# OIV-MA-AS4-01 Microbiological analysis of wines and musts- Detection, differentiation and counting of micro-organisms

**Type IV method**

**Objective:**

Microbiological analysis is aimed at following alcoholic fermentation and/or malolactic fermentation and detecting microbiological infections, and allowing the detection of any abnormality, not only in the finished product but also during the different phases of manufacture.

**Comments:**

All experiments must be carried out under normal microbiological aseptic conditions, using sterilized material, close to a Bunsen burner flame or in a laminar flow room and flaming the openings of pipettes, tubes, flasks, etc. Before carrying out microbiological analysis, it is necessary to ensure that the samples to be analyzed are taken correctly.

**Field of application:**

Microbiological analysis can be applied to wines, musts, mistelles and all similar products even when they have been changed by bacterial activity. These methods may also be used in the analysis of industrial preparations of selected microorganisms, such as dry active yeasts and lactic bacteria.

**Microbiological analysis techniques:**

1. Reagents and materials
2. Installations and equipment
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5. Microscopic techniques for the detection, differentiation of micro-organisms and direct counting of yeasts
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    4. *Yeast cell count – haemocytometry*
    5. *Yeast cell count – methylene blue staining of yeast cells*
6. Counting of micro-organisms by culture
    1. *Detection, differentiation and enumeration of microorganisms (plate count)*
    2. *Culture in liquid environment* - "Most Probable Number" (MPN).

## 1. Reagents and materials

Current laboratory equipment and apparatus, as listed in ISO 7218:2007 – Microbiology of food and animal feeding stuff – General rules for microbiological examinations.

The following ones are recommended:

- Common laboratory materials and glassware, sterile (sterilized or ready-to-use sterile).
- Tubes (16x160 mm or similar) containing 9 ml sterile peptone water (Tryptone: 1 g/l) or other diluents to be used for serial sample dilutions.
- Ethanol to flame spreaders and tweezers.
- Hydrogen peroxide 3% solution.
- Micropipette holding sterile tips: 1 ml and 0.2 ml.
- L-shaped or triangular-shaped bent glass rods (hockey sticks) or plastic spreaders.
- Stainless steel tweezers, with flat edges.
- Sterile cellulose ester membranes (or equivalent) porosity 0.2 and 0.45 µm, 47

mm or 50 mm diameter, possibly with a printed grid on the surface, and packed singularly.

- Sterile cylinders.
- 10 ml sterile pipettes.

## 2. Installations and equipment

Current laboratory equipment and apparatus, as listed in ISO 7218:2007 – Microbiology of food and animal feeding stuff – General rules for microbiological examinations.

The following ones are recommended:

- Microbiological cabinet or laminar flow cabinet. In the absence of this device, work in the proximity (within 50 cm) of a gas burner.
- Balance, with an accuracy of ± 0.01 g.
- Autoclave.
- Incubator with settings ranging from 25°C to 37°C.
- pH meter, with an accuracy of ± 0,1 pH units and a minimum measuring threshold of ± 0,01 pH units.
- Refrigerator(s), set at 5 ± 3°C, and freezer(s), which temperature shall be below –18°C, preferably equal to – 24 ± 2°C.
- Thermostatically controlled bath, set at 45 ± 1°C
- Microwave oven.
- Optical microscope.
- Gas burner.
- Colony-counting device.
- Equipment for culture in a modified atmosphere (a sealed jar in which anaerobiosis can be made).
- Filtering apparatus with 47 mm or 50 mm diameter filters.
- "Vortex" stirrer or equivalent.
- Incubator for dry heat sterilisation
- Centrifuge

- Pump

## 3. Sampling

The sample must reproduce the microbiology of the whole mass of must or wine to be analyzed. As far as possible, the mass must be homogenized before sampling, in order to resuspend microorganisms that tend to set down to the bottom of the container. In case the homogenization is undesirable, samples must be taken from where the microorganisms are likely (or suspected) to be present (i.e. when searching for yeasts lying in the bottom of tanks or barrels), but in this case results are not quantitative. Before taking a sample from a tap, this latter must be flamed, and 2-3 litres liquid must be flushed. The sample must be put in a sterile.

The sample must be kept refrigerated and analysed as quickly as possible.

The following amounts of samples are required for the microbiological examination:

Must, or fermenting must or wine in storage: not less than 250 ml;

Bottled or packed wine: not less than one unit, whateverthe capacity;

## 4. Quality tests

4.1. Objective

These tests are aimed at detecting the risk of microbial infection in advance.

*4.2.* Principle

This technique is based on organoleptic and appearance changes (clouds, films, deposits, unusual colors) shown by wine when subjected to certain aeration and temperature conditions which can bring about microbiological activity. The nature of the changes should be confirmed by microscopic examination.

4.3. Operating method

4.3.1. Air quality tests

A 50 mL wine sample after filtration on coarse sterile filter paper is placed in a 150 mL sterile conical flask stoppered with cotton and left at an ambient temperature for at least 3 days. The clarity, color and possible presence of clouds, deposits and films are examined over this time. A microscopic examination is carried out in the case of cloud, deposit or film or a color change.

4.3.2. Incubator quality tests

A 100 mL wine sample, after filtration on coarse sterilized filter paper, is placed in 300 mL sterile conical flask stopped with cotton, put in an incubator at 30°C and examined after at least 72 hours. Organoleptic or visible changes can be indicative of microbial development. A microscopic examination must therefore be made.

## 5. Microscopic techniques for the detection and differentiation of micro-organisms, and for the direct counting of yeasts

5.1. Microscopic examination of liquids or deposits

*Objective:*

Microscopic examination under cool conditions is aimed at detecting and differentiating the yeasts from the bacteria that might be present, in terms of their size and shape. Microscopic observation cannot distinguish between viable and non-viable microorganisms.

*Comment:*

With appropriate staining (see below), an estimation of the viable yeasts can be made.

*Principle:*

This technique is based on the magnification made by a microscope that allows the observation of micro-organisms, whose size is on the order of a micron.

*Operation method:*

Microscopic examination can be carried out directly on the liquid or on the deposit.

Direct observation of the liquid will only be useful when the population is sufficiently high (more than $5 \times 10^5$ cells/mL).

When wine shows a lower microorganism population, it is necessary to concentrate the sample. Thus, about 10 mL of homogenized wine is centrifuged at 3000 - 5000 rpm for 5 to 15 minutes. After decanting the supernatant, the deposit is re-suspended in the liquid remaining at the bottom of the centrifugation tube.

To carry out the microscopic observation, a drop of the liquid sample or the homogenized deposit is placed on a clean glass slide with a Pasteur pipette or a sterilized wire. It is covered with a cover glass and placed on a slide on the stage of the microscope. Observation is made in a clear field, or preferably in phase contrast, which allows a better observation of detail. A magnification of x400 - x1000 is generally used.

5.2. Gram staining for the differentiation of bacteria isolated from colonies (see paragraph 6)

*Objective:*

Gram staining is used to differentiate between lactic bacteria (Gram positive) and acetic bacteria (Gram negative) and also to observe their morphology.

*Comments:*

It must be remembered that Gram staining is not sufficient to reach a conclusion, as other bacteria in addition to lactic and acetic bacteria may be present.

*Principle:*

This color is based on the difference in the structure and chemical composition of the cell walls between Gram positive and Gram negative bacteria. In Gram negative bacteria, the cell walls that are rich in lipids have a much reduced quantity of peptidoglycan. This allows the penetration of alcohol and the elimination of the gentian-violet-iodine complex, forming when the colorless cell is left, which will then be re-colored in red by saffron. Conversely, the cell walls of Gram positive bacteria contain a large quantity of peptidoglycan and a low concentration of lipids. Thus, the thick peptidoglycan wall and the dehydration caused by the alcohol do not allow the alcohol to eliminate the coloring of the gentian-violet-iodine complex.

Gram staining loses its usefulness if it is performed on a culture that is too old. Thus, the bacteria must be in an exponential growth phase within 24 to 48 hours. Gram staining is carried out after isolating the colonies and liquid cultivation.

*Solutions:*

The water used must be distilled.

*1. Gentian violet solution*

*Preparation*: Weigh 2g of gentian violet (or crystal violet), and put into a 100 mL conical flask and dissolve in 20 mL of 95% vol. alcohol. Dissolve 0.8g of ammonium oxalate in 80 mL of distilled water. Mix the two solutions together and only use after a period of 24 hours. Filter through paper at time of use. Keep out of light in a dark flask.

*2. Lugol solution*

*Preparation*: Dissolve 2g of potassium iodide in a minimal quantity of water (4 to 5 mL) and dissolve 1g of iodine in this saturated solution. Make the volume up to 300 mL with distilled water. Keep out of light in a dark flask.

*3. Saffranin solution:*

Preparation: Weigh 0.5g of saffranin in a 100 mL conical flask, dissolve with 10 mL of 95% vol. alcohol and add 90 mL of water. Stir. Keep out of light in a dark flask.

*Operating method:*

Smear preparation

Make a subculture of the bacteria in liquid or solid medium. Collect the young culture bacteria from the deposit (after centrifugation of the liquid culture) or directly from the solid medium with a loop or wire and mix in a drop of sterilized water.

Make a smear on a slide, spreading a drop of the microbial suspension. Let the smear dry, and then carry out fixation, rapidly passing the slide 3 times through the flame of a Bunsen burner, or equivalent. After cooling, perform staining.

Staining

Pour a few drops of gentian violet solution onto the fixed smear. Leave to react for 2 minutes and wash off with water.

Pour in 1 to 2 drops of lugol solution. Leave to react for 30 seconds. Wash with water and dry with filter paper.

Pour on 95% vol. alcohol, leave for 15 seconds. Rinse with water and dry with filter paper.

Pour on a few drops of saffranin solution, leave to react for 10 seconds. Wash with water and dry with filter paper.

Place a drop of immersion oil on the smear.

With the immersion objective, observe through a microscope in clear field.

*Results:*

Lactic bacteria remain violet or dark blue colored (Gram positive). Acetic bacteria are red colored (Gram negative).

5.3. Catalase Test for the differentiation of bacteria isolated from colonies (see paragraph 6)

*Objective:*

This test is aimed at making a distinction between acetic and lactic bacteria. The yeasts and acetic bacteria have a positive reaction. Lactic bacteria give a negative response.

*Comments:*

It must be taken into account that the catalase test is insufficient as other bacteria in addition to lactic and acetic bacteria may be present.

*Principle:*

The catalase test is based on the property that aerobic micro-organisms have of decomposing hydrogen peroxide with release of oxygen:

$$2H_2O \xrightarrow{catalase} 2H_2O + O_2$$

*Reagent:*

12 Volume hydrogen peroxide solution (3%)

*Preparation*: Measure 10 mL of 30% by volume hydrogen peroxide in a 100 mL calibrated flask and fill with freshly boiled distilled water. Stir and keep in the

refrigerator in a dark flask. The solution must be freshly prepared.

*Operating method:*

Place a drop of 3% by volume hydrogen peroxide on a slide and add a small sample of young colony. If gas is released, it can be concluded that catalase activity is occurring in the culture . It is sometimes difficult to observe gas clearing immediately, particularly with bacterial colonies. It is therefore advisable to examine the culture through a microscope (objective x10).

5.4. Yeast cell count – Haemocytometry

5.4.1. Scope

Determination of yeast cell concentration in fermenting musts or wines, and ADY (Active Dry Yeast). A high cell concentration is required: at least $5 \times 10^6$ cells/ml. Fermenting musts and wines can be counted directly, ADY must be diluted 1000 or 10 000 times. Musts or wines containing fewer cells must be centrifuged (3000 g, 5 minutes) and the sediment resuspended in a known volume.

5.4.2. Principle

A drop of yeast cell suspension is placed on the surface of a slide with a counting chamber. The counting chamber has a defined volume and is subdivided in squares on the surface of the slide. Counting is made under a microscope in light field. Phase contrast is not indicated if cells are stained,

5.4.3. Reagents and materials

- -Haemocytometer, double chamber, preferably with clips: Bürker, Thoma, Malassez, Neubauer.
- Haemocytometer cover slip: common (0.17 mm width) cover slips are not suitable to this use, because they are flexible and do not guarantee that the chamber width is constant.
- Pipettes, fine tips, 1 and 10 ml volume.
- Volumetric flask, 100 ml.
- Beaker, 250 ml.

4. Installations and equipment

- Microscope with bright field illumination: magnification 250-500 x. Phase contrast is contraindicated.
- Magnetic plate and stirring bar.

Haemocytometers are available with different counting chambers: Bürker, Thoma, Malassez, Neubauer. Confirm the identity and the volume of the counting chamber to be used. Bürker, Thoma and Neubauer chambers have 0.1 mm depth, Malassez chamber is 0.2 mm deep.

Thoma chamber has one central large (1 $mm^2$) square, so its volume is 0.1 $mm^3$ ($10^{-4}$ ml). This large square is subdivided in 16 squares, themselves further divided in 16 smaller squares. Thes small squares each have 0.05 mm x 0.05 side and 0.1 mm depth, so that the volume of each small square is 0.00025 $mm^3$ (25 x $10^{-8}$ ml). It is also possible to count in the medium squares, each medium square having 16 small squares 0.2 x 0.2 mm, and 0.004 $mm^3$ area, or 4 x $10^{-6}$ ml volume.

Bürker chamber contains 9 large 1$mm^2$ squares, which are divided into 16 0.2mm sided medium squares, separated by double lines with a 0.05mm spacing. The area of the medium squares is 0.04$mm^2$ and the volume is 0.004$mm^3$. The area of the small squares formed by the double lines have an area of 0.025$mm^2$.

Big, medium and small squares of Neubauer, Thoma and Bürker chambers have the same size. Bürker chamber medium squares do not contain other lines inside; therefore they are probably the easiest to count.

5.4.5. Examination techniques

The counting chamber and the cover slip must be clean and dry before use. It may be necessary to scrub the ruled area, as dirty chambers influence the sample volume. Clean with demineralised water, or ethanol, and dry with soft paper.

If flocculent yeast has to be counted, the suspension medium must be 0.5% sulphuric acid, in order to avoid flocculation, but this impairs the possibility of methylene blue staining and the count of viable and dead cells. Resuspension can be carried out by sonification.

Put the sample on the slide using a fine tip pipette, following one of the two following procedures.

Procedure 1

Mix well the yeast suspension. If dilutions are required, make decimal dilutions, as usual. If a methylene blue stain is performed, make it on the most diluted sample and mix 1 ml sample with 1 ml methylene blue solution.

Constantly shake the yeast suspension. Take a sample with a fine tip pipette, expel away 4-5 drops of suspension and place a small drop of yeast suspension (diluted if necessary) on each of the two ruled areas of the slide. Cover it with the cover slip

within 20 seconds and press firmly with the clips. The counting area should be completely filled, but no liquid should extend to the moat.

Procedure 2

Place the rigid cover slip so that both counting chambers are equally covered. Use the clips to press the cover slip against the support areas until iridescence lines (the Newton rings) appear. When there are no clips, do not move the cover slip when filling the chamber.

Constantly shake the yeast suspension. Take a sample with a fine tip pipette, expel away 4-5 drops of suspension and allow a small drop of sample to flow between the haemocytometer and the cover slip. Do the same in the other part of the slip. The counting area should be completely filled, but no liquid should extend to the moat.

Let the prepared slide stand for three minutes for the yeast cells to settle, and place it under the microscope.

Count 10 medium squares in each ruled area, standardizing procedures must be set, in order to avoid counting twice the same square. Cells touching or resting on the top or right boundary lines are not counted, those resting on bottom or left boundary lines are counted. Budding yeast cells are counted as one cell if the bud is less than one-half the size of the mother cell, otherwise both cells are counted.

To obtain accurate cell counts, it is advisable to count 200 – 500 total yeast cells, on average. Counts from both sides of the slide should agree within 10%. If a dilution is used, the dilution factor must be used in the calculation.

5.4.6. Expression of results

If C is the average number of cells counted in one medium square with 0.2 mm sides, the population T total in the sample is :

Expressed as cells/mL

- $T = C \times 0.25 \times 10^6 \times$ dilution factor

If C is the average number of cells counted in one small square with 0.05mm sides, the population T total in the sample is:

Expressed as cells/mL

- $T = C \times 4 \times 10^6 \times$ dilution factor

5.4.7. References

European Brewery Convention. Analytica Microbiologica – EBC. Fachverlag Hans Carl, 2001

5.5. Yeast cell count – Methylene blue staining of yeast cells

5.5.1. Scope

This method allows a rapid estimation of the percentage of viable yeast cells, which are not stained, because dead cells are blue-stained. The method is applicable to all samples containing yeasts, except musts containing more than 100 g/l sugar. Bacteria are too small and their staining is not visible with this method.

*Note: a good focus should be achieved at various depths, in order to properly see their coloring with methylene blue.*

5.5.2. Principle

Methylene blue is converted into its colourless derivative by the reducing activity of viable yeast cells. Dead yeast cells will be stained blue.

Viability is calculated from the ratio between the number of viable cells and the total number of cells. The method overestimates “real” viability when viable cells are less than 80%, because it does not distinguish between “live” cells and their ability to reproduce (Viable But Not Culturable cells).

If the sugar concentration is higher than 100 g/l, most cells are light blue, therefore this method is not recommended.

If wine has low pH and is strongly buffered, the dye cannot work properly. In this case the count must be applied at least to the first decimal dilution.

5.5.3. Reagents and materials

- Solution A: Methylene blue distilled water solution, 0.1 g/500 ml.
- Solution B: $KH_2PO_4$, distilled water solution, 13.6 g/500 ml.
- Solution C: $Na_2PO_4$ x $12H_2O$ distilled water solution, 2.4 g/100 ml
- Solution D: 498.75 ml Solution B + 1.25 ml solution C.
- Solution E: Mix the 500 ml of solution D with 500 ml solution A to give final buffered methylene blue solution, with pH approximately 4.6.

5.5.4. Installations and equipment

Microscope, 250-500 x magnifications. Phase contrast is contraindicated.

Microscope slides and cover slips, or haemocytometer (Thoma, Bürker or Neubauer chamber).

Test tube and stirring rod.

Pipettes, fine tips.

5.5.5. Examination techniques

Viability determination

Dilute the suspension of yeast with methylene blue solution in a test tube until the suspension has approximately 100 yeast cells in a microscopic field. Place a small drop of well-mixed suspension on a microscope slide and cover with a cover slip.

Examine microscopically using a magnification of 400 x within 10 minutes contact with the stain.

Count a total of 400 cells (T), noting the number of blue coloured (C) dead, broken, shrivelled and plasmolyzed cells. Budded yeast cells are counted as one cell if the bud is less than one half the size of the mother cell. If the bud is equal or greater than one half the size of the mother cell, both are counted. Cell stained light blue should be considered alive.

5.5.6. Expression of results

If T is the total cell number and C the blue coloured cell number, then the percentage of viable cells is

$$\frac{T-C}{T} \times 100$$

5.5.7. References


- European Brewery Convention. Analytica Microbiologica – EBC. Fachverlag Hans Carl, 2001


6. Counting of micro-organisms by culture

*Objective:*

The purpose of counting of microorganisms by culture is to evaluate the level of contamination of the sample, that is to say, to estimate the quantity of viable microorganisms. According to the culture media used and the culture conditions, four types of microorganisms can be counted, namely, yeasts, lactic bacteria, acetic bacteria and mould.

Principle:

Enumeration by culture is based on the fact that micro-organisms are able to grow in a nutrient medium and incubation conditions suitable to form colonies on the medium solidified by agar, or turbidity in a liquid medium. On an agar medium a cell produces bv proliferation a cluster of cells visible to the naked eye called colony.

6.1. Detection, differentiation and enumeration of microorganisms (plate count).

6.1.1. Scope

This standard gives general guidance for the enumeration of viable yeasts, moulds and lactic or acetic bacteria in musts, concentrated musts, partially fermented musts, wines (including sparkling wines) during their manufacture and after bottling, by counting the colonies grown on a solid medium after suitable incubation. The purpose of microbiological analysis is to control the winemaking process and prevent microbial spoilage of musts or wines.

6.1.2. Terms and definitions

The terms “plate” and “Petri dish” are used as synonyms.

CFU = Colony Forming Units.

6.1.3. Method

The number of viable microorganisms present in musts or wines is determined by spreading a small known volume of sample on the surface of a culture medium or adding it as per the incorporation method (see par. ~~9.5~~ 6.1.7.4), and incubating the plates for the required time in the better conditions for the growth of the microorganisms. Each cell, or cluster of cells, divides and gathers into a cluster and becomes visible as a colony. The number of colonies found on the surface of a plate states for the cells occurring in the original sample so that the results are reported as CFU. If the number of cells in a sample is supposed to be high, suitable serial decimal dilutions are performed in order to obtain colonies ranging from ~~15~~ 10 to 300 per plate. If the number of CFU in a sample is supposed to be low, they are collected on the surface of a sterile 0.45 to 0.88 µm filter for yeasts of 0.22 to 0.45 µm and for bacteria, which is then placed in the Petri dish on the surface of the culture medium.

The measuring range of this method rises from < 1 CFU/(analyzed volume) to $10^9$ CFU/ml or $10^{10}$ CFU/g in the original sample.

6.1.4. Reagents and materials

As indicated in paragraph 1 of the resolution, plus:

Tubes (16x160 mm or similar) containing 9 ml sterile peptone water (Tryptone: 1 g/l) or other diluents to be used for serial sample dilutions (Appendix 4). An indicative number of tubes required for the following samples is reported below:

Unfermented musts: 4 / sample.

Fermenting musts:7 / sample.

Wines in storage: 2 / sample.

Micropipette holding sterile tips: 1 ml and 0.2 ml.

L-shaped or triangular-shaped bent glass rods (Drigalski rods) or plastic spreaders.

90-mm diameter Petri dishes (56 $cm^2$) (with 15-20 ml of growth medium) for pour plate technique, and 90-mm or 60-mm diameter plates (with 6-8ml of growth medium)for membrane filter technique, filled 18-24 h in advance with 15-20 ml of culture medium (simple or double dishes are required for each sample tested):

For yeasts counts use: YM, YEPD, WL Nutrient Agar, YM Agar or TGY Agar. If searching non-*Saccharomyces* yeasts, Lysine Agar and WL Differential Agar plates (AppendixAppendix 5, culture medium) or equivalent if validated.

For acetic acid bacteria counts use: GYC agar, G2 or Kneifel medium (AppendixAppendix 5, culture medium) or equivalent if validated

For lactic acid bacteria counts use: MRS plus 20% tomato (or apple- or grape-) juice, or modified ATB Agar (medium for *Oenococcus oeni*), or TJB plus agar, or Milieu Lafon-Lafourcade, milieu 104, MTB agar (AppendixAppendix 5 culture medium) or equivalent if validated

For filamentous fungi counts use Czapek-Dox modified agar, DRBC agar or MEA added with tetracycline (100 mg/l) and streptomycin (100 mg)l). (Appendix 5 culture medium) or equivalent if validated

Antibiotics must be added in order to make the counting selective since all the microorganisms are together in wine.(see Appendix I culture media)

6.1.5. Installations and equipment

As indicated in paragraph 2 of the resolution.

6.1.6. Sampling

As indicated in paragraph 3 of the resolution

The following amounts of samples are required for the plate counting:

Must, or fermenting must or wine in storage: not less than 250 ml;

Bottled or packed wine: not less than one unit,whatever the capacity;

6.1.7. Examination techniques

6.1.7.1. Preliminary requisites

All the materials and equipments used in the tests must be sterile, and aseptic condition must be kept during all operations.

The laminar flow cabinet must be switched on 5 minutes before starting the work, in order to have a sterile and stable air flow.

6.1.7.2. Sterilization

Culture media must be sterilized in autoclave at 121°C for at least 15 minutes (20 minutes for large volumes). Single-use sterile materials and glassware must be opened and used under laminar flow cabinet. Tweezers and spreading devices must be immersed in ethanol and flamed before use. Stainless steel funnels must be flamed with ethanol after each use, while glass- and polycarbonate funnels must be autoclaved before use, so these ones must be available in the same number as the tested samples.

6.1.7.3. Sample dilution (Appendix 1)

One ml of sample is pipetted in a sterile 9 ml peptone water tube. The tube is stirred with the aid of a “vortex” shaker for 20 seconds. This is the first (decimal) dilution, from which 1 ml is transferred to the next 9-ml sterile peptone water tube, which is the second dilution. After 20 seconds shaking, the operation is repeated until necessary.

The indicative number of serial dilutions required for the following samples is reported below:

Unfermented musts: 4 decimal dilutions.

Fermenting musts: 7 decimal dilutions.

Unfiltered wines during ageing (Yeast counts): 2 decimal dilutions.

Unfiltered wines during ageing (Lactic Acid Bacteria counts) : 6 decimal dilutions.

Filtered wines or packed (bottled) wines No dilution.

Concentrated musts Dilute 10 ml in 100 ml peptone water (or 100ml in 1000ml).

Bottled or filtered wines, and concentrated musts after dilution in sterile peptone water, are analyzed with membrane filter technique.

6.1.7.4. Plating

The necessary serial dilutions are prepared for the number of samples to be plated. Multiple serial dilutions can be prepared, if many samples have to be plated, but any dilution must be plated within 20 minutes.

Inoculate each plate with 0.1 or 0.2 ml of the three lowest dilutions prepared, as follows:

Unfermenting musts: dilutions -2; -3; -4.

Fermenting musts: dilutions -5; -6; -7.

Unfiltered wines during ageing :dilutions 0; -1; -2.

In doubt, inoculate a higher number of dilutions, never a lower.

Under aseptic conditions (preferably under a laminar flow cabinet) spread the sample on the surface of the culture media before the liquid is absorbed (usually within 1-2 minutes) with a sterile bent glass rod (Drigalski rods) or a single-use one. A separate "hockey stick" must be used for each plate, or the plate must be spread starting with the most diluted sample and proceeding to the least dilute ones. Leave the plates some minutes under sterile air flow, until the liquid is absorbed.

*Note 1: Plating 0.,2 ml instead of 0.1 ml, as frequently reported, allows an easier spreading and a delayed one. Calculations must consider this.*

*Note 2: For the enumeration of yeast Bacterial growth is avoided by adding 50 mg/l chloramphenicol (or equivalent if validated) to growth media, after autoclaving it, and the mold by adding biphenyl 150mg/L (or equivalent if validated).*

*Note 3: For the enumeration of lactic acid bacteria, yeasts growth is prevented by the addition of natamycin (pimaricin) (0.1 g/L) (or equivalent if validated) and acetic bacteria by anaerobic incubation.*

*Note 4: For the enumeration of acetic bacteria, the growth of yeast is prevented by the addition of natamycin (pimaricin) (0.1 g/L) (or equivalent if validated) and that of lactic acid bacteria with the addition of penicillin (12.5 mg/L) (or equivalent if validated).*

*The addition of antibiotics is done after the autoclave sterilization.*

If a specific research of non-*Saccharomyces* yeast is performed, inoculate as previously described, three Lysine Agar plates and three WL Differential Agar plates with the appropriate dilutions

Incorporation method (alternative method).

Prepare and sterilize 15 ml of medium in tubes, and keep the tubes in a water bath *(or equivalent if validated)* at 47 ± 1°C.

Pour 1 ml of sample or dilution in an empty Petri dish.

Add 15 ml culture medium and stir gently the Petri dish, so as to obtain a homogeneous distribution of microorganisms within the mass of the medium.

Allow to cool and solidify by placing the Petri dishes on a cool horizontal surface (the solidification time of the agar shall not exceed 10 min).

6.1.7.5. Enumeration with concentration by membrane filtration

Membrane porosity must be 0.45 or 0.8 µm for yeast counting; 0.2 or 0.45 µm for counting bacteria. Membrane surface must be preferably be cross-hatched, in order to facilitate the colony counting.

The plates, on which the membranes are put, can contain an agar nutrient medium or a pad, in which the dry medium is dispersed, that must be soaked with sterile water just before the use. Some suppliers give sterile plates containing a sterile pad, on which the content of 2-ml of single-use sterile liquid medium is poured just before the use.

Aseptically assemble the filtration equipment, sterilize the funnel according to 9.2, and connect to the vacuum-producing system.

Dip the tweezers in ethanol and flame them: when the flame is extinguished, wait some seconds and put the membrane, with the tweezers, on its holder of the filtration unit.

Before opening the bottle, shake it well; dip the bottleneck upside-down in ethanol (1-2 cm) and flame to sterilize it.

Of each sample sample three amounts: 10 ml with a sterile 10-ml pipette, 100 ml with a sterile cylindrical 100-ml pipette, and the rest direct from the bottle, if possible. To filter the wine, pour the wine into the funnel.

When the desired amount of wine has been filtered, release the vacuum, flame the tweezers, open the funnel, keep the membrane with the tweezers, put its opposite edge on the solid medium of a plate and make it adhere to the medium surface, avoiding bubble formation beneath.

6.1.7.6. Sample incubation

Incubate the plates, upside-down, aerobically 4 days at 25 ± 2 □C, for yeast or for acetic acid bacteria. If temperature is < 23°C extend incubation one more day, if temperature is < 20°C extend three more days. The maximum temperature must not exceed 28°C.

In case of performing *Brettanomyces* (or *Dekkera)* yeast counts, increase twofold the incubation time.

In case of performing LAB count, put the plates in an anaerobic jar or bag, and incubate the plates upside-down 10 days at 30 ± 2 □C. If temperature is < 28°C extend incubation one more day, if it is < 25°C extend three more days. The maximum temperature must not exceed 33°C.

6.1.8. Expression of results

6.1.8.1. Counting yeast colonies and bacteria.

Count the colonies grown in 4 days for the yeast and acetic acid bacteria (8 days for *Brettanomyces/Dekkera* yeasts), and 10 days for lactic bacteria, if necessary with the aid of a colony counter, ignoring the different colony morphology if performing a total yeast count, or considering it, if required.

The media and incubation conditions are specific enough for it to be possible to count the different types of micro-organisms in the colonies visible to the naked eye.

6.1.8.2. Calculation of results.

The most reliable results come from counting plates containing from 10 to 300 colonies (ISO 7218:2007 - Microbiology of food and animal feeding stuff - General rules for microbiological examinations).

Calculate the number N of microorganisms present in the test sample as a weighted mean from two successive dilutions using the following equation:

$$N = \frac{\sum C}{V \times 1{,}1 \times d}$$

where

- $\sum C$ is the sum of colonies counted on the two dishes retained from two successive dilutionns, at least one of which contains a minimum of 10 colonies.
- $V$ is the volume of the inoculum placed in each dish, in millilitres.
- $d$ is the dilution corresponding to the first dilution retained [$d$=1 when the undiluted liquid product (test sample) is retained].

In other words, if plates from two consecutive decimal dilutions contain 10-300 colonies, compute the number of CFU/ml for each dilution, and then the average of the two values: this is the CFU/ml value of the sample. If one value is greater than the double of the other, keep the lower one as CFU/ml.

Round off the results to two significant figures only at the time of conversion to CFU/ml, and express the results as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (ISO 7218:2007 - Microbiology of food and animal feeding stuff - General rules for microbiological examinations).

If samples were inoculate in duplicate series, and one or two plates, inoculated with the same dilution, contain colonies, compute the average of the number of colonies and multiply by the reciprocal of the dilution factor, to obtain the number of CFU/ml.

If there is no plate containing 10-300 colonies, and all plates contain more than 300 colonies, count the less crowded ones. If they contain less than 10 colonies/cm$^2$,

count 12 squares of 1 $cm^2$ and multiply the average by 56 (the area of a 90-mm diameter plate); if colonies are more crowded, count 4 squares of 1 $cm^2$ and multiply the average by 56. Express the results as "Estimated CFU/ml". Do not express the results as TNTC (Too numerous to count) whenever possible.

If the only plates containing colonies contains less than 10 colonies, but at least 4, calculate the result as given in the general case, and report it as "Estimated CFU/ml".

If the total is from 3 to 1, the precision of the result is too low, and the result shall be reported as "(the searched microorganisms) are present but less than 4 × $d$ CFU/ml".

If plates from all dilutions of any sample have no colonies, report the results as "less than 1/$d$ CFU/ml", but consider the possible presence of inhibitors in the sample.

When performing membrane filtration technique, express the results referring to the amount of filtered liquid, e.g. CFU/bottle, CFU/100 ml, or CFU/10ml.

6.1.9. Uncertainity of measure

6.1.9.1. Criteria of controlling the results.

For each lot of medium, one plate is used as sterility control after sterilization. One plate per each culture medium used during the tests, is left opened under laminar flow cabinet during all operations, as a sterility check of the working environment. That plate will be incubated as the inoculated ones.

Periodically, one sample is inoculated in double, and the experimental $K_p$ is calculated with the following equation:

$$Kp = \frac{|C_1 - C_2|}{\sqrt{C_1 + C_2}}$$

where $C_1$ and $C_2$ are the results of the two counts.

If Kp < 1.96 ≈ 2.,0 the results are acceptable: the average of the two counts can be used as the result.

If 2.0<Kp ≤ 2.576 ≈ 2.6 the difference of the two counts is critical, and must be carefully evaluated before accepting the results as the average of the two counts.

If Kp >2.6 the difference of the two counts is anomalous. The result is rejected and the test must be repeated. In such event the person in charge of the laboratory must examine all the results obtained after the last acceptable ones.

6.1.9.2. Uncertainty of measure

If the number of counted colonies in the countable plate is lower than 10, the result is acceptable, but the population of colonies is considered to follow the Poisson distribution. The 95% confidence level, and consequently the uncertainty of measure,

of the estimated count made on a single Petri dish, is reported in the following table.

| Number of. colonies | Confidence limit at 95% level | | Percent error of the limit * | |
|---|---|---|---|---|
| | Lower | Upper | Lower | Upper |
| 1 | <1 | 6 | -97 | 457 |
| 2 | <1 | 7 | -88 | 261 |
| 3 | <1 | 9 | -79 | 192 |
| 4 | 1 | 10 | -73 | 156 |
| 5 | 2 | 12 | -68 | 133 |
| 6 | 2 | 13 | -63 | 118 |
| 7 | 3 | 14 | -60 | 106 |
| 8 | 3 | 16 | -57 | 97 |
| 9 | 4 | 17 | -54 | 90 |
| 10 | 5 | 18 | -52 | 84 |
| 11 | 6 | 20 | -50 | 79 |
| 12 | 6 | 21 | -48 | 75 |
| 13 | 7 | 22 | -47 | 71 |
| 14 | 8 | 24 | -45 | 68 |
| 15 | 8 | 25 | -44 | 65 |
| * Compared to the microrganism count (1st column) | | | | |

If the colony count is >10, the confidence limit at a *p* probability level is calculated with the following equation:

$$C = C_i + Kp\sqrt{C_i}$$

where $C_i$ is the number of colonies on the plate, and $K_p$ is the coverage factor. Usually the coverage factor is 2, or 1.96. C value is calculated from each plate and multiplied by the number of dilutions, together with the result of the count.

6.2. Culture in liquid medium- "Most Probable Number" (MPN)

6.2.1. Objective

The purpose of this technique is to evaluate the number of viable microorganisms in wines having high contents of solid particles in suspension and/or high incidence of plugging.

6.2.2. Principle

This technique is based on the estimation of the number of viable microorganisms in liquid medium, starting from the principle of its normal distribution in the sample.

6.2.3. Diluents and liquid culture media (see Appendices 4 and 5)

6.2.4. Operating method

Several quantitative and successive solutions are prepared and following this, after incubation, a certain proportion of tests will not lead to any growth (negative tests), while others will begin to grow (positive tests). If the sample and the dilutions are homogeneous, and if the number of dilutions is sufficiently high, it is possible to treat the results statistically, using suitable tables (tables based on McCrady's probability calculations), and to extrapolate this result to the initial sample.

6.2.5. Preparation of dilutions

Starting from a sample of homogenized wine, prepare a series of decimal dilutions ($^1/_{10}$) in the diluent.

Take 1 mL of wine and add to 9 mL of diluent in the first tube. Homogenize. Take 1 mL of this dilution to add to 9 mL of diluent in the second tube. Continue this dilution protocol until the last suitable dilution, according to the presumed microbial population, using sterilized pipettes for each dilution. The dilutions must

be made until extinction, i.e. the absence of development in the lowest dilutions (*appendix 2*).

6.2.6. Preparation of inoculations

Inoculate 1 mL of wine and 1 mL of each of the prepared dilutions, mixed at the time, in, respectively, 3 tubes with the appropriate culture medium (*appendix 5*). Mix thoroughly.

Incubate the inoculated tubes in the incubator at 25°C for yeasts (3 days, up to 10

days), under aerobic conditions, and for lactic bacteria, under anaerobic or microaerophilic conditions (8 days, up to 10 days), making periodic observations up to the last day of incubation.

6.2.7. Results

All those tubes that show a microbial development leading to the formation of a whitish deposit, more or less evident and/or with a more or less marked disturbance are considered as positive. The results must be confirmed by observation through a microscope. Specify the incubation period.

The reading of the tubes is made by noting the number of positive or negative tubes in each combination of three tubes (in each dilution). For example, "3-1-0" signifies: 3 positive tubes in the 10 dilution (wine), 1 in the $10^{-1}$ dilution and zero in the $10^{-2}$ dilution.

For a number of dilutions higher than 3, only 3 of these results are significant. To select the results allowing for the determination of the "MPN", it is necessary to determine the "typical number" according to the examples in the following table:

Table:

| Number of positive tubes for each dilution | | | | | | Typical number |
|---|---|---|---|---|---|---|
| *Example* | 10 | 10 | 10 | 10 | 10 | 3-1-0 |
| a | 3 | 3 | 3 | 1 | 0 | 3-2-0 |
| a | 3 | 3 | 2 | 0 | 0 | 3-2-1 |
| a | 3 | 2 | 1 | 0 | 0 | 3-0-1 |
| a | 3 | 0 | 1 | 0 | 0 | 3-2-3 |
| b | 3 | 2 | 2 | 1 | 0 | 3-2-3 |
| b | 3 | 2 | 1 | 1 | 0 | 3-2-2 |
| c | 2 | 2 | 2 | 2 | 0 | 2-2-2 |
| d | 0 | 1 | 0 | 0 | 0 | 0-1-0 |

Example a : take the greatest dilution for which all the tubes are positive and the two following ones.
Example b : if a positive result is achieved for a dilution that is bigger than the last chosen dilution, it must be added.
Example c : if no dilution achieves three positive tubes, take the dilutions that correspond to the last three positive tubes.
Example d : instance of a very small number of positive tubes. Choose the typical number so that the positive dilution is in the ten's row.

Adapted from Bourgeois, C.M. and Malcoste, R. *in* : Bourgeois,

C.M. et Leveau, J.Y. (1991).

Calculation of the Most Probable Number (MPN)

Taking account of the typical number obtained, the MPN is determined through Table A (*Appendix 3*) based on McCrady's probability calculations, considering the dilution made. If the dilution series is 10 ; $10^{-1}$ ; $10^{-2}$ the reading is direct. If the dilution series is $10^{1}$; 10; $10^{-1}$ the reading is 0.1 times this value. If the dilution series is $10^{-1}$; $10^{-2}$; $10^{-3}$; the reading is 10 times this value.

*Comment:*

If there is a need to increase the sensitivity, a concentration $10^{1}$ of wine can be used. To obtain this concentration of microorganisms in 1 mL, centrifuge 10 mL of wine and take 1 mL of deposit (after having taken 9 mL of excess liquid) and inoculate according to the previously described method.

6.2.8. Expression of Results

The microorganism content of wine must be expressed in cells per mL, in scientific notation to one decimal place. If the content is lower than 1.0 cells per mL, the result must be presented as "<1.0 cells per/mL".

(See annexes on following pages)

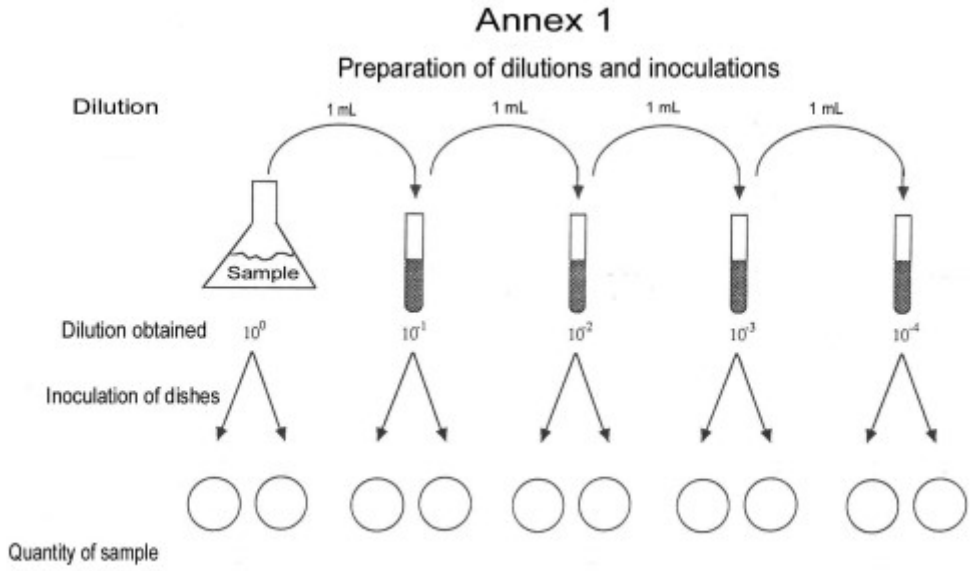
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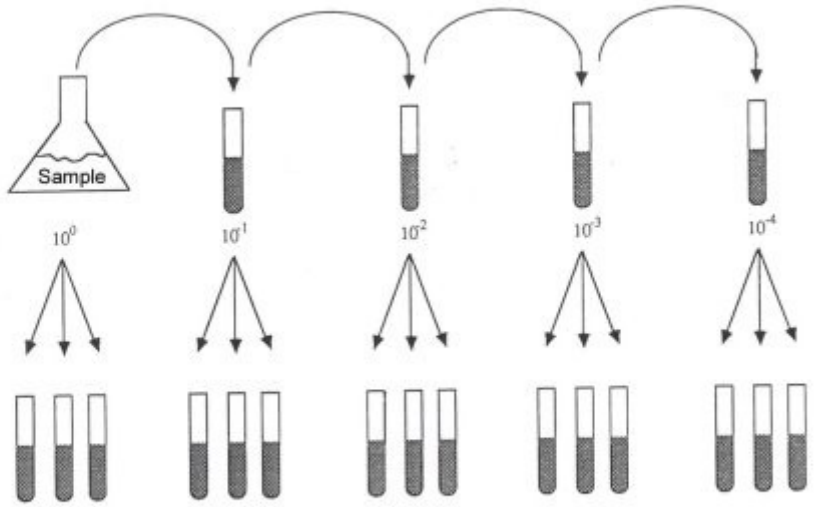
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Appendix 2 : Preparation of dilutions and inoculatons




Appendix 3 Table A
"Most Probable Number" (MPN) for 1 mL sample utilizing 3 tubes
with 1 mL, 0.1 mL et 0.01 mL

| Positive tubes | | | | Positive tubes | | | | Positive tubes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 mL | 0,1 mL | 0,01 mL | MPN 1 mL | 1 mL | 0,1 mL | 0,01 mL | MPN 1 mL | 1 mL | 0.1 mL | 0,01 mL | MPN 1 mL |
| 0 | 0 | 0 | 0,0 | 2 | 0 | 2 | 2,0 | 1 | 1 | 1 | 7,5 |
| 0 | 0 | 1 | 0,3 | 2 | 1 | 0 | 1,5 | 3 | 1 | 2 | 11,5 |
| 0 | 1 | 0 | 0,3 | 2 | 1 | 1 | 2,0 | 3 | 1 | 3 | 16,0 |
| 0 | 1 | 1 | 0,6 | 2 | 1 | 2 | 3,0 | 3 | 2 | 0 | 9,5 |
| 0 | 2 | 0 | 0,6 | 2 | 2 | 0 | 2,0 | 3 | 2 | 1 | 15,0 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0,4 | 2 | 2 | 1 | 3,0 | 3 | 2 | 2 | 20,0 |
| 1 | 0 | 1 | 0,7 | 2 | 2 | 2 | 3,5 | 3 | 2 | 3 | 30,0 |
| 1 | 0 | 2 | 1,1 | 2 | 2 | 3 | 4,0 | 3 | 3 | 0 | 25,0 |
| 1 | 1 | 0 | 0,7 | 2 | 3 | 0 | 3,0 | 3 | 3 | 1 | 45,0 |
| 1 | 1 | 1 | 1,1 | 2 | 3 | 1 | 3,5 | 3 | 3 | 2 | 110,0 |
| 1 | 2 | 0 | 1,1 | 2 | 3 | 2 | 4,0 | 3 | 3 | 3 | >140,0 |
| 1 | 2 | 1 | 1,5 | 3 | 0 | 0 | 2,5 | | | | |
| 1 | 3 | 0 | 1,6 | 3 | 0 | 1 | 4,0 | | | | |
| 2 | 0 | 0 | 0,9 | 3 | 0 | 2 | 6,5 | | | | |
| 2 | 0 | 1 | 1,4 | 3 | 1 | 0 | 4,5 | | | | |

Adapted from the “ Standard Methods for the Examination of Water and Waste Water ” (1976)

Appendix 4

Diluents:

Diluents are indicated by way of example. The water to be used must be distilled, double distilled or deionized, with no traces of metals, inhibitors or other anti-microbial substances.

1. *Physiological water*

Preparation: Weigh 8.5g of sodium chloride in a 1000 mL calibrated flask. After it has dissolved in the water, adjust the reference volume. Mix thoroughly. Filter. Distribute 9 mL in the test tubes. Stop with carded cotton and autoclave for 20 min at 121°C.

2. Ringer's solution 1/4

Preparation: Weigh 2.250g of sodium chloride, 0.105g of potassium chloride, 0.120g of calcium chloride (CaCl2.6H2O) and 0,050g of sodium hydrogen carbonate in a 1000 mL calibrated flask. After it has dissolved in water, make up to the mark. Mix

thoroughly. Distribute 9 mL in the test tubes. Stop with carded cotton and autoclave for 15 min at 121°C. (This solution is available commercially)

3. *Peptone water*

Preparation: Weigh 1g of peptone in a 1000 mL calibrated flask. After it has dissolved in the water, adjust the reference volume. Mix thoroughly. Distribute 9 mL in the test tubes. Stop with carded cotton and autoclave for 20 min at 121°C.

Appendix 5

Culture media

Culture media and antimicrobials are indicated by way of example.

The water to be used must be distilled, double distilled or deionized with no traces of metals, inhibitors or other antimicrobial substances.

Solid culture media

If not otherwise stated, pH of all media should be adjusted to pH 5.5 -6.0

1. Media for yeast count

1.1. YM

Glucose: 50 g

Peptone: 5 g

Yeast extract: 3 g

Malt extract: 3 g

Agar-agar: 20 g

Water: up to 1000 ml

If necessary add 100 mg chloramphenicol to suppress bacterial growth and 150 mg biphenyl to suppress mould growth.

1.2. YEPD

Glucose: 20 g

Peptone: 20 g

Yeast extract: 10 g

Agar-agar: 20 g

Water: up to: 1000 ml

If necessary add 100 mg chloramphenicol to suppress bacterial growth and 150 mg biphenyl to suppress mould growth.

1.3. WL Nutrient Agar
Glucose: 50 g
Peptone: 5 g
Yeast extract: 4 g
Potassium phosphate monobasic ($KH_2PO_4$):0.55 g
Potassium chloride (KCl): 0.425 g
Calcium chloride (Ca$Cl_2$):0.125 g
Magnesium sulphate (Mg$SO_4$): 0.125 g
Ferric chloride (Fe$Cl_3$):0.0025 g
Manganese sulphite (Mn$SO_4$):0.0025 g
Bromcresol green: 0.022 g
Agar bacteriological: 12 g
Water: up to: 1000 ml
pH: 5.5
WL Differential agar is made by adding 4 mg/l cycloheximide to WL Nutrient Agar.
If necessary add 100 mg chloramphenicol to suppress bacterial growth.
1.4. Lysine Agar ASBC
Solution A:
Yeast Carbon Bass: 2.35 g
Water: up to: 100 ml
*Sterilize by membrane filtration.*
Solution B:
Lysine-HCl: 0.5 g
Agar agar: 4 g
Water: up to: 100 ml
*Sterilize in 20 min. at 121□C.*
If necessary add 100 mg chloramphenicol to suppress bacterial growth.

**2. Media for lactic acid bacteria count**

2.1. M.R.S. + tomato (or apple) juice.
Glucose: 20 g

Peptone: 10 g
Beef extract: 8 g
Yeast extract: 4 g
Potassium phosphate, dibasic ($KH_2PO_4$): 2 g
Sodium acetate 3$H_2$O: 5 g
Ammonium citrate: 2 g
Magnesium sulphate □6$H_2$O: 0.2 g
Manganese sulphate □4$H_2$O: 0.05 g
"Tween 80": 1 ml
Agar agar: 12 g
Tomato (or apple, or grape) juice: 200 ml
Water up to : 1000 ml
Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, after autoclaving, just before use.

2.2. Tomato Juice Agar

Tomato juice (dry extract from 400 ml): 20 g
Peptone: 10 g
Peptonized milk: 10 g
Agar-agar: 14 g
Water : 1000 ml
pH: 6.1
Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, after autoclaving, just before use.

2.3. Modified ATB medium, or *Oenococcus oeni* medium (formerly *Leuconostoc oenos* medium).

Solution A:
Glucose: 10 g
Yeast extract : 5 g
Peptone : 10 g
Magnesium sulphate: 0.2 g
Manganese sulphate: 0.050 g
Tomato juice (or apple juice or grape juice): 250 ml

Agar agar: 12 g
Water: 750 ml
Sterilize by autoclaving 20 min. at 121□C.
Solution B:
Cysteine HCl: 1 g
Water: up to: 100 ml
pH : 4.8
Sterilize by membrane filtration.
Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, just before use.
Add 1 ml of solution B to 20 ml of solution A at the moment of use

2.4. Lafon-Lafourcade medium

Glucose: 20 g
Yeast extract: 5 g
Beef extract: 10 g
Peptone: 10 g
Sodium acetate: 5 g
Tri-ammonium citrate: 2 g
Magnesium sulphate □6$H_2$O: 0.2 g
Manganese sulphate □ 4$H_2$O 0.05 g
"Tween 80": 1 ml
Agar-agar: 20 g
Water: up to: 1000 ml
pH: 5.4
Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, after autoclaving, just before use.

2.5. Dubois medium (Medium 104)

Tomato juice: 250 ml
Yeast extract: 5 g
Peptone: 5 g
Malic acid: 3 g
Magnesium sulphate □ 6$H_2$O:0.05 g
Manganese sulphate □ 4$H_2$O: 0.05 g
Agar-agar: 20 g

Water: up to: 1000 ml

pH: 4.8

Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, after autoclaving, just before use.

2.6. MTb.

Glucose: 15 g

Lab-Lemco Powder (Oxoid): 8 g

Hydrolyzed casein: 1 g

Yeast extract: 5 g

Tomato juice: 20 ml

Sodium acetate: 3 g

Ammonium citrate: 2 g

Malic acid: 6 g

Magnesium sulphate: 0.2 g

Manganese sulphate: 0.035 g

"Tween 80": 1 mg

TC Vitamins Minimal Eagle, 100x (BD-Difco) 10 ml*

pH (con KOH): 5.0

Water up to: 1000 ml

* add after sterilization.

Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, after autoclaving, just before use.

3. Media for acetic acid bacteria count

3.1. GYC

Glucose: 50 g

Yeast extract: 10 g

Calcium carbonate ($CaCO_3$): 30 g

Agar: 25 g

Water: up to: 1000 ml

Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, and 12.5 mg/L of penicillin to eradicate the growth of lactic acid bacteria, after autoclaving, just before use.

3.2. Medium G2

Yeast extract: 1.2 g

Ammonium phosphate: 2 g

Apple juicE: 500 ml

Agar: 20 g

Water: up to: 1000 ml

pH: 5.0

Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, and 12.5 mg/L of penicillin to eradicate the growth of lactic acid bacteria after autoclaving, just before use.

3.3. Kneifel medium

Yeast extract: 30 g

EthanoL: 20 ml*

Agar: 20 g

Bromocresol green 2.2%: 1mL

Water: up to1000 ml

* to be added after sterilization.

Add 100mg / L natamycin (pimaricin) to inhibit the growth of yeasts, and 12.5 mg/L of penicillin to eradicate the growth of lactic acid bacteria after autoclaving, just before use.

Blue colonies: *Acetobacter*, *Gluconacetobacter*

Green colonies: *Gluconobacter*

## 4. Media for mould count

4.1. Czapek-Dox, Modified

Sucrose: 30 g

$NaNO_3$: 3 g

$K_2HPO_4$: 1 g

$MgSO_4$: 0.5 g

KCl: 0.5 g

$FeSO_4$ : 0.01g

Agar 15 g

Final pH (at 25°C) 7.3 ± 0.2

Add 10 mg/l cycloheximide to suppress yeast growth (cycloheximide-resistant yeast growth is usually slower than mould growth).

*Note: This medium allows the growth only of nitrate-growing moulds.*

Add tetracycline (100 mg/l) and streptomycin (100 mg/l) to suppress growth of bacteria.

4.2. Dichloran Rose Bengal Chloramphenicol Agar (DRBC Agar)

Glucose: 10 g

Peptone : 5 g

$KH_2PO_4$: 1 g

$MgSO_4$ :0.5 g

Rose Bengal: 0.025 g

Dichloran (2,6 dichloro-4-nitroaniline) : 0.002g

Chloramphenicol solution (0.1 g/10ml)*: 10 ml

Agar : 15 g

Final pH (at 25°C) 5.6 ± 0.2

* To be added after sterilization.

4.3. Malt Extract Agar (MEA)

Glucose: 20 g

Malt extract : 20 g

Peptone: 5 g

Agar : 15 g

Final pH (at 25°C) 5.5 ± 0.2

Add tetracycline (100 mg/l) and streptomycin (100 mg/l) to suppress growth of bacteria.

5. Liquid culture media

1. *For yeasts*

YEPD medium (Yeast Extract, Peptone, Dextrose) + chloramphenicol

*Preparation*: Weigh 10.0g of yeast extract (Difco or equivalent), 20g of peptone, 20g of glucose and 100 mg of chloramphenicol. Dissolve, make up to 1000 mL volume with

water and mix.

Distribute 5 mL portions of this medium in the test tubes and autoclave for 15 minutes at 121°C.

*5.2. For lactic bacteria*

*MTJ medium (50% MRS medium "Lactobacilli Man Rogosa and Sharpe*

*Broth" + 50% TJB medium "Tomato Juice Broth") + actidione*

*Preparation*: Weigh 27.5g of MRS "Lactobacilli Man Rogosa and Sharpe Broth" (Difco or equivalent). Add 500 mL of water, heat to boiling to permit complete dissolution and add 20.5g of TJB "Tomato Juice Broth" (Difco or equivalent). Add 50g of actidione. Dissolve with water in order to obtain 1000 mL of solution having first corrected the pH to 5 with 1N hydrochloric acid and mix.

Distribute 10 mL portions of this medium[3)] in the tubes and autoclave for 15 minutes at 121°C.

## 6. Appendix 6: Recognition of specific microorganisms

6.1. Yeast colony recognition on WL Nutrient Agar.

The use of this medium does not want to be a method to identify species, but can offer to non-specialized laboratories a quick and cheap way to predict the genus of viable and culturable yeasts. After 4-days incubation evaluate the colony morphology as follows (Pallman, C., J. B. Brown, T. L. Olineka, L. Cocolin, D. A. Mills and L. F. Bisson. 2001. Use of WL medium to profile native flora fermentations. American Journal of Enology and Viticulture 52:198-203; A. Cavazza, M. S. Grando, C. Zini, 1992. Rilevazione della flora microbica di mosti e vini. Vignevini, 9-1992 17-20):

*Saccharomyces* spp.: Colonies grow well in 4 days on WL Nutrient Agar giving circular cream-coloured to pale greenish colonies. Different colour shades do not necessary indicate the presence of different strains, but the presence of petite mutants; colonies are umbonated, smooth and dull surface, the consistency is butyrous. It doesn't grow on Lysine Agar.

*Torulaspora* spp.: the colonies are similar to those of *Saccharomyces* spp. It grows on Lysine Agar.

*Hanseniaspora* spp. *(Kloeckera* spp.*)* Grows on WL Nutrient Agar in 4 days, giving deep green flat, smooth and butyrous colonies. It grows on Lysine Agar and on WL Differential Agar.

*Candida stellata* Grows on WL Nutrient Agar in 4 days, giving pea-green, smooth and butyrous colonies, becoming darker in the centre with the age. It grows on Lysine Agar.

*Saccharomycodes* spp.Grows on WL Nutrient Agar in 4 days, giving light green, smooth and butyrous convex colonies. It grows on Lysine Agar, not on WL differential agar.

*Note: its cells, viewed under the microscope, are very large (up to 25 □m).*

*Schizosaccharomyces pombe* Grows on WL Nutrient Agar in 4 days, giving deep green pinpoint size, smooth colonies. It grows on Lysine Agar.

*Note: its cells, under the microscope are easily recognised because of typical scission division.*

*Rhodotorula spp.* Grows on WL Nutrient Agar in 4 days, giving deep pink, smooth and mucous surface and butyrous colonies. It grows on Lysine Agar.

*Metschnikowia* spp. Grows on WL Nutrient Agar in 4 days, giving clear, smooth and butyrous little colonies. A reddish pigment diffuses in the medium below the colonies. It grows on Lysine Agar.

*Pichia membranifaciens* Grows on WL Nutrient Agar in 4 days, giving greyish- or bluish-shaded rough and powdery convex colonies. It grows on Lysine Agar.

*Pichia anomala* (formerly *Hansenula anomala*) grows on WL Nutrient Agar in 4 days, giving cream-colored or bluish colonies, distinctly bluish after 8 days. Colonies are circular, the surface is smooth and the consistency is butyrous, but sometimes clearly mucous. It grows on Lysine Agar.

*Dekkera spp.* or *Brettanomyces spp.* Grows on WL Nutrient Agar in 8 days, giving small dome-shaped, cream-coloured, smooth and butyrous colonies. It produces high amounts of acetic acid, clearly perceivable by smell that turns the medium to yellow. It grows on Lysine Agar and on WL Differential Agar. The growth on this last medium makes it possible to distinguish it from *Zygosaccharomyces bailii*.

*Note: a confirmation is possible with microscopical examination: Dekkera has small cells, some of them have a typical ogival shape.*

*Zygosaccharomyces bailii* Grows on WL Nutrient Agar in 4 days, giving small circular cream-coloured, smooth and butyrous colonies. It grows on Lysine Agar but not on WL Differential Agar. A yellowish halo is often present around young colonies.

*Note: when grown on bottled wine it produces brown 0,5-1 mm clusters. Its cells do not have ogival shape.*

Acetic acid bacteria grow on WL Nutrient Agar with small to pinpoint-size deeply green and brilliant colonies that are strongly positive to catalase test. *(Note – This medium is not suitable for their count)*.

Lactic Acid Bacteria grow on WL Nutrient Agar in 10 days with pinpoint size clear catalase-negative colonies. *(Note – This medium is not suitable for their count).*

6.2. Lactic Acid Bacteria colony recognition.

LAB colonies are translucent and range in size from a pinpoint to a few mm in diameter. They are gram-positive and catalase-negative. *Oenococcus oeni* grow in short chains, pediococci form tetrads and diplococci, lactobacilli form long or short bacilli.

6.3. Acetic Acid Bacteria colony recognition.

AAB colonies are catalase positive and gram-negative, and are strong acid-producers: this can be seen by a clear zone around their colonies in media containing calcium carbonate or by a different colour if the medium contains a pH indicator. Their cells are cocci or bacilli, generally a little larger than LAB.

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[3)] The 10 mL volume is used instead of the 5 mL volume as with yeasts, due to the greater sensitivity of lactic bacteria to oxygen.