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Methanol release during fermentation of red grapes treated with pectolytic enzymes

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Red grapes of the Tinto fino (*Vitis vinifera*) variety were treated with four different commercial preparations of pectolytic enzymes, and methanol production during fermentation of the grapes was studied. Pectin content of the grapes and in the final wine were also quantified, to study the relation between methanol release and the extent of pectin degradation by the enzymes. The results showed that the enzymatic treatments enhanced the methanol content from day one of fermentation for three of the four enzymes, and from day three for all of them. Every enzymatic treatment produced higher methanol levels than the control in the final wine. During storage the methanol levels remained more or less constant. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Pectolytic enzymes play an important role in the wine-making process due to the fact that they improve the extraction of colour and aroma compounds, and the clarification and filtration processes of musts and wines (Martinière *et al.*, 1973; Brown and Ough, 1981; Fogarty and Kelly, 1983). They break up pectin and weaken the cell wall, reducing the viscosity of musts (Inama, 1994) and improving the extraction of the different compounds (Lecas, 1994). In addition, the pectolytic enzyme treatments can also modify the stability, the taste and the structure of red wines (Zent and Inama, 1992). Because the pectolytic activity of endogenous grape enzymes is lower than that necessary to hydrolyse all the pectin in the must, the addition of exogenous enzymes in the wine-making process has been widely practised in recent years (Colagrande *et al.*, 1994).

Pectolytic enzymes are usually classified into two groups (Baron, 1990, Brillouet *et al.*, 1990): de-esterifiers (pectin-methyl-esterase), which hydrolyse the methoxy group, and depolymerases which are themselves divided into another two groups: hydrolases which hydrolyse (1–4) bonds of pectin, and lyases which produce the β-elimination of one molecule of water when they break up the pectin.

Commercial preparations of pectolytic enzymes, usually isolated from microorganism cultures, normally contain both groups of enzymes: depolymerases and pectin-methyl-esterase. The second enzyme causes the main problem when using commercial preparations because this enzyme releases methanol which remains in musts and wines. Methanol is an alcohol toxic to humans. It produces lactic acidosis due to the fact that this compound interferes with liver metabolism where it is oxidised. Lactic acidosis is a metabolic disease caused by an increase in blood levels of lactic acid and its symptoms are weakness, vomiting and finally coma and death (Newsholme and Leech, 1986). The human oral lethal dose is 340 mg/kg of body weight.

Some authors have found that the addition of pectolytic enzymes induces an increase of methanol levels in different fermented products such as ciders (Massiot *et al.*, 1994) or wines (Brown and Ough, 1981; Servili *et al.*, 1992; Bosso, 1992; Bosso and Ponzetto, 1994). However Nicolini *et al.* (1994) did not find a significant increase in methanol levels in wines made with pectolytic enzymes. These authors pointed out that many other factors such as grape variety, some oenological practices and the yeast strain used, are able to influence methanol production. Delfini (1994) showed that the different types of yeast have different pectin methyl esterase activities and every grape variety has different levels and types of pectin; hence it is difficult to determine a general effect of pectolytic commercial preparations.

The aim of this work is to evaluate methanol production by different commercial preparations of pectolytic enzymes during the fermentation process of red

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grapes, Tinto fino (*Vitis vinifera*), the most important variety of Ribera de Duero D.O.C.

MATERIALS AND METHODS

Samples and treatment

Grapes of the Tinto fino (*Vitis vinifera*) variety cultured in one vineyard of the D.O.C Ribera de Duero (Burgos, Spain) were harvested at commercial maturity (23–25 °Brix). The damaged grape clusters (broken or with visual microbial alterations) were separated in order to eliminate undesirable contamination and degradation compounds. The intact clusters were weighed in groups of 5 kg and then processed.

Four different commercial preparations of pectolytic enzymes were used at the maximum doses suggested by the producers. The type of enzymes used and codes for them were: clarificant pectolytic enzymes: Zimopec PX1 (Perdomini SPA) 0.03 g litre^{-1} (Z.3) and Rapidase CX (Gist Brocades) 0.05 g litre^{-1} (R.5); and colour extracting enzymes: Pectinase WL Extraction (Wormser Oenologie) 0.01 g litre^{-1} (P.1) and Rapidase Ex. Colour (Gist Brocades) 0.05 g litre^{-1} (R.ex.5).

Control treatment

The grapes, once de-stemmed and crushed, were put into a 5-litre tank, then 0.04 g litre^{-1} of SO_2 and 0.1 g litre^{-1} of commercial yeast, *S. cerevisiae* and *S. bayanus* (Wormser Oenologie), previously hydrated, were added. The alcoholic fermentation was then carried out at 25°C. The end of fermentation was determined by the total consumption of reducing sugars.

At the end of the alcoholic fermentation the wine was strained off, the grape pomace was pressed, and the whole resulting wine was racked off into another tank and kept at 4°C for 24 hours to facilitate the settling process. Afterwards, the wine was centrifuged (10 500 ***g*** for 10 min at 5°C) and the clarified wine was bottled in 750 ml green glass bottles and stored at 15°C.

Enzymatic treatment

In these cases, the only difference from the control treatment was the addition of pectolytic enzyme one hour (the time suggested by the producers) before the inoculation of yeast, in order to improve the enzymatic action. During this time, the grape pomace was kept at 20°C.

Every treatment (control and enzymatic treatment) was performed in duplicate, and must-wine samples taken from each treatment were also analysed in duplicate, giving a total amount of four replicates for each day.

The analysed samples were part of the liquid extract 'must' formed after crushing the grapes and during the fermentation process. Samples of the initial must (control), and of the must one hour after the enzyme addition (enzymatic control) were collected. They were analysed, for the first time, at Day 0 of fermentation. During the fermentation process must-wine samples of every treatment were collected every day (1–8 days). In addition, samples were collected after the clarification process (Day 9), and after one month of storage in bottles at 15°C.

Analytical procedures

Total reducing sugars, ethanol °Brix, methanol and total pectin were evaluated. Reducing sugars was quantified by the Official Methods of Analyses (Ministerio de Agricultura, 1977). The methanol official method (OIVV, 1990) requires distillation as a previous step, then every must-wine sample of each treatment was distilled in duplicate. Afterwards, the chromotropic acid method was used to quantify the methanol content. This method was also carried out in duplicate in each distillation. °Brix was determined with a thermostatted refractometer, model CONVEX. Total pectin was measured in alcohol insoluble residues after their acid hydrolysis (Proctor and Peng, 1989). The Robertson method (Robertson, 1979) was used to quantify the total pectin content which was expressed as mg litre^{-1} of galacturonic acid.

Statistical analysis

The statistical analysis of the data was carried out by an analysis of variance (ANOVA). The statistical significance of each factor under consideration was calculated at the $\alpha = 0.05$ level using the *F*-test. At the same time, the LSD Fisher-test was employed to test for statistically significant differences between samples.

RESULTS AND DISCUSSION

The fermentation process was monitored by the decrease of reducing sugars (Fig. 1a), and the increase of alcohol content (Fig. 1b). The evolution of these parameters revealed the uniformity in the development of the fermentation process. The final alcoholic levels did not show statistically significant differences between treatments.

Methanol levels are showed in Fig. 2. The statistical analysis of experimental data revealed that, at initial time (Day 0), the methanol levels of the Z.3 and P.1 treatments were not statistically different from the control. However, methanol levels of R.5 and R.ex.5 were statistically different and higher than the control. This shows that the latter enzymes produced, in only one hour, an important increase of methanol levels.

Twenty four hours after of the yeast inoculation, the Z.3 treatment continued to be statistically equal to the control, while the other treatments were all statistically different from each other and the control and Z.3. The same phenomenon was observed 48 hours later.

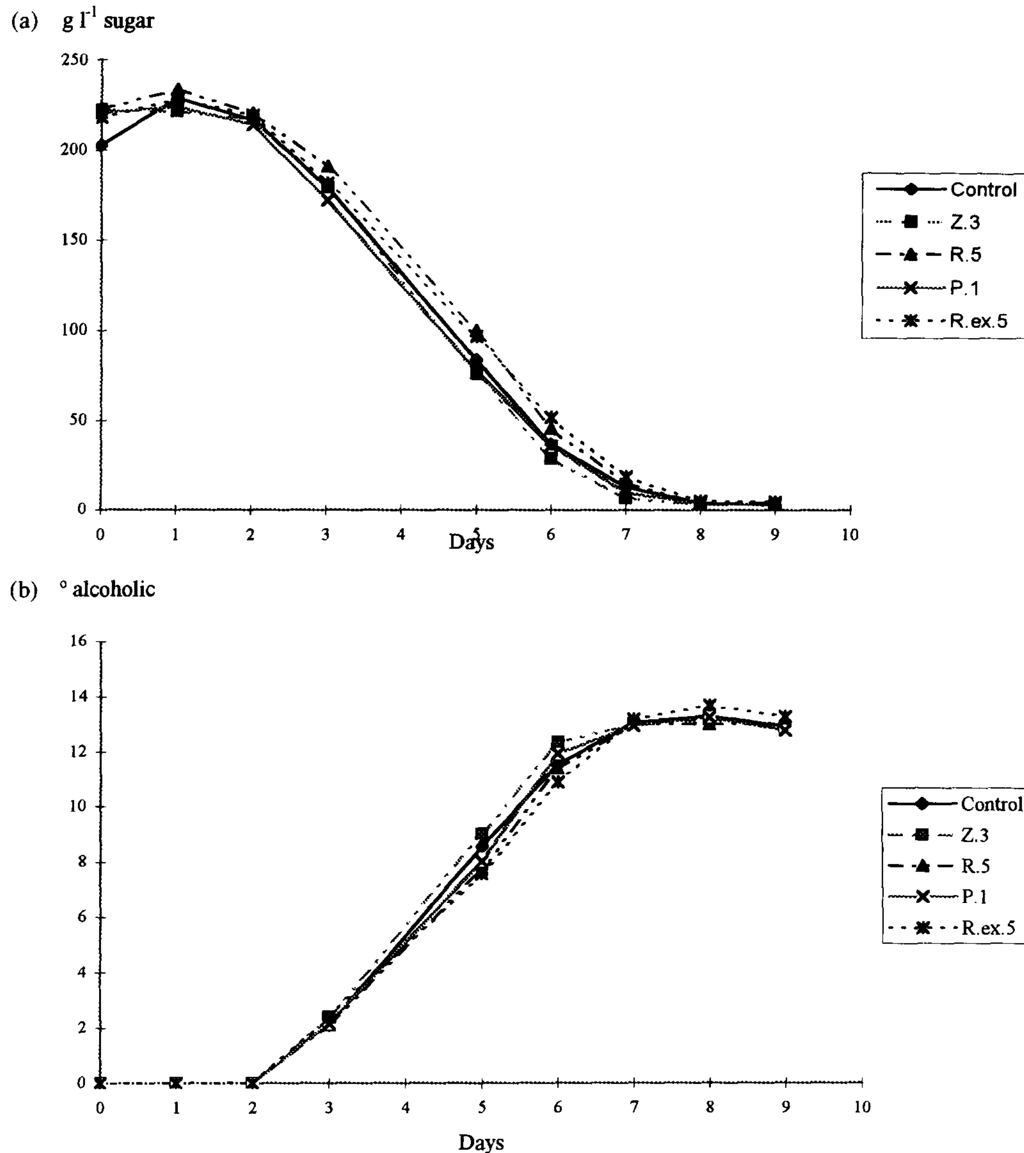

Fig. 1. (a) Reducing sugar contents during fermentation and in final wine. (b) Ethanol contents during fermentation and in final wine (data shown are the averages of four replicates).

However, from 72 hours until the end of fermentation all the treatments differed statistically from the control, showing ever higher levels of methanol.

It can be pointed out that, at the final stage of fermentation, Z.3 and P1 had statistically equal methanol levels, and at the same time, these values were lower than the R.5 and R.ex.5 methanol contents, which were statistically equal to each other.

Methanol levels in the final wine at the end of fermentation (Day 8) and in wine obtained after the clarification process (Day 9), revealed a statistical difference only in the control treatment. This can be explained by the localisation of endogenous pectolytic enzymes of grapes which are principally in the skin. These enzymes degrade the pectin of the nearest flesh, and the released methanol remains inside the berries until they are pressed.

During methanol production and accumulation, at least two different sets of events can be described:

1. the control and P.1 treatments showed a nearly parallel accumulation of methanol. The methanol levels were low on the first day, but they showed significant increases on the third day (34% and 33%, respectively). Afterwards, methanol levels increased slowly but continuously in both treatments, about 7% daily. This behaviour suggests that the Pectinase WL Extaction (P.1) commercial preparation contained enzymes with similar characteristics to endogenous grape enzymes, so the addition reinforced the action of endogenous enzymes producing quantitative but not qualitative changes.
2. the other three commercial preparations of pectolytic enzymes studied, Z.3, R.5 and R.ex.5, showed a rapid and important accumulation of methanol from the second to the fifth or sixth day of fermentation (Fig. 2). The rate of accumulation was

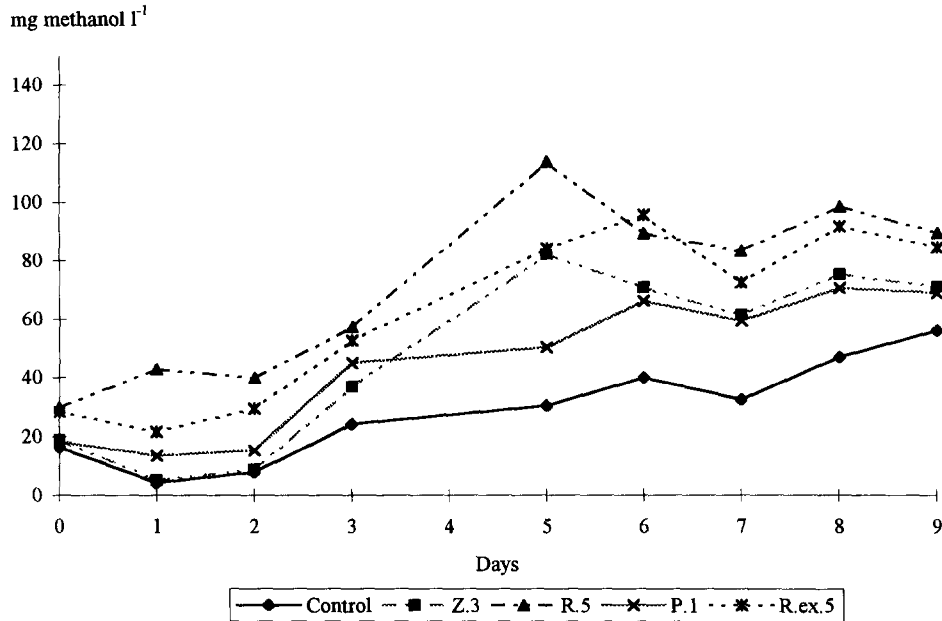

Fig. 2. Evolution of methanol levels for the control and enzymatic treatments during the fermentation and in final wine (data shown are the averages of eight replicates).

about 17% per day for R.ex.5, 21% per day for R.5 and 30% per day in the case of Z.3. Following this, a slight decrease in methanol levels was observed and after that, the methanol content remained more or less constant.

These results suggest that these preparations could be deactivated by ethanol due to the fact that these enzymatic preparations were very active at the beginning of the fermentation process, when the content of ethanol was not very high. However, when the alcoholic grade was higher than 8°, they did not show activity and methanol production was stopped. A total inhibition of pectolytic enzymes has previously been described in the literature for alcoholic contents greater than 17°, but this does not imply that a partial inhibition can not happen before, at a lower ethanol concentration.

On the other hand, the differences observed between production of methanol by the various enzymatic preparations could be the result of different kinds of activities. It is well known that pectic substances have different solubilities on aqueous dissolution according to their methoxylation degree and, in general, they are alcohol-insoluble. The pectic compounds become more water-insoluble when they lose the methoxy group. The enzymatic systems with high pectin-methylesterase activity degrade the pectic compounds with methanol release. The demethoxylated pectin formed precipitates, and so the enzymes lose the substrate on which they act. This could occur with enzymes R.5, R.ex.5 and Z.3. However, less active enzymatic systems or those with lower pectin-methylesterase activity, degrade less pectin, and produce less methanol and a lower amount of precipitate. Consequently, both substrate and enzyme remain in the medium and the enzymatic degradation of pectic compounds may continue during the entire fermentation process.

Previous work carried out in our laboratory indicates that there was no big difference between pectic levels of white musts obtained with a short actuation time of the enzymatic commercial preparations studied in this work (Pérez-Magariño, 1996). However, the final pectin content of wines was very different, depending on the enzymatic preparation used (Pérez-Magariño, 1996; Izcara, 1996).

Additional experiments were performed in order to evaluate the effects of enzymatic preparation added. Total pectin of grapes and wines was quantified. The initial pectin levels in the grapes was about 1600 mg litre^{-1}. Total pectin in the final wines (Table 1) was lower than in grapes and showed statistical differences from all the enzymatic treatments.

R.5 and R.ex.5 treatments gave a lower final pectin content while Z.3 and P.1 treatments gave higher pectin

Table 1. Total pectin contents (mg litre^{-1}) in final wine (Day 9)

Treatment	Tank	Rep1	Rep2	Mean	σ
Control	C1	286	319	312 c[a]	14.9
	C2	323	320		
Z.3	Z.3.1	396	374	376 d	21.6
	Z.3.2	356	380		
R.5	R.5.1	219	182	183 b	21.6
	R.5.2	165	167		
P.1	P.1.1	435	412	408 e	19.7
	P.1.2	380	404		
R.ex.5	R.ex.5.1	138	155	151 a	9.4
	R.ex.5.2	161	158		

[a]Different letter means statistically significant differences.

Table 2. (a) Methanol contents (mg litre^{-1}) in wine during storage. (b) Means of methanol contents (mg litre^{-1}) in wine during storage

	Treatment	Control				Z.3				R.5				P.1				R.ex.5			
	Tank	C1		C2		Z.3.1		Z.3.2		R.5.1		R.5.2		P.1.1		P.1.2		R.ex.5.1		R.ex.5.2	
(a)	Destillation	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Time	Rep.1	57.3	52.3	54.9	56.2	71.1	64.0	67.9	71.9	87.0	88.1	86.3	91.5	68.6	78.4	69.3	64.7	84.9	72.2	91.5	82.2
9 days (wine)	Rep.2	54.1	52.2	62.7	55.8	75.8	66.8	70.7	77.1	89.1	83.8	88.9	98.0	63.7	75.8	66.7	61.5	83.8	74.3	98.0	86.0
Time	Rep.1	54.7	50.3	63.8	52.5	71.6	66.1	78.2	62.0	105	90.6	97.7	97.8	78.2	63.2	86.0	77.5	99.0	83.5	99.0	88.3
1 month	Rep.2	58.6	55.5	61.2	48.9	76.9	72.4	86.0	70.4	110	91.8	95.1	96.6	79.5	76.6	84.7	66.8	91.2	94.2	96.4	89.5

	Control		Z.3		R.5		P.1		R.ex.5	
(b)	Mean	σ	Mean	σ	Mean	σ	Mean	σ	Mean	σ
9 days (wine)	55.7 a[a]	3.35	70.7 a	4.40	89.1 a	3.98	68.6 a	5.88	84.1 a	9.02
1 month	55.7 a	5.27	72.9 a	7.51	98.3 b	6.34	76.5 a	7.93	92.6 a	5.16

[a]Different letters in the same column means statistically significant differences.

levels. This result suggests that the former preparation could have a higher pectolytic activity: they were very active in degrading the pectín present in the medium and, at the same time, their pectin-methyl-esterase activity was greater than in other preparations, producing larger amounts of methanol (Fig. 2 and Table 2). The higher depolymerising activity could contribute to de-esterifying activity since the access to the methoxy groups was easier in less polymerised chains. However, Z.3 and P.1 preparations could have low depolymerizing activity and they were not able to degrade all the pectin (which was in larger amounts than in control wine because the enzymatic preparations improved the liberation of pectin from solid parts of grapes). Furthermore, it may be that they had lower pectin-methyl esterase activity, producing lower methanol concentrations.

The methanol content of wines after one month of storage remained more or less constant (Table 2). In general, enzyme-treated wines showed a slight increase in their methanol contents, but this was only statistically significant for the R.5 preparation. This results shows that, when pectolytic enzymes are used to elaborate wines, it is convenient to monitor methanol levels during wine storage.

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