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1. Assignment Topic

"Before taking up your duties in a new microbrewery capable of producing up to 50 hl of cask-conditioned beer per week, you have been asked to organize a small laboratory. Bearing in mind the limited funds available and the simple analyses required of the new laboratory, what equipment would you order and what analyses would you suggest? Explain your decisions."

2. Introduction to Cask-Conditioned Beer

Cask-Conditioned beer is beer that remains in contact with viable yeast throughout the fermentation, stillage, and serving cycles. $d0_2$ respiration, flavor maturation, and natural carbonation ("condition") are all achieved via secondary fermentation in a serving vessel, most commonly a cask. Clarification is usually achieved through the use of finings, added either at the brewery or pub. When the beer is judged to be "ready,"[1] the cask is vented of excess CO_2 and the beer is served – via gravity, or through a beer "engine" or pump.

(a) Methods of Secondary Fermentation

There are three methods of secondary fermentation:

Spunding. When the fermenting beer has reached a pre-determined level of attenuation, and a given amount of residual sugar and viable, suspended yeast is determined to remain, the fermentation vessel (FV) is "capped." As the fermentation progresses to its final stages, the CO_2 which evolves is unable to escape the FV, and condition is thus achieved. Typically, the FV is capped with 1 – 1.5% residual extract, and 1-4 million viable yeast cells/ml in suspension.

Krausening. A pre-determined volume of high-krausen, vigorously fermenting beer is introduced into fully attenuated beer. The FV is capped and secondary fermentation proceeds. Krausen volumes range from 5-10% of total beer volume.

Priming. The above two methods are more common among lager breweries. Priming is more common among ale breweries. It involves the introduction of a pre-determined amount of fermentable extract (typically dextrose or glucose, less commonly maltose or wort) to fully fermented beer, with residual or added suspended yeast populations in the range of 2.5×10^5 to 3.0×10^6 cells/ml.[2]

For the purposes of this assignment, it will be presumed that primary fermentation is carried out in a cylindrical-conical fermentor (CCV), despite its arguable drawback in terms of possible over-carbonation and flavor effects.[3] For each brand, it will also be presumed that

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[1] "Ready" varies with the product and brewery. *CAMRA Guide to Cellarmanship,* pp. 109-133.
[2] Ian S. Hornsey, *Brewing*, pg. 142. RSC Paperbacks, 1999.
[3] [?] F.G. Priest and I. Campbell, Eds., *Brewing Microbiology*, pg. 36. Chapman & Hall, London, 1996

key primary fermentation benchmarks (such as brand final gravity) are well known by empirical observation and history. Secondary fermentation is achieved by priming with glucose.

3. Concerns of the Laboratory

Given the economic constraint imposed by 50 hl/week of beer production, the range of equipment and analyses to be employed is fairly limited. Broadly, there exist six areas of concern for the laboratory of this cask-condition, small brewery:

(a) Microbiological Control;
(b) Brewhouse Analyses;
(c) Brewery Fermentations;
(d) Clarification.
(e) Sensory Evaluation Panel: Structure and Operation
(f) Equipment and Material List

(a) Microbiological Control

Introduction

Due to the relatively inhospitable environment which exists in finished beer for most micro-organisms, the range of possible brewery contaminants is fairly limited.[4] Nevertheless, at numerous key points throughout the production process, various classes of contaminating micro-organisms may flourish or, if they eventually die off, they may contribute substantial off-characteristics to the beer prior to their death.

The heart of the laboratory microbiological control regimen is the routine sampling program. Under "normal" conditions, when no product faults are detected, sterile samples would be routinely taken at key points throughout the production process. At this level, the goal is merely to detect early changes within the brewery yeast culture, and to rule out the growth of contaminant, spoilage species. Here, the widest possible "net" is cast to recover the broadest possible spectra of potential spoilers.

Classes of Brewery Contaminants and their Effects

Refer to Table 1 for a description of the common spoilage organisms and their characteristics.

Sampling And Laboratory Procedures

3.a.1. Routine Brewery sampling: "normal," when no faults are detected

Culture Media

The following is a breakdown of the media and culture methods used in the routine sampling program. For all media, culture samples undergo a serial dilution as described below, in order to facilitate colony population counts.

[4] W.J. Simpson, "Rapid Microbiological Methods in the Brewery," *Brewer's Guardian,* June, 1991, pg. 30.

Non-selective media on all sterile surfaces: On all surfaces that should be sterile, a non-selective media with the widest-possible recovery spectrum should be employed. With a very low microbial load, it may be necessary to utilize membrane filtration technique.[5] There is a host of non-specific media available, including malt-extract agar, Universal-Beer Agar (UBA), or WL Nutrient agar (WLN).

WLN contains brom-cresol green, a dye indicator, which gives useful additional information about the nature of any microorganisms that are recovered from the ostensibly sterile surface:

- **culture** yeast colonies tend to exhibit a light-green color, and large, dome-like morphologies;
- **bacteria** exhibit in small, dark-green, and often apiculate ("pointed") colonies;
- **Wild yeast** exhibit in white colonies.

Furthermore, acid production is marked by distinctive, yellow halos surrounding the colony. For the anaerobic recovery of lactics, especially Pediococcus, up to five days' incubation is required. It is slightly more expensive than UBA, but not prohibitively so.[6].

Any recovery of microorganisms from "sterile" surfaces warrants further tests, differential analysis and media. (See Figure 1), as well as an audit of the house cleaning/sanitation procedures. For all sterile surfaces (and bitter wort samples), WLN, cultured aerobically and anaerobically, will be used.

Inhibitory media for all other sampling: In cask ale production, once culture yeast has been pitched into the fermentor, it remains in contact with beer throughout the production process. Therefore, to detect contaminant bacteria and wild yeasts, it is necessary to use a culture-yeast inhibitory medium on all culture yeast and beer samples. Since the various types of bacterial contaminants and wild yeasts each have a particular set of growth conditions, there is no one medium which suppresses culture yeast while encouraging all possible types of contaminant growth.[7] Common inhibitory media include UBA with actidione (UBAA) or Schwartz's Differential Agar with Actidione (SDAA) for bacterial recovery.

Because UBA contains an aliquot of house beer, it recovers organisms that may thrive under actual brewery conditions. It is also vastly less expensive than other media such as SDA.[8] UBA with actidione (UBAA) will be used as a "workhouse" medium for recovery of bacteria on all beer surfaces and for all beer samples taken as part of the routine sampling program. Because some wild yeasts are not recovered on UBAA, Lin's Wild Yeast Medium and Lin's Cupric Sulfate Medium, in tandem, will be used at key points as described below for wild yeast recovery.

Media Preparation and Use: The various media to be used in routine sample culturing are given in Table 2 and Figure 1. The procedures for their preparation and use are as follows:

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[5] *ASBC Methods of Analysis*, *Microbiological Control*, 2C.
[6] $84.00/500g v. $67.50/500g for UBA. *Source:* Beckton-Dickinson (Fisher).
[7] F.G. Priest and I. Campbell, *op cit.*, pg. 194.
[8] SDA costs $84.00/250g (Crosby-Baker); UBA costs $67.50/500g (Beckton-Dickinson).

WL Nutrient Agar (WLN): 80g of WLN is suspended in 1 L of distilled water, and mixed with a magnetic stirrer on a stirrer-hotplate until boiling dissolves the media completely. The media is autoclaved in flasks for 15 minutes at 15 psi (121C). All sterile surfaces are swabbed and membrane filtered, then cultured on WLN anaerobically (via anaerobic, "Gas-Pak") pouches) and aerobically for up to 5 days at 28C. Any microbial growth should be noted for morphology and color, and microscopically evaluated for morphology and, if possible, motility. Recovered bacterias and yeasts should be further evaluated by reference to media and methods described below.

Universal Beer Agar with Actidione (UBAA). 62 grams of UBA is suspended in 750 ml of distilled water, and mixed with a magnetic stirrer on a stirrer-hotplate until boiling dissolves the media completely. 250 ml of beer is added, and the media is autoclaved in flasks for 15 minutes at 15 psi (121C). The media is cooled. To suppress most culture yeasts, cyclohexamide (actidione) is added to UBA and other media at a rate of 5-20 ppm.[9] Additionally, because actidione is extremely heat-labile, it must be added to a previously autoclaved media that has been cooled. The procedure for actidione dilution is as follows:

***Actidione Preparation*:** Dissolve 50 mg. cyclohexamide in 100 ml EtoH (95%). Add this solution at a rate of 1ml per 100 ml media, yielding 5 ppm actidione agar. Because cyclohexamide is extremely toxic, a mask and gloves should be worn at all times during its preparation and use. Because even culture yeasts may overcome actidione-inhibition if improperly diluted, all yeast culture samples should be diluted to at least 100X before plating.

Incubation is carried out anaerobically and aerobically at 28 C for up to five days (the longer period of anaerobic growth may be necessary for *Pediococcus* recovery).

Any recovery of microorganisms indicates non-culture growth, and further testing as diagrammed in Figure 1 is warranted.

Lin's Cupric Sulfate Medium (LCSM): Copper is toxic to culture S*accharomyces,* whereas many wild *Saccharomyces* spp. and non-*Saccharomyces* spp. are able to grow in the presence of copper. Therefore, LCSM is useful for the detection of many wild yeasts. **Lin's Wild Yeast Medium (LWYM)** inhibits culture yeasts by known effects of fuchsin-sulfite and crystal violet. Together, they provide for a wide range of wild yeast recoveries, although there are limitations to their inhibitory and differential effectiveness.[10] LCSM and LWYM are used as part of the routine sampling program in three key areas: at yeast propagations, weekly on the racking machine and its environs, and the cask itself.

Sampling Points And Frequencies

Table 2 includes key sampling points, the sample frequency, the media to be used, and other analyses to be performed as part of the routine sampling program. Figure 1 summarizes the decisions to be made based on the results obtained during routine sampling.

[9] Priest and Campbell, *op cit.*, pp. 185, 196-197.
[10] *Ibid.*, 5.E.

The sample points can be divided into six principal brewery areas. Sample frequencies are timed to coincide with key points in the brewery's production regimen. It is vital to obtain samples at readily distinguishable stages, in order to isolate where production faults may lie. For example, a UBAA fermentor culture testing positive for bacterial growth at 24 hours, where none existed in the pitching yeast or sterile wort, probably indicates that an infection was introduced into the fermentor midweek (after the last CIP fermentor sample was taken).

The sample areas and frequencies are:

Raw materials: Malt and hop storage areas are major depositories of various moulds, yeasts and bacterias. However, with proper storage (e.g., the avoidance of pooled water, separate storage facility), potential spoilage problems from malt or hop contamination is largely a thing of the past.[11] Only on gushing, a classic sign of *Fusarium* or *Aspergillus* infestation, would an audit of the raw material storage be instituted.

Yeast propagator vessels or yeast storage vessels (YPV's): Cleaned & sanitized YPV's are sampled once weekly with WLN to ensure the CIP regimen is doing its job. At each stage of propagation, and at each pitch or transfer, the culture yeast is cultured on UBAA to rule out likely bacterial contaminants, and at each propagation stage a culture is plated on LCSM and LWYM to rule out wild yeast contamination.

Post-brewhouse and wort transfer line(s): This includes the whirlpool, the whirlpool line, the heat exchanger, and wort transfer lines to fermentors. Cleaned, sanitized areas (CIP areas) are sampled once weekly (on rotating days) and cultured with WLN. Sterile wort from the brewhouse transfer line is sampled at each brewhouse transfer and cultured on WLN, prior to yeast pitching. The range of likely bitter wort spoilers is limited: although acetic acid and lactic bacterias are capable of growing in bitter wort, they are rare here and the only likely candidate is *E. coli* . especially if untreated rinse water is used, or manway shadows and "dead ends" in transfer piping fail to receive proper cleaning and sanitizing. [12]

Fermentors: CIP'd fermentors are sampled once weekly and cultured on WLN. Of the early fermentation spoilers, *O. Proteus* is the most likely, although other enterobacteriaceae may also flourish. Because the effect of early fermentation spoilers are very damaging (see Table 1), beer from every FV is sampled and cultured on UBAA at 24 hours, and every other day thereafter to rule out the lactic acid bacterias. Once weekly, a beer sample is taken and cultured on LCSM and LWYM to ensure no wild yeast has taken hold.

Priming Solution: *Zymomonas,* in particular, thrives in priming sugar solutions and primed beer. Each preparation of sterile priming solution is cultured on WLN to rule out contamination by this or other organisms.

Racking/cask washing/cask filling machines and casks: Acetic acid bacterias, *Zymomonas mobilis,* and wild yeasts tend to accumulate on racking equipment, casks, and immediate environs. Once weekly, sterile surfaces are tested with WLN, as well as LCSM and LWYM. Daily, with each filling run, cask washers and fillers (particularly brushes and other "retentive" areas), as well as a sample cask, are sampled and cultured on UBAA to rule out bacterial growth.

Procedures

[11] B. Hortshoj , "Quality Assurance: Microbiological Approach," pg. 67. United Breweries Ltd., Quality Services, Copenhagen.
[12] J.S. Hough, *et al*, *Malting and Brewing Science*, V. II: Hopped Wort and Beer, pp. 756-759. Chapman & Hall, 1999.

Sterile Sampling: Sterile sampling involves the use of two items: (1) swabbing tubes ("swubes") consisting of 3 ml sterile saline[13] and 15 cm swabbing sticks which have been autoclaved at 15 psi (121C) for 15 minutes; and twist-cap culture test tubes which have been autoclaved similarly.

The *swubes* are used to draw culture samples from all hard surfaces, e.g., the inside surface of a cleaned and sanitized vessel, butterfly valves, racking tubes, etc, by the following procedure:

Latex gloves are worn. 70% EtoH is sprayed liberally on gloved hands. Propane flame may be used to protect the sample environment by bathing the area in radiant heat, although care must be taken to avoid killing any sampled microorganisms by always keeping the swab further than 5 cm from the flame. Aseptically and quickly withdraw the swab from the tube, flame the tube mouth and rub swab over sample area, covering approximately 2 sq. inches. Again flame the tube mouth, quickly replace the swab in the culture tube (making sure it is immersed in the sterile saline), and label date and point of sterile sample. The sample is brought to the lab.

Sterile culture tubes are used to obtain samples of yeast, wort, or beer, by the following procedure:

A sampling rig, typically a sampling spigot and tri-clover assembly, is soaked in iodophor (concentration:12.5 ppm titratable iodine), for a period of at least one minute. Using latex gloves, the rig is assembled to the sampling port on the vessel in question. 95% EtoH is sprayed liberally on the spigot, ensuring that all interior surfaces are reached. The rig is then flamed, and while flaming the rig is opened, allowing sample to flow for approximately 15 sec. The culture tube is aseptically opened, the culture mouth is flamed, and sample is quickly taken. The mouth of the tube is again flamed and the tube cap is replaced. The tube is labeled with date and point of sterile sample. The sample is then brought to the lab.

Microscopic Analysis: As given in Table 1 and Figure 1, many morphological characteristics, reactions to differential stains, grouping and motility may be observed under the microscope, and this may give valuable clues as to the microorganism's identification.

- ***Yeast Culture Observation*:** As part of the routine sampling program, culture yeasts should be microscopically evaluated at each pitching (or shipment, when "remastering" a culture, and any changes (e.g., from round to elongate or pointed, or a markedly different budding pattern) should be further evaluated to eliminate potential contaminants.
- ***Isolation of Potential Contaminants; Limitations:*** On positive culture recovery on UBA or UBAA, microscopic observation may generally divide yeasts from bacterias by size, morphology, and motility, and further evaluation may proceed as indicated in Figure 1. There are limitations, however; motility is often confused with Brownian movement, and detritus is often confused for bacteria (dilute NaOH will help to cure this). Furthermore, staining and microscopic analysis to isolate bacterial contaminants are only effective if bacterial populations exceed 30,000/ml, and a distinction between viable and non-viable

[13] Saline is preferred as a buffer against potential osmotic shock.

bacterial populations cannot be made.[14] Nevertheless, in conjunction with the listed media and methods, microscopic analysis is an effective component of the total quality control picture.

Serial dilutions and microbial population counts: Although it is theoretically possible to obtain *total* bacterial colony counts by serial dilution and microscopic haemocytometry, it is extraordinarily difficult to distinguish cells from detritus, and this method will not distinguish viable from non-viable cells.

To obtain viable population counts, the following method is employed. Five tubes containing 9.0 ml sterile water are set up serially. Using a sterile pipette, 1.0 ml of culture is aseptically transferred into the first tube (10^{-1} dilution). This tube is mixed well, the pipette is discarded, and a new pipette draws 1.0 ml of this solution and places it into the next tube (10^{-2} dilution). The procedure is repeated until dilution down to 10^{-5} is achieved.

The ideal number of countable colonies on a spread plate are between 30 and 200.[15] To obtain this number, 0.1 ml of each dilution is uniformly spread on the desired media with a flamed, sterile glass spreader. The number of colonies formed on each plate is noted, and estimations of the original cell population density (expressed as "colony forming units," of CFU's, per ml) are noted.

EXAMPLE: 0.1 ml of culture is plated, beginning with 1/10 (10^{-1}) dilution and ending with 1/100,000 (10^{-5}) dilution. The plate with 1/10 dilution obtained too many colonies to count accurately. The 1/100 dilution plate obtained 45 colonies, the 1/1000 plate obtained 4 colonies, and the last two dilutions (1/10,000, and 1/100,000) obtained no colonies. The proper plate to use is the 1/100 dilution plate, and the cell density is 45,000/ml:

0.1 ml of 45 colonies = 450 colonies x 10^2 = 45,000 CFU's/ml.

Note: If the sample was originally drawn via membrane filtration, then the colonies reported must be in terms of the sample volume (e.g., if from 100 ml of sample beer, 45,000 CFU's were obtained via spread plate count, then the numbers reported must be reduced by 1/100, or 450 CFUs/ml).

Other Tests:

At this level, two additional tests provide an inexpensive and rapid means of detecting potential spoilage (and fermentation problems): The Sterile Wort Test and Rapid Fermentation Test (RF).

Sterile Wort Test: In addition to the sample drawn from the cooled, bitter wort for culturing, an additional sample is aseptically drawn into a culture tube. The cap is sealed and the tube is incubated for 2-3 days at 28C. Any visible signs of microbial growth (e.g., turbidity or surface slime/pellicles), bubble formation, or an audible "hiss" on opening the cap, is cause for concern. Any odors should be noted, and further evaluation is warranted.

Rapid Fermentation Test: The final gravity (apparent attenuation) that should be obtained from each brand is well known. The rapid fermentation test (RFT) acts as a

[14] Priest and Campbell, *op cit.,* pg. 184.
[15] J. Nicklin, K. Graeme-Cook, T. Paget, and R. Killington, *Instant Notes in Microbiology*, pg. 104. BIOS Scientific Publishers, Oxford, UK, 1999.

"check" against potential problems by comparing the final gravity of a laboratory sample with brewery fermentations. To perform the test, a supply of vacuum filtered, dry house culture yeast is needed. 200 ml of wort is aseptically transferred to a 500 ml Erlenmeyer flask, and the flask is incubated with 32 grams of the yeast on a stirrer/hotplate at elevated temperatures (up to 38C). After 5 hours, the sample is checked for final gravity, as it should be completely fermented. The RF sample may be a bit lower than normal brewery fermentations – the comparably higher pitching rate causes a proportionally higher respiration of fermentable extracts – but a variance from brewery fermentation final gravity of more than 2-3 tenths Plato is cause for concern. Many wild yeasts and bacterias are "superattenuators" (e.g., amylolytic *Saccharomyces*, formerly *S. diastiticus*; heterofermentative *Lactobacillus*; *R. aquatilis*), and many retard fermentation (*O. Proteus),* resulting in higher final gravities and therefore susceptibility to further contamination.

3.a.2. Abnormal Brewery Condition: Positive Recovery of Contaminants or Organoleptic Detection of Faults

It should be stated here that with an effective CIP regimen, tightly-monitored yeast management, and religious attention to the routine sampling program outlined above, most of the following media and methods should be unnecessary for the small brewery laboratory. Nevertheless, If bacterial or wild yeast contaminants are isolated by UBA, UBAA, LCSM, or LWYM plate cultures, or faults are organoleptically or analytically detected, the following materials and methods may be employed on an "as-needed" basis to differentially select the genera or species responsible.

Procedure

Gram Stain: On detection of bacteria, contaminants may be divided into "two houses" by the Gram stain. The Gram stain procedure is well known.[16] Gram positive cells posses a single, thick, net-like layer of peptidoglycan, which retains the Gram crystal violet/iodine stain and show as purple microscopically. Gram negative cells possess instead a multilayered cell membrane, and because the outer layer is hydrophobic, they do not retain the stain and appear pink or colorless microscopically.[17]

Catalase Test: The catalase test is an inexpensive and rapid detection test. Like the Gram stain, the catalase test divides classes of bacteria into "two houses." Those bacteria that are able to catalyze oxygen radicals to oxygen will react with hydrogen peroxide to immediately produce bubbles on a slide smear. One common scenario is to distinguish *Pediococcus*, a very harmful contaminant, from *Micrococcus*. Both appear similar under the microscope. Usually, a catalase positive result indicates *Micrococcus* and not *Pediococcus.*[18]

Gas Production from Glucose: A semi-solid medium composed of meat extract, tryptone, yeast extract, glucose and agar is stab inoculated with culture, and the tube is overlaid with sterile molten agar. Gas bubbles collecting beneath the surface indicate heterofermentative growth.[19] Homofermentation (producing one end product) is common among most lactic acid bacteria; heterofermentation is common among *Leuconostoc,* and some *Lactobacilli.* Heterofermentative, catalase negative bacteria are most likely *Leuconostoc* spp. Because the incidence of *Leuconostocs* is extremely rare in breweries, this test is included for identification purposes only and would not likely ever be utilized.

Acetic acid from EtoH: The acetic acid bacterias are strict aerobes, and are particularly found in faulty draft systems, and vented/tapped casks. Gram negative, catalase positive aerobic bacteria which produce a strong "vinegar" smell and sour bite are most likely *Acetobacter* or *Gluconobacter,* particularly if accompanied by pellicles/ropiness and turbidity.

Preparation and Use of Selective Media:

A. Hsu's Lactic Acid Pediococcus Medium (HLP): HLP is used to recover and differentially indicate for *Lactobacillus* and *Pediococcus* bacteria. Because of the oxygen-scavenging ability of sodium thioglycollate, the manufacturer indicates that there is no need for anaerobic incubation, nor autoclaving. 7.0 g HLP/100 ml H_2O is boiled for 2-3 minutes, then aseptically transferred to sterile culture tubes to 11 cm depth; the tubes are cooled to 40 C in water bath and 1 ml of diluted (100X) sample is injected into each tube. Invert to mix thoroughly (gently, to reduce oxygen uptake) and incubate at 28C for five days to ensure adequate time for *Pediococcus* differentiation.[20] *Pediococcus* tends to appear in a "snowball" pattern, while *lactobacillus* appears as a series of streaks or streaky teardrops. Although HLP contains actidione, a culture yeast inhibitor, as with all

[16] ASBC Methods of Analysis, Microbiological Control, 3.
[17] Priest and Campbell, *op cit*, pg. 128.
[18] Although not always. See note, Table 1.
[19] Priest and Campbell, *op cit*, pg. 156.
[20] *Ibid.*, pg. 150.

samples if the sample is not properly diluted the overabundance of culture yeast may cloud the medium and give a false positive result. On positive HLP recovery, further tests, such as Gram staining, microscopic evaluation, catalase reaction, and glucose respiration, are indicated.

B. Dadd's Medium[21]/Bacto-Sulfite Agar[22]: *Zymomonas* is a particularly pernicious bacteria for cask conditioned ale breweries. It has a distinct preference for high temperatures and pH, a high ethanol tolerance, an ability to readily ferment glucose or fructose to EtoH and CO_2 on an equimolar basis. It is commonly found in priming sugars and cask-washing apparatus.[23] Selection on glucose-containing Bacto-Sulfite agar is obtained by anaerobic growth at 30C for 2-3 days. H_2S production is indicated by reduction of sulfite, and displays as blackened areas in the medium. This method may be preferable to anaerobic growth in sugar-primed beer, which may give false positives for H_2S production due to Lactobacilli or wild yeasts.[24] Strong, "rotten apples" or "rotten egg" odor in priming solution or primed beer is a strong indicator for further testing to rule out this microbe.

C. MacConkey's Agar (Mac): This medium contains bile salts and crystal violet to differentiate for the enterobacteriaceae. *Citrobacter, Enterobacter,* and *Klebsiella* form red colonies, whereas *O. Proteus* and *H. Alvei* are pink to colorless. *O. Proteus* requires 36-48 hours incubation.[25]

D. Glucose/Peptone Broth: A 1% glucose/peptone broth, or 1%glucose/peptone nutrient agar, can select for *Megasphaera* if cultured in an anaerobic jar with an oxygen-scavenging "Gas-Pak" system.

E. LL: Tubes containing lactate-lead-lead acetate agar can select for *Pectinatus* if cultured in an anaerobic jar with an oxygen-scavenging "Gas-Pak" system.

F. Dextrin Agar: Many amylolytic wild yeasts of genera *Saccharomyces* (formerly, *S. diastiticus)* may be differentiated from culture *Saccharomyces* yeasts by using dextrin as the sole source of carbon.

Table 1 and Figure 1, pp. 2-4 summarizes the decision key(s) involved in isolating the most common bacterial and wild yeast spoilers. In this section, I have included many media and methods that will probably never be called upon, and there are many more tests, particularly many rapid detection methods (e.g., PCR), which are beyond the scope of the small brewery laboratory.

[21] Dadds, M.J.S., "The detection of Zymomonas anaerobia" in the Society of Applied Bacteriology: Technical Series No5, New York; Academic Press, 1971, p219.
[22] Becto-Dickinson Product No. 287210 (0972-17 Difco).
[23] Priest and Campbell, *op cit,* pg. 180.
[24] *Ibid.,* pg. 183.
[25] *Ibid.,* pg. 184.

(b) Brewhouse Analyses

Brewhouse analyses are more often carried out by staff brewers. Nevertheless, they are included here because in a small brewery, lab and brewhouse personnel often carry out overlapping missions.

Raw Materials: Grist analysis. A well-milled grist is critical to manageable lauter-tun runoffs and adequate brewhouse efficiencies. To much fine grist or flour in the mill, and the brewhouse extract efficiency is increased at the risk of lauter-tun blinding and turbid worts; too coarse a grind and although the lauter-tun runoff proceeds well, extract yields are compromised. The pan-sifter screens used for grist analysis are given in ASBC units: #'s 14, 30, 100, and pan-bottom. A 50 to 100 g of representative milled malt is drawn using the grist-sample device on the malt mill (the "trier"). The sample is weighed and transferred to the #14 screen and agitated in a smooth back-forth motion for a defined period. The grist retained on the #14 screen is weighed, divided by the total grist sample weight, and multiplied by 100 to give the % retained on the #14 screen. The process is repeated for the #30 and #100 screens, and the pan bottom. Ideally, for grists using well-modified, two-row pale malts as the mash basis, the following grist assortments should be obtained and noted:

#14 (%):	40
#30(%):	50
#100(%):	8
Pan (%):	2-3%.

Any radical deviation from this assortment warrants mill-gap adjustment.

Mash Starch Conversion. A useful test to ensure the completion of amlyosis in the mash tun is the iodine conversion test. A small sample of wort is drawn from the tun and placed on a clean, white ceramic plate. A small measure of prepared iodine media (.02 N iodine solution) is dropped onto the wort. A blue or purple color reaction indicates incomplete conversion (starch present). A very slight red color indicates that dextrins may be present. A true red color indicates that amylodextrins may be present, which may later be consumed by Pediococcus contamination. If a true red or purple color is obtained, a longer mash (or a higher rest, such as an α-amylase rest) is indicated. For very dark beers, the sample may be difficult to read and the beer should be mixed with a EtoH. After the precipitate has settled, the supernatant is discarded, the precipitate is drawn and blended with water, and the iodine proceeds as per normal.

Mash, last runnings, and sparge pH: Mash pH is important to obtain, as optimum starch conversion is obtained at a pH of 5.2-5.4. Last runnings should be monitored, as a high pH (above 6.0) may likely mean a great deal of husk tannins will have been extracted, and the brew is in danger of polyphenol-based haze. Similarly, to maintain a runoff pH within the proper range, the sparge water should be maintained at a pH of 5.7, whether via batch additions or dosing systems (if sparge is continuous). For these purposes, a non-temperature compensating meter (calibrated for 15.6C) is affordable, and will be ordered.

Specific Gravity: A hydrometer is used to measure the brewhouse yield, expressed in degrees Plato. The original gravity of the wort is noted and compared with historical yields, and any deviations addressed.

(c) Brewery Fermentations

Introduction: A consistent finished product depends in large part on consistent brewery fermentations, which in turn depends on a pure, healthy house culture, properly pitched, aerated and maintained. The following section outlines the materials and methods used to maintain such a culture, and to obtain consistent brewery fermentations.

As shown in Table 2, the house yeast culture is thoroughly monitored for contamination at numerous stages during production. In addition, the yeast is measured for its general state of health by several "markers." These materials and procedures are given below.

Yeast counting and Viability: Pitching rates. It is accepted brewery practice that 1 million viable yeast cells per ml of wort per degree Plato is a proper yeast pitching rate. This rate is determined by a direct viable cell count, using a microscope, a haemocytometer, methylene blue solution (despite its limitations, it still works well for the small brewery),[26] and serial dilution techniques.

Procedure: The haemocytometer is a glass counting chamber with a sample volume of 1×10^{-4} cm^3 (see Figure 2). Yeast slurry from the cone of a fermentor (or yeast propagation vessel) is drawn. A 1/100 dilution or 1/1000 solution is obtained by the serial techniques discussed above. The haemocytometer chamber is cleaned, using distilled water and lens paper. The coverslip (especially designed for the haemocytometer) is positioned on the haemocytometer, covering both chambers of the counting chamber (separated by a moat). The homogenously mixed, diluted sample is drawn by Pasteur pipette, a couple of drops are expelled, and the pipette tip is applied to the coverslip edge. The sample flows into the chamber, and care must be taken not to overrun the moat into the second chamber. Under 10X magnification, focus on one of the counting grids composed of 25 counting "squares" (Fig. 2A). Then, one of the four corner grids is isolated under higher magnification (typically, 45x) (Fig. 2B). Count each of the cells in the four corner grids, plus an additional grid (usually in the center), by the following counting rules:

- If more than 48 cells per (16) square counting area are obtained, the sample is not diluted enough and will have to be rediluted (e.g., from 1/100 to 1/1000) to obtain statistically correct results. No fewer than 75 cells per 1 mm^2 area (of 25 squares) should be obtained.
- Budding cells are counted as two cells *only* if daughter cells are more than one half the size of the mother cell
- Some standard reference of "line" cells must be used, to avoid double-counting cells. Cells touching or lying along the top and right boundary lines of each counting area

[26] The utility of methylene blue as a viability measure is debated. See "Improving Yeast Handling in the Brewery, Part 3." Erin O'Connor-Cox, *Brewer's Guardian*, March, 1998, pp. 20-21.

are counted. Those touching or lying along the bottom and left lines in the counting area are not counted.[27]

Referring to Figure 2, the cells counted within the 5 counted single-line grids (composed of 16 squares) number 75. This is multiplied by 5 to obtain an estimate of the $1mm^2$ (25 grid) cell count, or 75 x 5 = 375 cells. Assume this was obtained by 1/1000 dilution.

To obtain the cells/ml in the original sample, the cells counted in the central (25 square) area are multiplied by the dilution factor (100) and the haemocytometer volume (10^4 ml):

$375 \times 1000 \times 10^4 = 3.75 \times 10^9$ yeast cells/ml slurry.

To determine the pitching rate, the wort cast-out volume and original gravity must be known. Assuming a wort cast-out volume of 10 hl (1000 L) and an OG of 13 plato, the volume of yeast needed to be pitched is:

1 million cells/ml x 1000ml/l x OG(Plato) x wort volume =

1 x 10^6 Cells/ml x 1000 ml/L x 13P x 1000L = **1.3×10^{13} total cells needed**.

Since the yeast slurry cell density has been determined to be 3.75×10^9 cells/ml, the total pitching volume is:

1.3×10^{13} total cells/3.75×10^9 cells/ml to get 3466.67 ml, or **3.47 L** of yeast slurry.

***Methylene Blue and Viability*:** Above 90% viability, methylene blue is still considered to be an adequate medium for establishing the percentage of viable cells within a given yeast population, although it says nothing about "vitality," which is described by a host of markers, among them the ability to reproduce.[28] To know the total yeast pitching rate, the rate as given in the above paragraphs must be increased by the percentage of *non-viable* cells in the slurry.

Reagent: 0.01 g of methylene blue is dissolved with 2.0 g of sodium citrate in 100 ml of distilled water.

Procedure: After obtaining the yeast cell counts (above), 1 ml of methylene blue solution is mixed with approximately 8 ml of yeast slurry from the above sample. The sample is allowed to stand for 3-5 minutes, and the suspension is viewed under 60x objective on a haemocytometer. A total of ca. 500 cells should be counted. Cells which are dead will not be able to reject the stain, and will be marked blue; live cells will be clear or nearly so. The viability is given by:

Viable cells/dead cells x 100 = (%) viability.

The total pitching volume should be increased accordingly, e.g., if 94% viability is obtained, the pitching volume above should be increased by 6% (to offset the dead cells):

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[27] *ASBC Methods of Analysis*, *op cit*, Microbiology 4.

[28] *Ibid*, Microbiology 3; Erin O'Connor-Cox, "Improving Yeast Handling in the Brewery, Part 1," pg. 29. *Brewer's Guardian,* December, 1997. See also Takeo Imai, "The assessment of yeast vitality – the past and the future," Brewer's Guardian Lecture Series, *Brewer's Guardian,* June, 1999 pp. 20-22.

Original pitching volume: 3.47 L

After viability factor: 3.47 + (3.47 x .06) = **3.68 L.**

Propagation: The tendency of culture yeasts to become contaminated, undergo spontaneous mutations, or otherwise change in fermentation performance as noted by key "markers," increases over time. Such markers include:

Cell growth rates: Yeasts should multiply by approximately 4x from pitching to high-krausen. Viable yeast counts are taken at 24 hours to determine if optimal growth has taken place. Shortfalls in cell growth rates may indicate inadequate wort oxygenation, nutrient exhaustion, competition with early-fermentation spoilers, or a loss of vitality.

Fermentation rates and end fermentation: The fermentation rate, or time to final gravity, is a useful measure of the vitality of the house culture. Changes in fermentation rate may signal contamination (see *C.* freundii, Table 1), respiratory-deficient mutation, [29] or changes in vitality. Furthermore, changes in the culture's ability to consume the rate-limiting "end" fermentables maltose and maltotriose may signal changes to the culture which warrant repropagation (See Rapid Fermentation procedure, above).

To safeguard against these developments, it is common practice to repropagate culture yeast from a master (slant) culture every 8-10 generations.[30]

Procedure: YM agar, a synthetic nutrient medium, is especially suited for the propagation of fresh yeast subcultures. Serial dilution and plating is carried out as per normal, to obtain suitably isolated, morphologically "sound" colonies of normal size, shape, and color. Once colonies have been obtained, the choice can be made to select one CFU for slant storage, or several. A single colony likely emanates from one cell, with its associated genetic inheritance – for good or ill. If that CFU is of the same profile as the house yeast, which has thus far worked well, so much the better. However, there is also a chance that the single CFU may have undergone mutations in subculturing, which may not be desirable. To be safe, a selection of several distinct, sound colonies may be chosen for slant storage.

To move from the laboratory culture to the cellar, the yeast is incubated with successfully larger volumes. At the early stages, to ensure adequate cell density, it is common practice to "bump" the wort volume with small steps[31] - commonly no more than 4x per step increase e.g., 10 ml of sterile wort is inoculated with a slant loop; 16-24 hours later, at "high krausen," the fermenting starter is transferred to a 30 ml flask, yielding a total starter volume of 40 ml. The process is repeated until 1 Liter of starter is obtained, and the starter wort is then transferred to the yeast propagation vessel. At each stage, there is some evidence that constant agitation will increase the rate of cell growth, and thus cell density.

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[29] Graham G. Stewart, "Yeast Performance and Management," The Guild Review Paper, *The Brewer*, pg. 212.
[30] *Ibid;* Priest and Campbell, *op cit.,* pg. 82.
[31] Graham G. Stewart, *op cit*., pg. 212.

Once in the YPV, the starter steps may increase – as much as 10 fold.[32] Aerated, sterile wort is added at high-krausen stage so that the culture yeast is constantly maintained in its respiration and exponential growth phase. Given a final wort cast-out requirement of 10 hl (1000 L), the final "bump" before fermentation should bring the starter volume to 100 L. The yeast should be pitched within a very short period – no longer than 2-3 days, and it should be kept cold – as low as 2-4 C. It should also be maintained in an anaerobic environment, so as to bring all respiration and metabolic processes to a standstill. At each stage of the propagation and at pitching, the yeast culture is tested for viability and cell density, and screened for contaminants by the methods listed throughout this paper.

Condition – residual sugar and yeast: In the priming method of secondary fermentation, fermentation is allowed to come to completion in order to ensure that a consistent level of carbonation or condition is achieved. Retained dCO_2 levels from primary fermentation are best determined by empirical and historical observation for any given brand, since a dCO_2 meter is beyond the budget of the small brewery. At cellar temperatures of 10-13C, common dCO_2 levels at the end of primary fermentation range from 0.4-0.5 volumes. "Real ales" in the United Kingdom and United States are typically maintained at 1.0-1.2 volumes dCO_2. Adding dextrose at the rate of .37 kg/hl (3.7 kg for the 10 hl fermentor volume) will increase the CO_2 by approximately 0.8 volumes, yielding 1.2 volumes dCO_2. To ensure adequate stores of suspended yeast (2.5×10^5 to 3.0×10^6 cells/ml), a suspended yeast sample is drawn from the mixing tank prior to adding the dextrose or syrup. If lower yeast counts are obtained, pure yeast may be transferred from a YPV or fermentor to achieve the desired level.

(d) Clarification: The optimization of isinglass finings performance is dependent on a few factors, all readily measured in the laboratory:

pH: For optimization of the isinglass-yeast flocculation reaction, the pH of the isinglass solution should be 4.0 - 4.4.[33] Isinglass solution is treated with phosphoric acid and tested with a pH meter to ensure it falls within this range.

Temperature and Agitation: The isinglass solution should be thoroughly mixed with a magnetic stirrer over the course of two hours, and on storage should be maintained in the temperature range of 39-50C.

Suspended Yeast Counts: Suspended yeast counts in excess of 2 million/ml may overwhelm the fining ability of the isinglass.

Evaluation using Imhoff cones: The dosage rate should be adjusted according to how well the isinglass performs. Various dosage schemes can be evaluated using Imhoff cones and adjustments made accordingly.

(c) Sensory Evaluation Panel –Structure and Operation

Perhaps the least expensive, yet most formidable defense the cask-ale, small brewery laboratory can maintain against the host of problems that may develop, is a well organized, well trained, and motivated sensory evaluation panel. The panel is important, in any brewery. But a panel trained to detect the potential spoilers specific to cask-ales, via their off-flavors,

[32] Priest and Campbell, *op cit*, pg. 32.
[33] Hough, *et al, op cit.*

aromas, mouthfeel and visual effects, can be invaluable. In addition to the "regular" panel, which evaluates the beer against a host of known quality parameters for the brand, one additional scheme might be warranted: a panel devoted to tracking market aging for the brand. To do this, each brand is stored in a "library" which is maintained as close as possible to environmental market conditions as possible, e.g., warehouse temperatures, if they differ from cellar temperatures; cellar temperatures; 2 weeks stillage (or longer); and tasting over the course of 3-4 days after tapping. Certain off-products, such as sulfurous, acetaldehyde, and diacetyl flavors and aromas; ropiness and turbidity; viscosity, and others as noted in Table 1, should be especially evaluated. The data can then be entered into a spreadsheet which includes the batch number, the brew date, the date of testing, and all relevant ranking notes (e.g., out of 1-5 for "worty," a 4) and subjective evaluations (e.g., strong horse-blanket aroma).

(d) Equipment and materials list

Table 3 is a list of the main equipment and materials to be ordered, along with suppliers.

Table 1: Common Bacterial Spoilers and Their Typical Characteristics[34]

Organism	Morphology	Grouping	Gram	Motility	Aerobic/Anaerobic	Optimum pH	Catalase	Hop	Type of Spoilage
Lactobacillus (homofermentative)	Rods		+	-	Anaerobic /facultative aerobe	5.5-5.8	-	Yes	Mash Souring
Lactobacillus (heterofermentative)	Rods		+	-	Anaerobic /facultative aerobe	4.0-5.0	-	No	Acidity Diacetyl Superattenuation "Silky" Turbidity
Pediococcus	Cocci	Tetrads/Pairs	+	-	Microaerophilic	4.0-5.0	- *	No (*P. Damnosus)*	Diacetyl Ropiness/pellicle (polysaccharide)
Micrococcus (*M. varians)*	Cocci		+	-	Strict Aerobe	7.0	+	Yes	Non-Spoiler
Micrococcus (*M. Kristinae)*	Cocci	Tetrads (some strains)	+	-	facultative anaerobe	can grow at < 7.0	+	No	Under anaerobic conditions, "fruity aroma and atypical taste" in finished beer
Zymomonas (*Z. mobilis)*	Short, plump rods	Rosettes, chains; some display filamentous cells	-	1-4 polar flagella	Anaerobic /facultative aerobe	7.5	+	No	Esp. in priming solutions and primed beers, "rotten apples" (acetaldehyde) and prodigious H_2S.
Acetobacter	Ellipsoidal/ rod[ψ]	Singly, pairs, chains	-/var.	Motile: peritrichous or lateral flagella; also non-motile	Obligate aerobe	5.4-6.3	+	No	Acetic acid production Off flavors Ropiness/pellicle turbidity
Gluconobacter	Ellipsoidal/ rod	Singly, pairs, chains	-	If mot., 3-8 polar flagella	Obligate aerobe	5.5-6.0	+	No	Acetic acid production Off flavors Ropiness/pellicle turbidity

[34] Typical of the genera (or species, where included). As with Figure 1, the information contained in this Table is drawn primarily from Priest and Campbell, *op cit.*

[*] Some *Pediococcus* may give positive catalase results, esp. on agars with less than 1% glucose; they are often confused with micrococcus. If in doubt, distinguish by agar pH, and repeat catalase test by reculturing on >1% glucose medium. Priest and Campbell, *op cit*, pp. 151, 155.

[ψ] Pleomorphic forms also exist, and may display as spherical, elongated, swollen, club shaped, curved, or filamentous.

Table 1: Common Bacterial Spoilers and Their Typical Characteristics (Cont.)

Organism	Morphology	Grouping	Gram	Motility	Aerobic/Anaerobic	Optimum pH	Catalase	Hop	Type of Spoilage
Citrobacter (C. freundii) (rare)	Rods	Single/pairs	-	Peritrichous flagella	Facultative anaerobe		+	No	Increase fermentation rate Reduce yeast viability DMS
Rahnella (R. aquatilis)	Small rods		-	+ (25C), - (37C)	Facultative anaerobe		+	No	Overattenuation (in ales) Diacetyl DMS Acetaldehyde
Obesumbacterium (O. Proteus)	Short, fat rods		-	Peritrichous Flagella	Facultative Anaerobe	4.4-9.0 (in unhopped wort)	+	No	Under attenuation High beer pH DMS Diacetyl "parsnip" or "fruity" odor
Klebsiella	Rods		-		Facultative Anaerobe	7.2	+		Phenols, esp. 4-vinyl guaiacol DMS
Hafnia (H. alvei)			-						DMS
Pectinatus[35]	Rods	Single/pairs Short chains (rare)	-	One-sided flagella (comb-like)	Obligate anaerobe				Acetic acid H_2S
Megasphaera	Slightly elongated Cocci	Pairs/short chains	-	-	Obligate anaerobe	Sens. To low pH (and EtoH)	-	Yes	Fatty acid production (beer and wort) Acetic acid acetoin

[35] Fairly new genus. Lee, S.Y., et al., "Pectinatus: A new genus of Bacteria," Journal of the Institute of Brewing, v. 86, 1980.

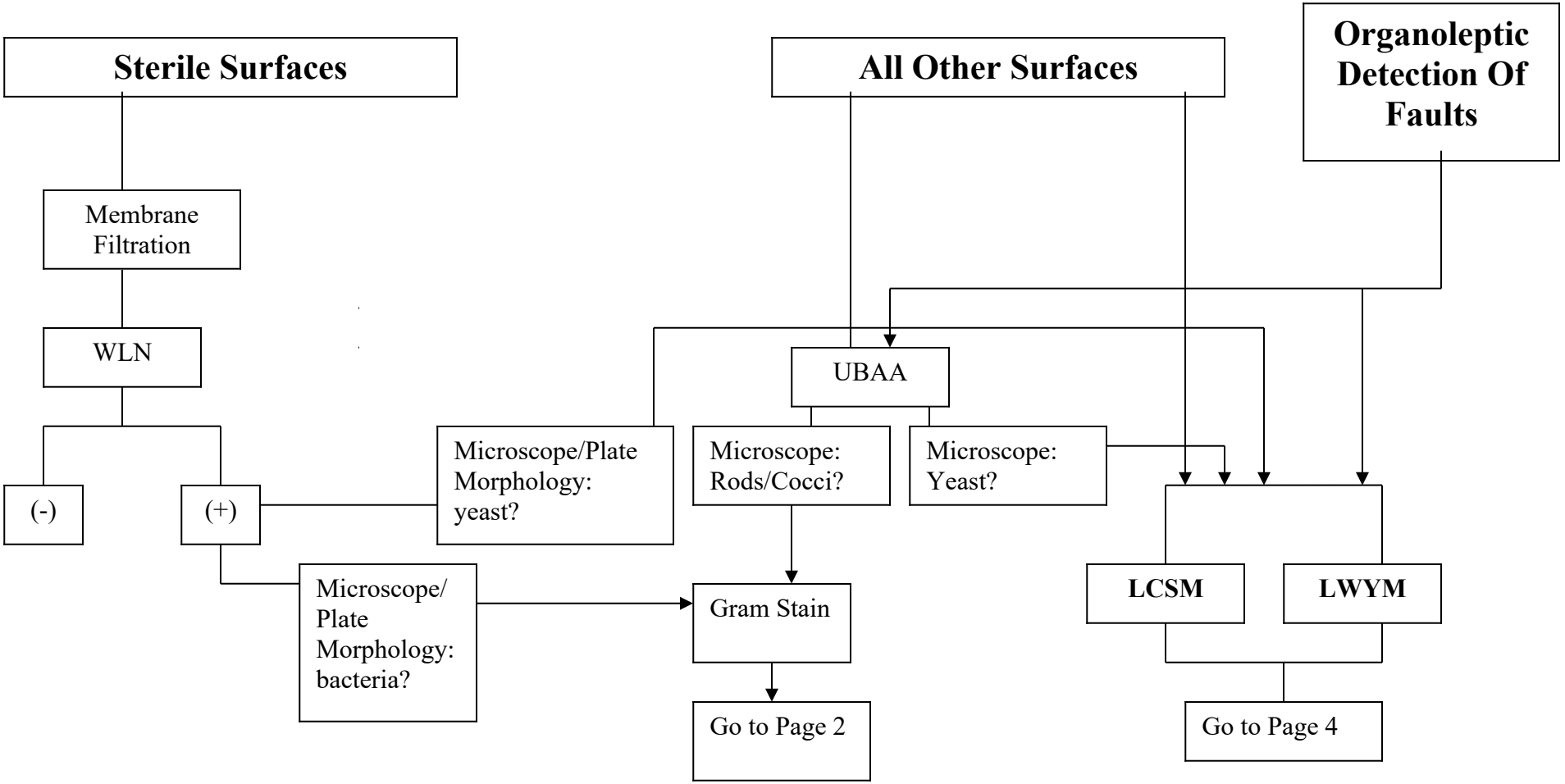
Table 2: Sample and Plating Schedule: "Normal" Conditions

Process	Sterile Sample Point	Test Media	Frequency of Sample	Additional Tests
Pitching Yeast	Yeast Propagation Vessel (YPV) Pitching Yeast	WLN* UBAA LCSM LWYM	Once weekly, via membrane filtration Every stage of prop., and at every pitch. Every stage of prop.	 Viability & Yeast Count, every stage of prop.
Brewhouse Wort "Cool-In"	WP; heat exchanger; transfer line Bitter Wort	WLN WLN N/A	Once weekly (w.p., transfer line), via membrane filtration Every transfer (bitter wort) Every transfer (bitter wort)	Original Specific Gravity (OG) pH Sterile Wort Test
Fermentation				
Fermentor	Manway/cone Pitched wort Beer Sample	WLN N/A UBAA/LCSM/LWYM	Once weekly, via membrane filtration At pitching Once weekly	 Rapid Fermentation Test (RFT)
24 Hours	Fermentor	UBAA		Specific Gravity (SG), Yeast Count
3 Days	Fermentor	UBAA		SG
5 Days	Fermentor	UBAA		SG/Racking Yeast Count[36]
Priming Sugar Solution	Solution Vessel	WLN	Every preparation	
Racking				
	Racking/Cask Filling Machine	WLN/LCSM/LWYM UBAA	Once weekly, via membrane filtration Every production run	Sensory Evaluation Panel on Finished Product, drawn from "Library."

* All WLN and UBAA plates are cultured aerobically and anaerobically.

[36] It will be presumed that 5 days fermentation is the normal period wherein primary attenuation and required suspended yeast populations of 2.5×10^5 to 3.0×10^6 cells/ml is achieved.

Figure 1: Brewery Contaminant Isolation Key: Media and Tests[37]



[37] Most of the material in Figure 1 is drawn from Priest and Campbell, *op cit.,* pp. 127-201.

Figure 1: Brewery Contaminant Isolation Key: Media and Tests (Gram Positive Bacteria)

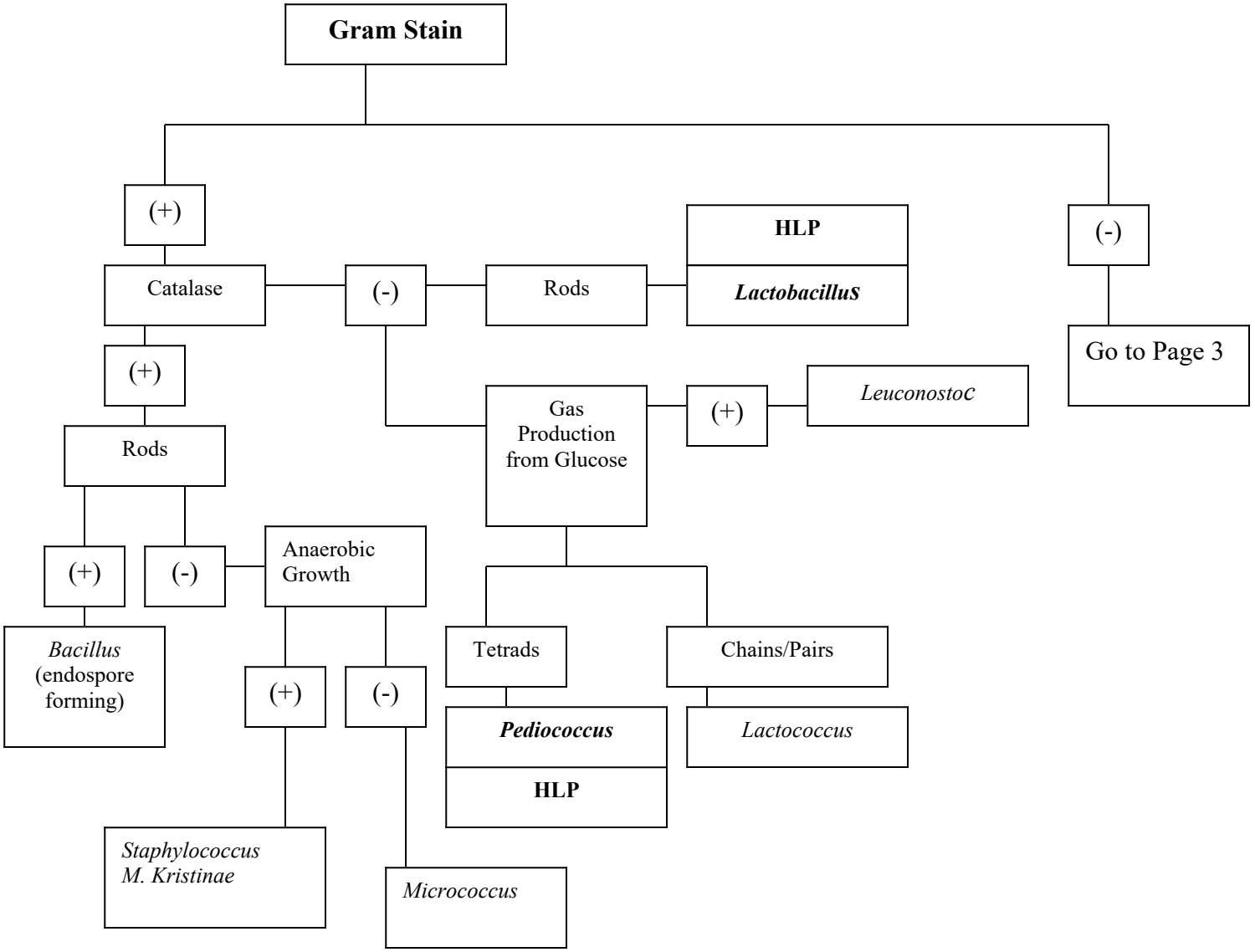


Figure 1: Brewery Contaminant Isolation Key: Media and Tests (Gram Negative Bacteria)

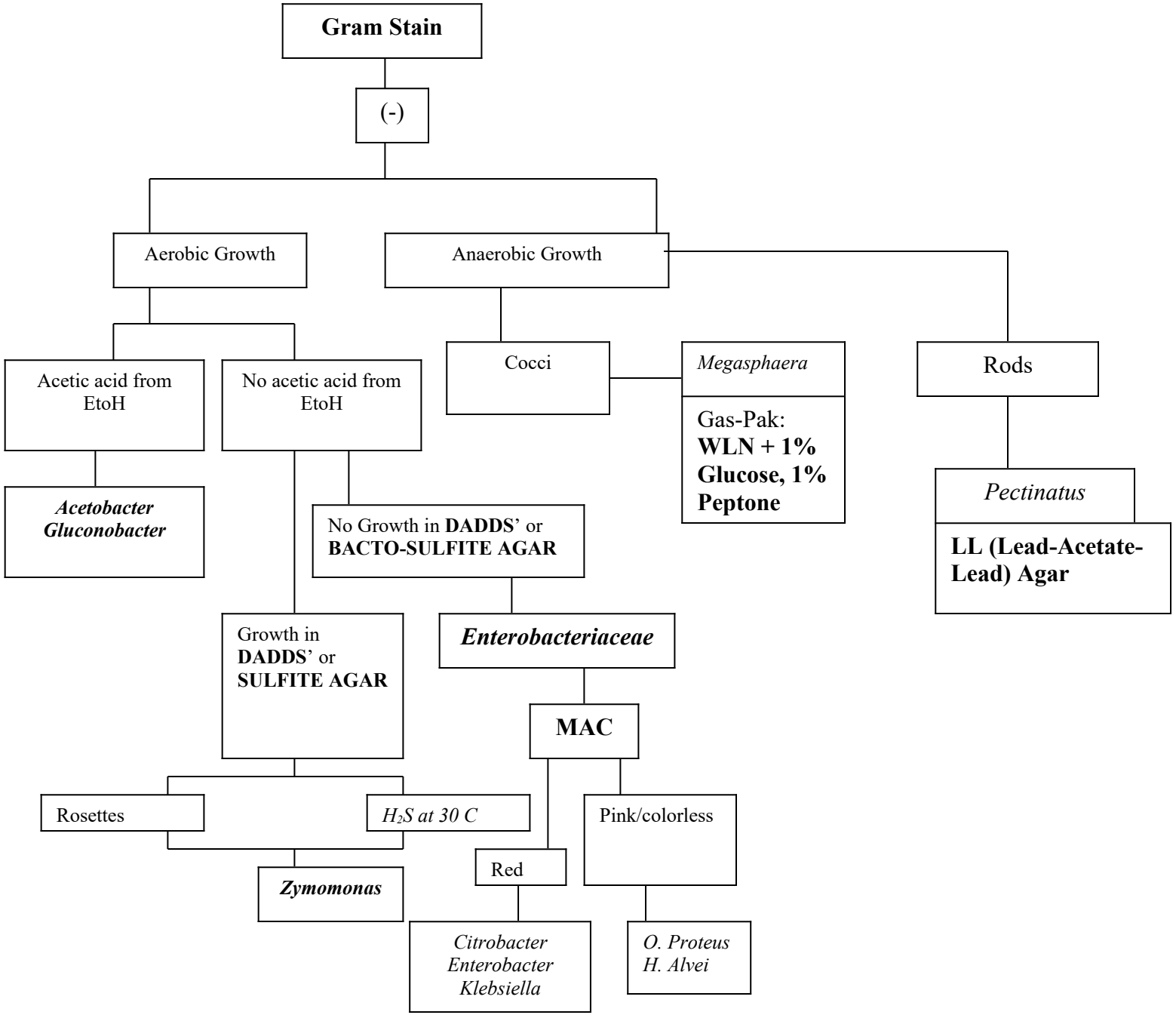
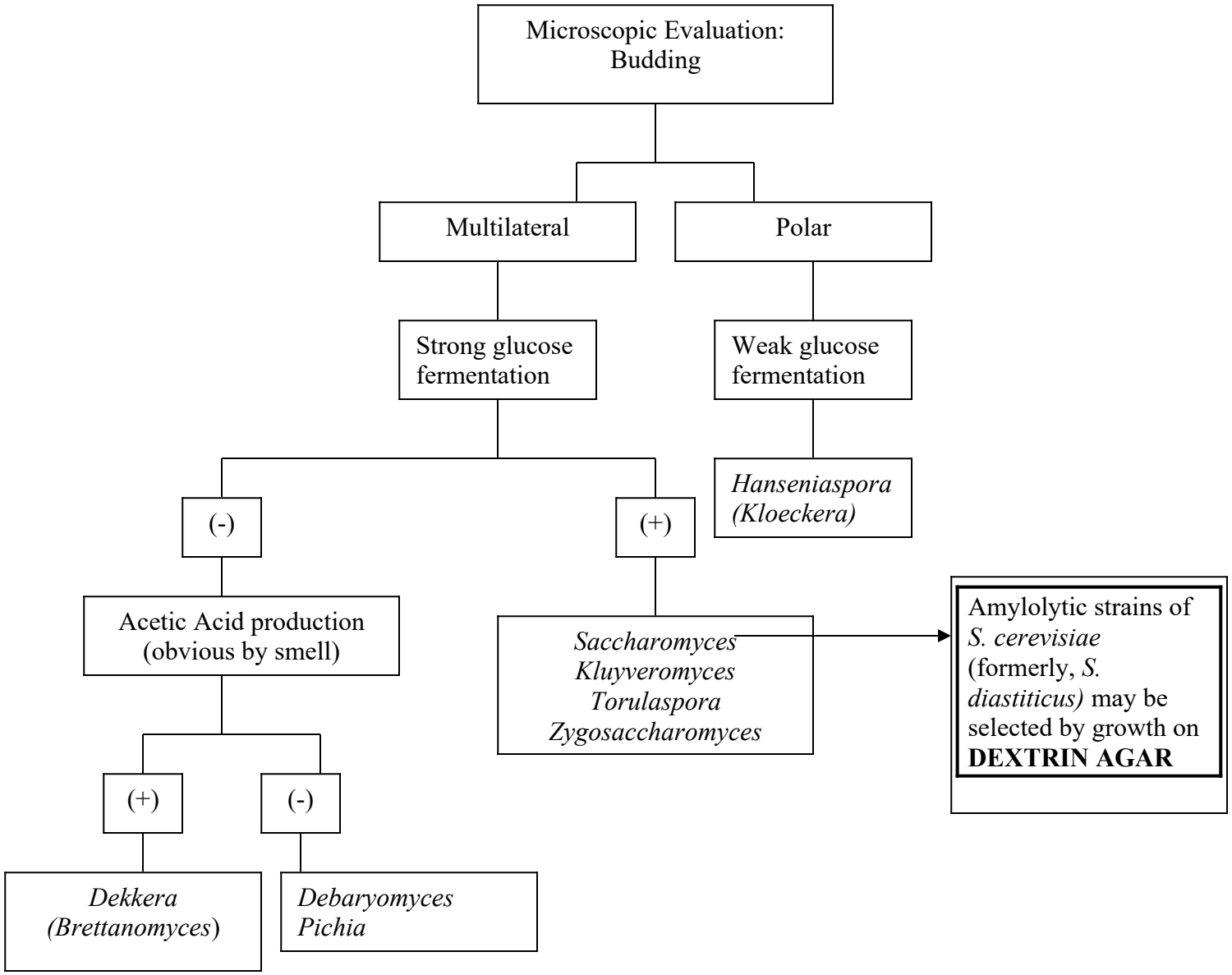


Figure 1: Brewery Contaminant Isolation Key: Media and Tests (Perfect, or spore-forming Wild Yeasts detected on LCSM/LWYM)*



* "Imperfect" non-sporing genera equivalents are given in parentheses.

Table 3 – Equipment and Material List

Equipment	
Equipment Type	**Supplier**
Microscope, 4-100X, binocular	Abbott Labs (used)
Slides/coverslips	Fisher Scientific
Haemocytometer/coverslip	Fisher Scientific
Pasteur Pipettes	Fisher Scientific
2.0 ml pipettes	Fisher Scientific
10.0 ml pipettes	Fisher Scientific
Pipette Bulb	Fisher Scientific
Immersion oil and lens paper	Fisher Scientific
Petri plates	Fisher Scientific
Test tubes, Kimax, screw cap	Fisher Scientific
Test tubes, straight slip cap	Fisher Scientific
Swabs	Fisher Scientific
Erlenmeyer Flasks, 100-500 ml	Fisher Scientific
Graduated Cylinders, nalgene	Fisher Scientific
Incubator	Abbott Labs (used)
Gas-Pak and pouches	Fisher Scientific
Laminar Flow Hood	Abbott Labs (used)
Bunsen burner	VWR Scientific
Hydrometers	Rascher & Betzold
pH Meter	Abbott Labs (used)
Balance, triple beam	Abbott Labs (used)
Autoclave	Goodman's (20 qt. pressure cooker)
Inoculating loops	Fisher Scientific
Magnetic stirrer/hotplate	Abbott Labs (used)

Table 3 – Equipment and Material List

Media, Stains, Reagents & Materials	**Supplier**
WLN	Fisher Scientific
UBA	Fisher Scientific
HLP	Crosby Baker (as needed)
LCSM	Crosby Baker
LWYM	Crosby Baker
Copper Sulfate	Fisher Scientific
Bacto-Sulfite Agar	Fisher Scientific (as needed)
MacConkey's Agar	Fisher Scientific (as needed)
Dextrin Agar	Fisher Scientific (as needed)
Glucose-Peptone broth	Fisher Scientific (as needed)
Methylene Blue	Fisher Scientific
Sodium Citrate	Fisher Scientific
Sodium Chloride	Fisher Scientific
pH Buffer 4.0	Fisher Scientific
pH Buffer 7.0	Fisher Scientific
Hydrogen Peroxide 3%	Local store
NaOH	Fisher Scientific
95% EtoH	Fisher Scientific
Membrane filtration kit	Fisher Scientific
Iodine solution	Fisher Scientific
Crystal Violet	Fisher Scientific
Safranin counterstain	Fisher Scientific
Priming Sugar	Crosby Baker
Isinglass	Savilles