The role of microbiology in winemaking

Introductory notes

Incorporating microbiological concepts and techniques into the winemaking process

Fermentation | **Storage** | **Packaging**

Planning

Some critical decisions

- the style of the target wine,
- whether to inoculate the ferment via an addition of yeast and, if so, with which yeast strain, and
- whether to inoculate the ferment/wine via an addition of bacteria for the conduct of malolactic fermentation (MLF) and, if so, with which bacterial strain and the timing of the inoculation.

Monitoring

Fermentation action/reaction

Action

- control fermentation conditions so that the primary alcoholic, secondary alcoholic (for sparkling wine production) and MLF are carried out efficiently and completely.

Reaction

- restart stuck fermentations.

Prevention/detection of spoilage

Yeasts and bacteria may grow in wine during storage and after packaging. Measures to prevent microbial spoilage include —

- treatments to ensure sanitised/sterilised conditions in storage vessels and in packaging line facilities,
- limiting ullage in storage vessels,
- the use of filtration operations, and
- the use of sulfur dioxide.

Risk assessment

The presence of microorganisms in packaged wine may or may not lead to spoilage. Specifications need to be set as to the types and numbers of organisms accepted in a packaged product — this will be determined by conditions such as the pH, sugar, alcohol and sulfur dioxide concentrations in the wine, the type of packaging and the expected storage conditions.

Operations, procedures and/or tests

(these might be used to monitor fermentation activity and the microbiological status of the juice or wine)

- Preparation of the yeast starter culture — either from a dried yeast product or a wet culture.
- Determination of the concentration of yeast cells in a yeast starter culture, and if necessary in the ferment, eg by use of microscopy and haemocytometry techniques.
- Preparation of the bacterial culture to conduct the MLF.
- Determination of the concentration of bacteria cells in the inoculum, eg by use of microscopy and a Petroff-Hausser counting chamber.

- Carrying out studies to determine the presence and types of yeasts and bacteria present at different stages of the ferment. This is achieved by plating a sample of the juice/must and/or ferment and conducting identification tests.
- Testing for the presence of spoilage organisms, eg *Dekkera/Brettanomyces* yeast or *Acetobacter* bacteria by plating a sample of the wine on selective media and by the use of specific tests.

- For sparkling wine production, determination of the concentration of yeast cells in the yeast culture intended to conduct the secondary alcoholic fermentation.
- If juice concentrate is to be added to a wine, plating a sample of the concentrate, diluted if necessary, can determine if there is any yeast or bacteria contamination in the juice concentrate.

- Conducting swabs on storage vessels or packaging line equipment and materials to determine the sterility status of the equipment/materials.
- Microscopic examination of a sample of packaged wine to assess the sterility status of the wine, eg by fluorescence microscopy techniques to determine the viability of any yeasts present in the packaged wine.

Sampling

Tank sampling

Sampling of juice/wine stored in a storage container, such as a tank, other containers and tankers is an essential part of all microbiological analysis. If the purpose of sampling is to obtain an assessment of the microorganism load, the samples must be taken aseptically. If a sample is not collected aseptically, a false high results may occur. The sample must be representative of the wine in the tank. Contamination can be introduced from using non-sanitised equipment or failure to properly sanitise the sample port or device.

Sampling devices

Devices for sampling tanks/tankers and other containers are bottles, cups or stainless steel containers attached to a chain or a cord. A chain is preferable to a cord as it is easier to sanitise.

Sanitising the sampling device

The sampling device is sanitised by rinsing with water and then immersing the whole apparatus in a bucket of 80% v/v ethanol between sampling each tank or tanker.

Whirl-pak® bags

The Whirl-pak® bag is a convenient means to collect samples. It is pre-sterilised and is disposable. It reduces preparation time, glassware sterilisation, saves taking heavy glassware out to the cellar and avoids breakages. However, when full they tend to fall over, and should be placed in a suitable sized bucket or plastic beaker.

Helpful hints

It is important to make sure that the sample port is cleaned when the tank is cleaned. If possible, it should be removed and disassembled for cleaning. Alternatively, the sample port should be opened and flushed with the cleaning agent and the rinse water while the tank is being cleaned.

Procedure for sampling from a sampling port

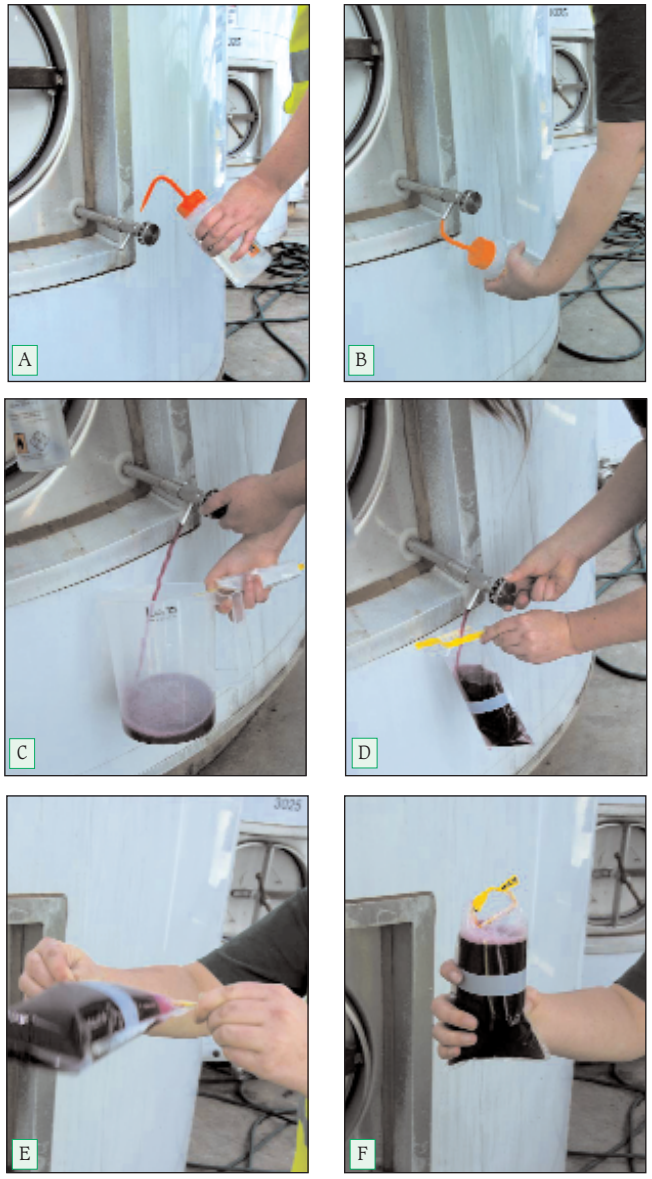
Equipment

Sterile, capped 100 mL sample bottles; Whirl-pak® bag; spray/wash bottles containing 80% v/v ethanol; bucket.

Procedure

1. The sampling port should be wiped if it is crusted with wine or foreign matter. If a water hose is available the sampling port and area can be rinsed out with water. Before taking the sample, thoroughly irrigate the external and internal surfaces of the sampling port with 80% v/v ethanol. A squirt bottle can be used for this purpose. Allow up to 2 minutes of contact time. If the tanks are clustered together, the port of each tank can be irrigated one after the other and then sampling commenced at the first tank.
2. Place a bucket or jug under the sampling port and run out at least 500 mL of wine to flush the ethanol from the sampling port before collecting the sample. Close the port slightly so that a steady stream of liquid is running from the port into the bucket or jug.
3. Aseptically remove the sample bottle cap, place the sample bottle under the port, fill the bottle and recap the bottle; or alternatively open the Whirl-pak® bag and collect the sample in it. Ensure that the details of the sample are recorded on the bottle or the bag.
4. Rinse and wash the inside and outside of the sampling port with water and then with 80% v/v ethanol.

Note: A piece of silicon or Tygon® tubing can be attached to the sampling port to aid in the delivery of the liquid into the sampling bottle. This tubing should be soaked in 80% v/v ethanol between samples.



An example of the sequence for sampling wine from a sampling port: a) and b) sterilising the sampling port with 80% v/v ethanol; c) running off some wine prior to sampling; d) collecting the sample in a Whirl-pak® bag; e) after closing the bag it is twirled to seal it and f) the filled and sealed Whirl-pak® bag.

Spread plate

Performing a spread plate

Procedure for performing a spread plate

The spreader is used to spread the inoculum across an agar plate, the surface of which should be dry. The inoculum needs to be diluted so that an appropriate number of colonies grow on the plate (see Precision below).

1. Label the bottom of two Petri dishes for each dilution to be inoculated with the sample identity and the decimal dilution.
2. Place the labelled Petri dishes onto the bench with the lids uppermost. Do not remove the lids. It helps to lay out the Petri dishes in the order of the dilutions corresponding to the order of the tubes in the rack.
3. Mix the diluted suspension to be spread before taking the aliquot (the inoculum) for spreading.
4. Lift the lid off the Petri dish just enough to add the inoculum to the agar. With a sterilised pipette tip, transfer 0.1 mL (100 µL) of inoculum onto the agar surface of the corresponding labelled Petri dish. Place the inoculum into the centre of the plate. Inoculate the plates for only 1 decimal dilution at a time to avoid all of the inoculum being absorbed into the agar before you have spread it out.
5. Sterilise the glass spreader by dipping it into 96% v/v ethanol and quickly passing it through a flame. Do not hold it in the flame. Allow the ethanol to burn off and the glass spreader to cool.
6. Use the spreader to spread the inoculum evenly over the entire surface of the agar plate to the edges of the Petri dish. It helps to rotate the agar plate whilst spreading the inoculum to achieve an even cover. Place the lid back onto the Petri dish.
7. Discard the plastic spreader after use or, if a glass spreader was used, flame sterilise it and allow it to cool. Repeat the spreading procedure for the second plate of the appropriate dilution.
8. Repeat the inoculation procedure (Steps 3 to 7) for all of the dilutions.
9. Leave the plates upright for several minutes to allow the inoculum to completely absorb into the agar.
10. Invert the plates to prevent condensation from dripping onto the agar surface during incubation.
11. Place the plates into an incubator set to an appropriate temperature for the target microorganisms and incubate for the recommended time.
12. Remove the plates from the incubator and examine for growth of organisms. Choose the appropriate plate to count, ie the plate that has between 30 and 300 colonies. It should not be necessary to count plates corresponding to more than one dilution. Count the number of colonies on both plates corresponding to the selected dilution and average the result. If one plate has obvious contamination, ie mould growth, or significantly different numbers of organisms, choose the next dilution that allows a good count to be achieved for both plates.
13. Calculate the numbers of Colony Forming Units (CFU)/mL using the formula below and 0.1 mL for the volume of inoculum.

$$\text{Colony forming units/mL} = \frac{\text{Average number of colonies x dilution factor}}{\text{Volume of inoculum (mL)}}$$

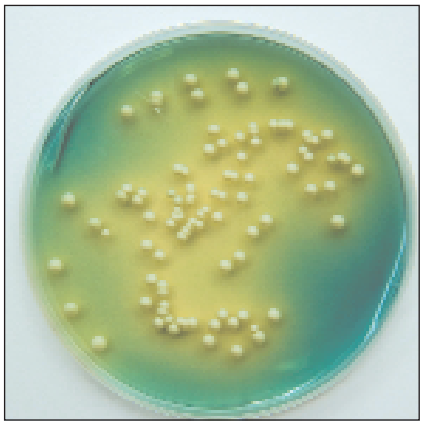
Precision

1. Greater than 300 colonies over the entire agar surface are considered to be too many to enable all organisms to grow due to limitations of space, nutrient competition and toxin accumulation. Choose another dilution to count.
2. Less than 30 colonies on the agar surface is considered to be too few to accurately represent the original sample and a smaller dilution should be counted.

Pipetting the inoculum into the centre of the plate

Using a spreader to spread the inoculum over the plate

An example of colonies on a spread plate



Yeast viability — staining methods

Methylene blue method

This is a commonly used method to estimate the viability of a population of yeast during culturing and fermentation. Methylene blue is a redox indicator that is a colourless compound when present in the reduced form and blue when in the non-reduced form. Viable, therefore metabolically active cells, can reduce the compound, and do not stain. Non-viable cells are not able to reduce the stain and so appear blue. The proportion of cells which do not stain, ie are colourless, is therefore an indication of the yeast viability in the sample.

The results of methylene blue staining should only be regarded as an indication of cell viability. At low culture viability the method becomes much less reliable when compared to plating methods. Under some conditions some cells, which are capable of dividing, still stain, while some dead cells fail to stain blue. Although there is some disagreement as to the appropriateness of the application of this method, it is commonly used for determining yeast viability in yeast propagation cultures and fermentation samples.

Equipment and materials

Microscope
Haemocytometer
Microscope slides and coverslips
Glass test tube(s)
Tally counter

Reagents

Distilled water and methylene blue solution

Methylene blue solution

Dissolve 0.3 g methylene blue chloride in 30 mL of 95% v/v ethanol. Prepare a citrate buffer by dissolving 2.4 g disodium hydrogen citrate and 2.1 g sodium dihydrogen citrate in approximate 50 mL distilled water and make to a final volume of 100 mL. Adjust the pH to 4.6 if necessary. Add the 30 mL of methylene blue chloride solution to the 100 mL of the citrate buffer. Mix well. Note, there are a number of methods for preparing a methylene blue stain solution.

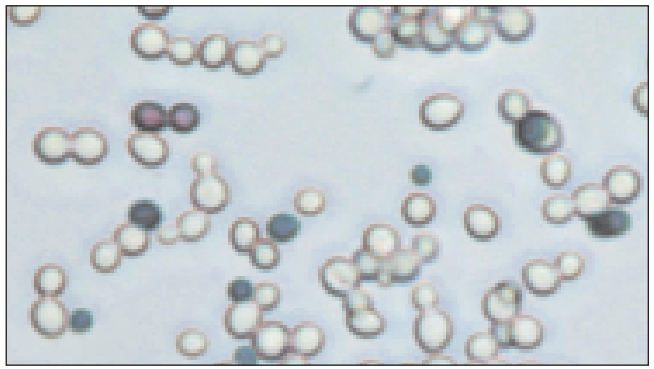
Sample Preparation

Samples should be tested within 30 minutes of staining as a result may not be valid for samples standing for a longer time period. The stain is toxic and overexposure will kill the cells.

Procedure for staining with methylene blue

1. Mix 0.5 mL of a sample of yeast suspension with 0.5 mL of methylene blue solution, and place a drop of the mixture on a haemocytometer or microscope slide. The yeast suspension may need to be diluted with 0.9% w/v sodium chloride, eg 1 to 50 for ferments or cultures.
2. After 5 minutes at a temperature of 20-22ºC, examine the sample via a microscope using 40x objective magnification.
3. Observe approximately 300 cells; some operators observe up to 600 cells. Record the exact number of cells observed. Record the number of cells which are colourless (viable cells). Score buds greater than one half the size of the parental cell as separate cells.
4. Report viability as the percentage of the total number of cells observed which are colourless (unstained) cells. Percentage budding can also be recorded to provide further indication of stage of growth and vitality of the culture.

An example of a methylene blue stain



Alternative staining methods

While methylene blue is the most widely applied yeast viability stain, there are alternative stains that offer better reproducibility and easier determination of viable versus non-viable cells.

Alkaline methylene blue or violet

This method is based upon the same principle as the commonly used methylene blue method. The use of alkaline conditions (pH 10.6) is believed to permit more rapid cell penetration of the methylene blue dye.

Reagents

Dissolve 0.01 g methylene blue or methylene violet 3 RAX in 10 mL distilled water. Dilute the methylene blue/violet solution 1:10 in 0.1M glycine buffer at pH 10.6. Filter through Whatman No 1 filter paper.

Procedure for staining with alkaline methylene blue or violet

1. Sample preparation and procedure is the same as the standard methylene blue method, with the modification of a 15 minutes incubation of the mixture of yeast culture and alkaline methylene blue. Dead cells stain medium to dark blue, live cells stain pale blue to unstained.

Notes for both methods

The alkaline methylene blue method is claimed to more closely approximate cell viability as determined by a slide culture method.

Note, staining techniques using methylene blue, alkaline methylene blue and other stains have not been verified with wine isolates of yeast, therefore operators need to assess their usefulness and interpretation in their particular situation.

It may be best to view the preparation under bright field rather than phase contrast.

Small buds may stain blue if they are not yet metabolically active.