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ORIGINAL PAPER

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Controlled formation of volatile components in cider making using a combination of *Saccharomyces cerevisiae* and *Hanseniaspora valbyensis* yeast species

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Abstract The effect of pure and mixed fermentation by *Saccharomyces cerevisiae* and *Hanseniaspora valbyensis* on the formation of major volatile components in cider was investigated. When the interaction between yeast strains of *S. cerevisiae* and *H. valbyensis* was studied, it was found that the two strains each affected the cell growth of the other upon inoculation of *S. cerevisiae* during growth of *H. valbyensis*. The effects of pure and mixed cultures of *S. cerevisiae* and *H. valbyensis* on alcohol fermentation and major volatile compound formation in cider were assessed. *S. cerevisiae* showed a conversion of sugar to alcohol of 11.5%, while *H. valbyensis* produced alcohol with a conversion not exceeding 6%. Higher concentrations of ethyl acetate and phenethyl acetate were obtained with *H. valbyensis*, and higher concentrations of isoamyl alcohol and isobutyl were formed by *S. cerevisiae*. Consequently, a combination of these two yeast species in sequential fermentation was used to increase the concentration of ethyl esters by 7.41–20.96%, and to decrease the alcohol concentration by 25.06–51.38%. Efficient control of the formation of volatile compounds was achieved by adjusting the inoculation time of the two yeasts.

Keywords *Saccharomyces cerevisiae* · *Hanseniaspora valbyensis* · Mixed culture · Volatile compounds · Cider fermentation

Introduction

Cider—a common alcoholic beverage in North American and European countries [11, 13]—is a natural product resulting from a number of biochemical reactions. As predominant microorganisms, the activity of yeasts has an important impact on cider production, including transformation of sugars to alcohols and formation of cider flavor compounds [11]. The common practice in the cider industry of using starter cultures greatly reduces the risk of spoilage and of unpredictable changes in cider flavor, but might also be responsible for the loss of characteristic aroma and flavor determinants. The main features discriminating between cider products made by traditional and modern process technologies are differences in aroma and flavor [9–11]. Apart from the principal yeast *Saccharomyces cerevisiae*, non-*S. cerevisiae* species have attracted much attention as additions to mixed fermentation in fruit wine production because of their abilities to provide individual flavor characteristics to the product. Wine and cider fermentations share many similarities in microbiological flora [5, 13, 15]. Although the literature contains numerous reports on combining different yeast strains for wine making [2, 4, 8, 16], little has been published about mixed fermentation of *S. cerevisiae* and non-*S. cerevisiae* strains in cider making, or on the influence of such mixed cultures on the formation of volatile components.

Several studies of the different ecological properties of non-*S. cerevisiae* yeasts and their influence on the final product in mixed culture with *S. cerevisiae* have been reported [11, 21]. It is widely observed that non-*S. cerevisiae* yeasts predominate in spontaneous fermentation of wine, and that a succession of yeasts take place. Non-*S. cerevisiae* species are not as ethanol-tolerant as *S. cerevisiae*. Some yeasts, such as those of the genera *Torulaspora* and *Kloeckera*, are less tolerant to low available oxygen conditions than *S. cerevisiae* [3, 18]. *S. cerevisiae* is capable of rapid growth under strictly anaerobic conditions, while other yeasts grow poorly under these conditions [12, 13, 15, 16]. The different growth temperature requirements of *S. cerevisiae* and non-*S. cerevisiae* genera affect their relative contribution to the acids, sugars, and ethyl acetate in cider; their ability to form acetate esters is in fact regarded as a

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criterion for selecting suitable yeast strains [1]. Much recent research on microbiology for wine fermentation has indicated that non-*S. cerevisiae* yeasts commonly include genera and species, e.g., *Hanseniaspora* (*Kloeckera*) *valbyensis*, *Kloeckera apiculata*, *Metschnikowia pulculate*, *Candida stellata*, *Phichia*, *Schizosaccharomyces* and *Torulaspora*, that are able to produce flavor compounds affecting final product quality and make a positive contribution to flavor quality in the modern wine industry [15, 16, 21]. Among non-*S. cerevisiae* yeasts that show a high capacity for aroma compound formation and alcoholic fermentation, the genera *Hanseniaspora*, which has a high ability to form acetates, is frequently used as the principal yeast [1, 15]. Understanding how to control mixed yeast fermentation by exploiting the individual physiological properties of the mixed yeasts involved is of extreme importance in the production of fruit wine [6, 17, 18].

In the present work, a laboratory-scale study was performed to investigate the growth of pure culture and mixed culture fermentation of *S. cerevisiae* and *H. valbyensis*, and the formation of major volatile ethyl esters and higher alcohols. The objective was to understand mixed fermentation of yeasts and their metabolic interaction in cider production so as to control the formation of major volatile components by combining species of *S. cerevisiae* and *H. valbyensis* yeasts for cider making.

Materials and methods

Yeast strains and medium

Saccharomyces cerevisiae (CCTCC M 201022, China Center for Type Culture Collection, Wuhan, China), a typical cider yeast strain previously selected in our laboratory, was used [19, 20]. *H. valbyensis* CECT 10122 from *Coleccion Espanola de Cultivos Tipo* (Valencia, Spain) for wine making was also used. Yeasts were stored before use at 4°C in YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2.5% agar, pH 6.5).

Fermentation conditions

Concentrated apple juice (70.8°Brix, bright and enzymatically treated) was supplied by the Changyu Pioneer Wine Company (Shandong, China), diluted with distilled water (1:3.2) to 22°Brix, and adjusted to a titration acid concentration of 5.0 g/L by addition of malic acid. Pure fermentations with *S. cerevisiae* and *H. valbyensis* were carried out at 15°C in pre-sterilized 5 L flasks with 4,200 mL diluted apple juice. The diluted apple juice was inoculated with each yeast species to an initial concentration of 4–5×10^6 cfu/mL. Yeasts was cultured in 500 mL flasks for 48 h at 30°C. Mixed fermentation tests were conducted by addition of one yeast into a pure cultivation of the other. Prior to sampling, flasks were stirred magnetically for a few minutes to ensure homogeneity. Supernatants for analysis were obtained by centrifugation at 1,000 *g* at 4°C for 10 min.

Sample preparation and analytical methods

Apple juice and cider samples were filtered through 0.45 μm pore size membranes. Yeasts were enumerated on malt agar plates in triplicate following incubation at 30°C for 48 h, which allows yeast colonies of *H. valbyensis* to be distinguished morphologically from those of*S. cerevisiae* [14]. Sugar and ethanol were determined according to Ough and Amerine [14]. Volatile components were determined by HS-SPME (headspace solid phase micro-extraction) manual analysis using the following extraction procedure: 75 μm Carboxen-PDMS fiber (Supelco, Bellefonte, PA) for 40 min equilibration at 45°C with 25% sodium chloride. Quantitative analysis was performed with 2-octanol as internal standard in a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a Supelcowax^{TM-10} column [30 m length, 0.25 mm ID, 1 μm film thickness (Restek, Bellefonte, PA)]. The column carrier gas was helium at a flow rate of 27 cm/s. The oven temperature was programmed for a 1 min hold at 45°C, and then increased at a rate of 4°C/min to 100°C, followed by an increase in temperature to 190°C at a rate of 5°C/min, finishing with a 10 min hold at 190°C. Injector and detector temperatures were 250°C [19].

Results and discussion

Interaction between *S. cerevisiae* and *H. valbyensis*

Yeast–yeast interaction could lead directly to an influence on strain growth, and thus impact both process efficiency and product quality. Indeed, an interaction between *S. cerevisiae and H. valbyensis* was observed when sequential fermentation was performed. Figure 1 shows growth of *H. valbyensis* in pure fermentation and growth of a mixed yeast fermentation. The maximal cell concentration reached by *H. valbyensis* in pure culture was 1.34×10^8 cfu/mL, while in mixed yeast culture, the maximal*H. valbyensis* concentration decreased gradually to only 0.85×10^8, 0.25×10^8 and 0.10×10^8 cfu/mL with inoculation of 2.0×10^6 cfu/mL *S. cerevisiae* on day 3, day 9 and day 15, respectively. It required 6–7 days for the *H. valbyensis* population in pure culture to reach a maximum, followed by a rapid decrease after day 18. However, the population in mixed fermentation decreased about 3–6 days earlier than that in pure culture of *H. valbyensis*. Thus, in accordance with our understanding of yeast natural ecology, growth of the non-*S. cerevisiae* yeast was inhibited by *S. cerevisiae* due to yeast interaction. The effect of yeast inoculation time of

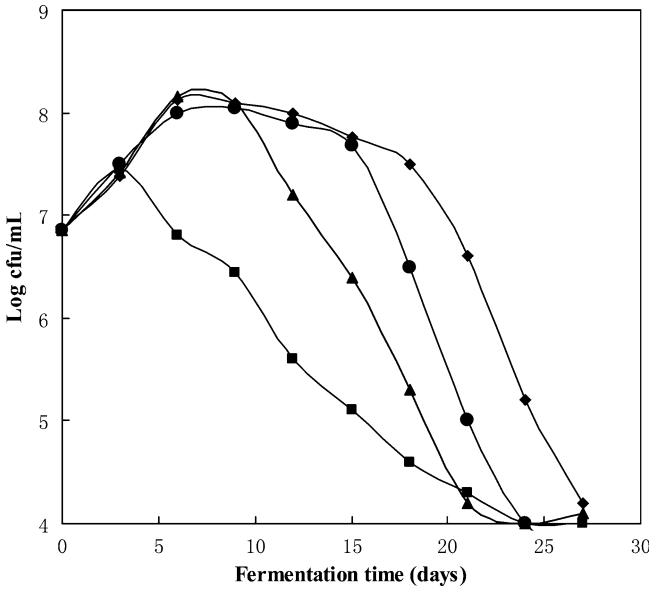

Fig. 1 Growth of *Hanseniaspora valbyensis* in pure culture (*filled diamonds*) and in mixed culture fermentations with *Saccharomyces cerevisiae* inoculated at 2×10^6 cfu/mL on day 3 (*filled squares*), day 9 (*filled triangles*), or day 15 (*filled circles*)

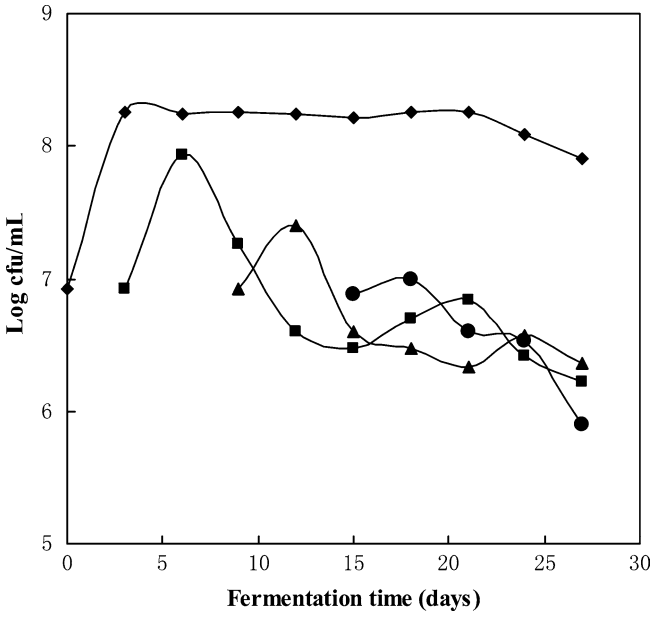

Fig. 2 Growth of *S. cerevisiae* in pure culture (*filled diamonds*) and in mixed culture fermentations with *H. valbyensis* inoculated at 2×10^6 cfu/mL with *S. cerevisiae* on day 3 (*filled squares*), day 9 (*filled triangles*), or day 15 (*filled circles*)

S. cerevisiae on yeast cell concentration during fermentation showed that the earlier *S. cerevisiae* is added, the greater the influence on non-*S. cerevisiae* yeast growth in the population, and the more quickly the lethal phase will occur. Although the population of *S. cerevisiae* in pure cultivation increased to a maximum of 1.36×10^8 cfu/mL during the 27 day fermentation, inoculation of *H. valbyensis* at a concentration of 2.0×10^6 cfu/mL likewise hampered the growth of *S. cerevisiae*, resulting in maximal *S. cerevisiae* cell concentrations of only 0.31×10^8, 1.45×10^8 and 1.26×10^8 cfu/mL (Fig. 2). Compared to *H. valbyensis*, *S. cerevisiae* in pure culture went through its logarithmic phase within 3 days, thereafter maintaining stationary phase until the 21st day. The results of this investigation differ from those of an earlier report in which growth of *S. cerevisiae* did not seem to be affected by the presence of *H. valbyensis* [1]. Although there are unanswered questions as to how *S. cerevisiae* interacts with non-*S. cerevisiae* yeasts, nutrient limitation probably plays a role [6].

Influence of pure and mixed fermentation on alcoholic fermentation and formation of volatile compounds

The effect of pure and mixed cultures on cider fermentation involves alcoholic fermentation and the formation of flavor compounds. In addition to its role in fermentation efficiency, alcoholic fermentation is the major activity of yeast contributing to cider flavor [7]. Table 1 shows a comparison of sugar reduction and alcohol formation in pure fermentation: *S. cerevisiae* converted sugar to alcohol with 11.5% alcohol content and 3.47% residual sugar after a 31-day cider fermentation, but *H. valbyensis* does not tolerate alcohol concentrations exceeding 6% and has 30.21% residual sugar after a 40-day cider fermentation, in accordance with other reports from wine fermentation [2]. Consequently, sequential fermentation, i.e., inoculating *S. cerevisiae* at a concentration of 2.0×10^6 cfu/mL on day 3 of a fermentation of *H. valbyensis*, resulted in a higher alcohol conversion efficiency, with 3.88% residual sugar following a 31-day cider fermentation.

This difference between fermentation with pure cultures of *S. cerevisiae* and *H. valbyensis* resides not only in their differential ability to reduce sugars and produce alcohol, but also in the concentration of major volatile compounds produced by each individual yeast. Table 2 lists the principle alcohols and esters in cider produced from pure yeast fermentation as determined by HS-SPME. Among the 13 major esters and 6 higher alcohols identified, the concentrations of ethyl acetate, phenethyl acetate, isoamyl alcohol and phenyl alcohol in cider fermentation by *S. cerevisiae* differed greatly from the amounts of those compounds produced by *H. valbyen-*

Table 1 Effects of pure and mixed culture on ethanol and residual sugar in cider fermentation

Fermentation	1[a]	2[b]	3[c]
Total residual sugar as glucose (g/L)	30.21	3.47	3.88
Ethanol (%, v/v)	6.0	11.5	11.5
Fermentation time (days)	40	31	31

[a]Addition of 4×10^6 cfu/mL *Hanseniaspora valbyensis* on day 1
[b]Addition of 4×10^6 cfu/mL *Saccharomyces cerevisiae* on day 1
[c]Inoculated with 1×10^2 cfu/mL *H. valbyensis* on day 1. Inoculated with 2×10^6 cfu/mL *S. cerevisiae* on day 3

Table 2 Effects of pure and mixed culture fermentation on major volatile alcohols and esters in cider. Data represent the average of three replicate analyses (RSD 2.7–6.0%)

Compounds	1[a] (mg/L)	2[b] (mg/L)	3[c] (mg/L)
Esters			
Ethyl acetate	87.74	15.40	48.09
Isoamyl acetate	0.68	0.44	1.53
2-Phenethyl acetate	18.46	3.17	6.43
Ethyl butyrate	0.00	0.46	0.00
Ethyl hexanoate	1.56	3.61	2.67
Ethyl lactate	1.20	2.20	1.90
Ethyl octanoate	0.04	1.02	0.23
Ethyl decanoate	0.21	0.47	0.61
Ethyl 9-decenoate	0.05	0.11	0.08
Diethyl succinate	0.15	0.40	0.31
Ethyl palmitate	0.20	0.30	0.25
Total esters	110.29	27.58	62.10
Alcohols			
1-Propanol	7.87	7.09	7.99
Isobutyl alcohol	24.90	42.43	48.80
1-Butanol	2.87	5.47	3.29
Isoamyl alcohol	45.35	232.05	125.36
1-Hexanol	2.10	3.05	2.40
2-Phenylethyl alcohol	7.15	38.01	2.13
Total alcohol	90.24	328.11	189.98
Total	200.53	355.69	252.08
A/E[d]	0.82	11.90	3.06

[a]Inoculated with4×10^6 cfu/mL *H. valbyensis* on day 1
[b]Inoculated with 4×10^6 cfu/mL *S. cerevisiae* on day 1
[c]Inoculated with 1×10^2 cfu/mL *H. valbyensis* on day 1. Inoculated with 2×10^6 cfu/mL *S. cerevisiae* on day 3
[d]Ratio of total concentration of alcohols to total concentration of esters

Table 3 Formation of the major volatile alcohols and esters in sequential mixed culture fermentation for cider production. Data represent the average of three replicate analyses (RSD 2.7–6.0%)

Compound	1[a] (mg/L)	2[b] (mg/L)	3[c] (mg/L)	4[d] (mg/L)
Esters				
Ethyl acetate	14.08	54.34	65.05	69.92
Isoamyl acetate	0.43	0.47	0.47	0.49
2-Phenethyl acetate	0.61	7.37	9.37	11.17
Ethyl butyrate	0.35	0.15	0.00	0.00
Ethyl hexanoate	3.89	2.50	2.00	1.98
Ethyl lactate	1.50	1.02	1.03	0.70
Ethyl octanoate	1.20	1.07	0.98	0.20
Ethyl decanoate	0.54	0.39	0.38	0.48
Ethyl 9-decenoate	0.13	0.07	0.08	0.09
Diethyl succinate	0.35	0.24	0.25	0.23
Ethyl palmitate	0.70	0.50	0.42	0.35
Total esters	23.78	68.12	80.03	85.60
Alcohols				
1-Propanol	4.50	5.43	6.00	6.20
Isobutyl alcohol	37.52	29.84	32.20	23.70
1-Butanol	5.98	3.60	3.35	2.97
Isoamyl alcohol	251.06	135.79	121.03	39.98
1-Hexanol	2.93	2.63	2.58	2.50
2-Phenylethyl alcohol	35.29	25.16	20.23	14.59
Total alcohols	337.28	202.45	185.39	89.94
Total	361.06	270.58	265.43	175.55
A/E[e]	14.18	2.97	2.32	1.05

[a]*S. cerevisiae* only
[b]*H. valbyensis* culture with addition of 2×10^6 cfu/mL *S. cerevisiae* on day 3
[c]*H. valbyensis* culture with addition of 2×10^6 cfu/mL *S. cerevisiae* on day 9
[d]*H. valbyensis* culture with addition of 2×10^6 cfu/mL *S. cerevisiae* on day 15
[e]Ratio of total concentration of alcohols to total concentration of esters

sia. As a result of mixed fermentation, the higher concentration of ethyl esters produced by *H. valbyensis* and the higher amount of alcohols formed by *S. cerevisiae* proves that the impact of volatile compounds in cider production can potentially be controlled by sequential fermentation with different mixed yeasts.

The diversity of yeast physiological characteristics and yeast interactions results in the formation of different volatile components contributing to cider flavor depending on the time of inoculation of *S. cerevisiae* in *H. valbyensis* fermentation. Table 3 shows that the concentration of major higher alcohols and esters in the cider differed depending on whether *S. cerevisiae* (at a concentration of 2.0×10^6 cfu/mL) was added to the *H. valbyensis* fermentation on day 3, day 9 or day 15. Compared to pure fermentation with *S. cerevisiae*, mixed fermentation resulted in an obvious increase in the total concentration of esters, from 23.78 mg/L in the pure fermentation cider to 68.12–85.60 mg/L in mixed fermentation—an increase of 7.41–20.96%. This was accompanied by a simultaneous decrease in the total concentration of alcohols, from 361.06 mg/L in pure fermentation to 175.55–270.58 mg/L in mixed fermentation—a reduction of 25.06–51.38%. In this study, the major contributors to variations in total esters were ethyl acetate and phenethyl acetate, which are considered to be the main impact aroma compounds in cider [9], and those for alcohols were isoamyl alcohol and

isobutyl alcohol. This change in the content of esters and higher alcohols, the major volatile flavor compounds, results in differences in the ratio of alcohol to esters (A/E), which affects cider product quality and balances the flavor components, although total alcohol and esters ($A + E$) was reduced greatly to 48.62 mg/L, or 74% of the 361.06 mg/L present in pure fermentation cider. Because of these changing levels in mixed fermentation, the formation of volatile compounds can be controlled and adapted for quality purposes by adjusting the inoculation time of the yeast strains. These observations have stimulated industrial interest in these yeasts with the aim of identifying differences between yeast strains and to combine them to direct formation of major flavor compounds in cider production.

Although the interactions that exist between yeast strains of *S. cerevisiae* and *H. valbyensis* may cause inhibition of cell growth for both strains, their interactive growth and biochemical activities, as well as their characteristics for formation of volatile flavor compounds such as higher alcohols and esters can be effectively exploited to control the formation of volatile components, i.e., an increase in volatile esters and a decrease in higher alcohols can be achieved by sequentially inoculating the two strains in cider fermentation. This difference from fermentation using pure cultures of *S. cerevisiae* and *H. valbyensis* is reflected not only in the conversion of sugar to alcohol, but also in the formation of the major volatile compounds produced by these yeasts. The higher concentration of ethyl acetate and phenethyl acetate produced by *H. valbyensis*, together with the higher concentration of isoamyl alcohol and isobutyl alcohol with *S. cerevisiae*, means that the combination of these two yeast species in sequential fermentation leads to increased ester and decreased alcohol concentrations, allowing efficient control of the formation of volatile compounds by adjusting the time of inoculation of the two yeasts.

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References

1. Bilbao A, Irastorza A, Duenas M, Fernandez K (1997) The effect of temperature on the growth of strains of *Kloeckera apiculata* and *Saccharomyces cerevisiae* in apple juice fermentation. Lett Appl Microbiol 24:37–39
2. Fleet GH (2003) Yeast interactions and wine flavor. Int J Food Microbiol 86:11–22
3. Hansen EH, Nissen P, Sommer P, Nielsen JC, Arneborg N (2001) The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. J Appl Microbiol 91:541–547
4. Heard GM, Fleet GH (1985) Growth of natural yeast flora during the fermentation of inoculated wines. Appl Environ Microbiol 50:727–728
5. Heard GM, Fleet GH (1986) Occurrence and growth of yeast species during the fermentation of some Australian wines. Food Technol Aust 38:22–25
6. Heard GM, Fleet GH (1988) The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. J Appl Bacteriol 65:23–28
7. Henschke P (1997) Wine yeast. In: Zimmerman FK, Entian KD (eds) Yeasts sugar metabolism, biochemistry, genetics, biotechnology and application. Technomic, Lancaster, UK, pp 527–560
8. Herrero M, Roza C, García LA, Díaz M (1999) Simultaneous and sequential fermentations with yeast and lactic acid bacteria in apple juice. J Ind Microbiol Biotechnol 22:48–51
9. Jarvis B, Forster MJ, Kinsella WP (1995) Factors affecting the development of cider flavour. J Appl Bacteriol Symp Suppl 79:5S–18S
10. Laplace JM, Apery S, Frere J, Auffray Y (1998) Incidence of indigenous microbial flora from utensils and surrounding air in traditional French cider making. J Inst Brew 104:71–74
11. Lea AGH (1995) Cider making. In: Lea AGH, Piggott JR (eds) Fermented beverage production. Blackie, London, pp 66–67
12. Mora J, Barbas JI, Mulet A (1990) Growth of yeast species during the fermentation of musts inoculated with *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. Am J Enol Vitic 41:156–159
13. Morrissey WF, Davenport B, Querol A, Dobson ADW (2004) The role of indigenous yeasts in traditional Irish cider fermentations. J Appl Microbiol 97:647–655
14. Ough CS, Amerine MA (1988) Methods for analysis of must and wines. Wiley, Davis, CA
15. Rojas V, Gil JV, Pinaga F, Manzanares P (2003) Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int J Food Microbiol 86:181–188
16. Romano P, Fiore C, Paraggio M, Caruso M, Capece A (2003) Function of yeast species and strains in wine flavour. Int J Food Microbiol 86:169–180
17. Swaffield CH, Scott JA, Jarvis B (1997) Observations on the microbial ecology of traditional alcoholic cider storage vats. Food Microbiol 14:353–361
18. Tahía B, Lucas DC, Andres A, Jaime C, Cerdáolmedo E (1983) Selection of wine yeasts for growth and fermentation in the presence of ethanol and sucrose. Appl Environ Microbiol 45:1429–1436
19. Wang LP, Xu Y, Zhao GA, Li JM (2004) Rapid analysis of flavor volatile in apple wine using headspace solid-phase microextraction. J Inst Brew 110:57–65
20. Wang D, Xu Y, Hu J, Zhao GA (2004) Fermentation kinetics of different sugars by apple wine yeast *Saccharomyces cerevisiae*. J Inst Brew 110:340–346
21. Zohre DE, Erten H (2002) The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. Process Biochem 38:319–324