UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

EUROPEAN UNION
European Structural and Investing Funds
Operational Programme Research, Development and Education

MINISTRY OF EDUCATION, YOUTH AND SPORTS

An overview of food, environmentally and industrially important microorganisms, their sources, isolation, identification, cultivation

Lecturer:

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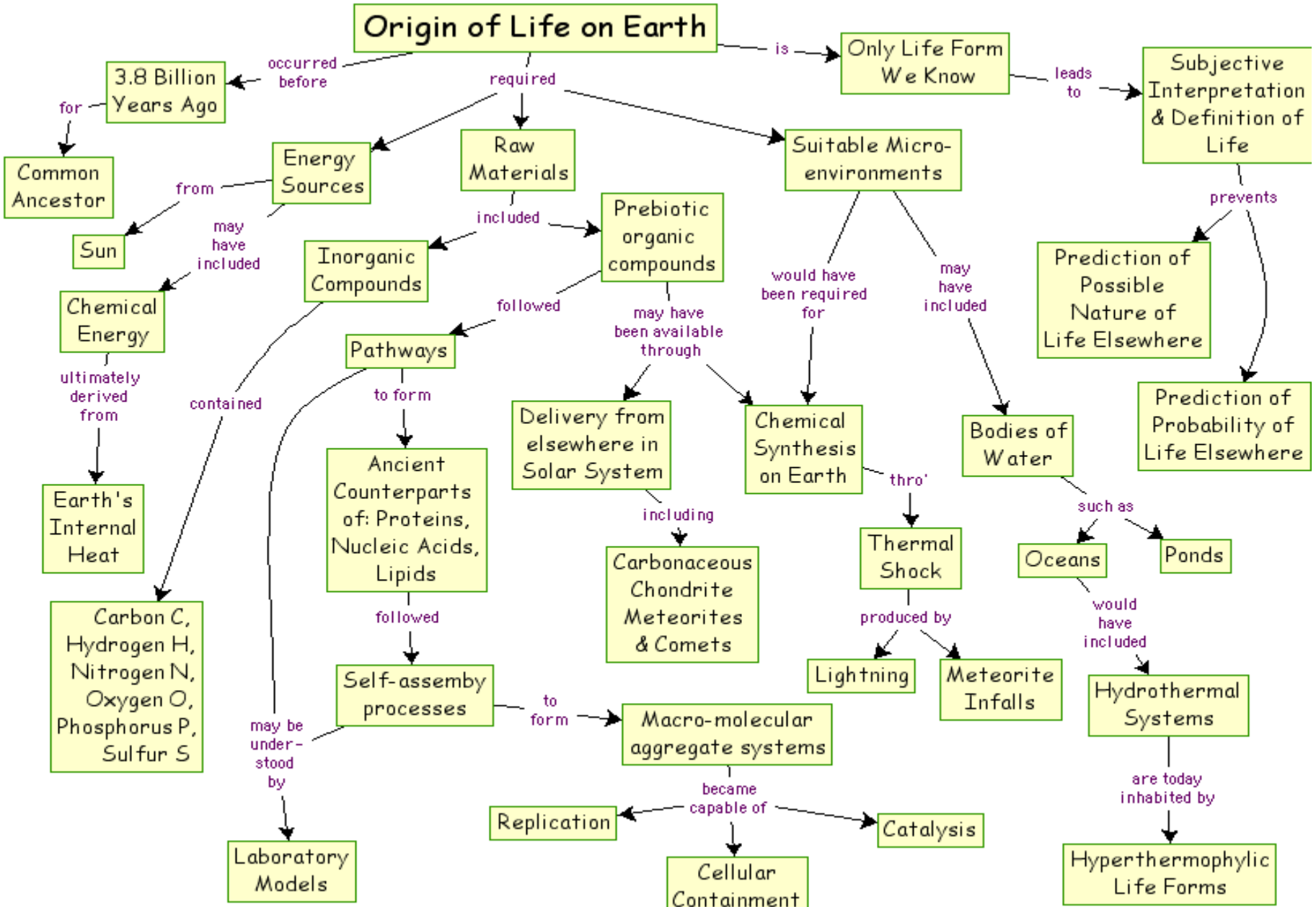

Origin of Life on Earth
occurred before
3.8 Billion Years Ago
for
Common Ancestor
required
Energy Sources
from
Sun
may have included
Chemical Energy
ultimately derived from
Earth's Internal Heat
Raw Materials
included
Inorganic Compounds
contained
Carbon C, Hydrogen H, Nitrogen N, Oxygen O, Phosphorus P, Sulfur S
Prebiotic organic compounds
followed
Pathways
to form
Ancient Counterparts of: Proteins, Nucleic Acids, Lipids
followed
Self-assemby processes
may be under-stood by
Laboratory Models
to form
Macro-molecular aggregate systems
became capable of
Replication
Cellular Containment
Catalysis
Suitable Micro-environments
would have been required for
may have included
may have been available through
Delivery from elsewhere in Solar System
including
Carbonaceous Chondrite Meteorites & Comets
Chemical Synthesis on Earth
thro'
Thermal Shock
produced by
Lightning
Meteorite Infalls
Bodies of Water
such as
Oceans
Ponds
would have included
Hydrothermal Systems
are today inhabited by
Hyperthermophylic Life Forms
is
Only Life Form We Know
leads to
Subjective Interpretation & Definition of Life
prevents
Prediction of Possible Nature of Life Elsewhere
Prediction of Probability of Life Elsewhere

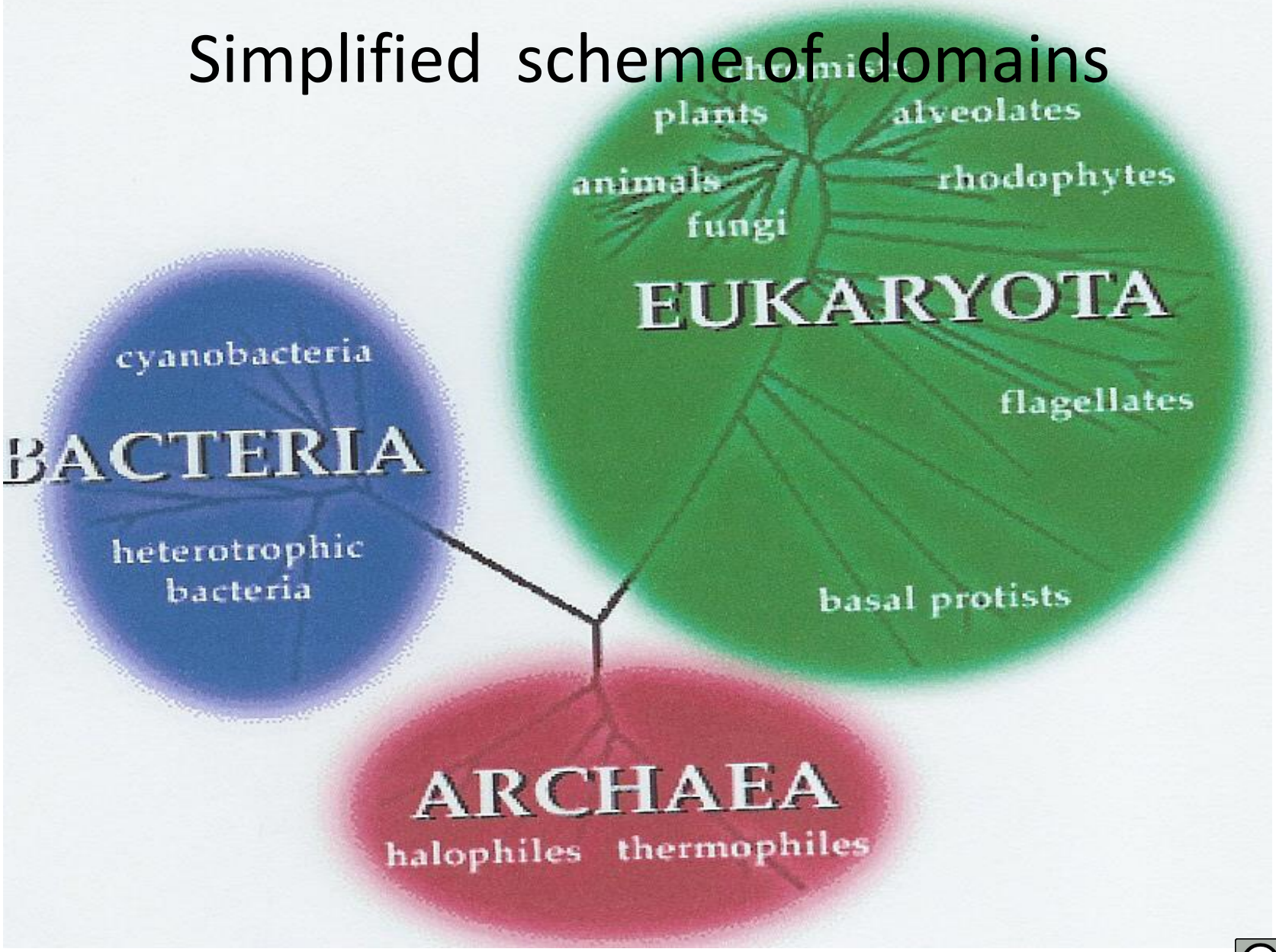
Definition of microorganisms

- Unicellular or multicellular organisms that do not form differentiated tissues.
- Typical attributes are very small dimensions- tenths of **µm** up to tenths of **mm**).

  Autotrophic + heterotrophic
- Two large groups: PROKARYOTES

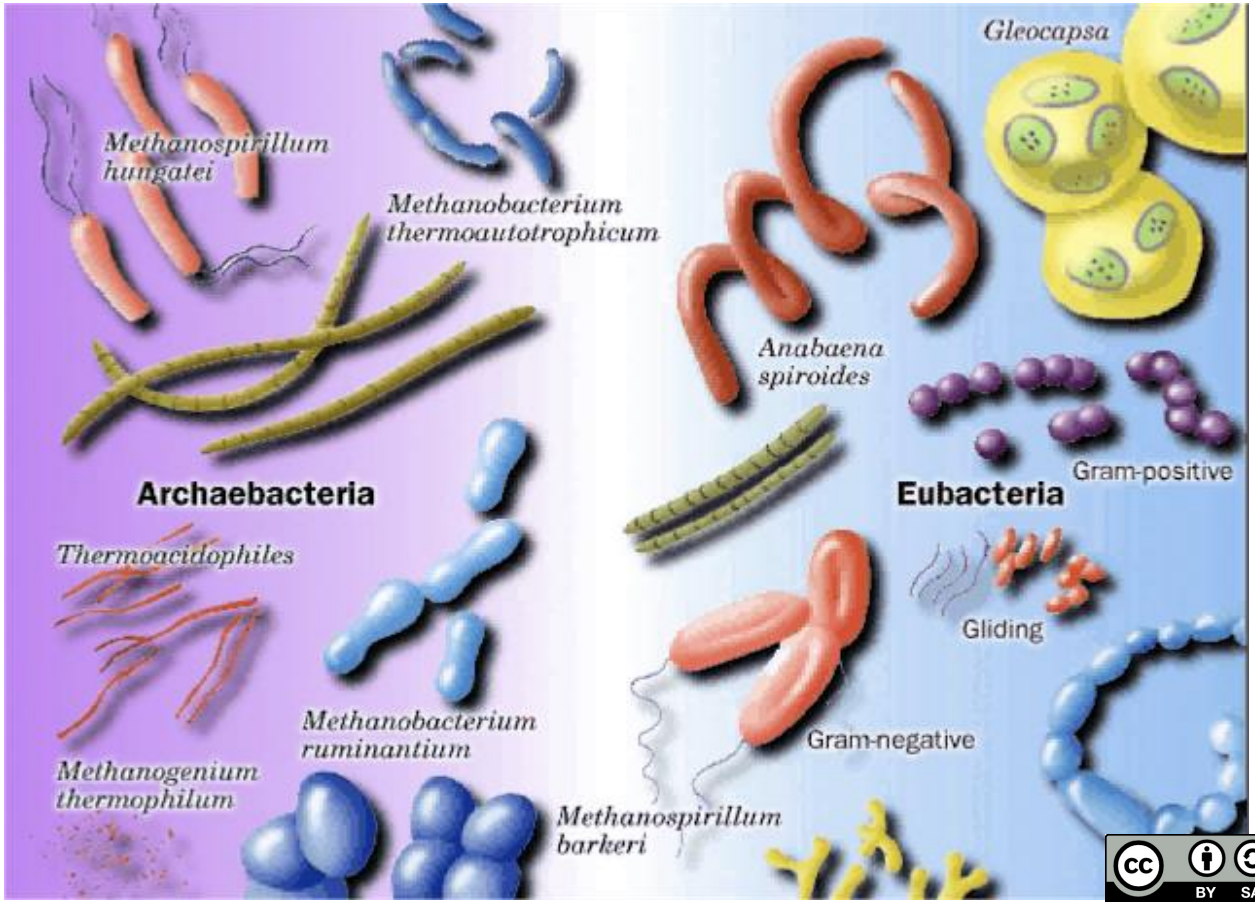
  EUKARYOTES

Simplified scheme of domains



PROKARYOTES

- **ARCHEA or ARCHEBACTERIA**
- **BACTERIA including CYANOBACTERIA**
- **VIRUSES**



- A few prokaryotic species are well described in terms of cell biology.
  - These represent only a tiny sample of the enormous diversity represented by the group as a whole.

- Many central features of prokaryotic cell organization are well conserved.

History of industrial microbiology

- Microbes have been used to produce products for thousands of years. Even in ancient times, vinegar was made by filtering alcohol through wood shavings, allowing microbes growing on the surfaces of the wood pieces to convert alcohol to vinegar.
- Likewise, the production of wine and beer uses another microbe — yeast — to convert sugars to alcohol. Even though people did not know for a long time that microbes were behind these transformations, it did not stop them from making and selling these products.

Microorganisms are **essential** to many processes**.**

- For example, the **nitrogen cycle** (where nitrogen is recycled in the ecosystem) or in the decomposition of animal and plant waste.
- Micro-organisms are increasingly important to industry, where they are used in a huge variety of processes ranging from food production to water treatment.
- Degradation of pollutants and xenobiotics.


Start
Fixes carbon?
Yes
No
Energy from light?
Yes
Photoautotroph
No
Energy from inorganic oxidation?
Yes
Chemoautotroph
No
Other autotroph
Energy from light?
Yes
Photoheterotroph
No
Energy from inorganic oxidation?
Yes
Chemoheterotroph
No
Other heterotroph

Types of products

- Industrial microbiologists study the production of
  (1) microbial biomass itself,
  (2) specific enzymes, or
  (3) metabolites.
- Enzymes that degrade polymers are especially important. The metabolites may be major metabolic products of catabolism, or compounds normally produced in trace amounts by natural isolates. **Industrial microbiology is an issue of scale**.
- Although industrial microbiologists culture organisms in many of the same ways as other microbiologists, the **goal is often to produce very large quantities,** sometimes measured in millions of liters at one time.

Industrial microorganisms

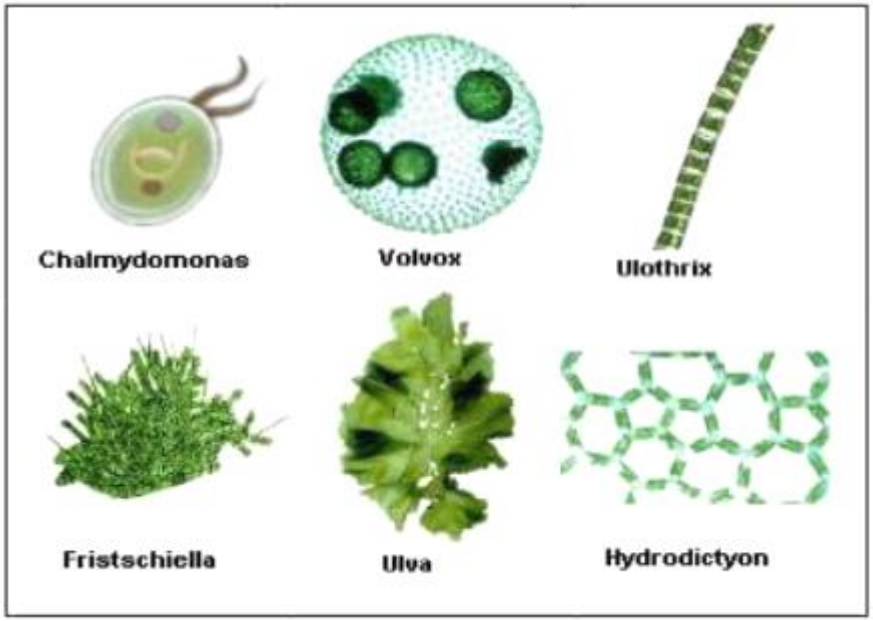
- Industrial microorganisms are initially selected from natural samples, or taken from a **culture collection** because they have been shown to produce a desired product.
- The **strain is** then **modified to improve** the product **yield**. This entails rounds of mutation, with careful selection for the rare clones that produce more or improved products.
- The selected strain is unlikely to survive well in nature, because the selection process has altered the regulatory controls in the cell to create metabolic imbalances.
- Other desirable characteristics are (1) rapid growth, (2) genetic stability, (3) non-toxicity to humans, and (4) large cell size, for easy removal from the culture fluid.

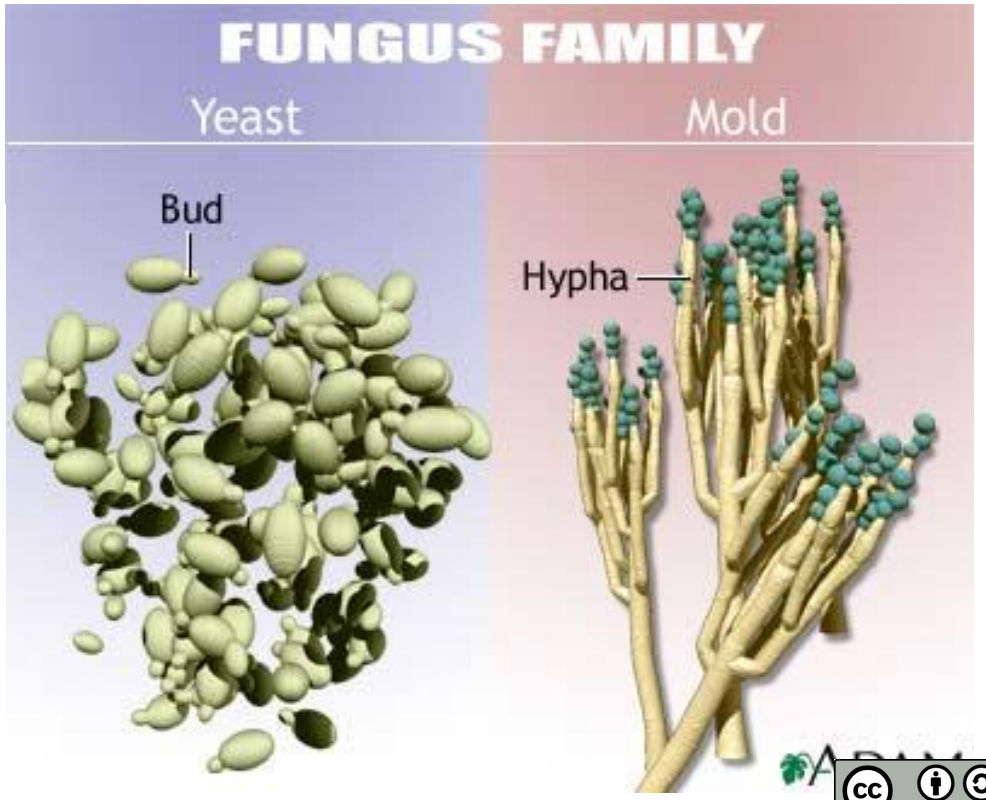
MICROBIAL EUKARYOTES

FUNGI - YEASTS and MOLDS/MICROMYCETES

PROTOZOA

ALGAE





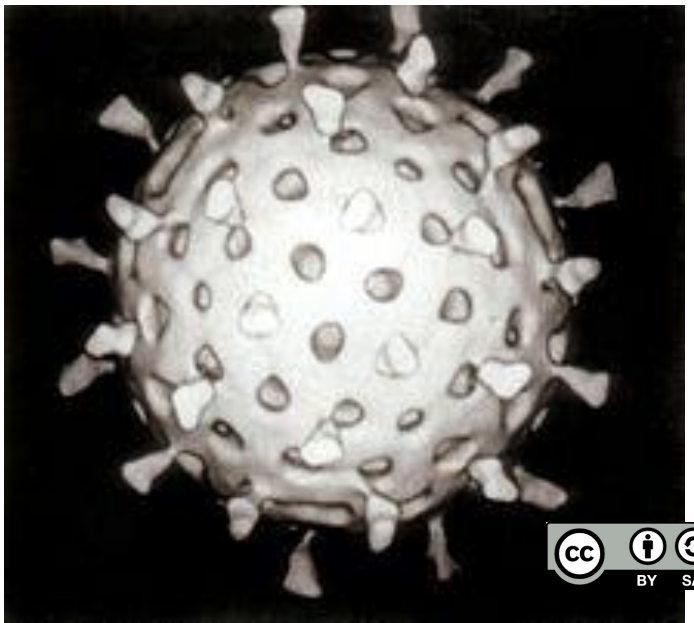
viruses

- A **virus** is a small infectious agent that can replicate only inside the living cells of an organism. Viruses can infect all types of organisms, from animals and plants to bacteria and archaea.
- Viruses are considered by some to be a **life form**, because they carry genetic material, reproduce, and evolve through natural selection.
- They lack key characteristics (such as cell structure) that are generally considered necessary to count as life.
- Because they possess some but not all such qualities, viruses have been described as

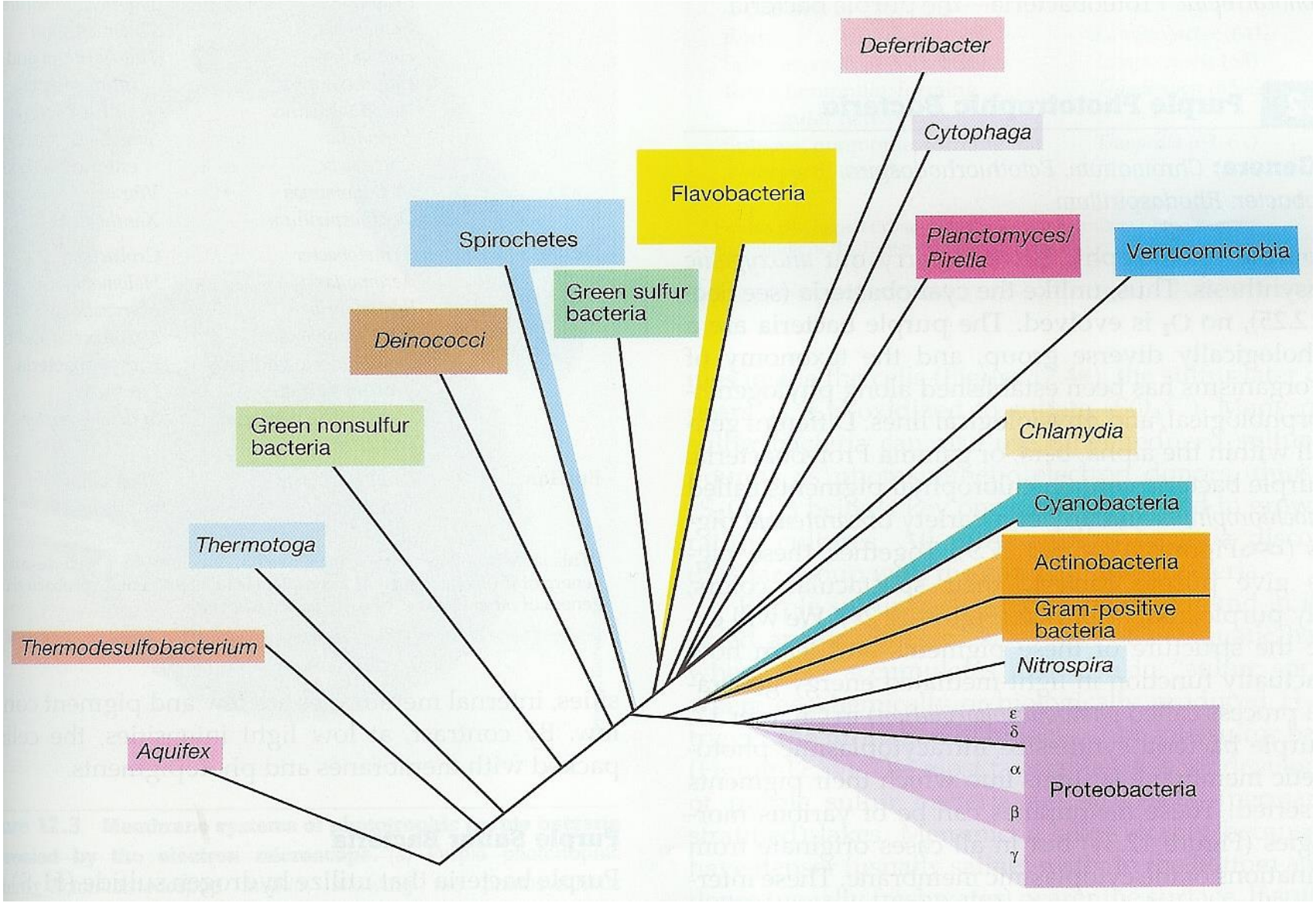
"organisms at the edge of life".

- Diameter of viruses : **20 – 300 nm**.
- A virus has either DNA or RNA genes and

is called a **DNA virus** or and **RNA virus**.



Phylogenetic trea - **BACTERIA**



Trea derived on the basis of DNA sequences for 16S ribosomal RNA (http:/rdp.cme.msu.edu)

Shapes of bacteria

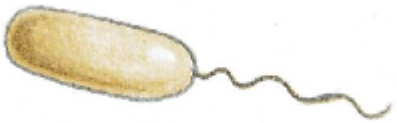
Three bacic shapes : coccoid
rod-shape
spiral

Coccoid cells (*Streptococcus*)

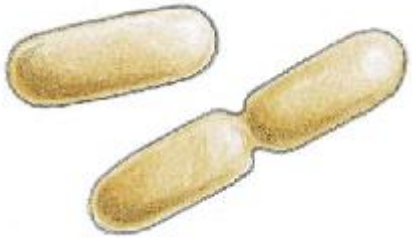
cocci

chains

Rod-shape cells *Salmonella*)



bacilus



Spiral cells (*Treponema pallidum*)

Molecular composition of bacteria

Bacterial cells contain 80-85 % water, the rest is dry weight

Chemical composition of typical bacteria (*Escherichia coli*) %**:**

proteins	**60 %**	
nucleic acids	**19 %**	DNA 3%, RNA 16 %
lipids	**15 %**	
polysaccharides	**3 %**	
low mol. compounds	**3 %**	

Proteins play in bacteria two roles : a) enzymes (active proteins)
b) structural proteins

Nucleic acids contain genetic information :

DNA (one/cell)
ribosomal RNA (5S, 16S a 23S)
mediator RNA (short life time)
transfer RNA tRNA

Lipids are of different structures : fats, waxes, phosphatides, lipopolysaccharides

Polysaccharides are in the forms of: peptidoglycans, teichoic acids and lipopolysaccharides.

Identification

- **Bacteria**. First classification only **shape** (vibrio, bacillus, coccus, etc.), presence of endospores, **gram staining**, effect of aerobic conditions, motility. **Cultivation, microscopy.**
- This system was changed after introduction of metabolic **phenotypes** study. **ENTERO-test**
- Recently, with the entrance of **molecular phylogenesis**, several chosen genes are used to **species** identification. Most relevant are genes coding 16S rRNA, next genes for 23S, **ITS** regions, **gyrB**.
- The most rapid identification of isolated strains to **genus** and **species** is performed by amplification of given **16S gene with universal primers** followed by amplicon sequencing. These data are sent to specialized database on web, namely to Ribosomal Database Project, which assigns sequences to next 16S sequences.

Division of bacteria according to metabolism

1) Autotrophic **- source of carbon is inorganic compounds** CO_2**, source of nitrogen** N_2, NH_4^+, NO_3^- **)**

2) Heterotrophic **- source of Carbon is organic mattters ethanol, saccharides**, fats

a) Prototrophic- simple organic molecules - sugars

b) Auxotrophic -**(demand complex organic compounds – e.g. vitamins, nucleic bases)**

Division according to oxygen demand

- **According to demands on oxygen, bacteria are divided into:**
- **1) Aerobic - demand oxygen (aerobic metabolism)**
- **2) Anaerobic - oxygen is inhibitor (anaerobic metabolism)**
- **3) Facultative anaerobic – (both types of metabolism)**
- **4) Microaerophilic – bacteria demand higher content of CO_2 and N_2, lower O_2.**

Fermentation

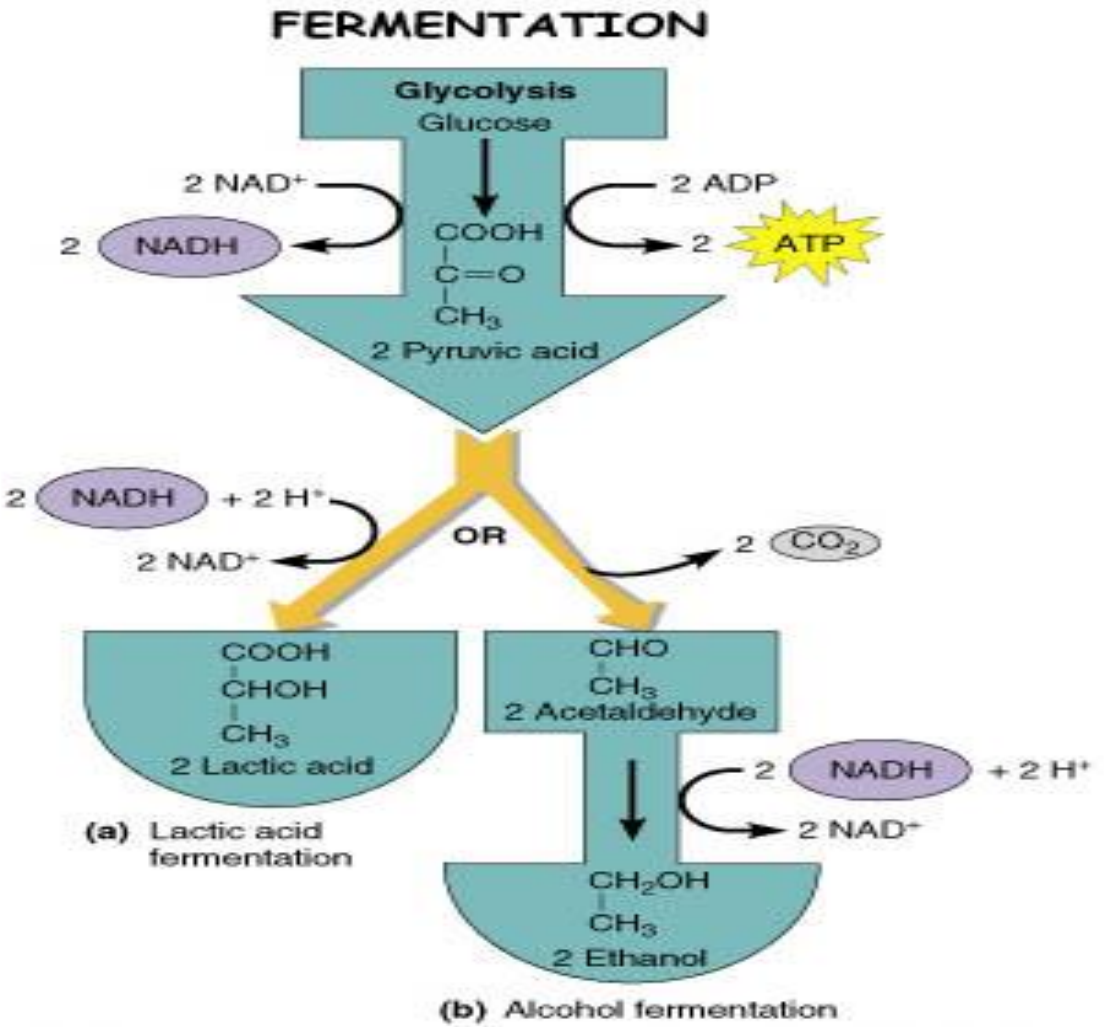
- One of the most well-known applications of micro-organisms in industry is fermentation, where carbohydrates (such as sugar) are converted into an acid or an alcohol.

  Foods that are produced using these methods include cheese, yoghurt, butter, beer, wine and bread.

“True“ fermentation

- The **most common yeast** that is used in the preparation of beer **is *Saccharomyces cerevisiae*,** commonly known as ‘baker’s yeast’ or ‘brewer’s yeast’.
- Beer is made from barley, wheat or rye grain, which is germinated to convert starch to sugar such as maltose. The grain is dried and crushed before hot water and yeast are added to initiate fermentation.
- In the fermentation process, **sugars** in the mixture are **converted to alcohol and carbon dioxide**.

Fermentation / lactic acid and alcohol fermentation



Other types of fermentations

- Aceton- butanol- ethanol ABE fermentation
- Propionic fermentation: the production of propionic acid by the action of certain bacteria on sugars or lactic acid.

Acetone–butanol–ethanol (ABE) fermentation

- is a process that uses bacterial fermentation to produce acetone, n-Butanol, and ethanol from starch.
- It was developed by the chemist Chaim Weizmann and was the primary process used to make acetone during World War I, such as to produce cordite.
- The process is anaerobic (done in the absence of oxygen), similar to how yeast ferments sugars to produce ethanol for wine, beer, or fuel.

ABE fermentation

- ABE fermentation is one of the oldest known industrial fermentation methods with a history of more than 100 years. In the first part of the 20th century, it ranked **second only to ethanol fermentation**. In 1861 butanol production through microbial fermentation was reported for the first time by **Pasteur**. This was followed by **Schardinger** in 1905 reporting production of acetone by fermentation.
- From 1912 to 1914 prolific strains of *Clostridium acetobutylicum* were isolated by **Chaim Weizmann**, showing the ability of fermenting starchy substrate. ABE fermentation was used to produce acetone during WW1 to make the explosive cordite and in the production of synthetic rubber.
- When **Weizmann's patent expired in 1936,** a flood of anaerobic fermentation plants were opened. Molasses fermentation processes grew in popularity as new microorganisms were isolated. Every company had its own unique patented microorganism which was able to produce acetone and butanol in great amounts from the molasses. In WW2, butanol was produced as fuel for fighter planes. The process disappeared in the second part due to the rise of the petrochemical industry.

Wine fermentation

- Wine is also produced by fermentation. Grapes are crushed to release sugars, and yeast is then added for fermentation and conversion of the sugars into alcohol.
- For sparkling wines, the carbon dioxide produced by the fermentation process is trapped to create bubbles.

Lactic acid bacteria - LAB

(LAB – it is not a taxonomic term)

This group includes various species with similar characteristics: **G^+, nonspore-forming, aerotolerant anaerobs,** catalase and oxidase negative

2 subgroups : homofermentative

heterofermentative

Lactic acid fermentation

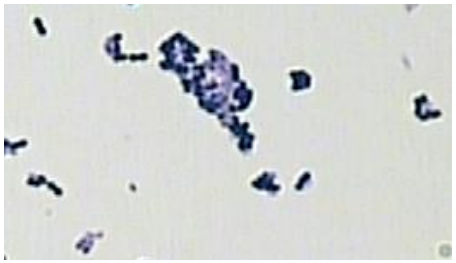
- Yoghurt-based drinks that contain **probiotic** bacteria (live micro-organisms that can give a health benefit to the host) are increasingly popular.

  Bacterial strains such as *Lactobacillus bulgaricus* **convert** lactose **sugar** into **lactic acid**. This lowers the pH and causes the milk to clot, creating the characteristic texture and taste of yoghurt.

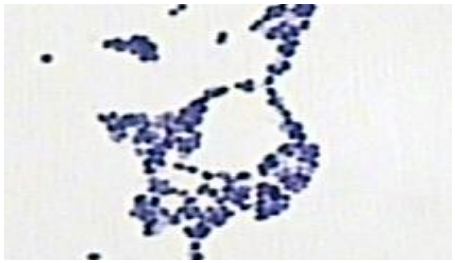
  The **live bacteria** in probiotic yoghurts are thought to restore the natural microbe population of the gut (flora), which can be depleted by antibiotics, for example.

Microscopic pictures of LAB

Lactococcus lactis, subsp.lactis



Lactococcus lactis, subsp.cremonis



Lactobacillus casei, subsp.casei

Lactobacillus delbrueckii, subsp. bulgaricus

Probiotic bacteria

- Probiotic (**for life**) bacteria can protect the host and prevent disease.
- They are live micro-organisms that provide a health benefit to the host.
- For example, antibiotics can kill off normal intestinal flora and the administration of probiotic bacteria can replenish the flora to normal levels.

Fermentor

- The term **fermentor** when used in the industrial microbiology context, includes any large scale microbial process carried out under aerobic or anaerobic conditions.
- Most recently, genetically engineered bacteria have been grown on an industrial scale to produce substances they do not normally make.

Two meanings of fermentation

- It should also be noted that microbiologists use the term **fermentation** in **two different contexts**.
- In the **context of metabolism**, fermentation refers to growth in the absence of an external electron receptor (*anaerobic conditions*)
- whereas in the **context of industrial microbiology,** the term refers to the growth of large quantities of cells (*aerobic and anaerobic*).

Collections of Microorganisms

- There are several important culture collections that maintain repositories of important microorganisms. In the United States, the **American Type Culture Collection** (**ATCC**) is probably the best known, however, the Northern Regional Research Laboratory (NRRL) is also well known.
- **World Federation for Culture Collections**
- Czech Collection of Microorganisms (CCM)
- Leibnitz-Institute DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen
- Leibnitz-Institute DSMZ –German Collection of Microorganisms and Cell Cultures

Secondary metabolites

- The pharmaceutical industry is an important user of microbes. For many years, **antibiotics** and **steroid hormones** have been produced by microbes. Genetic engineering has made it possible for bacteria to produce a wide variety of mammalian substances that are medically important.

Secondary metabolites

- Many of the most important industrial metabolites are **secondary metabolites**, produced in the stationary phase of the culture after microbial biomass production has peaked.
- These compounds are not essential for growth of the microbe. Their synthesis is usually tightly regulated by the cell. Therefore to obtain high yields, environmental conditions that elicit regulatory mechanisms such as repression and feedback inhibition must be avoided.
- In addition, mutant strains that **overproduce** the compound are selected. In secondary metabolism two phases are apparent: **trophophase** and **idiophase**. Trophophase is the growth phase of the culture; idiophase is the time when the secondary metabolites are formed. The success of the idiophase is dependent on the trophophase.

Secondary metabolites

- After a suitable microorganism has been identified from laboratory studies for an industrial process, there are still a number of **scale-up** problems to solve. These include provision of adequate aeration and mixing throughout the large fermentor.
- The difficulties involve the enormous volume of the vessel, areas where mixing is less efficient, and the high biomass content of the fermentor.
- High biomass is desirable to increase the product formed, but it creates an enormous demand for oxygen. Furthermore, a strain that worked well on a small scale may not be as efficient under the different conditions experienced in the large fermentor.

Industrial strains

- The organisms used in industrial processes must be **carefully preserved** so that their carefully selected attributes do not change because of mutation.
- This may involve frozen storage in liquid nitrogen or lyophilization.
- The inoculum for the fermentor must be built up from a **working strain**. Since inocula should be 5-10% of the culture volume, the inoculum for a production fermentor may be 10,000 liters.

Antibiotics

- Antibiotics are among the most important compounds produced by industrial microbes. The most useful ones are secondary metabolites produced by **filamentous fungi**, and bacteria classified as **actinomycetes**.
- New antibiotics are discovered by **screening** microbes isolated from natural samples for the production of chemicals that inhibit specific test bacteria. The test bacteria are related to bacterial pathogens.
- Most of the positive results are likely to be currently known antibiotics, but new ones are still discovered.
- The **new substance is tested for toxicity** and effectiveness in infected animals before commercial production is started. Most new substances are likely to be either toxic to the animal, or relatively ineffective in killing pathogens in the body.

Other products

- **Vitamins** and **amino acids** are used as supplements in human and animal feed. Some of these are produced most economically by bacteria, if high-yielding, overproducing strains can be developed. In general, this involves inactivating the regulatory mechanisms that keep biosynthesis of these substances in line with that of other cellular building blocks.
- One trick for doing so is to find mutants that grow in the presence of chemical analogs of the amino acid. These often **lack feedback** control of enzyme activity.
- In some industrial processes, microbes are only used to carry out a **specific biochemical reaction**; the remainder of product formation is accomplished by strictly chemical means. An example of this type of **bioconversion** is steps in the production of steroid hormones.

Microbial enzymes

- The largest use of microbial enzymes has involved extracellular **hydrolytic** enzymes that digest insoluble materials (amylases,proteases). They have been used in the laundry industry; one problem is that some people are allergic to these proteins.
- A suite of three enzymes is used to produce a sweet material from starch. **High-fructose corn syrup** is produced by hydrolyzing starch to glucose, and **isomerizing** glucose to a sweeter molecule, fructose. Another important microbial enzyme is microbial rennin that is widely used in the cheese production.

Food industry

- Vinegar can be produced by the **acetic acid bacteria** from an alcoholic fluid such as wine or cider, if oxygen is provided. There are several industrial methods for bringing these reactants together.
- **Citric acid** is produced by the fungus *Aspergillus niger*. Industrial fungal fermentations may occur on the surface of a medium or submerged in the liquid. For **surface processes**, the medium can be either solid or liquid.

Microbial biomass

- Microbes have potential as food supplements because many of them contain 50% or more protein. In most diets, protein is in shortest supply. However, there can be problems with toxicity and digestibility that limit the amount of **single-cell protein** in the human diet.
- Microorganisms are important in wastewater treatment for two reasons. First, one of the goals of treatment is to destroy all pathogenic microbes that are in the sewage. Second, microbial activity is used to oxidize the organic matter in wastewater to methane or carbon dioxide gas.

sewage treatment

- There can be three stages of sewage treatment.
- **Primary treatment** physically removes particulate material with screens and in a settling pond.
- **Secondary treatment** uses microorganisms to reduce the level of organic matter; the amount of residual organic matter is quantified as the **biochemical oxygen demand**.
- **Tertiary treatment** includes processes that remove inorganic nutrients, such as phosphate and nitrate, from the wastewater. Most sewage plants use only primary and secondary treatment of wastewater.

Anaerobic conditions

- Secondary wastewater treatment may involve either aerobic or anaerobic processes.
- Under **anaerobic conditions**, the microbial reactions are sequentional: macromolecules are hydrolyzed to soluble monomers; these are fermented by a series of bacteria to acetate, H_2, and CO_2.
- These substrates are converted to **methane** gas by **methanogens** – anaerobic respiration

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Relations between food and microorganisms

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Factors affecting microbial growth in food

- Intrinsic Factors
- Environmental Factors
- Implicit Factors
- Processing Factors

Intrinsic Factors

- Nutrients
- pH and buffering capacity
- Redox potential
- Water activity
- Antimicrobial constituents
- Antimicrobial structures

Nutrient content

- The concentration of key nutrients can, to some extent, determine the rate of microbial growth.
- The relationship between the two, known as Monod equation, is mathematically identical to the Michaelis-Menten equation of enzyme kinetics.
- It reflects the dependence of microbial growth on rate-limiting enzyme reaction.

Monod equation

- $\mu = \mu_m S / S + K_s$

- μ specific growth rate
- μ_m maximum specific growth rate
- S concentration of limiting nutrient
- K_s saturation constant

pH and buffering capacity

- As measured with the glass electrode, pH is equal to the **negative logarithm** of the hydrogenion activity.

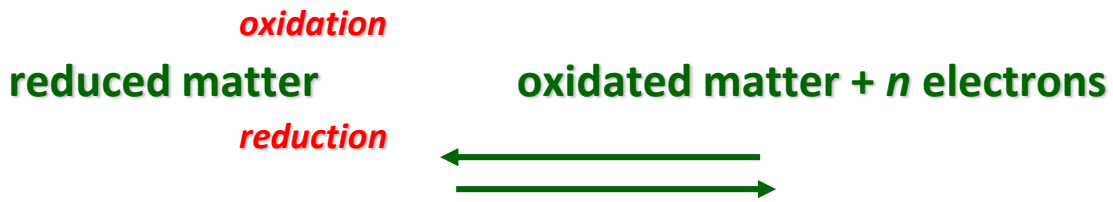
- For aqueous solutions **pH 7** corresponds to **neutrality**, pH values **below 7** are **acidic** and those **above 7** indicate an **alkaline** environment.

- In general, bacteria grow fastest in the pH range **6.0-8.0**, yeasts **4.5–6.0** and filamentous fungi **3.5-10.0**.

Redox Potential - E_h

An oxidation-reduction potential of environment

- Difference of platinum electrode potential placed into the given environment and the potential of standard hydrogen electrode.



Reduction of redox potential

- Addition of reducing compounds
- Growth of aerobic microorganisms
- Vacuum package , etc.

Water activity - a_w

Water activity

- Is defined as the ratio of the partial pressure of water in the atmosphere in equilibrium with the substrate p (*p),* compared with the partial pressure of the atmosphere in equilibrium with pure water at the same temperature (p_0).

$$a_w = \frac{p}{p_0}$$

- Amount of water available for microorganisms to metabolize
- Optimal value for majority of microorganisms **a_w>0,98**

Water activity - a_w

Lowered Water Activity (a_w)

Lowering a_w is one of the earliest methods of food preservation used by humans - (partial) drying, addition of salt or sugar

Still part of the modern diet - *e.g.* jams, hams, hard cheeses, pasta *etc.*

Many modern bakery goods, snack foods and cereals are preserved using the ancient methodology combined with modern processing techniques

a_w can be lowered by

- **removing water by (partial) drying)**
- **addition of solutes (preserving)**

Foods can be classified by their a_w value:

Fresh foods	**≥ 0.98**
"Wet foods"	**0.86 – 1.00**
Intermediate Moisture Foods (IMF)	**0.60 – 0.85**
"Dried foods"	**< 0.60**

Bacteria can grow down to a_w = 0.85 (*S. aureus* and some salmonellae) and moulds to a_w = 0.62

IMF contain 15 – 30% moisture and include dried fruits, jams, and some cakes and sauce

a_w - Microbial response

Microbial response to lowered a_w

- **Accumulation of Compatible Solutes**
- **Modification of Membrane Lipid Composition**

Compatible solutes (CS) are accumulated intracellularly

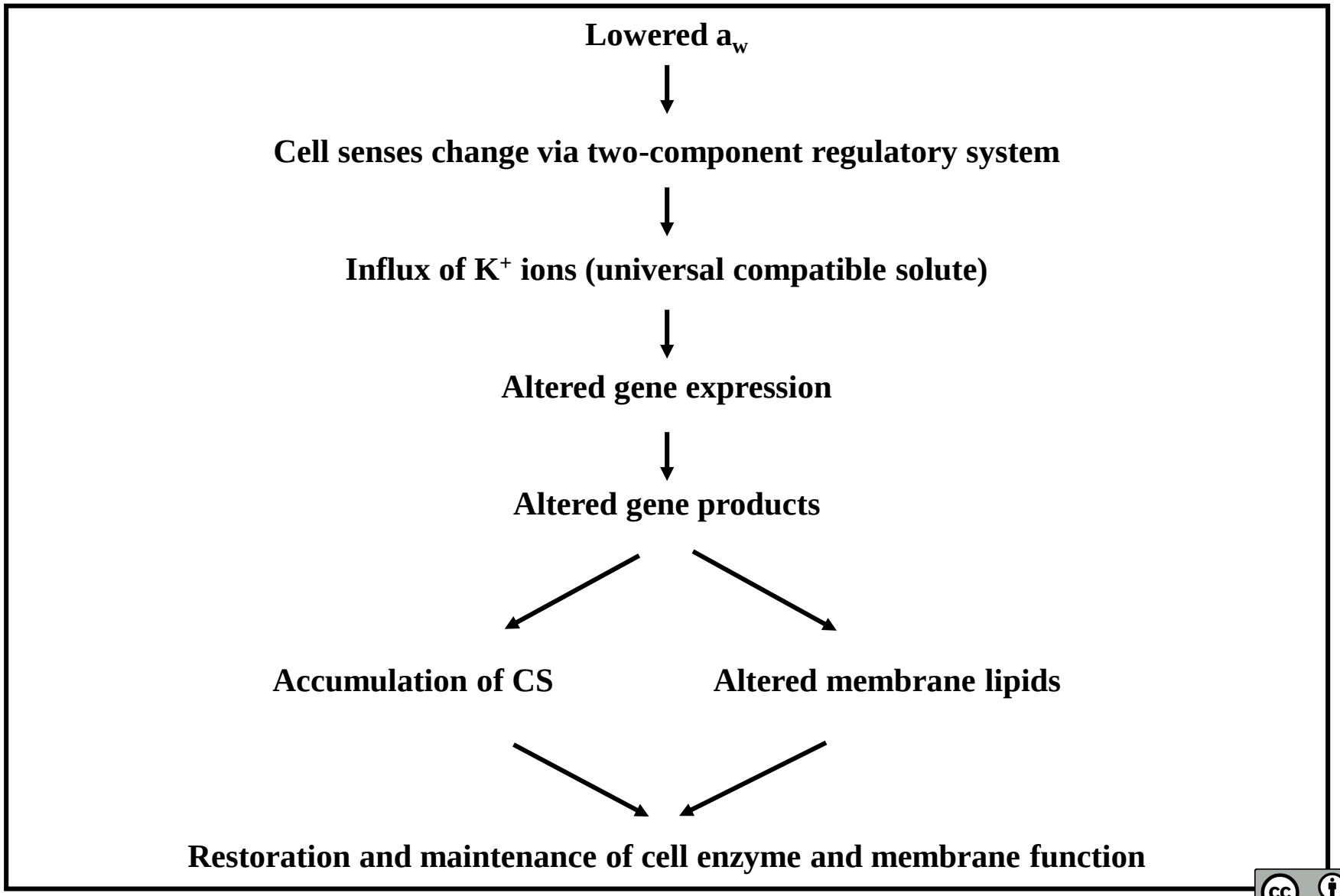
Typical CS accumulated by food-associated microbes include:

- **Betaine and derivatives**
- **Peptides, amino acids (*e.g.* glutamate, proline)**
- **Carnitine**
- **Trehalose, sucrose**
- **Mannitol, glycerol**
- **K^+**

There is no correlation between the ability to withstand low a_w and the type of CS accumulated

Bacteria tend to accumulate N-compounds, moulds mainly sugars/sugar alcohols

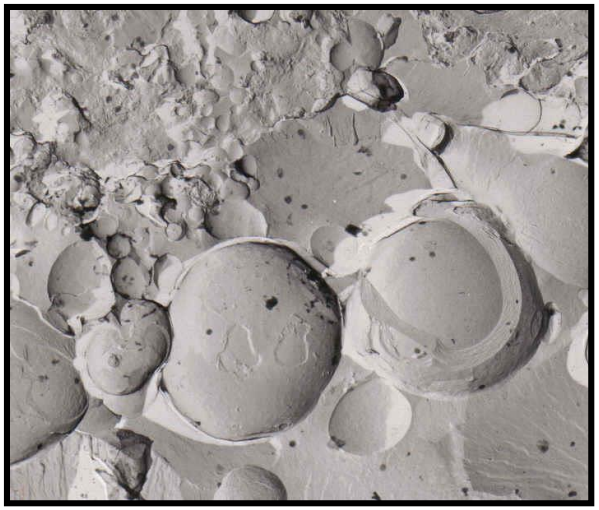
Lowered a_w - Microbial response



a_w - Membrane lipid response

When external OP increases due to the addition of preservative solutes, the head-groups of membrane phospholipids tend to become dehydrated (external solutes bind water)

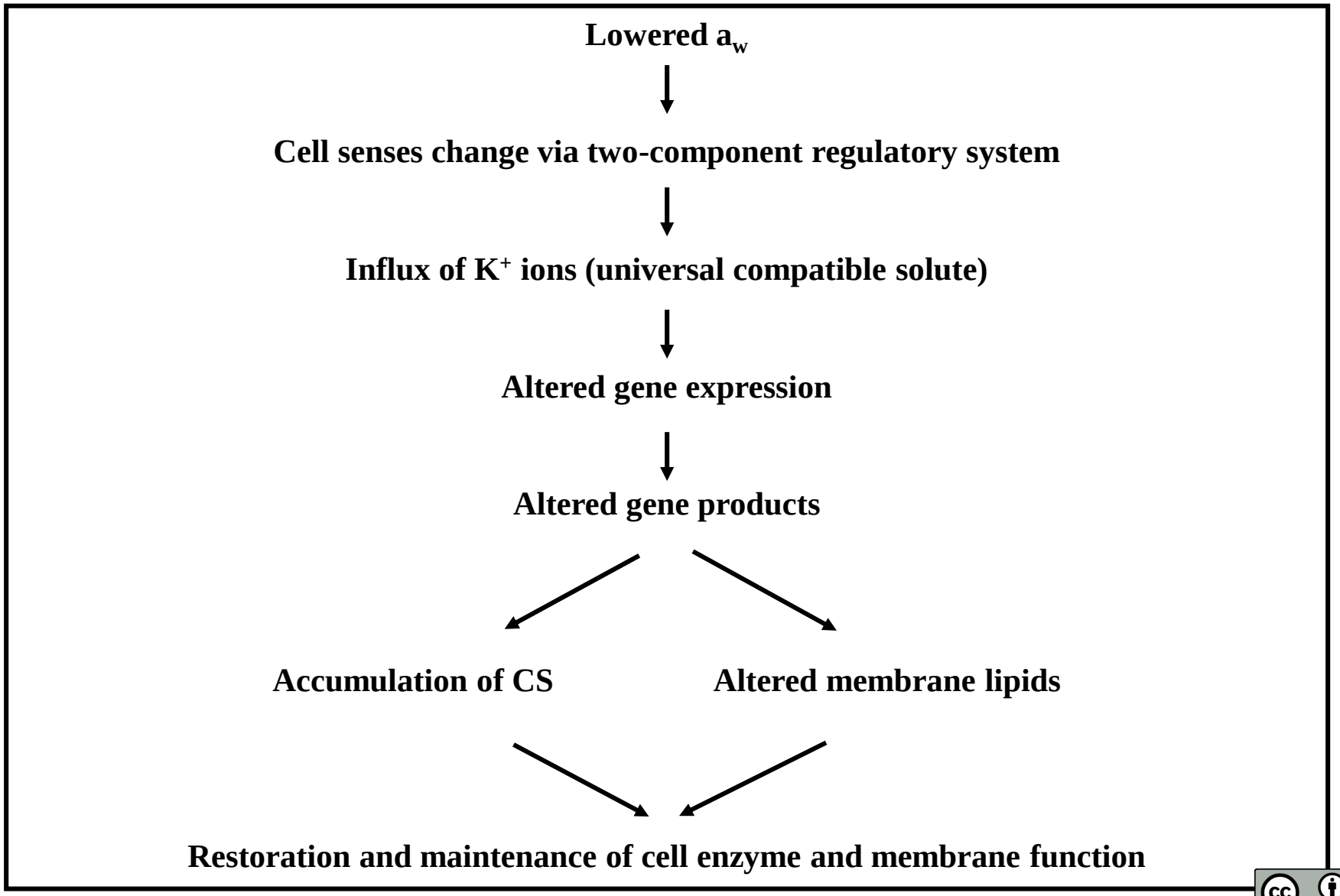
This disrupts the normal bilayer phase of the membrane and could lead to the formation of non-bilayer phases:



However, the microbial cell responds by synthesising more (anionic, negatively-charged) phospholipids that form non-bilayer phases less readily

This maintains the functional lipid bilayer

Lowered a_w - Microbial response



Hurdle Technology

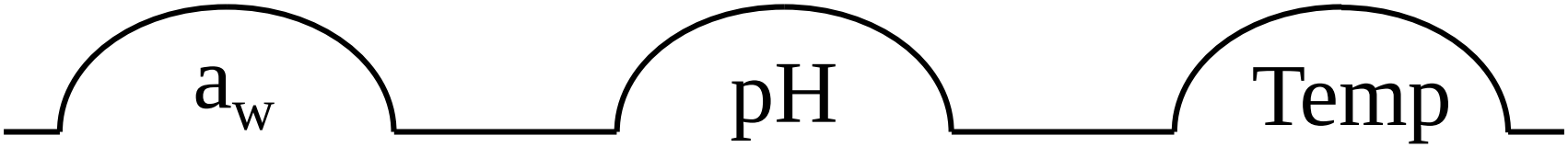
Hurdle technology is the use of (synergistic) combinations of preservation factors to enhance the preservative effect and give better organoleptic and nutritional qualities to foods

Driving force to its development is the public demand for more "natural" wholesome foods that taste/smell better and have improved nutritional quality

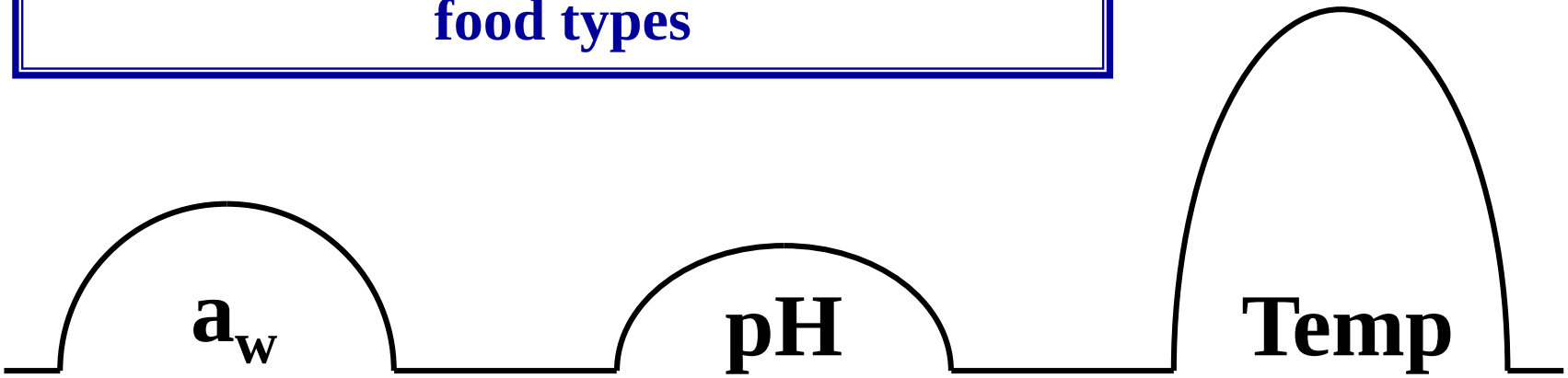
For example, if a limiting a_w of 0.85 or pH 4.6 alone prevents growth of a food-borne pathogen, similar protection might be given by a combined a_w of 0.92 and pH 5.2

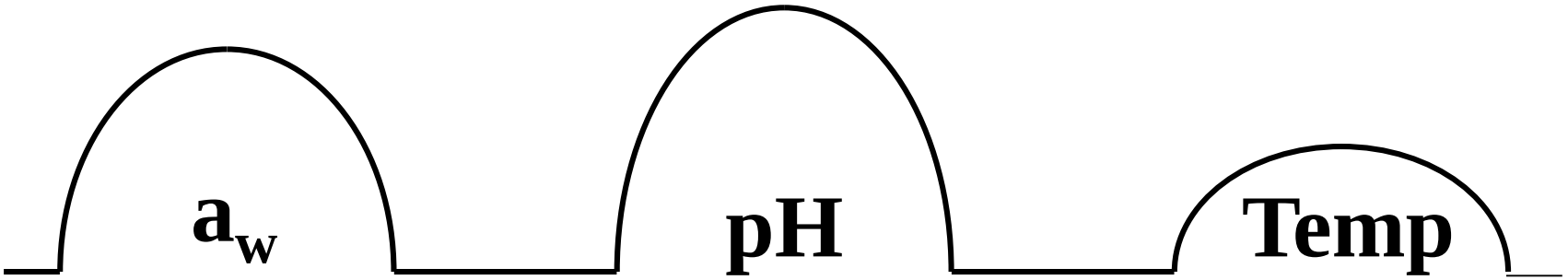
Chilling will further enhance the synergism

Each preservative factor is called a "hurdle"

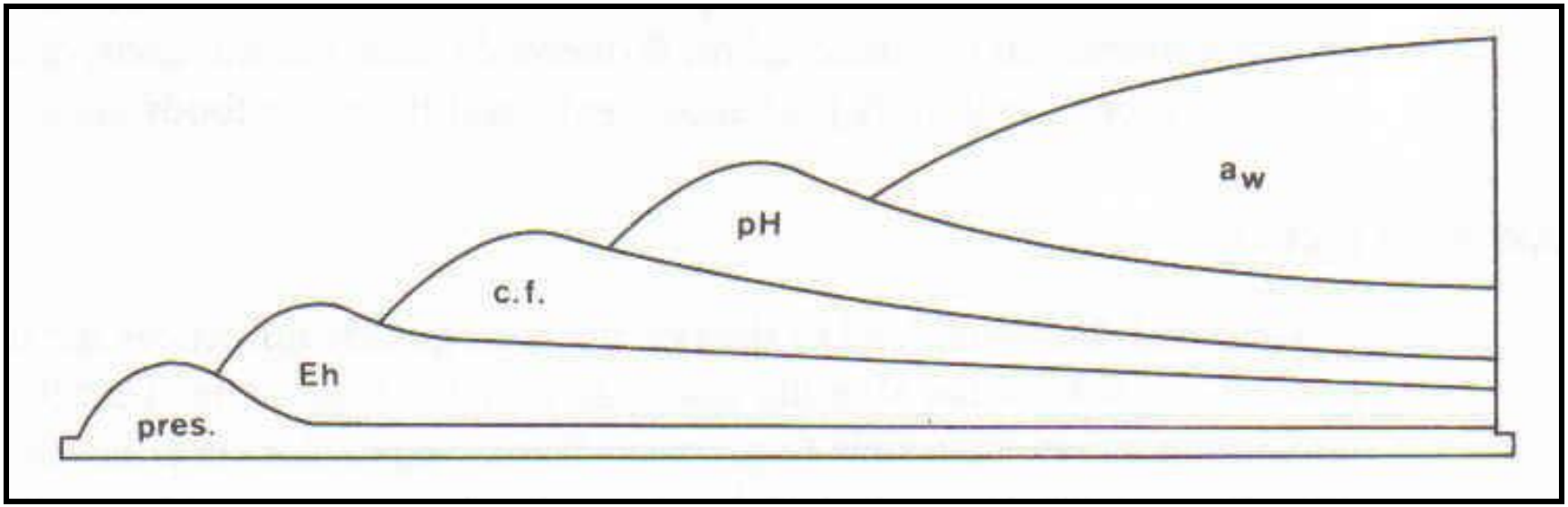


The sizes of hurdles can be varied in different combinations to suit different food types





Hurdle Technology - "The life of a salami"



The individual preservative factors of Hurdle Technology should target different homeostatic mechanisms in the food-associated microbes:

low a_w places demands on energy supply to accumulate CS

low pH places demands on energy supply to pump out H^+

low temperature demands energy to maintain membrane homeostasis

Because of the overlaps in the stress responses (*e.g.* via the RpoS system) this will drain the cell of energy, slow growth rate and even stop growth, leading eventually to cell death

Factors affecting microbial growth in food

- *Implicit factors:*
- Specific growth rate
- Synergism
- Antagonism
- Commensalism

Antimicrobial Barriers and Constituents

- **Physical barrier** to infection: skin, shell, husk or rind of the product. Usually composed of **macromolecules** relatively **resistant to degradation** and provides unhospitable environment for microorganisms by having low water activity, shortage of readily available nutrients and often **antimicrobial compounds** such as **short chain fatty acids** (on animal skin) or **essential oils** (on plant surfaces).

Effect of antimicrobial substances

Some substances present in environment display negative effect on microorganisms, based on their specific composition (antimicrobial).

Microbistatic - compounds stop division of microorganisms

Microbicidal – compounds killing microorganisms

Effect of concentration (stimulatory effect)

The main classes of antimicrobial agents are disinfectants, which kill a wide range of microbes on non-living surfaces to prevent the spread of illness, antiseptics and antibiotics.

Types of antimicrobial effects

Compounds damaging structure of cell or its function (cell wall, cytoplasmatic membrane, ribosomes)

Compounds affecting microbial enzymes (oxidative agents, chelating agents, heavy metals, antimetabolites)

Compounds reacting with DNA (chemical mutagenes – alkylating or deaminating agents, cytostatics)

Presence of antibacterial compounds (biocides) in food

- Some foods contain **natural antimicrobial compounds** (spices, mineral oils, garlic, mustard, honey)

- Raw cow milk contains lactoferrin, lactoperoxidase system, lysozyme, casein

- Eggs contain **lysozyme**, conalbumin, ovotransferrin, avidin

Antimicrobial enzymes

They are abundantly spread in nature and are very important in defensive reactions between living microorganisms.

Hydrolases – degrade basic structures of the cell walls (peptidoglycan)

Oxidoreductases – produce reactive molecules that degrade vital cell proteins.

Antimicrobial enzymes

Bacteriolytic

1. N-acetylhexosaminidases catalyze splitting of glycosidic bonds of saccharides in peptidoglycan
2. N-acetylmuramyl-L-alaninamidases catalyze splitting between saccharidic peptidic part of peptidoglycan
3. Endopeptidases hydrolyze peptidic bonds of peptidoglycan
4. Others – chitinases , ß-glucanases

Antimicrobial enzymes

Oxidoreductases

Glucosooxidases are produced by some molds, the principle of cytotoxicity lies in the formation of hydrogen peroxide (glucose oxidation on gluconic acid and H_2O_2)

Lactoperoxidases occur in milk and saliva

(thiocyanate oxidation on hyperthiocyanate)

Lactoferrin is a glycoprotein forming complexes with Fe ionts

Environmental factors

- Relative humidity
- Temperature
- Gaseous atmosphere

Relative humidity

- Relative humidity and water activity are interrelated, thus relative humidity is essential a measure of the water activity of the **gas phase**.
- When food commodities having a low water activity are stored in an atmosphere of high relative humidity - **water will transfer from the gas phase to the food.**

Temperature

Temperature Is one of the most important environmental factors , controlling the rate of cell division (multiplication) of microorganisms

- We recognize 3 basic temperatures

  minimal temperature

  optimal temperature

  maximal temperature

Cardinal temperatures for microbial growth

Microorganisms belong to groups according to optimal temperature for division and metabolism

Psychrophilic bacteria (12-15 °C)

Psychrotrophic bacteria (25-30 °C)

Mesophilic bacteria (30-40 °C)

Thermophilic bacteria (50-70 °C)

Effect of high temperatures

Killing effect of high temperatures (**lethal temperature**)

- is the **lowest temperature** at which in certain time are **all microorganisms killed** (70 °C/10 min)
- denaturation of proteins, enzyme inactivation, DNA and cytoplasmatic membrane disruption
- is dependent on: species of microorganisms
  its physiologic status
  cell concentration
  environment character

Thermoresistance

Degree of micoorganisms resistance depends on:

- physiologic status of bacteria
- their genetic properties
- amount of bacteria
- water content in substrate
- quantity of protective compounds (lipids, proteins, saccharides)

Appertization

appertization - French term for the process of destroying all the micro-organisms of significance in food, i.e. 'commercial sterility';

a few organisms remain alive but quiescent. Named after **Nicholas Appert** (1752–1841), a Paris confectioner who invented the process of canning; he opened the first vacuum bottling factory in 1804.

Processes where the only organisms that survive processing are non-pathogenic and incapable of developing within the product under normal conditions of storage.

- Appertized products have a long shelf-life even when stored at ambient temperature.

Pasteurisation

pasteurisation - partial sterilization of foods at a temperature that destroys harmful microorganisms without major changes in the chemistry of the food.

Properly done pasteurisation assures:

- **devitalisation of pathogen** microorganisms (*Mycobacterium tuberculosis*)
- devitalisation of substantial portion of saprophytic microflora (vegetative cells)
- conservation of original fysical, chemical, nutritive and sensoric qualities

PASTEURISED FOOD CONTAINS MICROORGANISMS!

Milk - Pasteurisation

There are 4 main types of heat treatment:

Low Temperature Holding (LTH)	63°C for 30 minutes
High Temperature Short Time (HTST)	72°C for 15 seconds
Ultra High Temperature (UHT)	135°C for 1 second
"Sterilised"	>100°C for 20 – 40 minutes

LTH has been superceded by HTST pasteurisation nowadays (shelf-life of 10 – 20 days) and membrane filtration is also being explored to produce quasi-sterile milk. UHT milk is essentially sterile and there is no problem with *Clostridium botulinum* as it is rare in milk and the oxygen levels are too high and the heat treatment sufficient. Pasteurisation is monitored by enzyme assay of alkaline phosphatase. Gram-negative psychrotrophs are easily killed by pasteurisation, but some "thermoduric" Gram-positives (*e.g. B. cereus*) do survive. Spoilage of milk is usually by Gram-negative rods (*Pseudomonas*, *Alcaligenes*, *Acinetobacter* and *Psychrobacter*), which are post-pasteurisation contaminants.

Sterilisation

- One-time application of temperature higher than 100 °C
- Sterilisation is defined as combination of temperature and time
- Sterilisation of food – survival of certain spores (genus *Bacillus* and *Clostridium*)
- Practical (commercial) sterility x absolute sterility

STERILISED FOOD MAY CONTAIN SPORES!

Quantifying the thermal death of microorganisms

- When the temperature is increased above the maximum for growth, cells are injured and killed as key cellular components are destroyed.
- The generally accepted view is that thermal death is a first order process, i.e. the rate of death depends upon the number of viable cells present.

$$dN/dt = -cN$$

dN/dt - is the rate of death, N – the number of present viable cells

C – proportionality constant

D - value

- The time required at a certain temperature to kill 90% of the organisms being studied. Thus after an organism is reduced by 1 D, only 10% of the original organisms remain. The population number has been reduced by one decimal place in the counting scheme.
- Generally, each lot of a sterilization-resistant organism is given a unique D-value.
- When referring to D values it is proper to give the temperature as a subscript to the D. For example, a hypothetical organism is reduced by 90% after exposure to temperatures of 60 degrees Celsius for 2 minutes, thus the D-value would be written as D_{60C} = 2 minutes.
- D-value determination is often carried out to measure a disinfectant's efficiency to reduce the number of microbes present in a given environment.

D value

Thus after an organism is reduced by 1 D, only **10%** of the **original organisms** remain. The population number has been reduced by one decimal place in the counting scheme. Generally, each lot of a sterilization-resistant organism is given a **unique D-value**.

When referring to D values it is proper to give the temperature as a **subscript** to the D.

For example, a hypothetical organism is reduced by 90% after exposure to temperatures of 60 degrees Celsius for 2 minutes, Thus the D-value would be written as

$D_{60}F$ = 2 minutes.

D-value determination is often carried out to measure a disinfectant's efficiency to reduce the number of microbes, present in a given environment.

Thermal Resistance

- **D-Value:**
  - Time at a given temperature needed to reduce a microbial population by 90%
- **Z-Value:**
  - Change in temperature needed to change D-value by 90%

Z - value

- **Z-value** of an organism is the temperature, in degrees Celsius or Fahrenheit, that is required for the D value to move(decrease) by **one log cycle**.
- It is the reciprocal of the slope resulting from the plot of the logarithm of the D-value versus the temperature at which the D-value was obtained.
- It may be simplified as the temperature required for one log reduction in the D-value.
- While the **D-value** gives us the time needed at a certain temperature to kill an organism, the **z-value** relates the resistance of an organism to differing temperatures.

Z - value

- So, the z-value allows us to calculate a thermal process of equivalency if we have one D-value and the z-value.
- So, if it takes an increase of 10°C to move the curve one log, then our z-value is 10.
- So then, if we have a D-value of 4.5 minutes at 65°C, we can calculate D-values for 65°C by reducing the time by 1 log.
- So, our new D-value for 65°C is 0.45 minutes. This means that each 10°C increase in temperature will reduce our D-value by 1 log. Conversely, a 10°C decrease in temperature will increase our D-value by 1 log.
- So, the D-value for a temperature of 55°C would be 45 minutes.

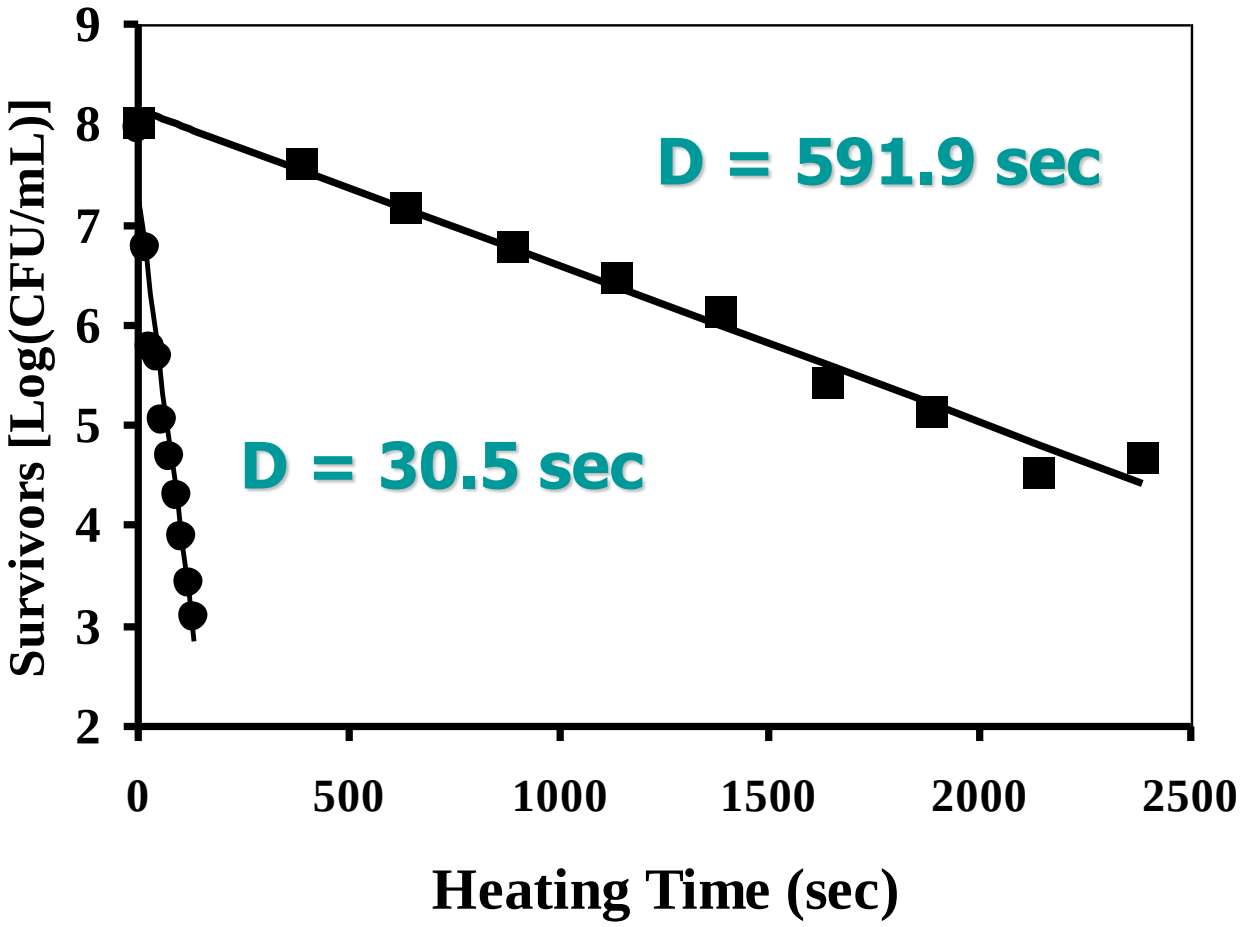
D value

- As the **temperature** is **increased** so the **D value decreases**. This is an exponential process over the range of temperatures used in the heat processing of food so that plotting **log D against temperature gives a straight line.**
- From this is possible to derive another important parameter in heat processing, **z:** the temperature change which results in a tenfold (1 log) change in **D.**

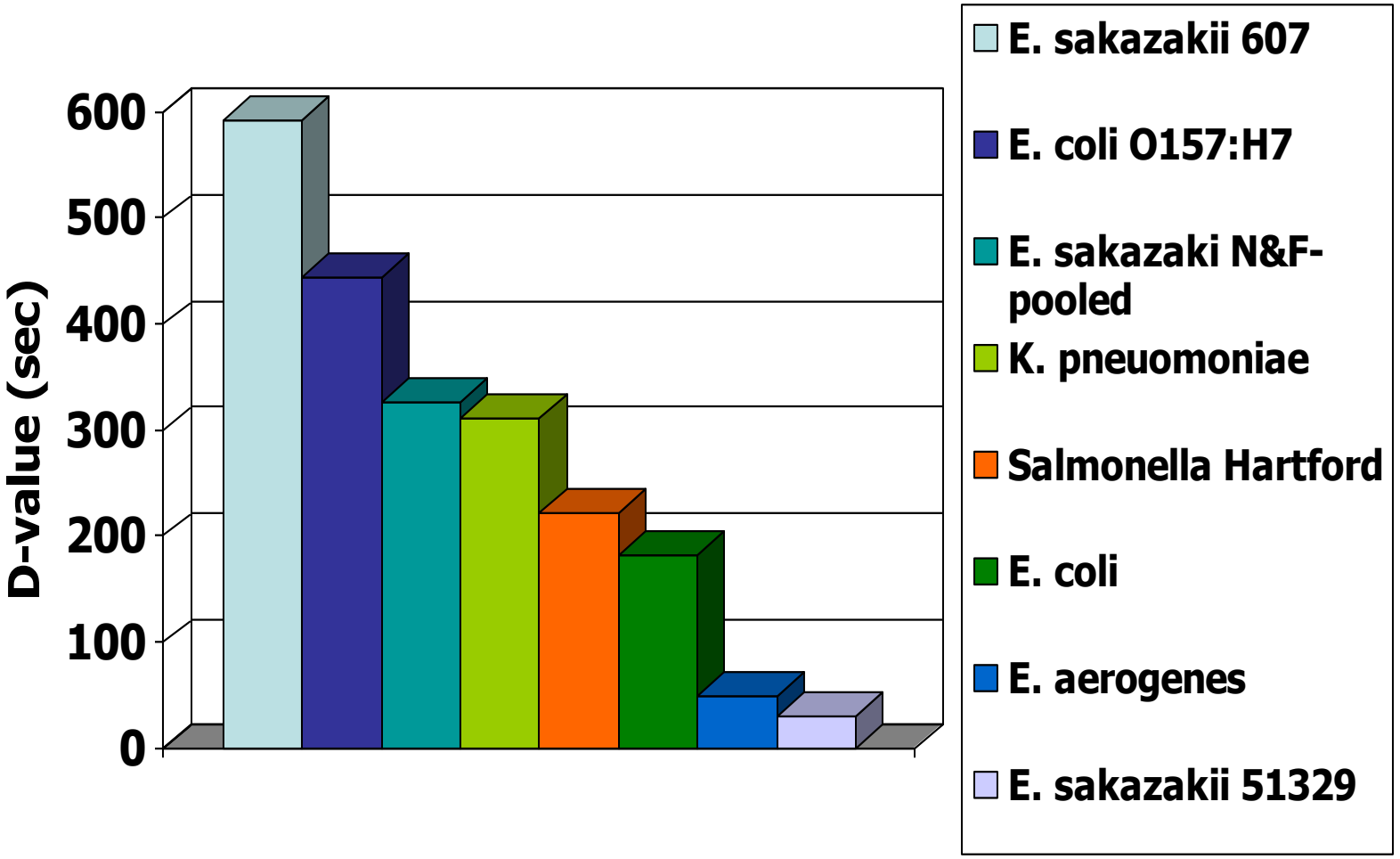
Microbial heat resistance

	D (mins)
Salmonella sp.	D_{65} 0.02 – 0.25
Staphylococcus aureus	D_{65} 0.2 – 2.0
Escherichia coli	D_{65} 0.1
Listeria monocytogenes	D_{60} 5.0 – 8.3
Campylobacter jejuni	D_{55} 1.1
Yeasts and molds	D_{65} 0.5 – 3.0

Thermal Death Time Curves for 2 *Enterobacter sakazakii* Strains Heated at 58°C



Comparison of $D_{58°C}$-Values for Different Enterobacteriaceae



Gaseous atmosphere

- Oxygen forms 21% of the Earth atmosphere.
- Effect of carbon dioxide is not uniform.

- Anaerobic microorganisms
- Aerobic microorganisms
- Facultatively anaerobic microorganism
- Microaerobic microorganisms

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Factors influencing microbial growth

Lecturer:

Jarmila Pazlarová

Department of Biochemistry and Microbiology

Factors affecting microbial growth in food

- Intrinsic Factors
- Environmental Factors
- **Implicit Factors**
- Processing Factors

Implicit factors

Factors affecting microbial growth in food

- *Implicit factors:*
- **Specific growth rate / μ**
- Synergism
- Antagonism
- Commensalism

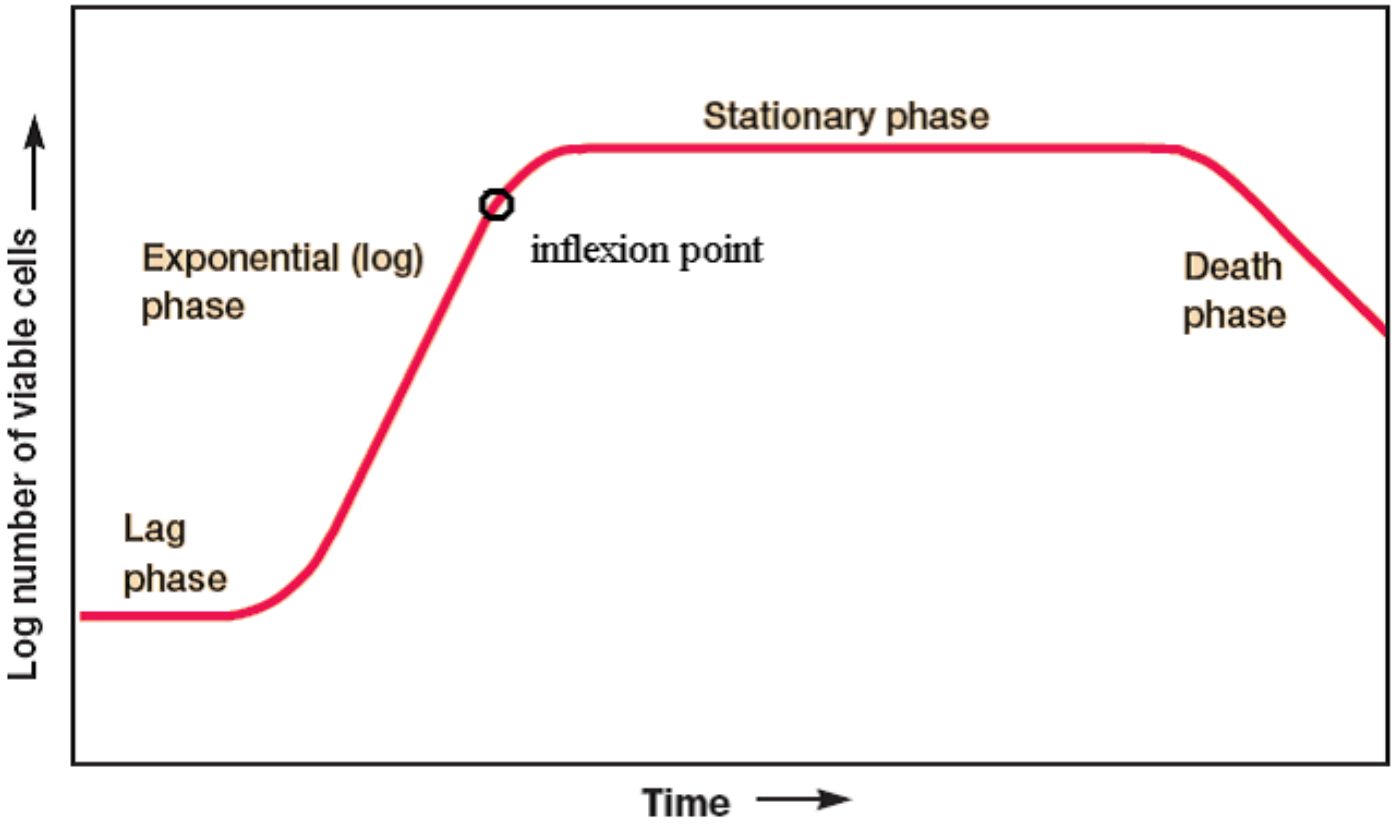
Implicit factors

- **Specific growth rate –** individual characteristic, geneticaly coded
- **Synergisms –** cooperation of more species
- **Antagonisms –** negative actions between various species
- **Commensalisms –** one population uses other one without demaging it – one member has advantage, the other is not infuenced

Specific growth rate / **μ**

- The ***rate*** of exponential growth of a bacterial culture is expressed as **generation time**, also the doubling time of the bacterial population.
- **Generation time** (**G**) is defined as the time (**t**) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time (below) derive.

Standard growth curve



Microbial Growth Curve in a Closed System.

Bacterial growth rate

- Growth of bacterial cultures is defined as an increase in the number of bacteria in a population rather than in the size of individual cells. The growth of a bacterial population occurs in a geometric or exponential manner: with each division cycle (generation), one cell gives rise to 2 cells, then 4 cells, then 8 cells, then 16, then 32, and so forth.
- The **generation time**, which varies among bacteria, is controlled by many environmental conditions and by the nature of the bacterial species. For example, *Clostridium perfringens* , one of the fastest-growing bacteria, has an optimum generation time of about 10 minutes; *Escherichia coli* can double every 20 minutes; and the slow-growing *Mycobacterium tuberculosis* has a generation time in the range of 12 to 16 hours.

Lag phase

Bacteria are placed in a medium that provides **all of the nutrients** that are necessary for their growth, the population exhibits **four phases** of growth that are representative of a typical bacterial **growth curve**. Upon inoculation into the new medium, bacteria do not immediately reproduce, and the population size remains constant. During this period, called the **lag phase**, the cells are **metabolically active** and **increase only in cell size**.

They are also synthesizing the enzymes and factors needed for cell division and population growth under their new environmental conditions.

Log phase

- The population then enters the **log phase** , in which cell numbers increase in a **logarithmic fashion**, and each cell generation occurs in the same time interval as the preceding ones, resulting in a balanced increase in the constituents of each cell.
- The log phase continues **until nutrients are depleted** or toxic products accumulate, at which time the cell growth rate slows, and some cells may begin to die. Under optimum conditions, the maximum population for some bacterial species at the end of the log phase can reach a density of **10 to 30 billion cells per millilitre.**

Stationary and death phases

- The **log phase** of bacterial growth **is followed** by the **stationary phase**, in which the size of a population of bacteria remains constant, even though some cells continue to divide and others begin to die.
- The **stationary phase** is followed by the **death phase,** in which the death of cells in the population exceeds the formation of new cells. The length of time before the onset of the death phase depends on the species and the medium. Bacteria do not necessarily die even when starved of nutrients, and **they can remain viable for long periods of time.**

Calculation of the growth rate I

- From the average rate of division it is possible to calculate the dependency of the number of created cells on time: $\mathbf{x = x_0.2^{r.t}}$
- This dependency shows that the number of newly created cells is an **exponential function of time.**
- From the average rate of division it is also possble to calculate **mean generation time**, which is the time necessary for the formation of one generation:
- $\mathbf{T = 1/r = T \,.\, log2 \,/\, log\, x/x_0}$

Calculation of the growth rate II

- Derivation of the dependence of number of created cells according to time interval (= immediate increase of cells) gives :
- $\mathbf{x = x_0.2^{r.t}}$

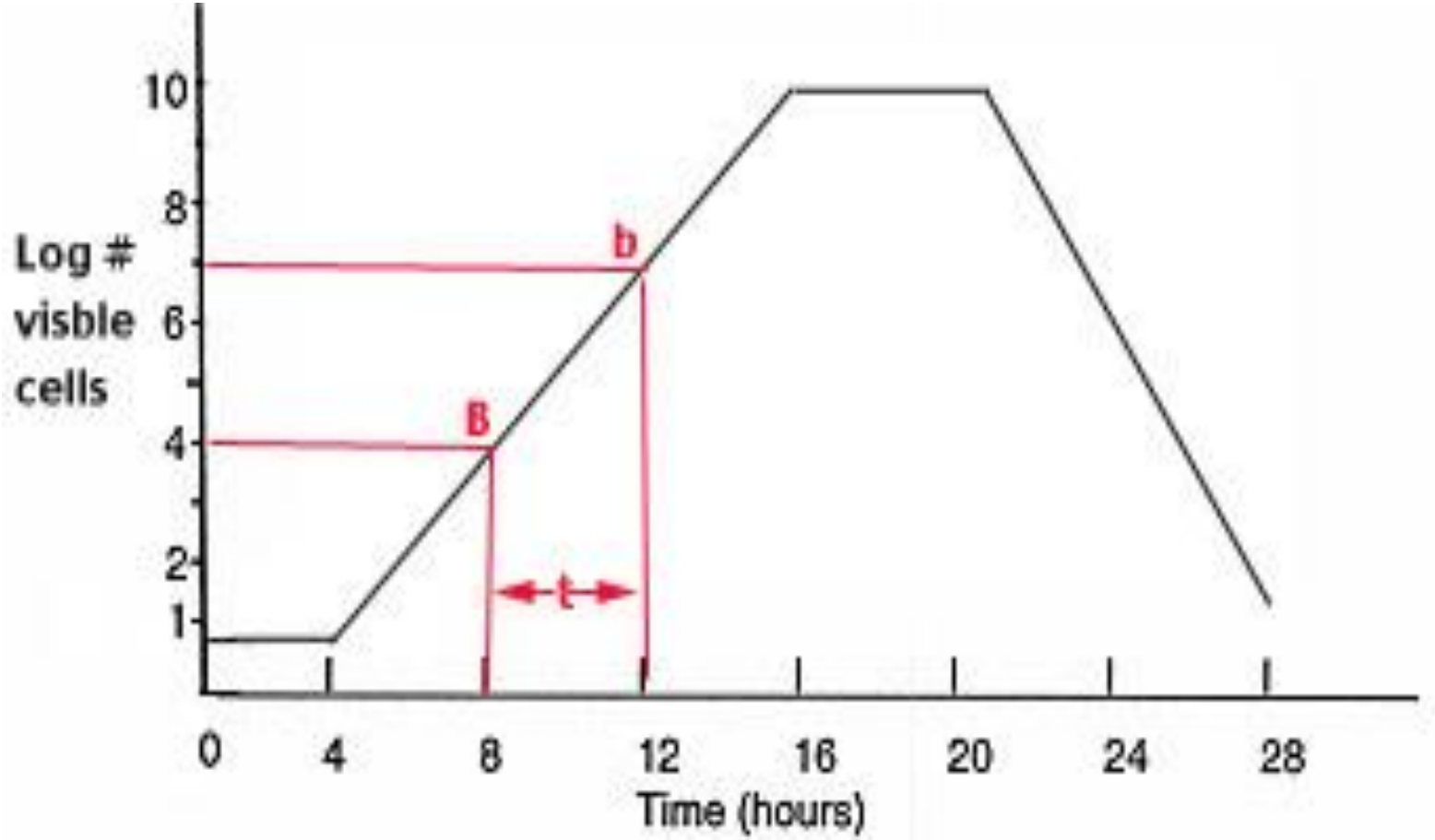
- This equation shows that the number of created cells is an **exponential function of time.**

$\frac{dx}{dt}$

Calculation of the growth rate III

- **$dx/dt = x_0.2^{rt}.r.\ln 2 = x.r.\ln 2$**
- When we define the specific growth rate as : **$\mu = r.\ln 2$** , we obtain a simple diferential equation :
- **$dx/dt = \mu.x$**
- Doubling time **T** is obtained from euqation
- **$\ln 2 = \mu . T$**
- **$T = \ln 2 / \mu = 0{,}693 / \mu$**

Growth rate calculation



Monod equation

$$\mu = \mu_m S / S + K_s$$

- μ specific growth rate
- μ_m maximum specific growth rate
- S concentration of limiting nutrient
- K_s saturation constant

Growth Kinetics

- **Growth curves** showing the **lag**, **exponential, stationary**, and **death phases** of a culture are normally plotted as the number of cells on a log scale, or Log 10 cell number versus time.
- These graphs represent the **state of microbial populations** rather than individual microbes. Thus, both the lag phase and the stationary phase of growth represent periods when the growth rate equals the **death rate** to produce no net change in cell numbers.
- **Food microbiology is the only area of microbiology where all four phases of the microbial growth curve are important**. Microbial inhibitors can extend the lag phase, decrease the growth rate, decrease the size of the stationary phase population, and increase the death rate.

Factors affecting microbial growth in food

Processing factors

- *Processing factors:*
- Slicing
- Washing
- Packing
- Irradiation
- Pasteurization

Mechanic effects

High mechanic resistance of microorganisms is caused by a rigid cell wall and small size.

Destruction of cells is caused by:

- repeated **slow freezing and thawing**
  (enzymes resistance)
- high pressure treatment
- mixing with abrasive materials (glass beads]

Radiance and radiation

Electromagnetic waves of different lengths display different effect on microorganisms

- Infrared – no direct lethal effect (heat!)
- Visible light – pozitive and negative effect on some activity of cells
- Ultraviolet – strong mutagenic and lethal effects (low penetration)
- Ionic radiation- strong mutagenic and lethal effects (high penetration)

Microwave radiation I

The microwave region of the e.m. spectrum occupies frequencies between 10^9 Hz up to 10^{12} Hz and so has a relatively low quantum energy.

Microwaves act indirectly on microorganisms through the generation of heat.

When a food containing water is placed in a microwave field, the dipolar water molecules align themselves with the field. As the field reverses its polarity 2 or 5 x 10^9 times each second, depending on the frequency used, the water molecules are continually oscillating.

This kinetic energy is transmitted to neighbouring molecules, leading to a rapid rise in temperature throughout the product.

Microwave radiation II

The principal problem associated with the domestic use of microwave is non-uniform heating of foods, due to the presence of **cold spots** in the oven, and the non-uniform dielectric properties of food.

These can lead to cold spots in some microwaved foods and concern over the risks associated with consumption of inadequately heated meals .

It has led to more explicit instruction on microwavable foods.

UV radiation

UV radiation has wavelengths below 450 nm and quantum energy of 3-5 eV (10^{12}).

The quanta contain energy sufficient to excite electrons in molecules from their ground state into higher energy orbitals making the molecules more reactive.

Chemical reactions thus induced in microorganisms can cause the failure of critical metabolic processes leading to injury or death.

UV radiation

The greatest lethality of UV radiation is shown by wavelengths around 260 nm, which corresponds to strong absorption by nucleic acid bases.

The photo chemical dimerization of thymine.

Generally, the resistance to UV irradiation follows the pattern: **Gram-negatives ‹ Gram-positives = yeasts ‹ bacterial spores ‹ mould spores ‹ ‹ viruses**

Hydrostatic pressure

Majority of microorganims multiply under normal atmospheric pressure.

Pressure increase at 10-20 MPa delays multiplication and 30-40 MPa completely stops it.

Some bacteria easily grow even at 60 MPa – barophilic x barotolerant (deep see)

High hydrostatic pressure acts primarily on non-covalent linkages, such as ionic bonds, hydrogen bonds and hydrophobic interactions.

Hydrostatic pressure

Vegetative bacteria and fungi can be reduced by at least one log cycle by 400 MPa applied for 5 min.

Bacterial endospores are more resistant to hydrostatic pressure, tolerating pressures as high as **1200 MPa**.

At present, commercial application of high-pressure technology has been limited to beverages products (juices, jams).

Ultrasound

Ultrasound

Sound waves with frequency higher than 20 kHz, lethal effect on bacteria is effective only at high intensity.

Kavitative ultrasound

Subsequent to vibration, an intensive pulsation of cell membranes and cytoplasm occurs (lethal effect)

Sensitive to ultrasound are rods and fibrils, cocci are more resistant.

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Food preservation

- Microbial growth in foods is complex. It is governed by genetic, biochemical, and environmental factors. Developments in molecular biology and microbial ecology will change or deepen our perspective on the growth of microbes in foods.
- Food preservation can be thought of as a race between the human and the microbe to see who gets to eat first.

Food preservation - sulphite

Sulphite is the ancient "brimstone" of "Fire and Brimstone"

Long history, *e.g.* ancient Romans and Greeks used sulphite to preserve wine

Gaseous SO_2 dissolves in water with similar equlibria to that of CO_2

$$\mathbf{SO_2 + H_2O \leftrightarrow H_2SO_3 \leftrightarrow H^+ + HSO_3^- \leftrightarrow 2H^+ + SO_3^{2-}}$$

Sulphite is mainly used as an antibacterial in drinks and wine; also used in fruit stored for making jams or in vegetables for processing (inhibits browning reactions)

Food Preservation - Nitrite

Nitrite also has a long history of use to flavour and colour cured meats and sausages. Now also used for fish, cheese, cereals and vegetable products

Broad antimicrobial activity (incl. clostridia and salmonellae), but LABs are poorly inhibited, so nitrite can be added during milk fermentations.

Prevention of *Cl. botulinum* growth in cured meats is probably a "hurdle" phenomenon - *i.e.* other factors also play a role, particularly low pH and a_w

Mode of action poorly understood: nitrous acid (HNO_2) is probably the reactive species; this reacts with Fe and –SH groups, which are both present in respiratory compounds - energy metabolism seems particularly sensitive to nitrite.

Concern over use of nitrite is the formation of carcinogenic N-nitrosamines by reaction with secondary amines (this reaction is inhibited by vitamin C)

Hurdle technology

- Many food products use **multiple-hurdle** technology to inhibit microbial growth. Instead of setting one environmental parameter to the extreme limit for growth, hurdle technology "**deoptimizes"** several factors.
- Hurdle technology assaults multiple homeostatic processes.

Hurdle technology

Hurdle technology is the use of (synergistic) combinations of preservation factors to enhance the preservative effect and give better organoleptic and nutritional qualities to foods

For example, if a limiting a_w of 0.85 or pH 4.6 alone will prevent growth of a food-borne pathogen, similar protection might be given by a combined a_w of 0.92 and pH 5.2

Chilling will further enhance the synergism

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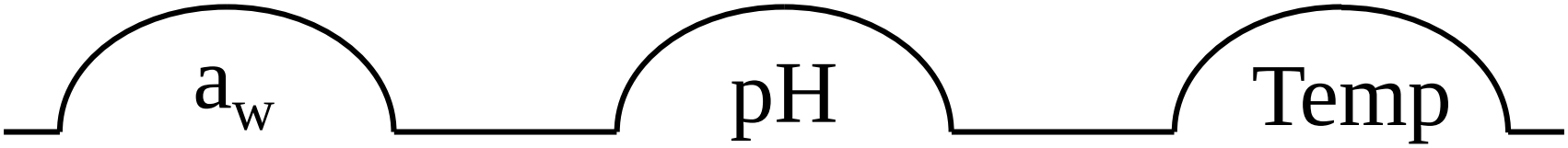
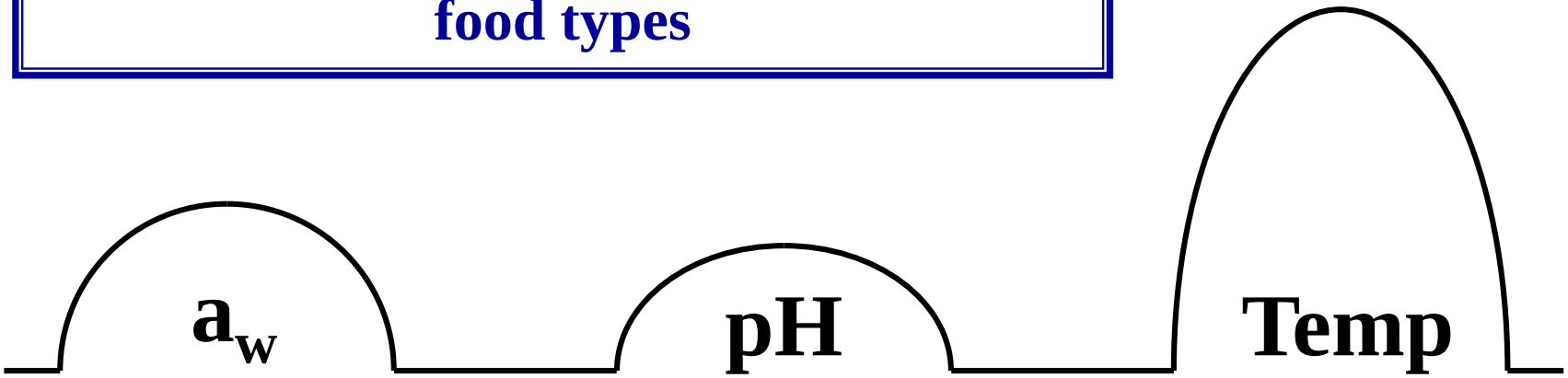
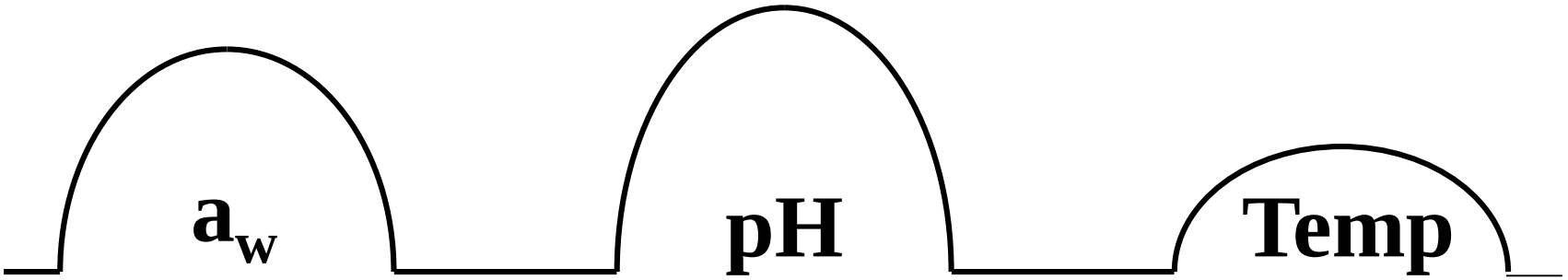
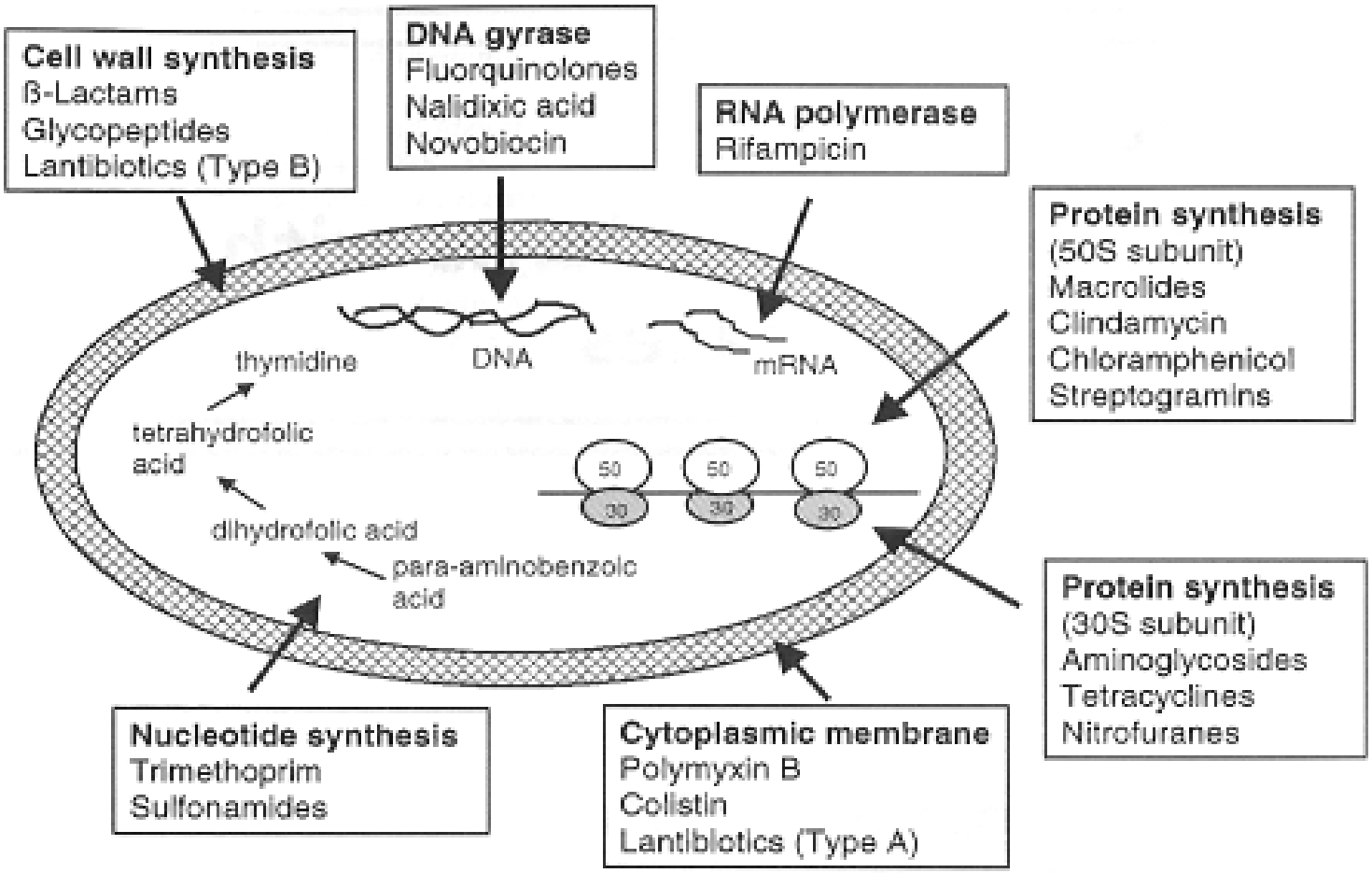


The sizes of hurdles can be varied in different combinations to suit different food types





Target points of AMC on bacterial cell



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Classic (standard) methods of microbial detection and quantification of microorganisms in food

Lecturer:

Jarmila Pazlarová

Department of Biochemistry and Microbiology

Food is not sterile

- Having access to safe and nutritious food is essential.
- Meat, fish, dairy products, eggs, shellfish, etc., **contain significant microbial flora** and this can affect our health. Therefore, it is important to identify the microorganisms to maintain food safety but the microbial flora can also affect, if not controlled, the quality of food and its shelf life.
- Whether you are a manufacturer or user of microbiological test methods, you want to ensure that your methods are fully tested, validated and fit for use.

Hygiene

- High hygiene with low occurrence of spoilage microbes and no occurrence of pathogens is a must in processing high quality food products with long shelf-life.
- Especially when there are problems in process hygiene the collection of process contaminants and their further characterization is needed to be able to define and establish the contamination routes.
- Thus knowledge of the domestic flora is an important background fact in solving process hygiene problems.
- The hygiene testing on food processing equipment surfaces is normally carried out using conventional culturing or contact agar methods.

History of isolation

- In microbiology, the term **isolation** refers to the separation of a strain from a natural, mixed population of living microbes, as present in the environment, for example in water or food flora, or from living beings with skin flora, oral flora, or gut flora, in order to identify the microbe(s) of interest.
- Historically, the laboratory techniques of isolation first developed in the field of bacteriology and parasitology (during the 19th century), before those in virology during the 20th century.
- Methods of microbial isolation have drastically changed over the past 50 years, from the labor perspective with increasing mechanization, in regard to the technology involved, and hence speed and accuracy.

Necessity of sample dilution

- Traditionally microbes have been cultured in order to identify the microbe(s) of interest based on its growth characteristics. Depending on the expected density and viability of microbes present in a liquid sample, physical methods to increase the gradient as for example **serial sample dilution** or centrifugation may be chosen.
- In order to isolate organisms in materials with **high microbial content**, such as sewage, soil or stool, **serial dilutions** will increase the chance of separating a mixture.
- More recently, microbes have been isolated without culturing them. Samples are inoculated into microtiter plates or cartridges extracting their particular genetic material (DNA or RNA) which can be used to identify them.

Streaking

- The modern streak plate method has progressed from the efforts of Robert Koch and other microbiologists to obtain microbiological cultures of bacteria in order to study them.
- The dilution or isolation by streaking method was first developed by Loeffler and Gaffky in Koch's laboratory, which involves the dilution of bacteria by systematically streaking them over the exterior of the agar in a petri dish to obtain isolated colonies, which will then grow into quantity of cells, or isolated colonies.
- If the agar surface grows microorganisms which are all genetically same, the culture is then considered as a microbiological culture.

Procedure

- When done properly, streak plate isolation thins out a specimen and enables individual bacterial cells to develop into isolated colonies.
- A microbiologist begins by sterilizing the inoculating loop in a flame. She cools the loop by touching it to the agar, then dips the loop into the sample and spreads it back and forth to cover a section of the plate.
- She sterilizes the loop, cools it, and inoculates a second, adjacent section of the plate by dragging the loop through the first section several times and covering the second section using a zigzag motion.
- This picks up a small number of bacteria from the first section and transfers them to the second section. The number of times this basic procedure is repeated depends on the streak plate method used. Regardless of the method, the original sample is used to inoculate the first section of the plate only.

Why we count the number of microbes

Microbiological evaluation of raw materials also provides important information about the heat-processing parameters that would be necessary to meet the microbiological standards, guidelines, or specifications of a product.

Microbiological evaluation of a food, food ingredient, and environment help determine **possible sources of a specific microbial type** in a food and, in the case of heated food, the source and nature of postheat treatment contamination.

The number of living organisms is deducted from the number of colonies- **CFU – colony forming unit.**

Viable plate count -CFU

The most common procedure for the enumeration of bacteria is the **viable plate count**. In this method, serial dilutions of a sample containing viable microorganisms are plated onto a suitable growth medium.

The suspension is either spread onto the surface of agar plates (**spread plate method**), or is mixed with molten agar, poured into plates,and allowed to solidify (**pour plate method**).

The plates are then incubated under conditions that permit microbial reproduction so that colonies develop that can be seen without the aid of a microscope. It is assumed that each bacterial colony arises from an individual cell that has undergone cell division. Therefore, by counting the number of colonies and accounting for the dilution factor, the number of bacteria in the original sample can be determined.

Importance

The total microbial population in a food varies greatly and depends on:

*The *level of sanitation* used at all phases.

*The *degree of abuse* that leads to microbial growth.

*The *processing and preservation methods* used to kill and prevent growth of microorganisms.

Contamination of a food by specific types or species of microorganisms depends on the presence of a source of these microorganisms, and their entrance into the food mostly due to poor sanitation during handling and processing.

Reliability of CFU count

- The plate count is linear for *E. coli* over the range of 30 - 300 CFU on a standard sized Petri dish.
- Therefore, to ensure that a sample will yield CFU in this range requires dilution of the sample and plating of **several dilutions**.
- Typically **ten-fold dilutions** are used, and the dilution series is plated in replicates of 2 or 3 over the chosen range of dilutions.
- The CFU/plate is read from a plate in the linear range, and then the CFU/g (or CFU/mL) of the original is deduced mathematically, factoring in the amount plated and its dilution factor.

Methods Used

These methods are either **qualitative or quantitative**.

Quantitative methods are designed to enumerate or estimate directly or indirectly the microbial load in a test material.

None of the quantitative methods used now enumerate or estimate **total microbes**, total bacteria, or **total viable population,** rather each method enumerates or estimates a **specific group** among the total microbial population normally present in a food and that grows or multiplies preferentially under the conditions or methods of testing.

These include composition of an enumeration medium, temperature, time of incubation, oxygen availability, pH, and treatments of a sample before enumeration and estimation.

Examples of **quantitative** methods

Aerobic Plate Count(APCs),or standard Plate Count(SPCs) for dairy products.

Anaerobic Counts, Psychotrophic Counts, Thermoduric Counts, Coliform Counts, *S.aureus* Counts, Yeast and Mold Counts.

Qualitative Methods:

They determine either a representative amount (a sample) of a food or a certain number of samples in a batch of a food contain a specific microbial species among the total microbial population.

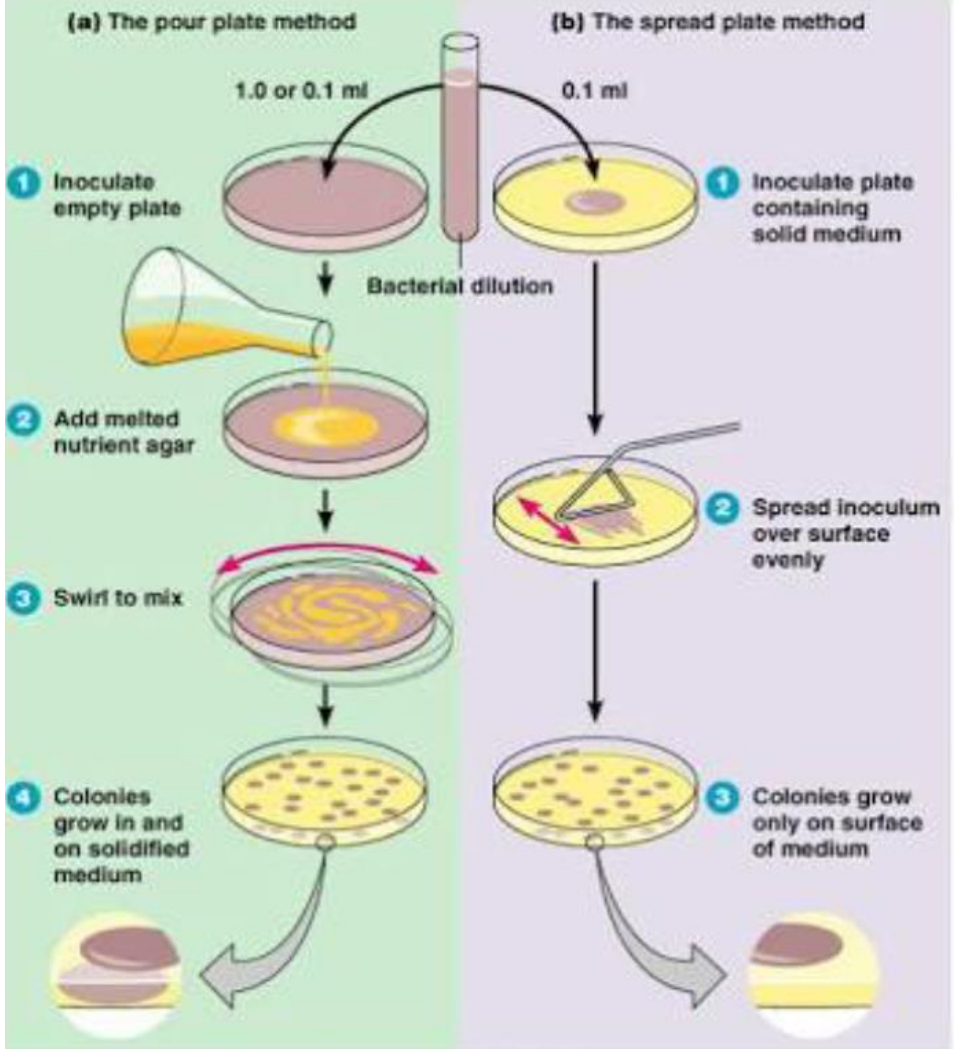
Importance of qualitative methods

Qualitative methods are used to detect the possible presence of certain **foodborne pathogens**, especially those capable of causing **high fatality rates** among consumers.

Salmonella*, *Clostridium botulinum*, *Cronobacter (Enterobacter*) *sakazakii*, *Escherichia coli* 0157:H7**, and probably ***Listeria monocytogenes in ready-to-eat food, are some that fall into this group.

These organism ought to be identified according standard normative protocols.

Differencies between pour plate and spread plate technique



The Pour Plate method wherein the sample is suspended in a petri dish using molten agar cooled to approximately 40-45 °C (just above the point of solidification to minimize heat-induced cell death). After the nutrient agar solidifies the plate is incubated.

The Spread Plate method wherein the sample (in a small volume) is spread across the surface of a nutrient agar plate and allowed to dry before incubation for counting.

Possibilities of CFU I.

The plate count is linear for *E. coli* over the range of 30 - 300 CFU on a standard sized **Petri dish**. Therefore, **dilution** of the sample and **plating of several dilutions are required** to ensure that a sample will yield CFU within that range . Typically ten-fold dilutions are used, and the dilution series is plated in replicates of 2 or 3 over the chosen range of dilutions. The CFU/plate is read from a plate in the linear range, and then the CFU/g (or CFU/mL) of the original is deduced mathematically, factoring in the amount plated, and its dilution factor.

Possibilities of CFU II.

A solution of bacteria at an unknown concentration is often serially diluted in order to obtain at least **one plate** with a **countable number of bacteria**. In this figure, the "x10" plate is suitable for counting.
An advantage to this method is that different microbial species may give rise to colonies that are clearly different from each other, both microscopically and macroscopically.
Shape and color of colony may help the identification.
What is the advantage of the plate count method? Counts only live bacteria, very accurate for bacteria with a wide range of concentrations, inexpensive (agar plates, dilution buffer solution).

Qualitative Methods

Isolation of Pathogens

The main objective of this method is to determine whether a sample contains viable cells or spores of a specific pathogen.

Foods are tested for several pathogens, such as *Salmonella, E.coli 0157:H7, L. monocytogenes, Vibrio cholerae, and Shigella spp.* by a specific isolation procedure.

For other pathogens, such as enteropathogenic *E. coli*, *Y. enterocolitica*, and *Campylobacter jejuni*, isolation procedures are not generally used; instead, enumeration procedures are used.

Conventional qualitative methods

- Qualitative methods are also termed presence/absence methods, as their primary aim is to find out if certain micro-organisms are present in the sample or not, and an actual level or count is not required. These methods are generally used to test for pathogens such as *Salmonella* spp., *Listeria* spp. and *Campylobacter* spp.
- Generally, there are four stages involved in a conventional pathogen presence/absence test which include:(**i)primary or pre-enrichment**,
- (**ii)selective enrichment**,
- **(iii)detection/plating and**
- **(iv)confirmation**.

Yeasts and Molds cultivation detection

- Many selective agars exist for the cultivation and determination of mold and yeast cultures, a majority of them do not depend on strict nutritive requirements for growth. Many fungal strains will grow on Sabouraud Dextrose Emmons Agar. Alternative agars for growth include Sheep Blood Agar, Nutrient Agar, Tryptic Soy Agar (Soybean Casein Digest), Potato Dextrose Agar, and Standard Methods Agar (Plate Count Agar) with an additional 24 hours of incubation.
- **Temperature:** 25°C
- **Atmosphere:** Aerobic
- **Growth Time:** 2 to 7 days

Yeasts and Molds

- The large and diverse group of microscopic foodborne yeasts and molds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile environmental requirements
- **Yeasts tend to grow within food and drink matrices** in planktonic form and they tend to ferment sugars, growing well **under anaerobic conditions.**
- **Molds,** on the other hand, tend to **grow on the surface** of objects in the shape of a visible ‘mycelium’ made up of many cells.

Spoilage yeasts I.

- **Spoilage yeasts** detection is the key to improve the quality of alcoholic fermentation beverages such as wine and cider.
- The metabolic activity of the spoilage yeast **causes irreparable damage** to many liters of final products every year. Therefore, winemakers and cider-house companies suffer a substantial economic impact.
- Thus, over the years, **many detection techniques** have been proposed **to control the occurrence of spoilage yeast**.
- Among all spoilage microorganisms found in wine and cider production, yeasts of the genus ***Brettanomyces*** or its teleomorph ***Dekkera***, are probably the most relevant microbes in the quality of final beverages.

Spoilage yeasts II.

- Traditional *Brettanomyces* detection methods (both **indirect** and **direct**) are too **slow** to prevent critical contamination of spoilage yeasts; more sensitive tests, such as **PCR**, are **expensive** and require a specialized team.
- New emerging spoilage yeast detection platforms, such as **biosensors**, and **microfluidic devices** (based on analysis of metabolic products) aim to alleviate these constraints. Novel platforms have already demonstrated great promise to be a real alternative for in situ and fast detection in the beverage industry.

Detection of molds - micromycetes

- Molds are widely distributed in nature and are common contaminants of agricultural commodities, foods, beverages, and feed.
- Traditional microbiological methods to detect molds in foods and feed are modified bacteriological methods.
- Accurate characterization and identification of food-spoilage mold species is becoming essential in order to control and prevent food contamination by fungi and potential mycotoxin production.
- A total plate count of mold colony- forming units (propagules) is still done the same way as in bacteriology.

Micromycetes detection in food

- **Chemical and biochemical methods**. These techniques are used to estimate fungal growth by **measuring specific components** of the fungus.
- These methods fall into the following categories: determination of **chitin**, **ergosterol**,and adenosinetriphosphate(**ATP**) by chemical and biochemical methods.
- **Immunological methods**. Fungal antigens are heat resistant and they can survive after the death of the fungal species and can be detected in thermally treated food. An enzyme-linked immunosorbent assay (**ELISA**) to detect mold in foods was developed.

Indirect Estimation

***Most Probable Number(MPN) in Selective Broths:**

Aliquots from a serially diluted sample are inoculated in a broth (in tubes) having one or more selective agents that facilitate growth of selected microbial groups present in a food.

Three or five broth tubes in each dilution and a minimum of three consecutive dilutions are used.

After incubating at recommended temperature and time, the broth tubes in each dilution are scored for the presence and absence of growth.

Dye Reduction Test

Some dyes such as methylene blue and resazurin are colored in oxidized states but colorless under reduced conditions.

This change can occur because of microbial metabolism and growth.

It is assumed that the rate of reduction during incubation of a specific concentration of methylene blue added to a food is directly proportional to the initial microbial load in the food.

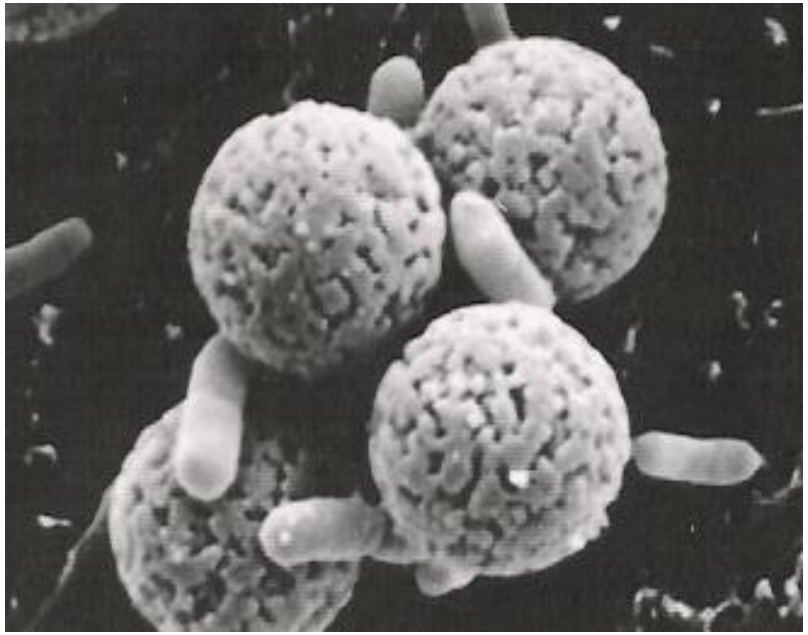
This method is generally used to determine the microbiological quality of raw milk.

Immunological methods

- **GLISA**
- Gold labelled immuno sorbent assay
- *Salmonella*
- *E.coli* O157
- *Campylobacter* sp.

1. Examples of the assembled rapid immunochromatog

Immunomagnetic separation



Bioluminescent ATP techniques in rapid microbiology

- Use of firefly luciferase to assay adenosine triphosphate (ATP) extracted from microorganisms provides an easy means to enumerate microbes within minutes. The small amount of light produced is proportional to ATP and thus microbial number.
- The average bacterium contains around 10^{-15} g ATP per cell. Present reagents permit detection of 10^{3} cells per tube. Luminometers currently on the market detect about 10^{-12} g ATP.
- Proper extraction of ATP from the microbes is an essential part of any protocol, as is the removal of non-microbial ATP from, for example, somatic cells also present in samples. The technique may be applied to a wide range of samples, for example food and beverages and clinical samples such as urine.
- The ATP assay gives a global measure of microbial numbers, i.e. it is not species specific unless a species separation step is included in the protocol.

Chemical reactions of the ATP-bioluminescence

$$\xrightarrow{Mg^{2+}}$$

D-luciferin + luciferase + ATP → luciferin adenylate complex +PPi

$$\xrightarrow{O_2}$$

- **Luciferil adenylate complex → Oxyluciferin + AMP+ CO_2 + light**
- PPi:inorganic pyrophosphate,
- Photons of yellow-green light (550 to 570 nm) are emitted.

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Rapid (modern) methods of the detection and quantification of microorganisms in food

Lecturer:

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Department of Biochemistry and Microbiology

Why rapid methods?

- Traditional methods of microbial detection tend to be labor-intensive and **take more than a day** to yield results. Rapid methods for microbial detection can be sensitive, precise, and quick.
- **Rapid microbial methods (RMM**s), also known as alternative microbiological methods, are the technologies that allow the user to get microbiology test results faster compared with traditional culture-plate methods.

RAPID METHODS

- **immunonological method** (based on antigen/ antibody-binding)
- **Based on molecular biological method** (based on PCR)
- **Others** (ATP Photometry, Direct Epifluorescent Filter Techniques (DEFT), Electrical impedance method, Flow cytometry, etc.)

Development of RMMs

- In general, rapid methods can be grouped into **three distinctive categories** in accordance with their application.
- These categories include **qualitative**, **quantitative,** and **identification** methods.
- Qualitative rapid methods provide a presence or absence result that indicates microbial contamination in a sample.
- Quantitative methods provide a numerical result that indicates the total number of microbes present in the sample.
- Identification methods provide us with a species or genus name for the microbial contaminant in a sample.

Qualitative RMMs

- **Qualitative RMMs detect the presence of microorganisms** in products. There are a number of tests currently available that measure changes in impedance, CO_2 (via colour change in media) or pressure (headspace pressure) that signal microorganism growth.
- There are also methods such as polymerase chain reaction (PCR), flow cytometry and endotoxin tests (Limulus ameobocyte assay test) that can be used to more rapidly detect the presence of microorganisms (or related bacterial endotoxins in the case of LAL).
- ATP measurements are also becoming more commonplace and have been used very successfully in hygiene monitoring to detect contamination. There is some difficulty perceived when comparing RFUs (relative fluorescence units) yielded by the ATP assays with CFUs (colony forming units) detected by traditional means, and how exactly these relate to one another[1].

Qualitative Microbial Detection

- I**mpedance**: measures ionic changes from metabolic byproducts due to growth
- **CO2 Detection**: measures carbon dioxide produced during growth
- **Headspace Pressure**: measures pressure change, positive or negative, due to production or consumption of gases
- **Nucleic Acid-based Technologies**: commonly PCR based
- **Flow Cytometry**: first applied to eukaryotic cells can also detect viable bacteria
- **Endotoxin**: LAL test for bacterial endotoxin
- **ATP Bioluminescence**: through surface swabs, membrane filtration, etc

Impedance

- There are several commercial instruments that use principles of **impedance or conductivity measurement** to detect bacteria. The relationship between capacitance at the electrode surface and conductance from ionic changes in the media from byproducts produced during bacterial growth allows calculation of impedance.
- Increases in capacitance and conductance result in decreased impedance indicative of bacterial growth. Each instrument type uses variable design principles that measure conductance based upon frequency and electrode quantity and type.

CO_2 Detection I.

- **Growth-based technologies** use either biochemical or physiological measures to reflect microorganism growth. Media formulations encourage **microbial proliferation** in test samples. A major advantage of these systems is the ability to recover microorganisms for failure investigations or identification after analysis.
- These systems use an **internal colorimetric CO_2 sensor** incorporated into **each media bottle during manufacture**. The sensor, separated from the media by a semi-permeable membrane, is impermeable to most ions and other media components but is freely permeable to CO_2.

CO_2 Detection II.

- **Carbon dioxide produced** by microbial metabolism diffuses across the membrane, dissolves in water in the sensor, and generates **hydrogen ions** which result in a color change detected by a **colorimetric detector.** Light emitted by the detector reflects off the sensor onto a photometer.
- The resulting voltage signal is proportional to the intensity of the reflected light and to the concentration of CO_2 in the bottle.

Quantitative Microbial Detection

- **Direct Laser Scanning**: detects microcolonies
- **ATP Bioluminescence**: detects microcolonies
- **Autofluorescence**: detects autofluorescence from viable organisms
- **Nucleic Acid-based Technologies**: commonly real-time PCR

Quantitative RMMs

- Quantitative RMMs are capable of providing a **numerical value** on the microorganisms present in a known sample unit. This is useful across the spectrum of microbiological tests.
- PCR is again useful as it is capable of quantifying as well as detecting the presence of microorganisms (commonly **RT-PCR**) in a matter of hours.
- There are also other methods that utilise direct detection of microorganisms using digital imaging of microcolonies (far too small to be seen with the naked eye) growing on solid media or filters as in direct laser scanning, light scattering, ATP bioluminescence and auto fluorescence methods.
- Methods such as flow cytometry and Raman spectroscopy are also coming to the fore in quantitative RMMs, providing a much quicker time to result than the standard incubation and detection procedures used in most microbiology laboratories

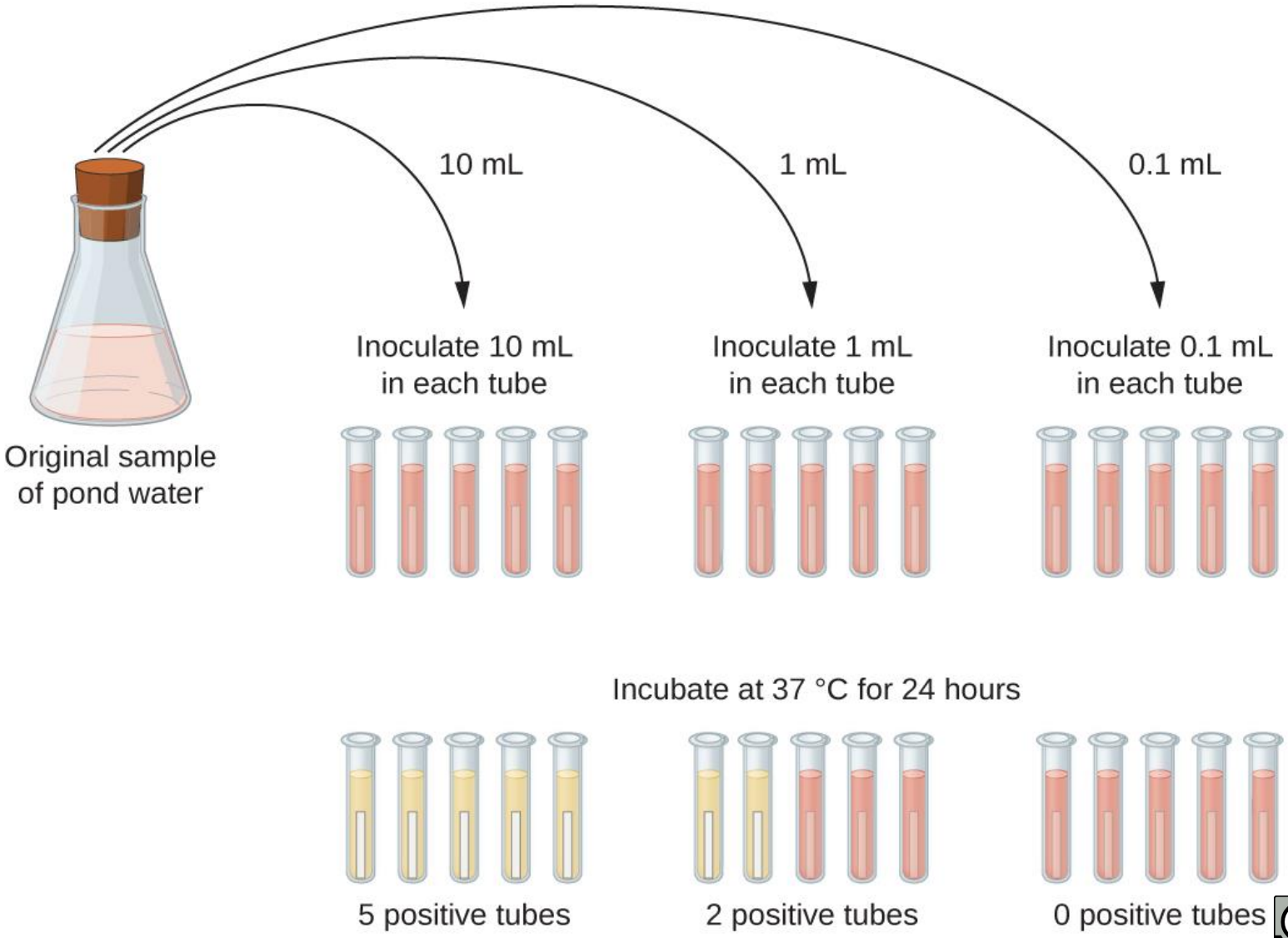
Most probable number

- The **most probable number** method, otherwise known as the method of **Poisson zeroes**, is a method of getting quantitative data on concentrations of discrete items from positive/negative (incidence) data.
- In microbiology, the cultures are incubated and assessed **by eye**, bypassing tedious colony counting or expensive and tedious microscopic counts.
- The major weakness of MPN methods is the need for **large numbers of replicates** at the appropriate dilution to narrow the confidence intervals. However, it is a very important method for counts when the appropriate order of magnitude is unknown *a priori* and sampling is necessarily destructive.

Most Probable Number Method (MPN)

- ❖ The most probable number (MPN) method is familiar to quality control (QC) microbiologists as part of the microbial limits tests.
- Its usefulness goes far beyond this one test, however.
- The theory behind the MPN method is central to the commonly used D-value determination by fraction-negative method, and a variant of this method has been suggested for trending of environmental monitoring data from the aseptic core.
- MPN can be adjusted to provide a sensitive method to determine differences between two qualitative microbiological methods. As such, it can be used as a tool in validation of rapid microbiological methods and for growth promotion testing of broth media.

Most probable number test



Nucleic Acid-based Technologies

- Qualitative nucleic acid-based detection technologies commonly use **polymerase chain reaction (PCR).**
- After extraction and purification of the **genomic DNA**, broad-range (universal) primers amplify highly conserved specific regions of the bacterial, mycoplasmal, or fungal genomes.
- Conventional PCR uses end-point detection on an agarose gel stained with ethidium bromide.

DNA based methods

- PCR or nucleic acid-based methods can be used to identify organisms based on the **16s rRNA gene** (or 16sDNA) in **bacteria** or the **26s rRNA gene**, **ITS** or **D2** region in **fungi.**
- They can provide a result in a **matter of hours**, most likely an overnight run, but can identify to a very distinct level (type or sub-type) so are used commonly in determining the exact source of sterility failures or serious contaminant route cause analysis.

PCR I.

Polymerase chain reaction (**PCR**) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of **DNA** across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Developed in 1983 by Kary Mullis, who was an employee of the Cetus Corporation, and also the winner of **Nobel Prize** in Chemistry in **1993**, it is an easy, cheap, and reliable way to repeatedly replicate focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences.

PCR II.

The majority of PCR methods rely on thermal **cycling**, which involves exposing the reactants to cycles of **repeated heating and cooling**, permitting different temperature-dependent reactions—specifically, DNA melting and enzyme driven DNA replication—to quickly proceed many times in sequence.

Primers (short DNA fragments) containing sequences complementary to the **target region**, along with a **DNA polymerase** (e.g. Taq polymerase), after which the method is named, enable selective and repeated amplification.

To check whether the PCR successfully generated the anticipated DNA target region **agarose gel electrophoresis** may be employed for size separation of the PCR products.

The size(s) of PCR products is determined by comparison with a DNA ladder, a **molecular weight marker** which contains DNA fragments of known size run on the gel alongside the PCR products.

Mass spectrometry

- An alternative to the more traditional microbiological tests is **matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-ToF).**
- This is **a different phenotypic identification** method used to determine the identity of a microorganism on the basis of a **protein profile or 'fingerprint'**.
- It is **relatively inexpensive** to run samples in the MALDI-ToF equipment and results are available in minutes, but there is a **large outlay cost** that is seen to be prohibitive for small throughput in smaller laboratories.

Flow Cytometry

- Flow cytometry, a well-established detection platform and investigative tool first applied to studies of eukaryotic cells, can also be adapted to detect the presence of viable bacteria in samples.
- The ability to **stain microorganisms with dyes** such as **propidium iodide,** which is impermeable to cells with intact membranes, and **thiozole orange**, which is permeable to all cells, allows a differentiation of viable and nonviable bacterial cells in fluid media.
- Routine point-of-use screening of blood components for microbial contaminants, such as platelets, is one application of flow cytometry that could readily be adapted to the needs of the pharmaceutical microbiology laboratory.

Endotoxin

- Automated **Limulus Amebocyte Lysate** (LAL) testing can provide results within minutes regarding the presence of **bacterial endotoxin** in raw materials, buffers, or in-process intermediates right in the warehouse or on the manufacturing floor.
- One such automated system uses the kinetic chromogenic method with endotoxin reagents contained in a plastic cartridge analyzed using a specialized reader to kinetically monitor the chromophore produced during the reaction.

Bioluminiscence

- Light-producing living organisms are widespread in nature and from diverse origins. The process of light emission from organisms is called **bioluminescence** and represents a **chemical conversion of energy into light.**
- The bioluminescence mechanism involving **Luciferase enzyme** is a multistep process which mainly requires **Luciferin substrat**, **Oxygen** (O_2), **Magnesium cation** (Mg++)and **ATP**. ATP-bioluminescence using luciferine/luciferase relies on luciferine oxidation by the luciferase and the integrated light intensity is directly proportional to ATP content

ATP Bioluminescence

- Industry has incorporated rapid detection of microbial contamination using **ATP bioluminescence** for many applications. One such application involves surface swabs dispersed into a liquid matrix for filtration. Addition of a substrate to the membrane surface yields fluorescence following exposure to microbial ATP. **Adenosine triphosphate (ATP)** is the main chemical energy source of **all living cells**.
- Detection systems based on bioluminescence exploit the chemical release of ATP from microorganisms. ATP reacts with **luciferase** and a **photon counting imaging tube** detects photons released by this reaction.
- A computer monitor then represents the photons detected. There are both qualitative and quantitative systems available.
- Instant checking of performed cleanup and disinfection.

Equation of bioluminiscence

The light emission is a consequence of a rapid loss of energy of the oxyluciferine molecule from an excited state to a stable one. This reaction induces the emission of photons with a efficient quantum yield of about 90%

D-luciferin + luciferase + ATP → **Luciferil adenylate complex +PPi** *in presence of* Mg^{2+}

Luciferil adenylate complex → **Oxyluciferin + AMP+** CO_2 + **light** *in presence of* O_2

Microbial Identification

- **Phenotypic and Biochemical Methods**: classical phenotypic/biochemical methods. May require preliminary characterization such as Gram Stain
- **Genotypic Methods**: most involve comparative DNA sequencing of the 16S rRNAgene in bacteria or 26S rRNAgene in fungi. Can reduce identification to an overnight procedure
- **Mass Spectrometry**: MALDI-TOF methodology can be available in minutes rather than days

Microbial Detection – Quantitative

- **Direct Laser Scanning**
- Visualization of colonies for slow growing microorganisms can require a number of days. Using lasers to scan microorganisms growing on membrane filters with optical imaging using a digital camera can greatly reduce this time.
- This technique allows detection and enumeration of **microcolonies** within a few days. An advantage of this technique is that the **microorganisms remain viable for identification** after colonies become visible.

ATP Bioluminescence

- ATP bioluminescence can also enumerate the microorganisms present in a sample. After filtering samples in a liquid matrix onto a membrane and spraying the membrane with an ATP-releasing reagent and substrate, enumeration of fluorescent microorganisms occurs by capturing a digital image and processing the fluorescent data.
- These results can be available within hours. Historically, the staining process would render the microorganisms non-viable, so isolates would not be available for identification. Newer techniques are available allowing retention of microorganism viability for subsequent identification.

Immunology based test I.

ELISA or **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay, a biochemical technique.

The prime **advantage of ELISA** applications is that the results are **quantifiable**. Performing an ELISA involves at least one antibody to detect a particular antigen or the presence of another antibody.

The **sample with an unknown amount of antigen** is immobilized on a solid support. The "detection " antibody is then added, forming a complex with the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal.

Immunology based test II.

- Instead of using radioactive materials, which have been utilized on a large scale in the beginning of molecular biology, to determine the results of a test, **the ELISA uses enzymes which react with antibodies to form colored products**.
- The **development of color in an ELISA test indicates a positive result**. The ELISA can be used to detect the presence of antigens that are recognized by an antibody (direct method) or it can be used to test for antibodies that recognize an antigen (indirect method).

ELISA

- Most of the commercially available immunoassays for bacteria detection are based on **standard sandwich immunoassay**, which involves the formation of an immunocomplex between an **immobilized antibody**, the **target bacteria**, and a **secondary labeled antibody**.
- Generally, the primary antibody is immobilized on beads (magnetic, silica, or gold particles), polystyrene microtiter plates, filter membranes, or directly on the surface of transducers, and the secondary antibody is labeled with enzymes or fluorescent molecules.
- The detection limits of immunoassays ranged between **10^3** and **10^6** colony-forming units (CFU)/mL, with assay times from 10 min to several hours

Multiplex ELISA

- **Multiplexed** format immunoassays suitable for the simultaneous evaluation of different bacteria in a sample was developed to increase the analytical productivity and drastically reduce analysis costs and sample and reagent consumption.
- Simultaneous detection of ***Escherichia coli*** O157:H7, ***Yersinia enterocolitica***, ***Salmonella typhimurium*** , and ***Listeria monocytogenes*** was proposed using **monoclonal antibodies** specific for each bacteria.
- This method can be used as a screening test to evaluate the presence of these pathogen bacteria in different foodstuffs.

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Microbial fermentation and production of biomass

Lecturer:

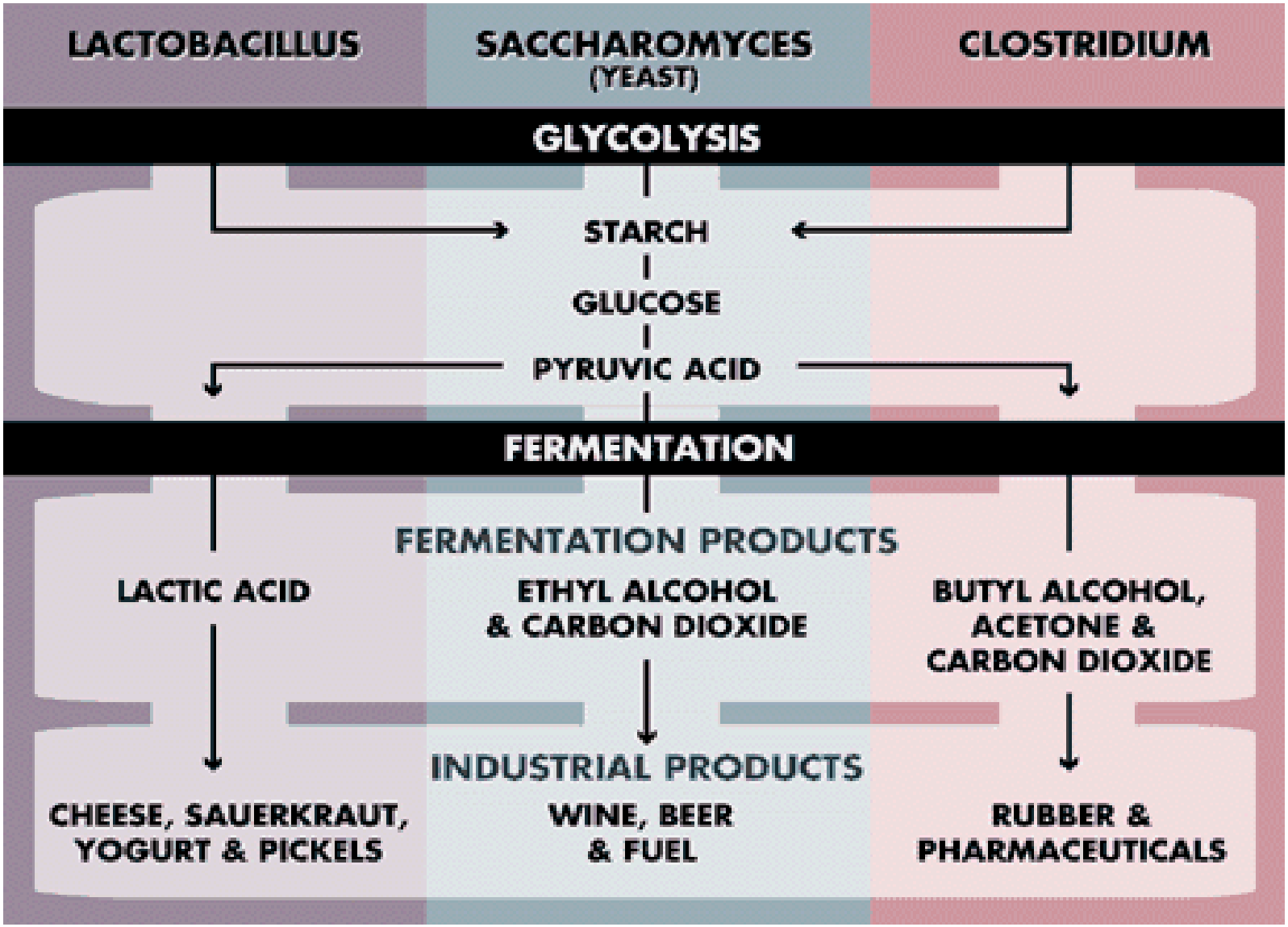
Jarmila Pazlarová

Department of Biochemistry and Microbiology

Meaning of “fermentation“

- **Fermentation** is a metabolic process that converts sugar to acids, gases or alcohol. (anaerobic)
- It occurs in yeast and bacteria, and also in oxygen-starved muscle cells, as in the case of lactic acid fermentation.
- **Fermentation is also used more broadly to refer to the bulk growth** of microorganisms on a growth medium, often with the goal of producing a specific chemical product. (aerobic conditions)

Distribution of fermentations



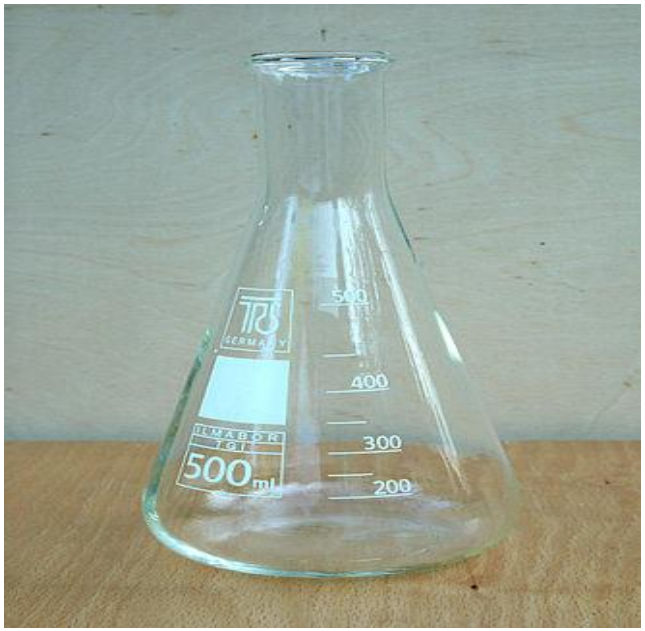
Definition of fermentation

- Production of **alcoho**l: grains and fruits are fermented to produce beer and wine. Preservation methods for food via microorganisms (general use).
- **Any process that produces alcoholic beverages** or acidic dairy products (general use).
- **Any large-scale microbial process** occurring with or without air (common definition used in industry).
- **Any energy-releasing metabolic process** that takes place only under **anaerobic conditions** (becoming more scientific).
- **Any metabolic process that releases energy from a sugar or other organic molecules, does not require oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor** (most scientific).

Fermenter design

- What should be the **basic points** of consideration while designing a fermenter?
- **Productivity** and **yield**
- Fermenter operability and reliability
- Product purification
- Water management
- **Energy requirements**
- **Waste treatment**

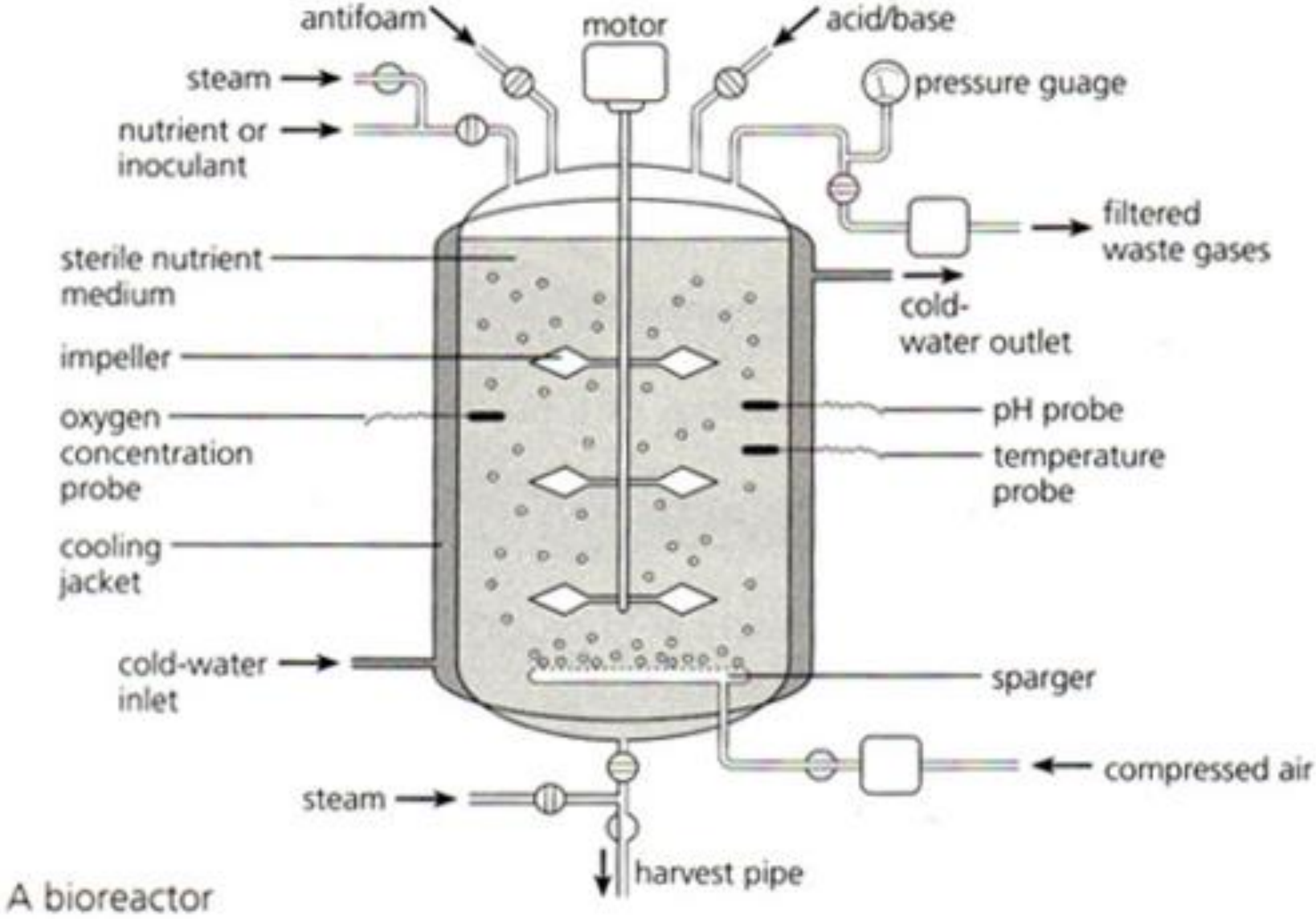
Cultivation vessels



Scale-up

Fermenter – bioreactor I.

- STIRRED TANK FERMENTER - tank reactor having the following functions: **homogenization,** suspension of solids, **dispersion** of gas-liquid mixtures, **aeration** of liquid and **heat exchange**.
- The stirred tank reactor is provided with a baffle and a rotating stirrer is attached either at the top or at the bottom of the bioreactor.
- The typical decision variables are: **type**, **size**, location and the number of impellers; sparger size and location. These determine the **hydrodynamic pattern** in the reactor, which in turn influence mixing times, mass and heat transfer coefficients, shear rates, etc.



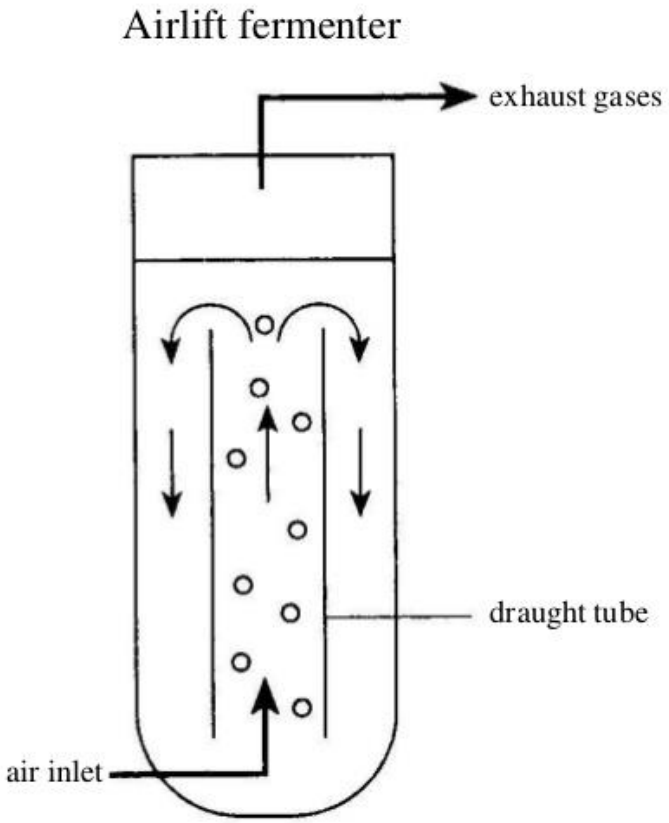
Fermentor

Fermenter – bioreactor II.

AIR-LIFT FERMENTER - Airlift fermenter (ALF) is generally classified as **pneumatic reactor** without any **mechanical stirring** arrangements for mixing. The turbulence caused by the fluid flow ensures adequate mixing of the liquid. The draft tube is provided in the central section of the reactor. The introduction of the fluid (air/liquid) causes upward motion and results in circulatory flow in the entire reactor. The air/liquid velocities will be low and hence the energy consumption is also low. ALFs can be used for both **free** and **immobilized cel**ls. There are very few reports on ALFs for metabolite production

Airlift fermenter

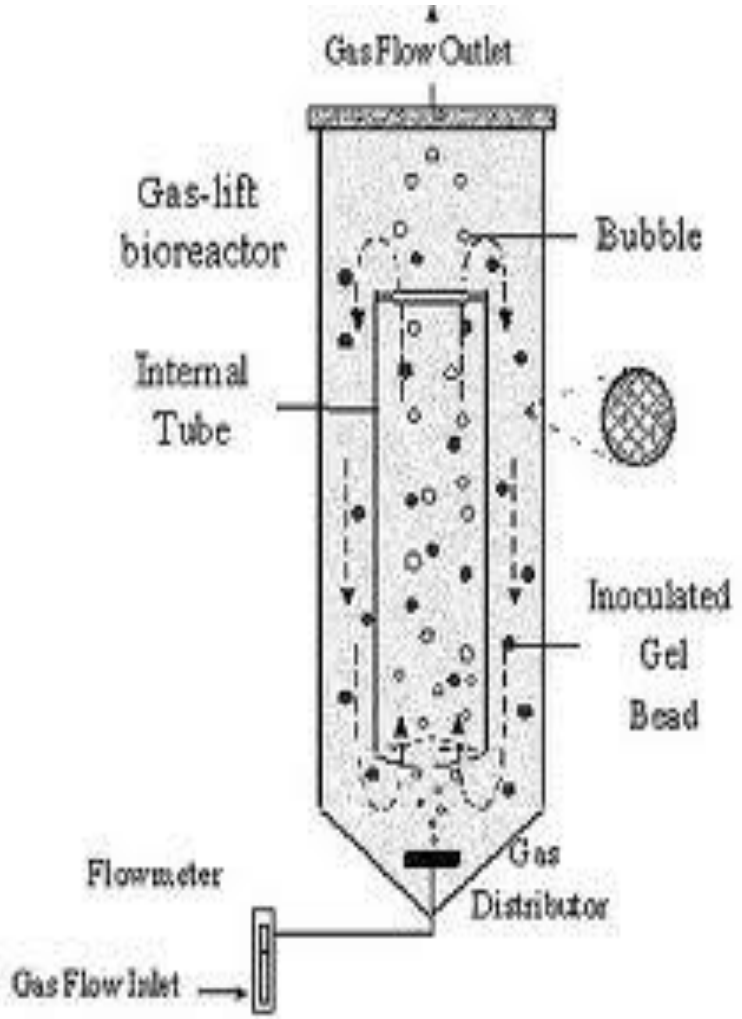
Fig. 9.12

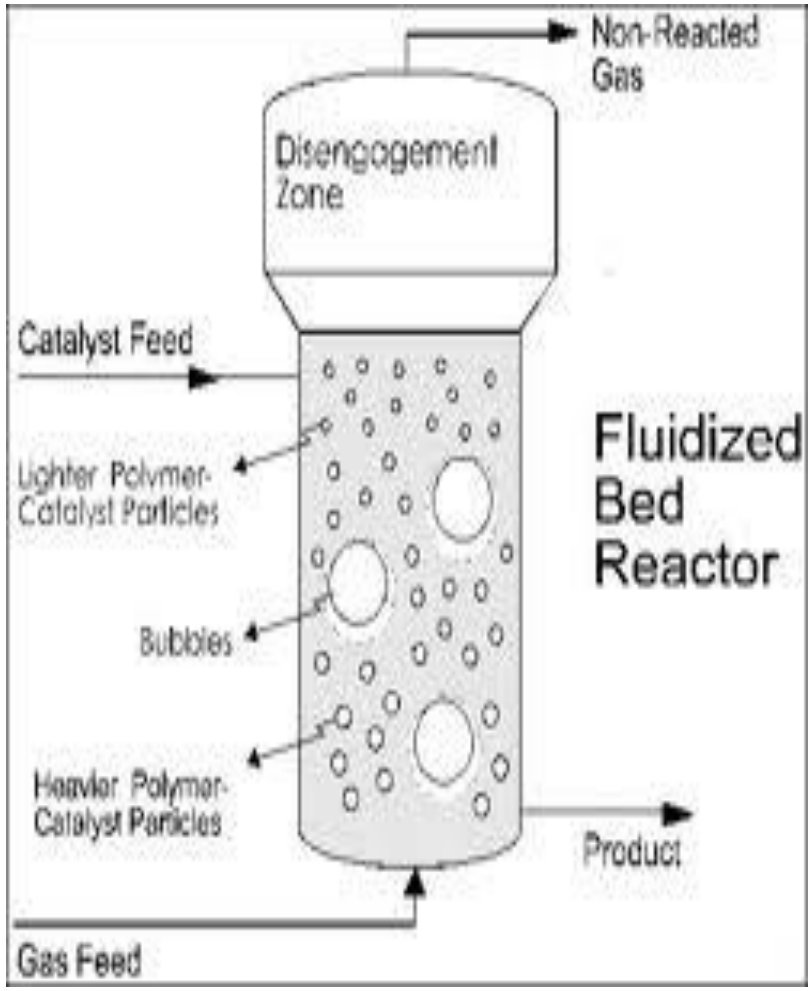


Fermenter – bioreactor III.

FLUIDISED BED BIOREACTOR - (FBB) have received increased attention in the recent years due to their advantages over other types of reactors. Most of the FBBs developed for biological systems involving cells as biocatalysts are **three phase systems** (solid, liquid & gas). The fundamentals of three phase fluidization phenomena have been comprehensively covered in **chemical engineering literature**. The FBBs are generally operated in co-current upflow with **liquid as continuous phase** and other more unusual configurations like the inverse three phase fluidized bed or gas solid fluidized bed are not of much importance. Usually fluidization is obtained either by external liquid re-circulation or by gas fed to the reactor

Gas-lift / fluidized bed reactors





Fermenter – bioreactor IV.

PACKED BED BIOREACTOR - Packed bed or fixed bed bioreactors are commonly used with attached **biofilms** especially in **wastewater engineering**. The use of packed bed reactors gained importance after the potential of whole cell immobilization technique has been shown. The immobilized biocatalyst is packed in the column and fed with nutrients either from top or from bottom. One of the disadvantages of packed beds is the changed flow characteristic due to alterations in the bed porosity during operation. While working with soft gels like alginates, carragenan etc the bed compaction which generally occurs during fermentation results in high pressure drop across the bed. In

Special demands on fermentors

- **In citric acid** fermentation where **pH** may be **1** to **2**, it will be necessary to use a stainless steel with 3-4% molybdenum (AISI grade 317) to prevent leaching of heavy metals from the steel which would interfere with the fermentation.
  AISI grade 304, which contains 18.5% chromium and 10% nickel, is used extensively for brewing equipment.
- With **plant** and **animal cell tissue culture**, a low-carbon version (type 316L) is often used.

Main types of products

- **Microbial cells** or **biomass** as the product, e.g. **single cell protein**, bakers yeast, lactobacillus, *E. coli*, probiotics,etc.
- **Microbial enzymes**: catalase, amylase, protease, pectinase, glucose isomerase, cellulase, hemicellulase, lipase, lactase, streptokinase, etc.
- **Microbial metabolites** :
  - **Primary metabolites** – ethanol, citric acid, glutamic acid, lysine, vitamins, polysaccharides etc.
  - **Secondary metabolites--** all **antibiotics** fermentation
- **Recombinant products**: insulin, hepatitis B vaccine, interferon, granulocyte colony-stimulating factor, streptokinase
- **Biotransformations:** phenylacetylcarbinol, steroids biotransfor mation, etc.

Biomass production

- Microorganisms such as algae, bacteria, yeasts and fungi have been considered as protein sources. Culture of microorganisms for their nutritional value started at the end of the First World War. The Germans developed yeast culture for use in animal and human diets. The term “fodder yeast” was coined by many scientists.
- After the Second World War, production of fodder yeast using the pentoses in sulfite liquor was developed in the USA, and other processes were developed in the United Kingdom. Carbon substrates such as molasses, pulp of the coffee and seed pulp of the palmyra palm were used.

Single cell proteins

- **Single-cell protein** (**SCP**) refers to edible unicellular microorganisms. The biomass or protein extract from pure or mixed cultures of algae, yeasts, fungi or bacteria may be used as an ingredient or a substitute for **protein-rich foods**, and is suitable for human consumption or as animal feeds.
- **SCP** is commonly grown on **agricultural waste products**, and as such inherits the ecological footprint and water footprint of industrial agriculture.
- SCP may also be produced entirely independent of agricultural waste products through **autotrophic** growth. Thanks to the high diversity of microbial metabolism, autotrophic SCP provides several different modes of growth, versatile options of nutrients recycling, and a substantially increased efficiency compared to crops.
- **The term SCP was coined in 1966 by Carroll L. Wilson of MIT.**

SCP I.

- Research on **Single Cell Protein** Technology started a century ago when Max Delbrück and his colleagues found out the **high value** of surplus **brewer's yeast** as a feeding supplement for animals.
- During World War I and World War II, yeast-SCP was employed on a large scale in Germany to counteract food shortages during the war. Inventions for SCP production often represented milestones for biotechnology in general: for example, in 1919, Sak in Denmark and Hayduck in Germany invented a method named, "Zulaufverfahren", (**fed-batch**). Sugar solution was fed continuously to an aerated suspension of yeast instead of adding yeast to diluted sugar solution once (batch).
- In post war period, the Food and Agriculture Organization of the United Nations (**FAO)** emphasized on hunger and malnutrition problems of the world in 1960 and introduced the concept of protein gap, showing that 25% of the world population had a deficiency of protein intake in their diet.

SCP II.

In the 1960s, researchers at British Petroleum developed what they called "**proteins-from-oil process**": a technology for producing single-cell protein by yeast fed by waxy **n-paraffins**, a byproduct of oil refineries.

Initial research work was done by A. Champagnat at BP's Lavera Oil Refinery in France; a small pilot plant there started operations in March in 1963, and the same construction of the second pilot plant, at Oil Refinery in BP "food from oil" idea became quite popular by the 1970s, with Champagnat being awarded the UNESCO Science Prize in 1976.

SCP III

Paraffin-fed yeast facilities being built in a number of countries. The primary use of the product was as **poultry and cattle feed.**

- The most important features resulting from research on SCP concerned the following topics:
- – **diversity of the substrates** used and their catabolism (renewable substrate, fossil mass);
- – **breeding and genetic improvement** of a great variety of microorganisms.

SCP IV

- And paraffin-fed yeast facilities being built in a number of countries. The primary use of the product was as **poultry and cattle feed.** The first oil crisis in the 70s increased the price of oil, and the process and economy of the production became inconvenient.
- To **reduce the cost** of production of SCP, it is essential to use biodegradable **agroindustrial** by-products and waste as a source of nutrients for the cultivation of micro-organisms.
- The most suitable waste products for the production of single-cell oils and divided them into **four groups**: mono and disaccharide-rich waste products; starch rich waste products; glycerol-rich waste products; lignocellulose-rich waste products.

SCP V

- Each agricultural waste group has its **own advantages** and **disadvantages** if used as substrate for SCP production.
- In general, the key considerations for choosing the most suitable waste product for SCP production are:
- (1) **local availability** of the particular waste product; (2) **pre-treatment costs** of the waste product before using it in fermentation;
- (3) the costs of **transportation** of the waste product; (4) **SCP concentrations in the final microbial biomass** after fermentation.

ALGAE BIOFUELS PHOTOBIOREACTOR

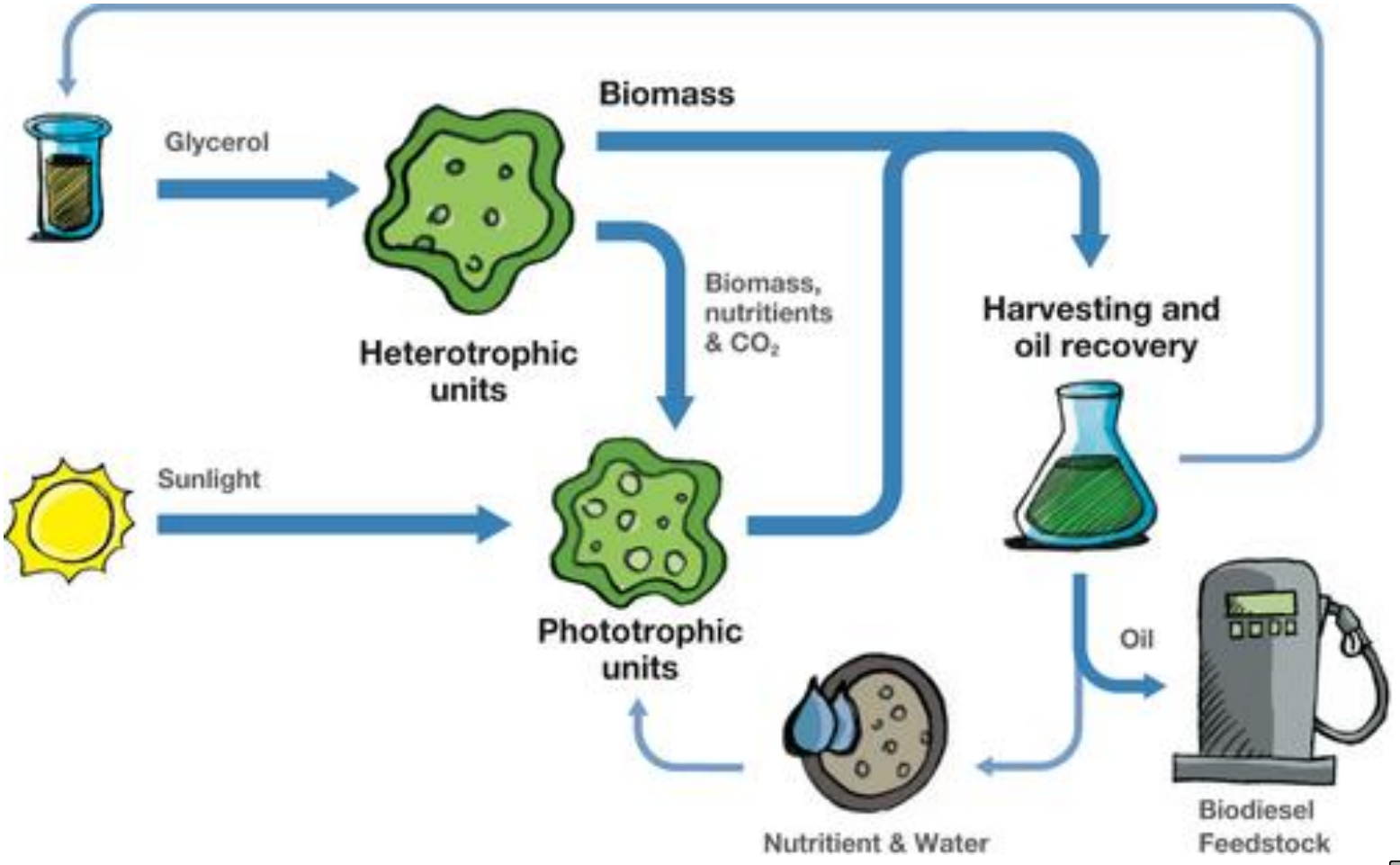
Ability of microalgae to mitigate CO_2 emission and **produce oil** with a high productivity show that it has the potential for applications of producing **the third-generation of biofuels**. For microalgae biofuel production there is a need of identification of preferable culture conditions for high oil productivity, development of effective and economical **microalgae cultivation systems**, as well as separation and harvesting of microalgal biomass and oil.

- Chisti in 2007 proposed that under suitable culture conditions, some microalgal species are able to accumulate up to **50–70%** of **oil/lipid per dry weight**.
- Chisti also proposed the major reason of using microalgal oil for **biodiesel production** which is the tremendous oil production capacity by microalgae, as per hectare, they could produce up to 58,700 L oil, which is **one or two magnitudes higher** than that of any other energy crop.

Algae cultivation

- **Autotrophic microalgae** are cultivated on land in large ponds, or in enclosed so-called **photobioreactors,** using enriched CO_2. The CO_2 can come in the form of flue gases from power plants or be obtained from other fossil fuel combustion and biological processes. They thus can help recycle this specific greenhouse gas, and can help reduce greenhouse gas emissions overall when the algal biomass is converted into biofuels.
- **Heterotrophic microalgae** are grown in large fermenters using **sugar** or **starch**, similar to the corn ethanol fermentation already providing almost 10 percent of our liquid transportation fuels.

Microalgae cultivation



Mushroom production

- Mushrooms are classified as vegetables in the food world, but they are not technically plants. They belong to the **fungi kingdom**.
- **Fungiculture** is the process of producing food, medicine, and other products by the cultivation of mushrooms.
- All **mushroom growing techniques** require the correct combination of humidity, temperature, substrate (growth medium) and inoculum (spawn or starter culture). Wild harvests, outdoor log inoculation and indoor trays all provide these elements.
- Mushrooms are rich in **B vitamins** such as riboflavin (B2), folate (B9), thiamine (B1), pantothenic acid (B5), and niacin B3). The B vitamins help the body to get energy from food, and they help form red blood cells.

Commercially cultivated fungi

- ***Agaricus bisporus***, also known as **champignon** and the button mushroom. This species also includes the portobello and crimini mushrooms.
- ***Auricularia polytricha*** or *Auricularia auricula-judae* (Tree ear fungus), two closely related species of jelly fungi that are commonly used in Chinese cuisine.
- ***Lentinus edodes***, also known as **shiitake**, oak mushroom. *Lentinus edodes* is largely produced in Japan, China and South Korea.
- ***Pleurotus*** species are the second most important mushrooms in production in the world, giving for 25% of total world production.
- ***Rhizopus oligosporus*** - the fungal starter culture used in the production of **tempeh**. In tempeh the mycelia of *R. oligosporus* are consumed.

Manufacturing of Yeast

In the commercial production of yeast, **molasses** is used to provide this sugar source. Molasses is a by-product of the refining of sugar beets and sugar cane. Either **cane molasses** or **beet molasses** can be used, however, some yeast manufactures prefere the mix of both.

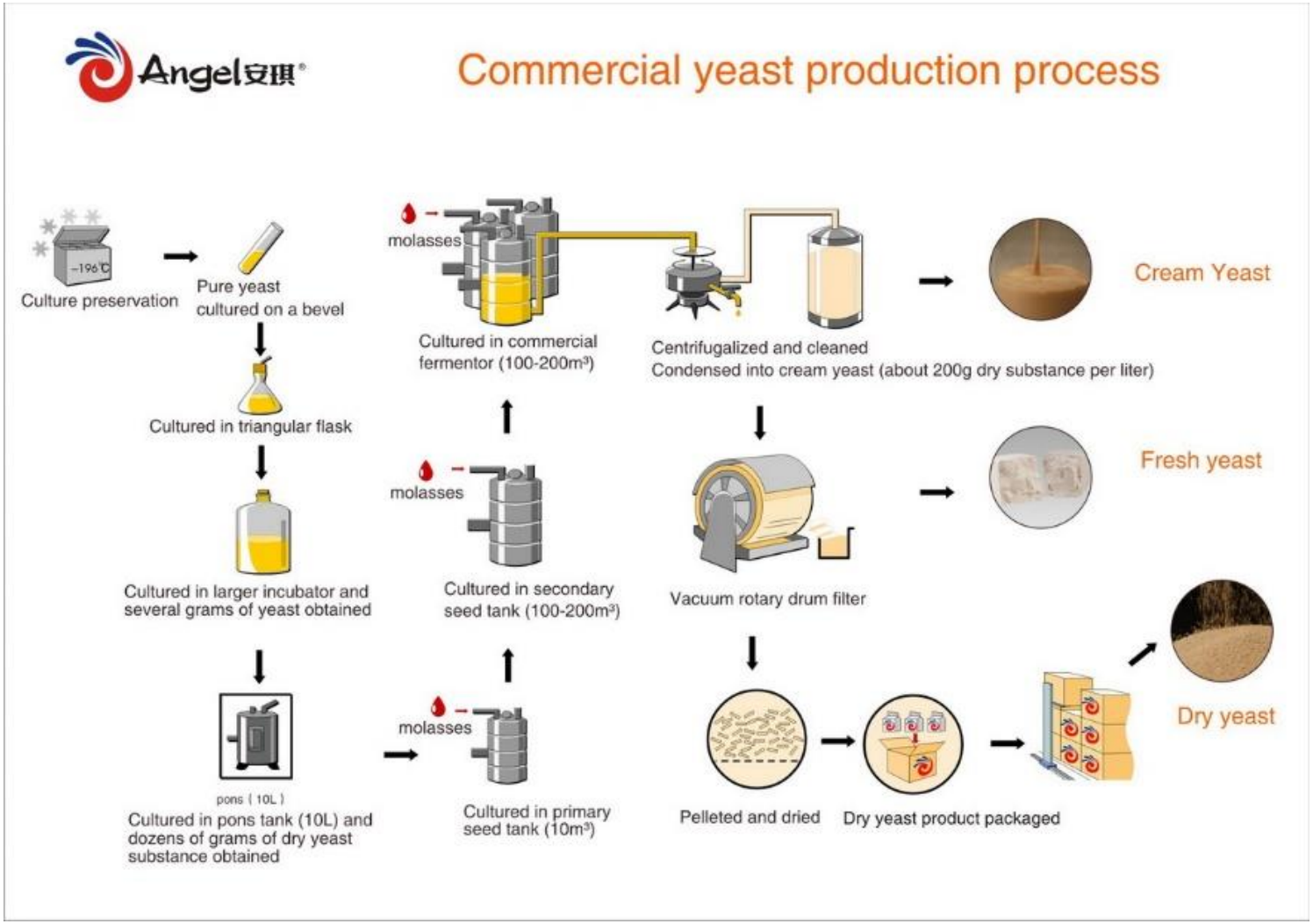
Before feeding molasses to the yeast cells, it must be clarified and **sterilized**. This is done in order to assure the final yeast color. The sterilizing also prevents bacteria and other organisms from being introduced during manufacturing.

The **seed yeast** is a carefully maintained laboratory culture so as to avoid contamination by “wild” yeast present in the air. Yeast seeds are selected with care according to the type of yeast to be produced and the specific characteristics desired. All cultures are laboratory pure; all transfers are made with absolute sterility; all vessels are completely sterilized.

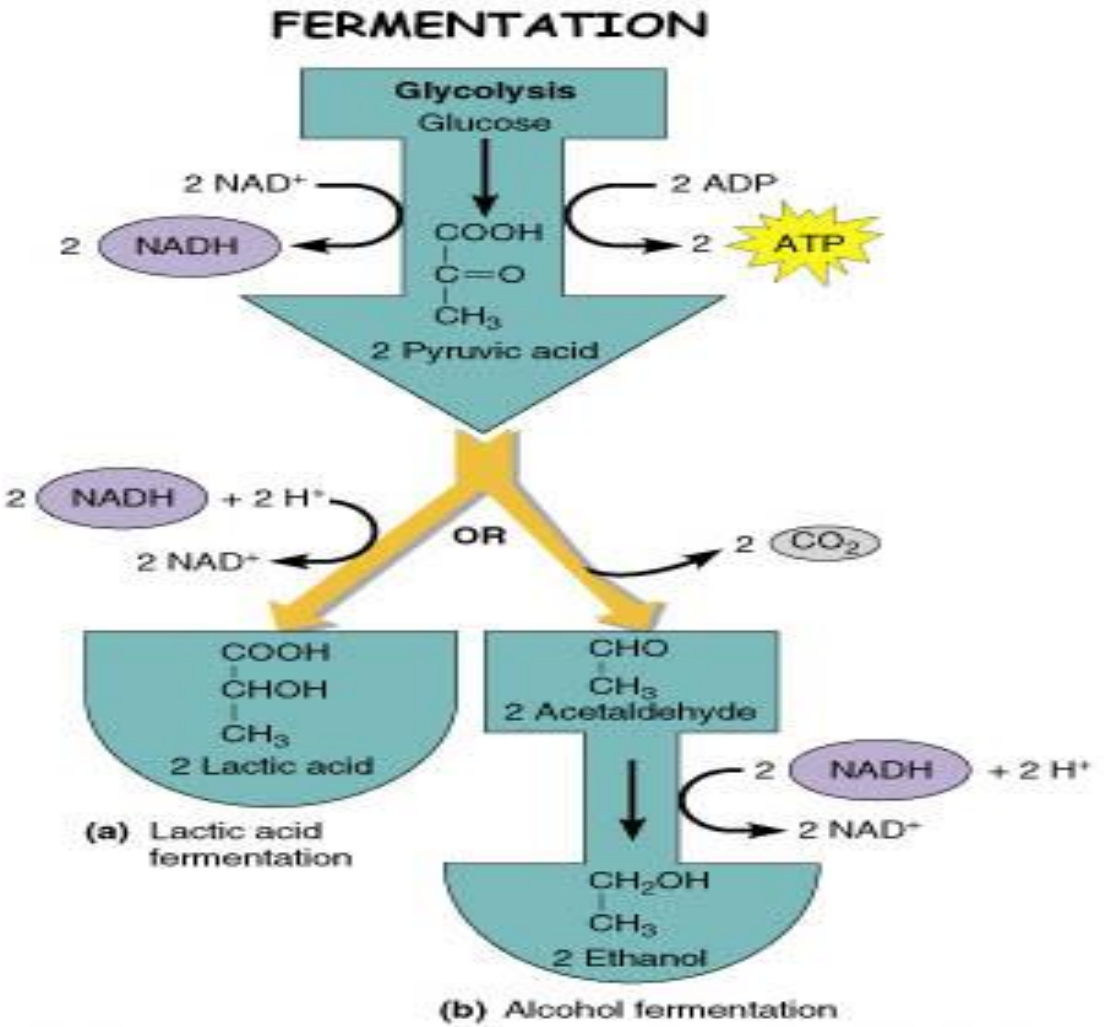
Manufacturing of Yeast II

- The **cultivation** or advancement of the fermentation process is accomplished in large several cubic metres vessels. It is impractical at this point to sterilize such large vessels but careful cleaning with steam assures cleanliness and quality.
- **The harvesting of yeast** is nothing more than concentrating the yeast cells by passing the fermented liquid through large centrifugal pumps called “separators”. This process is similar to spinning clothes dry in a washing machine. The result is an off-white liquid called “cream yeast”. Further **processing/ drying** is dependent on the type of yeast desired – cake yeast, active dry yeast or instant yeast.

Scheme of yeast production



Fermentation / lactic acid and alkohol fermentation



Alcohol fermentation

- **Alcoholic fermentation** converts one mole of glucose into two moles of **ethanol** and two moles of carbon dioxide, producing two moles of ATP in the process. Typical generator organisms are yeasts. The overall chemical formula for **alcoholic fermentation** is:
- $C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$.
- Sucrose is a dimer of glucose and fructose molecules. In the first step of alcoholic fermentation, the enzyme **invertas**e cleaves the glycosidic linkage between the glucose and fructose molecules.

Lactic acid fermentation

- Yoghurt-based drinks that contain **probiotic** bacteria (live micro-organisms that can give a health benefit to the host) are increasingly popular.

  Bacterial strains such as *Lactobacillus bulgaricus* **convert** lactose **sugar** into **lactic acid**. This lowers the pH and causes the milk to clot, creating the characteristic texture and taste of yoghurt.

  The **live bacteria** in probiotic yoghurts are thought to restore the natural microbe population of the gut (flora), which can be depleted by antibiotics, for example.

ABE fermentation

- ABE fermentation is one of the oldest known industrial fermentation methods with a history of more than 100 years. In the first part of the 20th century, it ranked **second only to ethanol fermentation**. In 1861 butanol production through microbial fermentation was reported for the first time by **Pasteur**. This was followed by **Schardinger** in 1905 reporting production of acetone by fermentation.
- From 1912 to 1914 prolific strains of *Clostridium acetobutylicum* were isolated by **Chaim Weizmann**, showing the ability of fermenting starchy substrate. ABE fermentation was used to produce acetone during WW1 to make the explosive cordite and in the production of synthetic rubber.
- When **Weizmann's patent expired in 1936,** a flood of anaerobic fermentation plants were opened. Molasses fermentation processes grew in popularity as new microorganisms were isolated. Every company had its own unique patented microorganism, which was able to produce acetone and butanol in great amounts from the molasses. In WW2, butanol was produced as fuel for fighter planes. The process disappeared in the second part due to the rise of the petrochemical industry.

Propionic acid fermentation

- Propionic acid (propionate) is a commercially valuable carboxylic acid produced through microbial fermentation.
- Propionic acid is mainly used in the food industry but has recently found applications in the cosmetic, plastics and pharmaceutical industries.
- First described by Albert Fitz in 1878, *Propionibacterium* species can ferment sugars into propionic acid as their main fermentation product.

Biochemistry of propionic acid fermentation

$$3\ C_6H_{12}0_6 = 4\ C_2H_5COOH + 2\ CH_3COOH + 2\ CO_2 + 2\ H_2O$$

The theoretical equation for the fermentation of

- glucose by propionibacteria is giving a propionic
- acid:acetic acid ratio of 2: 1.
- Bacteria of the genus *Propionibacterium* produce propionic acid as the end-product of their anaerobic metabolism. This class of bacteria is commonly found in the stomachs of ruminants and the sweat glands of humans, and their activity is partially responsible for the odor of both Swiss cheese and sweat.

UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

EUROPEAN UNION
European Structural and Investing Funds
Operational Programme Research, Development and Education

MINISTRY OF EDUCATION, YOUTH AND SPORTS

Industrial microbiology

Lecturer:

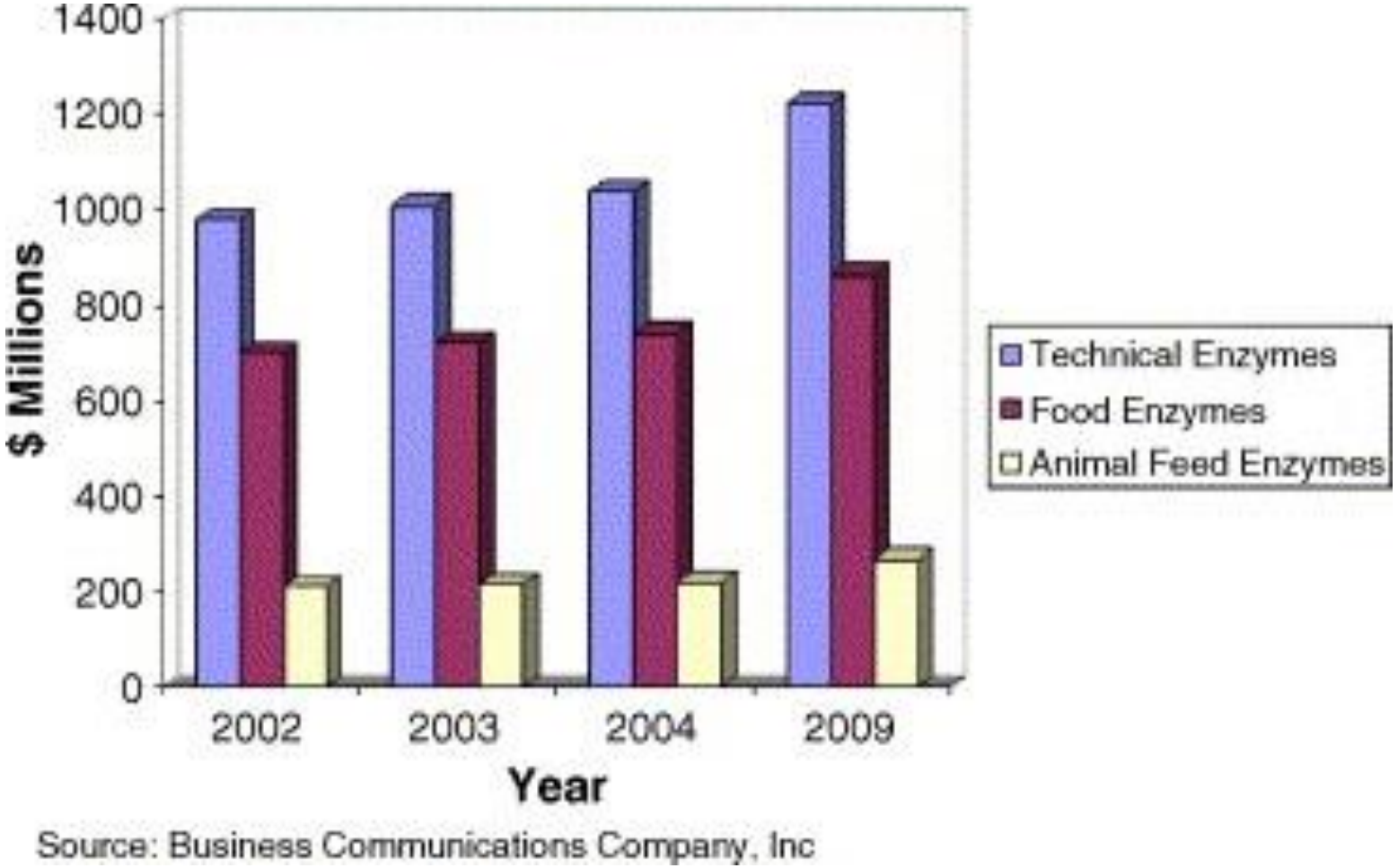
Jarmila Pazlarová

Department of Biochemistry and Microbiology

Microbial enzymes

In contrast to submerged (liquid state) fermentation, **Solid State Fermentation (SSF)** is the cultivation of microorganisms under controlled conditions in the absence of free water.

Examples of products of **S**olid **S**tate **F**ermentation include **industrial enzymes**, fuels and nutrient enriched animal feeds. The application of modern biotechnical knowledge and process control technologies can lead to significant productivity increases from this ancient process.



Global enzyme markets by application sectors, 2002 through 2009 ($ millions).

Enzyme and Microbial Technology, Volume 39, Issue 2, 2006, 235–251

http://dx.doi.org/10.1016/j.enzmictec.2005.10.016

Solid state fermentor

Solid state fermentor

Proteases

- **Commercially useful enzymes** (CUEs) are enzymes which have commercial uses.
- Microbial enzymes have well-known applications as biocatalysts in several areas of industry, such as biotechnology, agriculture, pharmaceuticals, etc.
- **Metagenomic data** provide a unique resource for discovering novel commercially useful enzymes (CUEs) from yet unidentified microbes belonging to complex microbial communities in diverse ecosystems.
- **Proteases** are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale.
- **The major uses of free proteases** occur in dry cleaning, detergents, meat processing, cheese making, and silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and the virulent wounds.

Neutral proteases

- Neutral protease is produced at industrial level using agro-industrial residues as substrate e.g. wheat bran, rice husk, rice bran, spent brewing grain, coconut oil cake, palm kernel cake, sesame oil cake, jackfruit seed powder and olive oil cake etc.
- For developing a production medium it is very important to monitor the **cost-effectiveness** of the medium so these agro-industrial residues mentioned above are very cheap and easily available.
- Seven fungal cultures, i.e. three strains of *Aspergillus oryzae* and four strains of *Penicillium* species e.g. *P. funiculosum*, *P. funiculosum*, *P. pinophilum*, *P. aculeatum* were evaluated using a plate assay for enzyme production, which showed a strain of *A. oryzae* NRRL 1808 as the most useful culture.
- Protease enzyme is produced in two fermentor systems, in **solid-state fermentors** (SSF) and **sub-merged fermentors** (SmF).

Amylases

- **Completely replaced chemical hydrolysis** of starch in starch processing industry. Although many microorganisms produce this enzyme, the most commonly used for their industrial application are ***Bacillus licheniformis***, ***Bacillus amyloliquifaciens*** and ***Aspergillus niger***.
- Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries.
- *Rhizopus oligosporous* 1HS13, *Aspergillus niger*, *Rhizopus oryzae*, and *Conidiobolus spp* have ability to produce proteases.

Glukoamylase

- ***Aspergillus*** sp. A3 is used for the production of glucoamylase under **solid state fermentation**.

  Different substrates like wheat bran, green gram bran, black gram bran, corn flour, barley flour, jowar flour, maize bran, rice bran and wheat rava are the best substrate and give best results among all these wheat bran showed the highest enzyme activity.

  The maximum enzyme activity under optimum conditions was 247 U/g of wheat bran.

- The optimum conditions are fructose as additive 1% w/w, urea as additive 1% w/w, incubation time of **120 h**, incubation temperature at **30 °C**, 2:10 (v/w) ratio of salt solution to weight of wheat bran, inoculum level 10% v/v, moisture content of solid substrate 80%, 1:50 ratio of substrate weight to flask volume and pH 5.0.

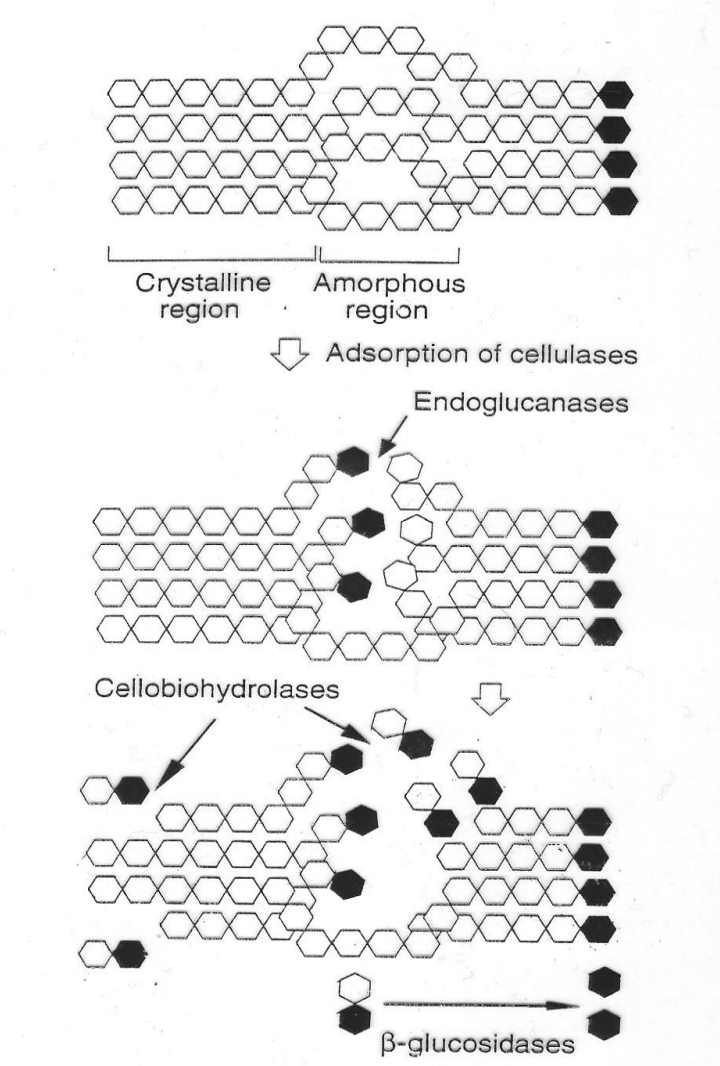
Cellulases

- **Cellulase** is any of several enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose of some related polysaccharides.
- The name is also used for any naturally occurring **mixture or complex of various such enzymes**, that act serially or synergistically to decompose cellulosic material.
- The specific reaction involved is the hydrolysis of the 1,4-beta-D-glycosidic linkages in cellulose,hemicellulose, lichenin, and cereal beta –D-glucans.
- Because **cellulose molecules bind strongly** to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides such as starch.

Synergy between endoglucanases , cellobiohydrolases and β-glucosidases

Hexagones - glucose

Reducing ends black



Cellullases I.

- Several different kinds of cellulases are known.
- Synonyms, derivatives, and specific enzymes associated with the name "cellulase" include
- endo-1,4-beta-D-glucanase (beta-1,4-glucanase,
- endoglucanase D, 1,4-(1,3,1,4)-beta-D-glucan 4-glucanohydrolase),
- carboxymethyl cellulase (CMCase), avicelase, celludextrinase, cellulase A, cellulosin AP, alkali cellulase, cellulase A 3, 9.5 cellulase, and pancellase SS.
- **Endocellulases (EC 3.2.1.4)** randomly cleave internal bonds at amorphous sites that create new chain ends.

Cellulases II.

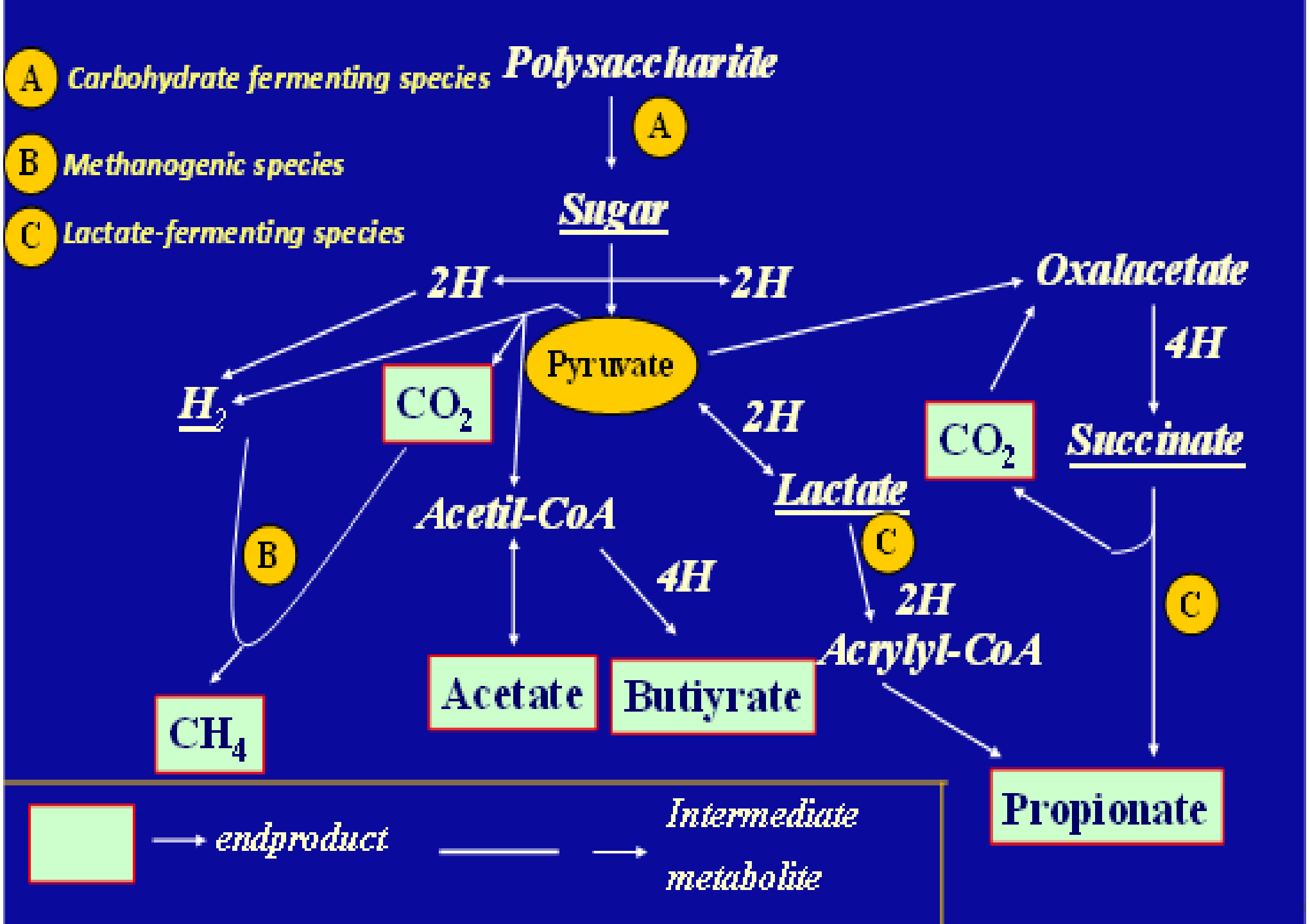
- Exocellulases or cellobiohydrolases (EC 3.2.1.91) cleave two to four units **from the ends of the exposed chains** produced by endocellulase, resulting in tetrasaccharides or disaccharides, such as **cellobiose**.
- Exocellulases are further classified into **type I,** that work processively from the **reducing end** of the cellulose chain, and **type II**, that work processively from the **nonreducing end**.
- **Cellobiases (EC 3.2.1.21)** or beta-glucosidases hydrolyse the exocellulase product into individual monosaccharides.

Lipases

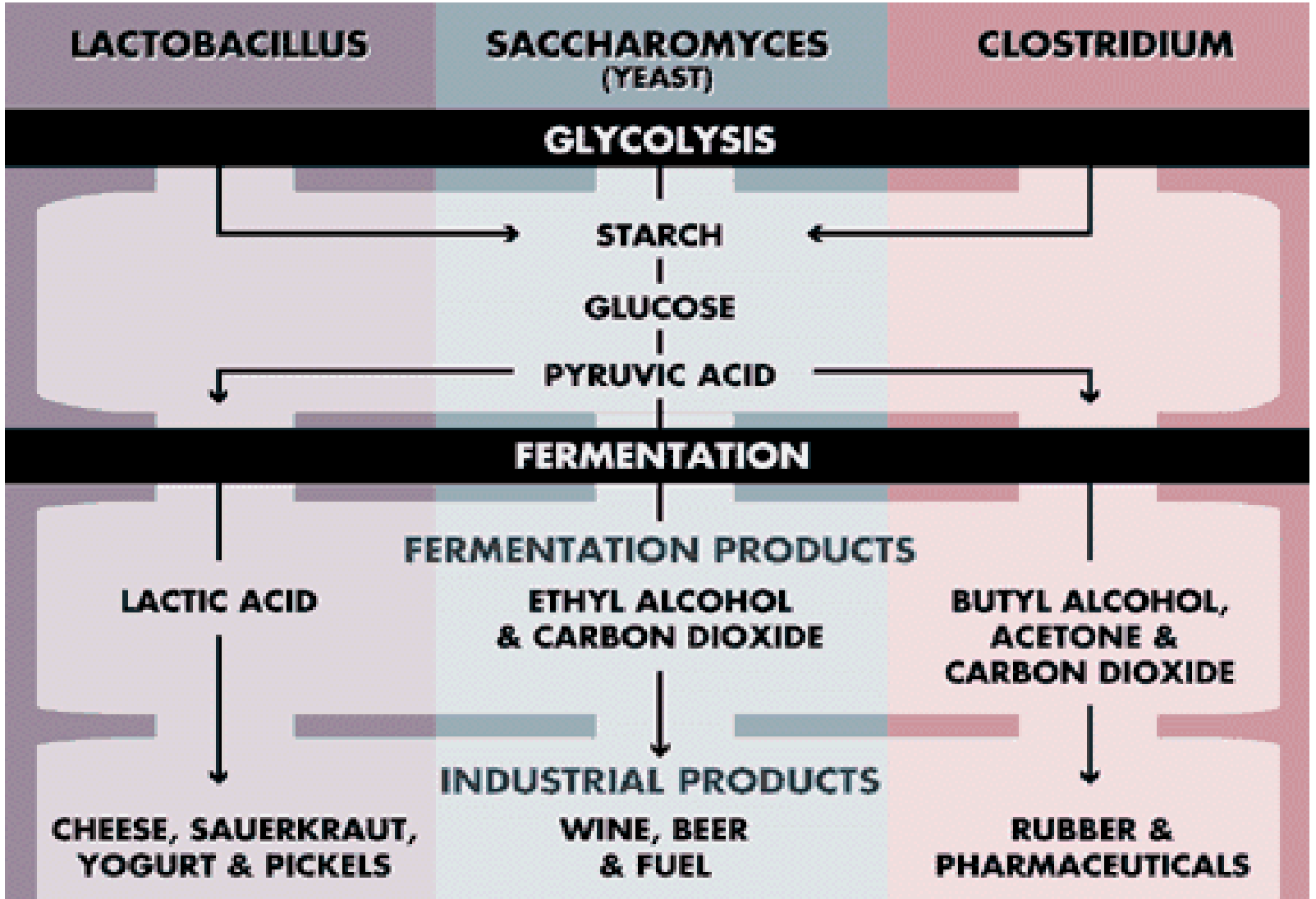
- Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes which catalyse the hydrolysis of long chain **triglycerides**. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology.
- The high-level production of microbial lipases requires not only the **efficient overexpression** of the **corresponding genes** but also a **detailed understanding of the molecular mechanisms governing their folding and secretion**. The optimization of industrially relevant lipase properties can be achieved by directed evolution.
- Numerous species of **bacteria**, yeasts and **molds** produce lipases. Taxonomically close strains may produce lipases of different types.

Microbial metabolites :
Primary products formation during exponential phase

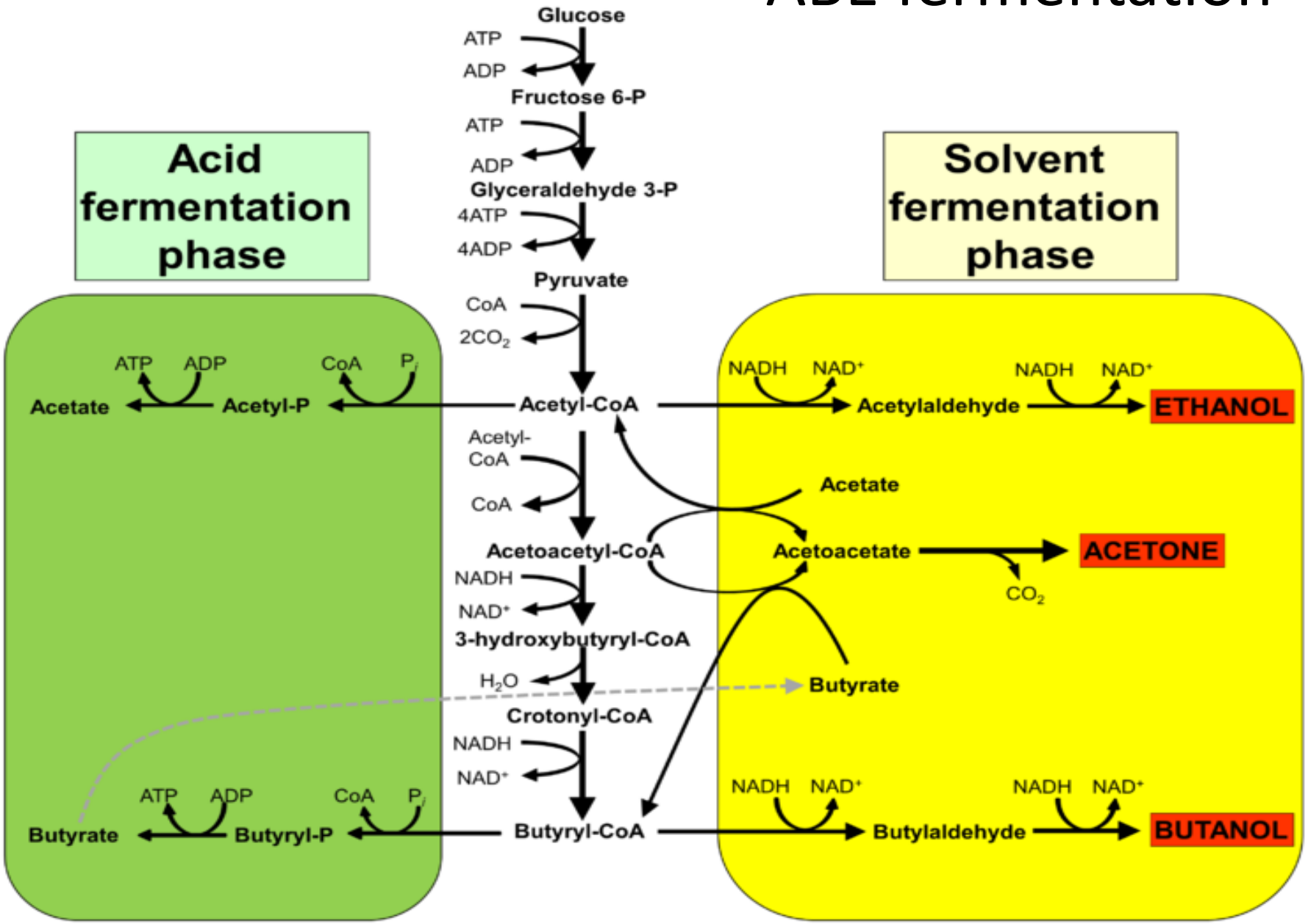
- **metabolites** – ethanol,
- citric acid,
- glutamic acid
- lactic acid
- lysine,
- vitamins,
- polysaccharides


A Carbohydrate fermenting species
B Methanogenic species
C Lactate-fermenting species
Polysaccharide
A
Sugar
2H
2H
Oxalacetate
4H
Pyruvate
H_2
CO_2
2H
CO_2
Succinate
Lactate
Acetil-CoA
B
C
4H
2H
Acrylyl-CoA
C
Acetate
Butiyrate
CH_4
Propionate
endproduct
Intermediate metabolite

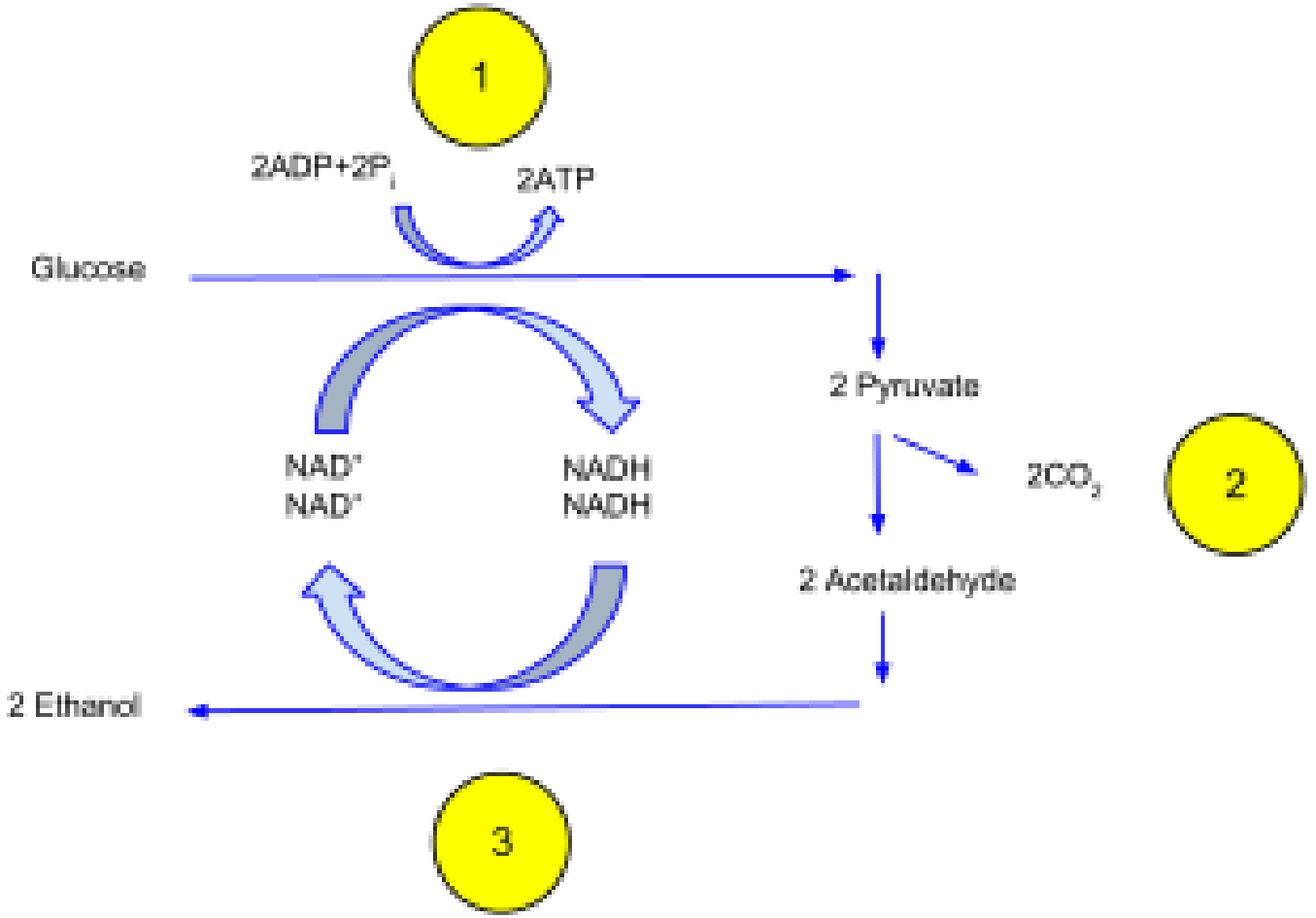
Distribution of fermentations



ABE fermentation



Ethanol fermentation



Lactic acid fermentation

- **Homolactic fermentation** (producing only lactic acid) is the simplest type of fermentation.
- The pyruvate from glycolysis undergoes a simple redox reaction, forming lactic acid.
- It is unique because it is one of the only respiration processes to not produce a gas as a byproduct. Overall, **one molecule** of **glucose** (or any six-carbon sugar) is converted to **two molecules of lactic acid**:

$$\mathbf{C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH}$$

Heterolactic fermentation

Bacteria of heterolactic fermentation do not have **aldolase** – direct oxidation of glucose by pentose cycle on **pentosa-5-phosphate** and **CO_2**.

In presence of inorganic phosphate it is split to **acetylphosphate** (CH_3-COO-PO_3H_2) and **glyceraldehyde- 3-phosphate**

acetylphosphate → **ETHANOL**

glyceraldehyde → **LACTATE + CO_2**

Pentose phosphate pathway

pentose phosphate pathway (also called the **phosphogluconate pathway** and the **hexose monophosphate shunt**) is a metabolic pathway parallel to glycolysis that generates NADPH and **pentoses** (5-carbon sugars) as well as ribose 5-phosphate, a precursor for the synthesis of nucleotides.

While it does involve oxidation of glucose, its **primary role is anabolic rather than catabolic.**

Amino acids fermentation

- Market development has been particularly dynamic for the **flavor-enhancer glutamate** and the **animal feed amino acids** L-lysine, L-threonine, and L-tryptophan.
- Amino acids are produced by fermentation processes using high performance strains of ***Corynebacterium glutamicum*** and ***Escherichia coli*** from sugar sources such as molasses, sucrose, or glucose.
- Four production methods for amino acids—extraction, synthesis, fermentation, and enzymatic catalysis—it is particularly the last two biotechnological processes, their economic and ecological advantages, that are responsible for this spectacular growth.

Overview of amino acids production

Amino acid (g/100 g scr.)	**Strain/mutant**	**Titer (g/l)**	**Estimated yield**
L-Lysine HCl	*C. glutamicum* B-6	100	40–50
L-Threonine	*E. coli* KY 10935	100	40–50
L-Tryptophan	*C. glutamicum* KY	58	20–25
L-Tryptophan	*E. coli*	45	20–25
L-Phenylalanine	*E. coli* MW	51	20–25
L-Arginine	*Brevibacterium flavum*	36	30–40
L-Histidine	*C. glutamicum* F81	23	15–20
L-Isoleucine	*E. coli* H-8461	30	20–30
L-Serine	*Methylobacterium* sp.	65	30–35
L-Valine	*C. glutamicum* VR 3	99	30–40

Amino acids production

- All 20 amino acids are sold, albeit each in greatly different quantities. ***Essential amino acids ?***
- Amino acids are used as **animal feed additives** (lysine, methionine, threonine),
- **flavor enhancers** (monosodium glutamic, serine, aspartic acid)
- **nutrients** in the medical field.

Glutamic acid, lysine and methionine account for the majority, by weight, of amino acids sold.

Glutamic acid and lysine are made by fermentation; methionine is made by chemical synthesis.

The major producers of amino acids are based in Japan, the US, South Korea, China and Europe.

How to increase production

- **Auxotrophic mutation-** lack of the formation of regulatory end product (i.e. repressor or regulatory effector). The intermediates of the metabolic pathways accumulate and get excreted.
- **Genetic recombination-** Protoplast fusion in certain bacteria is used for development of hybrids e.g. *Corynebacterium glutamicum* and *Bacillus flavum*.
- **Recombinant DNA technology-**E: coli and cloning vector pBR322 were used to increase the genes for the production of amino acids e.g. glutamic acid, lysine, phenylalanine, valine.

Secondary metabolites- all **antibiotics** fermentation

- **Penicillin** - Nobel prize 1945 Sir A. Fleming, H.W. Florey and E.B. Chain
- The **production of antibiotics** has been widespread since the pioneering efforts of **Florey** and **Chain** in 1938. The importance of antibiotics to medicine has led to much research into their discovery and production.
- Useful antibiotics are often discovered using a **screening process**. To conduct such a screen, isolates of many different microorganisms are cultured and then tested for production of diffusible products that **inhibit the growth of test organisms**.
- Most antibiotics identified in such a screen are already known and must therefore be disregarded. The remainder must be tested for their selective toxicities and therapeutic activities, and the best candidates can be examined and possibly modified

Antibiotics production

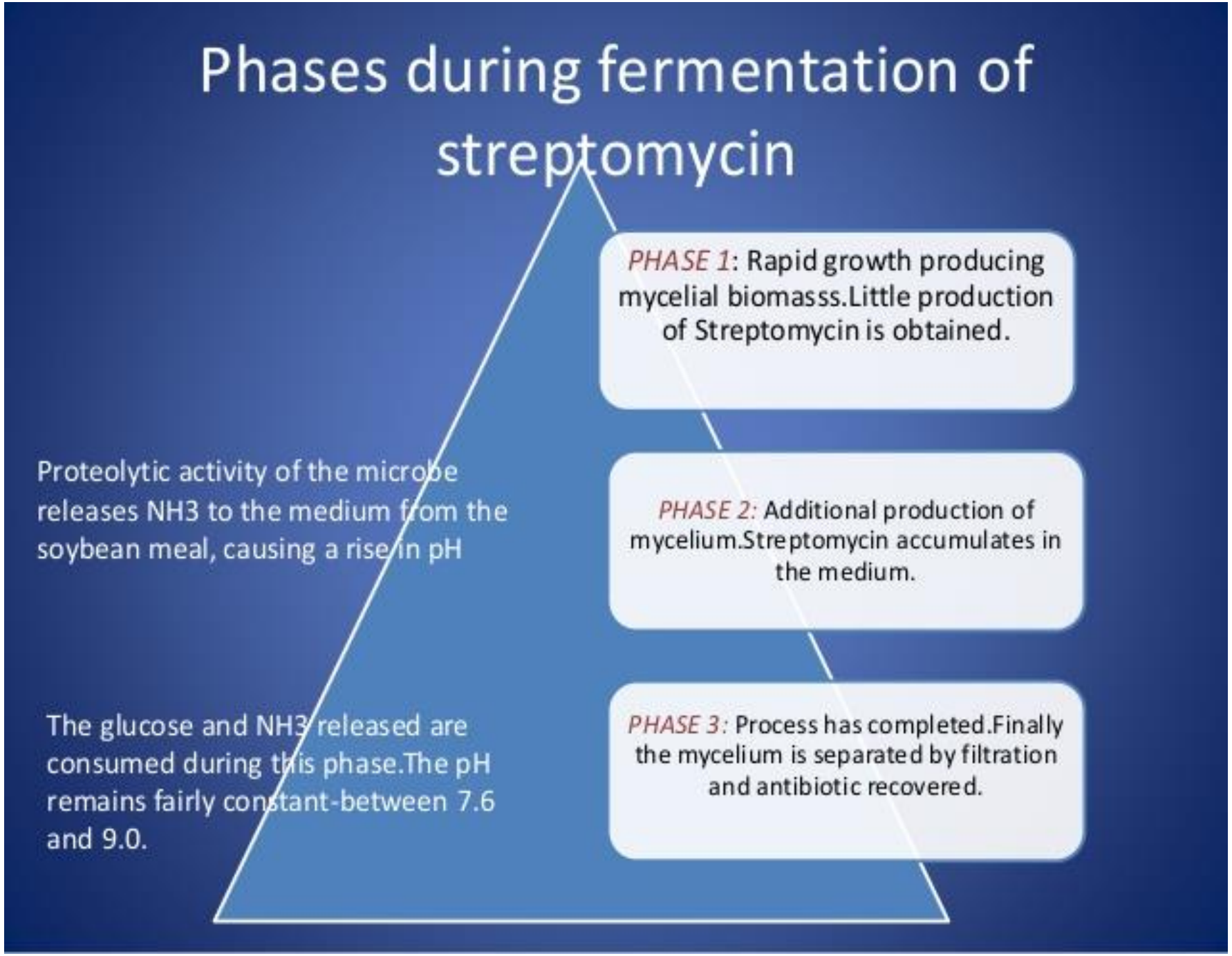
- **Source microorganism** is grown in large containers (100,000–150,000 liters or more) containing a liquid growth medium.
- **Oxygen concentration**, temperature, pH and nutrient levels must be optimal, and are closely monitored and adjusted if necessary.
- As antibiotics are **secondary metabolites**, the population size must be controlled very carefully to ensure that maximum yield is obtained before the cells die.
- Once the process is complete, the antibiotic must be **extracted** and **purified** to a crystalline product.
- This is easier to achieve if the antibiotic is soluble in organic solvent. Otherwise it must first be removed by ion exchange, adsorption or chemical precipitation.

Producers of antibiotics

- Microorganisms used in fermentation are rarely identical to the **wild type.**
- Species are often **genetically modified** to yield the maximum amounts of antibiotics.
- **Mutation** is often used, and is encouraged by introducing mutagens such as **ultraviolet radiation**, **x-rays** or certain chemicals.
- **Selection** and further reproduction of the higher yielding strains over many generations can raise yields by 20-fold or more.
- Another technique used to increase yields is **gene amplification** - copies of genes coding for enzymes involved in the antibiotic production can be inserted back into a cell, **via vectors** such as **plasmids**. This process must be closely linked with retesting of antibiotic production.

Typical producers

- **Actinomycetes** have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances.
- ***Streptomyces*** is the largest genus of Actinobacteria and the type genus of the family *Streptomycetaceae.* Over 500 species of ***Streptomyces*** have been described.
- As with the other Actinobacteria, Streptomycetes are **Gram-positive** and have genomes with high **GC-**content.
- *Streptomyces* sp. are widely recognized as industrially important organisms for their ability to elaborate different kinds of **novel secondary metabolites.**


Phases during fermentation of streptomycin
PHASE 1: Rapid growth producing mycelial biomasss.Little production of Streptomycin is obtained.
Proteolytic activity of the microbe releases NH3 to the medium from the soybean meal, causing a rise in pH
PHASE 2: Additional production of mycelium.Streptomycin accumulates in the medium.
The glucose and NH3 released are consumed during this phase.The pH remains fairly constant-between 7.6 and 9.0.
PHASE 3: Process has completed.Finally the mycelium is separated by filtration and antibiotic recovered.

Recombinant products

- insulin,
- hepatitis B vaccine,
- interferon,
- granulocyte colony-stimulating factor,
- Streptokinase
- and many others

Insulin I.

In the **1980s,** researchers used genetic engineering to manufacture a human insulin. In 1982, the **Eli Lilly Corporation** produced a **human insulin** that became the first approved **genetically engineere**d pharmaceutical product.

Without needing to depend on animals, researchers could produce genetically engineered insulin in unlimited supplies. It also did not contain any of the animal contaminants.

According to the Eli Lilly Corporation, in **2001 95% of insulin** users in most parts of the world take **some form of human insulin**. Some companies have stopped producing animal insulin completely. Companies are focusing on synthesizing human insulin and insulin analogs, a modification of the insulin molecule in some way.

Insulin II.

The insulin gene is coding a protein consisting of **two separate chains of amino acids**, an **A** above a **B** chain, that are held together with bonds. Amino acids are the basic units that build all proteins.
The insulin **A chain** consists of **21 amino acids** and the **B chain** has **30**.
Before becoming an active insulin protein, insulin is first produced as **preproinsulin**. This is **one single long protein** chain with the A and B chains not yet separated, a section in the middle linking the chains together and a **signal sequence** at one end telling the protein when to start secreting outside the cell.

Insulin III.

After **preproinsulin**, the chain evolves into **proinsulin**, still a **single chain** but without the signaling sequence. Then comes the active protein insulin, the protein without the section linking the A and B chains**. At each step, the protein needs specific enzymes to produce the next form of insulin.**

One method of manufacturing insulin is to grow the **two insulin chains separately**. This will avoid manufacturing each of the specific enzymes needed.

Manufacturers **need the two mini-genes**: one that produces the A chain and one for the B chain. Since the exact DNA sequence of each chain is known, they synthesize each mini-gene's DNA in an amino acid sequencing machine.

Interferon

- **Interferons** (**IFN**s) - a group of **signaling proteins** made and released by host cells in response to the presence of several **pathogens** (viruses, bacteria, parasites) and also **tumor** cells. In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses. First described in **1957.**
- IFNs belong to the large class of proteins known as **cytokines** , molecules used for communication between cells to trigger the protective defenses of the **immune system** that help eradicate pathogens.
- Interferons are named for their ability to "interfere" with viral replication. IFNs also have various other functions: they activate immune cells, such as natural killer cells and macrophages ; they increase host defenses by up-regulating **antigen presentation** by virtue of increasing the expression of major histocompatibility complex (MHC) antigens.

Interferon production

- In 1978 – 1981 the **purification** of the type I interferons IFN-α and IFN-β.
- By the early 1980s, **genes for these interferons** had been cloned, adding further definitive proof that interferons were responsible for interfering with viral replication.
- Gene cloning also confirmed that IFN-α was encoded by a family of many related genes.The type II IFN (IFN-γ) gene was also isolated around this time.[
- Interferon was **scarce and expensive until 1980**, when the interferon gene was inserted into bacteria using **recombinant DNA technology**, mass cultivation and purification from **bacterial cultures** or derived from yeasts.
- Interferon can also be produced by **recombinant mammalian cells**.

Streptokinase

- **Streptokinase** (**SK**) is an **enzyme** secreted by several species of streptococci (*Streptococcus equisimilis*) that can bind and activate human plasminogen
- SK is used as an effective and inexpensive **thrombolysis** medication in some cases of **myocardial infarction –MI** (heart attack) and pulmonary embolism.
- It is on the WHO List of Essential Medicines, the most important medications needed in a basic health system.
- It is no longer available in the United States. SK is used as thrombolysis for acute MI in about half of countries worldwide because it is affordable.
- "In countries where patients do not have rapid access to catheterisation, streptokinase is still important."

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Cultivation and molecular microbiology methods used for the identification of food-born pathogens

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Microbial cultivation

- When microorganisms are cultivated in the laboratory, a **growth environment** called a **medium** is used.
- The medium may be purely chemical (a chemically defined medium), or it may contain organic materials, or it may consist of living organisms such as fertilized eggs.
- Microorganisms growing in or on such a medium form a **culture.** A culture is considered a **pure culture** if only one type of organism is present and a **mixed culture** if populations of different organisms are present.
- When first used, the **culture medium should be sterile**, meaning that no form of life is present before inoculation with the microorganism.

General microbial media

- For the cultivation of bacteria, a commonly used medium is **nutrient broth**, a liquid containing proteins, salts, and growth enhancers that will support many bacteria.
- To solidify the medium, an agent such as **agar** is added. Agar is a polysaccharide that adds no nutrients to a medium, but merely solidifies it.

Types of media

Many media for microorganisms are complex, reflecting the growth requirements of the microorganisms. For instance, most **fungi** require extra **carbohydrate** and an acidic environment for optimal growth.

The medium employed for these organisms is **potato dextrose agar**, also known as **Sabouraud dextrose agar.**

For **anaerobic microorganisms**, the atmosphere must be **oxygen free**. To eliminate the oxygen, the culture media can be placed within containers where carbon dioxide and hydrogen gas are generated and oxygen is removed from the atmosphere. Commercially available products achieve these conditions.

Special microbial media I

- Certain microorganisms are cultivated in **selective media.**
- These media retard the growth of unwanted organisms while encouraging the growth of the organisms desired. For example, **mannitol salt agar** is selective for staphylococci because most other bacteria cannot grow in its high-salt environment.
- Another selective medium is **brilliant green agar**, a medium that inhibits Gram-positive bacteria while permitting Gram-negative organisms such as *Salmonella* species to grow.

Special microbial media II

- Still other culture media are **differential media**. These media provide environments in which different bacteria can be distinguished from one another.
- For instance, **violet red bile agar** is used to distinguish coliform bacteria such as *Escherichia coli* from noncoliform organisms. The coliform bacteria appear as bright pink colonies in this media, while noncoliforms appear a light pink or clear.
- Certain media are **both selective and differential**. For instance, **MacConkey agar** differentiates lactose-fermenting bacteria from nonlactose-fermenting bacteria while inhibiting the growth of Gram-positive bacteria.

Isolates – pure cultures

- In order to work with microorganisms in the laboratory, it is desirable to obtain them in pure cultures.
- Pure cultures of bacteria can be obtained by **spreading** bacteria out and permitting the individual cells to form masses of growth called **colonies.**
- One can then pick a sample from the colony and be assured that it contains only one kind of bacteria. Cultivating these bacteria on a separate medium will yield a pure culture.

Isolation methods I.

- To obtain separated colonies from a mixed culture, various **isolation methods** can be used. One is the **streak plate method**, in which a sample of mixed bacteria is streaked several times along one edge of a Petri dish containing a medium such as nutrient agar.
- **A loop is flamed** and then touched to the first area to retrieve a sample of bacteria. This sample is then **streaked several times** in the second area of the medium.
- **The loop is then reflamed**, touched to the second area, and streaked once again in the third area. The process can be repeated in a fourth and fifth area if desired. During incubation, the bacteria will multiply rapidly and form colonies.

Isolation methods II.

- A second isolation method is the **pour plate method.** In this method, a sample of bacteria is diluted in several tubes of melted medium such as nutrient agar or physiological saline.
- After dilution, the melted agar is poured into separate Petri dishes and allowed to harden. Since the bacteria have been diluted in the various tubes, the plates will contain various dilutions of bacteria, and where the bacteria are most diluted, they will form isolated colonies.

Isolation methods III.

- The biggest drawback in the culture-based method is the **slow growth** due to which excess time is lapsed to get the final result, which can turn out to be fatal. All **these media take up to 18-24 h** to give the exact result, indicating the slow turnaround time.
- One of the best known examples which shows high success rate and also shows that the method is highly cost-effective is the culture of *E. coli* O157:H7 on Sorbitol MacConkey agar (**SMAC**) which is based on the principle of fermentation of **sorbitol.**
- The drawbacks of the SMAC agar can be overcome by the use of chromogenic medium for STEC isolation which has increased specificity and sensitivity. The major advantage of this is the easier discrimination based on **colour.** Due to the use of the chromogenic substance, the medium is better known as **CHROMagar**

Evaluation of conventional methods

- **Conventional culture methods** remain the **most reliable** and accurate techniques for **food-borne pathogen detection.**
- Conventional methods include blending of the food product with a **selective enrichment** medium to increase the population of the target organism; plating onto selective or differential agar plates to isolate pure cultures; and examining the cultures by phenotypic analysis or metabolic fingerprinting (monitoring of carbon or nitrogen utilization).
- **A major drawback is that these methods are labor-intensive** and take 2–3 days for results, and up to 7–10 days for confirmation.

Sampling

- The ability of **microorganisms** to grow in **food,** and cosmetic products has been identified for many years. From the infectious point of view, the existence of pathogenic microbes in food products makes them hazardous.
- **Microbiological te**st is a vital part of analysis of any food in which microorganisms can survive and grow.
- Only a fraction of the batch is sampled for testing; and therefore, that **fraction must be representative** of the batch under consideration.
- **Microbiological sampling,** microorganisms may not necessarily be randomly distributed throughout the whole batch or product and random sampling scheme should not therefore work there. Hazard analysis and control of critical points (**HACCP**) regulate the sampling.

What method is more suitable?

- **Conventional methods – the gold standard**
- These methods are highly sensitive, reliable, low-priced and provide both qualitative and quantitative results on the bacterial populations present in the food sample.
- **Molecular methods – the upcoming rival**
- In contrast to the conventional methods, the identification and characterisation of unculturable and slow-growing pathogens is easier to implement. In addition, they can be performed and results interpreted by staff with no taxonomical expertise.

DNA-based methods

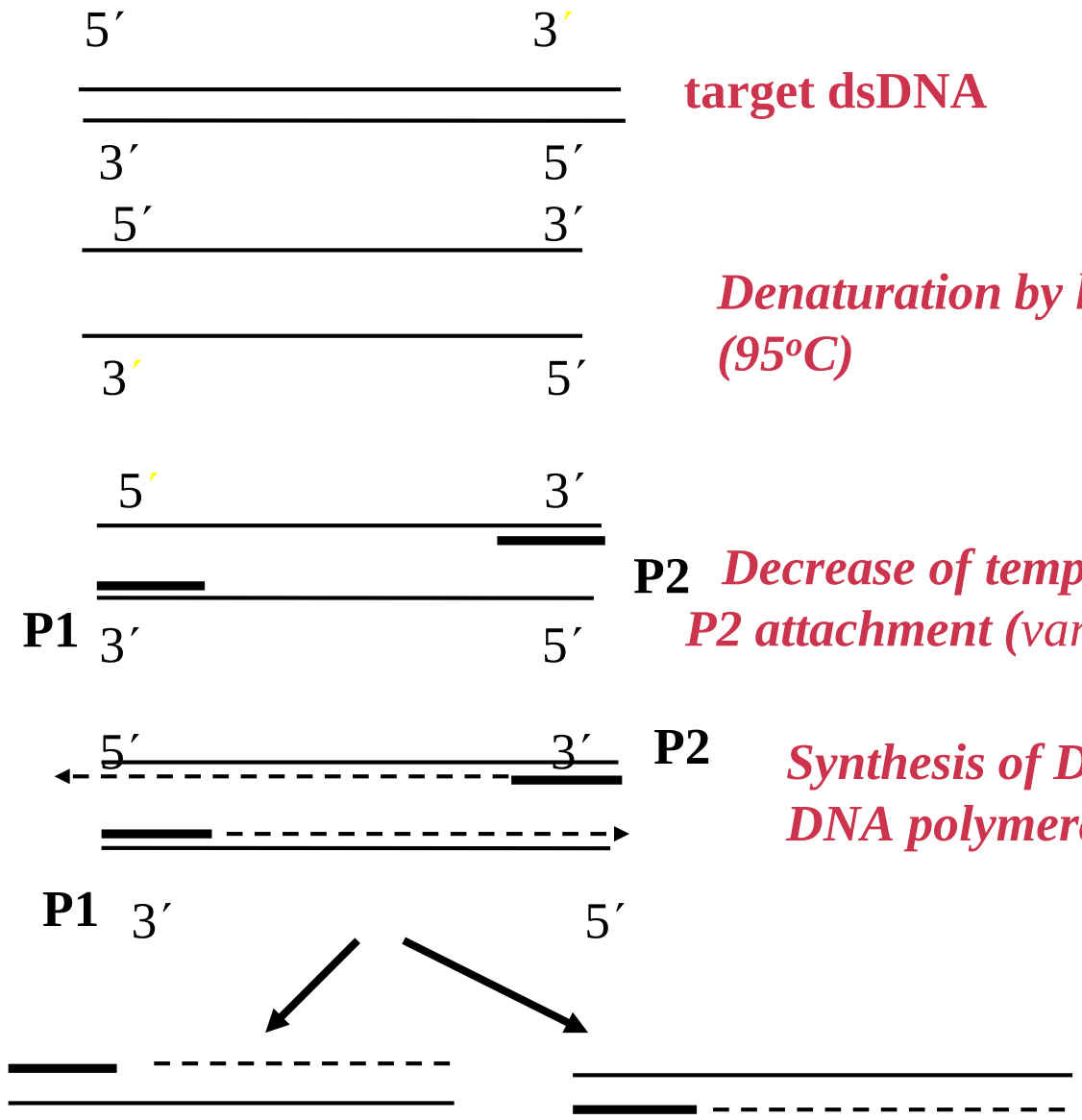
DNA-based methods, such as polymerase chain reaction (**PCR**), overcome some these limitations since they are generally faster and can provide more information than culture-based methods.

One limitation of traditional PCR-based methods is that they are normally limited to the analysis of a single pathogen, a small group of related pathogens, or a small number of relevant genes.

PCR

- **Nucleic acid-based meth**ods in pathogen detection are promising in their rapid results, high specificity, and low detection limits of up to, in theory, a single cell.
- Developed in the mid-1980s,nucleic acid-based technology quickly achieved widespread use in the field of pathogen detection, with a particular focus on **polymerase chain reaction** (**PCR**) assays that were developed to detect virtually every clinically relevant bacterial pathogen. In the past decades, our understanding of DNA has grown considerably, with currently 880 **fully sequenced microbial genomes**.

PCR



target dsDNA

cycle 1

Denaturation by heat (95°C)

***Decrease of temperature primers P1 a P2 attachment** (variabile temperature)*

***Synthesis of DNA by thermostabile DNA polymerase** (72°C)*

cycle 2

Denaturation by heat (95°C)

Repetition of cycles leads to exponential growth of starting DNA

Multiplex PCR (mPCR)

- Several sets of specific primers are used in mPCR assay whereas only one set of specific primers are used in conventional PCR assay. Primer design is very important for the development of mPCR, as the primer sets should have similar annealing temperature in order to produce a successful mPCR assay.
- mPCR is more advanced and it can detect up to five or more pathogens simultaneously.

Real-time or quantitative PCR (qPCR)

- Real-time PCR or quantitative PCR is different from simple PCR whereby it does not require agarose gel electrophoresis for the detection of PCR products. This method is able to monitor the **PCR products formation continuously** in the entire reaction by measuring the fluorescent signal produced by specific dual-labeled probes or intercalating dyes.
- The fluorescence intensity is proportional to the amount of PCR amplicons. **Several fluorescent systems** have been developed for qPCR and the most commonly used fluorescent systems include **SYBR green**.
- TaqMan probes and molecular beacons. SYBR green is a double-stranded DNA (dsDNA)-binding fluorescent dye . TaqMan probes and molecular beacons are the common alternatives to SYBR green.

Molecular biology methods

- **PCR** – polymerase chain reaction, specific and sensitive - **10^1-10^2** number of microbes.
- **Amplification** of **target DNA** by two **(15-30 bp)** synthetically prepa**red oligonucleotides - primers,** that hybridize with **complementary DNA chains** specific for wanted **microorganism.**
- Mostly the **specific genes coding virulence factors** are used.
- **Formed DNA** is visualized in **agarose gel** by **electrophoresis**

Molecular techniques

- The application of molecular techniques for detecting and typing of foodborne pathogens in surveillance studies provide reliable epidemiological data for tracing the source of human infections.
- A wide range of molecular techniques (including **pulsed field gel electrophoresis (PFGE)**, **multilocus sequence typing (MLST)**, **random amplified polymorphism deoxyribonucleic acid**, **repetitive extragenic palindromic, deoxyribonucleic acid sequencing, multiplex polymerase chain reaction** and many more) have been used for detecting, speciating, typing, classifying and/or characterizing foodborne pathogens of great significance to humans.

Whole-genome sequencing

Microbial **whole-genome sequencing (WGS)** is an important tool for mapping genomes of novel organisms, finishing genomes of known organisms, or comparing genomes across multiple samples. **Sequencing the entire microbial ge**nome is important for generating accurate reference genomes, for microbial identification, and other comparative genomic studies.

Recent advances in sequencing technologies and analysis tools have rapidly increased the output and analysis speed as well as **reduced the costs of WGS.**

The first whole genome to be sequenced was of the **bacterium *Haemophilus influenzae*.**

WGS

- **Proficiency testing**, as commonly used in routine diagnostic laboratories, is the logical next step. The Global Microbial Identifier (**GMI**) is an interlaboratory proficiency test (**PT**) for WGS in clinical settings (http://www.globalmicrobialidentifier.org/workgroups/about-the-gmi-proficiency-tests).
- The **GMI project** is a useful start for understanding and validating variations between laboratories that perform WGS out-break analyses.
- Nonetheless, the GMI PT is currently limited to three species, and therefore more such tests have to be conducted in order to cope with all hospital-acquired pathogen.

Microbial Detection – Qualitative

- **Impedance**
- There are at least four commercial instruments that use principles of **impedance** or **conductivity** measurement to detect bacteria.
- The relationship between capacitance at the electrode surface and conductance from ionic changes in the media from byproducts produced during bacterial growth allows calculation of impedance.
- **Increases** in capacitance and **conductance** result in decreased impedance indicative of bacterial growth.
- Each instrument type uses variable design principles that measure conductance based upon frequency and electrode quantity and type.

CO_2 Detection

- Growth-based technologies use either biochemical or physiological measures to reflect microorganism growth.
- Traditional or enhanced media formulations encourage microbial proliferation in test samples. A major advantage of these systems is the ability to recover microorganisms for failure investigations or identification after analysis.
- These systems use an **internal colorimetric CO_2 sensor** incorporated **into each media bottle during** manufacture. The sensor, separated from the media by a semi-permeable membrane, is impermeable to most ions and other media components but is freely permeable to CO_2.
- **Carbon dioxide produced by microbial metabolism** diffuses across the membrane, dissolves in water in the sensor, and generates hydrogen ions which result in a color change detected by a colorimetric detector.
- Light emitted by the detector reflects off the sensor onto a photometer.
- The resulting voltage signal is proportional to the intensity of the reflected light and to the concentration of CO_2 in the bottle.

Flow Cytometry

- Flow cytometry, a well-established detection platform and investigative tool first applied to studies of eukaryotic cells, can also be adapted to detect the presence of viable bacteria in samples.
- The **ability to stain microorganisms** with dyes such as propidium iodide, which is impermeable to cells with intact membranes, and thiozole orange, which is permeable to all cells, allows a **differentiation of viable** and **nonviable bacterial cells** in fluid media.

Endotoxin

- Automated **Limulus Amebocyte Lysate** (**LAL)** testing can provide **results within minutes** regarding the presence of bacterial endotoxin in raw materials, buffers, or in-process intermediates right in the warehouse or on the manufacturing floor.
- One such automated system uses the kinetic chromogenic method with endotoxin reagents contained in a plastic cartridge analyzed using a specialized reader to kinetically monitor the chromophore produced during the reaction.

Limulus polyphemus

- The **Atlantic horseshoe crab** (*Limulus polyphemus*), also known as the **American horseshoe crab**, is a species of marine and brackish chelicerate arthropod.
- The **blood of horseshoe cr**abs contains **one type** of blood cell, the **amebocytes**. These play an important role in the defense against pathogens.
- Amebocytes contain granules with a clotting factor known as **coagulogen**; this is released outside the cell when bacterial endotoxin is encountered.
- The resulting **coagulation** is thought to contain bacterial infections in the animal's semiclosed circulatory system.

LAL test

- There are three basic methodologies: **gel-clot**, **turbidimetric**, and **chromogenic**.
- The primary application for LAL is the testing of parenteral pharmaceuticals and medical devices that contact blood or cerebrospinal fluid.
- The LAL cascade is also triggered by (1,3)-β-D-glucan. Both bacterial endotoxins and (1,3)-β-D-glucan are considered "Pathogen-Associated Molecular Patterns", or **PAMPs**, substances which elicit inflammatory responses in mammals.

ATP Bioluminescence

Industry has incorporated rapid detection of microbial contamination using **ATP bioluminescence** for many applications.

One such application involves **surface swabs** dispersed into a **liquid matrix** for filtration.

Addition of a substrate to the membrane surface yields fluorescence following exposure to microbial ATP.

Adenosine triphosphate (ATP) is the main chemical energy source of all living cells. Detection systems based on bioluminescence exploit the chemical release of ATP from microorganisms. ATP reacts with luciferase and a photon counting imaging tube detects photons released by this reaction.

A computer monitor then represents the photons detected. There are both qualitative and quantitative systems available.

Principle of the test

- Mg^{2+}

Luciferin + ATP + O2 → oxyluciferin + AMP + PPi+ CO2+ light

- **luciferase**
- The reaction proceeds in two parts: the **adenylation of luciferin**, followed by the **oxygenation of adenyl-luciferin**. The adenylation step activates luciferin as an enzyme-adenyl-luciferin complex, which is analogous to the activation of fatty acids by acyl-CoA ligases (based on homology, beetle luciferases are thought to have evolved from acyl-CoA ligases, retaining their catalytic mechanism for adenylation). In the second step, **luciferase acts as an oxygenase on adenyl-luciferin** to produce oxyluciferin and carbon dioxide, the **decay of oxyluciferin producing a photon of light.**

Mass Spectrometry

- A new identification methodology uses Matrix Assisted Laser Desorption/Ionization – Time of Flight (**MALDI-TOF**) mass spectrometry. This method simultaneously screens molecular ions and charged fragments by analyzing their mass-to-charge ratios.
- Comparing the patterns with the patterns from known microorganisms establishes identity. Identifications can be available in minutes rather than days for the classical methods.

MALDI-TOF MS

- **Matrix-assisted laser desorption ionization-time** of flight mass spectrometry (MALDI-TOF MS) has recently emerged as a powerful tool for the routine identification of clinical isolates.
- MALDI-TOF MS based identification of bacteria has been shown to be more rapid, accurate and cost-efficient than conventional phenotypic techniques or molecular methods.
- Rapid and reliable identification of food-associated bacteria is also of crucial importance for food processing and product quality.

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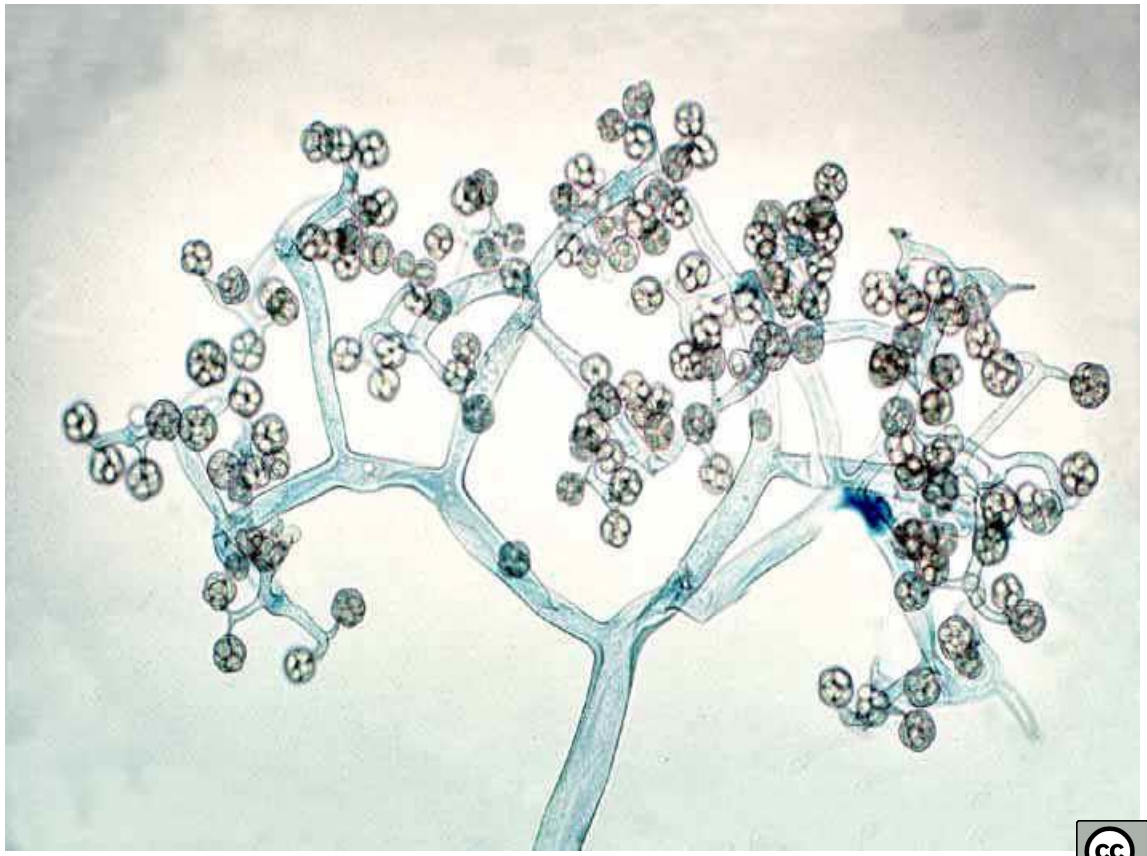
Micromycetes as food spoilage agents , mycotoxines

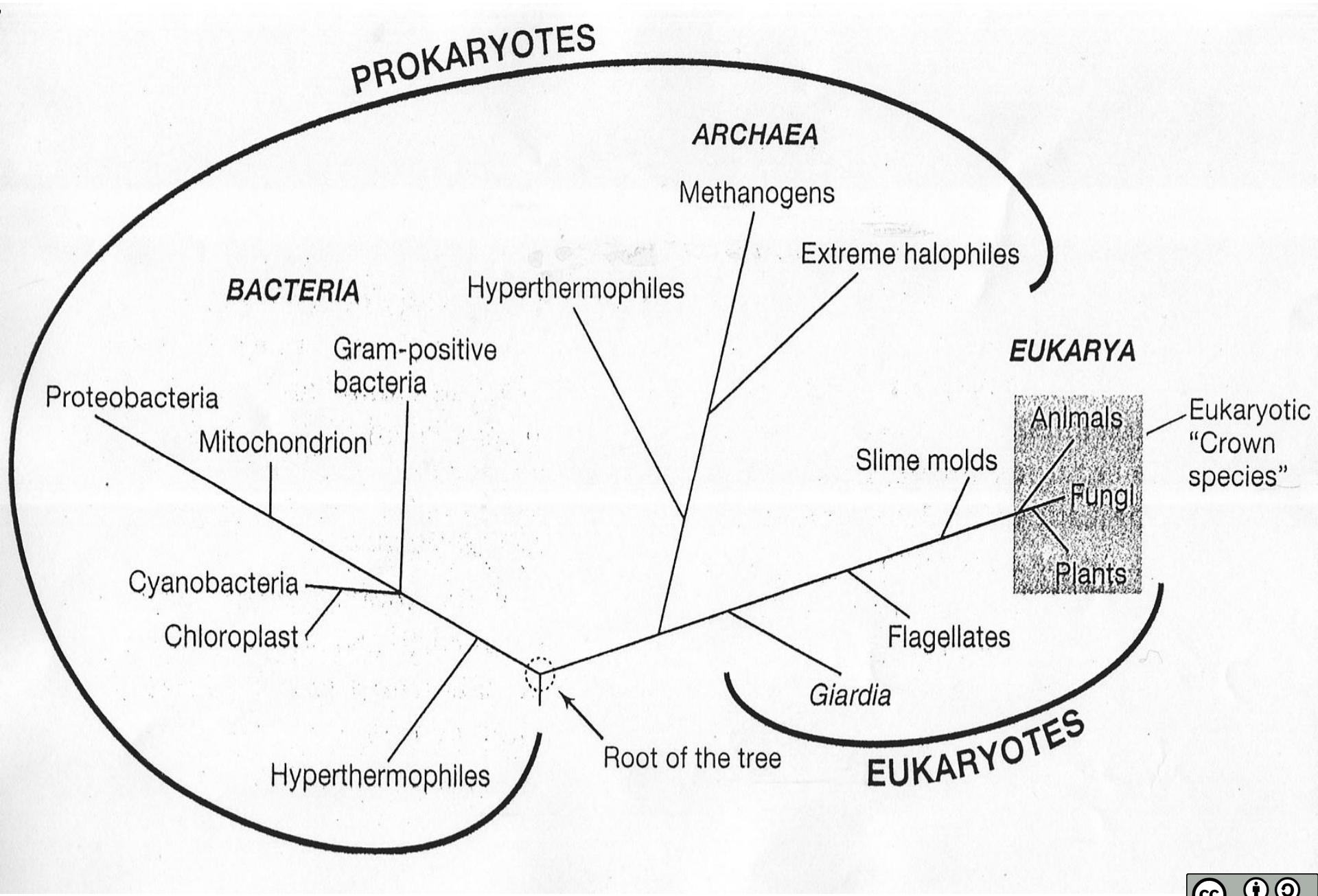
Lecturer:

Jarmila Pazlarová

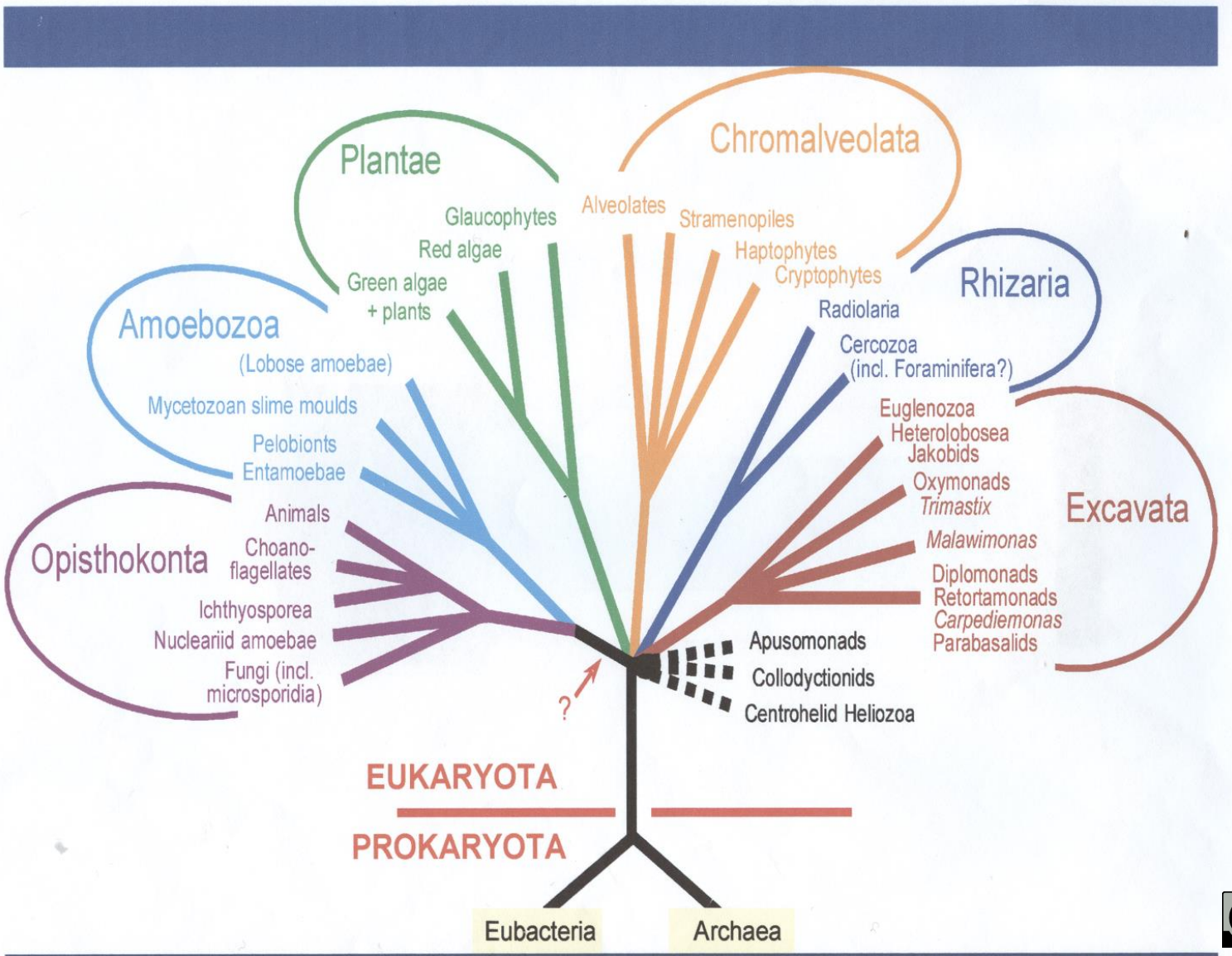
Department of Biochemistry and Microbiology

Micromycetes -
microscopic fungi - **molds**
microfungi




PROKARYOTES
ARCHAEA
Methanogens
Extreme halophiles
BACTERIA
Hyperthermophiles
Gram-positive bacteria
EUKARYA
Proteobacteria
Mitochondrion
Animals
Eukaryotic "Crown species"
Slime molds
Fungi
Plants
Cyanobacteria
Chloroplast
Flagellates
Giardia
Root of the tree
Hyperthermophiles
EUKARYOTES

Alternative system



FUNGI

-Kingdom of **Eukaryota**

-Eukaryotic organisms without plastids

-Nutrition absorptive (osmotrophic)

-Cell walls containing **chitin** and **β-glucans**

-**Mitochondria** with flattened cristae

-Unicelullar or filamentous

-Mostly non flagellate

-Reproducing **sexually** or **asexually**

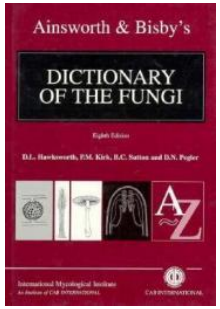
-The **diploid phase generally short-lived**

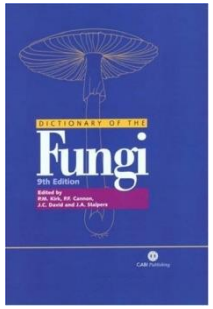
-Saprobic, mutualialistic or parasitic

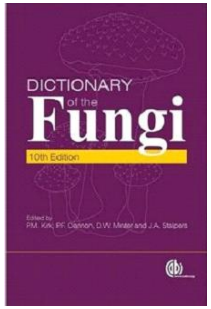
International Mycological Institute:

DICTIONARY OF THE FUNGI (Kirk, Cannon, Minter, Stalpers) - 2008

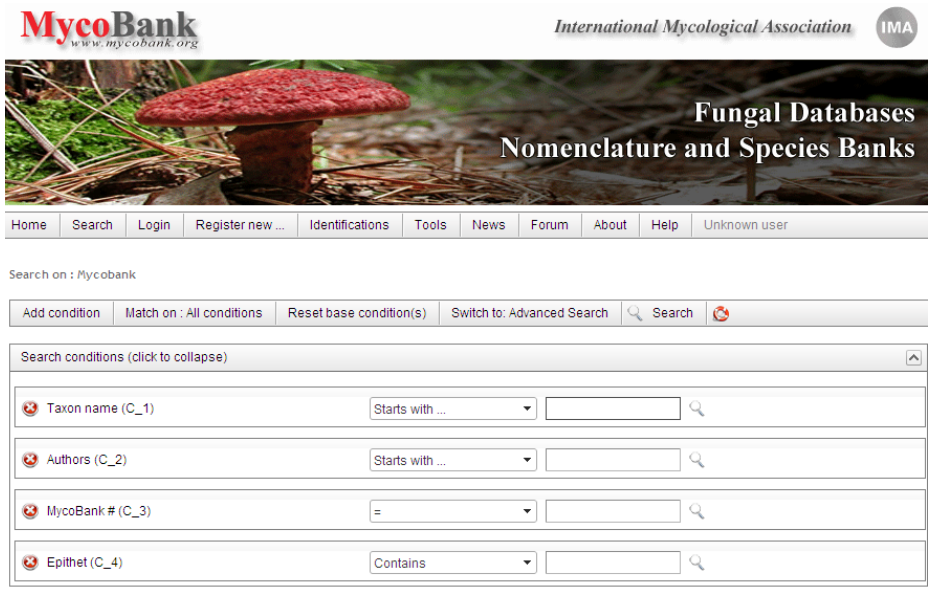
(1943, 1945, 1950, 1954, 1961, 1971, 1983, 1995, 2001, 2008)







http://www.indexfungorum.org/Names/Names.asp



http://www.mycobank.org/

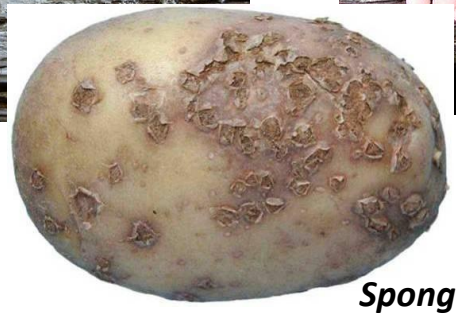
Size of micromycetes

- 1.5 milion species, only 5% of them were formally classified
- Great diversity of life cycles and morphology
- Recent taxonomy is based on DNA analysis
- Majority of micromycetes is growing as **fibre** (hypha), that are cylindrical, fibrous structures **2–10 µm** in diameter, and up to several centimeters long.

Fungi and pseudofungi

Kingdom: **PROTOZOA**

Myxomycota



Spongospora subterranea

Kingdom: **CHROMISTA**

Plasmodiophora brassicae

Phytophthora infestans

Fungi and pseudofungi

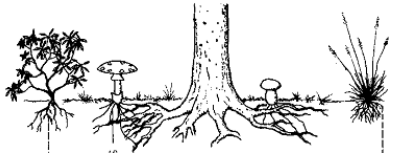
Kingdom: **PROTOZOA**	Division	Acrasiomycota
		Myxomycota
		Plasmodiophoromycota
Kingdom: **CHROMISTA**	Division	Labyrinthulomycota
		Peronosporomycota
		Hyphochytriomycota
Kingdom: **FUNGI**	Division	Chytridiomycota
		Microsporidiomycota
		Glomeromycota
		Zygomycota
		Ascomycota
		Basidiomycota

Fungal ecology

saprobic

Penicillium sp.

mutualistic symbioses

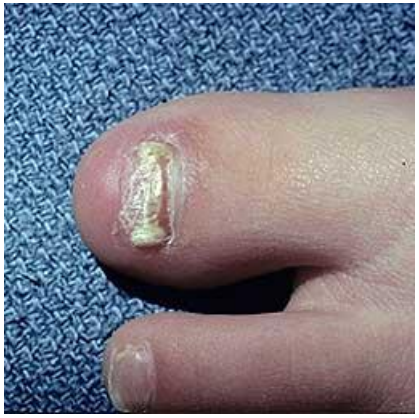


Mycorrhizal fungi

parasitic

lichens

+ endophyte



dermatophyte

Cordyceps lloydii

Mycosphaerella fragariae

Spinellus fusi

Kingdom: Fungi (Ophisthokonta)

- **Division**:

- Chytridiomycota
- Microsporidiomycota
- Glomeromycota
- Zygomycota
- Ascomycota
- Basidiomycota

Kingdom: Fungi (Ophisthokonta)

- **Division**:

- Chytridiomycota
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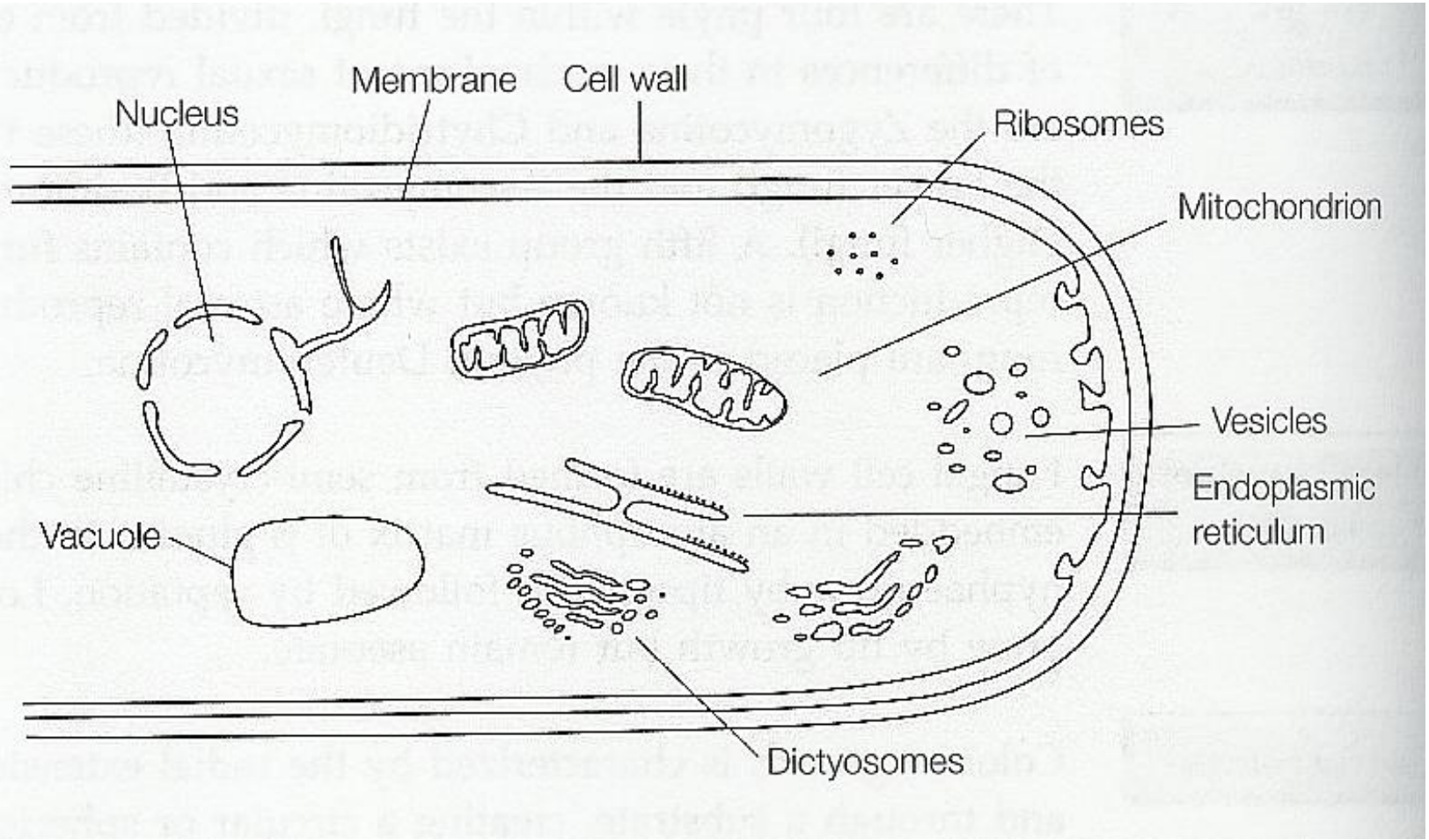
Kingdom: Fungi (Ophisthokonta)

- **Division**:

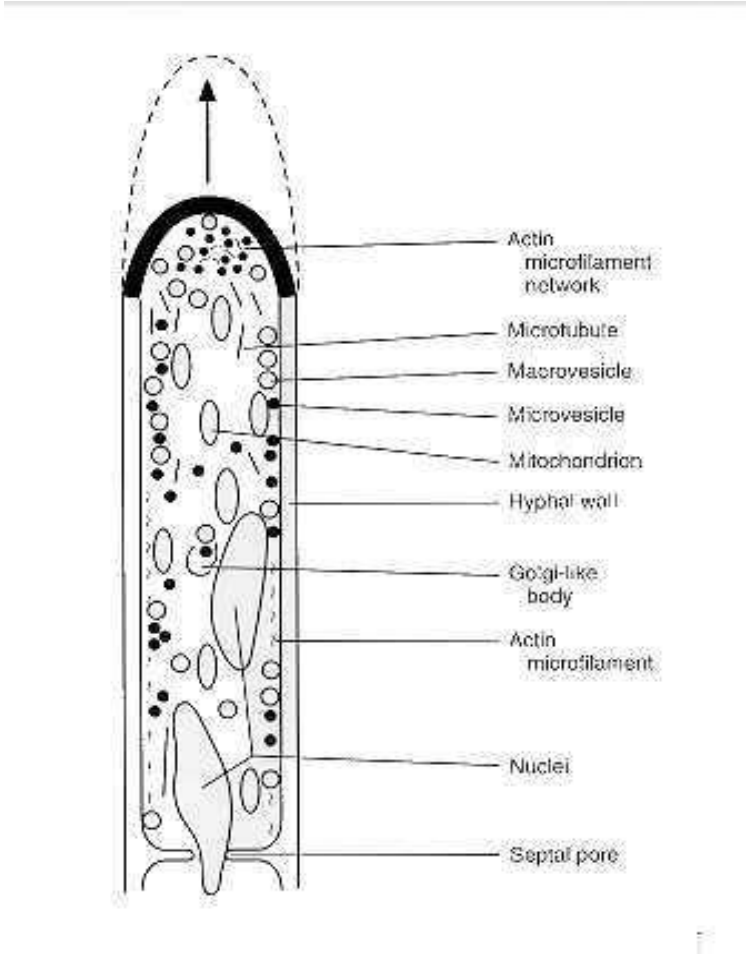
- Chytridiomycota
- Microsporidiomycota
- Zygomycota
- Ascomycota
- Basidiomycota

***Rhodotorula* sp.**

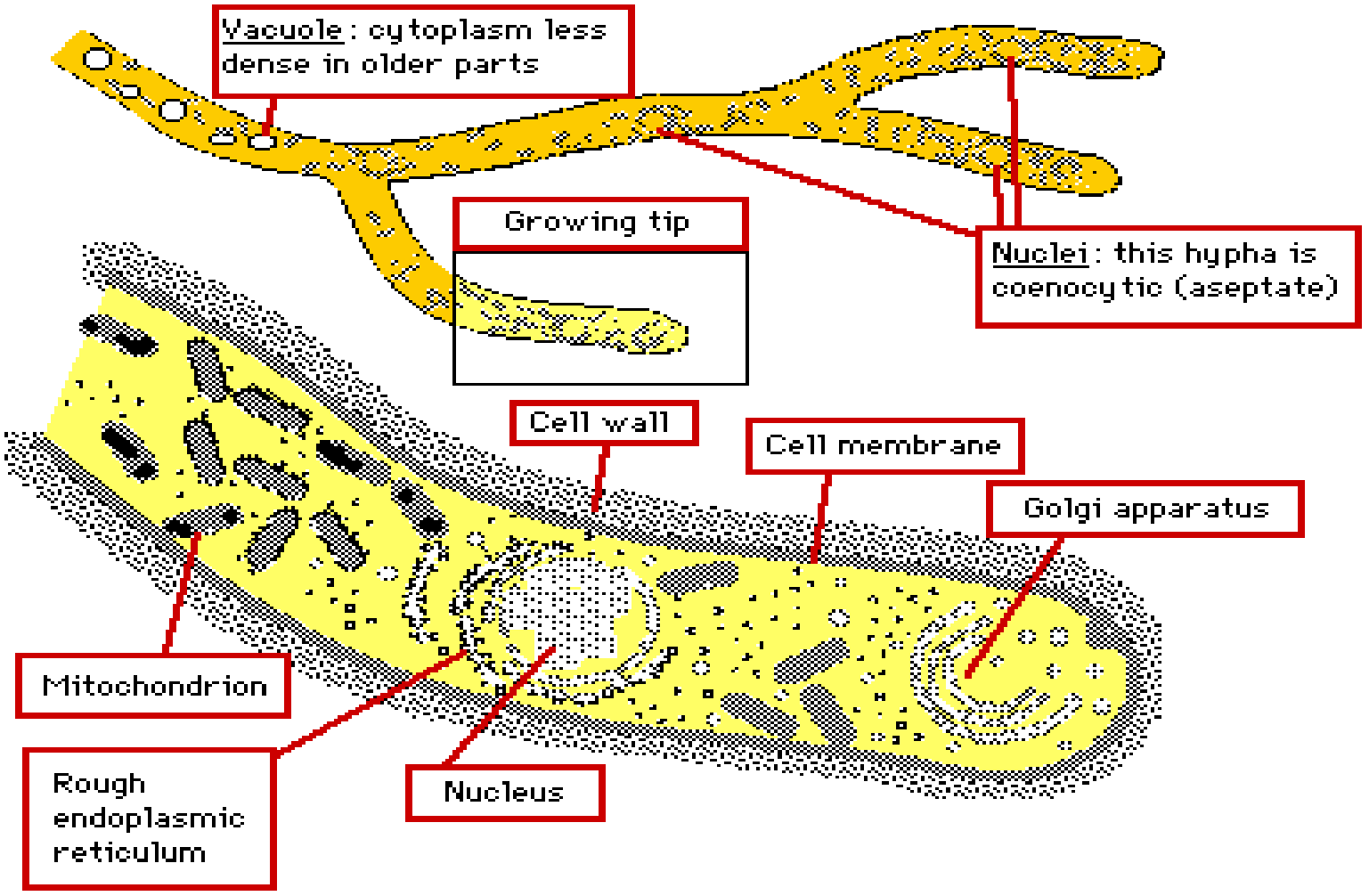
Structure of hyphae tip



Growth tip of hypha



Micromycetes – cell structure



Main organels

- Nucleus
- Mitochondria (mtDNA)
- Endoplasmatic reticulum
- Golgi apparat
- Vacuols

Cell wall – chemical composition

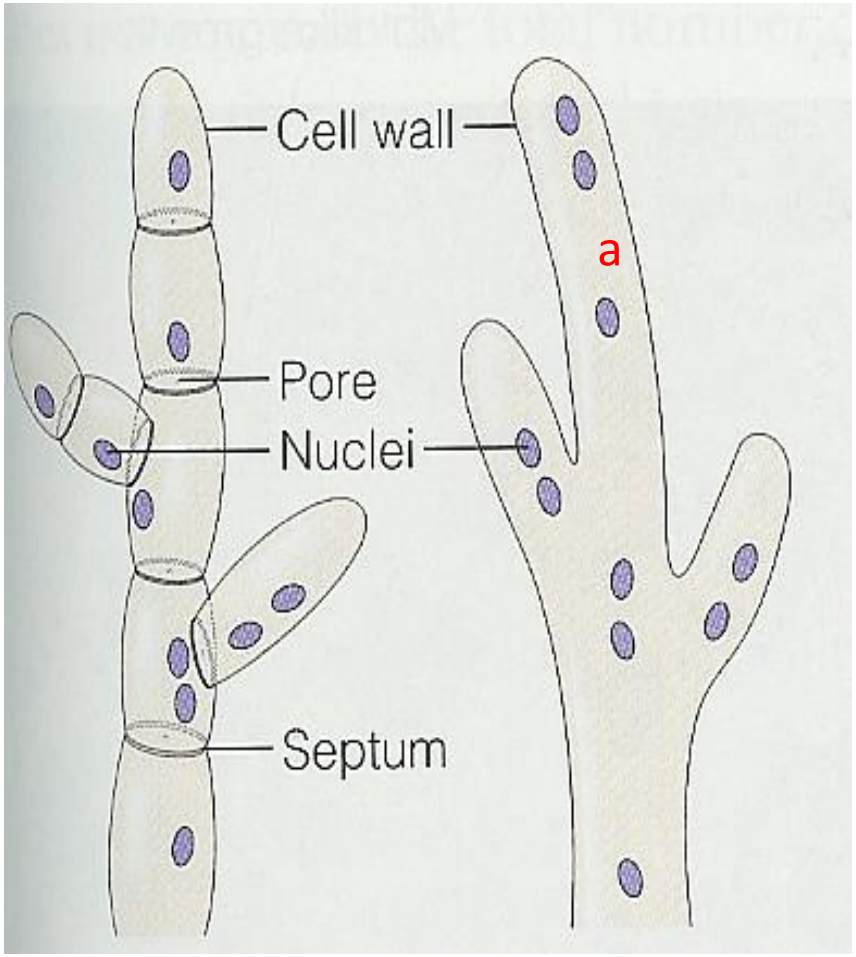
- **Polysaccharides**
  - **chitin**(N-acetylglucosamin)
  - **chitosan** (deacetylated chitin)
  - β-**glucans**, **mannans**
  - next polysaccharides formed from 6-deoxy-hexose (e.g. rhamnose -6-deoxy-L-mannosa, etc.)
  - **cellulose**
  - compounds resembling **lignin** (support the wall rigidity)
- **proteins**
- **fats**
- **waxes** (respond for minimal moistening)

Cytoplasmatic membrane

- Responsible for transport of nutrients and osmoregulation
- Location of some components of cell wall synthesis
- **Oxidative phosphorylation does not take place in membrane**
- Invaginations are not so common as in yeasts

Nucleus

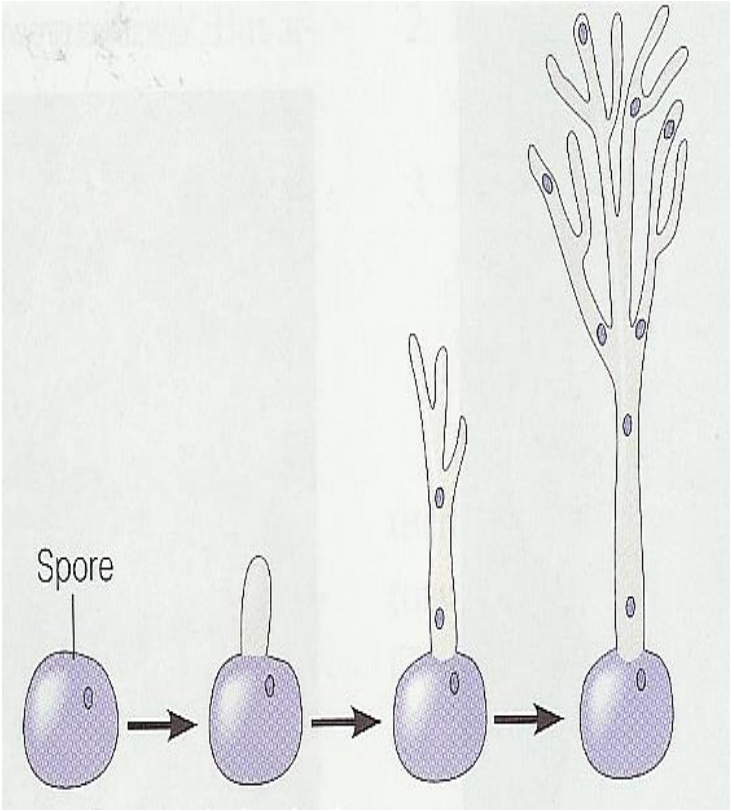
- Double membrane with huge pores
- Position in the centre of the cell
- Cells of some hyphae are **coenocytic** (multinuclear – a)
- Spores contain only one nucleus
- Number of chromosomes in haploid nucleus – 7 to 40



Basic cytoplasma

- **Composition and function does not differ from yeast cytoplasma**
- **Main storage compound are lipids**
- **Lipids are stored in vacuols, in cytoplasma**
- **In older cells the lipids may release from vacuols, may be considered for spores**

Mycelium

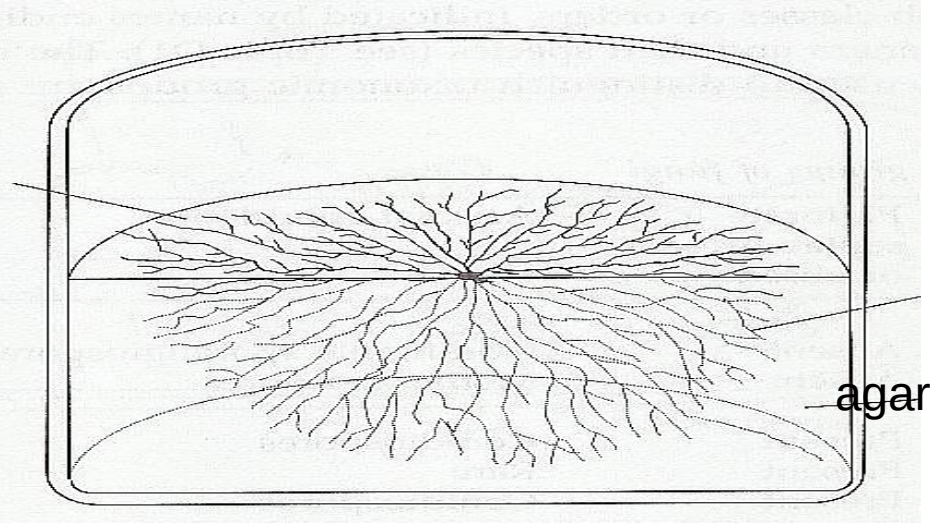


- Spore
- Hypha
- Progressive branching
- Mycelium – tangle of hyphae
- Scleromycelium – hard globular structure made from dense tangle of hyphae
- Stroma – leatherlike tangle of hyphae

Mycelium

substrate and aerial

Aerial mycelium



Substrate mycelium

Two types of hyphae

- Zygomycota and Chytridiomycota – vegetative mycelium **is not septated**

- Ascomycota and Basidiomycota – vegetative mycelium is septated, may be perforated (ascomycetes). Dikaryotic basidiomycetes are formed clamps.

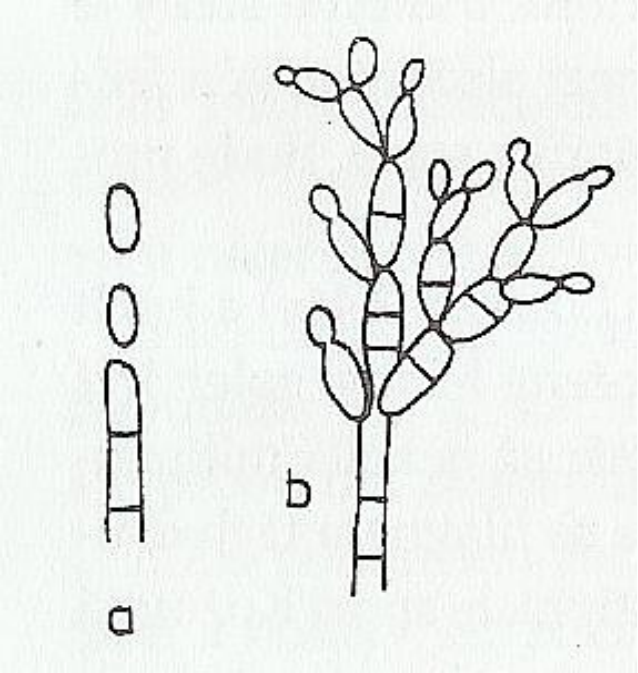
Reproduction of micromycetes

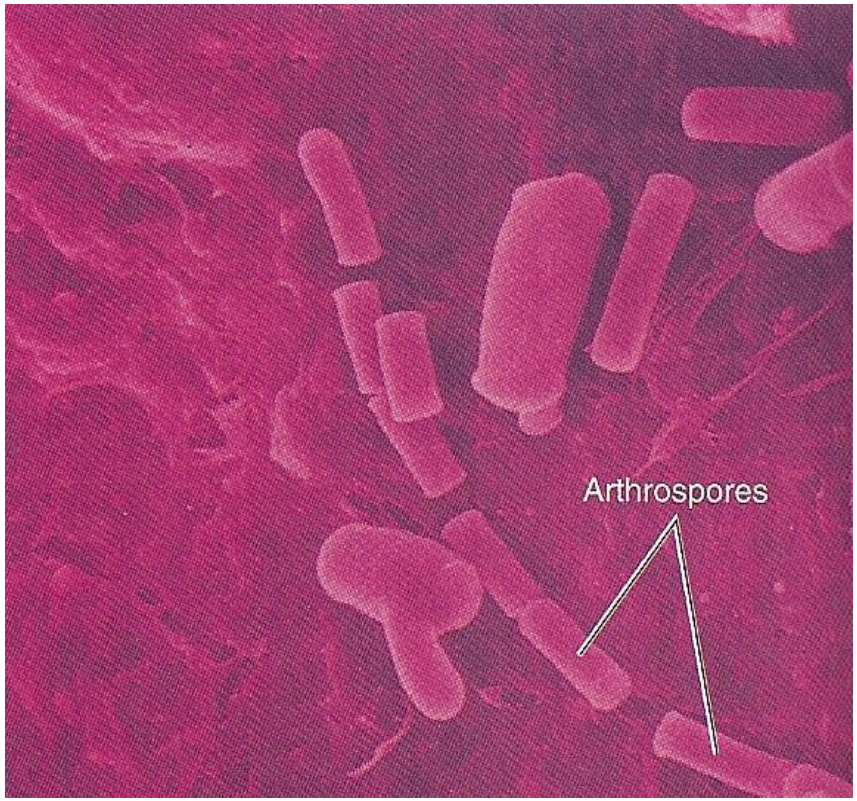
- **Vegetative** – more frequent, identification based on morphology of vegetative spores
- **Sexual** – less frequent, problems with names
- One organism – two names:
- One for vegetative reproduction
- **anamorpha**
- Second for sexual reproduction
- **teleomorpha**

Reproduction of micromycetes - **vegetative**

- Outgrowth of hyphae
- Vegetative spores are formed on
  - vegetative hyphae
  - fructification organs
    - -- exospores
    - -- endospores

Vegetative spores on
- vegetative hyphae

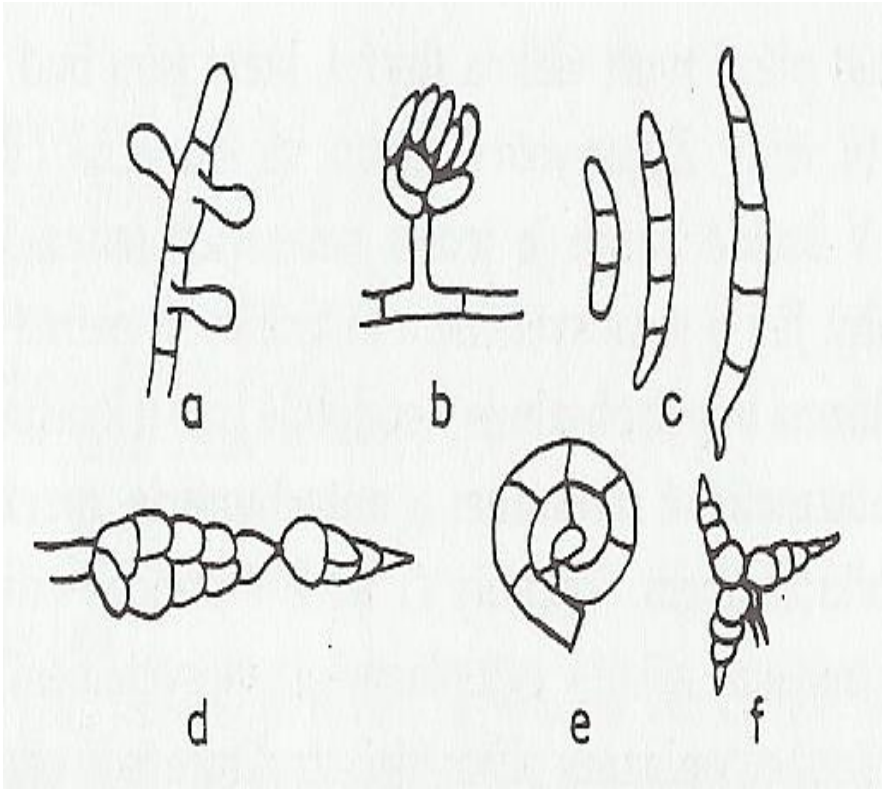




a – oidie (arthrospores) are formed by disintegration of hyphae

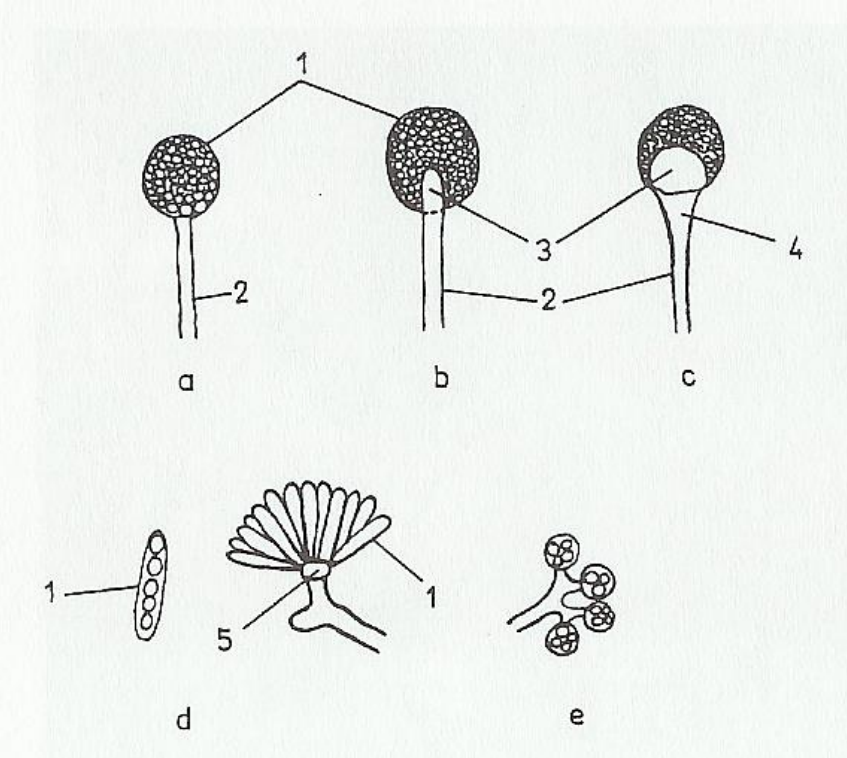
Shape and placement of exospores

Microconidia and macroconidia



- a – individual spores on septated mycelium (*Sporotrichum*)
- b – false bulb from chain of spores (*Cephalosporium*)
- c – halfmoon macroconidia (*Fusarium*)
- d – chain of septated macroconidia (*Alternaria*)
- e – spiral macroconidia (*Helicoma*)
- f – star shape macroconidia

Reproduction of micromycetes – vegetative – endospores in sporangium



1 - sporangium
2 - sporangiophore
3 - columela
4 - apophyse
5 – basal cell

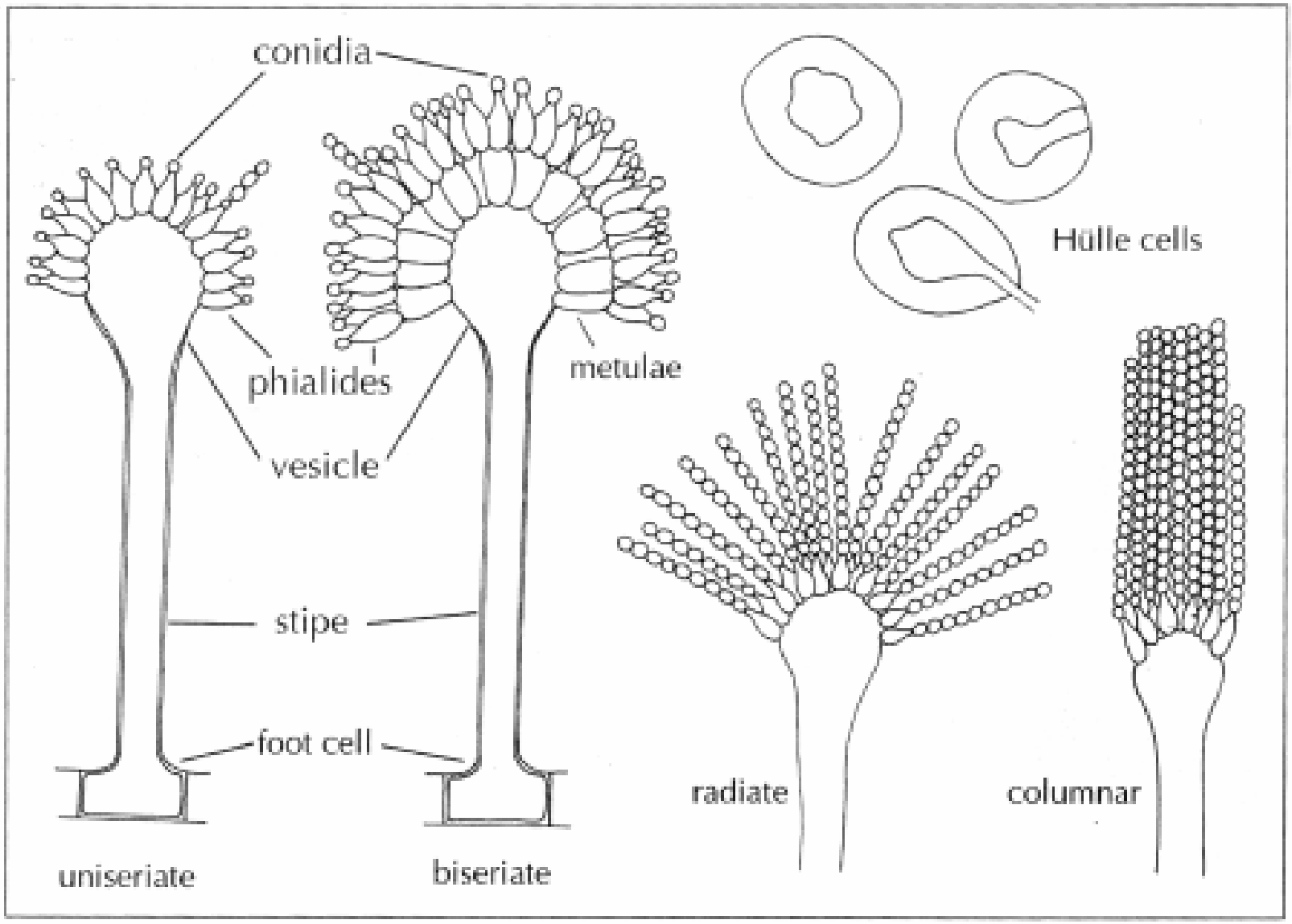
a –without columela (*Mortierella*)

b – with columela (*Mucor*)

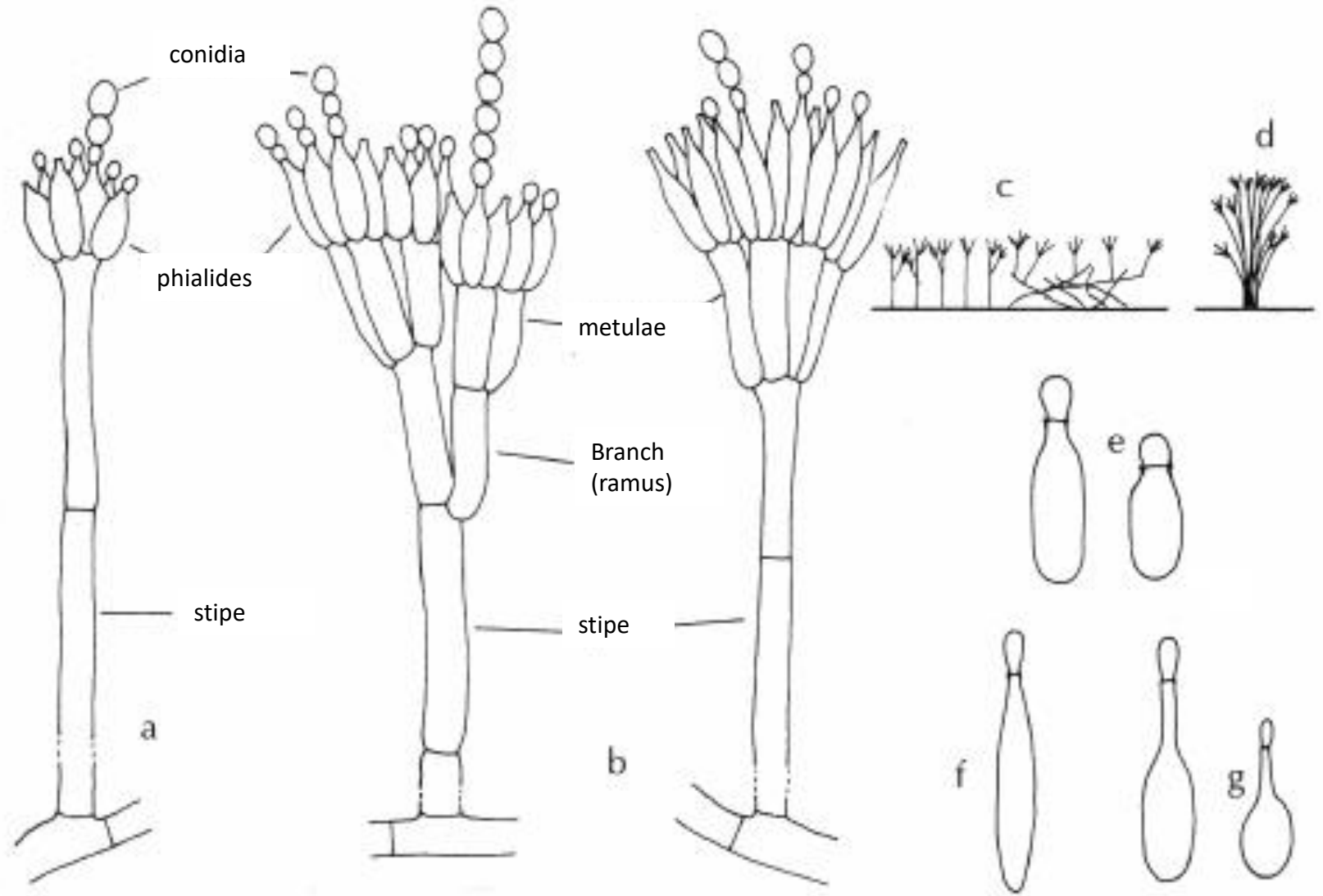
c – columela with apophyse (*Rhizopus*)

d – tubular sporangia (*Piptocephalis*)

e – sporangioles (*Thamnidium*)

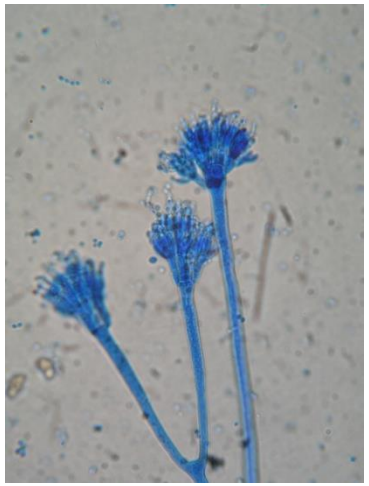


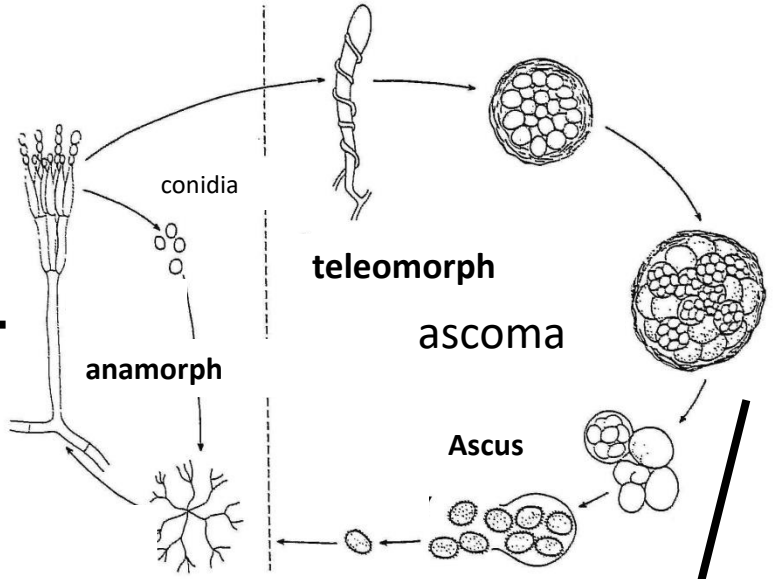
Morphological structures in *Aspergillus*.

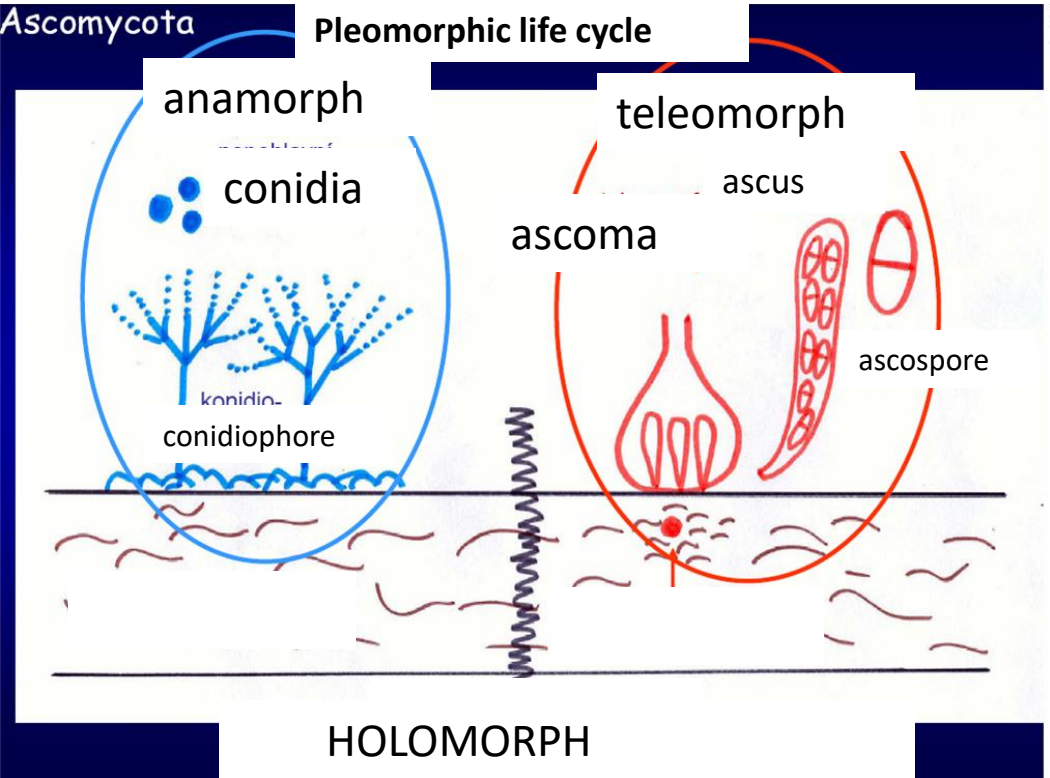


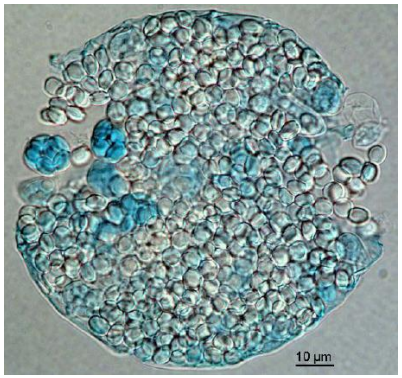
a-g. Morphological structures in *Penicillium*. a-b. Conidiophore structure; c. mononematous; d. synnematous; e. flask-shaped; f. lanceolate (= acerose); g. *Paecilomyces*-type.

Life cycle

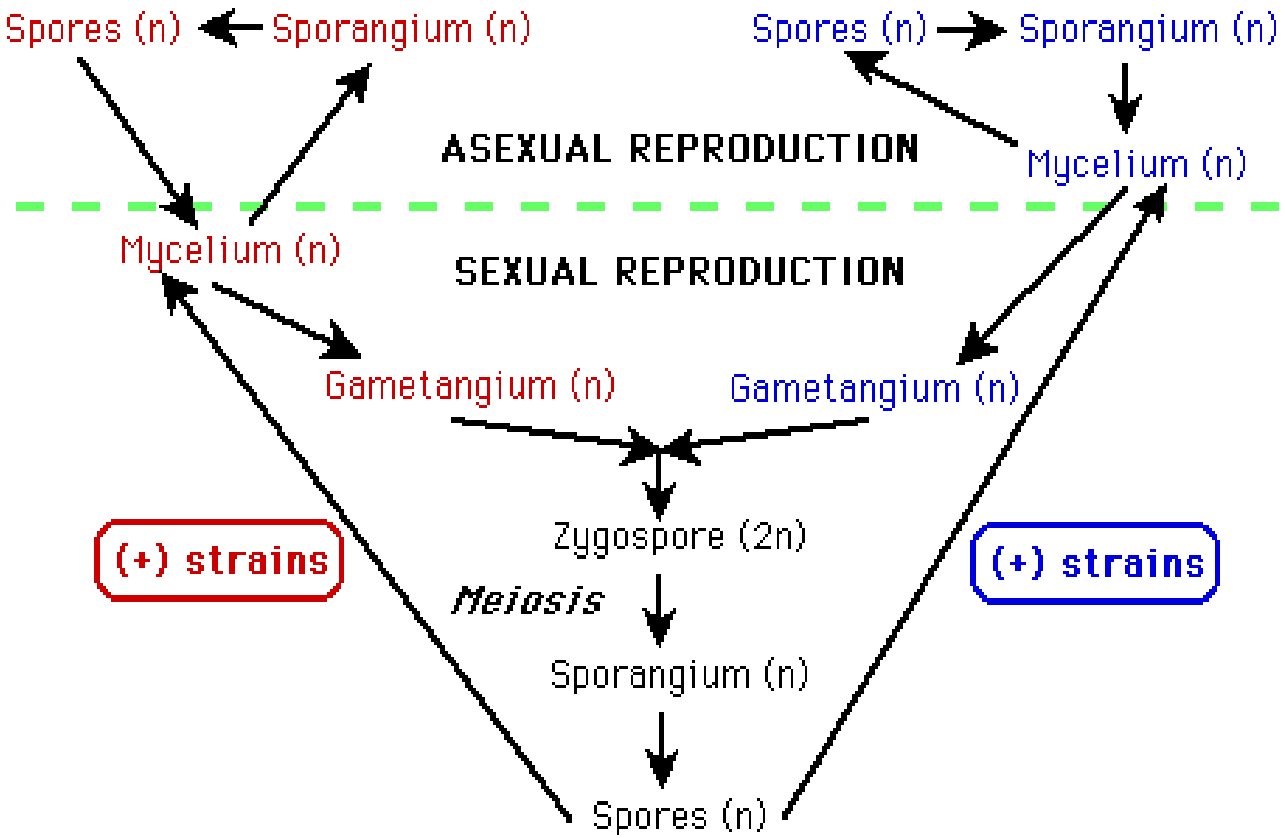




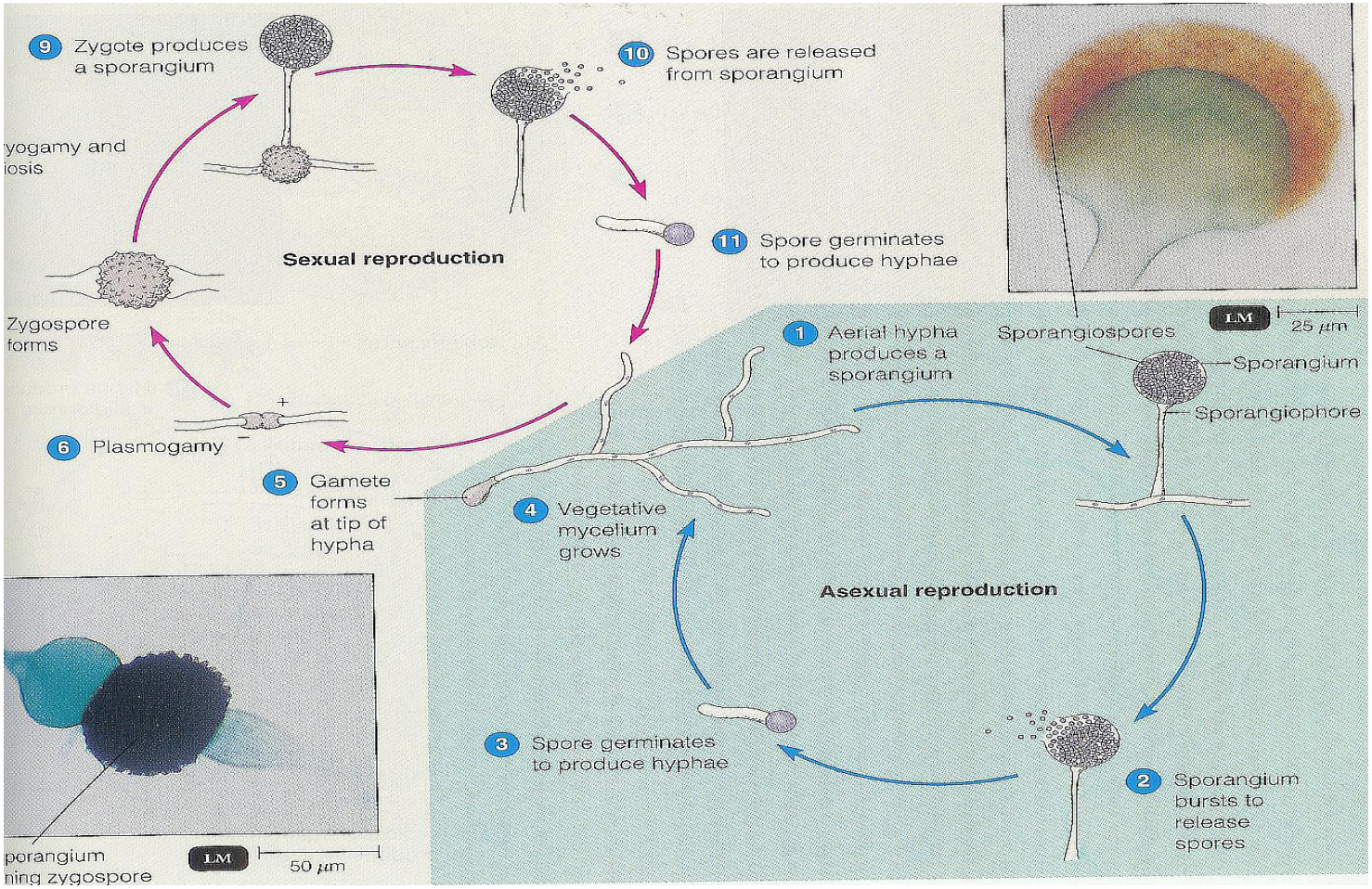




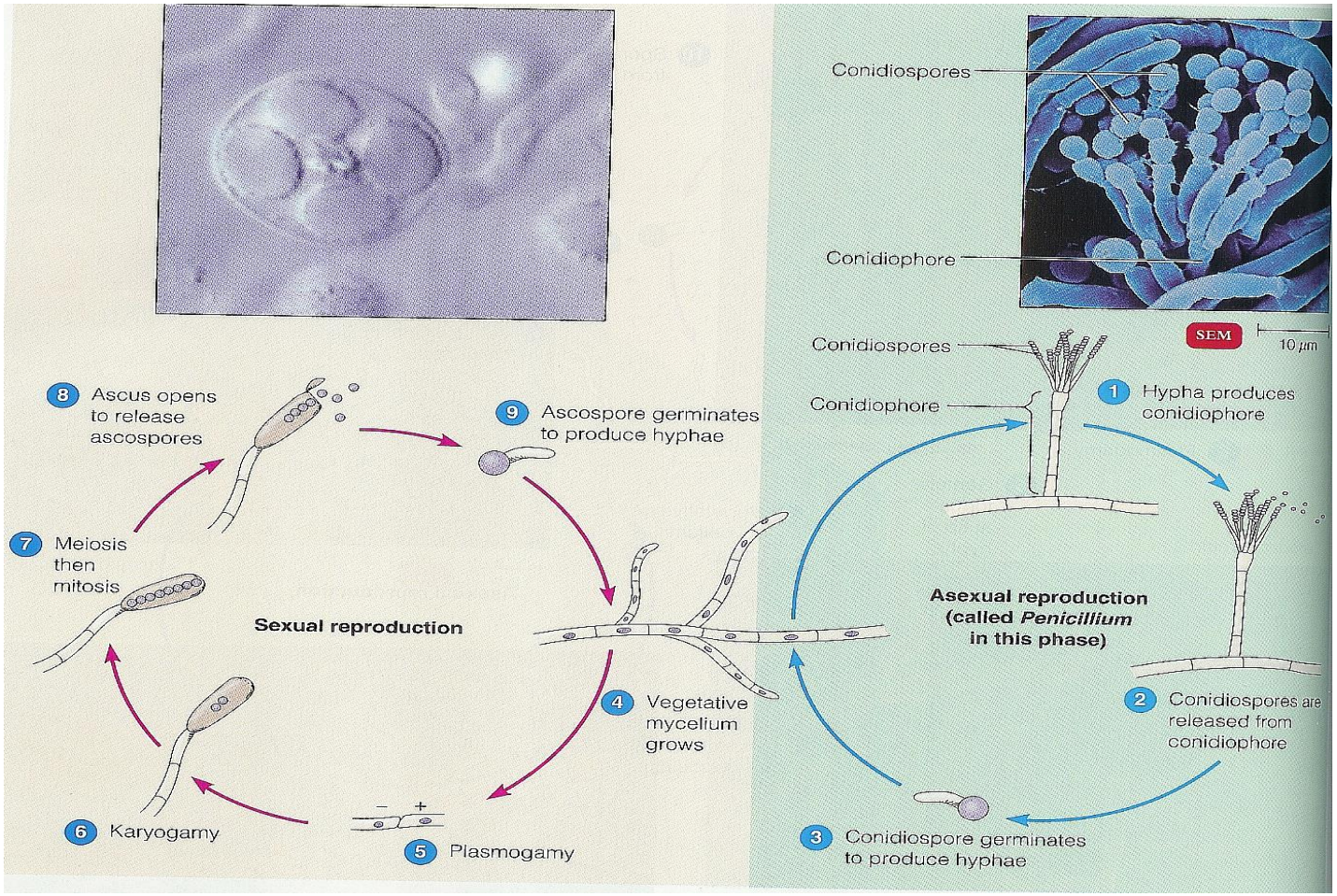
Life cycle



Life cycle of zygomycetes (*Rhizopus*)

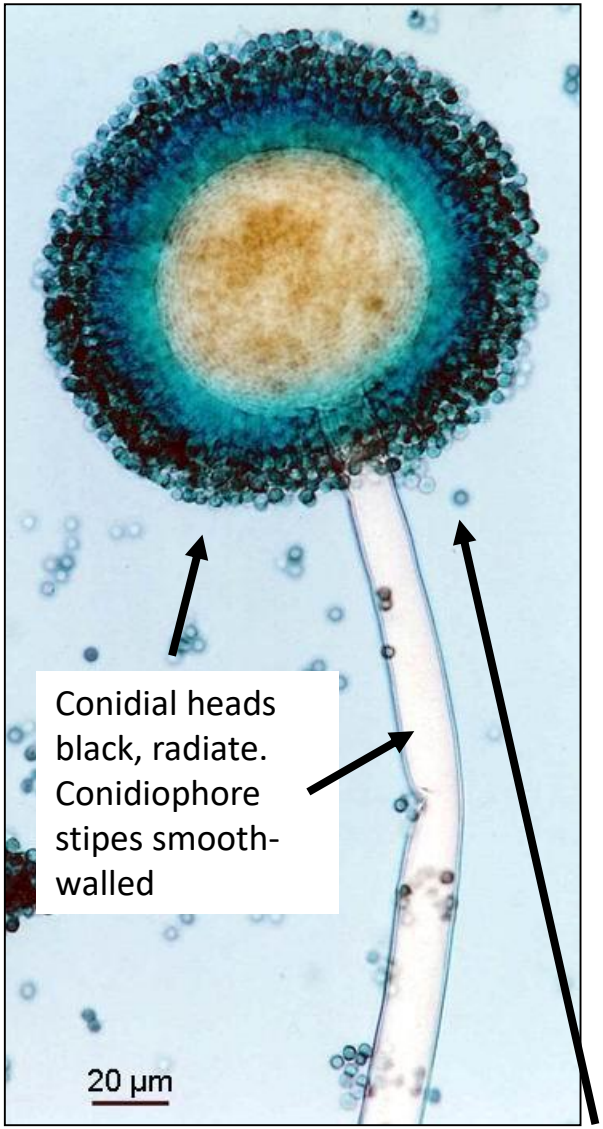


Life cycle of ascomycetes (*Eupenicillium*)



Aspergillus niger

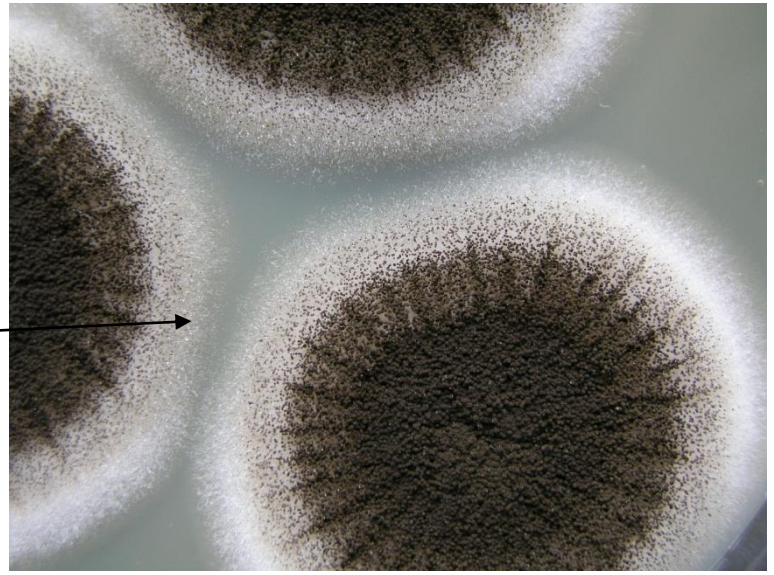
Black aspergilli (*Aspergillus* section *Nigri*)

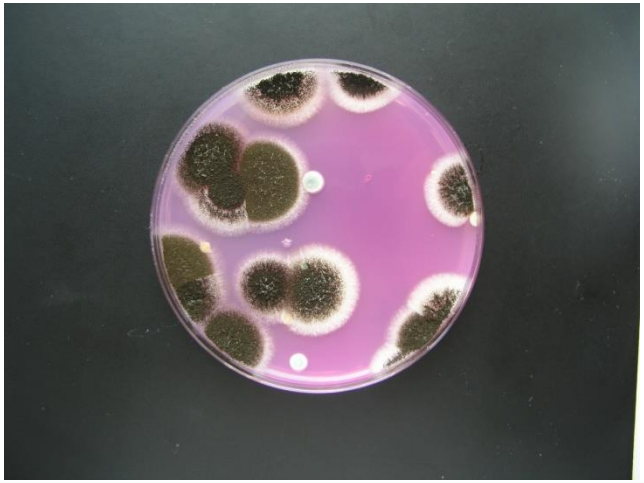


Conidial heads black, radiate. Conidiophore stipes smooth-walled

Conidia globose to subglobose

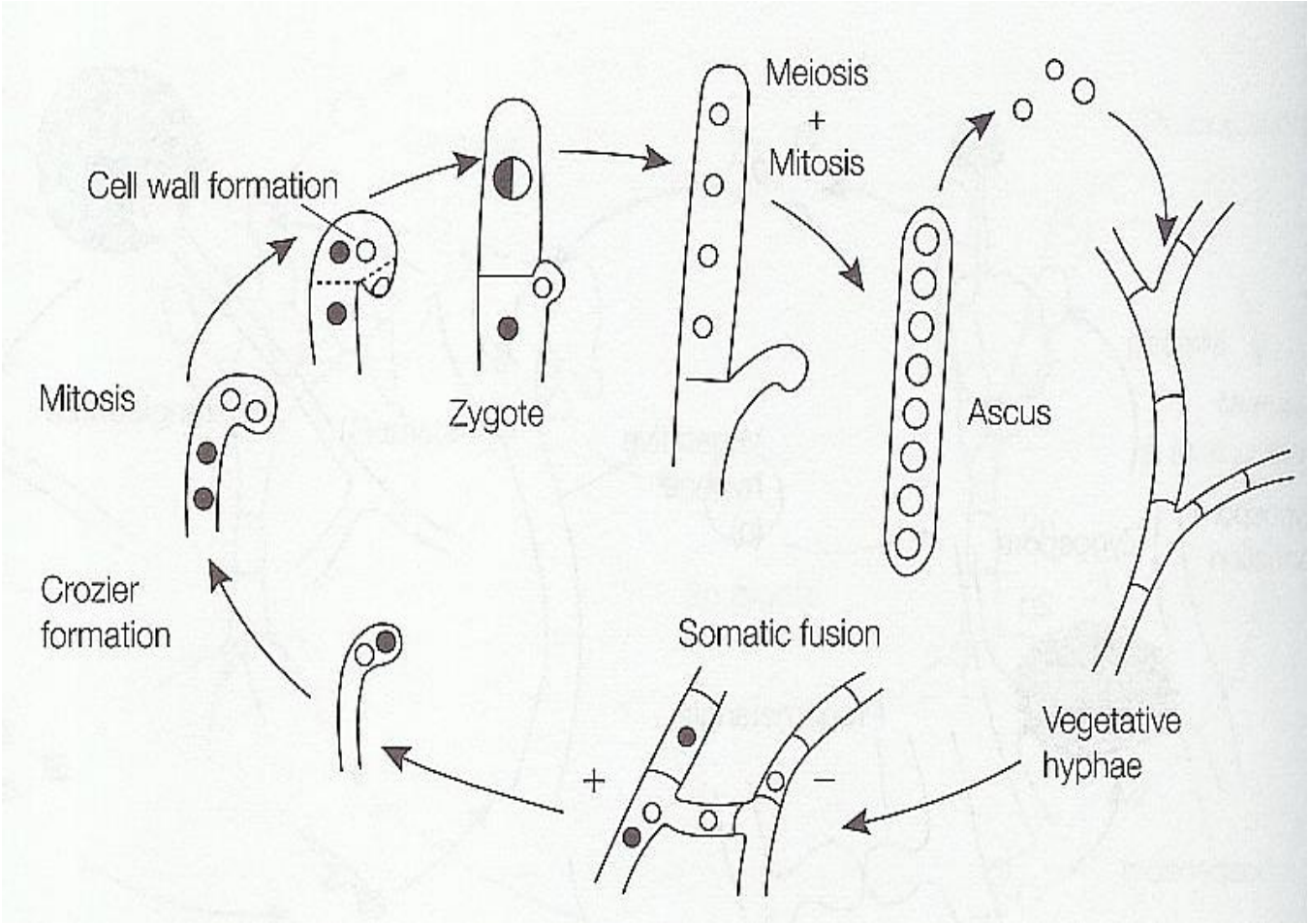
Colonies consisting of a compact white or yellow basal felt with a dense layer of dark brown to black conidiophores



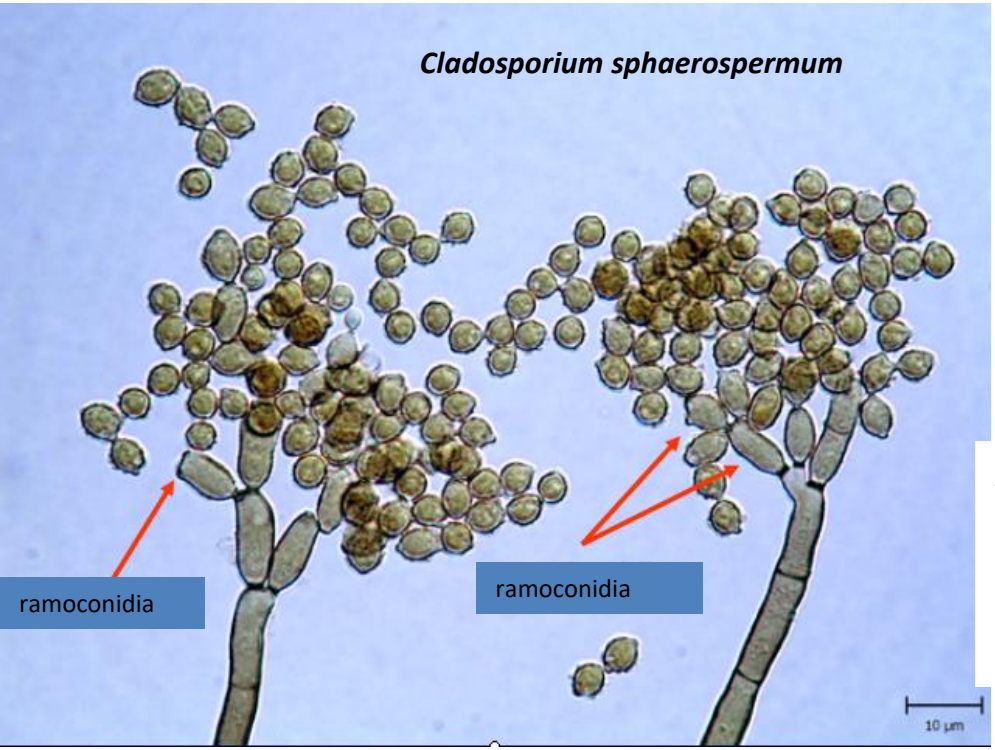


Important (toxic) metabolites: naphto-γ-pyrones, malformins, **ochratoxin A** (few isolates)

Life cycle of ascomycetes

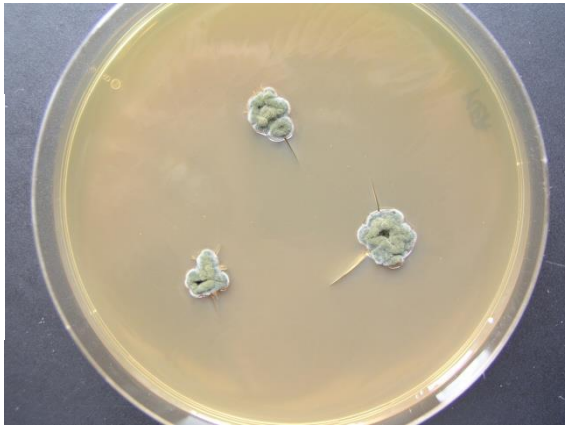


Cladosporium



Cladosporium herbarum
DBM 4073

Cladosporium sphaerospermum DBM 4282

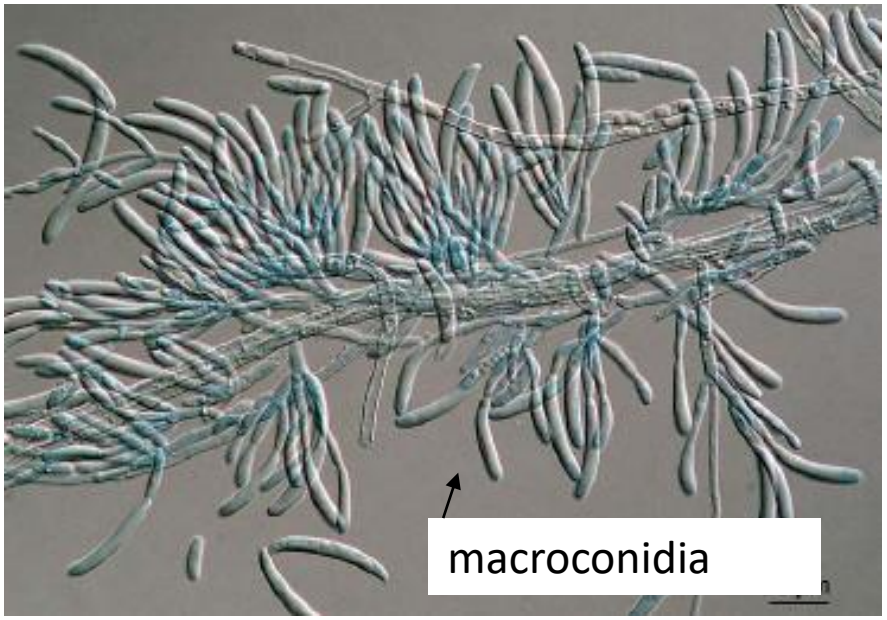


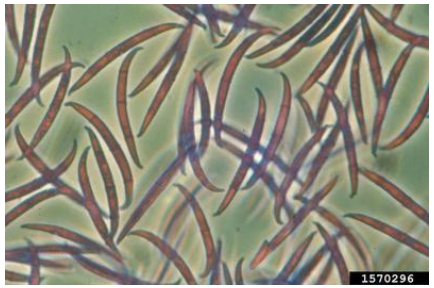
A very common species occurring on plant material and in soil.

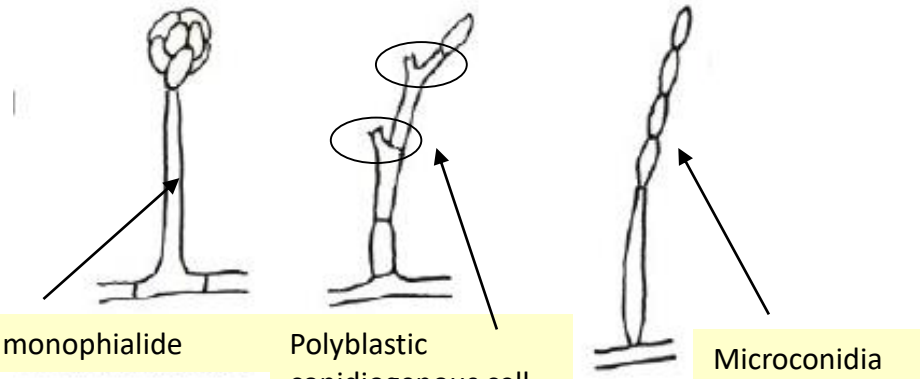
Reverse greenish-black

Cladosporium cladosporioides DBM 4145

Fusarium (tel. Giberella, Nectria)

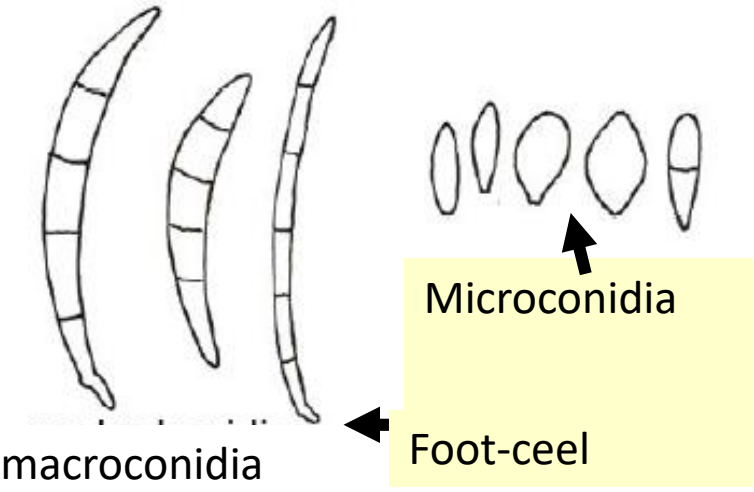




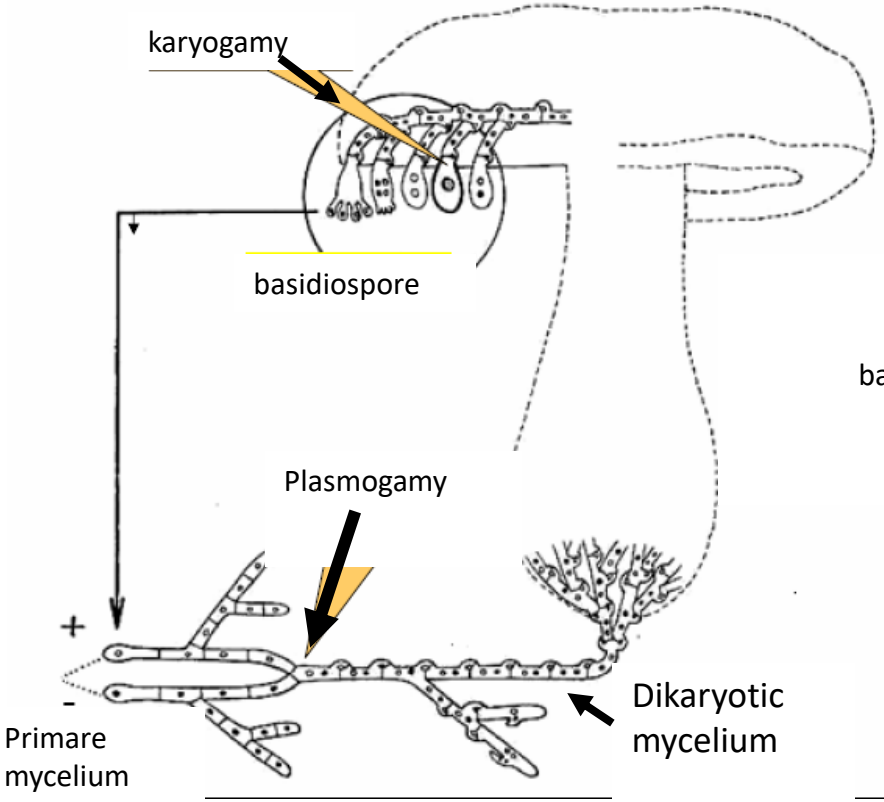


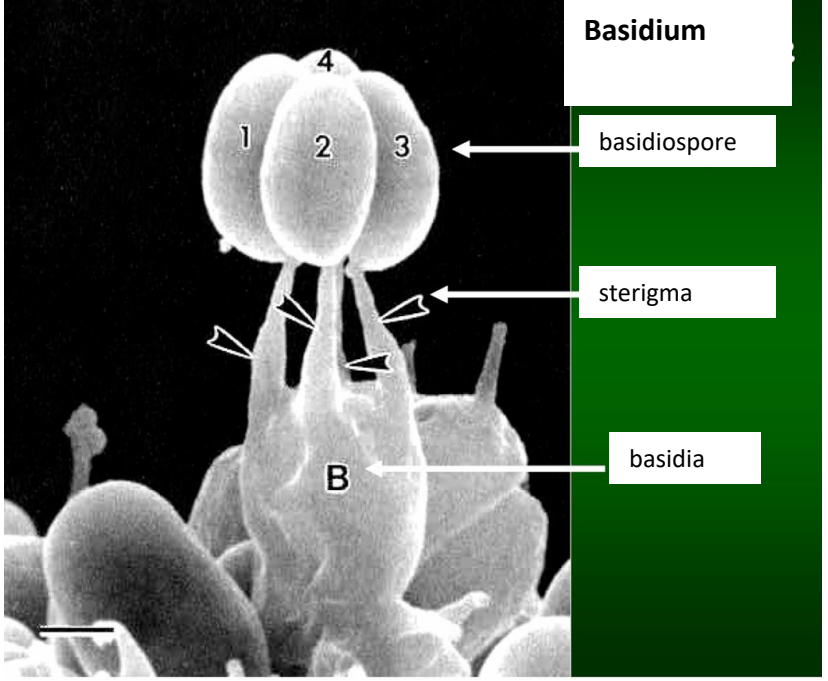
The most important mycotoxins

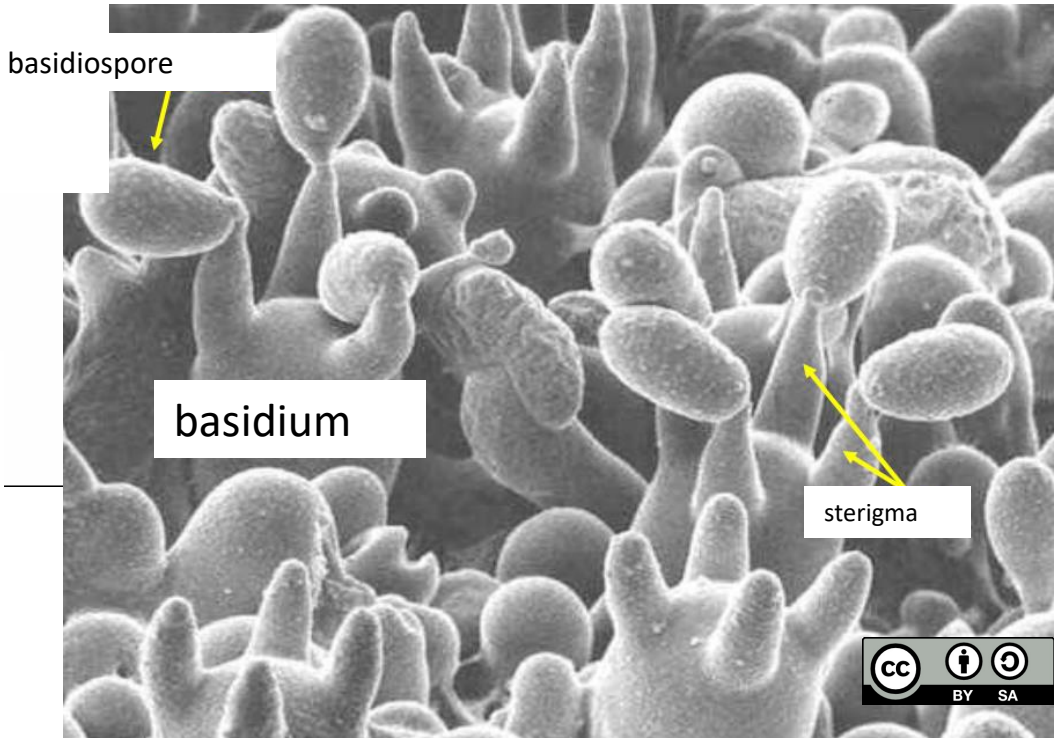
1.Aflatoxins B and G
2.Aflatoxin M1
3.Patulin
4.Ochratoxin A
5.Deoxynivalenol (DON)
6.Zearalenon
7.Fumonisins
8.T-2 toxin



Basidiomycetes







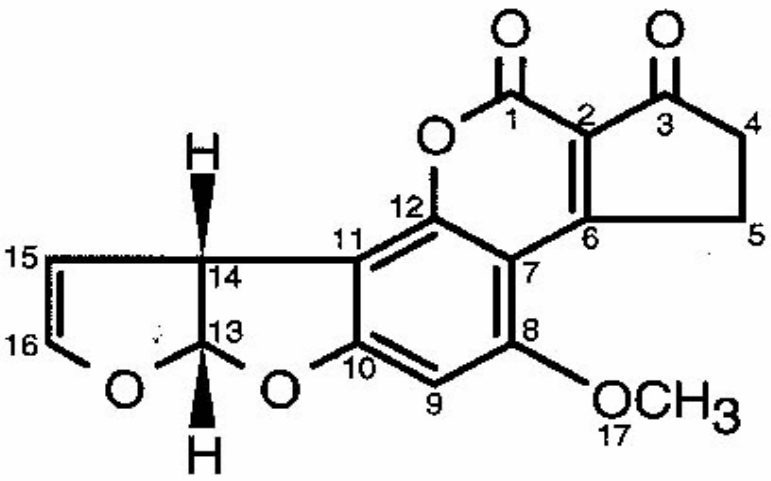
Mycotoxins

- Mycotoxins are toxic **secondary metabolites** of many species of microscopic filamentous fungi (molds), that can contaminate a broad spectrum of food and feed. Producers of these dangerous natural contaminants cause various toxic syndromes called **mycotoxicoses .**
- Action of mycotoxins depends on **type of toxin**, **duration** of its effect, dose and age, its nutrition and actual state of health.
- Target organs of mycotoxins are in the first place the cells of **liver**, **kidney, lungs**, and **nerves**, **endocrine glands** and **cells of immune system**. They can cause an acute toxic reaction and some can have mutagenic , terratogenic , carcinogenic and estrogenic effect.

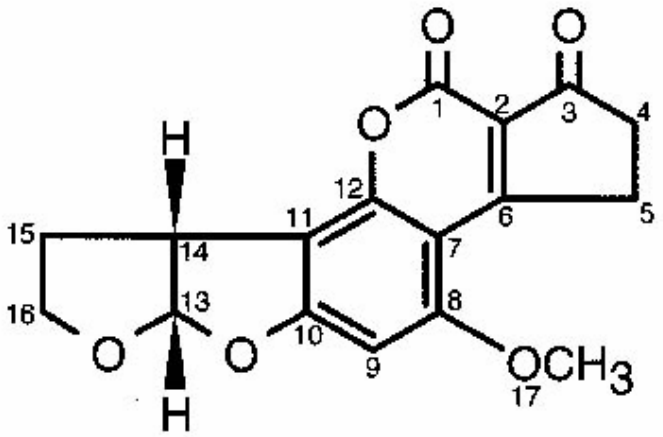
Surveillance of mycotoxins

- Numerous international authorities are trying to reach worldwide standardization of **regulatory limits for mycotoxins**. Recently, more than 100 countries set up regulatory limits for mycotoxins in feed industry.
- Today **13 mycotoxins** or groups of mycotoxins are recorded. Evaluation process necessary for regulation of mycotoxins includes great complexes of laboratory tests that use extraction, purification and separation techniques.
- Majority of official control methods is based on high pressure liquid chromatography (**HPLC**).
- **Many standards for mycotoxin analysis are guaranteed by European Committee for Standardization (CEN).**

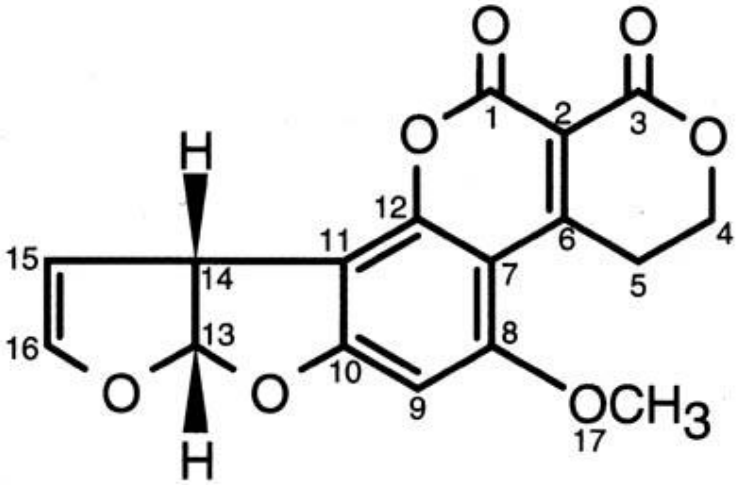
Structure of aflatoxins



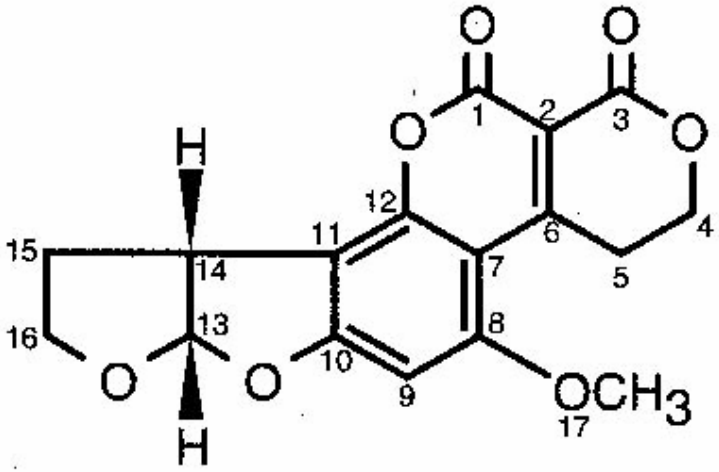
Aflatoxin B1



Aflatoxin B2



Aflatoxin G1



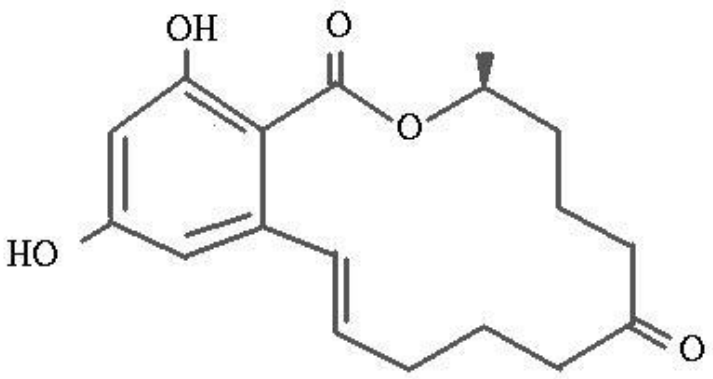
Aflatoxin G2

Fusarium mycotoxines

- Fusaria belong to noticeable originators of mycoses in humans and produce mycotoxins threatening the health. Among the most dangerous mycotoxins are **trichotecens, zearalenon, fumonizin.**
- **Zearalenon** ($C_{18}H_{22}O_5$) is a white or pale yellow crystallic compound without odour. Temperature of thawing about 161 - 164 °C. Insoluble in water, tetrachlormethane (CCl_4) and alcohols .
- Zearalenon is relatively lipophilic compound. Its content is reduced during technology of processing of cereals.

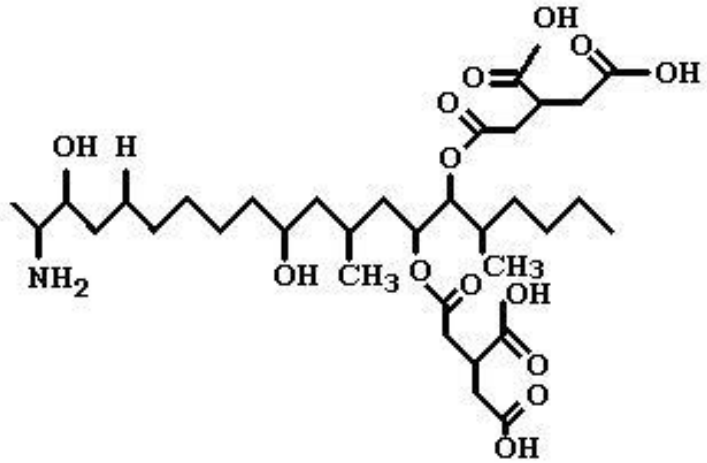
Zearalenon

- Isolated in 1966
- White or pale yellow crystallic compound
- Temperature of thawing 161 – 164 °C
- Common in flour and cereal products.
- Estrogenic effect



Fumonisins

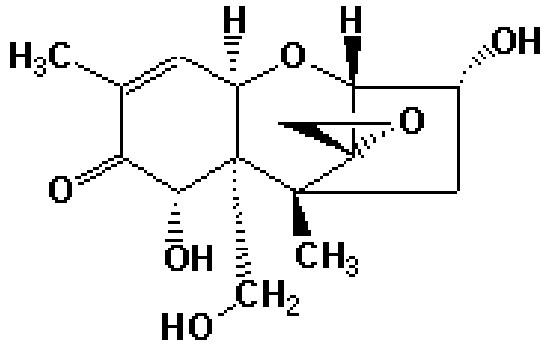
- Six structures: FB_1, FB_2, FB_3, FB_4, FA_1 a FA_2
- Derived from unsaturated fatty acids
- Disorders of sphingolipid metabolism
- Liver and kidney tumors in rodents



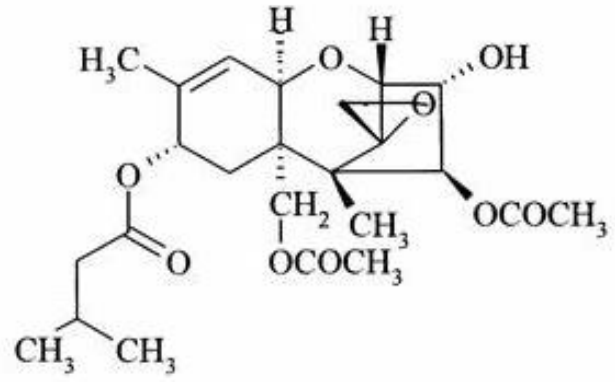
Fumonisin B_2

Trichothecenes

- 80 species (T-2 toxin, deoxynivalenol-DON…)
- Chemically variable
- Tricyclic sesquiterpenes
- Signs of intoxication: inflammation of gastrointestinal tract, nausea, diarrhea, inhibited proteosynthesis, impaired immune system



deoxynivalenol



T-2 toxin

Ergot alkaloids

An **ergot kernel**, called a ***sclerotium***, develops when a spore of fungal species of the genus *Claviceps* infects a floret of flowering grass or cereal. The infection process mimics a pollen grain growing into an ovary during fertilization.

Ergot alkaloids are produced as mixture of toxic alkaloids in sclerotium of some *Claviceps* species, which are common pathogens of grass.

After consumption of ergot sclerotium from infected cereals, usually as a bread baked from contaminated flour, ergotism takes place, human disease historically known as the **Saint Antony´s Fire.**

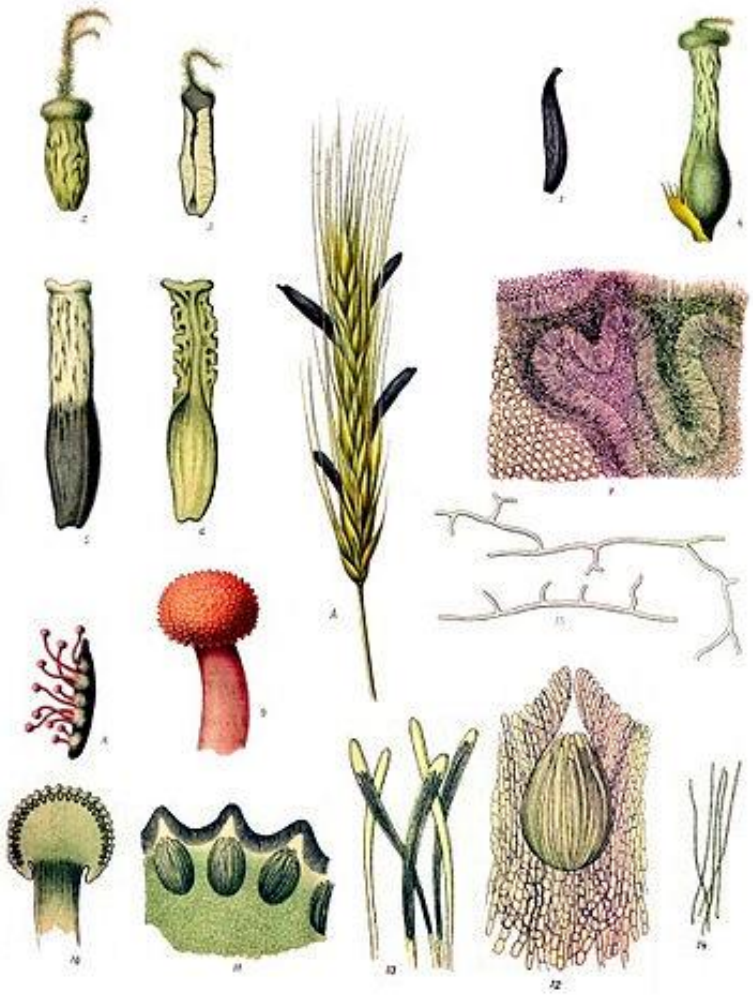
Two forms of ergotism

1) **gangrenous,** affecting the blood circulation in limbs
2) **spasmic**, affecting central nerve system.

Modern methods of cereal cleaning reduced significantly ergotism as human disesease, but it is still a veterinary problem.

Ergot alkaloids have pharmaceutical application.

Claviceps purpurea



Ergot alkaloids

- Ergot alkaloids are classified as:
- derivatives of 6,8-dimethylergoline and
- lysergic acid derivatives
- The neurotropic activities of the ergot alkaloids may also cause hallucinations and attendant irrational behaviour, convulsions, and even death.

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Characteristics of *Listeria monocytogenes* and coagulase positive staphylococci

Lecturer:

Jarmila Pazlarová

Department of Biochemistry and Microbiology

Listeria monocytogenes

- *Listeria monocytogenes* is a Gram-positive bacterium , in the division Firmicutes.
- Its ability to grow at temperatures **as low as 0 °C** permits multiplication at typical refrigeration temperatures, greatly increasing its ability to evade control in human foodstuffs.
- Motile via flagella at 30 °C and below, but usually not at 37 °C, *L. monocytogenes* can instead move within eukaryotic cells by explosive polymerization of actin filaments (known as comet tails or actin rockets).

Listeria monocytogenes

- ***Listeria monocytogenes*** is the species of **opportunistic pathogenic** anaerobic bacterium that causes the infection listeriosis.
- It is a facultative pathogen in the presence or absence of oxygen. It can grow and reproduce inside the host's cells and is one of the most virulent foodborne pathogens, with 20 to 30% of food borne listeriosis infections in high-risk individuals may be fatal.
- *L. monocytogenes* can act as a saprophyte or a pathogen, depending on its environment.
- The official method of **detection** is **EN/ISO 11290-1** (in food with zero tolerance) and **EN/ISO 11290-2** (for RTE foods with limit 100 cfu/g.)

Food safety criteria I.

- According to the **COMMISSION REGULATION** (EC) **No 1441/2007** of 5 December 2007
- Ready-to-eat foods intended for infants and ready-to-eat-foods for special medical purposes
- ***Absence in 25 g*** EN/ISO 11290-1
- Ready-to-eat foods able to support the growth of *L.monocytogenes*, other than those intended for infants and for special medical purposes
- **100 CFU/g** EN/ISO 11290-2

  products introduced to the market during shelf-life

Food safety criteria II.

- Ready-to-eat foods unable to support the growth of *L.monocytogenes,* other than those intended for infants and for special medical purposes
- **100 CFU/g** EN/ISO 11290-2

- It means there are two limits : zero and 100 CFU per gram

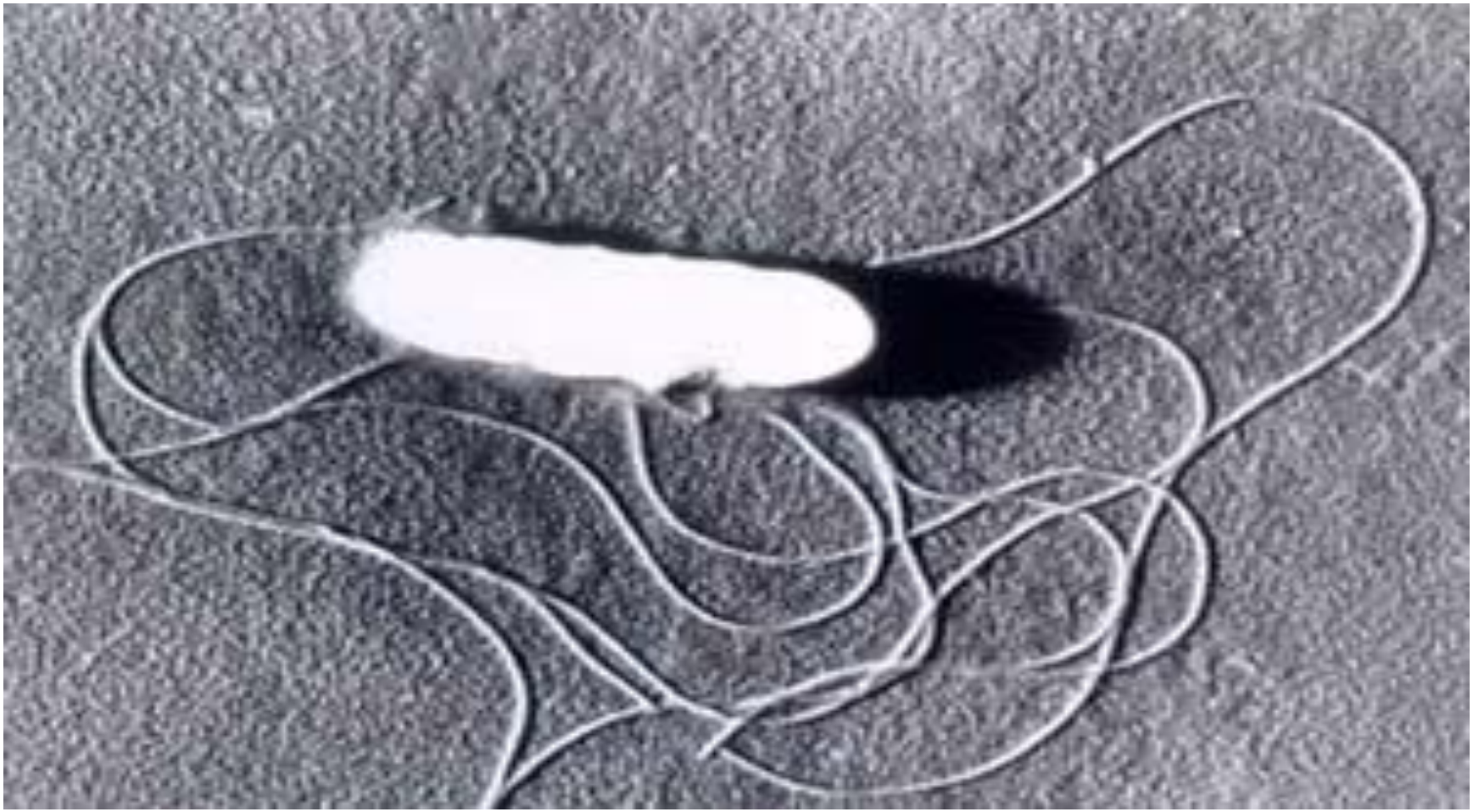
Routes of infection

- *Listeria monocytogenes* has been associated with such foods as raw milk , pasteurized fluid milk, cheese (particularly soft-ripened varieties), ice-cream, raw vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats (of all types), and raw and smoked fish (smoked salmon). Typical **ubiquitous** bacteria.
- Most bacteria can survive near freezing temperatures, but cannot absorb nutrients, grow or replicate.
- *L. monocytogenes* ability to grow at temperatures as **low as 0 °C** permits exponential multiplication in refrigerated foods.

Next sources of infection

- Due to its frequent pathogenicity, causing meningitis in newborns (acquired transvaginally), pregnant mothers are often advised not to eat soft cheeses such as Brie, Camembert, feta, and queso blanco fresco, which may be contaminated with and permit growth of *Listeria monocytogenes*.
- It is the **third-most-common cause** of meningitis in **newborns.** *Listeria monocytogenes* can infect the brain, spinal cord membranes and/or the bloodstream of the host through the ingestion of contaminated food such as unpasteurized dairy or raw foods.

Listeria monocytogenes Scanning EM showing Flagella



whole genome sequencing

- With **whole genome sequencing (WGS)**, *Listeria* outbreaks can be detected when as few as two people have gotten sick. Determining that the same strain of *Listeria* is making people sick is an indication that these illnesses may have come from the same source – for example, the same contaminated food or the same contaminated food processing facility.
- Thus, whole genome sequencing can help disease detectives solve outbreaks.
- The only disadvantage of such detection is the high cost of analysis.

Occurrence of listeriosis

- Listeriosis is **not very frequent** in Europe. During the years 2007-2011 there were documented in EU 1500-1600 cases a year, that is one case per 300 000 inhabitants. Those who suffer most frequently from listeriosis are **elderly people** (over 65 years old) and **newborn babies**.
- Incubation time is about few days to several weeks and depends on infection dose, virulence of bacteria and health condition of the patient.
- **Mortality** in case of listeriosis is generally **very high** and reaches **20-30 %.**

Endangered people

- Healthy adults are generally asymptomatic or develop only mild symptoms with simple gastroenteritis, however, infection in high-risk individuals, such as pregnant women, newborn infants and immunocompromised people may cause meningitis, encephalitis, septicemia or abortions with the mortality rate as high as 30 %.
- In pregnant women, listeriosis may lead to spontaneous late-term abortions, stillbirth or fetal death

Survival in biofilm I

- **Formation of biofilm** by *L. monocytogenes* results from a continuous process from **attachment** to a surface to the development of a bacterial community structure and detachment.
- The initial step for biofilm formation is the **bacterial adhesion** to the surface, which could occur within only 3 to 5 seconds for some *L. monocytogenes* strains.
- This reversible adhesion becomes irreversible through stronger bonds leading to the initiation of biofilm formation.

Survival in biofilm II

- For *L.monocytogens* is typical survival in **biofilm**. The growth in biofilm is mainly characterized by aggregate formation and **extrapolymer substances** (EPS) secretion.
- Biofilm maturation is reached when these three dimensional structures are crossed by water channels or pores which ensure nutrients exchange and removal of bacterial waste products.
- The bacteria involved in the biofilm formation undergo transitions from **planktonic (loss of flagella)** to **sessile** forms and the net accumulation of biomass in a mature biofilm depends on cell growth and cell detachment.

Survival in biofilm III

- The cell attachment to the surface is influenced by physico-chemical properties of the material including charge, liquid velocity (static/dynamic flow biofilms), rugosity, hydrophobicity (nonpolar surface such as teflon and other plastics) and hydrophilicity (glass or metals).
- **All types of processing environment may be covered by biofilm**.
- *L. monocytogenes* can act as a **saprophyte** or a **pathogen**, depending on its environment. When this bacterium is present within a host organism**, quorum sensing** causes the up-regulation of several virulence genes. Depending on the location of the bacterium within the host organism, different activators up-regulate the virulence genes.

Staphylococcus aureus and staphylococcal enterotoxins

- ***Staphylococcus*** is the main genus of the ***Staphylococcaceae*** family in the order of **Bacillales,** the class of ***Bacilli*** and the phylum of **Firmicutes.**
- A non spore-forming, non motile, facultative aeroanaerobic **Gram-positive coccus**, 0.5 to 1 micrometre in diameter, catalase- and **coagulase-positive**.
- *S. aureus* is a golden yellow colony-forming *Staphylococcus*. It produces **numerous toxins** including **staphylococcal enterotoxins** (SEs) responsible for staphylococcal food-poisoning outbreaks (**SFPO**).
- To date, **21 different serotypes** (SEA to SEE, SEG to SEV) have been described, six of them have been involved in SFPO: SEA (the most commonly detected in SFPO) to SEE, SEH.

Staphylococcus aureus

- **Directive No.1441/2007**, commands the absence of ***Staphylococcal* enterotoxins**, or the absence of coagulase –positive staphylococci in some food categories: cheeses made from raw milk, cheeses from milk that has undergone low heat treatment, fresh cheeses made from milk or whey that has undergone pasteurisation, milk powder and whey powder.
- There are **3 coagulase-positive *Staphylococcus* (CPS) species:** *Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus intermedius.*

Occurence of *S. aureus*

- *Staphylococcus aureus* is known as the third most important cause of disease in the world amongst the reported food-borne illnesses. A few decades ago, *S. aureus* was responsible for 25% of all food-borne illnesses in the US, however from 1988 to 1992 in the US, *S. aureus* was only responsible for 1.8%, 2.8%, 2.4%, 1.7% and 1.5% of the outbreaks of food-borne illnesses, respectively.
- During the same 5-year period, *S. aureus* caused 5.1% of the food poisoning outbreaks reported in Europe, and in Italy it caused 4 of the 233 outbreaks reported.
- This Gram-positive bacterium has no particular nutritional and environmental requirement for its growth and it can grow at an $\boldsymbol{a_w}$ **of 0.86**, **pH above 4.8** and its minimum growth temperature is **8/9 °C** .

Coagulase-positive staphylococci

- Cheeses made from raw milk - **10^4 cfu/g**
- If values **>10^5 cfu/g** are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
- Cheeses made from milk that has undergone a lower heat treatment than pasteurisation and ripened cheeses made from milk or whey that has undergone pasteurisation or stronger heat treatment **100 cfu/g**
- Unripened soft cheeses made from pasteurised milk- **10 cfu/g**
- Sampling plan - 5, c - 2

Multiplex-PCR Method for CoPS

- Coagulase-positive staphylococci (CoPS) other than *Staphylococcus aureus* have frequently been misidentified as being *S. aureus* strains, as they have several phenotypic traits in common. There has been no reliable method to distinguish among CoPS species in veterinary clinical and food microbiology laboratories.
- The thermonuclease (*nuc*) genes of staphylococcal species were used and devised a multiplex-PCR (M-PCR) method for species identification of CoPS by targeting the *nuc* gene locus.

Staphylococcus aureus and staphylococcal enterotoxins

- *S. aureus* is a **zoonotic bacterium**. However, the strains isolated in cases of SFPO are for the most part of **human origin** due to a human contamination of a food during the process.
- Staphylococci are ubiquitous bacteria found on the skin, nose and throat in warm-blood animals (mammals and birds) and especially in humans. They have also been isolated in the natural environment (soil, water, dust and air), at home (kitchen, fridge), in hospitals environment, in food processing plants and from foodstuffs.

Staphylococcal enterotoxins

- *S. aureus* produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. The staphylococcal enterotoxins (SEs) are recognized agents of the staphylo-coccal foodpoisoning syndrome and may be involved in other types of infections with sequelae of shock in humans and animals.
- First have been recognized nine major antigenic types of SEs and designated SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ.
- All these toxins exhibit superantigenic activity by interacting with antigen-presenting cells and T lymphocytes without regard for the antigen specificity of the cells.

Symptomatic forms

- The foodborne human disease is due to the ingestion of **preformed SEs in food**, in which *S. aureus* or any other SE-producing staphylococci has been able to develop in such a way as to produce its (or their) toxin(s).
- The **incubation period** and severity of symptoms observed depend on the amount of enterotoxins ingested and the susceptibility of each person. Initial symptoms, nausea followed by incoercible characteristic vomiting (in spurts), appear within **30 minutes to eight hours** (three hours on average) after ingesting the contaminated food.

Control measures in the food sector

- The prevention of **SFPO** is based on **hygiene measures** in order to avoid or limit the contamination of food by *S. aureus*.
- These must include **monitoring of the health of animals** (mastitis, for example), good manufacturing practices (**GMPs**) and cleaning and disinfection of equipment and environmental surfaces.
  Moreover for people involved in food processing, the hand care as well as the wear of hair protection covering the whole of the scalp are essential good hygiene practices that must be followed.
- Food handlers presenting skin lesions must be excluded from handling non-packaged foods, while the lesions are not correctly covered (gloves).

Occurence of staphylococci

The importance of staphylococci as pathogens for humans and animals has been recognized for more than 100 years.

There are about **40 species** of staphylococci that differ in their ability to cause a risk to human and animal health, ranging from non-pathogenic to dangerous pathogens causing severe infections and being resistant to the treatment by most of the commonly applied antibiotics.

The coagulase test usually correlates well with staphylococci pathogenicity. The two major pathogenic staphylococci, *Staphylococcus aureus* and *Staphylococcus pseudintermedius* are coagulase-positive.

Many healthy people may carry S. aureus as a part of their normal microflora in the nose, throat, perineum or skin.

Coagulase test

- The tubes with rabbit plasma are incubated at **37 °C** with the cell suspension of suspicious strains.
- After 3 hours the tubes are held at an angle to demonstrate coagulation of the rabbit plasma after incubation with cultures of *Staphylococcus aureus* or other coagulase-positive strain.
- Coagulase-positive strains hydrolyze the plasma gel to liquid phase.
- Recently, these bacteria have received increasing attention due to their potential role in the dissemination of antibiotic resistance markers.

Occurence of Staphylococci

- Staphylococci, in particular ***S. aureus***, are major **opportunistic pathogens** in humans resulting in large numbers of community and healthcare-associated infections. While some of these infections may be relatively minor skin and soft tissue infections, *S. aureus* also frequently causes life threatening invasive infections including septicaemia and endocarditis. ***Staphylococcus epidermidis***, while less virulent than *S. aureus*, is a frequent cause of infection in hospitalised patients, often associated with implanted medical devices. In both of these bacterial species increasing antimicrobial resistance, especially to the glycopeptide antibiotic vancomycin is making treating more difficult.

Staphylococcus epidermidis

- ***Staphylococcus epidermidis*** is a Gram-positive bacterium, and one of over 40 species belonging to the genus *Staphylococcus*. Coagulase-negative staphylococci (CoNS), particularly *Staphylococcus epidermidis*, are among the most frequently isolated microorganisms in clinical microbiology laboratories.
- It is part of the normal human flora, typically the skin flora, and less commonly the mucosal flora. It is a facultative anaerobic bacteria. Although *S. epidermidis* is not usually pathogenic, people with compromised immune systems are at risk of developing infection. These infections are generally hospital-acquired. *S. epidermidis* is a particular concern for people with catheters or other surgical implants because it is known to form biofilms that grow on these devices

The recklessness of *S. epidermidis*

The ability to form biofilms on plastic devices is a major virulence factor for ***S. epidermidis***. It is caused by surface proteins that bind blood and extracellular matrix proteins. It produces an extracellular material known as polysaccharide intercellular adhesin (PIA), which is made up of sulfated polysaccharides.

Such biofilms decrease the metabolic activity of bacteria within them. This decreased metabolism, in combination with impaired diffusion of antibiotics, makes it difficult for antibiotics to effectively clear this type of infection. *S. epidermidis* strains are often resistant to antibiotics, including rifamycin, gentamicin, fluoroquinolones, tetracycline, clindamycin, and sulfonamides

Resistant organisms are frequently found in the intestine, but organisms living freely on the skin can also become resistant due to routine exposure to antibiotics secreted in sweat.

Directive No 1441/2007 of 5 December 2007

- According to this regulation, the quantity of coagulase positive staphylococci must be examined in dairy products as milk, cheese, whey.
- In case that the **number of CFU /g** is higher **than 10^4** , the test for enterotoxines presence must be done.
- There is an urgent need to detect and identify SEs rapidly and accurately for governmental and non-governmental agencies, including the military, public health departments, and health care facilities.

How many SEs are known

- Staphylococcal enterotoxins (SEs) are members of a family of more than 20 different staphylococcal and streptococcal exotoxins, sharing a common phylogenetic relationship, structure, function, and sequence homology.
- Today, **23 enterotoxins** have been identified as distinct serological entities, including SEA, SEB, SEC, SED, and SEE. These toxins are basic proteins made up of approximately **220–240 amino acids** and have similar molecular weights of 25–30 kDa. The most common SEs are SEA and SEB. SEA is most frequently involved in food poisoning caused by staphylococcus.

SEs Identification

- **Serologic tests** are some of the earlier methods applied for SEs' detection, including the gel diffusion test and agglutination test. These tests are essentially reactions based on antigen/antibody binding. Because antibodies mainly exist in serum, *in vitro* antigen/antibody reactions have been regarded as serologic tests characterized by precipitation and agglutination reactions.
- Currently, **fluorescent immu**noassays are the most popular approach for the detection of SEs. A variety of labels have been used in the development of SE immunoassays including fluorescent dyes or fluorescent nanoparticles producing optical signals that can be correlated with the concentration of the analyte.
- Compared with enzyme-mediated immunoassays, it was determined that fluorescent-based immunoassays possessed greater potential to provide high-throughput analysis and increased sensitivity.

SEs detection

- Detection of SEs is paramount to food safety and protection of the food supply.
- SE detection methods rely on commercially available polyvalent enzyme-linked immunoassays (**ELISA)** or enzyme-linked fluorescent immunoassay (**EFLA**) with antibodies that detect SEA-SEE. Monovalent methods are specific for SEA-SEE and can be used to distinguish these types.
- The methods require extraction of enterotoxin from suspected food prior to analysis. The method sensitivity and selectivity is improved with dialysis concentration of the food extract, but time constraints may require preliminary testing without concentration. However, dialysis concentration should be performed on all dairy products prior to analysis.
- **Staphylococcal enterotoxins are highly toxic and procedures that may create aerosols should be performed in approved biological safety cabinet (BSC**).

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Characteristics of genus *Salmonella* sp., and pathogenic *Escherichia coli*

Lecturer:

Jarmila Pazlarová

Department of Biochemistry and Microbiology

Foodborn pathogens

- Implication of foodstuff in numerous outbreaks of foodborne illnesses caused by pathogens such as *Salmonella*, *Listeria*, and *Staphylococcus* is a serious threat to human health.
- *Salmonella* is one of the most common reasons of gastric illnesses.

Characteristics of *Salmonella* I.

- *Salmonella* is closely related to the *Escherichia* genus. They cause illnesses like typhoid fever, paratyphoid fever, and foodborne illness.
- *Salmonella* is also extremely dangerous, and like most diseases, weaker humans like the old and young could easily die from it.
- *Salmonella* can only be **killed** in food **by** cooking at **high temperatures**.

Characteristics of *Salmonella* II.

- ***Salmonella*** is a genus of rod-shaped (bacillus) Gram-negative bacteria of the family Enterobacteriaceae.
- *Salmonella* species are non-spore -forming, predominantly **motile** enterobacteria with cell diameters between about 0.7 and 1.5 µm, lengths from 2 to 5 µm, and peritrichous flagella (all around the cell body).
- The two species of *Salmonella* are *Salmonella enterica* and *Salmonella bongori. S. enterica* is the type species and is further divided into six subspecies that include over 2,600 serotypes.

Characteristics of *Salmonella* III.

- They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. They are also **facultative aerobes**, capable of generating ATP with oxygen ("aerobically") when it is available, or when oxygen is not available, using other electron acceptors or fermentation.
- *S. enterica* subspecies are found **worldwide in all warm-blooded animals** and in the environment. *S. bongori* is restricted to cold-blooded animals, particularly reptiles.
- On top of that majority of species show enormous ability to survive in many different types of natural environment (brooks, streams, ponds, sewerage systems, soil, manures).

Characteristics of *Salmonella* IV.

- Chemical antimicrobial agents such as chlorinated solutions, electro-activated water, **organic acids** and salts, alone or in combination with different hurdles demonstrated important reduction of microbial contaminations in food products.
- However, today's consumer also tends toward the green technologies used for food preservation; thus, the safety of chemical preservatives used to control those pathogens must be followed.

Detection, culture, and growth conditions

- Most subspecies of *Salmonella* produce hydrogen sulfide which can readily be detected by growing them on media containing ferrous sulfate, such as is used in the triple sugar iron test.
- **ISO 6579:002** standard norm for Salmonella detection in food – takes 5 days
- *Salmonella* can also be detected and subtyped using multiplex or real-time polymerase chain reactions (PCR) from extracted *Salmonella* DNA.

Salmonella - growth conditions I.

- **pH** 4 – 9.5

e.g. **3.99** in tomatoes

e.g. **9.5** in eggs rinsing water

After longer exposition to acids population may exert **ATR** Acid Tolerance Response

e.g. Some food prepared for immediate consumption

Salmonella - growth conditions II.

- $\mathbf{A_w}$ water activity
- **0.93** e.g. instant soup

The growth is inhibited by 3-4% NaCl. Salt is more efficient under lower temperature. Resistent to drought – **chocolate persistent.**

The bacteria are not destroyed by freezing, but UV light and heat accelerate their destruction. They are killed after being heated to 55 °C for 90 min, or to 60 °C for 12 min. To protect against *Salmonella* infection, heating food for at least 10 minutes to an internal temperature of 75 °C is recommended.

Salmonella - growth conditions III.

- **Temperature**
- capable of growth from **2** to **46** ^{o}C
- E.g. 2 ^{o}C (1-2 days) beef and chicken meat
- E.g. 4 ^{o}C (<10 days) on eggs shells
- 46 ^{o}C in creams, in chicken meat

(56 ^{o}C mutants in labotaratory)

Taxonomy of salmonells

- According to taxonomic study of Le Minor *et al.* (1982) genus *Salmonella* has only **one species** and **seven subspecies**, which are distinguishable by DNA-DNA hybridization, biochemical and serological characteristics. Each of the seven subspecies may be separated on **serovars** according to **composition** of somatic (O) and flagellar (H) antigens.

- The most common usage of serovars according to **Kauffmann-White** scheme:
- **family –** *Enterobacteriaceae*
- **genus –** *Salmonella*
- **species –** biochemical variants
- **serovar =** serotype – serological variants
- **fagotype –** lyzotype
- **typization of plasmid profiles**

Antigens

- Antigenic classification of salmonellas is based on **somatic antigens O** and **flagellar antigens H**.
- Somatic O-antigens posses lipopolysaccharidic character.
- Enormous types of these antigens results from differences in the **structure of specific saccharidic chain,** where are present various types of glycosidic bonds, both anomeric configurations and unequal acetylation .
- **Flagellar H-ant**igens possess protein nature and are thermolabile. H-antigens are of two types– antigens of the first and the second phase differ by a primary protein structure.

Directive 1447/2007

- According to this document the salmonella strains are the main danger in food poisoning.
- Absence in samples of 10 g (or 25 g) is required.
- Absence is demanded in all meat, milk and egg products.

Salmonella

Food :

1) Meat, poultry, see fish, and see fruits

2) Food of animal origine :

Both pasteurised and unpasteurised egg products

3) Other food with addition of 1 and/or 2

4) Live bivalve molluscs and live echinoderms, tunicates and gastropods.

Food safety criteria: Meat and meat products

Salmonella spp.

Negat./**25g**:
- minced meat intended for ready-to-eat consumption
- meat products intended for ready-to-eat consumption
- gelatine and collagen

Negat./**10g**:
- minced meat intended for thermal preparation

(c=1) - mechanicaly separated meat

- poultry products intended for thermal preparation

Food safety criteria
Milk products

Salmonella spp.

Negat./25 g: - cheeses from raw milk (< pasteurisation)
- dried milk and whey
- frozen products

Absence in 25 g : - Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age

Absence in 25 g : - **Sprouted seeds** (ready-to-eat)

Food safety criteria - Various food

Salmonella spp.

Negat/25 g: - eggs manufactures
- ready-to-eat food containing raw eggs

- Boiled shellfish and mollusca
- live bivalved mollusca and gastropods
- sprouted plant seed
- cut fruits and vegetable
- unpasteurised fruit and vegetable juices

Pathogenesis

Infection dose – healthy human approximately **10^2 -10^5** bacteria sometimes only **10^2**.

Incubation time usually between 6 to 36 hours. Its length depends on quantity of infection dose and individual susceptibility.

The **most serious course of salmonellosis** takes place in children, elderly people and immunocompromised patients.

Symptoms of illness are sickness, nausea, vomiting, abdominal pains, fever about **39 °C** and diarrhea.

Small children and **elderly persons** or diversly ill people there is danger of dehydratation followed by **blood circulation failure**.

Specific *Salmonella* pathogenesis

- A hallmark of *Salmonella* pathogenesis is the ability of the bacterium to survive and proliferate within **phagocytes.** Phagocytes produce DNA-damaging agents such as nitric oxide and oxygen radicals as a defense against pathogens.
- Some of the ways that *Salmonella* serotypes have adapted to their hosts include **loss of genetic material and mutation**.
- In more complex mammalian species, immune systems, which include pathogen specific immune responses, target serovars of *Salmonella* through binding of antibodies to structures such as flagella. Through the loss of the genetic material that codes for a flagellum to form, *Salmonella* can evade the host's immune system.

Salmonella taxonomy

Genus	**Species**	**Subspecies**	**Serovar**
Salmonella	*enterica*	*enterica 59%*	Typhimurium
		salamae 19%	Enteriridis
		arizonae 4%	Typhi
	bongori	*diarizonae 13%*	Virchov
		houtaneae 3%	Heidelberg
		indica 1%	

etc.

Definition of salmonellosis

Salmonellosis is a bacterial infection, bearing name after the genus of bacteria causing it. Salmonells belong to the family *Enterobacteriaceae*, which are facultative anaerobs able to live in intestinal tract. Bacteria are nutritionally undemanding, capable of multiplying outside the host body, primarily in foods of animal origin.

- Salmonells cause **3 types of illness**:
- **Typhoid fever** – the most serious salmonella disease.
  It is caused by ***S. typhi*** and represents < 2.5% of human cases.
- **Paratyphoid fever** – caused by ***S.paratyphi*** **A, B a C**, form only < 0.5% cases.
- **Gastroenteritis – the most common type of salmonellosis** known as '*Salmonella* food poisoning'. Gastroenteritis are caused by all other types of salmonells.
- **Source of gastroenteritis inf**ection caused by salmonells are most frequently animals and c**ontaminated food.**

Sources of salmonells I

- Typhoid and paratyphoid fever is an illness with cyclic continuous infection, long time fever.
- **Source of infection is human**, and ill person or carrier. Transfer of infection takes place by direct or indirect way through contacts with stool or urine of patient, next by water, milk, and food.
- Transfer from animals was detected only by serotype *S. paratyphi* B.
- **Transfer between humans is only exceptional.** People are infected nearly regularly by oral way, by food that is not prepared at high temperature.

Sources of salmonells II

- **Food plays a central role in spreading and propagation of salmonells**. The most common cause of epidemies are contaminated eggs, chicken, meat and milk products.
- Salmonellosis starts usually after **6 to 8 hours** after ingestion of contaminated food.
- Illness arrives by fever, nausea, watery diarrhea, vomiting, headache, sometimes bacteria attack the blood and lymphatic system.
- The disease proceeds for 5-10 days, excretion of bacteria may persists for several weeks.

Salmonella and food

- You can get a ***Salmonella*** **infection** from a variety of foods **(https:// www.cdc.gov/foodsafety/foods-linked-illness.htlm**
- *Salmonella* can be found in many foods including beef, chicken, eggs, fruits, pork, sprouts, vegetables, and even processed foods, such as nut butters, frozen pot pies, chicken nuggets, and stuffed chicken entrees.
- When you eat a food that is contaminated with *Salmonella*, it can make you sick.
- Contaminated foods usually look and smell normal, which is why it is important to know how to prevent Salmonella infection.

Environment and salmonella

- *Salmonella* species lead predominantly host-associated lifestyles, but the bacteria were found to be able to persist in a bathroom setting for weeks following contamination, and are frequently isolated from water sources, which act as bacterial reservoirs and may help to facilitate transmission between hosts.
- *Salmonella* is notorious for its ability to survive desiccation and can persist for years in dry environments and foods

Resistance of salmonells to internal and external factors.

- **optimal growth** temperature about 37 °C, minimal growth temperature 5 °C, maximal 47 °C,
- **critical value** a_w for reproduction of salmonells is 0.92
- range of **pH values** enabling salmonells reproduction takes place at **3.8 – 9.5**, optimum is at neutral pH
- concentration of salt over 9 % has bactericidal effect.
- salmonells are usually sensitive to majority of antimicrobial and disinfectional substances.

Effect of technologies

Salmonells do not survive sterilization nor pasteuration temperatures.

It is generally known that the effectivity of thermal treatment of food depends on the concentration of the present bacteria, on the reached temperature and the length of exposition.

Low temperatures do not destroy salmonells, but they retard or stop their multiplication. At 5 °C salmonells stop to divide, after exposition to temperatures below 0 °C salmonells are not destroyed, during long term storage under these temperatures sublethal damage may take place.

How is the differentiation of epidemiologicaly important isolates provided

- Phagotypization
- Biotypization (xyl, rha, ino, d-tar)
- ATB resistance
- pulsed-field gel electrophoresis (PFGE)
- random amplified polymorphic DNA (RAPD)

Pathogenic *Escherichia coli*

- ***Escherichia coli*** also known as ***E. coli*** is a Gram-negative, a facultative aerobic, rod-shaped, coliform bacterium of the genus *Escherichia,* commonly found in the lower intestine of warm-blooded organisms (endotherms).
- **Most *E. coli* strains are harmless**, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination.

Serotypes

- A common subdivision system of *E. coli*, but not based on evolutionary relatedness, is by serotype, which is based on major surface antigens (**O antigen**: part of lipopolysaccharide layer; **H: flagellin**; **K antigen: capsule**), e.g. **O157:H7**.
- It is, however, common to cite only the serogroup, i.e. the O-antigen. At present, about **190 serogroups** are known. The common laboratory strain has a mutation that prevents the formation of an O-antigen and is thus not typable

Dangerous serotypes

- Certain strains of *E. coli*, such as O157:H7, O104:H4, O121, O26, O103, O111, O145, produce potentially lethal toxins.
- **Food poisoning** caused by *E. coli* can result from eating unwashed vegetables or poorly butchered and undercooked meat. O157:H7 is also notorious for causing serious and even life-threatening complications such as hemolytic-uremic syndrome - **HUS**.

Virulence properties I.

- Enteric *E. coli* (EC) are classified on the basis of serological characteristics and virulence properties.

The major pathotypes of *E. coli* that cause diarrhea are listed below.

ETEC – Enterotoxigenic *E.coli* – these strains are non-invasive, they do not leave intestinal lumen. Leading cause of diarrhea in children and traveler´s diarrhea.

EPEC – Enteropathogenic *E.coli* – virulence factors are similar to those found in *Shigella*. Strains adhere the intestinal mucosa, are moderately invasive and elicit inflammatory response.

Virulence properties II.

- **EIEC** – Enteroinvasive *E.coli* – infection causes a syndrome nearly identical to shigellosis. They cause severe diarrhea and high fever. Serotype O157:H7 bloody diarrhea and no fever.
- **EHEC** – Enterohemorrhagic *E.coli* - cause hemolytic-uremic syndrome and sudden kidney failure. Use bacterial fimbriae for attachment and produce phage-encoded shiga-toxin.
- **EAEC** – Enteroaggregative *E.coli* -strains posses fimbriae that bind to intestinal mucosa, cause water diarrhea without fever.

Virulence properties III.

- **AIEC** - Adherent-invasive *E.coli* – strains are able to invade intestinal epithelial cells and replicate intracellulary. They proliferate more effectively in hosts with defective innate immunity. They are associated with the ileal mucosa in **Crohn´s disease**.
- Most of the above listed serotypes cause diarrhea not only in humans but also in pigs, sheep, goats, cattle dogs, horses, cats and rabbits.
- **A natural carrier and host of O157:H7 is cattle**.

German epidemy 2011 pt. I.

As of June 24, 2011, **834** cases of HUS and **2,967** non-HUS cases were reported by the German **Robert Koch Institute** (RKI);
30 of the HUS cases and **16** of the non-HUS cases resulted in death.
Furthermore, **100** additional infections have been identified in 12 other European countries and even in the United States and Canada (one case in the Czech Republic).

German epidemy 2011 pt. II.

Extensive efforts to identify the source implicated contaminated **sprouts**, which was later confirmed by isolation of the outbreak strain from the sprouts on June 12th.

Historically, ***E. coli* O104:H4** has been associated with very few HUS cases. To date, in Germany only one of 588 **EHEC** strains isolated from HUS patients in the National Consulting Laboratory for HUS and the Reference Laboratory for *Enterobacteriaceae* of the RKI belongs to serotype **O104:H4**, and this strain was isolated in 2001.

The *E. coli* O104:H4 isolate from this German HUS case is included in the HUS-associated *E. coli* (HUSEC) collection.

UNIVERSITY OF CHEMISTRY AND TECHNOLOGY PRAGUE

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MINISTRY OF EDUCATION, YOUTH AND SPORTS

Characteristics of *Cronobacter sakazakii, Bacillus cereus* and *Campylobacter* spp.

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Enterobacter (Cronobacter) sakazakii

- ***Cronobacter sakazakii***, which before 2007 was named ***Enterobacter sakazakii***, is an opportunistic, rod-shaped, Gram-negative pathogenic bacterium that can live in very dry places. The majority of *Cronobacter sakazakii* cases are adults but low-birth-weight preterm **neonatal** and older infants are highest at risk.
- The disease is associated with a rare cause of invasive infection of infants with historically high fatality rates (40–80%).
- Most neonatal *C. sakazakii* infections cases have been associated with the use of powdered infant formula **PIF** with some strains able to survive in a desiccated state for more than **two years**.

Occurence I.

- The bacterium is **ubiquitous** being isolated from a range of environments and foods, and the majority of *Cronobacter* cases are in the adult population. However, it is the association with intrinsically or extrinsically contaminated powdered formula which has attracted the main attention.
- Little is known about the ecology of *E. sakazakii*. However, *E. sakazakii* is considered to be an environmental organism and is likely to be present in both manufacturing facilities and domestic situations.

Occurence II.

- The taxonomic relationship between *E. sakazakii* strains has been studied using full-length 16S rRNA gene sequencing, DNA-DNA hybridization, multilocus sequence typing (MLST), f-AFLP automated ribotyping.
- This resulted in the classification of *E. sakazakii* as a new genus, *Cronobacter* within the Enterobacteriacaae, initially comprising four named species in 2007. The taxonomy was expanded to five named species in 2008, and more recently (2011) to **seven named species**.

Occurence III.

- Although powdered infant formula (**PIF) is not a sterile product**, risk of infection can be greatly reduced by correctly preparing and storing it.
- The WHO recommends that infants should be exclusively breastfed for the first six months of life to achieve optimal growth, development and health. **Mothers who are not breastfeeding for any reason as well as caregivers, both at home and in care settings, should always be alerted that powdered infant formula is not a sterile product and can be contaminated with pathogens that can cause serious illness**. Cleaning and sterilisation of feeding and preparation equipment is an essential prerequisite for safe preparation of powdered infant formula.

Occurence IV.

- *C. sakazakii* is an opportunistic and life-threatening pathogen. It has been known to be implicated in the development of bacterial infections in newborns since 1961. This pathogen has a **40-80% mortality rate** among the infected newborns and seems to be specifically associated with strains of *C. sakazakii* sequence type-4.
- It is considered an **emerging opportunistic pathogen**, responsible for cases of neonatal infections including **necrotizing enterocolitis** (NEC), meningitis, bacteraemia and septicemia.
- **NEC** is characterized by inflammation and intestinal tissue damages and is one of the most common reasons of gastrointestinal disorders in neonates, due to the use of contaminated newborn milk formula.

Cronobacter sakazakii I.

The bacterium is ubiquitous being isolated from a range of environments and foods, and the majority of *Cronobacter* cases are in the adult population.

However, it is the association with intrinsically or extrinsically contaminated **powdered formula** which has attracted the main attention. According to multilocus sequence analysis (MLSA) the genus originated ~40 MYA, and the most clinically significant species, *C. sakazakii*, was distinguishable ~15-23 MYA.

The first documented isolation of what would become known as *Cronobacter sakazakii* was from a **can of dried milk** in 1950, although these organisms have likely existed for millions of years.

Cronobacter sakazakii II.

- **EU Directive 1441/2007** on microbiological criteria for foodstuffs. According to this directive it is necessary to follow the presence of *Enterobacter sakazakii* in powdered infant formulae (PIF) and in similar dried products intended for sucklings up to 6 months age.
- The Directive was published **few months** before scientific renaming of the bacteria. Therefore in expert microbiological literature the organism exists as ***Cronobacter;*** in legislation it is called ***Enterobacter.***

How does powdered infant formula become contaminated with ***Cronobacter*****?**

- **Powdered infant formula (PIF) is not sterile**. Manufacturers report that, using current methods, it is not possible to produce sterile powdered infant formula.
- At the factory, *Cronobacter* could get into formula powder if contaminated raw materials are used to make the formula, or if the formula powder touches a contaminated surface in the manufacturing environment.

Horizontal method for the detection of *Cronobacter* spp.

- ISO 22964:2017(en)
- The essence of the test is nonselective enrichment cultivation in buffered peptone water. After 16 – 18 h at 37 C the suspension is inoculated to selective broth (CBS), and next day chromogenic *Cronobacter* isolation (CCI) agar is inoculated. Biochemical confirmation of suspected colonies has to follow.

Bacillus cereus

- ***Bacillus cereus*** is a Gram-positive, rod-shaped, aerobic, facultatively anaerobic, motile, beta hemolytic bacterium commonly found in soil and food.
- Some strains are harmful to humans and cause **foodborne illness** while other strains can be beneficial as probiotics for animals.
- It is the cause of "**fried rice syndrome**", as the bacteria are classically contracted from fried rice dishes that have been sitting at room temperature for hours.
- *B. cereus* bacteria are facultative anaerobes and, like other members of the genus *Bacillus*, can produce **protective endospores**. The **virulence factors** include **cereolysin** and **phospholipase C.**

Directive 1441/2007

- Commission Regulation (EC) No **1441/2007** of 5 December 2007 amending Regulation (EC) No **2073/2005** on microbiological criteria for foodstuffs.
- According to this directive the presence of *Bacillus cereus* is not allowable in milk, milk products and PIF.

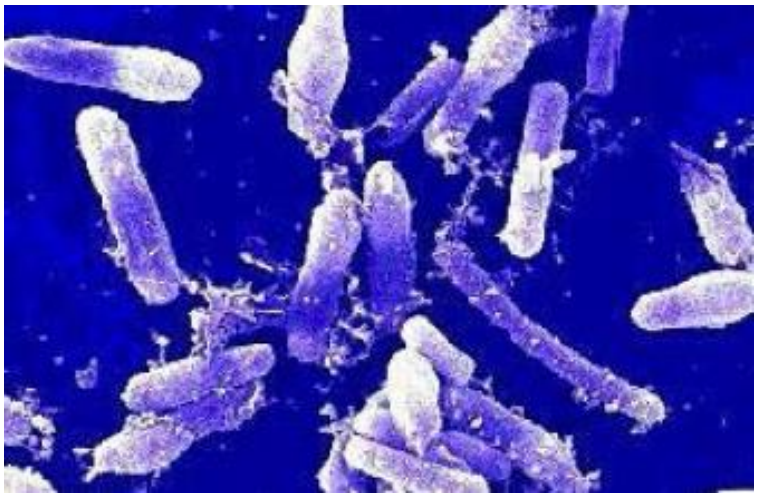
History

- *B. cereus* was first described in **1887,** as it was isolated from a cow shed.

- ***Bacillus cereus*** was recognized as an agent of food poisoning in **1955** by the Norwegian doctor Steinar Hauge

Some characteristics

- Scientific classification :
  - Family : Bacillaceae
  - Genus : *Bacillus*
  - Species : *B. cereus*
- Aerobe
- Gram positive
- Rod-shaped
- Spore former
- Mesophilic :
  Temperature range : 10°C-48°C
  → Optimum range: 28°C-35°C
  → Some psychotrophic strains can grow down to 4 to 5°C
- pH range : 4.9-9.3



Food poisoning in humans

Two distinct types of food poisoning have been attributed to the consumption of food contaminated with *B. cereus* :

	DIARRHOEAL FORM	EMETIC FORM
Incubation period	> 6 hours (8-16h)	< 6 hours (1-5h)
Symptoms	Diarrhea, nausea, abdominal cramps	Severe vomiting, nausea, abdominal cramps
Duration	Lasts 20-36 hours	Lasts 8-10 hours
Infective dose	10^5-10^7 (total)	10^5-10^8 (cells.g^{-1})
Implicated food	Meat, vegetables	Rice
Toxin	Heat-labile (heat unstable)	Heat-stable
Toxin produced	In the small intestine of the host	Preformed in foods

Virulence factors

Diarrheal syndrome

3 enterotoxins including :

- Hemolysin BL (HBL),
- Non-hemolytic enterotoxin (NHE), and
- Cytotoxin K

→The main virulence factor of *B.cereus* is HBL, which is made of the three proteins B, L1, and L2

Emetic syndrome

Cereulide peptide toxin

This toxin has a ring structure, dodecadepsipeptide, which consists of four amino acids, repeating three times, and oxy acids

→The mechanism of action of the emetic toxin still has to be elucidated

Diseases - ocular infection and other opportunistic infections

An opportunistic pathogen

Especially among immunocompromised patients, newborns, and patients with surgical wounds

Therefore, current research is being conducted to understand its pathogenicity and virulence factors in order to find potential targets for antimicrobial drugs

B. cereus can cause ocular infections such as keratitis, endophthalmitis, and panophthalmitis

For example : *E*ndophthalmitis can lead to blindness.

There is currently no universal treatment for this disease.

B. cereus can also cause bacteremia, pneumonia, cellulitis, infant death, septic meningitis, endocarditis…

However, these infections are less common

Prevention

To limit the presence of *B. cereus :*

- **Good Manufacturing Practices** (GMP) and **HACCP**
- Cool hot foods quickly
- Do not store perishable foods within the *Temperature Danger Zone* (5°C to 60°C)
- Always evenly reheat food to above 75°C.
- When reheating in a microwave, periodically stir the food to ensure even heating
- Do not store precooked foods under refrigeration for excessive periods of time

Food should be consumed immediately after preparation or refrigerated

Isolation and identification

Isolation of the organism in implicated food product or nonfecal specimens (e.g., eye, wound). Specimens are stored temporary at refrigerating temperature if necessary, diluted if necessary, and plated on selective agar media.
Incubation at 30 ± 1°C for 24 hours

.

→ **Mannitol egg yolk polymyxin agar (MYP)** is usually recommended :

- *Polymyxin* is the selective agent
- *Egg yolk* and *mannitol* are differential agents

Typical colonies are rough with a violet-red background, surrounded by a zone of precipitate.
2-4 mm

MYP AGAR

A medium for the enumeration of Bacillus cereus in food samples

ISO 7932:2004 – Standard Method

- Horizontal method for the enumeration of viable *Bacillus cereus* by means of the colony-count technique at 30 °C.
- Applicable to products intended for human consumption and the feeding of animals.
- In order to have a practicable test method, the confirmatory stage has been restricted to the typical aspect on MYP agar and the haemolysis test.
- "**PRESUMPTIVE**"

Distinction of *B. cereus* from other closely *Bacillus* species such as *B. anthracis, B. weihenstephanensis ?*

An additional motility test may help to differentiate *B. cereus* from *B. anthracis*

Conclusion and “Remember it !”

- *B. cereus* is a normal soil inhabitant and is frequently isolated from foods.
- Psychotrophic strains have become an increasing problem for the dairy industry.
- *B. cereus* food poisoning is not a reportable disease, and is therefore highly underestimated in official statistics.
- *B. cereus* causes two different types of food poisoning, one emetic due to a preformed small cyclic peptide, and one diarrheal due to enterotoxins.

Bacillus cereus

- Most emetic patients recover within six to 24 hours, but in some cases, the toxin can be fatal.
- In 2014, 23 neonates receiving **total parenteral nutrition** contaminated with *B. cereus* developed septicaemia, with three of the infants later dying as a result of infection.[

Taxonomy of *Campylobacter I.*

- The ***Campylobacter* genus** was established in 1963 following the renaming of *Vibrio fetus* to *Campylobacter fetus*, forming the type species of this genus .
- The *Campylobacter* genus belongs to the family *Campylobacteraceae*, the order *Campylobacterales*, the class *Epsilonproteobacteria*, and the phylum *Proteobacteria*. Since its first description, the genus has grown to include several important human and animal pathogens that are primarily classified through phylogenetic means.

Taxonomy of *Campylobacter II.*

- The genus *Campylobacter* consists of **26 species**, 2 provisional species, and 9 subspecies (as of December 2014).
- Molecular typing methodologies have been instrumental in enhancing epidemiological investigations aimed at tracking sources of sporadic infections with *Campylobacter* spp. by providing information on the **genetic subtypes** in circulation. Poultry products are frequently contaminated with *Campylobacter* spp. and molecular typing data has linked *Campylobacter* spp. on these products to human infections.

Campylobacter sp. I.

- *Campylobacter jejuni* is one of the **most common causes** of food poisoning in **Europe** and in the **United States**. The vast majority of cases occur as isolated events, not as part of recognized outbreaks.
- Active surveillance through the Foodborne Diseases Active Surveillance Network (FoodNet) indicates that about **14 cases** are diagnosed each year for each **100,000 persons** in the population. *Campylobacter jejuni* is in a genus of bacteria that is among the most common causes of bacterial infections in humans worldwide.

Campylobacter sp.II.

- There are many species of *Campylobacter* ; 11 of them cause human illness, including enteric and extraintestinal disease. **The major human pathogens are *C. jejuni* and *C. coli*** , which usually cause acute enteritis, and *C. fetus* , which is the major pathogen causing extraintestinal illnesses. *Campylobacter coli* is not so frequent,but is the second most common species.
- *Campylobacter* infection is the most commonly identified cause of **Guillan-Barré syndrome**.
- The bacteria colonize the small and large intestines, causing inflammatory diarrhea with fever. Stools contain leukocytes and blood. The role of toxins in pathogenesis is unclear.

Pathogenity I.

Campylobacter is a helical shape, nonspore-forming, Gram-negative, **microaerophilic**, nonfermenting bacterium forming motile rods with a single polar flagellum, which are also oxidase-positive and grow optimally at 37 to **42 °C.**

Exposed to atmospheric oxygen, *C. jejuni* is able to change into a coccal form. This species of pathogenic bacteria is one of the most common causes of human gastroenteritis in the world.

Food poisoning caused by *Campylobacter* species can be severely debilitating, but is rarely life-threatening. It has been linked with subsequent development of Guillain-Barré syndrome, which usually develops two to three weeks after the initial illness.

Individuals with recent *C. jejuni* infections develop Guillain-Barré syndrome at a rate of 0.3 per 1000 infections, about 100 times more often than the general population.

Pathogenity II.

Studies on the pathogenesis of *C. jejuni* show that for this organism to cause disease, the **susceptibility of the host** and the relative **virulence** of the infecting strain are important.

Infection results from the ingestion of contaminated food or water, and the **infective dose can be as low as 800 organisms**. To initiate infection, the organism must penetrate the gastrointestinal mucus, which it does using its high motility and spiral shape. The bacteria must then adhere to the gut enterocytes and can then induce diarrhea by toxin release.

C. jejuni releases **several different toxins**, mainly **enterotoxin** and **cytotoxins**, which vary from strain to strain and correlate with the severity of the enteritis. IgA immobilises organisms, causing them to aggregate and activate complement, and also gives short-term immunity against the infecting strain of organism.

Natural genetic transformation

C. jejuni is **naturally competent** for genetic transformation. Natural genetic transformation is a sexual process involving DNA transfer from one bacterium to another through the intervening medium, and the integration of the donor sequence into the recipient genome by homologous recombination.

C. jejuni freely takes up **foreign DNA** harboring genetic information **responsible for antibiotic resistance**. Antibiotic resistance genes are more frequently transferred in biofilms than between planktonic cells (single cells that float in liquid media).

Sources of infection I.

C. jejuni is commonly associated with **poultry,** and it naturally colonises the digestive tract of **many bird species**. All types of poultry and wild birds can become colonized with *Campylobacter*.

One study found that 30% of European starlings in farm settings in the United Kingdom were carriers of *C. jejuni*. It is also common in **cattle**, and although it is normally a harmless commensal of the gastrointestinal tract in these animals, it may cause campylobacteriosis in calves.

Contaminated drinking water and unpasteurized milk provide an efficient means for distribution. Contaminated food is a major source of isolated infections, with **incorrectly prepared meat and poultry** as the primary

Sources of infection II.

Surveys show that **20 to 100% of retail chickens are contaminated**. This is not overly surprising, since **many healthy chickens carry these bacteria** in their intestinal tracts. Raw milk is also a source of infection. The bacteria are often carried by healthy cattle and by flies on farms. Unchlorinated water may also be a source of infections.

However, properly cooking chicken, pasteurizing milk, and chlorinating drinking water kill the bacteria. *Campylobacter* is not, in contrast to *Salmonella*, transmitted vertically and therefore humans do not get infected by consuming eggs.

Prevention I.

Cook all poultry products thoroughly. Make sure that the meat is cooked throughout (no longer pink) and any juices run clear. All poultry should be cooked to reach a **minimum internal temperature of 74 °C**.

Wash hands with soap **before** preparing food.

Wash hands with soap **after handling raw foods** of animal origin and before touching anything else.

Prevent **cross-contamination in the kitchen** by using separate cutting boards for foods of animal origin and other foods and by thoroughly cleaning all cutting boards, countertops, and utensils with soap and hot water after preparing raw food of animal origin.

Prevention II.

Do not drink **unpasteurized milk** or **untreated surface water**.

Make sure that people with diarrhea, especially children, wash their hands carefully and frequently with soap to reduce the risk of spreading the infection.

Wash hands with soap after contact with pet feces.

One major possible complication that *C. jejuni* can cause is **Guillain–Barré syndrome**, which induces neuromuscular paralysis in a sizeable percentage of those who suffer from it. Over time, the paralysis is typically reversible to some extent; nonetheless, about **20% of patients with GBS are left disabled**, and around 5% die.

Another chronic condition that may be associated with *Campylobacter* infection is what was formerly known as Reiter's syndrome, a form of reactive arthritis.

Pathogen or commensals

- **Genomic** and **genetic analyses** of *C. jejuni* have revealed **mechanisms of pathogenicity** and chick colonization. Pathogenicity is multi-factorial and requires the **presence** of the flagella, **the capsule**, both ***O*-linked** and ***N*-linked protein glycosylation** and secreted proteins that facilitate host-cell invasion.
- *C. jejuni* is a **pathogen in humans**, but a **commensal species in chickens**. The immune responses and/or bacterial colonization features might therefore be host specific.

Legislation?

- The high incidence of *Campylobacter* diarrhea, as well as its duration and possible complications, makes it highly important from a socio-economic perspective. In developing countries, *Campylobacter* infections in children under the age of 2 years are especially frequent, sometimes resulting in death.
- **EU legislation does not include this pathogen**, because this organism can multiply only in living host.
- But its resistence to environment causes its **high survival** rate.

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MINISTRY OF EDUCATION, YOUTH AND SPORTS

Biogenous amines , food spoilage microflora

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Spoilage of food

Spoilage of food involves any change which renders food unacceptable for human consumption and may result from a variety of causes, which include:

- Insect damage
- Physical injury due to freezing, drying, burning, pressure, radiation
- Activity of indigenous enzymes in plant and animal tissues
- Chemical changes not induced by microbial or naturally occurring enzymes. These changes usually involve O2 and light and other than microbial spoilage are the most common cause of spoilage e.g. oxidative rancidity of fats and oils and the discolouration of cured meats
- **Growth and activity of microorganisms: bacteria, yeasts and moulds**

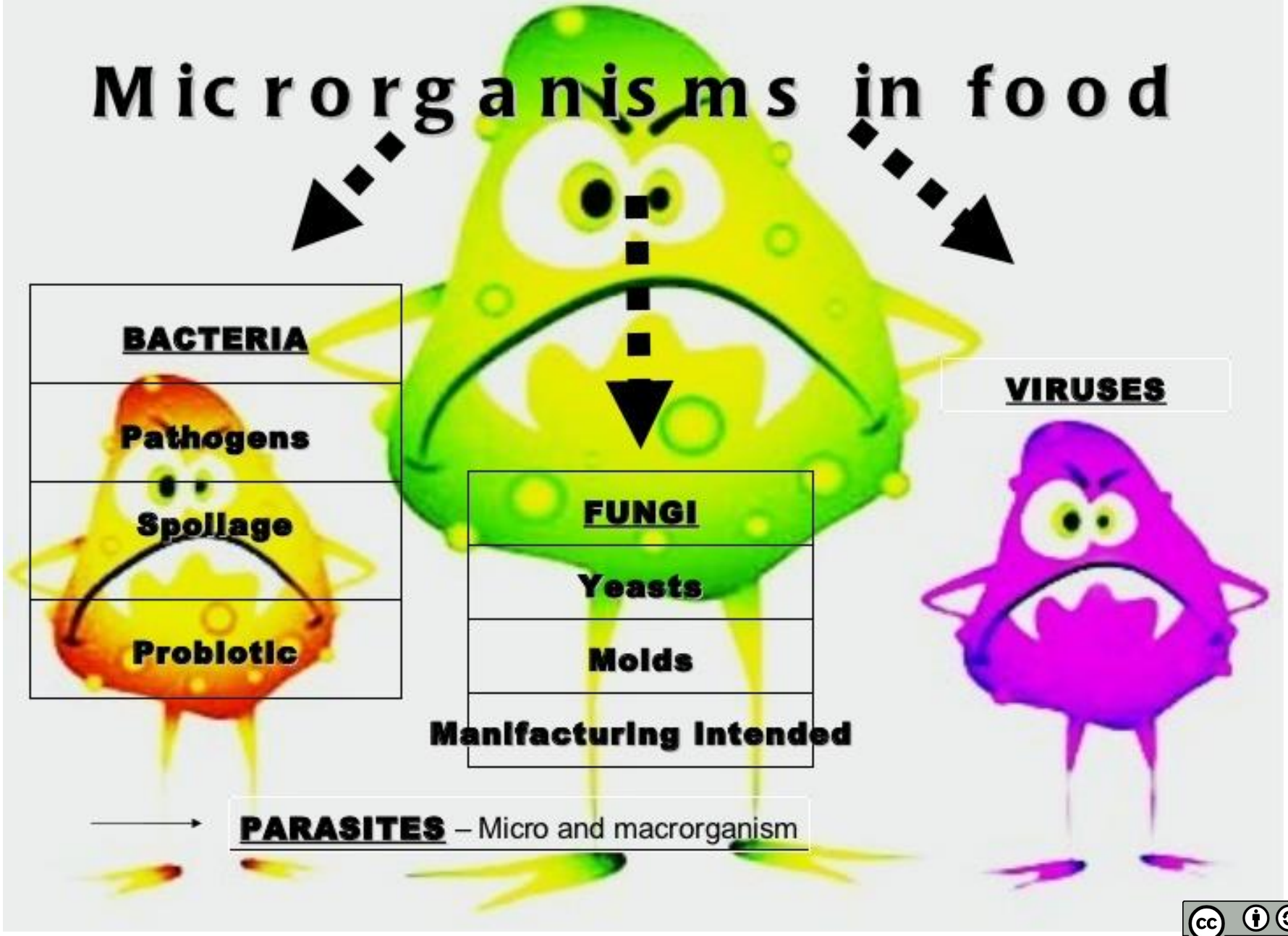
Spoilage microorganisms

Spoilage is a term used to describe the **deterioration** of a foods' texture, colour, odour or flavour to the point where it is unappetizing or unsuitable for human consumption. Microbial spoilage of food often involves the degradation of protein, carbohydrates, and fats by the microorganisms or their enzymes.

Microorganisms occur everywhere in the environment, and there is always a risk of spoilage when foods are exposed to unsuitable conditions. Microbial spoilage results from bacteria, molds, and yeast.

While microorganisms may or may not be harmful, the waste products they produce when growing on or in food may be unpleasant to taste.

Microrganisms in food



PARASITES – Micro and macrorganism

The cause of spoilage

- Spoiled Food
- Damage or injuries that make food undesirable for human consumption.
- Can be the result of:
  - insect damage
  - physical injury
  - enzymatic degradation
  - microbial activity
-

Classification of foods depending on stability I.

- **Highly perishable foods** – This group includes meat, poultry, fish, raw milk, eggs, many fruits and vegetables, and all cooked foods.
- **Semiperishable foods –** Slight processing is given to this type of foods. Examples are pasteurized milk and egg products, baked goods, hard cheese, and refrigerated foods.
- **Semi-shelf-stable foods**
- **Shelf-stable foods (nonperishable foods)**

Classification of foods depending on stability II.

- **Semi-shelf-stable foods –** two or more preservation processes (hurdle concept) can be used. The quality retention time of these foods ranges from1 to 4 months. Examples are vacuum-packaged cooked meat products and fermented dry sausages.

- **Shelf-stable foods (nonperishable foods)** Highly processed (such as heat-processed, sterilized and hermetically sealed) foods can be listed in this group. The quality retention time of these foods is up to 1 year.

Basic types of food spoilage

- Appearance: when a food “looks bad,” what is this referring to?
  - **Microbial growth**
- mycelia or colonies visible on surface
- development of cloudiness in liquids
  - Changes in food color due to heme or chlorophyll breakdown
- colony pigments, growth of mycelia, etc.

Other characteristics of spoilage

- Changes in taste and odor
  - Development of:
    - nitrogenous compounds (ammonia, amines, etc.)
    - sulfides
    - organic acids

The **numbers** and **types** of MO in food are largely determined by:

- Environment from which the food was obtained.
- Microbiological quality of the food in its raw or unprocessed state (intrinsic factors).
- Handling and processing sanitation.
- Effectiveness of packaging, handling and storage conditions in restricting microbial growth (extrinsic factors).

Microbial metabolism

- Some microorganisms can **synthesize polymeric compounds** as metabolic products. *Leuconostoc mesenteroides* can metabolize sucrose and dextran (polymer of glucose) to produce polymeric compounds.
- Microorganisms can also secrete **extracellular enzymes** to hydrolyze large molecular nutrients (polymers) in a food; for example, *Aspergillus niger* produces amylase to hydrolyze starch.
- Some microorganisms can **produce pigments** during growth in a food; for example, *Micrococcus luteus* produces yellow pigment, *Pseudomonas syncyanea* blue pigment,and *Serratia marcescens* red pigment.

Enzymes effects

- Textural changes (feel)
  - Slime formation
- due primarily to surface accumulation of microbial cells
- also a manifestation of tissue degradation
  - Tissue softening due to enzymatic degradation (e.g. soft rot in veggies)
- In milk, the microorganisms that are principally involved in spoilage are **psychotropic** organisms. Most psychotrops are destroyed by pasteurization temperatures, however, some like *Pseudomonas fluorescens, Pseudomonas fragi* can **produce** proteolytic and lipolytic extracellular **enzymes** which are **heat stable and capable of causing spoilage.**

Most frequent spoilage microorganisms

- **Gram positive**

Spormaking, nonsporulating, LAB

Bacillus *Micrococcus* *Lactobacillus*

Clostridium *Brocotrix* *Streptococcus*

Leuconostoc

Pediococcus

Gram negative

Coliform

Citrobacter

Serratia

Proteus

Escherichia

Enterobacter

Erwinia

Klebsiella

Hafnia

Pseudomonas

Acinetobacter

Aeromonas

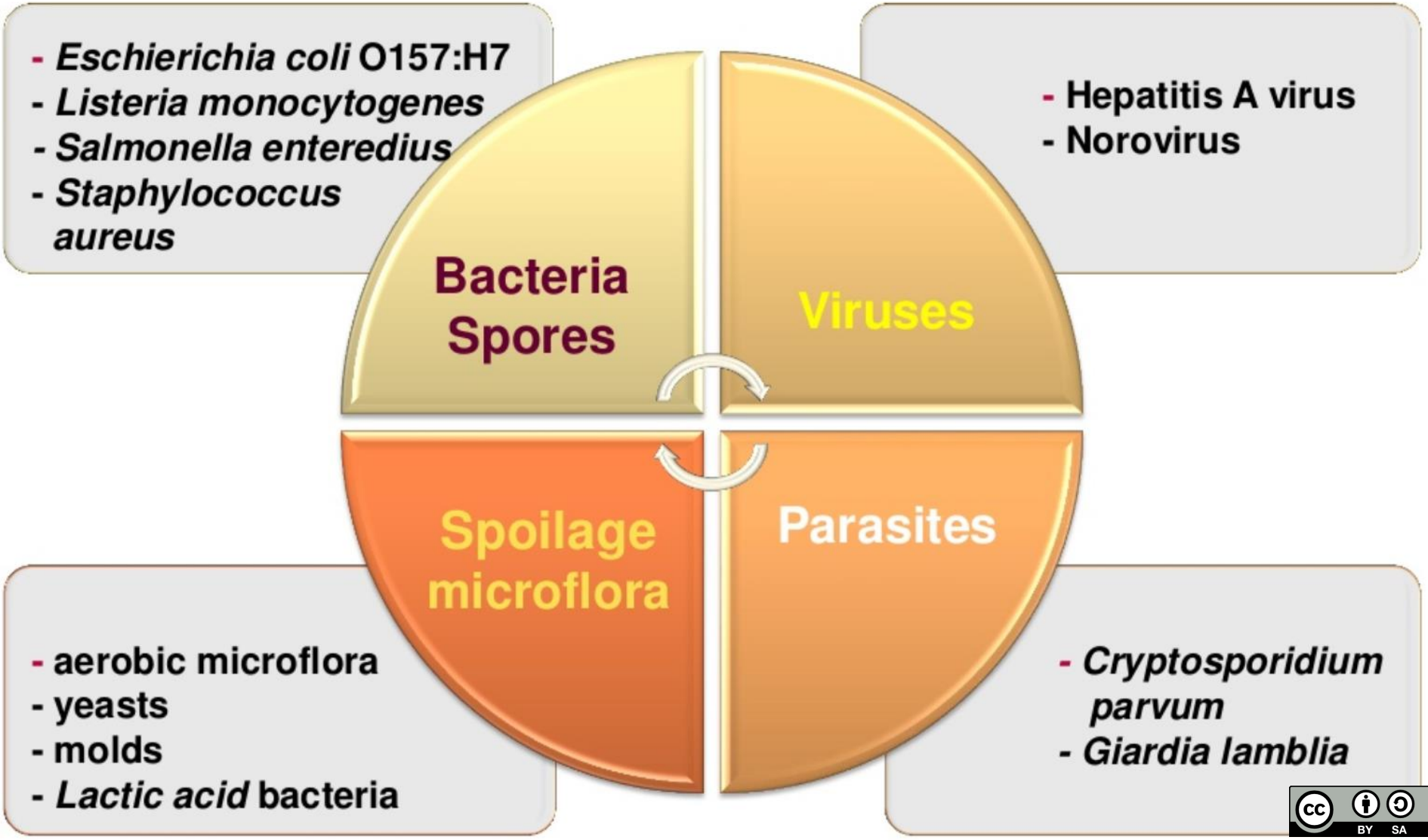
Alcaligenes

Moraxella

Altermonas

Flavobacterium

Effectiveness of UV light against microflora



Brief outline of spoilage micromycetes and yeasts

Yeasts :

Candida, Saccharomyces, Zygosaccharomyces, Torulospora, Rhodotorula, Pichia

Micromycetes :

Aspergillus, Mucor, Penicillium, Rhizopus, Geotrichum, Botrytis, Cladosporium, Byssochlamys

Wine-making

Production of **4-ethyl phenol** by *Brettanomyces/Dekkera* spp. in red wines is considered as spoilage only in case that this **secondary metabolite** is present in a quantity higher than **620 µg/l.**

- Less than **400 µg/l** contribute to improvement of the wine complexity, bacause it supports aromatic features of spice, leather, and other aromatic markers appreciated by consumers.
- **More than 620 µg/l**, for many consumers such wines are inferior (low class), but for some they are pleasant.
-

Main types of spoilage yeasts

Saccharomyces *cerevisiae,* ***Debaryomyces*** *hansenii*

Pichia *anomala, Pichia membranifaciens, Pichia fermentans, Pichia burtonii,* ***Candida*** *parapsilosis, Candida zeylanoides subpelliculosa, Candida guilliermondii, Candida albidus, Candida tropicalis,* ***Torulaspora*** *delbrueckii,* ***Issatchenkia*** *orientalis,* ***Zygosaccharomyces*** *bailii, Zygosaccharomyces bisporus,* ***Cryptococcus*** *spp., Zygosaccharomyces rouxii,* ***Kluyveromyces*** *marxianus,*

Saccharomyces exiguus, ***Sporobolomyces*** *roseus,*

Trichosporon *pullulans, Trichosporon cutaneum,*

Hanseniapora *uvarum*

Factors effecting spoilage microflora

- 1. Starting number of all microbes
- 2. Type of food / intrinsic characteristics
- - pH
- - a_w
- - Content of nutrients
- - Antimicrobial compounds
- - Eh Redox potential (oxidative – reducing potential)
- - Biological structures

Processing and storage conditions

- - heat treatment
- - acidification
- - reduction of water activity
- - usage of preservative agents
- - low temperature storage
- - atmosphere of storage
- - combination of all factors

SSO

- The main culprits are microbial organisms known as **specific spoilage organisms** (**SSOs**). The concept of SSOs arises from the fact that not all bacteria cause food spoilage; indeed, the degree of food spoilage is not proportional to the amount of microbes present in the food.
- SSOs are solely responsible for spoilage of the food and the typical characteristics associated with that spoilage. They are typically present in very low numbers and comprise a low percentage of the microflora present on the food.

Identification of SSOs

- Identification of **SSOs** is done by comparisons of the physical and chemical features of the collective spoiled products with the individual products left behind by each organism in the spoilage microflora.
- In particular, the qualitative ability of each organism to produce off-odors (spoilage potential) and the quantitative ability of each organism to produce spoilage metabolites (spoilage activity) are examined.
- This simple phenotypic identification scheme, along with a 16S rDNA gene sequencing to confirm results, allows scientists to discover which organism or organisms in the spoilage microflora are directly responsible for the spoilage.

Spoilage and pathogenic bacteria

- Each unique environment has its own unique SSOs, because each different environment selects for particular organisms to thrive.
- The spoilage domain for an SSO is identified based upon the conditions (pH, temperature, water activity, and atmosphere) under which that SSO can grow and produce the metabolites that cause spoilage.
- It is important to note that **spoilage bacteria** normally **outgrow pathogenic bacteria** during storage. Thus some foods may spoil before they become toxic.
- **Spoilage bacteria** and **pathogenic species** in spoiled food have different effects. Pathogens are responsible for the symptoms that result from eating spoiled food; SSOs may or may not have a direct harmful effect on the consumers.

Raw fish spoilage

- Raw fish spoilage is part of the **25% of food products** that is **lost yearly** due to microbial activity. Microbes are found on the outer body covering and the inner surfaces of fresh fish, such as the skin, gills, and GI tract.
- The poikilotherm nature of fresh fish allows a wide variety of bacteria to grow, including the Gram-negative, rod-shaped bacteria which belong to the genera *Pseudomonas,Moraxella*, *Acinetobacter* , *Shewanella*, *Flavobacterium, Aeroemonadaceae*, and *Vibrionaceae*, and Gram-positive bacteria such as *Bacillis, Micrococcus, Clostridium, Lactobacillus,* and *Corynebacterium*.
- Psychotrops are bacteria that can tolerate cold temperature and grow at 0 degree Celsius but **grow optimally around 25 degrees Celsius.**

Monitoring and detection of spoilage microorganisms

- Direct microbiological methods
- Chemical/ physical/ physicochemical methods
- Molecular biology methods - PCR
- Criteria of “acceptability“ of food – sensoric evaluation of colour, texture, smell, odour, taste and overall impression

Biogenic amines I.

- Biogenic amines are natural antinutritive factors and they are important as hygienic criterion.
- They are joined with many food poisoning and are able to initiate various pharmacological reactions.

Histamine, putrescin, cadaverine, tyramine, tryptamine, β-phenyletyl amine, spermine and **spermidine** are considered as the most important biogenic amines in food.

A **biogenic amine** is a biogenic substance with one or more amine groups. They are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones.

Biogenic amines II.

- Biogenic amines can be found in **all foods containing proteins or free amino acids** and are found in a wide range of food products including **fish products**, **meat** products, **dairy** products, wine, beer, vegetables, fruits, nuts and chocolate.
- In non-fermented foods the presence of biogenic amines is mostly undesired and can be used as indication for microbial spoilage. In **fermented foods** , one can expect the presence of many kinds of **microorganisms,** some of them **being capable of producing biogenic amines.**
- They play an important role as **source of nitrogen and precursor** for the synthesis of hormones, alkaloids, nucleic acids, proteins and food aroma components.
- However, food containing high amounts of biogenic amines may have toxicological effects.

Endogenous and exogenous biogenic amines

Endogenous amines are synthesized by many types of tissues (adrenalin in suprarenal medulla, histamine in mastocytes (cells of connective tissue) and liver. Amines are spread locally or through blood system.

Exogenous amines are absorbed from food in digestive tract. Alcohol may increase the rate of absorbance.

Monoaminoxidase (MAO) decomposes biogenic amines and prevents excess resorption.

MAO inhibitors (MAOI) are used as medication of depressions, to prevent MAO in decay of amines important by positive effects.

Some prominent examples of biogenic monoamines include

Monoamine neurotransmitters

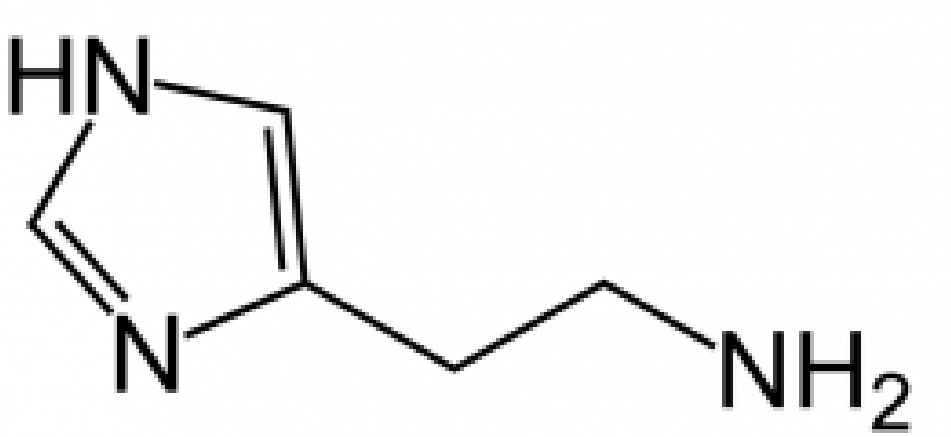
Histamine - a substance derived from the amino acid histidine that acts as a **neurotransmitter** mediating arousal and attention, as well as a pro-inflamatory signal released from mast cells in response to **allergic** reactions or tissue damage. Histamine is also an important stimulant of HCl secretion by the stomach through histamine H_2 receptors.

Serotonin – a **central nervous system neurotransmitter** derived from the amino acid tryptophan involved in regulating mood, sleep, appetite, and sexuality.

Biogenic amines

- **Histamine** – compound derived from amino acid histidine, acts as neurotransmitter mediating transfer of signal, and is also signal of inflammatory diseases.
- Histamine is also an important stimulant of HCl secretion in stomach, using histamine H2 receptors.
- **Tyramine** - compound present in many common food it is connected with higher blood pressure and headache.

histamine



It is a mediator in pathofysiological processes namely, in **allergic reaction of type 1 and inflammatory reaction**. It is active in some mechanisms of nausea and vomiting. Histamine released in skin causes the increase of capillary permeability, reddening of skin and support blood bruises.

Detection of biogenic amines producers

- **Detection of BA-producing** bacteria by cultivation techniques is slow with uncertain results. Many studies described the loss of BA synthesis in LAB after longer storage or after cultivation of isolates on synthetic media.
- Methods based on **molecular biology** are rapid, reliable and independent of cultivation.
- These methods provide information about potential risk of these compounds synthesis.

Why they are dangerous

- Toxicity of histamine seems to be strengthened by the presence of other amines, as **cadaverine**, **putrescin** and **tyramine**.

Biogenic amines can be also considered for carcinogens, because they can react with **nitrites** forming c**arcinogenic nitrosamines**.

Content of BA was followed in different food and feed. They were found in cheeses, fishes, meat products, eggs and mushrooms.

Origin of biogenic amines

- Foods prepared by **fermentative processes**, or those exposed to microbial contamination during ripening and storage, probably contain BA.
- **Alcoholic beverages** like beer can contain BA, as well as other fermented food e.g. **sauerkraut** and soya food.
- Amines are considered for **endogenous plant compounds;** some types of fruit and vegetable contain high concentrations of various amines.

Limits of biogenic amines content in food

- **Histamine- fish products from species known for a high histidine content.**
- These families: *Scombridae, Clupeidae, Coryfenidae, Pomatomidae, Scombresosidae*
- **Sampling plan : n (9), c (2), m 100mg/kg, M 200mg/kg**

- Fish products made by fermentation in brine, they are not made from species known for a high histidine content.
- **Sampling plan : n (9), c(2), m 200mg/kg, 400mg/kg**
- **Analytical method - HPLC**

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Lactic acid bacteria and production of cultured dairy products, probiotics, prebiotics

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Definition

- **Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.** Most probiotics are bacteria similar to those naturally found in the intestine. Common examples are ***Lactobacillus*** and ***Bifidobacterium*** **.**
- **FAO, WHO (2002)**
- **WGO (2008)**
- **Prebiotics** are food ingredients (mostly oligosaccharides) that induce the growth or activity of beneficial microorganisms.
- **Synbiotics = probiotics + prebiotics**

- synergism x not symbiotic!

Human microbiota – collection of microorganisms

- ***Resident microbiota*** permanently colonizes the host and is established during the first months-years of life; historical definition - **microflora**
- commensal microbiota
- ***Transient microbiota*** colonizes the host for a short period; do not persist in the body due to the host's defense system.
- ***Opportunistic pathogens*** – cause disease (also could be some of resident of transient microbes).
- Normal relationship between resident microbiota and human body:
- Mutualistic, symbiotic - a relationship in which both species benefit

Discovery of probiotics

- The root of the word *probiotic* comes from the Greek word ***pro***, meaning "promoting," and ***biotic***, meaning "life ".
- The discovery of probiotics came about in the early 20th century, when **Elie Metchnikoff**, known as the "father of probiotics," had observed that rural dwellers in Bulgaria lived to very old ages despite extreme poverty and harsh climate. He theorized that health could be enhanced and senility delayed by manipulating the intestinal microbiome with host-friendly bacteria found in sour milk.
- Since then, research has continued to support his findings along with suggesting even more benefits.

Main sources of probiotics

- The major consumption of probiotics by humans is in the form of dairy-based foods containing intestinal species of lactobacilli and bifidobacteria.
- Consumption of the probiotic affects the composition of the intestinal microflora. This effect of the probiotic on the intestinal ecosystem impacts in some beneficial way the consumer.

Human microbiota occurence

- **External and internal surfaces**
- skin, mucous membranes, **gastrointestinal tract** (**GIT**), upper respiratory system and distal portion of the urogenital system
- sterile areas include the body cavity, lungs, central nervous system, circulatory system and upper urogenital regions

Potential benefits of optimal composition of probiotics in GIT:

- increased resistance to infectious diseases, particularly of the intestine
- decreased duration of diarrhea
- reduction in blood pressure
- reduction in serum cholesterol concentration
- reduction in allergy
- stimulation of phagocytosis by peripheral blood leucocytes
- modulation of cytokine gene expression
- adjuvant effect
- regression of tumours
- reduction in carcinogene or co-carcinogene production

Microbiome of GIT

- Bacteria (60 %), yeasts, protozoa
- *“It is tempting to suggest that the role of the host is to function as an advanced fermenter, carefully designed to maximize the productivity of the microbiome.”*
- *Nicholson*
- Composition : 98% anaerobic MO 60-80% not cultivating,
- 500 – 1000 genes DNA analysis: ~ 1800 genes, 15 – 30 000 species! Bacterial metabolic products influence other part of our body!

Vaginal tract

- *Lactobacillus crispatus,, L. gasseri, L. iners, L. acidophilus*
- *L. jensenii, L. buchneri*
- Glycogen fermentation
- Acidic condition = inhibition of pathogenic MO
- **H_2O_2** production

Colonization of GIT I.

- **Birth**
- Sterile intestine, contact with vaginal and faecal microflora of mother
- First cultivated MO: *E. coli*, enterococci, lactobacilli, staphylococci
- Normal birth X caesarean section
- In term X premature delivery
- **1st phase of colonization – the main source = mother**
- Breast feeding - colonic MO = **90% bifidobacteria (*B. longum*) *Lactobacillus* spp.**
- Infant formula – Enterobacteriacae, *Clostridium* spp., Bacteroides, streptococci etc.

Colonization of GIT II.

- **2nd phase of colonization –** solid diet

- Reaching the “adult“ type of microbiota between 1st and 2nd year of life
- Coliform bacteria, enterococci and clostridia
- The number of bifidobacteria
- Differences: Influenced by: geographic - physiological factors
- ethnic - diet
- individual diet - iatrogenic factors

- Disbiosis = illness
- **Is there a core microbiome?**

Classification of microbiome members I.

- **Phylum Firmicutes 65%**
- Mainly G+ bacteria, not Actinobacteria
- Coccus, rods, many with endospores
- Big variability
- Low content of C+G
- **Lactic acid bacteria**
- Families:
- Bacilli
- Clostridia
- Erysipelotrichi
- More than 250 species

Classification of microbiome members II.

- **Phylum Bacteroidetes 16%**
- •Mutualistic microorganisms, important in all warm-blooded organisms
- •Gram negative, non-sporing, obligatory anaerobic rods
- •Both mobile and immobile
- •Pleomorphic morphology
- •Bile resistant
- •Bacteriocin producers
- •40-48% C+G
- •Often antibiotic resistant
- •Extracellular substances
- •Saccharides metabolism!!! – all glycoside bonds

Classification of microbiome members III.

- **Phylum Proteobacteria 9%**
- •Anaerobic or facultative anaerobic, Gram negative
- •Non-sporing rods
- •***Escherichia coli,*** coliforms

- •Non-sporing, Gram positive
- •Anaerobic
- •Pleomorphic
- •***Bifidobacterium* spp.,** *Propionibacterium* spp.

Classification of microbiome members IV.

- **Phylum Actinobacteria 5%**
- •Non-sporing, Gram positive
- •Anaerobic
- •Pleomorphic
- •***Bifidobacterium*** **spp.,** *Propionibacterium* spp.

Importance of human microbiome I.

- **I. Stimulation and training of the immune system** (GALT-90% of immune cells)
- Activate innate and adaptive immunity
- Cytokines, interleukins
- Hygienic hypothesis

- **II. Metabolic function**
- Digestion of non-digestible substances
- Source of vitamins, essential fatty acids
- Source of energy – mainly butyric acid (50% of energy for intestinal epithelial cells)

Importance of human microbiome II.

- **III. Microbial barrier against pathogens and undesirable microorganisms**
- Competition for a site on the intestinal mucosa
- Competition for nutrients
- Production of antimicrobial agents

- **IV. New findings** – the effect of microbiome on cognitive functions

Criteria for probiotic microoganisms

- I. Origin and properties of microorganism
- II. Health criteria – health claims
- III. Technological criteria
- **All probiotic properties are STRAIN SPECIFIC!**

Origin and properties of microorganism I.

- Isolates from human digestive tract x wild strains adaptable to GIT
- Safety – GRAS (**g**enerally **r**ecognized **a**s **s**afe), Qualified Presumption of Safety
- Exhibits no virulence factors
- No pathogenity
- No toxicity
- No haemolytic activity
- Tolerant to gastric juice – pH, bile

Origin and properties of microorganism II.

- Colonization of gut (biofilm formation)
- **No transferable genes of antibiotic resistance**
- Antimicrobial activity
- Ability to reduce adhesion of pathogens or exclude pathogens from the gut

- Good adhesion properties (fimbria, surface proteins)

Properties of microorganisms I.

- **Virulence** = the ability of an agent of infection to produce disease
- “degree of pathogenity“
- All members of the genus *Staphylococcus* have a slime layer - major virulence factor.
- *S. epidermis* – normally on human skin, but problematic when it grows in layers on plastic medical devices, but less virulent than *S. aureus.*
- *S. aureus* expresses many other potential virulence factors.

Properties of microorganisms II.

- **Antibiotic resistance**
- intrinsic resistance, a natural property of an organism, genes bearing resistance are localized both on chromosome and plasmids
- evolutionary stress on a population, consequence of evolution via natural selection
- acquired resistance, which results from mutation or via the acquisition of generic material (plasmid or transposon)
- **Horizontal transfer** - conjugative plasmid

- - transduction
- - transformation

Probiotic microorganisms III.

- **LACTIC ACID BACTERIA - LAB**
- *Lactococcus*
- *Streptococcus*
- *Pediococcus*
- ***Enterococcus***
- ***Lactobacillus***
- *Leuconostoc*
- *Carnobacterium*

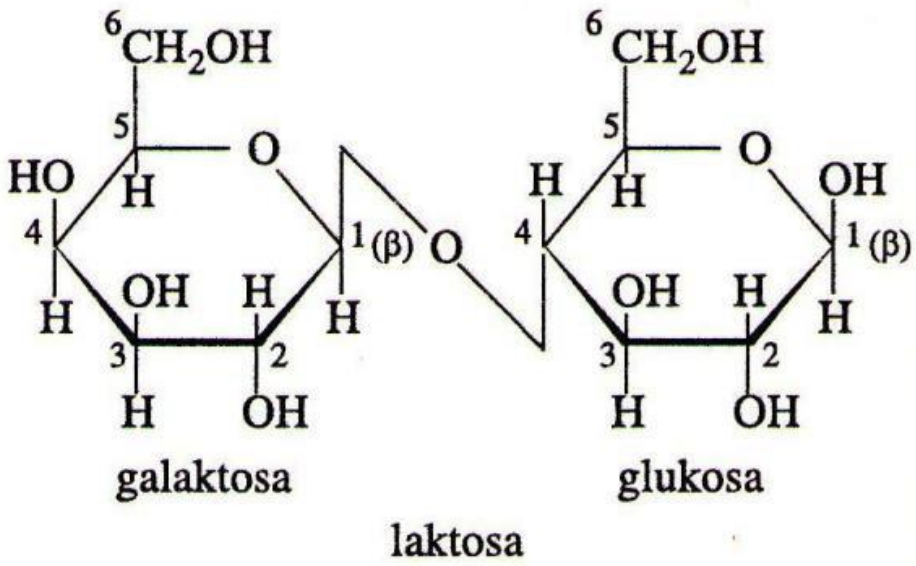
Lactic acid bacteria - characteristics

- Gram positive, non-sporing, no pigment
- Catalase negative, oxidase negative
- Coccus or rods
- Anaerobic, facultative anaerobic, but aerotolerant or microaerophilic
- Very demanding for nutrients (vitamins, amino acids, nucleic acids)
- Tolerant to acid environment
- Obligatory fermentative – lactic acid as main product
- Occurrence:
- Ubiquitous
- Food (milk, meat, vegetables, …), grass …
- Part of normal microbiota of animals

Antimicrobial compounds

- Organic acids
- Hydrogen peroxide – vaginal lactobacilli; oxidative agent (cell wall, DNA)
- Minority products – acetoin, diacetyl (citrate fermentation), acetaldehyde, ethanol
- Reuterin
- Bacteriocins – small peptides with pore activity (nisin, pediocin, acidocin, helveticin, etc.)

Yoghurt culture



- *Lactobacillus delbruecki* subp. *bulgaricus*
- *Streptococcus thermophilus*
- Symbiotic microorganisms
- β-galactosidase
- Lactose reduction in product, ?? in human body
- Often in combination with bifidobacteria of other lactobacilli

Prebiotics I.

A prebiotic is a special type of soluble fiber that is used mostly by the beneficial good bacteria as a fuel. The term was coined in **1995**.

So what are prebiotics? The ones with the most science behind them are **inulin** and **oligofructose**. Inulin, itself, is remarkable in that it has been around in the plant world for a very long time. It has been found in over 36,000 different plants, so it somehow has been a vital food source for plant-eating animals and humans for a very long time.

Prebiotic is **not digested** by the **small intestine,** is used as nutrition, or fermented by some **colon bacteria**, produces health benefits by objective measurements.

They were first identified and named by Marcel Roberfroid in **1995.**

Prebiotics II.

- Prebiotic research has focused on the effects that prebiotics confer on ***Bifidobacteria*** and ***Lactobacillus****.*
- These bacteria have been highlighted as key probiotics and beneficial gut bacteria as they may have several beneficial effects on the host in terms of **improving digestion** and the **effectiveness and intrinsic strength of the immune system.**
- Both *Bifidobacteria* and *Lactobacillus* have been shown to have **differing prebiotic specificity** and selectively to ferment prebiotic fiber based on the enzymes characteristic of the bacterial population.
- Thus, ***Lactobacilli*** prefers **inulin** and **fructooligosaccharides**, while ***Bifidobacteria*** displays specificity for inulin, fructooligosaccharides, **xylooligosaccharides** and **galactooligosaccharides.**

Prebiotics – mechanism of action

- *Bifidobacteria* and *Lactobacillus* are bacterial populations which use saccharolytic metabolism to break down substrates. *Bifidobacteria* contain specific metabolic pathways specialized for the fermentation and metabolism of plant-derived oligosaccharides, or prebiotics.
- These pathways in *Bifidobacteria* ultimately produce **short chain fatty acids**. An **endogenous source of prebiotics** in humans is human breast milk, which contains oligosaccharides structurally similar to GOS, referred to as **human milk oligosaccharides** (**HMOs**). They strengthen the **infant immune system.**
- **Exogenous sources** are found in **raw oats, unrefined barley**, yacon and **whole grain breakfast cereals**.

Gnotobiology

- **Gnotobiology** is the science of study of animals or other organisms raised in environments **free of germs** or those which contain only specifically known germs.
- Scientists compare gnotobiotic animals with ordinary animals whose bodies carry many germs, like bacteria, viruses and parasites.
- **Germ-free** mice and piglets, allow to study the effects of single microbiota components on the development of physiological and immunological responses in experimental models of human diseases.

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Control of microbiological quality of foods; EU Legislative rules valid for pathogens presence, GMP, HACCP

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Food safety

Food safety is a scientific discipline describing handling, preparation, and storage of food in ways that prevent food-borne illness. The occurrence of two or more cases of a similar illnesses resulting from the ingestion of a common food is known as a food-borne **disease outbreak**.

This includes a number of routines that should be followed **to avoid potential health hazards**. In this way food safety often overlaps with food defense to prevent harm to consumers.

The tracks within this line of thought are safety between industry and the market and then between the market and the consumer.

In considering industry to market practices, food safety considerations include the origins of food including the **practices relating to food labeling, food hygiene**, **food additives and pesticide residue**s, as well as policies on biotechnology and food and guidelines for the management of governmental import and export inspection and certification systems for foods.

The five key principles of food hygiene, according to WHO, are:

- **Prevent contaminating** food with **pathogens** spreading from people, pets, and pests.
- **Separate raw and cooked foods** to prevent contaminating the cooked foods.
- Cook foods for the appropriate length of time and at the **appropriate temperature to kill pathogens**.
- Store food at the **proper temperature**.
- Use **safe water** and safe raw materials.

Committee on the Environment, Public Health and Food Safety (EU)

- The committee is responsible for
- environmental policy and environmental protection measures,
- public health,
- **food safety issues**, in particular:
- (a) the **labelling and safety of foodstuffs**,
- (b**) veterinary legislation** concerning protection against risks to human health; public health checks on foodstuffs and food production systems,
- (c) the **European Food Safety Authority (EFSA)** and the European Food and Veterinary Office..

Regulation (EC) No 2073/2005

Regulation (EC) No 2073/2005 also provides that **food business operators** are to ensure that foodstuffs comply with the relevant microbiological criteria set out in Annex I to that Regulation.

Previous documents were extended for food safety criteria and process hygiene criteria regarding **dried infant formulae** and dried dietary foods for special medical purposes.

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COMMISSION REGULATION (EC) **No 1441/2007** of 5 December 2007

amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

Commission Regulation (EC) **No 2073/2005** of 15. November 2005 on microbiological criteria for foodstuffs lays down **microbiological criteria** for certain micro-organisms and the implementing rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to in Article 4 of Regulation (EC) No 852/2004.

Why new document

- The BIOHAZ Panel of **EFSA** issued an opinion on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs on 26 and 27 January 2005.
- It concluded that one of the **major control measures** is to control **temperature** and to establish a system based on **hazard analysis and critical control point principles**.
- Dehydrated foods, in which the presence of spores of pathogenic *Bacillus* spp. is frequent, might permit the growth of *Bacillus cereus* once rehydrated in warm water.
- In line with the **EFSA** opinion, the numbers of *Bacillus cereus* spores in dried infant formulae and dried dietary foods should be as low as possible during processing and a process hygiene criterion should be laid down in addition to good practices designed to reduce delay between preparation and consumption.

Definition I.

- **micro-organisms** means bacteria, viruses, yeasts, moulds, algae, parasitic protozoa, microscopic parasitic helminths, and their toxins and metabolites;
- **microbiological criterion** means a criterion defining the acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number of micro-organisms, and/or on the quantity of their toxins/metabolites, per unit(s) of mass, volume, area or batch;
- **food safety criterion** means a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market;

Definition II.

- **process hygiene criterion** a criterion indicating the acceptable functioning of the production process. Such a criterion is not applicable to products placed on the market. It sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law;
- **batch** means a group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period;
- **shelf-life** means either the period corresponding to the period preceding the ‘use by’ or the minimum durability date, as defined respectively in Articles 9 and 10 of Directive 2000/13/EC;

Definition III.

- **ready-to-eat food (RTE food)** means food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level micro-organisms of concern;
- **food intended for infants** means food specifically intended for infants, as defined in Commission Directive 91/321/EEC (14);
- **food intended for special medical purposes** means dietary food for special medical purposes, as defined in Commission Directive 1999/21/EC (15);

Definition IV.

- **sample** means a set composed of one or several units or a portion of matter selected by different means in a population or in an important quantity of matter, which is intended to provide information on a given characteristic of the studied population or matter and to provide a basis for a decision concerning the population or matter in question or concerning the process which has produced it;
- **representative sample** means a sample in which the characteristics of the batch from which it is drawn are maintained. This is in particular the case of a simple random sample where each of the items or increments of the batch has been given the same probability of entering the sample;

Definition V.

- **compliance with microbiological criteria** means obtaining satisfactory or acceptable results set in Annex I when testing against the values set for the criteria through the taking of samples, the conduct of analyses and the implementation of corrective action, in accordance with food law and the instructions given by the competent authority.

Testing against criteria

- **Food business operators** shall perform testing as appropriate against the **microbiological criteria** set out in Annex I, when they are validating or verifying the correct functioning of their procedures based on **HACCP p**rinciples and **good hygiene practice**.
- **Food business operators** manufacturing **ready-to-eat foods**, which may pose a *Listeria monocytogenes* risk for public health, shall sample the processing areas and equipment for *Listeria monocytogenes* as part of their sampling scheme.

Analysis of the trend

- **Food business operators** shall analyze trends in the test results. When they observe a trend towards unsatisfactory results, they shall take appropriate actions without undue delay to remedy the situation in order to prevent the occurrence of microbiological risks.

How to analyze

- Routine **microbiological test is** a *vital part of analysis* of any product in which microorganisms can survive and grow. Indeed, **microbiological tests are significant process** to determine the **safety and quality of these products**.
- One of the **most important steps** for this purpose is **sampling process**. Sample collection is the original stage of a process whereby data on the characteristics of a batch are collected for evaluation.
- **Practically, only a fraction of the batch** is sampled for testing; and therefore, that fraction must be representative of the batch under consideration.
- Since the fate of the batch depends upon the results generated from that first sample, **sample selection must be regarded as a critical process.**

Prevention

- The **purposes of microbiological quality** control, whether carried out by government inspection services or industry, are mainly twofold: **prevention of food-borne disease** and **retardation of microbial spoilage**.
- In addition, quality control must also meet general microbiological quality requirements, having no direct relationship to health or spoilage.

ISO 22000

- **ISO 22000** is a **standard** developed by the International Organization for Standardization dealing with food safety. This is a general derivative of ISO 9000.
- The ISO 22000 international standard specifies the requirements for a food safety management system that involves interactive **communication,** system management, prerequisite programs, **HACCP principles**.
- ISO 22000 was first published in 2005. It is the culmination of all previous attempts from many sources and areas of food safety concern to provide an end product that is safe as possible from pathogens and other contaminants. Every 5 years standards are reviewed to determine whether a revision is necessary, to ensure that the standards remain as relevant and useful to businesses as much as possible.

The choice of method

- **Microbiological test procedures** for the examination of foods and beverages have been standardized and regulated, but nearly every country has its own regulations.
- After this period, the system of normative procedures **ISO** was established.
- All parts of the analytical process (culture media, additives, dyes, and indicators) have to conform to ISO standards.

Sampling schemes employed in microbial quality control are based on an analysis of susceptible points in a system, known as **hazard analysis and control of critical points (HACCP**).

1. Conduct a hazard analysis
2. Determine the Critical Control Points (CCPs)
3. Establish critical limits
4. Establish monitoring procedures
5. Establish corrective actions
6. Establish verification procedures
7. Establish record keeping and documentation procedures

Microbiological criteria for foodstuffs

- Food safety criteria
- Process hygiene criteria
- In both cases the main responsibility for quality lies with food operators.
- Each product must have own HACCP document.

Content of the document

COMMISSION REGULATION (EC) **No 1441/2007**

- Meat and products thereof
- Milk and dairy products
- Egg products
- Fishery products
- Vegetable, fruits and products thereof
- Rules for sampling and preparation of test samples

Food safety criteria

Sampling plan : n – number of sample units (usually 5)

c – number of sample units giving values between **m** and **M**

Limits : m – tolerated number of target organisms

M – the highest number of target organisms in **c**

Size of sample : usually **10g,** in some cases **25g**

GMP

- Good Manufacturing Practice (**GMP**) is a system for ensuring that products are consistently produced and controlled according to quality standards. It is designed to minimize the risks involved in any pharmaceutical production that cannot be eliminated through testing the final product.
- There is a heavy emphasis on compliance with Good manufacturing practices (GMP) in all relevant **food legislation** and customer certification standards.

Why is GMP important?

- The food business has a **legal and moral responsibilit**y to produce and prepare food that will not harm the consumer.
- There can be a high cost to the food business if it does not implement adequate Good manufacturing practices (GMP).
- **All staff should be trained** in the food businesses GMP procedures. Good manufacturing practices (GMP) includes many basic operational conditions and procedures that are required to be met by the food business. These can include the following:

Good manufacturing practices (GMP)

- **GMP include the following**:
- The correct **construction** and **layout** of the food premises.
- The condition of the external environment of the food premises.
- The **adequate maintenance of equipment** and utensils used within the food business.
- The use of **suitable chemicals** within and around the food premises including **cleaning chemicals, pest control chemicals** and machine lubricants.
- The identification and storage of waste within and by the food business.
- The **cleanliness of the food premises**, equipment, utensils, floors, walls and ceilings. An **effective pest control program** implemented within the food premises and surrounds.
- The avoidance of foreign matter within the finished product. Sources of foreign matter can include wood, glass, metal, plastic, pests, paper, string, tape.

GMP

"**Food safety**" is a broader term which means an assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use.

Today we master food safety with different good practices which are the consequence of human culture, history and lifestyle.

Analysing good practices in the **broad spectrum** of food area we could arrange them in **three categories**.

First category of good practices is directly **connected with food technology (i.e., Good Manufacturing Practice – GMP**).

Second category is indirectly connected with food issues (i.e., Good Research Practice – GRP, **Good Educational Practice – GEP**, Good Training Practice – GTrP).

Third category deals with all activities regarding consumers, food handling (**Good Housekeeping Practice – GHKP)**

Good Laboratory Practice

- The phrase **good laboratory practice** or **GLP** specifically refers to a **quality system** of management controls for research laboratories and organizations to ensure the **uniformity**, **consistency**, **reliability, reproducibility, quality**, and **integrity** of chemical (including pharmaceuticals) non-clinical safety tests; from physio-chemical properties through acute to chronic toxicity tests.
- **GLP** was first introduced in New Zealand and Denmark in **1972.** GLP was instituted in US following cases of fraud generated by toxicology labs in data submitted to the FDA by pharmaceutical companies.

European Union I

- Since 1987 the European Council has adopted **two** basic **Directives** and a **Decision** relating to the application of the **GLP principles**. Directive 2004/10/EC has replaced Directive 87/017/EEC as of 11 March 2004; Directive 2004/9/EC has replaced Directive 88/320/EEC as of 11 March 2004.
- "Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances."

European Union II

- **Product Oriented Directives** referring to GLP obligations: **Feeding stuffs**; Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition **Foodstuffs**; Directive 89/107/EEC Novel Foods and novel food ingredients; Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel **foods and novel food ingredients.**
- In **academic** and **nonclinical research areas**, the phrase Good Laboratory Practice (GLP) specifically refers to a **system of management controls** ensuring that researchers' tests and operations provide reliable, consistent, and reproducible results under safe conditions.

JMBE – Journal of Microbiology &Biology Education

- *Establishing Good Laboratory Practice at Small Colleges and Universities*
- J Microbiol Biol Educ. 2017; 18(1): 18.1.10.
- Published online 2017 Apr 21. doi: 10.1128/jmbe.v18i1.1222
- **Susan M. Bornstein-Forst**
- Supplemental materials available at http://asmscience.org/jmbe

GLP

- **GLP** underpins the mutual acceptance of test data between countries, which avoids duplicative testing, is **beneficial to animal welfare**, and **reduces costs for industry and governments**.
- **Common principles for GLP** also facilitate the **exchange of information** and prevents the emergence of non-tariff barriers to trade, while contributing to the protection of human health and the environment.

WHO

- WHO Library Cataloguing-in-Publication Data
- **Handbook**: good laboratory practice (GLP): quality practices for regulated non-clinical research and development - 2nd ed.
- 1.**Laboratories** - organization and administration.
- 2.Laboratories - handbooks.
- 3.Laboratories techniques and procedures.
- 4.Manuals. I.UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.
- **ISBN 978 92 4 154755 0**
- This is the **second version of the WHO Handbook** on GLP. It is the result of experience gained since the first version was published. It also refers to material related to GLP developments over the last seven years.

WHO and FAO

- In 1963, the WHO and FAO published the **Codex Alimentarius,** which serves as a guideline to food safety.
- The ***Codex Alimentarius*** is a collection of internationally recognized standards, codes of practice, guidelines, and other recommendations relating to foods, food production, and food safety.
- The Codex Alimentarius is published in the **six official languages** of the **United Nations**: Arabic, Chinese, English, French, Spanish and Russian. Not all texts are available in all languages.