Romanian Biotechnological Letters *Vol. 18, No.1, 2013*

ORIGINAL PAPER

Researches concerning the use *Oenococcus oeni* in the malolactic fermentation

Received for publication, September 20, 2012
Accepted, December 20, 2012

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Abstract

During the recent six years, the collective of teaching staff and scientific research from the Microbiology discipline belonging to the Faculty of Horticulture, University of Craiova has chosen, as main scientific interest, to isolate and identify, from the spontaneous flora, some lactic bacteria strains able to transform, through their metabolic processes, especially in the case of red high quality wines, the malic acid into lactic acid. Through this controled malolactic fermentation, we should be able to obtain red wines, generous, fine, extremely equilibrated and with a great aging capacity. In the present work, our aim was to present some results we have obtained during the process of malolactic fermentation for red wines, under usual productive circumstances, by making use of some lactic bacteria strains we have previously isolated and identified. Due to the scientific results we have obtained, we are entitled to state that some lactic bacteria strains, isolated from the spontaneous flora, do own appropriate physiological and technological assets, thus they can be used, bearing adequate results, into the elaboration process of red high quality wines.

Key words: bacterial strain, data, identifying, malolactic fermentation.

Introduction

Nowadays, we are justly founded to state that each of the viticultural areas concerned has its own capacity of exerting a natural selection among its indigenous lactic bacteria strains which would be useful, especially among the ones which already exist within the respective area. This is why it is not necessary to make the effort of isolating, identifying, processing them and making use of them under precisely defined and controlled circumstances, in order to develop the red wines' malolactic fermentation. For most of the viticultural regions of the world, generous and fine red wines were perfected in the flow of time, wines which have brought a lot of fame for these regions. It is this significant fact which stands for supporting our opinion (Popa A., 2008; Baie S., and all., 2006; Cavin J.F., 2006; De Revel and all., 2005; Flanzy C., 1998; Renouf V. and all., 2008).

The perfecting of the elaboration process, for wines in general and for red wines especially, is nowadays, preferred to be realized in less time, but yet with no compromises made over the wines' quality. In order to attend this aim, the malolactic fermentation (which is compulsory in the case of red high quality wines) ought to be realized immediately after the end of the alcoholic fermentation, on in the shortest delay possible after this moment (Popa A., and all., 2004).

Yet, many times, this desideratum is impossible to be realized, due to some situations which inhibit the activity of the lactic bacteria existing within the spontaneous flora.

These might be: a small amount of the bacteria's population; an insufficient qualitity of nourishing substances; large amounts of tanning substances and of sulphur anhydride; an insuficient quantity of oxygen, and particularly-values under 2.8 of the concentration of the hydrogen ions (pH<2.8).

Teking into account all these considerations, the oenologist should have at his disposal selected cultures of lactic bacteria, which he would be able to inoculate within wine, in order to develop the malolactic fermentation. The researches we have achieved led us to the conclusion that, in all the viticultural zones of Oltenia, upon the grapes, but especially into the must and the wine, there are enough strains of lactic bacteria some of them endowed with outstanding assets. We have isolated and identified a large number of strains, belonging mostly to the *Oenococcus oeni* species. In the present study, we discuss of the behaviour under productive circumstances, of two lactic bacteria strains we have isolated and identified: CS 62 and CS8 II.

Material and methods

We have performed our researches at the S.C. Domeniul Coroanei Segarcea, using red wine Cabernet Sauvignon from the harvest of 2010. The red wine of Cabernet Sauvignon obtained in Segarcea is known and long science appreciated for its colours' intensity, for the shiny tonality of its colour, while its finesse and generosity have rendered it famous. At Segarcea, in time, such Cabernet Sauvignon wines have been obtained, but in order to shape up such qualities and especially to achieve the malolactic fermentation, 4-5 years of maturation within vessels were necessary. But nowadays when the circulation of merchandises, thus the one of wines, is bound to go through its briefest cycles, for the red wines we are also interesed by the grape flaours which in fact could be found especially during the first year of the wine-making process. Therefore, we have to cope with the necessity of releasing the red wines on the market after one year, a year and half at most. As a consequence, the delicate and unavoidable process of transforming the malic acid into lactic one should take place the soonest possible, after the end of the alcoholic fermentation.The 2010 Cabernet Sauvignon wine had an alcoholic strenght of 13.8 vol% an acid contents of 4.6 gl^{-1} (H_2SO_4) and 29 gl^{-1} of non-reducing extract.

The strains of *Oenococcus oeni* was isolated from the spontaneous microflora. The culture media used was MRS agar. The inoculation was realized using two strains of lactic bacteria which we had isolated and identified and which have also shown a poor metabolizing process in respect to the citric acid. This fact does constitute for us a hope of being exempted from the eventual unwented modifications of a microbiological nature, changes which do occur into the wine, after the achievement of the malolactic fermentation.

The Gram colouring has consisted in applying upon the preparations the following solutions: gentiane violet, Lugol, acetone-alcohol and fuchsine. Lactic bacteria become coloured in violet, as they are framed in the G (+) type. The metabolizing of acids and sugars was realized through spectral photo-metric procedures.

In order to find out wholt is the ratio between the malic and tartric acids, issued from the development of the malolactic fermentation, we have determined the initial contents in malic acid, then the one in resulted lactic acid.

The value of this ratio is also decided by other items, such as: the value of the pH; the fermentation temperature; the vessel's capacity; the presence or not of the sulphur anhidride.

We determined the value of this ratio and we determined the time duration of the respective fermentation process. The concentration of malic and lactic acid was determined through UV method on the R-BIOPHARM kits. The absorbance was read at 340 nm.

It is a known fact that, from 1 gram of malic acid, 0.67 g of lactic acid are issued, through transformation. This is haw we have evaluated the manner into which the two lactic bacteria strains (CS 62 and CS 8II) are metabolizing the malic acid. The performing strains should be the ones which could attend to a ratio as near possible to the one previously

mentioned, but under the circumstances offered by the winehouse and by the wine itself, in terms of: value of the pH; temperature; presence of oxygen and the one of the sulphur anhydride; amount of nutritive substances.

Results and discusions

In Table 1, we have presented all the lactic bacteria strains identified as belonging to the *Oenococcus oeni* species.

Table 1. Substrata metabolized by the bacterial strains belonging to the species *Oenococcus oeni*

No	Strains	Gram stain	Catalasis test	Sugars metabolized						*Acids metabolized*		
				Arabinosa	Xilose	Glucose	Fructose	Galactose	Saccharose	Malic acid	Tartric acid	*Citric acid*
1	CS 62	+	-	+	-	±	±	+	-	+	-	±
2	*CS 8II*	+	-	+	-	±	±	+	-	+	-	±

The two studied strains (CS 62 and CS 8II) are G(+) and catalasis (-), they do metabolize easily the main sugars which exist into must or wine, as well as the one issued from the lysis of the yeast cells. Yet, they do not metabolize the tartric acid (which is the most important acid within wine) and they have shown a slow metabolizing of citric acid, an effect which is extremely important in the wine's elaboration process.

Table 2 is presenting the obtained results which concern the ratio between the acids malic and lactic, issued from the achievement of the Cabernet Sauvignon wine's malolactic fermentation, which presents the following assets: value the pH 3.3; presence of sulphur anhydride in the free form at the level of 10 mgl^{-1}; fermentation temperature = 15ºC (frequently met into a wine cellar).

The two strains were inseminated imediately after the end of the alcoholic fermentation. The wine shares contained malic acid going from 2 to 4.5 gl^{-1} and they were stored in vessels with a capacity of 200 or 500 l, often employed for the wine's maturation. The ratio between the malic and lactic acids is, in all cases, depending upon: the capacity of the lactic bacteria strain to realize this transformation through the malolactic fermentation; the capacity of the fermentation's vessel.

Our data and observations have pointed out, for the strain CS 62, a stronger capacity of metabolizing the malic acid into the lactic one. As a function of the initial quantity of malic acid, this strain also presents the shortest duration of the malolactic fermentation (10-17 days). In the case of this bacteria strain (CS 62), the duration of the malolactic fermentation is influenced by the vessel's capacity. For the 500 l vessels, the duration is lorger (about 5-6 days more) than the one for the vessels with the capacity of 200 l.

The ratio between malic acid and lactic acid has a value around 0.64, pretty near to the standard theoretical value (0.67).

For the case of the strain CS 8II, with the wine is inocolated and bottled, the some phenomena happen and flow into the same sense as for the CS 62 strain. But this second strain has a smaller capacity of transforming the malic acid into lactic one, the ratio standing from 0.44 to 0.61 if compared to the standard value of 0.67. The duration of the fermentation is much larger, for the vessels of 500 l it attends even 28 days.

Table 2. Ratio between malic acid and lactic acid, resulted from the performing of malic fermentation. Cabernet Sauvignon wine, 2010, pH 3.3, temperature 15ºC, free SO_2 10 mgl^{-1}

The wines contents of malic acid (gl^{-1})	CS 62				CS 8 II			
	Vessel's capacity				Vessel's capacity			
	200 l		500 l		200 l		500 l	
	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)
2	1.29	10	1.29	15	1.21	19	1.23	23
2.5	1.60	14	1.61	17	1.47	20	1.41	24
3.0	1.92	16	1.92	19	1.61	24	1.59	25
3.5	2.25	16	2.26	19	1.95	24	1.96	26
4.0	2.57	17	2.57	21	2.10	25	2.00	26
4.5	2.90	17	2.91	21	2.00	25	2.00	28
Ratio malic acid/ lactic acid	0.64		0.64		0.44-0.60		0.44-0.61	

The maximal dosage of free sulphur anhydride employed was of 20 mgl^{-1} (Table 3). For this amount, the two strains have preserved their capacity of metabolizing the malic acid into lactic one, but this process takes more time to be achieved. The ratio between the two acids has, in this case, practically the same value. This aspect is extremely important for the productive use made of these bacterial strains.

Being aware of the influence exerted by the environment's pH (of the wine) upon the lactic bacteria, which is important and multilateral, as we have remarked a selective effect, the bacteria being naturally chosen, we have inoculated some wine lots of the some kind (Cabernet Sauvignon) but with a pH of 2.8 (very close to the limit at which the lactic bacteria become able to transform the malic acid into lactic one (Table 4). We are able to state that, in this situation too, the two strains are able to perform the malolactic fermentation, the strain CS 62 providing the best results, when the ratio between malic and lactic acids has the some value as for the Cabernet Sauvignon wine with a pH of 3.3 but the duration of the malolactic fermentation is some haw larger (0.62-0.64).

The strain CS 8II, at pH 2.8 allows a much smaller ratio between malic and lactic acids (0.39-0.59) for a fermentative duration of at most 31 days.

The Cabernet Sauvignon wine, with pH = 2.8 inseminated with the 2 strains (CS 62 and CS 8II), if it could dispose of a double contents in sulphur anhydride (20 mgl^{-1} instead of 10 mgl^{-1}), in the convenient circumstances and duration, would perform the metabolizind process of malic acid through the strain CS 64 only (Table 5). If the strain CS 8II should be used alone, the fermentation's duration would be considerably extended (attending even 36 days), while the ratio for the acids (malic and lactic) would presented much smaller values (0.39-0.58). In order to point out the best way possible the capacity of the CS 62 strain to transform malic acid into lactic acid, we have ensured for the inoculated wines a temperature of 18°C (Table 6).

Table 4. Ratio between malic acid and lactic acid, resulted from the performing of malic fermentation. Cabernet Sauvignon wine, 2010, pH 2.8, temperature 15ºC, free SO_2 10 mgl^{-1}

The wines contents of malic acid (gl^{-1})	CS 62				CS 8 II			
	Vessel's capacity				Vessel's capacity			
	200 l		500 l		200 l		500 l	
	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)
2	1.28	13	1.28	15	1.18	16	1.18	18
2.5	1.59	17	1.60	19	1.39	17	1.40	20
3.0	1.90	18	1.91	20	1.58	22	1.49	22
3.5	2.22	19	2.23	21	1.87	25	1.85	27
4.0	2.49	20	2.50	25	2.00	27	1.95	29
4.5	2.84	20	2.84	28	1.80	28	1.79	31
Ratio malic acid/ lactic acid	0.62 – 0.64		0.62-0.64		0.40-0.59		0.44 – 0.61	

Table 5. Ratio between malic acid and lactic acid, resulted from the performing of malic fermentation. Cabernet Sauvignon wine, 2010, pH 2.8, temperature 15ºC, free SO_2 20 mgl^{-1}

The wines contents of malic acid (gl^{-1})	CS 62				CS 8 II			
	Vessel's capacity				Vessel's capacity			
	200 l		500 l		200 l		500 l	
	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)	Amount of lactic acid resulted (g/l)	Duration of the malolactic fermentation (days)
2	1.28	15	1.26	19	1.17	19	1.17	24
2.5	1.57	19	1.55	22	1.39	24	1.40	28
3.0	1.88	21	1.88	26	1.41	27	1.42	29
3.5	2.20	22	2.19	29	1.82	29	1.83	31
4.0	2.47	25	2.46	30	1.94	30	1.93	32
4.5	2.83	26	2.83	30	1.75	31	1.76	36
Ratio malic acid/lactic acid	0.61 – 0.64		0.61 – 0.63		0.38 – 0.58		0.39 – 0.58	

In this situation, the ratio between the malic and lactic acids, resulted from the development of the malolactic fermentation, reaches to values that are extremely close to the theoretical value (0.67). Into the vessels with a larger capacity (500 instead of 200 l), the

fermentation takes longer here as well. But the wines' pH (2.8-3.3) and the vessels' capacity (200-500 l) do not matter for the acids' ratio.

Table 6. Ratio between malic acid and lactic acid, resulted from the performing of malic fermentation, Cabernet Sauvignon wine, temperature 18ºC, free SO_2 10 mgl^{-1}, strain CS 62

pH of wine	The wine's contents of malic acid (gl^{-1})											
	2.0		2.5		3.0		3.5		4.0		4.5	
	Vessel's capacity (l)											
	200	500	200	500	200	500	200	500	200	500	200	500
3.3	1.3	1.3	1.6	1.56	1.95	1.95	2.27	2.25	2.6	2.56	2.92	2.89
2.8	1.3	1.3	1.58	1.15	1.90	1.92	2.20	2.19	2.5	2.5	2.8	2.85

Conclusions

The researches performed under industrial conditions upon two lactic bacteria strains that we have isolated and identified (CS 62 and CS 8II) concerning their capacity have confirmed for them the assets that we had previously spotted under laboratory conditions. For these two strains of lactic bacteria, the value of the ratio between malic and lactic acids, resulted from the performing of the malolactic fermentation, depends upon: the physiological assets of the strain; the presence or not of sulphur anhydride (in its free form); the value of the pH; the environment's temperature (of the wine); the capacity of the vessel where the malolactic fermentation does take place.

For the ratio of the acids (malic and lactic), the nearest value, in respect to the theoretical one (0.67) is obtained when the strain CS 62 alone does perform the malolactic fermentation. The lactic bacteria strains that we have isolated and identified as belong to the *Oenococcus oeni* species (CS 62 and CS 8II) are able to produce good results, when they are used to perform the malolactic fermentation especially in the case of the CS 62 strain.

These strains are necessarily required to be multiplied and stored, so they could be used for the elaboration of high quality red wines. For this purpose, the necessary funds should be obtained. The money amount would not be substantial, if only we should take into account the immense contribution brought by these strains to the elaboration of our own native red wines, which are fine and generous, enabling us to eliminate the imports in this matter, which turn to be so expensive.

Acknowledgement

This work was supported by CNCSIS –UEFISCDI, project number 1094-PNII – IDEI code 436/2008.

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