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Analysis of yeast diversity during spontaneous and induced alcoholic fermentations

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M. SCHÜTZ AND J. GAFNER. 1993. The diversity of yeast species and strains was monitored by physiological tests and a simplified method of karyotyping of yeast chromosomes. During the first phase of investigated alcoholic fermentations, the yeast species *Metschnikowia pulcherrima* and *Hanseniaspora uvarum* were predominant, irrespective of the origin of the grape must. At the beginning of fermentation *H. uvarum* was even present in the case of induced fermentations with dried yeast. Middle and end phase of the alcoholic fermentation were clearly dominated by the yeast species *Saccharomyces cerevisiae*. In the case of spontaneous fermentations, several different strains of *S. cerevisiae* were present and competed with each other, whereas in induced fermentations only the inoculated strain of *S. cerevisiae* was observed. A competition of strains of *S. cerevisiae* also occurred during the fermentation with dried yeast product consisting of two different strains. An effect of *H. uvarum* on taste and flavour of wines can be postulated according to the frequency of its appearance during the first phase of fermentation. With the method of rapid karyotyping and supplementary physiological tests it was possible to make reliable assertions about the yeast diversity during alcoholic fermentation.

INTRODUCTION

Wine-making has turned increasingly to a well-controlled process with extended microbiological investigations on the alcoholic fermentation. The risk of wine spoilage has been reduced to an acceptable degree, even in the case of spontaneous fermentations (Schütz and Gafner 1992). The production of dried yeasts about two decades ago was a very helpful step in overcoming wine spoilage problems. Inoculated dried yeasts, however, can influence the natural microflora in musts so that certain desired metabolic substances are no longer produced in adequate amounts. Wucherpfennig and Bretthauer (1970) and Sponholz and Dittrich (1974) have shown that wines fermented with dried yeasts contained lower amounts of higher alcohols, isoamylacetate and ethylacetate than spontaneously fermented wines. As wine quality is also a consequence of the diversity and the composition of micro-organisms and their dynamics and frequency of appearance, it is very important to know more about the evolution of the entire microflora during the alcoholic fermentation process.

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Several investigations were done on the diversity and composition of the yeast flora in musts. Differences have been shown according to the age of the vineyards, grape variety and harvest technique (Cuinier 1976; Martini *et al.* 1980; Rosini *et al.* 1982). Longo *et al.* (1991) identified the yeast populations from two regions of Northwest Spain. The composition not only differed from region to region but also from year to year. Two very successful methods to distinguish between different strains are available to characterize strains of *Saccharomyces cerevisiae* during fermentation: the restriction endonuclease analysis of mitochondrial DNA and the karyotyping by chromosomal banding patterns (Lee and Knudsen 1985; Carle and Olson 1985; De Jong *et al.* 1986; Vezinhet *et al.* 1990). With these methods it has been shown that during spontaneous fermentations several different strains of *S. cerevisiae* could be involved simultaneously, and furthermore that inoculated dried yeasts could dominate the microflora until the end of fermentation (Dubourdieu and Frezier 1990; Hallet *et al.* 1990; Querol *et al.* 1992; Vezinhet *et al.* 1992).

Nevertheless, none of these investigations were done with respect to the diversity and the dynamics of yeast species and strains over the course of the whole alcoholic fermentation. Therefore, the aim of the present work was

to obtain more precise information to discover what happens at the microbiological level of the wine-making process.

MATERIALS AND METHODS

Yeasts and grapes

Yeast strains used in this work: *S. cerevisiae* strain YNN295 from Bio-Rad, Glattbrugg, Switzerland, *Metschnikowia pulcherrima* strain 152, *Hanseniaspora uvarum* strain 58 from Professor F. Radler, Universität Mainz, Germany, and the dried yeast strains Siha7 from Begerow, W27 from Lallemand and Oenoprox68-72 from Bioprox.

Investigations were carried out with musts from the grape varieties Nebbiolo (Sandrone, Barolo, Italy), Pinot Noir (Sponholz, Forschungsanstalt Geisenheim, Germany, and Marugg, Fläsch, Switzerland) and Muller-Thurgau (Forschungsanstalt Wädenswil, Switzerland). All musts were from the vintage 1992.

Fermentation procedure

Fermentations of the grape musts from Sandrone, Sponholz and Marugg were carried out under the normal large scale wine-making conditions without any special treatment according to our investigations. All fermentations were done spontaneously without inoculation of dried yeast.

The behaviour of the dried yeast strains Siha7, W27 and Oenoprox68-72 were tested by inoculation into 100 l of must from the grape variety Muller-Thurgau. The must was not treated with enzymes or chemicals before or during the alcoholic fermentations.

To investigate the influence of yeast species on fermentation products *H. uvarum* and *S. cerevisiae* were added to 25 l of pasteurized must (sugar content: 175 g l^{-1}) from the grape variety Muller-Thurgau. Fermentation trials were done in triplicate.

Isolation of yeasts

At the beginning, middle phase and end of fermentation samples from each fermentation process were taken and appropriate dilutions were plated on Phytone™ yeast extract agar (Becton Dickinson). In the case of the three spontaneous fermentations, the time points where samples were taken are not defined exactly. Otherwise beginning, middle phase and end of fermentation mean a conversion of total sugar of 0–10%, 45–55% and 90–100%, respectively measured by HPLC analysis. Incubation of the plates was carried out aerobically at 25°C. All yeast colonies from plates containing between 10 and 100 colonies were taken for further investigations.

Identification and characterization of yeasts

To distinguish between *S. cerevisiae* and other yeast species, physiological tests were done according to the keys, methods and descriptions published by Barnett *et al.* (1990). To distinguish between different strains of *S. cerevisiae* the chromosomal separation procedure by CHEF gel electrophoresis was used. Yeast chromosomes were isolated by a modified method described by Schwartz and Cantor (1984): cell material from separated colonies was suspended in 100 µl of 0·05 mol l^{-1} EDTA, pH 8·0. Then 50 µl of Zymolyase solution (1 mg ml^{-1} SEC buffer (1·2 mol l^{-1} sorbitol, 0·04 mol l^{-1} EDTA, 0·02 mol l^{-1} citrate-phosphate, pH 5·6)) was added and incubation was at 37°C for 45 min. Meanwhile 1% low melt preparative grade agarose from Bio-Rad (Glattbrugg, Switzerland), in 0·125 mol l^{-1} EDTA, pH 7·5, was prepared. Enzyme-treated cell suspension and agarose were mixed at 45°C and put into the 10 well sample plug mold (Bio-Rad). Incubation was at 4°C for 15 min. The solidified agarose samples were placed into 10 ml of buffer A (0·5 mol l^{-1} EDTA, pH 8·0, 0·01 mol l^{-1} Tris, pH 7·5, 7·5% β-mercaptoethanol). Incubation was at 37°C for 4 h. After washing three times with 0·05 mol l^{-1} EDTA, pH 8·0, 10 ml of buffer B (0·01 mol l^{-1} Tris, pH 7·5, 0·5 mol l^{-1} EDTA, pH 8·0, 1% laurylsarcosine, 1 mg ml^{-1} proteinase K) was added. Incubation was at 50°C overnight. After removing buffer B and washing again three times with 0·05 mol l^{-1} EDTA, pH 8·0, the agarose samples were stored in 0·05 mol l^{-1} EDTA, pH 8·0. The CHEF Mapper™ system (Bio-Rad) was used for gel electrophoresis. The yeast chromosomes were separated with the following parameters: angle, 120°; voltage gradient, 6 V cm^{-1}; block 1, pulse time 60 s, run time 15 h; block 2, pulse time 90 s, run time 8 h; buffer, 0·5 × TBE (1 × TBE: 10·8 g l^{-1} Tris base, 5·5 g l^{-1} boric acid, 4 ml of 0·5 mol l^{-1} EDTA, pH 8·0); agarose, 1% chromosomal grade agarose (Bio-Rad); temperature, 13·5°C.

Statistical accuracy

All samples were diluted so that 0·1 ml of the diluted sample plated on Phytone™ yeast extract agar (Becton Dickinson) gave 10–100 colonies. For further characterizations all colonies from the agar plates were taken. Although the total number of colonies examined per sample differed with this method the statistical accuracy of strain percentages is better than by taking only a selection of colonies from one plate. The distribution of strains in the samples (diluted or not) may be considered as homoge-

neous and therefore all colonies taken from one plate represent the real situation better than just a selection of colonies.

RESULTS

Yeast diversity of three different spontaneous fermentations

The yeast microflora during three different spontaneous fermentations performed with musts from the grape variety Nebbiolo and Pinot Noir was examined by physiological tests according to Barnett *et al.* (1990) and by the separation of the chromosomes of yeasts appearing during the time course of the fermentation processes. As the chromosomes of *M. pulcherrima* could not be separated appropriately under the experimental conditions, no strain characterization was possible. Colonies of *M. pulcherrima* were therefore recognized only as species.

The species *M. pulcherrima* (MpI), three strains of *H. uvarum* (HuI) and six of *S. cerevisiae* (ScI) were detected during the fermentation of the Nebbiolo grape must from Sandrone (Barolo, Italy). Figure 1a shows the banding patterns of the separated chromosomes of all yeast strains differing from each other and their frequencies of appearance during the fermentation. Strain differences were detectable by the comparison of the different chromosomal banding patterns. Of 10 colonies at the beginning of fermentation, three represented the species MpI (30%), two colonies strain HuI-1 (20%), one colony strain HuI-2 (10%) and another two colonies HuI-3 (20%). In the middle phase of fermentation where 11 colonies were examined these strains were not found. Strains of *S. cerevisiae* then began to predominate. Strains ScI-1 and ScI-2 were already represented with one colony each (10%) at the beginning. In the middle phase two colonies of strain ScI-1 (18%) and three colonies of strain ScI-2 (28%) were present. In addition, three new strains appeared, ScI-3, ScI-4 and ScI-5, and were represented with two colonies each (18%). At the end of fermentation, 20 of 40 colonies were strain ScI-1 (50%) while strain ScI-2 was represented by 10 colonies (25%). Another new strain, ScI-6, was also represented with 10 colonies (25%).

During the fermentation of the Pinot Noir grape must from Sponholz (Geisenheim, Germany), one *M. pulcherrima* (MpII), one *H. uvarum* (HuII) and four strains of *S. cerevisiae* (ScII) were characterized respectively (Fig. 1b). At the beginning of fermentation 91 colonies were examined; 83 represented MpII (91%) and eight the strain HuII (9%). In the middle phase of fermentation five of 12 colonies were MpII (41%). Strain HuII was not found whereas three strains of *S. cerevisiae*, ScII-1, ScII-2 and ScII-3 appeared with two (17%), three (25%) and again two (17%) colonies, respectively. Of 50 colonies examined at the end of fermentation, 40 (80%) represented strain ScII-2. The other 10 colonies (20%) represented the new strain ScII-4. Strains ScII-1 and ScII-3 were not found.

The spontaneous fermentation of the Pinot Noir grape must from Marugg (Fläsch, Switzerland) was carried out by *M. pulcherrima* (MpIII), three strains of *H. uvarum* (HuIII) and two strains of *S. cerevisiae* (ScIII) (Fig. 1c). At the beginning of fermentation, eight of 36 colonies were MpIII (22%), three were HuIII-1 (8%), 17 were HuIII-2 (47%) and eight were HuIII-3 (22%). In the middle phase of fermentation where 19 colonies were examined neither MpIII nor HuIII were found, but two strains of *S. cerevisiae*, ScIII-1 and ScIII-2, appeared and were represented by 17 (89%) and two (11%) colonies, respectively. At the end of fermentation only ScIII-1 was present.

Succession of dried yeast strains

To investigate the succession of inoculated dried yeasts the commercially-produced strains Siha7 (Begerow), W27 (Lallemand) and Oenoprox68-72 (Bioprox) were cultured in 100 l of fresh must of the grape variety Muller-Thurgau. At three different time points, beginning, middle phase and end of fermentation, samples were taken to examine the development of the microflora. Yeast species and strains were characterized as described. In all three cases the dried yeast strains began to predominate immediately after inoculation. In the fermentation with Siha7, the dried yeast strain was the only one detected throughout the course of fermentation. Among 30 colonies examined at the beginning, 17 in the middle phase and 13 at the end of fermentation, only strain Siha7 was present. At the beginning of the fermentations with W27 and Oenoprox68-72, strains of *H. uvarum* were also found (Fig. 2). Among 53 colonies from the fermentation with inoculated W27, one represented a strain of *H. uvarum* (2%) and among 25 colonies from the fermentation with inoculated Oenoprox68-72, two represented another strain of *H. uvarum* (8%).

Influence of yeast species on fermentation products

The end production of acetate, glycerol and ethanol from fermentations carried out with *H. uvarum* and *S. cerevisiae* was measured by HPLC analysis. Mean values of three independent fermentations are listed in Table 1. The acetate production by *H. uvarum* (0·5 g l^{-1}) was significantly higher than by *S. cerevisiae* (0·03 g l^{-1}). The values of glycerol and ethanol production did not differ significantly. *Hanseniaspora uvarum* produced a glycerol content of 5·47 g l^{-1} and *S. cerevisiae* 4·97 g l^{-1}. The ethanol production by *H. uvarum* was 78·3 g l^{-1}, and by *S. cerevisiae*

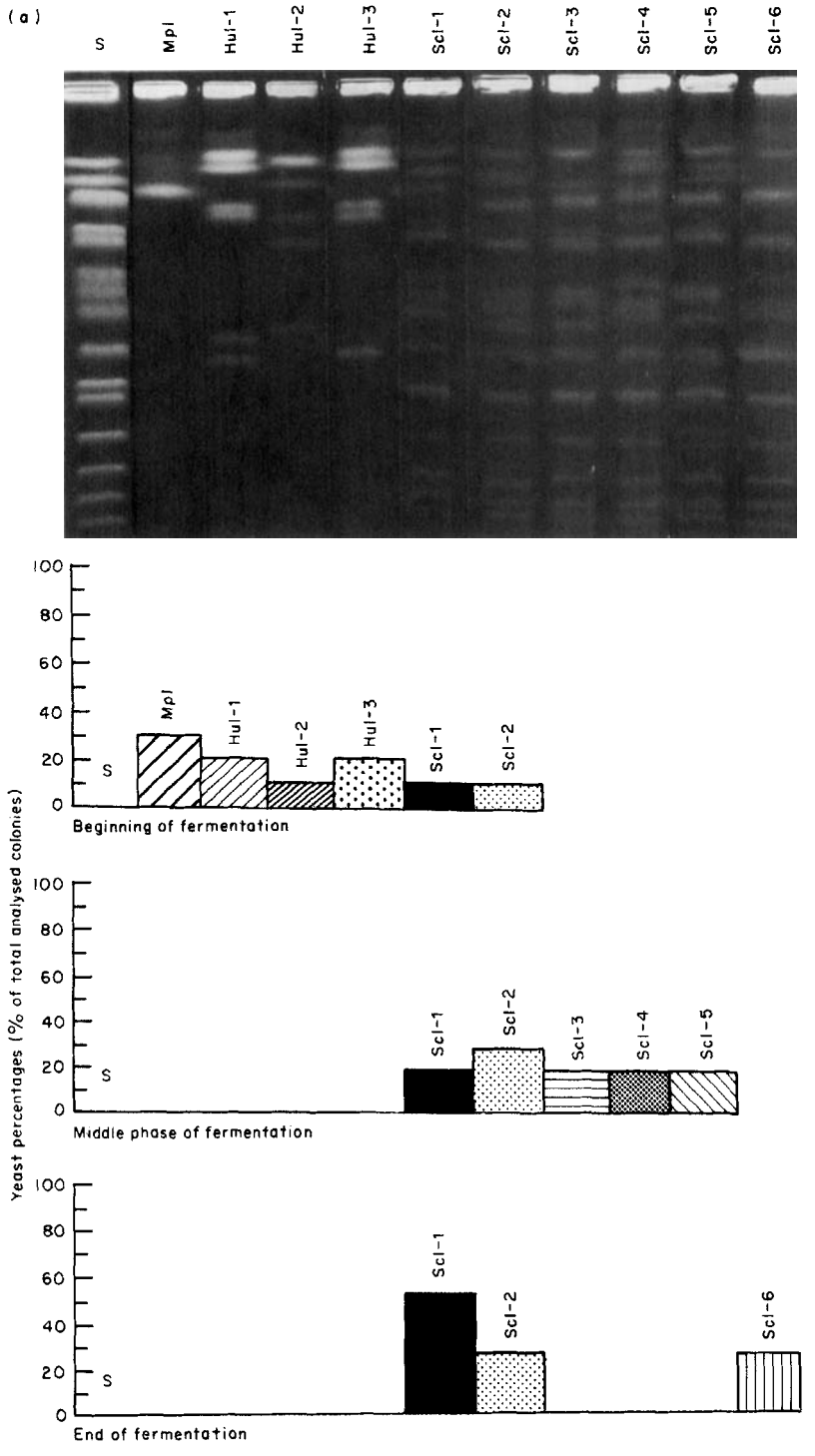

Fig. 1 Karyotype of different yeast strains and their frequencies of appearance during the spontaneous fermentations. S, Standard yeast strain YNN295; Mp, *Metschnikowia pulcherrima*; *Hu*, *Hanseniaspora uvarum*; Sc, *Saccharomyces cerevisiae*; I, Nebbiolo grape must from L. Sandrone (Fig. 1a); II, Pinot Noir grape must from W.R. Sponholz (Fig. 1b); III, Pinot Noir grape must from D. Marugg (Fig. 1c)

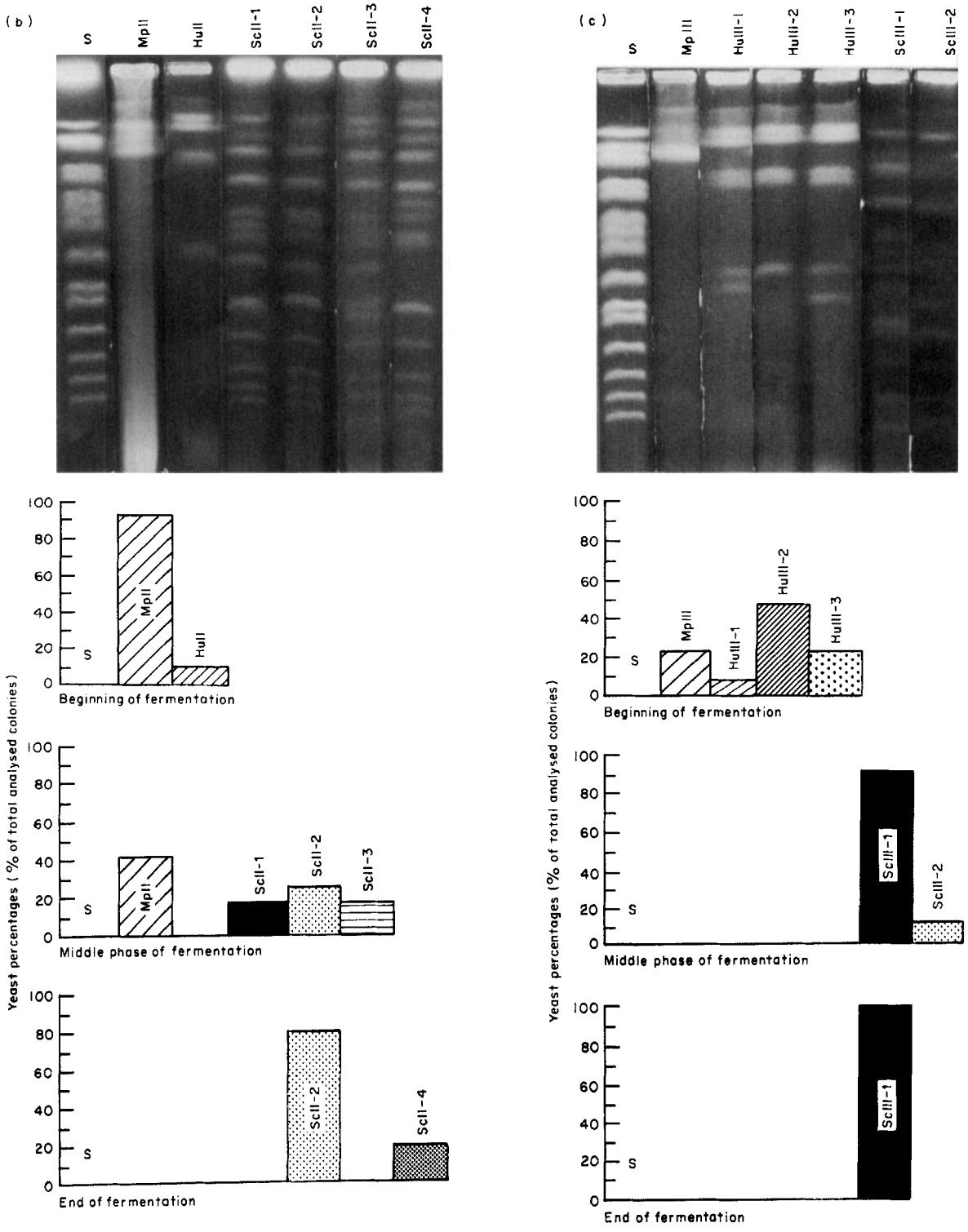
(b)
S
MpII
HuII
ScII-1
ScII-2
ScII-3
ScII-4
(c)
S
MpIII
HuIII-1
HuIII-2
HuIII-3
ScIII-1
ScIII-2
Yeast percentages (% of total analysed colonies)
Beginning of fermentation
Middle phase of fermentation
End of fermentation
0
20
40
60
80
100

Fig. 1

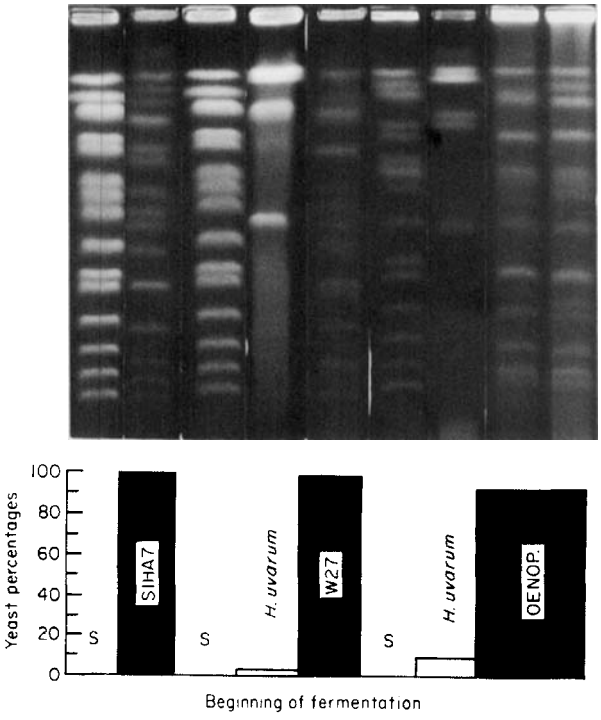

Fig. 2 Karyotype of yeasts detected during the first phase of fermentations (0–10% conversion of total sugar) with either Siha7, W27 or Oenoprox68-72 (two strains) and their percentages of total analysed colonies

79·3 g l^{-1} (*H. uvarum* could not completely ferment the sugar and the residual content was 8·2 g l^{-1}).

Competition of two strains of a dried yeast product during the fermentation

To monitor the competition of the strains OenoproxL2868 and OenoproxL2872 from the mixed dried yeast product Oenoprox68-72 samples were taken at three different time points, at the beginning, in the middle phase and at the end of fermentation and yeast colonies were prepared for karyotyping as described. In addition yeast colonies were prepared from a rehydrated sample before inoculation into the grape must (rehydration was done as recommended by the supplier). Of 13 colonies of the rehydrated sample three (23%) represented strain L2868 and 10 (77%) strain L2872. After inoculation, when fermentation began the ratio between L2868 and L2872 changed already: 14 of 25 colonies (56%) were L2868 and 11 (44%) were L2872. Strain L2872 was not detected after the middle phase of fermentation. The total number of colonies examined in the middle phase and at the end of fermentation were 19 and 12, respectively (Fig. 3).

Table 1 Quantities of acetate, glycerol and ethanol produced during the fermentation of a pasteurized must of the grape variety Muller-Thurgau by either *Hanseniaspora uvarum* or *Saccharomyces cerevisiae* (n = random sample)

$n = 3$	*Hanseniaspora uvarum* M (S.D.) (g l^{-1})	*Saccharomyces cerevisiae* M (S.D.) (g l^{-1})
Acetate	0·50 (0·06)	0·03 (0·05)
Glycerol	5·47 (0·48)	5·07 (0·34)
Ethanol	78·3 (1·89)	79·3 (1·25)

M, Mean values of three independent fermentation trials; S.D., standard deviation of mean values.

DISCUSSION

Specificity of the spontaneous yeast flora

The three spontaneous fermentations with musts from the grape varieties Nebbiolo and Pinot Noir from different regions showed similarities in the composition and dynamics of yeast species. At the beginning of each fermentation the yeast species *M. pulcherrima* and *H. uvarum* were

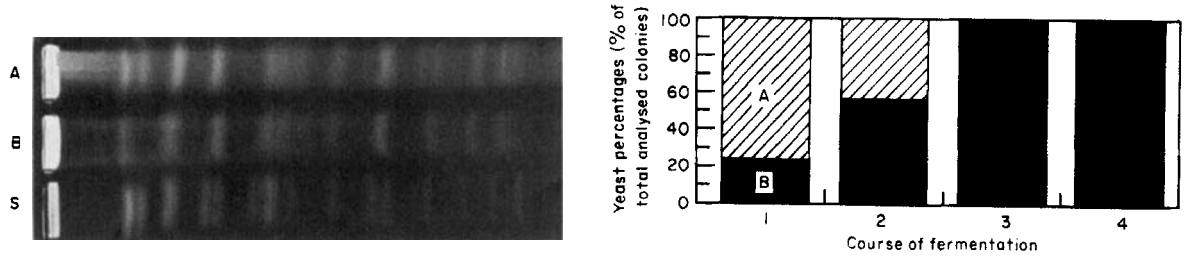

Fig. 3 Karyotype of the two strains of Oenoprox68-72 and their competition throughout the fermentation process. A, Strain L2872; B, strain L2868; S, standard yeast strain YNN295; 1, rehydrated yeast; 2, beginning of fermentation; 3, middle phase of fermentation; 4, end of fermentation

predominant. *Saccharomyces cerevisiae* began to predominate in the middle phase and end of fermentation. The presence of yeasts of the genera *Metschnikowia* and *Hanseniaspora* (*Klöckera*) in early phases of fermentations was also reported by Fleet *et al.* (1984). The genus- or species-specific pattern of yeast chromosomes during the alcoholic fermentation seems therefore to be relatively uniform irrespective of the origin of the grape must. However, differences occurred between the analysed fermentations in the composition and dynamics of yeast strains. The composition of the yeast strain population changed from one fermentation to another. Therefore the different strain-specific patterns can be postulated as typical for each fermentation.

Development of the microflora by inoculation of dried yeasts

According to the results obtained from induced fermentations the succession of the dried yeast strain Siha7 during the first phase of fermentation seems to be stronger in comparison with the other strains W27 and Oenoprox68-72 where strains of *H. uvarum* have also been detected. The presence of *H. uvarum* at the beginning of fermentation may be important with respect to the taste and flavour of wine. Benda (1982) and Lafon-Lafourcade (1983) reported that yeast species such as *H. uvarum* (*Klöckera apiculata*) can produce large amounts of volatile acids. Acetate and glycerol may also be produced at the expense of ethanol. It has been shown in this work that *H. uvarum* indeed produces more acetate than, for instance, *S. cerevisiae*, but not at the expense of ethanol. Examination of the influence of strains of *H. uvarum*, which are active during the first phase of fermentation, on the taste and flavour of wines will be the subject of further investigations.

Strain dynamics of the dried yeast product Oenoprox68-72

The investigation of the mixed dried yeast product Oenoprox68-72 showed that the present two strains were not able to develop simultaneously. Although strain L2872 was dominant in the rehydrated sample it could not succeed against strain L2868. According to the rapid decline of strain L2872, it may be concluded that its influence is rather weak. To increase its influence it would be necessary to increase the percentage of L2872 in the dried yeast product. Recent studies with mixed cultures have shown that it is very important to investigate the competition of component strains to produce mixed dried yeasts in which all strains are able to influence the fermentation process.

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