Spoilage micro-organisms and their effects

The alcohol content of distilled spirits is too high for microbial growth to occur, but being a food product, a high level of physical cleanliness is required at all stages of production. In addition, fermentation vessels and associated equipment must be free from microbial contamination in order to maintain product quality. In some brandy and rum distilleries microbial growth other than, or in addition to, culture yeast is encouraged for the flavour of the final product. That aspect, discussed in Module 1, is not contamination in the harmful sense that is the subject of this unit. The main microbial contaminants are listed here in alphabetical order rather than decreasing order of importance. The named genera are also important contaminants of the brewing industry, and the reading list at the end of this unit refers to brewing literature, but the spoilage effects are different in breweries and distilleries. It is important to be aware that the alcohol content of distilled spirits is too high for microbial growth to be possible, but contaminants introduced with the reduction (dilution) water at the time of bottling may be able to survive. Also, it is assumed that readers are already familiar with basic microbiological methods, including Gram’s stain to distinguish between the two main types of bacterial cell-wall structure.

*Acetobacter* and *Gluconobacter*. The most important feature of these strictly aerobic Gram-negative bacteria is their ability to oxidize sugars or ethanol to acetic acid, produced in such quantity that the flavour of the acid, and ethyl acetate produced by subsequent esterification, would be objectionable. Also, yeast growth is inhibited by acetic acid, and not just by the low pH, since acetate ion also has an inhibitory effect. The difference between the genera is that *Gluconobacter* can oxidize only as far as acetic acid; whereas *Acetobacter* can eventually complete the oxidation to CO₂. In addition to the well-known acetic acid spoilage of alcoholic beverages up to about 12% abv, they are common on plants, particularly sugary fruits. Being strictly aerobic, they are no longer able to grow after a blanket of CO₂ forms in the fermentor head space once fermentation is under way.
The normal habitat of the facultatively anaerobic Gram-negative genera *Escherichia* and *Enterobacter* is the human and animal intestine. Although some toxin-producing strains of *Escherichia coli* are capable of causing human disease, that is an unusual occurrence. The main significance of the normally non-pathogenic *E. coli* is that its presence in a water supply indicates contamination by domestic sewage or farm animals, so intestinal pathogens may also be present. If present as contaminants of a fermentation, *Escherichia* and *Enterobacter* are no longer able to grow when about 2% alcohol and/or pH below about 4.6 have developed, but in their short period of growth in fresh molasses or cereal wort they produce objectionable aromas which could taint the distilled spirit. *Obesum-bacterium*, named for its unusually fat cells, is related to *Escherichia* and *Enterobacter*, but is not known to have an intestinal habitat; it is assumed to live on plant surfaces. *Obesumbacterium* has similar off-flavour effects but is slightly more resistant to alcohol and low pH.

*Lactobacillus* is probably the most likely bacterial contaminant of brandy, rum or whisky fermentations. These Gram-positive non-sporing bacilli are common on plants in general and therefore on the three types of raw materials. Although tolerant of the presence of air, lactobacilli grow only by anaerobic fermentation to lactic acid, and possibly also to ethanol and CO$_2$, depending on the species (see unit 2.2). Troublesome strains can grow throughout fermentation, and also in the charger vessel for the still, being unaffected by anaerobic conditions, ethanol or low pH, and able to utilise a range of simple sugars and dextrins. Some rum and whisky distillers regard moderate contamination by lactic bacteria as good for flavour development, but excessive growth, over about $10^6$/ml, causes not only unacceptable flavour but also loss of spirit yield by utilisation of fermentable sugar. *Pediococcus* is also a possible contaminant of this group, with similar nuisance effect to *Lactobacillus*, but less common. Another coccus member of the lactic acid bacteria is *Leuconostoc*. *Leuconostoc mesenteroides* is capable of growth in concentrated molasses or syrup stocks of rum distilleries, using the glucose half of sucrose molecules to synthesise the viscous polymer dextran which blocks pipework and pumps. However, *Leuco. oenos*, is not a harmful contaminant in the malo-lactic fermentation, but quite the opposite, improving cider, wine and therefore brandy flavour by converting malic acid with its two –COOH groups to lactic acid, with only one.. A similar type of reaction may take place in prolonged rum and whisky fermentations with *Lactobacillus* spp converting di- or tri-carboxylic acids to lactic, again resulting in a more mellow spirit.

The Gram-negative rods of the genus *Zymomonas* also live on plant surfaces as their natural habitat, and like the lactic bacteria are tolerant of atmospheric oxygen but grow only by anaerobic fermentation: of fructose or glucose to ethanol and CO$_2$ (see Figure 2.2.6 of unit 2.2). Being unaffected by the final pH and alcohol concentration and more tolerant of the anaerobic conditions than *S. cerevisiae*, *Zymomonas* is capable of growth throughout fermentation. However, its production of flavour congeners is likely to be different from the culture yeast, and therefore unwelcome.

That problem of different congeners also applies to fermentative wild yeasts, although they may be a useful component of the natural inocula of some brandy and dark rum.
fermentations. Biochemically, there are two main types of wild yeasts. The facultative anaerobes can grow during part or all of a fermentation (depending on alcohol tolerance), certainly affecting flavour but possibly also spirit yield. Not only are their metabolic products important, being different from distillery yeast, but distillation of wild yeast cells of different chemical structure could also affect the flavour of the distillate. Other *Saccharomyces* spp., even different strains of *S. cerevisiae* itself, come into this category, as well as other fermentative genera, of which *Hanseniaspora* (*Kloeckera* if non-sporing) and *Schizosaccharomyces* are perhaps the most important. Aerobic wild yeasts are restricted to growth early in the fermentation, but some, particularly *Hansenula* and *Pichia* spp., are capable of producing significant amounts of esters in that time. Finally it is worth mentioning that a few wild yeasts which produce zymocin "killer factor" against culture yeast are a possible hazard of "natural" mixed culture grape and molasses fermentations but the risk is low.

**Detection of contaminants**

It is most unlikely that microbial contamination of culture yeast or a fermentation could be detected by routine microscopical examination. Wild and culture yeasts have similar appearance, and contamination by bacteria is difficult to detect against the background of a much greater number of larger yeast cells. Since yeasts stain Gram positive, Gram-negative bacteria are more likely to be noticed, but only in the high numbers of really serious contamination. Specific immuno-fluorescent staining can detect bacteria or wild yeasts in the presence of 100 – 1000 times as many culture yeast cells depending on the expertise of the operator, but requires special reagents and equipment (unit 2.1).

So culture methods are more effective, but require 2 – 3 days' incubation at 25°C (1 – 2 days at 30°C). Different methods are required for testing (a) grape juice or wort before inoculation (seldom done, but possibly required to trace the source of contamination) or (b) culture yeast or an active fermentation. Standard nutrient media are suitable for (a), and a spread-plate count with a 0.1 ml sample on malt extract agar (a useful medium for grape, molasses or cereal fermentations) can detect contaminants down to 10 cells/ml. Using an indicator medium, e.g. Wallerstein medium (basically a synthetic equivalent of malt extract agar with added pH indicator bromocresol purple) can distinguish by colour and shape the colonies of different yeasts or bacteria, i.e. distinguish that they are different, it is impossible to give a definite identification by colony morphology. The same media can be used to confirm effectiveness of sterilization of a vessel, to grow contaminants trapped on a membrane filter of 0.45 µm pore size after filtration of 250 ml of last rinse.

Samples (b) require a selective medium to allow any contaminants to grow but inhibit growth of the culture yeast that is known to be there. For detection of lactic acid bacteria and *Zymomonas*, and usually for the other bacteria listed above, "actidione agar", i.e. Wallerstein agar + 100 µg/ml of the anti-fungal antibiotic cycloheximide (also known as actidione) is most commonly used, but various other media on the same
principle are also available. Yeasts are unable to grow in the presence of the antibiotic; but bacteria are unaffected and grow as normal colonies. Lactic bacteria and Zymomonas grow by anaerobic metabolism and may require a reduced-O$_2$, high CO$_2$ atmosphere for first isolation. That is most easily provided by incubating plates in a sealed can, with a lit candle added just before closing. Completely anaerobic conditions are required only for Clostridium spp, which may be of interest to rum distillers.

No selective medium exists to suppress culture yeast but allow all other yeasts to grow. “Lysine agar” is the most useful, a synthetic medium of glucose, lysine, salts and vitamins, which depends on the inability of Saccharomyces spp to grow on lysine as sole source of nitrogenous compounds (lysine taken in by the specific transport system can not be converted to other amino acids). Most other yeast genera, and certainly all of the common non-Saccharomyces contaminants listed in unit 2.3, are able to utilize lysine, and grow to normal colonies. One problem with this medium is that it depends on starvation of the culture yeast, but even after thorough aseptic washing and centrifugation of the sample, sufficient intracellular N remains to allow growth of small colonies. More seriously, wild Saccharomyces yeasts, probably more likely contaminants than other genera, can not be detected. However, a modified actidione agar often works, with only 5 – 10 g/ml of antibiotic (whatever amount is found to be just sufficient to suppress the culture yeast) since many wild yeasts have sufficient antibiotic resistance to grow.

Rapid methods
Since traditional culture methods require several days’ incubation various rapid methods have been developed to perform urgently required microbiological checks. Accepted terminology for these methods is: “instant” = no growth required; “rapid” = shorter incubation than the traditional method. Two examples of instant methods (both involving brief incubation, but not for growth of the micro-organisms concerned) are immuno-fluorescence for detection of contaminants in a yeast culture (unit 2.3) and the polymerase chain reaction (PCA) which is capable of amplifying minute amounts of DNA from specific contaminants. A disadvantage of most instant methods, and certainly of these two, is inability to distinguish dead from living cells. Although PCA can detect contamination after a cleaning cycle, it can not confirm the effectiveness of sterilization since the DNA may be from killed organisms, so its usefulness is limited.

Rapid methods do recognise viable micro-organisms, but possibilities for distinguishing different types are limited. The two most convenient and widely used are detection of ATP by bio-luminescence, and detection of microbial metabolism by conductance, impedance or micro-calorimetry. The principle of the bioluminescence test is that light emission by fireflies (“glow-worms”) requires ATP, and the light intensity from purified extract of the insects in commercial kits of the luciferin/luciferase system is proportional to the amount of ATP. Although the literature supplied with the kit must be consulted for full details, the principle is that ATP is extracted from the sample, and the light emitted by its reaction with firefly extract is compared with known amounts of ATP which can in turn be related to numbers of micro-organisms.
Conductance, and its reciprocal measurement impedance, change in culture media during the lag phase with ion efflux, and the change is proportional to the number of living cells. Since these electrical effects are temperature-sensitive, incubation of the samples requires a water bath accurately attemperated to +/- 0.1°C. The cells of the instrument (sample sizes vary from 5 – 50 ml according to intention of the test) are each fitted with a pair of probes to detect and record electrical activity. Comparing the graphs with those from calibration cells with known numbers of commonly encountered yeasts or bacteria gives a reasonably accurate measure of numbers. Commercially available selective media allow different types of bacteria or yeasts to be recognised. Alternative instrumentation is available to detect the heat produced by microbial growth, but for a rapid method to detect small numbers of micro-organisms.

Cleaning and sterilization: definitions

Physical cleanliness - visually clean.

Chemical cleanliness – anything in contact with the cleaned surface suffers no contamination. In practice, clean water will completely wet the surface and drain as a continuous film without forming rivulets or droplets.

Detergent – a cleaning agent. By a combination of physical and chemical processes a detergent removes soil from a surface.

Biocide or Disinfectant – an agent for destruction of micro-organisms, but not necessarily 100%. In the food industries the words Sanitiser, Sanitisation are commonly used to imply both combined cleaning and disinfection.

Sterilant – an agent for complete destruction or removal of micro-organisms, which is unlikely to be achieved by chemical means, and so is often used wrongly as a synonym of disinfectant or sanitiser.

Cleaning in Place (CIP) – Cleaning (not necessarily automatic) without the need to dismantle equipment. Automatic CIP is now almost universal for cleaning and disinfection in the distilling industry, not least to avoid as far as possible the manual handling of hazardous chemicals. For cleaning vessels, there are two principal methods of applying detergents (a) low-pressure spray balls placed to cover the entire surface and (b) rotating high-pressure jets. The powerful stream of liquid from (b) gives the most effective cleaning, but the cleaning cycle can not be shortened in the case of light soiling. The jet must rotate itself through at least one complete cycle of coverage of the internal surface. Also, some external indication is required that the jet is actually rotating. The gentler spray from the ball relies solely on the chemical effect of the detergent, but the entire inner surface is sprayed continuously during the cycle. So for lightly soiled surfaces a shorter cleaning cycle is acceptable. It is essential to keep the holes in the ball clear, e.g. by a filter to remove solids from a recirculating system.
Cleaning of pipework is carried out by circulating detergent solution through the pipes. A flow rate of 2 m/s is accepted as providing the necessary turbulent flow for cleaning, without the risk of damage (e.g. water hammer) from higher flow rate (Table 2.4.1). Clearly, >100 mm requires impracticable pumping rates, although a lower flow rate would be acceptable for sterilization of an already cleaned line.

**Table 2.4.1. Flow rate of detergent to achieve cleaning velocity 2 m/s in pipes.**

<table>
<thead>
<tr>
<th>Pipe diameter (mm)</th>
<th>Flow rate (litres/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>62</td>
</tr>
<tr>
<td>50</td>
<td>220</td>
</tr>
<tr>
<td>75</td>
<td>550</td>
</tr>
<tr>
<td>100</td>
<td>890</td>
</tr>
<tr>
<td>150</td>
<td>1550</td>
</tr>
</tbody>
</table>

There are three basic types of CIP system: (a) a total-loss system, (b) a partial recovery system and (c) a full recovery system. Total-loss is best for plant with heavy soiling, where recovered detergent would be too contaminated for re-use. Recovery systems not only save detergent, which can be topped up and re-used, they also save water by using the final rinse as the pre-rinse of the next cycle. In all cases, the cleaning cycle involves a pre-rinse to remove loose soil, and detergent re-circulation to clean the vessel and pipework. After discarding or recovering the dilute detergent, the vessel is rinsed with clean water. A disinfectant may be added to this rinse water. A pulsed rinse cycle ("burst rinsing") gives more efficient use of rinse water.

Health & Safety aspects of distillery operation constitute one of the units of Module 3, but is is important to mention here that detergent and biocide preparations are labelled as hazardous chemicals. Therefore the storage, preparation, use and ultimate disposal of such materials is subject to the UK Control of Substances Hazardous to Health (COSHH) regulations or their equivalent in other countries, and according to the risk assessment and safety precautions literature provided with the products..

**Cleanliness and sterility requirements**

Fermentation is susceptible to microbial infection, so the risk is minimised by sterilization of the relevant equipment. Contamination at this stage is not a health hazard, since pathogenic micro-organisms or toxins are eliminated by the distillation process, but microbial contamination causes off-flavours and loss of product quality. Therefore vessels for preparation of pitching yeast, fermentation vessels, and pumps and pipework associated with these items absolutely require both cleaning and disinfection. Sterilization of the still charger vessel and associated pipework and equipment could also be considered, to reduce the risk of further microbial growth in fermented liquid awaiting distillation. For all other stages of the process, the physical cleanliness that is obligatory for a food product is sufficient.
How often is cleaning required? For stills the reduction of reactivity of copper surfaces and effect of accumulated soil on steam coil heat transfer (pot stills) or blockage of sieve plates (analyser column of a continuous still) are generally regarded as the most important factors, although the heated deposits could also generate off flavours. These effects develop slowly, so occasional cleaning is sufficient. However, plant for preparation of fermentable extract is much more sensitive. The equipment for the cereal mashes of whisky and grain neutral spirit is most susceptible to soiling, and cleaning (but not sterilization) after each use is essential, or various bacteria grow producing persistent off-flavours. For molasses and grape equipment, daily cleaning rather than after each use may be sufficient.

Process plant surfaces have an important effect on cleaning and sterilization. Comparing the smooth surfaces of stainless steel and copper with not only the roughness of cast iron and wood, but also the joints between the plates or planks, it is obvious that the latter two are extremely difficult to clean and disinfect (and probably absolute sterilization is impossible).

Cleaning and detergent action

Four distinct stages are involved in the actual cleaning, but easy rinsing is also important: 

- wetting of the surface to allow intimate contact between detergent and soil
- chemical action on the soil, e.g. solution of mineral scale by acids, hydrolysis of protein by acid or alkali, saponification of lipid material by caustic detergents.
- dispersion of large particles as finely-divided soil.
- suspension in solution of any removed soil.

Also, a good detergent allows efficient and complete removal of detergent and suspended soil by final rinsing.

Sodium hydroxide meets many of these requirements, and 2% NaOH used hot (> 70°C) is also an efficient disinfectant, but it has two main disadvantages.

(a) Precipitation of mineral salts, e.g. Ca, Mg associated with hard water, which is prevented by addition of sequestrants Na gluconate or Na hexametaphosphate to detergent formulations.

(b) Reaction with CO₂, forming much less efficient Na₂CO₃, requires fermentation vessels to be drained of CO₂ before cleaning.

So to avoid that lengthy delay, acid detergents have become common in the alcohol fermentation industries, with the additional advantage of action on mineral scale. Phosphoric acid with added surfactants makes a good CIP detergent. Some formulations also include nitric acid for greater detergency, also for disinfectant effect, but HNO₃ mixtures are destructively incompatible with copper or Cu-containing alloys. However, the stainless steels commonly used in the fermentation industries are
resistant. Various organic acids are also possible, but have no advantages over \( \text{H}_3\text{PO}_3 \) and are more expensive.

Quaternary ammonium compounds and “amphoteric” detergents are "surfactants" which are now widely used in the food industries. In a single compound they combine detergent and disinfectant activity: their powerful surface-active effect lyses microbial, particularly bacterial, cell walls and membranes. However, they have serious disadvantages for the fermentation industries. Foaming during CIP is a problem, they are difficult to rinse off, and residues cause foaming during fermentation, therefore they are not ideal for distillery use. On the other hand, that residual biocide on food-processing surfaces is an advantage.

**Biocides (disinfectants)**

All biocides are dangerous, since their purpose is to kill, so rigorous attention to safety is essential. With automatic CIP there should be no requirement for manual handling of biocides, except for preparing the working solution. Few of these agents kill all micro-organisms, but if fermentation vessels, yeast mixing vessels and associated pipework are cleaned and then treated to kill any remaining yeasts and the spoilage bacteria listed above, that is sufficient. Absolute sterility of plant is not justified when the fermentation medium itself is not sterile.

Killing of micro-organisms by a disinfectant is influenced by:

- Concentration of disinfectant
- Temperature of disinfectant
- Number of micro-organisms
- Time of contact between disinfectant and micro-organisms
- Amount of inert organic soil.

Disinfection is a chemical reaction between the agent and the target sites on the micro-organisms, therefore rate of reaction is increased by higher concentration and temperature. Longer time of contact can compensate for lower concentration and/or temperature. Disinfectant is diluted by increased microbial load, and for some disinfectants, halogens in particular, inactivated by organic soil in general.

The two most useful biocides for distillery fermentation vessels and associated plant are steam and peracetic acid.

Saturated (wet) steam is very effective for sterilizing cleaned equipment only. Steam bakes some types of soil on to surfaces, making it more difficult to remove, and may protect embedded micro-organisms. Steam kills all types of micro-organism, although to achieve sterility at atmospheric pressure requires continuous steaming for 1½ hours. Steam is freely available in a distillery, and no subsequent rinsing is required. However, radiated heat from a vessel being sterilized could affect a nearby fermentation if the vessels are too close.
Peracetic acid is a fast-acting effective disinfectant, even at ambient temperature. The concentrated acid has an unpleasant smell and is dangerous to handle because of its corrosive and strongly oxidising properties, so is unsuitable for manual use. However, it is a very effective final disinfectant in a CIP program.

\[
\begin{align*}
\text{CH}_3\text{C}=\text{O} \\
\quad \mid \\
\text{O–OH}
\end{align*}
\]

**Peracetic acid**

Peracetic acid breaks down first to acetic acid and hydrogen peroxide (which also have anti-microbial activity) and then the latter to water and oxygen. The small amount of acetic acid formed at use-dilution is insufficient to cause flavour taint so a final rinse is not required.

Rinsing after sterilization is best avoided; or UV-sterilized water should be used. However, the other possible final disinfectants mentioned here must be rinsed, for obvious reasons. Chlorine (as hypochlorite NaOCl) or iodine (as iodophor, a solution of I\(_2\) in a surfactant, which must be non-foaming for CIP) are not widely used because of the risk of flavour taint from residue, but are very effective oxidising disinfectants. Most NaOH detergent formulations give effective disinfection when used hot, but the alkaline residue must be rinsed off; and they are incompatible with residual CO\(_2\) in fermentation vessels.

**Further reading**

The following chapters in Brewing Microbiology, ed. F. G. Priest & I Campbell, collectively cover this unit: Gram-positive bacteria, Gram-negative bacteria, Wild yeasts, Rapid detection and identification of microbial spoilage, Cleaning and disinfection.

However, it is important to remember that (a) some of the spoilage effects described in beer do not exist in the distilling industry and (b) the problems caused by specific bacteria in beer and distilled spirits may be different.