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SELECTION AND BIOCHEMICAL CHARACTERISATION OF *SACCHAROMYCES CEREVISIAE* AND *KLOECKERA APICULATA* STRAINS ISOLATED FROM SPANISH CIDER

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The behaviour of different strains of *Saccharomyces cerevisiae* and *Kloeckera apiculata* in apple juice fermentation was studied. Ethanol production was higher for *Saccharomyces* strains while residual sugars and ethyl acetate content was higher for *Kloeckera* strains. *Kl. apiculata* fermented products showed the lowest amount of higher alcohols and the lowest content in organic acids with the exception of acetic acid, so this yeast produced an increment in volatile acidity. On the basis of ethyl acetate, hydrogen sulfide, and acetic acid production, fermentative ability, potassium metabisulfite resistance and sporulation percentage, one strain from *Sacch. cerevisiae* could be employed as starter for making cider.

Key Words: *Yeast, fermentation, cider, Kloeckera, Saccharomyces*

INTRODUCTION

Traditionally, cider fermentations depend on the activity and growth of indigenous yeasts of the apple or, as the season progresses, on the yeasts of the cider plant which are found in the crushers, on the belts, in the fermenting tanks, etc. Undesirable strains of yeasts often spoil alcoholic fermentation since fermentation usually occurs in an open system without sterilisation[4,5]. Under such conditions, it is difficult to maintain uniformity in cider production during successive seasons. The individual characteristics of spontaneous fermentations are determined by the different species that ferment the must and contribute to the flavour of the fermented products in question[9,24]. This occurs because yeasts metabolise apple juice constituents, principally the sugars, into a wide range of volatile and non-volatile end-products. The nature and concentration of these end-products are determined by the species of yeast that grow during fermentation, the extent to which they grow and fermentation factors, such as temperature, that may affect their growth and biochemical behaviour[6,20].

It is possible to isolate pure cultures of yeasts which could be supplied to cidermakers in the form of pure culture slants and then be propagated in the cider plant to provide starters. We will refer to these as "selected pure cultures" although it must be realised that we are not dealing with pure culture fermentations once the yeasts are grown in a non-sterile environment.

While the use of selected pure cultures is well established in the beer-brewing industry, it has not been adopted in cidermaking. Only the use of a pure yeast culture allows the fermentation rate and the formation of desirable compounds to be controlled in the manufacture of cider.

It was established, with few exceptions, that strains of *Sacch. cerevisiae* rapidly grew and dominated in fermentations. Consequently strains of *Sacch. cerevisiae* should be eventually selected for use as starter cultures in inoculated fermentations.

It is also well established that *Sacch. cerevisiae* is not the only species that grows during spontaneous cider fermentations. The early stages of the fermentation are characterised by the growth of *Kloeckera apiculata*[19], in cider this yeast remains through the later stages because of the low alcohol content of the product[12]. The authors stated a need for more detailed study of the technological and biochemical behaviour of individual yeast species, in order to found the appropriate selected pure culture for cider fermentation.

So far, there has been no systematic study of the yeasts species associated with the fermentation of Spanish cider.

EXPERIMENTAL METHODS

Cider fermentations

The must used during this work was prepared from a mixture of cider apples with different sensory properties: Collaos (mild sharp) 30%, Raxao (sharp) 40%, Durón Arroes (sweet) 10%, Coloradona (bittersweet) 15% and Meana (bittersharp) 5%; endowing the resulting juice with an overall mild sharp nature (titrable acidity 57.55 meq/l; volatile acidity 1.66 meq/l; density 1,064 g/l; pH 3.61; nitrogen 35.11 mg/l; phenolic compounds 1.08 g/l).

Fresh apple juice was first crossflow microfiltered using a 0.45 μm membrane filter and then sterilised by passing through a 0.2 μm filter (Millipore Ibérica S.A., Madrid, Spain). Fermentations were carried out in a 2 l sterilised fermenter reactor with periodical shaking (15 min shaking each 120 min) and an internal nitrogen atmosphere was created to avoid the undesirable presence of oxygen 24 hours after the fermentation began. Two litres of apple juice was pitched with yeast at a concentration of approximately 10^6 cells/ml. The fermentations were carried out at 12°C and 18°C. All experiments were performed in triplicate. Must and fermented products were analysed. The fermentations were carried out for 30 days at 18°C and for 45 days at 12°C.

Yeast strains

Sacch. cerevisiae and *Kl. apiculata* strains used in this study, belong to the collection of our laboratory. Yeast strains were obtained from the following sources: M267, T279, F486, F235 and M468 *Sacch. cerevisiae* strains were isolated by the author from different Asturian cider-makers; C27419 and C27520 *Sacch. cerevisiae* strains were isolated from our Institute pilot-plant; C0223K, C04224K and C0721K *Kl. apiculata* strains were isolated from our Institute pilot-plant[12].

Screening of yeast strains on the basis of technological characteristics

The production of H_2S was assessed by the blackening of lead acetate paper above a synthetic medium and the blackening of yeast streaks on BIGGY (Difco Laboratories, Detroit, Michigan, USA) agar after 24 h incubation at 25°C; SO_2 resistance according to Hara *et al.*[16]; yeast flocculation was determined according to Johnston and Reader[18]. Screening of strains for killer activity and killer sensitivity was performed according to Heard and Fleet[17]. The sporulation behaviour of yeast was tested according to Anderson and Martin[1]. Fermentation capacity was determined according to Benítez *et al.*[7].

Chemical analyses

Alcohols, organic acids and carbohydrates were determined by HPLC (Waters Associates, Milford, MA, USA) equipped with two Waters 510 pump, two Wisp 712 automatic injectors, a Waters 410 RI and 481 UV-Vis detectors and a Digital 380 data station was used to determine carbohydrates and alcohols (glucose, sucrose, fructose, glycerol, ethanol and sorbitol) and organic acids (quinic, shikimic, malic, lactic, acetic, succinic and fumaric). D-Lactic acid was determined by an enzymatic method (Boehringer Mannheim, S.A., Barcelona, Spain)[8].

The sugars and alcohols analysis was carried out by means of the Sugar Pak I column, (300 × 6.5 mm i.d.) (Waters Associates, Milford, MA, USA) which was operated under the following conditions: mobile phase, water containing 50 ppm $Ca(Na)_2EDTA$; flow rate 0.5 ml/min; column temperature 80°C; detector temperature 37°C; detector sensitivity 32; injected volume 10 µl, according to the method described by Blanco *et al.*[10].

The major organic acids were determined using a Spherisorb ODS-2 column (5 µm, 250 × 4 mm i.d.) (Teknokroma, Barcelona, Spain). The operating conditions were as follows: mobile phase KH_2PO_4/H_3PO_4 buffer, pH 2.25; ionic strength 10^{-2} M; flow-rate 0.5 ml/min; column temperature 25°C; injected volume 20 µl. Column effluents were monitored at a wavelength of 206 nm[11].

The volatile compounds with a boiling point <145°C (ethyl acetate, methanol, 1-propanol, 1-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol) were analysed employing a Perkin Elmer 8500 gas chromatograph (Perkin Elmer Corp., Norwalk, CT, USA) fitted with flame ionisation detector and 5 m micropacked column made from 0.85 mm stainless steel. Volaspher A-2, 120–140, was used as solid support with 4% liquid loading of a mixture of carbowax 300 + bis 2-ethyl-hexylsebacate (92 + 8). The column temperature was maintained at 70°C, injector and detector set at 170°C, and helium was used as carrier gas at a flow of 27 ml/min. Qualitative and quantitative analyses of major low-boiling-point components present in cider were carried out directly on the cider by injection of 1 µl of cider previously filtered through a 0.22 µm Millex membrane (Millipore Ibérica, Madrid, Spain). The volatile compounds were identified by comparing the relative retention times of each one with those of the standard reference compounds. Quantification of volatile compounds was carried out by the internal standard method on the basis of the peak areas according to the method described by Mangas *et al.*[22].

Total nitrogen was determined by the modified Hach method[15]. The concentrations and total and volatile acidity were determined according to standard methods (AOAC)[2].

Statistical analysis

Analysis of variance (SAS)[23] was used to determine significant differences between treatments, the factors considered were strain, species and temperature. In order to determine the relationships between strains and treatments, Tukey's HSD Test was also employed.

Results

Screening of yeast strains according to technological characteristics

The presence of killer activity and sensitivity to killer action was examined in 20 strains of *Sacch. cerevisiae* and 8 strains of *Kl. apiculata*. The latter yeast was examined because it is an important wild or indigenous species that grows during the early stages of cider fermentation. Each strain was tested for killer activity against all other strains, and each strain was tested for killer sensitivity against all other strains. Killer activity was not observed in any of the *Kl. apiculata* and *Sacch. cerevisiae* strains. Some strains of *Kl. apiculata* were resistant to killer activity.

In our results, 10/20 of the *Sacch. cerevisiae* strains and 2/8 of the *Kl. apiculata* strains tested produced high levels of H_2S. With regards to the flocculative abilities, the cider yeasts covered the spectrum from non-flocculent to very flocculent.

The fermentation activity of cider yeasts was examined and narrow differences were observed in *Sacch. cerevisiae* (0.5 g/l); fermentation activity is defined as the rate of carbon dioxide production in a sugar-containing medium expressed in absolute terms as grams of CO_2 per hour per gram of yeast solids. *Kl. apiculata* strains showed in all cases lower fermentation activity than the *Sacch. cerevisiae* ones.

Sporulation capacity was studied and wide differences were found. An examination of 20 strains of cider yeast of *Sacch. cerevisiae* gave amounts ranging from 10% to 99%. As is known *Kl. apiculata* is an asporogenous wild cider yeast. A high sporulation capacity is selected for further genetic studies.

Eleven of the twenty strains of *Sacch. cerevisiae* tested were resistant to 150 mg/l SO_2. Only three of the eight strains of *Kl. apiculata* tested were resistant to 100 mg/l SO_2.

Seven yeast strains of *Sacch. cerevisiae* (M267, T279, F486, F235, M468, C27419, C27520) were preselected for biochemical characterisation on the basis of their high fermentative ability and sporulation percentage, intermediate flocculation ability and low hydrogen sulfide production.

In relation to the strains of *Kl. apiculata*, we selected three strains (CO223K, CO424K and CO721K) on the basis of killer and SO_2 resistance and H_2S production.

Characteristics of selected yeast cultures for cider fermentation

Organic acids

Table I shows the organic acid contents in apple juice and fermented products. The variance analysis of these data shows that within our experimental conditions, a significant effect of the yeast strain could be detected on the amount of quinic acid, malic acid, shikimic acid, L-lactic acid, succinic acid, fumaric acid and acetic acid ($P = 0.0001$).

Apple juice fermented by the weakly fermenting yeast *Kl. apiculata* gave, in general, relatively low organic acid concentrations, except for acetic acid. The production of acetic acid was higher at 18°C (1.46 g/l for CO424K strain) than 12°C ($P = 0.0001$).

Yeast may either break down or form malate during fermentation; synthesis to the extent of 91% and 89% of the original malate content occurred in some cider fermentation at 12°C with *Sacch. cerevisiae* (7.71 g/l for C27419) and *Kl. apiculata* (7.63 g/l for CO721K).

The strain T279 of *Sacch. cerevisiae* formed the highest amount of fumarate during must fermentation (0.18 ppm) in relation to all the strains tested.

The highest amount of L- and D-lactic acid detected was produced by C27520 strain of *Sacch. cerevisiae* (0.55 g/l and 0.22 g/l at 12°C and 18°C, respectively).

Succinate, a component with a typical salty-bitter taste, has long been known to be excreted by yeast during fermentation. Its concentration in the final product varied widely (1.76–0.11 g/l). As should be expected, the highest amount of this acid occurred in the fermented products of *Sacch. cerevisiae*.

TABLE I. Organic acids content in fermented cider and the original must

Species	Code	°C	Malic (g/l) x	Malic (g/l) σ	Quinic (g/l) x	Quinic (g/l) σ	Shikimic (g/l) x	Shikimic (g/l) σ	L-Lactic (g/l) x	L-Lactic (g/l) σ	D-Lactic (g/l) x	D-Lactic (g/l) σ	Acetic (g/l) x	Acetic (g/l) σ	Succinic (g/l) x	Succinic (g/l) σ	Fumaric (mg/l) x	Fumaric (mg/l) σ
S. cerevisiae	M267	18	6.41^{b}	0.10	0.28^{b}	0.07	0.29^{b}	0.01	0.41^{a}	0.06	0.10^{b}	0.07	0.08^{d}	0.01	1.76^{a}	0.03	0.14^{b}	0.01
S. cerevisiae	T279	18	7.06^{a}	0.51	0.23^{b}	0.03	0.30^{b}	0.01	0.45^{a}	0.04	0.06^{c}	0.05	0.10^{d}	0.04	1.63^{ab}	0.11	0.18^{a}	0.02
S. cerevisiae	F486	18	6.53^{a}	0.17	0.21^{c}	0.01	0.36^{a}	0.03	0.28^{c}	0.01	0.21^{a}	0.01	0.14^{d}	0.01	1.57^{bc}	0.06	0.07^{c}	0.01
S. cerevisiae	F235	18	6.40^{b}	0.28	0.18^{c}	0.01	0.30^{b}	0.02	0.38^{b}	0.01	0.04^{c}	0.01	0.15^{d}	0.01	1.40^{c}	0.08	0.13^{bc}	0.01
S. cerevisiae	M468	18	5.06^{d}	0.28	0.27^{b}	0.05	0.02^{d}	0.01	0.34^{b}	0.02	0.05^{c}	0.07	0.36^{c}	0.30	0.36^{c}	0.01	0.02^{d}	0.01
S. cerevisiae	C27419	18	6.39^{b}	0.61	0.31^{b}	0.02	0.03^{c}	0.01	0.23^{c}	0.06	0.02^{c}	0.01	0.26^{c}	0.07	0.70^{d}	0.03	0.01^{d}	0.01
S. cerevisiae	C27520	18	5.61^{c}	0.99	0.23^{bc}	0.06	0.03^{c}	0.01	0.03^{d}	0.01	0.22^{a}	0.10	0.23^{c}	0.10	0.75^{d}	0.16	0.01^{d}	0.01
K. apiculata	CO223K	18	5.02^{d}	0.41	0.40^{a}	0.18	0.03^{c}	0.01	0.13^{cd}	0.03	0.05^{c}	0.02	1.31^{a}	0.14	0.37^{c}	0.14	0.10^{c}	0.01
K. apiculata	CO424K	18	4.70^{d}	0.16	0.41^{a}	0.03	0.02^{d}	0.01	0.13^{cd}	0.01	0.03^{c}	0.02	1.46^{a}	0.04	0.13^{f}	0.01	0.01^{d}	0.01
K. apiculata	CO721K	18	5.77^{c}	0.80	0.17^{c}	0.06	0.03^{c}	0.01	0.35^{b}	0.23	0.14^{b}	0.15	0.72^{b}	0.30	0.69^{de}	0.01	0.10^{c}	0.01
	MUST	—	4.04		0.12		0.18		0.11		0.01		0.01		0.01		0.02	

x, mean value; σ, standard deviation; ^{a,b,c,d,e,f}, Tukey's Grouping.

TABLE II. Sugars and alcohols content in fermented cider and original must

Species	Code	°C	Sucrose (g/l) x	Sucrose (g/l) σ	Glucose (g/l) x	Glucose (g/l) σ	Fructose (g/l) x	Fructose (g/l) σ	Glycerol (g/l) x	Glycerol (g/l) σ	Ethanol (g/l) x	Ethanol (g/l) σ	Sorbitol (g/l) x	Sorbitol (g/l) σ
S. cerevisiae	M267	18	0.38^{c}	0.06	0.06^{c}	0.07	0.06^{d}	0.08	5.87^{c}	0.45	66.27^{ab}	5.02	12.76^{c}	0.96
S. cerevisiae	T279	18	0.19^{c}	0.04	0.15^{c}	0.20	8.09^{b}	5.36	6.82^{cd}	0.90	65.68^{b}	3.05	13.14^{bc}	0.74
S. cerevisiae	F486	18	0.18^{c}	0.02	0.05^{c}	0.06	0.20^{cd}	0.18	6.35^{d}	0.08	70.99^{a}	2.43	13.27^{b}	0.38
S. cerevisiae	F235	18	0.18^{c}	0.01	0.09^{c}	0.05	12.47^{b}	0.62	6.65^{d}	0.14	64.08^{b}	3.83	13.35^{b}	0.10
S. cerevisiae	M468	18	nd	—	nd	—	8.17^{b}	0.64	9.51^{ab}	0.21	67.15^{a}	1.13	14.27^{a}	0.17
S. cerevisiae	C27419	18	nd	—	nd	—	15.60^{ab}	3.09	9.00^{b}	1.21	56.13^{c}	4.32	12.22^{d}	0.99
S. cerevisiae	C27520	18	nd	—	nd	—	8.11^{b}	1.75	8.65^{b}	0.50	62.32^{b}	2.95	12.68^{d}	0.47
K. apiculata	CO223K	18	2.07^{b}	0.04	2.66^{a}	1.02	23.95^{a}	2.90	9.53^{a}	0.45	49.38^{d}	3.26	14.45^{a}	0.15
K. apiculata	CO424K	18	1.92^{b}	0.04	2.46^{a}	0.78	14.90^{ab}	1.80	10.08^{a}	0.38	52.33^{c}	2.72	13.90^{ad}	0.24
K. apiculata	CO721K	18	6.89^{a}	5.97	0.63^{b}	0.09	5.54^{c}	7.25	7.72^{c}	0.06	63.76^{b}	0.27	13.03^{c}	0.35
	MUST	—	19.90		33.83		82.29		0.24		1.05		12.86	

x, mean value; σ, standard deviation; ^{a,b,c,d,e,f}, Tukey's Grouping.

All strains tested accumulated quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid) during the fermentation of apple juice. In relation to shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid), all strains of *Kl. apiculata* consumed this acid; on the contrary, some strains of *Sacch. cerevisiae* accumulated it (Table I).

As was expected, products fermented with *Kl. apiculata* showed low titrable acidity and high volatile acidity; these results are in accordance with the yeast organic acid metabolism.

Residual sugars

Table II shows the sugars and alcohols contents in apple juice and fermented products. The yeast strain was statistically significant for the rate of residual major sugars (glucose, fructose and sucrose) found in the fermented products ($P = 0.0001$, $P = 0.0019$, $P = 0.0001$).

Products fermented with *Kl. apiculata* contained he highest amounts of residual sugars (23.95 g/l of fructose for CO223K strain) which is due to the weak fermentative metabolism of this species.

Fermentation temperature was a significant factor for the amount of residual sucrose found ($P = 0.0004$). When fermentation was carried out by *Kl. apiculata*, the highest level of this carbohydrate was found at a low temperature (results not shown).

Glycerol and sorbitol production was significantly affected by the fermentation temperature and the yeast strain employed ($P = 0.0001$). In general, the glycerol content increased with the temperature (results not shown).

The variance analysis shows that within our experimental conditions, a significant effect of the yeast strain could be detected on the ethanol concentration ($P = 0.0001$). In the case of temperature, no more effect on the ethanol formation could be detected. Ciders fermented with *Kl. apiculata* showed lower ethanol concentration than products fermented with *Sacch. cerevisiae*.

Alcohols and esters

The results described in this paragraph can be observed in Table III. There was a significant effect of the yeast strain employed on ethyl acetate formation ($P = 0.0001$). *Kl. apiculata* strains gave greater quantities of ethyl acetate (214–340 mg/l) than *Sacch. cerevisiae* strains (65–105 mg/l). The fermentation temperature did not affect ethyl acetate formation.

The average production of methanol was significantly affected by yeast strain and fermentation temperature ($P = 0.0001$). The highest methanol level occurred at 18°C.

The previously carried out strain selection on the basis of H_2S production resulted in no differences in 1-propanol production. As is known, production of both is genetically linked.

The behaviour of the yeast with regard to the production of higher alcohols differed significantly ($P = 0.0001$); thus *Kloeckera* yielded little 2-methyl-1-butanol (18.92–26.50 mg/l), 3-methyl-1-butanol (48.37–86.59 mg/l), 1-butanol (16.03–20.12 mg/l) and 2-methyl-1-propanol (45.96–73.04 mg/l). However, the higher alcohol production in fermented products of *Sacch. cerevisiae* was: 2-methyl-1-butanol (36.55– 54.83 mg/l), 3-methyl-1-butanol (131.18–225.24 mg/l), 1-butanol (18.14–37.55 mg/l) and 2-methyl-1-propanol (36.74–171.11 mg/l). These results are due to the greater fermentative metabolism of *Sacch. cerevisiae*. The fermentation temperature increased the production of isoamyl alcohol (results not shown).

TABLE III. Major volatile compounds found in fermented cider and original must

Species	Code	°C	*Acetate (mg/l)		Methanol (mg/l)		1-Propanol (mg/l)		i-Butanol (mg/l)		1-Butanol (mg/l)		D-Amylic (mg/l)		i-Amylic (mg/l)	
			x	σ	x	σ	x	σ	x	σ	x	σ	x	σ	x	σ
S. cerevisiae	M267	18	68.90^{c}	4.38	101.47^{d}	8.17	149.61^{c}	23.39	67.28^{c}	14.23	19.59^{c}	1.38	44.34b	6.07	135.66^{d}	15.61
S. cerevisiae	T279	18	69.97^{c}	3.53	106.85^{bc}	6.37	136.29^{d}	1.86	50.05^{d}	4.59	20.93^{b}	0.67	54.83^{a}	6.70	142.79^{cd}	23.14
S. cerevisiae	F486	18	69.37^{c}	3.18	105.48^{c}	2.62	93.94^{f}	85.34	61.06^{c}	5.48	20.73^{b}	0.61	46.87^{b}	1.90	176.79^{b}	9.48
S. cerevisiae	F235	18	66.67^{c}	1.24	96.15^{d}	11.03	119.47^{e}	19.71	36.74^{f}	3.56	18.51^{c}	2.48	42.07^{b}	8.16	157.72^{c}	29.92
S. cerevisiae	M468	18	76.91^{b}	5.58	124.65^{a}	11.24	182.56^{a}	12.74	74.04^{b}	6.87	22.84^{ab}	1.09	53.36^{a}	3.98	225.24^{a}	16.55
S. cerevisiae	C27419	18	74.93^{b}	3.06	105.60^{c}	3.48	136.78^{d}	2.70	72.08^{b}	20.02	19.86^{c}	0.44	43.62^{bc}	3.03	152.27^{c}	5.14
S. cerevisiae	C27520	18	78.36^{b}	4.76	105.75^{c}	5.44	147.50^{c}	8.73	91.41^{d}	27.46	25.94^{a}	4.85	42.57^{c}	3.40	168.23^{b}	10.44
K. apiculata	CO223K	18	315.02^{a}	95.66	112.25^{b}	13.40	136.48^{d}	40.13	56.89^{d}	4.48	19.50^{c}	1.71	23.12^{d}	2.57	59.63^{f}	5.94
K. apiculata	CO424K	18	301.85^{a}	80.45	116.60^{ab}	2.95	151.49^{b}	28.63	45.96^{e}	14.16	20.12^{b}	0.34	24.23^{d}	1.56	58.95^{f}	7.01
K. apiculata	CO721K	18	322.51^{a}	87.97	100.86^{d}	6.75	135.07^{d}	21.56	58.65^{cd}	21.23	19.36^{c}	0.61	26.50^{d}	1.44	86.59^{e}	6.88
	MUST	—	0.00		29.21		0.85		0.00		18.96		15.35		10.67	

b.p., boiling point; *Ethyl Acetate; ^{a,b,c,d,e,f}Tukey's Grouping.

Discussion

In selecting yeast strains to use as starters, the initial selection is usually carried out by taking into consideration characteristics such as alcohol tolerance, H_2S production, volatile acidity production, and fermentation rate, with toxin production or toxin resistance as a secondary criterion for selection. The sporulation ability is an important test for further breeding of the strains. We believe that determining the presence of the killer factor and resistance to killing activity are also important criteria. *Kl. apiculata* was examined because it is an important wild or indigenous specie that grows during the early stages of cider fermentation. We found that some strains of *Kl. apiculata* were resistant to killer activity but killer activity was not observed in any of the strains tested, these findings are consistent with those reported elsewhere[3] and confirm the natural occurrence of both killer-resistant and killer-sensitive strains during spontaneous fermentation.

Ciders occasionally smell more or less strongly of hydrogen sulfide, research has revealed[27] that the strain of yeast is the basic cause of the aroma, and with proper selection of yeast strains this aroma can be completely inhibited. The large proportion of H_2S producing yeast strains observed in our study is unexpected since this production is undesirable in cidermaking and this character would have been selected against over the course of time. Non-production of H_2S can be caused by a leaky mutation of a gene affecting sulfite reductase and this property can be readily transferred by genetic techniques[29].

A high degree of flocculative ability is generally desirable in cider yeasts since it aids clarification, however it has been alleged that strong flocculation causes problems of slow and stuck fermentations, although conclusive evidence is still lacking[26].

Most of the strains of *Sacch. cerevisiae* tested were resistant to SO_2, this resistance might be expected in cider yeasts since this antioxidant and antiseptic is used extensively in cider production and presumably cider yeasts have been selected over many years for their ability of ferment effectively in the presence of SO_2 residues. The level of resistance is greater than the residual level of SO_2 permitted in ciders in Spain. *Kl. apiculata* was more sensitive to this antiseptic than *Sacch. cerevisiae*, which is according to the apple juice fermentation investigations carried out by Beech and Carr[4].

Apple juice fermented by the weakly fermenting yeast *Kl. apiculata* gave higher levels for acetic acid. Acetic acid is always the main volatile acid in cider, and high levels cause off-flavours.

We found synthesis of malate in cider fermentation with *Sacch. cerevisiae* and *Kl. apiculata*. Formation of L(−)malate in yeast fermentations was first observed in 1924, synthesis of malate was not observed in a natural product fermentation (pear juice) until 1952[28]. Due to the relatively high malic acid concentration in the apple juice employed for cider making in Spain, it is necessary to promote the development of malolactic fermentation when sterilised must is used.

The presence of quinic acid in cider is very important because certain hetero- and homofermentative *Lactobacillus* and also *Leuconostoc* can metabolise this acid. Generally this takes place in storage after malic acid has been converted to lactic acid. It was first reported by Carr *et al.*[14] and has been mentioned on subsequent occasions. Under our experimental conditions the strains tested accumulated quinic acid which could be linked to their capacity for hydrolising chlorogenic acid.

Shikimate plays an important role in the formation of a tainted flavour in cider, since its reduction to dihydroshikimate promotes the accumulation of acetic acid, furthermore, shikimate is a key component in volatile phenols formation, e.g. catechol[28].

As we can see in Table II, the production of acetic acid could be linked with the decrease of shikimate level; this fact is detected in the case of the *Kl. apiculata* strains and three strains of *Sacch. cerevisiae*, namely M468, C27419 and C27520.

Ciders fermented with *Kl. apiculata* showed lower ethanol concentration than products fermented with *Sacch. cerevisiae*. Therefore, ciders fermented with *Kl. apiculata* show high residual sugar concentration and low ethanol concentration, and hence these ciders have specific organoleptic properties[21].

The lower level of residual nitrogen products obtained from *Sacch. cerevisiae* agree with the greater fermentative metabolism of this yeast. For this species, the residual nitrogen content increased when the fermentative temperature was lower (results not shown), which obviously, is related to the greater fermentative activity that is obtained when the temperature increases.

There was an important effect of the yeast strain employed on volatile compounds formation. According to the literature, the greatest differences in production of aroma compounds correspond to different yeast species, while strains of the same species do not differ significantly[13,25].

For instance *Kl. apiculata* strains produced the highest level of ethyl acetate, which causes a typical ester taint. On the other hand, the highest methanol level occurred at 18°C, which is due to the increased enzymatic activity when the temperature increases. The high methanol concentration obtained in products formed from *Sacch. cerevisiae*, shows the higher pectolytic activity of *Sacch. cerevisiae* strains in relation to *Kl. apiculata* ones.

It is evident from the results of the pure-culture studies that both the strain of yeast and temperature strongly influence the amounts of the various compounds formed during fermentation and the control which could be achieved over these would depend on the choice of yeast and temperature conditions as well as the extent to which the inoculated strain dominates the indigenous microflora during fermentation.

On the basis of the results obtained, it is our opinion that the F486 strain could be employed as a starter for cidermaking, since this strain had an adequate potassium metabisulfite

resistance and fermentative ability, a high sporulation percentage, a low production of ethyl acetate and acetic acid, and hydrogen sulfide was not detected. On the other hand, we think it is also necessary to try the effect of *Kl. apiculata* on *Sacch. cerevisiae* population.

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