Influence of Enzymatic Clarification with a Pectin Methylesterase on Cider Fermentation

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ABSTRACT

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In this study, the effect of two apple juice treatments (clarification with a pectin methylesterase in the presence and absence of sulphiting) on the cidermaking process was investigated. Prefermentative clarification with the pectin methylesterase (Rapidase CPE) slowed the alcoholic fermentation in respect to traditional fermentation, the greatest effect being found for the combination of the enzymatic treatment with sulphur dioxide addition. With this treatment the start of the fermentation was delayed seven days. Enzymatic clarification delayed malolactic fermentation (MLF) and the interaction of both treatments produced a cider in which malolactic fermentation had not occurred. Both treatments ensured that the volatile acid levels during the maturation phase were significantly lower than in the traditional fermentation. Enzymatic clarification led to lower levels of yeasts during the active phase of alcoholic fermentation with respect to the control. When this treatment was combined with sulphiting, a decrease in apiculate yeast numbers during alcoholic fermentation was observed and a better survival of these species was noted after 59 days of fermentation.

Key words: Alcoholic fermentation, apple juice treatments, cider, malolactic fermentation.

INTRODUCTION

In cidermaking the use of enological techniques such as starter culture addition, temperature control or clarification treatments are not common practice in the Basque Country. The alcoholic and malolactic fermentations occur spontaneously and at the same time, without inoculation of selected yeast or lactic acid bacteria and large numbers of these bacteria are found. They cause alterations such as "piqûre lactique", which leads to an increase in the acetic acid content and the ciders obtained are characterized by high volatile acidity, an acidity higher than that permitted by Spanish regulations of 2.2 g acetic acid/L[4]. Another typical microbial disorder produced by lactic acid bacteria is "ropiness". This is produced by some *Lactobacillus* and *Pediococcus* spp. isolated from spoiled bottled ciders, which secrete an extracellular polysaccharide increasing the viscosity of the cider[5,7]. These disorders can be prevented by the use of several pre-fermentation treatments, among them, sulphiting, clarification with pectolytic enzymes and fining. The use of pectolytic enzymes is a technique which produces important advantages such as easier pressing with higher yield when added initially to the fruit pulp and a pectin-free juice and consequently a more complete clarification of the ciders is obtained.

According to the mechanisms of pectin degradation, the pectolytic enzymes are classified in two groups: polygalacturonases and pectin-lyases, which catalyse the breakdown of the polygalacturonic chain; and pectin methylesterases, which split methanol from esterified carboxyl groups and transform pectins into low-methoxy pectins and pectic acids. These react with the calcium salts to form a jelly, which causes a loss of cloudiness in turbid juices[17]. The results obtained largely depend upon the type of enzymes used, the pectic composition of the substrate and the combination with other treatments.

In previous work[6] we reported the effect of different treatments on the cider making process, including the enzymatic clarification with a polygalacturonase, in the presence and absence of sulphiting. Although sulphur dioxide is extensively employed in cidermaking to prevent enzymatic and non-enzymatic browning reactions in apple juice, as well as to control the development of wild yeasts and bacteria[3], in Basque Country this practice is rarely used.

With the aim of reducing the high levels of volatile acids in ciders obtained by traditional methods, in this study the effect on the cidermaking process of two apple juice treatments (sulphiting and clarification with pectin methylesterases) applied individually or together was examined. Malolactic and alcoholic fermentation, as well as the treatment effects on the population of the different yeasts species that occurred naturally in the apple juice were examined. Results presented are compared to those obtained when a polygalacturonase was added to apple juice[6].

MATERIALS AND METHODS

The source of apples, production of musts and sampling were as described previously[6]. In brief, a mixture of local washed cider apples were milled with a grating mill

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and pressed in a rapid cycle (2 h) pneumatic press. The juice was subdivided into 140-L poly(vinyl chloride) vessels. The treatments were carried out at temperatures between 10-15°C. After alcoholic fermentation the vessels were sealed to prevent aeration of the juices.

Apple juice treatments

Two different treatments were employed:

(i) *Sulphiting*. Potassium metabisulphite was used as the SO_2 source (50 mg/L of total SO_2). The treatment was applied immediately after pressing.

(ii) *Enzymatic clarification*. Pectolytic treatment was carried out with pectin methylesterase (15 mL/hL, Rapidase CPE; Gist-Brocades, S.A, France**)** and calcium salts (10 mM $CaCl_2$. $2H_2O$). Rapidase CPE was standardized on a minimum activity of 100 pectin esterase units per mL. The treatment lasted 48 h and after treatment, the juices were racked.

TABLE I. Experimental design of the project.

	Treatment[1]	
	SO_2	Rapidase CPE
I	-	-
II	+	-
III	-	+
IV	+	+

[1]Treatment: + present, - absent.

To determine the influence of apple juice treatment on different physicochemical and microbiological parameters on the cider making process the experimental design shown in Table I was used.

Microbiological and chemical analysis

In the four barrels, yeasts were enumerated by spread-plating 0.1 mL aliquots of Ringer solution serial dilutions on malt agar (Difco) and lysine agar (Oxoid) plates. Lysine agar was used for the enumeration of non-*Saccharomyces* species during the fermentation[15]. During the first stages of fermentation, 100 mg/L of streptomycin and 100,000 IU/L of penicillin G were added to the plating media to suppress the growth of acetic and lactic acid bacteria. After incubation from three to five days at 25°C, the different yeast colony types were counted and representative samples of each were purified and identified according to the descriptions and the keys of Barnett et al.[2] and Kreger-van Rij[12].

Lactic and acetic acid bacteria were enumerated as described previously[6].

Physicochemical parameters were determined in barrels III and IV, and were compared to those obtained in previous work for barrels I (traditional fermentation) and II (traditional fermentation + sulphiting)[6].

Glycerol, L(+) malic acid, L(+) and D(-) lactic acids were analysed by the enzymatic procedures developed by Boehringer Mannheim (Germany). Volatile acidity, pH

TABLE II. Colony counts of yeasts species during the cider making process.

		Yeast population per barrel (CFU/mL)			
Days	Species	Barrel I control	Barrel II SO_2	Barrel III Rapidase CPE	Barrel IV SO_2 + Rapidase CPE
0	Total yeasts	3.1×10^5	3.1×10^5	3.1×10^5	3.1×10^5
	K. apiculata	6.5×10^4	6.5×10^4	6.5×10^4	6.5×10^4
	C. pulcherrima	1.8×10^5	1.8×10^5	1.8×10^5	1.8×10^5
	S. cerevisiae	ND[a]	ND	ND	ND
4	Total yeasts	1.2×10^6	4.4×10^5	1.8×10^6	3.8×10^5
	K. apiculata	7.9×10^5	1.7×10^5	1.1×10^6	2.4×10^5
	C. pulcherrima	2.1×10^5	1.7×10^5	4.2×10^5	3.5×10^4
	S. cerevisiae	ND	ND	ND	ND
7	Total yeasts	4.5×10^6	6.4×10^6	2.5×10^6	2.8×10^6
	K. apiculata	3.4×10^6	4.5×10^5	2.7×10^6	2.9×10^6
	C. pulcherrima	3.5×10^5	1.3×10^5	2.0×10^5	3.0×10^5
	S. cerevisiae	8.0×10^5	5.0×10^4	1.0×10^5	3.0×10^5
14	Total yeasts	1.6×10^8	1.2×10^8	7.4×10^7	6.6×10^7
	K. apiculata	7.0×10^7	1.7×10^7	$2.3x 10^7$	2.5×10^7
	C. pulcherrima	ND	ND	ND	ND
	S. cerevisiae	9.2×10^7	1.0×10^8	4.8×10^7	4.1×10^7
23	Total yeasts	1.2×10^8	1.1×10^8	9.8×10^7	5.6×10^7
	K. apiculata	5.0×10^7	2.3×10^7	3.9×10^7	2.1×10^7
	C. pulcherrima	ND	ND	ND	ND
	S. cerevisiae	7.3×10^7	8.5×10^7	5.9×10^7	3.4×10^7
34	Total yeasts	3.6×10^7	5.6×10^7	5.5×10^7	5.5×10^7
	K. apiculata	8.0×10^6	7.5×10^6	2.8×10^7	1.7×10^7
	C. pulcherrima	ND	ND	ND	ND
	S. cerevisiae	2.8×10^7	4.8×10^7	2.4×10^7	3.7×10^7
59	Total yeasts	1.2×10^7	1.4×10^7	4.5×10^7	2.9×10^7
	K. apiculata	2.0×10^5	3.0×10^5	1.4×10^7	1.1×10^7
	C. pulcherrima	ND	ND	ND	ND
	S. cerevisiae	1.1×10^7	1.4×10^7	2.8×10^7	1.8×10^7

[a] ND, not detected.

and density at 20ºC were measured in accordance with O.I.V. methodology[16].

RESULTS AND DISCUSSION

Evolution of yeasts

In this study, the total yeast levels and the growth of yeasts not belonging to the *Saccharomyces* genus were studied. At the same time populations of *Kloeckera apiculata*, *Candida pulcherrima* and *Saccharomyces cerevisiae* were quantified, given that previous work showed that these three yeasts were the most abundant during the alcoholic fermentation[4].

The initial levels of yeasts in the fresh apple juice were high and the most frequent yeast species isolated were *K. apiculata* and *C. pulcherrima*, the latter being dominant (Table II). *S. cerevisiae* was not detected in this phase. Other yeast species such as *Rhodotorula rubra* and *Pichia membranaefaciens* were found occasionally. In all the tanks, the population of yeasts increased during the alcoholic fermentation reaching populations of 10^7-10^8 CFU/mL after 14 days. The initial number of *K. apiculata* increased to levels of the order of 10^7 CFU/mL. *S. cerevisiae* was not detected in the samples until day 7 when alcoholic fermentation was underway. Subsequently, the levels of this yeast increased, reaching populations of 10^7-10^8 CFU/mL and *S. cerevisiae* always was found at levels higher than *K. apiculata.* With respect to the evolution of *C. pulcherrima*, slight changes of this population in all the tanks were observed but this species disappeared during the active alcoholic fermentation. The rapid death and disappearance of this yeast from fermenting apple juices was consistent with their oxidative metabolism and sensitivity to ethanol[8]. After alcoholic fermentation, a decline phase was observed in the four barrels during which the cell density decreased to 10^2-10^4 CFU/ mL.

Regarding the influence of the different treatments, the addition of SO_2 to the apple juices provoked a slight growth inhibition of yeasts for 4 days (Table II, barrels II and IV).

During active alcoholic fermentation, this treatment did not have an important effect on the population of *Saccharomyces.* However, sulphiting affected the apiculate population; lower levels of these yeasts were detected in the samples analysed. This may be due to the sensitivity of this yeast to SO_2[11], although the dose of SO_2 used was not enough to eliminate this yeast completely.

On the other hand, as shown in Table II, the addition of Rapidase CPE in barrels III and IV led to reduced levels of yeasts during the active phase of the alcoholic fermentation in comparison to barrels I and II. This treatment affected the development of *S. cerevisiae* whose populations were slightly lower in presence of CPE than in the absence.

However, the behaviour of *K. apiculata* was different. The population of this yeast in the barrels treated with Rapidase CPE reached a maximum after 23 days of fermentation, this value was similar in all four barrels, but subsequently a better survival of this species was noted in barrels III and IV, with persisting populations of 10^7 CFU/mL after 59 days of fermentation. These populations were about 100 times higher that those found in barrels I and II.

Evolution of chemical parameters

Pre-fermentative clarification of the apple juices with the pectin methylesterase (Rapidase CPE), slowed down the alcoholic fermentation (Fig. 1) with respect to traditional fermentation (barrel I[6]). This is related to the lower levels of yeasts found during the active phase of the alcoholic fermentation which can be attributed to a decrease in the nitrogen concentration of the juice caused by the enzymatic clarification[1,10]. The addition of SO_2 to the cider musts caused the initial inhibition of yeast growth[19] that was detected in barrels II and IV, and consequently when enzymatic and sulphiting treatments were combined (barrel IV), the start of alcoholic fermentation was delayed for 7 days. After this delay the alcoholic fermentation developed in parallel in barrels III and IV (Fig. 1). The interaction of clarification with Rapidase CPE and sulphiting led to a very slow fermentation in barrel IV, the rate of

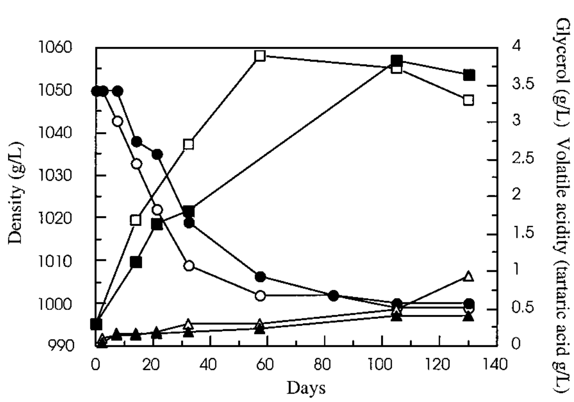

FIG. 1. Evolution of density (○), glycerol (□) and volatile acidity as tartaric acid (Δ) during the cidermaking process. Open symbols: barrel III (Rapidase CPE). Filled symbols: barrel IV (Rapidase CPE + SO_2).

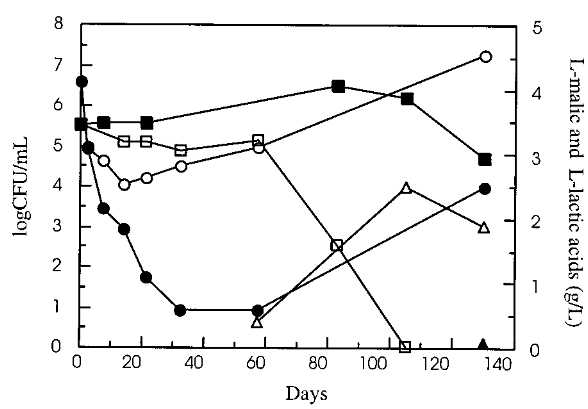

FIG. 2. Evolution of numbers of lactic acid bacteria (○), L-malic acid (□) and L-lactic acid (Δ) during the cidermaking process. Open symbols: barrel III (Rapidase CPE). Filled symbols: barrel IV (Rapidase CPE + SO_2).

fermentation being lower than that found in musts treated with Ultrazym G100 alone or in combination with SO_2 and fining[6]. The influence of these treatments on organoleptic properties requires further investigation and must include the analysis of the fusel alcohols, ester concentrations and sensory evaluation tests.

Previously[6] we reported that in traditional cidermaking, alcoholic and malolactic fermentations began simultaneously, even when other pectolytic treatments (Ultrazym 100) were used. However in comparison with traditional fermentation (barrel I) [6], the effect of Rapidase CPE addition (barrel III) was a delay of 60 days at the beginning of malolactic fermentation (MLF) (Fig. 2). This process was complete after 110 days, alcoholic and malolactic fermentations developing sequentially.

Enzymatic clarification with Rapidase CPE led to lower number of lactic acid bacteria (LAB) during the alcoholic fermentation compared to the level of LAB in the traditional process (Fig. 2). The delaying effect was most dramatically evidenced in barrel IV, subjected to enzymatic clarification and sulphiting. In this barrel, malolactic fermentation did not take place over the experimental time period.

The combination of sulphiting and enzymatic clarification with Rapidase CPE meant that the lactic acid bacteria were in a less favourable growth medium and the population fell dramatically during the alcoholic fermentation stage (Fig. 2).

Production of L-lactic acid began around 60 days in barrel III, but the concentration in barrel IV was negligible through to 140 days of fermentation. In the depectinized must (barrel III) L-lactic acid reached a maximum concentration at 110 days and subsequently a slight decrease was observed (Fig. 2). This could be due to degradation by the yeast *Saccharomycodes ludwigii*[3]. However, given the low level of this yeast in the cider of Gipuzkoa[11], this explanation alone is not adequate. Likewise, *Acetobacter* can degrade the two stereoisomers of lactic acid[13], but the population of acetic acid bacteria during the maturing period was similar in all barrels and lower than 10^3 CFU/mL. Moreover, surface films of these bacteria were not found and consequently their participation in the degradation of L-lactic acid can be rejected. Lactic acid bacteria may be a more probable cause of the degradation since they can use lactic acid as an energy source[21], or as a hydrogen donor in the reduction of quinic acid[3].

Production of glycerol took place in both barrels during the alcoholic fermentation due to glycerol-pyruvic fermentation[14,18] by the yeasts (Fig. 1). This production was slower in barrel IV than in barrel III, due to smaller population of yeasts, but after 100 days the glycerol content in both barrels was similar. Subsequently the glycerol level fell slightly, possibly through consumption by the high lactic bacterial population present in this phase[20]. This consumption was very feeble compared with traditional processes.

Volatile acidity increased slowly during alcoholic fermentation (Fig. 1), the levels being similar in barrels III and IV and of the order of 0.3 g/L (measured as tartaric acid). However, in the traditional cider making method, the volatile acid level after alcoholic fermentation is typically much higher (of the order of 1 g/L tartaric acid)[4] and in some cases reaching, up to 2.5 g/L tartaric acid after MLF. Later, during the maturation period, the two treatments ensured that the volatile acid levels were significantly lower than in traditional fermentations. The volatile acidity content was lower than those obtained by traditional cider making process and in accordance with Spanish health regulations. To evaluate the effect of these treatments on organoleptic properties, further investigations are required (fusel alcohol and ester analyses and sensory evaluations).

In conclusion, enzymatic clarification with Rapidase CPE slowed the fermentation while the combination of Rapidase CPE and sulphiting delayed by 7 days the initiation of alcoholic fermentation. With respect to malolactic fermentation, each treatment (applied separately) also delayed the occurrence of the MLF, which took place only after alcoholic fermentation. The combination of both treatments (sulphiting and enzymatic clarification) produced a cider in which malolactic fermentation had not occurred. The low levels of malolactic bacteria could explain the delay of malolactic fermentation during cidermaking, as well as the low content of volatile acidity in the ciders produced.

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