

Isolation and characterization of cryotolerant *Saccharomyces* strains

by

CATHERINE MASSOUTIER, H. ALEXANDRE, M. FEULLAT and CLAUDINE CHARPENTIER

Institut Universitaire de la Vigne et du Vin, Laboratoire d'Œnologie, Dijon, France

S u m m a r y : Cryotolerant *Saccharomyces* strains were isolated from grape must (Pinot noir) using the enrichment method (CASTELLARI *et al.* 1992). Eleven cryotolerant strains were collected which all belonged to the *S. uvarum* species (Melibiose test and karyotype). Fermentations were carried out at 10 and 25 °C, the level of fermentation products was compared with those produced by mesophilic yeasts. Regardless of temperature, cryotolerant yeasts (SYO55 and 12233) produced twice as many isobutyl and isoamyl alcohols as mesophilic yeast (FB) and 2-phenethyl alcohol was produced by cryotolerant yeasts at levels 4 times as high as by mesophilic yeasts. The potential of these yeasts for oenological application is discussed.

Key words: *Saccharomyces*, cryotolerance, low temperature, wine composition, isobutyl alcohol, isoamyl alcohol, 2-phenethyl alcohol.

Introduction

Among wine and beer yeasts, only some *Saccharomyces cerevisiae* strains are able to ferment well at low temperatures. Cryotolerant or cold-resistant strains ferment well between 6 and 30 °C (rather than between 12 and 36 °C for mesophilic) with an optimum <30 °C. WALSH and MARTIN (1977) reported that these cryotolerant *S. cerevisiae* strains belong more frequently to the p.r. *uvarum* (strains fermenting melibiose) but also to p.r. *bayanus* (strains not fermenting galactose) (CASTELLARI *et al.* 1992).

The purpose of this study was to isolate and collect cryotolerant yeasts which could be interesting from an oenological point of view. After identification the cryotolerant characteristics of these strains were checked by fermentation and growth tests in synthetic must. Then juice from Pinot noir was fermented to determine the differences in the composition of wine produced with the different strains. Those wines were produced with a mesophilic yeast (*S. cerevisiae*) or with a cryotolerant (*S. uvarum*) strain at two different temperatures (25 and 10 °C).

It has been reported that cryotolerant yeasts ferment well at low temperatures. However, there is no report on the produced metabolites of these yeasts at 10 °C. Thus, mesophilic and cryotolerant strains were compared in a biometric study analysing their action on the amount of glycerol, ethanol, higher alcohols, medium chain fatty acids and esters.

The study was also carried out at low temperature (10 °C) to compare the production of these compounds with those produced at an intermediate temperature (25 °C).

Materials and methods

Yeast strains

Sample collection: This study was carried out in 1996 with yeasts isolated from Pinot noir grapes in Bur-

gundy vineyards. The cryotolerant yeasts were isolated by enrichment from grape must according to CASTELLARI *et al.* (1992). Several samples consisting of 500 g of grapes were collected aseptically, placed in sterile plastic bags, stored at +2 °C for 1–2 d and then pressed. Fifty-four samples of must were collected and kept in sterile jars and incubated at +4 °C. Strain N°12233, cryotolerant yeast of the Diproval collection, and Esave and Fermol Bouquet (*S. cerevisiae*, LSA Pascal Biotech) were also used as references for cryotolerant and mesophilic strains, respectively.

Strain isolation: Isolation from must was performed as soon as the first signs of fermentation became visible, *i.e.* after 20–45 d. Eighteen jars out of 54 showed a positive fermentation and were examined. Two samples of 1 ml were withdrawn from each positive fermentation, diluted and then plated on Wickerham medium (10 g·l⁻¹ glucose, 5 g·l⁻¹ bactopectone Difco, 3 g·l⁻¹ yeast extract Difco, 3 g·l⁻¹ malt extract, 20 g·l⁻¹ agar agar) for single colony isolation. The plates were then incubated at 4 °C. Only cultures with clearly different cell morphology and colony traits were isolated from each plate. Nineteen colonies were isolated and stored on Wickerham agar for further investigations.

Strain identification: To identify strains, physiological tests were carried out according to KREGER VAN RIJ (1984) and BARNETT *et al.* (1990). The API 20C identification system (BioMérieux), the melibiose fermentation test, the NO₃⁻ assimilation and the sporulation test were used. The melibiose test was carried out in a chemically defined broth (Yeast Nitrogen Base, Difco 6.7 g·l⁻¹) with melibiose as sole carbon source, Bromothymol blue (0.01 g·l⁻¹) and a Durham tube for gas detection.

The nitrate assimilation test was carried out in liquid medium (Yeast Carbon Base, Difco 11.7 g·l⁻¹) containing 0.78 g·l⁻¹ of potassium nitrate. Pulsed-field electrophoresis: The TAFE system (Transverse Alternating Field Electrophoresis) was employed to separate the chromosomal DNA. Samples of chromosomal DNA were prepared by the method of VEZINHET *et al.* (1990) from yeast cells grown in 10 ml of

YEPD (20 g·l⁻¹ glucose, 20 g·l⁻¹ bacto-peptone Difco, 10 g·l⁻¹ yeast extract Difco) liquid medium. Electrophoresis was carried out at 150 mV for 18 h with a switching interval of 55 s and then for 6 h with a switching interval of 30 s.

Fermentation and growth tests

Culture conditions: Strains were grown and fermented at 10 and 25 °C, in a liquid synthetic medium containing Yeast Nitrogen Base Difco (6.7 g·l⁻¹), glucose (200 g·l⁻¹), tartaric acid (3 g·l⁻¹), malic acid (2 g·l⁻¹), citric acid (0.3 g·l⁻¹), asparagin (2 g·l⁻¹); the pH value was adjusted to 3.5. Culture fermentation was performed in 250 ml flasks containing 125 ml of culture medium which was inoculated with 10⁶ cells·ml⁻¹. The specific growth rates were calculated in the usual way from the linear parts of semi log plots of the absorbance of samples measured at 620 nm against time.

Fermentation tests: Fermentations were monitored by measuring the fermentor weight loss. The validity of this technique using the direct relationship between CO₂ release, sugar consumption, and ethanol production has been shown by EL HALOUI *et al.* (1989). The ethanol concentration was measured by gas chromatography, the final sugar concentration by the dinitrosalicylic acid method (MILLER 1959).

Fermentation: Pinot noir grape juice (1.4 l) with 218 g·l⁻¹ sugar, 7.7 g·l⁻¹ total acidity (as H₂SO₄), 1.12 g·l⁻¹ malic acid and 4 g·hl⁻¹ of SO₂ was inoculated with 10⁶ cells·ml⁻¹, with 3 different strains (SY055: *S. uvarum*, a cryotolerant yeast isolated as described above; 12233: *S. uvarum*, a cryotolerant yeast used as a reference for cryotolerant strain and Fermol Bouquet: *S. cerevisiae*, a mesophilic yeast).

Fermentations were conducted at 10 and 25 °C. Samples were taken during the alcoholic fermentation and analysed for the sugar concentration, glycerol and ethanol. The glycerol analysis was carried out enzymatically using specific kits (Boehringer Mannheim, Germany). Fermentations with cryotolerant yeasts were usually completed in 10-12 d at 10 °C and in 6-7 d at 25 °C. Extraction, analysis by gas chromatography and identification of major volatile compounds of the fermenting juice were carried out according to the method of BERTRAND (1988), with the exception that nonanoic acid was used instead of octan-3-ol as internal standard. Extraction and wine analysis was carried out in triplicate. Gas chromatography was performed with a Chrompack CP 9001, a flame ionisation detector and a capillary column Carbowax 20M (60 m x 0.25 mm x 0.2 µm). Oven temperature was raised from 40 to 200 °C at a rate of 2 °C·min⁻¹. Nitrogen was used (22 ml·min⁻¹) as carrier gas. Both, injector and detector were operated at 250 °C.

Results and Discussion

Strain isolation and identification: We were able to identify two yeast species, *Saccharomyces* (*S. cerevisiae*) in 18 cultures and *Kloeckera* (apiculate yeast) in one culture. The 18 *Saccharomyces cerevisiae* cultures were identified as *uvarum* 11 (Mel+), *cerevisiae* 4 (Mel-) and *bayanus* 3 (Mel-).

In order to confirm the identification of yeast strains performed with a biochemical test, karyotypes of the 18 isolated *Saccharomyces* were determined. Compared to those of *S. cerevisiae* (Mel-), the electrophoretic karyotypes of *S. uvarum* (Mel+) showed some differences in the number and mobility of the chromosomal bands. Thus we could distinguish between *S. cerevisiae* and *S. uvarum* by two specific chromosomal bands, a and b, indicated by arrows (Figure). Both bands are present only in *S. uvarum* (KISHIMOTO and GOTO 1995). An additional band c is characteristic for *S. cerevisiae* but this could not be found in *S. uvarum* (Figure).

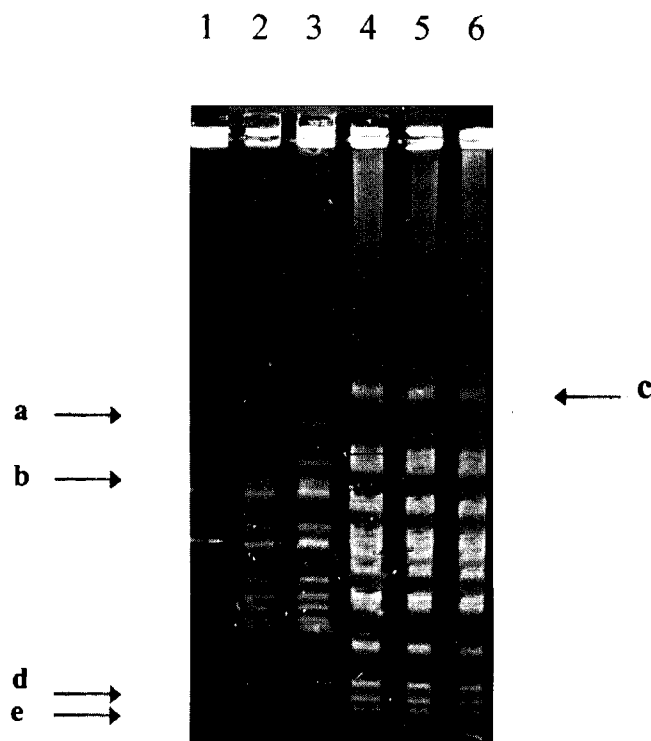


Figure: Electrophoretic karyotypes of *Saccharomyces uvarum* (lanes 1, 2, 3) and *S. cerevisiae* (lanes 4, 5, 6).

Another karyotype analysis highlighted the permanent presence of two well-defined trait bands in the low molecular weight area between 225 and 375 Kb (bands d and e) which differentiates them unequivocally from *S. cerevisiae* strains (GUIDICI *et al.* 1997). Among the yeasts only Fermol Bouquet, Fermol primeur, Fermol rouge and SY021 are *S. cerevisiae* (Tab. 1).

Fermentation and growth tests: To confirm the cryotolerant characteristic of isolated yeast the growth and fermentation tests of the 12 strains of *S. uvarum* were compared with 4 strains of *S. cerevisiae* (Tab. 1). To compare the behaviour of cryotolerant strains with mesophilic strains, fermentations were conducted at 10 °C (low temperature) and at 25 °C (intermediate temperature). 10 °C was chosen instead of 4 °C since at this temperature fermentation with *S. cerevisiae* mesophilic strain species stuck (data not shown).

Table 1

Growth and fermentation characteristics of selected cryotolerant wine yeasts at various temperatures

| Strains | Growth ^a | | Fermentation ^a | | | | |
|---------------------------------|-----------------------------------|-------|---|-------|--|-------|------|
| | Growth rate (h ⁻¹) at | | Fermentation rate (CO ₂ mg/100 ml for 0-12 days) ^b at | | Ethanol yield ^c (v/v, %) at | | |
| | 10 °C | 25 °C | 10 °C | 25 °C | 10 °C | 25 °C | |
| <i>Saccharomyces uvarum</i> | | | | | | | |
| Group I | SY054 | 0.31 | 0.16 | 136 | 86 | 10.3 | 3.2 |
| | SY046 | 0.41 | 0.15 | 152 | 137 | 10.1 | 5.2 |
| | SY043 | 0.21 | 0.17 | ND | 82 | ND | 8.9 |
| | SY045 | 0.21 | 0.11 | 75 | 301 | 7.5 | 10.6 |
| Group II | SY014 | 0.11 | 0.77 | 80 | 446 | 6.7 | 10.2 |
| | SY028 | 0.09 | 0.75 | ND | 344 | ND | 9.6 |
| | SY005 | 0.10 | 1.11 | 80 | 464 | 6.8 | 10.7 |
| | SY029 | 0.10 | 0.43 | 86 | 145 | 9.1 | 10.6 |
| | SY062 | 0.08 | 0.10 | 96 | 302 | 7.0 | 9.6 |
| Group III | 12233 | 0.24 | 0.43 | 144 | 256 | 10.7 | 11.3 |
| | SY066 | 0.21 | 0.42 | 120 | 446 | 10.2 | 10.8 |
| | SY055 | 0.20 | 0.76 | 112 | 416 | 9.9 | 10.5 |
| <i>Saccharomyces cerevisiae</i> | | | | | | | |
| Group II | Fermol Rouge | 0.11 | 0.60 | 64 | 285 | 8.7 | 10.8 |
| | Fermol primeur | 0.10 | 0.52 | 56 | 394 | 9.2 | 10.3 |
| | Fermol Bouquet | 0.18 | 0.77 | 72 | 362 | 8.7 | 11.2 |
| | SY021 | 0.12 | 0.43 | 96 | 432 | 9.6 | 10.8 |

a = Fermentation tests and growth were carried out in 125 ml synthetic medium containing 6.7 g·l⁻¹ Yeast Nitrogen Base Difco, 200 g·l⁻¹ glucose, 3 g·l⁻¹ tartaric acid, 2 g·l⁻¹ malic acid, 0.3 g·l⁻¹ citric acid, 2 g·l⁻¹ asparagin and pH was adjusted to 3.5.

b = Fermentation rates were expressed by the weight of CO₂ produced.

c = Ethanol concentrations were measured by gas chromatography at the end of fermentation.

ND = not determined because it took more than 130 days to finish fermentation.

Among the isolated yeasts, we can distinguish between three groups:

I: Yeasts which ferment well at 10 °C but which have a low production of ethanol at intermediate temperatures. At 10 °C they have a growth rate of 0.2-0.41 h⁻¹ and show a good fermentability at low temperature (strains SY054 and SY046). They are named 'cryotolerant' yeasts (KISHIMOTO *et al.* 1993) and correspond to group A according to WALSH and MARTIN (1977).

II: Yeasts which ferment well only at 25 °C. They have an optimum temperature range of 30-36 °C and grow with difficulty at low temperature (Fermol Bouquet, Fermol Rouge, SY021, Tab. 1). They are named mesophilic yeasts and correspond to group B according to WALSH and MARTIN (1977).

III: Yeasts fermenting well at 10 and 25 °C, with a normal production of ethanol at intermediate temperatures. They are named 'cryophilic' yeasts, too (strains 12233, SY066, SY055, Tab. 1).

Therefore, groups I and III include cryotolerant yeasts whereas group II includes mesophilic yeasts. At low tem-

perature (10 °C) the specific growth rate and fermentability of cryotolerant yeasts were superior to the mesophilic yeasts. During fermentation at 10 °C, the CO₂ evolution velocity of cryotolerant yeasts ranged from 1.7 to 1.9 mg CO₂·100 ml⁻¹ for 0-20 d. These figures were 2-2.5 times as high as those of *S. cerevisiae*.

On the other hand, the ethanol yield of cryotolerant yeasts is lower (1.9-3.2 %) when fermentation is conducted at 25 rather than at 10 °C. In contrast, *S. cerevisiae* showed higher yields of ethanol at 25 °C.

Among the isolated yeasts, the cryotolerant yeast SY055 was chosen. This strain fermented at both temperatures and its ethanol yield was similar to that of *S. cerevisiae*. We also used the strain N°12233 as a reference for cryotolerant strain.

Fermentations: To determine differences in the composition of wine produced with the mesophilic *S. cerevisiae* or with the cryotolerant *S. uvarum* at 25 and 10 °C the amount of glycerol and ethanol produced at the end of the fermentation was determined (Tab. 2): At 25 °C, the cryotolerant strains SY055 and 12233 produced higher

Table 2

Production of glycerol and ethanol at 10 and 25 °C with a mesophilic yeast (Fermol Bouquet) or with a cryotolerant yeast (12233 and SY055) grape must (Pinot noir)

| Strains | Glycerol g ⁻¹ | | Ethanol % | |
|----------------|--------------------------|-------|-----------|-------|
| | 25 °C | 10 °C | 25 °C | 10 °C |
| 12233 | 8.7 | 8.0 | 11.4 | 10.7 |
| SY055 | 8.2 | 6.3 | 11.8 | 10.8 |
| Fermol Bouquet | 7.4 | 5.1 | 12.8 | 8.7 |

amounts of glycerol (8.2 and 8.7 g⁻¹, respectively) than the non-cryotolerant *S. cerevisiae* (Fermol Bouquet). At 10 °C the cryotolerant strains also produced more glycerol than *S. cerevisiae* but the glycerol is produced in a larger amount at 25 °C, whatever yeast we used. At 25 °C, CASTELLARI *et al.* (1994) also observed a larger amount of glycerol in wine fermented with cryotolerant strains compared to those fermented with *S. cerevisiae*.

Determination of the medium-chain fatty acids and other volatile compounds produced at 10 and 25 °C in wine fermented with *S. cerevisiae* (Fermol Bouquet) and *S. uvarum* (12233 and SY055) indicate that higher-boiling esters (ethyl octanoate, 2-phenethyl acetate, ethyl decanoate) were produced in larger amounts in wine at higher fermentation temperatures with Fermol Bouquet (Tab. 3). Compared with both cryotolerant yeasts, Fermol Bouquet produced more medium chain fatty acids at both temperatures.

However, the most significant difference was observed with the formation of 2-phenethyl alcohol, the cryotolerant yeasts (12233 and SY055) producing 4 times as many of it than the mesophilic yeast (Fermol Bouquet) at both, low and intermediate temperatures. Isobutyl and isoamyl alcohols were also produced by cryotolerant strains at levels twice as high as those produced by mesophilic yeast, whatever the temperature (Tab. 3). These differences were observed in the analysis of three strains only.

2-phenethyl alcohol has a pleasant rose-like odor and at a low level may be regarded as a positive component. However, the large amounts produced by cryotolerant strains could affect wine quality in a negative way (BERTOLINI *et al.* 1996). Isobutyl and isoamyl alcohols which are regarded as unpleasant were produced at concentrations under the perception threshold (RANKINE 1967) and may not influence quality.

In contrast to our findings CASTELLARI *et al.* (1992) and CORTE *et al.* (1995) reported a greater production of isoamyl acetate and 2-phenethyl acetate when using *S. uvarum*. Our results show that cryotolerant strains could be interesting in wine making in terms of the production of glycerol and aromatic compounds.

Table 3

Production of esters, higher alcohols and medium-chain fatty acids by cryotolerant yeast (12233 and SY055) and non-cryotolerant strain (Fermol Bouquet, FB). The fermentation tests were carried out in triplicate in grape must (Pinot noir)

| Compounds (mg ⁻¹) | Strains | 25 °C | | 10 °C | | Difference (%) |
|-------------------------------|---------|---------|---------|-------|-------|----------------|
| | | 25 °C | 10 °C | 25 °C | 10 °C | |
| isobutyl alcohol | SY055 | 86±3 | 83±3 | | | -4 |
| | 12233 | 88±2 | 85±2 | | | -3 |
| | FB | 44±2 | 45±2 | | | +2 |
| isoamyl acetate | SY055 | 2.9±0.2 | 3.2±0.3 | | | +10 |
| | 12233 | 3.0±0.3 | 3.3±0.4 | | | +10 |
| | FB | 2.9±0.2 | 2.5±0.2 | | | -16 |
| isoamyl alcohol | SY055 | 245±25 | 245±27 | | | 0 |
| | 12233 | 280±30 | 265±28 | | | -6 |
| | FB | 146±12 | 179±15 | | | +18 |
| ethyl hexanoate | SY055 | 2.0±0.1 | 2.0±0.1 | | | 0 |
| | 12233 | 2.0±0.2 | 1.9±0.1 | | | -5 |
| | FB | 2.0±0.1 | 2.0±0.2 | | | 0 |
| ethyl octanoate | SY055 | 1.6±0.1 | 1.9±0.2 | | | +16 |
| | 12233 | 1.5±0.1 | 1.7±0.2 | | | +12 |
| | FB | 1.9±0.3 | 1.2±0.1 | | | -40 |
| ethyl decanoate | SY055 | 1.4±0.1 | 1.8±0.2 | | | +22 |
| | 12233 | 1.6±0.2 | 1.7±0.2 | | | +6 |
| | FB | 1.6±0.2 | 1.5±0.2 | | | -7 |
| 2-phenethyl acetate | SY055 | 1.6±0.2 | 1.8±0.3 | | | +11 |
| | 12233 | 1.6±0.2 | 1.7±0.2 | | | +6 |
| | FB | 1.6±0.2 | 1.4±0.2 | | | -14 |
| hexanoic acid | SY055 | 1.2±0.1 | 1.0±0.1 | | | -20 |
| | 12233 | 1.3±0.1 | 1.0±0.1 | | | -30 |
| | FB | 1.7±0.3 | 1.3±0.2 | | | -30 |
| 2-phenethyl alcohol | SY055 | 166±15 | 188±22 | | | +12 |
| | 12233 | 216±23 | 225±25 | | | +4 |
| | FB | 45±4 | 47±5 | | | +3 |
| octanoic acid | SY055 | 2.0±0.2 | 1.9±0.2 | | | -5 |
| | 12233 | 1.6±0.2 | 1.5±0.2 | | | -7 |
| | FB | 2.1±0.3 | 2.0±0.2 | | | -5 |
| decanoic acid | SY055 | 1.6±0.2 | 1.4±0.1 | | | -14 |
| | 12233 | 1.6±0.2 | 1.3±0.2 | | | -23 |
| | FB | 1.7±0.2 | 1.6±0.2 | | | -6 |

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