METABOLIC DIVERSITY OF *OENOCOCCUS OENI* STRAINS ISOLATED FROM SPANISH WINES

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Introduction

Malolactic fermentation (MLF) is the process converting L-malic acid to L-lactic acid and carbon dioxide. This process decreases the acidity and increases the pH of wine, which provides a softer mouthfeel. In addition, the exhaustion of L-malic acid contribute to the microbial stability of the wine (Davis *et al.,* 1988; Kunkee, 1991; Maicas *et al*., 1999; Ugliano *et al.,* 2003). Other positive effect of MLF is the generation of desirable aroma and flavor compounds by transformation of substrates present in wine. This fermentation takes place after alcoholic fermentation (AF), once yeasts have exhausted the majority of sugars. At this moment, the environmental conditions of wine are very harsh: low nutrient content, low pH, high ethanol concentration, presence SO_2, and low temperatures (Wibowo *et al*., 1985; Lonvaud-Funel, 1999; Coucheney *et al*., 2005). Although all species of the genera of lactic acid bacteria (LAB) associated to winemaking (*Leuconostoc*, *Lactobacillus, Pediococcus* and *Oenococcus*) are able to perform MLF, the best adapted to survive in wine environment is *Oenococcus oeni*. Several reports have demonstrated that the resistance to wine conditions and the ability to modify the organoleptic characteristics of wines are strain-dependent (Bartowsky, 2005).

The growth of BAL in wine depends largely on sugars, organic acids, amino acids and vitamins which are found in grape must or wine (Garvie, 1967a; Pardo *et al*., 1988; Lonvaud-Funel, 1999). Like most heterofermentative LAB, *O. oeni* is able to degrade hexoses, pentoses and other sugars from must (Unden and Zaunmüller, 2009).

The major residual sugars in wine after completion of AF are glucose and fructose, which may vary from 10 g/L to less than 0.5 g/L, depending on wine style (Bauer and Dicks 2004). Fructose is generally in higher concentrations than glucose. Another sugars as arabinose, xylose, ribose and trehalose may be present in wine although in lower levels than glucose and fructose (Cabanis *et al*., 2000).

LAB could also use organic acids of wine. In some cases the metabolism of organic acids is a way to gain energy (Poolman, 1993; Konings *et al*., 1997). Concentrations of organic acids in musts are variable in malic acid (0.7 - 8.6 g L^{-1}), tartaric acid (3.56 - 7.42 g L^{-1}) and citric acid (0.13 - 0.9 g L^{-1}). It depends on geographic location and climatic conditions (Cabanis *et al*., 2000; Swiegers *et al*., 2005). The malic acid metabolism through the malolactic enzyme is the basis of MLF (Davis *et al*., 1988; Lonvaud-Funel, 1999). Citric acid metabolism in LAB is initiated after the depletion of malic acid and results in the formation of one of the most important compounds associated with MLF, diacetyl (2,3-butandione). This compound is tolerated in wine only in low concentrations, because confers a 'buttery' character to wine (Bartowsky and Henschke, 2004). Other aromatic compounds arise from citric metabolism: acetoin, 2, 3-butanediol and acetic acid. Although tartaric acid is found in high quantities in wine, few LAB strains were reported as degrading.

The objective of this work was to study the diversity of indigenous wine *O. oeni* strains by analysing their abilities to metabolize several carbon sources and different organic acids present in wine. Relationship between carbohydrate fermentation profiles and performances to accomplish MLF in a synthetic wine was established.

Materials and Methods

Bacteria strains

Oenococcus oeni strains used in this study were obtained from ENOLAB (Universidad de Valencia) culture collection. They were isolated from several Spanish wines (Table 1). Working cultures were maintained at - 80 ºC in MLO (Zúñiga *et al*., 1993) supplemented with glycerol (30 % v/v) as cryoprotective agent. The strains were routinely cultivated in MLO broth at 28 ºC for 2 - 3 days.

Wine type	Strain
Red and Rose	245, 246
Red	118
Red and Rose	164, 165, 168, 169, 182, 188, 191, 193, 194,197, 202, 203, 225, 226, 228A, 229, 251
Red and Rose	235, 236
Red and Rose	248, 249, 254
Red and Rose	176A, 184, 185, 186
White	141,142, 234
Red and Rose	160B, 160C, 161, 237
Red	Z1, Z2, Z3, Z6, Z8
Rose	75, 255,
Rose and White	123, 124, 125, 129, 130, 133
Red and White	122, 128, 138, 139, 171, 196
Rose	146, 149,154,158, 205A, 206, 207, 208A, 209, 210, 213, 217B, 218A, 219, 222M, 222G, 223A, 224
Red, Rose and White	120, 134, 238, 239, 240, 242, 266

Table 1. Description of Spanish wine types from which *Oenococcus oeni* strains were isolated

Fermentative profile

Fermentation tests on different sugars were performed using the basal medium MBFC described by Garvie (1967b) which contained 2 % (v/v) of the following carbon sources: amylose, D-arabinose, L-arabinose, D-arbutin, D-cellobiose, dextrin, D-fructose, D-galactose, D-glucose, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-ribose, D-sucrose, D-salicin, D-trehalose, D-xylose and L-xylose.

Cells from MLO culture with an optical density at 600 nm ($O.D_{600}$) of 0.8 were harvested by centrifugation at 6000 rpm for 15 minutes (Heraeus Multifuge 1 SR), washed twice, resuspended in the same volume of sterile water and inoculated at 4 % in MBFC. The tubes were incubated at 28 ºC for 30 days. Positive fermentative results were recorded if tubes turned blue-green into yellow colour.

Preparation of wine-like medium to analyse organic acids metabolism

The wine-like medium was obtained by fermentation of a synthetic must as Guija (2011) described. Synthetic must was inoculated at 10^6 CFU ml L^{-1} *Saccharomyces cerevisiae* var. *bayanus* BY strain (Agrovin) and AF was performed at 28 ºC for 20 days. Then, yeasts were removed by centrifugation at 10000 rpm for 20 minutes

(BECKMAN COULTER Avanti J-E). Wine-like medium was adjusted at 1.5 g L^{-1} glucose, 1.5 g L^{-1} fructose, 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 1 mL L^{-1} vitamin solution (Liu 1993) and 10 % (v/v) ethanol, according to Cruz-Pio (2012). Three batches of this wine were separately added 5 g L^{-1} L-malic acid, 1 g L^{-1} L-citric acid and 5 g L^{-1} L-tartaric acid and pH of all of them was adjusted at 3.7. Finally the wine was sterilized by filtration through a 0.22-µm-pore size cellulose nitrate filter and was stored at 4 ºC until use.

To evaluate the ability to degraded organic acids in this synthetic wine medium, *O. oeni* strains were preadapted by growing in Medium 7 described by Pardo and Zúñiga (1992). After the pre-adaptation step, cultures were centrifuged at 10000 rpm for 10 min and the pellet was washed and resuspended in sterile water. Ten millilitres of synthetic wine were inoculated with this cell suspension at 2.0 % (v/v) and incubated at 28 ºC for 20 days. An inoculated tube was used as a control. To monitor the kinetics of acids degradation samples at 3, 6 and 20 days were taken. Assays were performed in duplicate.

Quantification of organic acid consumption

Acids organics presents in the samples at different moments were analysed using high performance liquid chromatography (HPLC). HPLC analysis were performed according to Frayne (1986). Before injection, the samples were centrifuged at 13000 rpm for 10 minutes (Prism R Microcentrifuge C2500-R), filtered through a 0.22 µm syringe filter and directly injected on HPLC.

Results

Table 2 shows the fermentation profile of 80 *O. oeni* strains. Strains were grouped in twelve profiles, which B and I were the most common. Profile I, is the less reactive, showing that only fructose and ribose are fermented. In addition to these two carbohydrates, profile B show activity on L-arabinose.

All strains degraded fructose and ribose. The consumption of D and L arabinose, arbutin, cellobiose, dextrin, glucose, mannose, salicin and trehalose were variable. None isolate fermented amylose, galactose, lactose, maltose, mannitol, melibiose, sucrose, D and L xylose (Table 3).

Strains 75, 255, 118, 161 and 139 belonging to F, E and H profiles had higher ability to ferment carbohydrates; they degraded seven and eight of them. The less common profiles were C, H and K profiles comprising only one representative strain

Table 2. Fermentation profile in *Oenococcus oeni* strains used in this work.

Profile	Strain
A	129, 146, 154, Z6, Z8
B	120,122, 124, 125, 128, 130, 133, 138, 141, 142, 171, 185, 196, 197, 217B, 223A, 224, 234, 238, 239, 246, 251, Z1
C	205A
D	218A, 240, 248, 254
E	118, 161
F	75, 255
G	158, 164, 169, 182, 222M
H	139
I	134, 149, 165, 168, 176A, 184, 186, 188, 191, 193, 194, 202, 203, 206, 207, 208A, 209, 210, 213, 219, 222G, 225, 228A, 235, 236, 237, 242, 245, 249, 266, Z2, Z3
J	123, 160B
K	160C
L	226, 229

Table 3. Carbohydrates fermented by *Oenococcus oeni*.

Carbohydrate	Fermentation profile											
	A	B	C	D	E	F	G	H	I	J	K	L
Amylose	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	+	-	-	-	-	-	-	-	-	-
L-arabinose	-	+	+	+	+	-	-	-	-	-	+	-
Arbutin	+	-	-	+	+	+	-	+	-	-	-	-
Cellobiose	-	-	-	-	-	+	-	-	-	-	-	-
Dextrin	-	-	-	-	-	-	-	+	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	-	-	-	-	+	+	-	+	-	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	n.d
Maltose	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	n.d
Mannose	-	-	-	-	-	+	-	-	-	-	+	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	+	+	-	+	-	-	-	-
Trehalose	-	-	-	-	+	+	+	+	-	+	+	-
D-xilose	-	-	-	-	-	-	-	-	-	-	-	n.d
L-xilose	-	-	-	-	-	-	-	-	-	-	-	-

Regarding malolactic performance 142, 149, 171, 182, 205A, Z2 and Z1 strains showed a more rapid decarboxylation of L-malic acid. All of them degraded 97 % of the initial malate in wine-like medium in three days. Strains 213, 197, 160B, 138, 133, 160C, Z6, 142, 158 and Z3 consumed a similar percentage of L- malic acid but in 6 days. Forty-three strains showed a consumption percentage between 84.4 to 98.2 % in twenty days. Reduction of L-malic acid was slower and lasted twenty days in the cases

of strains Z6, 239 and 202. Some of these results are shown in Figure 1. Citrate utilization was higher in strains 138M, 171, 197 and Z1, which completely depleted L-citric acid in three days. Twelve strains made it in six days. However, strain 128 showed lower citrate utilization, consuming only 37.6 % of initial citrate. Strain 202 was unable to metabolized citrate after twenty days of incubation. None strain degraded L-tartaric acid (Figure 1).

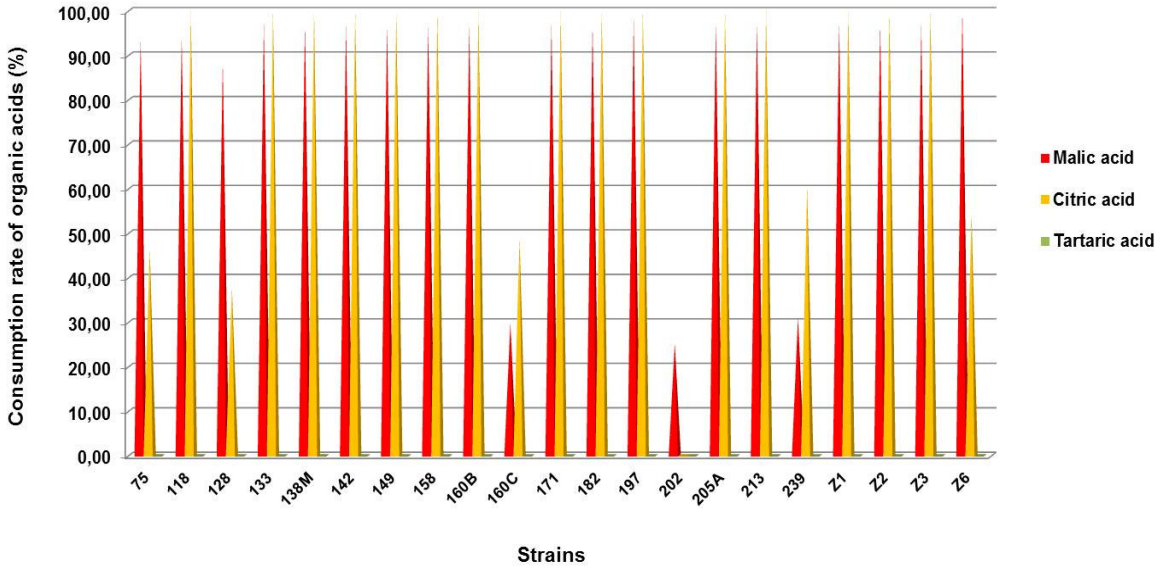

Figure 1. Consumption rates of L-malic acid, L-citric acid and L-tartaric acids after twenty days of incubation.

Discussion

O. oeni belongs to a heterogeneous group from the metabolic point of view, as evidenced Lafon-Lafourcade *et al*., (1983); Davis *et al*., (1988) and Edwards *et al*., (1991).

All the strains tested fermented both fructose and ribose, but Davis *et al*. (1988) found that only 55 % of the strains tested were able to ferment this last sugar.
Glucose is only fermented by 12.5 % of strains and at lower speed than fructose and ribose. This behaviour is probably related to cofactor re-oxidations problems; however co-metabolism of glucose and fructose resolves this problem and leads to maximize biomass (Maicas *et al*., 2002). A similar percentage of strains *O. oeni* that used both fructose and glucose as was found by Lafon-Lafourcade *et al*. (1983). Although Davis *et al*. (1988) found that 100 % strains of *O. oeni* were able to metabolize fructose and glucose.

According to our data L-arabinose is fermented by 38.7 % of strains. The ability to ferment pentoses was a criteria used by Peynaud and Domercq (1968) to differentiate two species of *Leuconostoc* found in wine (*Leuconostoc gracile* unable to ferment pentoses and *Leuconostoc oenos* able to ferment L-arabinose, L-xylose or both).
The fermentation of arbutin, cellobiose, mannose, salicin and trehalose was a variable character among our strains, in contrast with the results reported by Davis *et al*. (1988) the ability to degrade these carbohydrates were common among their strains.

These authors determined that 45 % of the strains studied fermented sucrose and 27 % fermented D-arabinose. In our case, none strain metabolized sucrose and only one used D-arabinose. None of our strains were able to ferment melibiose, mannitol, D and L-xylose, however Davis *et al*. (1988) found strains able to use these carbohydrates.

It is difficult to draw out good conclusions about *O. oeni* carbohydrate fermentation abilities by comparing different references. Different results from that same strain could be recorded because experiments have been performed in different media and with different procedures (tubes test, API Galleries, etc) (Garvie, 1967a and 1967b; Pardo *et al*., 1988; Jensen and Edwards, 1991; Fugelsang and Edwards, 2007)

As *O. oeni* is the main species associated to MLF the ability to degrade L-malic acid is a main character to select good malolactic starters. We have observed that our strains were different in efficiency and degradation speed of malate consumption. Henick-Kling *et al*. (1989) found that this differences were associated to wine pH.

Differences in the ability to use citric acid were also observed among our strains. Although the citric acid is present in low concentrations, its metabolism has considerable sensorial consequences. Citric acids could be transformed into acetic acid, increasing the volatile acidity of the wine, but also into diacetyl and other acetonic compounds that could improve the organoleptic profile of wine. Some researchers have considered that partial degradation of citric acids was better than a total degradation in order to limit that excessive accumulation of final products (Bartowsky and Pretorius, 2009). One positive character of our strains is that none of them were able to degraded tartaric acid. Generally, tartrate degradation leads to an alteration of wine named *tourne* which spoilt wine (Unden and Zaunmüller, 2009).

In conclusion, in this study, a high number of *O. oeni* strains were characterized from a metabolic point of view. Data showed that a high metabolic diversity among *O. oeni* strains exists. The majority of strains exhibit few fermentative abilities including strains having higher efficiencies to metabolize malic and citric acids. It seems that an extended ability to ferment many carbohydrates does not imply a superior performance to accomplish MLF. It is possible that strains showing ability to degrade fructose, ribose and L-arabinose degraded these sugars more efficiently that others able to use a higher number of carbohydrates. This can contribute to obtain more energy supporting growth and MLF performance.

Metabolic heterogeneity existing in *O. oeni* strains allows appropriate selection of strains to perform MLF in different types of wines and allows getting wines with diverse sensory profiles.

The use of selected strains can ensure a positive flavour contribution during MLF and reduce the risk of unknown wild strains that could produce undesirable or off-flavours which spoilt wine (Bartowsky, 2005).

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