobilization (Genisheva et al., 2011). Grape skins and grape stems together with the grape seeds are known with the common name of grape pomace. The grape pomace is the biggest solid waste of the wine industry and it is of interest that an alternative use for this byproduct is found. Another advantage of using

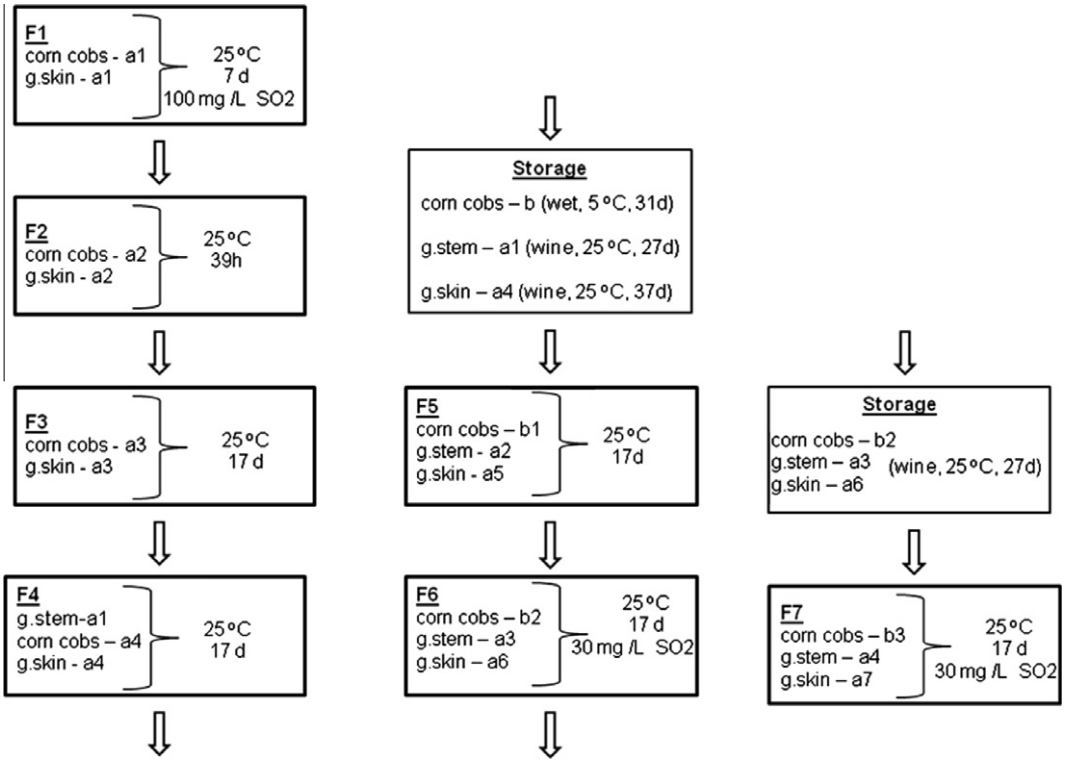

Fig. 1. Flowchart of the malolactic fermentation assays of white wine conducted with immobilized cells.

grape skins and stems as immobilization supports is that they are natural products coming from the prime material, and therefore, a lesser negative effect over the final product is expected.

In the present study, the increase of the concentration of support material from 10 g/l to 30 g/l, during the immobilization assays, had a positive effect on the quantity of immobilized cells $C_{i,biom}$ (Table 1), which had the concentration doubled or even tripled. This higher affinity of the cells to the support material when the amount of support is increased is due to the biocatalyst activities of these supports. According to Genisheva et al. (2011), these materials provide nutrients to the medium, improving the yeast bioconversion performance.

The highest immobilization efficiency values were recorded for assays with 30 g/l of corn cobs or grape skins, with values of 68.29% and 62.61%, respectively. Assays with grape stems showed significant differences ($p < 0.05$) in terms of immobilization efficiency (Y_i) for the two concentrations of support utilized, although there was not found statistical difference in the concentration of immobilized cells per mass of support, $C_{i,biom}$. All the immobilization assays did not show significant differences ($p < 0.05$) for the response of cell yield factor ($Y_{X/S}$). On the other hand, assays using 10 g/l support material achieved higher values of produced lactic acid ($C_{lac.ac}$) and productivity (Q_p).

Fig. 2 shows the results obtained during MLF assays carried out with and without immobilized cells. As can be seen in this figure, fermentations with immobilized cells were twice faster than fermentations with free cells. This is in agreement with our previous study, which demonstrated also that immobilized cells improved the fermentation rates as well as the efficiency of bioconversion (Genisheva et al., 2011). In the presence of the support material, the production of free biomass is higher than in the fermentations containing only free cells, demonstrating that the support contributes for a better performance of the bacteria (Genisheva et al., 2011). It can be seen in Fig. 2 that grape skin assays registered higher values for free biomass on the 4th hour; however, this point is an outlier which results from an anomalous value obtained for one of the replicates.

In summary, corn cobs and grape skins in amounts of 30 g/l were the best support materials for *O. oeni* immobilization, since they immobilized the highest amount of cells (111.0 mg/g and 108.8 mg/g, respectively). However, fermentation with cells immobilized on 10 g/l of corn cobs and grape stems gave the highest productivity in lactic acid, 4.06 g/(L h) and 4.03 g/(L h), respectively. As a whole, fermentations with bacteria immobilized on 10 g/l support achieved more significant concentrations of lactic acid than with bacteria immobilized in 30 g/l of support. Additionally, malic acid consumption was faster in the fermentations with immobilized cells compared to fermentations with free cells (Fig. 2).

Table 1
Multiple comparison analysis (Tukey's test; $p<0.05$) for the concentration of immobilized cells ($C_{i,biom}$) and lactic acid ($C_{lac.ac}$), immobilization efficiency (Y_i), cell yield factor ($Y_{X/S}$), lactic acid productivity (Q_p) and total produced cell (X_t) during the malolactic fermentation by *Oenococcus oeni*.

Support and concentration		$C_{i,biom}$ (mg/g)	Y_i (%)	$C_{lac.ac}$ (g/l)	$Y_{X/S}$ (g/g)	Q_P [g/(L h)]	X_t (g/l)
Corn cobs	10 g/l	32.8^{b}	22.21^{cd}	32.52^{a}	0.71^{a}	4.06^{a}	3.75^{ab}
Grape skins		40.75^{b}	42.44^{bc}	27.36^{b}	0.61^{a}	3.42^{b}	3.43^{ab}
Grape stems		31.0^{b}	9.48^{d}	32.27^{a}	0.79^{a}	4.03^{a}	4.26^{a}
Corn cobs	30 g/l	111.0^{a}	68.29^{a}	14.72^{c}	0.81^{a}	1.84^{c}	3.43^{ab}
Grape skins		108.8^{a}	62.61^{ab}	14.07^{c}	0.69^{a}	1.76^{c}	3.16^{ab}
Grape stems		40.7^{b}	38.87^{bc}	14.68^{c}	0.59^{a}	1.83^{c}	2.42^{b}

a, b, c, d – Values with the same letters mean no significant difference at 95% confidence level.

3.2. Consecutive malolactic fermentations

The bacteria cells previously immobilized on 30 g/l of corn cobs, grape skins or grape stems were used for conducting malolactic fermentation in white wine. Fig. 1 shows the MLF assays conducted

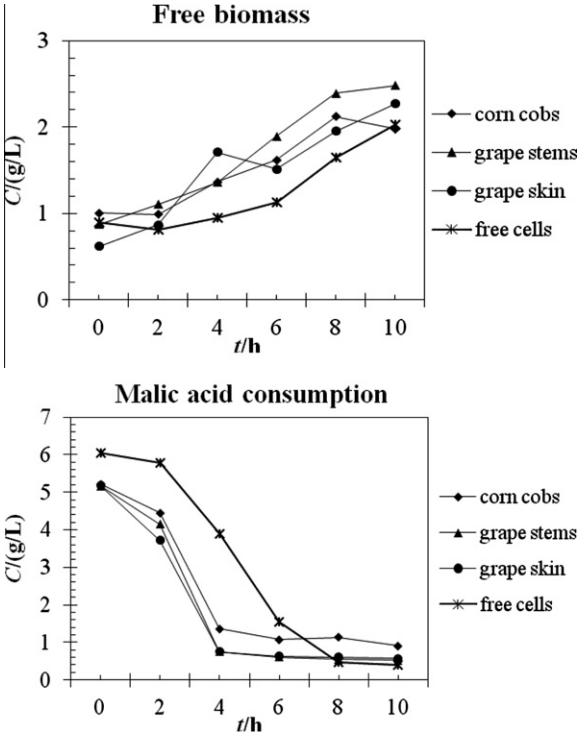

Fig. 2. Malic acid consumption ($C_{mal,ac}$) and concentration of free biomass (C_{free}) during the immobilization runs in presence of 30 g/l support material compared with free cell assays.

with the different immobilized supports. All fermentation assays were conducted in white wine with concentrations of malic acid around 3.5 g/l. In the total, seven series of MLF were done, which were named from F1 to F7 (Fig. 1). For the different supports, different numbers of consecutive batch fermentations were made. At the end of fermentation F4 new corn cobs-b (b-second immobilization of corn cobs) was immobilized and further used in the consecutive malolactic batch fermentations. Four consecutive batches were done with immobilized corn cobs-a (corn cobs-a1, a2, a3 and a4), and other three consecutive batches were made with immobilized corn cobs-b (corn cobs-b1, b2 and b3).

The bacteria activity may be affected by several parameters, being the most important the ethanol concentration and wine pH, fermentation temperature, and level of sulphur dioxide (Ribéreau-Gayon, Dubourdieu, Donéche, & Lonvaud, 2006). In order to test the influence of different inhibitory factors over the performance of *O. oeni* immobilized on corn cobs and grape skins (the materials that gave better results during the immobilization), the biocatalysts were placed into a white wine with the following growth inhibitors for the bacteria cells: ethanol 9% v/v, 20 mg/l free SO_2 and 100 mg/l total SO_2. The flasks were incubated at 25 °C since it has been reported that the growth of *O. oeni* is inhibited and the malolactic fermentation is slower at temperatures of 25 °C or above (Ribéreau-Gayon et al., 2006). Guzzo, Jobin, and Diviès (1998) demonstrated that in the presence of 15 mg/l of free SO_2 most of the cells of *O. oeni* died within 3 h. The growth of the bacteria is inhibited in environments richer in ethanol (above 6% v/v), being difficult at or above 13% v/v, 14% v/v (Ribéreau-Gayon et al., 2006). For evaluating the viability of the immobilized cells after their prolonged exposure to these conditions, the biocatalyst from assays F1 were separated aseptically from the liquid media, washed with distilled sterilized water and placed in a new wine without sulphites at 25 °C for 39 h (F2). Fermentation activity was noticed almost instantly. The conversion of malic acid in the MLF with immobilized corn cobs-a2 was of 6%, while in assays with immobilized grape skins were of 33% (Table 2). These results show that the immobilized cells of *O. oeni* are highly tolerant against inhibitors.

Once the malic acid conversions in fermentation assays from series F2 were relatively low, the fermentation time was fixed in 17 d in the subsequent batch series. During the series F3, corn cobs-a3 and grape skins-a3 were placed in a new wine for 17 d at 25 °C. The obtained results for the malic acid conversion were of 71% and 50% for assays in presence of corn cobs and grape skins, respectively (Table 2). F4 fermentation series were conducted with cells immobilized on grape stems-a1 (batch1), grape skins-a4 (batch4) and corn cobs-a4 (batch4). The fermentations lasted 17 d and the obtained malic acid conversion was 75%, 87% and 23%, respectively (Table 2). Concerning fermentations with immobilized cells an important aspect stands up, i.e. the storage of an immobilized support for a further use. To verify if the chosen supports are suitable for storage at different conditions and periods of time, at the end of F4 the three immobilized supports were stored at different conditions. Corn cobs were aseptically removed from the liquid and stored at 5 °C for 31 d. Grape skins and grape stems were stored in wine from the previous MLF at 25 °C for 37 and 27 d, respectively (Fig. 1). After the storage, all the supports were washed with sterilized water and placed in a new wine for a new series of fermentations (F5), which was maintained at 25 °C for 17 d. A slight decrease in the malic acid conversion in the assays with immobilized grape skins and grape stems was observed, while the malic acid conversion was practically maintained in the assays with immobilized corn cobs. These results reveal that storage slightly affected the fermentation performance of cells immobilized on grape skins and grape stems, but not of cells immobilized on corn cobs "hidden" in the porous like surface of the corn cobs, where the biggest loads of cells are found (Genisheva et al., 2011).

In the subsequent series of fermentations (F6) the immobilized supports were exposed to 30 mg/l of free SO_2. Assays with grape skins were not negatively affected by the sulphur dioxide but on the contrary, there was a noticed increase of the malic acid conversion attaining a value similar to that achieved in F4 assays. The level of SO_2 used in this experiment had no effect on malic acid conversion by *O. oeni* immobilized on grape stems, while assays with *O. oeni* immobilized on corn cobs showed strong decrease of the malic acid conversion, (Table 2). Then, in the next stage of the study it was decided to evaluate the combined effect of storage of the immobilized supports and presence of free SO_2 (30 mg/l) in the fermentation media. At the end of F6 series, all the supports were stored in wine at 25 °C for 30 d. After storage, the supports were separated from the liquid, washed with distilled sterilized water and placed in a new wine with 30 mg/l free SO_2, at 25 °C

Table 2
Malic acid conversion (%), ± standard deviation during the consecutive MLF by immobilized *O. oeni* on different support materials.

Support/batch	F1	±	F2	±	F3	±	F4	±	F5	±	F6	±	F7	±
Corn cobs	0	0	6	0	71	24	23	0	24	4	10	1	50	13
Grape skin	0	0	33	7	50	4	87	1	63	4	85	0	39	23
Grape stem	–	–	–	–	–	–	75	12	65	6	63	19	6	3

for 17 d (fermentation series F7). The obtained results for malic acid conversion were as follows: corn cobs > grape skin > grape stems (Table 2). It was then concluded that cells immobilized on corn cobs were more protected from the influence of the inhibitory conditions than cells immobilized in the other support materials, showing previous adaptation to the SO_2 present in the wine. Cells immobilized on grape skins and grape stems were strongly affected by the high doses of SO_2, combined with previous storage of the supports. The F7 fermentation assays were extended till 30 d of fermentations and the results showed a complete malic acid conversion (100%) in the assays with cells immobilized on corn cobs, 75% conversion for cells immobilized on grape skins and 83% conversion for cells immobilized on grape stems (results not shown). These results suggest that the combined effect of the factors storage of the support and the presence of SO_2 in the medium did not prevent the malic acid consumption, but just slowed it down.

The results obtained in this study reveal also that the support materials used for the cells immobilization without any previous treatment, have longer operation stability when compared to delignified cellulosic material (Agouridis et al., 2008), being also of lower cost due to not requiring treatment prior to their use in the fermentation. In summary, *O. oeni* cells immobilized on corn cobs-b were able to conduct consecutive MLF for a total period of 150 d (3 batches), on grape stems for 174 d (4 batches) and on grape skins for 192 d (7 batches). These results are of large interest since they allow a better control and conduction of the malolactic fermentation process.

Although MLF using immobilized cells present unambiguous advantages with respect to traditional systems with free cells, a more intense colour could be present for the first runs of fermentations. This aspect represents an apparent drawback. However, previous studies with immobilized yeasts used to conduct alcoholic fermentation on white winemaking (Genisheva, Macedo, Mussatto, Oliveira, & Teixeira, 2012), clearly showed that as the number of baths increased, the colour tend to stabilize. Additionally, the use of such immobilized systems could be difficult to implement on traditional winemaking systems. Nevertheless, since the final objective is the use of immobilized bacteria in continuous reactor systems, to control the extent of the MLF, the advantages will be obvious.

4. Conclusions

Corn cobs and grape skins, prepared in a culture media at the concentration (mass support per volume media) of 30 g/l, were the best support materials for *O. oeni* immobilization. Immobilized bacteria cells were more resistant against the inhibitory effect of high concentrations of ethanol, SO_2 and elevated temperatures. Cells immobilized on corn cobs were strongly affected from high concentration of free SO_2 (30 mg/l) present in the wine; however, once the cells were adapted to the presence of SO_2, there was not reduction of the malic acid conversion. Assays with cells immobilized on grape skin and grape stems were not negatively affected by the presence of 30 mg/l of SO_2 in the wine. Nevertheless, previous storage of the biocatalyst at 25 °C for 27 d, combined with the presence of 30 mg/l of SO_2 had a strong negative effect over the malic acid conversion. Bacterial cells immobilized on corn cobs, grape skin and grape stems are capable to perform consecutive MLF for long periods of time, at least for 5 months. The immobilized supports can be stored for at least 30 d to 37 d.

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