Isolation of yeasts and bacteria from food

Microbiology of Fermented Food and Beverages

PRACTICAL

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Bashir Aideh, Dennis Nielsen and Lene Jespersen, August 2024

Contents

I. Isolation and identification of yeasts and bacteria from food

Microorganisms play positive as well as negative roles in food. Some are essential for production of products such as wine, cheese, and sauerkraut. Others have the potential to spoil the food whereas some can play both roles (e.g. *Saccharomyces cerevisiae* is important for alcohol and flavour production during wine fermentations but has the potential to spoil sweet and semi-sweet wines after bottling). The aim of the laboratory exercise is to isolate yeasts and bacteria from different food products and subsequently identify selected isolates.

When examining food products for microorganisms several factors should be taken into consideration – e.g. composition of the food product (high/low salt; high/low sugar; pH etc.); manufacturing process; storage/handling; expected level of microflora (dilution/filtration/pre-enrichment) and the presence of accompanying microorganisms.

In this exercise different food products will be examined using a traditional culture-dependent approach. Table 1 shows the food products and microorganisms being the target of the group work.

Table 1: Products, target organisms and substrates

Group	Product	Organisms	Substrate	Temp	Atmosphere	Time
1-1,1-2	Sourdough (rye)	LAB	mMRS	30°C	Anaerobic	3-5 days
2-1,2-2	Kimchi	LAB	MRS	30°C	Anaerobic	3-5 days
3-1,3-2	Salami	LAB	MRS	30°C	Anaerobic	3-5 days
4-1,4-2	Kefir	LAB	MRS	30°C	Anaerobic	3-5 days
5-1,5-2	Red smear cheese (surface)	Coryneform/ *Staphylococcus*	TSA + 3% NaCl	25°C	Aerobic	3-5 days
6-1,6-2	Sourdough (rye)	Yeasts	MYGP	25°C	Aerobic	3-5 days
7-1, 7-2	Apple cider	Yeasts	MYGP	25°C	Aerobic	3-5 days
8-1, 8-2	Red smear cheese (surface)	Yeasts	MYGP + 4% NaCl	25°C	Aerobic	3-5 days
9-2, 9-2	Feta cheese	Yeasts	MYGP + 4% NaCL	25°C	Aerobic	3-5 days
10-1, 10-2	Serrano ham	Yeasts	MYGP	25°C	Aerobic	3-5 days

The culture-dependent approach includes a) sampling, b) pretreatment, c) plating, d) incubation, and e) determination of CFU. Subsequent to CFU determination, randomly chosen isolates will be identified by: i) picking and purification of isolates, ii) investigation of colony and cell morphology, iii) molecular typing using Rep-PCR $(GTG)_5$- fingerprinting, iv) grouping of isolates based on Rep-PCR-profiles (aided by Bionumerics), v) sequencing of rRNA genes (yeast, LAB) including database search, and vi) literature review of phenotypic tests and genotypic methods to confirm sequencing results.

Bashir Aideh, Dennis Nielsen and Lene Jespersen, August 2024

A preliminary work plan is shown in Table 2. See Fig.1 for illustration of the workflow.

Table 2: Work plan (adjust as needed in the groups)

Steps	Tasks
1	Sampling, pre-treatment, spread plating and incubation.
2	CFU determination. Picking and purification of isolates. Aim at 12 isolates in total. Select isolates **randomly**
3	Examination of purified isolates. If pure examine macro- and micro-morphology. If not pure repeat purification.
4	DNA extraction. Rep-PCR /(GTG)5 fingerprinting. Continue micro- and macro-morphological examination (if you have left over time)
5	Loading Rep-PCR samples Rep-PCR cluster-analysis. PCR for sequencing, gel loading, quality check of PCR products and sending for sequencing
6	BLAST search (database search) of sequences. Identification of isolates. Literature search for phenotypic tests.
7	Editing of presentations (product + lab work) and upload in Absalon (in lab groups)

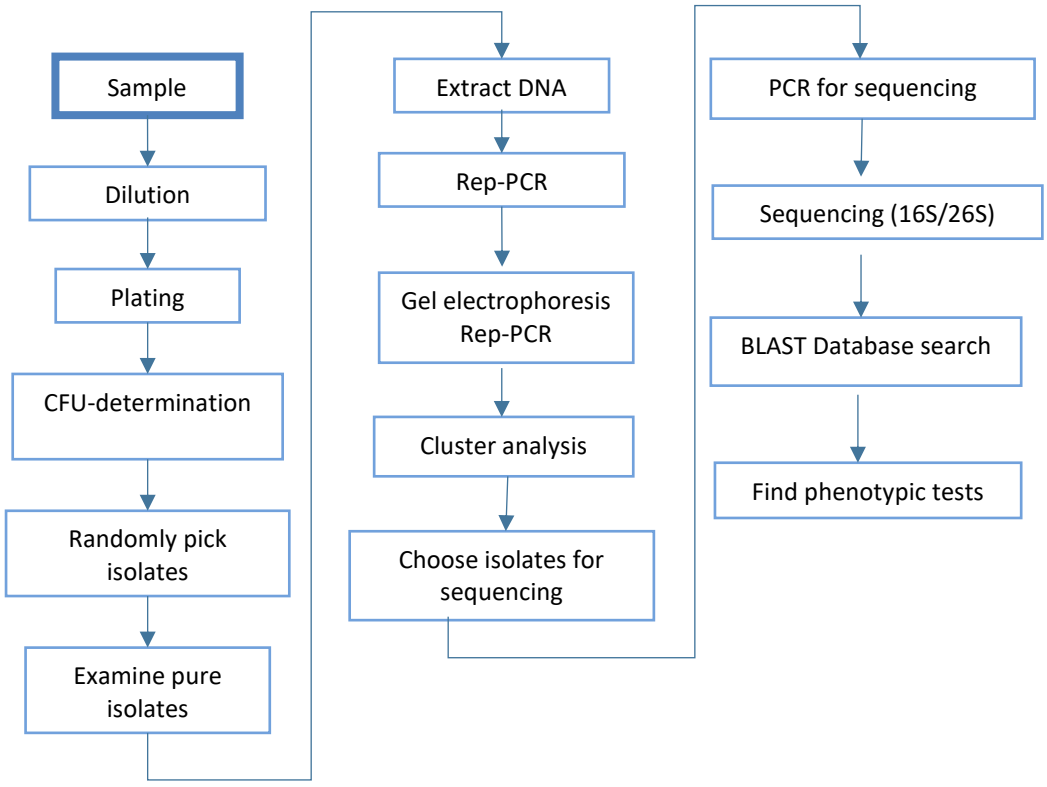


Fig 1. Workflow of sampling and identification

Day 1. Sampling, sample preparation and spread plating.

See appendix I for detailed step-by-step guide to sampling of the different food products. See appendix II for diluents and media.

1.1 Examination

Description of food product:

Prior to the examination a thorough description of the food should take place with respect to designation, packaging (intact?), manufacturer/importer, size of product and production and expiry dates. Date of examination should be registered as well.

Removal of sample:

The packaging, if there is one, is cleaned with 70% (v/v) ethanol. It is important to work aseptically to avoid contamination from the environment and e.g., scissors, scalpel and forceps should be sterilized before use (with 70% ethanol and flame).

Liquid food products:

If the sample is expected to contain only low levels of yeasts/bacteria 0.1 mL of the liquid may be spread plated directly after thorough mixing. Otherwise, serial dilutions are made by transferring 1 mL sample to 9 mL of SPO (pH 5.6 or 7.2, depending on products) to achieve a 10^{-1} dilution after mixing. This is repeated until a suitable dilution is reached.

Solid and semi-solid food products:

A 10 g sample of the food product is taken, so that both surface and interior is represented (unless otherwise specified). The weighing is done directly into a Stomacher bag into which the appropriate type of diluent (Table 2) is subsequently poured or pipetted until the weight reaches 10 times the weight of the sample (i.e., total weight should be 100 g if the sample size is 10 g). Homogenize for 1 min. at full speed or 2 min at medium speed according to food product, as specified in appendix I. After homogenization, sample + SPO constitute the 10^{-1} dilution.

Table 3: Type of SPO used for dilution series.

Product	Microorganism	Diluent
Sourdough (rye)	LAB	SPO pH 5.6
Kimchi	LAB	SPO pH 5.6
Salami	LAB	SPO pH 5.6
Kefir (milk)	LAB	2% Na_3-citrate pH 5.6 and SPO pH 5.6
Red smear cheese (surface)	Coryneform/*Staphylococcus*	2% Na_3-citrate pH 5.6 and SPO pH 5.6
Sourdough (rye)	Yeasts	SPO pH 5.6
Red smear cheese (surface)	Yeasts	2% Na_3-citrate pH 5.6 and SPO pH 5.6
Apple cider	Yeasts	SPO pH 5.6
Feta cheese	Yeasts	2% Na_3-citrate pH 5.6 and SPO pH 5.6
Serrano ham	Yeasts	SPO pH 5.6

1.2 Dilution

Serial dilutions of the 10^{-1} dilution are prepared as noted in Table 3, using SPO with appropriate pH for the product examined. See Fig. 1.

1.3 Inoculation

Either the pour plate or the spread plate techniques are chosen for isolation of microorganisms from food. In this exercise the spread plate technique will be used: An inoculum of 0.1 mL is spread at the surface of the relevant medium (Table 1): MYGP (Yeast), MRS-agar (LAB), mMRS (LAB from sourdough), and TSA (Coryneform).

Inoculate plates from three to five following dilutions based on Table 3, e.g., for LAB from sourdough (rye) spread plating of 10^{-5}, 10^{-6}, 10^{-7}.

All microorganisms: The ideal range for counting is 20-50 colonies/agar plate for yeast and 20-200 for bacteria. *Keep in mind that the inoculum used for spreading plating is 0.1 ml which means that a factor of 10 should be included in the CFU calculation.*

For yeasts: If the food is expected to contain a significant amount of LAB (or other bacteria capable of growing on the chosen medium/media) the MYGP agar plates should contain appropriate antibiotics.

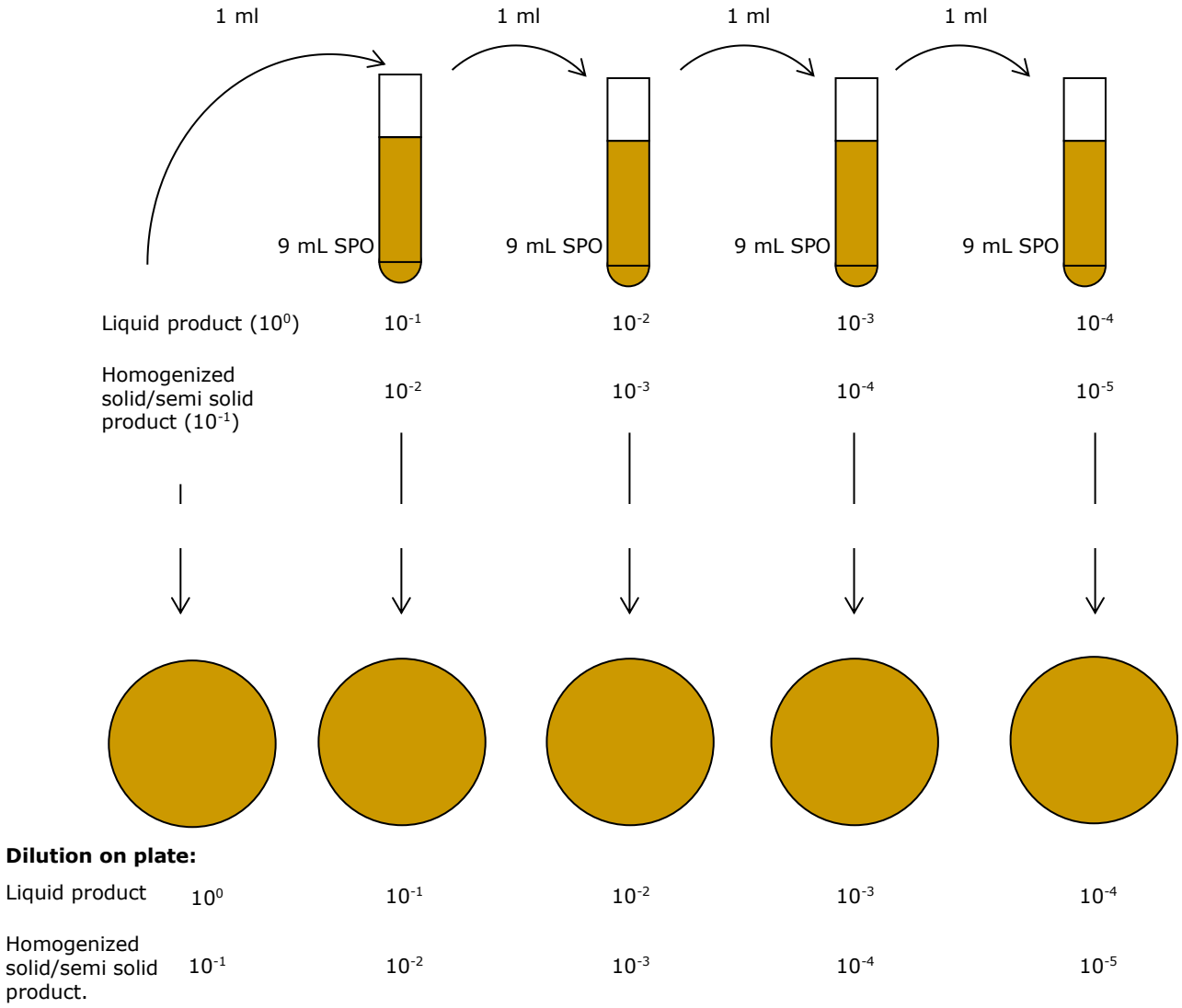


Fig 2. Example of dilution series and plating with the dilutions obtained on plates. Note the actual dilution on the plates, which will used for CFU calculations, section 2.1.

1.4 Incubation

The plates are inverted and incubated as follows:

Yeasts: aerobically at 25°C for 3-5 days

LAB: anaerobically at 30°C for 3-5 days, using anaerobic jars and anaerobic gas generator bags.

Day 2. CFU determination, randomly selection of isolates and purification

2.1 Reading results

For all products: Count the number of colonies from two suitable dilutions: 20-200 colonies. Calculate CFU/g or CFU/ml. Colony forming units pr. g (CFU/g) and ml (CFU/ml) are calculated as a weighted mean according to the following equation:

$$Weigthed\ mean\ (CFU) = \frac{c_1 + c_2}{n_1 + (n_2/10)} \times \frac{1}{V} \times \frac{1}{d_1}$$

Where C_1 = total count for dilution d_1 (the lowest dilution); C_2 = total count for the following dilution. V = volume inoculated (normally 1 ml for pour plate and 0.1 ml for spread plating).

Example for one replicate for spread plating:

Dilution	Replicates (n)	Colonies/plate	Total C
10^{-4}(d_1)	1 (n_1)	160	160 (c_1)
10^{-5} (d_2)	1 (n_2)	21	21 (c_2)

Calculation:

$$CFU/g = \frac{160 + 21}{1 + (1/10)} \times \frac{1}{0.1} \times \frac{1}{10^{-4}} = 1.65 \times 10^7$$

Example for two replicates for spread plating:

Dilution	Replicates (n)	Colonies/plate	Total C
10^{-4}(d_1)	2 (n_1)	158,170	328 (c_1)
10^{-5} (d_2)	2 (n_2)	18,23	41 (c_2)

Calculation:

$$CFU/g = \frac{328 + 41}{2 + (2/10)} \times \frac{1}{0.1} \times \frac{1}{10^{-4}} = 1.68 \times 10^7$$

See e.g., Adams & Moss, Food Microbiology, RSC, Cambridge for more details.

2.2 Random selection of colonies

1. Pick 8 colonies randomly, as shown in Fig 2 to be purified as described in section 2.3

Normally 20-25 colonies should be randomly picked and identified from a given sample. Due to time, space and consumable constrains 8 colonies are randomly picked in this exercise.

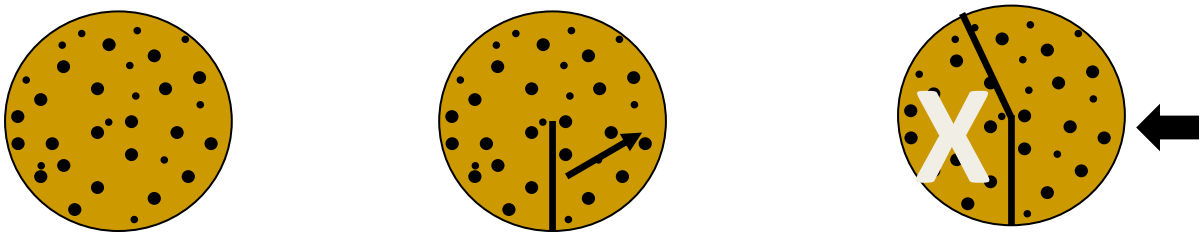


Plate with suitable number of colonies (25-200 for bacteria, 20-50 for yeasts). Preferably, use one petri dish for random isolation of colonies.

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

When reaching 20-25 colonies make a new line is from the centre 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.

Fig 3: Schematic presentation of the procedure for random picking of colonies in petri dishes.

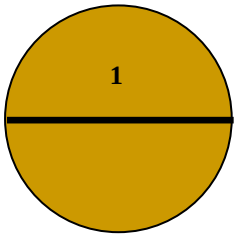
2.3 Purification of isolates

1. Divide agar-plates (choose the relevant agar plate to your microorganism) into two (= room for two isolates on each plate, see Fig. 3 below) and select test tubes with relevant broth
2. Then label plates and test tubes ("intelligent" numbering/naming of isolates is extremely important, remember to write your **group number** on the tubes). Each isolate must be traceable back to the product, dilution, and plate it was picked from. E.g., group no. and colony no.: Group 1-1 (1-1.1, 1-1. 2…….1-1.8), group 2-2 (2-2.1, 2-2. 2, ..2-2.8)
3. Pick a single colony and transfer it to 100 µL of sterile SPO in a sterile Eppendorf tube, vortex.
4. Streak a single streak (10 µl loop) along the side of the petri dish.
5. Use a new inoculation needle (10 µL loop) to streak on the remaining of the plate to obtain single colonies.
6. Transfer 25 µL of the suspension to a labelled test tube containing the relevant broth.

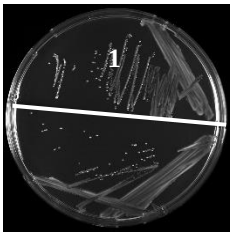
Incubation:

1. Incubate the plates as was done initially: 25°C aerobically for yeast and Coryneform/*Staphylococcus* and 30 °C anaerobically for LAB
2. Incubate the test tubes aerobically at 25 °C for yeast, Coryneform/*Staphylococcus*, and 30°C for LAB

Note. The identification can begin if the isolate is pure. Pure isolates have homogeneous colony characteristics on agar plates and are free from any contamination.



Divide plate into two.



Streak isolate onto half of the plate.

Transfer 25 µl from the same Eppendorf tube with SPO into test tube with relevant media for the microorganism.

Fig. 4: Purification of isolates

Day 3. Examination of purified isolates

3.1 Macro- and micro-morphological examinations

For the macro- and micro morphological examinations of the isolates use Appendix II and for:

- Yeasts: Jakobsen, M. and Jespersen, L (2000), revised 2012. Classifications of yeast 2, pp 1-28 or homepage: https://theyeasts.org/
- LAB: Bergey's Manual, 2nd edition, Volume 3 "The Firmicutes"

For LAB also perform the tests described below: (see appendix II)

- Gram test
- Catalase test
- Oxidase test
- CO_2 production test

Day 4. DNA extraction and Rep-PCR

4.1 DNA extraction

The Instagene kit is a rapid and cheap way of obtaining DNA of fairly high quality suitable for most PCR-based methods. If very long DNA fragments are needed more gentle DNA extraction methods should be used.

DNA is extracted from pure cultures (**only 5 colonies for each group**) using the Instagene kit (Bio-Rad) as following protocol:

1. Pick an isolated colony and resuspend it in 1 mL of autoclaved SPO in a microfuge tube.
2. Centrifuge for 1 minute at 10,000-12,000 rpm. Remove supernatant.
3. Add 100 µL of InstaGene matrix and dissolve the pellet.
4. Incubate at 56 °C for 20 minutes.
   NOTE: InstaGene matrix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. The pipet tip used should have a large bore, such as 1000 µL pipet tip.
5. Vortex at high speed for 10 seconds. Place the tube in a 100 °C heat block or boiling water bath for 8 minutes.
6. Vortex at high speed for 10 seconds. Spin at 10,000-12,000 rpm for 2 minutes.
7. Transfer the supernatant (the extracted DNA) to a new microfuge.
8. Store the extracted DNA at -20 °C. (the DNA can be stored at –18 °C for at least a year.)

NOTE: It is very important that the DNA extracted using Instagene is whirly mixed and subsequently centrifuged (13,000 x G, 3 min.) before use (this step MUST be repeated every time the DNA is used!!).

4.2 Rep-PCR/(GTG)5 fingerprinting.

Rep-PCR is molecular biology-based method very suitable for rapid grouping and tentatively identification of microorganisms. Eukaryotic and prokaryotic DNA contains so-called repetitive DNA elements distributed randomly over the genome. In rep-PCR primers that anneals to these repetitive elements are used. The PCR-products are separated using agarose gel electrophoresis and a species (sometimes strain)-specific pattern is obtained. These patterns can be analysed using e.g., BioNumerics. Isolates with similar patterns (i.e., belonging to the same species) will cluster together. Full identification can be achieved by e.g., sequencing a limited number of isolates from each group within the cluster. Various rep-PCR-primers have been developed. The primer GTG5 (5'GTG GTG GTG GTG GTG 3') seems to be very suitable for grouping of LAB and yeast at the species level, but other primers may prove better depending on the specific task (use the same overall approach, just change primers and possibly annealing and elongation temperature).

NOTE: Rep-PCR is only suitable for grouping isolates that have been partially characterised (e.g., catalase, Gram-reaction, and microscopy). A suitable group for Rep-PCR would for instance be Gram-positive, catalase-negative rods and cocci originating from MRS (i.e., presumptive LAB).

Protocol, rep-PCR using the GTG5-primer:

1. Extract DNA using Instagene (Bio-Rad) following the instructions of the manufacturer. Use only ½ volume of the Instagene mixture (100 µl instead of 200 µl).

2. Rep-PCR.
Rep-PCR mixture.

Compound	Volume 1 reaction
PCR Mastermix	13 µl
Primer (GTG5, 5 µM)	5 µl
Sterile MilliQ-water	4 µl
Sub-total	22 µl
DNA	3 µl
Total	25 µl

Prepare mixture (mastermix, primer, MQ-water) for appropriate number of reactions (calculate number of samples + 2 = e.g., 8 samples + 2 = 10 reactions). Add DNA to (labelled!!) PCR-tubes. Add 22 µl of mixture to each tube. Remember to include a blank (= no DNA).

NOTE: It is very important the DNA extracted using Instagene is whirli-mixed and subsequently centrifuged (13000 G, 3 min.) before use (this step MUST be repeated every time the DNA is used!!).

Use the following thermo cycling programme:

95 °C	5 min.	
95 °C	30 sec.	30 cycles
45 °C	60 sec.	
65 °C	8 min.	
65 °C	16 min.	
4 °C	24 hours	

3. Separate PCR-products by agarose gel electrophoresis: Prepare a 1.5 % agarose gel in 0.5×TBE. Fill running chamber with 0.5×TBE (10×TBE = 108 g Trisbase/l, 55 g boric acid/l and 40 ml of 0.5 M EDTA, pH 8.0). Load 10 µl of sample. Load 3 µl of marker (e.g., on a 30-well gel load marker in well 1, 15 and 30). Run electrophoresis (120 V, 5 h). (Done by students)

4. Stain gel with ethidium bromide (done by technicians)

5. Document gel using digital camera (done by technicians)

6. Carry out cluster analysis using BioNumerics (see guide in separate document). Use DICE alternatively Pearson. Use UPGMA as the clustering algorithm.

References for primers

Andrade, M. J., Rodriguez, M., Sánchez, B., Aranda, E., & Córdoba, J. J. (2006). DNA typing methods for differentiation of yeasts related to dry-cured meat products. *International Journal of Food Microbiology*, *107*, 48-58.
Gevers, D., Huys, G., & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiology Letters*, *205*, 31-36.

Nielsen, D. S., Schillinger, U., Franz, C. M. A. P., Bresciani, J., Amoa-Awua, W., Holzapfel, W. H., & Jakobsen, M. (2006). *Lactobacillus ghanaensis*; A novel motile lactic acid bacteria isolated from Ghanaian cocoa fermentations. *International Journal of Systematic and Evolutionary Microbiology.*, Submitted for publication-

Nielsen, D. S., Teniola, O. D., Ban-Koffi, L., Owusu, M., Andersson, T., & Holzapfel, W. H. (2007). The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture independent methods. *International Journal of Food Microbiology.*, *114*, 168-186.

Day 5. Bionumerics and PCR for sequencing

5.1 Cluster-analysis of Rep-PCR profiles

Cluster analysis normally is carried out using the Bionumerics software. As only a limited number of isolates (8 per team) are analysed, comparison is only done by eye. **Isolates with similar (GTG)5-profiles belong (generally) to the same species**.

Based on the fingerprints a few isolates are selected for sequencing. A good rule of thumb is to pick a few isolates from each "cluster" (= type of fingerprint) roughly corresponding to the square root number of isolates in the cluster (i.e., pick 3 isolates, if the cluster consists of 9 isolates). In this practical select **no more than 4 isolates** for sequencing.

5.2 PCR for sequencing

A rapid method for identification of microorganisms is by sequencing of specific genes (or fragments thereof). Today, the most widespread practice is to sequence a fragment of the genes encoding the ribosomal genes – often 16S in bacteria and 26S in yeasts. The hardware for carrying out sequencing reactions is quite expensive. Nowadays several companies offer sequencing as a service eliminating the need for investing in the expensive equipment.

5.2.1 For bacteria: Amplification and sequencing of the 16S rRNA gene

A PCR reaction is conducted using the DNA from Instagene isolation as a template and with the two primers 16S-27F (5'- AGAGTTTGATCMTGGCTCAG-3', which hybridises to the beginning of 16S rRNA gene) and 16S-1540R (5'-TACGGYTACCTTGTTACGACT-3', which hybridises to the 3' end of 16S rDNA). Using these two primers a product of approx. 1500 bp is expected.

Materials: PCR-mix (see below), PCR machine, 200 µl PCR tubes, pipettes

	Compound	Volume (1 reaction)
PCR-mix	PCR mastermix	25 µL
	Primer mix (27f+1540r (5 µM))	5 µL
	Sterile MilliQ-water	17 µL
	Sub-total	47 µL
	DNA	3 µL
	Total	50 µL

Procedure:
For each isolate belonging to a Rep-PCR group a PCR reaction is set up. You will sequence 1-2 isolat(s) per rep-seg group. The PCR reaction for sequencing is set up as described below:

1. Mark appropriate number of PCR tubes with group number and isolate number. Mark 1 tube extra as blank (=no DNA).
2. It is very important that the DNA extracted using Instagene is whirly mixed and subsequently centrifuged (13000 G, 3 min.) before use (this step MUST be repeated every time the DNA is used!!)
3. Prepare PCR-mix by mixing all volume of primer and all volume of MQ water into mastermix
4. Add 47 µl of mixture to each PCR-tube.
5. Add 3 µL DNA to (labelled!!) PCR-tubes
6. Add 3 µL sterile MilliQ-water to blank control PCR-tube.
7. Use the following thermocycling program:

95°C	5 min.	
95°C	30 s	Step 2-4: 35 cycles
60°C	30 s	
72°C	120 s	
72°C	10 min	
4°C	∞	

Reference for 16S rRNA primer sequences:
Jensen, M.P., Ardö, Y., Vogensen, F.K. (2009). Isolation of cultivable thermophilic lactic acid bacteria from cheeses made with mesophilic starter and molecular comparison with dairy-related *Lactobacillus helveticus* strains. Letters in Applied Microbiology 49, 396-402.

5.2.2 For yeast: Amplification and sequencing of D1/D2 region of the 26S rRNA gene

A PCR reaction is conducted using the DNA from Instagene isolation as a template and with the two primers: **NL1:** 5**'** GCA TAT CAA TAA GCG GAG GAA AAG 3' and **NL4:** 5' GGT CCG TGT TTC AAG ACG G 3', which amplify the D1/D2 region of the 26S rRNA gene in yeasts.

Materials: PCR mix (see below), PCR machine, 200 µl PCR tubes, pipettes

	Compound	Volume (1 reaction)
PCR mix	PCR mastermix	25 µL
	Primer mix (NL1+NL4 (5 µM))	5 µL
	Sterile MilliQ-water	17 µL
	Sub-total	47 µL
	DNA	3 µL
	Total	50 µL

Procedure:

For each rep-seq group choose 1 – 3 DNA extract and prepare separate PCR reactions in separate tubes. The PCR reaction for sequencing is set up as described below:

1. Mark appropriate number of PCR tubes with group number and isolate number. Mark 1 tube extra as blank (=no DNA).
2. It is very important that the DNA extracted using Instagene is whirli-mixed and subsequently centrifuged (13000 G, 3 min.) before use (this step MUST be repeated every time the DNA is used!!)
3. Prepare PCR-mix by mixing all volume of primer and all volume of MQ water into mastermix
4. Add 47 µl of mixture to each tube.
5. Add 3 µL DNA to (labelled!!) PCR-tubes
6. Add 3 µL sterile MilliQ-water blank control PCR-tube.
7. Use the following thermocycling program:

95 °C	5 min.	
95 °C	90 s	Step 2-4: 30 cycles
53 °C	30 s	
72 °C	90 s	
72 °C	7 min	
4 °C	ON/storage	

Reference for 26S rRNA primer sequences:

Jespersen, L., Nielsen, D.S., Hønholt, S., Jakobsen, M. (2005), Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. FEMS Yeast 5,441-453.

5.3 Agarose check of PCR products for sequencing

1. Prepare 1.5 % agarose gel (TBE-buffer) (done by lab technicians)
2. Load 5 µL of PCR product to a well in the gel
3. Load 3 µL 1kp marker to wells on the gel (done by lab technicians)
4. Run electrophoresis (2:30 min., 120 V) (done by lab technicians)
5. Stain gel with ethidium bromide (done by lab technicians)
6. Document gel using digital camera (done by lab technicians)
7. Results are uploaded to Absalon.

5.4 Selected isolates for 16S/26S rRNA gene sequencing

PCR products of isolates with “good” (i.e., strong, and single) bands of approx. 1500 bp for LAB and 600 bp for yeasts on agarose gels are sequened at MICRO using the Oxford Nanopore platform. Lab technicians will do the quality check of the PCR products before sending. Gels will be uploaded to Absalon. Technicians will conduct purification of PCR product, setup of sequencing reaction, purification of sequencing products, and analysis of sequencing products. Sequencing results will be uploaded to Absalon.

Day 6. Proof reading and BLAST search of sequence

Use the following workflow to identify the microorganisms:

1. Download relevant sequences from Absalon.
2. Use sequence to do BLAST -search at the homepage: https://blast.ncbi.nlm.nih.gov/Blast.cgi. Wait for answer (15-100 sec). Please be aware that there is no proofreading or review on the sequences uploaded in the database, therefore a critical mind is sometime useful! What does the result show?
3. For bacteria: After the initial NCBI BLAST then use www.eztaxon.org/ for BLAST against verified 16S rRNA gene sequences (compare results).

6. 1 Identification of isolates and description of relevant phenotypic tests

Identify the isolates based on the 16S/26S rRNA gene sequencing results, macro- and micromorphological characteristics. Remember that isolates with similar rep-seq profiles belong to the same species.

For isolates identified to species level, describe which phenotypic tests would be relevant to include to confirm the result obtained from the 16S/26S rRNA sequencing, macro- and micro morphological characteristics, e.g., fermentation of carbohydrates, growth at different temperatures, and further for yeast assimilation of carbohydrates and nitrogen etc.

For isolates only identified to genus level, describe additional tests which could be performed to to differentiate to species level including:

1. phenotypic tests (e.g., fermentation of carbohydrates, growth at different temperatures, for yeast assimilation of carbohydrates and nitrogen etc.)
2. genotypic methods

Relevant literature and webpages:

LAB:

Bergey's Manual of Systematic Bacteriology 2nd Edition, The Firmicutes.2009. Editors: Paul De Vos, George Garrity, Dorothy Jones, Noel R. Krieg, Wolfgang Ludwig, Fred A. Rainey, Karl-Heinz Schleifer and William B. Whitman.

For newer LAB species and more specific details on discriminative tests find relevant literature at List of Prokaryotic names withstanding in Nomenclature (LPNS): http://www.bacterio.cict.fr/. Please note that new reclassification of the *Lactobacillus* genus was introduced in 2020 and for the *Lactococcus* genus in 2021 see LPSN for standing names.

Yeasts:

http://www.theyeasts.org/

Kurtzmann, C. P., Fell, J.W. and Boekhout, T. (2010). The Yeasts - a taxonomic study. 5th Edt. Elsevier, Amsterdam, NL (can be downloaded in electronic form from REX).

Check the newest updates species descriptions of phenotypic characteristics for LAB and yeasts at: https://www.microbiologyresearch.org/content/journal/ijsem

In the powerpoint presentation, the following questions should be addressed:

1) Which methods (both pheno- and genotypical) are necessary for unambiguously identification of your yeasts/bacteria? Discuss the purpose of using them, i.e., why they are relevant. Include a discussion on what pheno- and genotypic tests you additionally could have performed in the lab to verify the identified species or differentiate to species/strain level.
2) Which yeasts/bacteria or genera and/or species did you based on your literature survey expect to find in the examined food product? Discuss how/if the CFU-values and microorganisms you found in your product correspond with what you expected, based on the literature. In case not, give suggestions for any differences observed.
3) Why is it relevant to determine the taxonomical identities of yeasts/bacteria isolated from fermented foods?

Appendix I –Step-by-step sampling, dilution and spread plating for each food examined.

Sourdough (rye), kimchi

Sourdough (rye) –LAB (group 1-1, 1-2)
Sourdough (rye) –yeast (group 6-1, 6-2)
Kimchi –LAB (group 2-1, 2-2)

Sampling
1- Use ethanol (70%) to sterilize tools.
2- Transfer 10.0 g aseptically to a sterile stomacher bag
3- Add 90.0 g of sterile SPO solution
4- Stomacher for 1 minute at high speed. This will homogenize microorganisms into the diluent and give you the 10^{-1} dilution.

Preparation of dilution row
1- From the 10^{-1} dilution transfer 1.0 ml into 9.0 ml sterile SPO solution to obtain 10^{-2} dilution
2- Mix by Vortex for 15 sec.
3- Repeat step 1 and 2. For LAB until you have obtained 10^{-6} dilution. For yeasts until you have obtained 10^{-5} dilution.

Plating of dilution for LAB enumeration:
1- Transfer 100 µl of 10^{-1} dilution to 10^{-6} dilution to MRS plates.
2- Spread dilutions using sterile spatula
3- Transfer plates to an anaerobic jar and insert a gas-generating kit.
4- Quickly close the jar and incubate at the required temperature (30°C) for 3-5 days

Note: Always incubate agar plates upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Plating of dilution for yeast enumeration:
1- Transfer 100 µl of 10^{-1} dilution to 10^{-5} dilution to MYGP + antibiotics agar plates
2- Spread dilutions on the surface using sterile spatula
3- Incubate aerobically at 25°C for 3-5 days

Note: Always incubate agar plates in a plastic bag upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Danbo cheese kefir (milk) and Feta Cheese

Red smear cheese surface –Coryneform (group 5-1, 5-2)
Red smear cheese surface –yeasts (group 8-1, 8-2)
Kefir (milk) –LAB (group 4-1, 4-1)
Feta cheeser –yeast (group 9-1, 9-2)

Sampling of cheese surface
1- Use an ethanol (70%) sterilized knife to scrape the surface from an area of 25 cm^2 (5*5 cm) and transfer to a sterile stomacher bag
2- Weigh the transferred amount of scraped cheese surface and add 9 times the weight of sterile 2% Na_3-citrate
3- Stomacher for 2 minutes at medium speed. This will homogenize microorganisms into diluent and give you the 10^{-1} dilution.

Sampling of kefir and Feta cheese
1- Use an ethanol (70%) to sterilize tools
2- Transfer 10,0 g aseptically to a sterile stomacher bag
3- Add 90.0 ml of sterile 2 % Na_3-citrate solution
4- Stomacher for 1 minute at high speed. This will homogenize microorganisms into diluent and give you the 10^{-1} dilution.

Preparation of dilution row
1- From the 10^{-1} dilution transfer 1.0 ml into 9.0 ml sterile SPO solution to obtain 10^{-2} dilution
2- Mix by Vortex for 15 sec.
3- Repeat steps 1 and 2. For LAB until you have obtained 10^{-6} dilution. For coryneform until you have obtained 10^{-8} dilution. For yeasts on Red smear cheese surface until you have obtained 10^{-7} dilution on and for kefir until you have obtained 10^{-5}

Plating of dilutions for coryneform and* Staphylococcus *enumeration
Plating is done on TSA + 3 % NaCl to select for coryneforme bacteria and *Staphylococcus.*
1- Transfer 100 µL of 10^{-4} to 10^{-9} dilution to TSA agar plates (6 per group).
2- Spread dilutions using sterile spatula
3- Incubate aerobically at 25°C in an incubator with light (or alternatively, on the table for 5-7 days

Note: Always incubate agar plates in a plastic bag upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Plating of dilution for yeast enumeration from surfaces of red smear cheese:
1- Transfer 100 µl of 10^{-4} dilution to 10^{-7} dilution to MYGP + 4% NaCl + antibiotics agar plates.
2- Spread dilutions on the surface using sterile spatula
3- Incubate aerobically at 25°C for 3-5 days

Note: Always incubate agar plates in a plastic bag upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Plating of dilution for yeast enumeration from Feta cheese:
1- Transfer 100 µl of 10^{-1} dilution to 10^{-5} dilution to MYGP + antibiotics agar plates.
2- Spread dilutions on the surface using sterile spatula
3- Incubate aerobically at 25°C for 3-5 days

Salami, serrano ham

Salami –LAB (group 3-1, 3-2)
Serrano ham –yeast (group 10-1, 10-2)

Sampling
1- Use an ethanol (70%) to sterilize tools
2- Transfer 10,0 g aseptically to a sterile stomacher bag
3- Add 90.0 g of sterile SPO solution
4- Stomacher for 1 minute at high speed. This will homogenize yeasts into the diluent and give you the 10^{-1} dilution.

Preparation of dilution row
1- From the 10^{-1} dilution transfer 1.0 ml into 9.0 ml sterile SPO solution to obtain 10^{-2} dilution
2- Mix by Vortex for 15 sec.
3- Repeat steps 1 and 2. For LAB until you have obtained 10^{-4} dilution. For yeasts until you have obtained 10^{-4} dilution.

Plating of dilution for LAB enumeration:
1- Transfer 100 µl of 10^{-1} dilution to 10^{-4} dilution to MRS plates.
2- Spread dilutions using sterile spatula
3- Transfer plates to an anaerobic jar and insert gas-generating kit.
4- Quickly close the jar and incubate at the required temperature (30°C) for 3-5 days

Note: Always incubate agar plates upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Plating of dilution for yeast enumeration:
1- Transfer 100 µl of 10^{-1} dilution to 10^{-4} dilution to MYGP agar plates.
2- Spread dilutions on the surface using sterile spatula.
3- Incubate aerobically at 25°C for 3-5 days.

Note: Always incubate agar plates in a plastic bag upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Apple cider

Apple cider –yeasts (group 7-1, 7-2)

Sampling

1- Homogenize apple cider before sampling
2- If the sample is expected to contain only low levels of yeasts, 0.1 mL of the liquid may be spread plated directly after thorough mixing (resulting in 10^{-1} dilution on plate). Otherwise, serial dilutions are made by transferring 1 mL sample to 9 mL of SPO to achieve a 10-1 dilution after mixing.

Preparation of dilution row

1- From the 10^{-1} dilution transfer 1.0 ml into 9.0 ml sterile SPO solution to obtain 10^{-2} dilution
2- Mix by Vortex for 15 sec.
3- Repeat steps 1 and 2 until you have obtained 10^{-7}.

Plating of dilution for yeast enumeration:

1- Transfer 100 µl of 10^{-4} dilution to 10^{-7} dilution to MYGP agar plates.
2- Spread dilutions on the surface using sterile spatula.
3- Incubate aerobically at 25°C for 3-5 days.

Note: Always incubate agar plates in a plastic bag upside (the lid) down to avoid condensation drips from the lid and to prevent drying out.

Appendix II – Diluents and media

All diluents and media are prepared in advance.

I.1 Saline peptone water (SPO)

NaCl (Merck 106404)	8.5 g
Bacto Peptone (Difco 0118-17-0)	1.0 g
Na_2HPO_4, $2H_2O$ (Merck 106580)	0.3 g
Distilled water is added up to	1.0 L.

pH = 5.6 ± 0.1 (yeast, LAB)/7.2 ± 0.1(bacteria)
Autoclave at 121°C for 15 min.

Optional:
SPO with 4% (w/v) NaCl: The amount of NaCl in SPO is adjusted to 40 g/L.
SPO with 10% (w/v) sucrose: SPO is added 100 g/L sucrose.

I.2 YPG broth

D (+)-Glucose, H_2O (Merck 104074)	10.0 g
Bacto Peptone (Difco 0118-17-0)	10.0 g
Yeast Extract (Difco 0127-17-9)	5.0 g
Distilled water is added up to	1.0 L.

pH = 5.6 ± 0.1

Distribute in tubes with 10 mL in each tube.
Autoclave at 121°C for 15 min.

I.3 MYGP agar

D (+)-Glucose, H2O	10.0 g
Bacto Peptone (Difco 0118-17-0)	5.0 g
Yeast Extract (Difco 0127-17-9)	3.0 g
Malt Extract	3.0 g
Bacto agar	20.0 g
Distilled water is added up to	1.0 L.

pH = 5.6 ± 0.1
Autoclave at 121°C for 15 min.

To prevent growth of bacteria 100 mg chloramphenicol and 50 mg chlortetracycline are added per litre of MYGP.

Chloramphenicol stock:

Chloramphenicol (Sigma C0378)	0.50 g
96% ethanol	10.0 mL

Before autoclaving 2 ml stock solution/litre substrate is added.

Chlortetracycline stock:

Chlortetracycline (Sigma C4881)	0.50 g
Distilled water is added up to	100 mL.

The solution is filter-sterilized through a 0.22 μm filter. 10 mL stock solution/litre substrate is added after autoclaving and cooling to 50°C.

I.4 MRS

Enzymatic digest of casein	10.0 g
Meat extract	10.0 g
Yeast extract	4.0 g
Tri ammonium citrate	2.0 g
Sodium acetate	5.0 g
Magnesium sulphate heptahydrate	0.2 g
Manganese sulphate tetrahydrate	0.05 g
Dipotassium hydrogen phosphate	2.0 g
Glucose	20.0 g
Tween 80	1.08 g
Agar	15.0 g
Distilled water is added up to	1.0 L.

To prevent growth of yeast 0,01% cyclohexamide is added.

pH is adjusted to 5,6±0.2 (for starter cultures in cheese, pH is adjusted to 6.2)
Autoclave for 10 min at 115°C

I.4a Modified-MRS for sourdough (Modified 638 LACTOBACILLUS MEDIUM III)

Tryptone	10.0 g
Meat extract	5.0 g
Yeast extract	5.0 g
Fresh yeast extract	15.0 mL
Glucose	7.0 g
Fructose	7.0 g
Maltose	7.0 g
Na-gluconate	2.0 g
Na-acetate x 3 H_2O	5.0 g
(NH_4)2-citrate	2.0 g

K_2HPO_4 x 3 H_2O		2.6 g
$MgSO_4$ x 7 H_2O		0.1 g
$MnSO_4$ x 4 H_2O		0.05 g
Cysteine-HCl x H_2O		0.5 g
Tween 80	1.0 mL	
Distilled water is added up to		1.0 L

Adjust pH to 5.6 with 20% lactic acid or HCl. Fresh yeast extract is prepared by autoclaving a 20% suspension of commercial baker's yeast in distilled water for 30 minutes at 121°C, allowing the suspension to settle overnight at 2 to 8°C, decanting and further clarifying the supernatant by centrifugation. The extract prepared in this manner contains 1.5% solids and if not to be used within a few days, should be frozen or freeze-dried immediately.

To prevent growth of yeast 0,01% cyclohexamide is added.

Appendix II – Preliminary phenotypic tests

Macro-morphology (Colony morphology on solid media), see Fig 5.

Isolate no./name								
Colony size Pinpoint = < 1 mm Small = 1-2 mm Medium = 2-4 mm Large = > 4 mm								
Surface (glistering, dull, smooth, rough, sectored, folded, finely hairy, ridged)								
Margin/rind (entire, undulate, crenulated, lobate, fringed)								
Profile/Elevation (smooth, smooth raised, smooth crateriform, flat, crateriform, convex wringled, flat wringled, fringed with pseudohyphae)								
Pigment/color								
Smell								
Mycelium -Yeasts only								

Liquid substrate (for yeasts only), see Fig 5.

Isolate no./name								
Sediment (weak, moderate, strong)								
Consistency of sediment (powdery, granulated, sticky)								
Pellicle/surface layer (creeping, ring)								
Flocculent/turbid growth								

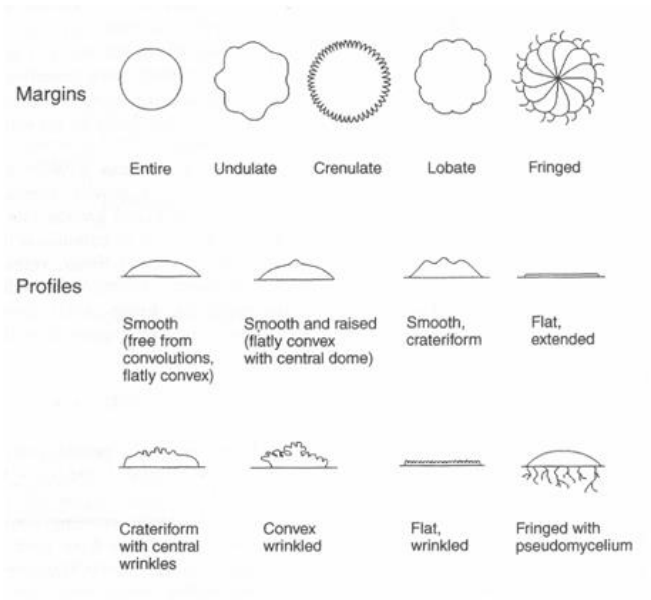
Other characteristics for LAB, see pp. 29-31

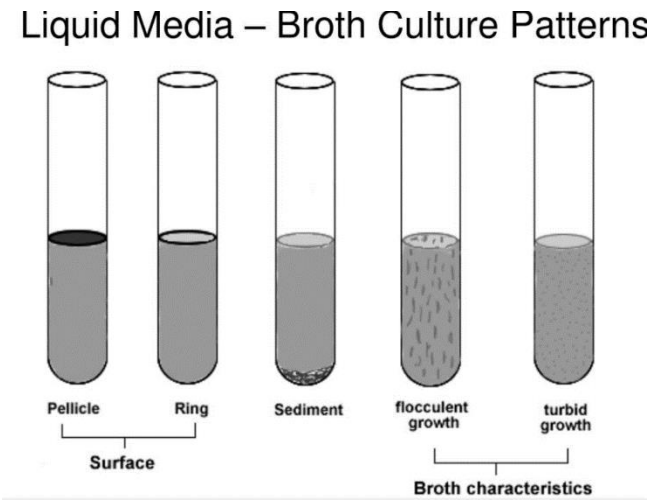
Isolate no./name								
Gram test (with KOH) • Positive reaction = ropy slime = G- • Negative reaction = no slime = G+								
Catalase activity (H_2O_2 test) Positive reaction = bubbles = C+								
Oxidase activity (Blue colour after max 10 sec = positive)								
CO_2 Air in Durham tube = positive/negative								

Micro-morphology/Cell morphology, see Fig 6-9.

	Isolate no./name								
	Shape: bacteria (coccus, rod, spore forming rod). **Yeasts:** (globose, spheroidal,ellipsoidal, ovoid, lemon shaped, elongated)								
	Cell arrangement (single, pairs, short chains (<5), long chains, tetraeds, clumps, branching)								
	Motility								
	Spore morphology								
FOR YEASTS ONLY	**Vegetative reproduction** (multilateral, mono- or bi-polar budding, fission, arthroconidia)								
	Mycelium (pseudo- or true hyphae)								

Surfaces: Glistering, dull, smooth, rough, sectored, folded, finely hairy, ridged





Pellicle: creeping (yeast growing up along the sides of the tube)/flat (on the surface)

Ring: Growth on the surface, but only close to the side of the tube

Sediment: weak/moderate/strong

Consistency of sediment: powdery/granulated/sticky.

Smell: esters/putrid/sulphuric, etc.

Fig. 5. Schematic presentation of macro-morphologies and their descriptive terms.

Catalase test

The catalase test is very important for differentiating lactic acid bacteria from other Gram-positive bacteria. LAB together with *Clostridium* are catalase negative, while all other Gram-positive bacteria are catalase positive. However, some strains of LAB may have a pseudo-catalase activity.

Catalase is an enzyme, which splits H_2O_2 into water and oxygen:

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

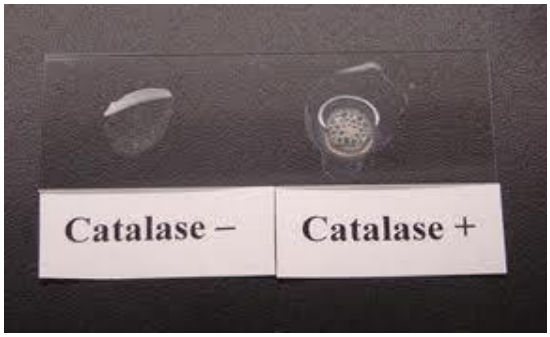
A fresh culture (1-2 days old) is used for the catalase test. Incubate at optimum temperature either on agar or in broth.

Reagent:
3 % Hydrogen peroxide (H_2O_2)
Add 90 mL distilled water to 10 mL 30 % H_2O_2. Store in tight-fitting flasks. Shelf life is 3-4 weeks.

Technique:
The catalase reaction can be analysed on a slide (you can conduct 2-3 analyses on the same slide). Mix a drop of 3% H_2O_2 with colony material or broth culture. The reaction is easier to see if you place a cover glass over the slide. Look for gas generation for 3-5 min. If necessary, follow the reaction under a microscope at low magnification.
You can also drip 1 drop of 3% H_2O_2 directly onto colonies (not colonies on blood agar though).

Colony:
Use a glass pipette or a disposable plastic inoculation needle to transfer a small amount of the colony material to a microscope slide and mix with one drop of 3 % H_2O_2.

Broth culture:
Transfer one drop of bacteria suspension to a slide and mix with one drop of 3% H_2O_2.



Reading:
Positive reaction (catalase$^+$) always causes small air bubbles (O_2). Negative reaction (catalase$^-$) does not generate any gas.

Errors:
The H_2O_2 used loses strength when left to stand. If you suspect that the H_2O_2 solution is too weak, test on known weak catalase$^+$ strains (e. g. *E. coli*). If these do not react as expected, then the H_2O_2 solution is not OK. Blood has a catalase activity. It is important, therefore, that blood is not extracted along with the colony material from a blood agar plate. You should also pay attention to production of pseudo-catalase in certain bacteria cultivated

on a medium containing blood. If you use a platinum inoculation needle to mix H_2O_2 and the colony material, you will cause a false positive reaction because the platinum will catalyse splitting of H_2O_2.

Gram test – Potassium hydroxide test

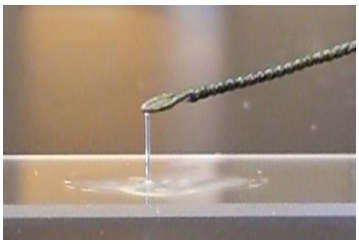
The KOH test can be used as a quick method of distinguishing Gram-positive and Gram-negative bacteria. The test is because freshly grown colonies of Gram-positive bacteria are not lysed by a 3 % KOH solution. The Gram-negative bacteria cell wall is destroyed, and DNA is freed, producing a ropy slime.

Reagent: Dissolve 3.0 g KOH in 100 ml distilled H_2O. Store airtight in filled plastic bottles (or plastic bottles from which air have been squeezed out) at room temperature. Dependent on the access of air the solution may work for 4-12 weeks.

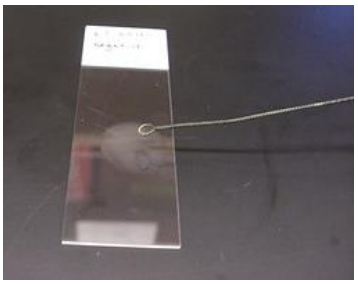
Technique: Put one drop of 3 % KOH on a slide. Mix a small amount of the colony material into the KOH-solution with a looped inoculation needle. After stirring a few times (5-10), lift the inoculation needle and observe whether ropy string occurs (positive reaction).

Reading:

Positive reaction: ropy string = Gram-negative bacteria.



Negative reaction: no ropy string = Gram-positive bacteria.



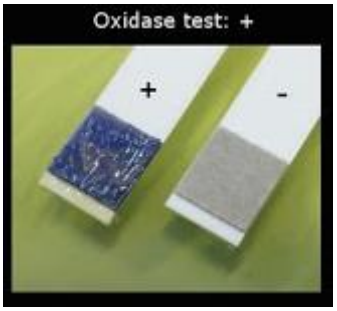
Comments: The KOH test agrees with the results of the Gram stain in most cases (> 80 %). It does not, however, make the Gram stain superfluous, as the KOH test is only effective with colony material. The Gram stain is still one of the best ways to assess the composition of the bacteria directly in food samples.

Oxidase test

The oxidase test is a test used to determine if a bacterium produces certain cytochrome c oxidases. It uses disks impregnated with the redox indicator reagent N, N-dimethy1,4-p-phenylene diammonium chloride. The reagent is a dark blue to maroon colour when oxidized, and colourless when reduced.

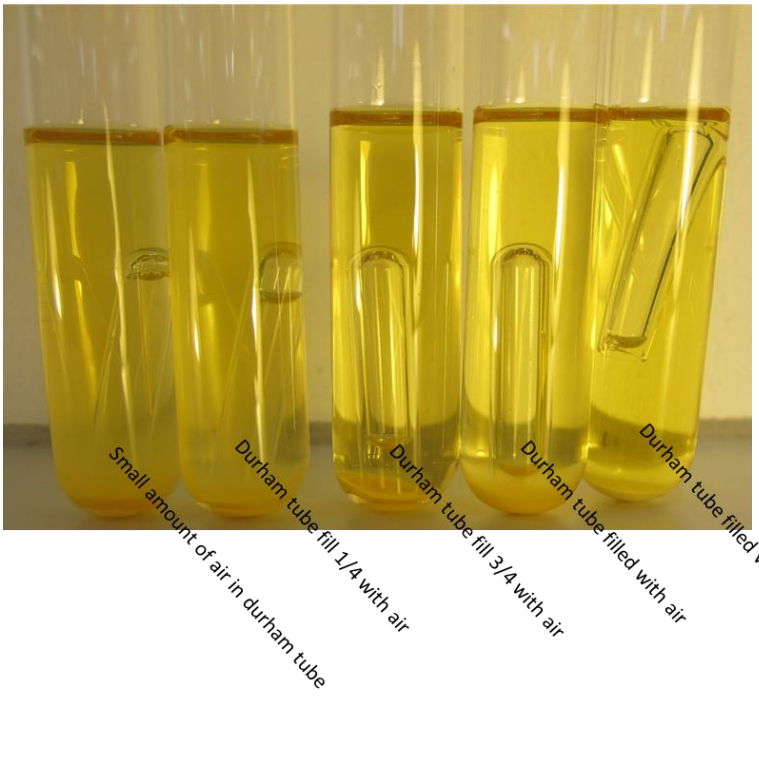
Technique:
Wet each disk with about 10 µL of deionized water. Use a loop to aseptically transfer a large mass of pure bacteria to the disk. Observe the disk for up to 3 minutes.

Reading:
Positive reaction: If the area of inoculation turns dark blue to maroon to almost black within 10-30 seconds.
Negative reaction: If a colour change does not occur within three minutes. The colour of the disk is light pink or absence of colouration.



CO_2 production test

Inoculate MRS-tube containing an inverted Durham tube with one colony. **Do not whirly mix!** Incubate 1-3 days (30 °C). If CO_2 is produced the Durham tube will be filled (or partly filled) with air and the isolate is obligate heterofermentative.



Cell shapes

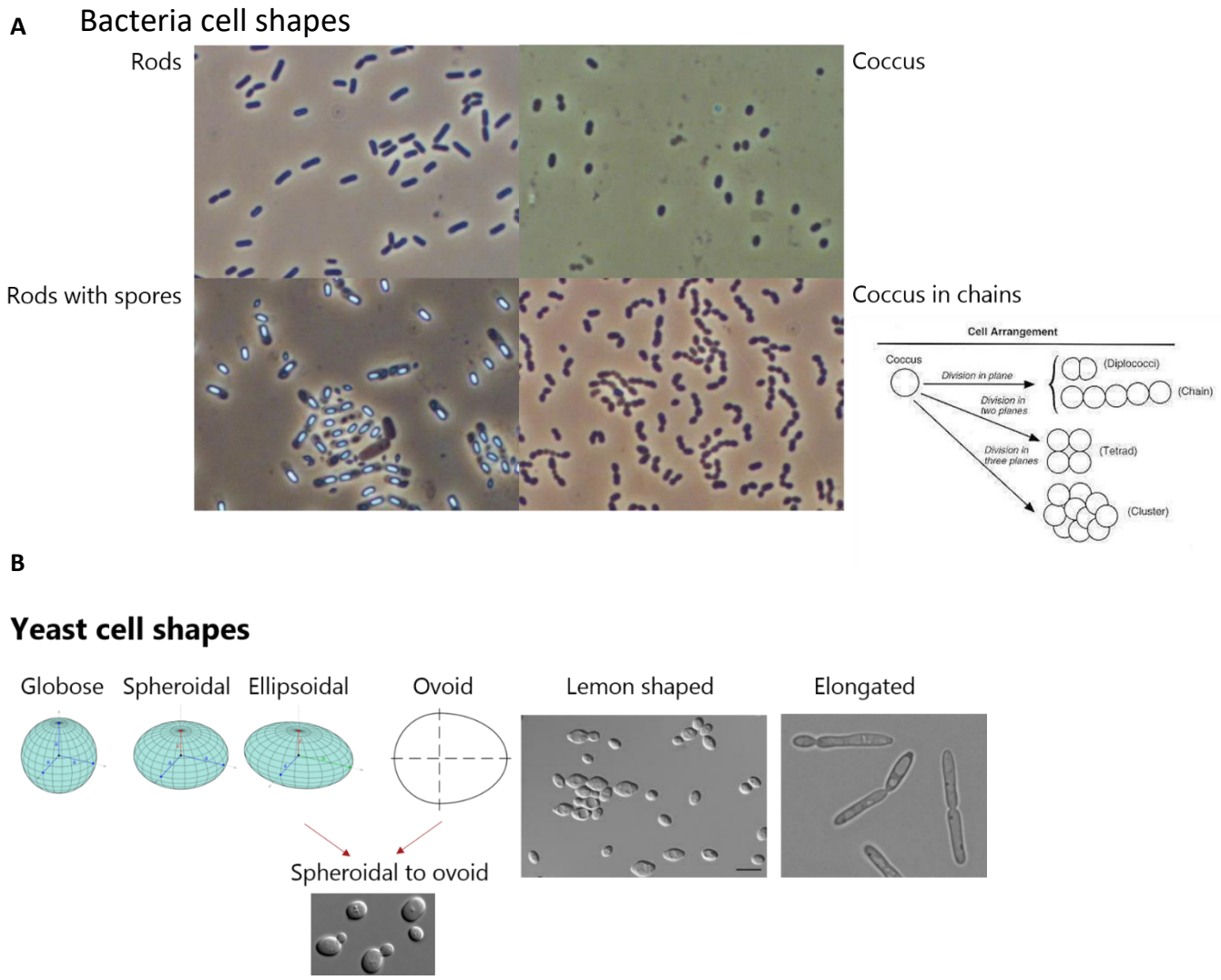


Fig. 6. Schematic presentation of micro-morphology and their descriptive terms for A) lactic acid bacteria, acetic acid bacteria, aerobic spore forming bacteria, coryneform and staphylococci as well as for B) selected yeasts.

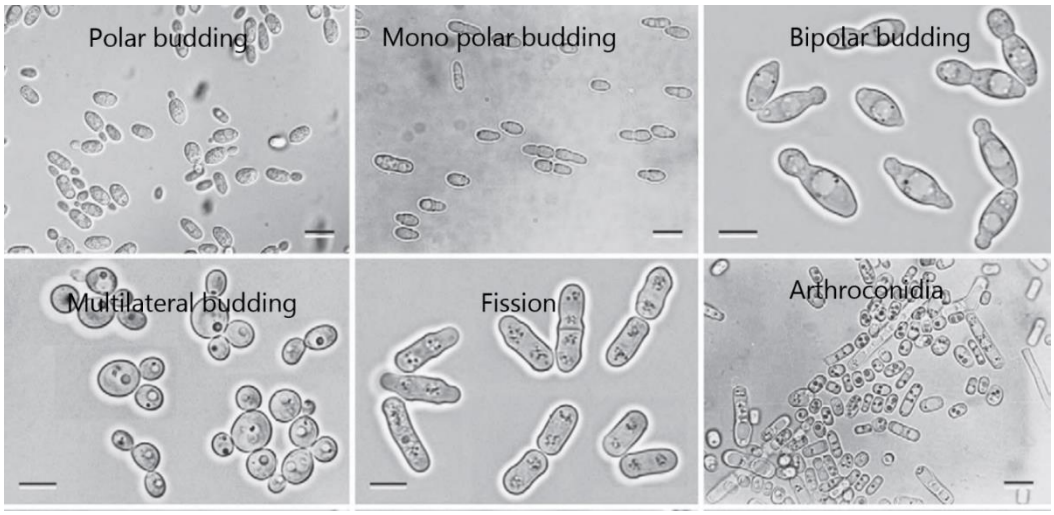
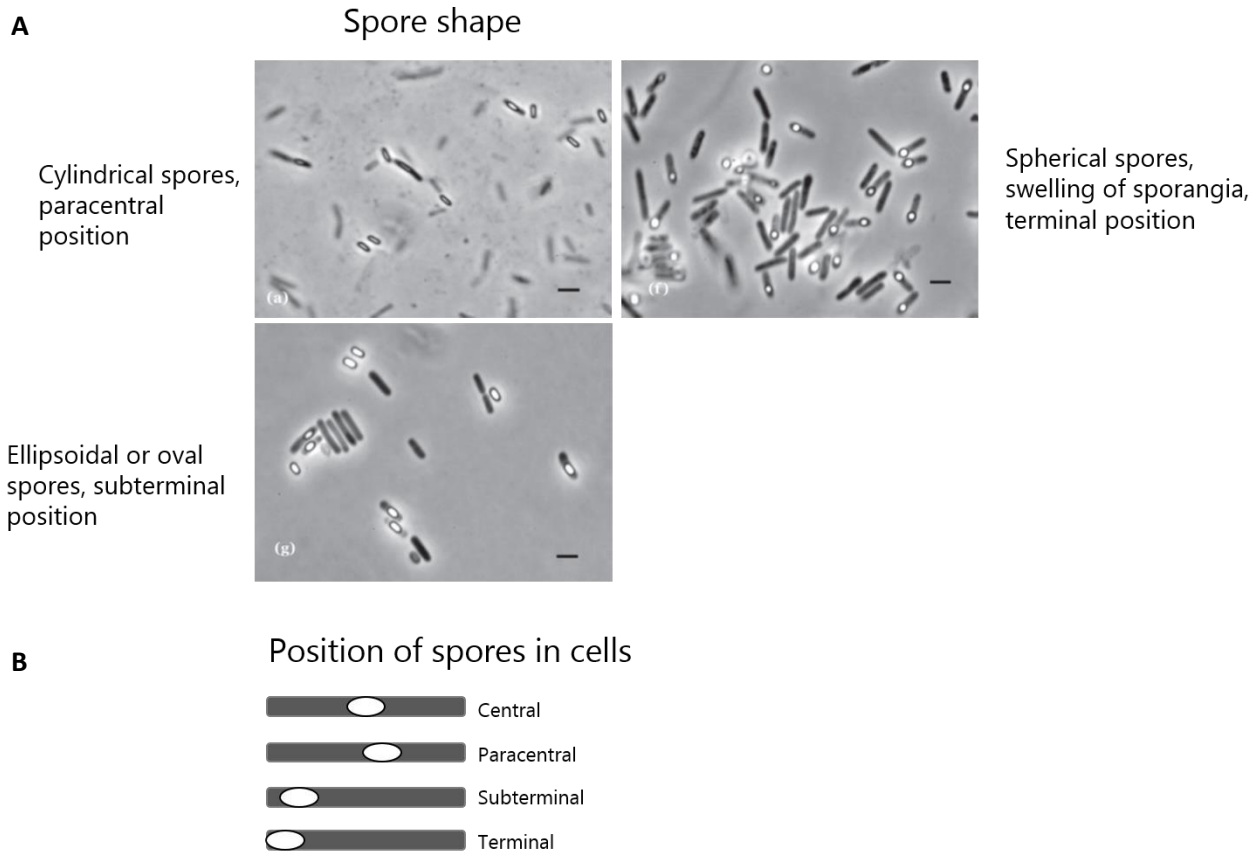


Fig. 7 Vegetative formation by yeasts (adapted from Kurtzman, C.P., Fell, J. W. and Boekhout, T. (2011) The Yeasts, a Taxonomic Study, 5th ed., Elsevier, Amsterdam).



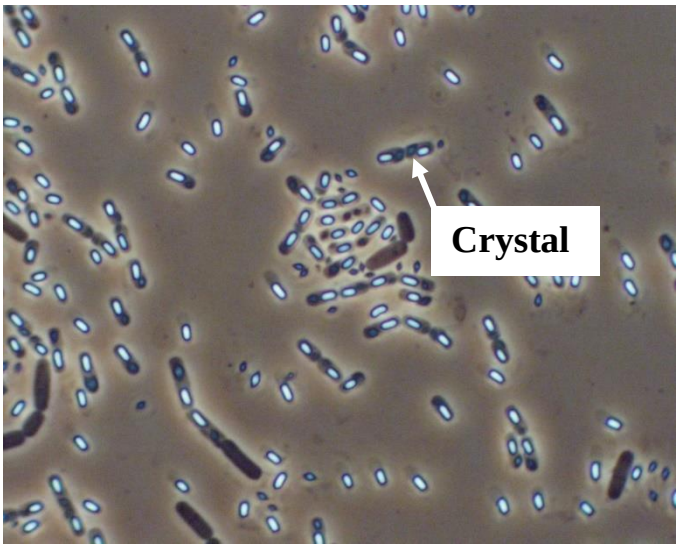


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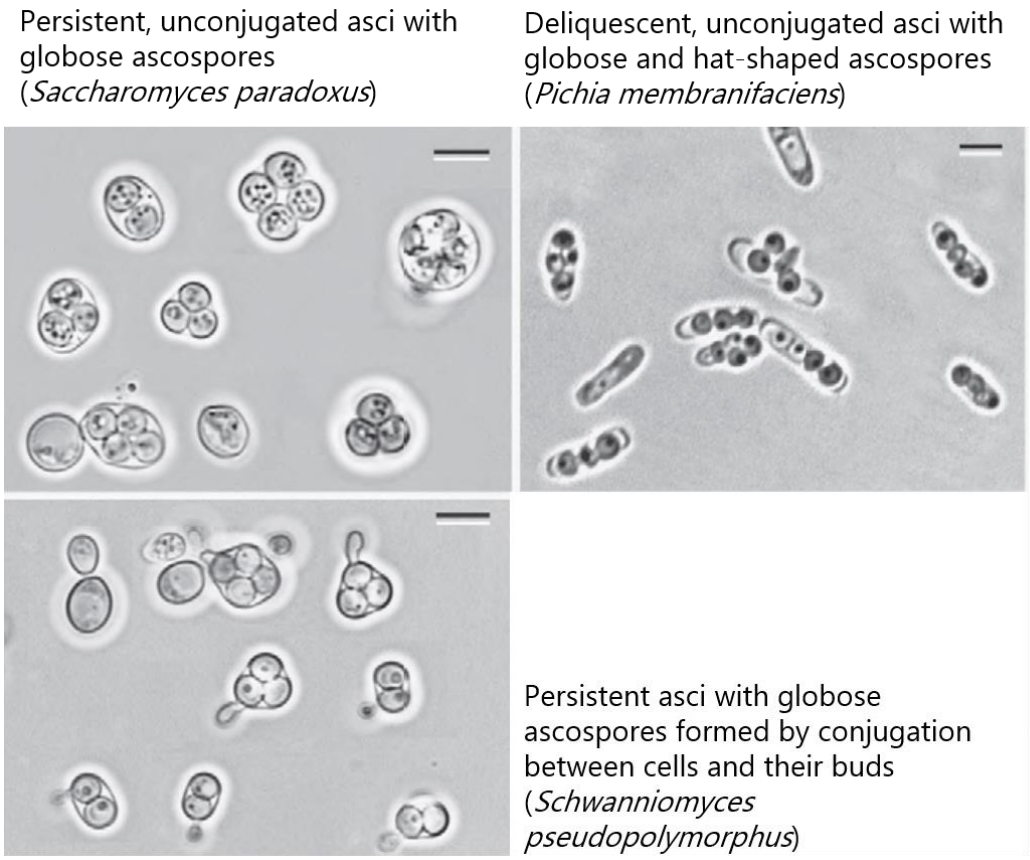
Fig. 8 A) Morphology of aerobic endo sporeforming species as viewed by phase-contrast microscopy. (Adapted from Fig, 10, Bergey's Manual of Systematic Bacteriology 2009: Volume 3: The Firmicutes). B) Sporepositions. C) Crystal toxin formation in sporulating *B. thuringiensis* cells (Michael Crafack, 2011).



Descriptive terms for spores in yeasts:
Formation of spores: none/conjugated/unconjugated.
Shape of spores: spherical/hat-shaped/ellipsoidal/saturn-shaped

For ascosporogenous (endogen spore-formation) yeast also note the following:
Number of spores per asci: 1,2,3,4
Formation of asci: on loose cells/on hyphae/on mycelium

Fig. 9. Morphology of some yeast spores (adapted from Kurtzman, C.P., Fell, J. W. and Boekhout, T. (2011) The Yeasts, a Taxonomic Study, 5th ed., Elsevier, Amsterdam).