Principles for isolation of microorganisms from fermented food and beverages

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Steps in isolating microorganisms from fermented foods:	**Things to consider:**
a. Product type: • Solid product • Semi solid product • Liquid product	Product types are highly influencing the initial steps (1-2) in a sampling plan.

1. Sampling (depending on product type)	• For solid/semi solid foods:
	  • Making a representative sample from surface or interior or a mix of both, according to the purpose of the isolation
	  • Size: Typically, sampling sizes are 10-100 g that aseptically are grated, cut into smaller cubes and mixed

2. Pretreatment (depending on product type)	• For solid/semi solid foods:
	  • Homogenization in Stomacher bag
	  • Type of diluent in homogenization, see p. 3
	• For liquid foods
	  • Homogenization (mixing)
	  • Membrane filtration (for fermented foods with low CFU)

3. Serial dilution (depending on food product and type of microorganism)	• Type of diluent, see p. 3 • What dilutions are needed (if you know approximate CFU)

4. Inoculation (depending on type of microorganism)	• Type of agar media (substrate) see p. 4-6
	• Optional, adding selective agents
	• Type of inoculation on agar media
	  • **Spread plating (100 µL on the surface)** (primarily used at MICRO)
	  • Pour plating (1 mL mixed into agar)

5. Incubation (depending on type of microorganism) see p. 4-6	• Temperature (25-42 °C) • Aerobic/anaerobic • Time (days) (2-7 days)
6. Counting colonies on plates	Count plates with 20-200 colonies, used for calculating CFU/mL for liquid products and CFU/g for solid/semi solid products. Take care to consider all dilution steps introduced from step 2-3, see also Fig 1.
7. Selection of representative colonies for identification and characterization, see p. 7	Select 20-25 colonies, randomly
8. Purification, see p. 7	Usually it is not necessary to add selective agents to these plates

A succesful isolation:

- Will ensure the growth of all relevant microorganisms
- Will inhibit the unwanted part of the microbiota (bacteria or filamentous fungi/yeasts)
- Can give a specific indication of selected microorganisms (selective and indicative media)

Standard isolation procedure (step 1-5):

1-2. For solid foods/semi-solid foods:

- 10.0 g of sample is weighed into stomacher bag with filter. The sample should be representative of the surface, interior or a mix of both, according to the purpose of the isolation
- Diluent is added to 100 g → This equals a 10^{-1} dilution
- Sample and diluent is homogenized in Stomacher for 2-3 min at medium speed or 1 min at high speed, depending on the structure of the food product. 1 mL of homogenized product is sampled for dilution series

For liquid foods: Homogenization (mixing) before sampling 1 mL for dilution series

3. Make 10-fold serial dilutions with diluent, see Fig 1. Notice –always include minimum 3 platings, in duplicate (to allow for calculating standard deviation to you CFU counts). In case you know the approximate CFU numbers you can decide which dilutions and platings you need, e.g. from a semi-solid product with estimated CFUs of 10^9/g use duplicate platings of 10^{-8}, 10^{-9}, 10^{-10} (in total 6 plates), corresponding to dilution tubes 10^{-7}, 10^{-8}, 10^{-9}.

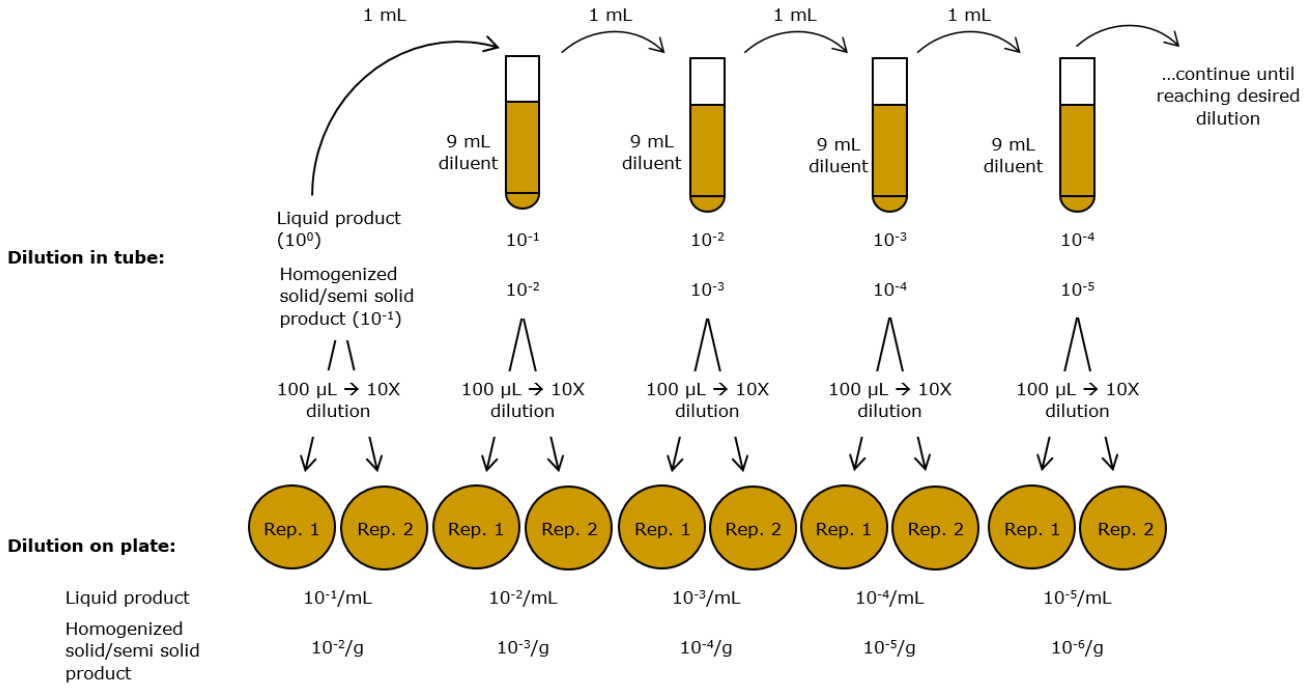


Fig 1. Example of dilution series and the dilutions obtained on plates. On the plate bottom, write the dilution on the plate, which can used for CFU calculations. Duplicate platings (Rep 1 and Rep 2) for each dilution. When estimated CFU numbers of a given product in known, these can be used for deciding which dilutions and platings to include in the isolation plan.

4. Spread plating 100µL on surface of agar (this corresponds to an extra 10-fold dilution), see Fig 1.
5. Incubate plate up-side down at appropriate temperature, days and atmosphere.

Diluents: (used in homogenization and serial dilutions, step 2 and 3)

Saline Peptone (SPO): NaCl 0.85 %, Bactopeptone 0.1 %, Na_2HPO_4 $2H_2O$ 0.03 % (primarily used at MICRO)

- pH =7.2 (bacteria)
- pH=5.6 (yeast)

May be added:

- Tween 80 =0,05% (a nonionic surfactant and emulsifier e.g. for lactobacilli and for molds)
- 20-60% (w/v) sucrose or 4% (w/v) NaCl (for yeast and molds originating from environments with high sugar or NaCl, respectively)

Saline: 0.9 % (w/v) NaCl

Ringer solution: 6.5 g NaCl, 0.42 g KCl, 0.25 g $CaCl_2$ and 0.2 g of sodium bicarbonate per liter

For cheese and fermented milk products:

- 2 % Na-citrate used in homogenization in Stomacher bag (dissolves cheese and fermented milk by binding Ca^{2+})

For LAB: Media, additional growth factors (used in inoculation, step 4)

LAB	Media (solid)	Extra growth factors, occasionally added)
lactobacilli*, *Pediococcus* and *Leuconostoc*	**MRS agar** (primarily used at MICRO) Rogosa or SL-agar	• Whey filtrate (*Leuconostoc* from dairy starters) • Orange juice (general growth promotion) • Tomato juice (general growth promotion) • Apple juice (Wine and juice spoilage organisms) • Un-hopped beer (Beer spoilage organisms) • Wine (ethanol)(Kefir isolates) • Wheat or rye bran extracts (sourdough isolates) • Fresh yeast extract (sourdough isolates) • 3-5% salt when isolating from high salt environments, e.g. cheese surfaces
Lactococcus, Streptococcus and *Enterococcus*	**M17 agar** (primarily used at MICRO) Elliker's agar	
Coryneform and *Staphylococcus*	**Tryptic soy agar (TSA)** (primarily used at MICRO) Plate count agar (PCA) Modified milk agar	
Carnobacterium and heterofermentative LAB	APT-agar	
Enterococcus	Bile Esculin agar Kanamycin Esculin Azise agar (both selective agar, used for isolating and identifying members of the genus *Enterococcus*)	

*Reclassification of the *Lactobacillus* genus was introduced by Zheng et al. (2020) introducing new genera. In this protocol lactobacilli is covering all of the new genera.

Useful selective agents for LAB (added to media, step 4)

Agent	Action
pH 5.7-4.5	Selects for lactobacilli*, Pediococcus, and Leuconostoc* against most other bacteria
10 mg/L 0.1% cycloheximide (primarily used at MICRO), approx. 0.2 % K-sorbate (sorbic acid)	Prevents growth of yeast and mould
1-2 mg/L 2-phenylethanol	Prevents growth of Gram negative bacteria
0.01-0.04 % Sodium-azide	Prevents growth of aerobic bacteria
50 µg/ml Vancomycin	Prevents growth of most other Gram-positive bacteria including homofermentative lactobacilli*, Lactococcus*, *Streptococcus*, and some *Enterococcus*
Calcium propionate (0.1-0.2%), Diphenyl, Thiabendazole, NaCl (4% (w/w), anaerobic incubation	Prevents growth of filamentous fungi

LAB incubation conditions (step 5)

Incubation 25-42°C for 3-5 days (depending on LAB)

Examples:

LAB	Temperature, days, atmosphere
lactobacilli, *Leuconostoc, Enterococcus, Carnobacterium*	30 °C, 3-5 days, anaerobic
Lactococcus, Pediococcus,	30 °C, 3-5 days, aerobic
Coryneform, Staphylococcus	25 °C, 3-7 days, aerobic, in light (to develop color)
Streptococcus *Streptococcus termophilus*	35-37 °C, 3-5 days, anaerobic 42 °C, 3-5 days, anaerobic

For Acetic acid bacteria: Media and incubation conditions (step 4 and 5)

Media	**Incubation conditions** **Temperature, days, atmosphere**
Glucose Yeast extract Carbonate (GYC) agar	25-30 °C, 3-6 days, aerobic
Deoxycholate-Mannitol-Sorbitol (DMS) agar	
Malt extract-yeast extract-acetic acid (MYA) agar	
Yeast extract-Peptone-Mannitol (YPM) agar	
Acetic acid-Ethanol (AE) agar	

For aerobic spore forming bacteria (e.g. *Bacillus*): Media and incubation conditions (step 4 and 5)

Media	**Incubation conditions** **Temperature, days, atmosphere**
Nutrient Agar (NA)	30 °C, 3-5 days, aerobic 80 °C for 10 min kills vegetative cells and induce spore germination
Plate count agar (PCA)	
Brain Heart Infusion (BHI) agar	
Blood agar (check for hemolytic activity)	

Useful selective agents for acetic acid bacteria and aerobic spore forming bacteria (added to media, step 4)

Agent	**Action**
10-20 mg/L cycloheximide (primarily used at MICRO), approx. 0.2 % K-sorbate (sorbic acid)	Prevents growth of yeast and mould
Calcium propionate (0.1-0.2%), Diphenyl, Thiabendazole, NaCl (4% (w/w), anaerobic incubation	Prevents growth of filamentous fungi

For yeasts: Media and incubation conditions (step 4 and 5)

Media	**Incubation conditions** **Temperature, days, atmosphere**
Malt Yeast Glucose Peptone (MYGP) agar (primarily used at MICRO)	25 °C, 3-5 days, aerobic
Yeast extract Peptone Glucose (YPG) agar	
Malt agar (MA)	
Wallerstein Laboratory Nutrient (WLN) agar	
Lysine agar a.o. (for non-Saccharomyces yeast)	

For stress tolerant yeast: Media and incubation conditions (step 4 and 5)

Media	**Selective for**	**Incubation conditions** **Temperature, days, atmosphere**
Yeast nitrogen base + NaCl (10 % (w/v)) + glucose (5 (w/v) %)	Moderately xerotolerant yeasts	25 °C, 3-10 days, aerobic
50 % (w/v) glucose-yeast extract agar	Xerotolerant yeasts	
Malt-extract agar + glucose (2, 20, 40, 50 % w/v)	Xerotolerant yeasts	
Wort agar + sucrose (3.5 % w/v) + glucose (1.0 % w/v)	Xerotolerant yeast from foods of high salt and sugar	
Potato-dextrose agar + sucrose (60 % w/v)	Sugar-tolerant yeast from concentrated orange juice	
Malt Yeast Glucose Peptone (MYGP) agar + NaCl 4% (w/v)	Halotolerant yeast	

Useful selective agents (added to media, step 4)

Agent	**Action**
MYGP, pH 5.6 + chlortetracycline and chloramphenicol (primarily used at MICRO)	Prevents growth of bacteria
Glucose yeast extract, pH 5.4-6.8 + oxytetraycline gentamycin	Prevents growth of bacteria
Glucose yeast extract, pH 5.4-6.8 + chloramphenicol	Prevents growth of bacteria
MYGP, pH 3.5	Prevents growth of bacteria

For molds: Media and incubation conditions (step 4 and 5)

Media	**Incubation conditions** **Temperature, days, atmosphere**
Malt-Extract agar (MEA)	25 °C, 3-5 days, aerobic
Potato Dextrose Agar (PDA)	
Dicloran 18% glycerol (DG18) medium (xerotolerant molds)	
Dicloran Rose Bengal Yeast Extract Sucrose agar (DRYES) (selective for *Penicillium* spp. And *Aspergillus* spp.)	

Useful selective agents (added to media, step 4)

Agent	**Action**
Rose Bengal, pH 5.4-6.8 + chlortetracycline	Prevents growth of bacteria
Dichloran rose Bengal, pH 5.4-6.8 + chlortetracycline	Prevents growth of bacteria
PDA, pH 3.5	Prevents growth of bacteria

Step 7: selection of representative colonies for identification and characterization

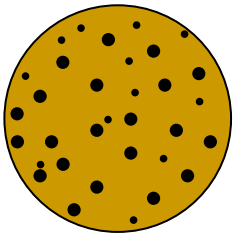
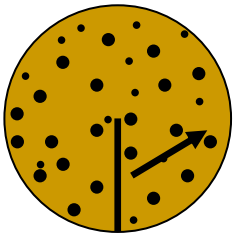
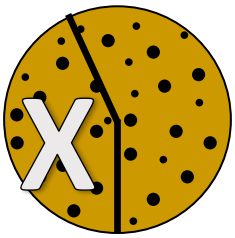


Plate with suitable number of colonies (25-200 for bacteria, 20-50 for yeasts). Preferably, use one petri dish for random isolation of colonies.

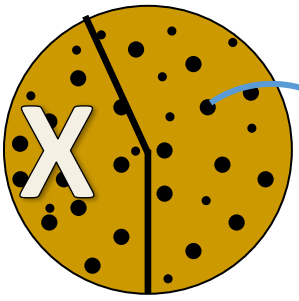


Using a ruler, make a line from the center to the edge of the plate. Count all colonies in the direction of the red arrow until reaching 20-25 isolates.

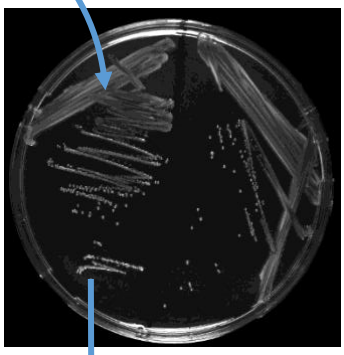


When reaching 20-25 colonies make a new line is made from the center to the edge. Pick/purify all colonies on the "right" side of the line (indicated by black arrow). The "left" side is here marked with X to indicate that no isolates are picked from this part of the petri dish.

Step 8: Purification of selected colonies

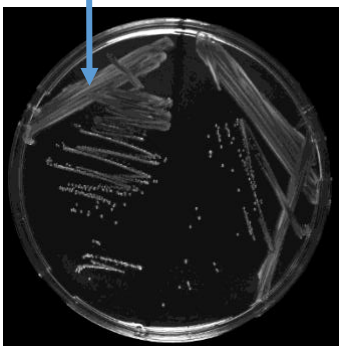


Transfer a single colony to a sterile tube with 100 µl diluent



Streak on the surface of appropriate agar media. Incubate at appropriate conditions.

Pure isolates have uniform colony characteristics and are free on any contamination.



One colony is streaked again on solid agar media to confirm the purity of the isolate.

Now you have a purified culture.

References

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Elliker's medium (Elliker et al. (1956), J. Dairy Sci. 39; 1611-1612)

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Rogosa or SL-agar (Rogosa et al. (1951), J. Bacteriol. 62; 132-133)

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