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CIDER PRODUCTION WITH IMMOBILIZED *LEUCONOSTOC OENOS*

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Cider obtained with *Saccharomyces cerevisiae* and immobilized *Leuconostoc oenos* attained the desired attenuation without residual sugars. Decarboxylation of L-malic to L-lactic acid by heterolactic bacteria and the formation of secondary products in cider were studied using immobilized cells of *L. oenos* IC11. Malic acid metabolism was influenced by the temperature at which the fermentation took place. The production of ethyl acetate and methanol was influenced by the type of *L. oenos* inoculation. Acetaldehyde was the most discriminating variable in the ciders obtained at different temperatures. The use of multivariate analysis allowed differentiation of the ciders obtained on the basis of the inoculation model followed.

Key Words: Leuconostoc oenos, *immobilization, cider, chemometrics.*

INTRODUCTION

Malolactic fermentation is the bacterial conversion of L-malic acid into L-lactic acid and carbon dioxide. A good correlation between malic acid decarboxylation and the corresponding concentration of the bacterial population has been demonstrated by Rice and Mattik[16].

Malolactic conversion is considered to be a desirable manufacturing step for three reasons: (a) to decrease the acidity of cider, (b) to stabilize cider by assuring that fermentation will not take place in the bottle, and (c) to increase the flavour complexity of cider. In cool areas with high contents of malate in cider, acid reduction is most important[10], while in areas with less acidic cider, biological stabilization must be assured in order that spontaneous growth of undesirable microorganisms does not occur. Several lactic acid bacteria carry out malolactic fermentation, but only *Leuconostoc oenos* and a few *Lactobacilli* strains have been judged to lead to the desirable organoleptic improvement of treated ciders[5]. Because the initial stages of the transformation are often hard to control, attempts have been made to stimulate this transformation through bacterial inoculation. Induction of malolactic fermentation with bacterial starter cultures eliminates long periods of time during which the fermented beverage is exposed to microbial spoilage. Furthermore, the use of selected strains of lactic acid bacteria offers some control over the type of flavours produced[12]. Strains of *L. oenos* are the most widely used but this inoculation method has been only partly successful as the metabolic activity of heterolactic bacteria may either improve or reduce cider quality. Immobilized microorganisms as catalysts have attracted increasing interest over the past decade as the immobilization of whole microbial cells eliminates the need for isolating and purifying enzymes and provides more stable activity[17]. Ca-alginate is an apparently suitable matrix because it is a non toxic immobilization reagent, there is no leakage of bacteria during the immobilization process, and fermented products present good organoleptic properties[9].

At the same time, the use of complex data bases requires multivariate treatment of data. One of the principal aims of chemometric techniques is to optimize analytical information. For instance, principal component analysis (PCA) has been used for classifying and characterizing alcoholic beverages[6,7].

The present research was undertaken to investigate the effect of *L. oenos* immobilization and fermentation conditions (temperature and timing of inoculation with lactic acid bacteria) on the chemical composition of the cider produced.

MATERIALS AND METHODS

Cell immobilization

Different amounts of bacteria cells grown in MRS (Man, Rogosa and Sharpe; Oxoid, England) were suspended in 2.5 mL of de-ionized water and mixed with 5 mL 2.5% sodium alginate. The suspension was added dropwise to 250 mL of 100 mM $CaCl_2$. Bead-type gels of 2–3 mm diameter were incubated in gel-inducing reagent for 2 h at 4°C, stored in 20 mM $CaCl_2$ at 4°C, and washed with de-ionized water before use, according to the procedure described by Spettoli *et al.*[18].

Raw materials

Apple juice was obtained from a mixture of apples with different sensory properties: Raxao (sharp) 40%, Collaos (slightly sharp) 30%, Coloradona (bitter-sweet) 15%, Durón Arroes (sweet) 10% and Meana (bitter-sharp) 5%. A grating mill and a double-tray hydraulic press were used in the extraction of the juice, which was subsequently kept at −20°C. Before being used, the juice was clarified by means of tangential micropore filtration, using a 0.33 µm sulfone polyester plaque and was then sterilized by microfiltration through a 0.22 µm polyvinylidene difluorure filter (Millipore, Spain).

Experimental design

The fermentations took place in previously sterilized 2-litre capacity sleeved reactors with periodic stirring for fifteen minutes every two hours. The inocula were allowed to grow in the presence of oxygen for the first twenty-four hours, an inert atmosphere being subsequently achieved by means of nitrogen. Two litres of apple juice were inoculated with a final concentration of 10^6 cfu/mL of *Sacch. cerevisiae*. Immobilized *L. oenos* was inoculated using two inoculation models: simultaneous to the addition of the fermentative yeast, and sequential when the cider density was 1,019 g/litre. The fermentations were conducted at 12°C and 18°C. All assays were carried out in triplicate. The analytical controls were carried out at the beginning and the end of the fermentation.

Analytical methods

A liquid chromatograph equipped with two Waters 510 pumps, a Wisp 712 automatic injector, two Waters 410 RI and 481 UV-Vis detectors and Millenium v 2.1 software was used to determine carbohydrates and alcohols (glucose, sucrose, fructose, glycerol, ethanol and sorbitol) and organic acids (quinic, shikimic, malic, lactic, acetic and succinic).

The analysis of sugars and alcohols was accomplished by means of a Sugar Pak I column (300×6.5 mm i.d.) (Waters Associates), which was operated according to the method

described by Blanco *et al.*[1] under the following conditions: mobile phase, water containing 50 µg/mL $Ca(Na)_2EDTA$; flow rate 0.5 mL/min; column temperature 80°C; detector temperature 37°C; injected volume 10 µL.

Organic acids were determined according to the method described by Blanco *et al.*[2] using a Spherisorb ODS-2 column (3 µm, 250×4 mm i.d.). The operating conditions were as follows: mobile phase 0.01 M KH_2PO_4/H_3PO_4 buffer, pH 2.25; flow-rate 0.5 mL/min; column temperature 37°C; injected volume 10 µL. Column effluents were monitored at a wavelength of 206 nm.

The following volatile compounds were analyzed: acetaldehyde, ethyl acetate, methanol, 1-propanol, 1-butanol, 2-methyl-1-propanol (isobutyl alcohol), amyl alcohols, ethyl lactate, 2-phenethanol and 2-phenethyl acetate; GC analyses were accomplished on a Hewlett-Packard 5970 gas chromatograph fitted with a TR-FFAP column (30 m×0.53 mm i.d., phase thickness 1 µm). Chromatographic conditions were as follows: initial temperature 40°C for 5 min; program rate, 4°C/min to 60°C; program rate 10°C/min; final temperature 220°C for 34 min. Injector and detector temperature were 240°C and 275°C respectively; the carrier gas was He at 3 psi; injected volume 2 µL. The sample was filtered across 0.45 µm pore size cellulose acetate membrane filters (Teknokroma, Barcelona, Spain) and was directly injected.

Microbiological counting

The evolution of the different populations of microorganisms was monitored every twenty-four hours during fermentation until a density of 1,000 g/litre was reached. Ringer serum was used for serial 1:10 dilutions and the selective media employed in the quantitative analyses for *L. oenos* was, MRS containing the antifungal antibiotic pimaricin at a concentration of 25 µg/mL. Surface seeding was carried out in duplicate and the plates were incubated in anaerobic flasks in a hydrogen and carbon dioxide atmosphere at 28°C for five days. For total yeasts, YMA (Yeast, Malt, Agar; Adsa-Micro, Spain) with 25 µg/mL of penicillin and 100 µg/mL of streptomycin. Samples were spread on plates and incubated for 48 h at 28°C.

Statistical analysis

The statistical study of the results consisted of a factorial variance analysis in which the following were considered as factors: the timing of the inoculation with the lactic bacteria and the temperature. ANOVA was carried out using the SPSS statistical software-package[19] and multivariate analyses were accomplished by means of the PARVUS statistical package[8].

Results

L. oenos IC11 features

The need to choose an appropriate local lactic bacterial strain to boost and drive malolactic fermentation has been widely demostrated[13,20]. The lactic bacteria used in this work was chosen on the basis of the following criteria: an ability to grow at high ethanol concentrations and low pH and temperature; resistance to 200 µg/mL SO_2, a high malolactic conversion rate, a low capacity for accumulating acetic acid and a low dextran producing activity.

Microorganism growth

The number of free cells of *L. oenos* previously entrapped is very small and this only occurs in the last step of the assay Malolactic fermentation was conducted by the entrapped cells of these bacteria.

Yeast growth was not influenced by the inoculation type or the lactic acid bacteria immobilization. This observation was contrary to Boidron's[3] work, which indicated that yeast growth and alcoholic fermentation rate were inhibited by the presence of lactic acid bacteria, but is in accord with other authors such as King and Beelman[11], and Scott and O'Reilly[17].

Analysis of variance

Sugars and Polyalcohols. As can be seen in Table I, glucose and sucrose were completely metabolized in all experimental units. In contrast, a residual level of fructose was detected in all cases studied. This was to be expected taking into account the fact that glucose and sucrose were the carbohydrates which were most readily metabolized by these microorganisms.

Glycerol and sorbitol production were affected by fermentation temperature ($p<1\%$), the greatest accumulation of these substances being observed at 18°C. The authors found similar results in previous assays with free lactic acid bacteria[4]. However, production of these polyalcohols was not influenced by the timing of inoculation with the lactic acid bacteria.

Ethanol production was not affected by the experimental variables. This result can be explained by the fact that the major sugars were almost completely consumed and the yeast *Sacch. cerevisiae* found no competition in the fermentation process.

Organic Acids. The malic acid initially present in the apple juice was fully metabolized at 18°C. Residual amounts of this acid were observed at 12°C (Table II) but this was about 10% of the initial concentration. The highest lactic acid concentration was obtained at 18°C. In this respect, it should be borne in mind that not all lactic acid produced during fermentation comes

TABLE I. Sugar and alcohol concentrations in apple juice and cider

Inoculation	°C	Sucrose		Glucose		Fructose		Glycerol		Ethanol		Sorbitol	
		µ	s	µ	s	µ	s	µ	s	µ	s	µ	s
SI*	18	ND	-	ND	-	0.20	0.05	5.17	0.05	42.16	2.03	6.63	0.05
	12	ND	-	ND	-	0.21	0.01	4.71	0.42	38.35	0.98	5.29	0.49
SE	18	ND	-	ND	-	0.22	0.05	5.63	0.49	36.57	8.65	6.49	0.25
	12	ND	-	ND	-	0.18	0.02	4.00	0.27	34.31	5.14	4.50	0.57
Apple juice		16.09		15.56		41.51		0.00		0.53		4.88	

*SI: simultaneous, SE: sequential, ND: not detected, µ: mean (g/litre), s: standard deviation.

TABLE II. Organic acid concentrations in apple juice and cider

Inoculation	°C	Quinic		Malic		Shikimic[1]		Lactic		Acetic		Succinic	
		µ	s	µ	s	µ	s	µ	s	µ	s	µ	s
SI*	18	0.15	0.01	0.01	0.01	0.10	0.01	3.79	0.02	0.46	0.04	1.48	0.13
	12	0.15	0.01	0.30	0.40	0.20	0.01	3.54	0.59	0.20	0.06	1.78	0.09
SE	18	0.14	0.05	0.01	0.01	0.10	0.01	4.26	0.18	0.39	0.15	1.47	0.38
	12	0.14	0.01	0.68	0.46	0.20	0.01	2.68	0.59	0.15	0.05	1.55	0.29
Apple juice		0.00		4.88		10.65		0.00		0.00		0.00	

*SI: simultaneous, SE: sequential, ND: not detected, µ: mean (g/litre), s: standard deviation,[1](µg/mL).

TABLE III. Volatile compounds concentrations in apple juice and ciders

Inocul.	°C	Acet-aldehyde		Methanol		1-Propanol		Ethyl acetate		Isobutanol		1-Butanol		Amyl alcohols		2-Phe. acetate		2-Phenetanol		Ethyl lactate	
		μ	s	μ	s	μ	s	μ	s	μ	s	μ	s	μ	s	μ	s	μ	s	μ	s
SI*	18	6.63	7.89	33.02	5.58	8.54	4.29	19.18	11.50	38.65	6.92	5.98	6.37	149.14	1.71	4.40	13.99	105.91	3.78	216.83	5.49
	12	49.78	5.73	26.60	15.06	7.07	5.29	17.00	8.50	25.45	4.88	5.55	4.42	136.10	3.26	4.50	29.14	111.74	2.85	118.75	42.30
SE	18	7.83	20.78	23.29	12.05	8.21	12.99	12.28	20.30	36.84	11.37	5.18	5.67	149.63	5.68	5.66	8.45	126.21	20.49	197.94	5.83
	12	47.58	35.68	24.94	4.92	7.48	1.89	14.31	24.70	24.02	9.95	5.27	5.71	129.84	11.37	3.76	21.83	109.09	7.75	130.83	11.11
Apple juice		11.30		16.23		2.52		0.00		2.22		5.29		5.56		0.00		0.00		0.00	

*SI: simultaneous, SE: sequential, ND: not detected, μ: mean (μg/mL), s: standard deviation.

from malolactic fermentation. These results are in accord with previous findings obtained with free cells of *L. oenos*[4].

Changes in the shikimic acid concentration during fermentation were influenced by fermentation temperature ($p<1\%$). The highest accumulation was recorded when the fermentation was conducted at 12°C.

The amount of acetic acid accumulated at the end of fermentation was low (maximum concentration 0.46 g/litre), but is double that obtained with free cells of *L. oenos*, especially at 18°C[4]. There was no significant influence of the experimental variables on succinic acid.

Volatile Compounds. Acetaldehyde, 1-propanol, isobutanol, ethyl lactate and amyl alcohols were clearly affected by the fermentation temperature ($p<1\%$). The production of acetaldehyde was higher at 12°C (Table III), but the concentration of the remainder was higher at 18°C. The production of high levels of amyl alcohols is particularly undesirable since these compounds have an objectionable odour as well as a strong burning taste[12]. The production of higher alcohols as important secondary constituents of ciders is linked to the yeast strain and fermentation temperature[14,15] and was not affected by the type of inoculation with immobilized *L. oenos*.

The accumulation of ethyl acetate and methanol was stimulated by simultaneous inoculation of yeast and lactic acid bacteria ($p<10\%$ and $p<1\%$, respectively). In contrast, 2-phenethanol and 2-phenethyl acetate were not affected by assay conditions.

The activity of the immobilized cells of *L. oenos* gave unaltered sensory properties to the final product. These findings support the use of immobilized lactic bacteria for the biological de-acidification of cider. Immobilization of viable bacteria may facilitate control of malolactic conversion and easy removal of the cells after fermentation.

Multivariate analysis

Data. A data matrix was constructed in which rows (twelve) corresponded to ciders produced by different fermentation patterns (two temperatures and two models of inoculation with immobilized lactic acid bacteria) and columns (twenty) corresponded to chemical variables, namely: fructose, polyalcohols (glycerol and sorbitol), organic acids (quinic, malic, shikimic, lactic, acetic and succinic) and volatile compounds (acetaldehyde, ethanol, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, amyl alcohols, ethyl lactate, 2-phenethanol and 2-phenethyl acetate). Data were

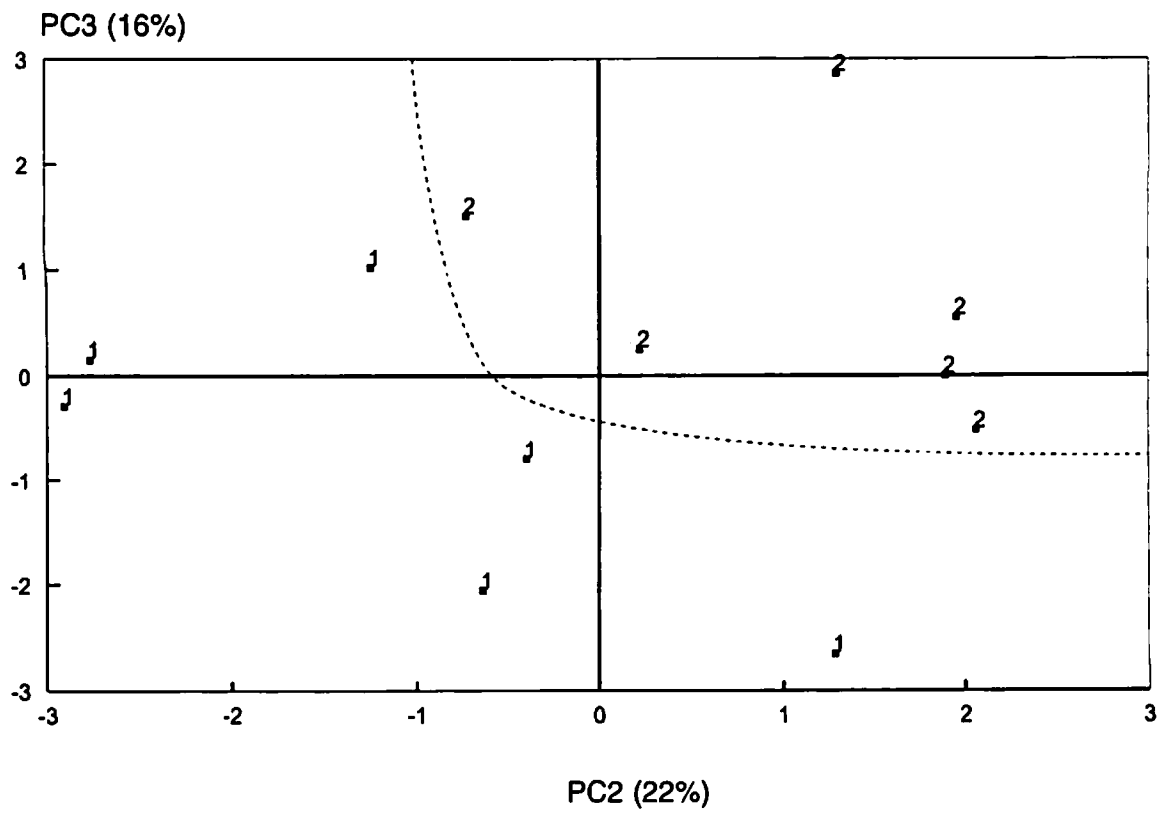

FIG. 1. Eigenvector projection of ciders. 1: sequential inoculation; 2: simultaneous inoculation.

categorized on the basis of the temperature of fermentation (T=18°C, High: H; T=12°C, Low: L) and the timing of inoculation with *L. oenos* (Simultaneous: Si; Sequential: Se).

Univariate Treatment. Fisher's test (FW: Fisher Weight) was carried out in order to ascertain if any one variable by itself would allow the differentiation of the fermented products categorized as H/L and Si/Se. The most discriminating variables found were acetaldehyde (FW: 16.96; $p<1\%$) and ethyl acetate (FW: 2.13; $p<1\%$) which were used for discriminating between High (H) and Low (L), and Simultaneous (Si) and Sequential (Se) classes, respectively. From the results obtained with the univariate treatment used, it was possible to differentiate the fermented products obtained at different temperatures using the most discriminating variable, namely acetaldehyde. In contrast, it was not possible to differentiate the ciders produced on the basis of the timing of inoculation with lactic acid bacteria using the most discriminating variable, namely ethyl acetate. In this case, multivariate treatment of the data was needed. At the same time, the correlation matrix was evaluated in order to ascertain relationships between chemical variables. Thus, variables with high correlations (lactic and acetic acid, glycerol, ethanol, 1-propanol and amyl alcohols) were removed from the data matrix so that the new matrix dimension was 12 ciders×14 variables.

Factor Analysis of the Internal Structure. The principal component technique was used to ascertain the structure and to reduce the dimension of the data and the number of principal components (PC) was determined. Four PC that accounted for 87.38% (eigenvalues>1.34) of the variance were chosen, once the data had been standardized by means of autoscaling. The highest correlations obtained between original variables and principal components were as follows: ethyl acetate and methanol with the second eigenvector (0.52 and 0.50, respectively) and quinic acid and succinic acid with the third eigenvector (0.59 and 0.57, respectively). In Figure 1, the apple fermented products are projected onto the factorial plane shaped by the second and the third eigenvectors. A structurization of the data is detected on the basis of the inoculation model followed. Ciders obtained from simultaneous inoculation are situated in the upper right-hand corner of the map, which corresponds to samples with high levels of ethyl acetate, methanol, quinic acid and succinic acid.

In conclusion, it may be affirmed that under these experimental conditions, cider obtained with immobilized *L. oenos* attained the desired alcoholic degree without residual sugars and the development of malolactic fermentation was not affected by the type of inoculation with immobilized lactic acid bacteria. The fermentation temperature influenced the development of malolactic conversion and the production of acetaldehyde. Chemical variables and chemometrics allowed differentiation of ciders on the basis of the fermentation model followed.

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