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Research Article

A laboratory yeast strain suitable for spirit production

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Abstract

Yeast strains of the species *Saccharomyces cerevisiae* currently in use for the production of consumable alcohols such as beer, wine and spirits are genetically largely undefined. This prevents the use of standard genetic manipulations, such as crossings and tetrad analysis, for strain improvement. Furthermore, it complicates the application of the majority of modern methods developed in yeast molecular biology. Here we used two haploid laboratory strains with suitable auxotrophic markers for the construction of a genetically well defined, prototrophic diploid production strain. This strain was tested for its fermentative and sensory performances in comparison to commercially available yeasts. Three different fruit mashes (cherries, plums and pears) were fermented in a 90 kg scale. These were then subjected to distillation and used for the production of spirits with a final ethanol content of 40% (v/v). Fermentation parameters assayed included growth, sugar utilization, ethanol production and generation of volatile compounds, higher alcohols and glycerol. The spirits were also tested for their sensory performances and the data obtained statistically consolidated. Our results clearly demonstrate that this laboratory strain does not display any disadvantage compared with commercial yeasts in spirit production for any of the parameters tested, yet it offers the potential to apply both classical breeding and modern molecular genetic techniques for adjusting yeast physiology to special production schemes. Copyright © 2004 John Wiley & Sons, Ltd.

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Introduction

Long before Pasteur's demonstration that yeasts are the agents that cause alcoholic fermentation, they had been in practical use for the production of beer, wine and spirits (reviewed in Barnett, 1997; Huxley, 1873). Whereas traditionally these yeasts were derived from the raw material employed (i.e. yeasts colonize the fruit to a minor extent and constantly increase in number after the onset of fermentation), modern industrial standards recommend the addition of cultured *Saccharomyces cerevisiae* strains to speed up fermentation and to avoid production of deleterious metabolites by biological contaminants (see Grossmann *et al*., 2000, and references therein).

The yeast strains commonly employed for alcohol production are genetically largely undefined and highly heterogenous (Benitez *et al*., 1996). Thus, little is known about their chromosomal constitution and aneuploidy is frequently observed (Vezinhet, 1981; Bidenne *et al*., 1992; Cardinali and Martini, 1994). Moreover, sporulation is extremely poor (if observed at all) and is accompanied by poor spore viabilities (Maraz, 2002). Therefore, such strains cannot be manipulated by most techniques developed for classical yeast genetics (i.e. sporulation, crossing, tetrad analysis) that would be analogous to breeding in plant and animal genetics. The methods usually employed to manipulate yeast in modern molecular genetics are limited to the use of dominant selectable markers due to the uncertainties in genomic composition and the lack of auxotrophic markers (Pretorius, 2000).

These features of industrial yeast strains explain the huge gap between the vast amount of knowledge gathered on the genetics and physiology of *S. cerevisiae* (for summaries, see e.g. Walker, 1998; Sherman, 2000) and its application in fermentation industries. The genome of *S. cerevisiae* was the first of a eukaryotic organism to be completely sequenced (Goffeau *et al.*, 1996; Zagulski *et al.*, 1998). Functional analysis resulted in the availability of single deletion mutants in more than 5000 chromosomal gene copies (e.g. available from EUROSCARF, Frankfurt, Germany; **http://www.srd-biotec.de/deeuroscarf.html**). In particular, the genetics and physiology of glycolysis and sugar transport, as a basis for alcoholic fermentation, have been extensively studied (reviewed in Heinisch and Hollenberg, 1993; Boles and Hollenberg, 1997). This basic knowledge has resulted in comparatively few attempts to engineer industrial yeast strains. These include the production of amylases for brewing purposes (Jansen and Pretorius, 1995) of peptide antibiotics in wine strains (Pretorius, 2000) and the reduction of the presumed cancerogenic compound urethane, by manipulating arginine metabolism in sake yeasts (Kitamoto *et al.*, 1991). Certain strains have also been genetically engineered to produce esterases in order to enhance the generation of volatile compounds (Hirata *et al.*, 1992; Lee *et al.*, 1995).

The use of laboratory yeast strains for industrial purposes has so far been prevented by the general belief that they are not as competitive as natural isolates. With microbiological contaminants inherent in the raw materials employed, they are thought to have lower fermentative capacities and to produce undesirable flavour compounds, due to differences in their secondary metabolism. Although it has been frequently claimed that laboratory yeast strains display these characteristics, comparatively few sound scientific studies have been published in this respect (Tuite, 1992; Bothast *et al.*, 1999; Gimren-Alcaniz and Matallana, 2001; Romano *et al.*, 2003). Recently, laboratory strains have been developed with improved genetic and physiological performances (van Dijken *et al.*, 2000). The latter work showed that one of these strains, CEN.PK122, was amenable to genetic, physiological and biochemical studies under controlled laboratory conditions. It performed sufficiently well in batch cultures as well as in steady-state chemostat cultures in defined mineral media and displayed growth rates, sugar utilization capabilities and biomass yields similar to the other strains tested.

Spirit production on a commercial basis differs from controlled laboratory conditions in various aspects. Due to the differences in fruit composition, yeast strains used for fermentation have to adapt to different environments (e.g. sugar compositions and concentrations, presence of organic acids, etc.). In addition, depending on the fruit of choice and varying climatic conditions, the yeast employed has to compete for sugar utilization with other microorganisms present in the mashes, e.g. other yeast species such as *Kloeckera apiculata* (= *Hanseniaspora uvarum*), and with bacteria such as various lactic acid bacteria (Pieper *et al.*, 1993; Narendranath *et al.*, 1997). Furthermore, a major quality of spirits lies in their flavour compounds, rather than merely the speed and amount of ethanol production. The most abundant esters and higher alcohols in fermented beverages are ethyl acetate, isoamyl acetate, amyl alcohols and isobutanol (Renger *et al.*, 1992). Interestingly, different yeast strains will usually produce individual ester- and alcohol profiles when fermenting similar media (Younis and Steward, 1998). On the other hand, the type of sugar being fermented also affects volatile compound production (Engan, 1972; Pollock and Weir, 1976; Gil *et al.*, 1996; Pretorius, 2000). Owing to the distillation following the fermentation process, secondary fermentation compounds produced by the yeast may be concentrated in spirits and preclude product consumption (Piggott, 1983; Postel and Adam, 1989; Meinl, 1995). Such profound differences between applied and laboratory growth conditions have to be taken into account when adjusting a laboratory yeast strain for production purposes.

We used here a derivative of the *S. cerevisiae* CEN.PK122 strain (van Dijken *et al.*, 2000) to test its performance on the fermentation of different fruit mashes under medium-scale production conditions. In contrast to the general opinion, our results demonstrate that the laboratory strain HHD1 performs in a similar manner to the industrial breeds for all parameters tested.

Materials and methods

Yeast strains employed

Apart from the laboratory strain (HHD1) constructed here (*MAT***a**/α *ura3-52/URA3 leu2-3,112/LEU2 MAL2-8^C/MAL2-8^C SUC2/SUC2*, obtained from a cross of CEN.PK113-5D with CEN.PK113-16B; Entian and Kötter, 1998), the haploid parents and two representative spirit production strains of *S. cerevisiae* were employed: *Siha Aktiv6* (manufacturer's trade name, further abbreviated herein as 'Siha') and *Uvaferm CGC62* (manufacturer's trade name, further abbreviated herein as 'Uvaferm'; both purchased from Begerow GmbH & Co., Langenlonsheim, Germany). The latter two strains were packaged as dried yeast in 500 g aliquots. First, experiments with these strains were performed in pilot scales (1.5 kg) to observe their general fermentation behaviour (data not shown). Based on these results, fermentations on a technical scale (90 kg) which are reported here were initiated.

Media, culture conditions and growth determinations

Rich media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with 2% glucose (YEPD). Haploid strains were also grown on YEPD and tested for markers on minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids, adenine and uracil (Sherman *et al*., 1986), with the omission of uracil or leucine as required. For growth on plates 1.5% agar was added to the described media. All strains were incubated at 30 °C.

For small scale experiments, strains were grown in YEPD and inoculated to $OD_{578} = 0.1$ in 1.5 kg apple mashes, cultivar Rubinette (Cox-Orange × Golden Delicious). Growth rates were determined in cherry mashes on an even smaller scale of 0.8 kg. For this purpose, the fruit was stoned and passed through a filter to obtain a homogenous medium. The mashes were inoculated with $OD_{578} = 0.05$ and samples regularly taken over a period of 120 h. Cells were plated from appropriate dilutions onto YEPD and after 2 days of incubation at 30 °C the number of colony forming units (cfu) was determined. Experiments for each strain were performed in duplicate.

For spirit production scales, strains were grown in 5 ml YEPD overnight on a rotor shaker (30 °C, 140 rpm), transferred into 500 ml shake-flask cultures with fresh YEPD, incubated for 12 h and harvested by centrifugation (3500 × *g* for 5 min at room temperature). Cell pellets were washed twice with 25 ml NaCl/peptone (0.85% NaCl, 0.05% peptone), resuspended in 25 ml of the same medium and transferred to 1.5 l YEPD in 3 l shake flasks. After 20 h incubation at 30 °C at 140 rpm, yeasts from each culture were again harvested by centrifugation (3500 × *g* for 10 min at room temperature), washed twice as described above and resuspended in 100 ml NaCl/peptone solution. The cell density was calculated by optical measurements at 578 nm in appropriate dilutions, assuming that 1 $OD_{578} = 10^7$ cells/ml. From this, the yeasts were added to the mashes at a final density of 10^6 cells/ml each.

Sugar utilization

All strains were tested for their ability to utilize different sugars. The utilization of glucose, fructose, galactose, maltose, sucrose and raffinose was tested on agar plates. The media were based on 1% yeast extract and 2% bacto peptone (Difco) and supplemented with the particular sugar (at 1% concentration). The strains were also incubated in the respective media in liquid at 30 °C. After 12 h at 140 rpm the cells were harvested, washed and resuspended in NaCl/peptone solution to $OD_{578} = 1$. Serial dilutions of 10^{-1}–10^{-5} in NaCl/peptone solution were prepared and 5 µl of each dropped onto rich medium containing the respective sugar and 3 ppm antimycin A (Sigma-Aldrich, Germany) to block respiration. Growth was assessed after 3 days of incubation at 30 °C by visual inspection. Each experiment was performed in triplicate.

Raw materials and mashing process

For pilot scale experiments (0.8 kg or 1.5 kg, as indicated) apple mashes, cultivar Rubinette (Cox-Orange × Golden Delicious) inoculated with the yeast strains listed above were used. Technical scale studies (90 kg) were performed on three different fruit mashes: cherries (cv. Dollenseppler), plums (cv. Ersinger Frühzwetschge) and pears (cv. Bartlett).

Mashes were prepared according to standard procedures. The pip fruits were washed and crushed

by passing them through a rasp mill (Wahler, Stuttgart, Germany). The stone fruits (with peduncles removed) were washed and subsequently chopped using a drill machine attached to a beater, so that the stones remained undamaged. Immediately after this, the mashes were adjusted to pH 3.0–4.0 with sulphuric acid (technical grade). After 24 h fermentation, the pH was again adjusted to 3.0. To increase the decomposition of pectin, pectinolytic enzyme was added to the pip fruit mashes (Pectinex Ultra SP-L; Novoenzymes, Denmark at 8 ml/l mash). No pectinolytic enzyme was added to the stone fruit mashes.

Fruit and mash qualities

The cherries were in an excellent condition, like fresh dessert fruit. No bruised or decayed fruit were present. This minimizes the risk of a bacterial contamination. The pears were generally in a faultless condition. Foul fruit were removed. They were stored at 15 °C for a few days to achieve a doughy consistence which facilitates the mashing process. The plums were windfallen and therefore, microbiologically, in a more critical condition. To reduce the amount of by-products formed by spontaneous fermentation (which had already begun in the plum mashes), and to cope with the presumed higher load of bacterial contamination, the plums were processed immediately.

The mashes were divided into approximately 90 kg lots and transferred into 120 l vessels. The vessels were then sealed with a fermentation bung and incubated with the various yeast strains. All experiments were performed at least in triplicate and different parameters, such as ethanol yields, extract, sugar utilization, sugar content, yeast metabolites and pH values, were determined at regular intervals during the fermentation period.

Fermentation

The mashes (prepared as described above) were inoculated with the selected commercial yeast strains or the laboratory strain HHD1 (all standardized to be in the same physiological state and cell density as described above) and fermented to completion at 15–17 °C. During fermentation, the mashes were agitated and samples were collected and analysed with regard to the different parameters, as indicated in Results.

Analytical methods

During fermentation, changes in the pH were monitored using a pH meter 521 (WTW, Weilheim, Germany). As a preliminary indication, the decrease in fermentable carbohydrates (% sugar) was determined with a hand refractometer (Carl Zeiss, Jena, Germany) and the synthesis of ethanol was determined using steam-distillation (Vapodest 20, Gerhardt, Bonn, Germany) and a density-meter DMA48 (Paar Physica, Graz/Strassburg) according to the standard procedures described in Chemisch-Technische Bestimmungen (1980). The total (titratable) acidity was measured by titration with NaOH and calculated in tartaric acid equivalents according to Adam *et al*. (1995).

For the determination of various yeast metabolites and the compounds ethyl acetate and 3-methylbutyl acetate, 2-methyl-1-propanol, 1-propanol and the isoamyl alcohols (3-methyl-1-butanol and 2-methyl-1-butanol), a headspace gas chromatograph (Perkin-Elmer HS40, GC 8420) equipped with a packed crossbond phenylmethylpolysiloxane column (Rtx-volatiles; 60 m × 0.32 mm, film thickness 1.5 μm; Resteck GmbH, Bad Homburg, Germany), a flame ionization detector and a CLASS VP 4.2 integrator (Shimadzu, Duisburg) were employed. As an internal standard, *n*-Butanol (200 mg/l; purchased from Merck, Darmstadt, Germany) was used. For mashes, the method described in Brautechnische Analysenmethoden (1996) and according to Boettger and Pieper (1994) was used. The following temperature profile was employed: 50 °C for 7 min, rising to 120 °C at 15 °C/min, held at 120 °C for 2 min, then rising to 250 °C at 20 °C/min. The profile for the spirit analyses was: 60 °C for 2 min, rising to 70 °C at 2 °C/min, further rising to 160 °C at 8 °C/min, held at 160 °C for 2 min, and rising further to 200 °C at 4 °C/min and to 250 °C at 15 °C/min, with a final hold at 250 °C for 10 min. Nitrogen was applied at a flow rate of 5 ml/min, hydrogen/synthetic air at 30 ml/min and helium as the carrier gas at 1.7 ml/min. All gases were supplied by Sauerstoffwerk GmbH (Friedrichshafen, Germany).

The exact decrease of the fermentable sugars (glucose and fructose) and the formation of the volatile compounds acetic acid, propionic acid and lactic acid, as well as the exact ethanol content, were determined by HPLC (Bischoff Modell 2200

HPLC using a Bischoff Modell 728 Autosampler; Bischoff, Leonberg, Germany), using an Aminex HPX-87 H column (Biorad, Munich, Germany), a RI detector ERC7510 (ERC, Altegolfsheim, Germany) and a McDAcq15 Integrator (Bischoff, Leonberg, Germany). 0.1 N sulphuric acid (technical grade) was used for elution.

Distillation

After 6–8 weeks of fermentation, the mashes were distilled using a 120 l copper pot (Jacob-Carl, Göppingen, Germany) fitted with an enrichment section consisting of three bubble plates, a dephlegmator and a cyan catalyst (Holstein, Markdorf, Germany). This modern plant allows for distillation under technical and standard conditions. The dephlegmator was run at a flow rate of 120 l/h. The catalyst was used for cherries and plums, but not for pears. Fermented cherry mashes were distilled with one plate in operation, pears and plums with two plates. The distillates were collected in fractions of volume 250–300 ml each. In the vicinity of the switching points (heads to product fractions and product fractions to tailings) smaller volumes of 150 ml were collected. The heads were identified with the detaching test determining acetaldehyde according to Pieper *et al*. (1987). The tailings were screened by organoleptic assessment.

Spirit fractions

The product fractions were stored for at least 1 week at 17 °C, then diluted with deionized water to an alcohol content of 40% (v/v), cold filtered at 4 °C (Macherey Nagel, Düren, Germany) and kept for another 4 weeks at 17 °C prior to further analysis and sensoric assessment. Heads and tailings were discarded.

Sensoric evaluations

The spirits produced with the three different yeast strains were tested and assessed for their characteristic flavour quality using order-of-precedence and triangle tests (Jellinek, 1981). Sensoric evaluation of the spirits was conducted with a panel of at least 10 judges, previously trained for their ability to correctly match spirits. To enhance statistical significance, larger panels (>20 persons) with a short introductory training were also employed.

Statistical analyses

Data were analysed by the statistical software SigmaStat (Jandel Scientific) using one-way ANOVA on ranks. This non-parametric test compares several different experimental groups which received different treatments (for the purposes of this study, the only parameter difference being the three yeast strains employed). To isolate the group or groups that differed, all pairwise multiple comparison procedures (according to the Student–Newman–Keuls method) were performed at 5% significance level (Fox *et al*., 1995). Means of mash samples were not compared because of an inferior replication extent ($n = 2$).

Results

Growth characteristics of commercial and laboratory yeast strains

The aim of this work was to obtain a strain of *S. cerevisiae* suitable for the production of various spirits that is amenable to both classical and modern genetic manipulations. For this purpose we chose two haploid segregants from an isogenic series previously shown to perform well in chemostat cultures (van Dijken *et al*., 2000). The diploid strain (HHD1) is prototrophic for all amino acid and base requirements but heterozygous for the *ura3-52* and the *leu2-3,112* markers, which are commonly used for genetic manipulations. First, we assessed the ability of this strain to ferment different carbon sources (glucose, fructose, maltose, galactose, sucrose and raffinose) in a serial dilution test on rich medium containing antimycin A to block respiration. Simultaneously, we also tested the two industrial strains (Siha and Uvaferm) on the same medium (Figure 1A). No significant difference was observed for any of the strains, indicating that the commonly available fruit sugars serve equally well as carbon sources (note that the Uvaferm strain seems to be more suitable for fermentation of raffinose than the other strains tested).

In order to assess the performance of the different strains under applied fermentation conditions, we inoculated cherry mashes at lower cell densities of $OD_{578} = 0.05$ (in a small scale of 0.8 kg) and determined growth at 30 °C by plate counts. Again, no significant differences in viabilities were observed between the commercial preparations and

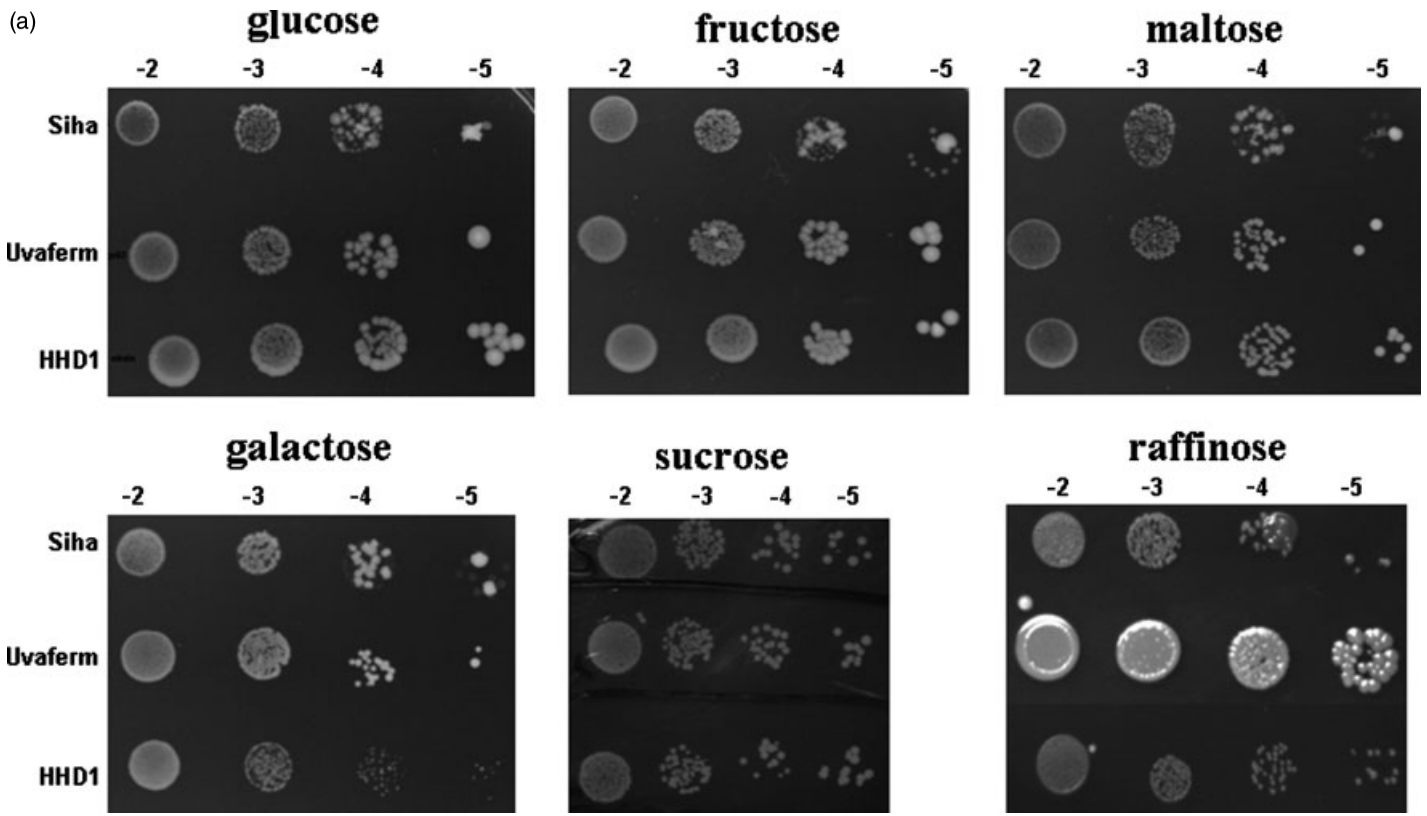

Figure 1. Sugar utilization and growth characteristics of commercial and laboratory yeast strains. (A) Strains were pregrown in liquid rich media containing the indicated sugars at 1% concentrations. Serial dilutions of 10^{-2}–10^{-5} were prepared in NaCl/peptone solution and 5 μl of each was dropped onto rich media containing 1% of the sugars, as indicated. Respiration was blocked by the addition of 3 ppm antimycin A to the plates. Growth was assessed after 3 days of incubation at 30 °C. (B) Cherry mashes, prepared and filtered as described in Materials and methods, were inoculated with the yeast strains indicated and incubated at 17 °C. Samples were taken at the indicated times, diluted and plated onto YEPD to give rise to approximately 100 colonies/plate. Viable cell densities in the mashes were calculated from these counts. CEN.PK113-5D and CEN.PK113-16B are the haploid parental laboratory strains from which the diploid strain HHD1 has been derived. cfu, colony-forming units

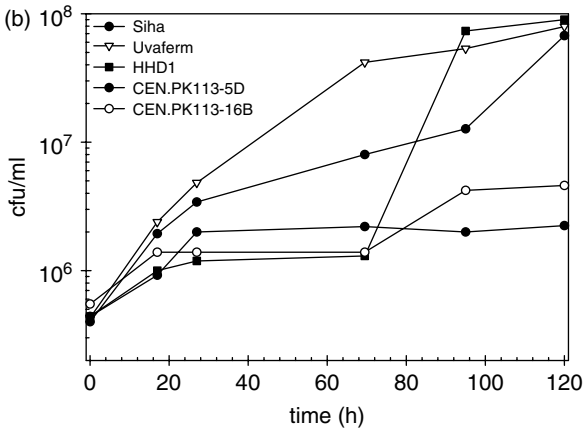

Figure 1. Continued

the diploid laboratory strain (Figure 1B). Although the latter showed an elongated lag in the initial growth phase, it reached the same cell density as the commercial strains after approximately 90 h of fermentation. It is generally assumed that yeast strains for industrial purposes need at least a diploid set of chromosomes to cope with the requirements of good fermentation capacity (Oda and Ouchi, 1989). This assumption was confirmed for our laboratory strain when we tested the haploid parental strains. Neither could compete for growth or for fermentation capacity with their diploid derivative or the industrial yeasts (Figure 1B).

Analyses of fermentation parameters in mashes

To assess the fermentation capacity of the laboratory yeast strain (HHD1) under real production conditions, different fruit mashes (cherries, plums and pears; 90 kg each) were inoculated in triplicate with 10^6 yeast cells/ml and allowed to ferment under semi-anaerobic, non-sterile conditions at low temperatures (see Materials and methods). As controls, both the Siha and the Uvaferm yeasts were tested under the same conditions. Samples were taken weekly for microscopic examination and it was confirmed that no significant bacterial contaminations were present within the mashes. However, growth of a layer of wild yeast contaminants on the surface, due to exposure to oxygen during sampling, was observed to a similar extent in all cases. To assess the amount of fermentable carbohydrates present in the mashes, we determined their refraction index with the assumption that % Plato corresponds to approximately 1 g sucrose/100 g mash liquid (Schobinger *et al*., 2001). The approximate alcohol content in different samples was determined by steam distillation. Table 1 summarizes the data obtained for the different fruit mashes, including the theoretical and practical alcohol yields. As expected from the higher initial sucrose content, the highest yields for all strains tested were obtained in the cherry mashes, as opposed to those of plums and pears. Comparison of the three yeast strains within each of the mashes did not reveal significant differences in the final alcohol concentrations.

As a more accurate measure of yeast metabolic activity, we determined the kinetics of glucose degradation and the increase in ethanol concentrations over a period of 48–55 days by HPLC (Figure 2; the kinetics of fructose degradation was also determined and behaved similar to the ones of glucose after a longer lag-phase; data not shown). A steady state was reached after a maximum of

Table 1. Sugar content in mashes and alcohol yields after fermentation

	% Plato[a]									
	Siha		Uvaferm		HHD1		Theoretical alcohol	Observed alcohol yield in mashes[c]		
Mash	Initial	Final	Initial	Final	Initial	Final	yield[b]	Siha	Uvaferm	HHD1
Cherries	20.4	4.1	20.4	4.2	20.4	4.3	7.33	7.45	6.69	7.84
Plums	12.5	1.8	12.5	2.0	12.5	1.8	4.26	4.62	4.92	5.28
Pears	11.6	2.2	11.6	2.4	11.6	2.2	4.13	4.67	4.70	4.47

[a] % Plato equals % mas sucrose/100 g mash liquid.
[b] The theoretical alcohol yield was calculated as l alcohol/100 l mash [(%Plato — non-fermentable substances) × 0.56 × TF], with TF values: cherries = 0.85, plums = 0.885, pears = 0.91. Non-fermentable substances: cherries = 5.0%, plums = 4.0%, pears = 3.5%.
[c] Calculated as total alcohol content after distillation (v/v) × l distillate/l mash

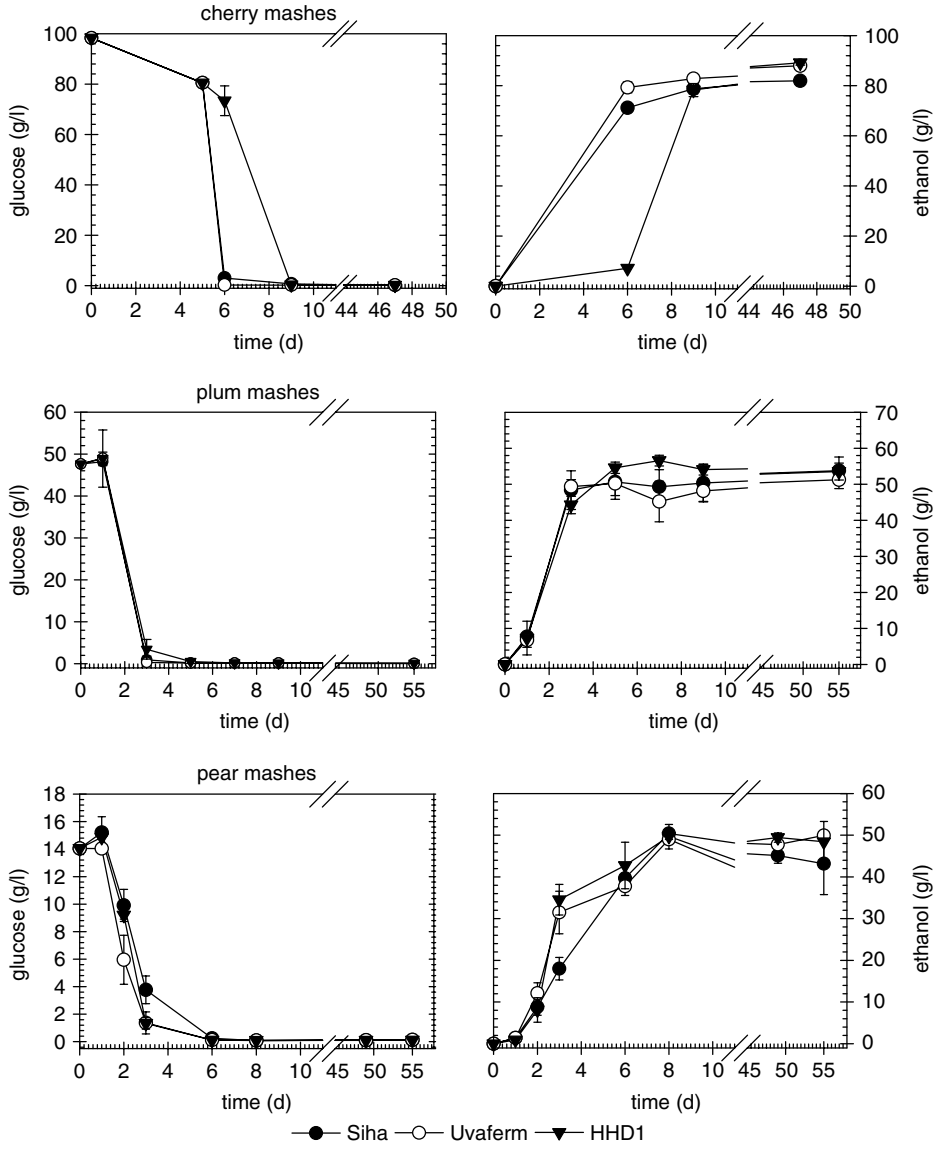

Figure 2. Glucose consumption and ethanol production in different fruit mashes by commercial and laboratory yeast strains. Mashes were prepared and inoculated with approximately 10^6 cells/ml of precultured yeasts, as described in Materials and methods. Fermentation at 15–17 °C was followed for a minimum of 48 days. All experiments were performed in triplicate, with bars indicating the standard deviation

10 days of fermentation in all cases. Supporting the data summarized in Table 1, no significant differences were observed between the performances of Siha, Uvaferm and HHD1 in this respect. In the case of the cherry mashes only, HHD1 displayed a longer lag-phase before the onset of fermentation, but then degraded the carbohydrates and produced ethanol to the same final levels as observed for the two industrial strains (Figure 2). Note that the total sugar content as deduced from the % Plato given in Table 1 was similar for plum and pear mashes, but the content of free glucose is considerably lower in pear mashes.

Organic acids and glycerol production during fermentation

Other important parameters of mash and product quality are the acidity of the mashes and

Table 2. Acidities of mashes before and after fermentation

	Acidity[a]								
	Siha		Uvaferm		HHD1		Final pH[b]		
Mash	Initial	Final	Initial	Final	Initial	Final	Siha	Uvaferm	HHD1
Cherries	8.60	6.50	8.60	7.50	8.60	7.90	3.90	3.76	3.80
Plums	10.30	8.03	10.30	7.03	10.30	7.43	3.56	3.50	3.50
Pears	2.60	5.43	2.60	6.56	2.60	5.10	2.96	2.90	3.00

[a] The total acidity was calculated as tartaric acid equivalents, according to Adam *et al.* (1995).
[b] The final pH values are the mean of all values measured during days 10–50 and did not vary by more than 0.2 within this period.

the compounds determining their viscosities. We therefore proceeded by determining the concentrations of some key organic acids and glycerol in the mashes. Our examinations concentrated at first on the changes of the mash pH during fermentation. After an initial decrease within the first 2 days of fermentation, values then reached a slightly higher constant pH, after approximately 5 days (data not shown). Whilst these did not change significantly over the elongated period of 48–55 days, an increase in the total titratable acidity (calculated as equivalents of tartaric acid) was observed for the pear mashes. This was most pronounced with the Uvaferm yeast but was observed in all fermentations. On the other hand, acidities decreased during fermentation of the plum and cherry mashes (Table 2).

Accurate determinations of specific organic acids and glycerol were obtained by HPLC measurements (Table 3). Although HHD1 seemed to produce a little less glycerol (approximately 14% lower) than the industrial strains in the pear and plum mashes, this was not observed for the cherry mashes. Likewise, the higher content of acetic acid detected for the laboratory strain in the pear mashes was not consistent with the other fruit mashes. Similarily, differences in concentrations of lactic and propionic acids did not generally correlate with any of the yeast strains employed for fermentation.

Table 3. Concentration of organic acids and glycerol (g/l) in fruit mashes after complete fermentation

		Yeast strain employed		
Fruit	Compound	Siha	Uvaferm	HHD1
Cherries	Acetic acid	0.26 ± 0.11	0.16 ± 0.09	0.11 ± 0.05
	Propionic acid	0.26 ± 0.08	0.20 ± 0.22	0.52 ± 0.20
	Lactic acid	2.25 ± 0.19	2.51 ± 0.08	2.40 ± 0.37
	Glycerol	6.54 ± 0.15	6.01 ± 0.17	6.58 ± 0.33
Plums	Acetic acid	1.66 ± 0.19	0.80 ± 0.22	1.07 ± 0.24
	Propionic acid	0.47 ± 0.21	0.57 ± 0.13	0.61 ± 0.29
	Lactic acid	1.73 ± 0.83	2.74 ± 0.69	1.91 ± 1.03
	Glycerol	4.57 ± 0.15	4.85 ± 0.32	3.94 ± 0.75
Pears	Acetic acid	1.02 ± 0.17	0.92 ± 0.11	1.45 ± 0.23
	Propionic acid	0.34 ± 0.17	0.06 ± 0.00	0.23 ± 0.04
	Lactic acid	0.22 ± 0.02	0.35 ± 0.01	0.21 ± 0.00
	Glycerol	4.28 ± 0.19	4.46 ± 0.12	3.71 ± 0.17

Secondary fermentation products

Other volatile compounds, such as esters, aldehydes and higher alcohols present in the mashes after fermentation, are of crucial importance for the quality of the final spirits. Therefore, we also quantified some of these key compounds in the mashes by headspace gas chromatography (Figure 3A).

Siha yeast consistantly produced the lowest amounts of acetaldehyde concentrations in the mashes, whilst Uvaferm and HHD1 produced approximately double (although at very low overall concentrations of 30–60 mg/l, i.e. below the threshholds for sensoric detection). In the case of 1-propanol concentrations, no consistency between the different mashes and the three yeast strains employed could be observed. However, HHD1 invariably correlated with the highest concentrations (again, all below sensoric threshholds), although the Uvaferm strain produced similar amounts in the case of the cherry mashes, whereas Siha led to the lowest 1-propanol amounts in cherry and plum fermentations (Figure 3A). 2-Methyl-1-propanol and isoamyl alcohol (3-methyl-1-butanol and 2-methyl-1-butanol) concentrations did not differ significantly between the yeast strains in the different mashes (with the exception of the cherry mashes, where the Siha strain produced

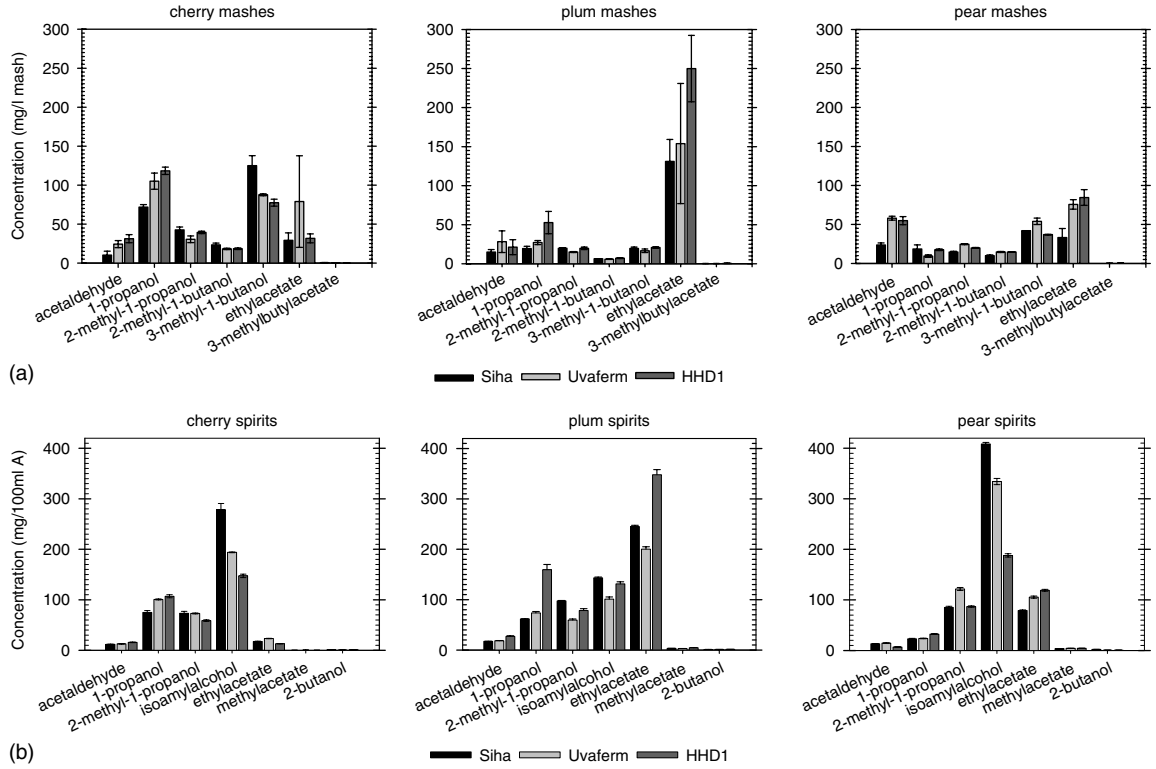

Figure 3. Concentration of volatile substances in mashes and spirits produced by fermentation with commercial and laboratory yeast strains. (A) Concentrations of key compounds found in the mashes indicated, as determined after complete fermentation (see Materials and methods for details). (B) Concentrations of key volatile compounds (in mg/100 ml alcohol = A) observed in the spirits produced from the mashes tested above. All experiments were performed in triplicate, with bars indicating the standard deviation. Note that concentrations in mashes and spirits are not directly comparable, since their references (per litre of mash and 100 ml alcohol, respectively) are different

Table 4. Methanol concentrations in different fruit mashes after complete fermentation and in drinkable spirits produced from these mashes

	Siha		Uvaferm		HHD1	
Fruit	**Mash[a]**	**Spirit[b]**	**Mash[a]**	**Spirit[b]**	**Mash[a]**	**Spirit[b]**
Cherries	327 ± 21	565 ± 13	307 ± 7	548 ± 3	355 ± 10	717 ± 8
Plums	241 ± 18	256 ± 4	269 ± 25	210 ± 20	319 ± 2	252 ± 72
Pears	252 ± 41	766 ± 39	339 ± 18	741 ± 22	380 ± 22	742 ± 12

[a] The methanol concentration in the mashes was calculated in mg/l mash.
[b] The concentration of methanol in the spirits was calculated in mg/100 ml alcohol.

approximately 30% more 3-methyl-1-butanol than Uvaferm and HHD1).

Ethyl acetate concentrations were considerably higher in the plum mashes than in the pear and cherry mashes, presumably due to the lower quality of the fruit employed (see Materials and methods). For the pear and plum mashes, fermentation with either Uvaferm or the laboratory strain yielded higher values than with the Siha yeast. This correlation was not observed for the cherry mashes, where HHD1 led to low values similar to the Siha preparation. 3-Methyl-butylacetate concentrations scarcely exceeded detectable levels. Finally, the methanol content (introduced by the pectinases

from the fruit) of the pear and plum mashes (Table 4), although slightly higher for the laboratory strain than for the two industrial yeasts, was within acceptable limits (i.e. below 400 mg/l). In the cherry mashes, variations between the three yeast strains with respect to the final methanol concentrations were much less pronounced (Table 4).

Distillation and spirit analyses

Although the composition of the mashes has a crucial influence on the final quality of the spirits produced, distillation leads to the elimination of a variety of volatile compounds and to thermal changes within others. Therefore, we examined all distillates with headspace gas chromatography for their aromatic compounds (partly repeating those already tested in the fermented mashes). The concentrations were calculated in mg/100 ml pure alcohol and are shown in Figure 3B. As expected for a successful distillation process, acetaldehyde levels were extremely low in the spirits. Generally, 1-propanol concentrations were higher in the spirits produced with the laboratory strain, especially in plum spirits (correlating with the higher values already observed in the mashes). The spirits produced from plums showed significantly higher levels of ethyl acetate when fermented with HHD1. This was not observed for pear and cherry spirits, for which ethyl acetate concentrations were generally lower than those of plums. 3-Methyl-1-butanol concentrations were considerably lower in pear and cherry spirits produced with the laboratory strain. All other compounds exhibited approximately the same concentrations for all strains and distillates tested. Moreover, differences in methanol concentrations observed in the mashes for the different yeast strains were generally levelled out by the distillation process (Table 4).

Sensoric evaluation

Despite the highly sensitive detection equipment employed above, it is not yet possible to predict the quality of spirits merely by their known chemical composition (Busch-Stockfisch, 2002; Jellinek, 1981; Koch, 1986; Neuman and Molnár, 1991). We therefore used two different approaches to determine the sensoric properties of the products.

In a first series of sensoric evaluations, we performed an 'order-of-precedence test', in which the three different spirits, fermented with the different yeast strains, were placed in order of decreasing quality (fruity character, smell and taste). With a panel consisting of 25 probationers, no statistical preference for any of the yeast strains could be established (data not shown).

Second, the so-called 'triangle test' was employed to determine whether the yeast strains had any detectable influence on the flavour or taste of the spirits produced. Up to 65 test persons participated in evaluating the laboratory strain against either Uvaferm or Siha products. Probationers were trained to detect the principle taste qualities (sweet, sour, salty, bitter) and then presented with three spirit samples, two of which were identical for each spirit. By simply asking each tester to identify the different sample, even small differences in taste or flavour of a spirit could be detected. The test persons were also asked to judge which of the samples was of better quality. For statistical reasons, only the answers of those able to identify the differing sample were used in our calculations for the latter (Koch, 1986). Table 5 shows the results and statistical analysis of this test. In the case of pear and cherry spirits, a significant statistical difference was observed between the spirits produced with the laboratory strain and those produced with the Siha or the Uvaferm yeast. In the case where the pear mashes were fermented and distilled, HHD1 was judged to produce a spirit of significantly higher quality. No differential quality judgement could be made for the cherry spirits. Spirits produced from plums using the three different yeast strains could not be distinguished at all by the panel.

Discussion

In the fruit fermentation industry, whether for the production of wines or spirits, the addition of a selected strain of *S. cerevisiae* to avoid bacterial contamination and to ensure a reproducible performance and product quality is common practice (Pretorius, 2000). Commercially available yeast preparations used for this purpose have not been sufficiently characterized regarding their life cycle and genetic composition (Heinisch and Hollenberg, 1993). Therefore, reproducible performance is threatened by strain evolution caused by sporulation and mating, mutations, gene conversions and genetic transpositions. Moreover, targeted genetic manipulations for strain improvement are quite

Table 5. Sensoric analyses ('triangle test') of spirits produced from different fruit mashes fermented with commercial and laboratory yeast strains

Fruit	Spirits	Number of test persons	Differences detected[a]				Preference[b]		
			Number	$\chi^2_{theoretical}$	$\chi^2_{calculated}$	Signific. ($\alpha = 5\%$)	$\chi^2_{theoretical}$	$\chi^2_{calculated}$	Preference yeast
Cherries	HHD1 vs. Siha	65	41	2.71	24.56	Yes	3.84	0.39	None
	HHD1 vs. Uvaferm	56	25	2.71	2.73	Yes	3.84	0.64	None
	Siha vs. Uvaferm	18	10	2.71	3.06	Yes	3.84	0.40	None
Pears	HHD1 vs. Siha	55	32	2.71	14.18	Yes	3.84	6.13	HHD1
	HHD1 vs. Uvaferm	56	34	2.71	17.68	Yes	3.84	7.53	HHD1
	Siha vs. Uvaferm	19	8	2.71	0.32	No	3.84	3.13	None
Plums	HHD1 vs. Siha	55	22	2.71	0.82	No	3.84	0.05	None
	HHD1 vs. Uvaferm	55	21	2.71	0.38	No	3.84	0.00	None
	Siha vs. Uvaferm	13	3	2.71	0.24	No	3.84	0.30	None

Spirits were subjected to triangle tests (see Materials and methods for details) to detect differences introduced by the yeast strain used for fermentation of the mashes (e.g. HHD1 vs. Siha = HHD1 vs. Siha).

[a] The numbers of test persons detecting a difference were subjected to χ^2 analyses and differences are given (yes = significantly different; no = not significantly different).

[b] If differences were detected, the test persons were asked to judge their preference. Again, these data were subjected to statistical analyses (using the forced-choice technique) and evaluated for the preference of the yeast strain employed in the fermentation of the mashes.

laborious. Thus, protoplast fusions between different *S. cerevisiae* strains, as well as with other yeast species, have been employed to circumvent the problems caused by the non-sporulating phenotype of most commercial strains (Spencer and Spencer, 1996). Genetic instability is an obvious by-product of the latter procedure. On the other hand, transformation with plasmids or DNA carrying heterologous genes (e.g. for the production of enzymes, vaccines, etc.) relies on the introduction of dominant genetic markers and does not find public acceptance where food production processes are concerned (Drewnoski and Rock, 1995; Danner, 1997; Nishiura *et al.*, 2002). These problems could be avoided by the use of a genetically well-defined yeast strain that can be easily sporulated and thus crossed to combine the desired properties. Furthermore, the availability of haploid segregants would ease phenotypic selection procedures.

In the study presented here, we confirmed that haploid laboratory yeast strains carrying auxotrophic markers are indeed not suitable for practical applications. Growth, sugar consumption and ethanol production were decreased in comparison to the commercial yeast preparations when tested in cherry mashes. However, this was not observed for the diploid derivative constructed from the two laboratory strains by simple mating. HHD1 showed similar fermentation rates and survived just as well as the commonly used Siha and Uvaferm yeasts. This observation substantiates its ability to ferment all sugars usually found in fruit mashes, i.e. glucose, fructose, sucrose and raffinose. Although the start of fermentation was delayed by 2–3 days in some cases when employing the laboratory strain, it caught up with the commercial yeasts within the first 10 days. Since mash fermentations usually last over a period of at least 1 month in spirit production (Pieper *et al.*, 1993), this does not present an obstacle. It has been suggested that the longer alcohol production is delayed, the greater the risk of bacterial contamination (Bayrock and Ingledew, 2001). Yet, this was not observed (either by microscopic examination or by the distribution of fermentation by-products) even in the case of the pear mashes, where we employed low-quality fruit in order to test the prevalence of the yeast preparations over bacteria being introduced by the raw material. It seems noteworthy that interactions between lactic acid bacteria and various yeast species are frequently inherent to fruit fermenting processes (Addis *et al.*, 2001; Corsetti *et al.*, 2001; Eliseeva *et al.*, 2001; van Beek and Priest, 2002). Metabolic activity of bacteria is usually indicated by increased amounts of acetic acid and lactic acid. These may in part explain the growth inhibitory effect of bacterial contaminations on the yeast population (Boidron, 1969; Thomas *et al.*, 2001). Conversely, bacterial growth is enhanced

by the presence of yeasts, as they serve as a nutrient source to provide essential compounds. Massive bacterial growth leads to competition for the sugars present in the mashes and may result in a considerable reduction in the alcohol yield (Thomas *et al*., 2001). None of these deleterious effects was observed in the mashes fermented in this work, indicating that the competitive fitness of the laboratory strain equals that of the industrial yeasts employed. This is also true for the competition with 'wild yeasts' commonly found on fruit, such as *Kloeckera apiculata* (= *Hanseniaspora uvarum*; Meyer *et al*., 1978).

A major by-product of yeast carbohydrate metabolism which also reduces the alcohol yield is glycerol. Therefore, this compound is of significant interest in the wine, beer and ethanol production industries (Cronwright *et al*., 2002). Moreover, overproduction of glycerol in an engineered *S. cerevisiae* strain leads to substantial changes in the formation of other by-products and to a stimulation of the fermentation rate in stationary phase cells (Remize *et al*., 1999). Again, the laboratory strain produced similar glycerol concentrations as compared to the two industrial strains (4–5g/l) in all fruit mashes tested in this work. In this respect, no negative effect could be observed.

For spirits and other alcoholic beverages a major factor for their application is the sensoric performance. This in turn is mainly influenced by a combination of higher alcohols and volatile compounds, such as organic esters (Goranov, 1983). Although some of the key substances may escape detection, we here employed both HPLC and GC methods to determine the exact concentrations of some of the major constituents, both in the mashes and the final products. In general, concentrations of higher alcohols and esters varied depending more on the raw material used than on the yeast strain employed for the fermentation. However, a fairly consistent higher production of 1-propanol, which was carried over into the distillates, could be observed for the laboratory strain as opposed to the two commercial preparations tested. However, this did not result in an altered sensoric performance. Likewise, methanol levels in the final spirit were largely independent of the yeast strain employed for fermentation and remained below the legal limits in all cases. It is most interesting to note that a difference between the spirits produced with the commercial strains and the laboratory strain could be detected, with statistical significance for two of the three fruit spirits tested (i.e. pear and cherry). The preference for the use of the laboratory strain ('triangle test') in the preparation of the pear spirits, although statistically significant, should be taken with care. Since tailings have to be excluded by organoleptic assessment in the distillation process, slight differences observed in the products may result from a relatively small amount of tailing included in one case and not the other (Postel and Adam, 1989). This result would have to be verified by the large-scale fermentation of higher numbers of pear mashes, which would exceed our possibilities with the equipment available to us. On the other hand, since none of the commercial strains were preferred, it seems safe to conclude that the laboratory strain at least does not have a negative influence on the sensoric properties of the final spirits. This conclusion is further supported by the order-of-precedence test, where none of the yeast strains employed was consistently associated with a preferred spirit.

Conclusions and outlook

The data presented here indicate that the diploid laboratory strain tested for the fermentation of various fruit mashes and spirit production is as suitable as commercially available yeast preparations with respect to both fermentation capacity and sensoric performance. However, while these preliminary experiments are very promising, a number of questions remain to be addressed. Thus, the laboratory strain should be adjusted for improved fermentation at lower temperatures. This may resolve the problem of delayed fermentation start observed in some cases. Furthermore, to be of commercial value, large-scale strain production and preparation of dry yeast would be desirable. Thus, growth and viability of the laboratory strain under industrial production conditions need to be tested. Given that these problems can be solved, the strain would be of great value for the production of spirits and may also be tested in other beverages and for bioethanol production. Its known genetic constitution will greatly simplify the adjustment to such different purposes.

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