

Brewers'

Laboratory Handbook:

BREWING WITHOUT THE BLINDFOLD™

Overview of Microbiological Methods Used in the Brewery

- 2 Aseptic Technique
- 2 Quick & Dirty "Film Test" for Bottled Beer
- 2 Microscopic Examination
- 2 Detecting Mixed Strains of Brewing Yeast
- 2 Detecting Bacteria, Wild Yeast & Petite Mutants with Selective Media
- 3 Simple Tests for Identifying Bacteria
- 3 Cell Counts and Determination of Proper Pitching Rate

Protocols

- 3 Where & When to Sample
- 3 Drawing Samples
- 4 Swabbing
- 4 Wort Stability Test
- 4 Pouring Plates
- 5 Plating Samples Directly
- 5 Plating Samples Using Filtration
- 6 Catalase & Oxidase Tests
- 6 Gram Staining
- 6 Streak-Plating for Single Colonies
- 6 Strain Purification
- 7 Yeast Harvesting & Storage
- 7 Cell Counting

Reference

- 8 Table of Brewing Bacteria
 - 9 Quick Reference
 - 9 Glossary
 - 10 Notes
 - 10 Laboratory Supplies
-

“Brewing without the blindfold” means keeping an eye on your microbes, be they brewing yeast or the myriad contaminants that plague brewhouses. This handbook helps you do just that, and respects the fact that you want to spend time in the brewery, not in the lab!

Overview of Microbiological Methods Used in the Brewery

- **Aseptic Technique**

The ability to transfer cultures without contamination is imperative to good microbiology. Keep in mind:

- hands must be washed and work areas must be cleaned with a 5% solution of disinfectant
- sterile vessels and liquids must be used (subject to 15# pressure for 15 minutes in a pressure cooker)
- sterile transfer tools must be used (dip in 70% ethanol or rubbing alcohol and flame)
- aerial fall-out and drafts must be minimized.

See *Protocol: Pouring Plates*, *Protocol: Drawing Samples*, *Protocol: Swabbing*, *Protocol: Plating Samples Directly* and *Protocol: Plating Samples Using Filtration*.

- **Quick & Dirty “Film Test” for Bottled Beer**

Bottled products can be checked for gross contamination without much difficulty. The following are undesirable:

- bottle necks with a rings at the beer/headspace interface
- cloudiness in any part of the body
- residue at the bottom of filtered beer
- excessive foaming of opened, chilled beer.

Odor and taste are indicators as well. What is most useful, however, is catching contamination well before it gets to taste, odor and visibility thresholds.

- **Microscopic Examination**

For yeast, use the 40x lens and make note of:

- percent viability.

For bacteria, use the oil immersion lens and make note of:

- whether cells are rods (bacilli) or rounds (cocci)
- the approximate length or diameter in microns
- the gram reaction.

Microscopic examination of samples has limited use. The microscope is not very helpful for detecting even moderate levels of contamination, whether it be in yeast, wort or beer. If enough bacteria or wild yeast is present to be seen while surveying only a few fields, contamination levels are very high. Also keep in mind that different yeast strains can have very similar appearances under the microscope, so it can be impossible to tell a wild strain from a brewing strain, or to tell one brewing strain from another. Petite mutants are so-named because of their small colony size. They cannot be differentiated from normal cells under the microscope.

- **Detecting Mixed Strains of Brewing Yeast**

If you use more than one strain of yeast in the brewery, you may wish to determine whether or not they are becoming intermingled. By plating the slurry for single colonies on wort agar and incubating for 5-7 days, you will be able to tell one from another after careful examination of colony shapes, sizes and colors. Any differences in these characteristics are solid indications of different strains. See *Protocol: Pouring Plates*, *Protocol: Streaking for Single Colonies* and *Protocol: Strain Purification*.

- **Detecting Bacteria, Wild Yeast & Petite Mutants with Selective Media**

If you know what type of contaminant you're encountering, you are better equipped to determine how serious a problem it presents and how to get rid of it. On a non-selective medium, such as wort agar, yeast colonies are relatively large, opaque, whitish and waxy-looking, while bacteria colonies are generally smaller, more translucent and slick-looking. Knowing this is fine, but a selective medium is needed for more specific identification.

We recommend the following selective media for the basic groups of brewing contaminants:

- brewing bacteria – SDA
- wild yeast – LCSM and LWYM
- petite mutants – wort agar containing or over-laid with TTC.

Each of these media are easy-to-use and allow for enumeration (counting the number of contaminants in the sample tested) and differentiation (knowing one type of organism on the plate from another), which is more than can be said for most testing media. See *Protocol: Pouring Plates*, *Protocol: Plating Samples Directly*, *Protocol: Plating Samples Using Filtration*, *Protocol: Streaking for Single Colonies* and *Protocol: Strain Purification*.

- **Simple Tests for Identifying Bacteria**

Make the following observations of any bacteria colonies before disturbing the colony with stain or other tests:

- odor (vinegary, rotten, sulfury, fruity)
- acid production (halo surrounding colony, with color change and/or clearing of medium)
- colony color, size, texture and shape.

Then determine the

- catalase and oxidase reactions
- gram reaction.

The gram stain takes advantage of certain differences in cell membrane properties of bacteria. All bacteria are divided into two categories: gram-positive (takes on crystal violet stain and turns blue) and gram-negative (takes on safranin stain and turns pink). Yeasts are gram-positive. Armed with all the above information, check *Table of Brewing Bacteria* for bacteria's identity. See [Protocol: Gram Staining](#) and [Protocol: Catalase & Oxidase Tests](#).

- **Cell Counts and Determination of Proper Pitching Rate**

A hemacytometer is essential for accurate cell counts (determination of number of yeast cells/ml in a slurry). The number of cells counted within a chosen grid is converted into cells/ml. This number is compared to the number of cells needed for healthy fermentation. See [Protocol: Cell Counting](#).

Protocols

Protocol: Where & When to Sample

Sample	Frequency	Sample Size	Common Contaminants	Tolerance
water supply	1/week	100ml, filtered	enteric, molds	≤10 cfu
wort	every brew	1.0ml	enteric, acetic & lactic, wild yeast	≤10 cfu; 0 cfu wild yeast
pitching yeast	every crop	1.0ml	enteric, acetic & lactic, wild yeast	≤10 cfu; 0 cfu wild yeast
fermenting beer, days 1-2	every tank	1.0ml	enteric, acetic & lactic, wild yeast	≤10 cfu; 0 cfu wild yeast
fermenting beer, days 3-5	every tank	1.0ml	acetic & lactic, wild yeast	≤10 cfu; 0 cfu wild yeast
storage tank	3/week	1.0ml	acetic & lactic	≤10 cfu
finishing tank	3/week	1.0ml	lactic	≤10 cfu
bottling tank	1/month	100ml, filtered	lactic	≤10 cfu
bottled beer	every batch	100ml, filtered	acetic & lactic	≤10 cfu
CIP'd surfaces	every CIP	swab	-	0 cfu

Notes

cfu = colony-forming units, or the number of colonies growing on the test plate

Protocol: Drawing Samples

Equipment

70% ethanol or rubbing alcohol
sterile sample tube

Procedure

Wash and dry hands thoroughly.
Sterilize intervening "outside" surfaces, such as valves, with 70% ethanol and flame.
Do not uncap tube until immediately before sampling.
Do not touch inside of tube or cap to any surface.
If possible, allow sample to flow or crumble directly into tube without using utensils.
Do not allow sample to overflow onto outside of tube.
Recap tube as soon as possible.

Protocol: Swabbing

Equipment

sterile swab tube

Procedure

Wash and dry hands thoroughly.

Unscrew swab/cap from tube without touching inside of tube or cap to any surface.

Firmly swab area to be tested, twisting swab to expose entire tip to area in question.

Immediately place exposed swab back into tube, tighten cap and label.

Notes

Exposed swabs can be tested by streaking across the surface of plated media (see [Protocol: Plating Samples Directly](#)), or by adding 10ml sterile wort to swab tube and allowing to grow in a warm area (~86°F/30°C) for 3 days (see [Protocol: Wort Stability Test](#)).

Protocol: Wort Stability Test

Equipment

70% ethanol or rubbing alcohol

sterile sample tube

Procedure

Wash and dry hands thoroughly.

Sterilize intervening "outside" surfaces, such as valves, with 70% ethanol and flame.

Do not uncap sample tube until immediately before sampling.

Do not touch inside of tube or cap to any surface.

If possible, allow cooled, aerated wort to flow directly into tube without using utensils; fill tube about halfway.

Do not allow sample to overflow onto outside of tube; recap loosely.

Allow to grow in a warm area (~86°F/30°C) for 3 days.

Notes

This test determines whether or not brewing organisms are present without allowing for their identification. Clear, bubble-free wort after 3 days of incubation at room temperature indicates the sample contains no viable brewing organisms. Cloudiness, bubbles or gas escaping when lid is loosened indicates that live organisms are present, which means either your sanitizing regime is inadequate, or sterile materials and surfaces are being exposed and contaminated.

Protocol: Pouring Plates

Equipment

5% disinfecting solution

sterile petri dishes

sterile prepared media

permanent marker

Procedure

Prepare media according to instructions.

Wash and dry hands thoroughly.

Clean work area with a 5% disinfecting solution such as Lysol or bleach.

Lay out, label and date underside of plates and turn each back over, covers up.

Allow media to cool until it is comfortably-warm to the touch (about 50°C).

Swirl media occasionally to keep any insoluble components of media suspended.

Pour 15ml (7ml for small plates) directly into each plate (about 5mm thick), lifting covers just enough to allow for pouring.

Do not disturb plates until media has solidified.

Invert and store plates refrigerated in a clean plastic bag.

Notes

Pouring media while too hot will result in condensate inside the plate, which increases the chances of contamination.

You can check plates for contamination by examining them after 48 hours of incubation in a warm area (~86°F/30°C).

Wort agar (instead of commercial nutrient agar) = 30ml house wort, 70ml water, 2g agar; sterilize for 15 minutes at 15psi.

Protocol: Plating Samples Directly

Plated media are generally used to test the purity of liquid samples such as wort, yeast slurry and bottled beer. It is important that the plates do not become contaminated with anything other than the sample you want tested, so store your plates refrigerated and upside-down in a clean plastic box until you are ready to use them.

Equipment

Starsan or a 5% Lysol or bleach solution
media plates
sterile pipettes
plastic box

Procedure

Choose an area that is enclosed or away from drafts and messy or dusty operations.
Wipe down countertop or table with Starsan or a 5% Lysol or bleach solution.
Wash and dry hands thoroughly.
Inspect each new plate for contamination; label underside with date, sample name, sample site and size of sample plated.
Liquid sample: place sample on media using a sterile pipette, gently spread over entire surface without gouging media and immediately replace plate lid.
Swab sample: streak swab tip over entire media, twisting swab to expose all of tip to media surface and immediately replace plate lid.
Wipe down a plastic box with Starsan or a 5% Lysol or bleach solution.
Place plates in box upside-down; loosely fit box lid and allow to grow in a warm area (~86°F/30°C) for 3 days.
Do not wrap plates or seal plates in any way.

Protocol: Plating Samples Using Filtration

If the entire sample is too large to be plated directly (>1.0ml), sample filtration is necessary. The purpose of filtration is to trap any organisms present onto a membrane filter by drawing the sample through it. The filter is then laid directly onto plated media.

Equipment

Starsan or a 5% Lysol or bleach solution
70% alcohol
gas burner
vacuum pump
sterile filtration apparatus
sterile 0.45 μ filters
tweezers
media plates
plastic box

Procedure

Choose an area that is enclosed or away from drafts and messy or dusty operations.
Wipe down countertop or table with Starsan or a 5% Lysol or bleach solution.
Wash and dry hands thoroughly.
Inspect each new plate for contamination; label underside with date, sample name and sample site.
Dip tweezers in alcohol and light with flame. Allow flame to extinguish on its own.
Use tweezers to place sterile filter into filtration apparatus; reassemble apparatus.
Flame mouth of sample vessel and add 100ml to the top receptacle.
Cap top receptacle and attach vacuum to bottom receptacle. Draw entire sample through filter.
Sterilize tweezers as above; remove top receptacle and retrieve filter¹.
Place filter face-up on agar, making sure membrane's entire underside is in contact with media.
To filter another sample, liberally spray alcohol into apparatus and draw through with vacuum; load with sterile filter.
Wipe down a plastic box with Starsan or a 5% Lysol or bleach solution.
Place plates in box upside-down; loosely fit box lid and allow to grow in a warm area (~86°F/30°C) for 3 days.
Do not wrap plates or seal plates in any way.

Notes

¹If another sample is to be filtered, assemble apparatus and liberally spray down interior of top receptacle with alcohol; empty using vacuum suction. Sterilize tweezers, put fresh filter in place and repeat filtration procedure.

Protocol: Catalase & Oxidase Tests

Equipment

3% hydrogen peroxide
glass slide
oxidase “dry slides”

Procedure: Catalase Test

Drop a little hydrogen peroxide onto the colony in question, or onto bacteria smeared on a clean glass slide.
A positive result is indicated by the formation of bubbles.

Procedure: Oxidase Test

Smear a little of the colony in question onto the slide.
A positive result is indicated by the bacteria turning a very dark “royal” purple color within 2 minutes.

Protocol: Gram Staining

Equipment

microscope with 100x objective
immersion oil
glass slide
gram stain kit

Procedure

Place a drop of water on a clean glass slide and barely touch a flamed and cooled inoculating loop to the colony¹.
“Wash” loop in drop of water and evenly smear the suspension around in a pea-sized area; allow to air-dry.
Hold glass slide about 3 inches above flame and hold for 4 seconds to kill and fix organisms to slide; allow slide to cool.
Cover smear with crystal violet stain and leave on for 1 minute.
Rinse and flood slide with iodine mordant; leave on for 3 minutes then drain.
Slowly drip decolorizing rinse onto smear while holding slide vertically; stop as soon as color ceasing flowing from smear².
Cover smear with safranin counterstain, leave on for 1 minute, then rinse gently with water and allow to air dry.
Examine under microscope using oil-immersion lens. Gram⁺ is blue or purple; gram⁻ is red or pink.

Notes

¹Adding too much bacteria to the water droplet will lead to too dense a smear, making good staining almost impossible.
The proper amount of bacteria will be a barely visible dot of material on the loop.
²The decolorizing rinse is the most critical step. If you continually get gram⁻ or variable results for known gram⁺ bacteria, you are over-rinsing. Gram⁺ results for gram⁻ bacteria can result from too dense a smear.

Protocol: Streak-Plating for Single Colonies

Equipment

sterile water (contact-lens saline works well)
wort agar¹ plate
yeast culture

Procedure

Put three drops of sterile water next to each other on the periphery of a wort agar plate.
Get a film of sample across an inoculating loop (for yeast slurry) or barely touch loop to a colony (for solid culture).
Dip loop into first water drop and “wash” around until material from loop is suspended in water drop.
Flame and cool inoculating loop.
Get “film” from first drop across loop and transfer to second water drop, “washing” loop as before.
Flame and cool inoculating loop.
Get “film” from second drop across loop and transfer to third water drop, “washing” loop as before.
Flame and cool inoculating loop.
Get “film” from third drop across loop and streak across surface of plate, avoiding the three drops.



Notes

¹Wort agar = 30ml house wort, 70ml water, 2g agar; sterilize for 15 minutes at 15psi.

Protocol: Strain Purification

Procedure

Select three or four average-sized colonies which are representative of most of the rest of the colonies.
Transfer together to wort agar plate and incubate for 2 days in a warm area (~86°F/30°C).
Wrap plate with parafilm and store upside-down in refrigerator; shelf life is 6 months.

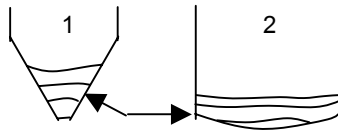
Notes

¹Wort agar = 30ml house wort, 70ml water, 2g agar; boil to dissolve, sterilize as usual and pour while still warm to touch.

Protocol: Yeast Harvesting & Storage

Equipment

70% ethanol or rubbing alcohol
sanitizer
storage container
cold water
sterile wort



Procedure:

Sterilize valves with 70% ethanol and flame, and sanitize any hoses, utensils and storage containers to be used. Catch the middle layer of yeast in a storage container as follows, discarding top and bottom layers:

¹CONICAL vessels require draining the bottom trub-filled layer until good color and consistency is seen.

²ROUND vessels require manual harvesting with a paddle (layers mix if allowed to drain through the valve).

Acidify about 100ml of water to ~pH 3 using a food-grade acid.

For every gallon of yeast to be treated, add 2ml sodium chlorite* to the acidified water.

When concoction turns a very pale yellow (at about 15 minutes) add to the yeast slurry, mixing well.

Allow to sit for a minimum of 30 minutes (longer reaction time ensures effectiveness).

Use yeast as needed or add an equal volume of sterile, aerated wort and store at 34°F/1°C for up to 2 weeks.

If stored, test for viability, cell count and contamination before pitching.

Notes

It is best to harvest yeast immediately after final gravity has been reached, before diacetyl rest. Even if it is kept very cold, yeast should not be stored in the cone or under beer. Finished beer is a nutrient-poor waste product to yeast, and forcing it to reside there will cause the viability to drop precipitously (over 50% within 48 hours), no matter how cold it is kept!

**DioxyChlor*TM from BIRKO at (800) 525-0476, or **Star-Xene*TM from Five Star at (800) 782-7019

Protocol: Cell Counting

Equipment

microscope with 40x objective
methylene blue, 1% aqueous
hemacytometer and cover slip, clean and dry

Staining

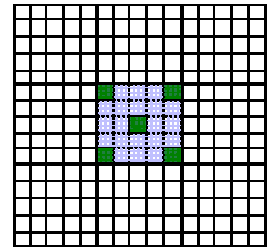
Obtain a few ml of a slightly-cloudy suspension of yeast, keeping track of any dilutions¹ made.

Dye with methylene blue dye, one drop at a time, until slurry is a cobalt blue.

Shake sample until color is uniform; allow to sit for 1 minute.

Place sample in one of the chambers using instructions that came with your hemacytometer.

View under microscope using 40x objective; blue cells are non-viable, clear are viable.



Counting

Locate the central square with 25 smaller squares which are gridded in a 4x4 pattern.

Count the total number of viable yeast in 5 of the squares², using pattern shown at right.

Calculating Pitch Rate

HOW MUCH YEAST DO YOU HAVE?

Slurry Density: _____ cells counted in 5 squares x 50,000 x _____ dilution factor¹ = _____ million cells/ml

Slurry Volume: _____ gal x 3800ml/gal = _____ ml

Total Cells Available: _____ million cells/ml x _____ ml = _____ Cells Available

HOW MUCH WORT DO YOU HAVE?

Pitch Rate: _____ °P x 1 million cells/ml = _____ million cells/ml

Wort Volume: _____ bbl x 31gal/bbl x 3800ml/gal = _____ ml

Total Cells Required: _____ million cells/ml x _____ ml = _____ Cells Required








DO YOU HAVE ENOUGH YEAST TO PITCH AT THE PROPER RATE?

Notes

¹Confused about making dilutions and calculating dilution factors? Consider a 1:10 dilution. The number on the left side of the colon is the volume (in ml, let's say) you begin with; the number on the right is the volume you end up with. A dense slurry may require a 1:100 dilution, namely, the addition of 1ml yeast to 99ml water, in order to render it countable. There is another way to get a 1:100 dilution. You may add 1ml yeast to 9ml water, mix, then remove 1ml of that suspension and add it to 9ml of fresh water. In other words, two 1:10 dilutions made in series are equivalent to a single 1:100 dilution. Similarly, three 1:10 dilutions made in series are equivalent to a single 1:1000 dilution. The dilution factor is the product of the numbers on right side of the colon. For example, a 1:10 dilution followed by a 1:4 dilution is equal to a 1:40 dilution and has a dilution factor of 40.

²Exclude buds and yeast lying on any two of the four borders of each of the 5 squares.

Table of Brewing Bacteria

GENUS	GRAM	ACID	CATALASE	OXIDASE
<i>Acetic Acid Bacteria</i> (smell sharp & vinegary) 				
Acetobacter	-	+	+	-
Acidomonas	-	+	+	+
<i>Enteric Bacteria</i> (smell rotten, sulfury or like a bad cold) 				
Citrobacter	-	-	+	-
Enterobacter	-	-	+	-
Hafnia	-	-	+	-
Klebsiella	-	-	+	-
Obesumbacterium	-	-	+	+
<i>Lactic Acid Bacteria</i> (smell sharp & fruity)				
Lactobacillus 	+	+	-	-
Pediococcus 	+	+	-	-
<i>Other Bacteria</i>				
Megasphaera 	-	+	+	-
Pectinatus 	-	-	-	-
Zymomonas 	-	-	+	-

Acetobacter, Acidomonas
from plant material
found in wort and sometimes bottled beer
contribute vinegary or cidery flavor

Acetic Acid Bacteria

Citrobacter, Enterobacter, Hafnia, Klebsiella, Obesumbacterium
from water, soil, plant material
found in wort, early stages of fermentation
Enterobacter and *Citrobacter* killed at pH 4.4 and 2% alcohol, others are not
contribute fusel alcohols, sulfur compounds, phenolics, diacetyl

Enteric Bacteria

Lactobacillus, Pediococcus
from grain dust
found throughout brewing process
contribute tart or sour flavor, diacetyl

Lactic Acid Bacteria

Megasphaera, Pectinatus, Zymomonas
occur rarely
sources vary
found in areas with little oxygen, such as CO2 recovery systems
Zymomonas tolerates up to 10% alcohol
contribute sulfur compounds, acetaldehyde

Other Bacteria

Quick Reference

- **Wort Aeration**
Aeration rates vary with wort volume, wort gravity and knockout time. In general, 8-10ppm of dissolved oxygen is desired for normal gravity worts. If aerating in-line with pure oxygen for the duration of a 1-hour knockout, this translates into 1.4LPM for every 10bbl of wort:
10ppm = 10mg/L = 117,800mg/10bbl
1.4mg O₂ = 1ml O₂
117,800mg O₂ = 84,142ml O₂ = 84.1L O₂
84.1L of O₂ delivered over 1 hour = 1.4L delivered over 1 minute
- **Sterilizing Time vs. Temperature**
The system (not the liquid running through it!) must be kept at: 160°F (71°C) for 45 minutes
170°F (77°C) for 30 minutes
180°F (82°C) for 25 minutes
- **Conversions**
l = liter
hl = hectoliter
bbl = beer barrel

3.8 l / gal
100 l / hl
118 l / bbl
31 gal / bbl

°C = 5/9 (°F - 32)
°F = (9/5 °C) + 32

° Plato = % sugar = 1/4 (last two digits of specific gravity reading)
5° Plato = 5% sugar = 1.020 sg

OE = original extract, ° Plato AE = apparent extract, ° Plato RE = real extract, ° Plato
% alcohol by weight = 0.42 (OE - AE)
% alcohol by weight = 0.52 (OE - RE)
% alcohol by weight = 2.22 (RE - AE)
% alcohol by volume = (% alcohol by weight x specific gravity of beer) / 0.791

Glossary

- aerobe:** an organism which needs oxygen to survive (obligate aerobes will die without air)
- anaerobe:** an organism which does not need air to survive (obligate anaerobes will die if exposed to air, while facultative anaerobes can grow with or without air)
- aseptic:** against or without infection
- contaminant:** a relative term referring to any organism which lends undesirable characteristics; what is “bad” in one kind of brew may be intentionally cultured in another
- inoculate:** to introduce an organism to a sterile environment, intentionally or not
- morphology:** shape or appearance
- petite mutant:** a yeast with deficient respiratory abilities, resulting in slow growth and small (petite) colony size
- strains:** subspecies or “races” of *Saccharomyces cerevisiae*; all brewing yeasts are strains of this species
- viability:** percent of live cells in a population; not to be confused with vitality, which refers to metabolic activity or vigor of live cells
- wild yeast:** undesirable, undomesticated, or non-brewing yeast

About The Brewing-Science Institute

BSI knows that the success of the brewing process depends upon the health and abundance of certain microorganisms and the rigorous limitation of others. Forgetting this is the most common cause of brewhouse failures.

We strive to help microbrewers understand and gain control over the biological processes so essential to their craft.

BSI is structured to serve microbrewers who do not have complete in-house laboratory facilities and staff. Among its services are fresh yeast supply, contamination testing and laboratory training.

www.brewingscience.com



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