



In-House
Microbiological
Testing of Wine in
Large Wineries

Simplifying Progress

SARTORIUS

Causes of Spoilage of Bottled Wine

Type and Origin of Microorganisms

A wide variety of microflora is present in freshly pressed grape must. These microorganisms consist of the most diverse yeasts, bacteria and molds, and come from the grape skin or the pulp, if the skin has been punctured by insects or animals, or is broken, and from the leaves and the vines of grape plants. Last but not least, these organisms originate from the soil, where they survive partly as spores.

However, yeasts far outweigh the other microorganisms at the onset of fermentation, especially “wild yeasts,” which have weak fermentation activity. However following the onset of fermentation, these yeasts are quickly dominated by *Saccharomyces* yeasts, provided that the must has not been inoculated with pure yeast cultures, which is standard practice. Alcohol resulting from fermentation inhibits the growth of molds. For this reason, they do not play a significant role as wine-spoilage microbes. Even the majority of bacteria are incapable of growth in aged wine. This leaves a small number of acid-tolerant bacteria from the group of lactic-acid bacteria for which the conditions are still sufficient for growth. However, alcohol is moderately significant in inhibiting bacterial growth as compared to the pH and unbound H_2SO_3 .

Completely fermented, dry wines entail few problems from a microbiological viewpoint. In dry wines yeasts do not find any substrate on which to grow, and the growth conditions for bacteria are not optimal either.

However, wines containing residual sugar and those sweetened by the addition of unfermented must are highly susceptible to spoilage by micro-organisms. The higher the sugar content and the pH, the greater the susceptibility of relatively sweet wines.

Causes of Spoilage of Bottled Wine

Errors during filling are a major source of problems affecting the healthy microbiology of wine. Often, these errors are not discovered until after weeks or even months have passed. The H_2HO_3 level in wine is frequently so high during filling that microorganism growth is delayed or microbes even do not grow at all on cultures prepared as part of a winery’s in-house microbiological testing. However, once the sulfurous acid has been used up within a bottle, any remaining microbes can resume growth and thus spoil the wine. To prevent such unpleasant surprises, stringent in-plant standards of hygiene must be implemented in addition to performing the conventional methods of must pasteurization, rapid fermentation and the specific dosage of sulfurous acid. This ongoing microbiological testing not only encompasses the bottling lines but also the entire winemaking process, from tanks to ready-to-sell bottles.

In addition to final quality control of bottled wine, the empty bottles must be microbiologically tested after cleaning. Microbiological testing must also include the sterilizer to check its sterilizing efficiency, the filling nozzles, the corking machine, the hoses and lines through which wine is pumped from the aging cellar, and, last but not least, the tanks into which the wine is pumped following prefiltration.

The primary objective of this standard operating procedure is not to detect winespoilage microbes in filled bottles – although this final quality control is an important part of microbiological testing. Rather, the main goal is to prevent microbes from entering the final bottling line right from the start. Since the storage conditions for the microbiological stability of wine are not always favorable – for example, wine bottles warehoused in wholesale beverage supply depots, in super markets and even at the end consumer’s home – very low microbe counts per mL of wine can cause wine to cloud over time. Therefore, it is better to avoid microbiologically contaminated wine bottles by implementing in-line control and in-plant hygiene measures than to discover what went wrong during bottling in order to eliminate the source of contamination and “salvage” the wine (this is highly cost-intensive).

Detection of Sources of Contamination

In-line Control

The first quality-control test stage takes place when wine is filtered after fermentation and fining. Although this stage involves only filter sheets or crossflow filtration that removes most microorganisms, but not all as in sterile filtration, enologists must ensure that high microbe counts can no longer occur when the wine is stored in tanks following this filtration step. Otherwise, this unnecessarily reduces the throughput of the filters used in final filtration. The storage tank, filling tank, inlet and outlet piping and hoses must have a low bioburden or, better yet, be sterile, in order to meet the requirements for high microbiological stability. Microbiological testing is done by taking samples at several points within the winemaking system and passing these samples through membrane filters to recover any microbes present.

The second stage is to test the empty bottles after cleaning. Even microorganisms that are not true winespoilage organisms can affect the shelf life of bottled wine because the preservative substances in the wine, such as sulfuric acid, are consumed, which reduces their protection against winespoilage microbes.

Flaming equipment sterilizes the bottle openings that might not be able to be reliably kept free of microbes. Unsterile openings carry over microbes into the wine when bottles are corked. This wine, in turn, can contaminate the filling machine. This is why the flaming equipment must be checked by microbiological testing as part of the third stage.

The filling machine itself is the fourth stage for microbiological testing. Since the product continuously splashes or sprays the machine, especially when a bottle breaks during filling under pressure, the risk of contaminating the filling machine is high. Contaminated filling stations also result in infections that regularly affect every nth bottle.

Parallel to microbiological testing of samples taken from the filling machine, the product entering the machine must also be tested. Product samples must be taken upstream of the prefilter, between the prefilter and the membrane filter, and, of course, downstream of the membrane filter as well. The purpose of testing these samples is to determine how high the bioburden is in the two sets of cartridge filters in the prefiltration and the final filtration systems.

The service life of membrane filter cartridges depends on the microbe count downstream of the prefiltration system. The higher the number, the faster the cartridge's retention capacity is depleted. Used-up retention capacity causes blockage of filter membranes and breakthrough of microbes in pad filters. This example clearly shows that the quality and the effectiveness of each step depends on that of each previous step.

Downstream of the filling machine, the corking machine is the most frequent cause of contamination. Its hygienic condition in terms of microbiology can be monitored using either the direct or indirect method. In the direct method, a sample is taken from the filler bells by wiping them with a sterile, cotton-tipped swab. Then the swab is placed in a liquid culture medium or streaked on a solid culture medium. In the indirect method, samples are taken from the originally corked bottles and microbiologically examined. The corks can be separately tested by shaking each one in sterile water, then membranefiltering the water.

The last quality control test stage must be carried out using the originally filled and corked bottles. If contamination is detected at this stage, its source must be traced step-by-step in the reverse winemaking sequence.

For the sake of simplicity, enologists will probably not want to perform each stage of in-house microbiological testing every day. However, in addition to testing the final product, it is advisable to test the most critical points daily, such as the filling machine, corking machine and empty bottles after cleaning. The time and expense of these microbiological tests are insignificant compared with the financial loss that can be incurred when bottled wine is contaminated.

Growth Phases of Microorganisms

Microbiological Stability of Wine

The microbiological stability of wine depends on three factors:

1. on the particular presence of microorganisms in wine
2. on the rate of propagation of these microorganisms in wine as a culture medium
3. on the time it takes for these microorganisms to alter the appearance or the taste of wine

An important parameter is the initial bioburden (Figure 1).

The growth curve of microorganisms always follows the same pattern of the curves plotted in Figure 1.

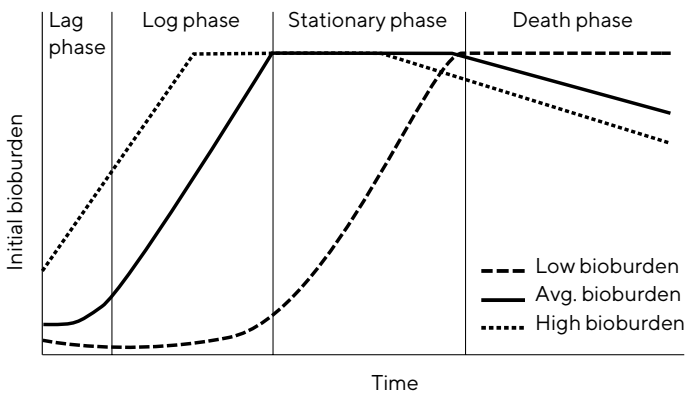


Figure 1: *Initial bioburden*

1. Lag Phase

During this time, the microorganisms activate their enzyme system and adapt to the available growth and nutrient conditions. They do not propagate during this phase. Instead, the microorganisms adversely affected by the medium die off.

2. Log Phase or Phase of Exponential Growth

During this time, microorganisms multiply very rapidly and intensively; this phase continues until the amount of nutrients begins to wane and/or the accumulation of metabolic products slows or inhibits growth.

3. Stationary Phase

During this time, the number of microorganisms that emerge by cellular division is equal to that of the microbes that die because of deteriorating growth conditions. Hence, the number of cells remains approximately constant.

4. Death Phase

The ambient conditions for the microorganisms have meanwhile deteriorated to the extent that these microbes successively die off. For this reason, the microbe count decreases.

The higher the initial bioburden in a product, the earlier the transition of the growth curve from the lag phase to the log phase will occur, since relatively high microbe counts increase the odds that microbes already specialized or adapted to the medium are present. However, the logarithmic growth phase does not begin until the product starts to spoil in the microbiological sense. Therefore, the objective of the entire winemaking process must be to minimize the initial bioburden to delay the onset of microbiological spoilage to such an extent that the product, wine, will have already been consumed by this time.

The following factors inhibit the propagation of microbes in wine:

- Cool storage temperatures
- High content of unbound SO_2
- High acid content
- Short storage times

The greatest risk of microbiological instability is a high residual unfermented must content. Earlier, wines had 2–4 grams of residual sugar per liter. In today's sweet varieties of wine, the amount of sugar contained can be many times more. As a result, the risk of instability is accordingly higher. Theoretically, a single yeast cell is all it takes to result in post-fermentation of a bottled wine that contains unfermented must. In practice, approximately 10 cells per bottle are necessary for this to occur, however.

In dry wines, a few yeast cells are no problem. Only if they number 100 to 300 per bottle will they pose a risk. This also applies to champagne and sparkling wine in which the CO_2 content inhibits yeasts.

Checking and Controlling Sources of Contamination During Bottling

Sources of Contamination

Sources of contamination include the following:

Bottles

Brand-new bottles delivered straight from the glass manufacturer are usually free of yeasts. By contrast, recycled glass bottles are highly at risk for yeast contamination because yeasts form spores in residual traces of wine. This is why it is essential that recycled glass bottles be treated properly by undergoing thorough mechanical cleaning, chemical disinfection and | or heat sterilization. In addition, this cleaning procedure must be carefully monitored by microbiological testing.

Method: Flush the inside of each sample bottle with 20 – 50 mL of sterile water or sterile physiological saline. Filter this water through a membrane, then incubate the membrane filter on a suitable culture medium.

The mouths of the bottles can be checked for contamination by pressing each one on an agar plate (contact plate method) or by swabbing them with a sterile Q-Tip, then washing this swab in a rinse solution and using the membrane filtration detection method, or by incubating the swab directly in a liquid culture medium.

Water used to rinse and sterilize the bottles can be membrane-filtered, just as can the disinfectant from CIP systems, and the membranes are incubated on Standard culture media for determining the CFU count.

Corks

Corks are dangerous source of fungal contamination (causes the wine to taste like cork); however, they can also harbor yeasts and bacteria spores.

Method: Prepare an enrichment culture using sterile, unfermented must or shake corks in sterile water and membrane-filter the rinse liquid.

Hoses and Fittings

Stainless steel piping and equipment are easy to clean and disinfect, unlike rubber components: hoses and gaskets are at risk because rubber cracks and becomes brittle as it ages. These cracks can harbor microbes that are able to survive. The same applies to plastic components. They must be well maintained and frequently changed. Filling and corking machines have a particularly large number of parts exposed to the risk of contamination.

Method: Swab area with a sterile Q-Tip, then incubate in liquid culture medium or streak on a solid culture medium. As an alternative, rinse swab in sterile liquid, then determine the colony count using the membrane filter method.

Surfaces (Bottom of Tanks, Walls, Material) and Air

Cellar walls can harbor fungi; old cellar facilities are often dusty or have difficult-to-clean floors. Dirty surfaces, which may even contain residual traces of the product and are frequently moist, breed microbial infection, a source of wine-spoilage microbes that survive in a mixture of fungal growth, for instance. This especially applies to filling rooms. If the route from the bottle sterilizer to the filling machine is long and the unprotected bottles have to traverse this route on conveyor belts, airborne microbes frequently contaminate these bottles as a consequence.

Method: Contact-plate or swab test. If sterile swabs are not available, perform the contactplate method using sterile membrane filters as a direct method of detection.

Personnel Hygiene

This is an important point that is often overlooked. Street or protective clothing with dried stains of residual wine can cause yeast infections or serve as a medium for carrying over spoilage bacteria into wine. In addition it is important that winemakers carefully follow hygienic procedures when tasting wine at the sampling valves. Otherwise, this can result in contamination of the piping system and tubing or false positive samples.

Method: The contact-plate or swab test is recommended for microbiological testing.

Example of a Microbiological Quality Control Test Series at a Winery (Table 1: page 6)

In a series of quality control tests at a winery to determine the cause of microbiological contamination of wine, the percentages for various sources of contamination were listed in the order of their frequency.

Therefore, special attention must be given to the major sources of contamination, the filling workstation and the corking machine, including the mouths of bottles, which are frequently re-infected after they have been removed from the bottle sterilizer.

Table 1: *Frequency of Occurrence of Sources of Contamination*

| Source of Contamination | Cause or Explanation | Frequency |
|-------------------------|---|-----------|
| Filling station | Contaminated filling valves or filler bells | 48% |
| Corking machine | Insufficient sterilization of the corking machine or filler bells | 28% |
| Bottle openings | Insufficient or lack of flaming equipment | 8% |
| Filtration system | Defective fittings, insufficient sterilization of the filters | 6% |
| Bottler sterilizer | Defective sterilizer | 10% |

Microbiological Testing of Filled Bottles

This is merely a final quality control test to allow any of the ongoing sources of contamination mentioned above to be detected.

Methods: a) Geisenheimer method, b) centrifugation or c) membrane filtration. High bioburdens can be determined using the first two methods cited. In the centrifugation method, dead cells are also counted. Trace infections can be detected only by the membrane filter method.

a) Geisenheimer method:

inoculate 3 test tubes, each containing 10 mL of sterile grape juice, with 10 ml each of the sample, and incubate the test tubes. After 3–5 days, check the test tubes for signs of cloudiness. If the results are negative, extend the incubation time to approx. 10 days.

b) Centrifugation:

Centrifuge a sample of approximately 10–20 mL, depending on the capacity of the centrifuge tube. Examine 1 drop of sediment under the microscope for the presence of yeasts, bacteria and molds.

c) Membrane filtration:

Filter the contents of one wine bottle through a membrane and incubate the membrane filter on a culture medium. Any microorganisms present form colonies that are visible to the unaided eye and can be counted. As an alternative, filter one half of the bottle contents through one membrane and the second half through a second membrane. Then incubate one membrane filter on a selective medium for yeasts and molds and the other on a special medium for detection of winespoilage microbes. This method is advantageous because inhibitors, such as alcohol and sulfurous acid, can be washed out of the membranes after subsequent flushing with 50 to 100 mL of sterile water following filtration.

This substantially enhances the growth conditions for microorganisms. Table 2 entitled “In-line Control Tests of a Filling Line” lists the main sites for in-house quality control tests as well as the method and recommended culture media.

Instead of conventional agar plates, nutrient pad sets can be used. These are ready-to-use, dehydrated, presterilized solid culture media in petri dishes that include membrane filters specially optimized for the specific application. To use them, all that is needed is to moisten them with 3.5 mL of sterile, preferably deionized, water. The user obtains the filters, culture media and petri dishes together as a single set. Nutrient pads can be conveniently stored up to 2 years at room temperature. Therefore, the user will not have any problems with preparing culture media from scratch, autoclaving and refrigeration, and can minimize laboratory equipment and the labor involved.

A membrane filter that has not been incubated can also be microscopically examined, provided it is stained with Loeffler’s methylene blue. Yeasts can be readily detected in this manner; bacteria are more difficult to detect; because they can be easily confused with inorganic particles.

In many cases, it is helpful to microbiologically examine the fermentation processes to enable the winemaker to intervene when necessary. Here, winemakers cannot afford to wait during the long incubation phase that it takes for colonies to grow. Rather, they need the results immediately. A quick test system offers considerable advantages in this respect because it does not merely determine the CFU count. Instead, it measures the metabolizing activity of the microorganisms.

With respect to this last feature, the instrument can be used for rapid detection of poor quality wines that are highly contaminated with spoilage microbes and have been delivered from a far-away region. In extreme cases, the instrument can be used to save costs by allowing immediate rejection of such wine shipments.

Outlook

Microbiological testing is increasing in importance during winemaking and bottling. The consumers' requirements for shelf life and quality are growing, and so are the volumes of wine being bottled. Microbiologically contaminated wine bottles mean tremendous financial losses for the winemaker, not to mention the image loss that these bad wine lots entail. A large winery simply cannot afford this any longer in today's tough times.

For this reason, it is indispensable to extend microbiological quality control in the wine industry. Only a good in-plant monitoring system can reliably protect wine from microbial spoilage.

Table 2: *In-line Control Tests of a Filling Line*


| Stage | Sampling Location | Test for | Method | Type of Agar or Nutrient Pad Set |
|-------|---|----------------------------------|---|---|
| 1 | Storage tank, filling tank, hoses, piping system | Fecal and wine-spoilage microbes | Membrane filtration of small sample volumes | Standard, Wort, possibly Orange Serum |
| 2 | Empty bottles removed from the cleaning machine or bottle sterilizer; rinse water, if necessary | Fecal microbes Yeasts | Rinse out with sterile water and filter this water. Also membrane-filter rinse water. | Standard |
| 3 | Bottle openings after flaming | Yeasts | Contact-plate or swab test | Wort |
| 4 | Filling machine | Wine-spoilage microbes | Swab filler bells | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |
| 5 | Prefilter inlet | Wine-spoilage microbes | Membrane filtration | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |
| 6 | Prefilter outlet | Wine-spoilage microbes | Membrane filtration | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |
| 7 | Final filter outlet | Wine-spoilage microbes | Membrane filtration | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |
| 8 | Corking machine | Wine-spoilage microbes | Swab filler bells | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |
| 9 | Corks | Molds | Flush in sterile water, then membrane-filter this water | Wort |
| 10 | Filled bottles | Wine-spoilage microbes | Membrane filtration | Orange Serum, Wort, Tomato Juice (Jus de Tomates) |

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