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Biometric Study of Acetoin Production in *Hanseniaspora guilliermondii* and *Kloeckera apiculata*

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Gas chromatographic analysis by direct injection of samples yielded quantitative data on acetoin content. Ninety-six strains of *Hanseniaspora guilliermondii* and *Kloeckera apiculata* were investigated for the ability to produce acetoin in synthetic medium and in must. High-level production of acetoin was found to be a characteristic of both species. In synthetic medium, the two species were not significantly different with respect to sugar utilization and ethanol or acetoin production. In grape must, the two species were significantly different ($P = 0.001$) in acetoin production and *K. apiculata* exhibited a significantly negative correlation between acetoin production and either sugar consumption or ethanol production. Use of selected apiculate yeasts in mixed cultures with *Saccharomyces cerevisiae* seems promising for optimization of wine bouquet.

It has been widely reported that the early stages of alcoholic fermentation are characterized by the growth of non-*Saccharomyces* species, which consist mostly of apiculate yeasts of the genera *Kloeckera* and *Hanseniaspora* (14, 19). In recent years, apiculate wine yeasts have become the object of ever-growing interest because, as well as being among the yeasts most frequently found on grapes and in the early stages of must fermentation, they have been found growing in relatively high numbers in competition with *Saccharomyces cerevisiae* (7). This growth is even more dramatic at cool fermentation temperatures (9).

The growth of apiculate yeasts is influenced by different vinification factors, such as temperature and pH (14), and is affected by ethanol concentrations above 5 to 6% (16, 18) and by sulfite (2, 26). It has been reported that initial proliferation of apiculate yeasts is reflected in the sensory quality of wine (8, 13). Strains of these yeasts are known to produce concentrations of acetic acid, esters, and glycerol higher than those produced by *S. cerevisiae* (3, 4, 17). In contrast to the general assumption, Romano et al. (25) found that *Kloeckera apiculata* and *Hanseniaspora guilliermondii* produced less than 1 g of acetic acid per liter and showed a low general production of higher alcohols in synthetic medium.

As regards other secondary products that affect the flavor of alcoholic beverages, acetoin is important in enology because of its involvement in the bouquet of wine. This compound, present in amounts ranging from 2 to 32 mg/liter in wines (22), is normally produced in high amounts by apiculate yeasts. Antoniani and Gugnoni (1) found that two apiculate yeast strains produced 100 and 200 mg of acetoin per liter in wine. Conversely, a study on 100 strains of *S. cerevisiae* (24) showed that this yeast generally produces small amounts (up to 12 mg/liter) of acetoin and that low production is the dominant pattern.

Taking into account the fact that the yeast strain represents a factor which can exert a marked effect on the formation of secondary products of fermentation (23, 27–29), we performed a biometric study of acetoin production by 96 apiculate wine yeasts in either synthetic medium or grape must. The relationships between acetoin produced, sugar consumed, and ethanol yielded are reported.

MATERIALS AND METHODS

Organisms. The following 96 apiculate yeasts, isolated from grape must at the early stages of fermentation, were used: 48 strains of *H. guilliermondii* and 48 strains of *K. apiculata*, as reported previously (25). The wine strain *S. cerevisiae* Ba 85, belonging to the collection of the Dipartimento di Protezione e Valorizzazione Agroalimentare, University of Bologna, Bologna, Italy, was used as a control.

Media. To determine the capacity of a strain to produce acetoin, a basal synthetic medium and grape must were employed. The composition of the synthetic medium used, similar to that recommended by Wickerham (33), has been reported by Romano et al. (25). Triplicate fermentations were done in 50 ml of the synthetic medium at 20°C for 20 days.

White grape must from the Trebbiano cultivar of the Emilia-Romagna region (16% [wt/vol] fermentable sugar, 0.65% [wt/vol] titratable acidity [pH 2.85]) was used. Triplicate fermentations were done with 150 ml of must sterilized by tangential-flow microfiltration (0.2-μm pore diameter). Each sample was inoculated with a 5% concentration of 48-h precultures in the same must and incubated at 20°C. Fermentation was followed by determination of weight loss caused by CO_2 evolution.

After fermentation, the samples were refrigerated for 2 days at 2°C and then racked and stored at −20°C until required for analysis.

Analytical determinations. Acetoin was analyzed by direct injection of 2 μl of fermented medium into a glass column (2 m by 6 mm [outer diameter] by 2 mm [inner diameter]) packed with 80-100 Carbopack C 0.2% Carbowax 1500 (Supelco Inc.). Nitrogen (20 ml/min) was used as the carrier gas. *N*-Butanol (100 mg/liter) was used as the internal standard. A Dani 86.10 gas chromatograph, equipped with a flame ionization detector and linked to a C-R6A Shimadzu integrator, was used. The oven temperature was programmed from 45 to 165°C (10°C/min).

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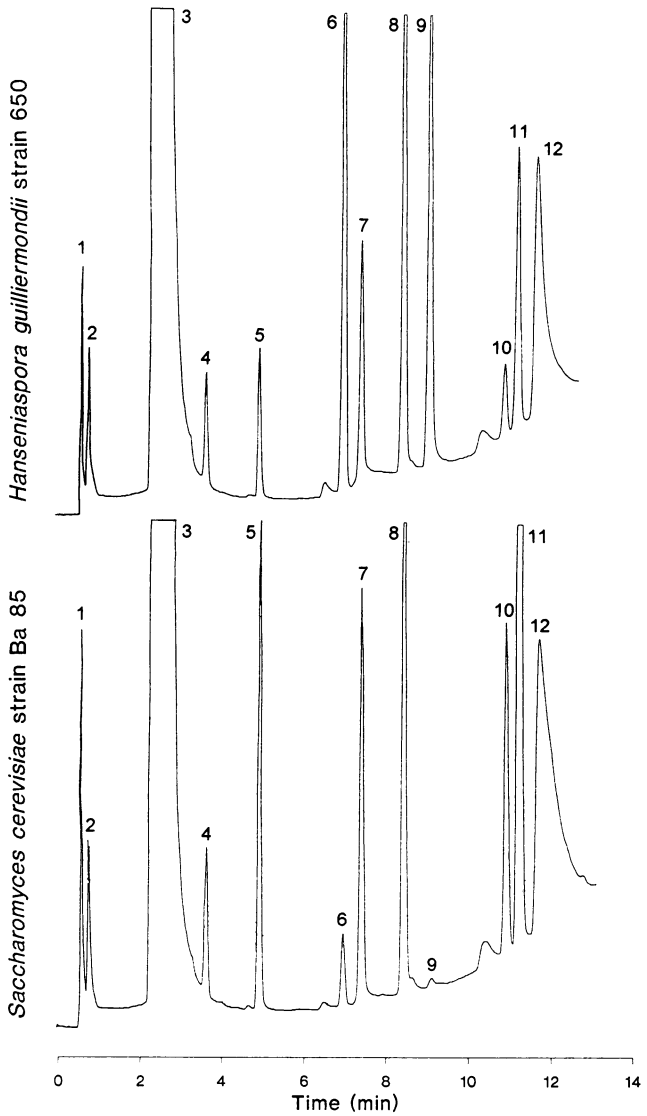

FIG. 1. Chromatograms of must fermented with *H. guilliermondii* (A) and *S. cerevisiae* (B). Peaks: 1, acetaldehyde; 2, methanol; 3, ethanol; 4, 2-propanol; 5, 1-propanol; 6, ethyl acetate; 7, isobutanol; 8, internal standard 1-butanol; 9, acetoin; 10, active amyl alcohol; 11, isoamyl alcohol; 12, acetic acid.

Identification of acetoin was done by gas chromatography-mass spectrometry. The analysis was done on a Varian Saturn high-resolution mass spectrometer directly interfaced to a Varian 3400 capillary gas chromatograph. The samples were analyzed by an ion trap detector to obtain mass spectra for correlation with the computerized National Bureau of Standards data base. A Carbowax 20M fused silica capillary column (1.00-μm film thickness; 30 m by 0.32 mm; Stabilwax; Resteck Corporation) was used, and helium (2 ml/min) served as the carrier gas. The temperature was programmed from 40 to 230°C (5°C/min). The temperature of the injector, transfer line, and manifold was 230°C. The filament emission current was 10 mA. Injection was in the split mode, at a ratio of 1:50.

Glucose and ethanol concentrations were determined enzymatically by using Test Combinations (Boehringer). Sugar concentrations in must were determined by the method of total reducing sugars as described by Zironi et al. (34).

Statistical analyses. Data were analyzed for statistical significance by a one-way analysis of variance and by regression and correlation analyses. Snedecor's *F* value was used to evaluate the significance of the analysis of variance. Pearson's correlation coefficient was used to evaluate the correlation between acetoin production and ethanol yield. The statistical analysis was done with the SPSS-X (1989) package for a VAX/VMS computer.

RESULTS

Acetoin was quantified by direct injection of the samples into the gas chromatography column. Apart from peaks normally found with this column, an unknown peak with a retention time of 9.30 min was detected at high concentrations in all of the samples fermented with apiculate yeasts (Fig. 1). In the samples fermented with the control strain of *S. cerevisiae*, the same peak was found to be of insignificant size (Fig. 1). The identity of the compound was confirmed by gas chromatography-mass spectrometry analysis to be acetoin.

Acetoin production in synthetic medium. In synthetic medium, all of the strains completely consumed the sugar and yielded ethanol at average concentrations of 2.06% for *K. apiculata* and 2.17% for *H. guilliermondii* (Table 1). Acetoin production varied from 22.3 to 49.7 mg/liter for *H. guilliermondii* and from 19.0 to 42.5 mg/liter for *K. apiculata*. Most strains of both species produced quantities ranging from 30 to 35 mg of acetoin per liter (Fig. 2).

Acetoin production in must. In must, the strains showed great variability in sugar consumption and ethanol production (Table 1). *K. apiculata* generally showed lower sugar consumption and ethanol production than *H. guilliermondii*. Acetoin production in must varied from 50.3 to 258.1 mg/liter for *H. guilliermondii* and from 55.8 to 187.4 mg/liter for *K. apiculata*. Most strains of *K. apiculata* (about 60%) produced quantities ranging from 110 to 170 mg of acetoin per liter, whereas *H. guilliermondii* strains showed greater variability, with maxima of 170 to 200 mg/liter (Fig. 3).

Statistical analyses. In synthetic medium, the analysis of variance showed nonsignificant acetoin production variability between the two species studied ($F = 1.263$; yeast species, df = 1.94; $P = 0.263$), whereas in must *H. guillier-*

TABLE 1. Sugar consumption and ethanol production in synthetic medium and must by *H. guilliermondii* and *K. apiculata*

Medium	Organism	Sugar consumption (% wt)			Ethanol production (% vol)		
		Avg	Range	SD	Avg	Range	SD
Synthetic	*Hanseniaspora guilliermondii*	99.987	99.976–99.998	0.003	2.17	1.13–2.98	0.45
	Kloeckera apiculata	99.995	99.989–99.998	0.002	2.06	1.27–2.56	0.25
Grape must	*Hanseniaspora guilliermondii*	80.17	52.98–98.58	11.94	7.10	4.80–7.91	1.07
	Kloeckera apiculata	56.37	44.72–73.50	5.60	5.28	4.04–6.83	0.57

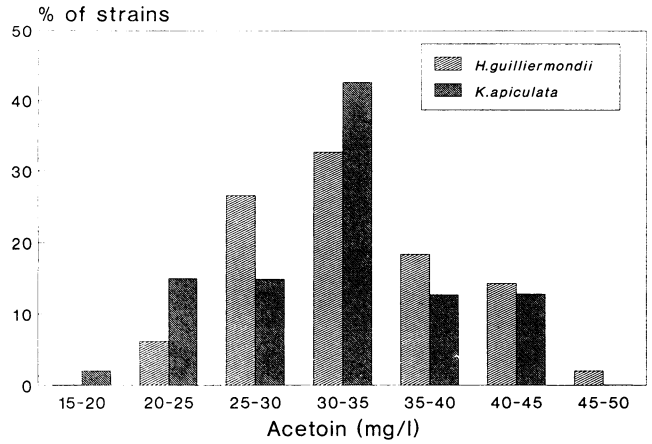

FIG. 2. Histograms of acetoin production in synthetic medium by 96 strains of apiculate yeasts.

mondii and *K. apiculata* strains were significantly different ($F = 12.215$; yeast species, df = 1.94; $P < 0.001$).

In must, acetoin production by *H. guilliermondii* was unrelated to sugar consumption and ethanol production. In contrast, *K. apiculata* showed a significant relationship between acetoin production and sugar consumption (Table 2) and an inverse correlation of acetoin production versus ethanol yield ($P = 0.007$).

DISCUSSION

Nutritionally, biochemically, and morphologically, the species of *Hanseniaspora* and the imperfect genus *Kloeckera* have been reported as a natural group (20). *H. guilliermondii* is the perfect form of *K. apis*, whereas *K. apiculata* is the imperfect state of *H. uvarum*. Our results show that on the basis of acetoin production, *H. guilliermondii* and *K. apiculata* are not as far apart as the two different genus names imply, because the levels of production for one species fall completely within the range of those of the other. In must, *H. guilliermondii* strains, consuming nearly all of the sugar present, produce a higher quantity of acetoin than do *K. apiculata* strains, which only partially utilize the available sugar. In a previous work (25), higher levels of

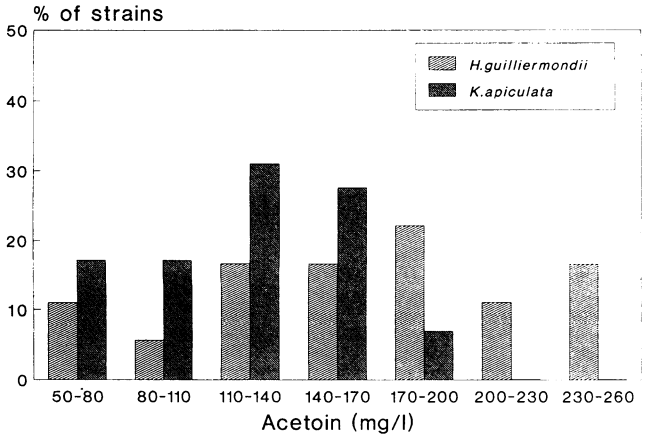

FIG. 3. Histograms of acetoin production in must by 96 strains of apiculate yeasts.

TABLE 2. Parameters of linear regression analysis of acetoin production[a] versus sugar consumption[b] in must by *H. guilliermondii* and *K. apiculata*[c]

Organism	*a*	*b*	r^2	*F*	*P*
Hanseniaspora guilliermondii	168.32	0.054	0.000	0.002	0.967
Kloeckera apiculata	290.64	−1.875	0.181	8.687	0.007

[a] In milligrams per liter.
[b] In grams per liter.
[c] *a*, constant; *b*, slope; regression, df = 1.46; *F*, Fisher; *P*, probability.

alcohol and acetic acid production by the same strains used in this study differed significantly in the two species of apiculate yeasts, showing that the formation of these secondary products is an individual and reproducible strain characteristic. The biometric study of acetoin production in the two species revealed great strain variability, indicating that formation of this compound is also a strain characteristic.

It is interesting that in a complex medium like grape must, *H. guilliermondii* and *K. apiculata* produced quantities of acetoin five times higher than those produced in synthetic medium. The noncorrelation between acetoin produced and sugar consumed, such as that between acetoin and ethanol, indicates that acetoin is produced not only by anabolic pathways (Fig. 4) but also by catabolic ones. On the other hand, it is well known that acetolactate is an intermediate in the biosynthetic formation of valine (10, 21). Acetolactate is highly labile, and lowering of the pH results in accelerated degradation to acetoin (6). Thus, part of the abnormally increased acetoin contents could be ascribed to the must pH.

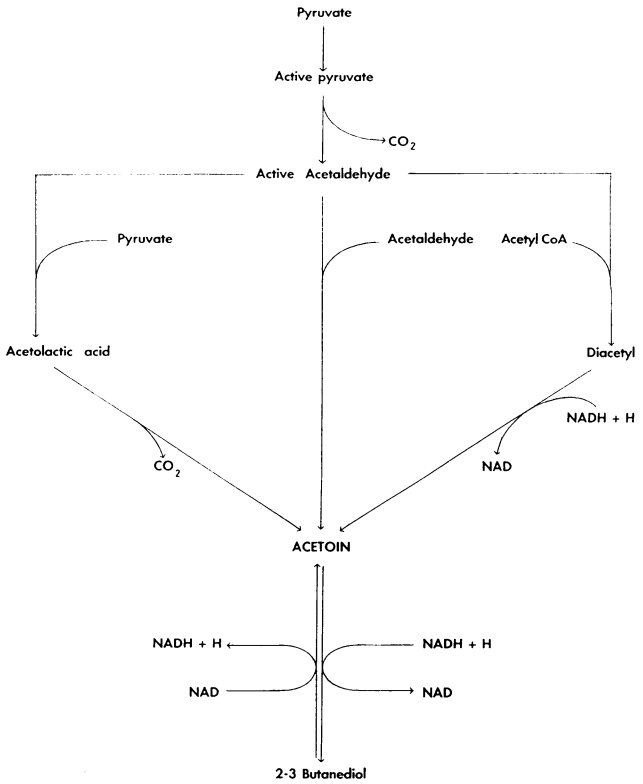

FIG. 4. Possible scheme of biosynthetic origin of acetoin in yeasts (from Wainwright [32] and Collins [5]).

In spontaneously fermented wines, acetoin is found in quantities averaging 5.9 mg/liter in white wine and 15.0 mg/liter in red wine (22). Wines fermented with pure cultures of *S. cerevisiae* generally present similar amounts. Romano and Suzzi (24), using 100 strains of *S. cerevisiae*, found that most strains produced acetoin from nondetectable amounts up to 12 mg/liter in wine. It is known that acetoin is produced by *S. cerevisiae* in the early phase of fermentation, but its content declines rapidly in the final stage of the process because of its reduction to 2,3-butanediol (11, 15). In contrast, in wines fermented with pure cultures of apiculate yeasts, we have always found high contents of acetoin in the final product.

Taking into account the facts that acetoin is considered an odorless compound and that its threshold value is about 50 mg/liter (31), the flavor significance of acetoin is greater in terms of its potential influence on aroma than in terms of the odor itself. In fact, diacetyl and 2,3-butanediol, which are known to develop off flavors in wine, can be derived from acetoin: the former by chemical oxidation, the latter by yeast reduction (12, 30). In addition, acetoin produced by apiculate yeasts seems to be utilized by *S. cerevisiae* to form 2,3-butanediol (1). In this way, our results indicate that acetoin plays a more important role in vinification than has been hitherto supposed.

ACKNOWLEDGMENTS

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