COEI-1-NOSACC Non-Saccharomyces selected yeasts

1. Object, origin and field of application

Non-*Saccharomyces* yeasts can be used for the inoculation of grapes, musts and wine according to resolution OENO-MICRO 14-546. As addition of non-*Saccharomyces* yeast might not result in the completion of alcoholic fermentation, inoculation with non-*Saccharomyces* may be followed by or carried out at the same time as inoculation with *Saccharomyces* spp.

Yeasts with desirable oenological properties must be isolated from grapes, musts or wine or result from hybridisation of grape/must/wine strains, or have been derived from other wine yeasts.

Prior to the use of genetically modified oenological yeasts authorisation by competent authorities is required.

2. Labelling

The following information must be indicated on the packaging :

- the genus name, the species name, the name of the strain(s) and all elements that can guarantee the traceability of the product,
- the physical form of the products as described in point 3,
- the name of the selector,
- the name and contact address of the manufacturer or marketer or distributor,
- operating instructions recommended by the manufacturer,
- a recommended rate of inoculation,
- the minimum number of viable cells per gram of product (CFU as determined in 4.6) guaranteed by the manufacturer, with a recommended storage temperature,
- the manufacturing batch number, the expiration date and storage conditions,
- where relevant, the indication that the yeast strain(s) were obtained through genetic modifications and their modified character(s),
- all additives present.

3. Characteristics

The formulation is a pure culture or a blend of non-*Saccharomyces* strains, or a blend of strains of *Saccharomyces* and non-*Saccharomyces*.
Non-*Saccharomyces* selected yeasts can be used in the following forms:

- Active Dry Yeast (ADY) with a minimum dry matter of 92% and a level of viable yeasts equal or above to 10^{10} CFU/g of dry matter,
- Active Frozen Yeast (AFY) with a range of dry matter from 40 to 85% and a level of viable yeasts equal to or above 10^{10} CFU/g of dry matter,
- Compressed Yeast (COY) with a range of dry matter from 30 to 35% and a level of viable yeasts equal to or above 10^{10} CFU/g of dry matter,
- Cream Yeast (CRY) with a range of dry matter from 18 to 25% and a level of viable yeasts equal to or above 10^{10} CFU/g of dry matter,
- Encapsulated (beads) or Immobilised Yeasts (ENY) with alginate and/or other products admitted by the OIV, with a minimum of dry matter of 86% and a level of viable yeasts equal to or above 10^{9} CFU/g of dry matter,

4. Limits and methods of analysis

4.1. Humidity

Measured by the weight loss of 5 g of product dried at 105 °C until it reaches a constant weight

Content should comply with the characteristics of humidity or water level described in point 3.

4.2. Lead

Proceed with the determination according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 2 mg/kg of the suitable preparation described in point 3.

4.3. Mercury

Proceed with the determination according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 1 mg/kg of the suitable preparation described in point 3.

4.4. Arsenic

Proceed with the determination according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 3 mg/kg of the suitable preparation described in point 3.

4.5. Cadmium

Proceed with the determination according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 1 mg/kg the suitable preparation described in point 3.

4.6. Total viable yeasts

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*. Content should comply with the characteristics described in point 3.

4.7. Yeasts of genera/species/strains other than the genera/species/strains indicated on the label

The genera, species and strains indicated on the packaging should be at least 95% of the total yeast population.

See Annex 1.

4.8. Moulds

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 10^3 CFU/g the suitable preparation described in point 3.

4.9. Lactic acid bacteria

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 10^5 CFU/g of the suitable preparation described in point 3.

4.10. Acetic acid bacteria

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*.

Content should be less than 10^4 CFU/g of the suitable preparation described in point 3.

4.11. Salmonella

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*.

Absence should be checked on a 25 g sample.

4.12. Escherichia coli

Proceed with counting according to the method in Chapter II of the *International Oenological Codex* using the selective differential medium for *Escherichia coli.*

Absence should be checked on a 1 g sample.

4.13. Staphylococci

Proceed with counting according to the method in Chapter II of the *International Oenological Codex*. The presence of staphylococci is evaluated by an enrichment culture in a liquid Giolitti and Cantoni medium followed by a confirmation on a solid Baird Parker medium.

In the case that the Giolitti and Cantoni medium gives positive results, the presence of staphylococci is confirmed by isolation on a solid Baird Parker medium. A positive culture medium loop is used to inoculate solid BP mediums to obtain isolated colonies.

Absence should be checked on a 1 g sample.

4.14. Coliforms

Proceed with counting according to the method in Chapter II of the *International Oenological Codex* using a selective differential medium for coliforms, desoxycholate gelose.

Number should be less than 10^2 CFU/g of the suitable preparation described in point 3.

5. Additives

These must be in conformity with regulations in force.

6. Storage conditions

Products must be stored and propagated under conditions which favour their genetic stability.

Always refer to manufacturer's recommendations.

7. Product documentation

Product documentation should specify guidelines about storage, transportation, handling and application conditions (temperature, activation, rehydration when needed, possibly in suitable must or wine suspensions, etc.).

Annex 1

1. Obtention of colonies

Sample 1 g, and suspend it under sterile conditions in 100mL sterile saccharose 5%. Homogenise and allow standing at 25-30 °C for 20 min.

After adequate serial decimal dilutions, spread 0.1 mL of the diluted sample onto the surface of a nutrient YEPD agar plate (Glucose 20 g, Peptone 20 g, Yeast extract 10 g, 100 mg chloramphenicol to suppress bacterial growth and 150 mg biphenyl to suppress mould growth, Agar-agar 20 g, Water q.s.p. 1000 mL). Incubate for 6 days at 25 °C in aerobiosis. All yeast can grow, regardless of the species present.

2. Identification of contaminant genera/species/strains

Identification is carried out on the colonies isolated on plates.

As required in the characteristics, the contaminant population (which is neither the pure strain nor the different strains in case of mixed strains) should be less than 5% of the total population. After the dilutions needed to obtain individual colonies, if 20 colonies out of 300 are identified, a contaminant at 5% (ideally) should represent 1 colony out of 20.

The contaminant is identified based on the species, and therefore the genus, by D1/D2 sequencing (see 2.1). If all of the colonies are of the same species, it is possible to verify that a contaminant strain corresponds to less than 5% through analysis of 20 colonies, using SSR (see 2.2).

If the preparation is a blend of 2 or 3 species/strains, the least represented is 15% of the total. The verification of the composition of the mix by identification of colonies is not appropriate. Indeed, for 2 strains in the blend, the less represented should produce 3 colonies out of 20 identified, picked up out of 400 on the plate.

Therefore it can be suggested that the checking for 2 or even more species in blend (proportion of the different species), use the quantitative specific PCR with probes targeting each of the expected species. In this case there is no preliminary plate culture. DNA is extracted directly from the sample.

For controlling blends of same species strains (proportion of the different strains) the only possibility up to date cannot exclude the plate culture is and identification of colonies to the strain level; the result needs to be interpreted with precaution since the representation of each strain on the plates is affected by the growth ability on the one hand and on the other by the excessively low number of colonies that can reasonably be identified.

2.1. Identification of the species

The species is identified by DNA sequencing of the variable domain D1/D2 of26S ribosomal region obtained by PCR amplification. It is the “method of choice” for yeast species identification: strains with more than a 1% sequence divergence of the domain D1/D2 of 600 nucleotides are not of the same species.

- Suspend separately, colonies directly in the PCR mixture, or previously in water (about 50 µL depending on the size of the colony) and add a sample to the PCR mixture;
- PCR mixture (final volume 50 µL): 10 mM Tris HCl pH 8, 50 mM KCl, 0.1% Triton X100 v/v, 0.2 mg/mL BSA, 3.12% v/v glycerol, 1.5 mM MgCl2, 200 µM dNTPs, 0.1 U/ µL Taq polymerase;
- primers: NL1/NL4. NL 1 (5'-GCATATCAATAA GCGGAGGAAAAG) and NL 4 (5'-GGTCCGTGTTTCAA GACGG);
- amplification is performed, after 10 min at 95 °C to make accessible DNA, by 30 cycles comprising the steps, 95 °C for 1 min, 55 °C for 45 s, 72 °C for 1 min, then a final step at 72 °C for 7 min;
- PCR product is purified by any “PCR purification kit” and sequenced using the primers used for the amplification;
- sequences obtained are compared to those available in the Genebank database (www.ncbi.nih.gov./Genbank).

2.2. Identification of a strain

When the species is identified, it is possible to identify the strains. For most wine yeast species, at least the main ones used as starters, the most reliable and accurate method for identification is based on the analysis of sequence repeats (microsatellites SSR). Strains differ by the number of repetitions of short sequences at certain point of their genome. These loci are delimited by conserved regions that are chosen as primers for PCR amplification. The analysis consists in PCR amplification of several loci, with suitable primers for each yeast species, and measurement of their length by capillary electrophoresis for sequencing (with a degree of resolution of a single nucleotide).

Note:

- at the time of writing strain typing is not possible for all yeast species;
- in order to further the advances in knowledge, suitable primers for each yeast species are chosen by referring to studies published in international peer-review scientific journals;
- for some species about 9-12 loci are analysed; some loci are more discriminant than others;

- the analysis can be simplified by considering first a smaller number of loci chosen for their better discriminating power, and continuing the analysis in case of ambiguity;
- amplification can be done in multiplex, which shortens and simplifies the analysis.