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Les milieux de culture et leur utilisation en
contrôle qualité

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Wort VS Beer : Culture media for contaminants ?

WORT	BEER
<ul style="list-style-type: none">• Rich in sugar• Oxygen• pH 5.5	<ul style="list-style-type: none">• 3-5% Alcohol• No O₂• SO₂• Hops• Low pH (4.5)

A beer contaminant must
be resistant to this harsh
environment !

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Contaminants

What are they?

- Bacteria
- Yeast
- Fungi

Where do they come from?

- Raw materials
- Water
- Environment
- Airborne
- Insects
- Repitched yeast
- Etc.

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Contaminants detection :

Why is it so important to have QC procedures?

- Direct competition with culture yeast for nutrients during fermentation
- Affect product quality / consistency in different ways:
 - Off flavors
 - Filtration problems
 - Beer sediments
 - Haze
 - pH drop
 - Super attenuation -> gushing

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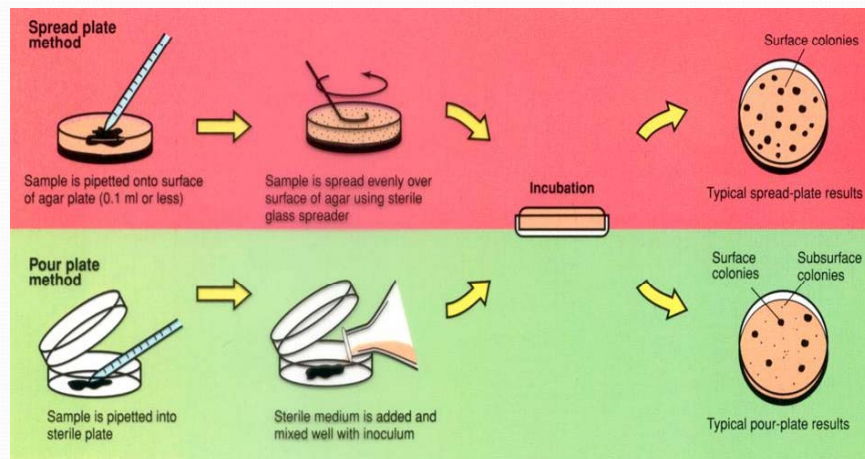
Detection & Identification

1. Aseptic sampling & techniques
2. Choose the right media (duplicate or triplicate)
3. Choose the appropriate plating technique
 - A. Spread plate method
 - B. Pour plate method
 - Both methods recommended for samples in which high levels of contamination is expected : yeast, beer and ingredients such as process water
 - Dilutions might be required (30-300 colonies /plate = significantly representative)
 - C. Membrane filtration technique
 - Recommended for samples with low contamination levels such as beer, rinse water
4. Incubate under appropriate conditions: w/ or w/o O₂, temperature, time
5. Detection, quantification & characterization
6. Identification

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Plating techniques



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Different types of media

- **Nutritive medium:** Synthetic, complex medium prepared in the lab that contains various amounts of different chemicals that are known to support growth of many microorganisms.
- **Selective medium (S):** One that encourages the growth of some organisms while suppressing the growth of others.
- **Differential medium (D):** One that will cause an observable change in the medium when a particular biochemical reaction occurs.
- **Enriched medium (E):** One that contains special nutrients that encourages growth of a particular organism that might not be otherwise present at an high enough level to allow it to be isolated and identified.

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Different types of media...

- **Inhibitory medium (I):**
One which inhibits certain microorganisms from growing while allowing others to grow – usually by adding an inhibitory substance (like antibiotics).

Cycloheximide = yeast inhibitor
Chloramphenicol = bacteria inhibitor



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General guidelines on how to prepare media?

- Follow manufacturer's instructions!
- Using an appropriate container, weight the appropriate amount of dehydrated powder based on the needed volume
- Add the appropriate exact volume of distilled water
- Heat to boiling until complete dissolution
- Sterilize by autoclaving (15 min) at 121C and 15 pounds of pressure
- Let media cool down to approx. 50C

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General guidelines on how to prepare media...

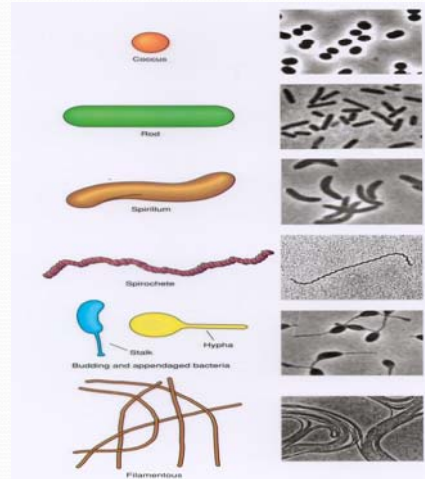
- Add inhibitory agents if necessary – sterile stock solutions!
- Pour 15-20ml /sterile petri dish
- Let it solidified
- Good practice to incubate before use (2-3 days)
(unless restrictions apply)
- Plates can be stored upside-down at 4C for about 1 month
(unless restrictions apply)
- Don't use commercial media that is beyond shelf-life!

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Bacteria

- Unicellular organisms
- Gram (+) or (-)
- Various morphology
 - Coccus
 - Rod
- Multiple arrangements
 - Single cells
 - Pairs
 - Tetrads
 - Chains

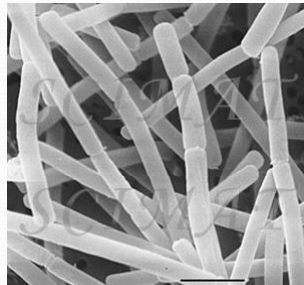


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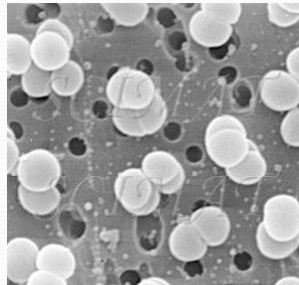
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Lactic Acid Bacteria (LAB)

Lactobacillus



Pediococcus



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Lactic Acid Bacteria (LAB)

Lactobacillus

- Gram (+) **rods, chains**
- **Aerotolerant** anaerobe
- Hop resistant
- RISK : fermentation to product
- SPOILAGE:
 - Make beer sour by fermenting glucose, maltose and maltotriose into lactic acid
 - Silky turbidity
 - Diacetyl

Pediococcus

- Gram (+) **cocci, tetrads**
- Anaerobe
- **Slow growing**
- RISK: late fermentation onwards
- SPOILAGE:
 - Diacetyl
 - Ropiness

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Acetic Acid Bacteria (AAB) - *Acetobacter & Gluconobacter*

- Gram (-)
- Pleomorphic : ellipsoidal to rods, pairs to chains
- **Obligate aerobes**, microaerophilic capacity
- RISK: beer dispenser, cask beers
- SPOILAGE:
 - Ropiness, haze
 - Gluconobacter : ETOH -> Acetic acid
 - Acetobacter: ETOH -> Acetic acid + CO₂ + H₂O
- CONTROL: tolerant to alcohol and low pH, sensitive to anaerobic conditions

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Enterobacteriaceae « coliforms »

- Gram (-), short straight rods
- Facultative anaerobe
- RISK: Wort, early fermentation
- SPOILAGE:
 - Diacetyl
 - Organic acids
 - Phenolics
- CONTROL:
 - Sensitive to pH < 4.4 (beer pH 4.5)
 - Sensitive to ETOH > 2%
 - Heat sensitive (pasteurization)

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Strictly anaerobic bacteria

Pectinatus

- Gram (-) **slightly curved rods**
- SPOILAGE:
 - Turbidity
 - Acetic acid
 - H₂S
- CONTROL:
 - Growth pH 4.5-6,
slow at pH 4.0
 - Oxygen

Megasphaera

- Gram (-) to variable , **very large cocci**
- SPOILAGE:
 - Turbidity
 - Short chain fatty acid
 - Butyric & Caproic acid
 - H₂S
- CONTROL:
 - Can't grow at pH < 4.1
 - Can't grow if ETOH > 3.5%
 - Oxygen

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Anaerobic bacteria - *Zymomonas*

- Gram (-), short straight rods, **rosettes**
- Anaerobic, but **oxygen tolerant**
- Ferment glucose & fructose, not maltose
- SPOILAGE:
 - H₂S
 - Acetaldehyde (green apple)
 - Alcohol tolerant at very high levels (ETOH > 13%)
- CONTROL:
 - Hygiene
 - CIP

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General Culture Media for the brewery

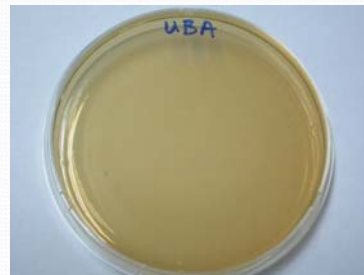
UBA (Universal Beer Agar)

Included in the ASBC « Methods of Analysis »

Most culture & nonculture yeast, Gram (-) rods commonly found in breweries will grow well under standard (aerobic) conditions.

LAB often found in breweries (*Lactobacillus*, *Pediococcus*) will grow well under anaerobic incubation at 28°C.

Cycloheximide can be added to suppress yeast growth.



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General Culture Media for the brewery

LMDA / SDA

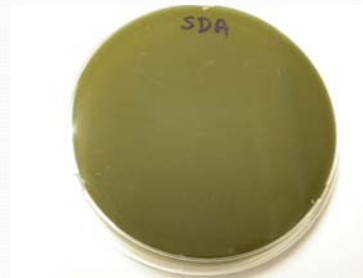
(Lee's Multi-Differential Agar
/ Schwartz Differential Agar)

Included in the ASBC « Methods of Analysis »

Nutrient and differential medium that will detect most organisms commonly encountered in a brewery and that provides, just by visual observation of colonies, some identifiable characteristics of the microorganisms growing on it.

Actidione may be added to the medium to suppress yeast growth.

Good as or usually even better recovery of brewery bacteria than UBA.



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General Culture Media LMDA / SDA ...

- Aerobic and/or anaerobic incubation at 28°C :
 - 2-3 days incubation for coliforms
 - 2-7 days incubation for LAB
- Calcium carbonate will help to identify acid producing bacteria colonies around which a clear zone develops. Media will turn yellow.
- Further identification of colonies is facilitated by the characteristic color reactions (BG).
- Use within 2 weeks of preparation



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General Culture Media for the brewery

WLN

(Wallerstein Nutrient Agar)

- WLN is a good media to grow brewer's yeast.
- Differential medium that can be used to evaluate macroscopic characteristics / differences of yeast colonies (variants).
- For yeast growth, incubate at 28-30°C for 24-48 hours and up to 4 weeks for the giant colonies assay.

WLD

- WLN that has been made selective for bacterial contaminants by the addition of actidione.
- Incubate aerobically 2-3 days for acetic acid bacteria
- Incubate anaerobically for 4-6 days for LAB investigation

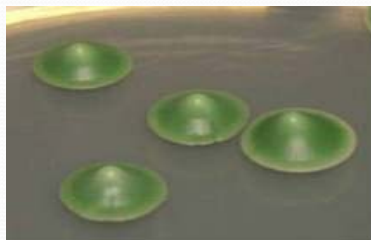
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General Culture Media WLN

Lager yeast strain

(Powell & Diacetis, 2007)



Ale yeast strain

(Powell & Diacetis, 2007)



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How to detect LAB ?

HLP

(Hsu's Lactobacillus/Pediococcus)

Semi-solid medium that enables selective detection & quantification of lactic acid bacteria.

Many LAB can be detected in 2 days (28°C). Differentiation of *Lactobacillus* and *Pediococcus* can be made after 5-7 days.

Yeast growth is suppressed by cycloheximide already in the medium.


Simple, quick test for the most common beer spoiling bacteria, requiring minimal lab equipment. Anaerobic incubation and autoclave are not required.

Included in the ASBC « Methods of Analysis »

Free video on Siebel's website & YouTube

HLP PLATES

- Add 1.5-2.0g agar
- Anaerobic incubation needed !



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How to detect LAB?


HLP (Hsu's Lactobacillus / Pediococcus)


Lactobacillus sp.

- Inverted tear drop shape colonies

Pediococcus sp.

- Comet shape colonies
- Rain drop shape colonies
- Snowballs shape colonies





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How to detect LAB ?

RRLM

(Raka-Ray Lactic Acid Bacteria Medium)

- Selectively detects LAB in beer and brewing process by encouraging larger colonies of this bacterial group to grow in a shorter time.
- Presence of LAB is also emphasized by suppressing, in varying degree, the growth of facultative nonlactic acid (wort) bacteria.
- Included in the ASBC « Methods of Analysis »
- Incubate anaerobically at 28°C
- Most LAB will develop within 3 days
- *Pediococcus* will require 5-7 days incubation

How to detect LAB ?

BMB

(Barney-Miller Brewery Medium)

- Developed by Michael Barney when at Miller
- Under patent, a few manufacturer
- Time required for incubation has been reduced compared to other selective media for LAB detection
- Anaerobic incubation required at 28°C
- Growth in +/- 3 days for most LAB but up to 7 days for slow-growing lactics
- Included in the ASBC « Methods of Analysis »

How to detect LAB ?

MRS

(De Man Rogosa Sharpe)

- Formulated to support good growth of *Lactobacilli* in general
- Apt for LAB detection too, including beer spoilers
- Anaerobic incubation required at 28°C for 5-7 days
- Beer can be added to make it more selective
- Included in the ASBC « Methods of Analysis »



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How to detect enterics ?

MacConkey Agar

- Selective medium for Gram (-) enterics because crystal violet and bile salts inhibit Gram (+) bacteria.
- Differential because lactose and pH indicator will identify lactose fermenters as red colonies and nonfermenters as light pink colonies.
- Coliform colonies, which are lactose fermenters, will therefore be red.
- When lactose is fermented, a local pH drop around the colony causes a color change of the pH indicator (neutral red) and bile precipitaton.
- Aerobic incubation at 35°C for 18-48 hours
- Various formulations – be careful !

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How to detect *Megasphaera* and *Pectinatus* ?

SMMP (Selective Medium *Megasphaera* and *Pectinatus*)

- Included in the ASBC « Methods of Analysis »
- Complex liquid medium not commercially available composed of a basal medium (reduced-incubation environment) and selective solution.
- The reagents of the selective stock solution (sodium fusidate, cycloheximide and crystal violet) inhibit or restrict significantly the growth of other bacterial species and yeasts.
- Anaerobic incubation at 28-30°C for 14 days.
- Visible turbidity should be reported as presumptive positive and should be followed by microscopic examination.

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Megasphaera and *Pectinatus* SMMP...

Megasphaera

- Coccoid cells, large
- Medium may change from purple to yellow after a longer incubation period
- Produces butyric, isovaleric, valeric, caproic and caprylic acids

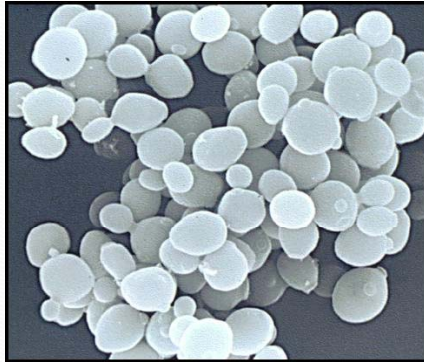
Pectinatus

- Rods
- Medium remain purple with sediments
- Produces acetic and propionic acids

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WILD YEASTS



Any yeast that has not been deliberately introduced in the wort, including other production strains...


- 1) Non-Saccharomyces
- 2) Saccharomyces

Important to use a combination of media for optimal detection of wild yeasts.

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Non-Saccharomyces Wild Yeasts

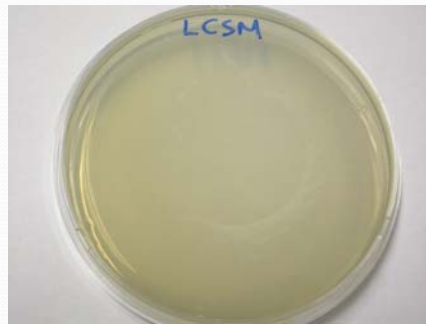
- Ellipsoidal to brick
 - Aerobes
 - Various genus: *Pichia sp.*, *Hansenula sp.*,
Candida sp., *Brettanomyces sp.*,
Schizosaccharomyces sp.
- 
- RISK: Fermentation onwards
 - SPOILAGE:
 - Form films in the presence of air
 - Haze
 - Off flavors
 - *Pichia* & *Candida*: reduction in ETOH concentration : ETOH -> acetic acid
 - *Brettanomyces*: acetic acid, mousy flavor

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How to detect non-Saccharomyces Wild Yeasts?

LCSM
(Lin's Cupric Sulfate Medium)



Selective medium for the detection and quantitative determination of wild yeast populations in brewer's culture yeast.

The growth of culture yeast is suppressed by cupric sulfate. Wild yeast grow as larger distinct colonies.

This medium is designed to encourage the growth of non-*Saccharomyces* yeast, but a few *Saccharomyces* yeast may show some growth.

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How to detect non-Sacch Wild Yeasts ? LCSM...

- Important to use the CS solution from the same lot than the dehydrated LCSM powder
- The media must be used within 3 days of preparation
- Sample should contain approx. 1 million yeast cells
- Aerobic incubation at 28C for 4-6 days

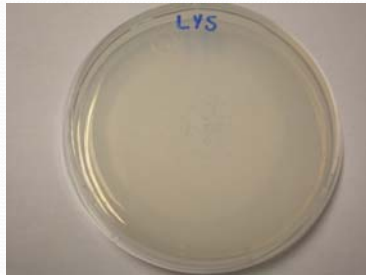


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How to detect non-Saccharomyces Wild Yeasts?

LYSINE MEDIUM



Selective medium in which lysine is the sole source of nitrogen. While most *Saccharomyces* sp. are lysine-negative, many other yeasts can utilize it and will therefore grow on this medium.

Useful for yeast slurries, process beer, and rinse waters.

Aerobic incubation 2-6 days at 25-28C

A background haze can often be seen on this medium (culture yeast). Only distinct colonies should be considered as WY.

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How to detect non-Saccharomyces Wild Yeasts?

CLEN medium

- ASBC recommended
- Multinutrient source medium that utilizes cadaverine, lysine, ethylamine, and nitrate as nitrogen sources.
- Suitable for the detection of some wild yeast
- It has been observed that some species of WY developed as larger colonies on CLEN than on the other WY media
- It has also been observed that CLEN medium exhibited the best recovery (highest count) for some WY species than the other WY media
- Aerobic incubation at 27C for 4 days or longer
- Distinct colonies may be considered as WY

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Saccharomyces Wild Yeasts

- Ellipsoidal, discrete or chain forming
- Facultative anaerobes
- RISK: fermentation onwards

SPOILAGE – *S. ellipsoidus*:

- Slow sedimentation
- No interaction with finings (no clumping)
- Resist pasteurization

SPOILAGE – *S. diastaticus*:

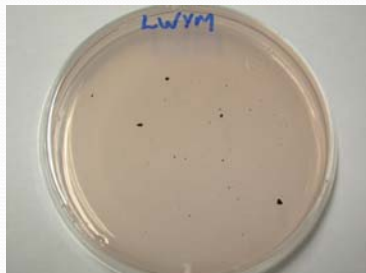
- Superattenuation: breaks down maltotriose and dextrans to produce ETOH and CO₂
- POF

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How to detect Saccharomyces Wild Yeasts?

LWYM
(Lin's Wild Yeast Medium)



Selective medium for the detection and quantitative determination of wild yeast populations in brewing culture yeast.

This medium is designed to encourage the growth of *Saccharomyces* wild yeast, but some non-*Saccharomyces* will also grow.

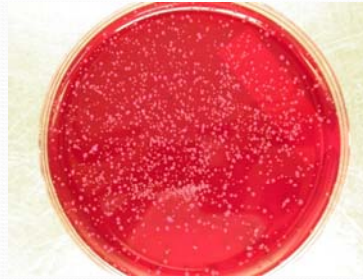
The growth of most culture yeast is suppressed or markedly reduced by fuschin-sulfite and crystal violet.

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How to detect Sacch Wild Yeasts? LWYM...

- Wild yeast will show vigorous growth and grow as larger distinct colonies
- Some strains of brewer's yeast may show weak growth – Only medium available for Saccharomyces WY detection
- Sample should contain approx. 1 million yeast cells
- Plates should be used within 5 days of preparation
- Aerobic incubation for 4-6 days. For optimal detection, 1 set of plates at 25C and 1 set at 30C
- Important to use CV of the same lot then of dehydrated LWYM powder



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Saccharomyces Wild Yeasts...

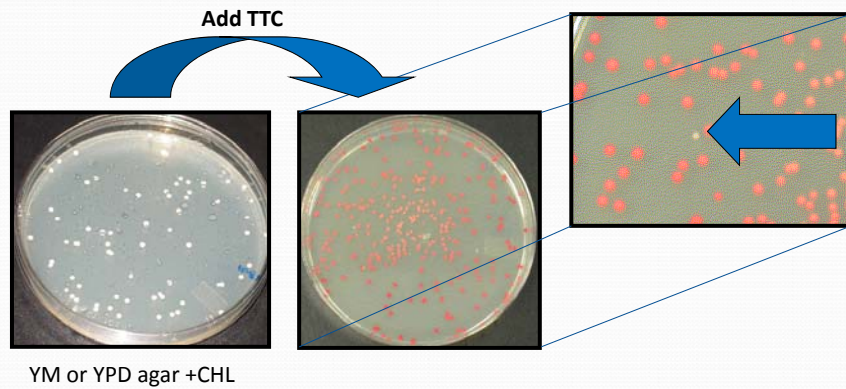
- **SPOILAGE – Petite / RD mutants :**
 - Result from genetic drift within culture yeast population
 - Lack certain respiratory enzymes
 - Associated with poor growth, lower yeast viability and unfavorable flavor production
 - Poor flocculation
 - Slow fermentation
- **SPOILAGE – Killer yeasts:**
 - Toxin -> disrupts plasma membrane of sensitive yeasts = DEATH !
 - Poor flocculation
 - POF
 - Superattenuation

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How to detect Saccharomyces Wild Yeasts?

Respiratory Deficient Mutants : TTC Overlay technique



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Identification of contaminants... briefly

BACTERIA

- Gram stain : (+) or (-)
- Microscopic evaluation:
 - Cell shape, arrangement
 - Gram
- Catalase test
- Oxydase test
- C sources assimilation/ fermentation tests
- Genus & Species ID
 - Various api galleries
 - Biolog
 - Genetic ID methods

YEAST

- Microscopic evaluation
 - Cell shape
 - Budding
- C sources assimilation / fermentation test
- Genus & Species ID
 - Api galleries (20C AUX, ID32)
 - Biolog
 - Genetic ID methods

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CONCLUSION & Suggestions

- Which media should you choose?
- Not necessary to have all the fancy equipment and to spend a lot of money to have a lab...
- Lab work doesn't necessarily have to monopolize all your time...
- Not confident about your results? Rely on an external lab to do the work for you or to confirm your results from time to time

Useful References

- ASBC: <http://www.asbcnet.org/>
- Difco / Becton Dickinson (BD) : <http://www.bd.com/>
- EMD: <http://www.emdchemicals.com/>
- Fisher Scientific:
<http://www.thermofisher.com/global/en/home.asp>
- HiMedia: <http://www.himedialabs.com/abouthimedia.aspx>
- Neogen / Acumedia: <http://www.neogen.com/acumedia/>
- OXOID: <http://www.oxid.com/uk/blue/index.asp>
- Siebel Institute : <http://www.siebelinstitute.com/>
- VWR Canlab: <https://www.vwrsp.com/>