Microbiology of Fermented Foods & Beverages - MFFB

Notes for the course NFOK14019U, at the University of Copenhagen September-November 2024

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Chapter 1
Course description

1.1 Course content

The course has major focus on the microorganisms involved in the processing of various fermented foods and beverages. The course includes the taxonomy of important microorganisms especially lactic acid bacteria (LAB) and yeasts covering both phenotypic characteristics and molecular typing techniques for their identification. Fermentation is introduced as a sustainable green technology and innovative technologies to improve fermented foods and beverages are considered.
Methodologies for isolation will be covered, including both culture dependent and culture independent techniques. Techniques such as high-throughput sequencing and digital programmes for bioinformatics are applied.
Various fermentation techniques is introduced covering the use of starter cultures and other fermentation techniques such as back-slopping. Further, the role of fermentation in sustainable food production and in prevention of food waste will be discussed.
An introduction to various fermented foods and beverages will be given including products such as cheese, bread, wine and beer as well as a number of traditional indigenous fermented food and beverages. Focus will additionally be on microbial interactions including topics such as quorum sensing, bacteriocin formation, etc.

1.1.1 Learning objectives

The objective of the course is to give the students a thorough knowledge on the microbiology behind production of fermented food and beverages and to give the students skills within isolation and identification of microorganisms occurring in these products. Additionally the students will be able to evaluate the functionalities and applications of microbial starter cultures.

Knowledge

- Show overview of fermented food and beverages in general and the microorganisms involved in their production
- Describe important groups of microorganisms identified from fermented food and beverages
- Comprehend microbial taxonomic systems
- Describe microbial interactions and their importance in food systems
- Reflect on microbial cytology and physiology
- Define molecular techniques for identification and typing to species and strain level

Skills

- Apply procedures for isolation and identification of the predominant microorganisms in fermented food and beverages
- Explain at the molecular level the behaviour and interactions between various groups of microorganisms
- Assess the most important parameters leading to optimal product quality and food safety
- Apply food fermentation to develop innovative food products

Competences

- Predict the composition of the microbiota of specific fermented food and beverages
- Discuss presumed functionalities of microorganisms in fermented food or beverages related to product quality and food safety
- Communicate and work independently on own data and discuss the results in relation to existing literature

1.1.2 Teaching and learning methods

Lectures, theoretical and laboratory practicals. The lectures will introduce issues of importance for the understanding of microbial behaviour during production of fermented food and beverages. The theoretical and laboratory practicals will give the students practice on how to identify various microorganisms from fermented food and beverages including skills within various molecular techniques and digital tools for bioinformatic purposes. Knowledge on food innovation will be obtained throughout the course.

1.1.3 Exam

At the time of the oral examination, 20 min, one theoretical question is drawn, and the examination proceeds without preparation time. Two weeks before the exam, the questions will be given to the students. At the oral exam, the drawn question and the curriculum will account for 75% of the grade. The discussion of the laboratory work and the experimental results will account for 25% of the grade.

Chapter 2
Lecture Notes

2.1 04.09.24 - Microscope

2.1.1 Parts of the microscope

The Microscope is put together by different components, as shown in Figure 2.1.

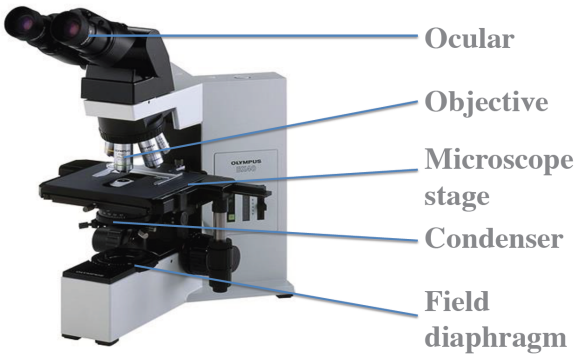


Figure 2.1: The different components of a microscope.

The main components are:

- Ocular

  This is the set of lenses that you peer into, when you use a microscope. The "only" purpose of this part is to relay the intermediate image created by the objective to the retina of our eyes, i.e. to make us see an image. The ocular also magnifies the intermediate image with the factor of the ocular (usually around 10x); however, increasing the magnification of the ocular does not increase the resolving power (finer details of the specimen will not be revealed)

- Objective

  Resolves the structure of the specimen, creating an intermediate image inside the microscope. The image is magnified with the factor depicted on the objective (often 10X, 40X and 100X). The objectives can be changed by rotating the objective turret, but this must be done cautiously to avoid mechanical damage. Objectives fitted for phase contrast will also be labelled with the size of the phase ring (usually this is depicted on the objective as either PH1, PH2 or PH3)

- Microscope stage

  The (translation) microscope stage holds the specimen firmly with a specimen holder, and allows the specimen to be moved in fine increments in two dimensions

---

- Condenser

  * The condenser is located underneath the microscope stage. The purpose of this optical part is to illuminate our specimen optimally

  * Our microscopes for microbiological work have a condenser with a rotating turret that enables the use of phase contrast imaging, as a specific annular ring in this turret must be matched with the size of the phase ring in the objective. There is a cut-out on the front of the turret that depict the actual selection. In this picture the Ph2 ring has been selected

  * Apart from the phase rings, the turret also includes a blank opening that allows for traditional brightfield observation depicted with a 0, and a setting for darkfield depicted with a DF

2.1.2 Contrast methods

Brightfield

This simply magnifies the specimen. However it is almost impossible to see cells, without a staining procedure, that selectively stains parts of the cells. Quite often, a stained specimen is also fixed, which means that the cells have been killed, so for live specimens (e.g. live bacteria), we need a different contrast method.

Darkfield

This is only useful for 10X objectives or smaller, so for observing virtually all living cells.

Phase contrast

This is the best method. The theory behind phase contrast is a little complicated (but hey - the guy won the Nobel Prize), but you can utilize phase contrast without knowing the theory. All you have to remember is:

- The number on the phase objective and the number on the condenser turret must match to obtain a proper image (e.g. Ph 2 for the 40 x objective)
- The Field diaphragm in the bottom of the microscope should be completely open

2.1.3 How to use the microscope

Preparing the microscope

Set the ocular distance to match YOUR interpupillary distance; The distance between the eyes of people differs a lot. Our microscopes are equipped with two eyepieces, in order for you to look down the microscope in the same way as you look through a binocular, and ultimately the same way as you look on the world as such. Therefore, the first thing to do when you approach a microscope is to adjust the ocular distance to your individual interpupillary distance. The ocular distance can be read on a mm-scale (commonly 50-75) between the oculars.

The interpupillary distance is extremely easy to obtain, because you simply ask a friend to measure the distance between your pupils with an ordinary ruler. Memorise this value (because it is the same for the rest of your life), and adjust the oculars to your distance before you do anything else. This increases the comfort of using a microscope tremendously.

2.2 04.09.24 - Yeast: Cytology, taxonomy and physiology

2.2.1 Background

Yeast are quite different from bacterias, first of all, they are bigger than bacterias. Yeast are also eukaryotic, which means that they have a nucleus, and they have a lot of organelles. The yeast *Saccharomyces cerevisiase* has a pair of 16 chromosomes, which is quite a lot for a yeast and thus making it a non-haploid. The many differences from bacteria will influence their identification, their growth characteristics as well as the interactions with the food matrix. Proper identfication of yeast is therefore important for the correct use of starter cultures as well as for spoilage yeasts.

2.2.2 Eukaryotes

Since yeast are Eukaryotes, here are some key features of Eukaryotes:

- Eukaryotes contain far more DNA than prokaryotes (x 100-1000). Yeast genome sizes range from 10-15 Mb and encode 5000-10000 genes
- The DNA is organised in a number of chromosomes
- The chromosomes are organised in a nucleus
- The presence of mitochondria
- The presence of different organelles such as: the Golgi apparatus and the endoplasmic reticulum
- Besides partly being involved in killer toxin production, the function of plasmids in yeasts is not really known

2.2.3 Organelles

- **CW: Cell wall (physical protection, adhesion etc.)**
- P: Periplasm
- **CM: Plasma membrane (transport in/out of the cytoplasma)**
- CMI: Invagination
- BS: Bud scar
- C: Cytoplasm
- **N: Nucleus (DNA in chromosomes)**
- **M: Mitochondrion (ATP synthesis, enzymes)**
- S: Secretory vesicles
- **V: Vacuole (protein trafficking, storage)**
- PER: Peroxisome
- ER: Endoplasmic reticulum
- G: Golgi apparatus

In this course, we will focus on the cell wall, the plasma membrane, the nucleus, the mitochondrion and the vacuole. Though the other listed organelles are important, they are not as important for the physiology of yeast.

Cell wall

The cell wall gives the yeast physical protection against the environment. It also maintains the shape of the yeast cell, and it is important for the osmotic stability of the yeast cell. The cell wall also has permeability barriers, which means that solutes larger than 620-760 Da can not pass the cell wall. The cell wall also has enzyme support, cation binding, cell-cell recognition and adhesion to other surfaces.

Plasma membrane

The primary functions of the yeast plasma membrane are to dictate what enters and what leaves the cytoplasma i.e. to be a primary barrier for passage of hydrophilic molecules and prevent cytoplasmic contents to mix with the environment. The plasma membrane is also important for the uptake of nutrients, the excretion of waste products, the maintenance of the cell shape and the cell wall, and the cell division.

Nucleus

2.2.4 Methodologies for species identification of yeast

There are various methods for species identification of yeast, including:

- Micro- and macromorphological identification added phenotypic characterisation
- Sequencing of the D1/D2 domain of the 26S rRNA gene (> 99% homology = belong to the same species)
- Sequencing of multiple genes
- Sequencing of the ITS region (mostly moulds and yeasts identified by metagenomics)
- Identification of protein patterns by MALDI-TOF MS
- Analysis of biomas by Fourier Transform Infrared Spectroscopy (FTIR)
- Others...

2.2.5 Yeast taxonomy

The taxonomy of yeast is quite complex, but it is important to know the different levels of taxonomy. The taxonomy of yeast is as follows:

- Kingdom: Fungi (Both yeast and moulds are fungi)
- **Phylum:** e.g Ascomycota // Basidiomycota
- **Class:** e.g *Saccharomycetes*
- **Order:** e.g *Saccharomycetales*
- Family: e.g *Saccharomycetaceae*
- Genus: e.g *Saccharomyces*
- Species: e.g *Saccharomyces cerevisiae*

The following is not universally recognized, but are used in some contexts:

- Subspecies: e.g *Saccharomyces cerevisiae* subsp. *cerevisiae*
- Variety: e.g *Saccharomyces cerevisiae* var. *cerevisiae*
- Strain: e.g *Saccharomyces cerevisiae* subsp. *cerevisiae* strain *S288C*

2.2.6 Substrates for yeasts

Following are some examples of some substrates which are greatly used for the propagation of yeasts:

- Malt yeast extract Glucose Peptone agar (MYGP)
- Yeast extract Peptone Glucose (YPG) agar
- Malt Agar (MA)
- Lysine agar a.o. (for non-Saccharomyces yeasts)
- WLN medium (allows separation by coloration)
- Others...

There are som stress tolerant yeasts where high concentrations of substrates are needed in the media for the growth of specific yeast. Some examples of medias for stress tolerant yeasts can be seen in 2.2.

Purpose	Media
Moderately xerotolerant yeasts	Yeast nitrogen base + sodium chloride (10% (w/v)) + glucose (5 (w/v)
Xerotolerant yeast	50% (w/v) glucose-yeast extract agar
Xerotolerant yeast from foods of high salt and sugar	Wort agar + sucrose (3.5% w/v) + glucose (1.0% w/v)
Zygosaccharomyces rouxii	Potato-dextrose agar + sucrose (60% w/v)
Sugar-tolerant yeast from concentrated orange juice	Glucose-citric acid-tryptone agar
Isolation and cultivation of xerotolerant yeast	Malt-extract agar + glucose (2, 20, 40, 50% w/v)

Figure 2.2: Examples of medias for stress tolerant yeasts.

2.2.7 Conventional methods for identification of yeast

There are various methods for identification of yeast, here are a list of some of the most common methods:

- By their cultural characteristics
  - Solid media: colony morphology, color, size, shape, surface, margin, elevation, texture
  - Liquid media: turbidity, sediment, pellicle, ring, flocculation
- By their cell morphology and arrangement
  - Cell form and size
  - Vegetative reproduction by budding or fission
  - Pseudomycelium or true mycelium
- By their sexual characteristics
  - Perfect stage (*teleomorph*) or imperfect stage (*anamorph*)
  - By their formation and arrangement of spores
- Biochemical characteristics
  - Assimilation of carbon or nitrate sources (e.g. API20c, ID32c)
  - fermentation of carbohydrates
- Physical and chemical tolerance
  - Growth in the presence of 100 or 1000 ppm cycloheximide
  - Growth in the presence of 1% acetic acid
  - Growth on 50% glucose-yeast extract agar
  - Etc.

2.2.8 Macro-morphological characteristics

There are many different macro-morphological characteristics of yeast, some of the ones we will be using in this course laboratory exercises can be seen in figure 2.3.

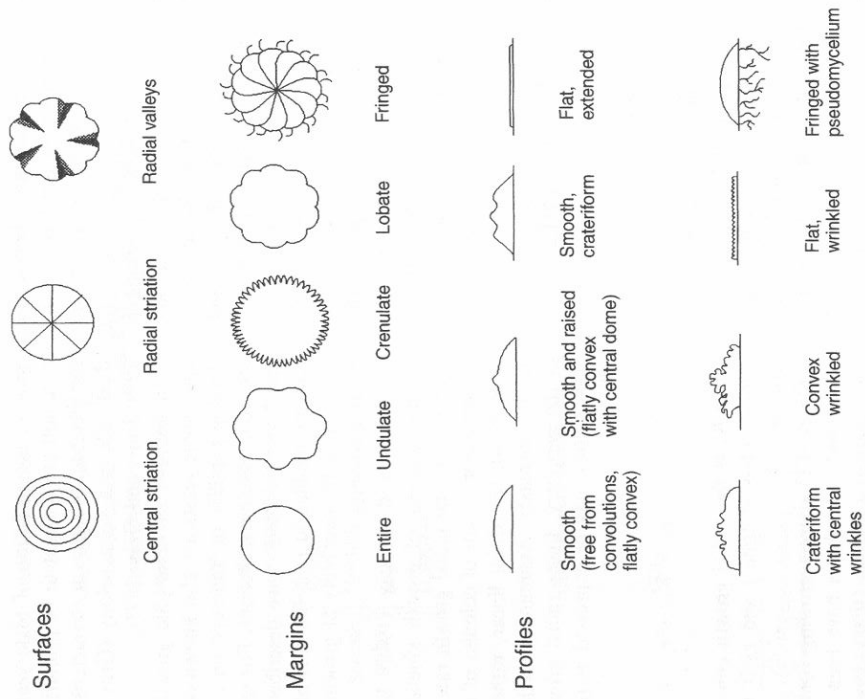


Figure 2.3: Examples of macro-morphological characteristics of yeast that will be evaluated in the laboratory exercises.

Furthermore, the lecture included a list of some key aspects to keep in mind while looking at the macro-morphological characteristics colonies. These are:

- Tecture (mucoid, viscous, matted, coherent etc.)
- Color
- Size
- Surface (glistening, smooth, verrucose, rough etc.)
- Elevation
- Margin (entire, rhizoid etc.)
- Mycelium (x100 magnification)

2.2.9 Micro-morphological characteristics

Micro-morphological characteristics are also important when identifying yeast. Some of the key aspects are listed below:

- Cell form (spheroidal, ellipsoidal, ovoid, lemon-shaped, elongated, triangular etc.)
- Cell size (length, width)
- Cell arrangement (pairs, aggregates)
- Vegetative reproduction
  - Budding (monopolar, bipolar, multilateral)
  - Fission
- Mycelium
  - Pseudomycelium (not distint septa)
  - True mycelium (distint septa)

2.2.10 Assimilation and fermentation

Assimilation refers to the process by which an organisms takes in and incorporates nutrients from the environment. The assimilation of carbon compounds is important for the growth of yeast. Yeast assimilates carbon compounds by various pathways, like Krebs cycle (TCA pathway) or the glycolysis.
Fermentation is the process by which yeast converts sugars into alcohol and carbon dioxide. The fermentation of carbohydrates typically happens under anaerobic conditions. The fermentation process is a less efficient energy source than respiration. Nevertheless, without it, wine and beer could not be made. Brewers therefore exploit the yeasts fermentative capability by introducing yeast into an anaerobic environment.

Assimilation of carbon compounds

To propagate yeast while having assimilation in mind, the growth medium Yeast Nitrogen Base from "Difco" can be used. This medium contains:

- Hexoses
- Trisaccharides
- Pentoses
- Organic acids
- Disaccharides
- Polysaccharides
- Alcohols
- Glycosides

Fermentation of carbon compounds

Here are a list of some of the carbon compounds that yeast can ferment:

- Glucose
- Maltose
- Trehalose
- Galactose
- Raffinose
- Melibiose
- Saccharose
- Lactose
- Inulin

Assimilation of nitrogen compounds

To propagate yeast while having assimilation in mind, the growth medium Yeast Carbon Base from "Difco" can be used. This medium contains:

- Nitrate
- Ethylamine hydrochloride
- L-lysine
- Creatine
- Nitrite
- Cadaverine dihydrochloride
- Creatinine

2.2.11 Vegetative reproduction (genera)

Vegetative reproduction is the process by which a plant or fungus reproduces asexually. Yeast can reproduce vegetatively by budding or fission. Some examples of yeast vegetative reproduction is:

Bipolar

- *Hanseniaspora (Kloeckera)*
- *Rhodotorula**
- *Saccharomycodes*
- *Trichosporon*

Fission

- *Schizosaccharomyces*

Arthroconidia

- *Galactomyces (Geotrichum)*
- *Saccharomycopsis**

Enterobalstic Budding

- *Phaffia*

Multilateral

- *Candida*
- *Debaryomyces*
- *Dekkera (Brettanomyces)*
- *Kazachstania*
- *Kluyveromyces*
- *Pichia*
- *Rhodotorula**
- *Saccharomyces*
- *Saccharomycopsis**
- *Torulaspora*
- *Yarrowia*
- *Zygosaccharomyces*

*Two types of vegetative reproduction my be present

2.2.12 Lecture conclusion

- Yeasts are predominantly unicellular eukaryotes
- Yeasts might have both imperfect and perfect names
- Yeast taxonomy is changing continuously especially due to new molecular techniques
- Yeasts are currently mostly identified by sequencing of the D1/D2 region of the 26S rRNA gene – however, never forget to verify by examining morphology and include phenotypic tests
- Remember to consult the newest scientific literature in order to use up dated taxonomic names

2.3 09.09.24 - Taxonomy and the species concept

2.3.1 Lecture content

During this lecture the following topics will be the focus point.

- Definitions of taxonomy and phylogeny
- Phylogeny
- Microbial taxonomy: from domain to species
  - Species concept in Bacteria
  - Species concept in Yeast and Moulds
- Subspecies, variant and strains

2.3.2 Taxonomy and phylogeny

From figure 2.4 we can see that the common ancestor of Bacteria, Archaea and Eukaryota is the root of the tree, and the branches of the three domains is what sums up the tree.

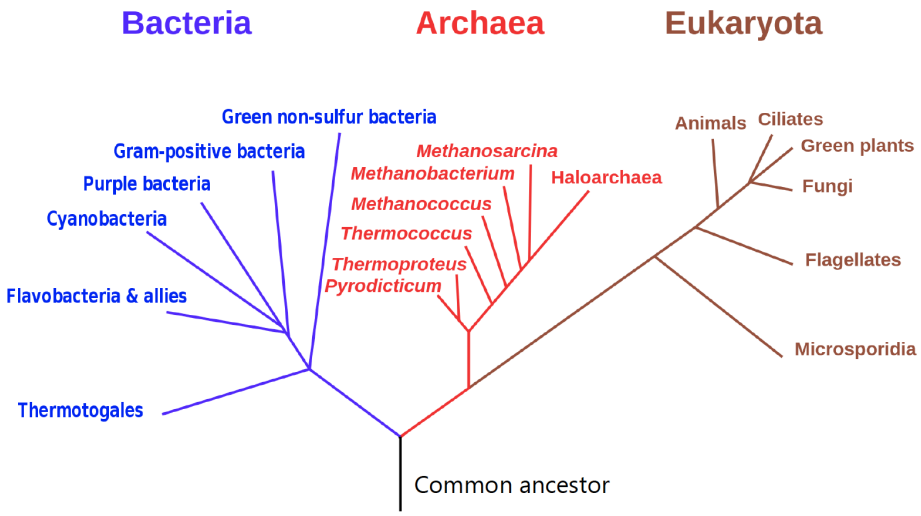


Figure 2.4: The comon ancestor of Bacteria, Archaea and Eukaryota and the som of their respective branches

A definition **Taxonomy**, this the naming and classification of organisms based on shared properties, which can be based on

- Genotypic properties
- phenotypic properties
- Phylogeny - evolutionary relationships

While working with taxonomy and phylogeny in a broder sense, some of the following terms are important to know:

- **Isolation:** The separation of a pure culture from a mixed culture
  Sampling: The collection of a representative part of a population
  Colony purification: The isolation of a single colony from a mixed culture
  DNA extraction: The isolation of DNA from a pure culture
- **Identification:** The determination of the taxonomic position of an organism
  Microscopy: The determination of the morphology of an organism through a microscope
  Sequencing: The determination of the sequence of a gene or a genome

While looking at the phylogeny we can look deeper into the classification, in table 2.1 we can see an example of the classification of a Bacteria and Eukarya where we stop at species. This can be further divided into subspecies, variant and strains.

Table 2.1: An example of the classification of a Bacteria and Eukarya

Domain	Bacteria	Eukarya
Kingdom	None assigned	Fungi
Phylym	Firmicutes	Ascomycota
Class	Bacilli	Saccharomycetes
Order	Lactobacillales	Saccharomycetales
Family	Lactobacillaceae	Saccharomycetaceae
Genus	*Lactobacillus*	*Saccharomyces*
Species	*L. acidophilus*	*S. cerevisiae*

In the following table, there will be listed some differences between prokaryotes and eukaryotes.

Table 2.2: Differences between prokaryotes and eukaryotes

	Prokaryotes	**Eukaryotes**
DNA	DNA is naked DNA is circular Usually no introns	DNA is bound to protein DNA is linear Usually has introns
Organelles	No nucleus No membrane-bound 70S robossoms	Has a nucleus Membrane-bound 80S ribosomes
Reproduction	Binary fission Single chromosomes (haploid)	Mitosis & meiosis Chromosomes paired (diploid or more)
Average size	Smaller (1-5 μm)	Larger (10-100 μm)

2.3.3 Gram-positive & -negative bacteria

(Almost) all bacteria can be divided into two groups, Gram-positive and Gram-negative bacteria. The difference between the two groups is the cell wall. The cell wall protects the bacteria e.g against osmotic stress. The cell wall differs where the Gram-positive bacteria have a thick cell wall, while the Gram-negative bacteria

have a thin cell wall. The difference in the cell wall is due to the peptidoglycan layer, which is thicker in the Gram-positive bacteria than in the Gram-negative bacteria. The peptidoglycan layer is also the reason why the Gram-positive bacteria are more resistant to antibiotics than the Gram-negative bacteria. Here are some bulletpoints of the two groups:

- **Gram-positive bacteria**
  Lactic Acid Bacteria (LAB)
  Bacillus
- **Gram-negative bacteria**
  Most Acetic Acid Bacteria (AAB)

2.3.4 Differentiating between bacterial species

There are several methods to differentiate between bacterial species, some of the most common methods are:

- **DNA:DNA hybridization (DDH)**:
  The DNA of two organisms is mixed and the degree of hybridization is measured.
  If DDH > 70% the organisms are considered to belong to the same species
- **16S rRNA gene sequencing**:
  The 16S rRNA gene is sequenced and compared to a database, if the sequence is > 97.5-98.5% identical to a known species, the organism is considered to belong to the same species
- **Whole genome sequencing**:
  Average nucleotide identity (ANI) > 95-96% is considered to belong to the same species
  Digital DNA-DNA hybridization (dDDH) > 70% is considered to belong to the same species

2.4 11.09.24 - Methodologies for identification and typing

2.4.1 Intended leaning outcomes

After this lecture, the students should be able to:

- Get to know how different microbial identification and typing methods works
- Apply this knowledge to comprehend microbial identification and taxonomy
- Reflect on the application possibilities of the different methods for microbial identification and typing

2.4.2 Background

- When you want to know what microorganisms are in a fermented food
  IDENTIFICATION
- Often identification is coupled with quantification (predominant/minor microorganisms)
- Many different methods are available
  Make an overview of the most used methods
  Elaborate the steps of the methods → insights into the structure/mechanistics
  Show how the methods are applied
- All methods have strengths and limitations

Note: typing is identification of isolates below species level

2.4.3 Methodology overview

Culture dependent methods

This is the most common method for identification of microorganisms. The method is based on the growth of the microorganisms on a solid or liquid medium. Here are an example of a method which is based on this:

- Plating
- Isolation
- Purification (The next 4 steps are methods for identification)
- Finger print based[1]
  rep-PCR, RAPD, RFLP or PFGE (All these are based on PCR)
- Sequence based[2]
  rRNA gene, MLST or MLSA
- Biochemical identification[3]
  MALDI-TOF or FTIR
- Classical[4]
  Macro/micro morphology, phenotypic tests (fermentation, assimilation or growth conditions)

Culture independent methods

- Direct extraction of DNA or RNA
- Molecular methods (the next two steps are both molecular methods methods)
- PCR[1]
  Species specific PCR
- qRT-PCR[2]
  Next generation sequencing

2.5 16.09 - From 1st to 3rd generation DNA sequencin strategies

DNA is quite small, 2 nm thick. Though, if folded out, the DNA would be around 1.8 m long.

2.5.1 Sanger Sequencing of DNA 1st generation sequencing

5′- TACAACTGAGCGACT -3′
3′- ATGTTGACTCGCTGA -5′
Here we have a blue sequence and a black complimenting sequence. If we want to sequence the blue sequence, we can use the black sequence as a primer. The primer will bind to the blue sequence, and the DNA polymerase will start to build a new strand of DNA. The DNA polymerase will build the new strand by adding nucleotides to the 3′ end of the primer. The nucleotides are added in a 5′ to 3′ direction. The DNA polymerase will continue to add nucleotides until it reaches the end of the DNA strand. The DNA polymerase will then stop, and we will have a new strand of DNA.
Though with the Sanger sequencing, we will have a mix of DNA strands, where each strand will end with a different nucleotide. The DNA strands will then be separated by size, and the sequence can be read by the size of the DNA strands.
With Sanger sequencing, the -A, -C, -G and -T nucleotides will cut the DNA strand at different lengths, because it cuts at the respective letter. An example of how the Sanger sequencing works can be seen in figure 2.5.

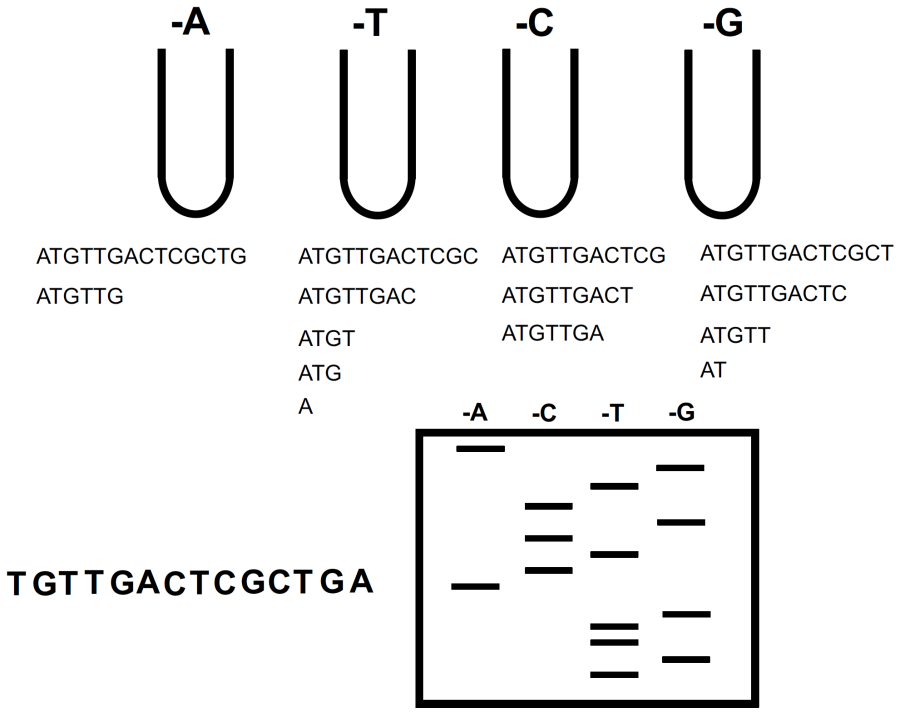


Figure 2.5: An example of how the method of Sanger sequencing of DNA works with the given blue sequence

2.5.2 Next generation sequencing

Next generation sequencing (NGS), has been evolving a lot since extensive research started in the 90's. Today we have a lot of different NGS methods, one of the most common methods is the Illumina sequencing. The

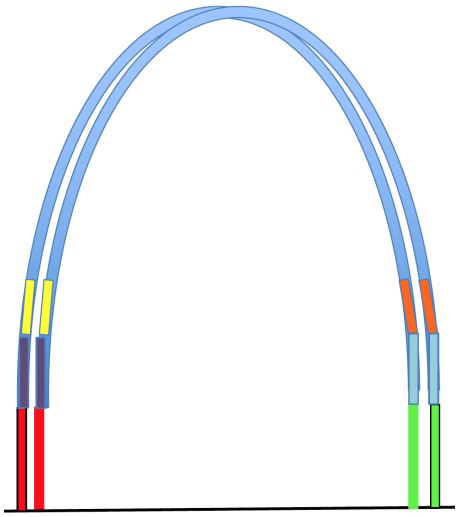
Illumina sequencing is based on the Sanger sequencing, but with a few modifications. Their HiSeqX can sequence around 50 human genomes in a day with a cost sub 1000 USD/genome.

Sequencing by synthesis

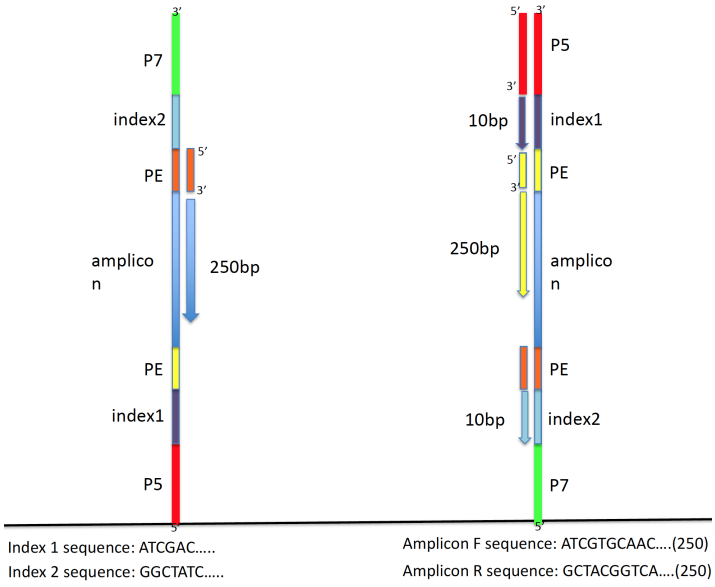
The Illumina sequencing is based on the sequencing by synthesis. It needs a primer, but these a fairly simple to make. After the primer has been added, the DNA polymerase will start to build a new strand of DNA. The DNA polymerase will add nucleotides from -A, -T, -G, anc -C, to the 3′ end of the primer, and the nucleotides will be added in a 5′ to 3′ direction. The DNA polymerase will continue the flashing with nuclouetides until it reaches the end of the DNA strand. The DNA polymerase will then stop, and we will have a new strand of DNA.
The maximum read out length of the Illumina is between 75 bp to 350 bp, but the average read length is around 150 bp.

Bridge amplification

A piece of DNA is attached to a glass slide, and the DNA is then amplified. The DNA is then denatured, and the DNA is then amplified again. The DNA is then denatured again, and the DNA is then amplified again. This process is repeated until the DNA is amplified enough. The DNA is then sequenced, and the sequence is read by the computer. This method grows exponentially like PCR, though the DNA is amplified in a half circle, and not in a straight line.
An example of how the Illumina bridge amplification works can be seen in figure 2.6.



(a) CHere an example of how the DNA is attached to the glass slide on both ends forming the bridge.



(b) After heating the bridge opens up and the DNA is amplified.

Figure 2.6: An example of how the Illumina bridge amplification works.

2.5.3 3rd generation sequencing

Chapter 3
Laboratory Exercises

Chapter 4
Lecture Exercises

4.1 11.09.24 - Exercise 1 - Replication, Transcription and Translation in prokaryotes

Figure 4.1 shows a DNA sequence which will used in Exercise 1 and its sub tasks. The given DNA sequence can be seen in figure 4.1

Figure 4.1: Sequence of DNA which is used in Exercise 1

4.1.1 Replication in prokaryotes

Replicate the DNA sequence based on the anti-sense (template) strand written below using the following primer **5′- AUCCAUU -3′** (This RNA complementary to the DNA sequence and is the defined -35 region, which will be used in the transcription subsection).

- While replicating the DNA, identify the leading and lagging strands. Furthermore remember that G and C are the most stable base pairs, A and T are the least stable base pairs
- The DNA sequence is replicated from the 3′ to 5′ direction
- Use the primer as to define where to start from

5′- X X X **A T C C A** **T T G A C A** C T A A G C A T G C A T T G C A C T G C **T A T A A T** C G T A G G T G G C G T **A T G** T C T G C T A T A C G T A T C G A C T A G T A **T A G** C G G -3′ (This is the replicated)

3′- *G A T* **T A G G T A A** C T G T G A T T C G T A C G T A A C G T G A C G A T A T T A G C A T C C A C C G C A T A C A G A C G A T A T G C A T A G C T G A T C A T A T C G C C-5′ (This is the given)

So, to begin with, we will look at the given RNA primer, and convert it to a DNA primer. 5′-AUCCAUU-3′ will be converted to 5′-ATCCATT-3′ (since RNA has Uracil instead of Thymine).
Afterwards we will look at the given DNA sequence and find the complementary strand to it. The complementary strand will be the anti-sense strand. 5′-ATCCATT-3′ will be converted to 3′-TAGGTA-5′ (since A pairs with T, T pairs with A, C pairs with G, and G pairs with C).

Now we will highlight the given sequence in yellow and start translating the DNA sequence from the 3′ to 5′ direction. We will start from the primer and move towards the end of the sequence.

This concludes this part of the exercise.

4.1.2 Transcription in prokaryotes

Identify the Pribnow (-10 region) and (-35) regions in the above DNA double-helix (sense strand). Transcribe DNA into a Messenger-RNA (mRNA).

In this course the Pribnow box (-10 region) is defined as the region 5′-TATAAT-3′ and the -35 region is defined as 5′-TTGACA-3′. Wil will therefore
We found the Pribnow box sequence and highlighted it in green. The mRNA strand will be transcribed from the anti-sense strand, so we will use the antis sense strand to transcribe the mRNA until the -35 region which we highlighted in turquoise.
We can now go to the right end (10-ish letters) of the pribnow box and start transcribing the mRNA. In this course, the start codon is defined as the first AUG codon after the Pribnow box. The mRNA strand will be transcribed from the 3′ to 5′ direction.
The start codon is highlighted in apricot starting from the 13^{th} letter to the right. The stop codon is highlighted in bittersweet.
The Pribnow 10 region is **5′- ATCCATT -3′** and the -35 region is **5′- CGATAGG -3′**.
Now that we have identified both the start- and stop codon, we can start to transcribe the mRNA strand. The first codon will start from number 10 after the the Pribnow box. The mRNA strand will therefore be:
5′- GUA UGU CUG CUA UAC GUA UCG ACU AGU AUA GCG -3′

4.1.3 Translation in prokaryotes

Translate the mRNA strand from transcription step into amino acids.

The mRNA strand is written in the last subtask, and have already been translated into codons. It's now possible to translate the codons to amino acids.
H_2N- Val - Cys - Leu - Leu - Tyr - Val - Ser - Thr - Ser - Iso - Ala -COOH

4.2 11.09.24 - Exercise 2 - PCR and Restriction Enzymes

A part of DNA fragment shown below is to be amplified using PCR and the primers F and R. Subsequently the PCR product is cut with restriction enzyme HpyCH4V and the resultant products are run on a gel. The

Figure 4.2: Sequence of DNA which is used in Exercise 2

The two given primers are:

- Primer F: 5′- AACCT-3′
- Primer R: 5′-TGGAT-3′ (3′-TAGGT-5′)

4.2.1 What is the size (in base pairs) of the PCR-product?

The Primer F starts from the 3′ end of the DNA sequence, and the Primer R starts from the 5′ end of the DNA sequence. The PCR product will therefore be the DNA sequence between the two primers.
Primer F has to fit to the upper strand, and Primer R has to fit on the lower strand. The primners sequence are therefore:

- Primer F: 5′-AACCT-3′
- Primer R: 5′-TGGAT-3′ -> 3′-TAGGT-5′

Primer F starts from the 15^{th} to the right and ends at the 19^{th} base pair. Primer R starts from the 64^{th} from the right and ends at the 68^{th} base pair. The PCR product will therefore be the DNA sequence between the 19^{th} and 64^{th} base pair.
The size of the PCR-product is therefore 68-14 = 54 base pairs.

4.2.2 How many copies of DNA do you have after 4 PCR cycles if you start with 3 copies of the DNA- fragment to be copied (considering ideal conditions)?

After each PCR cycle, the amount of DNA doubles. After 4 PCR cycles, the amount of DNA will be 2^4 = 16 times the original amount of DNA. Though, we are starting with 3 copies of the DNA fragment, so the amount of DNA after 4 PCR cycles will be $3 \cdot 2^4$ = 48 copies of the DNA fragment.

4.2.3 What is the size of 2 fragments after cutting with HpyCH4V?

The restriction enzyme HpyCH4V recognizes the sequence 3′-ACGT-5′. The DNA sequence is cut at the recognition site, and the two fragments will be the DNA sequence before the recognition site and the DNA sequence after the recognition site.
5′- TGTCGGTGTG CCATAACCTA AGTCATAACG ATCCGTGAAT GCATACCCGT TTTGAATAAT TGAATC-CAAG AAACTTGTAT CTGTACTTGA-3′
3′- ACAGCCACAC GGTATTGGAT TCAGTATTGC TAGGCACTTA CGTATGGGCA AAACTTATTA ACT-TAGGTTC TTTGAACATA GACATGAACT-5′

HpyCH4V will therefore cut the DNA sequence at number 41, and the two fragments will be:
5′- AACCTA AGTCATAACG ATCCGTGAA**T GCA**TACCCGT TTTGAATAAT TGAATCCA-3′
3′- TTGGAT TCAGTATTGC TAGGCACTT**A CGT**ATGGGCA AAACTTATTA ACTTAGGT-5′

There will therefore be 27 base pairs in the first fragment and 27 base pairs in the second fragment.

4.2.4 Place the bands that you would observe after running your product on the an agarose gel (path 1 before cutting with enzyme, path 2 after cutting)

When you run the PCR product on a gel electroforesis, you will see the DNA fragments separated based on their size. The smaller fragments will move faster through the gel than the larger fragments. This means that both the DNA fragment before and after cutting with the restriction enzyme will be visible on the gel since they doesn't have the same fragment size .
Therefore you will see two bands on the gel, one at 54 base pairs (before cutting) and one at 27 base pairs (after cutting). This can be seen in Figure 4.3.

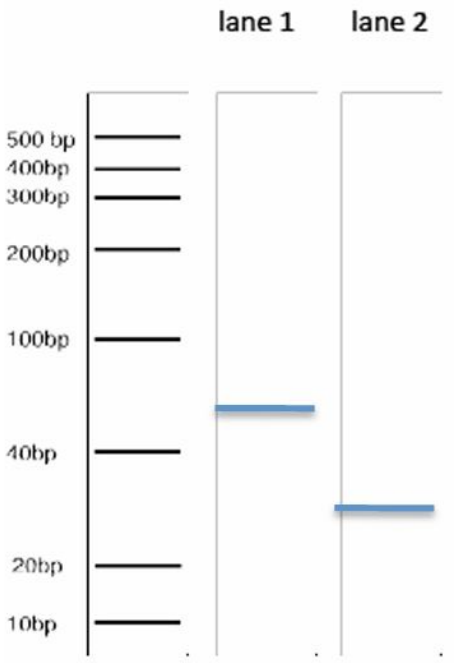


Figure 4.3: Gel electrophoresis of the DNA fragments and the PCR products' bands

4.3 11.09.24 - Exercise 3 - Primer3plus

Based on the sequence below design primers that fulfill following conditions:
The following information is given:

- size (22-24bp)
- Tm (55-57)
- GC% (35-70)
- amplicon size (300-400bp)

The following sequence is given:

```
TTGCAAGCCCTGATTTAAGCATTACACCAGCATCATCAAAGTAATACGCATTTCCATTTTGATCAGTTAT
ATATTGGTCAAAAACTTGATGACCTTTCTTATCGAAATAAACAATTGTACCATCTTCGTTTTGTAAGAAG
CTCTTGACCAGTTCGATACCATTAGGTAAGAAGAAGTAGGTATGGTTATTAATCTTTTGCAAACCTGTTA
CTAAATGACCTGTTTTATCAAAATAGTAATAGTTATTATCATCATCTTGAATAAATGCATTTTGGGCACG
ATAACCACTTAATGTATAATAGATGATTCCTTTATCATCGTGTGTAAAGCCAGTTTCTGACAGATCATTA
GTTAACTGTTTTGGTAAGTAGTCACCATCCTCAGTGTTCGAAACGACCTTAAAGTACTTATCAGAACCCA
TATCTTTCAATACGTATCCAGCACCTTTACCTTGGATGTTAGAGCCATTGAAGTACTTAGCCGCCCACTC
AGTAATCTTTACACTGCCATCAATTGGAACGCCAGTTGAGATTTGATTCACTTTAAATAGGGATGGATAG
AGTGCCTGTAACTCTTCTAAGAAGGCACCACCATACATCTCTTGATATTGACCACCCCCACGACTTTGTA
CAACATATAAGGCATTGTCAATATCAGAATCTGTATCGTCATCTCCAAATGAATTTGTTCTTGTGACAGT
AGCTAATTCTTGCTCTGGCAAATTATAAATTTGGTCCGGCACCCAATCGGCAATGGCTTGAATACCGCTA
GCATGTAAGGCTTTAATAGCATCGCGCAACTGATCAGCAGTTCCATATTTTGTCGGTGTGCCATAACCTA
AGTCATAACGATCCGTGAATGCATACCCGTTTTGAATAATTGAATCCAAGAAACTTGTATCTGTACTTGA
ACGATATTGTGGTGCCAATTGGAAGCTTGTCACACCCCATTGCTTAAATTGGTCCGCATTCTGAGCGATG
```

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ACTACGTTTGTATATTCACTGCTGTCTGTAGCAAATGCTTGGAAGTTTGAGAAACCTTCGTAGATGACTT
GAGAATCAAGAGCAGCGTTTGAATGGAACACTTTATCACTCGTGTTTGTTGTTGTATCAGAGGCCGTTCG
TGCATCTTGATCTTGTTGCGCACCTACCGGAACCCAAACTGCCAAGTAACCAGAAACTTGTGGATTTTGT
ACACCATAAATTGATTCATTCGTAAAAATCAAATCGCCGTTAGCATCTGTGTACGCCACAGGTGCATTTT
CATCAGTATCATAATAAGCTAATCCATCTGCAGTTGTTGATAACAAAGCACGATAAGCTTGGTTCTTATG
AGCTGCCCCCATATGCAATGTGACAGTATGCCCATCCTCTAATTGTAGCTCCGCGTTATTGCTGACGATG
ACTCCAATACCTTCAGTACGCGTCTCAGATGTTCCAGTGTCAGAAGCCGTCATGGCATCTTTACCATAGC
GAACACTTGTTAACACGTCATTACTATCAACGGACATCGATTGGCCACCAGCAACATATTGGACTCTAGC
CTTCAGCAAAGTGTTAATCGCATCATAGTATGGTGACTTTGTTGCCATATATTGACCATCATCTGTATAT
AAATCGCCATAATAGACACGAGGAACAGTATCCTTGTTGGTTAGCAACATCGCATAAGCACTAGCCATAT
TATATTGTGTGTACTTTTTGTCTGCTAATTTTTCATCTTCATTGTATACTTTGAAAGCAGCTGCCAATTG
```

Use Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)

While setting the conditions, it's important to remember that the amplicon size is the size of the DNA fragment that will be amplified by the primers.This is named "Product range size" on the web-side under general settings.

Write answers in the table bellow:

Table 4.1: The given table which should be filled out with the information from the Primer3Plus web-side

Primer	Sequence (5′-3′)	Primer size (bp)	Tm (°C)	GC content%
Forward	TCAGTAATCTTTACACTGCCATC	23	56.1	39.1
Reverse	TTATGACTTAGGTTATGGCACAC	23	56.1	39.1

The second part of this exercise was to use this information to deduct which bacteria this sequence belongs to. The sequence is from the bacteria *Leuconostoc mesenteroides*.

4.4 11.09.24 - Exercise 4 - Primers check and restriction enzymes

The following information is given:

- Primer F: 5′- GGAACCCAAACTGCCAAGT-3′
- Primer R: 5′-CCTCGTGTCTATTATGGCGATT-3′

Using **"In silico PCR amplification"** (http://insilico.ehu.es/PCR/) answer following questions:

4.4.1 What *Leuconostoc* strain will the primers align to?

We start by going to the web-side. Then we choose *Leuconostoc* and press "Next step". Afterwards we paste the primer sequences into the "Forward primer" and "Reverse primer" fields. We then choose "APPLY TO ALL Leuconostoc" and press "Amplify".
Then we can see 2 bands where the primers has traveled at 485 and 485 base pairs. The primers will therefore align to the 2 *Leuconostoc mesenteroides* strains.

4.4.2 What would be the PCR product size (bp) if we use those primers against that species?

Based on the bands that was produced the PCR product size will be 485 base pairs.

For the next subsections, the following information is given:
Using NEBcutter V2.O http://tools.neb.com/NEBcutter2/ retrieve the amplicon from “In silico PCR amplification” and answer following questions:

4.4.3 What will be the size of DNA products after digesting the amplicon with Hind III restriction enzyme?

To use the web-side you need to copy the DNA sequence from the "In silico PCR amplification" web-side and paste it into the "Enter DNA sequence" field and press sumbit. Then you look at the sumary and find the restriction enzyme HendIII and hold the mouse over it to see where it cuts.

For the two *Leuconostoc* strains the HINDIII cuts at:

- Cuts at bp 167 (Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293)
- Cuts at bp 167 (Leuconostoc mesenteroides subsp. mesenteroides J18)

This means that the DNA products will be 167- and 318 base pairs long after digesting the amplicon with HindIII restriction enzyme. This makes sense since the total sequence, for both, is 485 base pairs long. Thus 167+318 = 485 base pairs.

4.4.4 What are the reaction conditions requires for ApeKI?

To answer this, the following table has to be filled out. The information can be found on the NEBcutter web-side by pressing "Enzyme list" and then "ApeKI". Then in the bottom right corner of the first table, there is a button called "NEV Restriction Enzyme - Activity/Performance Chart". Then find the enzyme ApeKI and press on the buffer, there all the needed information is found.

Table 4.2: The given table which should be filled out with the information from the NEBcutter web-side

Buffer	**NEBuffer^{TM} r3.1**
Salt	100 mM NaCl
Main	50 mM Tris-HCl
pH	7.9 at 25°C
Mg	10 mM $MgCl_2$
rAlbumin	100 µg/ml Recombinant Albumin
Reaction temp. (°C)	75°C (Obtained from the solutions)

4.4.5 What is the digestion pattern of AciI?

The AciI enzyme cuts at the following base pairs:
Primer F 5′-...AA$^{\downarrow}$CGTT...-3′
Primer R 3′-...TTGC$_{\uparrow}$AA...-5′
This information can be found on the page before you press "NEV Restriction Enzyme - Activity/Performance Chart".

4.4.6 Using ENDMEMO

For this exercise we need to use this web-side (http://www.endmemo.com/bio/egel.php) In the graph on the right place bands you would receive after digesting the amplicon with HindIII, ApeKI and AciI in one reaction.

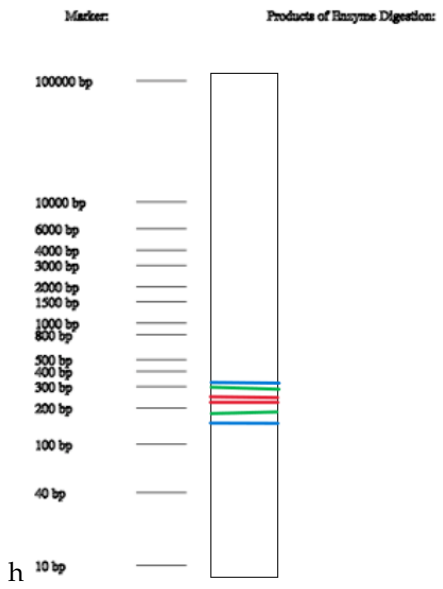

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Figure 4.4: Gel electrophoresis of the DNA fragments and the PCR products' bands

Here we simply need to insert a sequence and choose the right restriction enzymes. The bands will then be shown on the gel.

- HindIII cuts at 167 bp; bands will be at 167- and 318 bp
  Blue color
- ApeKI cuts at 184 bp; bands will be at 184- and 301 bp
  Green color
- AciI cuts at 233 bp; bands will be at 233- and 252 bp
  Red color

Chapter 5
Litterature resumees

5.1 1. Lecture

5.1.1 Article 1 - Fermented Foods as Experimentally Tractable Microbial Ecosystems

Introduction

The study of microbial communities faces significant challenges, largely due to their complexity and the difficulty in understanding how these multi-species communities are assembled, organized, and function. As a result, many aspects of microbial ecology remain unexplored.
The article highlights that the vast diversity and complexity of microbial communities are key factors contributing to our limited understanding. Additionally, the difficulty in isolating the majority of species from natural environments and cultivating them in vitro presents another notable challenge [2].
One approach to addressing these challenges is the development of simplified and controlled environments to study microbial communities. These controlled systems allow for reproducible results through measurable substrates, microbial growth, and community formation [2].
Several models with these properties have already been developed. These range from synthetic mixtures of microbial strains to more complex models composed of culture-able strains from free-living or host-associated communities, as well as naturally occurring communities that have been extensively sampled or perturbed in situ [2].

Fermented Foods as Experimentally Tractable Ecosystems

Fermented foods have been around for thousands of years and are an excellent example of how humans have optimized conditions to promote various properties of foods. Here are some of the main reasons why fermented foods have been produced for so long [2]:

- Good preserving effects
- Flavour can be improved
- Aroma can be enhanced
- Texture can be changed

Some factors for manipulating microbial growth are listed below [2]:

- Temperature
- Salinity
- Moisture

The article describes some micorbial communities of fermented foods (MCoFFs) and some examples of these, which will be listed bellow [2]:

- Multi-species biofilms associated with surfaces
  e.g. cheese rinds
- Suspended biofilms in liquid
  e.g. kombucha, kefir, and vinegar
- Dispersed growth in liquid
  e.g. lambic beers, natural wines, and yogurt
- Semi-solid substrates
  e.g. kimchi and miso

Many MCoFFs have been studied in detail, and the article highlights that some of these communities have been shown to have bacterial- and fungi species that have co-evolved over time. The microbial diversity within and across fermented foods is vast, and the article emphasizes that these communities are excellent models for studying microbial ecology. In the article, cheese rind (the MCoFFs on the surface of the cheese) was used as an example of the characterization and development of MCoFFs, since on the surface of cheese the microbial communities form as it ages [2].

Using Fermented Foods to Link Patterns, Processes, and Mechanisms of Microbial Community Assembly

In order to understand how species come together to form a community within a given environment microbiologists have developed several approaches for both plant- and animal communities. The ones named in the article is listed below [2]:

- **Dispersal**
  Contributions from the amount and timing of microbial propagules colonizing a habitat
- **Biotic selection**
  Interactions between species
- **Abiotic selection**
  Interactions between a species and the environment
- **Drift**
  Stochastic (random) changes in the relative abundances of species within communities
- **Diversification**
  Evolution of new species within communities

MCoFFs are ideal for linking microbial patterns with ecological processes. For instance, many fermented

foods exhibit clear microbial successions where early colonizers are replaced by other species. This process can be studied to understand how environmental changes and species interactions drive these transitions. New techniques like RNA sequencing and transposon mutagenesis have proven valuable in uncovering the molecular mechanisms behind these interactions. Additionally, tools like imaging mass spectrometry are enhancing our understanding of how microbial species communicate chemically [2].

Taste of Place? Microbial Biogeography of Fermented Foods

Though there are many different types of MCoFFs, many share some similarities. If a similar MCoFFs is used in two different cheese factories in two different locations, the microbial communities will be different, and with some distinct properties which can affect the texture, taste or aroma composition of the final product. This also enables the study of microbial biogeography, which is the study of how microbial communities are distributed across different locations. MCoFFs can therefore help linking patterns in microbial diversity from place to place. This is possible because each community has clear species abundance distributions [2].

Microbial Evolution in a Community Context

MCoFFs offer valuable systems for studying both ecological processes and microbial evolution. Microorganisms used in beer, wine, dairy, and sake production have been useful in understanding how microbes adapt to fermentation environments. Research suggests that these organisms often lose genes not needed in nutrient-rich settings, gain new traits through gene transfer, and adjust their metabolism to fit the fermentation niche [2].

Looking ahead, linking ecological and evolutionary processes could provide further insights. For example, studying long-term microbial communities could help explore how species interactions influence evolution. Experimentally testing coevolution within these communities could offer useful data on microbial adaptation over time [2].

Translation to Other Microbial Communities and Potential Applications

The study of microbial community assembly in traditional fermented foods (MCoFFs) can significantly enhance food quality and safety. Insights from these ecosystems can be applied to other microbial communities, such as the human microbiome. For example, the competitive mechanisms of *Lactobacillus reuteri*, found in both sourdough and human gut microbiomes, illustrate how patterns observed in MCoFFs can be relevant to other systems [2].

Research on cheese rind microbial diversity has revealed similarities with the human skin microbiome, suggesting that moisture is a major driver of surface biofilms in both environments. This indicates that ecological selection processes may be conserved across different microbial communities [2].

MCoFFs can directly impact human health as they are consumed and can interact with the human gut microbiome. While the probiotic effects of simplified MCoFFs like yogurt have been extensively studied, the broader microbial diversity in MCoFFs could introduce additional beneficial microbes and metabolites to the human digestive system. Studies have shown that MCoFFs can remain viable during passage through the human digestive tract [2].

Furthermore, MCoFFs offer valuable opportunities for designing synthetic microbial communities for applications in medicine, industry, and agriculture. Understanding the interactions within these communities can inform the construction of synthetic microbial ecosystems. By studying pre-existing MCoFFs, researchers can

learn about design principles and explore the potential for combining microbes from different MCoFFs into new, functional compositions [2].
Ultimately, research on food microbial communities could result in safer, more delicious foods and contribute to the development of essential principles for microbial community design [2].

5.1.2 Article 2 - Review: Diversity of Microorganisms in global fermented foods and beverages

Introduction

The article describes how rice with fermented and non-fermented legumes is a staple diet in many counties in Asia. Wheat/barley-based breads/loaves followed bt milk and fermented milk products, meat and fermented meats are more common in the western world (West Asia, Europe and North America). The staple diet in Africa and South America comprise of sorghum and maize with wild legume seeds, meat and milk products [1]. Consortia of microorganisms are found naturally in uncooked plant and animal materials, utensils, food containers, earthen pots and in general in the environment. When producing fermented foods, it is normal to introduce a starter culture, which contains functional microorganisms [1]. The microorganisms in fermented food has converted the chemical composition of the raw food, which results in a change in the nutritional value of the food, making it more enriched [1].

Microorganisms in fermented foods

Lactic acid bacteria (LAB) are one of the most important groups of microorganisms in fermented foods. They are used in the production of fermented foods and beverages. Some of the major genra of the LAB are listed below [1]:

- *Alkalibacterium*
- *Carnobacterium*
- *Enterococcus*
- *Lactobacillus*
- *Lactococcus*
- *Leuconostoc*
- *Oenococcus*
- *Pediococcus*
- *Streptococcus*
- *Tetragenococcus*
- *Vagococcus*
- *Weissella*

Bacillus has ben found in alkaline-fermented foods in Asia anf Africa. The species of *Bacillus* that are found in abundance in legume-based fermented foods are listed bellow [1]:

- *Bacillus amyloliquefaciens*
- *Bacillus circulans*
- *Bacillus coagulans*
- *Bacillus firmus*
- *Bacillus licheniformis*
- *Bacillus megaterium*
- *Bacillus pumilus*
- *Bacillus subtilis*
- *Bacillus subtilis variety natto*
- *Bacillus thuringiensis*

There has been reported several species of *Kocuria, Micrococcus* and *Staphylococcus* in fermented foods. The species of *Kocuria* are found in abundance in fermented milk products, fermented sausages, meat-, and fish products [1].

Yeasts are also associated with fermentation of foods and alcoholic beverages. The yeasts named in the article are listed bellow [1]:

- *Brettanomyces*
- *Candida*
- *Cryptococcus*
- *Debaryomyces*
- *Dekkera*
- *Galactomyces*
- *Geotrichum*
- *Hansenula*
- *Hanseniaspora*
- *Hyphopichia*
- *Issatchenkia*
- *Metschnikowia*
- *Saccharomyces*
- *Pichia*
- *Kazachstania*
- *Rhodotorula*
- *Saccharomycodes*
- *Kluyveromyces*
- *Rhodosporidium*
- *Saccharomycopsis*
- *Schizosaccharomyces*
- *Sporobolomyces*
- *Torulaspora*
- *Torulopsis*
- *Trichosporon*
- *Yarrowia*
- *Zygosaccharomyces*

Filamentous molds are also found in fermented foods. They hold a major role in various fermented products in resprect of enzyme production and in the degradation of anti-nutritive factors. The listed filamentous molds are listed bellow [1].

- *Actinomucor*
- *Amylomyces*
- *Aspergillus*
- *Monascus*
- *Mucor*
- *Neurospora*
- *Paracilomyces*
- *Penicillium*
- *Rhizopus*
- *Ustilago*

Taxonomic tools for identification of microorganisms from fermented foods

A commonly used method for profiling both culturable and non-culturable microbial populations from fermented foods is DNA extraction. The technique is based on the separation of PCR-amplified DNA fragments by denaturing gradient gel electrophoresis (DGGE). This method is both used for profiling bacterial populations and yeast populations in fermented foods. Also, in fermented foods a combination of Propidium MonoAzide (PMA) treatment, which is done before DNA-extraction, and molecular quantifying methods can be used to enumerate the viable microorganisms accurately [1]. Random amplification of polymorphic DNA (RAPD) is a typing method based on the genomic DNA fragment profiles amplified by rep-PCR, and is commonly used for differentiation of LAB strains from fermented foods and other bacteria or yeast in the consortia. This technique is able to differentiate between strains of the same species [1]. Amplified fragment length polymorphism (AFLP) is another technique which is used to identify and discriminate LAB strains. The technique is based on the selective amplification and separation of genomic restriction fragments [1].
DGGE techniques and temperature gradient gel electrophoresis (TGGE) are used to study the microbial diversity in fermented foods. The techniques are based on the separation of PCR-amplified 16s rDNA and 26s rDNA gene fragments by denaturing gradient gel electrophoresis. The techniques are used to study the microbial diversity in fermented foods. DGGE is quite time consuming and is not able to determine relative abundance of the dominant species, nor is it able to distinguish between viable and non-viable cells [1].
Next generation sequencing (NGS) are effective tools for studying the microbial diversity in fermented foods. The techniques are able to sequence all the genetic material present in a sample, providing a comprehensive view of the microbial community's diversity and functional potential. These techniques require extensive training and a well-equipped molecular laboratory [1].
In table 5.1 some of the NGS which is listed in the text are described. These NGS are used to study the microbial diversity in fermented foods [1].

Table 5.1: Some NGS techniques with their respective key-points

Metagenomics	**Phylobiomics**	**Metatranscriptomics**
• Sequences all the genetic material present in a sample, providing a comprehensive view of the microbial community's diversity and functional potential. • Allows the study of microbial communities without the need to culture individual species, overcoming limitations of traditional microbiology methods. • By sequencing entire genomes, metagenomics can identify functional genes involved in processes like metabolism, antibiotic resistance, or nutrient cycling.	• Is primarily concerned with analyzing the evolutionary relationships between microbial species, often using 16S rRNA or other conserved genes. • It helps characterize the diversity and phylogenetic structure of microbial communities, determining how different species are related within an ecosystem. • This technique is useful for identifying species and classifying them taxonomically based on their evolutionary lineage.	• Focuses on sequencing the RNA transcripts present in a microbial community, providing a snapshot of active genes and gene expression levels. • Reveals what genes are actively being expressed in a community at a given time. • It helps assess how microbial communities respond to environmental changes, stress, or disease by analyzing shifts in gene expression.

Bibliography

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Chapter A
Appendix