RESOLUTION OIV-OENO 370-2012

GUIDELINES FOR THE CHARACTERIZATION OF WINE YEASTS OF THE GENUS SACCHAROMYCES ISOLATED FROM VITIVINICULTURAL ENVIRONMENTS

THE GENERAL ASSEMBLY

IN VIEW OF ARTICLE 2, paragraph 2 IV of the Agreement of 3 April 2001, by which the International Organisation of Vine and Wine was founded,

CONSIDERING the Strategic Plan of the OIV

On proposal of the group of experts "Microbiology"

DECIDES to adopt the following guidelines on criteria for characterization of Saccharomyces yeasts from vitivinicultural environments:

Guidelines for the characterization of wine yeasts of the genus Saccharomyces isolated from vitivinicultural environments

RESOLUTION OIV-OENO 370-2012

GUIDELINES FOR THE CHARACTERIZATION OF WINE YEASTS OF THE GENUS SACCHAROMYCES ISOLATED FROM VITIVINICULTURAL ENVIRONMENTS

Guidelines for the characterization of wine yeasts of the genus Saccharomyces isolated from vitivinicultural environments

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In each paragraph of the sections 1 and 2 standard criteria for the characterization of

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wine yeast (S. cerevisiae) are included indicating, where it is necessary, the wine technology style

PREAMBLE

There is no doubt that the evolution of enological core techniques have been advancing rapidly over the last decades, due to a greater demand for wine quality and hygienic production conditions (safety).

New biotechnologies have been developed over the years to explain the importance of microorganisms and their effects on the sensorial characteristics of wine. Most of the wine makers use yeast starters selected according to various criteria which also assure control in the alcohol fermentation process.

For many years, these starters have been selected with a preference for Saccharomyces yeasts among the natural grape microflora, owing to their more suitable characteristics, mainly the high alcohol-tolerance. Specifically, fundamental enological reasons highlight the importance to indicate geographical origin and/or isolation area and/or grape variety origin of yeasts strains selected as starter cultures.

The genus Saccharomyces and some of its species (S. cerevisiae, S. bayanus), seem ideal for carrying out alcoholic fermentation as a result of an evolutionary adaptation to high must sugar concentrations and subsequent conversion into ethanol, carbon dioxide and numerous organoleptic compounds. The variation between and within species gives the possibility to amplify strain characterization, with some being very useful in industrial environments.

The progress in analytical techniques of researching the chemical compounds in grape must fermentations allows for constant advances in the understanding of strain functionality under different fermentation conditions. This without forgetting that molecular biology has not been indifferent to the goals that the industry has set for modern enology and will include targeted usage of "-omics" technologies (genomics, transcriptomics,proteomics or metabolomics).

In this sense the understanding of the complete genome of some species of Saccharomyces and the specific functions of thousands of genes will make possible new scientific advances, especially on their physiology and functionality during fermentation. All research on yeast as a complex entity will noticeably make characterization studies easier and deliver success in modern enology with the application of new selected yeast strains.

In addition, the following characterization criteria concentrate mainly on the characterizations of Saccharomyces yeasts, keeping in mind that there is a growing interest in isolating, characterizing and using yeasts belonging to the group of "Non-Saccharomyces" yeasts, which form a large part of the natural yeast flora on the

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grapes and are active at the begin of alcoholic fermentations. Although they often influence fermentability and the resulting wine flavour and taste negatively, some of these yeasts also add positively addressed aroma compounds that enrich the wine complexity. Guide lines for the characterization of Non-Saccharomyces wine yeasts will be presented in another specific resolution.

This resolution covers the yeast genus Saccharomyces and respective hybrids and contains a compilation of yeast characterization criteria which are helpful in the process of isolating and characterizing wine yeasts for quality oriented wine production. They are grouped into technological, organoleptical and also those criteria that have an impact on human health. According to the expression of different varietal characters or wine styles, these criteria might be of different importance for different musts, therefore they are not mandatory. As wine styles change with time, the current list should be seen as an open list that might be enlarged in future.

During the characterization process, the testing of some criteria involves the usage of complex or synthetic growth media. These media are helpful to gain first information about yeast properties; however they are only to be seen as indicators as the yeast strain might react in a different way in grape must during fermentation and so experiments in musts, have to be also performed.

The process to elaborate the active dry yeast (as nutrition, drying) could influence the performances of the yeast and so experiments with the dry form must also be performed.

Characterizations of yeasts include the aspects of producing high quality wines as well as the fulfillment of legislation in terms of food safety. Criteria relevant for safety aspects are not indicated in a special way as national and/or international legislation might differ between the member states. The selecting person or company is therefore obliged to take these legal requirements into consideration.

The guidelines can be also applied testing dried pure yeast cultures. These dried yeasts have to be reactivated and inoculated according to the manufacturer's instructions.

1. CHARACTERISTICS INFLUENCING VINIFICATION

1.1. GENERAL TECHNOLOGICAL CHARACTERISTICS

1.1.1. FERMENTING POWER

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1.1.1.1. Field of application

Standard criterion for strains with fermentation capability

1.1.1.2. Principle

Determining the maximum quantity of sugar that a yeast strain is capable of fermenting in a rich medium.

1.1.1.3. Equipment

- Sterilizer
- Analytical balance, precision to within 0.1 g
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

1.1.1.4. Reagents and materials

- The fermentation medium is prepared by the dilution of a concentrated grape must until the sugar concentration reaches 250 g·l–1 (yeasts for white vinification), or 300 g l-1 (for yeast strains to be used in red vinification especially in warm areas). In order to determine a general and comparable strain behaviour, synthetic media of similar composition are used instead (Appendix 1 for synthetic media)
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

1.1.1.5. Procedure

Yeast cell populations are obtained by inoculating 100 µl from the mother culture into 5 ml of fresh medium and incubating for 48 hours three times. The final culture should contain 108 CFU·ml–1. Then 1 ml of yeast inoculum is transferred to a 100 ml Erlenmeyer flask containing 50 ml of the fermentation medium. After inoculation, the flask is sealed with a Müller trap. Microvinification is monitored by gravimetric analysis at 16-18 °C for white wine production and at 24-28 °C for red wine

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production. The wine yeasts must achieve complete fermentation of musts containing 200-300 g/L sugar (approximately 11.5-14.5 % v/v ethanol).

1.1.1.6. Calculations

Fermenting power = 2.5 ·Δ weight

1.1.1.7. Bibliography

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1.1.2. FERMENTATIVE VIGOUR AND FERMENTATION KINETICS AT CERTAIN TEMPERATURES

1.1.2.1. Field of application

Standard criterion for wine yeasts

1.1.2.2. Principle

The fermentative vigour, expressed as the speed for which yeast starts the fermentation, is expressed as grams of CO_2 produced 2- 3 days after the beginning of fermentation. Monitoring fermentation kinetics uses a curve grams of CO_2 produced or grams of fermented sugar vs. time. In order to select strains showing fast fermentation start up, no peaks due to temperature variations and good performance towards the end of fermentation. Good fermentation kinetics mean lower energy consumption due to temperature control. Temperature is one of the factors that vary from one winemaking process to another. It influences the production of volatile acidity and metabolites during fermentation, but also yeast viability.

1.1.2.3. Equipment

- Sterilizer
- Analytical balance, precision to within 0.1 g
- Laminar flow cabinet
- Pipettes with sterile tips

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- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

1.1.2.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 250-300 g·l–1, depending on white or red wine making. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium to obtain mother culture.

1.1.2.5. Procedure

Yeast cell populations are preculturedby inoculating 100 µl from the mother culture into 5 ml of fresh medium and incubating for 48 hours three times. The final culture should contain 108 CFU·ml^{-1}. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106. living cells/ ml^{-1}.

Microvinification is monitored by gravimetric analysis as described in 1.1.1.5 but at different fermentation temperatures (15 °C, 20 °C, 25 °C and 30 °C). For every temperature a curve must be prepared showing the variations in CO_2 released per day in order to study the effect of temperature on fermentation speed.

1.1.2.6. Calculations

Using the curves the following parameters are studied for each strain: length of the latent (lag) phase, curve slope in the log phase, length of the stationary phase, and length and curve slope for the final phase. Find the optimum range for the sample strain by comparing the curves obtained at different temperatures.

1.1.2.7. Bibliography

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1.1.3. RESISTANCE TO SO_2

1.1.3.1. Field of application

Standard criterion for wine yeasts

1.1.3.2. Principle

Sulphur dioxide (SO_2) is an antioxidant and a bacteriostatic agent used in winemaking in order to prevent oxidation, to obtain microbiological stabilization and to enable the characterization of fermentative yeasts in spontaneous fermentations. Strains ensuring complete fermentation when the concentrations of free sulphur dioxide and total sulphur dioxide are over 30 mg·l^{-1}-1 and 50 mg·l^{-1} respectively are suitable for wine yeast characterization. Higher sulphur dioxide concentrations up to 100-150 ml^{-1} are recommended according to some authors for special wines (Ribérau-Gayon et al. 2003)

1.1.3.3. Equipment

- Sterilizer
- Analytical balance, precision to within 0.1 g
- Laminar flow cabinet
- Pipettes with sterile tips
- Sterile Petri dishes
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

1.1.3.4. Reagents and materials

1.1.3.4.1.

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar

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composition may be used instead (Appendix 1). The pH is adjusted to 3.5 with tartaric acid.

- Potassium metabisulfite
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium to obtain mother culture. Yeast cell populations are precultured by inoculating 100 µl from the mother culture into 5 ml of fresh medium and incubating for 48 hours three times. The final culture should contain 108 CFU·ml–1. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106. living cells/ ml^{-1}.

1.1.3.4.2.

- Medium composed by sterile agarized grape must. Synthetic media of similar composition may be used instead (Appendix 1). Grape must, sterilized in autoclave at 100°C for 20 minutes, is added with an equal amount of aqueous sterile solution containing 4% agar. The final pH of the medium is 3.5.
- Potassium metabisulphite
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

1.1.3.5. Procedures

1.1.3.5.1.

50 ml sterile must (pH 3.5) are treated with potassium metabisulfite at the rate of 50 mg·l^{-1} total SO_2. Prepare a control broth without potassium metabisulfite. Inoculate both broths with 1 ml of the synchronised culture of the selected strain. Monitor the production of CO_2 per day during fermentation and prepare growth curves. Calculate the fermentation power. Compare the fermentation power of the yeast in the presence and absence of SO_2. Analyze curve deviations in the presence of SO_2. Studying the influence of a higher SO_2concentration and that of temperature is highly recommended.

1.1.3.5.2.

The medium is supplemented prior to pouring into the Petri dishes with variable amounts of sterile solution containing sulphur dioxide, as potassium metabisulphite,

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in function of doses of sulphur dioxide tested, ranging from 50 until 300 ppm. Positive control is represented by the same medium, without sulphur dioxide. The strains are grown for 24 h on YEPD medium, after that they are inoculated on the medium in concentration of about 104 cells/ml. After incubation at 26°C for 48 h, yeast growth (positive/negative) at the different concentrations tested is examined in confront to the positive control. Resistance degree of yeast to sulphur dioxide is reported as the maximum dose at which the yeast exhibits a significant growth.

1.1.3.6. Bibliography

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1.1.4. COPPER RESISTANCE

1.1.4.1. Field of application

Standard criterion for wine yeasts

1.1.4.2. Principle

Copper occurs in must and in wine as a consequence of the use of compounds containing copper for pest control of the vineyard. High copper concentrations in must have toxic effects on growth and fermentative activity of yeast cells which, anyway, have developed some defence mechanisms

1.1.4.3. Equipment

- Sterilizer
- Analytical balance, precision to within 0.1 g

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- Laminar flow cabinet
- Pipettes with sterile tips
- Sterile Petri dishes
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

1.1.4.4. Reagents and materials

1.1.4.4.1.

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar composition may be used instead (Appendix 1).
- Copper sulphat
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium to obtain mother culture. Yeast cell populations are precultured by inoculating 100 µl from the mother culture into 5 ml of fresh medium and incubating for 48 hours three times. The final culture should contain 108 CFU·ml–1. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106. living cells/ ml^{-1}.

1.1.4.4.2.

- Medium composed by sterile agarized grape must. Grape must, sterilized in autoclave at 100°C for 20 minutes, is added with an equal amount of aqueous sterile solution containing 4% agar. The final pH of the medium is 3.5. Synthetic media of similar composition may be used instead (Appendix 1).
- Copper sulphate
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

1.1.4.5. Procedures

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1.1.4.5.1.

The copper resistance of yeast strains is evaluated by microvinifications performed according to procedures in the section 1.1.1.5 and by using grape must containing copper sulphate at different levels in order to reach copper concentrations ranging from 20 to 500 μmol/L.. Microvinifications not containing copper sulphate are performed as control tests. After inoculation, flasks are sealed with Müller traps and weighted daily to follow the weight loss caused by CO_2 evolution. The copper concentration causing a significant decrease of CO_2 evolution in comparison with the control test indicates the copper resistance level in assayed yeast strain.

1.1.4.5.2.

The copper resistance may be determined also by plate assay, as reported at 1.1.3.5.2 paragraph. Copper doses, added as copper sulphate, ranged from 20 until 500μmol/L, in comparison to the control without the antimicrobial compound.

1.1.4.6. Bibliography

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1.1.5. KILLER CHARACTER, K AND N PHENOTYPES

1.1.5.1. Field of application

Standard criterion for wine yeasts characterization.

1.1.5.2. Principle

Killer yeasts can grow to the detriment of other yeasts under winemaking conditions. The strains can produce the killer toxin (M+R+ phenotype) and are resistant to it. It has been suggested that using killer strains may enhance yeast implantation in winemaking. However, the killer toxin is buffered under winemaking conditions compared to the toxicity in vitro. This is especially true for red wines.

1.1.5.3. Equipment

- Sterilizer

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- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture.
- Petri dishes

1.1.5.4. Reagents and materials

1.1.5.4.1.

- Solid medium for yeast culture (YEPD) buffered
- Methylene blue
- Sensitive (S) and killer (K2) strains

1.1.5.4.2.

- Potate Dextrose Agar (PDA) synthetic medium, buffered at pH 5.6
- Bromophenol blue

1.1.5.5. Procedure

1.1.5.5.1.

The test strain is streaked and grown on a lawn of a sensitive (S) yeast strain obtained in solid medium buffered and containing methylene blue. The test strain is also streaked and grown on a lawn of a killer (K2) yeast strain.

1.1.5.5.2.

A loopful of tested yeasts is inoculated on PDA medium, after that the plates are incubated at 26°C for 3-5 days. Yeast strains forming blue-violet colonies are considered killer strains, whereas strains forming grey colonies are sensitive-strains.

Killer activity may be evaluated also by agar plate diffusion. In details, yeast sensitivity to killer toxin is evaluated by spreading about 105 cells of the yeast to be tested for sensitivity on plates containing 25 ml of Low-pH Blue medium (YEPD agar added with 0.003% (w/v) methylene blue, buffered at pH 4.5 with citrate/phosphate 0.1 M). After

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spreading sensitive-strain, killer activity is tested by inoculating in liquid (as a drop containing 108 cells/ml) or solid (streaking) killer strain on sensitive strain. The plates are incubated at 25°C for 4-5 days. Killer activity is measured on the basis of presence and dimension of the inhibition zone.

1.1.5.6. Bibliography

1. Suárez-Lepe, J. A. Levaduras vínicas. Funcionalidad y uso en bodega. Ed Mundiprensa, Madrid, 1997, 71-91.1.
2. Rosini, G. The occurrence of killer characters in yeasts. Can. J. Microbiol., 1983, 29, 1462–1464..

1.1.6. MODE OF GROWTH IN LIQUID MEDIUM

1.1.6.1. Field of application

Standard criterion for wine yeasts

1.1.6.2. Principle

The mode of growth of yeasts during fermentation is an important technological characteristic as it can affect the management of the fermentative process. Indeed, yeast cells may show a different mode of growth (such as single or aggregate cells) depending on cell wall hydrophobicity. Generally, dispersed growth and rapid sedimentation are required in wine yeast strains selected for standard winemaking process

1.1.6.3. Equipment

- Autoclave
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- Spectrophotometer
- vortex

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1.1.6.4. Reagents and materials

- Liquid medium YEPD

1.1.6.5. Procedure

After incubation in YEPD at 25°C for 3 days, optical density of yeast cell cultures is measured at 620 nm soon after a strong mixing (D0) and after 10 minutes (D1). Values of the ratio R = D1x100/D0 allow to identify not flocculent yeast strains (R = 100%) or sliglhtly flocculent yeast strains (R ranging from 70 to 100%)

1.1.6.6. Bibliography

1. Romano P., Fiore C., Capece A. Metodi per la caratterizzazione fenotipica di lieviti vinari In: Microbiologia del vino. Eds. Vincenzini M., Romano P., Farris G.A. Casa Editrice Ambrosiana - Milano, Italia, 2005. 435-450.

1.1.7. FOAM PRODUCTION

1.1.7.1. Field of application

Standard criterion for wine yeasts

1.1.7.2. Principle

The foam formation by yeasts is a strain-dependent characteristic that occurs during wine fermentation depending on cell wall hydrophobicity. The absence of foam production or low foam formation in wine fermentations is considered as a positive trait.

1.1.7.3. Equipment

- Autoclave
- Laminar flow cabinet
- Sterile Membranes 0,2 μm
- Analytical balance
- Graduated cylinder (50 ml)

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- Infrared light

1.1.7.4. Reagents and materials

- Synthetic medium (Palmieri et al, 1996)

1.1.7.5. Procedure

The ability of yeasts to produce foam is evaluated by flotation measure. The synthetic medium is inoculated at a concentration of 0.2 mg cell dry weight/ml and then it is put into the graduated cylinder whose top is connected to a glass vessel suitable to contain the foam. Hence, air flux (6-7 ml/s) is introduced onto the bottom of the cylinder. In order to evaluate the ability of yeast strain to produce foam, cell concentration is measaured at the beginning (C_p), after foam formation (C_r) and inside the foam (C_s) drying samples (1 ml each) by infrared light until constant weight is reached. The ability of yeast strain to produce foam is calculated by the following formula

$$Flot\% = \left[(C_p - C_r)/C_s \times 100\right]$$

1.1.7.6. Bibliography

1. Palmieri M.C., Greenhalf W., Laluce C. Efficient flotation of yeast cells grown in batch culture. Biotechnology and Bioengineering, 1996, 50: 248-256.
2. Romano P., Fiore C., Capece A. Metodi per la caratterizzazione fenotipica di lieviti vinari In: Microbiologia del vino. Eds. Vincenzini M., Romano P., Farris G.A. Casa Editrice Ambrosiana - Milano, Italia, 2005. 435-450.

1.2. TECHNOLOGICAL CHARACTERISTICS FOR WHITE VINIFICATION

1.2.1. CAPABILITY TO FERMENT HIGHLY CLARIFIED MUSTS WITH LOW LEVELS OF ASSIMILABLE NITROGEN

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1.2.1.1. Field of application

Producing young white wine using grape varieties with a neutral aroma.

1.2.1.2. Principle

In order to improve the wine's aroma when using grape varieties with a neutral aroma, selected yeasts producing esters during fermentation are commonly used. The production of these aromatic compounds may be enhanced by using highly clarified musts. However, this may result in stuck fermentation. Those strains capable of fermenting with around 100 mg·l–1 of assimilable nitrogen are suitable for characterization.

1.2.1.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

1.2.1.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. YAN / FAN is adjusted to 100 mg·l^{-1}. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}

1.2.1.5. Procedure

Monitor fermentation kinetics under small-scale winemaking conditions and with known low concentrations of FAN / YAN. Since there is a risk of stuck fermentation, the final phase is to be studied in the curves.

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1.2.1.6. Bibliography

1. Suárez-Lepe, J. A. Levaduras vínicas. Funcionalidad y uso en bodega. Ed Mundiprensa, Madrid, 1997, 28-40.

1.3. TECHNOLOGICAL CHARACTERISTICS FOR RED VINIFICATION

1.3.1. PERFORMANCE AT THE END OF FERMENTATION IN MUSTS RICH IN SUGARS. RESISTANCE TO STRESS DURING FERMENTATION

1.3.1.1. Field of application

strain characterization dedicated to produce red wines with high alcohol content

1.3.1.2. Principle

Determining the maximum quantity of sugar which a yeast strain is capable to ferment in a rich medium. When producing red wines, particularly in warm regions and with high polyphenolic quality, sugar concentration requirements may result in wines containing 14.5 15.5 % v/v alcohol. Yeast stress includes difficulties at the end of fermentation mainly due to a lack of essential nutrients and to the toxic synergy caused by ethanol. It occurs frequently during the last phases of fermentation of red wines with high sugar content. This process may be reproduced in the laboratory by adding short-chain (C10) saturated fatty acids. These compounds stiffen biological membranes creating a situation, like that observed in sluggish fermentation final phases, in which the yeast has difficulties in obtaining nutrients.

1.3.1.3. Equipment

- Sterilizer
- Analytical balance, precision to within 0.1 g
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap

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1.3.1.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar composition may be used instead (2.1 Appendix 1.)
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106. living cells/ ml^{-1} n-decanoic acid

1.3.1.5. Procedure

Fermentation under small-scale winemaking conditions and with high concentrations of n decanoic acid (150 200 mg·l^{-1}) is monitored by gravimetric analysis. Kinetics are studied by using control strains.

1.3.1.6. Bibliography

1. Morata, A.; Calderón, F.; Colomo, B.; González, M. C.; Suberviola, J.; Suárez, J. A. Mejora de la cinética fermentativa en la vinificación en tinto: Levaduras resistentes a estrés fermentativo. Tecnología del vino, 2004, 15, 39-45.

1.3.2. AUTOLYSIS AND RELEASE OF YEAST CELL WALL POLYSACCHARIDES

1.3.2.1. Field of application

Criterion for strain characterization to produce red wines aged on lees by releasing polysaccharides.

1.3.2.2. Principle

The use of yeast strains selected to produce red wines on lees with increased autolysis rates and release of polysaccharides. In wines, these polysaccharides enhance colloidal and color stabilities.

1.3.2.3. Equipment

- Sterilizer
- Laminar flow cabinet

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- Pipettes with sterile tip
- Test-tubes for culture synchronisation
- Erlenmeyer flasks
- Bioreactor
- Freeze dryer
- Orbital shaker
- Centrifug
- HPLC-IR apparatus
- Column for size-exclusion chromatography

1.3.2.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used (Appendix 1.)
- Model medium for autolysis assay (alcohol-water buffered solution)
- Eluant: $NaNO_3$(0.1 M)
- 96 % v/v ethanol
- Concentrated HCl
- Enzyme assay kit for determination of glycerol

1.3.2.5. Procedure

The polysaccharides contained in yeast autolysates are extracted by precipitation in an acid non-polar medium (ethanol: HCl). Wash with ethanol and centrifuge at 9000 rpm. Remove the supernatant. The sample is then mixed with 0.1 M $NaNO_3$, filtered (0.45 µm) and stored refrigerated until HPLC IR analysis. Select yeasts releasing high amounts of polysaccharides in a short time.

1.3.2.6. Bibliography

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1. Suárez-Lepe, J. A.; Morata, A.; Calderón, F.; Somolinos, S.; González, M.C.; Colomo, B. Utilización de levaduras seleccionadas en la crianza sobre lías de vinos tintos. Nuevo método de crianza sobre lías. Tecnología del vino, 2005, 26, 57 -61
2. Suárez-Lepe, J. A.; Morata, A.; Calderón, F.; Somolinos, S.; González, M.C.; Colomo, B. Utilización de levaduras seleccionadas en la crianza sobre lías de vinos tintos. Nuevo método de crianza sobre lías. Alimentación, Equipos y Tecnología, 2005, 207, 39-43.

1.3.3. SUGAR ALCOHOLS PRODUCTION

1.3.3.1. Field of application

Criterion for strain characterization to produce red wines.

1.3.3.2. Principle

2,3 butanediols are the most abundant sugar alcohols in wines besides glycerol. They also play an important role in improving structure and smoothness.

1.3.3.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap
- GC-FID apparatus

1.3.3.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium. Yeast cell concentration at the time of inoculation in the given test media should be approx.

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106 living cells/ ml^{-1}.

1.3.3.5. Procedure

Measuring 2,3 butanediols production during small-scale fermentation using a single yeast strain under isothermal (25 °C) conditions. Method of analysis: gas chromatography with flame ionization detection (GC-FID). Select strains producing 1 $g{\cdot}l^{-1}$ or more.

1.3.4. CELL WALL ANTHOCYANIN ADSORPTION

1.3.4.1. Field of application

Criterion for strain characterization designed to produce red wines, particularly in regions where warm climatic conditions result in poor anthocyanin synthesis.

1.3.4.2. Principle

Anthocyanins are adsorbed to a great extent by yeast cell walls during fermentation and maceration, according to the anthocyanins' polar nature and cell wall structure. Yeasts with a low cell wall anthocyanin adsorption rate to minimize the loss of anthocyanins and the possible influence on the color of the wine.

1.3.4.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- Centrifuge
- HPLC-DAD-MS apparatus
- C18 reversed phase HPLC column

1.3.4.4. Reagents and materials

- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

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- Crushed red wine grapes, or model media plus anthocyanin extracts.
- Methano
- Formic acid
- Milli-Q water

1.3.4.5. Procedure

A red wine grape must or a model media containing an anthocyanin extract is fermented using a single yeast strain under standard small-scale fermentation conditions. The biomass of yeast is obtained by centrifugation. Anthocyanins adsorbed by cell walls are extracted, quantified and identified by HPLC-PDA. Yeast cell wall anthocyanin adsorption varies from 2% to 6%.

1.3.4.6. Bibliography

1. Morata, A.; Gómez-Cordovés, M. C.; Suberviola, J.; Bartolomé, B.; Colomo, B.; Suárez, J. A. Adsorption of anthocyanins by yeast cell walls during the fermentation of red wines. J. Agric. Food Chem. 2003, 51, 4084-4088.
2. Morata, A.; Gómez-Cordovés, M. C.; Colomo, B.; Suárez, J. A. Cell wall anthocyanin adsorption by different Saccharomyces strains during the fermentation of Vitis vinifera L. cv Graciano grapes. Eur. Food Res. Technol. 2005, 220, 341-346

1.3.5. PRODUCTION OF ACETALDEHYDE AND PYRUVIC ACID TO ENHANCE VITISIN PRODUCTION

1.3.5.1. Field of application

Criterion for strain characterization designed to produce red wines, particularly those that will be aged in wood, using highly stable pyranoanthocyanins.

1.3.5.2. Principle

Vitisins are highly stable pigments. They can withstand sulphur dioxide, hydration, and oxidation reactions. Vitisins are not found in grapes but are produced during fermentation by condensation reactions involving certain metabolites (acetaldehyde and pyruvic acid). Structurally, vitisins are pyranoanthocyanins. The use of selected yeasts which produce acetaldehyde and pyruvic acid at optimum rates enhances the production of these highly stable pigments during fermentation.

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1.3.5.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- HPLC-DAD-MS apparatus
- C18 reversed phase HPLC column

1.3.5.4. Reagents and materials

- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- Crushed red wine grapes, or model media plus anthocyanin extracts. Synthetic media of similar composition may be used instead (Appendix 1)
- Methanol
- Formic acid
- Milli-Q water

1.3.5.5. Procedure

A red must is inoculated with a precultured yeast strain (approx. 106 living cells/ ml^{-1}) and fermentation under small-scale winemaking conditions is monitored. The formation of vitisins is studied by HPLC-PDA and HPLC-ESI-MS. The assay is performed in triplicate to increase reliability.

1.3.5.6. Bibliography

1. Morata, A.; Gómez-Cordovés, M. C.; Colomo, B.; Suárez, J. A. Pyruvic acid and acetaldehyde production by different strains of Saccharomyces cerevisiae: Relationship with vitisin A and B formation in red wines. J. Agric. Food Chem. 2003, 51, 7402-7409
2. Morata, A.; Calderón, F.; González, M. C.; Gómez-Cordovés, M. C.; Suárez, J. A.

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Formation of the highly stable pyranoanthocyanins (vitisins A and B) in red wines by the addition of pyruvic acid and acetaldehyde. Food. Chem. 2007, 100, 1144-1152.

3. Morata, A.; Calderón, F.; González, M. C.; Colomo, B.; Suárez, J. A. Formación de vitisinas durante la fermentación de vinos tintos. Tecnología del vino, 2004, 21, 61-65.

1.3.6. HYDROXYCINNAMATE DECARBOXYLASE POSITIVE STRAINS AND FORMATION OF VINYLPHENOLIC PYRANOANTHOCYANINS

1.3.6.1. Field of application

Criterion for strain characterization designed to produce red wines, particularly those that will be aged in wood, using highly stable pyranoanthocyanins.

1.3.6.2. Principle

Vinylphenol anthocyanin derivatives are pigments of pyranoanthocyanin nature. They have similar properties to vitisins. They are not found in grapes, but are produced during fermentation by condensation reactions involving vinylphenols, which are formed from hydroxycinnamic acids. These are produced by hydroxycinnamate decarboxylase positive strains of Saccharomyces. hydroxycinnamate decarboxylase positive yeast strains enhance the production of these highly stable pigments during fermentation.

1.3.6.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- HPLC-DAD-MS apparatus
- C18 reversed phase HPLC column

1.3.6.4. Reagents and materials

- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium. - Crushed

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red wine grapes, or model media plus anthocyanin extracts. Synthetic media of similar composition may be used instead (Appendix 1)

- Methanol
- Formic acid
- Milli-Q water
- p-coumaric acid, ferulic acid and caffeic acid.

1.3.6.5. Procedure

A red must is inoculated with a precultured yeast strain . Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml−1. Fermentation under small-scale winemaking conditions is monitored by gravimetric analysis as described in 2.1.1.1.5. The formation of vinylphenol anthocyanin adducts is studied by HPLC-PDA and HPLC-ESI-MS. The assay is performed in triplicate to increase reliability.

1.3.6.6. Bibliography

1. Morata, A.; Calderón, F.; González, M. C.; Colomo, B.; Suárez, J. A. Protección de color y aroma en vinos tintos mediante la formación de derivados vinilfenólicos de antocianos. Tecnología del vino, 2005, 24,32-36
2. Morata, A.; Gómez-Cordovés, M. C.; Calderón, F.; Suárez, J. A. Effects of pH, temperature and SO2 on the formation of pyranoanthocyanins during red wine fermentation with two species of Saccharomyces. Int. J. Food Microbiol. 2006, 106, 123-129.
3. Morata, A.; González, C.; Suárez, J. A. Formation of vinylphenolic pyranoanthocyanins by selected yeasts fermenting red grape musts supplemented with hydroxycinnamic acids. Int. J. Food Microbiol. 2007, 116, 144-152.

1.3.7. β-GLYCOSIDASE ACTIVITY

1.3.7.1. Field of application

Criterion for strain selection designed to produce red wines.

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1.3.7.2. Principle

Anthocyanins are found as glucosides in grapes. Some strains with extracellular □ glycosidase activity can hydrolyze anthocyanins into aglycones, which are more unstable. This may influence color stability. For red wines, β -glycosidase negative strains are selected.

1.3.7.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation

1.3.7.4. Reagents and materials

- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- API ZYM test strip for extracellular enzyme activity (bioMérieux SA, Lyon, France).

1.3.7.5. Procedure

In vitro detection of extracellular □ glycosidase activity. Use synchronised pure cultures containing 108 CFU·ml^{-1} (24-48 hours). Test □ glycosidase activity under winemaking conditions. Method: API ZYM test strips (bioMérieux SA, Lyon, France). Select β glycosidase negative strains.

1.3.7.6. Bibliography

1. Suárez-Lepe, J. A. Levaduras vínicas. Funcionalidad y uso en bodega. Ed Mundiprensa, Madrid, 1997, 110-111.
2. Delcroix, J.; Gunata, Z.; Sapis, J. C.; Salmon, J. M. ; Bayonave, C. Glycosidase Activities of Three Enological Yeast Strains During Winemaking: Effect on the Terpenol Content of Muscat Wine. Am. J. Enol. Vitic. 1994, 45, 291-296.

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2. CHARACTERISTICS INFLUENCING ORGANOLEPTIC WINE QUALITY

2.1. PRODUCTION OF VOLATILE ACIDITY

2.1.1. Field of application

Standard criterion for wine yeasts

2.1.2. Principle

Volatile acidity is expressed as g/L of acetic acid. Acetic acid is a fermentation by-product which causes wine defects when present in high concentration. It is an indicator of microbiological problems. Most regulations in wine-growing areas put a limit on the amount of volatile acidity in finished wine.

In order to enhance maceration, fermentation temperatures used are often higher for red wines than for white wines (25-32 °C). The production of volatile acidity is directly proportional to fermentation temperature. The sensory qualities of red wines may be enhanced by yeasts that are able to ferment within these temperature ranges with low production of volatile acidity.

2.1.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap
- Steam distillation and titration apparatus

2.1.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used instead (Appendix 1.).

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- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- Reagents for determination of acetic acid [1]

2.1.5. Procedure

Measuring volatile acidity produced during small scale fermentation using a pure yeast strain under isothermal and temperature-controlled conditions. Values of temperature are chosen according to the white or red vinification. .Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. Method of analysis: OIV reference method, steam distillation and acid-base titration [1]. Alternatively, the amount of acetic acid produced by yeasts subject to characterization can be determined using the enzymatic method. Alternatively, acetic acid may be determined by liquid chromatography (HPLC) with UV detection.

The assay must be carried out in triplicate for higher statistical significance. It is recommended that the production of volatile acidity be studied in relation to temperature, according to type of winemaking.

2.1.6. Bibliography

1. Recueil des methodes internationales d'analyse des vins et des mouts. OIV. MA-F-AS313-02-ACIVOL. 2006
2. Calderón, F.; Gutierrez-Granda, M. J., Suárez-Lepe, J. A. Isolation, identification and physiological characterization of indigenous yeast of Chardonnay grapes from Somontano. Am. J. Enol. Vitic. 1994 45, 368.

2.2. GLYCEROL PRODUCTION

2.2.1. Field of application

Criterion for strain characterization designed to produce red wines.

2.2.2. Principle

Glycerol is polyalcohol. It is the most abundant compound in wine after water and ethanol. Glycerol plays a major role in wine structure as it increases density and softness. The amount of glycerol that is produced depends on the initial sugar concentration, fermentation conditions and the yeast strain.

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The use of glycerol-overproducing strains plays a double role in improving red wine structure. On the one hand, it increases structure,density and tannin integration, thus mitigating harshness and astringency.

2.2.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- 100 ml Erlenmeyer flasks with Müller trap
- UV/vis spectrophotometer
- Cuvettes

2.2.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- Enzyme assay kit for determination of glycerol

2.2.5. Procedure

50 ml samples for determination of glycerol are obtained under small-scale winemaking and isothermal conditions at 25 °C using synchronised yeasts for inoculation Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. After fermentation, the samples are analyzed using enzyme assay for the determination of glycerol in foodstuffs. Alternatively, glycerol may be determined by liquid chromatography (HPLC) with IR detection. The assay should be performed in triplicate and using a highly productive strain for control.

Saccharomyces normally produces from 5 to 8 g·l^{-1} of glycerol.

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2.2.6. Bibliography

1. Eggstein, M.; Kuhlmann, E. In Methods of enzymatic analysis. Bergmeyer, H. U., Ed.; 3er ed.; Academic Press, Inc.: New York, 1974; Vol IV, pp 1825-1831
2. Recueil des methodes internationales d'analyse des vins et des mouts. OIV. MA-F-AS312-05-GLYENZ. 2006
3. Walter, E.; Kohler, P. Ringversuch für dieenzymatische bestimmung von glycerin. Z. Lebensm. Unters. Forsch. 1985, 180, 121-125

2.3. PRODUCTION OF HIGHER ALCOHOLS

2.3.1. Field of application

Criterion for strain characterization designed to produce red wines.

2.3.2. Principle

Main higher alcohols found in wine are: n-propanol, isobutanol, active amyl alcohol, isoamyl alcohol, 2-phenylethanol. These alcohols are produced during carbohydrate catabolism or by catabolism of corresponding amino acid (threonine, valine, isoleucine, phenylalanine). They are important compounds for wines' alcoholic fermentation aroma. In low amounts these compounds influence positively wine aroma, whereas high concentrations (>350 mgl^{-1}) affect adversely wine bouquet, in particular isoamyl alcohol.

In the case of production of young white wines starting from aromatic cultivars, yeast strains with lowproduction of higher alcohols are preferred.

In the case of production of young white wines starting from neutral cultivars, it is necessary to minimize the higher alcohols production (<400 mgl^{-1})

The amount of higher alcohols in high quality red wines must be limited by using selected yeasts producing less than 300 mgl^{-1} to prevent fermentation aromas hiding varietal or wood aging aromas.

2.3.3. Equipment

- Sterilizer
- Laminar flow cabinet

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- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap
- GC-FID apparatus

2.3.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h synchronised in YEPD medium.

2.3.5. Procedure

Measuring higher alcohols production during small-scale fermentation using a single yeast strain under isothermal conditions (25 °C). Method of analysis: gas chromatography with flame ionization detection (GC-FID).

2.4. ACETALDEHYDE PRODUCTION

2.4.1. Field of application

Standard criterion for wine yeasts

2.4.2. Principio

Acetaldehyde is a product of alcoholic fermentation and it represents 90% of total aldehydes of the wine. Generally, high amounts of acetaldehyde in the wine is unfavourable, in consequence of sharp smell and oxidized and herbaceous taste when the concentration reaches 100-125 mgl^{-1}.

2.4.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips

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- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap
- GC-FID apparatus

2.4.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l–1. Synthetic media of similar composition may be used instead (Appendix 1.).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium

2.4.5. Procedure

Monitor the production of esters under small-scale winemaking and isothermal conditions at 25 °C using yeasts for inoculation. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. Method of analysis: GC FID

2.5. PRODUCTION OF ESTERS

2.5.1. Field of application

Criterion for strain characterization designed to produce red wines.

2.5.2. Principle

Esters are produced by yeasts during alcoholic fermentation; these compounds originate by condensation of acetic and fatty acids with ethanol or other alcohols present in the wine. Main wine ester is ethyl acetate which contributes principally to wine aroma conferring typical vinegar smell. Concentrations ranging from 50 to 80 mgl^{-1} are desirable for wine aroma, higher concentrations result in unpleasant flavor.

In order to improve the wine's nose when using varieties with a neutral aroma, yeast-producing esters during fermentation are selected, thus increasing the secondary aromatic compounds responsible for the fruity character (isoamyl acetate, isobutyl acetate, etc.).

In order to improve the wine's nose when using aromatic varieties rich in terpenes, yeasts producing minimum volatile compounds during fermentation are used. Volatile

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compounds produced during fermentation usually hide or reduce some varietal aromas which are crucial from the sensory point of view.

Yeast strains suitable for winemaking should produce different ester amounts in function of vinification technique.

2.5.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller tra
- GC-FID apparatus

2.5.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l–1. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

2.5.5. Procedure

Monitor the production of esters under small-scale winemaking and isothermal conditions at 25 ºC using yeasts for inoculation. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ml^{-1}. Method of analysis: GC FID.

2.5.6. Bibliography

1. Calderón, F.; Gutiérrez-Granda, M. J., Suárez-Lepe, J. A. Isolation, identification and physiological characterization of indigenous yeast of Chardonnay grapes from Somontano. Am. J. Enol. Vitic. 1994, 45, 368.
2. Morata, A.; Calderón, F.; Colomo, B.; González, M. C., Uthurry, C.; Varela, F.;

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Yeramian, N.; Suárez, J. A. Primeros criterios de selección de levaduras para la vinificación en tinto. Semana Vitivinícola. 2005, 3057, 806-809.

2.6. PRODUCTION OF VOLATILE SULPHUR COMPOUNDS (HYDROGEN SULPHIDE AND MERCAPTANS)

2.6.1. Field of application

Standard criterion for wine yeasts characterization. The production of reduced volatile compounds is enhanced by certain grape varieties.

2.6.2. Principle

Unpleasant volatile compounds derived from sulphur metabolic by-products of yeast fermentation. Strains producing the least H2S and sulphur compounds such as mercaptans during fermentation are preferred. This applies especially to red wines.

2.6.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture assa
- Capoteck caps
- Sterile Petri dishes

2.6.4. Reagents and materials

2.6.4.1.

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used instead (Appendix 1.). The pH is adjusted to 3.5 with tartaric acid
- Lead acetate saturated solution.

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- 10 cm × 1 cm cellulose strip

2.6.4.2.

- BiGGY Agar synthetic medium
- Prior to inoculation, yeast cultures are cultured in YEPD medium

2.6.5. Procedures

2.6.5.1.

Inoculate test-tubes containing 5 ml of fermentation medium with 100 µl of culture. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. Place a cellulose strip soaked in lead acetate on top of the tube. Comparative quantification of the H_2S produced in small-scale winemaking fermentations by single strains. Semi-quantitative assay. The H_2S produced reacts with the lead acetate to form lead sulphide which appears as a black stain. The amount of lead sulphide is directly proportional to the quantity of H_2S released. The more H_2S the bigger and darker the stain.

2.6.5.2.

The yeast strains are inoculated (108 cells/ml) on BiGGY Agar medium and the plates are incubated at 26°C for 24 h. Yeast strains will develop colonies with colour ranging from white-cream until brown-black in function of increasing amounts of hydrogen sulphide produced.

2.6.6. Bibliography

1. Morata, A.; Calderón, F.; Colomo, B.; González, M. C., Uthurry, C.; Varela, F.; Yeramian, N.; Suárez, J. A. Primeros criterios de selección de levaduras para la vinificación en tinto. Semana Vitivinícola. 2005, 3057, 806-809.

2.7. ACTIVITY ON MALIC ACID

Yeast strains possessi ability to degrade or to produce malic acid. In cool or moderate climate areas with grape musts characterized by high acidity and low pH values, strains possessing high ability to degrade malic acid are favoured. On the contrary, in

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presence of grape musts characterized by low acidity and high pH values, wines characterized by sufficient acidity are favourable, so yeast strains producing malic acid are preferred.

2.7.1. Fields of application

- Producing white wines from grapes coming from regions with high must acidity.
- Producing white wines from raisins coming from warm regions,with low must acidity.
- Criterion for strain characterization designed to produce red wines.

2.7.2. Principle

Most strains of Saccharomyces cerevisiae can degrade malic acid, but only some can do it in great quantities, thus helping to get rid of it. This may be of special interest to reduce acidity in wines produced in regions where ripening is poor.

Some strains of Saccharomyces cerevisiae produce malic acid as a by-product of alcoholic fermentation. In musts produced in warm regions, pH levels may be reduced and acidity improved by using yeasts capable of producing large quantities of malic acid.

The use of malic acid-degrading strains enhances malolactic fermentation, which is accomplished by bacteria.

2.7.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture
- 100 ml Erlenmeyer flasks with Müller trap
- UV/VIS spectrophotometer
- Cuvettes

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2.7.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l–1. Synthetic media of similar composition may be used instead (Appendix 1.).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- Enzyme assay kit for determination of malic acid

2.7.5. Procedure

50 ml samples for determination of malic acid are obtained under small-scale winemaking and isothermal conditions at 25 ºC. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. After fermentation, the samples are analyzed using enzyme assay for the determination of malic acid in foodstuffs. The assay should be performed in triplicate to increase reliability.

2.7.6. Bibliography

1. Calderón, F.; Gutiérrez-Granda, M. J., Suárez-Lepe, J. A. Isolation, identification and physiological characterization of indigenous yeast of Chardonnay grapes from Somontano. Am. J. Enol. Vitic. 1994, 45, 368.
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4. Klopper, W. J.; Angelino, S. A.; Tuning, B.; Vermeire, H. A. Organic acids and glycerol in beer. J. Inst. Brew., 1986, 92, 225-228
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2.8. β GLYCOSIDASE ACTIVITY TO ENHANCE TERPENE RELEASE

2.8.1. Field of application

Producing young white wines using aromatic varieties.

2.8.2. Principle

In most aromatic varieties, terpene-based aromas usually exist as glycosides, thus reducing their volatility. Some strains of Saccharomyces cerevisiae have □ glucosidase and β xylosidase activities and can be used to optimize terpene release.

2.8.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture

2.8.4. Reagents and materials

- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.
- API ZYM test strip for extracellular enzyme activity (bioMérieux SA, Lyon, France).
- Qualitative determination:
- YNB (Yeast Nitrogen Base) agarized medium containing xylose as carbon source, added to specific solution, such as MUX (4-methylumbelliferyl-β-D-xyloside), for β-D-xylosidase activity. Medium containing arbutin as carbon source, added to ferrum ammonium citrate, for β-D-glucosidase activity.
- Quantititave determination. The β-glucosidase activity was determined in a medium containing 1.7 g l^{-1} Yeast Nitrogen Base (Difco) (without amino acid and ammonium sulphate), 5 g l^{-1} ammonium sulphate, 5 g l^{-1} yeast extract, 5 g l^{-1} peptone, 10 g l^{-1} glucose, pH 5.5. For the determination of β- xylosidase activity the same medium was used, substituting glucose with xylose.β-glucosidase activity was measured using ρ-nitrophenyl-β-D-glucopyranoside (ρ-NPG); β-

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xylosidase activity was measured using ρ-nitrophenyl-β-D-xylopyranoside (ρ-NPX) as substrate. Before the inoculum, yeasts were grown overnight in GPY medium (glucose 40 g l-1; peptone 5 g l-1; yeast extract 5 g l-1; pH 5.5) at 25°C in an orbital shaker at 200 rpm.

2.8.5. Procedure

In vitro detection of extracellular β glycosidase activity. Use pure cultures containing 108 CFU·ml^{-1} (24-48 hours). Test α glycosidase activity under winemaking conditions. Method: API ZYM teststrips ..

2.8.5.1. Qualitative determination

Fresh cells of yeast strains are inoculated on the plates, which are incubated at 26°C for 24 h (for β-D-xylosidase activity) or for 2-4 days, in the case of β-D-glucosidase activity

The β-D-xiylosidase activity is determined by UV fluorescence. Strain colonies showing fluorescence are considered positive for this activity: in fact, β-D-xylosidase enzyme determines hydrolysis of MUX, releasing MU (4-methylumbelliferyl), visualized by UV exposition.

Yeast strains positive for β-D-glucosidase activity show brown colonies on medium containing arbutin as carbon source.

2.8.5.2. Quantitative determination

After pre-grown in GPY medium, yeast strains are grown in the medium above indicated at 26°C for 3 days in an orbital shaker at 200 rpm. The upper phase, harvested by centrifugation, was added to 0,2 mL of ρNPG o ρNPX; after 1 h incubation at 30°C, absorbance was measured at 404 nm in order to determine the amount of ρ-nitrophenol released by enziymatic activity.β-D-glucosidase and β-D-xiylosidase activities are expressed as μmoles of μNP/h/mL of upper phase.

2.8.6. Bibliography

1. Suárez-Lepe, J. A. Levaduras vínicas. Funcionalidad y uso en bodega. Ed Mundiprensa, Madrid, 1997, 110-111.
2. Delcroix, J.; Gunata, Z.; Sapis, J. C.; Salmon, J. M. ; Bayonave, C. Glycosidase Activities of Three Enological Yeast Strains During Winemaking: Effect on the Terpenol Content of Muscat Wine. Am. J. Enol. Vitic. 1994, 45, 291-296

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3. Manzanares, P., Ramón, D., Querol, A. (Screening of non-Saccharomyces wine yeasts for the production of □-D-xylosidase activity. Int. J. Food Microbiol. 1999, 46, 105-112.

4. Manzanares, P., Rojas, V., Genovés, S., Vallés, S. A preliminary search for anthocyanin-β-D-glucosidase activity in non-Saccharomyces wine yeasts. Int. J. Food Sci. Technol. 2000, 35, 95-103.

3. CRITERIA FOR HEALTHY QUALITY OF WINE

3.1. ETHYL CARBAMATE PRODUCTION BY ARGINASE NEGATIVE STRAINS

3.1.1. Field of application

Criterion for strain characterization designed to produce red wines.

3.1.2. Principle

Ethyl carbamate (urethane) is a toxic compound that has been proved to be carcinogenic. The occurrence of ethyl carbamate in wines is related to microbial activity (of both yeasts and bacteria) at certain pH values and at certain concentrations of nitrogenous precursors. The use of selected yeasts is a good means of controlling the levels of this compound. It is important to remember that certain countries have set limits on the concentration of ethyl carbamate in wines suitable for import.

3.1.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- Erlenmeyer flasks
- GC-MS apparatus
- Capillary column
- Column extraction apparatus

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- Rotary evaporator

3.1.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used instead (Appendix 1.).
- Dichloromethane

3.1.5. Procedure

Ethyl carbamate is first obtained by concentration and extraction from 50 ml of the fermented sample (produced in small-scale winemaking conditions and using a selected yeast strain) and then determined by GC MS in SIM mode.

3.1.6. Bibliography

1. Uthurry, C.A.; Varela, F.; Colomo, B.; Suárez Lepe, J. A.; Lombardero, J.; García del Hierro, J. R. Ethyl carbamate concentrations of typical Spanish red wine. Food Chemistry, 2004, 88, 329-336
2. Uthurry, C.A.; Suárez Lepe, J. A.; Lombardero, J.; García del Hierro, J. R. Ethyl carbamate production by selected yeasts and lactic acid bacteria in red wine, Food Chemistry, 2006, 94, 262-270

3.2. HISTAMINE PRODUCTION AT HIGH pH

3.2.1. Field of application

Criterion for strain characterization designed to produce red wines.

3.2.2. Principle

Biogenic amines, such as ethanolamine, phenylethylamine, methylamine, agmatine, histamine, putrescine, cadaverine and tyramine, are low molecular weight organic bases that can be found in wine, in which they are mainly produced by microbial decarboxylation of amino acids. These compounds affect adversely wholesomeness of wine.

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Biogenic amines are molecules produced in nitrogen metabolism. They may produce toxic effect depending on concentration and individual sensitivity. They are a real nuisance to histamine-sensitive people. More generally they cause headaches and other adverse effects. Furthermore, ethanol present in the wine strengthens toxic effects of biogenic amines. Yeasts are involved in the production of ethanolamine, agmatine and phenylethylamine, whereas wine yeasts are low producers of histamine, the amine characterized by highest toxicity.

The ability to produce biogenic amines should be inserted among the criteria for wine yeast characterization.

Setting of limits for the concentration of biogenic amines, in particular for histamine are under discussion.

3.2.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- Erlenmeyer flasks
- HPLC FDA apparatus
- C18 column

3.2.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 $g \cdot l^{-1}$. Synthetic media of similar composition may be used instead (Appendix 1).

3.2.5. Procedure

Determine the amount of histamines produced by a selected yeast under standard small-scale winemaking conditions. Detection and quantification are performed by HPLC FDA after derivatization with dansyl chloride or o-phthaldehyde (OPA).

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3.2.6. Bibliography

1. Galgano F., Caruso M.,Favati F., Romano P. HPLC determination of agmatine and other amines in wine. Journal International des Sciences de la Vigne et du Vin, 2003, 74, 237-242
2. Caruso M., Fiore C., Contorsi M., Salzano G., Paparella A., Romano P. Formation of biogenic amines as criteria for the selection of wine yeasts. World Journal Microbiol. Biotechnol., 2002, 18, 159-163.
3. Granchi L., Romano P., Mangani S., Guerrini S., Vicenzini M. Production of biogenic amines by wine microorganisms, Bulletin dell'OIV, 2005, 78, 595-609.

3.3. YEAST ACTIVITY ON OCHRATOXIN A (OTA)

3.3.1. Field of application

Standard criterion for wine yeasts characterization.

3.3.2. Principle

Ochratoxin A (OTA) is a mycotoxin found in the wine as a consequence of metabolic activity of moulds present on the grapes. Because OTA showed nephrotoxic, hepatotoxic, and teratogenic properties to humans and animals, it was classified as an agent possibly carcinogenic to humans (International Agency for Research on Cancer, group 2B, 1993). Europe has recently fixed the maximum residue level of OTA in wines at 2 μg/l. Some yeasts are able to reduce OTA content of wine; in consequence of this, strains possessing this activity should be selected.

3.3.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- Erlenmeyer flasks with Müller trap
- Analytical balance, precision to within 0.1 g

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- HPLC apparatus

3.3.4. Reagents and materials

Synthetic fermentation medium (6.7% yeast nitrogen base (YNB), 20% glucose, and 0.1% diammonium phosphate, adjusted to pH 3.6 with tartaric acid) is used.

3.3.5. Procedure

Yeast precultures were prepared in 50 ml of synthetic fermentation medium on a rotary shaker at 250 rpm for 24 h at 25 °C.

Inoculated suspension with a concentration of 1 x 106 cells/mL is inoculated in100 ml Erlenmeyer flasks with Müller trap, containing synthetic fermentation medium added with 10 µg/l of OTA. The samples were incubated without shaking at 26°C. The weight loss is monitored every day until no variation is observed.

An aliquot of 5 mL of the culture media inoculated with different strains is transferred into a glass tube and centrifuged at 4000 rpm for 5 min. One ml of the aqueous layer, previously separated from the yeasts, is transferred in a vial and it is injected for HPLC analysis. The solid phase (yeasts) is extracted with 5 mL of ethyl acetate in a Vortex mixer for 30 s. The extracts are combined, and 2 mL of the resulting solution is gently evaporated to dryness with nitrogen. The residue is dissolved in 1 mL of bidistilled water and injected for HPLC analysis.

A LaChrom-Merck-Hitachi liquid chromatograph (Hitachi Ltd., Tokyo, Japan) consisting of a D-7000 System Manager, a L-7100 pump, a L-7200 autosampler, and a L-7485 Fluorescence Detector is used. A Spherisorb ODS2 column (4.6 mmx 250 mm; 5 μm; Waters, Milan, Italy) is employed. The injection volume is 50 μL, and the flow rate was 1 mL/min. OTA elution is with ammonium buffer (A) and acetonitrile (B) as eluting mixture, with the following program: t=0 A 90% isocratic until 15 min, t=30 A 75% with gradient. In this chromatographic condition, the retention time for OTA is about 28 min.

The fluorescence detector is set at 333 and 440 nm for the excitation and the emission wavelengths, respectively. Quantitative determinations are performed following the external standard method measuring peak area vs concentrations. The calibration graphs are constructed by injecting standard solutions prepared in bi-distilled water at five concentration levels. The limit of detection (LOD) calculated is 0.1 ¤g/L, and the limit of quantification (LOQ) is 0.2 μg/L.

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3.3.6. Bibliography

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3.4. METHANOL PRODUCTION

3.4.1. Field of application

Standard criterion for wine yeasts characterization.

3.4.2. Principle

Methanol (CH_3OH) is the simplest alcohol. It derives from the degradation of grape cell wall by enzymatic activities also of yeasts during vinification.. Characterization of yeasts determining less than 150 mg/L of methanol is encouraged.

3.4.3. Equipment

- Sterilizer
- Laminar flow cabinet
- Pipettes with sterile tips
- Test-tubes for culture synchronisation
- 100 ml Erlenmeyer flasks with Müller trap
- GC-FID apparatus

3.4.4. Reagents and materials

- The fermentation medium is prepared by dilution of a concentrated grape must until the sugar concentration reaches 212 g·l^{-1}. Synthetic media of similar composition may be used instead (Appendix 1).
- Prior to inoculation, yeast cultures are grown for 24 h in YEPD medium.

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3.4.5. Procedure

Measuring methanol production during small-scale fermentation using a single yeast strain under isothermal and temperature-controlled (25- °C) conditions. Yeast cell concentration at the time of inoculation in the given test media should be approx. 106 living cells/ ml^{-1}. Method of analysis: OIV reference method, gas chromatography with flame ionization detection (GC-FID).

3.4.6. Bibliography

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APPENDIX 1

Synthetic media of similar composition to grape must:

1- The medium 1 reported in the table 1 is the chemically-defined grape juice medium reported by Henschke and Jiranek (1993) but containing a lower amino acid quantity to give a nitrogen concentration of 200 mgL-1, a higher sugar amount (230 gL-1 instead of 200 gL-1) and one-fifth of the vitamin amount. The medium is sterilized by filtration

Table 1 Composition of the synthetic medium 1

	Component	Amount per litre
Carbon source	Glucose	115 g
	Fructose	115 g
Organic acid	Potassium tartrate	5.00 g
	Citric acid	0.20 g
	L-malic acid	3.00 g

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Salts	K_2HPO_4	1.14 g
	$MgSO_4 \cdot 7H_2O$	1.23 g
	$CaCl_2 \cdot 2H_2O$	0.44 g
	$MnCl_2 \cdot 4H_2O$	198.2 µg
	$ZnCl_2$	135.5 µg
	$CuCl_2$	13.6 µg
	$FeCl_2$	32.0 µg
	H_3BO_3	5.7 µg
	$Co(NO)_3 \cdot 6H_2O$	29.1 µg
	$NaMoO_4 \cdot 2H_2O$	24.2 µg
	KIO_3	10.8 µg
Vitamins	Myo-inositol	20 mg
	Pyridoxine·hydrocloride	0.40 mg
	Nicotinic acid	0.40 mg
	Calcium pantotenate	0.20 mg
	Thiamine hydrocloride	0.10 mg
	p-Aminobenzoic acid	0.04 mg
	Riboflavin	0.04 mg
	Biotin	0.03 mg
	Folic acid	0.04 mg

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Amino Acids	Aspartic acid	89 mg
	Glutamic acid	126 mg
	Alanine	26 mg
	Arginine	188 mg
	Asparagine	39 mg
	Phenylalanine	39 mg
	Glycine	14 mg
	Glutamine	51 mg
	Isoleucine	51 mg
	Histidine	39 mg
	Leucine	76 mg
	Lysine	63 mg
	Methionine	39 mg
	Proline	126 mg
	Serine	101 mg
	Tyrosine	6.00 mg
	Threonine	89 mg
	Tryptophan	26 mg
	Valine	51 mg
Nitrogen Sources	$(NH_4)_2HPO_4$	100 mg

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Lipids	Ergosterol	10 mg
	Tween 80®	0.5 mL
pH		3.2 - 3.5

Table 2 Composition of the synthetic medium 2

Components		**Amount per litre**
Carbon	D-Glucose	133 g
	D-Fructose	67 g
	myo-Inositol	0.5 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	K_2HPO_4	1.14 g
	$MgSO_4.7H_20$	1.23 g
	$CaCl_2.2H_2O$	0.44 g

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Nitrogen	$(NH_4)_2HPO_4$	300 mg
	L-Alanine	70 mg
	L-Arginine	525 mg
	L-Asparagine	105 mg
	L-Aspartic acid	245 mg
	L-Cysteine*	7 mg
	L-Glutamine	140 mg
	Glycine	35 mg
	L-Histidine. $HCl.H_2O$	105 mg
	L-Isoleucine	140 mg
	L-Leucine	210 mg
	L-Lysine. HCl	175 mg
	L-Methionine	105 mg
	L-Phenylalanine	105 mg
	L-Proline	350 mg
	L-Serine	280 mg
	L-Threonine	245 mg
	L-Tryptophan	70 mg
	L-Tyrosine	14 mg
	L-Valine	140 mg
	Glutamic acid	350 mg

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Trace elements	$FeSO_4.7H_2O$	20 mg
	$MnSO_4.H_2O$	7.56 mg
	$ZnCl_2$	135 µg
	$CuCl_2.2H_2O$	15 µg
	H_3BO_3	5 µg
	$Co(NO_3)_2.6H_2O$	30 µg
	$Na_2MoO_4.2H_2O$	25 µg
	KIO_3	10 µg
Vitamins	Pyridoxine.HCl	2 mg
	Nicotinic acid	4.5 mg
	Ca Pantothenate	5 mg
	Thiamin.HCl	0.5 mg
	PABA.K	0.2 mg
	Biotin	0.125 mg
pH		3.5

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